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Efficient synthesis of influenza virus hemagglutinin in mammalian cells with an extrachromosomal Epstein–Barr virus vector

(Recombinant DNA; glycoproteins; gene expression; cytomegalovirus; HeLa, CV-1 and 293 cells; sandwich hybridization)

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SUMMARY

The capability of an Epstein–Barr virus hybrid vector (EBV–CMV), containing the cytomegalovirus (CMV) immediate early enhancer and simian virus 40 promoter, to produce large amounts of authentic mammalian proteins was studied. The cDNA of influenza virus hemagglutinin (HA), a cell surface glycoprotein, was inserted into this vector and the EBV-CMV-*HA* plasmid was transfected into two human and two monkey cell lines. Southern-blot analysis revealed that the EBV-CMV-*HA* plasmid was maintained in extrachromosomal state and the recombinant cell clones contained on the average three copies (range 1–24) of the transfected DNA. The recombinant HA polypeptides from different cell clones, selected either randomly or by fluorescence-activated cell sorter, were analysed using immunological techniques. Three of the four cell lines expressed recombinant HA on the cell surface in glycosylated form. The highest production levels, 11.5 $\mu g/10^6$ cells, were obtained in HeLa cells containing only two copies of EBV-CMV-*HA* DNA per cell. The protein levels correlated with the mRNA levels in Northern-blot analysis. A corresponding vector, containing the same regulatory signals for *HA* expression, but lacking the EBV sequences, yielded clones with significantly lower expression levels. The results confirm that the extrachromosomal EBV–CMV vector is very useful in the production of apparently authentic mammalian glycoproteins.

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Abbreviations: aa, amino acid(s); ATCC, American Type Culture Collection; bp, base pair(s); BPV, bovine papillomavirus; CAT, chloramphenicol acetyltransferase; *cat*, gene coding for CAT; CMV, cytomegalovirus; EBV, Epstein–Barr virus; EBV-CMV and EBV-CMV-*HA* vectors, hybrid vectors (see Fig. 1); ELISA, enzyme-linked immunoadsorbent assay; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HA, hemagglutinin; *HA*, gene coding for HA; HSV, herpex simplex virus; IU, international unit(s); kb, kilobase(s) or 1000 bp; MEM, Eagle's minimum essential medium; moi, multiplicity of infection; NET, see MATERIALS AND METHODS, section **g**; nt, nucleotide(s); *ori*, origin of DNA replication; PBS, phosphate-buffered saline; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; SSPE, see MATERIALS AND METHODS, section **d**; SV40, simian virus 40; *tk*, thymidine kinase-coding gene; TRITC, see MATERIALS AND METHODS, section **f**.

INTRODUCTION

Extrachromosomal vectors based on viral *ori* have become important tools in the expression of cloned genes in mammalian cells. The main advantage is that in the extrachromosomal vector the transfected gene is free of rearrangements and interferences from chromosomal DNA.

Bovine papillomavirus (BPV) vectors have been extensively used in the expression of secretory or membrane-bound animal cell proteins, such as influenza virus hemagglutinin (Sambrook et al., 1985), tissue plasminogen activator (Sambrook et al., 1986) and factor VIII (Sarver et al., 1987). The BPV vector system is powerful, but its use is limited to rodent C127 and NIH3T3 host cells.

Epstein–Barr virus (EBV) vectors, having a large spectrum of host cell lines, offer a good alternative. Vectors based on an EBV *Bam*HI-C fragment that contains the proposed *ori* in the *oriP* segment (Yates et al., 1984; Sugden et al., 1985) and the *Bam*HI-K fragment that contains the nuclear antigen EBNA-1-encoding gene (Lupton and Levine, 1985) are capable of autonomous replication in several mammalian cell lines, such as the human HeLa, 293, K562, monkey CV-1, MA134 and dog D17 cells (Yates et al., 1985; Jalanko et al., 1988a,b; Young et al., 1988). Successful expression of the class-I HLA antigens in LCL721 cells (Shimizu et al., 1986) and tumor necrosis factor in 143/EBNA cells (Kioussis et al., 1987) and γ-interferon in 293 and CV-1 cells (Young et al., 1988) using EBV vectors has been reported.

Influenza virus HA is a well-characterized, integral membrane protein. Its synthesis and posttranslational processing by proteolysis and glycosylation has been intensively studied (Gething et al., 1980; Wilson et al., 1981; Schlesinger and Schlesinger, 1987). Thus HA is a good model to study production of authentic mammalian cell protein by expression vectors (Gething and Sambrook, 1981; Sambrook et al., 1985).

We have recently obtained a high level of expression of bacterial *cat* gene in human and monkey cells with an extrachromosomal EBV-based recombinant vector (EBV–CMV), where the expression of *cat* is directed by CMV immediate early enhancer and SV40 promoter (Jalanko et al., 1988a,b). Here we report the insertion of the influenza virus *HA* cDNA (H2 subtype) into this vector to study its expression potential with a glycosylated animal cell protein. The recombinant EBV-CMV-*HA* DNA was introduced into two human and two monkey cell lines and the expression of *HA* was studied.

MATERIALS AND METHODS

(a) Plasmids and bacterial strains

Recombinant plasmids pKTH539 (Jalanko et al., 1988a) and pJHA, obtained from M.-J. Gething, Cold Spring Harbor Laboratory (U.S.A.), were propagated in *Escherichia coli* B2. M13mp18 and M13mp19 were propagated in *E. coli* JM103.

(b) Cell culture and transfections

HeLa cells (human cervical carcinoma, ATCC CCL2), CV-1 cells (African green monkey kidney, ATCC CCL70), 293 cells (transformed human embryonal kidney; from U. Pettersson, University of Uppsala, Sweden), B-Vero cells (monkey kidney; from P. Halonen, University of Turku, Finland) and MDCK cells (canine kidney, ATCC CCL34) were cultured in MEM supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml), and 10% (v/v) FCS. DNA was transfected to cell monolayers as described previously (Jalanko et al., 1988a).

(c) Isolation and analysis of DNA

High-M_r DNA was isolated as described (Maniatis et al., 1982) and DNA of low M_r was isolated by the selective extraction method of Hirt (1967). 20 μg of total cellular DNA or equivalent of 5×10^6 cells of Hirt's extract, either nondigested on digested with *Bam*HI, was analyzed by electrophoresis in 0.8% agarose gel in Tris · acetate buffer (40 mM Tris-base/20 mM Na · acetate/26 mM acetic acid/1 mM EDTA pH 8.05). Southern-blot analysis (Maniatis et al., 1982) was then performed with ^{32}P-labeled nick-translated EBV-CMV-*HA* probe.

(d) Isolation and analysis of RNA

Polyadenylated RNA from transfected cells was isolated by the method of Schwab et al. (1983). RNA

samples (10 μg) were incubated for 15 min at 55°C in 2.2 M formaldehyde, 50% formamide in MOPS buffer (40 mM morpholinopropane-sulfonic acid pH 7.0/5 mM Na · acetate/1 mM EDTA) and then fractionated in a 1% agarose gel containing 2.2 M formaldehyde (Maniatis et al., 1982). Transfer of the RNA to nylon membrane (Hybond N, Amersham) and hybridization with nick-translated pJHA [^{32}P] DNA probe was done as described by Thomas (1980), except that SSPE buffer (180 mM NaCl/10 mM Na · phosphate pH 7.7/0.1 mM EDTA) was used instead of SSC.

(e) Sandwich hybridization

Copy numbers of the transfected DNA in different host-cell lines were determined by the sandwich hybridization assay as described (Korpela et al., 1987; Ruohonen-Lehto et al., 1987). For the detection of EBV–CMV-*HA* copies, the capture reagent was constructed from pJHA by cleaving with *Hin*dIII + *Bam*HI. After treatment with PolIk, the 1.8-kb *HA* fragment was isolated and ligated into M13mp18 and M13mp19 that had been digested with *Eco*RI + *Hin*dIII and treated with PolIk. The detector probe reagent for EBV–CMV-*HA* copies was the 5.6-kb *Eco*RI-*Hin*dIII fragment of EBV–CMV DNA, containing the EBNA-1, *oriP* and the CMV enhancer-SV40 promoter sequences. The copies of the integrating CMV-HA DNA were determined by the same capture reagent, but the detector probe reagent was pKTH537 DNA, from which the *HA* sequences had been deleted.

(f) Immunofluorescence staining

Non-confluent monolayers of cells grown on coverslips were washed once with PBS (0.14 M NaCl/0.01 M Na · phosphate buffer pH 7.4) and then fixed with 3% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 at room temperature for 15 min. Cells were double-stained using an indirect immunofluorescence method as described (Kuismanen et al., 1982). Cells were treated with 1:100 diluted rabbit polyclonal antiserum against Japanese strain influenza virus H2 subtype (a generous gift from M.-J. Gething, Cold Spring Harbor Laboratory, U.S.A.). The second antibody was swine anti-rabbit IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC; Dako). The fluorescence was examined with a Reichert & Jung Polyvar microscope fitted with a × 100 oil immersion objective and filter for TRITC fluorescence.

(g) Labeling of cellular proteins

The cells were washed twice with PBS, 48 h after seeding, and incubated for 30 min in methionine-free MEM supplemented with 2% of FCS. The cells were labeled for 3 h with 1.5 ml of 100 μCi/ml of [^{35}S]methionine (1160 to 1500 Ci/mmol, Amersham International) per 10-cm dish. After the labeling the cells were washed twice with ice-cold PBS and lysed with 1 ml of NET buffer (1% Nonidet P-40/0.005 M EDTA/0.05 M Tris · HCl pH 8.0)/0.4 M NaCl/100 IU of aprotinin (Trasylol, Bayer) per ml. Cell extracts were collected from the dishes and centrifuged at 10000 × *g* at +4°C for 5 min and the supernatant was used for immunoprecipitation. For labeling in the presence of tunicamycin, the cells were pre-incubated for 1 h with 5 μg/ml of tunicamycin (Sigma) and [^{35}S]methionine labeling was performed in the presence of 5 μg/ml of tunicamycin. Influenza-virus-infected MDCK cells were used as a control. The MDCK cells were infected with influenza virus (H2 subtype) with an moi of 10. The cells were labeled 3 h post infection with [^{35}S]methionine, as described above.

(h) Immunoprecipitation and SDS-gel analysis

For immunoprecipitation, 10^7 cpm of each labeled cell extract was diluted into 0.5 ml of NET buffer containing 50 IU of aprotinin. A total of 5 μl of influenza virus antiserum was added, and the mixtures were incubated at +4°C overnight. 200 μl of 10% (v/v) protein A Sepharose (CL-4B, Pharmacia Fine Chemicals) was added and the incubation continued at room temperature for 1 h. The precipitate was washed eight times with NET buffer and once with 10 mM Tris · HCl pH 6.8, containing 20% glycerol. Samples were then boiled for 2 min in electrophoresis sample buffer and the supernatants were analyzed by SDS–10% polyacrylamide gel electrophoresis according to Laemmli (1970). After electrophoresis the gels containing radioactively labeled material were treated for fluorography (Bonner and Laskey, 1974) and exposed on Konica medical A2

films. The [^{14}C]methylated M_r markers were myosin (200 kDa), phosphorylase (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa) (all from Amersham).

(i) FACS analysis

The expression of *HA* on the cell surface was examined by FACS IV (Becton-Dickinson). The cell monolayers were grown for 48 h at 37°C, detached from the dishes and incubated for 30 min at 0°C with a 1:80 dilution of a rabbit antibody specific to influenza A (H2 subtype). After repeated washings, the cells were incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit IgG antibody (Dako) at 0°C for 30 min. After repeated washings the cell suspensions were analyzed and cloned or fractionated with the FACS.

(j) Enzyme immunoassay for hemagglutinin

Microtiter plates for ELISA (Nunc) were coated with a 150-μl volume of IgG (10 μg/ml in PBS) from rabbit polyclonal antiserum against influenza virus (H2 subtype) overnight at room temperature and saturated with 1% bovine serum albumin in PBS for 2 h at 37°C. After coating the plates were washed twice with PBS containing 0.05% Tween-20 (PBS-Tween) and kept dry at +4°C until used. 50 μl of the HA samples (all diluted into 0.5% Triton X-100 in PBS-Tween) were incubated in antibody-coated wells overnight at room temperature. After two washings with PBS-Tween, the second antibody, 50 μl (10 μg/ml in PBS-Tween) of IgG from mouse monoclonal antibody against HA (a generous gift from J. Skehel, Imperial Cancer Research Fund, London), was added for 2 h at 37°C. The plates were again washed with PBS-Tween and alkaline-phosphatase-labeled swine antibodies against mouse IgG (Orion Diagnostica) were added in 50 μl of PBS-Tween (1:200 dilution) and incubated for 3 h at 37°C. The plates were washed twice with PBS-Tween and once with distilled water and 100 μl of 0.2% substrate, 4-nitrophenyl-phosphate in diethanolamine-$MgCl_2$ buffer (Orion Diagnostica) was added for 30 min and the intensity of color was read at 405 nm. The HA standard used for the assay was a partially purified hemagglutinin from influenza virus H2 subtype; the virus growth, purification and the HA purification were done as described by Phelan et al. (1980), modified by Julkunen et al. (1983). HA samples from the recombinant cell clones were taken two days after seeding by lysing the cells with 1% Triton X-100 in 0.15 M NaCl for 15 min on ice. Thereafter the nuclei were spun down for 5 min in an eppendorf microcentrifuge, and the clear supernatant was used for the HA assay.

RESULTS AND DISCUSSION

(a) Construction of vectors expressing influenza virus *HA* and their transfection to various cell lines

The 1.8-kb *HA* fragment, containing the HA-coding sequence flanked with short segments of non-coding DNA, as well as about 100 bp of plasmid pAT153 sequences at the 3′ end, was excised from plasmid pJHA (Wilson et al., 1981) with *Hin*dIII + *Bam*HI. The *HA* fragment was isolated and blunt-ended with PolIk and inserted into the EBV-CMV vector (Jalanko et al., 1988a). The *HA* fragment was also inserted into a vector (pKTH537), lacking the EBV sequences (this vector integrates into the host chromosome). The resulting extrachromosomal vector construct EBV-CMV-*HA* and the integrating vector construct CMV-*HA* are shown in Fig. 1. The EBV-CMV-*HA* construct was transfected into human HeLa, 293, monkey CV-1 and B-Vero cells, and the CMV-*HA* construct was transfected into HeLa and 293 cells. After two weeks of selection, resistant single-cell clones of each transfected cell type were isolated manually or by FACS.

(b) Selection of HeLa cell clones with FACS

The transfected and hygromycin-B-selected HeLa cell pool was analyzed by a FACS after staining the HA on the surface of the living cells with HA antibody and fluorescein conjugate. The transfected cell pool contained 35% of HA-positive cells and the fluorescence varied extensively. The 5% portion of the cells with highest fluorescence in the pool were collected and divided into single-cell clones by the cell sorter to obtain high-producer cell clones (Fig. 2). The FACS-selected cell clones showed 85–90%

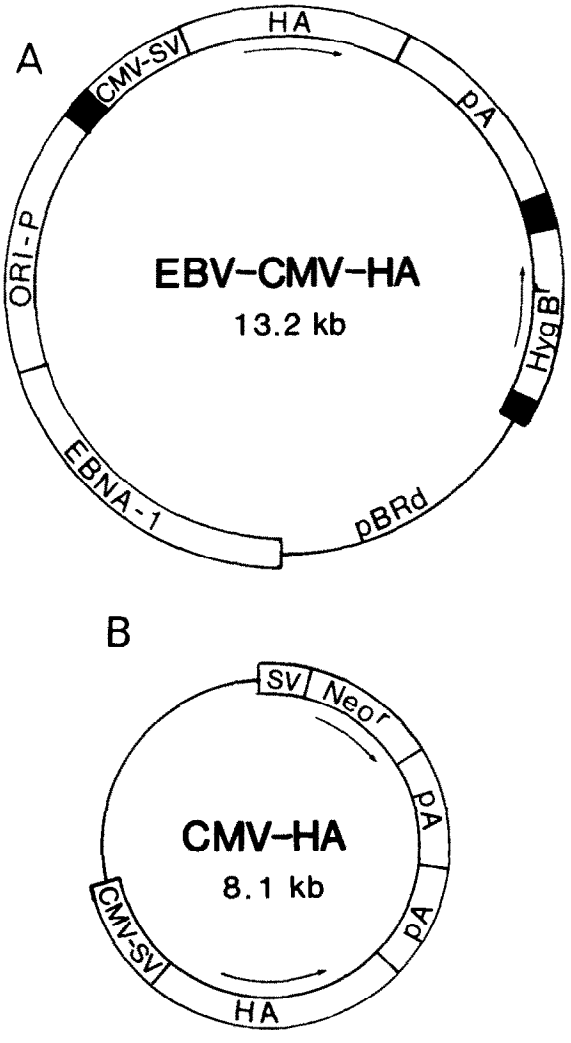

Fig. 1. Vector constructs EBV-CMV-*HA* (**A**) and CMV-*HA* (**B**) (see RESULTS AND DISCUSSION, section **a**). CMV-SV denotes the CMV enhancer-SV40 promoter, and pA is the intron and polyadenylation signal from the SV40 early region. Other genetic elements are as labeled; arrows show the direction of transcription. The thin lines indicate pBRd sequences and the black bars HSV *tk* sequences.

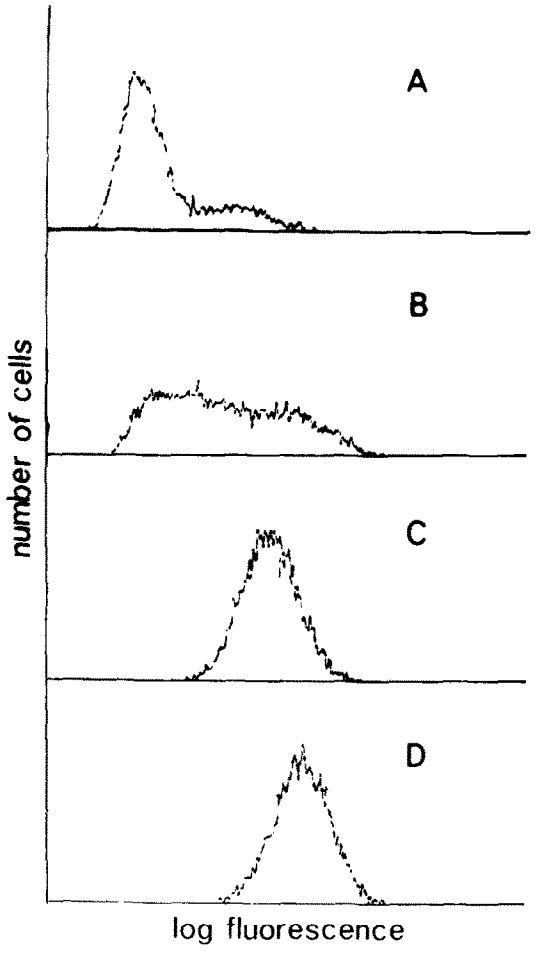

Fig. 2. Analysis of surface HA on EBV-CMV-*HA*-transfected HeLa cells by immunofluorescence staining followed by analysis in FACS (see MATERIALS AND METHODS, sections **f** and **i**). Nontransfected HeLa cells (**A**), transfected HeLa cell pool (**B**), FACS-selected clone F4 (**C**) and clone F4S (**D**), which is sorted from clone F4.

of HA-positive cells and had a clearly higher fluorescent intensity than the positive cells in the original cell pool. One of these clones, HeLa EBV-CMV-*HA* clone F4, was further analyzed with FACS and 10% of the most fluorescent cells were sorted to obtain HeLa clone F4S. This clone had 97% HA-positive cells and the fluorescent intensity was again clearly higher than in its parent clone (F4) (Fig. 2). The FACS analysis was repeated after three months of cultivation and the FACS-selected cell clones had retained the same amounts of HA-positive cells.

(c) Expression of *HA* in transfected cells

Transcription of *HA* in the EBV-CMV-*HA* vector is assumed to be under the control of CMV enhancer-SV40 promoter and it should be terminated at the RNA processing signals from SV40. To verify this postulate, the HA (poly)A$^+$ RNA preparations isolated from different cell lines were analyzed by Northern blotting using ^{32}P-labeled pJHA plasmid as a probe. mRNA derived from HeLa, 293 and CV-1 cells contained a HA transcript

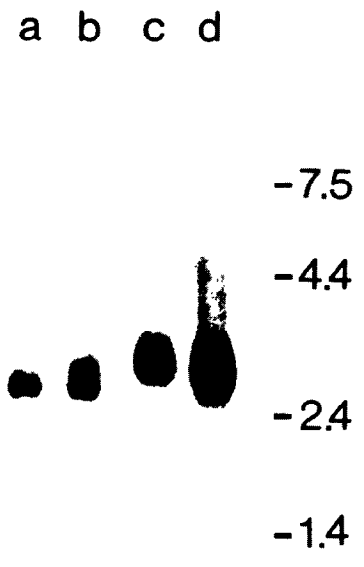

Fig. 3. Northern-blot analysis of poly(A)$^+$mRNA from EBV-CMV-*HA*-transfected cell clones. Polyadenylated RNA was prepared from transfected cells by the method of Schwab et al. (1983). Cells from ten 10-cm plates were harvested and resuspended in 20 ml of 10 mM Tris · HCl pH 7.4/100 mM NaCl/2 mM EDTA; SDS was added to 1% and the mixture was homogenized in an Ultra Turrax homogenizer for 1–2 min. Proteinase K (200 μg/ml) was added and the homogenate was incubated for 1 h at 37°C. 1.4 ml of 5 M NaCl was added and the sample was incubated with 250 mg of oligo(dT)-cellulose for 4–6 h at 22°C. The oligo(dT)-cellulose was washed first with the above resuspension buffer, then with 0.1 M NaCl/10 mM Tris · HCl pH 7.4/2 mM EDTA/0.2% SDS and eluted with 3 ml of 10 mM Tris · HCl pH 7.5/1 mM EDTA and ethanol-precipitated. The ethanol precipitate was dissolved in distilled water and the RNA concentration was measured by A_{260}. RNA (10 μg) was treated with formamide and formaldehyde and electrophoresed in 2.2 M formaldehyde/1% agarose gel (Maniatis et al., 1982). After electrophoresis the RNA was blotted to nylon membrane (Hybond N) and hybridized with a nick-translated probe as described by Thomas (1980). The [^{32}P]dCTP nick-translated pJHA DNA probe contained 10^8 cpm/μg DNA. The filter was exposed to x-ray film for 12 h at −70°C. Lanes: **a**, 293 clone 4; **b**, 293 clone F8; **c**, HeLa clone F4; **d**, HeLa clone F4S. Numbers on the right margin indicate the RNA ladder molecular size markers (BRL) in kb.

of about 2700 nt (Fig. 3), which is the expected length from the vector construct when the termination has occurred at the SV40 polyadenylation signal and assuming about 200 nt for the poly(A) tail. The *HA* RNA from 293 cells was slightly smaller than RNA from HeLa cells. The reason for this has not been assessed, but most likely the RNAs differ in length of their poly(A) tail.

To analyze the synthesis of the HA polypeptide in transfected cells, the cells were grown for 48 h after seeding, fixed and the HA was localized at the cell surface by indirect immunofluorescence. The intensity of fluorescence, as well as the proportion of fluorescent cells varied from clone to clone (not shown). Transfected HeLa cells showed most intense surface fluorescence of HA. The analytical FACS results shown above correlated well with the results obtained by indirect immunofluorescence staining of the fixed cells. Also the intensity of the mRNA bands in the Northern blots (Fig. 3) correlated well with the fluorescence intensity.

To analyze the HA polypeptides produced in the various cell lines, the cells were labeled with [^{35}S]methionine and immunoprecipitates of cell extracts were analyzed by SDS–polyacrylamide gel electrophoresis. HA synthesized from the EBV vector in HeLa cells is mainly in the form of glycosylated HA 0 (75-kDa precursor protein) and it is identical in size with the HA derived from infected MDCK cells (Figs. 4 and 5). The major band, based on the mobility in the gel, represents the terminally glycosylated HA 0 and the minor band is the core-glycosylated form (73 kDa) (Gething and Sambrook, 1982). Part of the HA 0 is processed to HA 1 (and HA 2). Also in CV-1 and 293 cells a band of HA 0 was clearly detectable whereas in B-Vero cells HA was not observed by any detection methods (data not shown). When the cells were labeled in the presence of tunicamycin, a drug which inhibits the primary glycosylation of the polypeptide, the EBV-HA proteins were identical in size (60 kDa) with influenza virus HA produced by tunicamycin-treated cells (Fig. 5). The clones transfected with the vector lacking the EBV sequences (CMV-*HA*) had no detectable HA polypeptides by immunoprecipitation.

The recombinant HA from the different cell lines was quantitated by enzyme immunoassay from cell extracts after 48 h of seeding. The EBV-vector-transfected HeLa cells showed the highest amounts of HA, up to 11.5 μg per 10^6 cells (Table I), which is equivalent to 10^8 molecules per cell. This is in the same range as the HA produced by lytic SV40 recombinant virus infection (Gething and Sambrook,

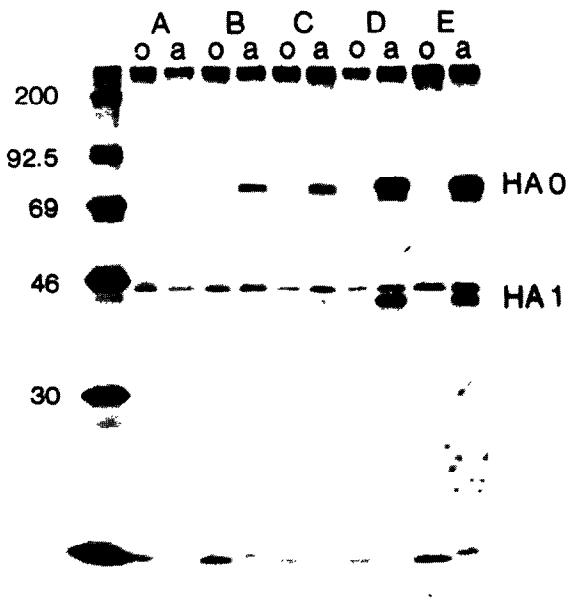

Fig. 4. Analysis of recombinant HA produced by EBV-CMV-*HA*-transfected HeLa cells. Cells were labeled with [^{35}S]methionine as described in MATERIALS AND METHODS, section **g**. Sample aliquots from 5×10^5 cells were precipitated with preimmune (o) or immune serum (a), and analyzed by electrophoresis in SDS–10% polyacrylamide gel (see MATERIALS AND METHODS, section **h**). Lanes: **A**, nontransfected HeLa cells; **B** and **C**, transfected, randomly isolated clones 2 and 3; **D**, FACS-selected clone F4, and **E**, clone F4S which is re-sorted by FACS from clone F4. ^{14}C-labeled molecular size markers (in kDa) are shown on the left margin.

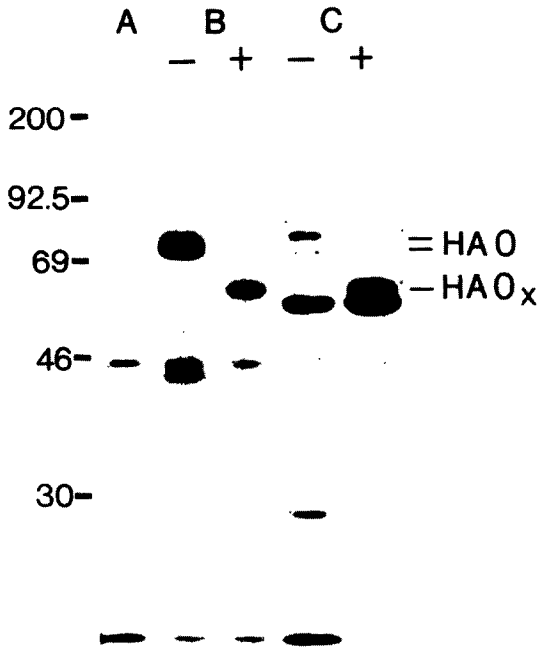

Fig. 5. Comparison of HA synthesized by EBV-CMV-*HA* transfected HeLa cells (**B**) and influenza-virus-infected MDCK cells (**C**). The cells were labeled with [^{35}S]methionine either in the presence (+) or absence (–) of tunicamycin, immuno-precipitated with influenza virus antiserum and electrophoresed (see MATERIALS AND METHODS, sections **g** and **h**). HA 0 designates the glycosylated forms and HA 0_X the non-glycosylated form. Sample aliquots are from 5×10^5 cells. Lane **A** contains nontransfected HeLa cells; ^{14}C-labeled M_r markers are shown on the left margin.

1981). The efficiency of FACS selection was well demonstrated in the quantity of HA produced in HeLa cells. The FACS-selected clones produced 3–11.5 μg HA per 10^6 cells, whereas the randomly selected clones produced 0.8–3 μg/10^6 cells. 293 cells showed lower HA quantities and in CV-1 cells only half of the clones showed detectable activities, e.g., produced more than 30 ng/10^6 cells. From the CMV-*HA* vector transfected HeLa clones only one showed detectable HA activity of 60 ng/10^6 cells.

(d) The state of DNA in transfected cells

The DNA extracted from the transfected cell lines was analyzed by Southern hybridization. Two different extraction methods were used; one which yields predominantly high M_r DNA (Maniatis et al., 1982) while the other, Hirts (1967) extraction method, yields extrachromosomal DNA. The EBV-CMV-*HA* plasmid was detected both in high- and low-M_r DNA preparations due to its high molecular mass (13.2 kb). Nondigested samples of Hirts extracts showed a covalently closed plasmid of the size of EBV-CMV-*HA* (Fig. 6). The digestion of the low- and high-M_r DNAs from the cell clones with *Bam*HI, which linearizes the vector DNA, yielded identical bands with the EBV-CMV-*HA* vector DNA in Southern hybridization (Fig. 7). This indicates that the transfected EBV-CMV-*HA* DNA resides mainly as a circular monomer in CV-1, HeLa and 293 cells (Fig. 7). In two B-Vero cell clones both plasmid monomers and possibly oligomers or rearranged forms existed (Fig. 6).

The EBV-CMV-*HA* copy numbers in the trans-

TABLE I

Enzyme immunoassay quantitation of recombinant HA expressed by EBV-CMV-*HA* or CMV-*HA* vector

EBV-CMV-*HA*[a]		CMV-*HA*[a]	
Cell line	HA (μg/10^6 cells)[b]	Cell line	HA (μg/10^6 cells)[b]
HeLa/1	1.45	HeLa/1	<0.03
2	0.80	2	0.06
4	0.90	3	<0.03
5	3.00	4	<0.03
F2[c]	3.00		
F4[c]	6.70		
F4S[c]	11.50		
293/ 1	0.08	N.D.	
2	0.05		
3	0.03		
4	0.47		
5	0.39		
CV-1/ 1	0.03	N.D.	
2	<0.03		
3	<0.03		
4	0.17		

[a] See Fig. 1. N.D., not done.

[b] See MATERIALS AND METHODS, section **j**.

[c] F2, F4, FACS-selected clone; F4S, FACS-selected clone, from which 10% of most positive cells have been sorted (see RESULTS AND DISCUSSION, section **b**).

Fig. 6. Southern-blot hybridization of Hirt (1967) extracts of EBV-CMV-*HA*-transfected cells. The DNAs were electrophoresed in a 0.8% agarose gel, blotted to a nitrocellulose filter and hybridized with nick-translated EBV-CMV-*HA* probe (see MATERIALS AND METHODS, section **c**). Lanes: **a** and **f**, EBV-CMV-*HA* DNA (0.1 ng); **b** and **c**, transfected HeLa cell clones F2 and F4, respectively; **d** and **e**, 293 clones F3 and F8; **g**, CV-1 clone 3; **h** and **i**, B-Vero clones 1 and 4.

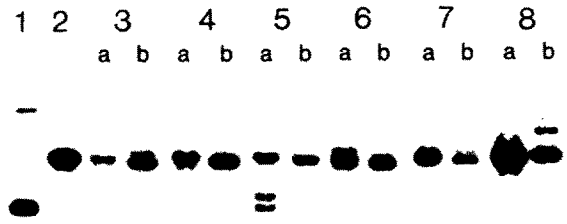

Fig. 7. Southern-blot hybridization of *Bam*HI-digested total cellular DNAs and Hirt (1967) extracts isolated from EBV-CMV-*HA*-transfected cell clones. The DNAs were electrophoresed in a 0.8% agarose gel, blotted to a nitrocellulose filter and hybridized with nick-translated EBV-CMV-*HA* DNA (see MATERIALS AND METHODS, section **c**). Lanes: **1**, nondigested EBV-CMV-*HA* DNA (0.1 ng); **2**, *Bam*HI digested EBV-CMV-*HA* DNA; **3**, HeLa clone F2 DNA; **4**, HeLa clone F4; **5**, 293 clone F3; **6**, 293 clone F8; **7**, CV-1 clone 1; **8**, CV-1 clone 3. **a**, total cellular DNA; **b**, Hirt extract DNA.

fected cell clones were determined by sandwich hybridization assay with the specific probes to EBV-CMV-*HA* (Table II). The copy numbers in HeLa, 293, CV-1 and B-Vero cells varied from one to 24. The HeLa cells contained the lowest copies: one to two and, significantly, the high expression HeLa clones, which were selected by FACS, did not show increased copy numbers. In 293, CV-1 and B-Vero cells the vector copy numbers varied from three to five with the exception of one B-Vero clone with 24 copies per cell. This is in agreement with the results obtained by others (Sugden et al., 1985; Yates et al., 1985; Young et al., 1988). As a comparison, we also analyzed the copy numbers of the CMV-*HA* DNA in transfected 293 and HeLa cells. The copy number of the CMV-*HA* construct was only 1 or less copies per cell in each cell clone (Table II).

(e) Conclusions

(1) HA was synthesized effectively with the EBV-based vector (EBV-CMV) in human HeLa, 293 and

TABLE II

Copy numbers of EBV-CMV-*HA* and CMV-*HA* in various cell lines

Cell line	EBV-CMV-*HA*[a] (copies/cell)	Cell line	CMV-*HA*[a] (copies/cell)
HeLa/ 1	2	HeLa/1	<1
2	2	2	1
3	2	3	1
4	5	4	<1
5	4		
F2[b]	2		
F4	1		
F4S[b]	2		
293/ 1	3	293/ 1	1
2	5	2	<1
3	2	3	<1
4	2	4	1
5	5	5	1
CV-1/ 1	3	N.D.[c]	
2	1		
3	4		
4	1		
B-Vero/1	4	N.D.[c]	
2	24		
3	2		

[a] See MATERIALS AND METHODS, section e.

[b] See Table I, footnote c, and RESULTS AND DISCUSSION, section b.

[c] Not done.

monkey CV-1 cells and was transported onto the cell surface. Selection by FACS of the high-producing cell clones was highly effective and fast. The FACS-selected HeLa cell clones produced five- to ten-fold the amounts of HA compared to the randomly selected HA-positive clones.

(2) Northern-blot analysis of HA-specific transcripts suggested that appropriate transcription initiation and termination signals of the vector were used.

(3) Immunoprecipitation of the labeled cell extracts revealed a HA polypeptide comigrating with the HA from virus-infected cells. The HA polypeptide was apparently glycosylated, as indicated by the mobility in SDS–polyacrylamide gels and by tunicamycin experiments.

(4) DNA analysis of individual cell clones showed that in all cell clones the EBV-CMV-*HA* existed mainly as an extrachromosomal, monomeric plasmid.

(5) Significantly higher expression levels of HA protein were obtained by the extrachromosomal EBV-based vector than by the respective vector lacking the EBV sequences. The EBV-vector-transfected HeLa cells were the best producers (0.8–11.5 μg Ha/10^6 cells).

(6) The vector copy numbers in the EBV-CMV-*HA*-transfected cell clones were one to five copies per cell (one clone with 24 copies). Significantly, the high-expression HeLa cell clones, which were selected by FACS, had only one to two copies per cell.

(7) The extrachromosomal EBV-CMV vector can be efficiently used for production of apparently authentic mammalian glycoproteins. The advantages of the EBV-CMV system is that it can be used in human and monkey cells and moreover that it does not cause phenotypic transformation of transfected cells as BPV vectors do.

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