Establishment of a Rat Cell Line Inducible for the Expression of Human Cytomegalovirus Immediate-Early Gene Products by Protein Synthesis Inhibition

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Upon transfection of Rat-2-TK⁻ cells with plasmid pES, containing the cloned 7.0-kilobase (kb) *EcoRI-SalI* fragment (0.063 to 0.089 map units) of the human cytomegalovirus genome, major immediate-early antigen expression was obtained in 1 to 2% of the nuclei of the transfected cells, as determined by immunofluorescence with the E3 monoclonal antibody. Cotransfection of pES with the cloned herpes simplex virus type 1 thymidine kinase gene resulted in the establishment of a hypoxanthine-aminopterin-thymidine-resistant cell line which expressed a major immediate-early antigen in approximately 1% of the cells at early passages, with expression gradually declining to less than 0.1% upon subculturing. Southern blot analysis of DNA extracted from this cell line revealed the presence of multiple integration events of pES DNA sequences into cellular DNA, including a head-to-tail tandem array of approximately 10 copies of pES. The integration pattern was stable for at least 80 passages. Metaphase chromosomes prepared from this cell line showed, upon *in situ* hybridization, a strong hybridization signal in both sister chromatids of a large submetacentric chromosome which is considered to have harbored the tandemly integrated pES molecules. Whereas in most cells of the population, immediate-early expression seemed to be repressed, this repression could be overcome by protein synthesis inhibition, resulting in a massive induction of human-cytomegalovirus-specific transcripts of 2.1 and 1.9 kb and a minor species of 2.9 kb. After release from protein synthesis inhibition, approximately 20% of the cells showed nuclear fluorescence when the E3 monoclonal antibody was used.

After infection of permissive cells *in vitro*, expression of the human cytomegalovirus (HCMV) genome is subjected to temporal regulation. Three main phases have been described: immediate-early (IE), early, and late (3, 9, 45, 53, 54). By definition, viral RNAs synthesized in the presence of inhibitors of protein synthesis are referred to as IE RNAs; the proteins synthesized after the removal of the inhibitor but in the presence of inhibitors of transcription are referred to as immediate-early antigens (IEAs). IE transcription originates from only limited parts of the viral genome located on the long unique segment (19, 30, 45, 53-55). Early gene products are defined as those which are synthesized before virus DNA synthesis or when viral DNA synthesis is blocked (32, 35, 44, 45, 49). In this early phase, the genome is extensively transcribed, and it is believed that the switch from restricted IE transcription to extensive early transcription is mediated through one or more IEAs.

By immunofluorescence (IF), IEAs can be detected in the nuclei of infected permissive and nonpermissive cells within a few hours after infection (13, 15, 32, 35, 45, 48). By immunoprecipitation, at least four IEAs can be detected in cells infected with HCMV, the predominant (major) IEA being a phosphorylated protein showing strain heterogeneity in apparent molecular weight (3, 5, 14, 31, 45, 48).

The structures of the major IE gene of HCMV strains AD169 and Towne have recently been investigated by nucleotide sequence analysis (2, 42, 50). These studies have shown an almost identical structural organization of the major IE coding region in both strains. The major IE mRNA

is a spliced molecule of 1,735 nucleotides, and the upstream regulatory region contains numerous short direct and inverted repeats extending approximately 500 base pairs from the IE transcriptional start site. Recently it has been shown that this upstream region contains a strong promoter-enhancer (4, 46).

As yet, little information is available concerning the function of the HCMV IEAs. They are likely to be involved in increased transcriptional activity and alterations of chromatin structure in the infected cell and in the switch from restricted IE to extensive early transcription (21-23).

Here we report that expression of a major IEA was obtained after transfection of Rat-2-TK⁻ cells with a cloned fragment of the HCMV genome. By cotransfection, using the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene as a selectable marker, we established a rat cell line containing several copies of the major IE gene stably integrated into chromosomal DNA. As determined by IF with the E3 monoclonal antibody (E3 MAb) (15), expression of the major IE gene seemed to be repressed because only 1% of the cells showed nuclear fluorescence. This repression was overcome, however, by inhibition of protein synthesis, which led to a dramatic increase of HCMV-specific transcripts. After reversal of protein synthesis inhibition, nuclear fluorescence was observed in approximately 20% of the cells.

The cell line described in this paper is likely to be a valuable tool in understanding IE gene regulation and IE functions and may provide an *in vitro* model for studies concerning latency and reactivation.

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MATERIALS AND METHODS

Cells. The Rat-2-TK⁻ cell line (51) is a TK-negative derivative of the established Rat-1 cell line and was a gift from W. C. Topp. Rat-2-TK⁻ cells were cultured in Eagle BME medium (GIBCO Laboratories, Grand Island, N.Y.), supplemented with Earle salts, 8% fetal calf serum (GIBCO), and antibiotics (100 IU of penicillin and 100 µg of streptomycin per ml) at 37°C in a humidified 5% CO₂ atmosphere. Cells transformed to the TK-positive phenotype were selected in the growth medium described above supplemented with hypoxanthine, aminopterin, and thymidine (HAT medium [27]).

Restriction enzymes and chemicals. *Bam*HI, *Sal*I, and *Pvu*II were from Boehringer GmbH, Mannheim, Federal Republic of Germany. *Bgl*II and *Xba*I were isolated as described by Skare and Summers (39). Puromycin, emetine, anisomycin, and cycloheximide were from Sigma Chemical Co., St. Louis, Mo.; stock solutions of these inhibitors were prepared in 50 mM Tris hydrochloride (pH 7.4) at 5, 1, 1, and 5 mg/ml, respectively, and frozen in small aliquots until use.

IF and antisera. Standard procedures for the indirect IF assay were used for the detection of HCMV IEA. Cells grown on glass cover slips were washed twice with phosphate-buffered saline, fixed with cold (−30°C) acetone for 10 min at room temperature, air dried, and frozen (−30°C) until used for IF. The E3 MAb (15) was generously provided as ascites fluid by Genetic Systems Corp., Seattle, Wash., and was used in a 1/1000 dilution in phosphate-buffered saline. Fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Miles Yeda, Israel) was used in a 1/40 dilution in phosphate-buffered saline.

Recombinant DNA. All recombinant plasmids were propagated in *Escherichia coli* K-12 strain HB101. Plasmid DNA was isolated from overnight cultures essentially as described by Ish-Horowicz and Burke (18), followed by column chromatography with Sepharose C12B (Pharmacia, Inc.) and ethanol precipitation. The concentration of plasmid DNA was determined in a spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 260 nm. Plasmid pAGO is a pBR322 recombinant containing the HSV-1 TK gene as a 2-kilobase (kb) *Pvu*II fragment and was kindly provided by F. Colbère-Garapin (6). Plasmid pJN201 is a recombinant containing the 21-kb *Hind*III E fragment of HCMV strain AD169 and was provided by J. Nelson (33). pJN201 was used as starting material for the construction of pEJ, pES, and pSS. pEJ contains the 10.3-kb *Eco*RI J fragment (41) cloned at the *Eco*RI site of pBR328; pES contains the 7.0-kb *Eco*RI-*Sal*I subfragment of the *Eco*RI J fragment cloned at the corresponding sites of pBR328; pSS contains the 5.0-kb *Sph*I-*Sal*I subfragment of the *Eco*RI J fragment cloned at the corresponding sites of pBR328 (Fig. 1).

Isolation of high-molecular-weight cellular DNA. High-molecular-weight total cellular DNA was isolated essentially as described by Venema et al. (52).

DNA transfection. Subconfluent monolayers of Rat-2-TK⁻ cells were transfected by using the calcium phosphate coprecipitation technique (16). Four hours after the addition of the DNA precipitate (500 µl per 60-mm petri dish), the monolayers were rinsed twice with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline (16) and shocked for 4 min with 15% glycerol in HEPES-buffered saline (vol/vol) at room temperature. Thereafter the glycerol-HEPES-buffered saline mixture was replaced by Eagle BME medium containing 8% fetal calf serum. In the case of HAT selection, HAT medium was

added 17 h after transfection. The establishment of the Rat-IEA⁺-9X cell line (see Results) resulted from a transfection experiment in which the precipitate contained 200 ng of pAGO, 10 µg of pES, and 10 µg of salmon testis DNA per ml.

Agarose gel electrophoresis and Southern blotting. DNA fragments were fractionated by size by electrophoresis through horizontal 1% (wt/vol) agarose slab gels. The gels were electrophoresed at room temperature with the buffer system described by Aaij and Borst (1) containing 1 µg of ethidium bromide per ml. DNA fragments were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) essentially as described by Southern (40).

Labeling and hybridization. To detect HCMV DNA sequences, the nitrocellulose filters were hybridized with in vitro ³²P-labeled DNA essentially as described by Jeffreys and Flavell (20). Hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) (wt/vol)–10% dextran sulfate (wt/vol)–heat-denatured salmon testis DNA (50 µg/ml) at 68°C for 16 h. Final washings were in 0.1× SSC–0.1% SDS at 68°C for 30 min. The 7.0-kb *Eco*RI-*Sal*I insert of pES (Fig. 1) was purified from pES by two cycles of gel electrophoresis followed by isolation as described by Yang et al. (56); it was then frozen in small aliquots until use. The purified fragment (500 ng) was labeled in vitro with ³²P-labeled deoxynucleoside triphosphates (specific activity, 2000 to 3000 Ci/mmol; Amersham International) by nick translation (36) to a specific activity of 1 × 10⁸ to 2 × 10⁸ cpm/µg. Under the hybridization conditions described above, essentially no hybridization was detected with pAGO or pBR328 DNA.

Chromosome spreads. Exponentially growing Rat-IEA⁺-9G cells and Rat-2-TK⁻ cells were treated for 5 h with vinblastin sulfate (2 × 10^{−7} g/ml of medium), and mitotic cells were shaken off and washed twice with phosphate-buffered saline. The cells were swollen in 75 mM KCl (30 min at 37°C), spun down, and fixed with 25% acetic acid in methanol (vol/vol) for 30 min at room temperature. The fixed cells were dropped onto cold (4°C) wet slides and flame dried. The slides were stored at 4°C until use.

In situ hybridization of chromosome spreads. Conditions for 2-acetylaminofluoren (AAF) modification of probe DNA, in situ hybridization, and reflection contrast microscopy were as described by Landegent et al. (24–26). Similar results were obtained with either pEJ or a single-stranded M13 probe containing the 5.2-kb *Bam*HI subfragment of pEJ (Fig. 1).

Induction of cells with protein synthesis inhibitors. Rat-IEA⁺-9G cells were seeded at 2 × 10⁵ cells per 60-mm petri dish in 4 ml of HAT medium. Forty-eight hours after seeding, 40 µl of a stock solution of the inhibitor (see above) or the solvent of the inhibitor alone (mock treatment) was added, and the cells were incubated for the period indicated in Results. To release the block, the growth medium was removed, the monolayers were washed three times with phosphate-buffered saline, and fresh HAT medium was added.

Isolation of total cellular RNA and Northern blotting. Total cellular RNA was isolated essentially as described by Frazier et al. (12). The final ethanol precipitate was washed twice with 3 M sodium acetate (pH 6.0) and dissolved in RNA sample buffer (29). The RNA samples were electrophoresed through 1% agarose gels containing formaldehyde. After electrophoresis, the gels were soaked in an equal volume of 20× SSC for 30 to 60 min and the RNA was transferred to nitrocellulose in 10× SSC. Hybridization was

FIG. 1. Restriction endonuclease cleavage map of the cloned 10.3-kb *EcoRI* J fragment (pEJ) and subclones derived therefrom. The top line represents the 236-kb DNA genome of HCMV strain AD169 and shows the location of the 10.3-kb *EcoRI* J fragment cloned in pEJ. pES and pSS indicate the cloned *EcoRI*-*Sall* (7.0 kb) and *SphI*-*Sall* (5.0 kb) fragments, respectively. The arrows show the map locations and direction of transcription of the major 1.95-kb, middle-abundant 2.15-kb, and minor 1.7-kb IE mRNAs observed upon viral infection of human diploid cells, as determined by Wilkinson et al. (55). P, *PvuII*; E, *EcoRI*; B, *BamHI*; Bg, *BglII*; Sa, *Sall*; Sp, *SphI*. *PvuII* and *SphI* sites are only indicated for sequences present in pES.

done overnight at 42°C in $5\times$ SSC–50% recrystallized formamide–0.1% SDS–salmon testis DNA (20 µg/ml)–10mM EDTA (pH 8.0)–10% dextran sulfate. Final washings were done at 68°C in 0.1% SDS–0.1 $\times$ SSC.

RESULTS

Expression of a major IEA of HCMV after transfection with cloned DNA fragments. Within a few hours after infection of permissive and nonpermissive cells by HCMV, IEAs can be detected in the nuclei by IF (13, 32, 35, 48). Because IE transcription is not dependent on preceeding viral protein synthesis, it was expected that after transfection of cloned HCMV DNA fragments, expression of IEAs might be obtained.

When the cloned *HindIII* E fragment (pJN201) (33) was used to transfect Rat-2-TK[−] cells (51), a nuclear antigen was detected within 4 h after transfection by IF with the E3 MAb, which has been shown to be directed against the major IEA of HCMV (15, 48). By testing the cloned *EcoRI* subfragments of the *HindIII* E fragment, the gene which codes for the nuclear antigen could be located within the 10.3-kb *EcoRI* J fragment (data not shown).

A restriction enzyme cleavage map of the cloned *EcoRI* J fragment (pEJ) and two subclones derived therefrom are shown in Fig. 1. When Rat-2-TK[−] cells were transfected with increasing amounts of pES (containing the 7.0-kb *EcoRI*-*Sall* fragment) and screened by IF 17 h after transfection, strong nuclear fluorescence was obtained in a linear dose-dependent fashion when the E3 MAb was used (Fig. 2). Strong nuclear fluorescence was observed as early as 4 h after the addition of the DNA precipitate, and the highest percentage of positive nuclei (usually 1 to 2% when 10 µg of pES per ml of precipitate was used) was seen at 14 to 18 h after transfection (data not shown).

No nuclear fluorescence was obtained after transfection with pSS containing the 5.0-kb *SphI*-*Sall* subfragment of pES (Fig. 1) or after transfection with salmon testis DNA.

From the above data it can be concluded that after transfection with pES, expression of a major IEA of HCMV strain AD169 was obtained in Rat-2-TK[−] cells. This is in good agreement with recent IE mRNA mapping data (19, 55) showing that the 1.9-kb major IE mRNA of strain AD169 originates from DNA sequences encompassed by pES.

Establishment of a rat cell line expressing a major IEA of HCMV. To establish a rat cell line expressing one or more

HCMV IEAs, Rat-2-TK[−] cells were cotransfected with pAGO (a pBR322 recombinant containing the HSV-1 TK gene) and a molar excess of pES followed by HAT selection. This resulted in the isolation of four HAT-resistant colonies growing in separate dishes. When tested by IF with the E3 MAb, one of these colonies (Rat-IEA⁺-9X) showed nuclear fluorescence in approximately 1% of the cells. To determine whether the limited expression of the nuclear antigen in Rat-IEA⁺-9X cells was a result of a genetically heterogeneous population of cells, six subclones were isolated by limiting dilution under HAT selective pressure. When tested by IF, all the subclones showed the same low level of expression of the E3 nuclear antigen as was observed with the parental Rat-IEA⁺-9X cell line. This indicated a stable association of the IE gene(s) with the genome of the parent and with the clones derived therefrom.

To confirm this expectation, the parental population and

FIG. 2. Induction of nuclear fluorescence in Rat-2-TK[−] cells after transfection with pES. Rat-2-TK[−] cells growing on cover slips were transfected with the indicated concentrations of pES (micrograms per milliliter of precipitate; 500 µl of precipitate were used to transfect approximately 4×10^5 cells in a 60-mm petri dish containing the cover slips; carrier salmon testis DNA was added to reach a final DNA concentration of 20 µg/ml of precipitate). The cells were fixed 17 h after transfection and prepared for IF with the E3 MAb. The data reflect the percentage of cells positive for nuclear fluorescence; at least 3,000 cells were scored for each data point.

FIG. 3. Southern blot analysis of Rat-IEA⁺ cell lines. *Bam*HI-cleaved total cellular DNA (4 µg) isolated from Rat-IEA⁺-9X cells (lane 6) and six subclones derived therefrom (lanes 7 through 12) was electrophoresed through a 1% agarose gel, transferred to nitrocellulose, and probed with the ³²P-labeled 7.0-kb *Eco*RI-*Sal*I fragment purified from pES. Marker lanes contained digestions of pES (*Sal*I, 10.0 kb; *Eco*RI-*Sal*I, 7.0 kb; *Eco*RI-*Bam*HI-*Sal*I, 4.8 and 2.2 kb; *Pvu*II, 6.9, 2.7, and 0.6 kb) at 3, 1, and 0.1 copies of each fragment per cell (lanes 1, 2, and 3, respectively) in a background of 4 µg of *Xba*I-digested Rat-2-TK⁻ cellular DNA. *Xba*I-digested Rat-2-TK⁻ DNA (4 µg) only (lanes 4 and 5) was also included.

the six subclones were grown to mass cultures under HAT selective pressure and total cellular DNA was isolated and tested for the presence of HCMV DNA sequences by Southern blot analysis with the purified *Eco*RI-*Sal*I insert of pES as a probe (ES probe).

The hybridization pattern obtained after *Bam*HI cleavage of DNA isolated from the parental Rat-IEA⁺-9X cell line is shown in Fig. 3, lane 6. The pattern was identical to those obtained for the *Bam*HI-cleaved DNAs from each of the six subclones (Fig. 3, lanes 7 through 12). In these *Bam*HI digestions, 16 DNA fragments could be detected. Because *Bam*HI cleaved only once in pES (Fig. 1), a minimum of eight integration sites were present. A supramolar 10.0-kb fragment, which comigrated with the linear form of pES (see marker lanes 1 through 3 in Fig. 3), indicated either the presence of an integrated head-to-tail tandem repeat of pES or the presence of multiple copies of episomal pES. However, when the unrestricted DNAs were subjected to Southern blot analysis to test for the presence of free pES, no hybridization was obtained other than in the high-molecular-weight (>40 kb) region at a level of detection of 0.1 copy of *Eco*RI-*Sal*I fragment per cell (data not shown; see also Fig. 4, lane 4). The integration of pES DNA sequences was further studied by Southern blot analysis of one of the subclones, Rat-IEA⁺-9G. To estimate the number of HCMV DNA integrations, Rat-IEA⁺-9G total cellular DNA was digested with *Xba*I. This restriction enzyme has no recognition site within pES; therefore, the number of hybridizing fragments generated is a reflection of the minimum number of integration events of HCMV DNA sequences. In the *Xba*I digest (Fig. 4, lane 5) five fragments which hybridized to the ES probe could be detected in the original autoradiograph, indicating a minimum of five integration sites. Interestingly, most of the hybridization seemed to be confined to one large (>45 kb) *Xba*I fragment, indicating that most of the integrated pES DNA sequences were present at a single chromosomal location.

Upon digestion with *Hind*III which, like *Xba*I, has no recognition site within pES, a similar, strongly hybridizing

high-molecular-weight fragment was observed. In addition, seven minor bands were visible; this was compatible with a minimum of eight integration sites (data not shown).

It is likely that the high-molecular-weight fragment generated by either *Xba*I or *Hind*III digestion contained the tandemly repeated pES molecules. Upon digestion with either *Eco*RI or *Bam*HI (Fig. 4, lanes 6 and 7, respectively), a supramolar 10.0-kb pES linear fragment was generated, consistent with the presence of a tandem repeat. From the intensity of the 10.0-kb fragment it was estimated that approximately 10 copies of pES were present in this tandem. Doubly digested Rat-IEA⁺-9G DNA yielded the expected supramolar 7.0-kb *Eco*RI-*Sal*I fragment, the 4.8-kb *Bam*HI-*Sal*I fragment, and the 2.2-kb *Bam*HI-*Sal*I fragment of pES (data not shown).

When chromosome spreads from metaphase-arrested Rat-IEA⁺-9G cells were hybridized in situ by using AAF-modified pEJ, a strong hybridization signal was observed at a distinct location in both sister chromatids of a large submetacentric chromosome, confirming the chromosomal location of pES DNA sequences (Fig. 5). Control chromosome preparations from the parental Rat-2-TK⁻ cell line did not hybridize to the probe (data not shown).

Induction of expression of a major IEA by inhibition of protein synthesis. The results described above show a stable association of integrated pES DNA with the cellular genome. No loss or major rearrangements were observed over more than 80 passages of the Rat-IEA⁺-9G cell line (data not shown). It is therefore likely that any cell present within the population carried the genetic information to express one or more proteins encoded for by pES. Yet only a small fraction of the cells expressed the E3 nuclear antigen.

To test whether protein synthesis was involved in the repression phenomenon, protein synthesis in Rat-IEA⁺-9G cells was inhibited by cycloheximide (50 µg/ml of medium) for various periods. The cells were fixed 24 h after the release from the protein synthesis block and scored for nuclear fluorescence by using the E3 MAb. A massive induction of the nuclear antigen was obtained, even upon a

short treatment (1 h) with the inhibitor (Fig. 6 and 7). With increasing duration of the protein synthesis block, a further increase in the percentage of cells expressing the nuclear antigen was observed, reaching a maximum (16% positive nuclei) when inhibition was for approximately 5 h. As expected, no increase in nuclear fluorescence was observed when cells were fixed immediately upon release from the protein synthesis block. Rat-IEA⁺-9G cells that received a mock treatment did not show increased nuclear fluorescence. The parental Rat-2-TK⁻ cell line remained negative for nuclear fluorescence after protein synthesis inhibition when tested by IF with the E3 MAb (data not shown).

To address the question of whether the observed induction was due, directly or indirectly, to the inhibition of protein synthesis or to some side effect of cycloheximide, three other inhibitors of protein synthesis (anisomycin, puromycin, and emetine) were tested for their potential to induce E3 nuclear fluorescence in Rat-IEA⁺-9G cells. In this context it is noteworthy that puromycin, an analog of aminoacyl-tRNA, inhibits protein synthesis by effecting the separation of the growing peptide chain from the tRNA-mRNA-ribosome complex. Cycloheximide and emetine, on the other hand, act by immobilizing ribosomes, thereby blocking chain elongation. These inhibitors were all effective (Table 1). It thus appears that the induction of nuclear fluorescence was due to the inhibition of protein synthesis rather than a

FIG. 4. Southern blot analysis of Rat-IEA⁺-9G cellular DNA. Rat-IEA⁺-9G total cellular DNA (1.5 μ g) was electrophoresed through a 1% agarose gel either undigested (lane 4) or digested with *Xba*I (lane 5), *Eco*RI (lane 6), or *Bam*HI (lane 7), transferred to nitrocellulose, and probed with the purified ³²P-labeled 7.0-kb *Eco*RI-*Sal*I fragment purified from pES. Marker lanes contained digestions of pES (10.0-kb pES linear, 7.0-kb *Eco*RI-*Sal*I insert, and 4.8-kb *Eco*RI-*Bam*HI fragment) at 1 (lane 1) or 0.1 (lane 2) copy of each fragment per cell in a background of *Xba*I-digested Rat-2-TK⁻ cellular DNA (1.5 μ g per lane). *Xba*I-digested Rat-2-TK⁻ cellular DNA (1.5 μ g) only (lane 3) was also included.

FIG. 5. Chromosomal location of integrated HCMV-specific DNA sequences in Rat-IEA⁺-9G cells. Chromosome spreads from metaphase-arrested Rat-IEA⁺-9G cells were hybridized in situ by using AAF-modified pEJ. Hybridization was visualized by reflection contrast microscopy.

side effect of the inhibitor. Upon subculturing of the Rat-IEA⁺-9G cell line, the spontaneous level of E3 nuclear fluorescence gradually decreased (to less than 0.1% at passage level 40), whereas the genotype with respect to the integrated pES DNA sequences remained virtually unchanged, as did the cycloheximide-mediated inducibility of the nuclear antigen (data not shown).

Induction of HCMV-specific transcripts upon protein synthesis inhibition in Rat-IEA⁺-9G cells. When total cellular RNA isolated from noninduced Rat-IEA⁺-9G cells was subjected to Northern blot analysis with pES as a probe, no hybridization was obtained (Fig. 8, lanes 1 and 2), even when very large amounts of RNA (200 μ g per lane) were tested (data not shown). RNA isolated from cycloheximide-treated cells, however, revealed the presence of high levels of HCMV-specific transcripts of 2.1 and 1.9 kb and a minor species of 2.9 kb (Fig. 8, lanes 3 through 10). Because RNAs of the same lengths were also detected with polysome-associated RNA (data not shown), it is likely that these transcripts represented mRNAs which originated from integrated pES DNA sequences. No hybridization was obtained when pBR328 DNA was used as a probe, suggesting that none of the transcripts observed upon hybridization with pES as a probe was the result of transcriptional readthrough into vector DNA sequences (data not shown).

With increasing duration of the protein synthesis block, increases in the amounts of the three pES-specific transcripts were observed. It is also clear that quantitative differences occurred, depending on the duration of the block (Fig. 8). Whereas a 4-h block showed the presence of two major species of 2.1 and 1.9 kb in about equal abundance, longer blocks showed a higher amount of the 1.9-kb

FIG. 6. Induction of E3 nuclear fluorescence: dependency on the duration of the protein synthesis block. Rat-IEA⁺-9G cells growing on cover slips were either treated with cycloheximide (CH, ●—●) or mock treated (○—○) for the indicated times. The cells were fixed 24 h after release from the block and prepared for IF with the E3 MAb. At least 1,000 nuclei were scored for fluorescence for each datum point.

species relative to that of the 2.1-kb species, suggesting a differential stability of these major transcripts.

Our current research is directed toward the identification by Western blot analysis of virus-specific proteins synthesized upon induction and toward the identification of the map locations of the induced transcripts with respect to the pES template.

DISCUSSION

Upon transfection of Rat-2-TK⁻ cells with the cloned 7.0-kb *EcoRI-SalI* fragment of HCMV strain AD169 (pES; 0.063 to 0.089 map units, Fig. 1), rapid and abundant expression of a novel nuclear antigen was detected by IF in a linear dose-dependent fashion (Fig. 2) when the E3 MAb was used. The E3 MAb has previously been shown to be directed against a nuclear antigen synthesized shortly after infection of human embryonic fibroblasts with HCMV strain AD169 or Towne. This MAb specifically immunoprecipitates a 72,000-molecular-weight (72K) IE protein from AD169-infected cells (15). By in vitro translation studies, it has been shown that RNA selected by hybridization to a HCMV DNA fragment that codes for the major IEA of strain Towne directs the synthesis of a protein recognized by the E3 MAb (42, 47).

TABLE 1. Induction of E3 nuclear fluorescence upon release from protein synthesis inhibition

Treatment ^a	% E3-positive nuclei ^b
Cycloheximide	20
Puromycin	22
Emetine	10
Anisomycin	17
Mock	1

^a Rat-IEA⁺-9G cells growing on cover slips were treated for 3 h with inhibitors of protein synthesis (cycloheximide, 50 µg/ml; puromycin, 50 µg/ml; emetine, 10 µg/ml; or anisomycin, 10 µg/ml) or mock treated with the solvent of the inhibitors.

^b Cells were fixed 19 h after the release from the protein synthesis block and prepared for IF with the E3 MAb. At least 1,000 nuclei were scored for the presence of nuclear fluorescence.

FIG. 7. Induction of E3 nuclear fluorescence after release from protein synthesis inhibition. Rat-IEA⁺-9G cells growing on cover slips were either mock treated (A) or treated with cycloheximide (50 µg/ml of medium) (B) for 3 h. The cells were fixed 18 h after release from the protein synthesis block and prepared for IF with the E3 MAb.

FIG. 8. Induction of HCMV-specific transcripts upon treatment with cycloheximide: dependency on the duration of the protein synthesis block. Rat-IEA⁺-9G cells growing in 60-mm petri dishes were either left untreated (lanes 1 and 2) or treated for 2 h (lanes 3 and 4), 4 h (lanes 5 and 6), 9 h (lanes 7 and 8), or 12 h (lanes 9 and 10) with cycloheximide (50 μ g/ml of medium). Total cellular RNA was then isolated, electrophoresed through a 1% agarose-formaldehyde gel, blotted onto nitrocellulose, and probed with ³²P-labeled pES. RNA was isolated from two separate dishes for each time point, and half of the RNA was loaded onto the gel. Autoradiographic exposure was for 17 h.

From the above considerations we conclude that the nuclear antigen detected by the E3 MAb upon transfection with pES represents a major IEA of HCMV. This conclusion is in good agreement with recent IE mRNA mapping data (19, 55) and DNA sequence analysis of the major IE cistron (2), showing that a major, spliced IE mRNA originates from DNA sequences encompassed by pES. These data are also consistent with the absence of nuclear fluorescence upon transfection of Rat-2-TK⁻ cells with the cloned 5.0-kb *SphI-SalI* fragment (pSS in Fig. 1) because pSS is deleted for the upstream IE regulatory elements, the leader, and part of the first intron of the major IE transcript relative to pES.

From the data presented in this paper, it cannot be determined whether the nuclear antigen detected upon transfection with pES represents the major 72K IEA or, due to alternative splicing, a variant IE nuclear antigen with an epitope in common with the 72K IEA. However, preliminary data obtained by Western blot analysis with the E3 MAb indicate that the nuclear antigen detected upon release from protein synthesis inhibition in Rat-IEA⁺-9G cells comigrates with the major 72K IEA synthesized upon viral infection of human diploid cells.

Cotransfection experiments using pES and the HSV-1 TK gene as a selectable marker resulted in the establishment of a HAT-resistant cell line, Rat-IEA⁺-9X, which expressed the E3 nuclear antigen in approximately 1% of the cells. Six subclones isolated from this cell line (among which is the Rat-IEA⁺-9G cell line) showed the same low level of expression of the nuclear antigen as the parental line (Fig. 7). Moreover, Southern blot analysis revealed that both the parental Rat-IEA⁺-9X cell line and the six subclones had identical integration patterns for HCMV DNA sequences (Fig. 3). These results suggest a stable integration of these HCMV DNA sequences within the genome of any cell present within the population of the parental cell line and the subclones derived therefrom. Chromosome spreads obtained from metaphase-arrested cells of one of the subclones (Rat-IEA⁺-9G) showed, upon in situ hybridization, a strong

hybridization signal at a distinct location on both sister chromatids of a large submetacentric chromosome (Fig. 6). It is likely that the tandemly repeated pES molecules are located in this chromosome. In addition to this tandem integration, at least seven other integration events occurred (Fig. 3 and 4), the nature of which was not further investigated. It should be noted that, upon subculturing, the spontaneous (versus induced) expression of the nuclear antigen gradually decreased (to less than 0.1% at passage level 40). However, no loss or major rearrangements of the integrated HCMV DNA sequences were observed for over 80 subculturings, either in the presence or absence of HAT selective pressure (data not shown).

Clearly, the genetic information to express one or more proteins encoded for by pES was present in all cells within the population, yet the E3 nuclear antigen was only detectable in a small fraction of the cells. It is therefore likely that major IE expression was repressed in most cells. A possible clue to the mechanism of the repression came from the experiments in which Rat-IEA⁺-9G cells were treated with inhibitors of protein synthesis. Within 4 h after the addition of the inhibitor, a massive increase in steady-state levels of pES-specific transcripts was observed (Fig. 8). Such an increase in steady-state levels is likely to have resulted from an increased transcription rate or decreased breakdown of transcripts (stabilization) or both in the presence of the inhibitor. Recent experiments measuring transcription rates before and after the addition of the inhibitor have provided evidence that the increase in steady-state levels of pES-specific transcripts is mainly the result of an increased transcriptional rate, indicating that the repression is at the transcriptional level (J. L. Geelen et al., manuscript in preparation).

After release from protein synthesis inhibition, a similar massive increase in the number of cells expressing the E3 nuclear antigen was observed (Fig. 6 and 7). Because inhibitors of protein synthesis with different modes of action showed similar levels of induction of nuclear fluorescence (Table 1), it can be concluded that inhibition of protein synthesis rather than a side effect of the inhibitor led to the induction phenomenon.

Enhancement of steady-state levels of viral and cellular transcripts mediated by protein synthesis inhibition has been known for several years. Stabilization of transcripts plays a major role in the accumulation of histone and *c-myc* RNA in the presence of the inhibitor (8, 28, 38, 43); on the other hand, increased transcription rates have been shown to be responsible for the increased amounts of actin, beta interferon, and early adenovirus transcripts (7, 11, 34, 37). Models to explain the stimulatory effect of protein synthesis inhibition at the transcriptional level assume the existence of short-lived repressor proteins (7, 34). For HCMV, accumulation of viral transcripts in the presence of protein synthesis inhibitors (and their products upon release) has been widely used to study IE gene expression (3, 10, 45); however, the mechanism of accumulation is still largely unknown.

Total cellular RNA derived from inhibitor-treated Rat-IEA⁺-9G cells showed strong increases in the amounts of two major pES-specific transcripts of 2.1 and 1.9 kb and a minor 2.9-kb transcript in Northern blot analysis (Fig. 8). It is likely that these transcripts represented mRNAs because transcripts of the same lengths were also detected with polysomal rather than total cellular RNA (data not shown). The lengths of two of the induced major transcripts are in good agreement with published lengths and map locations of IE mRNAs (a 1.95-kb major transcript and a 2.15-kb middle-

abundant transcript [55]). We did not detect the 1.7-kb IE transcript synthesized in permissive cells upon viral infection (55); instead a 2.9-kb transcript was observed.

HCMV, like other members of the herpesvirus group, can exist in a latent state (17). It has been proposed that the IE proteins of HCMV control the transcriptional program of the viral genome (47, 53). It is therefore likely that the first event leading to a properly programmed productive cycle (reactivation) requires viral IE expression, which may have been previously maintained in a repressed state (latency). The cell line described in this paper may therefore provide an *in vitro* model to study events crucial in latency and reactivation.

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