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Mapping of Herpesvirus Saimiri Proteins on the Viral Genome: Proteins Dependent and Not Dependent on Viral DNA Synthesis

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Hybrid selected translation was used to map the genome of herpesvirus saimiri, a lymphotropic and oncogenic herpesvirus. RNA extracted from virus-infected cells was hybridized to cloned genomic fragments, and the hybrid selected mRNAs were translated in vitro in a rabbit reticulocyte lysate. Forty-five virus-induced polypeptides were identified and correlated to their coding regions on the herpesvirus saimiri genome. Inhibition of the replication of viral DNA with phosphonoacetic acid showed that 22 of these polypeptides belong to the early group of herpesvirus saimiri gene products.

Herpesvirus saimiri can be isolated from populations of the New World primate *Saimiri sciureus* (squirrel monkey) in which it is ubiquitous without causing any detectable disease (17). However, infection of related primate species such as marmosets and owl monkeys leads to the development of fatal lymphoproliferative diseases (6, 8, 18). The availability of permissive cell lines, e.g., owl monkey kidney (OMK) cells, the possibility to produce tumors in artificial hosts, and the existence of various transformed cell lines which were isolated from those tumors permit the analysis of the lytic cycle of herpesvirus saimiri replication and also the investigation of the molecular mechanisms of viral oncogenesis.

The regulation of herpesvirus saimiri gene expression has been studied on the basis of virus-induced proteins appearing in the lytic cycle (11, 20, 22, 26). The time-ordered cascadelike synthesis of more than 30 viral proteins, various protein modifications, and proteins with specific functions, e.g., formation of structural components or binding of DNA, could be detected (2, 21). The infectious herpesvirus saimiri genome consists of two different regions: a 110-kilobase-pair unique sequence (L-DNA) which seems to be the only coding part of the genome (30) and two flanking sequences (H-DNA) composed of multiple repeats (3, 7, 9). Physical mapping of the genomic DNA with different restriction endonucleases revealed variations among the herpesvirus saimiri strains (5). At least one deletion has been linked to a biological property, the inability to induce fatal diseases in marmosets (13, 28).

In this study we present another approach of mapping the herpesvirus saimiri genome. We used in vitro translation of hybrid selected viral mRNA to correlate herpesvirus saimiri proteins with their coding regions. Inhibition of the virus-specific DNA polymerase with phosphonoacetic acid (PAA) allowed the differentiation of early and late transcription products.

MATERIALS AND METHODS

Cells and viruses. OMK cells (line 637) were grown at 37°C in minimal essential medium (Earle salts) supplemented with 10% heat-inactivated fetal calf serum, 20 mM glutamine, and 100 U of penicillin per ml in 32-oz. (960-ml) glass prescription bottles. Herpesvirus saimiri 11 was originally obtained from B. Fleckenstein, Institut für Klinische Virologie, University of Erlangen, Erlangen, Federal Republic of Germany. OMK cells were infected with herpesvirus saimiri 11 at 1 to 2 PFU per cell and kept at room temperature for an adsorbtion period of 2 h. The virus solution then was removed, and the cell cultures were replenished with minimal essential medium containing tetradecanoyl phorbol acetate at a concentration of 20 ng/ml (15). For preparation of early virus-induced mRNA, PAA was added to the infection medium at a concentration of 200 μg/ml.

RNA extraction. Medium was removed after 15 to 20 h of incubation, and the cell monolayers were washed with ice-cold phosphate-buffered saline. Cells were lysed with 4 M guanidine rhodanide–0.5 M mercaptoethanol–50 mM sodium acetate (pH 5.5) (4, 16). This method was chosen to efficiently inactivate the cellular RNases and to yield a high amount of translatable mRNA. The cell debris was removed by centrifugation at 20,000 rpm for 1 h in an SW41 rotor, and the cell lysate was layered on a cesium chloride cushion (1.8 g/cm^3 in 10 mM triethanolamine–1 mM EDTA [pH 7.4]). RNA was separated from cell lysates by centrifugation for 18 to 20 h at 35,000 rpm (SW41 rotor), extracted once with chloroform–4% isoamylalcohol, and precipitated with ethanol (at −20°C overnight). After washing twice with 70% ethanol, RNA was lyophilized and resolved in sterile bidistilled water.

In vitro translation. Purified RNA probes were translated in vitro by a rabbit reticulocyte system (25). The reticulocyte lysate was prepared similarly to the method described previously by W. C. Merrick (19). About 1 to 1.5 μl of RNA solution (concentration, 3 to 5 mg/ml) was heated to 70°C for 90 s and cooled on ice; then the translation mixture (9 μl of reticulocyte lysate, 0.4 μl of cocktail containing 800 mM HEPES [*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 400 mM creatine phosphate, 40 mM ATP, 2 mM GTP, 40 mM dithiothreitol, 20 mM spermidine, 2 mM cysteine), 0.3 μl of amino acid mixture, 1.5 μl of [^{35}S]methionine (specific activity, >800 Ci/mmol; final concentration in the translation mixture, approximately 1.5 mCi/ml), 0.5 μl of 2 M potassium acetate, 0.8 μl of 20 mM magnesium acetate, and 1.0 μl of calf liver tRNA (2 mg/ml) were added; the probes were incubated at 30°C for 2 h.

The translation products were suspended in 50 mM Tris (pH 7.0)–2% sodium dodecyl sulfate (SDS)–5% mercaptoethanol–3% sucrose–bromphenol blue, heated for 5 min at

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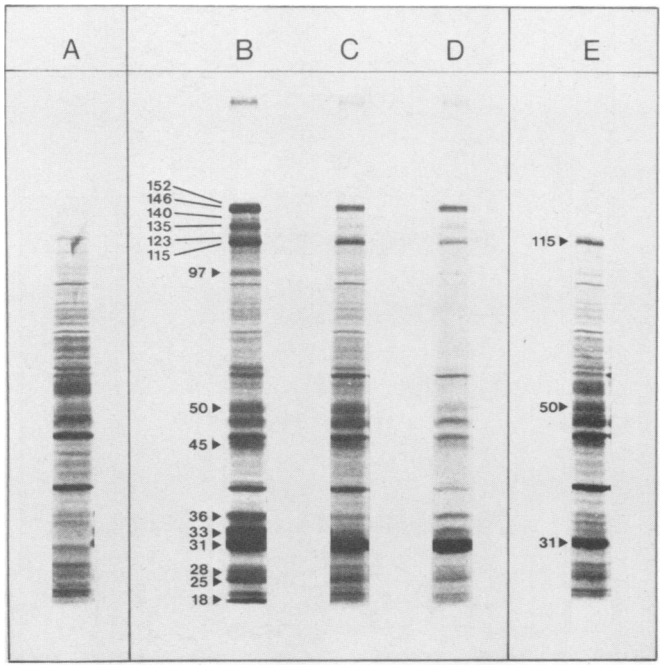

FIG. 1. In vitro translation of total RNA isolated from mock-infected and herpesvirus saimiri-infected OMK cells. RNA from mock-infected cells (A) and from herpesvirus saimiri-infected cells extracted 20 (B), 25 (C), and 30 (D) h postinfection. (E) RNA extracted 15 h postinfection from herpesvirus saimiri-infected OMK cells inhibited with 200 μg of PAA per ml. RNA samples were in vitro translated by using a rabbit reticulocyte system and [^{35}S]methionine as the radioactive marker. Translation products were separated on a 12.5% SDS–polyacrylamide gel. Virus-induced polypeptides are marked with black arrows and with their molecular weights.

100°C, and separated directly in SDS-polyacrylamide gels (14). SDS-polyacrylamide gel electrophoresis was performed as described previously (20).

Hybridization selection. For hybridization we used a set of *Eco*RI and *Kpn*I restriction fragments representing the entire nonrepetitive part of the herpesvirus saimiri genome (12). Plasmids pACYC 184 (containing the *Eco*RI B, D, E, F, G, H, I, K, L, M, and O fragments), pJC 81 (containing the *Kpn*I B, C, D, and F fragments), and pWD 7 (containing the terminal L-DNA *Kpn*I fragments E and G), and the lambda Charon 4A phage containing the *Eco*RI C fragment were obtained from the laboratory of B. Fleckenstein and propagated in *Escherichia coli* as described previously by E. Knust et al. (12). Cloned DNA fragments (8 to 10 μg) were bound to nitrocellulose filters (5 by 5 mm; BA 85; Schleicher & Schuell). The filters were washed with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), air dried, baked at 80°C for 2 h, boiled twice in 300 μl of bidistilled water for 90 s each, rapidly cooled on ice, and dried again in a desiccator. Approximately 10 to 20 μg of purified cellular RNA was hybridized to two filters in 100 μl of 0.4 M NaCl–20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid; pH 7.0]–45% formamide. The reactions were incubated for 2 h at 50°C. After hybridization, filters were washed in 1× SSC–0.5% SDS at 60°C and then in 2 mM EDTA. Hybridized RNA was eluted from the filters by heating in 300 μl of H_2O for 75 s at 100°C and subsequently precipitated with ethanol (at −70°C overnight). The RNA was pelleted, washed twice with 70% ethanol, and then lyophilized. The dried RNA was suspended in water, and portions of these probes were translated in vitro as described above.

RESULTS

In vitro translation of total RNA. Total RNA was extracted from OMK cells infected with herpesvirus saimiri 11 at different times after infection, from infected cells which were inhibited by PAA, and from mock-infected cells. At 15 h (infection plus PAA) and at 20, 25, and 30 h postinfection, RNA was prepared by the guanidine rhodanide method modified from the method of Chirgwin et al. (4). The RNA samples were translated in vitro with a rabbit reticulocyte system (25) with [^{35}S]methionine for radioactive labeling of the resulting proteins. Translation products were separated on SDS-polyacrylamide gels (Fig. 1). Virus-induced polypeptides could be identified by comparing the in vitro translation products of mRNA from noninfected cells with those of RNA from infected cells. In translating RNA extracted 20 h postinfection we found 15 virus-specific products with molecular weights ranging from 152,000 to 18,000. Proteins of higher molecular weight were not detected by in vitro translation. Viral proteins are conspicuous against the cellular background, especially in the region between 160 and 90 kDa. In addition, similarly, the predominant 31-, 33-, and 36-kDa proteins and the 25-kDa protein are clearly detectable, whereas others cannot be discerned because cellular proteins comigrate with viral products of the same molecular weight or because translation of a viral mRNA is so low that the corresponding protein is not visible on the gel. When RNA was extracted later in the infection cycle (25 or 30 h postinfection), the amount of some of the viral products decreased, e.g., the 135-, 97-, 36-, and 31-kDa proteins. The cellular background proteins also were reduced. RNA extraction at a later stage in lytic infection was not possible because most of the cell monolayer was destroyed by the virus.

The addition of 200 μg of PAA per ml to the culture medium during the infection of OMK cells with herpesvirus saimiri inhibits the virus-specific DNA polymerase (23). Proteins or mRNAs which are independent of viral DNA synthesis are still produced in these cells. Therefore, this inhibition led to a reduced number of virus-specific proteins identified by in vitro translation (Fig. 1E). Only the 115-, 50-, and 31-kDa proteins could still be detected. Transcription of those genes was not restricted to viral DNA synthesis. Additional early proteins which were not found on the late level did not occur. The cellular background remained unchanged during inhibition with PAA.

Translation of hybrid selected late viral mRNA. A set of 18 *Eco*RI and *Kpn*I restriction fragments cloned in plasmids (pACYC184, pJC81, and pWD7) or in lambda Charon 4A (12) were used for hybridization selection of specific viral mRNAs. These fragments covered the entire nonrepetitive part of the herpesvirus saimiri genome. Total cellular RNA (10 to 20 μg) was hybridized to 16 to 20 μg of plasmid or phage DNA bound on small nitrocellulose filters. Incubation with 45% formamide at 50°C (corresponding to 77°C without formamide) for 2 h was found to be the best condition for hybridization due to the relatively low G+C content of herpesvirus saimiri L-DNA (36% G + C; calculated T_m in 0.1 M aqueous salt, 82.9°C [29]). Higher formamide concentrations decreased the hybridization temperature too much. Extension of the incubation time led to decreasing production of large viral proteins, presumably due to the reduced stability of larger mRNA molecules.

Analysis by hybrid selected translation of the late viral

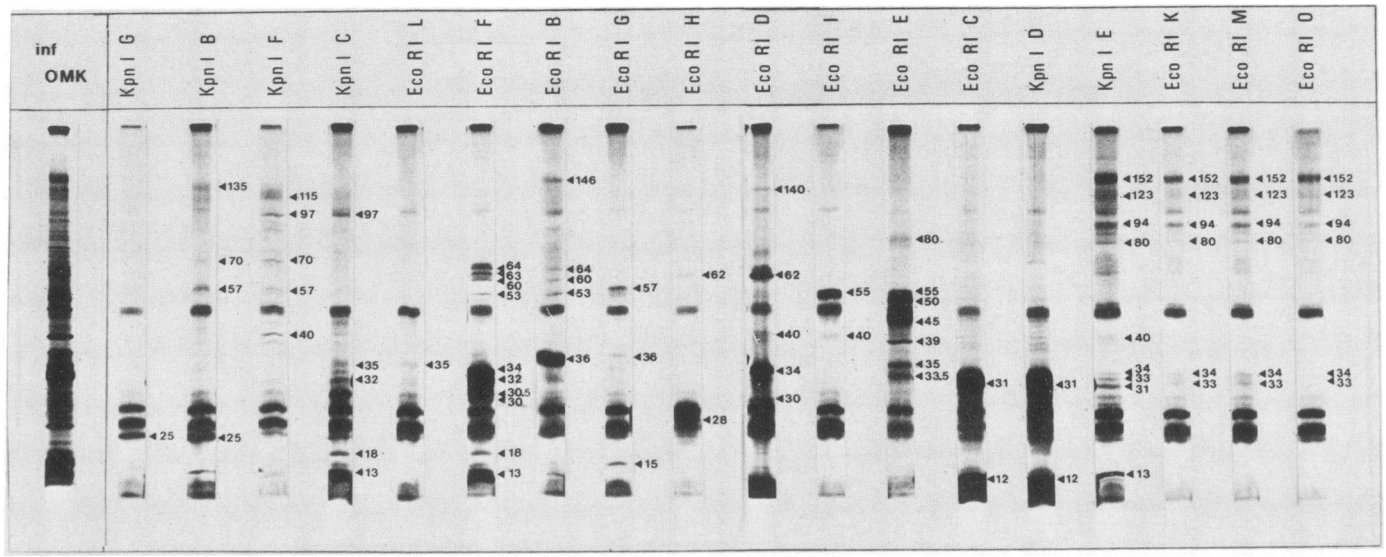

FIG. 2. In vitro translation of hybrid selected late mRNAs. Total RNA was extracted from OMK cells 20 h postinfection with herpesvirus saimiri and then hybridized to cloned DNA fragments representing the entire herpesvirus saimiri genome. Hybrid selected mRNAs were in vitro translated, and the [^{35}S]methionine-labeled translation products were analyzed on a 15% SDS–polyacrylamide gel. The leftmost lane shows in vitro translation products of total RNA.

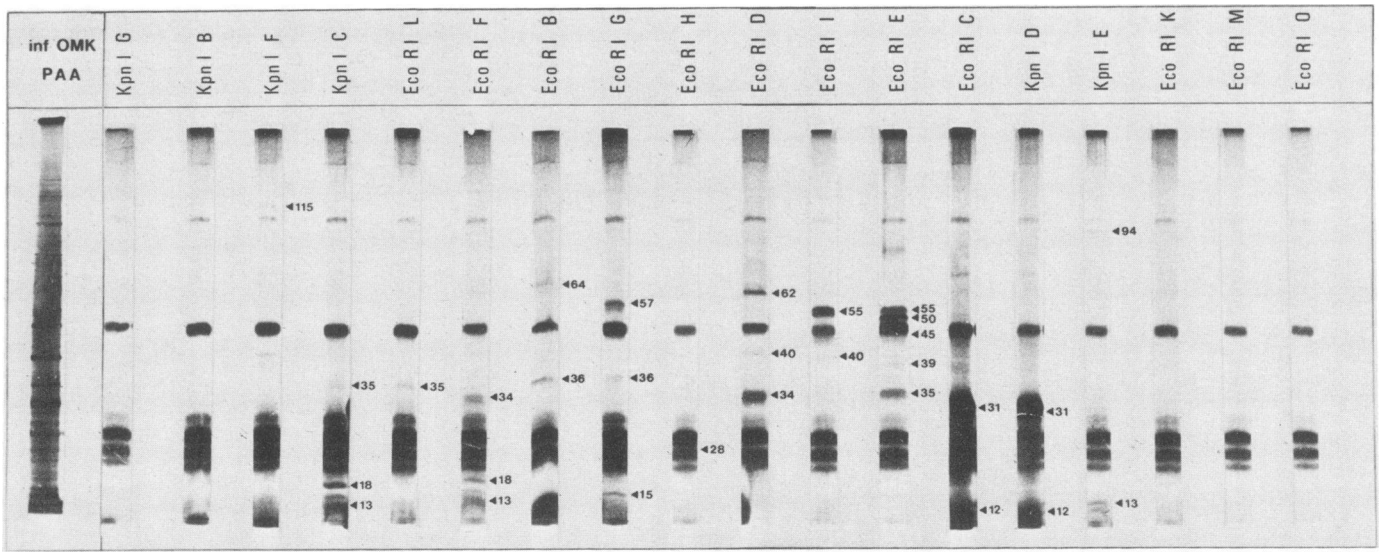

FIG. 3. In vitro translation of hybrid selected early mRNAs. OMK cells were infected with herpesvirus and inhibited with 200 μg of PAA per ml. RNA was extracted 15 h postinfection and hybridized to the same viral genome fragments as those in Fig. 2. Hybrid selected mRNAs were in vitro translated, and the resulting polypeptides were separated on a 15% SDS–polyacrylamide gel.

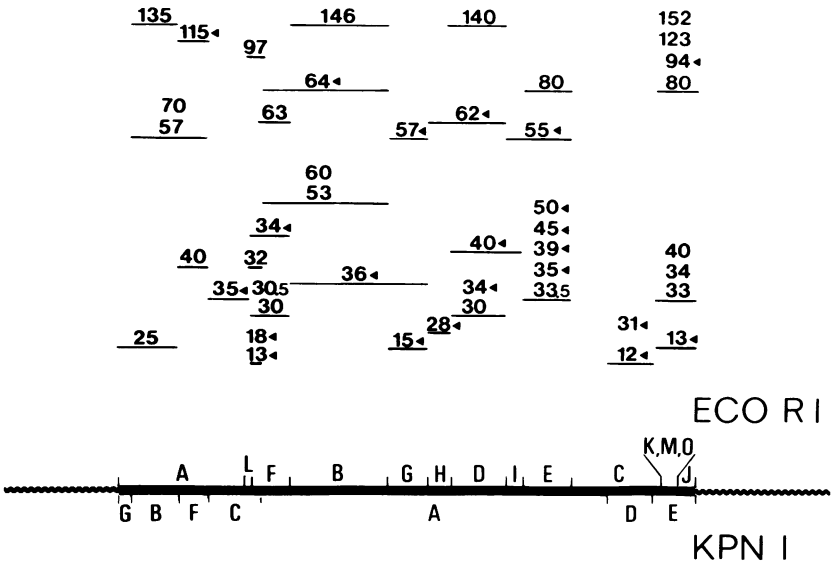

FIG. 4. Map of the viral polypeptides detected by hybrid selected translation. The herpesvirus saimiri L-DNA is shown with the *Eco*RI and *Kpn*I restriction enzyme sites. The potential coding regions of the viral proteins identified by in vitro translation of hybrid selected late and early mRNAs are presented as black lines with the molecular weights of the peptides. Viral proteins of the early stage of the lytic infection cycle are marked with black arrows.

mRNA extracted at about 20 h postinfection allowed the correlation of 45 virus-specific polypeptides to the different regions of the herpesvirus saimiri genome (Fig. 2). This number is significantly higher than the one detected by in vitro translation of total RNA. All the viral proteins identified by in vitro translation of total RNA were also found by hybrid selected translation. The molecular weights of the proteins ranged from 152,000 to 12,000. The coding genes were distributed over the whole L-DNA, and distinct regions for late transcription were not evident.

Hybrid selected translation of early mRNA. Hybridization selection with the RNA samples prepared after inhibition with PAA also was done with the same restriction fragments. The mapping of the proteins translated from early messengers is presented in Fig. 3. Only 22 translation products could be identified; most of the large proteins (152, 146, 135, and 123 kDa) were absent. Those large proteins were previously shown to be structural products (20, 21). The coding regions of the early proteins also were distributed over the entire genome, but specific early regions were not identified. All of these early gene products are found among the late viral proteins (Fig. 2). However, the existence of intermediate viral proteins whose synthesis is shut off in late phase of infection could not be demonstrated by in vitro translation because an appropriate synchronization of the infection cycle in OMK cells could not be achieved.

DISCUSSION

In vitro translation of hybrid selected RNA is a powerful method to map the different coding regions of viral proteins on the genome (27, 28a). In our experiments we used this method to analyze the L-DNA of herpesvirus saimiri and thus to correlate the observations at the protein level described in previous publications (11, 20, 26) with specific regions of the herpesvirus saimiri genome. The H-DNA was not investigated because there is no evidence for gene expression in this repetitive part of the genome (30). Figure 4 summarizes the results of the hybrid selected translation experiments. A total of 45 virus-induced polypeptides ranging from 152 to 12 kDa were identified and mapped on the genome. The number of polypeptides was higher than the protein number detected by in vivo labeling experiments (20) and by in vitro translation of total RNA. However, at some of the genomic fragments, e.g., *Eco*RI-E, *Eco*RI-F, and *Kpn*I-E, clusters of polypeptides were found, and those clusters may represent degradation products of defined viral proteins or may be the result of a truncated translation of the same messenger. The coding capacity of especially the *Eco*RI E, K, M, and O and the *Kpn*I E fragments would be too small to encode such a number of different proteins if these are encoded by linearly linked reading frames without overlaps.

Compared with the in vitro translation of total RNA and the in vivo labeling of proteins, the identification of virus-specific proteins is facilitated by hybrid selected translation because the cellular background is avoided except for a few proteins derived from the translation system and because the different viral proteins of similar molecular weights are normally separated from each other. For example, the three double bands of 152-146, 140-135, and 123-115 kDa were split by hybridization selection of the corresponding mRNAs. Also in the lower-molecular-weight range of 40,000 to 30,000, some proteins which were produced in smaller amounts could be detected when the dominating 31-, 33-, and 36-kDa proteins were removed. Another effect is the enrichment of viral products which cannot be seen in vivo due to their low rate of synthesis.

A direct correlation of in vivo and in vitro data without immunoprecipitation by monoclonal antibodies is only possible for those viral proteins that are not processed in vivo. Posttranslational modifications such as glycosylation, sulfatation, and phosphorylation are not included in the in vitro translation system, and cleavage of proteins in vitro does not necessarily reflect the in vivo situation. Proteins of 152, 146, 140, 135, and 123 kDa are found both in vitro and in vivo, and they seem to correlate because processing of those large proteins may not be dominant (152 and 146 kDa) or is lacking (140, 135, and 123 kDa) (22). Comparison of

proteins in the molecular weight region below 100,000 is speculative. An exact correlation would be possible only by immunoprecipitation with monospecific antisera or monoclonal antibodies.

In vivo labeling of virus-induced proteins revealed a regulated way of lytic expression of herpesvirus saimiri, which is reflected by the successive appearance of virus-induced proteins. This is similar to the situation in herpes simplex virus (10) or in Epstein Barr virus (1). The time-ordered synthesis is also seen on the mRNA level. By using PAA as an inhibitor of the virus-specific DNA polymerase (23), two classes of herpesvirus saimiri gene products can be distinguished: the early mRNAs or proteins which are synthesized without prior DNA replication and the late mRNAs and proteins. Synthesis of late products is turned on when viral DNA is produced. Most of them are structural proteins, and they are not needed when viral DNA is not obtained to build up new virions. There are more early proteins found by hybrid selected translation than by in vivo labeling experiments (24). This may have an explanation in a higher sensitivity of the in vitro system; alternatively, it could be due to translational rather than transcriptional control of some of these proteins.

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LITERATURE CITED

1. **Bayliss, G. J., and H. Wolf.** 1981. The regulated expression of Epstein-Barr virus. III. Proteins specified by EBV during the lytic cycle. J. Gen. Virol. **56**:105–118.
2. **Blair, E. D., and R. W. Honess.** 1983. DNA-binding proteins specified by herpesvirus saimiri. J. Gen. Virol. **64**:2697–2715.
3. **Bornkamm, G. W., H. Delius, B. Fleckenstein, F.-J. Werner, and C. Mulder.** 1976. Structure of herpesvirus saimiri genomes: arrangement of heavy and light sequences in the M-genome. J. Virol. **19**:154–161.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry **18**:5294–5299.
5. **Desrosiers, R. C., and L. A. Falk.** 1982. Herpesvirus saimiri strain variability. J. Virol. **43**:352–356.
6. **Falk, L. A.** 1980. Biology of herpesvirus saimiri and herpesvirus ateles, p. 813–832. *In* G. Klein (ed.), Viral oncology, Raven Press, New York.
7. **Fleckenstein, B., G. W. Bornkamm, and H. Ludwig.** 1975. Repetitive sequences in complete and defective genomes of Herpesvirus saimiri. J. Virol. **15**:398–406.
8. **Fleckenstein, B., and R. C. Desrosiers.** 1982. Herpesvirus saimiri and herpesvirus ateles, p. 253–332. *In* B. Roizman (ed.), The herpesviruses, vol. 1. Plenum Publishing Corp., New York.
9. **Fleckenstein, B., and C. Mulder.** 1980. Molecular biological aspects of herpesvirus saimiri and herpesvirus ateles, p. 799–812. *In* G. Klein (ed.), Viral oncology, Raven Press, New York.
10. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
11. **Keil, G., B. Fleckenstein, and W. Bodemer.** 1983. Structural proteins of herpesvirus saimiri. J. Virol. **47**:463–470.
12. **Knust, E. S. Schirm, W. Dietrich, W. Bodemer, E. Kolb, and B. Fleckenstein.** 1983. Cloning of herpesvirus saimiri DNA fragments representing the entire L-region of the genome. Gene **25**:281–289.
13. **Koomey, J. M., C. Mulder, R. L. Burghoff, B. Fleckenstein, and R. C. Desrosiers.** 1984. Deletion of DNA sequences in a nononcogenic variant of herpesvirus saimiri. J. Virol. **50**:662–665.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Luka, J., B. Kallin, and G. Klein.** 1979. Introduction of Epstein-Barr virus cycle in latently infected cells by n-butyrate. Virology **94**:228–232.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Melendez, L. V., M. D. Daniel, R. D. Hunt, and F. G. Garcia.** 1968. An apparently new herpesvirus from primary kidney cultures of the squirrel monkey (saimiri sciureus). Lab. Anim. Care **18**:374–381.
18. **Melendez, L. V., R. D. Hunt, M. D. Daniel, F. G. Garcia, and C. E. O. Fraser.** 1969. Herpesvirus saimiri. II. Experimentally induced malignant lymphoma in primates. Lab. Anim. Care **19**:378–386.
19. **Merrick, W. C.** 1983. Translation of exogenous mRNAs in reticulocyte lysates. Methods Enzymol. **101**:606–615.
20. **Modrow, S., and H. Wolf.** 1983. Herpesvirus saimiri induced proteins in lytically infected cells. I. Time ordered synthesis. J. Gen. Virol. **64**:37–46.
21. **Modrow, S., and H. Wolf.** 1983. Characterization of herpesvirus saimiri and herpesvirus ateles structural proteins. Virology **125**:251–255.
22. **Modrow, S., and H. Wolf.** 1984. Characterization of herpesvirus saimiri and herpesvirus ateles induced proteins, p. 105–126. *In* G. Wittmann, R. M. Gaskell, and H.-J. Rziha (ed.), Latent herpesvirus infections in veterinary medicine. Martinus Nijhoff Publishers, Boston.
23. **O'Hare, P., and R. W. Honess.** 1983. Evidence for a herpesvirus saimiri-specified DNA polymerase activity which is aphidicolin-resistant and phosphonoacetate-sensitive. J. Gen. Virol. **64**:1013–1024.
24. **O'Hare, P., and R. W. Honess.** 1983. Identification of a subset of herpesvirus saimiri polypeptides synthesized in the absence of virus DNA replication. J. Virol. **46**:279–283.
25. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
26. **Randall, R. E., R. W. Honess, and P. O'Hare.** 1983. Proteins specified by herpesvirus saimiri: identification and properties of virus-specific polypeptides in productively infected cells. J. Gen. Virol. **64**:19–35.
27. **Ricciardi, R. P., J. S. Miller, and B. E. Roberts.** 1979. Purification and mapping of specific mRNAs by hybridization-selection and cell-free translation. Proc. Natl. Acad. Sci. USA **76**:4927–4931.
28. **Schaffer, P. A., L. A. Falk, and F. Deinhardt.** 1975. Attenuation of herpesvirus saimiri for marmosets after successive passage in cell culture at 39°C. J. Natl. Cancer Inst. **55**:1243–1246.
28a. **Seibl, R., and H. Wolf.** 1985. Mapping of Epstein-Barr virus proteins on the genome by translation of hybrid-selected RNA from induced P3HR1 cells and induced Raji cells. Virology **141**:1–13.
29. **Thomas, M., R. L. White, and R. W. Davis.** 1976. Hybridization of RNA to double-stranded DNA: formation of R-loops. Proc. Natl. Acad. Sci. USA **73**:2294–2298.
30. **Tracy, S., and R. C. Desrosiers.** 1980. RNA from unique and repetitive DNA sequences of herpesvirus saimiri. Virology **100**:204–207.