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# WIE VERURSACHT DAS EPSTEIN-BARR-VIRUS TUMORE?

# EIN NEUES MOLEKULARES MODELL DER ENTSTEHUNG DES BURKITT LYMPHOMS

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ZUSAMMENFASSUNG

Ausgangspunkt dieser Experimentalarbeit war die Charakterisierung zentraler regulatorischer Elemente latent persistierender Genome des Epstein-Barr-Virus in lymphoiden Zellinien. Im Verlauf der Arbeit ergaben sich aber Erkenntnisse, die weitaus weitergehende Aussagen zur Entstehung von viruskorrelierten Krebserkrankungen und der spezifischen Rolle des EBV bei diesem Prozess erlaubten und zur Etablierung eines neuen Modells führten. Die Chromatinstruktur von zentralen Regulator-Elementen, Ursprüngen der DNA-Replikation und Promotoren der Transkription des EBV, wurde in einer Reihe von Zellinien mit Nukleotidauflösung in vivo analysiert. Im einzelnen wurden oriP, orilyt, EBER1p, EBER2p, Qp, Cp, LMP1p, LMP2Ap, L1-TRp, BARTp, Zp, Rp, und vIL10p/rep* untersucht. Dabei wurde als Methodik hauptsächlich das Genomische Footprinting zur Analyse der Protein-DNA-Bindung verwendet. Zusätzlich wurde das Bisulfit-Sequenzieren zur Bestimmung des Methylierungsgrads von CpG-Dinukleotiden verwendet. Zur weiterführenden Identifizierung und Charakterisierung von Bindungsstellen und regulatorischen Proteinen wurden Gelretardationsanalyse, Reportergen-Versuche und Chromatin-Immunpräzipitation eingesetzt.

In den meisten Promotoren konnten neue Bindungsstellen für Replikations- und Transkriptionsfaktoren kartiert werden. Andererseits konnte gezeigt werden, daß Bindungsstellen, die in früheren Analysen durch in vitro Techniken definiert wurden, in vivo auf latenten EBV-Genomen nicht wirklich von Proteinen besetzt sind. Untersuchungen von hier erstmals entdeckten Bindungsstellen führten zur weiteren Charakterisierung regulatorischer Proteine. Diese Kartierungsarbeit führte daher zur Modifikation und Korrektur bestehender in vitro Modelle.

Von allgemeinem molekularbiologischen Interesse war die Identifizierung und funktionelle Charakterisierung einer telomerartigen DNA-Sequenz, die im Initiator der DNA-Replikation des oriP in regelmäßigem Abstand dreimal vorliegt. Da der oriP wie ein chromosomaler Replikationsursprung des menschlichen Genoms funktioniert, hat die Entdeckung dieses neuen Replikationselements im oriP des EBV generelle Konsequenzen für die Erforschung der DNA-Replikation.

Eine Beobachtung, deren Bedeutung über ein allgemeines molekularbiologisches Interesse hinausgeht, gelang im EBER-Locus des EBV. Im Promotor des EBER1-Gens befindet sich eine funktionelle Bindungsstelle für das Onkoprotein und den Transkriptionsfaktor c-Myc. Diese Bindungsstelle liegt innerhalb einer DNA-Sequenz

von 19 Basenpaaren Länge, die mit identischer Basenabfolge ein einziges Mal auch im menschlichen Genom vorliegt. Dort liegt sie am 5´-Ende des Immunglobulin Lambda Locus, exakt vor den Genen für die variablen Abschnitte der Immunglobuline. Diese Bindungsstelle stellt die erste direkte Verbindung zwischen der Translokation und Überexpression des c-myc-Gens und dem EBV-Genom beim Burkitt Lymphom dar und erlaubt die Etablierung eines neuen molekularen Modells für die Entstehung des Burkitt Lymphoms. Die Entdeckung einer Bindungsstelle für das Onkoprotein c-Myc an einem zentralen Ort des viralen Genoms wurde daher zum neuen Schwerpunkt der Arbeit. Der gewählte Titel lautet daher „Wie verursacht das Epstein-Barr-Virus Tumore?“ zusammen mit einem Lösungsvorschlag „Ein neues molekulares Modell der Entstehung des Burkitt Lymphoms“. Dieses Modell berücksichtigt im Gegensatz zu bisher bestehenden Modellen virale Expressionsmuster und Eckdaten aus EBV-Forschung und Hämatologie, kombiniert diese auf einfache Weise, und weist dem EBV eine kausale Rolle bei der Tumorentstehung zu, die über eine Rolle als opportunistischer Trittbrettfahrer hinausgeht. Eine Immunsuppression ist bei diesem Modell keine Voraussetzung für die Lymphomentwicklung. Die durch EBV zur lymphoblastoiden Zelle transformierte B-Zelle ist in diesem Modell nicht die Vorläufer-Zelle der Lymphomzelle. Die Fähigkeit des EBV, B-Zellen zu immortalisieren und morphologisch zu transformieren, spielt nicht die wesentliche Rolle.

Die Entstehung des Burkitt Lymphoms läuft nach diesem neuen Modell folgendermaßen ab: Die Translokation des c-myc-Gens geschieht als Nebenprodukt der physiologischen Keimzentrumsreaktion, auch beim Gesunden. Praktisch alle translozierten Zellen werden jedoch durch den im Keimzentrum stark ausgeprägten Schutzmechanismus der Apoptose eliminiert. Normalerweise befinden sich in den lymphoiden Keimzentren des Gesunden keine EBER-positiven bzw. EBV-infizierten Zellen. Im Keimzentrum sind EBV-infizierte Zellen erst unter den Bedingungen der Überstimulierung des Immunsystems anzutreffen, wie sie z. B. bei der Malaria, bei frühen AIDS-Stadien und generell bei Parasitosen beobachtet werden. Wenn unter diesen speziellen Bedingungen die Translokation des c-myc-Gens in einer EBV-infizierten Zelle geschieht, dann aktiviert c-Myc direkt über seine Bindungsstelle im EBER1-Promotor die anti-apoptotische Funktion der EBERs. Das deregulierte c-myc-Gen kann durch die chromosomale Translokation nicht mehr abgeschaltet werden. Da die pro-apoptotische Funktion von c-Myc durch die starke Expression der anti-

apoptotisch wirkenden EBERs ausgeglichen wird, wird die translozierte Zelle mit höherer Wahrscheinlichkeit die Schutzmechanismen des Keimzentrums überleben, wenn sie EBV-infiziert ist. Als Folge kann sich in überlebenden translozierten Zellen das onkogene Potential des überexprimierten c-Myc-Proteins frei entfalten.

Die anti-apoptotischen Effekte der viralen LMP2A- und LMP1-Expression könnten, zusammen mit der EBER-Expression, in analoger Weise die Entstehung der EBV-positiven Subtypen des Hodgkin Lymphoms in Keimzentren erklären. Wenn zusätzlich zur Translokation und Deregulierung von zellulären Onkogenen und zur transienten Expression von anti-apoptotischen viralen Proteinen noch hit and run-Mechanismen in dieses Modell einbezogen werden, ist es denkbar, daß das EBV auch an der Entstehung von EBV-negativen Keimzentrums-Lymphomen kausal beteiligt ist, daß EBV aber im Lauf des Tumorwachstums aus den Lymphomzellen verloren geht. Die Erhaltung des EBV in der Burkitt Lymphom-Zelle dürfte durch die nuclear matrix attachment-Funktion der c-Myc-Bindung erst möglich werden. Der Normalfall wäre dann der sekundäre Verlust des viralen Genoms. Das klassische Burkitt Lymphom wäre unter den Keimzentrums-Lymphomen dadurch ein Sonderfall, daß das EBV im Tumor erhalten bleibt. Die Entwicklung transgener Tiermodelle soll in nächster Zukunft dazu beitragen, die Tragweite dieses molekularen Modells der Lymphomentstehung zu untersuchen.

Es bleibt die Frage bestehen, ob das EBV hauptsächlich durch seine anti-apoptotischen Funktionen zur Tumorentstehung beiträgt, oder ob zusätzlich noch virale Mutagenese-induzierende Funktionen dabei helfen. Die Bindungsstelle für c-Myc stellt eine Homologie zwischen dem EBV und den Immunglobulinloci dar. Über die c-Myc Bindungsstelle hinaus zeigen die EBERs Homologien mit den V-Segmenten, ist der oriP funktionell homolog zum intronischen Immunglobulin-Enhancer, und wurden in den W-repeats des EBV Signale gefunden, die homolog zu den switch-Signalen der konstanten Abschnitte der Immunglobuline sind. Damit wurde eine starke Kolinearität von strukturellen und funktionellen Elementen des EBV-Genoms und der Immunglobulin-Loci sichtbar, die sich teilweise in Sequenzhomologien widerspiegelt. Möglicherweise ist das EBV-Genom mit den Immunglobulin-Loci evolutionär verwandt. Deshalb könnten die EBERs mit der zellulären Maschinerie für die Somatische Hypermutation interferieren, diese fehlsteuern oder in nicht-B-Zellen aktivieren, zur größeren Mutationshäufigkeit in EBV-infizierten Zellen führen und damit zur Tumorentstehung beitragen.

ABKÜRZUNGEN

| | |
|---|---|
| AID | activation-induced cytidine-deaminase |
| BART | BamH1 A region transcripts, auch CST |
| BL | Burkitt Lymphom |
| BRLF1 | BamH1 R fragment left frame 1 |
| Cp | C-Promotor, für alle EBNA-Transkripte |
| CST | complementary strand transcripts, auch BART |
| DS | dyad symmetry |
| EBER | Epstein-Barr encoded small RNAs |
| EBNA | Epstein-Barr nuclear antigen |
| EBV | Epstein-(Achong-)Barr-Virus |
| FR | family of repeats |
| Ig | Immunglobulin |
| IM | Infektiöse Mononukleose |
| HL | Hodgkin Lymphom |
| L1-TRp | terminal repeat Promotor für LMP1 |
| LCL | Lymphoblastoide Zellen, Zellinien |
| LMP1p | bidirektionaler LMP-Promotor für LMP1 und LMP2B |
| LMP | Latenz-Membranproteine |
| NHL | Non Hodgkin Lymphom |
| NPC | Nasopharynxcarcinom |
| orilyt | Ursprung der DNA Replikation des EBV Genoms im lytischen Zyklus |
| oriP | Ursprung der DNA Replikation des EBV Genoms in der Latenz |
| p | -Promotor |
| PTLD | Post-Transplant Lymphoproliferative Disease |
| Qp | Q-Promotor für EBNA1 |
| Rp | R-Promotor, BRLF1-Promotor |
| SHM | Somatische Hypermutation |
| rep* | rep-star, überlappt mit vIL10p, hypothetisches Hilfselement für oriP |
| vIL10p | Promotor für das virale Interleukin 10 Homolog BCRF1 des EBV |
| Wp | W-Promotor, für alle EBNA-Transkripte |
| Zp | Z-Promotor, BZLF1-Promotor |

**1. Biologie und Klinik der EBV-Infektion** [zur Übersicht siehe 169, 170]

Das Epstein-Barr-Virus (EBV, HHV4, human herpesvirus 4) ist eines der acht Herpesviren des Menschen. Es wurde in die Gattung Lymphocryptovirus der Subfamilie γ-Herpesvirinae eingeordnet. Die Herpesviren sind große DNA-Viren mit einem linearen doppelsträngigen DNA-Genom von etwa 170 Kilobasen in einem membranumhüllten ikosaedrischen Capsid. EBV ist zu virtuell 100% mit den jeweils endemischen Subtypen der Malignome Burkitt Lymphom (BL) und Nasopharynx-Carcinom (NPC), zu niedrigeren Prozentsätzen zwischen 10 und 80% mit weiteren Malignomen, wie Untergruppen der Hodgkin Lymphome (HL), von T-Zell Lymphomen, Magen-, Mamma- und Leberkarzinomen vergesellschaftet (5, 18, 23, 69, 74, 95, 201-204, 216, 217, 224, 242). Die Assoziation des EBV mit Mamma- und Leberkarzinomen ist allerdings umstritten (76, 77). Wegen seiner Assoziation mit dem BL steht das EBV seit seiner Entdeckung im Jahr 1964 im Verdacht, an der Entstehung spezifischer Tumore kausal beteiligt zu sein (24, 25, 43). Da allerdings fast die gesamte erwachsene Weltbevölkerung mit EBV infiziert ist (72), ist es schwierig geblieben, die Diskrepanz zwischen extrem hoher Durchseuchung und niedrigen Tumorinzidenzen zu erklären. Die molekularen Mechanismen und die spezifische Rolle des EBV bei der Tumorentstehung sind bis in die jüngste Zeit nicht völlig geklärt worden. Die Primärinfektion des Menschen findet in den weniger entwickelten Ländern durch engen Eltern-Kind-Kontakt meist in den ersten Lebensjahren, in den weiter entwickelten Ländern durch enge Kontakte zwischen Jugendlichen oft auch erst in der Adoleszenz statt. Zu Beginn einer Primärinfektion werden wahrscheinlich oberflächlich sitzende B-Zellen in den Tonsillen und anderen lymphoiden Organen des Waldeyerschen Rachenrings durch EB-Viren infiziert, die in den Epithelzellen der Speicheldrüsen des Überträgers gebildet und mit dem Speichel ausgeschieden wurden. Hier läuft der lytische Zyklus der Virusvermehrung mit der Freisetzung von Nachkommenviren und der lytischen Infektion weiterer B-Zellen und Epithelzellen zeitlich geordnet ab. Am Beginn (immediate early) des lytischen Zyklus steht die starke Expression zweier viraler Schalter-Gene, BRLF1 und BZLF1, die für Transkriptionsfaktoren kodieren (15, 28, 34). Diese aktivieren zahlreiche frühe (early) virale Gene, die hauptsächlich für Transkriptionsfaktoren, Signalmoleküle oder Enzyme des viralen DNA-Stoffwechsels kodieren, und zelluläre Gene. Nach der frühen Phase folgt die virale DNA-Replikation, darauf die Expression der späten (late) Gene, die hauptsächlich für virale Strukturproteine kodieren. Die frühkindliche

Primärinfektion wird im allgemeinen vom Immunsystem symptomlos überwunden, die verspätete Primärinfektion in der Adoleszenz führt häufig zu einer Infektiösen Mononukleose (IM, auch „kissing disease“) (73). Die bei der IM namengebenden, im Blutbild beobachteten großen mononukleären Zellen sind, neben den aktivierten B-Zellen, hauptsächlich aktivierte Immun-Effektorzellen, die die EBV-infizierten B-Zellen eliminieren (210). Über die spezifische Immunabwehrreaktion hinaus sind diese atypischen „Monozyten“ auch Folge einer unspezifischen Stimulierung des Immunsystems. Die IM ist normalerweise eine selbstlimitierende Erkrankung, die innerhalb weniger Wochen ausheilt. Sie kann aber auch ernsthafte Komplikationen verursachen, und bei den Trägern des Duncan-Syndroms, eines seltenen X-chromosomal vererbten genetischen Defekts, sogar regelmäßig letal enden (12, 161). Nach einer, gleichgültig ob mit oder ohne klinische Symptomatik, durchgemachten Primärinfektion persistiert das Virus in einem latenten Zustand lebenslang im Organismus. Der am besten bekannte Ort der Viruslatenz im infizierten Organismus sind die Gedächtnis-B-Zellen des Immunsystems (11, 139, 140). Das Virusgenom liegt in multiplen Kopien als zirkuläres Plasmid etwa in einer von 20.000 bis einer von 1.000.000 B-Zellen des peripheren Bluts vor. Im Falle einer Zellteilung wird das virale Plasmid mittels seines Replikationsursprungs oriP, des viralen Replikationsfaktors EBNA1 und der zellulären Replikationsmaschinerie an die Tochterzellen weitergegeben. Die meisten der etwa 100 viralen Promotoren sind stillgelegt. Die Stillegung umfaßt sämtliche sehr frühen, frühen und späten Promotoren des lytischen Zyklus und auch die meisten Latenzpromotoren. Das virale Expressionsprogramm, bei dem keine wesentliche lytische Replikation in Blut, Knochenmark und lymphatischem System stattfindet, ist in den B-Zellen auf nur wenige Gene beschränkt (11, 29, 162). Lediglich in den Epithelien der Speicheldrüsen wird das Virus episodisch vermehrt und mit dem Speichel ausgeschieden. Durch den Rückzug in die Epithelzellen und auf das minimale Latenzprogramm in den Gedächtnis-B-Zellen bleibt das EBV für das Immunsystem des Gesunden unsichtbar (116).

**2. Molekularbiologie der EBV-Latenz** [zur Übersicht siehe 19, 100, 170]

Zur Untersuchung von Latenzformen des EBV in B-Zellen existieren zwei gebräuchliche Zellkultursysteme. 1.) Durch die in vitro-Kultivierung von Tumorzellen aus BL-Biopsien erhält man BL-Zellinien (44, 160). 2.) Die Infektion von

menschlichen B-Zellen mit EBV in vitro führt zur Entstehung von lymphoblastoiden Zellinien (LCL), immortalisierten und morphologisch transformierten B-Zellen. (134, 135, 158). Darüber hinaus existieren weltweit nur vereinzelte NPC-Zellinien, die das virale Genom nicht verloren haben (33). In den beiden gebräuchlichsten Zellkultursystemen, in LCL und BL-Zellen, persistiert das EBV-Genom in einem latenten Zustand als zirkuläres Plasmid in mehreren Kopien mit jeweils charakteristischen viralen Transkriptionsmustern. In BL-Biopsien und in kultivierten BL-Zellen ist das virale Latenzprogramm 1 aktiv. Bei diesem werden nur die beiden EBER-Transkripte (EBER, Epstein-Barr encoded RNAs), die Transkripte der BamH1 A Region (BART, BamH1 A region transcripts) und das nukleäre Protein 1 (EBNA1, Epstein-Barr nuclear antigen 1) exprimiert. In LCL und bei BL-Zellen, die in Kultur ihren Phänotyp zu LCL-artigem Wachstum umgeschaltet haben, wird das virale Latenzprogramm 3 gefunden. Bei diesem werden neben den EBERs und den BARTs alle sechs EBNA-Proteine und die Latenz-Membranproteine (LMP) exprimiert. Die Expression sämtlicher viraler Latenzgene in den LCL stellt ein B-Zell-Wachstumsprogramm dar, da die Latenzproteine für die Immortalisierung und Proliferation der LCL benötigt werden. Das Expressionsmuster des Latenztyps 3 wird auch bei lymphoblastoiden Zellproliferationen schwer immunsupprimierter Patienten nach Organtransplantation (PTLD, post-transplant lymphoproliferative disease) oder bei Lymphomen in fortgeschrittenen AIDS-Stadien gefunden. Es existiert noch ein intermediäres Latenzprogramm 2, bei dem zusätzlich zu den EBERs und BARTs die LMPs und EBNA1 exprimiert werden. Beim HL, bei T/NK-Zellymphomen und beim NPC wird Latenztyp 2 gefunden.

Die EBER-Gene (Abbildung 1) kodieren für zwei kleine RNAs, von denen aufgrund von Sequenzhomologien und von in vitro Untersuchungen vermutet wird, daß sie von der RNA-Polymerase 3 transkribiert werden (9, 92). Die Promotoren der EBER-Gene enthalten jedoch auch Bindungsstellen für Transkriptionsfaktoren des RNA-Polymerase 2-Komplexes (81). Daher ist die Frage noch nicht geklärt, welche Polymerase für die Transkription der EBERs zuständig ist oder ob unter verschiedenen Bedingungen beide Polymerasen eine jeweils spezifische Rolle spielen könnten. Den EBERs wurden anti-apoptotische und tumorigene Funktionen zugeschrieben (148, 176, 189). Sie binden an die dsRNA-abhängige Proteinkinase DAI (36, 188, 189) und blockieren dadurch den Interferon $\alpha$-Signaltransduktionsweg,

der normalerweise die Apoptose einer virusinfizierten Zelle auslösen würde (36, 148, 189). Über die Hemmung der Apoptose hinaus wurden den EBERs zusätzliche, noch nicht definierte Mechanismen zur Erhöhung der Tumorigenität zugeschrieben (176).

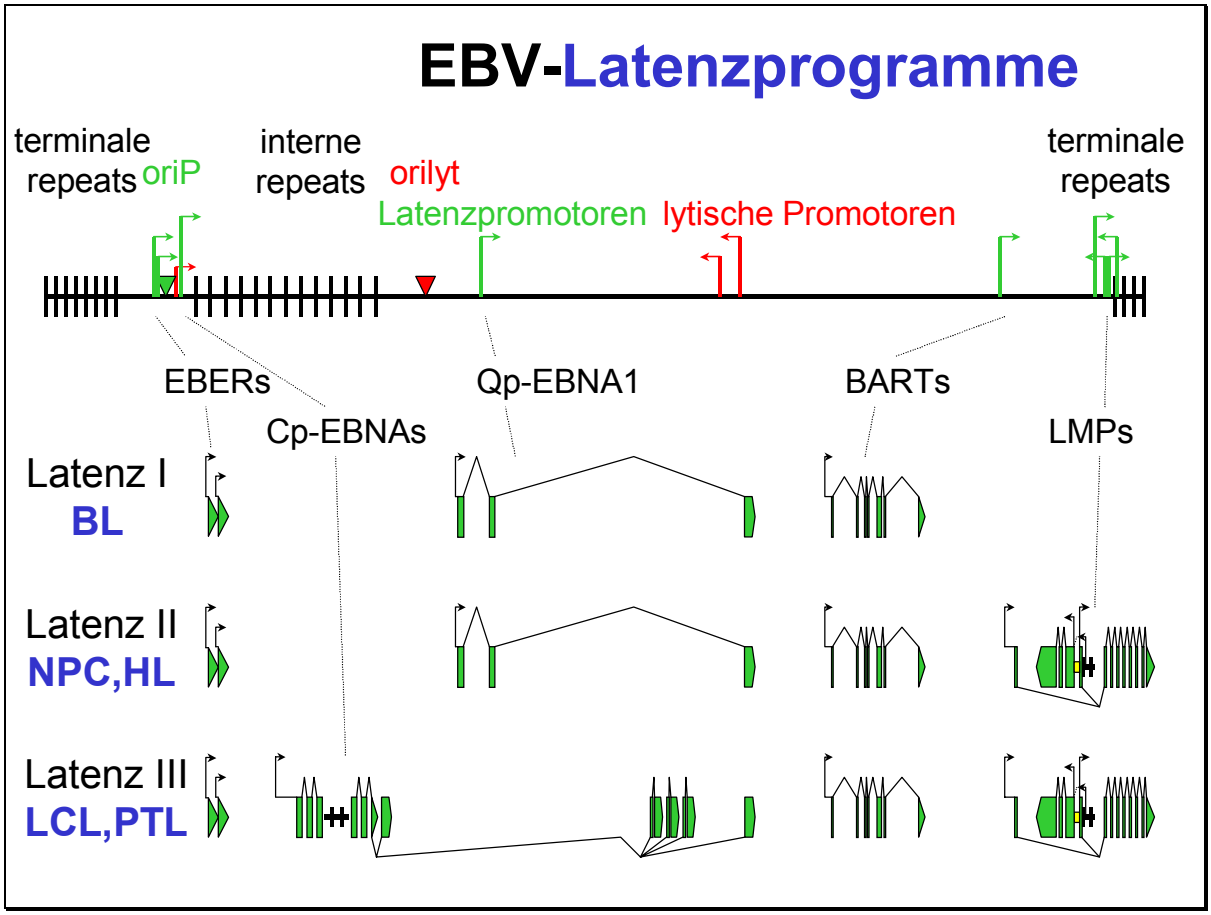


Abbildung 1: Hier sind die Genomstruktur des EBV, die Latenzpromotoren (grün), einige zentrale lytische Promotoren (rot), die drei Latenzprogramme in Zellkultur und die Transkriptions- und Spleißmuster der Latenzgene dargestellt.

Die EBNA-Proteine (Abbildung 1) sind Transkriptionsfaktoren, die für das Wachstumsprogramm in transformierten B-Zellen benötigt werden. Beim Latenztyp 3 werden alle EBNA-Proteine vom C-Promotor (Cp), beim Latenztyp 1 wird nur EBNA1 vom Q-Promotor (Qp) aus transkribiert (17, 154). EBNA1 hat eine besondere Rolle. Neben seiner Funktion als Transkriptionsfaktor ist EBNA1 auch ein nuclear matrix attachment-Faktor (132, 133) und außerdem der einzige virale Replikationsfaktor, der für die Replikation des oriP in der Latenz benötigt wird (124, 168). Der wesentliche Unterschied zwischen den Latenzprogrammen 1 und 2 einerseits und

dem Latenzprogramm 3 andererseits ist, daß bei den ersteren die EBNA-Expression auf den Qp und EBNA1 beschränkt ist, und beim letzteren die gesamte EBNA-RNA vom Cp aus transkribiert wird. Zu Beginn der Transformation von B-Zellen durch EBV wird die gesamte EBNA-RNA transient vom W-Promotor (Wp) aus transkribiert. Durch die ansteigende EBNA2-Expression wird die Transkription vom Wp auf den nahe upstream gelegenen Cp umgeschaltet (3, 206, 222, 223).

Die BART, auch CST (complementary strand transcripts) genannt, werden vom BART-Promotor (BARTp) kontrolliert (Abbildung 1). Sie sind bei allen drei Latenztypen und auch in Tumorbiopsien exprimiert, ihre Funktion ist aber noch weitgehend unbekannt (21, 79, 98, 179, 196, 197).

Bei den LMPs (Abbildung 1) handelt es sich um Membranproteine, die die Funktion von konstitutionell aktiven Rezeptormolekülen übernehmen können. LMP2A kann die Funktion des B-Zellrezeptors ersetzen und mit dessen Signalkaskade interferieren (26, 27, 130). LMP1 kann die Funktion des CD40 Signalwegs ersetzen (101). Die EBV-infizierte, defekte Zelle kann durch die Expression der LMPs im lymphoiden Keimzentrum unabhängig vom Antigenkontakt und vom Kontakt mit den T-Helferzellen überleben. Beide Membranrezeptoren können daher in einer kritischen Phase der B-Zellentwicklung zur Apoptoseresistenz der EBV-infizierten Zelle beitragen. LMP2A wird vom LMP2A-Promotor (LMP2Ap), LMP1 vom terminal repeat-Promotor für LMP1 (L1-TRp) aus gesteuert. Außerdem wurde der LMP1-Promotor (LMP1p) als bidirektionaler Promotor für LMP1 und für LMP2B, eine verkürzte Spleißform des LMP2A beschrieben (50, 89, 112). Das LMP2B-Protein konnte aber bisher nie in der lebenden Zelle nachgewiesen werden, ist also ein hypothetisches Konstrukt geblieben (19).

Die Transkription der viralen Latenzgene ist bemerkenswert, da die längsten RNA-Transkripte etwa 60% des gesamten 170.000 Basenpaare langen viralen Genoms umspannen, und wegen der komplizierten multiplen Spleißmuster dieser Transkripte. Durch die komplizierten Spleißvorgänge ergibt sich bei der Untersuchung der Transkriptionsmuster des EBV in der Latenz das Problem, daß nur mit hohem Aufwand mit Sicherheit bestimmt werden kann, von welchen Promotoren aus die Latenztranskripte exprimiert werden. Daher wurden auch erst in jüngster Zeit zwei

Latenzpromotoren entdeckt, die in der Zellkultur aktiv sind. Es sind dies BARTp für die Familie der BARTs (79, 179, 197) und L1-TRp, der in den terminalen Repeats gelegene Promotor für das LMP1-Protein (180). Ein weiteres, allgemeines Problem der Promotoranalyse, das unter anderem auch für Promotoranalysen im menschlichen Genom zutrifft, ergibt sich aus Unterschieden zwischen der in vitro und in vivo Analyse. Im allgemeinen wurden Promotoren durch Computeranalyse (2, 221), Reportergenstudien (40), Gelshifts und in vitro Footprints (75) untersucht. Bei diesen in silico- und in vitro-Ansätzen wird jedoch vernachlässigt, daß die DNA im Zellkern nicht nackt, sondern als Chromatin verpackt vorliegt. Das Chromatin ist eine komplexe Struktur, die durch die An- und Abwesenheit von Proteinen, wie Histonen und Transkriptionsfaktoren, und kovalenter Modifikationen, wie Acetylierung von Histonen und Methylierung von CpG-Dinukleotiden der DNA, gekennzeichnet ist (16, 96, 97). Daher wurden bereits Fälle beschrieben, bei denen Konsensus-Bindungsstellen für Transkriptionsfaktoren, die in vitro mit nukleären Extrakten auf nackter DNA eine klare Bindung zeigten, in vivo auf der genomischen DNA nicht besetzt waren (138).

**3. Experimentalansatz Chromatin-Analyse**

Ein experimenteller Ansatz zur allgemeinen Problematik der Promotoranalyse und zur Analyse latenter EBV-Genome ergibt sich aus der Möglichkeit, Protein-DNA-Wechselwirkungen nicht nur in vitro, sondern auch in vivo zu untersuchen (58, 128, 145). Die Feinkartierung der Protein-DNA-Wechselwirkungen in den Promotoren latenter EBV-Genome erbringt Informationen darüber, welche Bindungsstellen und Transkriptionsfaktoren für die Expression der viralen Gene verantwortlich sind. Durch die Untersuchung der Chromatinstruktur wird auch die Wissenslücke verkleinert, die im allgemeinen zwischen den Daten zur Signaltransduktion und zur RNA-Expression besteht. Daher habe ich die Methodik des Genomischen Footprintings etabliert und auf eine Serie zentraler regulatorischer Elemente des EBV angewandt. Ergänzend zur Analyse der Protein-DNA-Bindung wurde in Zusammenarbeit mit der Gruppe von Prof. Janos Minarovits vom National Center of Epidemiology in Budapest zusätzlich die Bestimmung des Methylierungsgrades von CpG-Dinukleotiden durchgeführt (35, 54, 147). Es wurden folgende regulatorische Elemente des EBV untersucht: Die beiden Ursprünge der DNA-Replikation, oriP für die Latenz und orilyt, der neben seiner Replikationsfunktion im lytischen Zyklus auch ein früher bidirektionaler

Promotor ist, die Latenzpromotoren EBER1p, EBER2p, Qp, Cp, LMP1p, LMP2Ap, L1-TRp und BARTp, die sehr frühen Promotoren Zp und Rp, und der späte Promotor vIL10p, der mit dem hypothetischen Replikationselement rep* weitgehend überlappt. Neben den Promotoren der Viruslatenz mit Ausnahme des Wp wurden exemplarisch also auch einige zentrale Promotoren des lytischen Zyklus untersucht. Für die Analyse wurde eine Reihe von Standard-Zellinien verwendet, in denen EBV-Genome in strikt latenter Form persistieren und die entweder den Latenztypen 1 oder 3 angehören. Im einzelnen handelte es sich um die Zellinien Raji [BL, Typ 3, (215)], 721 [LCL, Typ 3, transformiert mit dem EBV-Stamm B95-8, (131)] Rael [BL, Typ 1, (45)], CBM1-Ral-STO [LCL, Typ 3, transformiert mit dem EBV-Stamm Rael, (45)], zwei genetisch identische Subklone der BL-Zellinie Mutu (62), Mutu BLI-Cl216 (BL, Typ 1) und Mutu BLIII-Cl99 (BL, Typ 3) und C666.1, eine einzigartige NPC-Zellinie, die das EBV-Genom nicht verloren hat (33).

## 4. Ergebnisse und Diskussion

Die Ergebnisse dieser Untersuchungen erfüllten weitgehend unsere Erwartungen. Es wurden zahlreiche neue Bindungsstellen für Transkriptionsfaktoren gefunden (59, 153, 183). Andererseits konnten vormals postulierte Regulationsmechanismen nicht bestätigt werden (152, 153, 183). Bestehende Modelle der Regulation von Promotoren müssen daher modifiziert oder revidiert werden. Durch die Entdeckung des Telomer-Repeats im oriP (150, 213) und der c-Myc-Bindungsstelle im EBER-Locus (151) wurden unsere Erwartungen aber auch auf überraschende Weise übertroffen. Alle Ergebnisse sind ausführlich und im Detail in den beiliegenden Veröffentlichungen dargestellt und diskutiert. An dieser Stelle sind die wichtigsten Daten und Folgerungen daher nur kurz dargestellt.

### 4.1 Promotoren des lytischen Zyklus

In der Latenz ist der lytische Zyklus der Virusvermehrung abgeschaltet. Die lytischen Promotoren und Replikationsursprünge sind weitgehend stillgelegt. Es war also zu erwarten, daß lytische Regulator-Elemente in strikt latenten Zellinien nicht mit aktivierenden, sondern mit inhibitorischen Proteinen besetzt sind oder nur histonverpackt in einem geschlossenen Zustand vorliegen und in diesem Fall keine sequenzspezifischen Proteinbindungen aufweisen.

### 4.1.1 Rp, Zp und orilyt

Rp und Zp sind die Promotoren der beiden viralen immediate early Gene BRLF1 und BZLF1, zweier Schaltergene, deren starke Expression am Beginn des lytischen Zyklus benötigt wird (165, 174, 198, 209, 233). Orilyt ist der Replikationsursprung, der die multiplikative Vermehrung des viralen Genoms im lytischen Zyklus steuert (65). Vor allem für Zp, aber auch für orilyt und in geringerem Maße für Rp existiert eine Fülle von in vitro Daten, die zur Definition von zahlreichen Bindungsstellen für Transkriptions- und Replikationsfaktoren führte (13, 28, 34, 38, 51, 59, 63, 65, 70, 107, 118, 123, 125, 142, 143, 166, 185-187, 190, 191, 231, 232, 234). Es ist aber unbekannt, ob die betreffenden Bindungsstellen in der lebenden Zelle tatsächlich besetzt sind, mit welchen Proteinen sie besetzt sind und ob die angenommenen Bindungen eine Bedeutung für die Biologie des EBV besitzen.

Diese drei zentralen lytischen Promotoren des EBV wurden daher einer detaillierten in vitro und in vivo Analyse unterzogen. Für Rp wurden im Reportergen-Assay erstmals in einem relevanten Zellkultursystem Aktivitäten gemessen (59). Dadurch wurde im distalen Bereich von Rp eine neue Bindungsstelle für NF1-Proteine entdeckt, die diesen Promotor inhibiert. Eine Bindungsstelle für NF1 mit der gleichen Konsensus-Sequenz haben wir auch in unmittelbarer Nachbarschaft einer Bindungsstelle für Sp1 im distalen Abschnitt des Zp gefunden, der zuvor als inhibitorisch beschrieben worden war (142, 143, 187). Unsere eigene Analyse (153) ergab, daß die vielen in vitro beschriebenen Aktivator-Bindungsstellen in Rp, Zp und orilyt in vivo keine sequenzspezifische Proteinbindung aufweisen. Dies ist völlig im Einklang mit unserer Erwartung für inaktive Promotoren, relativiert aber die vormals beschriebenen in vitro Bindungsstellen. Deren Bedeutung liegt wohl am ehesten in einer Phase des lytischen Zyklus, in der replizierende virale Genome bereits an die nukleären ND10-Domänen angedockt haben (14). Nur auf dem distalen Sp1-NF1 Locus des Zp fanden wir eine kräftige Proteinbindung in allen Zellinien. Dies bestätigte, daß NF1-Proteine und GC-Box bindende Proteine, wie Sp1 an der Repression des lytischen Zyklus in der Viruslatenz beteiligt sind.

### 4.1.2 rep*

Rep* wurde mittels des üblichen in vitro-Ansatzes als Hilfselement für oriP, den Replikationsursprung in der Latenz vorgeschlagen (102). Rep* überlappt größtenteils mit dem Promotor für BCRF1, eines EBV-Gens, das zum humanen Gen für

Interleukin-10 homolog ist (144). Da es sich bei vIL10p um einen späten viralen Promotor handelt, der in der Latenz stillgelegt ist (88, 141, 211, 235), erschien es als sehr unwahrscheinlich, daß dieser gleichzeitig als aktives Replikationselement fungiert. Daher wurde die Chromatinstruktur von rep*/vIL10p untersucht.
Es konnten in keiner unserer Zellinien enge Protein-Basen-Kontakte gefunden werden. Rep* ist also frei von sequenzspezifischen Bindungen, wie sie für die meisten Replikations- und Transkriptionsfaktoren typisch sind. Geringfügige Spuren von Proteinbindungen deuten auf histonartige Verpackungsproteine hin. Im allgemeinen waren die CpG-Dinukleotide in rep*/vIL10p hochgradig methyliert, was wiederum die Inaktivität des Elements reflektierte. Aufgrund unserer Untersuchung ist es daher eher unwahrscheinlich, daß rep* in vivo als Hilfselement für den oriP fungiert. Es bleibt also bis auf weiteres bei den beiden Elementen FR (family of repeats) und DS (dyad symmetry) des oriP als Replikator in der EBV-Latenz.

## 4.2 Promotoren der Viruslatenz

Wenn das EBV in latentem Zustand in B-Lymphozyten persistiert, sind die meisten seiner Promotoren stillgelegt. Je nach Programm sind nur die Latenzpromotoren und der oriP oder spezifische Subgruppen dieser Elemente aktiv. Korrelierend mit der Aktivität eines Promotors in Abhängigkeit vom Latenztyp der untersuchten Zellinie waren Unterschiede in der Chromatinstruktur dieser Promotoren zu erwarten.

### 4.2.1 oriP

Der oriP ist der Ursprung der DNA-Replikation des latenten EBV-Genoms in B-Zellen (228, 230). Die viralen Genome werden durch die Replikationsfunktion des oriP in der Synthesephase des Zellzyklus verdoppelt, und durch die nuclear matrix attachment-Funktion des oriP im Zellkern festgehalten und in der Mitose auf die Tochterzellen verteilt (1, 64, 66, 91, 219, 229). Die Funktionen des oriP werden durch dessen cis-Elemente, FR und DS, den viralen Replikationsfaktor EBNA1 und die zelluläre Replikationsmaschinerie vermittelt (124, 168). Im Gegensatz zu lytischen Replikationsursprüngen, die zur multiplikativen Vermehrung der viralen Genome führen, gilt der oriP wegen seiner Abhängigkeit vom Zellzyklus als virales Modell für die chromosomale DNA-Replikation. FR enthält zwanzig Bindungsstellen für EBNA1 und wirkt als nuclear matrix attachment-Element und als Replikations- und

Transkriptionsenhancer. DS enthält vier Bindungsstellen für EBNA1 und fungiert als Initiationsort der DNA-Replikation im oriP (85, 124, 167, 168).
Durch unsere Analyse des oriP in der Standard-Zellinie Raji entdeckten wir ein bis dahin unbekanntes regulatorisches cis-Element im Initiator DS, das in Abhängigkeit vom Zellzyklus proteinbedeckt ist. Es handelt sich um die 9 Basen lange DNA-Sequenz TTAGGGTTA, die homolog zur Telomersequenz $[TTAGGG]_n$ der Vertebraten ist und die im DS-Element in regelmäßigem Wechsel mit den EBNA1-Bindungsstellen dreimal vorhanden ist. Zusätzlich fanden wir im Bereich der Telomer-Repeats DNA-5´-Enden, die nahelegten, daß innerhalb der Repeats die Initiation der DNA-Replikation stattfindet. Diese Entdeckung markiert die erste Spur von zellulären Replikationsproteinen im oriP, nachdem seit der Entdeckung des oriP nur das virale Protein EBNA1 relevant war (150).
Um das neugefundene Element funktionell charakterisieren zu können, wurde vor allem durch Herrn Dipl. Biol. Matthias Vogel ein neuartiger Reportergen-Assay entwickelt, der auf der Transfektion von Plasmiden mit dem green fluorescent protein als Reportermolekül und dem Sortieren grün fluoreszierender Zellen durch fluorescent activated cell sorting beruht. Durch den Vergleich von Wildtyp-Plasmiden mit solchen, die im Telomer-Repeat mutiert waren, konnte festgestellt werden, daß sich die Erhaltung der mutierten Plasmide in der Zelle deutlich verschlechterte. Damit war dem Telomer-Repeat eine Funktion bei der Replikation des oriP zugewiesen (213). In der Folge konnte von einer anderen Arbeitsgruppe unsere ursprüngliche Vorhersage bestätigt werden, daß Telomer-bindende Proteine an diese Repeats im oriP binden und eine funktionelle Rolle bei der Replikation übernehmen (42). Da der oriP als Modellsystem für die chromosomale Replikation gilt, legte die Entdeckung der Telomer-Repeats nahe, daß Telomer-bindende Proteine oder sogar die Telomerase selbst Teil des normalen DNA-Replikationskomplexes sein können.

### 4.2.2 Qp und Cp

Diese beiden Promotoren nehmen eine Schlüsselstellung bei der Unterscheidung der Latenzklassen 1 und 2 auf der einen Seite und Latenzklasse 3 auf der anderen Seite ein. Bei den Latenzklassen 1 und 2 wird von allen EBNA-Proteinen nur EBNA1 exprimiert. Das zugehörige Transkript wird vom Qp aus kontrolliert (154). Bei Latenzklasse 3 werden nicht nur EBNA1, sondern sämtliche EBNA-Proteine exprimiert. Die Transkription der zugehörigen RNA wird vom Cp aus kontrolliert, der

Qp ist inaktiv. Die mRNAs für die einzelnen EBNAs werden durch mehrfache Spleißvorgänge aus dem multicistronischen Cp-Transkript, das etwa 60% des viralen Genoms umspannt, erhalten (17).

Qp enthält zwei Bindungsstellen für sein eigenes Genprodukt, den viralen Transkriptionsfaktor EBNA1, und eine Anzahl von Bindungsstellen für zelluläre Transkriptionsfaktoren. Aufgrund von Reportergen-Experimenten wurde für Qp eine negative Autoregulation durch EBNA1 und eine zellzyklusabhängige Aktivierung durch E2F in der Synthesephase beschrieben (177, 184, 207). EBNA1 würde nach diesem Modell zur Repression des Qp in Zellen der Latenzklasse 3 beitragen, in der Latenzklasse 1 jedoch nicht.

Unsere in vivo Analyse ergab zahlreiche Proteinbindungsstellen in Qp, die in allen Zelltypen kräftig besetzt waren (183). Die beiden Bindungsstellen für EBNA1 waren in allen Zellinien gleichermaßen deutlich besetzt, auch in Zellen der Klasse 1, in denen der Qp aktiv ist. Die postulierten Bindungsstellen für E2F zeigten keine charakteristischen Proteinbindungsmuster (243). Geringe Unterschiede der Proteinbindung fanden wir um den Transkriptionsstart, im Bereich des Q regulatory element 2 (QRE2), das als Bindungsstelle für interferon regulatory factors (IRFs) in Frage kommt. Diese Bindungsunterschiede korrelierten mit der Latenzklasse und waren vereinbar mit der Bindung eines Repressors in Zellen der Klasse 3. Unsere Analyse des Methylierungsstatus des Qp ergab ein vollständig unmethyliertes Promotorelement. Zusammengenommen deuten unsere Daten auf einen relativ einfachen Promotor hin, der durch einige stark DNA bindende Aktivatoren, wie EBNA1 und Sp1 methylierungsfrei gehalten wird (67, 84). Durch seine offene Chromatinstruktur ist Qp damit der schnellen Regulation durch die An- oder Abwesenheit eines Repressors zugänglich. Die Situation ist analog zu bakteriellen Operons, die durch wenige Aktivatoren und einen dominanten Repressor reguliert werden.

Für Cp waren im wesentlichen fünf Bindungsstellen von Transkriptionsfaktoren bekannt: CBF1, CBF2, Sp1 und zwei CCAAT-Boxen (55, 56, 93, 110, 122, 159, 172). CBF1 wurde die Hauptrolle bei der Aktivierung des Cp in der Latenzklasse 3 zugeschrieben. CBF1 ist ein Repressor, der unabhängig von der Methylierung seiner Bindungsstelle DNA binden kann. Promotoren, die durch CBF1 reprimiert sind, können durch die Bindung des viralen Aktivators EBNA2 oder des zellulären

Aktivators Notch angeschaltet werden (87). Daher wurde eine positive Autoregulation von Cp durch sein eigenes Genprodukt EBNA2 beschrieben (86).
Unsere Untersuchung der Proteinbindung in Cp ergab zahlreiche neue Bindungsstellen für Transkriptionsfaktoren, die in Abhängigkeit vom Latenztyp besetzt waren (183). Erwartungsgemäß war der aktive Cp beim Latenztyp 3 proteinbesetzt, der inaktive Cp beim Latenztyp 1 unbesetzt. Ebenso erwartungsgemäß korrelierte der Methylierungsstatus vor allem im proximalen Bereich des Cp, der die meisten Bindungsstellen enthält, invers mit der Promotoraktivität. Der inaktive Cp war stark methyliert, der aktive war methylierungsfrei. Durch die Entdeckung von zahlreichen weiteren Aktivator-Bindungsstellen im Cp muß die bisherige zentrale Rolle für CBF1 bei der Umschaltung vom Latenztyp 1 zum Latenztyp 3 relativiert werden. Für die CBF1-Bindungsstelle selbst ergab sich ein unerwartetes Bild: Im Gegensatz zum einfachen in vitro-Modell, das eine Besetzung dieser Bindungsstelle unabhängig vom Aktivitätszustand postuliert (173), war die Stelle beim aktiven Promotor besetzt, beim inaktiven unbesetzt. Selbst in Mutu1-Zellen, wo sich die CBF1-Bindungsstelle des inaktiven Promotors ausnahmsweise inmitten einer Methylierungslücke befand, war trotz der Präsenz dieses Faktors im Zellkern keine typische DNA-Bindung für CBF1 zu erkennen. Daher muß das in vitro Modell für die CBF1-Promotorbindung und -regulation wohl noch weiter modifiziert werden.

### 4.2.3 LMP1p und LMP2Ap

Die Latenz-Membranproteine LMP1 und LMP2 werden, wie die EBNA-Proteine beim Latenztyp 3 exprimiert, beim Latenztyp 1 jedoch nicht. Sie sind aber im Gegensatz zu den EBNAs auch beim Latenztyp 2 exprimiert und unterliegen daher einer anderen Regulation als der Cp, der unter den viralen Promotoren das Wachstumsprogramm des EBV für B-Zellen repräsentiert. Für LMP2Ap ist die Regulation durch zwei CBF1-Bindungsstellen als zentraler Mechanismus beschrieben worden (80, 89, 113, 129, 239, 240). Für LMP1p waren über seine beiden CBF1-Bindungsstellen hinaus zahlreiche weitere Regulationselemente beschrieben worden (46-48, 50, 94, 110-112, 121, 192-195, 236).
Für LMP2Ap ergab sich das erwartete Bild: Beim Latenztyp 3 war die Methylierung des aktiven Promotors niedriggradig, die Bindungsstellen zeigten ein charakteristisches Muster, beim Latenztyp 1 war die Methylierung des inaktiven

Promotors hochgradig, die Bindungsstellen waren unbesetzt (182). Über die CBF1-Bindungsstellen hinausgehend fanden wir noch weitere Bindungsstellen, deren Besetzung mit der Promotoraktivität korrelierte. Wie beim Cp zeigte sich daher auch am LMP2Ap, daß CBF1 ein für die EBV-Latenz wichtiger Transkriptionsfaktor ist, aber noch weitere Faktoren berücksichtigt werden müssen.

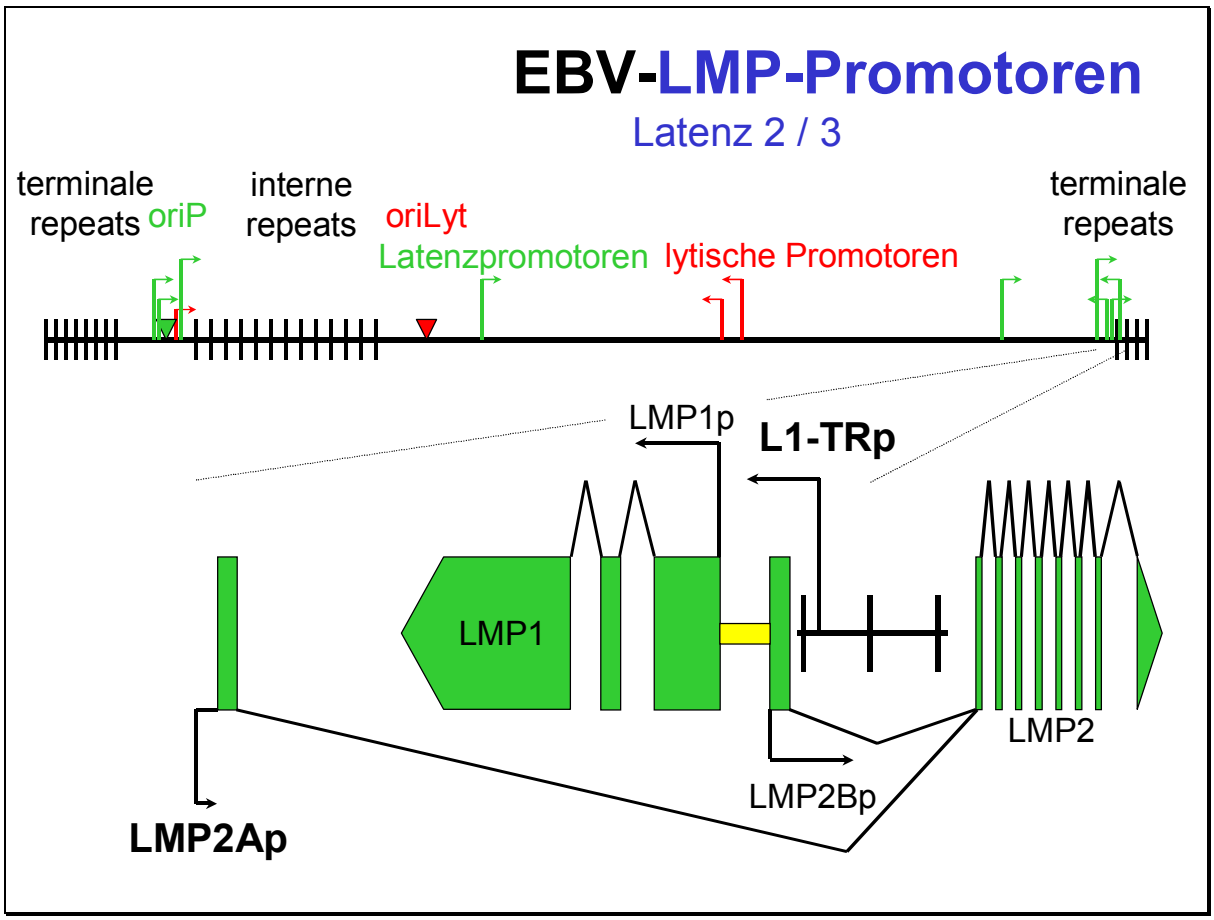


Abbildung 2: Hier sind die Genomstruktur des EBV mit dem vergrößerten LMP-Locus dargestellt. Es wurden zwei Promotoren LMP1p und L1-TRp für das LMP1-, und zwei Promotoren LMP2Bp und LMP2Ap für das LMP2-Protein beschrieben. LMP1p/LMP2Bp (gelber Balken) wurde als bidirektionales Promotorelement beschrieben.

Für den bidirektionalen LMP1p ergab sich eine gänzlich andere, völlig unerwartete Situation: Der Methylierungsstatus korrelierte zwar mit der Aktivität des Promotors, deutliche sequenzspezifische Proteinbindungen oder gar Unterschiede der Proteinbindung in Abhängigkeit vom Latenztyp wurden hingegen nicht gefunden (183, 208). Die beiden Promotoren LMP2Ap und Cp sind unter den nahen

Verwandten des EBV hochkonserviert (53, 56), LMP1p ist aber eher schwach konserviert (171). Da das LMP2B-Protein bisher nicht in vivo nachgewiesen werden konnte (19) und ein alternativer Promotor für LMP1, L1-TRp gefunden wurde (180), erhebt sich der Verdacht, daß LMP1p nur unter sehr speziellen Bedingungen aktiv sein könnte, oder daß es sich bei LMP1p gar um ein in vitro-Artefakt handeln könnte. Neben der Nicht-Nachweisbarkeit von LMP2B besteht in der EBV-Biologie folgendes Paradoxon. EBNA2-Domänen, die für die Transaktivierung des LMP1p benötigt werden, sind für die Transformierung von B-Zellen zu LCL verzichtbar (194, 226). Da die Transaktivierung des LMP1p durch EBNA2 eine wichtige Rolle in allen Transformierungsmodellen spielt, ist dies nur schwer erklärlich. Wenn LMP1p jedoch als in vitro Artefakt ersatzlos gestrichen wird, lösen sich beide Paradoxa zwanglos auf. Einen einfachen Ersatz für den bidirektionalen LMP1p könnten der oben genannte LMP2Ap und der kürzlich entdeckte terminal repeat-Promotor für LMP1, L1-TRp bieten (Abbildung 2).

### 4.2.4 L1-TRp und BARTp

Bei diesen beiden Promotoren stehen wir noch am Anfang unserer Analyse. Die vom L1-TRp ausgehende Expression führt zu einem geringfügig längeren Transkript für LMP1, als das für den LMP1p angenommene (180). Die mögliche Kontrolle des L1-TRp durch Proteine der Jak/STAT-Familie scheint biologisch sinnvoll und erklärt die Aktivität des Promotors nicht nur in Latenzklasse 3, sondern auch in Latenzklasse 2 (30, 31). Ähnlich zum Cp und zum LMP2Ap ergab unsere Untersuchung eine Proteinbindung in Zellen der Latenzklasse 3 und eine abgeschwächte Bindung in Zellen des Latenztyps 1 (Niller, unveröffentlichte Daten). Im Gegensatz zur in vivo-Struktur des LMP1p paßt dieses Bindungsmuster zum Aktivitätsprofil des Promotors. Dieser Befund bestärkt uns wiederum in der Annahme, daß LMP1p ein in vitro-Artefakt sein könnte.

In Zusammenarbeit mit Prof. Paul Farrell vom Ludwig Institute in London werden Aktivität und Chromatinstruktur von BARTp (179) untersucht. Bisher wurden Bindungsstellen für AP1, STAT-Faktoren und NF-κB gefunden (39). NF-κB ist ein Transkriptionsfaktor, der durch seine Regulation des κ-Immunglobulin- (Ig-) Locus bekannt wurde. Dies ist bemerkenswert, da es auf eine B-zellspezifische Regulation der BARTs hindeutet. Da eine Regulation durch STAT-Proteine auch für Qp und L1-

TRp diskutiert wird (30, 31), könnte dies auf eine gemeinsame Regulation hindeuten, die für die Molekularbiologie des EBV relevant ist.

### 4.2.5 Der EBER-Locus

Zwei wesentliche Funktionen wurden in Zusammenhang mit dem EBER-Locus gebracht: 1.) Die EBERs entfalten durch die Hemmung des Interferon $\alpha$-Signaltransduktionsweges in BL-Zellen eine anti-apoptotische Wirkung (105, 106, 127, 148, 189). 2.) Zusammen mit dem oriP gehört der EBER-Locus zur nuclear matrix attachment-Region des viralen Episoms (91, 219). Am EBER-Locus befindet sich das EBV-Chromatin, wie beim unmittelbar benachbarten oriP, in einem geöffneten Zustand, was sich in einer erhöhten Zugänglichkeit für Nukleasen äußert (218). Methylierungsstudien haben ergeben, daß der EBER-Locus zusammen mit dem oriP regelmäßig in Zellinien aller Latenztypen hypomethyliert ist (49, 137). Der Methylierungsstatus korreliert daher exzellent mit der Aktivität der EBER-Promotoren und des oriP (137, 181). Die beiden Promotoren der EBER-Region sind in allen drei Latenzklassen hochaktiv (8).

Unsere Untersuchung der Protein-DNA-Interaktionen im EBER-Locus ergab zahlreiche neue Bindungsstellen in beiden EBER-Promotoren (151). Die aufgrund von in vitro Experimenten postulierten Bindungsstellen, Box A und Box B, für den RNA-Polymerase 3-Komplex waren hingegen unbesetzt (81, 82, 92). Dies ist ein Hinweis darauf, daß in etablierten Zellinien die Transkription durch RNA-Polymerase 3 möglicherweise eine untergeordnete Rolle spielt. Unter den neu gefundenen in vivo-Bindungsstellen im EBER1p war auch eine sehr prominente Bindungsstelle (X-Box) von 19 Basenpaaren Länge vertreten. Diese X-Box enthielt zwei Konsensus-Bindungsstellen für E-Box-Faktoren. Eine der beiden E-Boxen, CACGTG bot eine perfekte Konsensussequenz für die Bindung des Transkriptionsfaktors, nuclear matrix attachment-Faktors und Onkoproteins c-Myc, die andere, CAGATG einen etwas verminderten Konsensus für c-Myc, aber einen perfekten Konsensus für die Bindung des Stammzell-Leukämie-Faktors SCF, auch Tal1 genannt (2, 221). Gelshift-, Reportergen-, und Chromatin-Immunpräzipitations-Experimente zeigten, daß die X-Box durch c-Myc gebunden und aktiviert wird (151). Durch die verschiedenen Ansätze konnte belegt werden, daß sich im EBER1p des EBV-Genoms eine funktionelle in vivo-Bindungsstelle für c-Myc befindet.

## 5. Ein neues molekulares Modell der Entstehung des Burkitt Lymphoms

Die chromosomale Translokation und Deregulierung des c-myc-Gens ist die molekulare Signatur des Burkitt Lymphoms, eines NHL, das in lymphoiden Keimzentren entsteht. Die Translokation des c-myc-Gens und anderer Onkogene wird auch beim Gesunden regelmäßig beobachtet (119, 120, 146). Meistens ist sie ein fehlerhaftes Nebenprodukt der Keimzentrumsreaktion, die durch Somatische Hypermutation (SHM) und Ig-Klassenwechsel im Normalfall zur Reifung des Antikörper-Repertoires führt (61). Da in lymphoiden Keimzentren die Apoptose als Schutzmechanismus gegen derartige tumorigene Mutationen sehr stark ausgeprägt ist, ja auch c-Myc selbst stark Apoptose-fördernd wirkt (32, 136), wird eine Zelle mit Translokation des c-myc-Gens beim Gesunden eliminiert (114, 126). Über die Expression des c-Myc-Proteins in den Zentroblasten des Keimzentrums existieren widersprüchliche Angaben (104, 126). Möglicherweise ist c-Myc wegen seiner übermäßig starken pro-apoptotischen Wirkung im Keimzentrum doch nicht exprimiert (104). In Analogie zu einem kürzlich beschriebenen Pankreastumormodell in der transgenen Maus kommt aber in beiden Fällen das onkogene Potential des c-Myc-Proteins durch das Übergewicht der Apoptose im Keimzentrum nicht zur Ausprägung (156, 157). Es ist gezeigt worden, daß die durch Interferon $\alpha$ ausgelöste Apoptose über einen c-Myc-abhängigen Signalweg läuft (227). Lymphoide Keimzentren sind normalerweise frei von EBER-positiven Zellen, selbst während einer IM (6, 108, 109, 149). Unter Überstimulierung der Keimzentren, wie sie für Entwicklungsländer mit erhöhter Inzidenz des BL und des HL charakteristisch ist, werden jedoch EBER-exprimierende Zellen mit großer Häufigkeit in tonsillären Keimzentren gefunden (6). Die direkte Aktivierung der anti-apoptotischen EBERs im Keimzentrum durch die Bindung des pro-apoptotischen c-Myc-Proteins an seine Bindungsstelle im EBER1p verschiebt das Gleichgewicht in der translozierten Zelle hin zum Überleben dieser Zelle. Da eine solche Zelle mit höherer Wahrscheinlichkeit nicht den Apoptose-Mechanismen des Keimzentrums zum Opfer fällt, kann sich dadurch das onkogene Potential des c-Myc-Proteins freier entfalten. Dabei spielt es zunächst keine Rolle, ob im hyperaktiven Keimzentrum RNA-Polymerase 3 oder 2 die EBERs transkribiert. Denn c-Myc kann nicht nur Polymerase 2, sondern auch Polymerase 3 transkriptionell aktivieren (60).

Die Bindungsstelle für c-Myc im EBV-Genom stellt das fehlende Bindeglied zur Etablierung eines einfachen molekularen Modells dar, das zur Lösung des EBV-Problems geeignet ist (151). Die bisher postulierten Modelle gründeten meist auf der Annahme von schwerer Immunsuppression und dem Auswachsen lymphoblastoider Zellen der Latenzklasse 3 als Vorstufen der Entwicklung eines BL (100, 103, 115). Beide Voraussetzungen konnten im lebenden Organismus experimentell jedoch nicht als regelmäßige Vorstufen des BL nachgewiesen werden und sind daher problematisch geblieben. Hauptproblem des alten Modells ist, dass es eine Umschaltung der Latenzklasse 3 zur Latenzklasse 1 bei der Entwicklung des BL postuliert, diese Umschaltung jedoch in vivo nicht nachgewiesen und auch in vitro nicht nachvollzogen werden konnte (155). Im hier beschriebenen neuen Modell ist nicht die Immunsuppression, sondern im Gegensatz dazu die Überstimulierung der lymphoiden Keimzentren eine notwendige Voraussetzung. Dies stimmt mit den epidemiologischen Daten besser überein, als die Annahme einer schweren Immunsuppression (115). Der eigentliche Effekt der Immunsuppression ist wohl die Erhöhung der Viruslast im Organismus (10). Die Betonung der Immunsuppression in der Literatur und die hervorstechende Fähigkeit des EBV, B-Zellen zu transformieren, scheinen die einfache Lösung des EBV-Problems verschleiert zu haben. Das neue Modell kommt ohne Immunsuppression und Transformation von B-Zellen durch EBV aus. Die LCL ist hierbei nicht die Vorläuferzelle des BL.

Kürzlich wurde ein Subtyp des endemischen BL beschrieben, der sich durch die Deletion des EBNA2-Gens auszeichnet (99). Die Autoren nehmen die Beschreibung dieses Subtyps zum Anlaß, das alte Modell der Tumorentstehung zu unterstützen, das die durch EBV immortalisierte lymphoblastoide Zelle als Vorläufer für die BL-Tumorzelle annimmt (103, 115, 170). Die Entstehung dieses Subtyps läßt sich aber einfach und zwanglos auch durch unser neu entwickeltes Modell erklären. Das Auswachsen von lymphoblastoiden Zellen aus dem Tumor des BL-Patienten wird durch schwere Anfälle von Immunsuppression ermöglicht, wie sie bei der Malaria tropica beschrieben werden (220). Wenn der Patient seine Immunkontrolle wieder gewinnt, werden die LCL durch zytotoxische T-Zellen eliminiert. Falls das Leseraster für EBNA2 aber in einer solchen LCL-Zelle durch Deletion spontan verloren gegangen ist, nimmt diese Zelle einen BL-Phänotyp an und unterliegt nicht mehr der Immunkontrolle durch T-Zellen (199). Daher besteht nicht mehr die Notwendigkeit,

solche Zellen zu eliminieren. Die spontane Deletion des EBNA2-Leserasters wurde in Zellkultur bereits beobachtet (20, 78, 163). Es handelt sich also bei diesem EBNA2-negativen BL um einen seltenen Subtyp, der in unserem Modell durch sekundäre und tertiäre Mechanismen erklärbar ist, deren Vorkommen in der Literatur seit langem belegt ist.

Wenn hit and run-Mechanismen (4) in das neue Modell einbezogen werden, ist es denkbar, daß EBV an der Entstehung auch anderer Keimzentrums-NHL beteiligt ist, aber häufig im Lauf des weiteren Tumorwachstums aus den Lymphomzellen verloren geht. Das endemische BL wäre dann ein Sonderfall, da hier das EBV meist im Tumor erhalten bleibt. Die Erhaltung des EBV in der BL-Zelle dürfte durch die nuclear matrix attachment-Funktion der c-Myc-Bindung vermittelt sein (91, 212, 219). Da c-Myc sehr stark pro-apoptotisch wirkt, ist bei der Translokation anderer Onkogene als des c-myc-Gens die transiente Expression anti-apoptotischer viraler Funktionen wahrscheinlich völlig ausreichend für das Überleben der translozierten Zelle. Die bei anderen Lymphomen fehlende nuclear matrix attachment-Funktion des c-Myc-Proteins führte jedoch häufiger zum sekundären Verlust des EBV-Genoms, nachdem der Tumor bereits etabliert ist. Tatsächliche Anhaltspunkte für hit and run-Mechanismen beim EBV ergeben sich aus der Epidemiologie von HL bei Post-Transplant-Patienten. Das Risiko für ein HL ist nach Knochenmarkstransplantation signifikant erhöht (175), was durch die antigene Stimulierung der Keimzentren durch das Spenderorgan erklärbar ist. Das HL in der Post-Transplant-Phase ist aber dadurch auffällig, daß es zu 80 bis 100%, das HL in der Normalbevölkerung nur zu etwa 40% EBV-positiv ist. Da nicht-transplantierte HL-Patienten nicht unter schwerer Immunsuppression leiden, läßt sich dieser Unterschied am einfachsten durch einen häufigen sekundären Verlust des EBV-Genoms aus den HL-Zellen bei der Normalbevölkerung erklären. Bei Knochenmarks-Transplantierten fällt durch die Gabe von Immunsuppressiva die Immunselektion gegen EBV-positive HL-Zellen weg. Die Möglichkeit eines sekundären Verlusts des EBV Genoms konnte für HL und in NHL bereits verifiziert werden (41, 68).

Um dieses neue BL-Modell einer Überprüfung zu unterziehen, soll ein transgenes Tiermodell etabliert werden. Dazu müssen die EBERs im B-Zellkompartiment transgener Mäuse exprimiert werden. Da durch die anti-apoptotische Funktion der

EBERs dann alle B-Zellen zum Überleben hin tendieren dürften, ist zu erwarten, daß eine höhere Zahl von translozierten B-Zellen als bei normalen Mäusen überlebt. Dadurch sollte auch die Häufigkeit verschiedener Keimzentrums-Lymphome deutlich über der normalen Inzidenz liegen. Wenn alle hier skizzierten Vorhersagen des neuen Modells zutreffen, könnte es die molekularen Entstehungsmechanismen von bis zu 10 Prozent menschlicher Tumoren erklären. Sollten die Vorhersagen dieses Modells im transgenen Tiermodell zutreffen, könnte auch getestet werden, ob sich durch eine Impfung gegen EBV die Inzidenz des BL und anderer Lymphome beim Menschen verringern läßt (225).

**6. Das EBV und die Immunglobulin-Genloci sind verwandt**

Die X-Box des EBER1p mit ihren 19 Basenpaaren kommt mit identischer Basensequenz auch ein einziges Mal im Humangenom vor. Der entsprechende Genort ist das 5´-Ende des V-Genabschnitts im Immunoglobulin-λ-Locus (GenBank accession number D86993). Wir vermuten daher, daß die X-Box des EBER1p durch das EBV aus dem λ-Locus aufgenommen wurde und daß die EBERs möglicherweise aus den V-Genen des λ-Locus hervorgegangen sind, aber im Lauf der Geschichte stark mutiert wurden. Dazu passend ist, daß die beiden EBER-Gene mindestens ein durchgehendes offenes Leseraster besitzen (Niller et al., unveröffentlichte Daten) und die EBERs mit den Polysomen assoziiert sind (178). Mindestens ein ununterbrochenes Leseraster findet man auch in den EBER-Genen des Herpesvirus (H.) papio, im EBER2-Gen des Rhesus Lymphocryptovirus, den Genen für die kleinen U-RNAs 3 und 4 von H. saimiri, in allen acht tRNA-Genen des Mäuseherpesvirus (MHV) 68, und den Genen für die VA-RNAs von Adenovirus Typ 5. Die Zufallswahrscheinlichkeit für ein solches Leseraster-Ereignis liegt für EBER-Gene (170 bis 190 Nukleotide) bei etwa 15 bis 20%, für tRNAs (70 bis 90 Nukleotide) bei etwa 50 bis 70%. Alle hypothetischen Peptide, die von diesen kleinen RNAs abgeleitet werden können, haben Segmente, für die man starke Homologien in Ig-V-Segmenten finden kann (http:/www.ncbi.nlm.nih.gov/BLAST). Das beste Beispiel hierfür liefert das EBER1-Gen von H. papio, eines Virus, das dem EBV beim Pavian entspricht (Niller et al., unveröffentlichte Daten). Dieses hypothetische EBER1-Peptid kann nahezu komplett aus Ig-V-Segmenten zusammengesetzt werden (Abbildung 3). Da V-Segmente verschiedene Sequenzen annehmen können, kann diese Ähnlichkeit

auch zufällig sein. Daher ist dies kein zwingender Beweis, aber ein möglicher Hinweis auf eine Verwandtschaft zwischen den EBERs und den V-Genen.


| | | | | |
|---|---|---|---|---|
| AREEIGAAY<br>AREAIGVAY<br>77%<br>Gadus morhua<br>AJ274748 | RPGYKSREVTVASVFALYQ<br>RPG E APVFVLYQ<br>52%<br>Heterodontus francisci<br>X15315 | VYSCSLAVA<br>IYYCSLAVA<br>77%<br>H. sapiens<br>AJ253035 | VAAISRLRSD<br>VAEI RLRSD<br>80%<br>M. musculus<br>AF242215 | SL AV AAISRLRSDASWWS<br>SLTCAVSGASIS DGDWWS<br>50%<br>H. sapiens<br>AJ406673 |
| | | | | |
| AREEIGAA<br>ARDEIAAA<br>75%<br>H. sapiens<br>AF235653 | RCPSGSARE<br>RSPSGSTKE<br>66%<br>H. sapiens, Ig-ähnlich<br>T09402 | YAARPGY<br>YCARPGY<br>85%<br>H. sapiens<br>AF150995 | | |
| | | | | |
| | PSGSARE<br>PSGASRE<br>71%<br>H. sapiens, TCR β-Kette-V<br>CAB59410 | | | |

Abbildung 3: Hier sind das hypothetische EBER1-Peptid von H. papio mit einer Länge von 62 Aminosäuren und seine Homologien mit Segmenten von Immunglobulin-V-Segmenten dargestellt. Die erste Zeile in jedem Block zeigt ein EBER-Segment, die zweite das entsprechende Peptid aus einem V-Segment, die dritte die Prozentzahl identischer Aminosäuren, die vierte den Speziesnamen, die fünfte die Zugangsnummer für die GenBank/EMBL-Datenbank. Alle Segmente mit einer Ausnahme stammen von Ig-V-Ketten, die Ausnahme stammt aus einem Ig-ähnlichen Protein bzw dem V-Abschnitt der T-Zell-Rezeptor-β-Kette.

Ein weiterer Hinweis auf eine Verwandtschaft zwischen den EBERs und den V-Genen kommt aus der Nukleotidzusammensetzung und Sekundärstruktur der EBERs. Die Sequenzmotive RGYW und WRCY wurden als Zielsequenzen der SHM charakterisiert. Die Häufigkeit dieser Zielsequenzen ist in den V-Genen der Ig-Schwerketten überzufällig auf das 1,3-fache erhöht (52). Im EBER1-Gen des EBV sind beide Motive 1,2- bzw. 1,4-fach häufiger vertreten, als zu erwarten ist. Dies könnte ebenfalls nur Zufall sein, aber interessanterweise sind beide Motive in der Sekundärstruktur des EBER1-Moleküls immer in ungepaarten Regionen, oder an

Übergängen zwischen gepaarten und ungepaarten Abschnitten gelegen (Abbildung 4). Daher sind diese Sequenzmotive für Protein- oder Basenkontakte mit anderen Molekülen grundsätzlich zugänglich. Die RNAs der Ig-V-Gene ergeben ganz ähnliche Sekundärstrukturen, in denen beide Motive ebenfalls exponiert vorliegen, wenn man sie mit dem mfold-Programm des HUSAR-Pakets (http://genome.dkfz-heidelberg.de) darstellt.

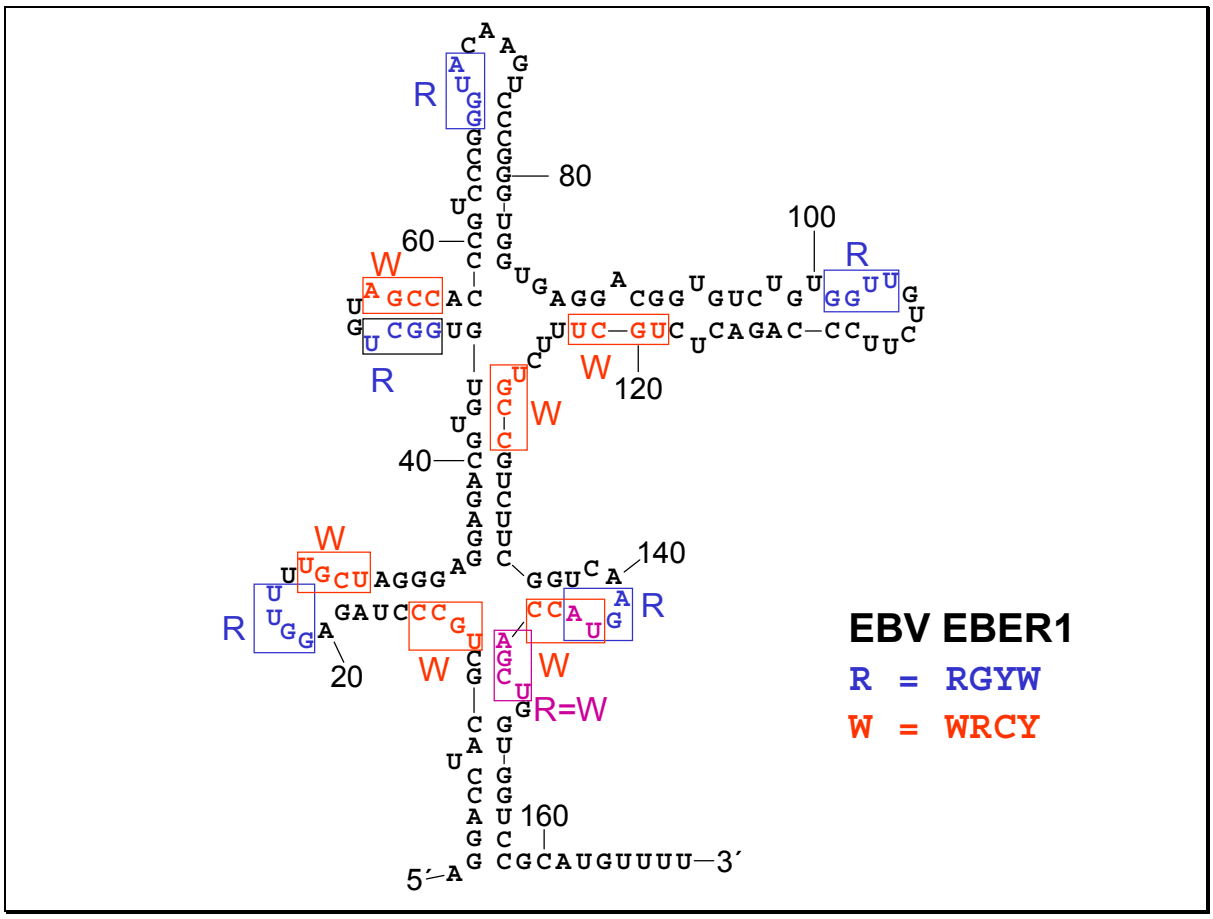


Abbildung 4: Sekundärstruktur der EBER1-RNA (214). Die Zielsequenzen der Somatischen Hypermutation RGYW and WRCY (52) sind durch Boxen markiert. R steht für die Basen A oder G, Y für C oder T, und W für A oder T.

Insgesamt scheinen die EBERs also mit den V-Genen verwandt zu sein. Der Intron-Enhancer ($E_i$/MAR) der Ig-Loci ist ein Zellltyp-spezifischer Transkriptionsenhancer, der nuclear matrix attachment-Elemente (MAR) (37) und einen Ursprung der DNA-Replikation mit einschließt. Die Initiationsfunktion des Replikationsursprungs in $E_i$/MAR ist an dessen 3´-Ende gelegen (7). Der oriP des EBV weist eine ähnliche

Größe, Lage und Orientierung auf, und besitzt Enhancer-, MAR- und Replikator-Funktionen, die homolog zu $E_i$/MAR ebenfalls mit B-Zellpräferenz oder -spezifität wirken (57, 66, 91, 219, 228). Die W-Repeats im EBV-Genom enthalten Sequenzmotive, die auch in den Switch-Elementen enthalten sind, die für die genomische Rekombination beim Ig-Klassenwechsel benötigt werden. W-Repeats und Switch-Elemente binden die gleichen regulatorischen Proteine. Im EBV-Infektionszyklus werden die viralen Genome in den W-Repeats rekombiniert (22, 117, 205). Darüber hinaus könnte auch der orilyt des EBV ein Funktionshomolog des 3´-gelegenen Replikationsursprungs im Ig-Genlocus sein, der nicht mit Zelltypspezifität wirkt (237, 238). Insgesamt ist daher der linke Abschnitt des EBV-Genoms stark kolinear mit den Ig-Genloci (Abbildung 5).

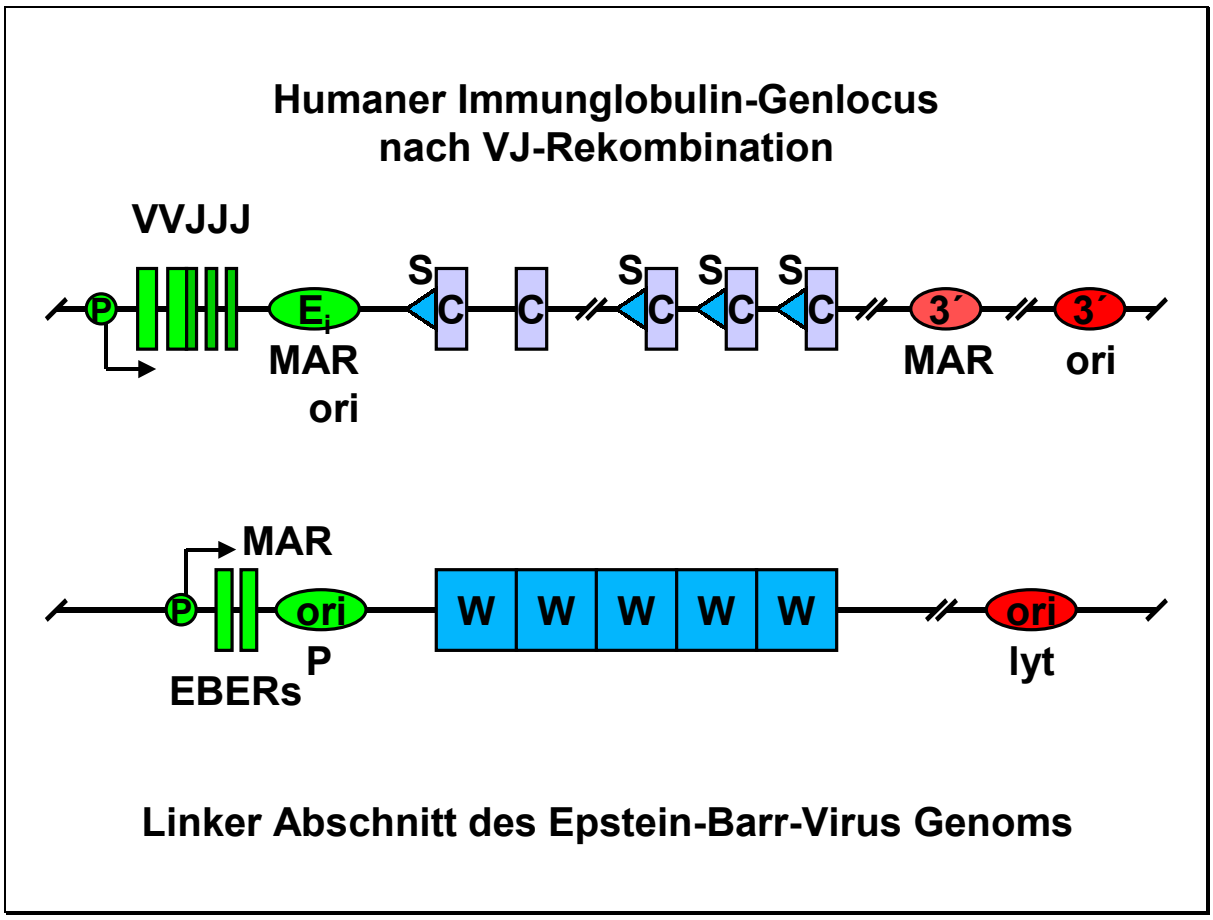


Abbildung 5: Kolinearität zwischen einem menschlichen Immunglobulin-Genlocus nach V(D)J-Rekombination und dem linken Abschnitt des EBV-Genoms. P stellt die 19-Basenpaar-Identität zwischen dem EBER1p und einer Sequenz am 5´-Ende des Lambda Ig-Genlocus dar. V, J, S and C stehen für die Variable-, Joining-, Switch- und Constant-

Elemente der Ig-Gene, $E_i$/MAR für den Intron-Transkriptionsenhancer mit seiner nuclear matrix attachment-Region und seiner B-Zell-spezifischen Initiationsfunktion für die DNA-Replikation, 3´-MAR und 3´ori für 3´-gelegene regulatorische Elemente des Ig-Locus (237). W steht für die the BamHI-W-Repeats des großen internen Repeats im EBV-Genom. Die Positionen der Replikationsursprünge oriP und orilyt im EBV-Genom sind ebenfalls gekennzeichnet.

All dies weist auf eine evolutionäre Verwandtschaft zwischen EBV und den Ig-Genloci hin. Das Ur-EBV könnte sogar ein notwendiges Werkzeug in der Evolution des Ur-Ig-Gens gewesen sein, beide könnten sich in Abhängigkeit voneinander zu ihren gegenwärtigen Formen entwickelt haben. Wenn dies der Fall ist, dann mag die asymptomatische Primärinfektion mit EBV eine unterstützende Wirkung auf die Entwicklung des B-Zellsystems und Antikörper-Repertoires eines Menschen ausüben. Sogar die IM könnte dieselbe Wirkung entfalten, wenn auch unter Nebenwirkungen. Die durch EBV verursachte Tumorigenese kann dann als molekularer Unfall einer normalerweise physiologischen Reaktion gesehen werden, dessen Auftreten durch die übermäßige Einwirkung von verstärkenden zusätzlichen Faktoren neben EBV gefördert wird. Solche Verstärker sind die Malaria in Äquatorialafrika oder Phorbolester in südchinesischen Kräutertees. EBV könnte mit Komponenten der SHM zu bestimmten Zeitpunkten im Leben einer Zelle interferieren, indem es diese Mutationsmaschine auf nicht-physiologische Ziele, wie auf Onkogene oder den T-Zellrezeptor lenkt, oder sie in nicht-physiologischen Zelltypen, wie Epithelzellen generell aktiviert. Ein Hinweis auf eine Aktivierung der Mutationsmaschine findet sich bereits in der Literatur. EBV-negative Ramos BL-Zellen erhöhten nach einer EBV-Infektion die Aktivität ihrer SHM auf das mehr als zweifache, wenn sie ihren Latenztyp 1 beibehielten (Abbildung 2A in 71). Ein weiterer möglicher Hinweis auf die unspezifische Aktivierung der SHM könnte das deutlich erhöhte Auftreten von charakteristischen und unspezifischen Mutationen beim NPC und beim EBV-assoziierten Magen-Karzinom sein (90, 241).
Weiterhin könnte EBV daher auch Hinweise auf die noch ungeklärten molekularen Mechanismen der SHM liefern. Die EBERs sind hauptsächlich im Zellkern gelegen (83), wurden aber auch im Zytoplasma gefunden (188). Bemerkenswerterweise ist das Schlüssel-Enzym der SHM, activation-induced cytidine deaminase (AID) im Zytoplasma lokalisiert (164), obwohl die Mutationen in der genomischen DNA des Ig-

Genlocus im Zellkern gesetzt werden. Da AID zu einer Familie von RNA-editierenden Enzymen gehört, ist es wahrscheinlich, daß in Analogie zum Reversen Transkriptase-Modell der SHM ein RNA-Intermediat eine Rolle spielt (200). Ein mögliches molekulares Modell würde postulieren, daß die V-Gene zuerst durch RNA-Polymerase 3 transkribiert werden, diese RNAs ins Zytoplasma wandern, um editiert zu werden, die editierten RNAs anschließend wieder in den Zellkern zurücktransportiert werden, um dort ihre neue Sequenzinformation auf die Ig-Genloci zu übertragen. Das gesamte editierte Ig-Gen würde dann durch RNA-Polymerase 2 transkribiert und als Rezeptor an der B-Zellmembran exprimiert oder als Antikörper sezerniert.

Weitere theoretische Möglichkeiten sind, daß die V-Gene von den tRNAs abstammen, und die SHM anfänglich als allgemeines molekulares Werkzeug für die Evolution vieler Gene, nicht nur der V-Ketten gedient hat. Es könnte sich damit herausstellen, daß Viren notwendige molekulare Werkzeuge in der Entstehung der Arten waren. Einige gegenwärtige Viren könnten immer noch wichtige Funktionen in der Definition, Entwicklung und Erhaltung von Grenzflächen der einzelnen Organismen haben, wie sie z. B. das Immunsystem oder Oberflächenepithelien darstellen.

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DANKSAGUNG

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# Nucleoprotein Complexes and DNA 5′-Ends at *ori*P of Epstein-Barr Virus*

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**Understanding protein-DNA interactions *in vivo* at origins of DNA replication throughout the cell cycle may shed further insight on the mechanisms of initiation and replication control. The Burkitt's lymphoma cell line Raji harbors multiple copies of latent Epstein-Barr virus. Once per cell cycle the origin of plasmid replication of Epstein-Barr virus provides replication function *in cis* for the viral DNA. Here we examined *in vivo* nucleoprotein complexes on the initiator element of the origin before and after DNA synthesis. For this purpose Raji cells were synchronously growth arrested in $G_1$ phase by mimosine and in mitosis by colchicine, respectively. The association of the initiator element with proteins was visualized by footprinting with dimethyl sulfate and ligation mediated polymerase chain reaction. Methylation patterns indicated a novel binding activity within each element of a nonamer repeated three times at the initiator element. This activity was strongly diminished in mitotic cells. Furthermore, 5′-ends of Epstein-Barr virus DNA were mapped to the nonamers by ligation mediated polymerase chain reaction, suggesting potential initiation sites for replication from DS.**

Plasmid replication of Epstein-Barr virus (EBV)[1] may serve as a viral model system for chromosomal DNA replication in higher eukaryotes (DePamphilis, 1988). EBV persists in lymphoid cells, such as the Raji cell line derived from an African Burkitt's lymphoma, in a tightly latent state as a circular plasmid in multiple copies (Adams and Lindahl, 1975; Lindahl *et al.*, 1976; Nonoyama and Pagano, 1972). Like cellular chromosomes, each viral plasmid is replicated once per cell cycle in the early synthesis (S) phase (Adams, 1987; Gussander and Adams, 1984; Hampar *et al.*, 1974; Yates and Guan, 1991). The replication of EBV-derived plasmids is dependent on *ori*P, the viral protein EBNA1, and on a set of mostly unknown cellular proteins (Lupton and Levine, 1985; Reisman *et al.*, 1985; Yates *et al.*, 1984, 1985). *Ori*P consists of two sequence elements, the family of repeat element (FR) and the dyad symmetry element (DS), separated by a stretch of about 960 base pairs of unique DNA dispensable for *ori* function (Lupton and Levine, 1985; Reisman *et al.*, 1985). FR contains 20 times a 30-base pair repeat in tandem (Lupton and Levine, 1985; Reisman *et al.*, 1985), each containing an EBNA1 binding site (Hsieh *et al.*, 1993; Rawlins *et al.*, 1985). In EBNA1 expressing primate cells, FR is essential for the stable maintenance of EBV plasmids (Lupton and Levine, 1985; Reisman *et al.*, 1985; Wysokenski and Yates, 1989). The element contains the termination site for replication (Dhar and Schildkraut, 1991; Gahn and Schildkraut, 1989) and works as a replication enhancer (Wysokenski and Yates, 1989). Furthermore, it functions as a transcriptional enhancer (Reisman and Sugden, 1986; Wysokenski and Yates, 1989). DS contains four EBNA1 binding sites and a region of dyad symmetry encompassing EBNA1 binding sites four and three (Hsieh *et al.*, 1993; Lupton and Levine, 1985; Rawlins *et al.*, 1985; Reisman *et al.*, 1985). In EBNA1 expressing primate cells DS most likely functions as the physical origin of bidirectional replication of EBV-derived plasmids (Gahn and Schildkraut, 1989; Platt *et al.*, 1993; Wysokenski and Yates, 1989). Since initiation of DNA replication from *ori*P is a strictly regulated and dynamic event, we asked if there are variations in the proteins associated with the initiator element of *ori*P *in vivo* before and after S phase. Therefore, we decided to examine the state of *in vivo* protein-DNA interactions at the initiator element of *ori*P in synchronously before and after S phase growth-arrested Raji cells. Methylation protected and sensitive nucleotides were visualized using the technique of ligation mediated polymerase chain reaction (LMPCR) (Mueller and Wold, 1989). The exact physical initiation points or areas of EBV plasmid replication are not yet known at nucleotide resolution. Therefore, we decided to use the same method to visualize 5′-ends of viral DNA within the initiator element of *ori*P.

## EXPERIMENTAL PROCEDURES

*Tissue Culture*—Raji cells were maintained in suspension cultures of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 units of penicillin/ml, and 50 μg of streptomycin/ml under 5% $CO_2$ and 37 °C.

*Flow Cytometric Analysis*—For flow cytometric analysis cells were harvested, fixed with 70% methanol for at least 1 h, resuspended in 1 ml of phosphate-buffered saline (PBS) (Saluz and Jost, 1990), digested with 100 μg of RNase A for 1 h at 37 °C, stained with 50 μl of propidium iodide (1 mg/ml), and scanned on a Becton Dickinson FACScan analyzer using Cellfit software.

*DMS in Vivo Footprinting*—For each footprint $10^7$ cells were harvested by centrifugation, washed with PBS (Saluz and Jost, 1990), resuspended in 1 ml of PBS, and incubated at room temperature for 5 min with 10 μl of dimethyl sulfate (DMS). The reaction was stopped by the addition of 5 ml of ice-cold DMS stop solution (1% of bovine serum albumin and 100 μM β-mercaptoethanol dissolved in PBS) (Saluz and Jost, 1990). Cells were pelleted and washed once more with DMS stop solution and two more times with PBS. Cells were resuspended in 1 ml of PBS. Genomic DNAs were isolated according to standard methods (Saluz and Jost, 1990; Sambrook *et al.*, 1989), either before sequencing reactions or after DMS treatment of cells. DMS-treated purified DNA was subjected to piperidine treatment (Maxam and Gilbert, 1980). For visualization by LMPCR, 2 μg of sequenced or footprinted DNA were

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[1] The abbreviations used are: EBV, Epstein-Barr virus; *ori*P, origin of plasmid replication; S phase, synthesis phase; EBNA1, Epstein-Barr viral nuclear antigen 1; FR, family of repeats; DS, dyad symmetry; LMPCR, ligation mediated polymerase chain reaction; PBS, phosphate-buffered saline; FACScan, fluorescence-activated cell scan; DMS, dimethyl sulfate; dNTP, deoxynucleotide triphosphate; M, mitosis.

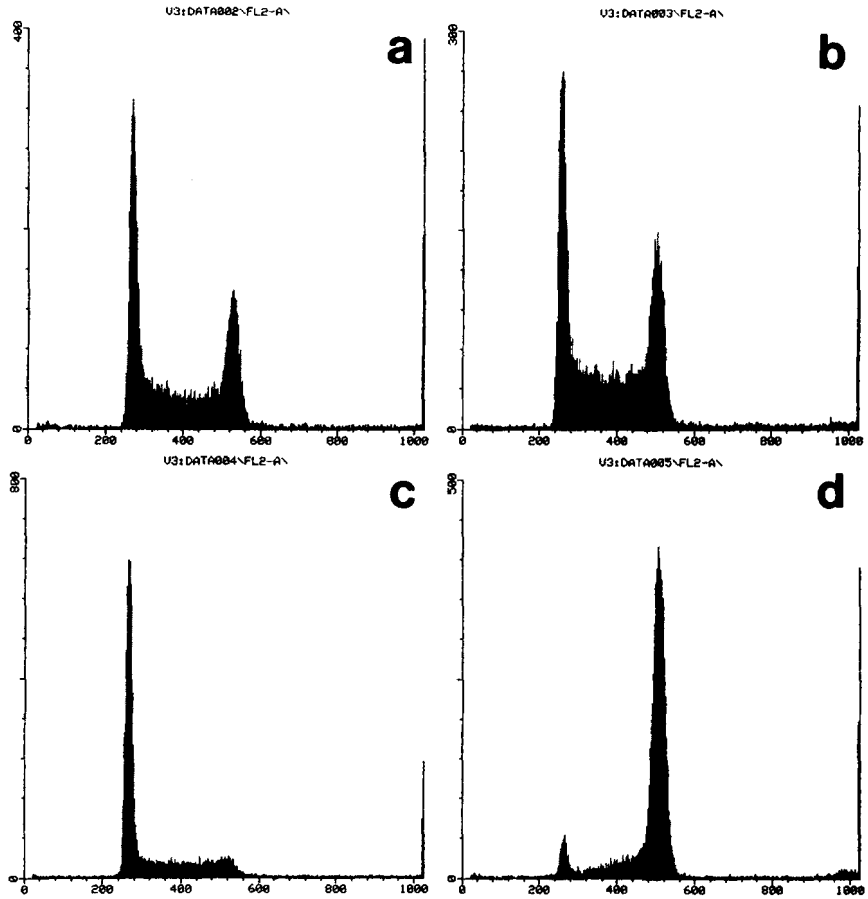


FIG. 1. **Histograms of flow cytometric analyses of propidium iodide-stained Raji cells.** The relative amount of measured DNA is depicted on the *abscissa*, the relative amount of cells is shown on the *ordinate* of each panel. 20,000 events each were gathered and are presented as ungated data. *A*, untreated, exponentially growing Raji cells at the time of drug addition. The left peak of the histogram represents cells in $G_1$, the right peak cells in $G_2$/M, cells at various stages of the S phase are in between the two peaks. *B-D*, 14 h after drug addition: *B*, untreated control; *C*, cells treated with 400 μM mimosine; *D*, cells treated with 1 μM colchicine.

analyzed according to the protocol of Garrity and Wold (1992) with some modifications. The following sets of oligonucleotides i, ii, and iii for the LMPCR assay of both strands were used, 5′ to 3′: lower strand (i) 8927-GGTTCACTACCCTCGTGGAATCCTG-8951, (ii) 8931-CACTACCCTCGTGGAATCCTGACCC-8955, (iii) 8969-CCGTGACAGCTCATGGGGTGGGAGATATC-8997; upper strand (i) 9229-GGCTACACCAACGTCAATCAGAGGG-9205, (ii) 9205-GGCCTGTGTAGCTACCGATAAGCGG-9181, (iii) 9195-GCTACCGATAAGCGGACCCTCAAGAGG-9169. The first strand primer extension reaction was done in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8, 25 °C (Vent buffer, New England Biolabs), containing 0.3 pmol of primer i, 240 μM each dNTP, and 1 unit of Vent (exo-) DNA polymerase (New England Biolabs) for 5 min at 94 °C, 30 min at 60 °C, and 10 min at 72 °C. For ligation of the common linker, the sample was transferred to ice and 5 μl of PCR linker mixture as in Mueller and Wold (1989), 2 μl of ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5, 20 °C, Boehringer Mannheim), 1 μl of T4 DNA-ligase (5 units/μl, Boehringer Mannheim), and 12 μl of water were added. After an overnight incubation at 4 °C the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. The PCR amplification was done in 100 μl of Vent buffer containing 10 pmol each of primer ii and the longer linker primer, 240 μM each dNTP, and 3 units of Vent (exo-) DNA polymerase for 20 cycles using 1 min at 94 °C, 1.5 min at 60 °C, and 3 min at 72 °C. For labeling, the sample was transferred to ice, 5 pmol of T4 kinase [$\gamma$-$^{32}$P]ATP labeled primer iii, 2.5 nmol each dNTP, and 0.5 units of Vent (exo-) DNA polymerase in a volume of Vent buffer not exceeding 15 μl were added. Then the sample was heated to 94 °C for 1.5 min, subjected to 5 cycles of 2 min at 94 °C, 2 min at 62 °C, and 5 min at 72 °C, and kept at 72 °C for 5 more minutes. Samples were transferred to ice, phenol/chloroform extracted, ethanol precipitated, and resuspended in loading dye. One-fifth of each sample was separated on a standard 6% sequencing gel (Sambrook *et al.*, 1989), and the gels were dried and autoradiographed for 15 h at room temperature with Kodak X-Omat LS film.

*Visualization of 5′-Ends*—Genomic DNA was harvested from unsynchronized Raji cells, and from Raji cells treated with 30 μM aphidicolin for up to 24 h (Saluz and Jost, 1990). 5′-Ends of DNA were visualized by LMPCR on 2 μg of genomic DNA using the same reaction conditions and primer sets as for the *in vivo* footprints.

## RESULTS

*Synchronous Growth Arrest of Raji Cells*—Initial efforts were focussed on the synchronization of Raji cells. The cells used in our experiments were growing fast, having a doubling time of about 24 h under standard cell culture conditions. For the purpose of arresting cells before and after S phase, we used the drugs mimosine and colchicine, respectively. Colchicine arrests mitotic cells in the metaphase by blocking the spindle apparatus for the separation of the chromosomes (Inoué, 1981). Mimosine, a plant amino acid, has the potential to reversibly block the mammalian cell cycle close to the $G_1$/S boundary (Lalande, 1990). The cell cycle block by mimosine may be due to the inhibition of initiation of DNA replication (Mosca *et al.*, 1992). Drugs were added to exponentially growing cells. The flow cytometric analysis of untreated, propidium iodide-stained cells showed a relatively high DNA synthesis rate and a relatively large fraction of the cells in $G_2$/M (Fig. 1*A*). 14 h later, when the cells were harvested for the footprints, a control sample analyzed by flow cytometry showed essentially the same pattern (Fig. 1*b*). Cells treated with 400 μM mimosine or with 1 μM colchicine for 14 h, respectively, showed a rather different distribution over the cell cycle (Fig. 1, *C* and *D*). In the mimosine-treated sample, S phase cells and $G_2$/M cells were strongly depleted and a large majority of the cells were in $G_1$ (Fig. 1*C*). In the colchicine-treated sample, $G_1$ cells and S phase cells were strongly depleted and the vast majority of the cells were in $G_2$/M (Fig. 1*D*). These two cell cycle arrests seemed sufficiently quantitative for our intended studies of protein-DNA interactions at DS of *ori*P before and after S phase.

*EBNA1 Binding to DS Throughout the Cell Cycle*—Cells treated with mimosine and colchicine were harvested and genomic DMS footprints on the initiator element (DS) of *ori*P of EBV were performed (Fig. 2). The footprinting was done on

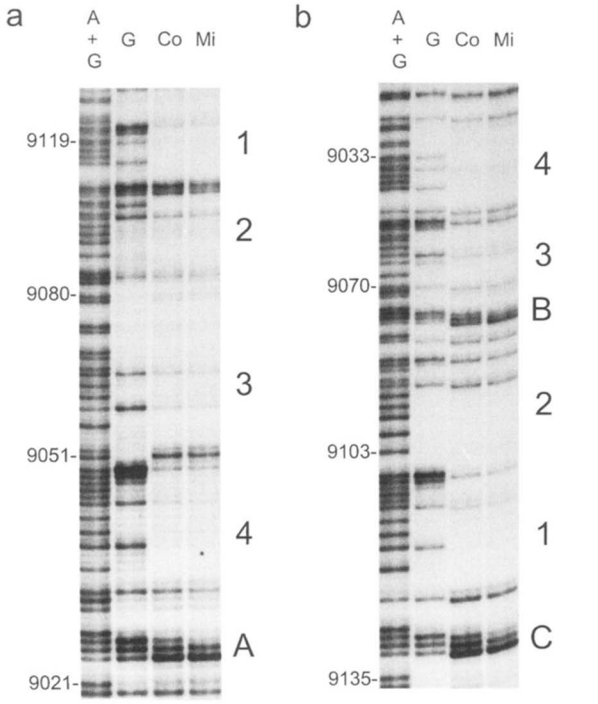


FIG. 2. **Genomic footprinting on both strands of the dyad symmetry element of *ori*P with dimethyl sulfate.** *Panel a* shows data for the lower strand, *panel b* for the upper strand. *Lanes A+G* and *G* refer to Maxam and Gilbert (1980) sequencing reactions using purified genomic DNA. *Lanes Co* and *Mi* are DMS footprints done on live cells treated with colchicine and mimosine, respectively, as shown in Fig. 1. Experiments were done several times from independent synchronous growth arrests with the same results. The *numbers* on the *left* of each panel refer to the EBV sequence (Baer *et al.*, 1984), the *numbers* on the *right* of each panel refer to the EBNA1 binding sites within DS (Rawlins *et al.*, 1985), nonamers are indicated by the *letters A, B,* and *C*.

both strands of DS according to standard methods (Garrity and Wold, 1992; Maxam and Gilbert, 1980; Mueller and Wold, 1989; Saluz and Jost, 1990; Sambrook *et al.*, 1989) with some modifications. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized in Fig. 4. The footprint patterns for mimosine-treated cells and colchicine-treated cells were similar, in general, and consistent with the concept of EBNA1 binding to its four *in vitro* binding sites (Rawlins *et al.*, 1985) within DS also *in vivo* (Hsieh *et al.*, 1993). Nuclear proteins other than EBNA1 might also cause the *in vivo* footprints on the EBNA1 binding sites at some phase of the cell cycle (Oh *et al.*, 1991; Wen *et al.*, 1990). However, since the footprints spanning the EBNA1 binding sites of DS were the same in mimosine- and colchicine-treated cells and also in nontreated, exponentially growing cells (data not shown), we concluded that EBNA1 binding to DS is unchanged throughout the cell cycle (Hsieh *et al.*, 1993). Since EBNA1 binds on only one face of the double helix within DS (Frappier and O'Donnell, 1992; Kimball *et al.*, 1989), the protections of guanines 9037, 9044, 9065, and 9119 are not readily explained solely by the binding of EBNA1. These protections may be caused by structural alterations of the DNA induced by the binding of EBNA1 (Frappier and O'Donnell, 1992), or also by the presence of nuclear proteins other than EBNA1 in the nucleoprotein complexes on DS (Oh *et al.*, 1991; Wen *et al.*, 1990).

*Novel Protein Binding at DS*—We observed differences between previously published DMS footprints on DS from nonsynchronized cells (Hsieh *et al.*, 1993) and this data. First, there was a guanine instead of an adenine at position 9062 in *ori*P of our Raji cells, that was methylation protected. This transitional point mutation is unlikely to have any effects on the function of *ori*P (Ambinder *et al.*, 1990). Second, the adenine at position 9051 was strongly DMS reactive. This difference may be due to variations in technical procedures. The methylation sensitive adenine at 9051 has also been observed in *in vitro* methylation studies on the distortion of *ori*P by EBNA1 (Frappier and O'Donnell, 1992). Third, and more important, we observed a novel pattern of DMS reactivity within DS that was not reported by the earlier paper (Hsieh *et al.*, 1993). This discrepancy may be explained by variations in technical procedures or also by differences between Raji cells that have been passaged for many years.

*Cell Cycle Dependence*—The novel pattern was observed in nonsynchronized cells (data not shown) in a way similar to mimosine-treated cells. However, the pattern was different between mimosine- and colchicine-treated cells. The pattern was found at each nonamer, 5′-TTAGGGTTA-3′, repeated three times within DS. The nonamers are located at 9021 to 9029 (A), 9073 to 9081 (B) and 9127 to 9135 (C), each location being outside of the EBNA1 sites. Nonamer A points to one direction, nonamers B and C point to the other direction. The first guanine of each nonamer was protected stronger in mimosine than in colchicine-treated cells. The third guanine each was slightly stronger methylation sensitive in mimosine- than in colchicine-treated cells. These differences in the footprint patterns between mimosine- and colchicine-treated cells most likely reflect the differential interaction of the nonamer with nuclear protein or, alternatively, a more general change of the nucleoprotein complex on DS. An earlier experimental hint for protein binding to the nonamer sequence came from *in vitro* footprints on DS. Nuclear proteins from HeLa cells yielded distinct DNase I protected areas within DS that spanned the nonamers (Oh *et al.*, 1991). Therefore, distinct nucleoprotein complexes on DS probably exist in Raji cells *in vivo*.

*DNA 5′-Ends at the Nonamers*—In order to correlate more the protein binding data at DS with initiation function, we tried to locate the initiation points of DNA replication within DS at nucleotide resolution. Therefore, we performed LMPCR on purified genomic DNA from untreated Raji cells to visualize DNA 5′-ends. Exponentially growing Raji cells were harvested, genomic DNA was purified, and LMPCR was performed using the same sets of primers as for the genomic footprints. On the lower strand 5′-ends were found mainly at nonamers B and C, on the upper strand mainly at nonamers A and B (Fig. 3, *lanes N*). The pattern of 5′-ends at the nonamers in untreated DNA is also summarized in Fig. 4. In addition to 5′-ends at the nonamers, 5′-ends were also found upstream of nonamer C for the lower strand (*horizontal arrow above C* in Fig. 3*a*) and upstream of nonamer A for the upper strand (*two horizontal arrows above A* in Fig. 3*b*) These upstream 5′-ends are less abundant than 5′-ends at the nonamers. They occur at pyrimidine-rich stretches of the respective strands. Since aphidicolin blocks DNA chain elongation (Huberman, 1981), we did the same experiment on genomic DNA from Raji cells treated with aphidicolin. In this experiment the signal for 5′-ends at the nonamers increased strongly in a time dependent fashion (Fig. 3, *lanes 24*) (only data for 24 h shown). In addition to this strong signal increase at the nonamers, there was also an increased background and an increased abundance of 5′-ends downstream of nonamer C on the upper strand (*nicked arrow below* nucleotide *9118* in Fig. 3*b*). These 5′-ends cannot be readily explained by some mechanism, but they may correspond to damaged DNA, resulting from the prolonged treatment of cells with aphidicolin. The increased background of *lanes 24* may also result from DNA damage.

DISCUSSION

The EBV latent origin of plasmid replication serves as a model for the controlled replication of eukaryotic chromosomes (DePamphilis, 1988). The only viral protein required for the replication of *ori*P is EBNA1 (Lupton and Levine, 1985; Yates *et al.*, 1985). Purified EBNA1 lacks detectable ATPase and helicase activity (Frappier and O'Donnell, 1991; Middleton and Sugden, 1992), that both should be needed for the initiation of replication of DNA. Thus, it is likely that at least one cellular protein cooperates with EBNA1 to initiate DNA synthesis at *ori*P. An important step toward the characterization of cellular proteins required for the initiation of DNA replication from *ori*P is the examination of *in vivo* protein-DNA interactions at the initiator element of *ori*P. A further step is the investigation of *in vivo* protein binding in dependence on the cell cycle. Therefore, we analyzed *in vivo* interactions of nuclear proteins with the dyad symmetry element (DS) of *ori*P of Epstein-Barr virus in Raji cells that were synchronously growth arrested at two points of the cell cycle by the use of mimosine and colchicine. Nucleoprotein complex formation was probed with DMS and visualized by LMPCR. In order to gain a closer view on replication initiation, we tried to map initiation points within DS at nucleotide resolution.

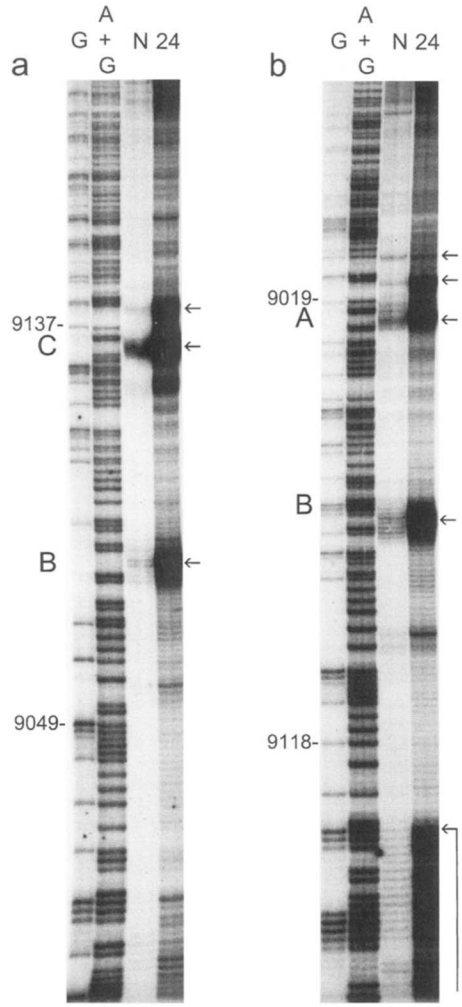


FIG. 3. **Visualization of 5′-ends on both strands of DS in genomic DNA from Raji cells by LMPCR.** *Panel a* shows data for the lower strand, *panel b* for the upper strand. *Lanes A+G* and *G* refer to Maxam and Gilbert (1980) sequencing reactions. *Lanes N* show DNA 5′-ends occurring in nontreated, nonsynchronously growing cells. *Lanes 24* show accumulated 5′-ends in cells treated with 30 $\mu$M aphidicolin for 24 h. Experiments were done at least twice with the same results. The *numbers* on the sides of each panel refer to the EBV sequence, locations of nonamers are indicated by the *letters A, B,* and *C*. *Horizontal arrows* show 5′-ends in genomic DNA from nontreated cells, the *nicked arrow* indicates an accumulation of 5′-ends in genomic DNA from aphidicolin-treated cells.

Our results suggest that EBNA1 binds to its 4 binding sites within DS (Lupton and Levine, 1985; Rawlins *et al.*, 1985; Reisman *et al.*, 1985) throughout the cell cycle in an unaltered manner. The same conclusion was made by Hsieh *et al.* (1993) who performed a study of *in vivo* protein-DNA interactions on *ori*P in nonsynchronous Raji cells. In addition, we obtained three novel findings. First, there is *in vivo* binding activity at three homologous nonamers within the initiator element of *ori*P of EBV that is apart from EBNA1 binding. Second, the formation of distinct nucleoprotein complexes on DS is possible. The binding at the nonamer may be cell cycle dependent. An analogous situation was recently described for yeast origins of replication. In that case the prereplicative state also showed a more extended footprint than the postreplicative state (Diffley *et al.*, 1994). Third, at the nonamers there are free 5′-ends in untreated genomic DNA which are accumulating under aphidicolin treatment. These 5′-ends may correspond to replication initiation points within DS. A possible replication model would postulate the start points for the leading strand replication for both strands around nonamer B, and the closest lagging strand initiations at and upstream of nonamer A for the upper strand and nonamer C for the lower strand, respectively. In this model the nonamer binding protein would bind before S phase and serve some kind of initiator function. Interestingly, the nonamer sequence 5′-TTAGGGTTA-3′ is resembling the human telomeric repeat sequence d(TTAGGG)$_n$ (Brown, 1989; Cross *et al.*, 1989). Telomeric DNA is able to bind nuclear proteins (Price and Cech, 1987, 1989). Thus, there could be some function for telomere binding proteins or relatives thereof

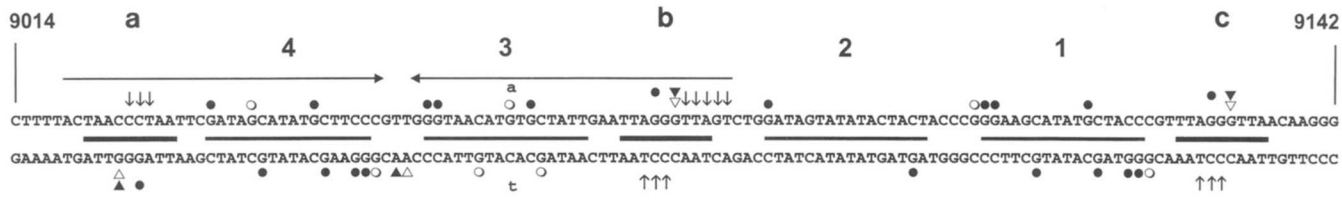


FIG. 4. **Summary of genomic footprinting (Fig. 2) and free 5′-ends (Fig. 3) on the dyad symmetry element of *ori*P of Epstein-Barr virus in Raji cells.** The positions of protections and enhanced cleavage sites as presented in Fig. 2 are shown here for $G_1$ and $G_2$/M cells. EBNA1 binding sites (Ambinder *et al.*, 1990) and nonamers (see text) are indicated by *horizontal lines* drawn between the DNA strands. Numbering of the sequence and the EBNA1 sites refers to Baer *et al.* (1984) and Rawlins *et al.* (1985), respectively, the dyad symmetry (Lupton and Levine, 1985; Reisman *et al.*, 1985) is indicated by *horizontal arrows*, nonamers are *labeled A, B,* and *C*. Differences between this genomic sequence and the published B95–8 sequence (Baer *et al.*, 1984) are indicated by *small letters above* and *below* the respective DNA strand. Guanines strongly protected from methylation by DMS are indicated by *filled circles*, weakly protected guanines are indicated by *open circles*, enhanced reactivity to DMS is shown by *filled* and *open triangles*, analogously. The pattern of DMS reactivity for colchicine-treated cells is shown for the entire sequence, the different reactivities of the nonamer in mimosine-treated cells for the upper and lower strand, respectively, are indicated *above* and *below* the symbols for reactivities of colchicine-treated cells. Free 5′-ends at nonamers in genomic DNA from untreated cells visualized by LMPCR are indicated by *small vertical arrows above* and *below* the respective strand.

in the replication and nuclear maintenance of *ori*P containing episomes.

The role of the nonamers has not yet been systematically addressed by functional assays that use plasmids containing scanning mutations in elements A, B, and C of *ori*P. However, slight deletions into nonamer A disturbed the extrachromosomal maintenance of unrearranged plasmids containing *ori*P in a plasmid maintenance assay in D98/Raji cells (Chittenden *et al.*, 1989). This functional hint together with our novel data strongly suggest that the nonamer and its binding protein carry out some function in the extrachromosomal maintenance of *ori*P containing plasmids. A more complete answer, however, on the function of the nonamer elements awaits a thorough mutational analysis of DS, including the nonamers and a biochemical and genetic characterization of the nonamer binding protein.

To our knowledge, this study is the first to use nucleotide resolution genomic footprinting to show a specific, probably cell cycle dependent alteration of the nucleoprotein complex at a viral origin of DNA replication. In addition, the study uses LMPCR to map at nucleotide resolution replication initiation sites from total genomic DNA. This line of research may provide one more step toward the detection of a general mechanism for the replication of chromosomal DNA or for a unique and novel mechanism for the plasmid replication of EBV.

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# Plasmid Maintenance Assay Based on Green Fluorescent Protein and FACS® of Mammalian Cells

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Commonly, the ability of plasmids to replicate in vivo in cell culture is determined by transient plasmid replication assays and plasmid maintenance assays (1,2,4,5,7,8). To examine the function of a replication element using the plasmid maintenance assay, plasmid DNA bearing the origin in its wild-type or mutated form is transfected into cells. At several time points, plasmid DNA is extracted from the cells and digested with suitable restriction enzymes, and the level of plasmid maintenance is measured by Southern blotting with an appropriate probe. To separate plasmid-carrying cells from the overall cell population, plasmids are usually carrying a resistance gene, and transfected cells are kept under selective pressure.

This procedure is laborious and suffers drawbacks. First, plasmid maintenance curves cannot be generated easily because Southern blotting and clonogenic assays are too laborious for this purpose. Second, in many cases selective force is applied for several weeks at the beginning of the experiment or for the complete experiment, until DNA is isolated. Through antibiotic pressure, the maintenance of the antibiotic marker can be selected for by mechanisms not related to purely replicative functions; for example, by selection for integration events. Therefore, plasmid maintenance might be caused in part by the selection mechanisms and in part by the plasmids' purely replicative functions.

To partially overcome these drawbacks and to complement the existing method, we designed a novel plasmid maintenance assay based on green fluorescent protein (GFP) from *Aequorea victoria* and cell sorting. The assay may principally be applied on plasmids containing widely different replicative elements and on all kinds of animal cells. Because practical plasmid maintenance assays are of relevance for many gene therapy trials, this novel type of assay (if not the specific plasmids used) might be helpful for gene therapy approaches as well as for basic replication research. In this paper, plasmids containing Epstein-Barr viral replication elements and an expression cassette for GFP but no resistance marker are transfected into human cell lines. Two to three days after transfection, cells expressing GFP are isolated under sterile conditions on a fluorescence-activated cell sorter (FACS®; Becton Dickinson, Heidelberg, Germany). By sorting the cells, an over 90% GFP-expressing culture is routinely obtained. The percentage of

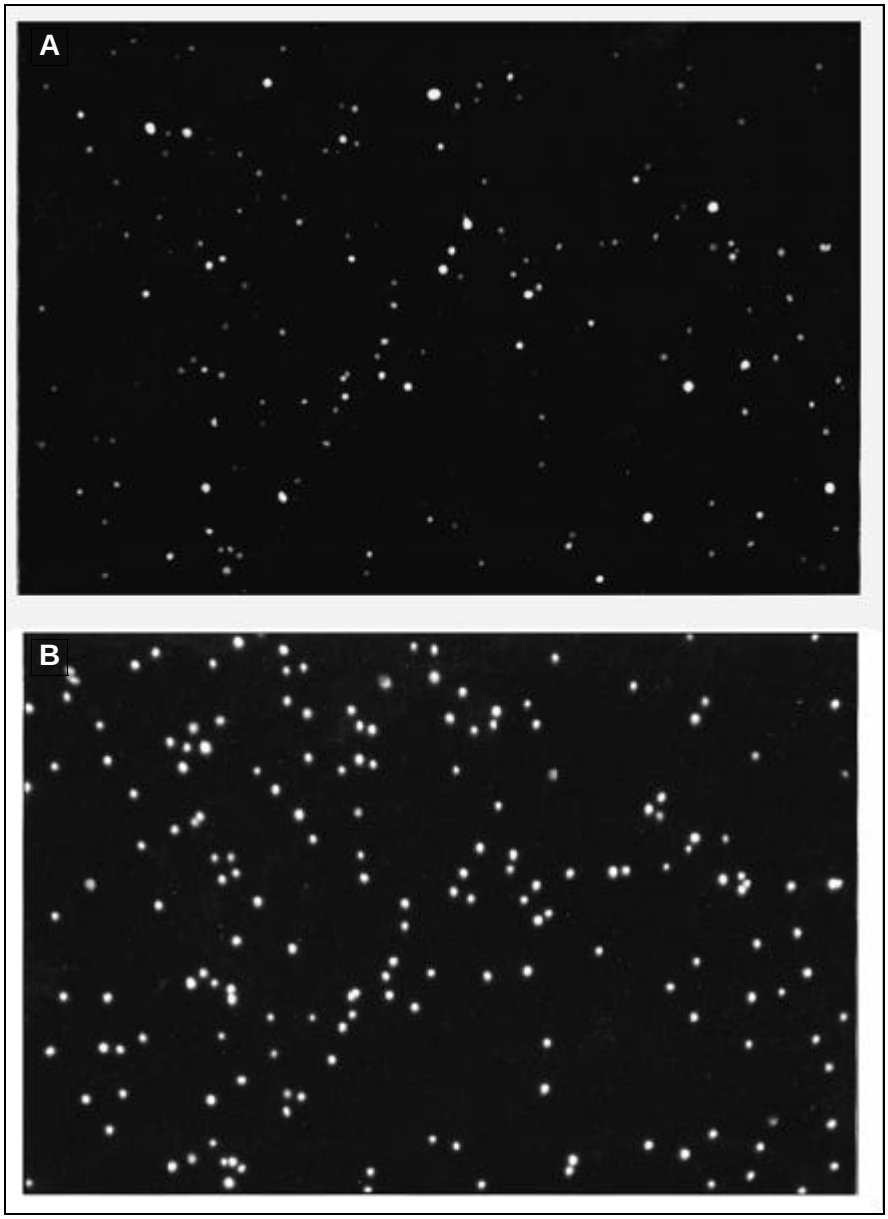


**Figure 1. Transfection and sorting of green fluorescing cells.** Raji cells transfected with a plasmid containing an expression cassette for GFP were sorted for green fluorescence on a FACStar$^{PLUS}$. (A) Cells after sorting with epifluorescence only. (B) DNA stain Hoechst 33342 was used to visualize all cells in the same sector of the culture dish, demonstrating a very high sorting purity. Very few cells show up in the DNA stain that do not also show up under GFP fluorescence. Fluorescence microscopy was performed on an ARISTOPLAN® (Leica) using standard filter sets (excitation band-pass filter 450–490 nm and emission long-pass filter 520 nm for GFP; excitation band-pass filter 330–385 nm and emission long-pass filter 420 nm for Hoechst 33342).

GFP-expressing cells at specific time points in this experiment is an indirect parameter for plasmid maintenance in these cells. The percentage of green fluorescing cells is determined at several time points after sorting by reanalyzing small aliquots of the sample on a FACScan™ (Becton Dickinson). Advantages of this system are that the sampling and measuring are less time-intensive than Southern blotting, sample size can be smaller, plasmid maintenance can be monitored by simply looking at the cells by fluorescence microscopy, the method is nonradioactive per se, and the assay can be taken down to the single-cell level. A further advantage of the system is that it generally avoids antibiotic pressure and therefore avoids a possible positive selection for integration events. One possible disadvantage is that the readout of the assay is an indirect one because it relies on a reporter gene.

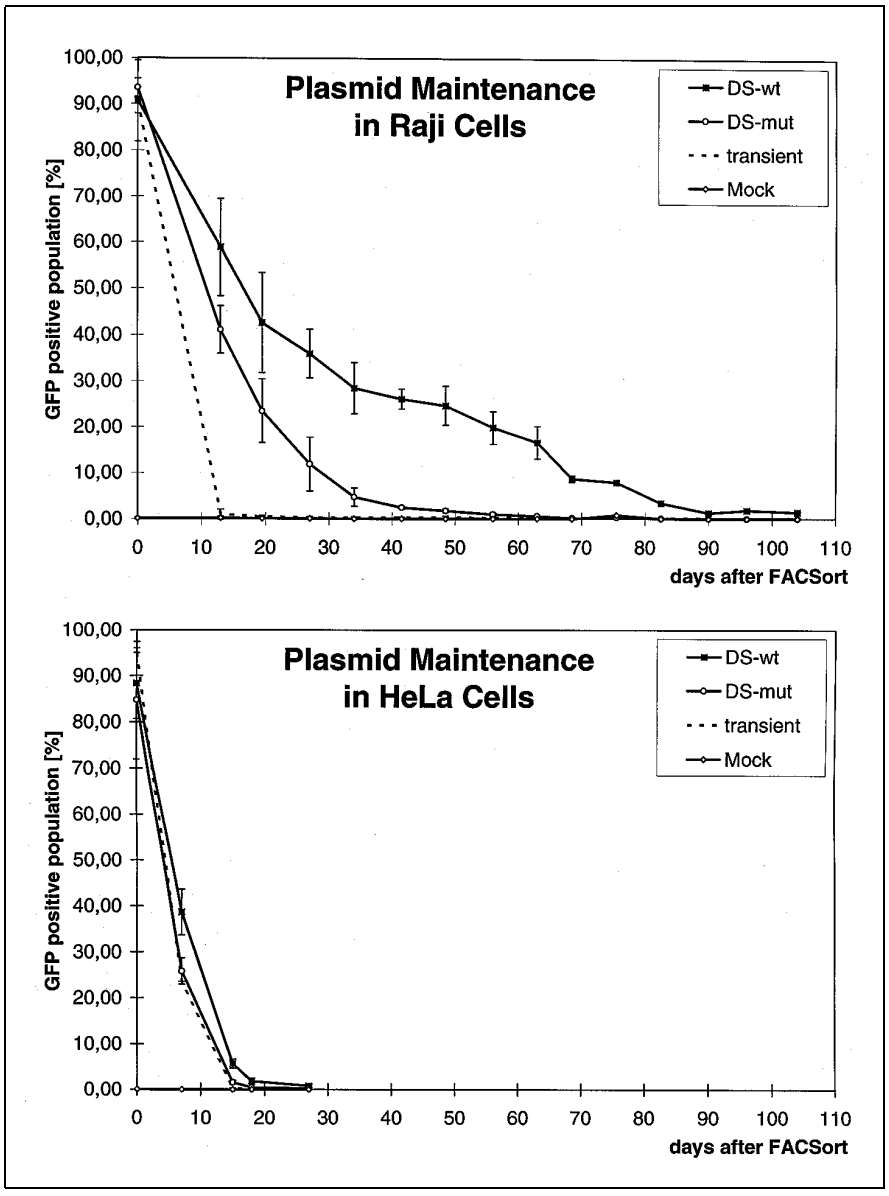


**Figure 2. Graphical representation of a set of plasmid maintenance assays.** The fraction of GFP-expressing cells is shown on the ordinate; the time points of sampling are depicted on the abscissa. After transfection of the cells with the respective plasmids, approximately 90% of the green fluorescent populations were routinely obtained through sterile sorting with a FACStar$^{PLUS}$. The sorted populations were maintained in culture without selective force applied. Each experiment was done at least in triplicate. The upper panel shows results for Raji cells, the lower panel for HeLa cells.

Plasmids based on the pBS(-) Phagemid Vector (Stratagene, Heidelberg, Germany) carried an expression cassette for GFP, an expression unit for the viral replication factor EBNA1 and the origin of plasmid replication (oriP) of Epstein-Barr virus (EBV). GFP was under the control of the major immediate early promoter/enhancer unit of human cytomegalovirus (hCMV). The gene for the S65T redshift mutant of GFP (3), exclusively using human codons, was obtained from Brian Seed (Harvard University, Cambridge, MA). The origin under investigation in our lab is oriP of EBV. Our own experiments (6) and earlier results (1) pointed to a possible replication function of a nonamer sequence within the dyad symmetry (DS) element of oriP. To test the replication assay, plasmids were constructed that contained oriP in its wild-type and in a mutated form. The only difference between the wild-type and the mutated plasmid was the replacement of the three telomere-like nonamers 5′-TTAGGGTTA-3′ within the DS element of oriP with the sequence 5′-CGTCTCGAG-3′. Another plasmid containing the expression cassette for GFP only, but neither the family of repeats (FR) element nor the DS of oriP, served as a control for transient GFP expression.

For the plasmid maintenance assays, the two cell lines Raji (Burkitt's lymphma, EBV-positive, EBNA1-positive, lymphoid) and HeLa (cervix carcinoma, EBV-negative, EBNA1-negative, epithelial) were used. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) (HeLa) and RPMI-1640 (Raji) containing 10% fetal calf serum, 2 mM glutamine, 50 U penicillin and 50 µg streptomycin per milliliter under 5% $CO_2$ and at 37°C. (All cell culture media were from Life Technologies, Eggenstein, Germany.) The three plasmids were purified with NUCLEOBOND® cartridges (Macherey-Nagel, Düren, Germany) and introduced into the cells by the Gene Pulser® II Electroporation System (Bio-Rad, München, Germany) using a setting of 250 V and 960 µF. Between $1 \times 10^7$ and $5 \times 10^7$ cells were electroporated in a volume of 0.5 mL of phosphate-buffered saline (without $Mg^{++}$ and $Ca^{++}$), using an electrode spacing of 4 mm. For the cells

that survived electroporation, up to 60% transfection efficiency was routinely obtained for HeLa cells and up to 50% for Raji cells, which was determined by both propidium iodide staining and green fluorescence. Two to three days after transfection, green fluorescing cells were isolated under sterile conditions on a FACStar$^{PLUS™}$ (Becton Dickinson) using 488 nm excitation from an argon-ion laser (60 mW output). GFP fluorescence was recorded with a 633-nm band-pass filter (bandwidth 22 nm) and a 525-nm band-pass filter (bandwidth 30 nm) (Leica, Wetzlar, Germany). Sorting was performed at a rate of approximately 5000 cells per second. An example for sorting purity is shown in Figure 1. Green fluorescent cells were kept in culture for several weeks, and the percentage of GFP-expressing cells, i.e., plasmid-carrying cells, was determined every five to ten days on a FACScan. In this way, plasmid loss over time was monitored indirectly. The graphical representation of a set of experiments is shown in Figure 2. An acceptable maintenance of the oriP plasmid was only obtained in the EBV-positive cell line Raji but not in the EBV-negative cell line HeLa. Because replication of oriP is known to be dependent on the viral protein EBNA1, the failure of plasmid maintenance in HeLa cells may be related to some problem with the transient expression of EBNA1 from the plasmid as compared with the stable expression of endogenous EBNA1 in EBV-positive cells. Plasmids carrying a mutant oriP, where the three nonamer sequences were replaced with an unrelated sequence, were lost in both cell lines at a rate significantly higher than wild-type plasmids, in HeLa cells as fast as in transient plasmids carrying no origin at all. However, plasmids carrying wild-type oriP were maintained better than plasmids carrying mutant oriP or no replication origin. At this point, we would like to point out that the actual rates of plasmid loss for Raji are approximately 4% per generation for wild-type and 7% per generation for mutant oriP. We conclude that the nonameric sequences have some function for the oriP-mediated maintenance of plasmids. Because the composition of the three nonamers in this specific case was changed from 66% AT to 66% GC, the ease of DNA unwinding might have been changed. Whether this nonamer-related function is connected to the initiation of replication or to other mechanisms such as the ease of unwinding of the plasmid or structural changes of DS will be the subject of future examinations.

The seemingly minor mutation of wild-type oriP, replacing the three nonamers with an unrelated sequence, leads to a significant difference in the maintenance of the two plasmids. The differences between the constructs give credibility to the usefulness of this novel replication assay combining GFP technology with cell sorting. Even not knowing the exact function of the nonamer repeats for plasmid maintenance at this point, the results demonstrate the possible usefulness of the GFP assay for detecting differences in plasmid maintenance rates and for an easy way to establish a time course of plasmid loss. The combination of GFP and cell sorting should prove useful also for further applications, such as the selection of eukaryotic cell clones obtained by transfection of bicistronic plasmids expressing the gene to be cloned and GFP.

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# Regulation of the Epstein-Barr viral immediate early BRLF1 promoter through a distal NF1 site

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**Summary.** The immediate early BRLF1 and BZLF1 promoters of Epstein-Barr virus are crucial for triggering the replicative cycle of the virus. To better understand the cell type dependence of the lytic cycle we conducted an analysis of the BRLF1-promoter in the epithelial cell line HeLa and the lymphoid cell line IM9. To analyze promoter activities, transient transfections with 5′-deletions of the BRLF1-promoter in front of luciferase as reporter gene were conducted. Besides the already known *cis*-acting elements of the promoter close to the TATA-box, more distal elements were located and functionally tested. A nuclear factor 1 consensus site was found to act positively in HeLa cells, but did not in lymphoid IM9 cells. The NF1 site was shown to bind protein by electrophoretic mobility shift assays, antibody-supershifts and in vitro footprinting. Thus, a protein belonging to the nuclear factor 1 family of proteins was identified as additional cellular *trans*-acting factor for the BRLF1-promoter besides the already described factors Sp1, Zta and Zif268.

## Introduction

Epstein-Barr virus (EBV) is a tumor associated human herpesvirus that is able to infect and replicate in epithelial cells. B lymphocytes, on the other hand, are generally infected latently, although it is possible to trigger the reproductive cycle of EBV also in B-cells [31, 36, 51, 58]. Activation of the lytic cycle can be achieved through the overexpression of the EBV immediate early [2] protein Z-transactivator (Zta) [6, 7, 29, 30, 43, 44, 50]. In some lymphoblastoid cell lines, though, the viral immediate early [2] R-transactivator (Rta) may play a bigger role in breaking EBV latency than Zta [3]. More recent observations suggest that Rta is sufficient to disrupt latency in an epithelial cell specific manner [56]. The immediate early protein Zta can be expressed from both the BZLF1 and

the BRLF1 promoter (Zp and Rp) [34]. Zp directs the transcription of a 0.9kb message encoding Zta and a spliced variant thereof [32, 34]. Rp directs transcription of two bicistronic messages that code for Zta and Rta and a spliced message for a negative regulator of Zta called RAZ [16, 34]. Since the proteins expressed from Rp, Zta, Rta and RAZ, are EBV key regulators, understanding the cellular regulation of Rp may yield valuable insight into the cellular mechanisms involved in the disruption of viral latency and the immediate early to early transition of the EBV productive cycle. Currently, only limited information is available on the cell specific regulation of Rp. In the proximal region of Rp several regulatory elements for cellular transcription factors and the viral transactivators Rta and Zta have been mapped. An indirect auto-upregulatory loop has been postulated for Rp [49] that is activated by Rta in several different cell types [54]. However, there are contradictory results on the activity of Rp that is induced by the viral transactivator Zta. Sinclair et al. [49] showed a clear induction of Rp by Zta through the two Zta binding sites of Rp in lymphoid cells and a weak induction in epithelial cells. Zalani et al. [54], however, did not observe significant activation of Rp by Zta in several cell types. The ubiquitous cellular factor Sp1 [8] has been shown to contribute to the constitutive activity of Rp in an epithelial cell line [54]. The only inducible cellular transcription factor with a possible role in the activation of Rp that has been described so far, is Zif268 [55]. This factor has been shown to induce Rp through two Zif268 binding sites in epithelial cells and in an epithelial/lymphoid fusion cell line. Zif268 expression can be induced by TPA and antiimmunoglobulin antibody [47], two pathways which are also able to induce the lytic cycle of EBV in latently infected B-cells [10, 14, 15, 33, 51].

Contrary to the well studied proximal region of Rp the more distal region of the promoter (up to nt −962) has not been examined in detail. Therefore, we performed a functional analysis of Rp using a series of 5′-deletion constructs of Rp in front of the very sensitive reporter gene luciferase [11]. In addition we analyzed protein-DNA interactions in the respective promoter area by electrophoretic mobility shift assays, supershift experiments and in vitro footprinting.

## Materials and methods

### *Reagents*

Enzymes were from Boehringer Mannheim. Radiochemicals from Amersham, cell culture media from Gibco BRL, Life Technologies.

### *Tissue culture*

IM9 cells (an EBV positive, immunoglobulin secreting B myeloma cell line) were obtained from ECACC, and HeLa S3 cells from B. Moss (NIH). Both HeLa and IM9 cells were maintained in suspension cultures of RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 50 units penicillin per ml and 50 μg streptomycin per ml under 5% $CO_2$ and 37 °C.

### *Bacteria and plasmids*

For cloning and plasmid preparations bacterial strains DH5α and JM109 were used. To obtain a full length construct of Rp in front of luciferase (pRLUC5′ −962) the DraI-fragment (−962 to +5) of Rp was taken from plasmid pRpUC (kindly provided by Manfred Marschall, Munich) and inserted into the SmaI site of p19LUC [11]. To obtain 5′-deletion constructs of Rp, plasmid pRLUC5′ −962 was opened with restriction enzymes SalI on one side and with SmaI, StuI and BstXI on the other side, blunted with Klenow enzyme and religated. A series of six more deletion clones (pRLUC5′ −445 to pRLUC5′ −899) was constructed by PCR amplification of Rp subfragments, cutting PCR products with BstXI and SalI and directed cloning of the fragments into equally cut pRLUC5′ −962. One additional deletion plasmid was made by cutting with SmaI and StuI and religation. The deletion constructs contained promoter sequences between nucleotides (nts) −962 and +5 (full length), −899 and +5, −864 and +5, −847 and +5, −687 and +5, −511 and +5, −445 and +5, −385 and +5, −276 and +5, −42 and +5. In construct pRLUCΔ −385/−276 sequences between nts −276 and −385 were deleted. As a positive control for luciferase assays the plasmid p19LUCLTR containing the Rous sarcoma virus LTR in front of the luciferase gene was used. Mock transfected cells and p19LUC served as negative controls.

For electrophoretic mobility shift analyses (EMSA) the following doublestranded oligonucleotides were cloned into the BamHI site of plasmid pBS−(Stratagene): GATCCCTCAGTGGTCATTGGATGTCTGCCACGGGCAACCCCCTGCCTGCA//GATCTGCAGGCAGGGGGTTGCCCGTGGCAGACATCCAATGACCACTGAGG (plasmids pBS-NF$1_1$/R and pBS-NF$1_2$/R), GATCTGGCACTGTGCCAAG//GATCCTTGGCACAGTGCCA [20], (plasmid pBS-NF1/C), GATCTTTATTCTCAAGATTACATAACACAAGACTCCAG//GATCCTGGAGTCTTGTGTTATGTAATCTTGAGAATAAA [46] (plasmid pBS-H1/R).

### *Transfections*

25 μg of each test plasmid were transfected into $1 \times 10^7$ log phase HeLa cells by electroporation (BioRad gene pulser, setting 960 μF, 250 V, $\tau \sim 20$). 30 μg of each test plasmid were transfected into $5 \times 10^7$ log phase IM9 cells by both electroporation and a modified DEAE Dextran method [40] with identical results. For internal standardisation 20 μg of pSVβ-Gal (Tropix, Serva) were included in the transfections [24]. Cells were incubated under standard conditions for two to three days before harvesting them and assaying cell extracts for luciferase activity. Total protein was harvested from the cells using a commercial cell lysis buffer (Promega), and luciferase activity was determined with a Berthold luminometer (EG&G Lumat LB 9501) as specified by the manufacturer (Promega). Each transfection experiment was done five to eight times for HeLa and three to six times for IM9 with at least three separate luminometric measurements for each transfection with consistent results.

### *Electrophoretic mobility shift assays*

Preparation of nuclear extracts was essentially based on the standard of Dignam et al. [12]. Nuclei were prepared using a combination and modification of two methods [5, 22] as described [40]. Nuclear extracts were prepared as described [22]. For enrichment of DNA binding factors crude nuclear proteins from IM9 cells were passed over a heparin agarose resin (Sigma, H6508) at a ratio of 100 μg protein per μl of heparin agarose. After extensive washing of the resin with nuclear extract dialysis buffer, proteins were eluted with 1 M KCl. Eluted proteins were dialyzed as described [22] and stored at −70 °C. DNA fragments containing binding sites were excised from clones pBS-NF$1_1$/R and pBS-H1/R with restriction enzymes EcoRI and HindIII and from clones pBS-NF1/C and pBS-CRE with restriction enzymes EcoRI and BamHI, respectively, and purified via polyacrylamide gel electrophoresis.

Gel retardation assays were performed as described [22] with the modifications described: 5 to 10 μg of crude nuclear proteins or 0.5 to 1 μg of heparin agarose enriched extract (as indicated) was incubated with 1 μg poly(dI-dC)-poly(dI-dC), 1 ng of Klenow $^{32}$P-labeled fragment and the indicated excess of unlabeled competitor fragment in 25 μl bandshift buffer (10 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 80 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 12.5% glycerol, 0.1% Triton X-100) for 20 min. Protein complexes were resolved by electrophoresis on native 4% polyacrylamide gels (29+1) in 6.7 mM Tris/HCl, pH 7.5, 3.3 mM NaOAc, pH 7.0, 1 mM EDTA at 20 mA for several hours. For antibody supershifts the procedure was identical, except that the indicated antiserum was added for 20 more minutes before loading the gel.

*DNAseI protection analysis*

Footprinting was performed as described [40]. Plasmid pRpUC was cut with appropriate restriction enzymes, endlabeled with Klenow enzyme and α-$^{32}$P-dATP or -dCTP, recut and fragments containing the regulatory sequences of Rp purified from a native 5% polyacrylamide gel. For footprinting 10 to 15 μg of nuclear extract or 5 to 7.5 μg of heparin fraction was incubated with 1 to 1.5 μg of poly(dI-dC)-poly(dI-dC) at room temperature (RT) for 15 min, then for 15 more min with 8 ng of endlabeled fragment (20000 cpm) in 12 mM Hepes/KOH, pH 7.9, 10% glycerol, 1.6 mM DTT, 0.12 mM EDTA, 60 mM KCl and 6 mM $MgCl_2$ in a reaction volume of 50 μl. The probe was digested with 1 U of DNAseI (Boehringer) at RT for 1 min. The digestion was stopped by addition of 100 μl of stop solution (100 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS, 10 μg proteinase K, 1 μg *E. coli* DNA), incubation for 15 min at 37 °C, and denaturation at 90 °C for 2 min. Digestion products were phenol extracted, ethanol precipitated and separated on a 6% sequencing gel. Maxam and Gilbert A + G sequencing markers [35] of the corresponding probes were used to locate the footprints.

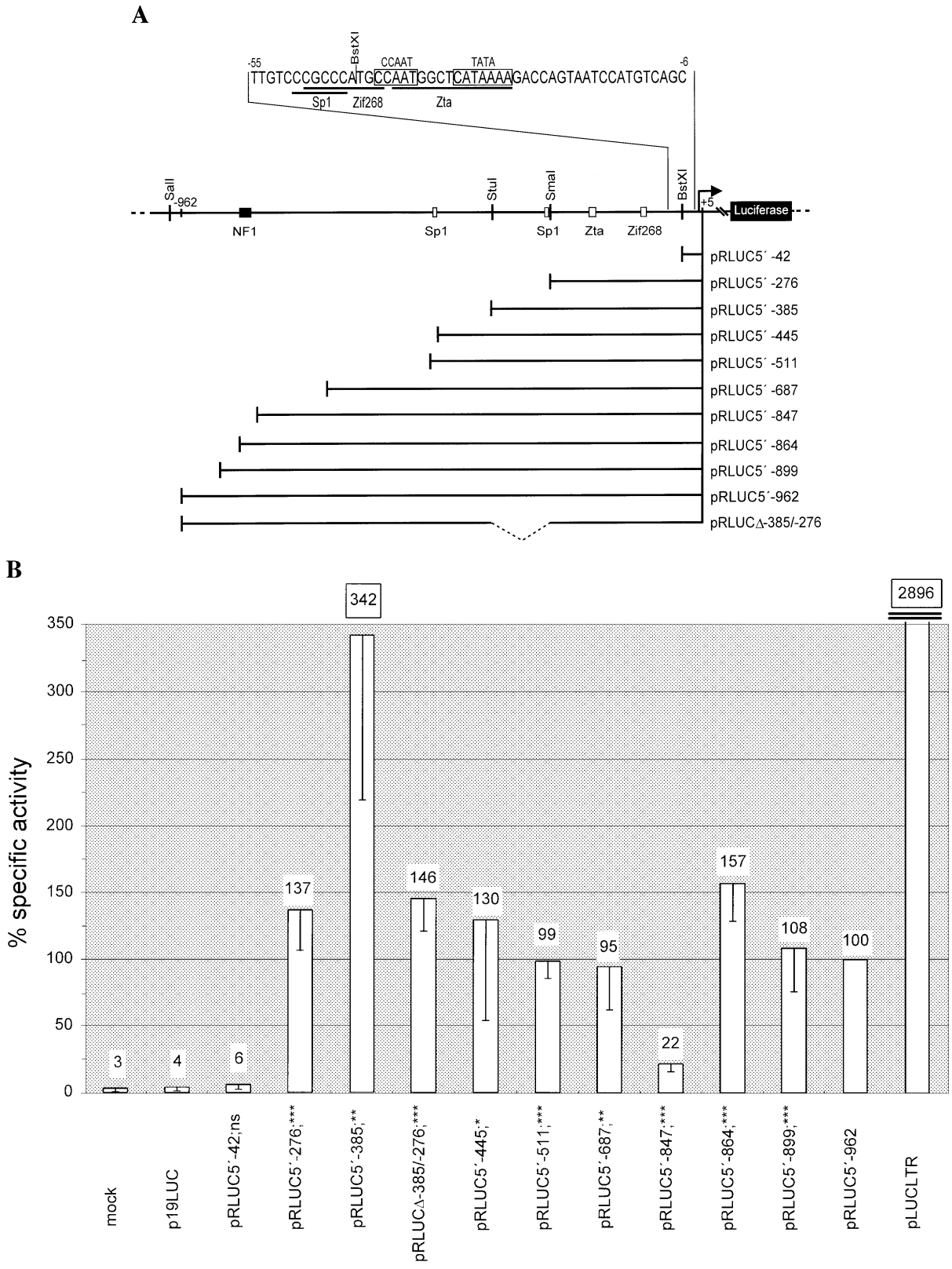
## Results

### *Profiles of Rp-activities in epithelial and lymphoid cells – identification of a functional NF1 binding site within Rp*

Previous functional analyses of the BRLF1-promoter using chloramphenicol acetyl transferase as a reporter [49, 54, 55] were mostly focused on the proximal promoter region and demonstrated a very low overall constitutive activity of Rp. We conducted our analysis with emphasis on the distal promoter region and using the very sensitive reporter gene firefly luciferase [11]. When the 5′-deletion constructs were made, we took into account the existence of both the distal Sp1 binding site of Rp and an NF1 consensus site within Rp. This NF1 site (5′-TGGN$_6$GCCAC-3′) was located at nucleotides −850 to −863 of Rp and presented a single nucleotide mismatch (underlined) to the described NF1 consensus (5′-TGGN$_6$GCCAA-3′), [17]. NF1 has been described as a cellular protein essential for adeno virus replication [18, 19, 39]. Binding sites for NF1 are widespread among cellular [13, 38, 42, 57] and viral [1, 21, 27, 40, 41] promoters. In most cases, NF1 is a potent transcriptional activator [4, 26]. Although NF1 is a ubiquitous factor, some of the examined genes show a highly tissue type dependent expression pattern [1, 23]. Therefore, we wanted to find out, if the distal promoter region including the NF1 site of Rp has any function for the promoter activity in dependence on the cell type. Chimeric constructs were made in which the 1 kb

upstream region of Rp, or deletions thereof were ligated to the luciferase reporter gene (Fig. 1a). Using electroporation and the DEAE dextran method plasmids were transfected into the epithelial cell line HeLa and the lymphoid cell line IM9. The cell line IM9 was comparable to Raji cells in its inducibility by TPA and butyric acid as tested by immune fluorescence experiments using patients' sera (data not shown). To delineate functional regions of the promoter, luciferase activities of the full length promoter construct pRLUC5′−962 were normalized to β-Gal activities and arbitrarily designated 100%. Comparisons were then made to the activity of the deletion mutants of this region as well as of controls p19LUC [11] and mock, using the same procedure to normalize values.

The level of transient expression from Rp was considerably lower in IM9 cells (5 to 6 times) than in HeLa cells, but it was possible to detect significant activity of Rp in IM9 cells (5 to 6 times above mock), as well as in HeLa cells (about 30 times above mock). For HeLa cells negative and positive controls yielded values in an expected and reasonable range (Fig. 1b). The full length promoter construct showed an activity of about 15 times above the basal promoter construct. The activity of the basal promoter was not significantly different from the negative controls. Adding the promoter region up to nt −276 considerably increased the constitutive activity (about 20-fold). Addition of the next segment up to nt −385 increased the constitutive activity approximately 60-fold. A construct with an internal deletion of Rp between nts −385 and −276 had an activity of about 20-fold above the basal promoter. This data indicates that there are strong constitutive promoter elements in Rp between nts −42 and −385. The strongly positive activity is gradually turned down by adding the more upstream sequences up to nt −445, nt −511, nt −687, and nt −847. The loss of activity is strongest between nts −385 and −445 and nts −687 and −847, so that plasmid pRLUC5′−847 yields an activity only about 4 times above the basal promoter construct. This data indicates that the promoter region between nts −385 and −847 contains negative regulatory elements repressing transcription from Rp in HeLa cells. The distal Sp1 site of Rp between nts −445 and −511 also seems to have no positive effect. The strongest repression is located between nts −687 and −847, which contains among other sequences an element (nts −808 to −815) with similarity to the H1-element, previously described as a repressor of Zp [46]. Adding the segment between nts −847 and −864, that essentially contains the NF1 consensus site of Rp, the activity increases about 7-fold to a level of about 25-fold above baseline level. This increase in activity may be attributed to a member of the nuclear factor 1 family, which may have a central function for the constitutive activity of Rp in epithelial cells besides the promoter proximal region up to nt −386. Adding the following segments up to nts −899 and −962 (full length) gradually decreases the activity again to a level of about 15-fold (100%) above baseline. This may be a hint for additional weak down regulatory elements within that segment, especially in the smaller segment between nts −864 to nt −899, because the difference in activity between constructs pRLUC5′−899 and pRLUC5′−962 is not significant. Since the full length construct of Rp has very little activity in lymphoid cells [54] it has been even more difficult to quantitatively examine the activity of


A
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BstXI
CCAAT
TATA
-6
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Sp1
Zif268
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-962
StuI
SmaI
BstXI
+5
Luciferase
NF1
Sp1
Sp1
Zta
Zif268
pRLUC5´-42
pRLUC5´-276
pRLUC5´-385
pRLUC5´-445
pRLUC5´-511
pRLUC5´-687
pRLUC5´-847
pRLUC5´-864
pRLUC5´-899
pRLUC5´-962
pRLUCΔ-385/-276
B
% specific activity
350
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50
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6
137
342
146
130
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95
22
157
108
100
2896
mock
p19LUC
pRLUC5´-42;ns
pRLUC5´-276;***
pRLUC5´-385;**
pRLUCΔ-385/-276;***
pRLUC5´-445;*
pRLUC5´-511;***
pRLUC5´-687;**
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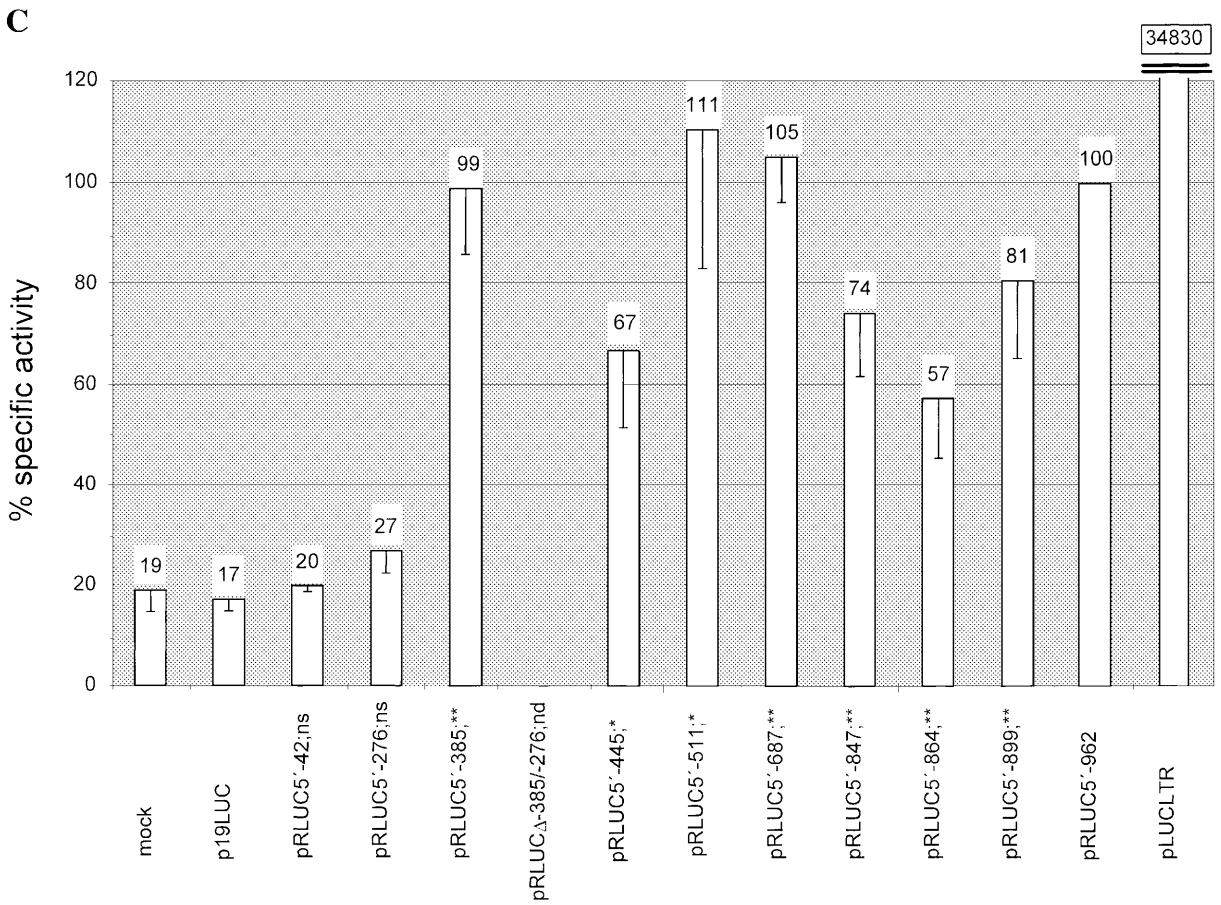


**Fig. 1.** **A** Schematic representation of the EBV Rp-Luciferase chimeric plasmids. pRLUC5′ −42 contains the basal promoter including the presumable TATAA-box of Rp, pRLUC5′ −276 contains a more extended promoter proximal region including recognition sites for Zta, Sp1 and Zif268. pRLUC5′−445 excludes the distal Sp1 site which is included by pRLUC5′−511. pRLUC5′−847 excludes the NF1 consensus site of Rp which is included by pRLUC5′−864. **B** Graphical representation of luciferase activities in cell lysates of HeLa cells after transfection with the Rp constructs indicated. All cells were cotransfected with plasmid pSVβ-Gal and the light units were normalized to β-Gal levels. Activities are presented as the mean ±SE for four to eight separate experiments. Asterisks indicate a significant statistical difference when compared with the activities expressed by pRLUC5′−42 (*ns* not significant; *P<0.05; **P<0.01; ***P<0.001). **C** Graphical representation of luciferase activities in cell lysates of IM9 cells after transfection with the Rp constructs indicated. All cells were cotransfected with the plasmid pSVβ-Gal and the light units were normalized to β-Gal levels. Activities are presented as the mean ±SE for three to six separate experiments. Asterisks indicate a significant statistical difference when compared with the activities expressed by pRLUC5′−42 (*ns* not significant; *P<0.05; **P<0.01; ***P<0.001)

5′-deletion constructs of Rp in cells of the B-lymphoid lineage. For IM9 cells, again, negative and positive controls yielded values in an expected and reasonable range (Fig. 1c). The activity of the basal promoter construct was almost identical to the negative controls. Contrary to HeLa cells, by adding the promoter region up to nt −276, promoter activity was increased only in an insignificant way. This may be a hint for the lack of activating factors for this promoter segment or a localized repression of Rp in lymphoid versus epithelial cells. Addition of the next segment up to nt −385 increased the promoter activity approximately 4-fold which constitutes the strongest single jump in the activity profile in IM9 cells. This indicates that there are positively acting constitutive promoter elements of Rp located between nts −276 and −385 just like in HeLa cells. In analogy to HeLa cells, the addition of the next segment up to nt −445 lead to a decrease in promoter activity, whereas, contrary to the situation in HeLa, the addition of the distal Sp1 site (nts −445 to −511) lead to a slight increase of promoter activity of up to more than full length level. The promoter activity is gradually turned down by adding the upstream sequences up to nts −687, −847, and −864, which shows that the NF1 site does not have a positive activity in IM9 cells, contrary to HeLa cells, where it has a strongly positive effect. Adding the following segments up to nts −899 and −962 (full length) gradually increases the activity to a level of about 5fold above baseline. This may be a hint for additional weakly positive promoter elements within this segment.

This functional promoter analysis, by using luciferase as a reporter, is the first one to quantitatively show constitutive activities of Rp of about 15-fold and about 5fold above baseline level in epithelial cells and in lymphoid cells, respectively, and the corresponding profiles of promoter activities using 5′-deletions. These activities could be measured without cotransfecting expression constructs coding for transcriptional activators of Rp. In sum, there seem to be three major differences in the regulation of Rp between HeLa cells and IM9 cells. The first difference is the overall much lower activity of Rp in the lymphoid than in the epithelial cell. The second difference relates to the promoter segment between nts −42 and −276. In HeLa cells this segment contributes strongly to the activity of Rp, whereas in IM9 cells this segment contributes almost no activity. The different regulation of this promoter segment and the overall lower activity in IM9 cells might be causally related. The third difference relates to the consensus NF1 site of Rp. In HeLa cells this site acts strongly activating, whereas in IM9 cells it does not.

### *Differential binding of nuclear protein to the NF1 site of Rp*

The different use to the NF1 site of Rp in the two cell lines led us to investigate differences of the nucleoprotein complexes on this site formed with proteins from the two cell types. Using the DNAseI protection assay we identified a clear protection of about 25 nucleotides on both strands of the NF1 consensus of Rp with crude nuclear proteins from HeLa cells (Fig. 2a, only one strand shown). The shape and extension of the protection is compatible with the notion of NF1 binding to this site in HeLa cells [20]. In order to identify the HeLa factor binding

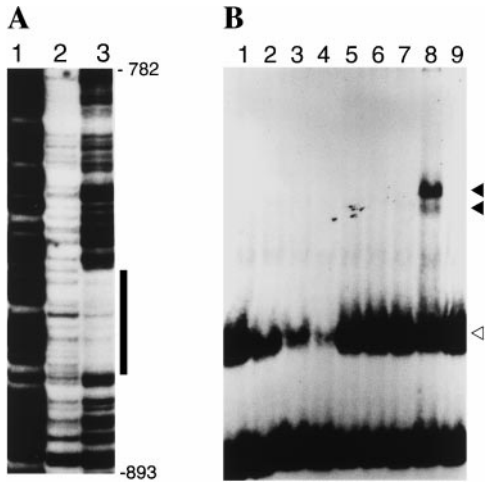


**Fig. 2.** **A** DNAseI protection analysis of the upper strand of the NF1 region of Rp with HeLa cell nuclear protein. *1* Maxam and Gilbert A+G sequencing markers of the footprint probe, *2* unprotected probe, *3* probe protected with crude nuclear extracts from HeLa cells. **B** Antibody supershift of HeLa nucleoprotein complexes with antiserum against NF1. For supershifting 1.25 μg of antiserum was added to the EMSA reaction. *1–9* Nuclear proteins added to a DNA fragment containing twice the NF1 binding site of Rp, *2–4* increasing amounts of identical unlabeled restriction fragment added as competitor, *2* 30-fold molar excess, *3* 75-fold molar excess, *4* 150-fold molar excess, *5–7* increasing amounts of unlabeled H1 fragment added as competitor, *5* 30-fold molar excess, *6* 75-fold molar excess, *7* 150-fold molar excess, *8* αNF1 polyclonal rabbit antiserum added, *9* rabbit control serum added

to the NF1 site of Rp we conducted antibody supershift experiments using a rabbit antiserum against NF1 which has been proven to be suitable for supershift experiments [42] (kindly provided by Anja Krause, Munich) (Fig. 2b). The retarded complex was generated with a DNA fragment containing two times the NF1 site of Rp in tandem (lane 1). Sequence specificity of binding was demonstrated by competition experiments. The addition of increasing amounts of identical unlabeled competitor clearly decreased the retarded complex (lanes 2 to 4). The hot probe without proteins added did not generate a retarded complex (data not shown). An excess of unlabeled nonrelated fragment (H1-site [46] of the R-promoter) could not compete for the complex (lanes 5 to 7). The addition of increasing amounts of antiserum against NF1 generated gradually increasing amounts of two additional complexes of higher molecular weight (lane 8, only one concentration [1.25 μg αNF1 antibody] of antiserum shown). The control serum was not able to generate the supershift, even at high concentrations (lane 9, only one concentration [1.25 μg control antibody] of antiserum shown). This supershift experiment established the identity of the HeLa protein binding to the NF1 site of Rp as belonging to the NF1 family of transcription factors.

Using the DNAseI protection assay on the NF1 consensus site with nuclear proteins from lymphoid IM9 cells we got an entirely different picture: Using crude extract, it was not possible to obtain a protection at the NF1-site under the same

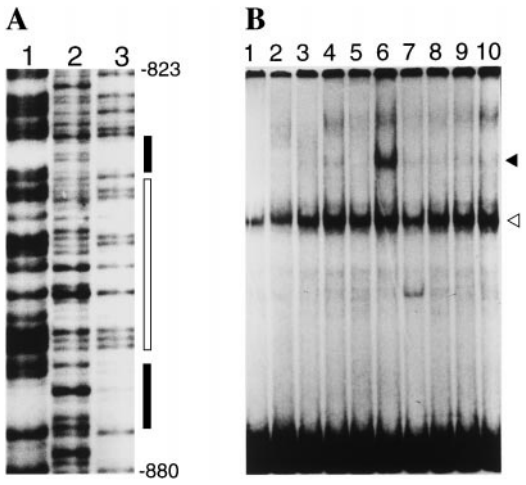


**Fig. 3.** **A** DNAseI protection analysis of the upper strand of the NF1 region of Rp with the heparin fraction of IM9 cell nuclear proteins. *1* Maxam and Gilbert A+G sequencing markers of the footprint probe, *2* unprotected probe, *3* probe protected with heparin fraction of nuclear proteins from IM9 cells. **B** Antibody supershift of IM9 nucleoprotein complexes on an NF1 consensus site with antiserum against NF1. EMSA was done with the heparin fraction of IM9 nuclear proteins, for antibody supershifting variable amounts of antiserum were added to the reactions, as indicated. *1–10* Heparin fraction added to a DNA fragment containing one copy of a consensus NF1 binding site as described [20], *1* and *2* addition of a polyclonal rabbit serum against the HIV V3 loop, *1* dilution 1/10, *2* dilution 1/100, *3–5* addition of rabbit control serum, *3* dilution 1/10, *4* dilution 1/100, *5* dilution 1/1000, *6–9* addition of αNF1 serum, *6* 12.5 μg, *7* 1.25 μg, *8* 0.25 μg, *9* 0.125 μg, *10* basic shift

experimental conditions. Therefore, we enriched our extracts for DNA binding factors by chromatography on heparin agarose. Using the heparin fraction there was a protection solely on the lower strand which was different from the situation in HeLa (Fig. 3a). Only a few individual bands spread over the NF1 site were protected, but no protection typical for an NF1 protein was visible. Closely flanking the NF1 element, however, there were two protected sites visible. Since the EMSA is a more sensitive assay than the DNAseI protection analysis, we also conducted an EMSA analysis with nuclear protein from IM9 cells on a consensus NF1 site. In this way we wanted to exclude the lack of NF1 protein in IM9 cells. A DNA fragment containing an NF1 consensus [20] was excised from plasmid pBS-NF1/C and incubated with the heparin fraction of IM9 nuclear proteins for supershift analyses (Fig. 3b). The sequence specificity of the generated single complex (lane 10) was tested by competition experiments (data not shown). The addition of increasing amounts of antiserum against NF1 was able to generate one additional complex of higher molecular weight (lane 6). This supershift appeared only at the highest concentration of specific antiserum used (lanes 9 to 6). Two control sera were not able to generate a supershift, even at the highest concentrations (lanes 1 to 2 and lanes 3 to 5). This supershift experiment established the identity of the IM9 protein specifically binding to the NF1 consensus site as

belonging to the NF1-family of transcription factors. From these experiments and previous data [40] we concluded that there is functional NF1 protein in IM9 cells that is able to bind to its corresponding isolated recognition sites. Therefore, it is very likely that the IM9 proteins binding around the NF1 site of Rp are not NF1 and not NF1 related. The two IM9 footprints flanking the NF1 site of Rp may correspond to protein that excludes the binding of the already low amounts of NF1 protein to its recognition site in Rp through steric hindrance. Therefore, this protein may contribute to the repression we observed in this cell line as opposed to the activation we saw in HeLa.

## Discussion

In epithelial cells EBV tends to a productive infection, in B cells to a strictly latent state that makes it hard to trigger the lytic cycle of virus production. Rp of EBV is unique in the regulation of expression of both major viral immediate early genes BZLF1 and BRLF1 [34]. Therefore, the examination of the cellular regulation of Rp may be one clue to explain the different behaviour of EBV in epithelial and lymphoid cells. Since the overall activity of Rp is very low in epithelial cells [49, 54, 55] and even lower in B cells [49, 54], it has been difficult to visualize Rp activities using the chloramphenicol acetyltransferase reporter assay. Therefore, we decided to use the more sensitive reporter system firefly luciferase. The experimental data published so far was focused on the inducibility of Rp by cotransfecting expression plasmids coding for transcription factors and analysed the proximal 500bp of Rp. In this way Zta, Rta, Sp1 and Zif268 were found to have positive regulatory effects on Rp activity [49, 54, 55]. The cellular environment used for some of the induction studies was the Drosophila cell line Schneider (SL2) which may be of limited significance for the actual regulation of Rp in the context of the switch from latency to the productive cycle and also a fusion cell line of an epithelial and a lymphoid cell line (D98HR1). So far no profile of constitutive activities of Rp in epithelial cells existed and there was only sporadic data in lymphoid cells [49, 54]. Therefore, we undertook to first establish a profile of Rp activities using a series of constructs containing 5′-deletion of Rp covering proximal as well as distal promoter segments in front of the luciferase gene in the epithelial cell line HeLa and the latently EBV-infected lymphoid cell line IM9. These cell lines seemed suited as a model system closer to the actual in vivo regulation of Rp than the artificial system SL2. Second, we wanted to focus on the more distal promoter segments that had not been examined much so far.

Our transient reporter gene analysis allowed measurements of significant Rp activities well above background activity in lymphoid and epithelial cellular background. The experiments demonstrated an overall lower activity level of Rp in IM9 than in HeLa cells. This is consistent with earlier transfection data using a comparative set of different cell lines [54] that showed an extremely low activity of full length Rp in several lymphoid cell lines as compared with several epithelial cell lines, where the activity was little, too. Contrary to the earlier experiments, however, activities of truncated promoter constructs could be visualized on a

meaningful scale. Since the positive control pLUCLTR in the IM9 cell had an excellent activity, we conclude that the low Rp activities are not due to inefficient transfections, but that they reflect actual Rp activities. Since there are binding sites in Rp for ubiquitously expressed and inducible transcription factors, this indicates that Rp may be in a repressed state in lymphoid cells. Alternatively, some constitutive activating factors that stimulate transcription from Rp in epithelial cells might be missing in IM9 and other lymphoid cells. The widely different activities of deletion construct pRLUC5′−276 in the two cell lines compared to the corresponding basal promoter activities suggest that the promoter segment between nts −42 and −276 may be the most important one for the difference in constitutive activities observed between the two cell lines. The only other analysis demonstrating constitutive activities of a series of in this case site directed mutations in the epithelial cell line C33 [54] showed that the most proximal Sp1 site of Rp was important for promoter activity. Since Sp1 is ubiquitous [8], and the level of Sp1 binding to the BRLF1-promoter is essentially the same in Raji, HeLa, and C33 extracts [54], it is likely that the low activity of Rp in IM9 cells is at least partly due to repression. The lack of binding of a positive factor, however, is not excluded at this point. A thorough examination of protein-DNA-interactions of this promoter segment using crude nuclear extracts and not only purified proteins should be conducted in the future. In addition, using highly sensitive reporter assays, a more detailed functional map of constitutive activities in the respective area of Rp is feasible.

The adjacent upstream promoter segment between nts −276 and −385 had a similarly positive effect on the constitutive activities in both cell lines. Since this segment contains the middle Sp1 binding site of Rp, this site might be connected with positive regulation in both cell types. The existence of additional positive factors in this segment cannot be excluded and should again be clarified through an analysis of protein-DNA-interactions and more detailed functional experiments.

The next segment between nts −385 and −445 is acting negatively in both cell lines. Since there are no binding sites for transcription factors described in this promoter section, the analysis of protein-DNA-interactions may yield more information on a possible repressive mechanism which seems active in both cell types. The distal consensus Sp1 binding site in the next construct pRLUC5′−511 seems to activate slightly in IM9 cells, whereas there was no clear effect in HeLa. Since Sp1 binding sites can also be recognized by a ubiquitously expressed negatively regulatory factor [28] the distal Sp1 binding site of Rp may be regulated differently through the competition of the GC box factor with Sp1 for binding. The binding of the negatively regulatory GC box binding protein [28] certainly is a possibility for the more proximal Sp1 consensus sites of Rp in IM9 cells, too. The different regulation of the distal Sp1 site is an indication for the limited value of insect cell lines for the analysis of Rp, where the distal Sp1 site strongly contributed to Rp activity.

Another segment of Rp that was contributing negatively to the overall activity of the promoter in both cell lines was between nts −687 and −847. One candidate binding site we found in this segment by using the Transfac database [52, 53],

was a consensus binding site for E4BP4, a negative regulatory cellular protein that was originally found in the regulation of adeno viral promoter E4 [9] and, overlapping with the E4BP4 site, a one mismatch consensus for the H1 element that was described as a negative regulatory element for Zp of EBV in lymphoid cells [46]. Since we also found DNAseI footprints at the E4BP4-H1 element of Rp in both of our cell lines (data not shown), these binding sites are good candidates for negative regulation of Rp in both cell lines. Further functional experiments, like the site directed inactivation of these binding sites in reporter constructs should reveal more information about their function in the promoter context.

The addition of the upstream fragment containing an NF1 binding site makes a difference for both cell types. In HeLa cells this site serves as a strongly activating element whereas in IM9 cells it acts sligthly repressive. This difference in function has a correlate in the nucleoprotein complexes found on the site with nuclear proteins from both cells. In HeLa cells there is NF1 or a member of the NF1 family of transcription factors binding to the site, as evident from DNAseI protection, electrophoretic mobility shift and supershift analyses, whereas in IM9 cells, despite the presence of NF1 in this cell type, there is no binding of protein to this site, but binding to two flanking regions of the NF1 site. Since in HeLa cells the promoter segment between nts −687 and −847 is able to repress Rp activity down to almost baseline levels and the next upstream segment between nts −847 and −864, containing essentially the NF1 site, more than compensates for this repression, the promoter area around and downstream of the NF1 site seems to be the second hotspot of regulation besides the proximal segment between nts −42 and −276. Although transcriptional repression may be mediated through NF1 sites [1, 25, 45] this mechanism seems not to play a major role in keeping EBV latent in IM9 cells. First, the NF1 site only marginally contributes in a significant way to transcriptional repression from Rp, and second we did not find typical binding of NF1 protein to this site in Rp. Rp may present a case of negative regulation by competition of factors for closely spaced binding sites. In the context of Rp the exclusion of NF1 from its recognition site through two neighboring factors might therefore play a role in keeping EBV latent in lymphoid cells.

Since the constitutive activity of the NF1 site of Rp was so clearcut in HeLa, we reviewed the sequence of Zp of EBV and found a one mismatch only NF1 binding site at nts −360 to −373 of Zp (5′-TGGN$_6$GCCAC-3′), that had the same recognition determinants as the NF1 site in Rp. The respective promoter region of Zp has been subject to extensive experimentation [37, 46] but the NF1 site of Zp has not found attention so far. A 5′-deletion construct (p386) containing this NF1 site showed strong constitutive activity in HeLa and in Balbc/3T3 cells, whereas a shorter construct (p222) lacking the site had no constitutive activity in HeLa cells [37]. DNAseI protection analyses conducted by the same investigators [37] indicated that the NF1 site of Zp was protected by nuclear proteins from HeLa cells. Another parallel to Rp we found in Zp with regard to the H1 elements described as repressors in lymphoid cells [46]. In close proximity of the NF1 sites of both immediate early promoters there are three and one H1 elements located,

respectively. This is one more hint to a similar regulation of both promoters. The presence of an NF1 binding site in both main immediate early promoters of EBV suggests that regulation through NF1 may contribute in an essential way to the maintaining and breaking of viral latency. Therefore, regulation through NF1 seems even more widespread among viral key promoters (CMVs major immediate early enhancer/promoter region, adenoviruses) than assumed so far.

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# Protein-DNA Binding and CpG Methylation at Nucleotide Resolution of Latency-Associated Promoters Qp, Cp, and LMP1p of Epstein-Barr Virus

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**Epstein-Barr viral (EBV) latency-associated promoters Qp, Cp, and LMP1p are crucial for the regulated expression of the EBNA and LMP transcripts in dependence of the latency type. By transient transfection and in vitro binding analyses, many promoter elements and transcription factors have previously been shown to be involved in the activities of these promoters. However, the latency promoters have only partially been examined at the nucleotide level in vivo. Therefore, we undertook a comprehensive analysis of in vivo protein binding and CpG methylation patterns at these promoters in five representative cell lines and correlated the results with the known in vitro binding data and activities of these promoters from previous transfection experiments. Promoter activity inversely correlated with the methylation state of promoters, although Qp was a remarkable exception. Novel protein binding data were obtained for all promoters. For Cp, binding correlated well with promoter activity; for LMP1p and Qp, binding patterns looked similar regardless of promoter activity.**

Epstein-Barr virus (EBV) infection is the cause of infectious mononucleosis and is most closely associated with tumor diseases Burkitt's lymphoma (BL) and nasopharyngeal carcinoma. EBV infection of human B lymphocytes in vitro results in B-cell proliferation and transformation into continuously growing lymphoblastoid cell lines (LCL) (for a review, see reference 42). In latently infected cells, viral genomes are maintained as multiple circular episomal copies which are replicated once per cell cycle (2, 103). Several classes of latency have been described depending on the gene expression pattern (41, 77, 78). In strict type I latency, represented by BL cells, viral gene expression is restricted to the two RNA polymerase III-transcribed EBER RNA genes and the EBNA1 gene (78) that is transcribed from the Q promoter (Qp) (68). The EBNA1 protein is required for the maintenance of the viral plasmid in dividing cells (45, 58). In type III latency, in addition to the EBERs, EBNA-LP, -2, -3A, -3B, -3C, and -1 are expressed from the C promoter (Cp) (6), whereas LMP-1 and -2B are expressed from the bidirectional LMP1 promoter (46), and a larger splice variant of LMP-2, LMP-2A, is expressed from the TP1 promoter (36). Qp generally is supposed to be silent in type III latency (82, 105), although there is also a different view (93). Among the viral proteins expressed in latency type III, EBNA2 plays a central role in switching EBNA transcription from Wp to Cp (W to C switch) (102, 104) and in the establishment and maintenance of B-cell transformation (11, 28), as EBNA2 transcriptionally activates the expression of the six nuclear antigens from the C promoter (Cp) and the membrane proteins LMP-1 and -2B from the LMP1 promoter (LMP1p), LMP-2A from the TP1 promoter, and a number of cellular proteins associated with the LCL phenotype (1, 12, 18, 39, 44, 72, 76, 90, 95, 98, 99, 100, 101, 102, 104, 110, 111). A crucial mechanism involved in the silencing of Cp and LMP1p in type I latency has been shown to be methylation of CpG dinucleotides (3, 15, 35, 54, 60, 61, 70, 73, 74, 75, 84, 91, 94). In LCL, the EBV genome is mostly free of CpG methylation, whereas in BL cells, EBV genomes are highly methylated. An essential step in understanding the differences between latency types I and III is to elucidate the patterns of methylation and in vivo protein binding of the latency promoters of EBV at nucleotide resolution. Therefore, we decided to examine Qp, Cp, and LMP1p in cells of both latency types.

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## MATERIALS AND METHODS

**Cell lines and tissue culture.** LCL 721 is a B95-8-transformed LCL with type III phenotype (40, 52, 57). Rael (15, 43, 61) is a group I BL cell line. Mutu BLI-C1216 is a subclone of the BL line Mutu, representative of latency type I (27). Mutu BLIII-C199 is a subclone of the BL line Mutu, representative of latency type III (27). Raji cells express all the type III latency genes but use a thus far unknown promoter, other than Cp, for the EBNA transcripts (29, 96). All cells were maintained in suspension cultures of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml at 5% $CO_2$ and 37°C.

**Electrophoretic mobility shift assay.** Preparation of nuclear extracts from Mutu I cells was essentially based on the standard of Dignam et al. (14). Nuclei were prepared using a combination and modification of two methods (8, 30) as

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TABLE 1. Primers for methylation mapping[a]

| Primer type | No. | Sequence (nucleotides) |
|---|---|---|
| Outer primers for modified Qp[b] | 1 | GGTTAGTTTGATTAAGGGTGAGGT (62179–62202) |
| | 2 | CCATTACCCCCAATACATTCC (62626–62606) |
| Inner primers for modified Qp[c] | 3 | Univ-CTCCCAACTACCCAAAATACCA (62504–62483) |
| | 4 | Biotin-GTTAGTTTGATTAAGGGTGAGGTTATAA (62180–62207) |
| Outer primers for modified Cp[d] | 5 | GGGTTTAGGTTTTGTAGGGTAGA (10585–10607) |
| | 6 | CCCTACRATAAAAACTCTAAAAATCTT (11392–11366) |
| Inner primers for modified Cp[e] | 7 | Univ-GTTAGGTTGATAAGGGGATAAG (10610–10631) |
| | 8 | Univ-TGAGAGGTTAGTGTTTTAAATATGT (10878–10902) |
| | 9 | Univ-GGATTATAGTTAATAAGAGAGTTTAAGA (11066–11093) |
| | 10 | Biotin-ATCCTTATCTCTATACCATCTAATCTA (11361–11335) |

[a] Primer positions refer to the nucleotides of the B95-8 sequence (4). Univ indicates the M13 universal primer sequence GTAAAACGACGGCCA. Primers were purchased from Metabion (Martinsried, Germany). Each PCR was cycled 30 times at the temperatures and times indicated. Primers for LMP1p, PCR conditions, and the CpG methylation maps of LMP1p are described in detail by Takacs et al. (91a).
[b] Cycled at 95°C for 40 s, 60°C for 40 s, and 72°C for 70 s.
[c] Cycled at 95°C for 40 s, 58°C for 40 s, and 72°C for 60 s.
[d] Cycled at 95°C for 40 s, 58°C for 40 s, and 72°C for 90 s.
[e] Cycled at 95°C for 40 s, 52°C for 40 s, and 72°C for 90 s (7 to 10), 70 s (8 to 10), or 60 s (9 to 10).

already described (64). Complementary double-stranded DNA oligonucleotides (Metabion) containing a consensus binding site for CBF1, 5′-GGATCCGCCGTGGGAAAAAGTCGAC-3′, and a mutant binding site disabled for CBF1 binding, 5′-GGATCCGCCGTGTTAAAAAGTCGAC-3′, (51) were kinase labeled, annealed, and spin column purified for a gel shift probe. Gel retardation assays were performed as described (30, 64): 1 μg of crude nuclear protein was incubated with poly(dI-dC)as indicated, 1 ng of $^{32}$P-labeled probe, and a 50-fold excess of unlabeled competitor fragment in 25 μl of bandshift buffer (10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 80 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 12.5% glyceroi, 0.1% Triton X-100) for 20 min. Protein complexes were resolved by electrophoresis on native 4% polyacrylamide gels (29+1) in 6.7 mM Tris-HCl (pH 7.5)–3.3 mM sodium acetate (pH 7.0)–1 mM EDTA at 20 mA for several hours.

**DNA sequences.** Oligonucleotides (Metabion, Martinsried, Germany) corresponding to EBV nucleotides (4) 10595 to 10614 and 11364 to 11342 were used for sequencing Cp, and others corresponding to nucleotides 62146 to 62165 and 62548 to 62524 were used for sequencing Qp. Both strands of the two promoters were sequenced from the genomic DNA of all five cell types on an ABI 377 DNA sequencing system using dye-labeled dideoxynucleoside triphosphates (ddNTPs). In the analyzed region of the C promoter (nucleotides 10615 to 11341), a few sequence polymorphisms were noted in Rael (GenBank accession number AJ297541) and in the Mutu subclones, whereas the sequences of LCL 721 and Raji were identical to the standard B95-8 sequence (4). The sequence of Cp between nucleotides 10615 and 11046 in the Mutu subclones is at GenBank numbers AJ000877 and AJ000878 (91), and the 3′ part between nucleotides 11047 and 11341 was identical with the standard sequence (4). The sequence of Qp between nucleotides 62166 and 62523 did not show any deviation from the B95-8 standard sequence in all cell types (4). Sequences of LMP1p have been described (91a).

**Automated genomic sequencing of sodium bisulfite-treated DNA.** We used the method of Frommer et al. (22) and Clark et al. (10) adapted for an automated DNA sequencer (63). A total of 5 μg of genomic DNA in 50 μl of water was denatured by adding 5.5 μl of freshly prepared 3 M NaOH and incubating for 15 min at 37°C. Then 30.5 μl of freshly prepared 10 mM hydroquinone (Sigma), and 530 μl of 3.6 M sodium bisulfite, pH 5 (Sigma), were added to the denatured DNA, mixed gently, divided into five 0.5-ml PCR tubes, overlaid with paraffin oil, and cycled five times at 95°C for 3 min and 55°C for 57 min. After this treatment, the modified DNA was purified using a GeneClean kit (BIO 101) according to the manufacturer's instructions. Then the DNA was desulfonated by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M and incubating the mixture for 15 min at 37°C. After desulfonation, the DNA was ethanol precipitated and dissolved in water. Then 100 ng of freshly modified DNA was used for PCR amplification with the strand-specific outer primer pairs (22) designed for the promoter regions (Table 1). The 50-μl PCR contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 40 pmol each of the primers, 0.2 mM each of the four dNTPs, and 2 U of *Taq* polymerase (Promega). Then 3 μl of a 1:100 dilution from the first PCR was amplified in a second nested PCR using the primers listed in Table 1. One of the nested primers was biotin labeled, and the other carried 15 bases of the M13 universal primer at its 5′ end. The reaction mixture for the nested PCR was the same as for the first PCR except that the amount of inner primers was 10 pmol each. The product of the second PCR was bound to streptavidin-coated magnetic beads (Dynal), and the purified biotin-labeled strand was sequenced using the AutoRead DNA sequencing kit (Amersham Pharmacia Biotech) and a fluorescein-labeled M13 universal primer as described by Myöhänen et al. (63). The reaction products were separated on acrylamide gels using an automated DNA sequencer (Amersham Pharmacia Biotech). The degree of methylation was estimated as described earlier (63). The bisulfite conversion reaction was complete, since all cytosines outside CpG dinucleotides were converted to uracil and therefore sequenced as thymine instead of cytosine after PCR (see reference 80 and Takacs et al. [submitted] for examples).

**DMS in vivo footprinting.** Genomic footprinting was performed essentially as described (65). For each footprint reaction, $10^7$ exponentially growing cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in 1 ml of PBS, and incubated at room temperature for 1 min with 5 μl of dimethyl sulfate (DMS). The reaction was stopped by the addition of 5 ml of DMS stop solution, containing 1% bovine serum albumin and 100 μM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in 1 ml of PBS, and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment (55). For visualization of footprints by ligation-mediated PCR (LM-PCR), 2 μg of sequenced or footprinted DNA was analyzed as described (26, 62) with modifications (65). The primers for LM-PCR are listed in Table 2. The first-strand primer extension reaction was done in 10 mM KCl–10 mM $(NH_4)_2SO_4$–20 mM Tris-HCl–2 mM $MgSO_4$–0.1% Triton X-100 (pH 8.8) at 25°C (Vent buffer; New England Biolabs), containing 0.3 pmol of primer i of each set, 240 μM each dNTP, and 1 U of Vent (exo-) DNA polymerase (New England Biolabs) for 5 min at 94°C, 30 min at 60°C, and 10 min at 72°C. For ligation of the common linker, the sample was transferred to ice, and 5 μl of PCR linker mix as in Mueller and Wold (62), 2 μl of ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP [pH 7.5] [20°C], Boehringer Mannheim), 1 μl of T4 DNA ligase (5 U/μl; Boehringer Mannheim), and 12 μl of water were added. After overnight incubation at 4°C, the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. The PCR amplification was done in 100 μl of Vent buffer containing 10 pmol of each primer ii and the longer linker primer, 240 μM each dNTP, and 1 unit of Vent (exo-) DNA polymerase for 20 cycles using 1 min at 94°C, 1.5 min at 60°C, and 3 min at 72°C. For labeling, the sample was transferred to ice, 5 pmol of T4 kinase [γ-$^{32}$P]ATP-labeled primer iii, 2.5 nmol of each dNTP, and 0.5 U of Vent (exo-) DNA polymerase in a volume of Vent buffer not exceeding 15 μl were added. Then the sample was heated to 94°C for 1.5 min, subjected to eight cycles of 2 min at 94°C, 2 min at 62°C, and 5 min at 72°C, and kept at 72°C for 5 more min. Samples were phenol-chloroform extracted, ethanol precipitated, ethanol washed, and resuspended in loading dye. One fifth of each sample was separated on a 5% sequencing gel, and the gels were dried and autoradiographed at room temperature with Kodak BioMax MR film.

TABLE 2. Primers for LM-PCR[a]

| Promoter | Primer set | No. | Sequence (nucleotides) |
|---|---|---|---|
| Qp | A | i | GCTATAACGCAGGTCCTGTTCCGGG (62201–62225) |
| | | ii | GCGGTGGATAGAGAGGAGGGGGATC (62229–62253) |
| | | iii | GAGGGGACCACTAGGTCGCCGGAGG (62256–62280) |
| | B | i | CCCCAAACATACACCGTGCGAAAAG (62548–62524) |
| | | ii | CCGTGCGAAAAGAAGCACCCCCATC (62535–62511) |
| | | iii | CCGCCTCCCAGCTGCCCAAAATGCC (62508–62484) |
| Cp | A | i | GTCCCAATTAGAAACCCAAGCGCAG (10845–10869) |
| | | ii | CCCAAGCGCAGAAATTAGTTGAGAGG (10859–10884) |
| | | iii | AACMTGCACCCTAGGCCAGCCAGAG (10896–10920) |
| | B | i | ACTTTGCGAGCCCTGCGTCTTGAG (11110–11087) |
| | | ii | TATTGGCTATAATCCGTCGCTCCTCCC (11080–11054) |
| | | iii | CCGTCGCTCCTCCCAGATAAGGCGT (11067–11043) |
| | C | i | CTCAAGACGCAGGGCTCGCAAAGT (11087–11110) |
| | | ii | GTATAGTGGCCCCGTGGGACCTTAG (11109–11133) |
| | | iii | TTAGAGGTGGAGCAACGTCTAAAGTGG (11130–11156) |
| | D | i | GGGCCTACATGGCCGCATGGTAAG (11418–11395) |
| | | ii | GGTAAGAACCCTGCGATGAGGGCTC (11400–11376) |
| | | iii | GATGAGGGCTCTGGGGGTCTTCGGTG (11386–11361) |
| | E | i | GTGCGTCGAGTGCTATCTTTGGAAC (10981–11005) |
| | | ii | ACCTTGTTGGCGGGAGAAGGMATAAC (11019–11044) |
| | | iii | ACGCCTTATCTGGGAGGAGCGACGG (11043–11067) |
| LMP1p | A | i | CCCCTCTCAAGGTCGTGTTCCATCC (169452–169476) |
| | | i var | CCCCTCTCAAGGTCCAGGTCCATGC (169452–169476) |
| | | ii | TCAGGGCAGTGTGTCAGGAGCAAGG (169477–169501) |
| | | ii var | TCAGGGCAGTGTGTCAGGAGCCAGG (169477–169501) |
| | | iii | AGGCAGTTGAGGAAAGAAGGGGGCAG (169489–169524) |
| | B | i | CTTAGCCCTCTTAGCCGCCTCACC (169966–169943) |
| | | ii | TACGGTTACCCCACAGCCTTGCCTC (169933–169909) |
| | | ii var | TACGGTGAACCCACATCCTTGCCTC (169933–169909) |
| | | iii | GCCTCACCTGAACCCCCCTAAAGCAC (169913–169888) |
| | | iii var | GCCTCACCTGAACCCCCCTAAAACMC (169913–169888) |
| | C | i | GCGCCTCTTTGTGCAGATTACACTG (169843–169819) |
| | | ii | CCGCTTCCCACAACACTACGCACTC (169818–169794) |
| | | iii | CCTTCTGATTGCCGCACTGCCTTTCC (169791–169716) |
| | D | i | GTACGGGYRCAGATTTCCCGAAAG (169621–169644) |
| | | ii | GATTTCCCGAAAGCGGCGGTGTGTG (169632–169656) |
| | | iii | CGGCGGTGTGTGTGTGCATGTAAGCG (169645–169660) |
| | E | i | AGAGGAGGAGAAGGAGAGCAAGG (169375–169397) |
| | | ii | CCCCTCTCAAGGTCGTGTTCCATCC (169452–169476) |
| | | ii var | CCCCTCTCAAGGTCCAGGTCCATGC (169452–169476) |
| | | iii | TCAGGGCAGTGTGTCAGGAGCAAGG (169477–169501) |
| | | iii var | TCAGGGCAGTGTGTCAGGAGCCAGG (169477–169501) |
| | F | i | CACACGCTTYCTACTTCCCCTTTYTAC (169696–169670) |
| | | ii | CGCTTACATGCACACACACACCGCC (169670–169646) |
| | | iii | CACACACCGCCGCTTTCGGGAAATC (169656–169632) |

[a] Primer positions refer to nucleotide numbers of the B95-8 sequence (4). Primers were purchased from Metabion (Martinsried, Germany). Variant primers or primers with wobble bases were used because of minor sequence deviations between EBV strains. With these primer sets, both strands of each promoter were visualized in their entire length, and several promoter parts were seen with more than one primer set.

## RESULTS

**Methylation patterns at CpG dinucleotides.** The methylation data solely reflected the status of tightly latent EBV circular genomes, but not the presence of linear genomes from a possible small amount of lytic replication as was tested by terminal repeat analysis through southern blotting (59, 91a) and for LCL 721 and Raji cells through Gardella gels, in addition (52). Early antigens or their coding mRNAs associated with productive EBV replication could not be detected either in the above-mentioned cell lines and clones (54, 57; J. Minarorits, unpublished data). In addition, specific segments of the EBV genome were found to be completely methylated

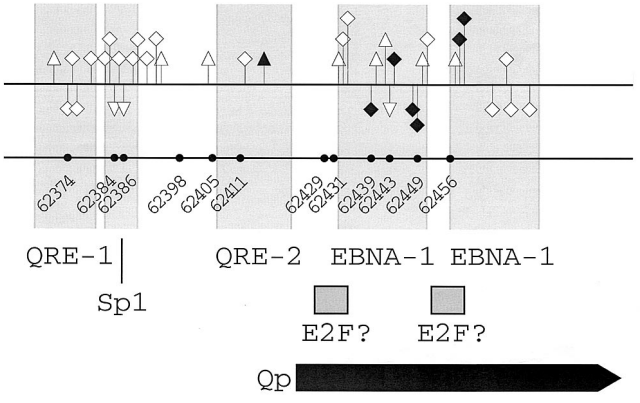



FIG. 1. Summary of genomic footprinting and methylation patterns of Qp. Numbers and circles on the lower line indicate positions of cytosines within CpG dinucleotides and show that all CpG dinucleotides within Qp are totally unmethylated in all cell types. On the upper line, guanines protected from methylation by DMS are indicated by squares and enhanced reactivity to DMS is shown by triangles. Guanines that showed a different reactivity to DMS between cell types are indicated by solid symbols. The upper strand is shown above, and the lower strand is below the line. The positions of important *cis* regulatory elements are indicated by columns and boxes. The transcription initiation site of Qp is shown by a thick arrow.

in all five cell types (data not shown), another indication that there was no lytic cycle viral DNA in the cell lines examined. Cytosines between nucleotides 62264 and 62482 of the Qp region were completely unmethylated in all cell types (Fig. 1). The methylation data on Qp were in agreement with previous observations (84, 93), completed these observations for Mutu I, and extended them to the additional cell types LCL 721 and Mutu III. Previous methylation analyses of Cp (3, 54, 61, 74, 75, 84, 91, 94) could be largely confirmed and extended by our present work. Overally, Cp was nonmethylated in class III cell lines, but highly methylated in class I BL cell lines and Raji (Fig. 2). However, in Mutu I there was a methylation gap of about 100 bp of complete demethylation around the crucial CBF1 and CBF2 binding sites (16, 37, 74, 75), and a further adjoining gap of about 100 bp of partial demethylation (Fig. 2). For Mutu III there was a small contradiction to our earlier work (91), where CpG dinucleotides 10702 and 10799 were found to be highly methylated. Methylation at these two CpGs could not be confirmed anymore. The discrepancy was most likely due to a sequencing artifact in the earlier work. Methylation of LMP1p in the five cell lines has been recently examined by Takacs et al. (submitted). Overall, LMP1p was hypo- or nonmethylated in class III cell lines, as well as Raji, but highly methylated in class I BL cell lines (Fig. 3).

**In vivo protein binding. (i) Q promoter.** The DMS footprinting was done on both strands of Qp (Fig. 4) according to standard methods (26, 62, 65). The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized in Fig. 1. The footprint patterns on Qp from the five cell lines were generally identical, with four remarkable features. First, in Mutu I cells the EBNA1 binding sites were more weakly protected than in the other cell lines. Second, there was a hypersensitivity in the type III cell lines on the upper strand at guanine 62416 within QRE2, but not in the type I cells. Third, there was a strong protein-DNA interaction at a potential Sp1 binding site around nucleotides 62382 to 62394 (67). Fourth, footprints with a typical protection pattern indicative of E2F binding (112), at two previously characterized unconventional E2F sites, interspersed with the EBNA1-sites and the transcriptional start site (13, 89), were not found.

**(ii) C promoter.** Previous in vitro binding and reporter gene experiments have charted a CBF1 site, a CBF2 site, and two CCAAT boxes as transcriptional elements of Cp and identified CBF1/RBP-Jκ and AUF1 as the respective binding proteins for the CBF1 and CBF2 sites (23, 25, 37, 47, 50, 71, 75). These binding sites and an additional Sp1 site have been shown to be highly conserved between EBV and two related lymphocryptoviruses of monkeys (24). The promoter area examined here is shown in Fig. 5. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized together with the methylation data in Fig. 2. There were two Sp1-like sequences around nucleotides 11176 and 11197 that were protected in all cell lines despite methylation (31). The Sp1 site at 11029 showed slight signs of protein-DNA interaction in all cells. Further, we found a series of sites protected only in 721 and Mutu III cells, but not protected in type I and Raji cells. The two CAAT boxes and several novel protections belonged to this category. The CAAT box at nucleotide 11075 was strongly protected, and the CAAT box at nucleotide 11268 was strongly protected in LCL 721 cells and weakly in Mutu III cells. A novel interaction, called X, was found around nucleotide 11222. Another novel interaction, called Y, carrying no familiar consensus sequence for transcription factor binding, was found around nucleotide 11062. An extended interaction was found between 11120 and 11140, with a methylation interference pattern characteristic of CBF1 binding (56) in the 5′ part and a protection, called Z, in the 3′ part. Binding at the CBF2 site in LCL 721 and Mutu III cells was weak at best, since there were slight signs of protein-DNA interaction only (Fig. 5). A characteristic protein-DNA interaction was found at the previously described CBF1 site at nucleotide 10959. Since the CBF1 site was unmethylated but the protection pattern was not typical in Mutu I, we performed electrophoretic mobility shift experiments. The gel shifts showed that a CBF1-like binding activity was present in Mutu I and was able to bind to its consensus site in a sequence-specific manner (Fig. 6).

**(iii) LMP1 promoter.** Many transcriptional elements of LMP1p have been characterized so far by in vitro binding and reporter gene experiments. Among these elements were binding sites for CBF1 (38, 51); PU.1, also called Spi-1, and Spi-B (38, 47, 48, 85, 108); AML1, also called LBF1, and several LMP1p binding proteins, named LBF-2 to -7 (38); negative regulatory element NRE (18); the E box carrying a USF binding site (88); a *cis*-inducible element (SIE) (86, 87); and an ATF/CRE that, depending on the distinct proteins binding, was able to activate LMP1p both independently and dependent on EBNA2 (20, 87, 88). The relevant footprinted promoter area is shown in Fig. 7. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation is summarized together with the methylation data in Fig. 3. Footprints were generally identical for all cell types. Signs for protein-DNA interactions, mostly of low or intermediate strength,

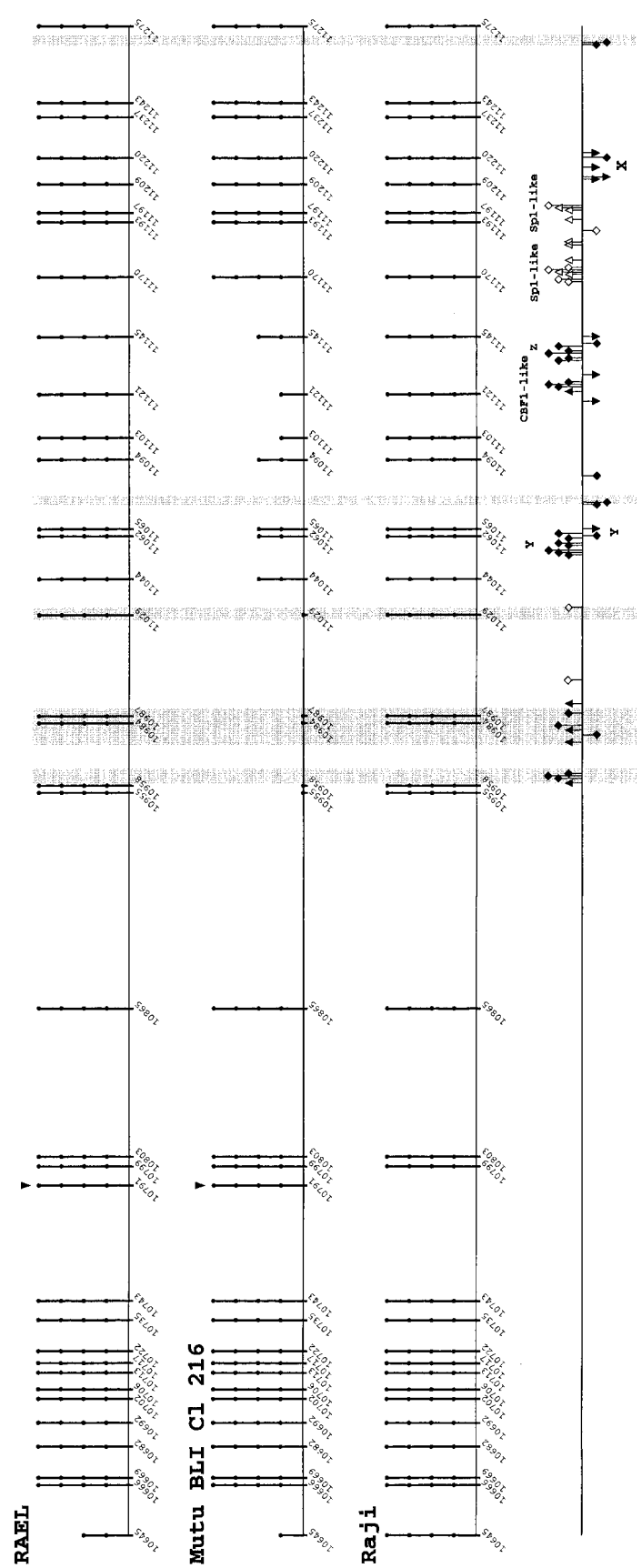



FIG. 2. Summary of genomic footprinting and methylation patterns in the sequenced region of the BCR2 promoter (Cp). Numbers and lollipops indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence (4). Triangles above the lollipops mark additional target cytosines for DNA (cytosine-5) methyltransferase (5, 69) present in the cell lines studied. The degree of methylation of cytosines is indicated by the height of the lollipops as follows: spot only, 0%; one lollipop unit, 0 to 25%; two units, 25 to 50%; three units, 50 to 75%; four units, 75 to 100%. The bottom line shows a summary of genomic footprints for the upper (above the line) and lower (below the line) strand of Cp. Guanines protected from methylation by DMS are indicated by squares, and enhanced reactivity to DMS is shown by triangles. Guanines that showed a different reactivity to DMS between cell types are indicated by solid symbols. Novel footprints (X, Y, Z, CBF1-like, and Sp1-like) are indicated above and below the footprint marks. Faint columns represent already published relevant transcription factor binding sites in Cp.

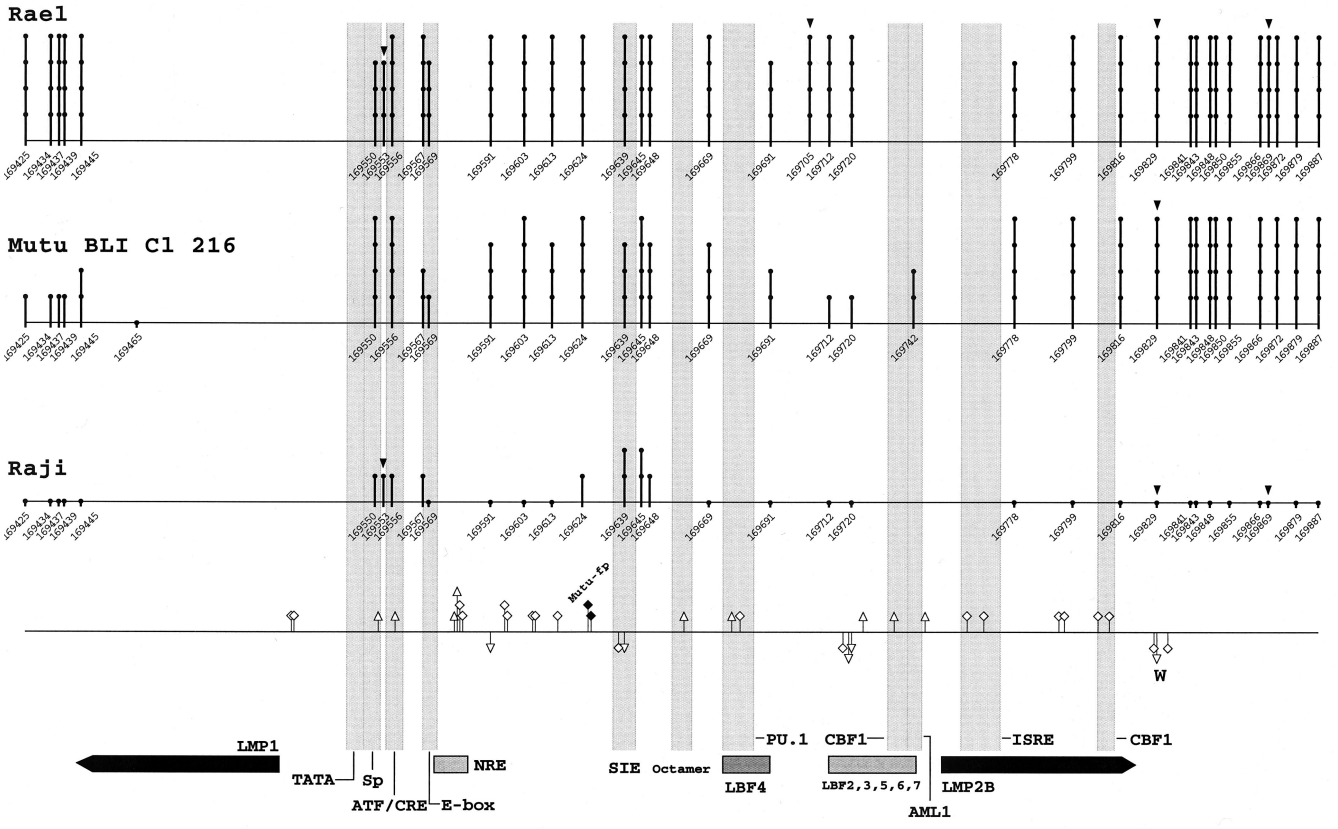



FIG. 3. Summary of genomic footprinting and methylation patterns in the sequenced region of the LMP1 promoter. Numbers and lollipops indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence (4). Triangles above the lollipops mark additional target cytosines for DNA (cytosine-5) methyltransferase present in the cell lines studied. The degree of methylation of cytosines is indicated by the height of the as defined in the legend to Fig. 2. The bottom line shows a summary of genomic footprints for the upper (above the line) and lower (below the line) strand of LMP1p. Guanines protected from methylation by DMS are indicated by squares, and enhanced reactivity to DMS is shown by triangles. Some novel footprints (Mutu-fp with solid symbols and W) are indicated above and below the footprint marks. Faint columns represent important transcription factor binding sites in LMP1p. The transcription initiation sites of LMP1 and LMP2B are shown by thick arrows.

were found on either one or both strands of the PU.1, AML1, LBF-3, 5, 6, 7, Oct, ISRE, SIE, NRE, and Sp1-like site, the two CBF1 sites, and the ATF/CRE site. The PU.1/LBF4 binding activity carried the typical methylation interference pattern described for these factors in vitro (38). The CBF1 footprints did not carry the typical methylation interference pattern (56) that was found in type III cells except Raji on Cp. In addition to protein-DNA interactions at already charted elements, we found a footprint at nucleotides 169520 and 169521 around the initiation site of the LMP1 transcript, a hypersensitive site at nucleotide 169591, a footprint at 169596 and 169597, a footprint at 169606 and 169607, a footprint at 169615, and a strong footprint, called W, at nucleotide 169833 for all cell types examined. In addition, we found a footprint, Mutu-fp, specific for both Mutu clones at nucleotides 169626 and 169627. These binding factors await further identification. However, there were no differences in the in vivo binding pattern, with the possible exception of the ATF/CRE-Spl locus. At this locus we found slight differences in the reactivity to DMS between cells.

## DISCUSSION

Although under some conditions Mutu I may drift to type III latency, Mutu BLI-C1216 of this study represents a type I cell, because of its phenotype and EBNA-2 protein could not be found by Western blotting (data not shown). Still, we cannot entirely rule out the possibility that an extremely small proportion of Mutu I cells were drifting towards type III latency. Because of this and because of the limited number of cell types in this study, final conclusions regarding the two latency types may only be drawn after the examination of a larger panel of cell lines and subclones.

**Qp behaves like a bacterial promoter.** Qp was unmethylated and extensively protein protected in all cell types, regardless of the activity of the promoter (Fig. 1 and 4). The protein binding pattern was generally in congruence with the in vitro (9, 66, 67, 83, 89, 106) and in vivo (33) data described earlier. It is clear now that the Sp1-like sequence just downstream of QRE1 is strongly protein bound. This site has been discussed as a potential unconventional E2F site, but has been shown not to compete for the in vitro binding of E2F-like proteins (79). The overall protection pattern was identical in the five cell types, with minor exceptions: at the QRE2 element there was a hypersensitivity indicative of closer protein binding at QRE2 for the silent promoter state in LCL 721, Mutu III, and Raji cells (Fig. 4). Since Qp is unmethylated and heavily protein protected, the QRE2-bound protein may be key to the silencing of

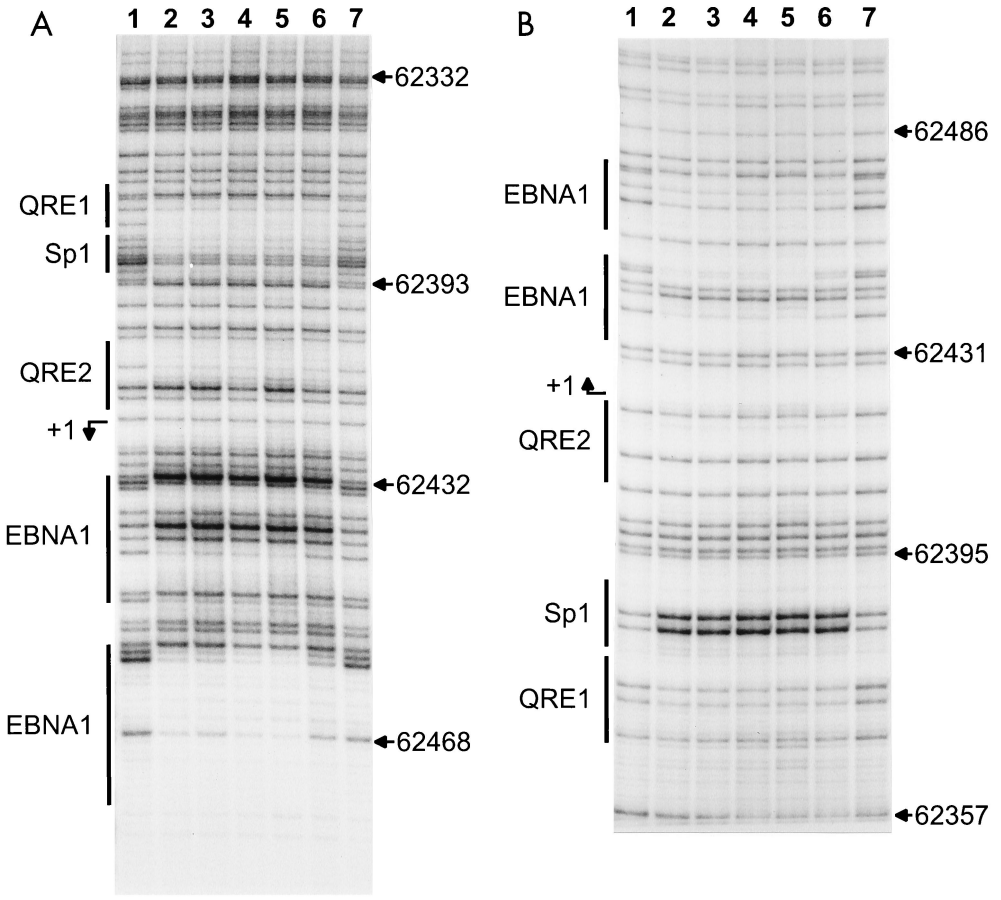



FIG. 4. Genomic footprint analyses of Qp. (A) Upper strand, (B) Lower strand. Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I DNA. At the left of each panel, the locations of in vivo footprints and previously described in vitro binding sites are indicated by vertical bars; at the right of each panel, nucleotide numbers are given according to the EBV sequence of Baer et al. (4).

Qp in type III latency. Candidate factors for this binding activity are IRF-7, a Qp-repressive factor described previously (66, 106), and IRF-2, although there are contrary views on the repressive nature of IRF-2 (83, 107). The EBNA1 binding sites were protected in all cells, in agreement with Hsieh et al. (33), who have already demonstrated the same in vivo EBNA1 site protection in Qp for Raji cells. The weaker EBNA1 binding in Mutu 1 may be interpreted in terms of promoter activity in type I cells and a repressive function for EBNA1. However, in Rael, where Qp is active, the EBNA protection is as strong as in the type III cells. A clear protection pattern indicating E2F binding (112) could not be found at the sites previously described as in vitro E2F binding sites (79, 89). Therefore, the repressive role for EBNA1 (81) and the activating role for E2F in Qp transcription that have been postulated (79, 89) may have to be modified. The constant strong binding of EBNA1 together with Sp1 may cause the constitutive hypomethylation of Qp (7, 32, 49, 53, 80). In summary, Qp activity is likely to be regulated in a comparably simple way, as in bacterial promoters, by the binding or not of a few key transcription factors and a repressor.

**Cp is regulated by methylation and protein binding.** Cp was unmethylated in the activity promoter state, but methylated in the inactive state (Fig. 2). The inverse correlation between methylation status and promoter activity was best in the promoter-proximal part, where Cp was completely methylated in the cell types not using Cp (Fig. 2). Therefore, extented alterations in overall CpG methylation seem to be more important than methylation of particular CpG dinucleotides in Cp (75). In agreement with earlier observations (16, 24, 70, 74), additional protein determinants of Cp activity besides CBF1 and CBF2 may play a role (Fig. 2 and 5). In addition to a couple of footprints at Sp1-like sequences that were common to all cells, there were several prominent footprints only found at active Cp that were completely lacking from inactive Cp. Differential footprinting was found at two sites for CBF1 and two CAAT boxes and three sites preliminarily named X, Y, and Z. The identity of these presumably activating transcription factors has yet to be established. CBF1 site protection patterns of Cp were remarkable because they were not identical in all cells, but correlated with promoter activity. Even in Mutu I cells, where the CBF1 binding sites are hypo- or unmethylated, there is the protection pattern of inactive Cp. This pattern is different from the typical CBF1 binding pattern, as demonstrated by methylation interference analysis (56). The difference is not due to the lack of CBF1 binding activity in Mutu I (Fig. 6).

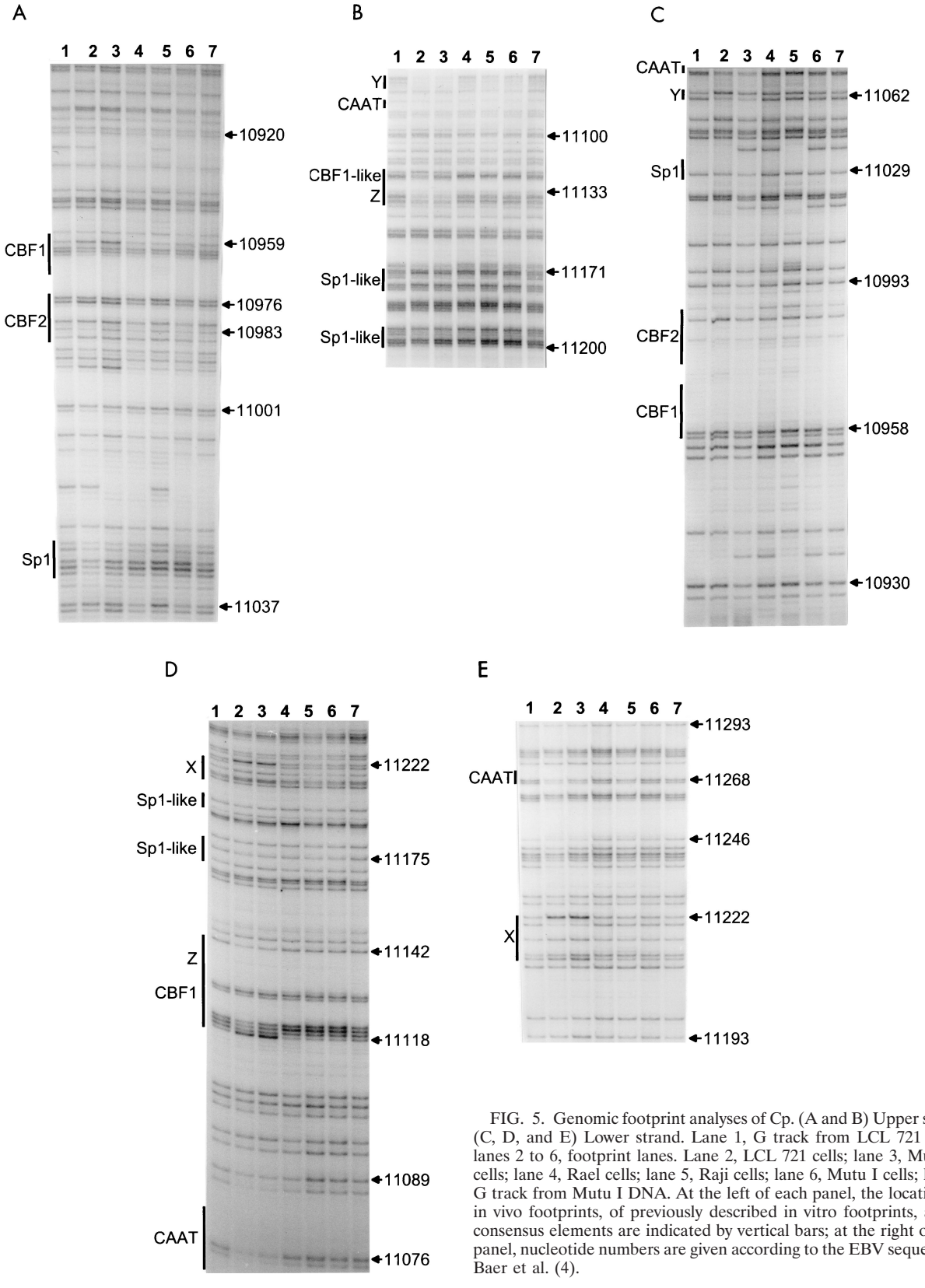



FIG. 5. Genomic footprint analyses of Cp. (A and B) Upper strand. (C, D, and E) Lower strand. Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprint lanes. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7; G track from Mutu I DNA. At the left of each panel, the locations of in vivo footprints, of previously described in vitro footprints, and of consensus elements are indicated by vertical bars; at the right of each panel, nucleotide numbers are given according to the EBV sequence of Baer et al. (4).

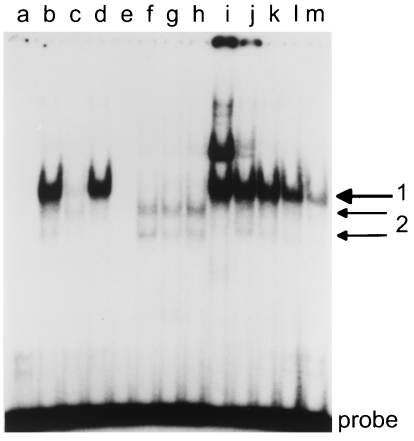


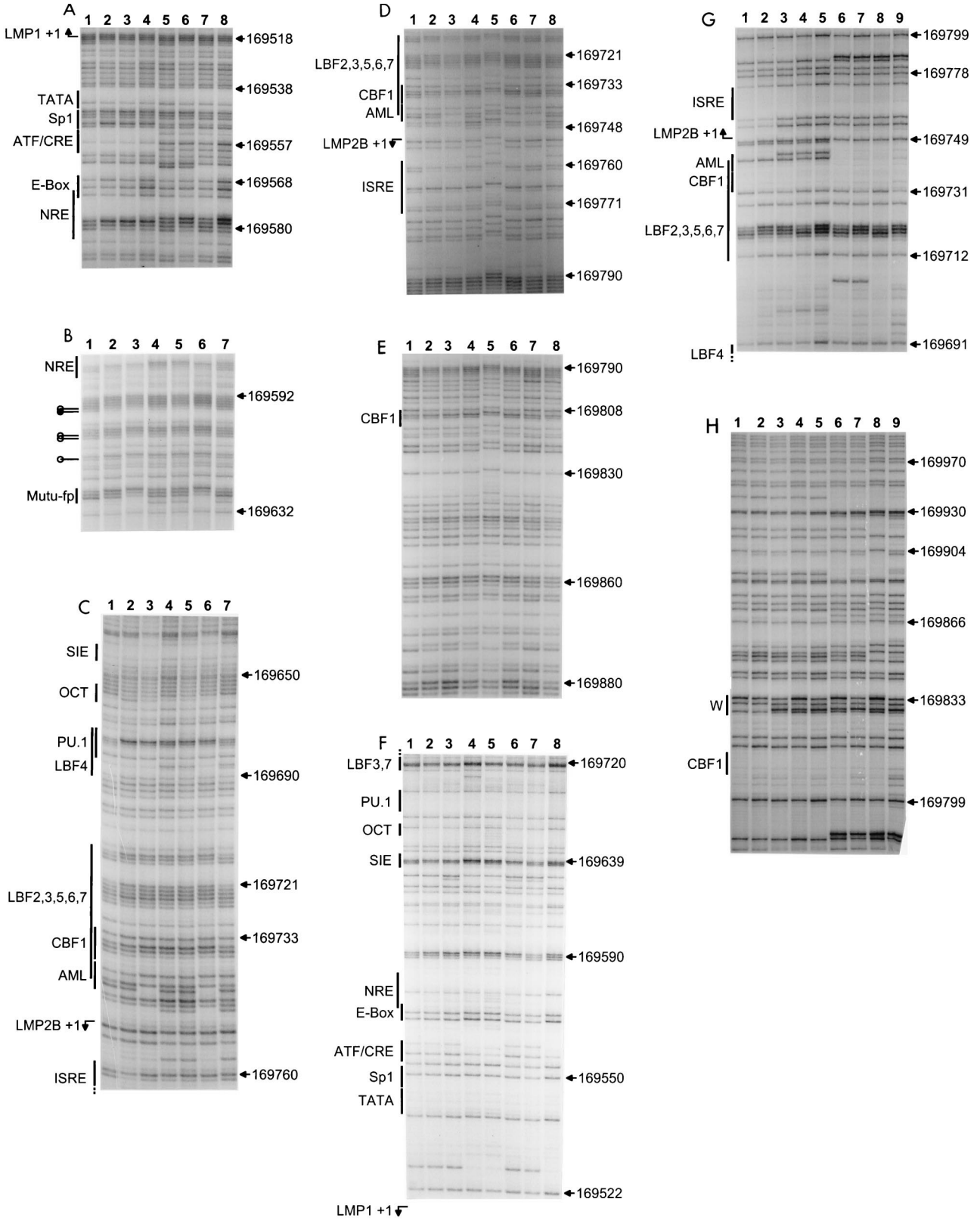

FIG. 6. Electrophoretic mobility shift assay: binding of nuclear proteins from Mutu I cells to the CBF1 site and to a mutant control oligonucleotide. Labeled double-stranded oligonucleotides containing a CBF1 consensus site and a mutant site disabled for CBF1 binding were incubated with 1 μg of crude nuclear extract from Mutu I cells with the oligonucleotides, amounts of poly(dI-dC), and unlabeled competitor oligonucleotides as indicated. The resulting protein-DNA complexes were separated in a 4% polyacrylamide gel. Lanes a to h and k, 1 μg of poly(dI-dC) added; lanes a to d and i to m, labeled CBF1 oligonucleotide as a probe; lanes e to h mutant oligonucleotide labeled as a probe. Lane 2, no protein added; lane b, nuclear extract; lane c, shift competed with a 50-fold excess of unlabeled CBF1 oligonucleotide; lane d, competition with a 50-fold excess of unlabeled mutant oligonucleotide; lane e, no protein added; lane f, nuclear extract; lane g, competition with a 50-fold excess of unlabeled CBF1 oligonucleotide; lane h, competition with a 50-fold excess of unlabeled mutant oligonucleotide; lane i, 0.1 μg of poly(dI-dC) added; lane j, 0.5 μg of poly(dI-dC); lane k, 2 μg of poly(dI-dC); lane m, 5 μg of poly(dI-dC) added.

Previously published models (73, 109) supposed that the repressive factor CBF1 is constitutively bound to its binding sites independent of the CpG methylation status of the binding site. In that model, promoter activation occurs when activating transcription factor EBNA2 binds to already promoter-bound repressor CBF1, thereby covering the transcriptionally repressive domain of CBF1 (34). Therefore, the nontypical CBF1 site pattern of inactive Cp may be due to the activity of an additional negative regulator, like KyoT2 (92), or the typical pattern (56) was caused by CBF1 and additional protein. Alternatively, we might assume that CBF1 is not at all bound at the inactive Cp. The major differences in protein binding and methylation may be a hint for a restructuring between the active and inactive Cp (17).

**LMP1p is regulated by methylation.** LMP1p has recently been examined in the five cell lines by Takacs et al. (submitted). LMP1p was hypo- or unmethylated in the active promoter state, but methylated in the inactive state. The detailed methylation maps (Fig. 3) were adapted from Takacs et al. (91a). Falk et al. (21) described comparable levels of methylation for both Mutu I and Mutu III cells. Our results for Mutu, however, were quite different. We found zero methylation in Mutu III and a high overall methylation in Mutu I cells. However, methylation in Mutu I was medium or less from nucleotides 169425 to 169465, at 169567 and 169569, and from 169691 to 169742. The discrepancy between the data of Falk et al. (21) and our data may be due to the use of a different clone of Mutu I and a different passage of clone BLIII-C199 for Mutu III. However, our methylation data on LMP1p fit to the promoter activities in the subclones of Mutu. In the previous literature on LMP1p, many binding sites have been characterized (19, 20, 38, 47, 48, 51, 56, 85, 87, 88, 97). Almost all previously characterized in vitro binding sites also carry signs of protein binding in vivo. However, this in vivo protection is visible regardless of LMP1p activity (Fig. 3 and 7). Therefore, since promoter protection patterns are identical in all cell types, the CpG methylation status seems to be the major determinant of promoter activity for LMP1p. Promoter activation is likely to be regulated by CpG demethylation and by alterations at the protein level that are transparent to genomic footprinting. Alternatively, there might be differences in promoter binding at other relevant promoter areas that we did not locate. Another possibility is that binding differences, especially at the ATF/CRE-Sp1 locus, are invisible to in vivo footprinting by DMS alone. These differences were very weak at best and were difficult to evaluate because of several sequence polymorphisms in this part of LMP1p. In vivo differences might be seen with the use of additional reagents for footprinting. We conclude that binding of all the factors involved in promoter activation at their respective binding sites is not sufficient to activate or repress LMP1p in vivo.

In summary, the contributions of CpG methylation and protein binding to promoter activity are in each case different for the three EBV latency-associated promoters Qp, Cp, and LMP1p. It would come as no surprise if the promoters for TP1, W, and the EBER RNAs also presented different pictures.

FIG. 7. Genomic footprint analyses of LMP1p. (A, B, C, D, and E) Upper strand. (F, G, and H) Lower strand. (A) Lane 1, G track from LCL 721 DNA; lane 2, footprint from LCL 721 cells; lane 3, footprint from Mutu III cells; lane 4, footprint from Mutu I cells; lane 5, G track from Rael DNA; lane 6, footprint from Rael cells; lane 7, G track from Raji DNA; lane 8, footprint from Raji cells. (B and C) Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Raji cells. (B) Protection of guanines against methylation by DMS indicated by lollipops. (D and E) Lane 1, G track from LCL 721 DNA, lanes 2 to 6, footprints; lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I cells; lane 8, G track from Rael cells. (F) Lane 1, G track from LCL 721 DNA; lanes 2 to 6, footprints. Lane 2, LCL 721 cells; lane 3, Mutu III cells; lane 4, Rael cells; lane 5, Raji cells; lane 6, Mutu I cells; lane 7, G track from Mutu I DNA; lane 8, G track from Raji DNA. (G and H) Lane 1, G track from LCL 721 DNA; lane 2, footprint from LCL 721 cells; lane 3, footprint from Mutu III cells; lane 4, G track from Mutu I DNA; lane 5, footprint from Mutu I cells; lane 6, G track from Rael DNA; lane 7, footprint from Rael cells; lane 8, G track from Raji DNA; lane 9, footprint from Raji cells. At the left of each panel, the locations of in vivo footprints, of previously described in vitro footprints, and of consensus elements are indicated by vertical bars, and at the right of each panel, nucleotide numbers are given according to the EBV sequence of Baer et al. (4).


A
1 2 3 4 5 6 7 8
LMP1 +1
TATA
Sp1
ATF/CRE
E-Box
NRE
169518
169538
169557
169568
169580
B
1 2 3 4 5 6 7
NRE
Mutu-fp
169592
169632
C
1 2 3 4 5 6 7
SIE
OCT
PU.1
LBF4
LBF2,3,5,6,7
CBF1
AML
LMP2B +1
ISRE
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169721
169733
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Short Communication

# Epigenetics of Latent Epstein-Barr Virus Genomes: High Resolution Methylation Analysis of the Bidirectional Promoter Region of Latent Membrane Protein 1 and 2B Genes

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**We analysed the methylation patterns of CpG dinucleotides in a bidirectional promoter region (LRS, LMP 1 regulatory sequences) of latent Epstein-Barr virus (EBV) genomes using automated fluorescent genomic sequencing after bisulfite-induced modification of DNA. Transcripts for two latent membrane proteins, LMP 1 (a transforming protein) and LMP 2B, are initiated in this region in opposite directions. We found that B cell lines and a clone expressing LMP 1 carried EBV genomes with unmethylated or hypomethylated LRS, while highly methylated CpG dinucleotides were present at each position or at discrete sites and within hypermethylated regions in LMP 1 negative cells. Comparison of high resolution methylation maps suggests that CpG methylation-mediated direct interference with binding of nuclear factors LBF 2, 3, 7, AML1/LBF1, LBF5 and LBF6 or methylation of CpGs within an E-box sequence (where activators as well as repressors can bind) is not the major mechanism in silencing of the LMP 1 promoter. Although a role for CpG methylation within binding sites of Sp1 and 3, ATF/CRE and a sis-inducible factor (SIF) cannot be excluded, hypermethylation of LRS or regions within LRS in LMP 1 negative cells suggests a role for an indirect mechanism, *via* methylcytosine binding proteins, in silencing of the LMP 1 promoter.**

*Key words*: Bisulfite modification / Burkitt's lymphoma / Lymphoblastoid cell line / Promoter methylation / Viral latency.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is associated with a number of malignant diseases and transforms primary B cells very efficiently *in vitro* (reviewed by Klein, 1996; Longenecker, 1998; Liebowitz, 1998). EBNA 2, one of the EBV-encoded nuclear proteins, is indispensable for the latter process, which leads to outgrowth of continually proliferating lymphoblastoid cell lines (LCLs; Hammerschmidt and Sugden, 1989). EBNA 2 transactivates promoters of latent, growth-transformation-associated EBV genes coding for three membrane and six nuclear proteins (LMP 2A, LMP 1, LMP 2B and EBNA 1 – 6) and upregulates the expression of the cellular CD23 gene (encoding an autocrine growth factor for B cells). EBNA 2 does not bind DNA directly, but interacts with a cellular DNA-binding protein, CBF1 (C promoter binding factor 1), which has recognition sequences in EBNA 2 responsive enhancers localised upstream of the LMP 2A promoter, the bidirectional LMP 1/LMP 2B promoter region (Figure 1), the BCR2 promoter (also called Cp or C promoter, where transcripts for EBNA 1 – 6 are initiated) and the cellular CD23 promoter (Henkel *et al*., 1994; Ling *et al*., 1994; Laux *et al*., 1994a, b; Meitinger *et al*., 1994). We described earlier that in clones of the Burkitt's lymphoma (BL) cell line Mutu, which differ in phenotype and BCR2 promoter usage, silencing of the BCR2 promoter correlates with the presence of a hypermethylated region localised -639 to -440 bases upstream from the beginning of the TATA box, but does not correlate with methylation patterns of CpG dinucleotides adjacent to attachment sites of cellular proteins CBF1 and CBF2 (Takacs *et al*., 1998).

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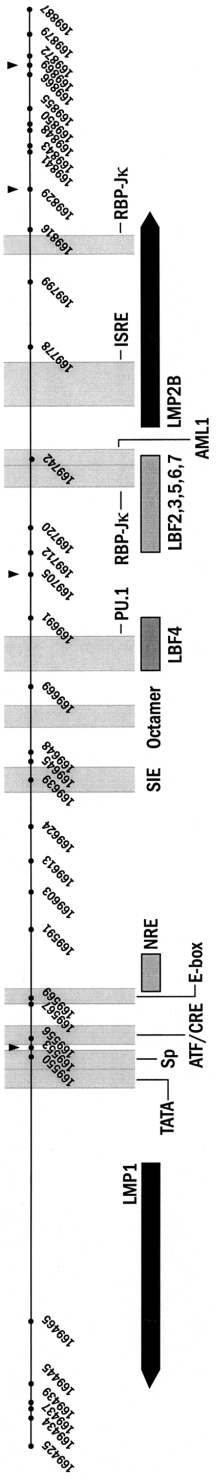


**Fig. 1** Positions of Cytosines within CpG Dinucleotides and Important *cis* Regulatory Elements in the the Bidirectional LMP 1/LMP 2B Promoter Region (LRS) of EBV.
Numbers and dots indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence (Baer *et al*., 1984). Positions of CpG dinucleotides which were absent from B95-8 are indicated by triangles above the dots. The initiation sites and direction of transcription of the LMP1 and LMP2B genes are indicated by black arrows. The positions of important *cis* regulatory elements are shown by shaded boxes based on the following references: Johannsen *et al*. (1995), Sjöblom *et al*. (1995a, b, 1998), and Sjöblom-Hallén *et al*. (1999).

In the present study we wished to establish high resolution methylation maps of the bidirectional promoter region of LMP 1 and LMP 2B genes (also called LRS, LMP 1 Regulatory Sequences, see Figure 1). Sharing of the same transcription control region results in coupled transcription of LMP 1 and LMP 2B genes in nasopharyngeal carcinomas (Chen *et al*., 1995). The LMP 1 protein affects the transmission of signals through TNF family receptors (which may result in malignant transformation), while the functional activity of the LMP 2B protein remains to be elucidated (Longenecker, 1998).

Since lytic EBV replication may alter methylation patterns of the viral genomes (Minarovits *et al*., 1991), as a first step we analysed the fused terminal fragments (Raab-Traub and Flynn, 1986) of the EBV genome in the cell lines and clones included in the present study (except Raji, a strictly latent BL line which has been analysed earlier; Hatfull *et al*., 1988; Minarovits *et al*., 1994b). This assay permits the detection of latent genomes as well as ongoing productive EBV replication. A single terminal fragment was detected in each case (Figure 2), indicating that these cell lines and clones carry predominantly latent episomal EBV genomes and can be regarded as clonal proliferations of cells infected with EBV on a single occasion.

As a next step we determined the nucleotide sequence of the bidirectional promoter region of LMP 1 and LMP 2B genes included in our study. The sequence of LCL

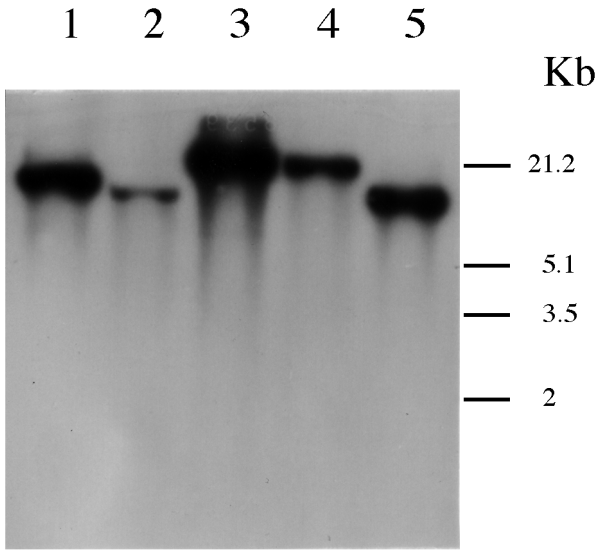


**Fig. 2** Clonotypic Analysis of DNAs from B Cell Lines and Clones.
Cells were maintained in suspension cultures and genomic DNA was isolated as described in an earlier work (Minarovits *et al*., 1991). DNA was digested with *Bam*HI and the resulting fragments were separated on a 0.8% agarose gel, blotted to a Hybond N membrane and hybridised with a [$^{32}$P]dCTP-labelled 0.8 kb *Bgl*I subfragment of the *Bam*HI N fragment of the B95-8 prototype EBV genome. The following DNAs were analysed: Rael, lane 1; CB-M1-Ral-STO, lane 2; Mutu BLI Cl 216, lane 3; MutuBLIII Cl 99, lane 4; LCL 721, lane 5. Positions of size markers are indicated in kb.

721, which has been transformed by the prototype EBV strain B95-8 (Baer *et al*., 1984), was identical to the prototype sequence in this region, while the other sequences differed from that of the B95-8 strain (see Table 1 for EMBL/GenBank accession numbers). CpG dinucleotides [potential targets for mammalian DNA (cytosine-5)-methyltransferases] present in the variant LRS sequences but absent from the B95-8 genome or *vice versa* are shown in Table 1. The majority of potential regulatory elements shown in Figure 1 were unaffected by mutations. These include the TATA box, binding sites for Sp1 and 3, an E box, a binding site for the sis-inducible factor (SIF) designated as sis-inducible element or SIE, an octamer motif, a PU.1 binding site, overlapping binding sites for LBF2, LBF3 and LBF7, two binding sites for CBF1/RBPJκ and an interferon-stimulated response element (ISRE). The ATF/CRE recognition site was found to be different, however, from the B95-8 sequence in two positions in Rael, CBM1-Ral-STO and Raji, while a negative regulatory element (NRE) and a binding site for LBF4 differed from the prototype sequence at a single position in the same cell lines. An AML1/LBF1 binding site was altered in a single (but variable) position in all cell lines and clones studied except for LCL 721. In Mutu BLI Cl 216 and Mutu BLIII Cl 99 the AML1/LBF1 mutation also affected the overlapping binding sites for LBF 5 and LBF 6.

The results of our methylation analysis are shown in Figure 3. In bisulfite-treated DNAs all cytosines outside CpG dinucleotides were converted to uracils and were sequenced as T instead of C after PCR amplification. Cytosines within CpG dinucleotides were also recovered as T if they were originally unmethylated but appeared as C in the final genomic sequence if they were methylated.

In the BL line Rael all of the cytosines located in CpG dinucleotides of the sequenced region were highly methylated. The same region was completely unmethylated in CBM1-Ral-STO, a lymphoblastoid cell line which carries the same EBV strain as Rael (Table 1). The methylation pattern of LRS was more complex in Mutu BLI Cl 216 (a clone of the BL Mutu), where cytosines showing moderate or low degree of methylation or no methylation at all (sites 169425 – 169465, 169567 and 169569, 169691 – 169742) were found adjacent to or between highly methylated sites (169550 and 169556) or regions (169591 – 169669, 169778 – 169887) (see Table 1 and Figure 3). On the contrary, another Mutu clone (Mutu BLIII Cl 99) was completely unmethylated in the sequenced region (Table 1, Figure 3). Raji, an LMP 1 expressing BL cell line, also carried predominantly hypomethylated sequences within LRS (Table 1). LRS was completely unmethylated in LCL 721, a lymphoblastoid cell line transformed by the prototype B95-8 EBV strain (Table 1), while there was a CpG dinucleotide with low degree of methylation within the LMP 1 coding sequence (position 169445) (Table 1).

LRS, the bidirectional promoter region of LMP 1 and LMP 2B genes is a mosaic of positive and negative transcriptional *cis* elements, and binding of a variety of transcription factors has been demonstrated to subfragments of LRS by electrophoretic mobility shift assay (EMSA) *in vitro* (see Figure 1; Fahraeus *et al*., 1990, 1993; Laux *et al*., 1994a, b; Johannsen *et al*., 1995; Sjöblom *et al*., 1995, 1998; Sjöblom-Hallén *et al*., 1999). Although in B cells the major transactivator of LRS is EBNA 2 (Wang *et al*., 1990; Fahraeus *et al*., 1993), the viral oncogene LMP 1 can be expressed in neoplasms of epithelial and T cell origin even in the absence of EBNA 2 (Fahraeus *et al*., 1988; Minarovits *et al*., 1994b). On the contrary, BCR2 (Cp), another EBNA 2-regulated latent EBV promoter, is practically inactive in non-B cells and EBNA 2 is unable to transactivate it in non-B cells or in a B cell/non-B cell hybrid (Contreras-Brodin *et al*., 1996).

Treatment with 5-azacytidine, a drug inhibiting cytosine methylation, can induce EBNA 2 to 6 and LMP 1 in Rael (Masucci *et al*., 1989), a finding confirmed as to LMP 1 induction by Sjöblom-Hallén *et al*. (1999). Our data, demonstrating hypermethylation of LRS in Rael and the absence of methylation in LMP 1 expressing CBM1-Ral-STO cells (which carry the same EBV strain as Rael), also suggest a role for DNA methylation in silencing of the LMP 1 promoter. The LRS in Rael was found to be highly methylated by Falk *et al*. (1998) as well, based on sequencing of individual clones of bisulfite-modified, PCR amplified DNA samples.

CpG methylation within the recognition sequence of a transcription factor may block transcription by interfering with factor binding. The presence of highly methylated CpG dinucleotides within binding sites for Sp1 and 3, ATF/CRE and SIE both in Rael and Mutu BLI Cl 216 cells which do not express LMP 1 is consistent with the idea that CpG methylation may directly inhibit binding of these proteins to their recognition sequences (Table 1, Figure 1). In contrast, the absence of highly methylated cytosines within the E-box of the LMP 1 negative Mutu BLI Cl 216 suggests that methylation of these sites in the vicinity of the negative regulatory element is not necessary for silencing of the LMP 1 promoter. Similarly, low level methylation of cytosines within binding sites of LBF2 (a factor expressed in epithelial cells but not in B cells) and the ubiquitous factor LBF3 (position 169712 and 169720; cytosine 169720 is located within the recognition site of LBF7 as well) suggests that strong methylation at these positions is not necessary for silencing. We described earlier that *in vitro* methylation of a reporter plasmid carrying LMP 1 enhancer-promoter sequences in three positions (corresponding to cytosines 169591, 169613 and 169720) significantly reduces the promoter activity after transfection into Raji cells (Minarovits *et al*., 1994a). Cytosines 169591 and 169613 are located close to the negative regulatory element (NRE, as defined by Sjöblom-Hallén *et al.*, 1999), but outside of the known nuclear factor binding sequences of LRS, while cytosine 169720 lies within the LBF2, 3, and 7 binding site. This implies that methylation within the LRS can silence the LMP 1 promoter even if CpG dinucleotides within certain factor binding sites (Sp1 and 3, ATF/CRE, E-box, SIE, AML1/LBF1) remain unmethylated.

**Table 1** Methylation Patterns of the Bidirectional Promoter Region (LRS) of EBV in Lymphoid Cell Lines and Clones.

| | 169425 | 169434 | 169437 | 169439 | 169445 | 169465 | 169550 | *169553* | 169556 | 169567 | 169569 | 169591 | 169603 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RAEL | ++++ | ++++ | ++++ | ++++ | ++++ | | +++ | +++ | ++++ | ++++ | +++ | ++++ | ++++ |
| MUTU BLI | + | + | + | + | ++ | – | ++++ | | ++++ | ++ | + | +++ | ++++ |
| RAJI | – | – | – | – | – | | + | + | + | + | – | – | – |
| MUTU BLIII | – | – | – | – | – | – | – | | – | – | – | – | – |
| CB-M1 | – | – | – | – | – | | – | – | – | – | – | – | – |
| LCL 721 | – | – | – | – | + | – | – | | – | – | – | – | – |

| | 169613 | 169624 | 169639 | 169645 | 169648 | 169669 | 169691 | *169705* | 169712 | 169720 | 169742 | 169778 | 169799 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RAEL | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++++ | ++++ | ++++ | | +++ | ++++ |
| MUTU BLI | +++ | ++++ | +++ | ++++ | +++ | +++ | ++ | | + | + | ++ | ++++ | ++++ |
| RAJI | – | + | ++ | ++ | + | – | – | | – | – | | – | – |
| MUTU BLIII | – | – | – | – | – | – | – | | – | – | – | – | – |
| CB-M1 | – | – | – | – | – | – | – | – | – | – | | – | – |
| LCL 721 | – | – | – | – | – | – | – | | – | – | – | – | – |

| | 169816 | *169829* | 169841 | 169843 | 169848 | 169850 | 169855 | 169866 | *169869* | 169872 | 169879 | 169887 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RAEL | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| MUTU BLI | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | | ++++ | ++++ | ++++ |
| RAJI | – | – | – | – | – | | – | – | – | | – | – |
| MUTU BLIII | – | – | – | – | – | – | – | – | | – | – | – |
| CB-M1 | – | – | – | – | – | – | – | – | – | – | – | – |
| LCL 721 | – | | – | – | – | – | – | – | | – | – | – |

Numbers in the Table designate positions of cytosines within CpG dinucleotides. Numbers in italics designate positions of cytosines which were absent from or not within a CpG dinucleotide in B95-8. Empty boxes indicate the absence of CpG dinucleotides in certain positions. The degree of methylation of cytosines is indicated as follows: 0%: –; 0 – 25%: +; 25 – 50%: ++; 50 – 75%: +++; 75 – 100%: ++++. The cells studied included latently infected, well characterised group I Burkitt lymphoma (BL) cells (Rael and Mutu BLI clone 216) which maintain the phenotype of BL biopsy cells and express only a single EBV encoded protein, EBNA 1 (Gregory *et al*., 1990; Minarovits *et al*., 1991; Altiok *et al*., 1992). LMP 1 expressing cells with an immunoblastic phenotype were group III BL cells (Raji, Mutu BLIII clone 99) and lymphoblastoid cell lines (CB-M1-Ral-STO, abbreviated as CB-M1; LCL 721) established *in vitro* (Ernberg *et al*., 1989; Gregory *et al*., 1990; Metzenberg, 1990; Altiok *et al*., 1992, Minarovits *et al*., 1994a,b). DNA sequence polymorphisms were noted, compared to the prototype B95-8 EBV sequence in Rael (EMBL/GenBank accession no.: AJ278794), CB-M1-Ral-STO (EMBL/GenBank accession no.: AJ278795), Raji (EMBL/GenBank accession no.: AJ278796), Mutu BLI Cl 216 (EMBL/GenBank accession no.: AJ278797) and Mutu BLIII Cl 99 (EMBL/GenBank accession no.: AJ278798). In the region analysed in this study, the sequence of LCL 721 was identical to the prototype B95-8 sequence (Baer *et al*., 1984).

In addition to direct blocking of transcription factor binding, DNA methylation may also silence promoter activity in an indirect manner, by attracting methyl-CpG binding proteins which recruit the nucleosome-remodelling complex SIN3 or NuRD (reviewed by Ahringer, 2000). Histone deacetylase components of these complexes promote the formation of a repressive chromatin structure (Jones *et al*., 1998; reviewed by Tyler and Kadonaga, 1999). MeCP2, one of the methyl-CpG-binding proteins, can silence transcription from a promoter located as far as several hundred base pairs away (for a review see Bird and Wolffe, 1999).

We noted that there are two hypermethylated sites (position 169550 and 169556) and two hypermethylated regions (cytosines 169591 – 169669 and 169778 to 169887) within the LRS of Mutu BLI Cl 216. Such densely methylated regions might favour binding of components of the MeCP1 repressor complex (Boyes and Bird, 1992; Ng *et al*., 1999) which is also associated with histone deacetylase (Ng *et al*., 1999). A similar densely methylated region was observed 5' of the silent BCR2 promoter in the same Mutu clone (Takacs *et al*., 1998).

In the present study the LRS region was found to be completely unmethylated in Mutu BLIII Cl 99 (Figure 3). In contrast, Falk *et al*. (1998) found unmethylated CpG sites mixed with moderately or highly methylated ones in the LRS of the same clone. The reason for this discrepancy remains to be elucidated.

In conclusion, our data are consistent with the idea that direct interference with transcription factor (SP1 and 3, ATF/CRE, SIF) binding may contribute to CpG methylation-mediated silencig of the LMP 1 promoter. The presence of a series of hypermethylated cytosines in the LRS of group I BL cells suggests, however, a role for an indirect mechanism (mediated by methyl-CpG binding proteins) in repression.

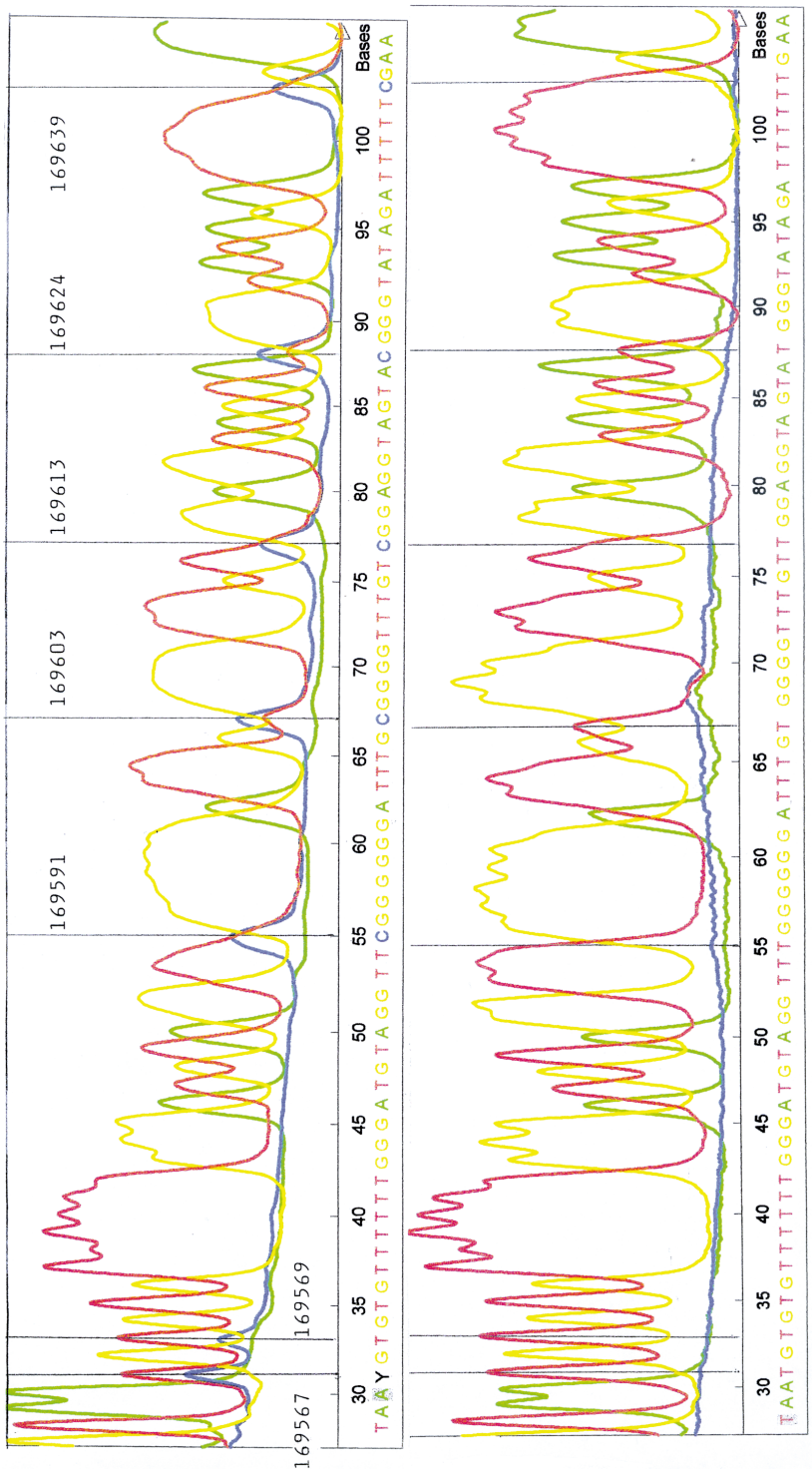


**Fig. 3** Nucleic Acid Sequence of Bisulfite-Modified DNAs in the Bidirectional Promoter Region (LRS) of Latent Epstein-Barr Virus Genomes.
Vertical lines indicate positions of cytosines within CpG dinucleotides. Differentially methylated cytosines located between nucleotides 169564 and 169642 in Mutu BL I Cl 216 (top), Mutu BL III Cl 99 (bottom) are shown.
Automated fluorescent genomic sequencing of sodium bisulfite-treated or control unmodified DNA samples was performed as described by Frommer *et al.* (1992) and Clark *et al.* (1994) adopted for an automated DNA sequencer (Myöhänen, 1994) as in our earlier studies (Takacs *et al.*, 1998; Salamon *et al.*, 2000) using the primers and PCR conditions summarised in Table 2. The degree of methylation of cytosines within CpG sequences was estimated as described earlier (Myöhänen *et al.*, 1994).

**Table 2** Primers (5'→3') (Part A) and PCR Conditions (Part B) Used for the Amplification of Bisulfite-Modified and Unmodified EBV Promoter Regions.

Part A

Outer primers for the modified LMP 1 promoter:
(1) GTT AAG AGG AGG AGA AGG AGA GTA (169371-169394)
(2) CAC CTC ATT CTA AAA TTC CCA TAT (170021-169998)

Inner primers for the modified LMP 1 promoter:
(3) Univ-TTT AGG GAA GAG GAG AGG GG (169398-169417)
(4) Univ-GAA GGG GGT AGA GTA GTG TGA GA (169514-169536)
(5) Biotin-CCC ACA ACC TTA CCT CAC CT (169924-169905)
(6) Univ-CCA CAA CCT TAC CTC ACC TAA ACC (169923-169900)
(7) Biotin-GGA GAA GGA GAG TAA GGT TTA GGG A (169381-169405)

Primers for the unmodified LMP 1 promoter:
(8) AGA GGA GGA GAA GGA GAG CAA GG (169375-169397)
(9) CTT AAC CCT CTT AAC CAC CTC ACC (169966-169943)

Primer positions in the B95-8 sequence are given in parentheses. 'Univ-' indicates the M13 universal primer sequence (GTAAAACGACGGCCA).

Part B

| Primer pairs | PCR conditions |
|---|---|
| 1 – 2 | 95 °C 40", 56 °C 40", 72 °C 1'20" |
| 3 – 5 | 95 °C 40", 56 °C 40", 72 °C 1'10" |
| 4 – 5 | 95 °C 40", 56 °C 40", 72 °C 1' |
| 6 – 7 | 95 °C 40", 61 °C 40", 72 °C 1'10" |
| 8 – 9 | 95 °C 40", 60 °C 40", 72 °C 1'10" |

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# Protein-DNA Interaction and CpG Methylation at rep*/vIL-10p of Latent Epstein-Barr Virus Genomes in Lymphoid Cell Lines

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**The viral interleukin-10 promoter (vIL-10p), overlapping the rep* element in the Epstein-Barr virus (EBV) genome, is a promoter element active mostly in the late phase of the lytic cycle and immediately upon infection of B cells. rep* was, through transfection experiments with small plasmids, characterised as a *cis* element supporting *ori*P replicative function. In this study, *in vivo* protein binding and CpG methylation at rep*/vIL-10p were analysed in five cell lines that harbour strictly latent EBV genomes. Contrary to the invariably unmethylated dyad symmetry element (DS) of *ori*P, rep*/vIL-10p was highly methylated and showed only traces of protein binding in all examined cell lines. This result is in agreement with vIL-10p being an inactive promoter of EBV genomes, and makes it less likely that rep* functions as a replicative element of latent EBV genomes.**

*Key words:* Bisulfite sequencing / Dimethyl sulfate / Genomic footprints / Latency / Replication.

## Introduction

EBV infection of human B lymphocytes *in vitro* results in B cell proliferation and transformation into continuously growing lymphoblastoid cell lines (LCL; for a review see Kieff, 1996). In latently infected cells, viral genomes are maintained as multiple circular episomal copies which are replicated once per cell cycle through *ori*P (Yates *et al*., 1984, 1985, 2000; Reisman *et al*., 1985; Adams, 1987; Yates and Guan, 1991). Several classes of EBV latency in cell culture have been described depending on the gene expression pattern (Rowe *et al*., 1986, 1992; Kerr *et al*., 1992; Kieff, 1996). In type I latency, the viral gene expression program is more restricted; among the EBNA proteins only EBNA1 is expressed from the Q promoter (Nonkwelo *et al*., 1996). In type III latency, all the EBNA proteins are expressed from the C promoter (Bodescot *et al*., 1987). In EBV latency, the immediate early, early and late genes for the lytic cycle of virus production are not expressed. EBV carries the BCRF1 reading frame, a viral gene homologous to the cellular IL-10 gene (Moore *et al*., 1990). The vIL-10 gene is expressed immediately after the infection of B cells and predominantly late after the induction of the lytic cycle from latency. As a late gene, vIL-10 is not expressed during latency (Hudson *et al*., 1985; Miyazaki *et al*., 1993; Touitou *et al*., 1996; Zeidler *et al*., 1997). The upstream promoter region for the major RNA species for vIL-10, an 0.8 kb mRNA, largely overlaps rep*, a 298 bp sequence element between EBV nucleotides 9370 and 9668 (Kirchmaier and Sugden, 1998). It is located just downstream of the minimal replicator of *ori*P, the dyad symmetry element (DS) that is approximately located between EBV nucleotides 9000 and 9150 (Reisman *et al*., 1985; Harrison *et al*., 1994; Shirakata and Hirai, 1998; Yates *et al*., 2000). rep* has been shown to support replication from *ori*P in transfection assays with small plasmids (Kirchmaier and Sugden, 1998). It has been suggested that rep* may contain binding sites for cellular proteins that are involved in the replication of chromosomal DNA (Kirchmaier and Sugden, 1998). In order to further clarify the potential functions of this element, we decided to establish a comprehensive map of *in vivo* protein-DNA-binding and CpG-methylation at rep*/vIL-10p.

## Results

### Overall High Degree of Methylation at CpG Dinucleotides of rep*/vIL-10p in All Cell Lines

For our analysis we chose a set of five cell lines representative for both latency types I and III. Rael and Mutu BLI-Cl216 cells represent type I latency (Klein *et al*., 1972; Ernberg *et al*., 1989; Gregory *et al*., 1990; Minarovits *et al*., 1991), Raji, LCL721, and Mutu BLIII-Cl99 cells represent type III latency (Kavathas *et al*., 1980; Gregory *et al*., 1990; Metzenberg, 1990; Walls and Perricaudet, 1991; Little and Schildkraut, 1995). Between the examined nucleotides 9301 – 9726 of the rep*/vIL-10p area were a total of 7 CpG dinucleotides in EBV strains Raji and LCL 721. In Rael and Mutu cells there were only 6

CpG dinucleotides, due to a C to T transition at nucleotide 9518. There was a high overall degree of methylation in all cells between nucleotides 9517 and 9580 with a few exceptions (Figure 1 for an overview, Figure 2A): CpG dinucleotide 9580 showed a medium degree of methylation in 721 and Raji cells. There was no detectable methylation at CpG dinucleotide 9446 in all cells except in Mutu III and Rael cells, where a medium degree of methylation and high methylation was found, respectively (Figure 2B). At CpG dinucleotide 9681 we found a high degree of methylation except in Mutu I, where only a low level of methylation was found (Figure 2C). All cytosines outside of CpG dinucleotides between nucleotides 9301 and 9726 were found to be unmethylated in all cell lines. All the upstream CpG dinucleotides from DS down to the CpG dinucleotide 9217 have been shown to be unmethylated in a large panel of cell lines (Salamon *et al*., 2000). Between nucleotides 9217 and 9300 there was no methylated cytosine either (data not shown). The CpG dinucleotides 9517 and 9519 of a potential E2F binding site (Black and Azizkhan-Clifford, 1999; Wingender *et al*., 2001) were highly methylated in the five cell lines of this study. Therefore, the majority of CpG dinucleotides of rep*/vIL-10p showed a high degree of methylation in five examined cell lines, whereas the upstream area towards DS of *ori*P was completely unmethylated in a large panel of cell types (Salamon *et al*., 2000; D. Salamon, unpublished data). These methylation data solely reflected the status of tightly latent EBV circular genomes, but not the presence of linear genomes from a possible low amount of lytic replication, as was tested by terminal repeat analysis by Southern blotting (Minarovits *et al*., 1994; Takacs *et al*., 2001) and for LCL 721 and Raji cells also through Gardella gels (Gardella *et al*., 1984; Little and Schildkraut, 1995). The high degree of methylation within rep*/vIL-10p is a further indication that there was no lytic viral replication in the cell lines examined. Selected early antigens or their coding mRNAs associated with productive EBV replication could not be detected in the above mentioned cell lines and clones

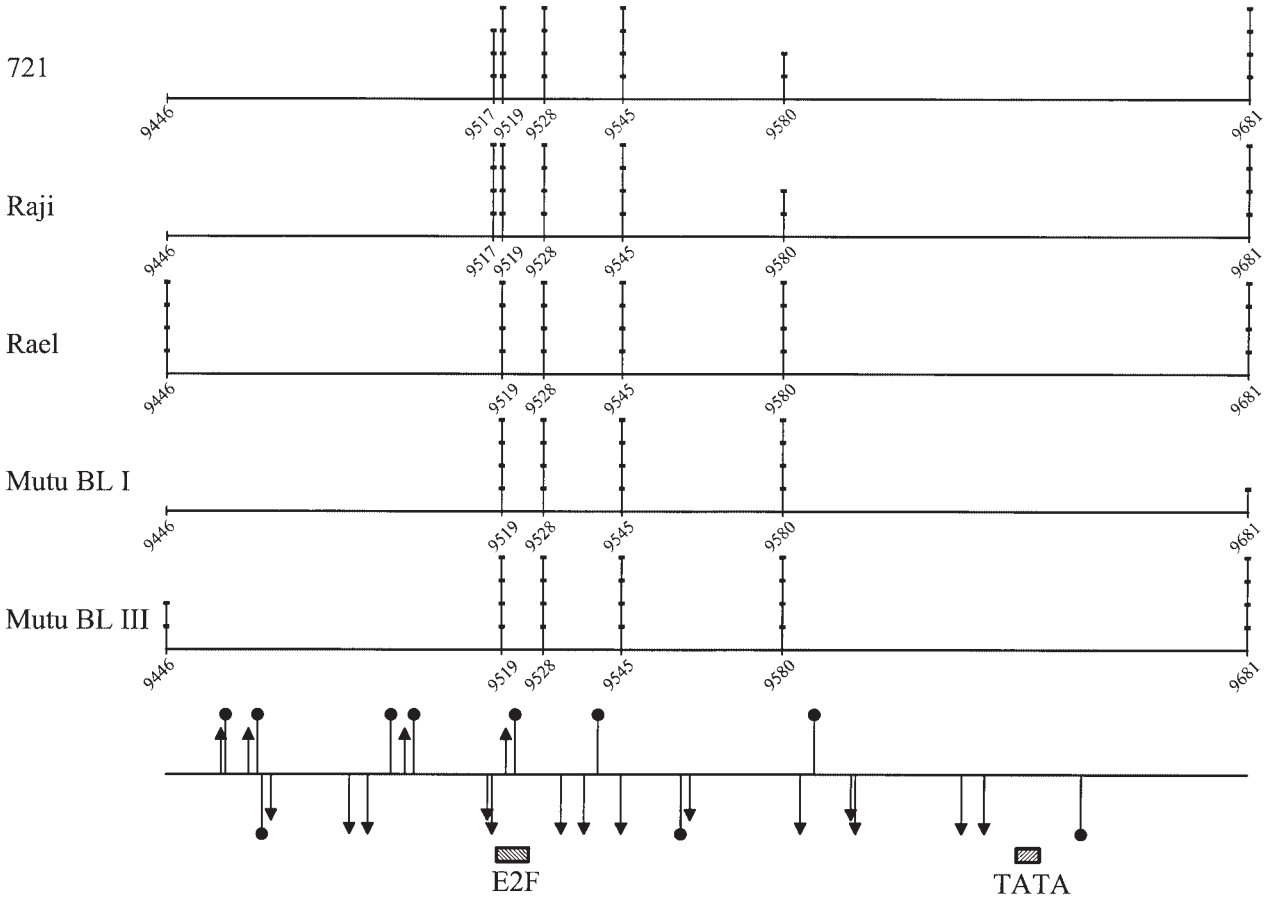


**Fig. 1** Summary of Genomic Footprinting Results and Methylation Patterns in the Sequenced Region of rep*/vIL-10p.
Numbers and columns indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence (Baer *et al*., 1984). The degree of methylation of cytosines is indicated by the height of the columns as follows: number only: 0%; one unit: 0–25%; two units: 25–50%; three units: 50–75%; four units: 75–100%. The bottom line shows the summary of genomic footprints for the upper (above the line) and lower (below the line) strand of rep*/vIL-10p. Guanines protected from methylation by DMS are indicated by lollipops, enhanced reactivity to DMS is shown by arrows. The positions of the TATA box and a potential E2F binding site are indicated by boxes. The lack of CpG dinucleotide 9517 in Rael and Mutu cells is explained by a point mutation at nucleotide position 9518 in these cell lines and subclones.

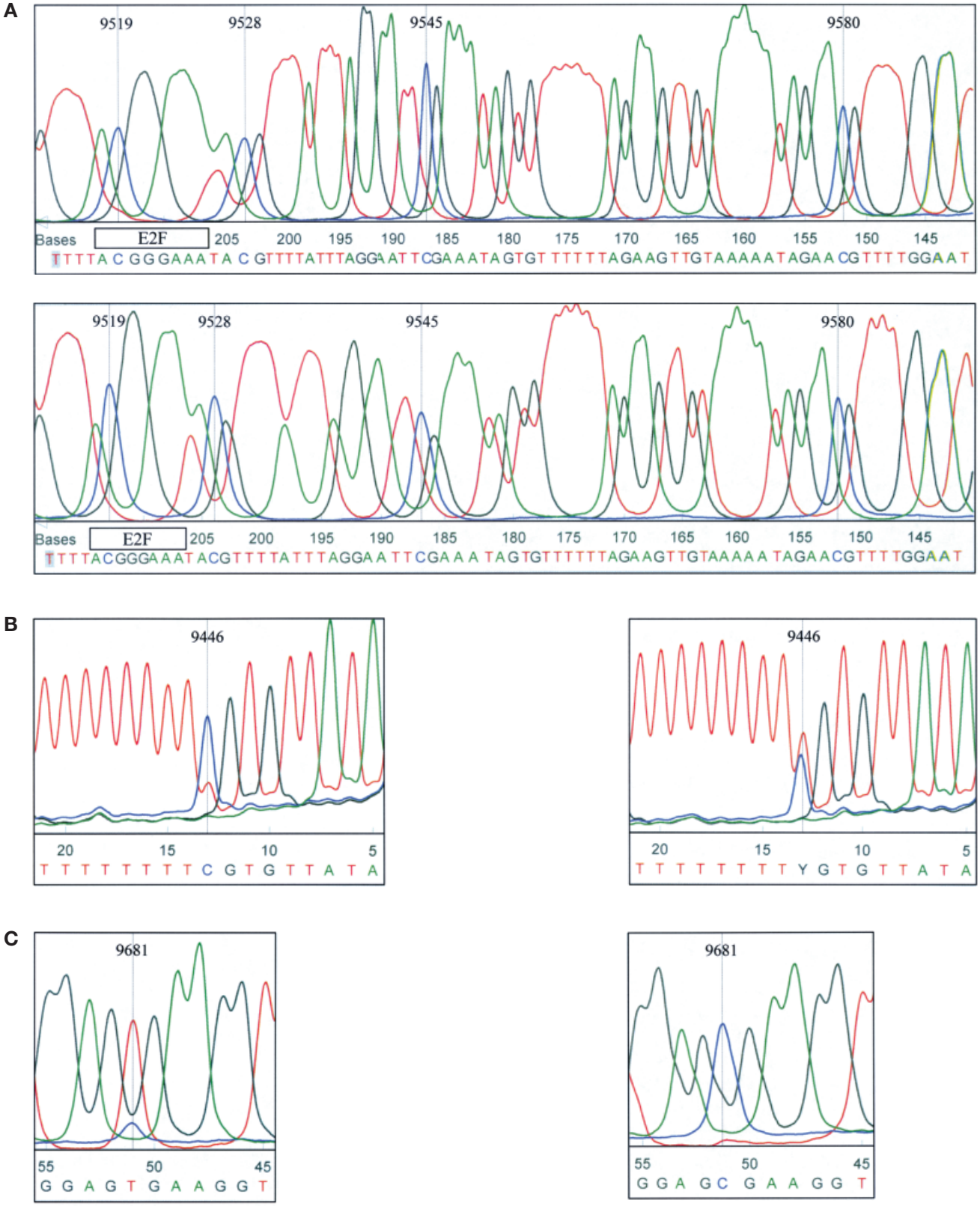


**Fig. 2** Nucleic Acid Sequence of Bisulfite-Modified DNAs in rep*/vIL-10p of Latent Epstein-Barr Virus Genomes Carried by Lymphoid Cell Lines.

Numbers adjacent to vertical lines indicate positions of cytosines within CpG dinucleotides. The numbering at the bottom of each panel indicates the distances from the sequencing primers used. In (A), a highly methylated region between nucleotides 9514 and 9590 containing a potential E2F binding site (open box) in Mutu BLI-Cl216 (top panel) and Mutu BLIII-Cl99 (bottom panel) is shown. In (B) one differentially methylated CpG between nucleotides 9438 and 9454 in Rael (left panel) and Mutu BLIII-Cl99 (right panel) is shown. In (C) one CpG dinucleotide between nucleotides 9677 and 9687 hypomethylated in Mutu BLI-Cl216 (left panel) and highly methylated in Mutu BLIII-Cl99 (right panel) is indicated.

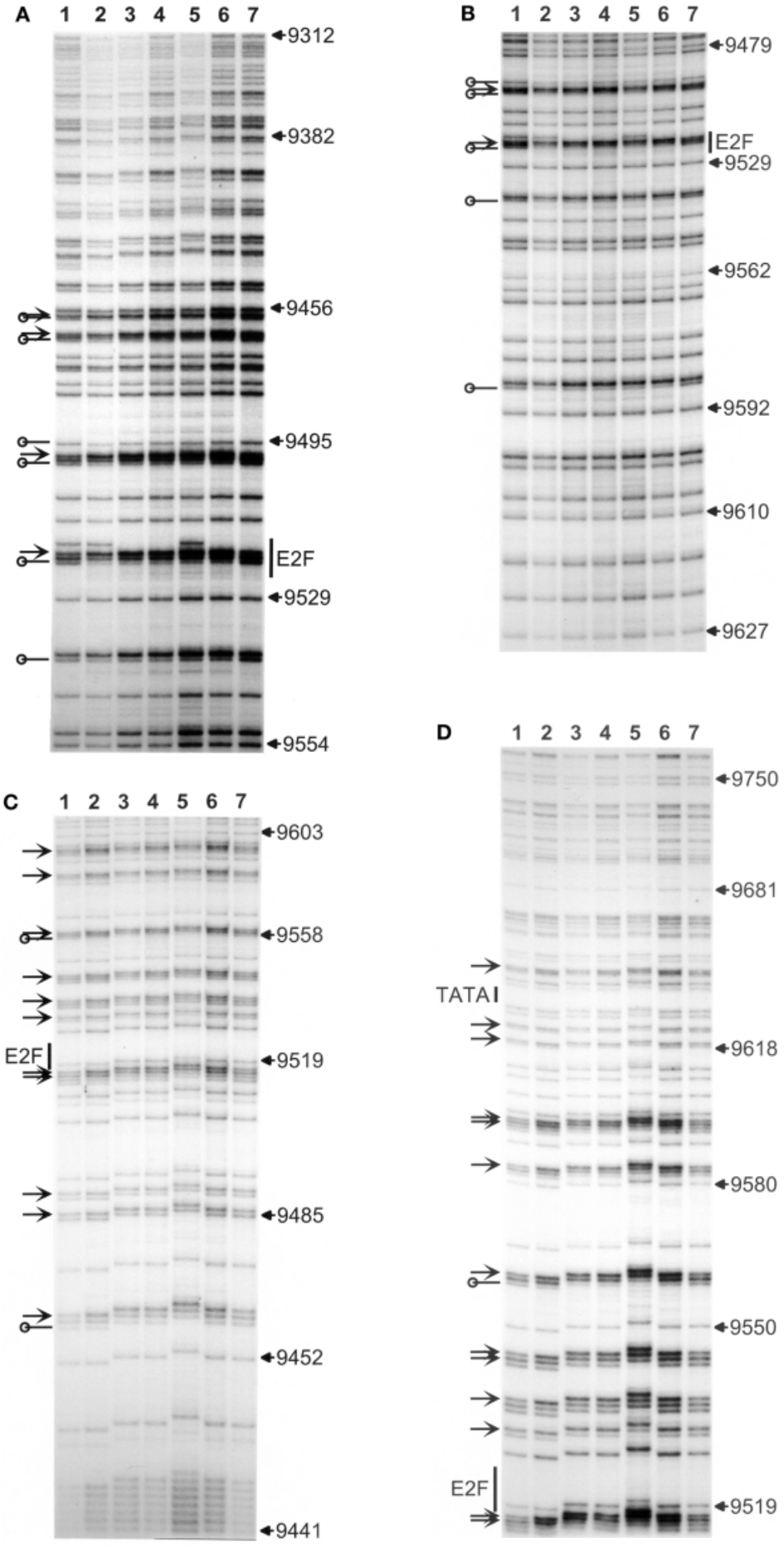
A
1 2 3 4 5 6 7
9312
9382
9456
9495
E2F
9529
9554
B
1 2 3 4 5 6 7
9479
E2F
9529
9562
9592
9610
9627
C
1 2 3 4 5 6 7
9603
9558
E2F
9519
9485
9452
9441
D
1 2 3 4 5 6 7
9750
9681
TATA
9618
9580
9550
E2F
9519

**Fig. 3** Genomic Footprinting Analyses of rep*/vIL-10p. Panels (A, B): upper strand (primer set F); panels (C, D): lower strand (primer set B). Lane 1: G-track from LCL 721 DNA; lanes 2 to 6: footprints, lane 2: LCL 721 cells; lane 3: Mutu III cells; lane 4: Rael cells; lane 5: Raji cells; lane 6: Mutu I cells; lane 7: G-track from Mutu I DNA. At the left side of each panel the location of *in vivo* footprints is indicated by arrows (hypersensitive bases) and circles (protected bases), and potential transcription elements are indicated by vertical bars; at the right side of each panel nucleotide positions for some of the G residues are given according to the EBV sequence of Baer *et al*. (1984).

(Masucci *et al*., 1989; Metzenberg, 1990; J. Minarovits, unpublished data).

### Protein Binding to rep*/vIL-10p

Protein binding at rep*/vIL-10p has previously not been examined. We analysed genomic protein-DNA interactions at rep*/vIL-10p by *in vivo* footprinting on rep*/vIL-10p. Genomic footprint patterns were at least extremely similar for all cell types. Indications for weak protein-DNA interactions were found on both strands of rep*/vIL-10p (Figure 1 for an overview, Figure 3). At one position these protections could be correlated with the location of a putative consensus binding site for the cellular transcription factor E2F (Figure 3) (Di Fiore *et al*., 1999; Wingender *et al*., 2001).

## Discussion

Episomal genomes of EBV represent a suitable viral model system for mammalian chromosomes to study replication and transcription in relation to CpG methylation and protein binding. We examined rep*/vIL-10p of EBV because of its potential dual role as replicative and transcriptional element. A role as supportive replication element has been proposed for rep* (Kirchmaier and Sugden, 1998). Incidentally, rep* overlaps the upstream region of the TATA-box at nucleotide 9631 for the major 0.8 kb mRNA for vIL-10 (Touitou *et al*., 1996). The *in vivo* protection and CpG methylation patterns found in our experiments represent data for the non-activated state of EBV latency and do not represent EBV genomes undergoing the lytic cycle of virus production. In agreement with the non-expression of the major 0.8 kb mRNA for the vIL-10 gene in latency (Hudson *et al*., 1985; Touitou *et al*., 1996), rep*/vIL-10p showed about 50 to 100% methylation in five examined cell lines. The C promoter has been shown to be unmethylated in 721 and Mutu III cells where it is active, and was found to be highly methylated in Raji, Rael and Mutu I cells where it is inactive (Takacs *et al*., 1998; Salamon *et al*., 2001). DS was shown to be unmethylated in a large panel of cell lines (Salamon *et al*., 2000). Therefore, rep*/vIL-10p may constitute an island of methylation between the unmethylated DS and C promoter sequences in 721 and Mutu III cells, or the beginning of a larger methylated stretch downstream of DS for Raji, Rael, and Mutu I cells (Takacs *et al*., 1998; Salamon *et al*., 2000, 2001). The inverse correlation between promoter activity and methylation is in agreement with previous observations on promoter activities and methylation states (Jones, 1999).

All analysed cell types presented essentially the same genomic footprint patterns at rep*/vIL-10p, regardless of type I or type III latency. There were no strong signs of genomic protein-DNA interactions. The absence of prominent footprints is not due to technical problems, since the same sets of footprinted DNAs were used for the present analyses that were used before (Salamon *et al*., 2001). In previous analyses of the major latency promoters of EBV a series of strong footprints was found (Salamon *et al*., 2001). The same was the case for DS where strong sequence-specific footprints were found in several cell lines (Niller *et al*., 1995, H. H. Niller, unpublished data). The identities of the proteins causing the slight footprints within rep*/vIL-10p are at present unknown. The footprints may be caused by general chromatin packaging factors. Therefore, we can only speculate whether E2F protein family members actually play a role in rep*/vIL-10p binding. However, the C to T transition at nucleotide 9518 makes this sequence less suitable for E2F binding in Rael and Mutu cells (Wingender *et al*., 2001), while at least one methylated CpG-motif remains at nucleotide 9519. CpG methylation of the two E2F binding sites in the retinoblastoma tumor supressor gene (Rb-1) promoter has been shown to prevent E2F binding (Di Fiore *et al*., 1999). Furthermore, this genomic footprint is not a typical E2F footprint (Zwicker *et al*., 1996). Therefore, other repressive factors, like MeCP2, are candidates for protein binding at this E2F site as well as other methylated CpGs (Antequera *et al*., 1989; Di Fiore *et al*., 1999).

CpG islands have been suggested to be genomic footprints of promoters that are associated with cellular replication origins (Delgado *et al*., 1998; Antequera and Bird, 1999). However, rep*/vIL-10p is strongly CpG depleted, a consequence of constitutive CpG methylation, and thus does not qualify as a CpG island. In summary, contrary to upstream DS and to the downstream located C promoter, rep*/vIL-10p is highly methylated and shows only weak specific protein binding in latency (Niller *et al*., 1995; Takacs *et al*., 1998; Salamon *et al*., 2000, 2001). This correlates well with an inactive promoter element. It was demonstrated that rep* functions as a supportive replicative element for *ori*P on transfected small plasmids that consist of naked and unmethylated DNA. On the other hand, the situation in strictly latent EBV genomes that are protein-packed and highly methylated at specific loci like rep*/vIL-10p may be entirely different. The rather sharp border between unmethylated DS and methylated rep*/vIL-10p might possibly play a role in facilitating the assembly of the replication initiation complex on DS.

## Materials and Methods

### Cell Lines and Tissue Culture

LCL 721 is a B95-8 transformed lymphoblastoid cell line (LCL, latency type III; Kavathas *et al*., 1980; Metzenberg, 1990; Little and Schildkraut, 1995). Rael is a group I Burkitt´s lymphoma (BL) cell line (Klein *et al*., 1972; Ernberg *et al*., 1989; Minarovits *et al*., 1991). Mutu BLI-Cl216 is a subclone of the BL line Mutu, representative for latency type I (Gregory *et al*., 1990). Mutu BLIII-Cl99 is a subclone of the BL line Mutu, representative for latency type III (Gregory *et al*., 1990). Raji cells express all the type III latency genes, but use a thus far unknown promoter, other than the C promoter, for EBNA transcripts (Walls and Perricaudet, 1991). B95-8, Mutu, and Raji EBV strains represent A-type virus that does not enter the lytic cycle as easily as B-type EBV (Buck *et al*., 1999). All cells were maintained in suspension cultures of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 units penicillin per ml and 50 µg streptomycin per ml under 5% $CO_2$ and 37 °C.

### DNA Sequences

Oligonucleotides corresponding to EBV nucleotides 9266 to 9290 and 9750 to 9727 for sequencing rep*/vIL-10p were purchased from Metabion (Martinsried, Germany). Both strands of the two promoters were sequenced from the genomic DNA of all five cell types on an ABI 377 DNA sequencing system using dye-labeled ddNTPs. All DNA sequences were identical to the B95-8 sequence (Baer *et al*., 1984) with only few exceptions. There was an A to G transition in Raji cells (GenBank accession number AJ307024) at nucleotide 9408, an addition of one cytosine in Rael cells (GenBank acc. no. AJ307025) and Mutu clones (Mutu BLI-Cl216, GenBank acc. no. AJ307026 and Mutu BLIII-Cl99, GenBank acc. no. AJ307027), of two cytosines in Raji to the cytosine run between nucleotides 9439 and 9446, and a G to A transition in Rael and Mutu cells at nucleotide 9518.

### Automated Genomic Sequencing of Sodium Bisulfite-Treated DNA

We used the method of Frommer *et al*. (1992) and Clark *et al*. (1994) adapted for an automated DNA sequencer (Myöhänen *et al*., 1994). Five µg of genomic DNA in 50 µl water was denatured by adding 5.5 µl of freshly prepared 3 M NaOH and incubating for 15 minutes at 37 °C. 30.5 µl of freshly prepared 10 mM hydroquinone (Sigma), and 530 µl of 3.6 M sodium bisulfite, pH 5 (Sigma), were added to the denatured DNA, gently mixed and divided into five 0.5 ml PCR tubes, overlaid with paraffin oil, and cycled 5 times at 95 °C for 3 minutes and 55 °C for 57 minutes. After this treatment the modified DNA was purified using a GeneClean kit (BIO 101) according to the manufacturer´s instructions. Then the DNA was desulfonated by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M and incubating the mixture for 15 minutes at 37 °C. After desulfonation the DNA was ethanol precipitated and dissolved in water. One hundred ng of freshly modified DNA were used for PCR amplification with the strand-specific outer primer pairs (Frommer *et al*., 1992) designed for the promoter regions (Table 1). The 50 µl PCR reaction contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 40 pmol each of the primers, 0.2 mM each of the four dNTPs, and 2 units of *Taq* polymerase (Promega). Three µl of a 1:100 dilution from the first PCR reaction were amplified in a second nested PCR reaction, using the primers listed in Table 1. One of the nested primers was biotin-labeled and the other carried 15 bases of the M13 universal primer at its 5´-end. The reaction mixture of the nested PCR was the same as the first one, except that the amount of the inner primers was 10 pmol each. The product of the second PCR reaction was bound to streptavidin-coated magnetic beads (Dynal) and the purified biotin-labeled strand was sequenced using the AutoRead DNA sequencing kit (Amersham Pharmacia Biotech) and a fluorescein-labeled M13 universal primer as described by Myöhänen *et al*. (1994). The reaction products were separated on acrylamide gels using an automated DNA sequencer (Amersham Pharmacia Biotech). The degree of methylation was estimated as described earlier (Myöhänen *et al*., 1994). The bisulfite conversion reaction was complete, since all cytosines outside CpG dinucleotides were converted to uracil and therefore sequenced as thymine instead of cytosine after PCR (see Takacs *et al*., 1998; Salamon *et al*., 2000, for example).

### DMS *in Vivo* Footprinting

Genomic footprining was performed as described earlier (Niller *et al*., 1995; Salamon *et al*., 2001). For each footprint reaction $10^7$ exponentially growing cells were harvested, washed with phos-

**Table 1** Primers and PCR Conditions Used for the Amplification of the Bisulfite-Modified EBV rep*/vIL-10p Region.

| |
|---|
| Outer primers for modified rep*/vIL-10p: |
| (1) TGGGTTTTTGGGAGGTATATG (9244 – 9264) |
| (2) CCTAATTTTCAACCTATAACATAACTTC (9957 – 9930) |
| Primers 1 – 2: 95 °C 40 seconds, 58 °C 40 seconds, 72 °C 90 seconds. |
| Inner primers for modified rep*/vIL-10p: |
| (3) Univ-CACATTAATCTATACCTCCACACTC (9756 – 9732) |
| (4) Univ-ACAACTTACCAACTAAACCCTATTC (9483 – 9459) |
| (5) Biotin-TGTAAAGTTTGTTTTAGGATGAAAG (9338 – 9362) |
| (6) Univ-ATTGGTGTAAGAGTTTTAGTTAAGAGT (9274 – 9300) |
| (7) Biotin-CACATTAATCTATACCTCCACACTC (9756 – 9732) |
| Primers 3 – 5 and 6 – 7: 95 °C 40 seconds, 57 °C 40 seconds, 72 °C 70 seconds. |
| Primers 4 – 5: 95 °C 40 seconds, 57 °C 40 seconds, 72 °C 50 seconds. |
| Primer positions refer to the nucleotides of the B95-8 sequence (Baer *et al*., 1984). 'Univ-' indicates the M13 universal primer sequence GTAAAACGACGGCCA). Primers were purchased from Metabion (Martinsried, Germany). Each PCR reaction was cycled 30 times at the temperatures and times indicated. |

**Table 2** Primer Sets Used for *in vivo* Footprinting of rep*/vIL-10p by LM-PCR.

| | |
|---|---|
| Primer set A: | |
| i) | CCCCCAGCATTGGTGTAAGAGCTTC (9266 – 9290) |
| ii) | GCTTCAGCCAAGAGTTACACATAAAGG (9286 – 9312) |
| iii) | GGCAATGTTGTGTTGCAGTCCACAGAC (9311 – 9337) |
| Primer set B: | |
| i) | TCAGTGTTGGCAAATGTGCACATCC (9367 – 9391) |
| ii) | GGATGTCARCTACAGTCAGAGAACC (9390 – 9424) |
| III) | CAGAGAACCCCTTTGTGTTTGGTCC (9416 – 9440) |
| Primer set C: | |
| i) | TCAGTGTTGGCAAATGTGCACATCC (9367 – 9391) |
| ii) | GGATGTCARCTACAGTCAGAGAACC (9390 – 9424) |
| iii) | GTGTCACATGTGGAACAGGGCCCAG (9447 – 9471) |
| Primer set D: | |
| i) | GTGTCACATGTGGAACAGGGCCCAG (9447 – 9471) |
| ii) | CAGGGCCCAGTTGGCAAGTTGTACC (9462 – 9486) |
| iii) | CCAACTGAAGGGATTACATGCACTGCC (9489 – 9515) |
| Primer set E: | |
| i) | GGTCTGTACCTCCACACTCAGGTG (9750 – 9727) |
| ii) | CAGGTGCCAGGTAAAGCAGCACCAG (9732 – 9708) |
| iii) | GTGACCACTAACCTTCGCTCCATACC (9697 – 9672) |
| Primer set F: | |
| i) | GACCACTAACCTTCGCTCCATACC (9695 – 9672) |
| ii) | CTAACCTTCGCTCCATACCTAAGG (9690 – 9667) |
| iii) | GTGCAGGCCTACCTGAGATAGGGAAG (9665 – 9640) |
| Primer set G: | |
| i) | CTGCATTGCACAGTGGGGCAGTTCC (9610 – 9586) |
| ii) | GGCGTTCTATTTTTACAGCTTCTGGG (9583 – 9558) |
| iii) | GGGAAACACTGTTTCGGGTTCCTGGG (9560 – 9535) |
| Primer set H: | |
| i) | GTGCACATTTGCCAACACTGAGTGG (9387 – 9363) |
| ii) | CAACACTGAGTGGCTTTCATCCTGG (9375 – 9351) |
| iii) | CCTGGAGCAGACTTTGCAGTCTGTGG (9355 – 9330) |

Primer positions refer to nucleotide numbers of the B95-8 sequence (Baer *et al*., 1984). Primers were purchased from Metabion (Martinsried, Germany). Because of single nucleotide mismatches between EBV strains, primer ii) of primer sets B and C contained a wobble base. With these 8 primer sets, both strands of rep*/vIL-10p were visualised in their entire length, respectively. The results for primer sets B and F only are shown in Figure 3. Due to the closely interspersed location of the primer annealing sites, some of the views on rep*/vIL-10p were redundant with identical results.

phate buffered saline (PBS), resuspended in 1 ml of PBS, and incubated at room temperature for 1 minute with 5 µl of dimethyl sulfate. The reaction was stopped by the addition of 5 ml DMS stop solution containing 1% bovine serum albumin and 100 µM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in 1 ml of PBS and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment (Maxam and Gilbert, 1980). For visualisation of footprints by ligation-mediated PCR (LM-PCR), 2 µg of sequenced or footprinted DNA were analysed as described (Mueller and Wold, 1989; Garrity and Wold, 1992) with modifications (Niller *et al*., 1995). The primers for LM-PCR are listed in Table 1. The first strand primer extension reaction was done in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 [25 °C] (Vent buffer, New England Biolabs), containing 0.3 pmol of primer i) of each set, 240 µM of each dNTP, and 1 unit of Vent (exo-) DNA polymerase (New England Biolabs) for 5 minutes at 94 °C, 30 minutes at 60 °C, and 10 minutes at 72 °C. For ligation of the common linker, the sample was transferred to ice and 5 µl of PCR linker mix as described by Mueller and Wold (1989), 2 µl of ligation buffer [660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5 (20 °C), Boehringer Mannheim], 1 µl of T4-DNA-ligase (5 U/µl, Boehringer Mannheim), and 12 µl water were added. After an overnight incubation at 4 °C the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. The PCR amplification was performed in 100 µl Vent buffer containing 10 pmol each primer ii) of each primer set and the longer linker primer, 240 µM each dNTP, and 1 unit of Vent (exo-) DNA polymerase for 20 cycles using 1 minute at 94 °C, 1.5 minutes at 60 °C, and 3 minutes at 72 °C. For labeling, the sample was transferred to ice, 5 pmol of T4-kinase [$\gamma$-$^{32}$P]ATP-labelled primer iii) of each set, 2.5 nmol each dNTP, 0.5 units Vent ($exo^-$) DNA polymerase in a volume of 10 µl Vent buffer were added. Then the sample was heated to 94 °C for 1.5 minutes, subjected to 8 cycles of 2 minutes at 94 °C, 2 minutes at 62 °C, and 5 minutes at 72 °C, and then kept at 72 °C for 5 more minutes. Samples were phenol/chloroform extracted, ethanol precipitated, ethanol washed, and resuspended in loading dye. One tenth of each sample was separated on a standard 5% sequencing gel, and the gels were dried and autoradiographed with Kodak BioMax MR film.

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# Nucleoprotein Structure of Immediate-Early Promoters Zp and Rp and of oriLyt of Latent Epstein-Barr Virus Genomes

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**Genomic footprints across Rp, Zp, and oriLyt of Epstein-Barr virus (EBV) have been conducted in a panel of latently infected B-cell lines. Close protein-base contacts were found about 360 nucleotides upstream of the Zp initiation site. Gel shifts and transient transfection assays indicated that an Sp1-NF1 locus may serve as a repressive transcriptional element against Zp induction from latent EBV genomes.**

Although the lytic cycle of latent Epstein-Barr virus (EBV) is strongly repressed in B cells, activation can be achieved through overexpression of the viral immediate-early proteins Rta (also called BRLF1 or R) (34, 49) and Zta (also called BZLF1, Z, EB1, or ZEBRA) (35, 43, 45), which is also the viral origin binding protein for oriLyt (12, 38). Currently, a cooperative model of mutual activation between Rta and Zta is favored (1, 5, 32). The molecular architecture of the immediate-early locus of EBV resembles that of the major immediate-early locus of human cytomegalovirus, with the exception that there is no strong enhancer in EBV. Therefore, Zta can be expressed from both the BRLF1 and BZLF1 promoters (Rp and Zp) (24). Zta and Rta coactivate oriLyt, a key *cis* regulatory element for the progress of the lytic cycle that is essential for virus production (7, 9, 12, 15, 16, 38, 39). Many transcriptional elements important for the induction of the three EBV regulators (3, 8, 13, 14, 21–23, 33, 39, 41, 42, 47, 48, 50) and also some elements important for the repression of Zp in latency (19, 22, 25, 26, 40) have been characterized so far by in vitro binding studies and reporter gene experiments (for review, see reference 43). However, there is a fundamental difference between naked DNA as used for in vitro analyses and chromatin as it occurs in the living cell. Therefore, understanding in vivo protein-DNA interactions at Zta, Rta, and oriLyt on latent genomes may shed some light on the mechanisms through which EBV is kept latent in B cells. Since they have not been examined on the chromatin of latent EBV genomes at nucleotide resolution, we undertook a survey of in vivo protein binding at these regulatory elements in latent EBV genomes in a panel of B-cell lines.

**Little overall in vivo protein binding to lytic cycle regulatory elements.** The cell lines Raji, LCL 721, Mutu BLI-Cl216, Mutu BLIII-Cl99, and Rael are in a strictly latent state, as documented by terminal repeat analysis (44) and by the absence of early antigens or their coding mRNAs (J. Minarovits, unpublished data). All cell lines belong to latency type I (Rael and Mutu I) or III (LCL721, Mutu III, and Raji), as described before (37). Ligation-mediated PCR (LM-PCR) (27) was applied under the same conditions as described earlier (29, 30, 37) in order to obtain a comprehensive set of genomic footprints of those regulatory elements from the five cell lines. For each footprint reaction, $10^7$ exponentially growing cells were treated with 5 μl of dimethyl sulfate for 1 min at room temperature. Reactions were stopped, footprinted DNAs were isolated and subjected to piperidine treatment, and footprints were visualized from 2 μg of sequenced or footprinted DNA by LM-PCR, under the same reaction conditions as described earlier (29, 30, 37). LM-PCR primers are listed in Fig. 1. One-fifth of each sample was separated on a standard 5% sequencing gel and autoradiographed. Footprints were mostly identical in all cell types. Subtle signals for protein-DNA interactions were found at many locations on both strands of Zp and Rp, and for oriLyt, footprint lanes were mostly similar to the G-tracks (data not shown). These subtle protections and hypersensitivities were generally not correlated with previously described in vitro binding sites at the promoters. The nature of the proteins causing faint signs of in vivo protein-DNA interactions at immediate-early promoters Zp and Rp and at oriLyt is unclear; they await further identification. Possibly, they might be caused by general chromatin packaging factors that do not contact the nucleotide bases, but the phosphate backbone. The absence of strong genomic footprints was not due to methodical problems, since the same sets of footprinted DNAs were used for the present analyses that had been used previously (37), and all experiments were performed several times with independent preparations of genomic DNAs with identical outcomes. Furthermore, strongly positive control footprints on the DS element of oriP comparable to the ones in Raji cells (30) have been obtained from the same set of genomic DNAs (H. H. Niller, unpublished data). These data most likely document the absence of activating transcription factors from lytic regulators in EBV latency. Activating factors for Zp, Rp, and oriLyt characterized through previous in vitro binding and reporter gene assays may play a crucial role at the second phase of the lytic cycle, after latency is interrupted, and viral genomes have been moved to the ND10 domains for lytic replication (4).

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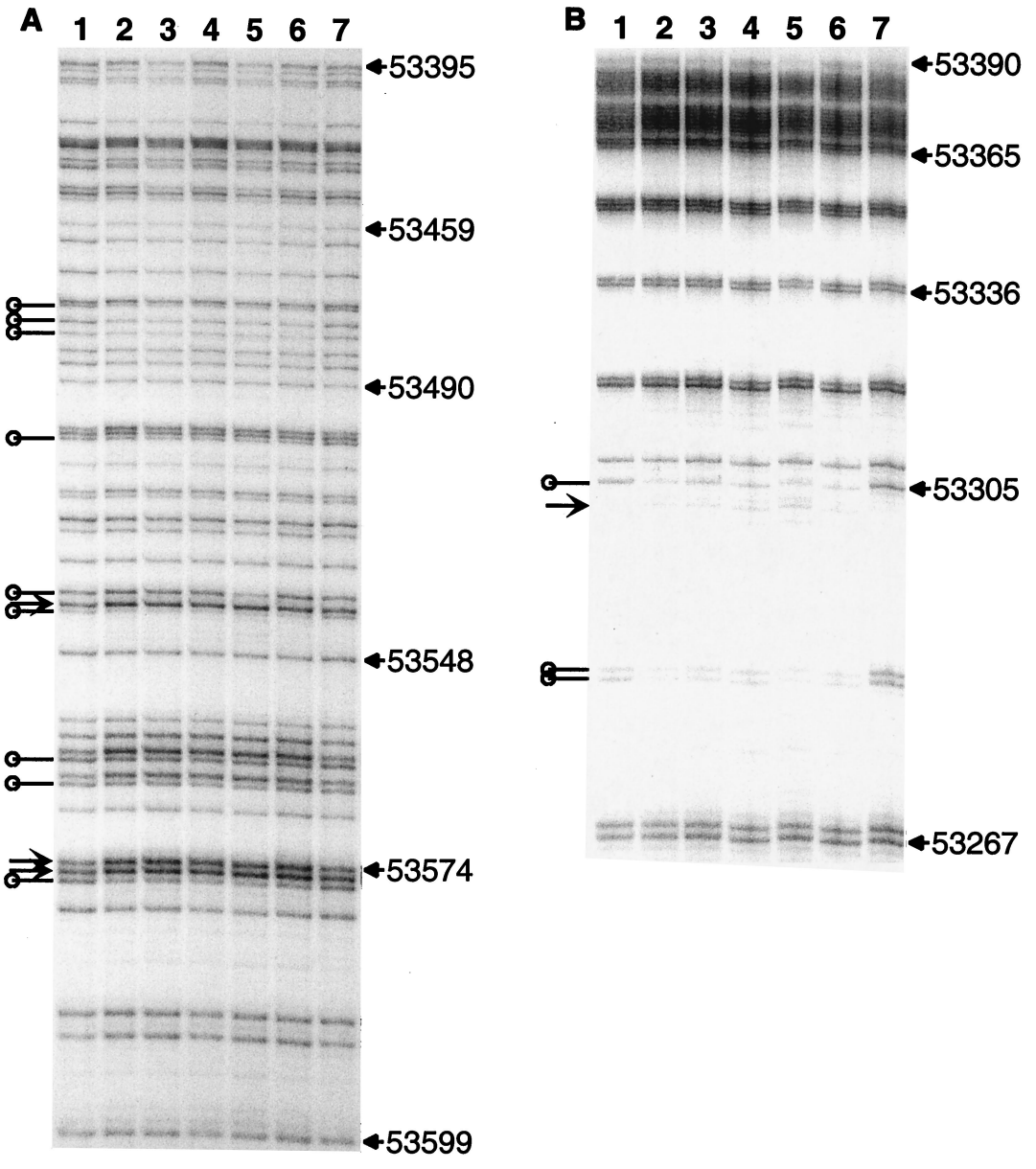


FIG. 1. Genomic footprint analyses of oriLyt (A and B) and Zp (C and D). Panels A and C show the upper strands, and panels B and D show the lower strands. Lanes: 1, G-track from LCL 721 DNA; 2 to 6, footprint lanes; 2, LCL 721 cells; 3, Mutu III cells; 4, Rael cells; 5, Raji cells; 6, Mutu I cells; 7, G-track from Mutu I DNA. To the left of each panel are indicated the locations of protected (lollipops) and of hypersensitive (arrows) guanines. To the right of each panel are given nucleotide numbers according to the EBV sequence of Baer et al. (2). Solid symbols indicate strong footprints, and open symbols indicate subtle signals. The LM-PCR primer coordinates are given in reference to the nucleotide numbers of the viral sequence (2) as follows: primer set A, primer 1, 53176 to 53200; primer 2, 53202 to 53226; and primer 3, 53224 to 53248; primer set B, primer 1, 53680 to 53657; primer 2, 53668 to 53644; and primer 3, 53629 to 53605; primer set C, primer 1, 103698 to 103674; primer 2, 103690 to 103666; and primer 3, 103666 to 103641; and primer set D, primer 1, 103403 to 103427; primer 2, 103453 to 103478; and primer 3, 103468 to 103492. In the case of a single nucleotide polymorphism at nucleotide 103654, a wobble base was included in primer 3 of primer set C.

**Protein binding at oriLyt.** In contrast to the overall absence of close protein-base contacts, there were some closer contacts at the upper strand of the downstream element of oriLyt (Fig. 1A); however, again more subtle footprints were found on the lower strand (Fig. 1B). Like in Zp and Rp, footprints on oriLyt were mostly identical for all cell types. Footprints generally did not correlate with previously characterized elements. There were no signs of protein binding visible at the Sp1 boxes (3, 14) on the parts of oriLyt examined. However, the protein contact around nucleotide 53539 was located at RRE1 (36), a previously characterized transcriptional and replicative element of oriLyt. Since oriLyt and both the BHLF1 and BHRF1 genes are silent in EBV latency, protein binding in the absence of Zta hints at cellular repressors being bound to oriLyt in latency.

**Specific protein binding at Zp.** A strong genomic footprint was found at a GC box, 360 nucleotides upstream of Zp tran-

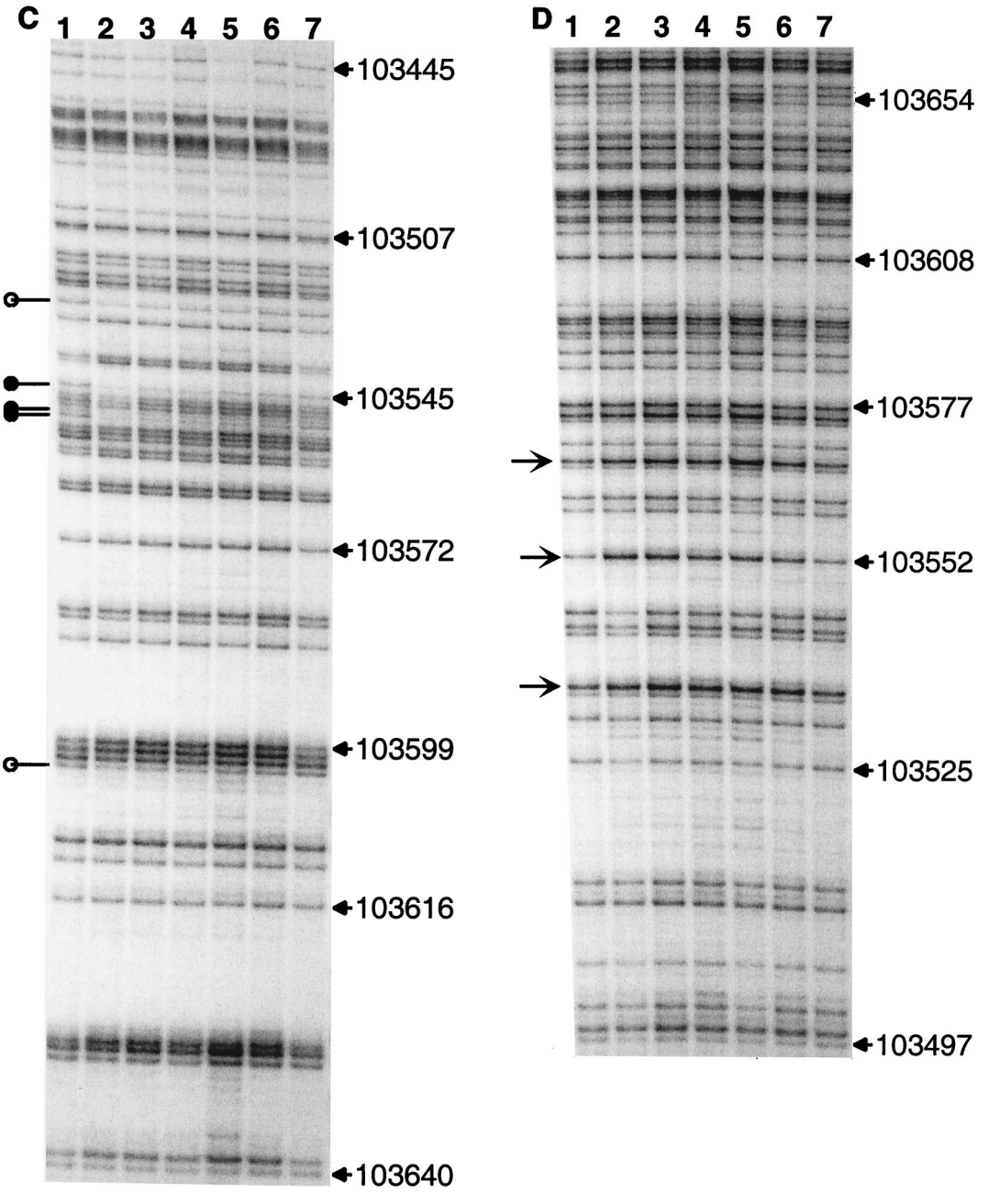


FIG. 1—*Continued.*

scription initiation (Fig. 1C and D). The GC box was located in the vicinity of YY1 site D (25, 26), HI elements γ and β (40), and an NF1-like site (13) and has not been described previously (see Fig. 3). However, it might correspond to a footprint previously seen by in vitro DNase I analyses on Zp with HeLa cell nuclear protein (26). For the GC box locus of Zp, we characterized candidate binding proteins through electrophoretic mobility shift experiments. Mutu I nuclear extracts were made according to the standard of Dignam et al. (10), with modifications as described previously (37). For gel shifts, 1 ng of T4 kinase-radiolabeled double-stranded oligonucleotides was incubated with 0.5 μg of poly(dI-dC)-poly(dI-dC), 5 μg of nuclear protein, and a 50-fold excess of unlabeled double-stranded competitor oligonucleotide, as indicated, in 25 μl of bandshift buffer (10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 80 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 12.5% glycerol) for 20 min. Protein complexes were resolved by electrophoresis on native 5% polyacrylamide gels (29 + 1) in 0.25× Tris-borate-EDTA (TBE) buffer at 20 mA for several hours. The shift experiments demonstrated that the four binding sites, YY1, Sp1, NF1, and HI, contributed to specific in vitro protein binding at this locus (data not shown). Sequence-specific binding of NF1 and Sp1 proteins from Mutu I cells to their respective binding sites is shown in Fig. 2. Specific binding was found

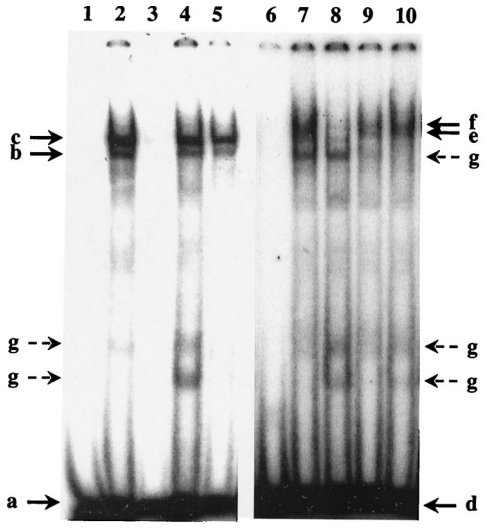


FIG. 2. Electrophoretic mobility shift assay demonstrating specific protein binding activities in Mutu I cell nuclear protein to the Sp1 (lanes 1 to 5) and NF1 (lanes 6 to 10) sites from Zp. Double-stranded (only one strand given) labeled oligonucleotides for shifts and unlabeled oligonucleotides for competition of complexes were either taken from Zp sequences or contained consensus binding sites for the respective transcription factors (46). Lanes: 1, ZpSp1 (GGATCCACG AGGGGGCGGGTGCCATG; Zp nucleotides 103542 to 103557) probe only, no protein added; 2 to 5, nuclear extract added; 3, competition with unlabeled Sp1 consensus oligonucleotide (GGATCCAC GAGGGGCGGGGTG CCATG); 4, competition with NF1 consensus oligonucleotide (GATCGGGTGGCACTGTGCCAAGGATC); 5, competition with ZpHI oligonucleotide (CATGGCGGTCCATCTGT CCACGATCC; Zp nucleotides 103563 to 103577); 6, ZpNF1 (GATCGGGTGGCTCAGGTCCATCCATG; Zp nucleotides 103553 to 103570) probe only, no protein added; lanes 7 to 10, nuclear extract added; lane 8, competition with unlabeled NF1 consensus oligonucleotide; 9, competition with Sp1 consensus oligonucleotide; 10, competition with ZpHI oligonucleotide. Lowercase letters with arrows represent unshifted probes (a and d), specific Sp1-related complexes (b and c), specific NF1-related complexes (e and f), and nonspecific complexes (g).

not only with nuclear protein from Mutu I cells, but also with protein from the other cell lines used in this study (data not shown). The in vivo protection together with the in vitro binding of this locus are a hint that NF1 and Sp1 proteins really play a role in the nucleoprotein structure of silent Zp. To elucidate further a possible functional role of the major footprinted area of Zp, transient transfection assays were done in DG75 cells, an EBV-negative Burkitt's lymphoma cell line, with luciferase reporter constructs. ZpLuc contained promoter sequences between EBV coordinates 103194 and 103730 in front of the luciferase gene of plasmid pGL2 (Promega). This corresponds to Zp sequences from nucleotide positions −536 to +1 relative to the transcriptional start site. Construct ZpLucΔ1, which contains a deletion of both the Sp1 and NF1 sites of Zp from nucleotides 103542 to 103570, was made with the QuikChange mutagenesis kit (Stratagene) from ZpLuc by using the mutant oligonucleotide GGTCAGTTCGTCCAAA TGGCTGTCCACATATGGCTGCTTC and the corresponding opposite-strand oligonucleotide. The mutant construct (Fig. 3) was confirmed by sequencing both strands of the deleted promoter area. Twenty micrograms of each double-cesium chloride-purified plasmid was transfected into DG75 cells by the DEAE-dextran method as described previously (28). Four hours after transfection, cells were induced with tetradecanoyl phorbol acetate (TPA) at 40 ng/ml for 20 h, and relative light units from cellular extracts were measured with the Promega dual luciferase reporter assay system on a Berthold luminometer (Lumat LB 9501). Since Renilla luciferase control constructs were themselves strongly induced by TPA, standardization was not done with Renilla luciferase, but by using equal amounts of protein extract for luciferase assays and repeating the experiment several times. Wild-type reporter construct ZpLuc and deletion construct ZpLucΔ1 yielded the same low baseline activities. Upon induction through TPA, luciferase

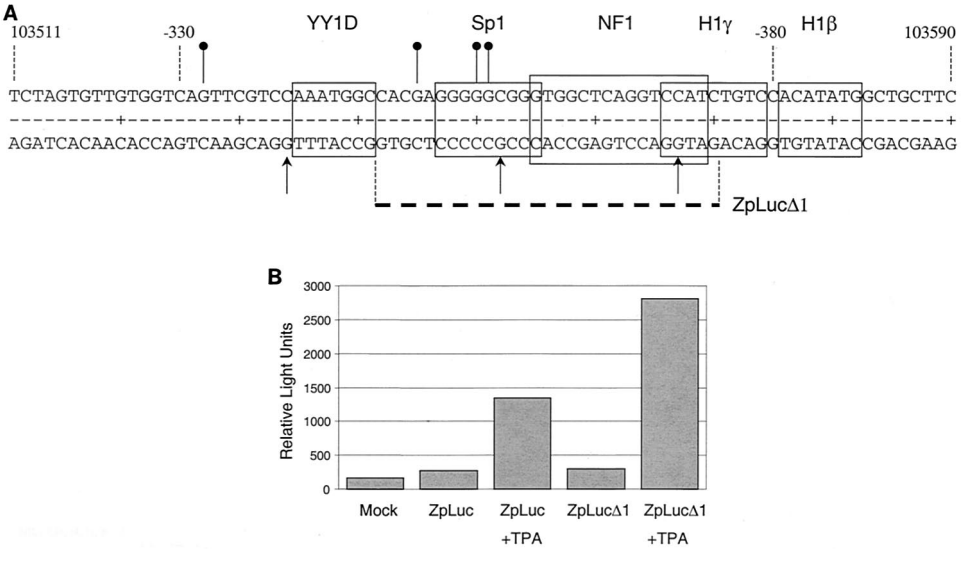


FIG. 3. (A) Sequence around the −360 upstream area of Zp. Previously described promoter elements YY1D (25, 26), HIγ and HIβ (40), and the newly found Sp1 and NF1 sites are indicated by boxes. The deleted sequence of mutant ZpLucΔ1 is indicated by a dashed line. The location of protected (lollipops) and hypersensitive (arrows) guanines from Fig. 1C and D is indicated. (B) Activities of ZpLuc and ZpLucΔ1 transcriptional elements in DG75 cells treated with TPA. The data from a representative transfection experiment are given in relative light units on the ordinate, and the plasmids and treatment of cells are indicated on the abscissa.

activity increased strongly for both constructs. However, inducibility of the deletion construct through TPA was on average 1.6-fold higher than that of the wild-type construct (Fig. 3B). The GC locus protection in the promoter-distal part of Zp together with the absence of activator binding in lytic regulators might contribute to keeping EBV tightly latent. NF1 and Sp1 may, together with YY1 and the HI binding factor, form an inhibitory protein complex on Zp. Previously, endogenous Zp was found to be so efficiently silenced that it is not active even under conditions that activate exogenously transfected Zta under the control of Zp (18, 20). Therefore, the Sp1-NF1 locus might contribute to a stronger inhibition of Zp on endogenous genomes compared to the transient effect from transfection experiments in DG75 cells. Rp, Zp, and oriLyt of latent EBV genomes are efficiently silenced in latency through the absence of activator binding, through the presence of repressive factors, and through a chromatin structure that favors inactivity. The latent state is stabilized through DNA methylation and histone deacetylation (6, 11, 17, 31). It will be interesting to examine the function of the newly found protein-DNA interactions on the Sp1-NF1 locus of Zp in an experimental system closer to latent EBV genomes, possibly through the construction of a mutant virus.

**Nucleotide sequence accession numbers.** Rp promoter sequences may be found under GenBank accession no. AJ422215, AJ422216, AJ422217, and AJ422218, and Zp promoter sequences may be found under GenBank accession no. AJ422219, AJ422220, AJ422221, and AJ422222 for the cell lines Rael, Raji, Mutu BLI clone 216, and Mutu BLIII clone 99, respectively.

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# The in vivo binding site for oncoprotein c-Myc in the promoter for Epstein-Barr virus (EBV) encoding RNA (EBER) 1 suggests a specific role for EBV in lymphomagenesis

**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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## Summary

**Background:** Epstein-Barr virus (EBV) was isolated in the 1960s from the African childhood tumor, Burkitt's Lymphoma (BL), characterized by the translocation of the c-myc gene into one of the immunoglobulin loci. Due to the extreme discrepancy between the widespread dissemination of EBV infection and the overall rarity of EBV-related tumors, it remains an open question whether EBV is really a human tumor virus, and if so, what specific contribution EBV may have to tumorigenesis.

**Material/Methods:** Protein binding at the EBER locus of EBV was analyzed by genomic footprinting electrophoretic mobility shift, reporter gene assay, and chromatin immunoprecipitation in a panel of six B-cell lines.

**Results:** Several novel in vivo protein binding sites were found in the EBER locus. Among those, a prominent binding site, 130 base pairs upstream of the EBER1 gene, contains two E-boxes providing a consensus sequence for binding of the transcription factor and oncoprotein c-Myc to the EBV genome.

**Conclusion:** Based on the discovery of a binding site for c-Myc in the EBV genome, a new molecular model for the specific role of EBV as a causal factor in the origin of endemic Burkitt's Lymphoma is proposed. Translocated and deregulated c-myc directly activates and maintains the antiapoptotic functions of the EBER locus in a single EBV-infected B cell undergoing the germinal center (GC) reaction. With the balance shifted towards cell survival, the oncogenic potential of the pro-apoptotic c-Myc protein is unmasked in the translocated GC cell. This single translocated and surviving cell is the founder cell of an endemic BL. The new model reinstitutes EBV as a real human tumor virus.

**key words:** **Burkitt's lymphoma • genomic footprints • germinal center • Hodgkin's Disease • Non-Hodgkin lymphoma**

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## BACKGROUND

EBV infection is the viral cause of infectious mononucleosis. The viral DNA can also be detected in a variety of malignant neoplasms, including endemic Burkitt's Lymphoma (BL), Hodgkin's Disease (HD), and Nasopharyngeal Carcinoma (NPC). In endemic BLs, both the cellular and latent episomal EBV genomes are monoclonal, as indicated by analysis of Ig/myc translocation and viral terminal repeats, respectively [1–3]. This suggests a role for EBV as a cofactor in lymphomagenesis. However, it has proven difficult to specify the role of EBV in lymphomagenesis, because more than 90% of the adult population worldwide carries the virus, but manifest EBV-positive BL is rare. Several classes of EBV latency have been described in cell culture and tumor materials, depending on the gene expression pattern [4–6]. In strict type I latency, represented by BL cells, viral gene expression is restricted to the two EBER genes, the BART gene, and the EBNA1 gene [2,5]. The EBERs are expressed also in type II latency, represented by EBV-positive HD and NPC, and in type III latency, represented by lymphoblastoid cell lines (LCL) and Post-Transplant Lymphoproliferative Disease in the severely immunocompromised patient. The EBERs have been reported to be transcribed by RNA polymerase III in vitro and in Raji cell nuclei [7–11]. With only a few exceptions they are mostly expressed abundantly in cell lines and in EBV-positive clinical materials [12–17]. Specific regulation of EBER1 and EBER2 transcription has been observed in different settings and cell types [7, 18–20]. The EBERs have been shown to upregulate bcl-2, to inhibit apoptosis by binding protein kinase DAI [21], blocking interferon-α signaling, and to induce colony growth of cells in agar. Therefore, the EBER transcription units have been implicated in EBV-related tumorigenesis [22–26]. Besides increasing resistance to apoptosis, additional mechanisms for increasing the tumorigenicity of BL cells have been attributed to the EBERs [27]. Another important function ascribed to the EBER locus is the nuclear matrix attachment of the viral episome [28,29]. The EBER locus is not packaged by histones, but shows a variant chromatin structure, most likely involved in EBER regulation and matrix attachment [30]. Because of the potentially essential function of the EBER locus in BL-pathogenesis, we established a comprehensive map of in vivo protein-DNA interactions at the EBER locus.

## MATERIAL AND METHODS

### Cell lines and tissue culture

LCL 721 is a B95-8 transformed LCL with type III phenotype [31,32]. Rael [33,34] is a group I BL cell line. CBM1-Ral-STO is a class III LCL transformed by the Rael EBV strain [33,34]. Mutu BLI-Cl216 and Mutu BLIII-Cl99 are subclones of the BL line Mutu, representative for latency types I and III, respectively [34]. Raji cells express all the type III latency genes, but use a thus far unknown promoter, other than Cp, for the EBNA transcripts [36]. DG75 is an EBV-negative BL cell line [37]. All cells were maintained in suspension cultures of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 units penicillin per ml and 50 µg streptomycin per ml at 5% $CO_2$ and 37°C.

### DNA sequences

Oligonucleotides corresponding to EBV nucleotides [38] 6236 to 6256 and 7213 to 7190, respectively, were used for sequencing of the EBER locus. Both strands were sequenced from the genomic DNA of all cell types on an ABI 377 DNA sequencing system using dye-labeled ddNTPs. Sequences of the EBER locus are at GenBank entries AJ315772 for Raji, AJ315775 for Mutu I clone 216, AJ315776 for Mutu III clone 99, AJ315773 for Rael, and at AJ315774 for CBM1-Ral-STO.

### Dimethyl sulfate in vivo footprinting

Genomic footprinting was performed essentially as described [39]. For each footprint reaction $10^7$ exponentially growing cells were harvested, washed with phosphate buffered saline (PBS), resuspended in 1 ml of PBS and incubated at room temperature for 1 minute with 5 µl of dimethyl sulfate (DMS). The reaction was stopped by the addition of 5 ml DMS stop solution containing 1% bovine serum albumin and 100 µM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in 1 ml of PBS and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment [40]. For visualization of footprints by LMPCR, 2 µg of sequenced or footprinted DNA were analyzed as described [41] with modifications [39]. The coordinates of the LM-PCR primers are listed in Figure 1. The first strand primer extension reaction was done in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 [25°C] (Vent buffer, New England Biolabs), containing 0.3 pmol of primer 1) of each set, 240 µM each dNTP, and 1 unit of Vent (exo-) DNA polymerase (New England Biolabs) for 5 minutes at 94°C, 30 minutes at 60°C, and 10 minutes at 72°C. For ligation of the common linker, the sample was transferred to ice and 5 µl of PCR linker mix, 2 µl of ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5 [20°C], Boehringer Mannheim), 1 µl of T4-DNA-ligase (5 U/µl, Boehringer Mannheim), and 12 µl of water were added [41]. After overnight incubation at 4°C the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. PCR amplification was done in 100 µl Vent buffer containing 10 pmol of each primer 2) and the longer linker primer, 240 µM of each dNTP, and 1 unit of Vent (exo-) DNA polymerase for 20 cycles for 1 minute at 94°C, 1.5 minutes at 60°C, and 3 minutes at 72°C. For labeling, the sample was transferred to ice and 5 pmol of T4-kinase [$\gamma$-$^{32}$P]ATP labeled primer 3), 2.5 nmol of each dNTP, and 0.5 units Vent (exo-) DNA polymerase were added in a volume of Vent buffer not exceeding 15 µl. Then the sample was heated to 94°C for 1.5 minutes, subjected to 8 cycles of 2 minutes at 94°C, 2 minutes at 62°C, and 5 minutes at 72°C, and kept at 72°C for 5 more minutes. Samples were

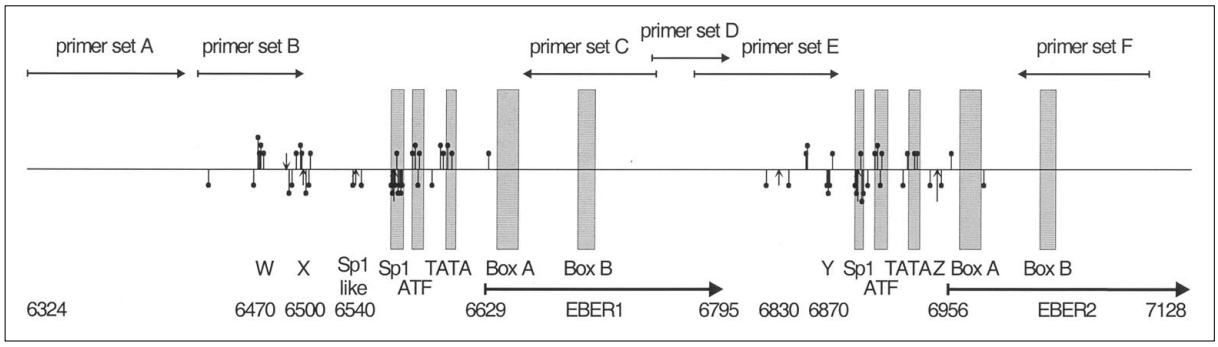



**Figure 1.** Genomic footprint analyses of the EBER promoters. Summary of genomic footprints on EBER1p and EBER2p. The line in the middle represents the double stranded DNA between EBV nucleotides 6320 and 7128 [38]. EBER1 and EBER2 transcripts are represented by thick horizontal arrows. Previously known transcription factor binding sites are labeled with Sp1, ATF, TATA, Box A, and Box B under grey rectangular boxes, respectively. Newly detected binding sites from this study are indicated by Sp1-like and the letters W, X, Y, and Z. The location of the binding sites and transcript coordinates are indicated by EBV nucleotide numbers. Primer sets A to F used for LM-PCR are indicated by thin horizontal arrows. In the following, LM-PCR primer coordinates refer to nucleotide numbers of the viral sequence (38). Primer set A: 1) 6324-6348; 2) 6353-6377; 3) 6391-6415. Primer set B: 1) 6425-6449; 2) 6496-6470; 3) 6475-6499. Primer set C: 1) 6749-6725; 2) 6732-6708; 3) 6690-6666. Primer set D: 1) 6746-6770; 2) 6767-6791; 3) 6776-6800. Primer set E: 1) 6776-6800; 2) 6832-6856; 3) 6852-6877. Primer set F: 1) 7098-7074; 2) 7064-7040; 3) 7030-7006. In the case of a single nucleotide polymorphism at nucleotide 6370, a G/T wobble base was included in primer 2) of primer set A. The positions of nucleotides hypersensitive to methylation and protected from methylation by dimethyl sulfate are indicated by small vertical arrows and lollipops, respectively. Symbols below the central line show reactivities of lower strand guanines, symbols above the DNA show reactivities of upper strand guanines.

phenol/chloroform extracted, ethanol precipitated, ethanol washed, and resuspended in loading dye. One tenth of each sample was separated on a 5% sequencing gel, and the gels were dried and autoradiographed at room temperature using Kodak BioMax MR film.

**Plasmids**

Plasmid pCMVp-Luc contained the minimal CMV immediate early promoter sequences from –55 to +7 relative to the transcriptional start site [42]. The promoter, together with parts of the pBS(-) (Stratagene) multilinker, was cloned between the HindIII and SmaI sites in front of the luciferase gene of plasmid pGL3-basic (Promega). Plasmid pX-CMVp-Luc contained the doublestranded oligonucleotide CGA CCG CGC C**AC CAG ATG GCA CAC GTG GG**G GAA AT (X-Box, EBV nucleotides 6481-6515, only one strand displayed, two E-box sequences underlined, 19 base pair homology to the 5'-end of human immunoglobulin lambda locus [GenBank entry D86993] in bold print) in the SmaI site in front of the CMV minimal promoter. Plasmids were confirmed by sequencing.

**Electrophoretic mobility shift assays**

The preparation of nuclear extracts was essentially based on the standard of Dignam et al. [43]. Nuclei were prepared as described [44,45]. Nuclear extracts were prepared as described [46]. A DNA fragment of 66 bp containing part of the multilinker and the X-Box was cut out with enzymes BamHI and Asp718 from the multilinker of plasmid pX-CMVp-Luc, labeled with T4-kinase and $\gamma$-$^{32}$P-ATP, and purified by polyacrylamide gel electrophoresis and gel elution. Gel retardation assays were performed as described [46] with the modifications described: 1 µg of crude nuclear proteins were incubated with 1 µg poly(dI-dC)-poly(dI-dC), 1 ng of labeled fragment and the indicated excess of unlabeled double stranded competitor oligonucleotide in 25 µl bandshift buffer (10 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 80 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 12.5% glycerol) for 20 min. For antibody shifts, 3.2 µg of each antibody (Santa Cruz Biotechnology) was pre-incubated with 1 µl of nuclear extract for 1 hour at 30°C. Immediately before loading the gel, the binding reaction was centrifuged for 2 minutes at 14.000 rpm in an Eppendorf 5417 bench top centrifuge at room temperature. Protein complexes were resolved by electrophoresis on native 5% polyacrylamide gels (29+1) in 0.25× TBE buffer at 20 mA for 1.5 hours. The following blunt ended, double stranded oligonucleotides (only one strand displayed) were used for competition experiments. The numbers in brackets for each oligonucleotide pair refer the to nucleotide numbers of the B95-8 sequence [38]. Oligo EBER2p-Z containing the Z binding site from the EBER2 promoter (EBER2p) TTA CGG TTC GCT ACA TCA AAC AGG (6935-6958) was used for non-specific competition, oligo EBER1p-X containing the X-box from EBER1p CGA CCG CGC CAC CAG ATG GCA CAC GTG GGG GAA AT (6481-6513) for specific competition.

**Transfection of cells and luciferase assays.**

20 µg of double cesium chloride gradient purified plasmid DNA from bacterial strain DH5a were transfected into $5x10^6$ DG75 cells by electroporation. Electroporation of DNA into cells was done in 500 µl PBS with a twin pulse setting of 750V, 2310Ω, 25µF and 100V, 2310Ω, 3000µF in 4 mm cuvettes with an Easyject Plus device from Eurogentec. 5 µg of an expression construct for the c-myc gene were included as indicated. Expression constructs for human c-Myc under the control of

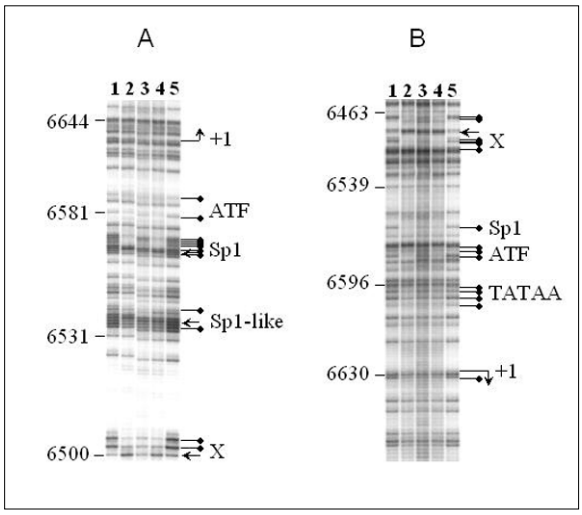



**Figure 2.** Sample of genomic footprint analyses of the EBER promoters. Panel A: lower strand of the EBER1p (primer set B), panel B: upper strand of the EBER1p (primer set C), Lanes 1: G-track from Raji DNA, lanes 2 to 4 inclusively: footprints, lane 2: Raji cells, lane 3: CBM1-Ral-STO cells, lane 4: Rael cells, lane 5: G-track from Rael DNA. At the right of each panel the location of in vivo footprints is indicated by arrows (hypersensitivities) and circles (protections), at the left of each panel nucleotide numbers are given according to the EBV sequence of Baer et al. [38]. On both panels, the initiation start sites of the EBER genes are shown at +1 with an arrow. Protein binding sites are labeled with ATF, Sp1, Sp1-like, TATAA, and X.

the CMV or the SV40 enhancer were obtained from Dirk Eick and Csaba Kiss, respectively. After transfection, cells were incubated under standard conditions for 24 hours. Cells were harvestet and relative light units from cellular extracts were measured with the Dual Luciferase Reporter Assay System (Promega) on a Lumat LB 9501 luminometer (Berthold). Standardisation was done by using equal amounts of protein extract for luciferase assays and repeating the experiment several times.

**Chromatin immunoprecipitation (ChIP).**

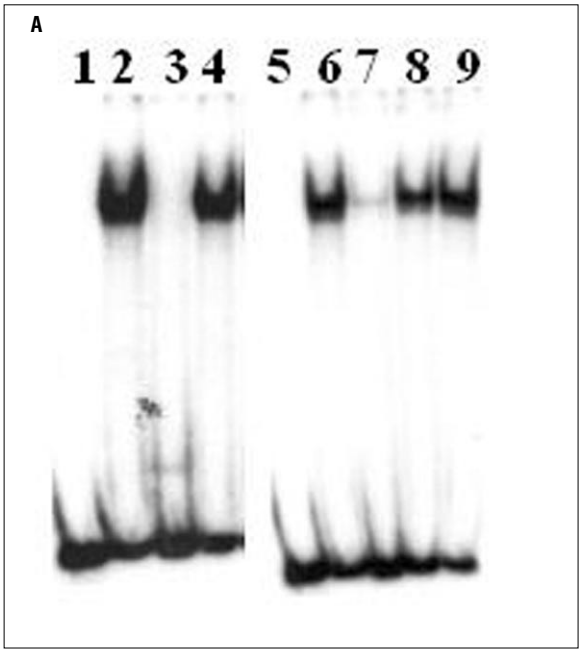
ChIP was performed on Raji cells exactly according to the protocol of the Farnham lab as published in the internet [47] with one exception: Chromatin was not sheared by sonication, but by pressing it 5 times through a 27G syringe needle. PCR primers used were B2 and C2 for EBER1p (Figure 1), primers with sequences CTT TTA AGA AGG CCA CCA ACC TTA TCT CC and GTA GTT CAC CAT CCA TCA TCT CTG TTC AC for the human lambda locus, and primers GAT AGC AAA GGT GGC CGG CAA GGT G and CCC CGA GGC AAG TCA TCT GTT GGA G for the EBV BZLF1 promoter. Briefly, crosslinking of $10^9$ Raji cells was done with 1% formaldehyde for 10 minutes at 37°C. Incubations were always overnight as suggested by the protocol. The remaining steps were precisely as desribed in the protocol [47]. The PCR reaction was done with 20 pmol each primer, 200µM each dNTP, 1 µl template and 1 µl genomic Red Taq (Sigma) for 35 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

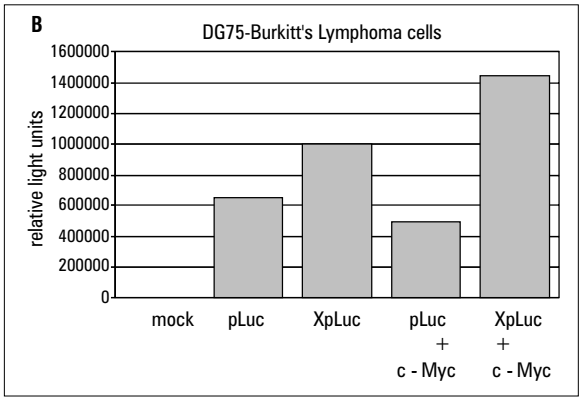
## RESULTS

The cell lines in this study were the same ones used in previously published sets of experiments [48–52]. The EBERs are expressed in all cell lines [48]. All cell lines were truly latent, without any lytic background replication. This was tested by terminal repeat analysis through southern blotting for 721, CBM1-Ral-STO, Rael, Raji, and Mutu subclones [51, 53] J. Minarovits, unpublished data]. Furthermore, selected early antigens or their coding mRNAs associated with productive EBV replication could not be detected in any cell type [32, 54]. J. Minarovits, unpublished data]. In addition, specific segments of the EBV genome were found to be highly methylated in most cell types [49] D. Salamon, unpublished data]. EBV gene expression patterns adhered to the expected latency class patterns. This was tested by Western blot experiments with a monoclonal antibody against EBNA2 on total cellular extracts (data not shown). Furthermore, a Northern blot showed that LMP2A mRNA was only expressed in class III, not in class I cells (D. Salamon, unpublished data).

**In vivo protein binding at both EBER promoters**

A map of the EBER locus is shown in Figure 1. Previous in vitro binding, in vitro transcription and reporter gene experiments charted pol II regulatory elements, an Sp1-site, an ATF-site and a TATAA-Box upstream of both EBER1p and EBER2p, and pol III regulatory elements Box A and Box B downstream of the start sites of both EBERs [10,11,55]. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation from our in vivo analysis is summarized in Figure 1. An exemplary selection of genomic footprints is shown in Figure 2. Footprints generally appeared identical between cell types in class I and class III latency, slight differences in CBM1-Ral-STO (footprint Z, see Figure 1 for location) seemed to be the exception. The footprint patterns for 721, Mutu BLI-Cl216 and Mutu BLIII-Cl99 cells were mostly identical with the patterns for Raji and Rael cells (Figure 2, remaining data not shown). In EBER1p there were protections on both strands of the previously described Sp1- and ATF-sites and on one strand of the TATAA-Box. An additional novel Sp1-like binding site was found at nucleotide 6540. Two novel interactions, named W and X, were found around nucleotides 6470 and 6500, approximately 160 and 130 nucleotides upstream of the transcriptional start site, respectively. Likewise, in EBER2p there were protections on both strands of the previously described Sp1- and ATF-sites and on one strand of the TATAA-Box. A novel binding site, named Y, was found around nucleotide 6870, approximately 80 nucleotides upstream from the transcriptional start site for EBER2. Another binding site named Z was identified immediately upstream of the transcriptional start site of the EBER2 gene. Further protections of one DNA strand only, upstream of the transcription initiation sites of both EBERs, were found



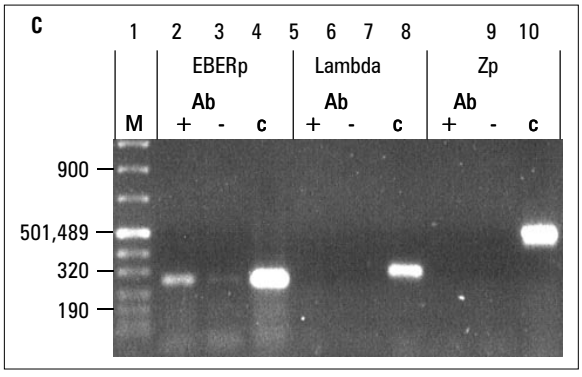




**Figure 3. A.** Electrophoretic mobility shift assays. Gelshift demonstrating specific protein binding activities in Mutu I cell nuclear protein to the X-Box of EBER1p. Lanes 1 and 5: probe only, no protein added; lanes 2 to 4 and 6 to 9: nuclear extract added; lane 3: competition with unlabeled X-Box oligo; lane 4: competition with unlabeled Z-Box oligo; lane 7: anti-c-Myc antibody A-14 added; lane 8: anti-c-Myc antibody 9E10 added; lane 9: anti-E2F1 antibody C-20 added.
**B.** Luciferase assay demonstrating activities of pCMVp-Luc and pX-CMVp-Luc in DG75 BL cells. The data from a representative electroporation transfection experiment is given in relative light units on the ordinate, the used plasmids are indicated on the abscissa.
**C.** Chromatin immunoprecipitation assay on Raji cells with anti-c-Myc antibody N262 (Santa Cruz Biotechnology). Lane 1 shows molecular size marker VIII (Roche), lanes 2–4 EBER1p PCR, lanes 5–7 human lambda immunoglobulin locus PCR, lanes 8–10 EBV BZLF1 promoter PCR. Lanes 2, 5, 8 with antibody, lanes 3, 6, 9 without antibody, lanes 4, 7, 10 total input chromatin as positive control.

around nucleotides 6835, 6855 and 6420. Regulatory cis-elements typical for pol III transcription, described earlier at locations downstream from the transcriptional initiation sites, did not carry prominent in vivo protection patterns, as far as they could be visualized with the primer sets used.

### c-Myc is able to bind the and activate the Myc-box of EBER1p

The identity of most factors binding at both EBER promoters was unclear. However, we noted that the DNA sequence of the X-Box of EBER1p contained two E-Box sequences, CAGATG and CACGTG, compatible with binding of the transcription factors and oncoproteins Tal-1 and c-Myc [56]. Interestingly, there was a 19 base pair sequence containing both E-boxes that was identical with the 19 base pair sequence at the very 5'-end of all the variable chain genes of the human lambda immunoglobulin locus (GenBank D86993). Genomic footprint analyses of the 19bp site in the lambda locus showed no protein protection in our cell lines (data not shown). In order to analyze the binding properties of the X-Box of the EBER locus, we conducted electrophoretic mobility shift experiments. Nuclear extract from Mutu I cells bound the X-Box in a sequence specific manner (Figure 3). Nuclear extracts from 721 LCL, Raji, Mutu III and Rael cells yielded exactly the same specific protein binding pattern as Mutu I cells (data not shown). In order to determine whether c-Myc was part of the shifted nucleoprotein complex, we added antibodies to the gelshift experiments. The specific complex was weakened by addition of anti-c-Myc antibodies A-14 and 9E10, but left unchanged by anti-E2F1 antibody C-20 (Figure 3A). Therefore in these experiments c-Myc was part of the shifted in vitro nucleoprotein complex.

Transfection assays with luciferase as a reporter gene showed that the isolated X-Box could be activated by c-Myc in BL cells (Figure 3B). Furthermore chromatin immunoprecipitation studies on Raji cells showed that the EBER1p bound c-Myc in vivo, while a human genomic control locus and the EBV BZLF1 promoter did not bind c-Myc (Figure 3C). This data convincingly demonstrated that there is an in vivo binding site for c-Myc in the EBV genome.

## DISCUSSION

### Novel protein binding at the EBER locus

The EBER promoters were extensively and mostly equally protected with protein in all cell lines examined (Figures 1, 2). Protein binding partially coincided with previously described in vitro data [11,55]. It is now clear that the upstream regulatory sites described earlier are actually protein-bound in vivo also. However, the pol III transcriptional elements, Box A and Box B, did not carry prominent signs of in vivo protection, to the extent visualized here. Since differences have been described between in vitro and in vivo transcription efficiency on the part of the EBER promoters [10], the question of the EBER transcripts being made by pol III or possibly through pol II in specific settings in live cells might be worth a second look. Further, there are several novel in vivo binding sites in both promoters (Figures 1, 2). Since the EBER promoters are hypomethylated and the EBERs are strongly expressed in many different cell types and tumor materials that carry latent EBV genomes, all footprints are most likely caused by transcriptional activators or chromatin opening factors [48].

### c-Myc and the X-box

The X-Box of EBER1p contained two E-Box sequences compatible with binding of the transcription factor and oncoprotein c-Myc [56]. Because of the great role of the translocation and deregulation of the c-myc gene in BL, we examined the X-Box further. We demonstrated through gelshifts and reporter gene assays that c-Myc is really able to bind and activate this site in vitro (Figure 3A and 3B). Chromatin immuno precipitation experiments showed that c-Myc is really bound to EBER1p in vivo (Figure 3C). The above-mentioned 19 base pair identity tempts us to speculate that EBV may have picked up this regulatory sequence from the human immunoglobulin lambda locus, and the EBERs may be derived from variable chain immunoglobulin genes. Since a novel type of EBV latency without expression of the EBER genes has recently been found in liver tumors [16], cell type specificity may play a role in transcription factor binding.

### Role of the c-Myc site of EBER1p in lymphomagenesis

The Myc-Box turns out to be crucial for our understanding of the development of endemic BL. BL is considered a tumor of the GC B cell [57]. The translocation of the c-myc gene to one of the immunoglobulin genes has been suggested to occur as a byproduct of the GC reaction either through somatic hypermutation of the variable immunoglobulin chain genes or through class switching of the heavy chain genes in a mutating GC B-cell [58]. At the extremely high mutation rates of the GC reaction, the vulnerability of GC B-cells to apoptosis is considered a protection mechanism against the accumulation of tumorigenic mutations through the immunoglobulin maturation process. Normal GC cells are vulnerable to apoptosis, because they express a panel of pro-apoptotic genes, e.g. c-myc, p53, fas, bax, but not the survival gene bcl-2 [59,60]. Another important discovery has been made by Araujo et al. [61]: EBER-positive cells are normally not found in lymphoid GC [61-63] However, EBER-positive B cells are highly enriched in the GC of children with hyperstimulation of the GC system [61].

### Proposal of a new scenario for the development of BL

Against this background the newly discovered binding site for c-Myc in EBER1p suggests a scenario for the specific role of EBV in the generation of endemic BL. In healthy individuals, spontaneously occurring translocations in EBV negative cells are eliminated by apoptosis. Under conditions of GC hyperstimulation, EBER-positive cells are frequently found in the GC reaction. In the EBV-positive B-cell that undergoes a Burkitt's translocation in the GC reaction, pro-apoptotic c-Myc [64] directly helps opening and upregulating the anti-apoptotic EBER transcription units. At later steps in B-cell development, c-myc is normally switched off again [65]. However, in the translocated B-cell, c-myc can no longer be shut down. Consequently, in the EBER-positive translocated GC B-cell, the balance between apoptosis and anti-apoptosis is permanently shifted in favor of cell survival. The normally masked oncogenic potential of the c-Myc protein can then unfold, as described in a transgenic model system for pancreatic oncogenesis [66]. Therefore, this crucial single cell has a greater chance of not being eliminated by apoptosis. The cell gains time to accumulate additional tumorigenic mutations, or, on the other hand, it may at this stage and without any further mutation already constitute a genuine BL cell, awaiting only an appropriate growth stimulus in order to expand. As the direct transcriptional effects of c-Myc have generally been found to be rather weak [67], another functional aspect of c-Myc binding to the EBV genome may be found in its role as nuclear matrix protein [68] and chromatin remodeling factor [69]. c-Myc may play a crucial role in keeping the viral genome in the nucleus and, therefore, EBV latency class I functions other than the EBERs may be provided to the myc-translocated cell through the continuous presence of the EBV genome. Therefore, the Myc-Box in EBER1p explains in a straightforward way the survival of one single EBV-infected translocated B cell that is the founder cell of a future BL. The order of events in our new scenario is a c-myc translocation occurring in a single EBV infected cell that carries a latency I viral expression pattern and undergoes the somatic hypermutation or class switch reaction in a GC. Hyperstimulation of the GC reaction is also the basis for the development of HD, of which there is a high incidence in children of developing countries who are continuously and heavily infested with parasites [3,61,70]. Occasional cells with type II latency, but no cells with type III latency have been found in the hyperstimulated tonsillar GC among the numerous type I cells [61]. The rare latency II expression pattern of an EBV-infected GC B cell may be a prerequisite for the rise of HD in hyperstimulated GC [61].

### No need for severe immunosuppression and LCL outgrowth

The new model explains the monoclonality of both the cellular and the viral genome in endemic BL and may explain the origin of all EBV-positive BL. However, the assumption of severe immunosuppression and the expansion of LCL as an intermediate stage in the development of BL is not necessary in this model. This is important, because the common assumption of immunosuppression and a consequential expansion of LCL as conditions for the development of BL seems to have confused the issue [2]. Although BL can very occasionally develop in transplant patients, severe immunosuppression has been more or less excluded as the common necessary condition from which BL develops [70]. Therefore, immunosuppression may help in the development of BL through an increase of the overall viral load [71]. However, the necessary condition for EBV-positive BL has been shown to be the hyperstimulation of the immune system caused by HIV, malaria, or other parasites [61,70]. Interestingly, BL-like lymphomas are a complication of the early stages of AIDS, when the GCs are still hyperactive, whereas LCL-like lymphomas are a complication of the later stages, when the immune system is already defunct [3]. Remarkably, BL cell lines can be easily switched from EBV latency I to latency III in culture, but attempts to switch type III latency back to type I latency have been largely unsuccessful [72,73].

In summary, analyzing the in vivo structure of EBV key elements regulating lytic cycle, latency, transformation and tumorigenesis with nucleotide resolution has already yielded many new and exciting data [39, 49–52,74,75]. It will be interesting to identify also the in vivo binding sites and transcription factors of the remaining EBV latency promoters, and to extend these studies to EBV-positive NPC cell lines and clinical material of different latency types. Further, it would be of interest to test in a transgenic mouse model whether the EBERs unfold their antiapoptotic effects not only in BL cell lines [26], but also in the lymphoid GCs where both BL, HD, and several other types of NHL originate [57,61,76,77]. The incidence of NHL other than BL may also be higher in such transgenic animals. Furthermore, it is most important to learn more about EBER expression and the cell type and differentiation stage specific regulation of their promoters, because we cannot really say with any confidence whether normal GC are mostly free of EBV [61-63] or merely free of EBER expression.

## CONCLUSIONS

The finding of a binding site for c-Myc in the EBV genome attributes a specific causal role to EBV in the generation of Burkitt's Lymphoma. Discussion on immunosuppression and the outgrowth of transformed lymphoblastoid cells have been confusing the issue, as both seem not to be required for BL development. The development of transgenic mouse models with the EBER expression targeted to the B cell compartment may be able to verify this new BL-model. Cell type specific regulation of EBER transcription and the mechanisms of EBER action should be further clarified.

### Acknowledgements

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# Updated Epstein–Barr virus (EBV) DNA sequence and analysis of a promoter for the BART (CST, BARF0) RNAs of EBV

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Two sequences required for activity of the Epstein–Barr virus BART RNA promoter in transfection assays have been identified by site-directed mutagenesis. One contains a consensus AP-1 site; the other has some similarity to Ets and Stat consensus binding sites. Candidate sequences were suggested by mapping a region of unmethylated DNA in EBV around the BART promoter followed by *in vivo* footprinting the promoter in the C666-1 nasopharyngeal carcinoma cell line, which expresses BART RNAs. The data are presented in the context of a revised EBV DNA sequence, known as EBV wt, that is proposed as a future standard sequence for EBV.

## INTRODUCTION

Epstein–Barr virus (EBV) contains about 85 genes but only a few of these are expressed in the EBV-associated human cancers that occur in immunocompetent patients. Since it has become clear that many of the established immortalizing proteins of EBV are not expressed in human tumours, attention has turned to those viral genes that are expressed in cancers for interpretation of the oncogenic properties of EBV. The BART/CST (*Bam*HI A rightward transcript/complementary strand transcript) RNAs were originally identified in nasopharyngeal carcinoma (NPC) samples (Hitt *et al.*, 1989), although the transcripts have also been detected at low level in some Burkitt's lymphoma and B lymphoblastoid cell lines (Brooks *et al.*, 1993; Chen *et al.*, 1992; Gilligan *et al.*, 1990; Griffin & Xue, 1998; Karran *et al.*, 1992; Raab-Traub *et al.*, 1991; Zhang *et al.*, 1993). There is also evidence for BART RNAs in EBV-positive gastric cancer (Sugiura *et al.*, 1996), Hodgkin's Disease (Zhang *et al.*, 2001) and in normal EBV persistence (Chen *et al.*, 1999; Gilligan *et al.*, 1991; Kienzle *et al.*, 1998), although the BART region can be deleted from the viral genome without any notable effect on B cell immortalization by EBV. The protein products from the BART RNAs have not yet been fully characterized but several potential products of the various spliced forms of BART RNA have been analysed (Smith *et al.*, 2000) including RPMS1, A73 and BARF0 (RK-BARF0). Biochemical activities of these proteins have been identified that could be relevant to the role of the virus in cancer (Kusano & Raab-Traub, 2001; Smith *et al.*, 2000; Zhang *et al.*, 2001).

The splicing of the BART RNAs is complex (Sadler & Raab-Traub, 1995), with at least 16 different, partly overlapping exons identified already in cDNA. The main full-length cDNA isolated so far (Smith *et al.*, 2000) was able to express the RPMS1 protein when transfected and there was evidence that such RNA constitutes a significant proportion of the BART RNA expressed in the C15 NPC xenograft tumour, which has relatively high expression of the BART RNAs. The transcription start was determined and a plasmid named SK containing EBV sequences from 442 nt upstream of the transcription start, through the first exon and some of the first intron was found to express the correctly initiated and spliced first exon of the BART RNA (Smith *et al.*, 2000).

We now identify genomic sequences around the BART RNA first exon which are protected from DNA methylation in C15 NPC tumour cells, characterize sequences required for transcription from the promoter and demonstrate *in vivo* footprinting of those sites in a cell line derived from NPC that maintains episomal EBV. Description of exons of the BART RNAs has been complicated by the fact that the gene spans the region of EBV deleted in the B95-8 strain that was sequenced initially (Baer *et al.*, 1984). A revised EBV

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sequence called EBV wt, renumbered to include the B95-8 deletion and various other corrections, is henceforth used to describe the virus and is proposed as a new standard reference sequence for EBV. The revised EBV wt sequence numbering is used to describe all EBV features in this paper.

## METHODS

**Cell lines.** 293 and C666-1 cells (Cheung *et al.*, 1999) were cultured in DMEM or RPMI with 10 % foetal calf serum. Transfection into 293 cells using the calcium phosphate method and RPA analysis were as described previously (Spender *et al.*, 2001; Wensing *et al.*, 2001).

**Plasmid construction.** Plasmid SK (containing EBV 137908–138989 in pCAT) was made by cloning an *Sph*I to *Kpn*I EBV restriction fragment between the *Sph*I and *Hinc*II sites of pBluescript (Stratagene), the *Kpn*I site having been made blunt with T4 DNA polymerase. The EBV fragment was excised using the *Hin*dIII and *Xba*I sites in the Bluescript polylinker and cloned between the *Hin*dIII and *Xba*I sites of pCAT-Basic (Promega). Plasmid BK (containing EBV 138173–138989 in pCAT) was made by cutting SK with *Hin*dIII and *Bgl*II, blunting the ends with T4 DNA polymerase and religation. Plasmid CK (containing EBV 138289–138989 in pCAT-Basic) was made by *Pfu* polymerase PCR from SK and cloned between the *Sph*I and *Xba*I sites of pCAT-Basic. Plasmid SS (containing EBV 137908–138720 in pCAT) was made by cloning an *Sph*I to *Ssp*I EBV restriction fragment between the *Sph*I and *Hinc*II sites of pBluescript, the *Ssp*I site having been made blunt with T4 DNA polymerase. The EBV fragment was excised using the *Hin*dIII and *Xba*I sites in the Bluescript polylinker and cloned between the *Hin*dIII and *Xba*I sites of pCAT-Basic. Mutations were introduced into BK and CK using the Quikchange kit (Promega). The A site was changed from TGAGTCA to TGAGGCA, the B site was changed from TACCCGGAA to TACCCGGGC and the N site was deleted. For this, EBV nucleotides 138926–138941 were deleted resulting in a sequence CAGTGTGC. All plasmids were sequence verified in the BART promoter region.

**DMS interference footprinting and methylation analysis.** *In vivo* dimethyl sulphate (DMS) interference footprinting was performed as described previously (Niller *et al.*, 2002). The C15 tumour was propagated in nude mice (Busson *et al.*, 1988) and DNA was extracted by proteinase K digestion and phenol extraction. For methylation analysis, C15 tumour DNA was digested with either *Hpa*II or *Msp*I, electrophoresed on a 1 % agarose gel and Southern blots were hybridized with the probes indicated in the legend to Fig. 1, labelled by random priming.

**Gel retardation assay (EMSA).** Double stranded oligonucleotides used were as follows. A site probe, CTAAATGAGTCATTCCTAA; mutated probe, CTAAATGAGGCATTCCTAA. B site probe, GCCATACCCGGAAGAGGAG; mutated probe, GCCATACCCGGGCGAGGAG. N site probe, GTAGGGGCCTCCACCTAGGT. Oligonucleotides were end-labelled with T4 polynucleotide kinase. To prepare nuclear extracts, cells were scraped and washed in PBS then resuspended in Buffer A [10 mM HEPES, 1·5 mM $MgCl_2$, 10 mM KCl, 0·5 mM DTT, 0·5 mM PMSF, 0·1 % NP40, 1 × protease inhibitor cocktail (Boehringer Mannheim)] and left on ice for 5 min. After brief centrifugation the supernatant was removed and the nuclei resuspended in Buffer B (25 % glycerol, 20 mM HEPES, 420 mM NaCl, 1·5 mM $MgCl_2$, 10 mM KCl, 0·2 mM EDTA, 1 mM DTT, 0·5 mM PMSF, 1 × protease inhibitor cocktail) and mixed at 4 °C for 15 min. Cell debris was removed by centrifugation and protein concentration determined using the Bio-Rad DC protein assay. For each reaction, 5 μg of nuclear extract was incubated at 25 °C for 5 min with a mixture of 2·5 μl BSA (2 mg/ml), 2 μl poly(dI:dC) (2 mg/ml) (Sigma), 0·5 μl 200 mM DTT and 5 μl Buffer D (20 mM HEPES pH 7·9, 100 mM KCl, 0·2 mM EDTA, 0·5 mM PMSF, 0·5 mM DTT, 20 % glycerol, 1× protease inhibitor cocktail. When competitor oligonucleotides were used, they were added at this point. The relevant $^{32}$P-labelled double stranded oligonucleotide was then added (0·4 ng per reaction) and the mixture was incubated at 25 °C for 30 min. Samples were electrophoresed on 4 % polyacrylamide gels in 0·3 × Tris/borate/EDTA; the gel was then dried and data were collected on the phosphorimager.

## RESULTS

### New reference sequence for EBV

The prototype DNA sequence for EBV has been the B95-8 strain, accession no. V01555 (Baer *et al.*, 1984). The revised EBV wt map and sequence file (accession no. AJ507799) used here have several advantages over the Baer *et al.* sequence. The B95-8 deletion sequence determined from Raji EBV has been inserted to give a more wild-type genome and the number of major internal repeat copies has been reduced from 11 to 7, which is more representative. A recently discovered single nucleotide error in the BcRF1 open reading frame (W. Amon & P. J. Farrell, unpublished) has been corrected and the annotation has been improved and brought into line with current standards. This sequence, known as EBV wt, is available with corrected and updated annotation from data libraries with accession no. AJ507799 and a corresponding genome map can also be downloaded from the http://www.med.ic.ac.uk/ludwig/ebv.htm website. The continuous sequence will allow a simpler description of the BART RNAs which cross the B95-8 deletion. The promoter for these RNAs is the topic of this paper.

### EBV DNA is unmethylated around exon I of the BART gene

Several studies have shown that most of the EBV genome DNA is methylated in tumour cell lines that have a latent infection with EBV (e.g. Robertson *et al.*, 1996; Salamon *et al.*, 2001). Absence of DNA methylation in a region of the EBV genome can be an indicator of locations where transcription factors required for promoter activity may be bound during EBV latency. Comparison of restriction digestion by *Hpa*II and *Msp*I on Southern blots of C15 tumour EBV DNA indicated a region of mostly unmethylated DNA extending from about 138200 to 139200 on the EBV wt map (Fig. 1). For example, probes 4 and 5 in the hypomethylated region give mostly the same sized bands with the two enzymes whereas with probes 1 and 2 show no digestion with the methylation-sensitive *Hpa*II enzyme. The region of hypomethylation extends from just upstream of the transcription start to a significant distance downstream of exon 1 (Fig. 1). We previously showed (Smith *et al.*, 2000) that promoter activity could be observed in the plasmid SK (Fig. 1) in transfection assays.

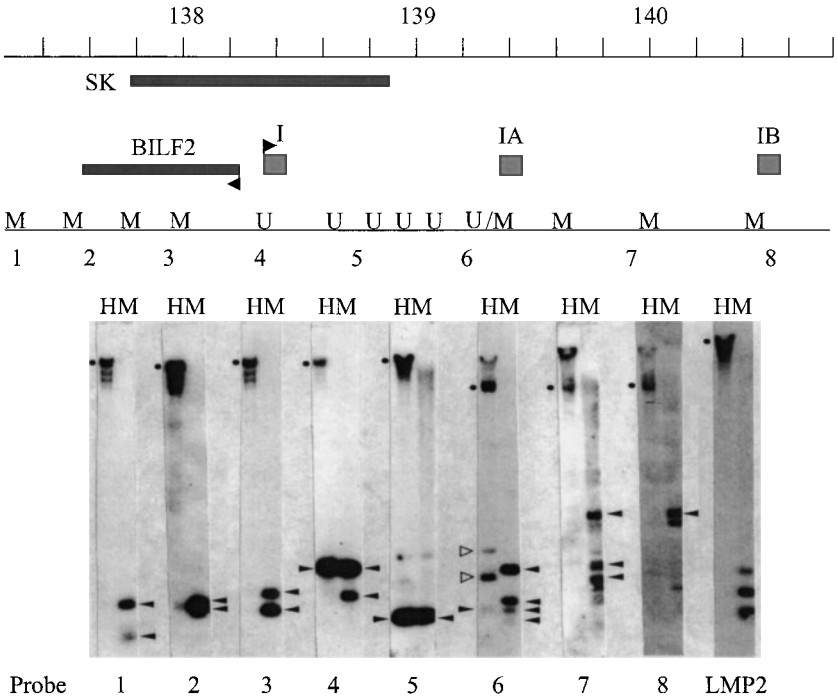


**Fig. 1.** Methylation of C15 EBV DNA spares the pBART promoter. DNA from C15 tumour was digested with either *Hpa*II (H) or *Msp*I (M) and analysed by Southern blotting using probes corresponding to the indicated regions 1–8 or a probe for the LMP2 gene region (LMP2). The positions of *Msp*I/*Hpa*II sites interpreted according to the EBVwt sequence to be either unmethylated or methylated are indicated as either U or M respectively or U/M in case of substantial partial methylation. Sites that are very close together are indicated by a single U or M for clarity; these are bracketed together in the following list of positions of the sites in the EBVwt genome: M137349, M137570, M(137802, 137857), M(138058, 138072), U(138335, 138350), U138733, U(138848, 138875, 138894), U139013, U139148, U/M(139320, 139413), M 139714, M140071. Probes were approximately 1 (137000–137640), 2 (137640–137920), 3 (137920–138180), 4 (138180–138740), 5 (138740–139010), 6 (139010–139750), 7 (139530–140430), 8 (140430–140850); LMP2 is an LMP2A cDNA probe. The map positions of the BILF2 open reading frame, exons I, IA and IB of the BART RNAs and the EBV content of the SK plasmid are shown above against a scale in kb corresponding to the EBV wt map. The predicted *Msp*I fragments are marked with filled arrowheads, high molecular mass DNA resistant to *Hpa*II is marked with filled circles and the partially methylated fragments are marked with open arrowheads.

## C666-1 cells express BART RNAs – genomic footprinting the BART promoter

Most of our previous investigation of EBV gene expression in NPC has used the C15 xenograft because there has been a lack of NPC cell lines that retain their EBV. The recently described C666-1 line (Cheung *et al.*, 1999) is derived from an NPC, retains its EBV and has been shown to have a restricted latent pattern of EBV gene expression. The cells make EBNA-1 protein but not EBNA-2 or LMP1 (Cheung *et al.*, 1999). Using similar RPA probes to those applied previously in C15 (Smith *et al.*, 2000), BART RNA expression was also readily detected in C666-1 RNA. Correctly spliced exon I and the boundary between exons VIIA and VIIB were demonstrated (Fig. 2). In each case, RNA that was not spliced at the splice junction was also detected; this might reflect partly spliced nuclear RNA (total cell RNA was used for these RPA experiments) or may indicate a heterogeneity of splicing in the BART RNAs. The unspliced signal could not be derived from viral DNA contaminating the RNA because the 200 nt band in the exon I RPA corresponds to the length of correctly initiated RNA unspliced at the exon I 3′ end rather than the whole EBV content of the probe (379 nt), which would be protected by viral genomic DNA.

The C666-1 line is unusual in the sense that there has been great difficulty in obtaining an NPC cell line that retains its EBV, so we also checked that the EBV genome was in the normal episomal state and had not suffered major deletions. Southern blotting *Bam*HI digests of C666-1 DNA (data not shown) revealed the normal *Bam*HI fragments C, W, K and A, which are widely distributed along the genome, indicating no obvious major deletions. Gardella gel analysis (Gardella *et al.*, 1984) showed the typical episomal EBV found also in a B95-8 lymphoblastoid cell line (LCL C) and in the Akata Burkitt's lymphoma cell line (Fig. 2). The lymphoid lines have a small fraction of cells spontaneously in the productive cycle giving some linear EBV DNA but this was only present at a very low level in C666-1, consistent

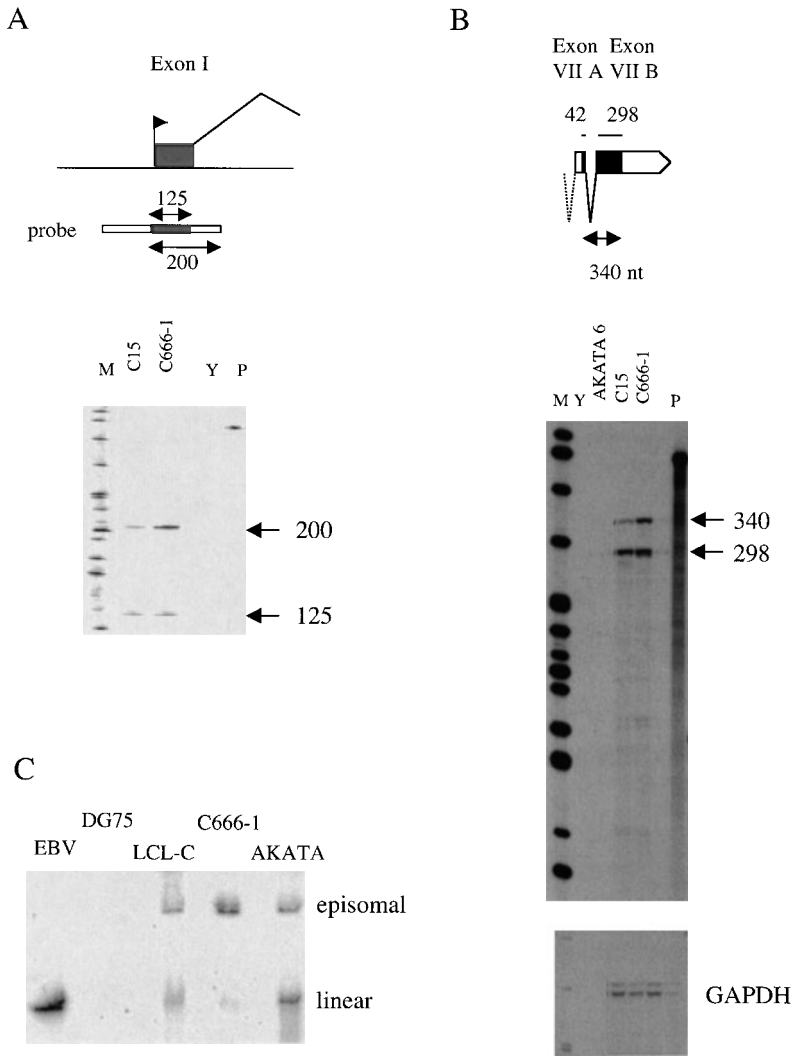


**Fig. 2.** RPA analysis of C666-1 RNA for (A) BART RNA exon I. The 125 nt protected fragment corresponding to exon I is marked with an arrow. (B) The exon VIIA to VIIB splice is shown by the 340 nt protected fragment. Probes were as described previously (Smith *et al.*, 2000); track P is input probe, Y is yeast RNA negative control. (C) Gardella gel analysis (Gardella *et al.*, 1984) of EBV DNA in C666-1, Akata and LCL-C (a B95-8 EBV LCL) cell lines using a *Bam*HI W probe. Track EBV contains B95-8 EBV virions as a marker for linear EBV DNA; DG75 is an EBV negative control line.

with the latent cycle protein expression pattern reported previously (Cheung *et al.*, 1999).

To identify likely binding sites for transcription factors within C666-1 EBV corresponding to the SK plasmid region, DMS interference *in vivo* footprinting was applied to the C666-1 cells. The interpretation of this type of data is sensitive to sequence variation relative to the prototype EBV-wt sequence so this region of C15, C666-1 and Akata EBV was first sequenced. A few sites of variation were detected, summarized in the legend to Fig. 3, but there was no variation from the EBV wt sequence in the sites identified by the footprinting. The footprinting showed several sites of either protection or enhanced cleavage relative to the equivalent naked plasmid DNA. These are shown in Fig. 3 and were named A (includes an AP1 consensus binding site TGAGTCA), B (sequence similar to an Ets or Stat consensus site) and N (some similarity to an NF-$\kappa$B site), the latter being downstream of exon 1. The B site showed protection, the NF-$\kappa$B site had enhanced cleavage and the A site had both protection and enhanced cleavage. The positions of these sites relative to the transcription map are shown in Fig. 4(A). The footprinting was done in C666-1 cells but subsequent transfection assays for BART promoter activity (Fig. 4) were performed in 293 cells because of their higher transfection efficiency, so extracts of both 293 and C666-1 cells were tested for binding oligonucleotides containing the A, B or N site. Clear binding of the A and B sites was observed by EMSA (Fig. 3B) with both 293 and C666-1 extract and this was specific since it was competed by an excess of the same oligonucleotide but not by an oligonucleotide in which some nucleotides had been mutated (the same mutations as used below in functional assays of the promoter). A single major A site complex was observed with C666-1 extract but several complexes were seen with 293 extract (Fig. 3B). The most specific of these (arrowed), as determined by competitor oligonucleotides, migrated close to the position of the C666-1 complex. It was already well established, e.g. Kirch *et al.* (1999), that 293 cells contain AP-1 activity which can bind to the same sequence that is present in site A. An

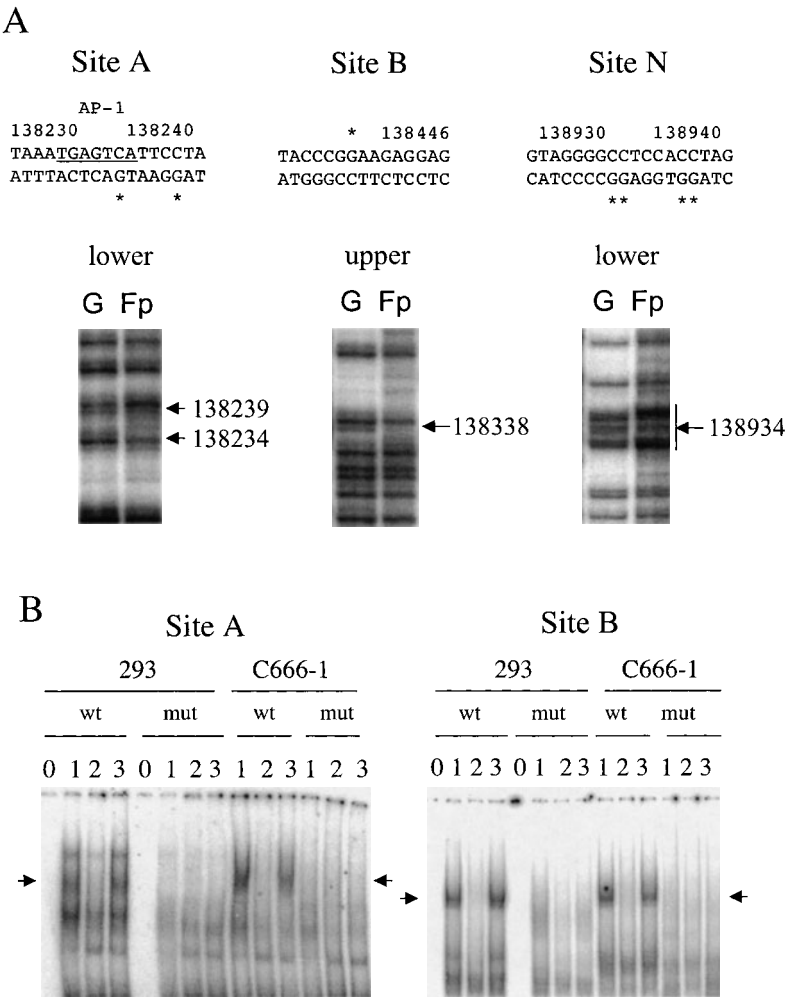


**Fig. 3.** (A) DMS interference footprints corresponding to Site A, B and N. Track Fp is the footprint and track G is the naked DNA control. The positions of the protection or enhanced cleavage are indicated by * on the local EBV sequences. Sequence differences between EBVwt and C666-1 were at 138283 (T to C), 138577 (T to G), 138580 (A to C), 138699 (G to T), 138723 (T to C), 138813 (T to G), 138870-138872 (TCC deleted) and 138987 (C to A). Akata and C15 also had these changes from EBV wt except for the TCC deletion. (B) Gel retardation assay of protein binding to the A or B site using wild-type probe (wt) or mutated site probe (mut) with extract from 293 or C666-1 cells, as indicated. The mutation was the same as in the expression assays (Fig. 4). Tracks 0 are probe alone; 1–3 have cell extract. Tracks 2 also included 100-fold excess of the unlabelled wild-type site oligonucleotide and tracks 3 had 100-fold excess of the unlabelled mutant site oligonucleotide. Specific complexes are indicated by the arrows.

oligonucleotide containing the N site was also tested with extracts from 293 and C666-1 cells but no specific binding was observed in the EMSA (data not shown).

To determine more precisely the sequences required for BART promoter activity, deletions were made in the SK plasmid and site-directed mutations were made at the locations identified by the *in vivo* footprinting (Fig. 4A). Mutations were introduced into the A and B sites and the N site was deleted from the BK plasmid. The plasmids were transfected into 293 cells and resulting RNA was assayed by RPA for exon 1 of the BART RNAs (Fig. 4B). The results were normalized relative to an RPA assay for GAPDH (Fig. 4B) and the results quantified (Fig. 4C) from the phosphorimager data. The results show that truncation of the plasmid down to BK or CK gave about twice the amount of exon 1 RNA as plasmid SK. Mutation of either the A or B site within BK caused modest reductions in expression but mutation of both A and B sites substantially reduced expression (about 10-fold). Consistent with this, mutation of the B site reduced CK expression (the A site is not present in the CK plasmid). Deletion of the N site either by truncation in the SS plasmid or by localized deletion in BK caused only a small reduction in exon 1 expression.

## DISCUSSION

The RNA mapping data shown here have further confirmed that the BART exon I starting transcription at 138350 is a significant point of initiation of BART family RNAs. The previously reported 5′ end was confirmed in C666-1 cells (Fig. 2A). One surprising feature of the DNA methylation study of C15 EBV DNA reported here is that the unmethylated region of DNA extends significantly downstream of exon I. This suggests that proteins may be bound to this region during latent persistence of the virus in the C15 tumour cells, preventing DNA methylation. The downstream unmethylated region could represent components of the BART promoter, other promoters so far unmapped or other genetic functions within this region of DNA. Previous RNA mapping in B95-8 cells (Farrell, 1989) recorded poorly characterized leftward RNAs that might originate from this region and it remains unclear whether the A73 type of BART RNA (Smith *et al.*, 2000) initiates at the normal BART exon I, so there are candidate RNAs that might come from a novel promoter in this region yet to be characterized. On the other hand, there is some evidence that could be consistent with downstream promoter elements in the BART promoter. To analyse the BART

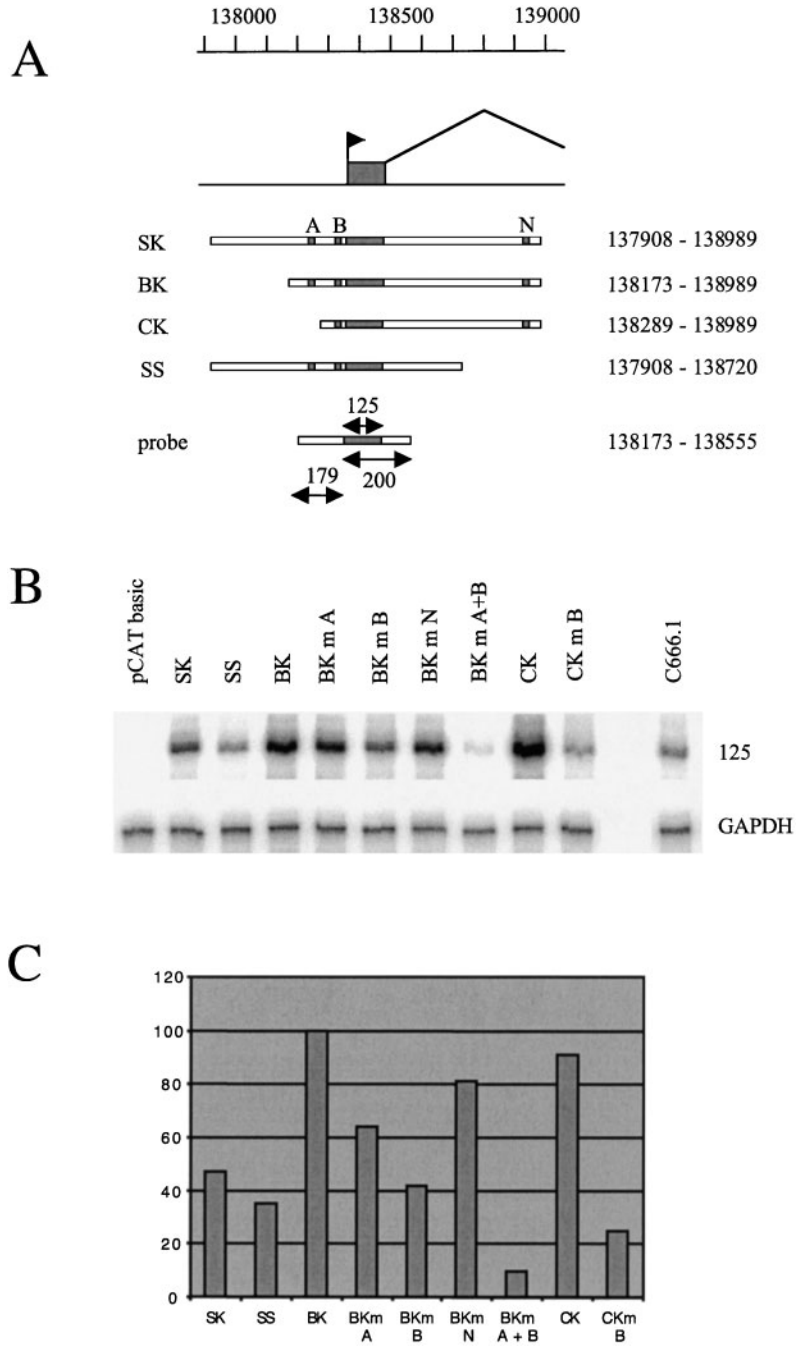


**Fig. 4.** (A) The EBV content of the plasmids SK, BK, CK and SS is shown beneath a map of BART exon I. The positions of the A, B and N sites are shown. (B) The indicated plasmids (mutated at the A, B or N sites, where indicated) were transfected into 293 cells and RNA was harvested 24 h later. RPA results, as in Fig. 1, for the 125 nt exon I protected fragment and a GAPDH control are shown. (C) The data from part B and another similar experiment were quantified from the phosphorimager. The exon I values, normalized for transfection efficiency (using a cotransfected $\beta$-galactosidase reporter) and for RNA amount using the GAPDH values are plotted as a percentage of the BK value. The mean values of the two experiments are shown in the histogram.

promoter, we used RPA assays on the SK, BK and CK constructs because simple fusion of the upstream region (which would normally be expected to contain the promoter sequence) to a CAT reporter gave very little CAT activity (data not shown). Downstream promoter elements are one possible explanation of this. DMS interference footprinting in the C666-1 cell line showed enhanced cleavage at site N in the downstream region, indicating a distortion of the normal DNA structure at that point but no other protein binding was observed directly. These possibilities therefore remain to be resolved.

Although the cells grow relatively slowly, the C666-1 cell line seems to be a valuable system for studying EBV gene expression in epithelial cells. It appears to contain a relatively wild-type EBV, retains the EBV in culture and expresses the BART RNAs. DMS interference footprinting suggested Sites A and B upstream of exon I that might be involved in BART expression and mutation of both of these sites substantially reduced activity of the BART promoter in a transfection assay. Site A contains a perfect match to the AP-1 consensus site that has been shown previously to bind c-Jun/c-Fos and the mutation we introduced to site A is

known to prevent c-Jun/c-Fos from binding (Risse *et al.*, 1989). A common specific EMSA band was obtained with both 293 and C666-1 cells so it is likely that this contributes to activity of the promoter in both cell lines. The factor that binds to the B site is less certain. Scanning the sequence with the TFMATRIX transcription factor binding site database (Heinemeyer *et al.*, 1998) suggests imperfect matches to NRF-2 (93 %), c-Ets (87 %) and a STAT consensus, STATx (86 %). These are widely expressed factors with several family members and overlapping binding specificities; it is difficult to be certain which factors are the functional ones on the B site but we have demonstrated that there is a single major complex detected in EMSA analysis with this site using 293 and C666-1 cells and that the mutation of the site that prevented activity of the promoter also prevented complex formation. A factor containing NRF-2 is perhaps the most likely since this is expressed in many cell types (NRF-2 has been purified from HeLa cells; Virbasius *et al.*, 1993). NRF-2 was originally studied as part of GABP, a factor involved in herpes simplex virus immediate early gene expression (LaMarco *et al.*, 1991), but it is involved in expression of the cytochrome *c* oxidase gene and the binding site in the rat cytochrome *c* oxidase gene is almost identical to site B in the EBV BART promoter. Antibodies to NRF-2 are not available to test this directly. It has been proposed that Stat 3 is a major regulator of EBV latent cycle promoters in epithelial cells (Chen *et al.*, 2001) based on the Qp and LMP1 promoters but we could find no evidence for Stat 3 binding to the B site or for binding of phosphorylated Stat 1 (data not shown).

These results are the first detailed analysis of sequences required for expression of the BART RNAs. They will provide an opportunity to identify cell factors that control expression of the BART RNAs and we have also shown that C666-1 will be a valuable system in which to investigate the BART genes since it contains an apparently normal episomal EBV genome and expresses the BART RNAs.

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# High-resolution Methylation Analysis and *In Vivo* Protein–DNA Binding at the Promoter of the Viral Oncogene LMP2A in B Cell Lines Carrying Latent Epstein–Barr Virus Genomes

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**Abstract.** Latency protein LMP2A of Epstein–Barr virus (EBV) has been implicated in EBV related tumorigenesis. To understand the host cell dependent expression of the LMP2A gene, it is necessary to analyse the regulatory mechanisms of the LMP2A promoter (LMP2Ap). By transient transfection and *in vitro* binding analyses two CBF1 sites have previously been shown to be involved in the regulation of LMP2Ap. However, the promoter structure has not been examined at the nucleotide level *in vivo*. Therefore we undertook a comprehensive analysis of *in vivo* protein binding and of CpG-methylation patterns at LMP2Ap in a panel of B cell lines carrying latent EBV genomes. The presence of characteristic footprints on two CBF1 and further binding-sites, together with overall hypomethylation of CpG dinucleotides correlated well with promoter activity. In contrast, the absence of several genomic footprints, as well as the presence of patches of highly methylated CpG dinucleotides were characteristic of silent LMP2Aps.

**Key words:** CpG methylation, Epstein–Barr virus, genomic footprints, viral latency

## Introduction

Epstein–Barr virus (EBV) is a ubiquitous gammaherpesvirus associated with a wide variety of neoplasms including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, posttransplant lymphoproliferative disease and Hodgkin's disease [1,2]. The virus is able to transform B lymphocytes to continuously growing lymphoblastoid cell lines (LCLs) *in vitro* [3]. Only a subset of viral genes is transcribed from latent episomal EBV genomes in EBV-associated neoplasms and LCLs. BL cell lines which maintain the phenotype of BL biopsy cells express only EBV nuclear antigen 1 (EBNA1), two protein non-coding RNAs (EBER1 and 2) and the *Bam*HI-A region transcripts (type I latency) [1]. A different pattern of viral gene expression is characteristic for LCLs and for BL cell lines which changed their phenotype during cultivation to type III latency. Five nuclear antigens and three EBV encoded integral latent membrane proteins (LMP) are expressed in addition to the type I latency gene products in these cells (type III latency) [3]. LMP1 mimics CD40 function and stimulates proliferation of B cells *in vitro* [4]. Both LMP1 and LMP2A have transforming effects, e.g. loss of anchorage dependence and altered cell morphology [5–7], inhibit epithelial cell differentiation [6,8] and affect signal transduction [9,10]. LMP1 transformed rodent cells grow as malignant tumors when inoculated into suitable hosts and transfection of the LMP2A gene into an immortalized human keratinocyte cell line

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confers tumorigenicity in nude mice. LMP2A expressing tumor cells are highly metastatic [6]. Further, LMP2A interferes with B cell signalling, acts as a constitutively active surrogate B cell receptor, and provide survival signals that prevent B cells from undergoing apoptosis [11–13].

We and others established earlier that host cell dependent activities of the major EBV latency promoters, W promoter (Wp) and C promoter (Cp) where transcripts for the EBNAs are initiated and the bidirectional LMP promoter (LMP1p) for transcription of LMP1 and LMP2B messages, are regulated by DNA methylation [14–20]. Since the expression of the LMP2A protein depends on the host cell phenotype as well [21], we established a high-resolution map of CpG methylation of its promoter in representative lymphoid cells and clones.

An essential step in understanding the regulation of key EBV genes involved in tumorigenesis is the analysis of the nucleoprotein structure of the respective latency promoters at nucleotide resolution. Therefore we also wished to analyse *in vivo* binding of putative regulatory proteins to the LMP2A promoter (LMP2Ap) control sequences using the method of genomic footprinting.

## Materials and Methods

### *Cell Lines and Tissue Culture*

LCL-721 is a B95-8 transformed LCL with type III phenotype [22]. Rael is a group I BL cell line [23]. CBM1-Ral-STO is a type III LCL transformed by the Rael EBV strain [23]. Mutu-BL-I-Cl-216 is a subclone of the BL line Mutu, representative for latency type I [24]. Mutu-BL-III-Cl-99 is a subclone of the BL line Mutu, representative for latency type III [24]. DG75 is an EBV negative Burkitt's lymphoma cell line [25]. All of the cell lines were maintained in suspension cultures of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 units penicillin per ml and 50 µg streptomycin per ml at 5% $CO_2$ and 37°C.

### *Northern Blot*

Total cellular RNA was isolated from cell cultures with TRI Reagent (Sigma) according to the manufacturer's instructions. From total RNA poly-A-RNA was enriched using the µMACS mRNA Isolation Kit (Miltenyi Biotec) according to the manufacturers instructions. Three microgram of poly-A-RNA per lane was subjected to electrophoresis through a 1% agarose gel containing formaldehyde and transferred to a Biodyne Plus membrane (Pall). The filters were hybridized to $\alpha$-$^{32}$P-CTP labeled RNA probes, then washed and autoradiographed according to standard methods [26]. The probe for the LMP2A transcript was hybridized first, and after two weeks of autoradiography at −80°C, the film was developed. Then the filter was rehybridized with the β-actin probe and autoradiographed for 9 h at room temperature. Absence of the LMP2A signals was confirmed by autoradiography for 2 days at room temperature before hybridization with the β-actin probe. The first exon probe for the LMP2A transcript was generated by PCR amplification of B95-8 genomic DNA with the primers 5′-TCCCTCTCGCCTTGTTTCTCAGAG-3′ (EBV nucleotides 166500–166523) and 5′-CCTCTG-CCCGCTTCTTCGTATATG-3′ (EBV nucleotides 166913–166890) [27]. The PCR product was cloned into the pCR4-TOPO vector (Invitrogen) with the TOPO TA Cloning Kit for Sequencing, Version E2 (Invitrogen). The LMP2A construct was verified by sequencing. The β-actin control construct [28] was obtained from T. Dobner. After digestion with *Spe*I (LMP2A probe) or with *Eco*RI (β-actin probe), the constructs were *in vitro* transcribed by T7 (LMP2A probe) or SP6 (β-actin probe) polymerase, respectively, with the Riboprobe *in vitro* Transcription System (Promega) to generate $\alpha$-$^{32}$P-CTP-labeled antisense RNA probes. The labeled RNA probes were immediately purified with Centri-Spin-20 columns (Princeton Separations) before hybridization.

### *DNA Sequences*

Oligonucleotides (Metabion, Martinsried, Germany) corresponding to EBV nucleotides [27] 165869–165893 and 166590–166566 were used for sequencing LMP2Ap. Both strands were sequenced from the genomic DNA of all cell types on an ABI 377 DNA sequencing system using dye-labeled ddNTPs. In the analysed region of LMP2Ap (nucleotides 166132–166557) a few sequence polymorphisms were noted in the Mutu subclones (GenBank accession numbers AJ414534 and AJ414535 for Mutu-BL-I-Cl-216 and Mutu-BL-III-Cl-99) and in the Rael and CBM1-Ral-STO cell line (GenBank accession numbers AJ414532

and AJ414533), whereas the sequence of LCL 721 was identical to the standard B95-8 sequence [27]. There was one G less in the G-run between 166208 and 166214 and a G to T transversion at nucleotide 166523 in the Mutu subclones, a G to C transversion at nucleotide 166397 and a T to C transition at nucleotide 166423 in the Mutu subclones, Rael and CBM1-Ral-STO cell lines. The latter two polymorphisms created two additional CpG dinucleotides that are absent from the B95-8 sequence (Fig. 3).

### *Automated Genomic Sequencing of Sodium Bisulfite-treated DNA*

We used the method of Frommer et al. [29] and Clark et al. [30] adopted for an automated DNA sequencer [31]. Five microgram of genomic DNA in 50 µl of water was denatured by adding 5.5 µl of freshly prepared 3 M NaOH and incubating for 15 min at 37°C. Freshly prepared 30.5 µl of 10 mM hydroquinone (Sigma), and 530 µl of 3.6 M sodium bisulfite, pH5 (Sigma), were added to the denatured DNA, mixed gently and divided into five 0.5 ml PCR tubes, overlaid with mineral oil, and cycled five times at 95°C for 3 min and 55°C for 57 min.

After this treatment, the modified DNA was purified using a GeneClean kit (BIO 101) according to the manufacturers instructions. Then the DNA was desulfonated by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M and incubating the mixture for 15 min at 37°C. After desulfonation the DNA was ethanol precipitated and dissolved in water. One hundred nanogram of freshly modified DNA was used for PCR amplification with the strand specific [29] outer primer pairs designed for the LMP2Ap region (see Table 1). The 50 µl PCR reaction contained 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 40 pmol each of the primers, 0.2 mM each of the four dNTPs, and 2 units of *Taq* polymerase (Promega). Three microliter of a 1 in 100 dilution from the first PCR reaction was amplified in a second nested PCR reaction, using the primers listed in Table 1. One of the nested primers was biotin-labeled and the other carried 15 bases of the M13 universal primer at its 5′-end. The reaction mixture of the nested PCR was the same as the first one, except that the amount of inner primers was 10 pmol each. The product of the second PCR reaction was bound to streptavidin coated magnetic beads (Dynal) and the purified biotin-labeled strand was sequenced using the AutoRead DNA sequencing kit (Amersham Pharmacia Biotech) and a fluorescein-labeled M13 universal primer as described by Myöhänen et al. [31]. The reaction products were separated on acrylamide gels using an automated DNA sequencer (Amersham Pharmacia Biotech). The degree of methylation was estimated as described earlier [31]. The bisulfite conversion reaction was complete, since all cytosines outside CpG-dinucleotides were converted to uracil and therefore sequenced as thymine instead of cytosine after PCR (see Fig. 2).

*Table 1.* Primers and PCR conditions used for the amplification of bisulfite modified EBV LMP2A promoter region

| |
|---|
| Outer primers for modified LMP2Ap |
| (1) TATGAAGGAAAAGGATGGGAGT (166028–166049) |
| (2) ATAACACTCTTACCTTCCTCTACCC (166929–166905) |
| Inner primers for modified LMP2Ap |
| (3) Univ-GGGAAAAATGATAAGGAGGAAG (166110–166131) |
| (4) Univ-GTGTGAAGATTGTTATAGTTGTTGGT (166302–166327) |
| (5) Biotin-ACCATTTCTAAAAACCCCATAAC (166580–166558) |
| (6) Biotin-GGGAAAAATGATAAGGAGGAAG (166110–166131) |
| (7) Univ-ACCATTTCTAAAAACCCCATAAC (166580–166558) |

Primer positions are referring to the nucleotides of the B95-8 sequence [27]. 'Univ-' indicates the M13 universal primer sequence (GTAAAACGACGGCCA). Primers were purchased from Metabion (Martinsried, Germany). Each PCR reaction was cycled 30 times at the temperatures and times as follows: for primer pairs (1–2) 95°C for 40 s, 56°C for 40 s, 72°C for 75 s; for primer pairs (3–5 and 6–7) 95°C for 40 s, 54°C for 40 s, 72°C for 60 s; for primer pair (4–5) 95°C for 40 s, 54°C for 40 s, 72°C for 50 s.

### *Dimethyl Sulfate In Vivo Footprinting*

Genomic footprinting was performed essentially as described [32]. For each footprint reaction $10^7$ exponentially growing cells were harvested, washed with phosphate buffered saline (PBS), resuspended in 1 ml of PBS and incubated at room temperature for 1 min with 5 µl of dimethyl sulfate (DMS). The reaction was stopped by the addition of 5 ml DMS stop solution containing 1% bovine serum albumin and 100 µM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in

1 ml of PBS and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment [33]. For visualization of footprints by LMPCR, 2 µg of sequenced or footprinted DNA were analysed as described [34,35] with modifications [32]. Coordinates of LM-PCR primers are listed in the legend of Fig. 4. The first stand primer extension reaction was done in 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 [25°C] (Vent buffer, New England Biolabs), containing 0.3 pmol of primer (1) of each set, 240 µM each dNTP, and 1 unit of Vent (exo-)DNA polymerase (New England Biolabs) for 5 min at 94°C, 30 min at 60°C, and 10 min at 72°C. For ligation of the common linker, the sample was transferred to ice and 5 µl of PCR linker mix as in Mueller and Wold [35], 2 µl of ligation buffer (660 mM Tris–HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5 [20°C], Boehringer Mannheim), 1 µl of T4-DNA-ligase (5 U/µl, Boehringer Mannheim), and 12 µl of water were added. After an overnight incubation at 4°C the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. The PCR amplification was done in 100 µl Vent buffer containing 10 pmol of each primer (2) and the longer linker primer, 240 µM of each dNTP, and 1 unit of Vent (exo-)DNA polymerase for 20 cycles using 1 min at 94°C, 1.5 min at 60°C, and 3 min at 72°C. For labeling, the sample was transferred to ice and 5 pmol of T4-kinase [$\gamma$-$^{32}$P]ATP labeled primer (3), 2.5 nmol of each dNTP, 0.5 units Vent (exo-)DNA polymerase in a volume of Vent buffer not exceeding 15 µl were added. Then the sample was heated to 94°C for 1.5 min, subjected to 8 cycles of 2 min at 94°C, 2 min at 62°C, and 5 min at 72°C, and kept at 72°C for five more minutes. Samples were phenol/chloroform extracted, ethanol precipitated, ethanol washed, and resuspended in loading dye. One tenth of each sample was separated on a 5% sequencing gel, and the gels were dried and autoradiographed at room temperature using Kodak BioMax MR film.

## Results

### *Cell Type-specific Expression of LMP2A*

For our analysis we chose a set of five cell lines representative for both latency type I (Mutu-BL-I-Cl-216 and Rael) and latency type III (Mutu-BL-III-Cl-99, CBM1-Ral-STO and LCL-721). All of these cell lines are in a strictly latent state as documented by terminal repeat analysis (J. Minarovits, unpublished data) [36] and by the absence of early antigens (J. Minarovits, unpublished data). In addition, highly methylated specific segments of the EBV genome were found in most of these cells (J. Minarovits, unpublished data) [20,37] indicating the absence of lytic cycle viral DNA. Western blot experiments with a monoclonal antibody against EBNA2 on total cellular extracts were consistent with the classification of the analysed cell lines into class I and class III latency (D. Salamon, unpublished data). Northern blotting of mRNA from the cells and hybridization with a probe containing the first exon of the LMP2A transcript, showed signals in all type III cells, while in type I cells LMP2A transcripts could not be detected (Fig. 1).

### *Cell Type-specific Differences in Methylation Patterns of LMP2Ap*

To create a high-resolution methylation map of LMP2Ap we used automated genomic sequencing of sodium bisulfite-treated DNA in all cell lines. Fig. 2 shows typical nucleic acid sequences of bisulfite modified DNAs with direct sequencing of the PCR products. The summary of methylation patterns of LMP2Ap is shown in Fig. 3.

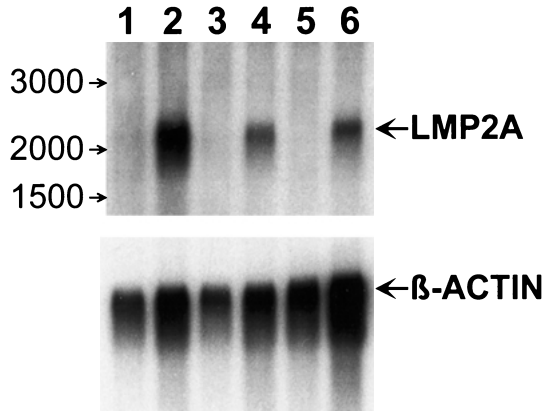


*Fig. 1.* LMP2A northern blot. Poly-A-mRNA from B cell lines was consecutively hybridized with probes for LMP2A and β-actin, respectively. Lane 1: Mutu-BL-I-Cl-216, lane 2: Mutu-BL-III-Cl-99; lane 3: Rael, lane 4: CBM1-Ral-STO, lane 5: DG75, lane 6: LCL-721. Numbers at the left of the panel indicate size marker positions.

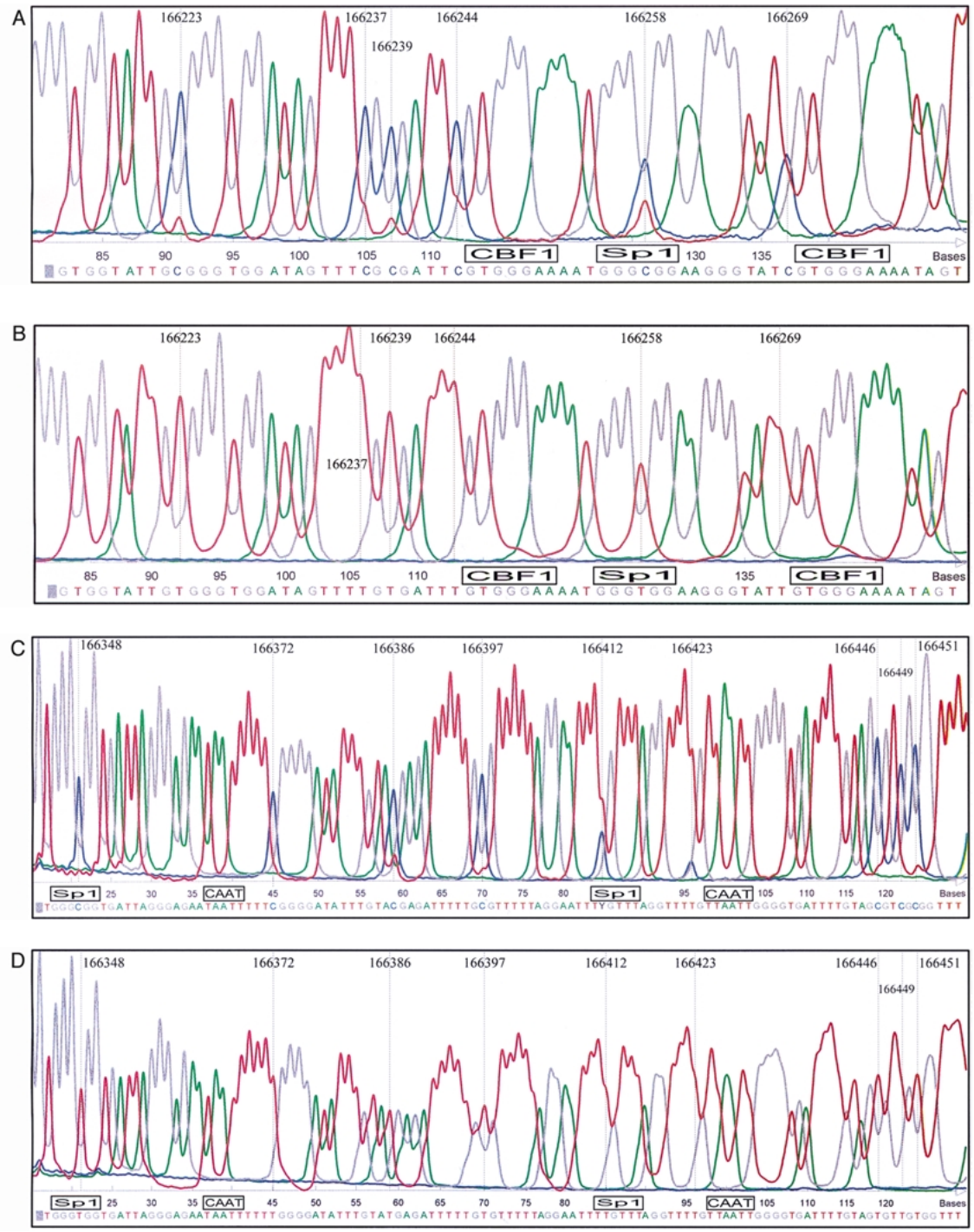


*Fig. 2.* Nucleic acid sequence of bisulfite-modified DNAs in LMP2Ap of latent EBV genomes carried by lymphoid cell lines. Numbers adjacent to vertical lines indicate positions of cytosines within CpG dinucleotides. Numbering at the bottom of each panel indicates distances from the sequencing primers used. In panel A, a highly methylated region around the CBF1 binding and Sp1 binding sites (open boxes) in Mutu-BL-I-Cl-216 is shown. In panel B, the same sequence area is shown from unmethylated CBM1-Ral-STO DNA. In panel C, the region around the CCAAT and Sp1 binding sites (open boxes) in Rael DNA is demonstrated. In panel D, the same region is demonstrated from completely unmethylated CBM1-Ral-STO DNA.

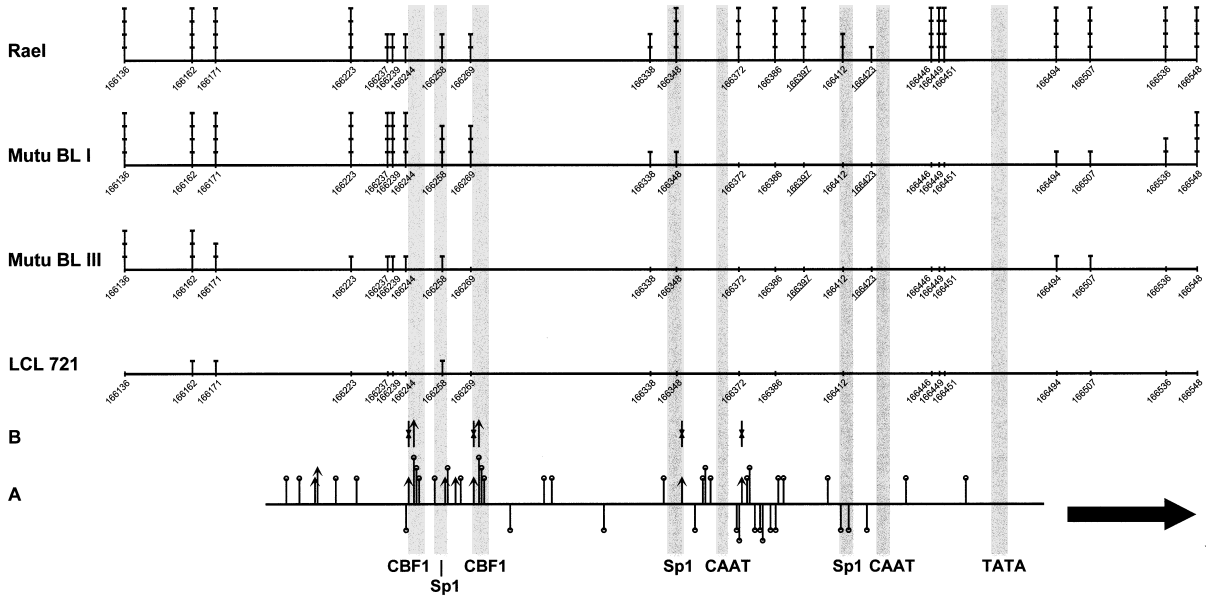


*Fig. 3.* Summary of genomic footprinting results and methylation patterns in the sequenced region of LMP2Ap. Numbers and 'lollipops' or spots indicate positions of cytosines within CpG dinucleotides, based on the prototype B95-8 sequence [27]. Underlined numbers mark additional CpG dinucleotides that are absent from the B95-8 sequence. The degree of methylation of cytosines is indicated by the height of the 'lollipops' as follows: spot only: 0%; one lollipop-unit: 0–25%; two units: 25–50%; three units: 50–75%; four units: 75–100%. CBM1-Ral-STO DNA was completely unmethylated in the examined area, and is not represented in the figure. Line A shows the summary of genomic footprints for the upper and lower strand of LMP2Ap (symbols above and below the line, respectively). Guanines protected from methylation by dimethyl sulfate are indicated by lollipops, enhanced reactivities to DMS (hypersensitivities) are shown by arrows. Arrows and crossed out lines at Level B indicate hypersensitivities instead of protections or absence of hypersensitivities in type I cells compared to type III cells. Faint columns represent already published or hypothetical *in vitro* binding sites. The transcription initiation site of LMP2A is shown by a thick arrow.

All cytosines outside CpG dinucleotides were found to be unmethylated in all cell lines. In Rael, three regions (nucleotides 166136–166223; 166348–166397 and 166446–166548) carried highly methylated (>50%) CpG dinucleotides and one region (nucleotides 166237–166338) carried moderately methylated (25–50%) CpG dinucleotides. Cytosine 166412 was also moderately methylated while cytosine 166423 was minimally (0–25%) methylated in Rael. In Mutu-BL-I-Cl-216 one region (nucleotides 166136–166269) contained highly methylated CpG dinucleotides, cytosine 166548 was highly methylated and cytosine 166536 was moderately methylated. One region (nucleotides 166338–166507) in Mutu-BL-I-Cl-216 carried only minimally methylated or completely unmethylated CpG dinucleotides. In Mutu-BL-III-Cl-99 cytosines 166136 and 166162 were highly methylated, cytosine 166171 was moderately methylated, while all other CpG dinucleotides were minimally methylated or completely unmethylated. In LCL-721 all the CpG dinucleotides were minimally methylated or totally unmethylated. In CBM1-Ral-STO cells LMP2Ap was completely unmethylated.

## In Vivo *Protein Binding at LMP2Ap*

In order to elucidate the *in vivo* protein binding at LMP2Ap, DMS *in vivo* footprinting combined with LM-PCR was done on both strands of the promoter in all cell lines. The pattern of guanines protected from methylation and nucleotides hypersensitive to methylation through DMS *in vivo* is summarized in Fig. 3.

There were two Sp1-like sequences at nucleotides 166258 (Fig. 4A) and 166413 with protections at nucleotides 166411 and 166414 that showed signs of protein binding in all cell lines regardless of methylation and two CCAAT-boxes at nucleotides 166364 and 166425 without protein protection (data not shown). There were protections in all cell lines at nucleotides 166371, 166372, 166378, 166380, 166381, 166384, 166386, and 166421 of the lower strand (data not

shown), and at several loci outside the above mentioned nucleotide positions and transcription factor binding sites (Figs 3 and 4). The methylation interference patterns obtained *in vitro* with oligonucleotides and HeLa nuclear extracts at L2BF2 and L2BF3 were not seen in this analysis [38]. Further, we found several sites that showed a different, mostly stronger footprint pattern in class III latency cells than in class I latency cells. The two CBF1 sites (Fig. 4A), and two hypersensitivities at nucleotides 166350 and 166373 (Fig. 4B) belonged into that category. The difference was clearest at the two CBF1 sites. There was a characteristic CBF1 footprint in class III cell types, whereas the CBF1 site protection patterns of inactive promoters were less intensive and different from the typical CBF1 methylation interference pattern that has been established with nuclear extracts from M-ABA, a marmoset LCL [39].

## Discussion

A crucial mechanism involved in the silencing of EBV latency promoters has been shown to be methylation of CpG dinucleotides [14–20,23,36]. Therefore an essential step in understanding the regulation of LMP2A is to elucidate the patterns of methylation of LMP2Ap at nucleotide resolution in a set of cell lines representing latency types I and III.

Altogether there were 15 highly methylated CpG dinucleotides in Rael and 10 in Mutu-BL-I-Cl-216, while there were only two in Mutu-BL-III-Cl-99 and none in CBM1-Ral-STO and LCL-721 (Fig. 3). Thus,

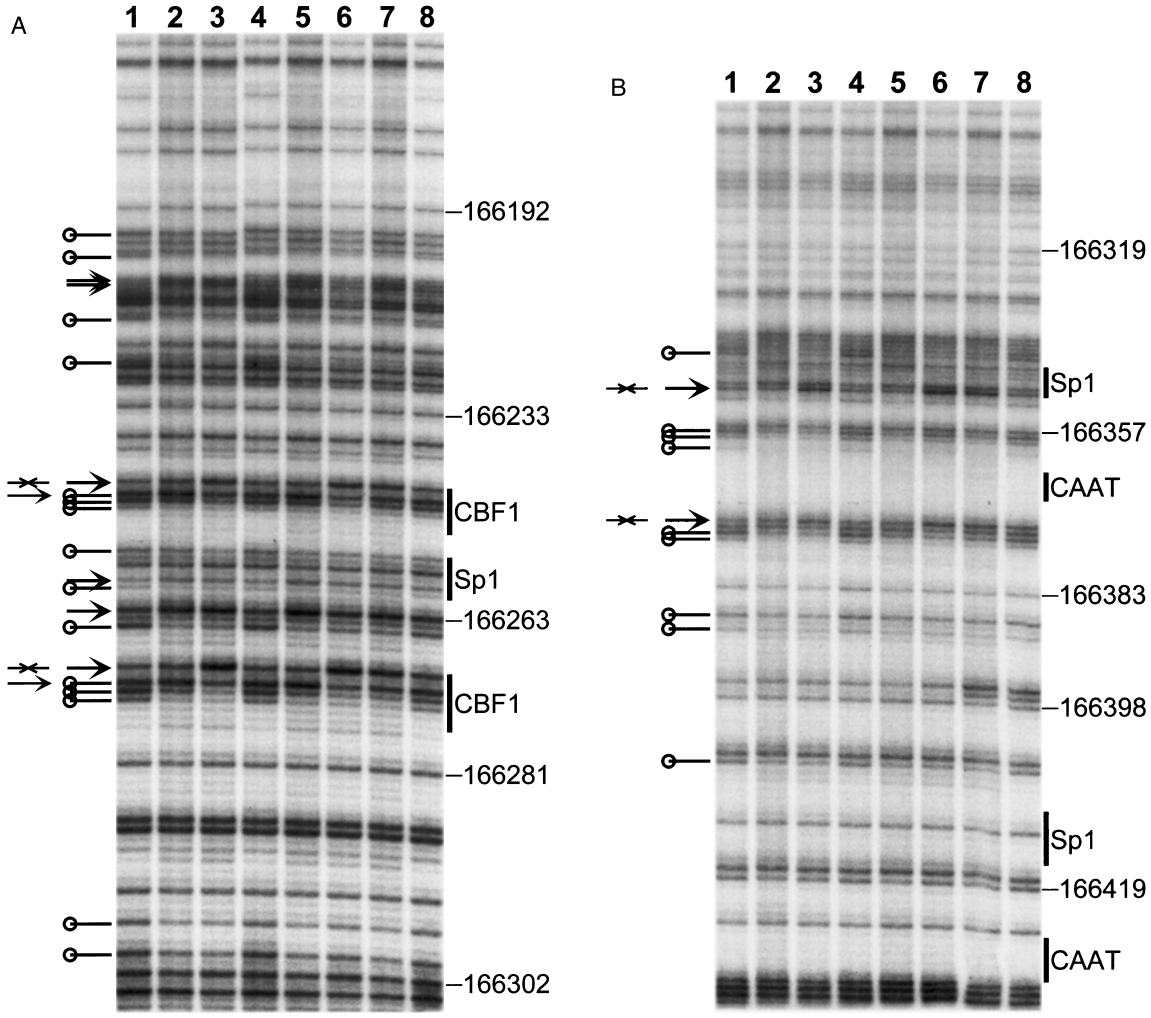


*Fig. 4.* Continued.

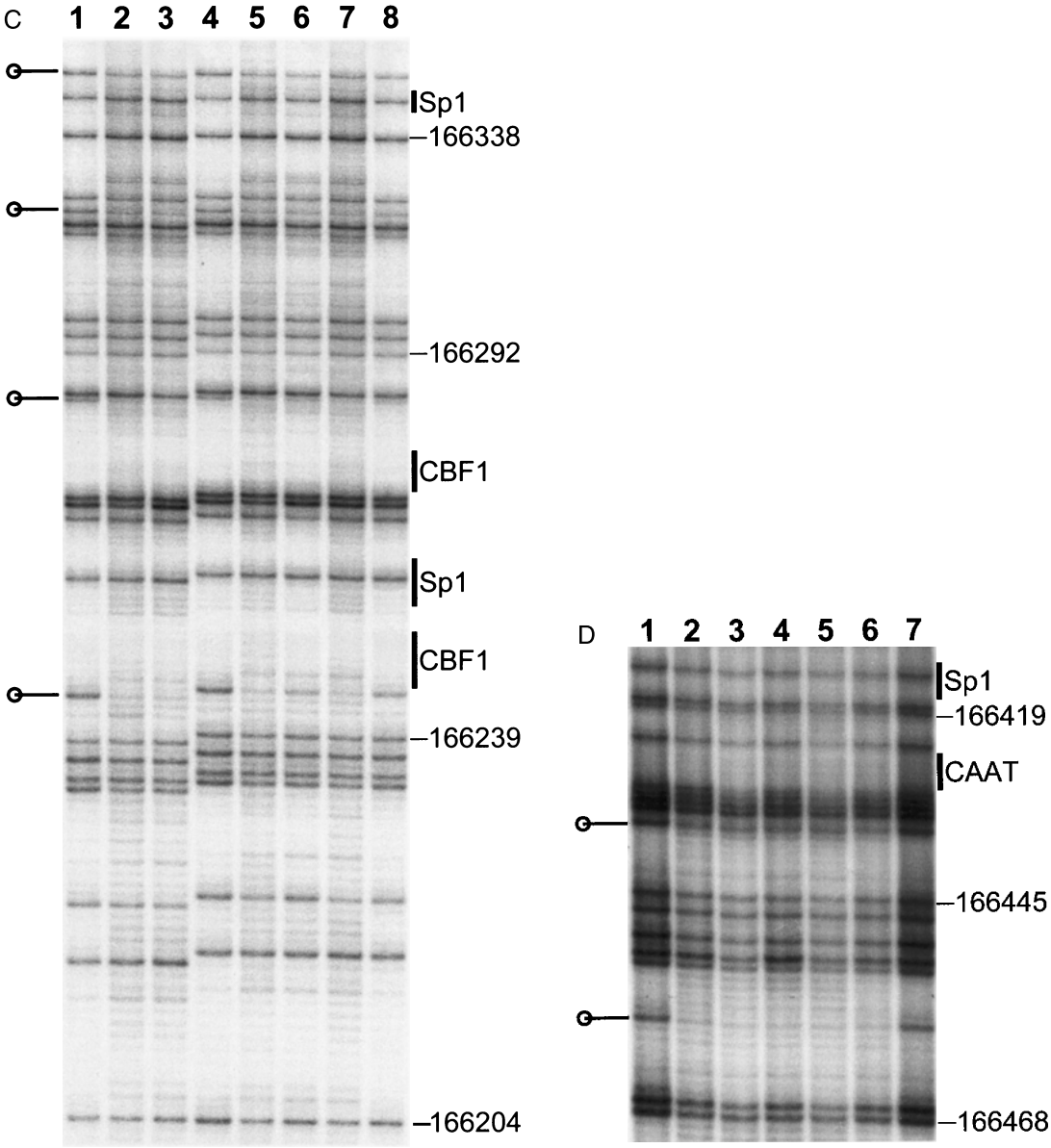


*Fig. 4.* Genomic footprinting analyses of LMP2Ap. Panels: A, B, and D, each: upper strand, panel C: lower strand. Panels A, B, and C, each: lanes 1: Mutu-BL-I-CL-216 G-track, lanes 2: Mutu-BL-I-CL-216 footprint, lanes 3: Mutu-BL-III-CL-99 footprint, lanes 4: Rael G-track, lanes 5: Rael footprint, lanes 6: CBM1-Ral-STO footprint, lanes 7: LCL-721 footprint, lanes 8: LCL-721 G-track. Panel D: lane 1: Mutu-BL-I-CL-216 G-track, lane 2: Mutu-BL-I-CL-216 footprint, lane 3: Mutu-BL-III-CL-99 footprint, lane 4: Rael footprint, lane 5: CBM1-Ral-STO footprint, lane 6: LCL-721 footprint, lane 7: LCL-721 G-track. At the left of each panel the location of *in vivo* footprints is indicated by arrows for hypersensitivities and lollipops for protections. Arrows and crossed out lines in the very left row indicate hypersensitivities instead of protections or absence of hypersensitivities in type I cells compared to type III cells. At the right of each panel nucleotide numbers are given according to the B95-8 sequence [27]. Previously described *in vitro* footprints or sequence consensus elements are indicated by vertical bars. In the following, LM-PCR primer coordinates are referring to nucleotide numbers of the B95-8 sequence [27]. Primers were purchased from Metabion (Martinsried, Germany). Primer set α: (1) 166128–166152; (2) 166163–166189; (3) 166198–166222. Primer set β: (1) 166128–166152; (2) 166163–166189; (3) 166163–166189. Primer set γ: (1) 166285–166309; (2) 166305–166331; (3) 166328–166352. Primer set δ: (1) 166410–166387; (2) 166390–166366; (3) 166365–166341. Primer set ε: (1) 166577–166553; (2) 166551–166526; (3) 166515–166490. Because a G was deleted from the G-stretch around nucleotide 166210 in Mutu DNA, a variant primer 3 of primer set α was used for Mutu cells.

LMP2Ap was generally less methylated in active promoters than in inactive promoters. However, there were only three individual cytosines at positions 166171, 166223, and 166548 of LMP2Ap which showed a clear inverse correlation between the methylation status and promoter activity. Position 166548, however, resides in the first exon of LMP2A and probably does not play a major role in the regulation of the promoter. There were no important *cis* regulatory elements shown to reside at positions 166171 and 166223 either. Yet, in Rael there were partially demethylated regions around the crucial CBF1 sites and around the promoter proximal Sp1 site, while in Mutu-BL-I-Cl-216 cells the region around and upstream from the CBF1 sites was highly methylated but the whole 3′ region of the promoter downstream from the CBF1 sites was hypomethylated. Therefore, patches of methylation in the 3′ region (Rael), or in the 5′ region (Mutu-BL-I-Cl-216) seem to be sufficient for promoter inhibition. Previously, we observed a mosaic of highly methylated and unmethylated sequences upstream of Cp in Mutu-BL-I-Cl-216. Cp is also silent in these cells due to two hypermethylated regions located upstream and downstream of hypomethylated attachement sites for CBF1 and other important transcription factors [20]. Overall, this data suggests that patches of methylation rather than methylation of particular CpG dinucleotides may play a role in the host cell dependent regulation of LMP2Ap activity.

Previous *in vitro* binding and reporter gene experiments charted two CBF1 sites and two more elements L2BF2 and L2BF3 immediately downstream of the CBF1 sites [21,38–41]. Furthermore, based on sequence homology, two Sp1 binding sites and two CCAAT boxes have been suggested [40]. Our DMS *in vivo* footprinting analysis of LMP2Ap showed that several additional protein binding sites besides the two CBF1 sites and the two Sp1 sites may play a role in the regulation of LMP2Ap activity (Figs 3 and 4). There were several footprint differences that identified the active and inactive promoter, respectively. Differential footprinting was found at the two binding sites for CBF1 and two more sites at nucleotides 166350 and 166373. The identity of these presumably activating transcription factors should be established. The CBF1 site protection patterns of inactive LMP2Ap were remarkable, because they were different from the CBF1 methylation interference pattern that has been established with nuclear extracts from M-ABA, a marmoset LCL [39], while in active LMP2Ap the CBF1 sites carried this *in vitro* pattern from M-ABA [39]. Therefore, it is unclear at present whether CBF1 is actually bound at the inactive promoter. It should be also noted that the CBF1 footprint pattern of the inactive LMP2Ap were similar to the CBF1 footprint pattern observed on the LMP1p independently of LMP1p activity and on the inactive Cp [20]. Further *in vivo* experiments are required to answer these questions and to elucidate exactly the role of additional proteins in the regulation of LMP2Ap, Cp, and LMP1p through CBF1.

The mechanisms contributing to the establishment of *de novo* DNA methylation patterns and regulating DNA demethylation are unknown at present. We detected a series of protein–DNA interactions in the regulatory region of LMP2Ap of latent EBV genomes. The majority of these footprints could be observed both in cells carrying highly methylated and hypomethylated LMP2Ap regulatory sequences (Fig. 3). This suggests that most protein–DNA interactions revealed by DMS *in vivo* footprinting do not influence the actual DNA methylation patterns in this region. The other way round, in Rael cells the presence of a series of methylated CpGs does not prevent the majority of protein–DNA interactions charted in the LMP2Ap regulatory region (Fig. 3).

In this study, we present the first high-resolution methylation maps and genomic footprints of LMP2Ap. Our data suggest a role for DNA methylation in the regulation of LMP2Ap activity. This is consistent with the known cell type dependent, methylation-mediated silencing of two other EBNA2 regulated EBV latency promoters, Cp and LMP1p [15–17,20,36]. Besides the key regulatory protein CBF1, further protein determinants also play a role in activating LMP2Ap, as has been described for Cp [20,42]. Based on *in vivo* footprinting experiments recently we identified c-Myc as a protein binding to EBER-1 regulatory sequences [43]. It will be interesting to use a similar approach to establish the identity of additional transcription factors for LMP2Ap as well.

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