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Short Communication

Truncated versions of the two major Epstein–Barr viral glycoproteins (gp250/350) are secreted by recombinant Chinese hamster ovary cells

(Recombinant DNA; membrane proteins; eukaryotic expression vector; amplification; nasopharyngeal carcinoma)

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SUMMARY

The expression of the two Epstein–Barr virus (EBV) major membrane proteins gp250/350 (MA-BLLF1) on the surface of recombinant CHO clones cannot be amplified by methotrexate (MTX) selection, perhaps due to toxic effects of these membrane proteins. After removal of sequences encoding the part of the glycoproteins responsible for membrane anchorage, the gp250/350 is secreted into the medium. Following selection with MTX, this construct allows the amplification of the expression products. Besides the possible use of these proteins in protection experiments, they can also be used as antigens for diagnosis, which opens an efficient approach for control of EBV-related neoplasias by early therapy.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous human γ herpesvirus which causes mononucleosis after primary infection and persists lifelong in man. The virus is associated with two neoplasias: the EBV-positive Burkitt lymphoma and an undifferentiated form of NPC, which has a remarkably high incidence in some areas of South-East Asia.

There are two possibilities for control of these neoplasias: either by early diagnosis and treatment, or by vaccination in an attempt to delay or modify

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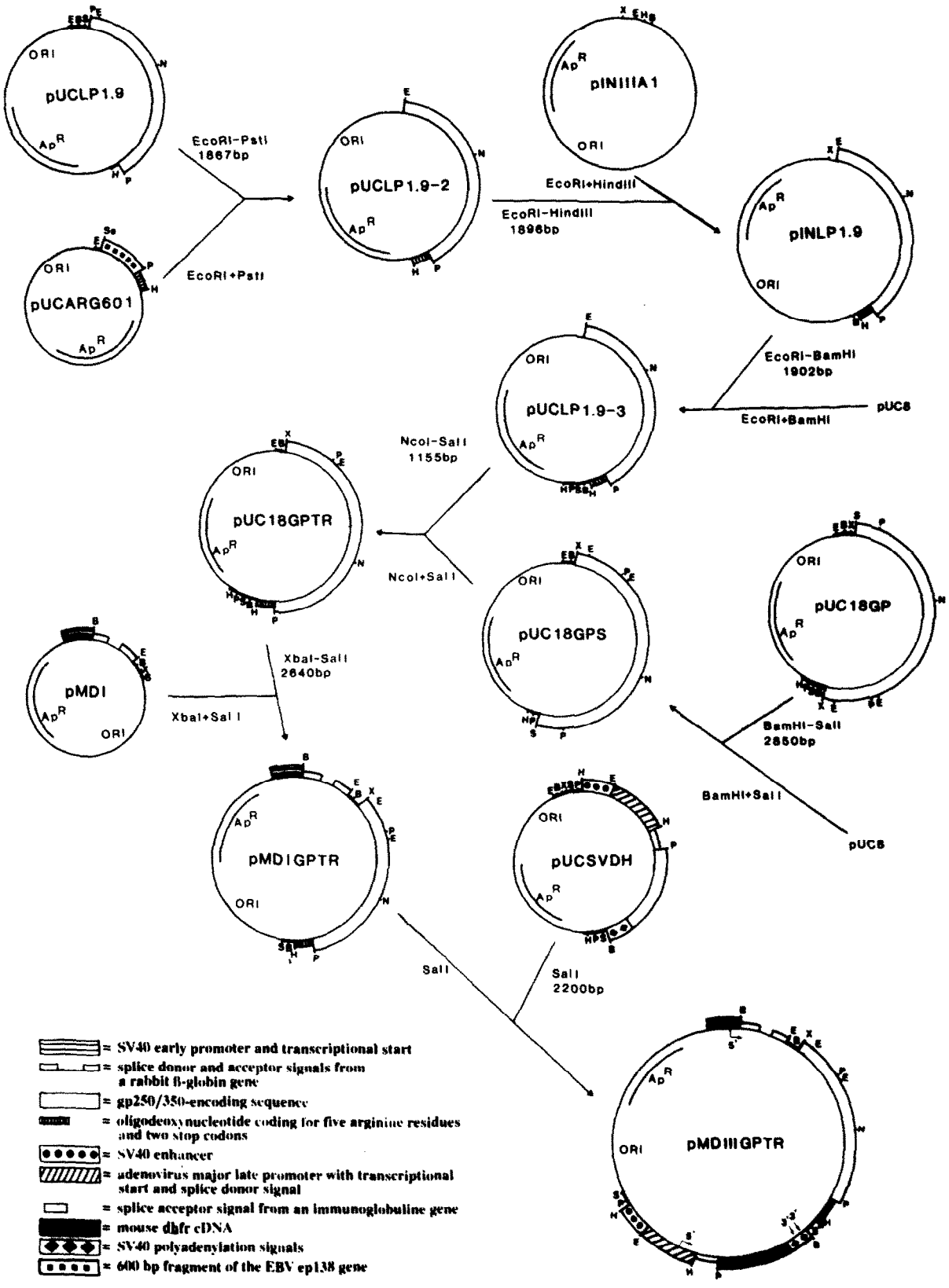
Abbreviations: Ad, adenovirus; BLLF1, first leftward reading frame on the EBV *Bam*HI-L fragment; bp, base pair(s); CHO, Chinese hamster ovary cells; DHFR, dihydrofolate reductase; *dhfr*, gene coding for DHFR; EBV, Epstein–Barr virus; IgG, immunoglobulin G; kb, kilobases or 1000 bp; MA, membrane antigen; MEM α^-, minimal essential medium lacking all nucleotides; MTX, methotrexate; NPC, nasopharyngeal carcinoma; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na_3·citrate, pH 7.5; SV40, simian virus 40; [ ], designates plasmid-carrier state.


pUCLP1.9
ORI
Ap^R
EcoRI-PstI
1867bp
EcoRI+PstI
pUCARG601
pUCLP1.9-2
pINIIIA1
EcoRI+HindIII
EcoRI-HindIII
1896bp
pINLP1.9
EcoRI-BamHI
1902bp
EcoRI+BamHI
pUC8
pUCLP1.9-3
NcoI-SalI
1155bp
NcoI+SalI
pUC18GPTR
pUC18GPS
pUC18GP
BamHI-SalI
2850bp
BamHI+SalI
pMDI
XbaI-SalI
2640bp
XbaI+SalI
pMDIGPTR
pUCSVDH
SalI
SalI
2200bp
pMDIIIGPTR
= SV40 early promoter and transcriptional start
= splice donor and acceptor signals from a rabbit ß-globin gene
= gp250/350-encoding sequence
= oligodeoxynucleotide coding for five arginine residues and two stop codons
= SV40 enhancer
= adenovirus major late promoter with transcriptional start and splice donor signal
= splice acceptor signal from an immunoglobuline gene
= mouse dhfr cDNA
= SV40 polyadenylation signals
= 600 bp fragment of the EBV ep138 gene

natural infection by EBV and possibly lower incidence rates.

Candidates for a possible EBV subunit vaccine are two viral envelope glycoproteins with M_rs of 250 000 and 350 000 (Epstein et al., 1985). Both proteins are encoded by the same viral reading frame, BLLF1 (Beisel et al., 1985), and are frequently addressed as MA-BLLF1 or gp250/350. The size differences are caused by a partial splice event which removes an internal part of the mRNA encoding gp350 to yield a smaller transcript, which in turn encodes gp250 in the same frame. More than half of the M_r is contributed by glycosyl residues. Recently we reported the synthesis of these glycoproteins in CHO using a vector which combines a *dhfr* gene together with the MA gene and eukaryotic regulation signals (Motz et al., 1986c).

The produced MA proteins were anchored in the membranes of the CHO and have an M_r slightly lower than that coded by the EBV-producing B95-8 cells. This may result from a different extent of glycosylation.

We attempted to amplify the recombinant sequences by MTX selection and, by doing so, increase expression. However, in our hands it was not possible to obtain clones which were able to overcome the MTX inhibition by amplification of the *dhfr* gene. This finding and the very low rate of successfully transfected cells made us assume that the integration of the foreign viral glycoproteins into the CHO cell membrane may become toxic when reaching certain levels.

EXPERIMENTAL AND DISCUSSION

(a) Construction of a eukaryotic expression plasmid coding for a truncated version of gp250/350

To prevent the integration of the glycoproteins into the cell membrane we removed gp250/350 sequences responsible for the membrane anchorage. Chou and Fasman (1978) predictions of the secondary structure, superimposed with values for hydrophilicity or hydrophobicity, predict a hydrophobic stretch with a β-sheet structure near the C terminus of the protein (Motz et al., 1986a). This part of the protein is believed to be responsible for membrane anchorage (Beisel et al., 1985) and the corresponding sequences were removed to allow secretion of gp250/350.

A *Pst*I site immediately 5′ of the transmembrane region was used for the construction of the truncated version shown in Fig. 1. Downstream from this *Pst*I site a short oligodeoxynucleotide encoding a trans-

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Fig. 1. Construction of the expression plasmid pMDIIIGPTR. The plasmid pUCLP1.9 (upper part, left; Motz et al., 1986a) contains the 1.9-kb *Pst*I fragment of the MA-BLLF1-gp250/350 coding region (a detailed map is given in Motz et al., 1986c); the 3′ end is located next to the *Hin*dIII site of the pUC-vector. By insertion of the 1867-bp *Eco*RI-*Pst*I-fragment from this plasmid into the *Eco*RI + *Pst*I-cut vector pUCARG601 (containing a fragment of the ep138 EBV gene and a short synthetic oligodeoxynucleotide; Motz et al., 1986b), the gp250/350-coding region lacking the sequences coding for membrane anchorage is terminated now by the oligodeoxynucleotide coding in frame for five arginine residues and two stop codons. For insertion into the eukaryotic expression vector pMDI (Motz et al., 1986c), it was necessary to generate a *Sal*I site at the 3′ end of the gene. This was achieved by insertion of the 1896-bp *Eco*RI-*Hin*dIII fragment from pUCLP1.9-2 into the *Eco*RI + *Hin*dIII-cut vector pINIIIA1 (Masui et al., 1984), isolation from pINLP1.9 as 1902-bp *Eco*RI-*Bam*HI fragment and ligation with *Eco*RI-*Bam*HI-cut pUC8 (Vieira and Messing, 1982). The resulting plasmid pUCLP1.9-3 now contains the 3′ part of the MA gene followed by the synthetic oligodeoxynucleotide and a *Sal*I site. The complete MA gene in pUC18GP (Motz et al., 1986c) was inserted into pUC8 as a *Bam*HI-*Sal*I fragment and the resulting plasmid pUC18GPS was cut with *Nco*I + *Sal*I which removes the 3′ part of the MA gene. This part was replaced by the 1155-bp *Nco*I-*Sal*I fragment from pUCLP1.9-3 to obtain pUC18GPTR now containing a truncated MA gene.

This truncated version of the MA gene was combined with the vector pMDI as a 2640-bp *Xba*I-*Sal*I fragment. In the resulting plasmid pMDIGPTR the MA gene is now located downstream from the SV40 early promoter and β-globin splice donor and acceptor sites. In a final step, pMDIGPTR was linearized by *Sal*I and combined with a 2200-bp *Sal*I fragment from pUCSVDH carrying the *dhfr* gene and transcriptional regulation signals (SV40 enhancer, major late Ad promoter and splice donor signal; a splice acceptor signal and the SV40 polyadenylation signals; Kaufman and Sharp, 1982). In the resulting vector pMDIIIGPTR the *dhfr* cassette has an orientation such that the SV40 polyadenylation signals are next to the MA gene and should terminate both the MA and *dhfr* transcripts. Abbreviations: ORI, origin of replication; Ap^R, β-lactamase gene. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; S, *Sal*I; Ss, *Sst*I; X, *Xba*I.

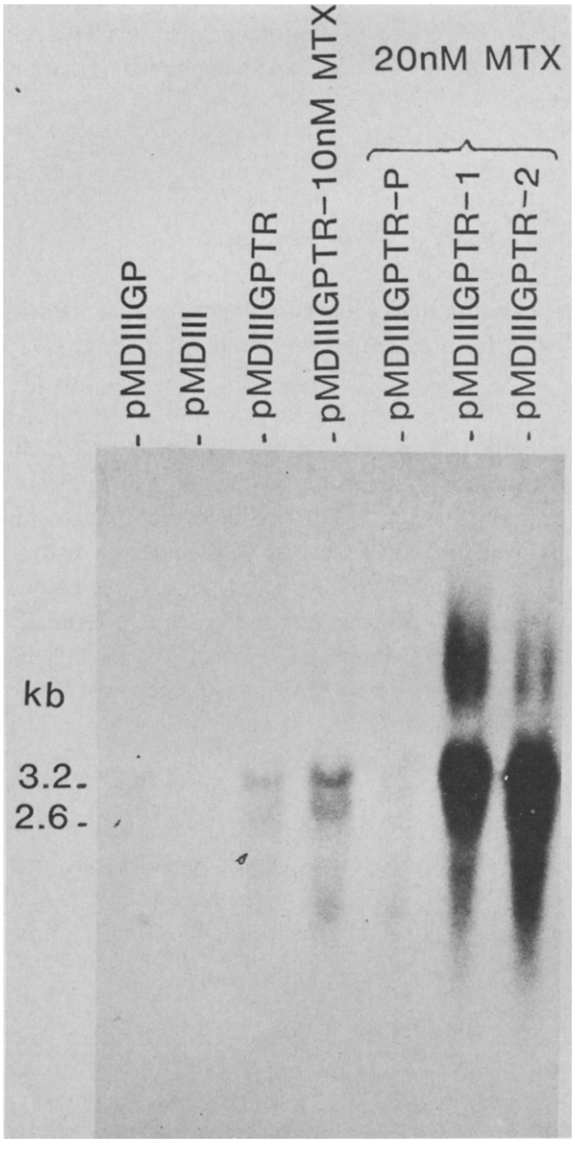

Fig. 2. Northern blot analysis of transcripts in recombinant CHO clones. 10 μg RNA obtained according to Chirgwin et al. (1979) and pelleted through CsCl (1.8 g/ml) was electrophoresed on a 1% agarose gel in MOPS buffer and in the presence of 6% formaldehyde. After transfer to nitrocellulose, EBV-specific transcripts were hybridized with [^{32}P]pUC18GP in 50% formamide, 5 × SSC and 2 × Denhardt's solution at 50°C for 20 h. The filter was washed 4 × 5 min with 2 × SSC and 0.1% SDS at room temperature and 2 × 15 min with 0.1 × SSC and 0.1% SDS at 68°C and exposed to an x-ray film filter for 48 h. Lanes 'pMDIIIGP' and 'pMDIII' contain RNA of the CHO clones with the membrane-anchored version (barely visible; Motz et al., 1986c) and the negative control. pMDIIIGPTR originates from a clone which secretes the EBV proteins into the medium. 'pMDIIIGPTR–10 nM MTX' shows the transcripts of a clone after amplification with 10 nM MTX. The following three lanes show the gp250/350-specific RNA from clones amplified with 20 nM MTX. 'pMDIIIGPTR-1' and '-2' were clones individually isolated after 20 nM MTX selection; 'pMDIIIGPTR-P' was obtained by combining all remaining clones from amplification and maintenance in culture for two months. As in CHO[pMDIIIGP], the truncated MA versions are also partially spliced and have nearly the same size, indicating that the SV40 polyadenylation signal is used for termination. The sizes of EBV-specific transcripts are indicated in kb on the figure.

lational stop codon was inserted. The sequences were combined in the steps shown in Fig. 1 to obtain the plasmid pMDIIIGPTR.

In comparison with the vector used for membrane-anchored expression (Motz et al., 1986c), the *dhfr* gene and its regulation signals are now inverted. This brings the SV40 polyadenylation signals next to the 3′ end of the EBV glycoprotein sequences. The polyadenylation of the early and late transcripts are located in the same area in reverse orientation on the SV40 genome. For that reason both transcripts, from the *dhfr* gene as well as from the glycoprotein sequence, should now be terminated by the same SV40 fragment.

Following transfection (Graham and Van der Eb, 1973) and selection of CHO in MEM α^- medium, many clones have been isolated without difficulty, as was seen with the membrane-anchored MA clones. None of the clones showed membrane fluorescence, but in six of eight culture supernatants tested the membrane protein was detectable.

(b) Sequence amplification

Amplification through MTX selection was now possible. After selection with 10 nM MTX, colonies were isolated and further amplified with 20 nM MTX. From these colonies two individual clones were isolated and the remaining colonies were pooled.

After two months in culture, the mRNA of these cell lines was analyzed in Northern blots (Fig. 2). With labeled gp250/350-specific probes, two bands of 2.6 kb and 3.2 kb can be detected, resulting from a partial splice event and corresponding to both gp variants. Compared to CHO cells without MTX selection a strong increase of the gp250/350-specific mRNA can be observed through the amplification procedure. The transcripts have nearly the same size as the mRNA from the CHO clone with the mem-

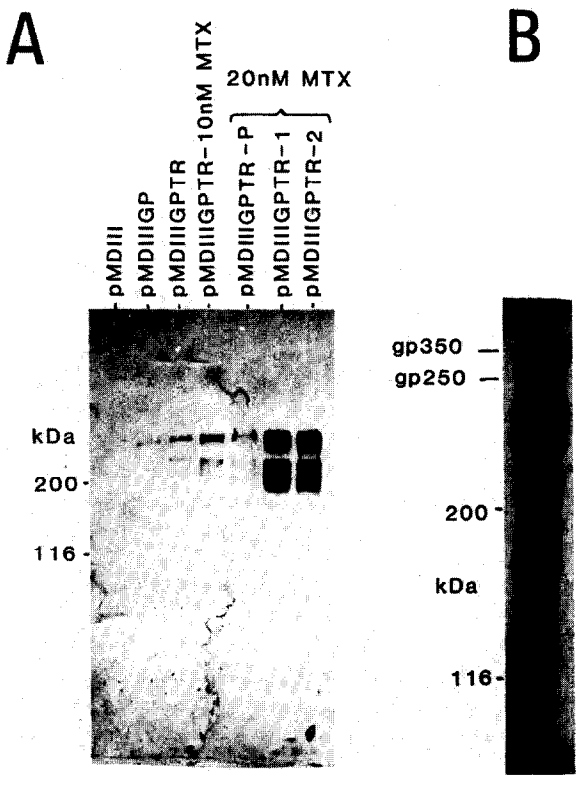

Fig. 3. Secretion of the truncated gp250/350 versions. (Panel A) Immunostained Western blot showing secreted gp250 and gp350 in the supernatant of the recombinant CHO clones. Proteins of 30 ml of culture medium (MEM α^- (Gibco); 10% fetal calf serum) from cells grown to confluence and maintained for 3 days were precipitated by 70% ammonium sulfate and dissolved in 3 ml of 20 mM Tris · HCl pH 7.5. 15 μl were loaded onto an SDS–10% polyacrylamide gel, the proteins electrophoresed and transferred to nitrocellulose. EBV-related proteins were detected by incubation with an NPC serum pool overnight, followed by incubation with anti-human IgG peroxidase-conjugated rabbit antibodies and staining with diaminobenzidine and peroxide. Lane 'pMDIII', negative control; lane 'pMDIIIGP', membrane-anchored version (the weak gp350-band may result from degraded cells); 'pMDIIIGPTR' and the following lanes, CHO clones with secreted MA versions and amplified as indicated with '–10 nM MTX' and '–20 nM MTX.' As described in the legend to Fig. 2, the last three lanes correspond to cells which were isolated individually (–1, –2) or derived from pooled colonies (–P). Amplification through MTX results in a strong enhancement of MA expression, but only in cells derived from individually isolated clones. (Panel B) A Coomassie blue-stained SDS–10% polyacrylamide gel with a gp250/350-positive fraction was derived through molecular sieving chromatography. Proteins from 300 ml culture medium from CHO[pMDIIIGPTR-1–20 nM MTX] maintained for three days were concentrated by a 70% ammonium sulfate precipitation, dissolved in 30 ml 20 mM Tris · HCl pH 7.5 and separated by molecular sieving through Sepharose 2B-CL (Pharmacia). MA-positive fractions were pooled, concentrated, an aliquot was analyzed by SDS–10% PAGE, and proteins were visualized by Coomassie blue staining. Positions of the 200-kDa and 116-kDa markers (myosin and β-galactosidase) are indicated as are the positions of gp250 and gp350. Due to their extensive glycosylations they appear as diffuse bands. From this figure it can be estimated that the glycoprotein production is in the range of 1–10 μg per 10^6 cells per day.

brane-anchored versions, indicating that the SV40 polyadenylation signals were used. Besides, additional larger transcripts can be detected in the lanes with the MTX-amplified clones. Probably they originate from an alternative termination downstream from the SV40 polyadenylation signal. Compared to the authentic transcripts from EBV-producing B95-8 cells these transcripts have slightly larger sizes as a result from different 5′-untranslated regions. An additional 140 nt were contributed by SV40 and globin sequences.

After selection in 20 nM MTX the two isolated clones have a strong amplification of the gp250/350 transcripts whereas in cells derived from the pooled (20 nM MTX) colonies transcripts were almost undetectable.

Fig. 3A shows an immunostained Western blot prepared from culture supernatant. The cells secrete increasing amounts of EBV proteins throughout the MTX selection process. The control experiment, a Western blot with NP40 cell extracts, allows the identification of the glycoproteins only in cells with the anchored versions, indicating that the truncated EBV products were totally secreted.

(c) Toxicity of the MA product

Despite MA secretion, it seems that their high-level production still has negative effects on the cells. Cells derived from pooled colonies after 20 nM MTX selection show almost no MA production after a two-month period in culture (Fig. 3A), as was already suggested by Northern blots (Fig. 2). This suggests that during selection in 20 nM MTX only the *dhfr* sequences were amplified in some of the pooled cells. These cells now have a growth advantage and displace the MA-producing cells. The assumption of an inhibitory effect is also supported by our inability to obtain a yet higher amplification level through increased MTX concentrations.

Quantification of the gp250/350 production is difficult. Fig. 3B shows a Coomassie blue-stained SDS–polyacrylamide gel with proteins of a culture supernatant. The glycoprotein yield corresponds to the production of 5×10^4 cells in three days and is estimated to be in the range of 1–10 $\mu g/10^6$ cells per day.

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