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Transcriptional Regulation of the Human Cytomegalovirus Major Immediate-Early Gene Is Associated with Induction of DNase I-Hypersensitive Sites†

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Human teratocarcinoma cells were used to examine structural features associated with expression of the major immediate-early (IE) gene of human cytomegalovirus. By immunofluorescence, comparison of RNA levels, and in vitro transcription of nuclei, we showed that the major IE gene is inactive in undifferentiated but active in differentiated cells. Therefore, the block in human cytomegalovirus replication in teratocarcinoma cells appears to be at the transcriptional level, in one of the initial genes transcribed. In addition, the in vitro transcription experiments demonstrated that in permissive infections the gene was transcriptionally inactive late in infection. A comparison of the structural features of the promoter region with the active and inactive IE genes showed the presence of constitutive and inducible DNase I-hypersensitive sites. The majority of the constitutive sites existed at −175, −275, −375, −425, and −525 relative to the cap site in an area which has been shown to be capable of simian virus 40 enhancer function. In contrast, the inducible DNase I sites were located outside this region at −650, −775, −875, and −975.

Teratocarcinoma cell systems have provided a unique in vitro tool to dissect molecular events involved in the regulation of viral genes after cellular differentiation. Teratocarcinoma cells are derived from embryonal carcinoma tumors which can be stimulated to differentiate into a wide variety of somatic cell types. Murine embryonal carcinoma cells are resistant to infection by retroviruses (16, 44), papovaviruses (42, 43), and a herpesvirus (11). However, these viruses were able to replicate when the teratocarcinoma cells were stimulated to differentiate. The lack of viral replication is attributed to multiple causes, including blocks in transcription (15) and inefficient RNA processing (36). A recent report describes a model for the replication of human cytomegalovirus (HCMV), a human herpesvirus, in a human teratocarcinoma cell line (17). HCMV was able to replicate in differentiated cells, but there was an unidentified block in viral replication in undifferentiated cells. We used this virus-cell system to examine important features of HCMV gene expression and to determine the reason for the block in replication.

The genome of HCMV is a large, linear, double-stranded DNA molecule (235 kilobase pairs), which contains two unique stretches of DNA, U_L and U_S, bordered by inverted terminal repeats (10, 49). Similar to the herpes simplex viruses, HCMV displays a cascade of RNA synthesis which can be divided into three separate phases (9, 26, 47). The RNA species detectable before protein synthesis are designated immediate-early (IE) RNAs and are transcribed in restricted areas of the genome (9, 47). After the onset of viral protein synthesis another class of RNA species, early RNA, originates from regions throughout the genome but primarily from the inverted terminal repeats. The late RNAs are transcribed after viral DNA replication. The most abundant mRNA transcribed during the IE period codes for a 72,000-dalton protein (41). This protein is preferentially associated with chromatin (S. Michelson, personal communication) and is proposed to play a major role in influencing transcription of other viral genes (41). A structural analysis indicates that the mRNA coding for this protein is transcribed as a 2.6-kilobase (kb) nuclear transcript containing four exons which are spliced into a 1.9-kb cytoplasmic transcript (40). This transcript is not detectable at late times during permissive infection, suggesting temporal regulation (22).

Actively transcribing eucaryotic genes are preferentially sensitive to DNase I (48). In addition to general DNase I sensitivity, local regions of altered chromatin conformation are also detectable by DNase I and by other nucleases (6, reviewed in reference 12). These DNase I-hypersensitive sites have also been found in enhancer elements of viruses (3, 7, 35) and eucaryotic genes (31) controlling active transcription and are thought to represent structural changes in the chromatin, perhaps because of the complexing of regulatory proteins with specific sequences of DNA (see review in reference 6).

To determine the level of control of expression of HCMV in human teratocarcinoma cells, we examined the chromatin structure and transcription of the region encoding the 1.9-kb major IE RNA in both differentiated and undifferentiated cells. Since DNase I-hypersensitive sites are often associated with regions involved in the regulation of gene expression, HCMV chromatin was examined for these sites in both permissive and nonpermissive states. Our results demonstrated a major area of DNase I hypersensitivity in the 5′ flanking region of this gene, a region which contains an HCMV DNA fragment capable of enhancerlike function (2a). In addition, an altered pattern of DNase I hypersensitivity was observed which correlated with active transcription of this gene as measured by nuclear transcription runoff experiments. From the chromatin and transcription studies, the inability of the major IE gene to function in undifferen-

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tiated teratocarcinoma cells represented a transcriptional block. Transfection experiments confirmed the lack of function of the IE gene in undifferentiated cells and demonstrated that the block in expression resides in the upstream control region of this gene.

MATERIALS AND METHODS

Virus and cell culture. HCMV strain AD169 was propagated by following standard methods (29). All cells were infected at 80% confluence with 3 to 5 PFU per cell. The human teratocarcinoma cell line Tera-2 clone D1 was isolated (1) and kindly provided by P. Andrews (Wistar Institute, Philadelphia, Pa.). This cell line was maintained in Dulbecco modified essential medium (high glucose formulation; Flow Laboratories, Inc., McLean, Va.) with 10% fetal calf serum and passaged at 1:3 before confluence. Unless otherwise indicated, Tera-2 cells were stimulated to differentiate in media containing 10^{-5} M retinoic acid (RA) (Sigma Chemical Co., St. Louis, Mo.) diluted from a 10^{-2} M stock solution in dimethyl sulfoxide. These cells were grown for 7 days in the presence of RA before use in experiments. For isolation of IE RNA, cells were pretreated for 1 h, and then they were infected and maintained in the presence of 100 μM anisomysin (Sigma). Total cell RNA was extracted by the guanidinium isothiocyanate-cesium chloride method (5). The cell line VA-13 was cloned from simian virus 40 (SV40)-transformed WI38 cells and kindly provided by J. Sorge (Scripps Clinic and Research Foundation).

DNase I digestion of nuclei. Nuclei isolation and DNase I digestion was performed as previously described (48). Briefly, cells were scraped and washed two times in phosphate-buffered saline. Nuclei were isolated by lysis of cells in reticulocyte standard buffer (0.01 M Tris hydrochloride [pH 7.4]; 0.01 M NaCl, 3 mM $MgCl_2$) containing 0.5% Nonidet P-40 (British Drug House). After Dounce homogenization, the nuclei were pelleted at 3,000 × *g* for 10 min, suspended in 5 ml of ice-cold reticulocyte standard buffer with 10 mM $CaCl_2$, and divided into 10 tubes at 500 μl each. DNase I (Worthington Diagnostics, Freehold, N.J.) was added to each tube in increasing concentrations, 0 to 5 mg/ml, to determine optimal enzyme concentration. Nuclei were digested for 10 min at 37°C, and the reaction was stopped with the addition of 500 μl of a solution containing 400 μg of protease K per ml, 1% sodium dodecyl sulfate, 0.6 M NaCl, 20 mM Tris hydrochloride (7.5), and 10 mM EDTA and digested at 37°C overnight. DNA was then isolated by phenol and chloroform extraction and ethanol precipitation.

DNA transfection. Transfection assays involved precipitation of various concentrations of plasmids containing the neomycin gene supplemented up to 20 μg with calf thymus carrier DNA (Worthington) by the calcium phosphate technique (18) onto subconfluent monolayers of Tera-2 or VA-13 cells. Each monolayer of cells contained 5×10^5 cells per 60-mm-diameter dish. The medium (Dulbecco modified essential and 10% fetal calf serum) was changed 4 h after precipitation, and the cells were allowed to incubate for 48 h at 37°C. After incubation, cells were passaged 1:3 and put into medium containing 200 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. The medium plus drug was changed every 3 days and colonies were counted after 14 days. Plasmids pSV2-neo and pSV_{tk}neo were generously donated by P. Southern (Scripps Clinic and Research Foundation) and plasmid pJTE201 was given by J. Elder (Fred Hutchinson Cancer Research Center).

RNA and DNA blot hybridization. Restriction enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.), and digestions were performed in the recommended buffers. Agarose gel electrophoresis was done in a buffer containing 90 mM Tris (pH 8.3), 90 mM boric acid, and 1 mM EDTA. DNA was transferred to nitrocellulose according to the method of Southern (38). RNA dot blots were prepared according to the method of White and Bancroft (50) with a dot blot apparatus from Schleicher & Schuell, Inc. (Keene, N.H.). Probes isolated from low-melting-point agarose gels (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were nick repaired to a specific activity of greater than 10^8 cpm of DNA per μg with [^{32}P]dATP and [^{32}P]dCTP (3,200 Ci/mM; New England Nuclear Corp., Boston, Mass.) according to the method of Rigby et al. (34). Procedures for prehybridization, hybridization and washing of Southern blots, and RNA dot blots have been described previously (29). Autoradiographic exposures were done at −80°C with XAR-5 X-Omat film (Eastman Kodak Co., Rochester, N.Y.) Lightning-Plus intensifier screens (Du Pont Co., Wilmington, Del.).

Immunofluorescence techniques. For these experiments, a mouse monoclonal antibody to the 72,000-dalton IE protein was previously described (33) and generously donated by M. Oldstone (Scripps Clinic and Research Foundation). Cells were plated onto glass slides and grown and infected with HCMV for 8 h at a multiplicity of infection of 3 to 5. Cells were then dried and fixed for 10 min in ethanol. After the monoclonal antibody was allowed to react with the cells, bound antibody was determined by indirect immunofluorescence with fluorescein-labeled pepsin-digested goat antibody to mouse immunoglobulin with fluorescence microscopy.

Transcription in isolated nuclei. Nuclei were isolated as described for the DNase I digestion of nuclei and were then suspended at a concentration of 2×10^8 nuclei per ml in nuclear-freezing buffer (40% glycerol, 50 mM Tris hydrochloride [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA). The nuclei were either used directly or were frozen at −70°C. Transcription assays were done as described by Groudine et al. (20) with modifications described by Greenberg and Ziff (19). Each reaction mixture consisted of 210 μl of nuclei and 60 μl of 5× runoff buffer (5× runoff buffer consists of 25 mM Tris hydrochloride [pH 8.0], 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM ATP, 1.25 mM GTP, and 1.25 mM CTP). A 30-μl volume (approximately 450 μCI) of [α-^{32}P]UTP (3,000 Ci/mM) was then added, and the nuclear suspension was incubated at 30°C for 30 min, after which time 15 μl of a solution containing 5 μg of DNase I per ml in 10 mM $CaCl_2$ was added. After 5 min at 30°C, 1× SET (1% sodium dodecyl sulfate, 5 mM EDTA, 10 mM Tris hydrochloride [pH 7.4]) was added to the reaction mixture, and proteinase K was added to a concentration of 200 μg/ml. After incubation at 37°C for 45 min, the solution was extracted with an equal volume of phenol-chloroform, and the interphase was reextracted with 100 μl of 1× SET. A volume of 10 M ammonium acetate was added to the combined aqueous phases (original plus reextraction) to a final concentration of 2.3 M, an equal volume of isopropyl alcohol was added, and nucleic acid was precipitated at −70°C for 15 min. The precipitate was centrifuged in a microcentrifuge for 10 min, and the pellet was suspended in 50 μl of TE (10 mM Tris hydrochloride, 1 mM EDTA). The sample plus 50 μl of TE used to wash the centrifuge tube was centrifuged through a G-50 (coarse) spin column. The eluate was made 0.2 M NaOH, and after 10 min on ice *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was added to 0.24 M. Then 2.5 volumes of ethanol was added, and the solution

was precipitated overnight at −70°C. After centrifugation in a microcentrifuge of 5 min, the pellet was suspended in hybridization buffer which consisted of 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.4), 0.2% sodium dodecyl sulfate, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt solution, and 250 μg of *Escherichia coli* RNA per ml.

Nitrocellulose filters containing plasmids of interest were prepared with a Schleicher & Schuell slot blot apparatus under conditions suggested by the manufacturer. A total of 5 μg of each plasmid containing the appropriate inserts was loaded per slot. A plasmid containing cDNA from chicken actin was generously donated by Nancy Hutchinson. These filters were prehybridized in the hybridization solution described above for a minimum of 2 h at 65°C. After prehybridization, the filters were hybridized to the runoff products in hybridization solution for 36 h. Equal numbers of counts per minute of runoff products were added per filter for nuclei that were being directly compared. A typical reaction contained 2 ml of hybridization solution with 2.5×10^6 to 5×10^6 counts per ml. After hybridization, the filters were washed for 1 h in 2× SSC with 10 mg of RNase A per ml for 30 min and were subsequently washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C for 1 h. Filters were then exposed to XAR film (Kodak) in cassettes containing Lightning-Plus screens (Du Pont) at −70°C for various times.

RESULTS

Mapping of DNase I sites in the promoter region of the HCMV major IE gene. To determine whether the structure of viral chromatin paralleled expression of particular genes, HCMV chromatin from productively infected human fibroblast cells was examined for the presence of DNase I-hypersensitive sites. In a representative DNase I experiment, HCMV-infected cell nuclei were digested with various concentrations of enzyme to determine the optimal concentration of DNase I to generate subbands. We examined the region encoding the 1.9-kb major IE transcript which is active immediately upon viral adsorption and uncoating (22, 41). The coding sequence of this gene is contained within a 2.6-kb section of HCMV DNA between a *Bam*HI site and an *Sst*I site (Fig. 1) (40). To map the hypersensitive sites in this region, DNA purified from DNase I-treated infected cell nuclei was double digested with *Bam*HI and *Eco*RI transferred to nitrocellulose and then hybridized with a *Bgl*II-*Bam*HI fragment, which served as an indirect end-labeled probe (51). A predicted 4.7-kb parental DNA band generated by the restriction endonucleases was observed in both the control and DNase-treated DNA lanes (Fig. 1). A major subband covering approximately 350 bases was observed in the DNase lane starting 100 bases upstream from the 5′ end of the coding sequence for the transcript. Because of the high degree of DNase I hypersensitivity in the 5′ flanking region, subbands could not be resolved with these restriction enzymes and this probe.

To examine the major area of DNase I hypersensitivity in the 5′ flanking region in greater detail, DNA was isolated from DNase I-treated infected cell nuclei and was redigested with *Pst*I to generate smaller fragments and to expand this region on a higher-percentage gel. Probing of a Southern blot with a *Hinc*II-*Pst*I end fragment 5′ to the start of the RNA demonstrated a 1.8-kb *Pst*I parental band with at least 13 reproducible subbands generated by DNase I (Fig. 2). The positions of these sites were confirmed by probing from the 3′ end relative to the start of the message with a 235-bp

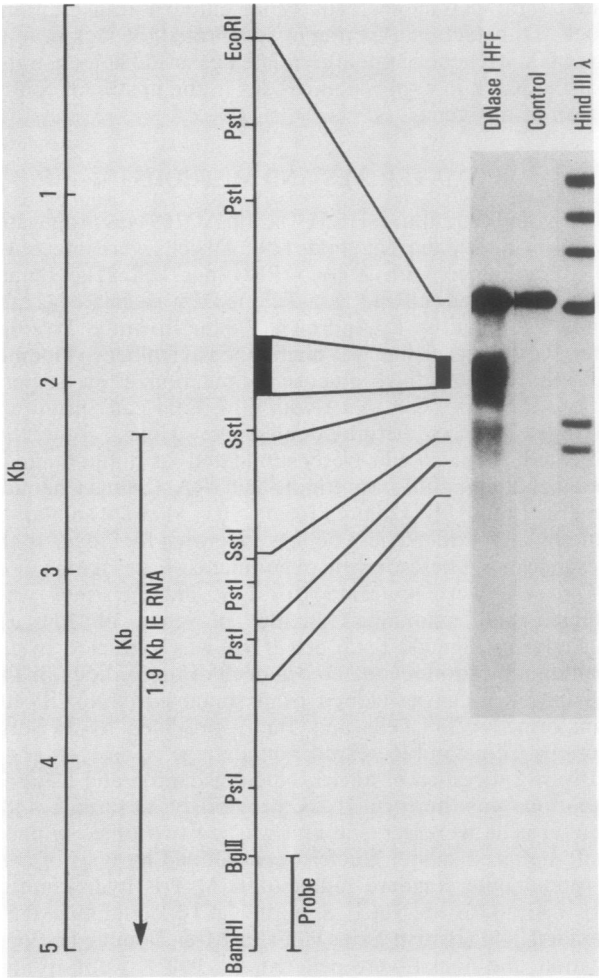


FIG. 1. Map of DNase I-hypersensitive sites in the 1.9-kb IE gene of HCMV. DNA from DNase I-digested nuclei derived from HCMV-infected HFF cells was double digested with *Eco*RI and *Bam*HI, electrophoresed in a 1% agarose gel, blotted, and probed with a *Bgl*II-*Bam*HI fragment located at the 3′ end of the 1.9-kb IE gene. The control lane represents DNA from nuclei undigested with DNase I. The positions of the DNase I-generated subbands are indicated on the restriction endonuclease map of the region.

*Pst*I-*Sst*I end-labeled fragment (data not shown). DNase I sites 1 and 2 are located well into the coding region of the gene, corresponding to an intron mapped in this area (40), while DNase I sites 3 and 4 are near the cap site and the TATAA box, respectively. DNase I sites 5 through 13 span a large area of the 5′ flanking region which is known to have multiple families of repeats (2a, 45). Specific cleavage sites were not seen when naked viral DNA was treated with DNase I under varying conditions (unpublished results).

Inducible DNase I-hypersensitive sites in the 5′ flanking region correlate with expression in teratocarcinoma cells. A human teratocarcinoma cell line, Tera-2 clone D-1 (1), was used to examine control of expression of the major IE gene of HCMV. These embryonal carcinoma cells are pluripotent cells which can be differentiated into a wide variety of somatic cell types when dilute amounts of RA are supplemented in the media. In a recent report, HCMV was shown to nonproductively infect these undifferentiated Tera-2 cells (17). However, infectious virus could be recovered from cells stimulated to differentiate with RA. This cell line

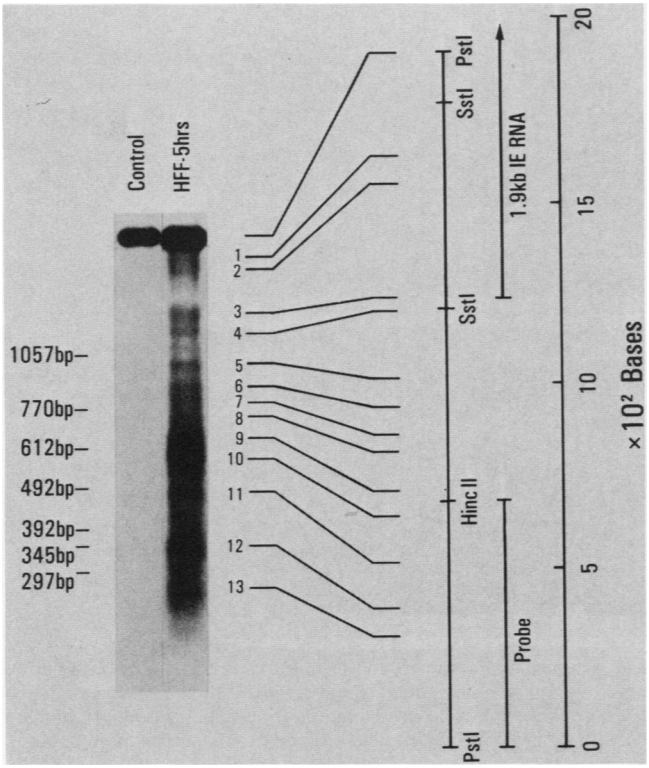


FIG. 2. Higher-resolution mapping of the DNase I-hypersensitive sites in the promoter region of the HCMV 1.9-kb IE gene. DNA from DNase I-digested nuclei derived from HFF cells infected with HCMV in the presence of 100 μM anisomycin was digested with *Pst*I, electrophoresed in a 1.5% agarose gel, blotted, and probed with a *Hinc*II-*Pst*I fragment located at the 5′ end of the promoter region relative to the cap site of the RNA. The positions of DNase I-hypersensitive sites 1 through 13 are indicated in relation to key restriction endonuclease sites in this region. The DNA markers are ^{32}P-end-labeled *Hinc*II restriction fragments of ϕX174.

provided an inducible system to examine expression of HCMV genes and its relationship to the structure of the viral DNA. The initial experiments examined the expression of the product of the 1.9-kb IE RNA, the 72-kilodalton polypeptide, by immunofluorescence with the monoclonal antibody L14 directed against the protein (33). By this method less than 1% of the infected undifferentiated cells demonstrated nuclear fluorescence. However, infection of cells stimulated to differentiate with increasing concentrations of RA (10^{-7} M to 10^{-5} M) resulted in a concomitant increase in nuclear fluorescence up to 100% at the highest concentration (Fig. 3). The few undifferentiated cells positive for the 72-kilodalton protein may represent a population which spontaneously differentiated to allow viral replication.

To determine if the lack of viral protein was caused by transcriptional or translational controls, the relative RNA levels of HCMV-infected differentiated and nondifferentiated Tera-2 cells were examined by RNA dot blot with a small 475-base-pair (bp) *Bgl*II-*Bam*HI fragment located at the 3′ end of the 1.9-kb IE coding region. The results shown in Fig. 4 indicate that only the normally permissive human foreskin fibroblast (HFF) and differentiated Tera-2 cells at 5 h postinfection contained high levels of viral RNA. However, all samples demonstrated equal hybridization to an actin probe, showing that all samples were capable of hybridization. Little or no RNA was detected in the undifferentiated Tera-2 cells, and the levels of RNA decreased at 48 h in the HFF and differentiated cells. The decreased levels of the 1.9-kb IE RNA over time during a permissive infection have been previously reported by Jahn et al. (22).

To determine whether a structural change of the viral chromatin paralleled levels of RNA detected by dot blot, DNase I-hypersensitive sites were mapped in the 1.9-kb IE RNA 5′ flanking region in the undifferentiated and differentiated Tera-2 cells. DNA from DNase I-treated DNA from the nuclei of HCMV-infected permissive (HFF and differentiated Tera-2 cells at 5 h postinfection) and nonpermissive cells (undifferentiated Tera-2 cells at 5 h postinfection and differentiated Tera-2 cells at 48 h postinfection) was digested with *Pst*I, electrophoresed in agarose, and probed with a *Hinc*II-*Pst*I probe at the end of the 5′ flanking region. Different DNase I-hypersensitive site patterns during permissive and nonpermissive situations are shown in Fig. 5A. The DNase I sites which are present in all the lanes are at +275 (site 1) and +175 (site 2) (corresponding to an area where an intron has been mapped), −50 (site 4), −175 (site 5), −275 (site 6), −375 (site 7), −425 (site 8), and −525 (site 9) relative to the start of the mRNA. These sites are designated as constitutive since they are present during both permissive and nonpermissive periods. Although the DNase I hypersensitivity of the constitutive sites in the differentiated Tera-2 cells at 5 h postinfection was greatly diminished, these sites were present on longer exposure of the gel. In the permissive HFF and differentiated Tera-2 cells at 5 h postinfection five additional DNase I sites (inducible) appear near the cap site (2a) and at −650 (site 10), −775 (site 11), −875 (site 12), and −950 (site 13) relative to the start of the mRNA. A map of these sites is shown in Fig. 5B. Clearly, the distal inducible DNase I sites at 10, 11, 12, and 13 correlate with high levels of RNA. In addition, the molarity of sites 10 to 13 in the permissive cells appears much greater than that of the more proximal sites 4 to 9; however, in the nonpermissive cells the opposite situation exists.

Examination of the major IE gene by in vitro transcription of infected nuclei. The lack of viral protein and RNA and the change in hypersensitive sites 5′ to and within the 1.9-kb IE gene in the undifferentiated teratocarcinoma cells led us to examine the transcription of this gene in both permissive and nonpermissive states. Interestingly, since hypersensitive sites change at both the 5′ end of the gene and in a region that corresponds to an intron, the control of expression of this gene could be based on the lack of polymerase initiation at the 5′ end of the gene or perhaps on premature termination or attenuation at the region defined by the change in hypersensitive sites within the gene. To examine these possibilities, we performed nuclear runoff transcription experiments and assayed for the presence of polymerase molecules in both the 5′ region and in coding regions of the IE 1.9-kb gene in human foreskin fibroblasts and in undifferentiated and differentiated human teratocarcinoma cells infected with HCMV. As described previously (20), the technique of nuclear runoff transcription measures the presence of elongating polymerases along a specific gene. Essentially, isolated nuclei were incubated with [α-^{32}P]UTP for 30 min under specific conditions (see Materials and Methods) and the resultant ^{32}P-labeled nascent RNA was hybridized to an excess of DNA bound to nitrocellulose. In the experiments illustrated in Fig. 6, a 0.75-kb *Sst*I fragment containing 5′ coding sequences and a *Bgl*II-*Bam*HI 450-bp fragment containing 3′ coding sequences were immobilized on nitrocellulose and the transcription of corresponding regions was assayed by hybridization of nascent RNA to these frag-

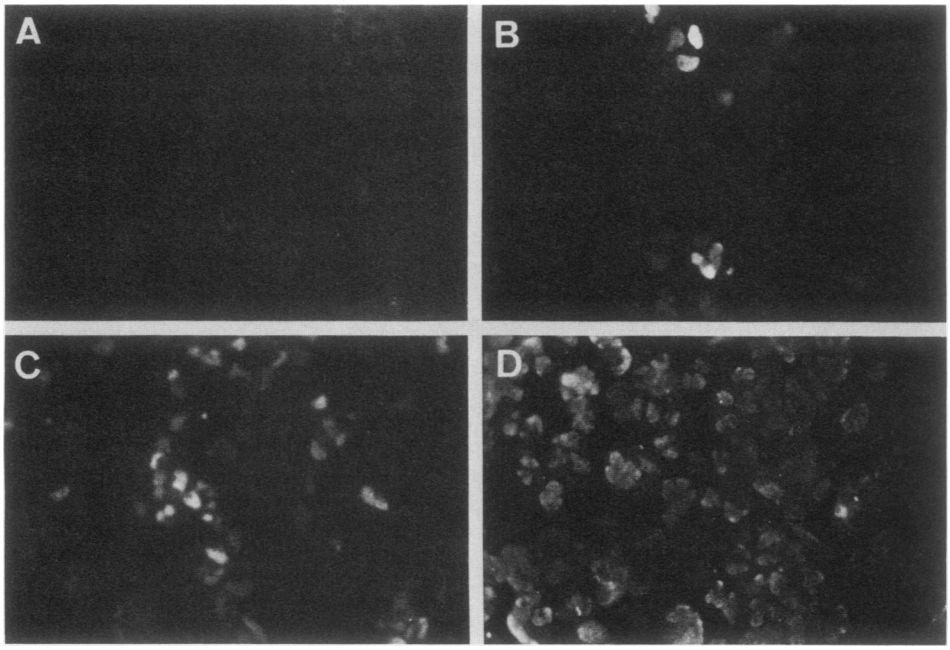


FIG. 3. Indirect immunofluorescence of HCMV-infected Tera-2 cells with a monoclonal antibody to the major IE protein. At 7 days before infection Tera-2 cells were exposed to RA concentrations of (A) 0, (B) 10^{-7} mg/ml, (C) 10^{-6} mg/ml, and (D) 10^{-5} mg/ml in the media to stimulate differentiation. The cells were then grown, infected, and fixed on glass cover slips as described in Materials and Methods. IE antigen was probed with monoclonal antibody L14 by indirect immunofluorescence with fluorescein-labeled goat antibody to mouse immunoglobulin.

ments. As shown in these experiments, no detectable signal from any region of the 1.9-kb gene was found in mock-infected HFF or teratocarcinoma cells. However, significant signals were detected in the 5′ and 3′ coding regions of this gene 5 h after infection of HFF and differentiated teratocarcinoma cells with cytomegalovirus. In contrast, no signal was detectable by 48 h postinfection of the HFF or differentiated teratocarcinoma cells. The ratio of the signal detectable in the HFF cells 5 h postinfection in the 5′ and 3′ coding sequences is proportional to the length of the fragments containing sequences from these regions. In addition, the results presented in Fig. 6 were unchanged when the nuclear runoff transcription experiments were conducted in the presence of 0.5% sarcosyl (data not shown). This compound removes histones and most other chromosomal proteins from chromatin, but leaves initiated polymerases bound to DNA and capable of elongation, but not of new initiation. Under these conditions, RNA polymerases which might normally be inhibited from elongation by the presence of histones or other proteins would be expected to elongate. Since sarcosyl has no effect on the transcription of any of the regions of the 1.9-kb HCMV gene, initiated but blocked polymerases do not appear to be contributing to regulation of expression of this IE gene.

Construction and transfection of the HCMV-*neo* plasmid. To study the regulation of the HCMV major IE promoter without the influence of viral factors or adjacent viral sequences, a 1.2-kb *Sst*I-*Pst*I fragment containing the regulatory signals for the transcription of this gene was cloned into a plasmid containing the bacterial gene (*neo*) conferring resistance to the antibiotic G418 (8). The *neo* gene without the SV40 early promoter was subcloned from pSV-2neo as a 2.5-kb *Hin*dIII-*Bam*HI fragment into the *Bam*HI site of the polylinker of pUC18. This fragment contains 847 bases of SV40 3′ to the *neo* gene to properly terminate the message (39). The 1.2-kb *Pst*I-*Sst*I promoter region was cloned into pUC12, released from the plasmid with *Sst*I, and cloned in the proper orientation 5′ to the *neo* gene in the *Sst*I site HCMV-*neo* (Fig. 7). The sequence of this promoter region indicates that the TATAA box is 3 bases 5′ to the *Sst*I site (2a, 45).

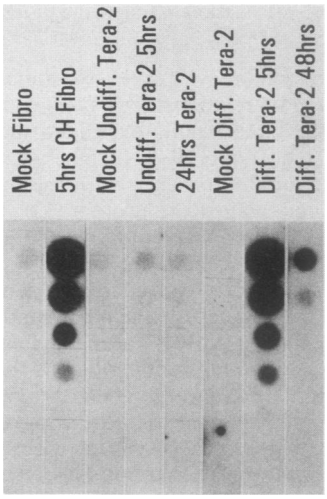
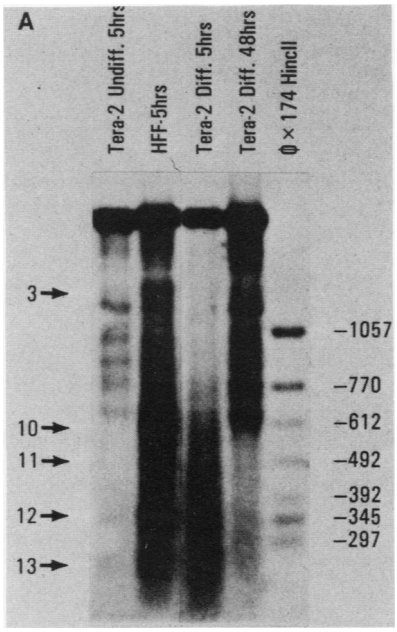


FIG. 4. Major IE RNA levels of HCMV-infected HFF and Tera-2 cells. RNA dot blot of whole cell RNA extracted from HCMV-infected HFF cells (5 h postinfection with 100 μM of anisomycin and 48 h postinfection), from undifferentiated Tera-2 cells (5 h postinfection with 100 μM of anisomycin and 24 h), and from Tera-2 cells exposed to 10^{-5} RA 7 days before infection (5 h postinfection with 100 μM of anisomycin and 48 h). Each well represents a fivefold dilution from an initial quantity of 25 μg of RNA. The blot was probed with a *Bgl*II-*Bam*HI fragment located at the 3′ end of the gene.



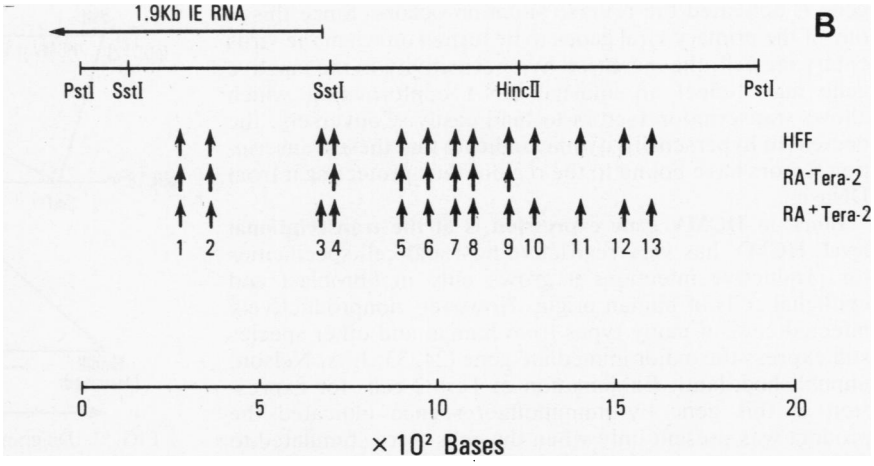


FIG. 5. Comparison of DNase I-hypersensitive sites in permissively and nonpermissively HCMV-infected cells. DNA from DNase I-digested nuclei derived from HCMV-infected HFF cells (5 h postinfection with 100 μM of anisomycin), undifferentiated Tera-2 cells (5 h postinfection with 100 μM of anisomycin), and differentiated Tera-2 cells (5 h postinfection with 100 μM of anisomycin and 48 h postinfection) was digested with *Pst*I, electrophoresed in a 1.5% agarose gel, blotted, and probed with a *Hinc*II-*Pst*I fragment (A). The positions of the inducible subbands which appear in permissively infected cells are indicated (arrows). The relative positions of the DNase I-hypersensitive sites in relation to the 1.9-kb IE RNA coding region in permissively (HFF and RA^+ Tera-2 cells at 5 h postinfection with 100 μM of anisomycin) and nonpermissively infected (RA^- Tera-2) HCMV cells are diagrammed (B).

To test the activity of this promoter, undifferentiated Tera-2 cells (nonpermissive) and SV40-transformed WI38 cells (permissive) were transfected with the HCMV-*neo* plasmid, and stably transformed colonies were selected for resistance to G418. The efficiency of colony formation with the HCMV-*neo* plasmid was tested with the neomycin gene attached to (i) the SV40 early promoter enhancer-SV2 *neo* (39), (ii) a 250-bp fragment containing the TK promoter (30), and (iii) the SV40 early promoter with 232 bp deleted from this area removing the SV40 72-bp repeats (14), plasmid pJTE201. The pUC-neo plasmid contains the neomycin gene with no known eucaryotic promoter. The results shown in Table 1 indicate that the HCMV promoter region worked as efficiently as the SV40 promoter-enhancer (SV2-*neo*) and better than the HSV1 TK promoter or SV40 promoter with the enhancer deleted. Similar results were observed in mouse L cells transfected with these clones (data not shown). However, when the same plasmids were transfected into the undifferentiated Tera-2 cells, a significant number of colonies resistant to G418 were observed only with the neomycin gene controlled by the herpes simplex virus 1 TK promoter. These results lead to the following conclusions. (i) The inability of the 1.9-kb IE gene to express in undifferentiated Tera-2 cells appears to reside in the promoter, and (ii) in permissive cells this HCMV promoter works as effectively as the SV40 promoter-enhancer.

DISCUSSION

Constitutive DNase I-hypersensitive sites correlate with an HCMV enhancer. We demonstrated that an area of DNase I hypersensitivity exists in the 5′ flanking region of the major IE gene of HCMV. The hypersensitive sites were composed of two types, constitutive and inducible, based on their appearance in permissive and nonpermissive cells. Recently, Boshart et al. (2a) identified a short segment of HCMV DNA which would substitute for the SV40 enhancer by cotransfection of HCMV DNA and enhancerless SV40 DNA and by isolation of SV40-HCMV recombinants. These segments fulfilled all the requirements of a *cis*-acting enhancer element by their ability to increase the transcription of β globin when linked to the gene in either orientation. These segments were located between −118 and −524 relative to the start of the major IE RNA, within the area we identified as DNase I hypersensitive. In this enhancer region we detected five constitutive DNase I sites at −175, −275, −373, −425, and −525 which were shown to be present whether or not the gene was actively transcribing. The fact that these sites were constitutive may reflect the presence of four families of repeat elements ranging in size from 17 to 21 bp (2a, 45). These repeat elements may confer a permanent DNA conformation which is DNase I hypersensitive. However, within the limits of our mapping there seems to be no correlation between the location of the DNase I sites with any particular repeat family. The above observations are consistent with the findings with the enhancers of SV40 and polyomavirus which are also found in areas of DNase I hypersensitivity (3, 23). In addition, it is interesting to note that the enhancer region of SV40 contains five DNase I-hypersensitive sites, with the first four sites at the beginning and the end of each of the two 72-bp repeats (3). The essential core region(s) of the HCMV enhancer element between −118 and −524 has not been identified, so the relationship to the DNase I sites remains uncertain.

The major IE gene has been hypothesized to be the first gene activated in the cascade of transcription for HCMV (41). We found that the constitutive DNase I sites in the enhancer are in greater molar excess relative to the distal DNase I sites when the gene is inactive. However, when the

gene is activated the reverse situation occurs. Since this is one of the primary viral genes to be turned on when the virus enters the cell, the increased hypersensitivity in the inactive gene may reflect an unusual DNA conformation which allows transcription factors to bind easily. Conversely, the decreased hypersensitivity may indicate that these transcription factors have bound to the region, thus protecting it from DNase.

Block in HCMV gene expression is at the transcriptional level. HCMV has very restrictive host and cell specificities for productive infection; it grows only in fibroblast and epithelial cells of human origin. However, nonproductively infected cells of many types from human and other species still express the major immediate gene (24, 33; J. A. Nelson, unpublished data). Examination of Tera-2 cells for expression of this gene by immunofluorescence indicated the product was present only when the cells were stimulated to differentiate. To our knowledge, the undifferentiated Tera-2 cells are the only cells in which the gene appeared to be inactive upon infection. Therefore, these cells offered an attractive model for studying cellular factors which regulate expression of this gene. By examination of RNA levels and in vitro transcriptional properties of undifferentiated and differentiated Tera-2 cells, we demonstrated that expression of this gene was regulated at the transcriptional level. The inability of the HCMV promoter-enhancer attached to the neomycin gene to induce colony formation in undifferentiated Tera-2 cells supports this observation. At least two different models may explain how the HCMV IE gene is regulated in teratocarcinoma cells. In the first model one or several cellular protein(s) necessary for activation of the enhancer-promoter region is absent in undifferentiated cells. Herpes simplex virus types 1 and 2 enhancers require a viral *trans*-acting factor for efficient activation (25; L. Whitton, personal communication), whereas the HCMV enhancer operates as efficiently as the SV40 enhancer without the presence of a viral protein. Therefore, HCMV may be more dependent on cellular factors to activate the element. When

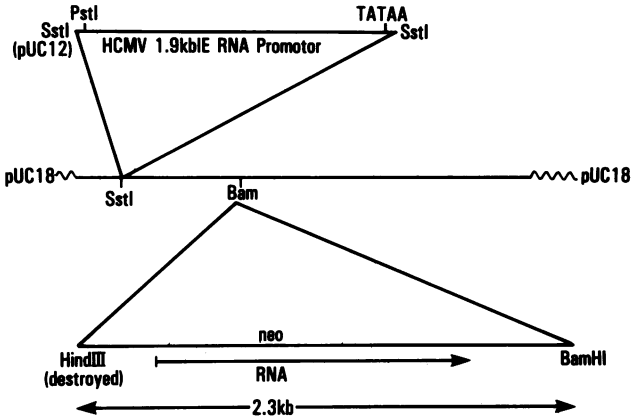


FIG. 7. Diagram of the HCMV-*neo* plasmid. A 2.3-kb *Hin*dIII-*Bam*HI fragment containing the *neo* gene with the *Hin*dIII site removed was cloned into the *Bam*HI site of pUC18. The direction of transcription for the *neo* gene is indicated (arrow). The promoter region for the HCMV major IE gene was released from pUC12 and cloned in the proper orientation in the *Sst*I site 5′ to the start of the *neo* transcript.

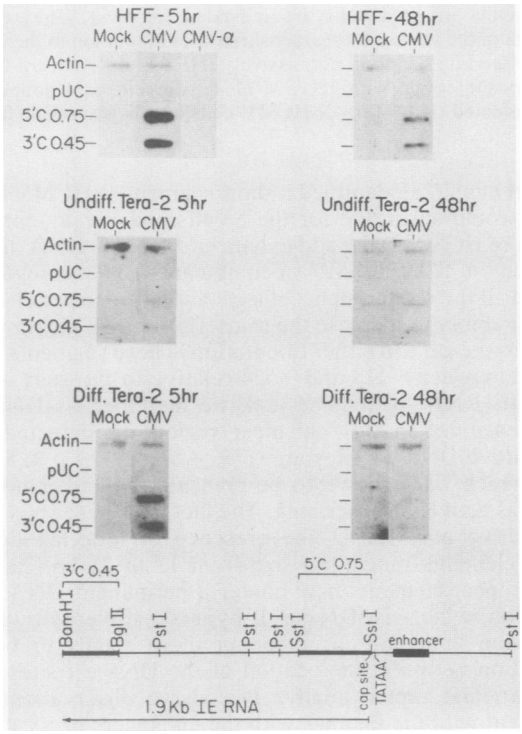


FIG. 6. Transcription of the 5′ noncoding and 5′ and 3′ coding regions of the 1.9-kb IE HCMV gene in HFF and teratocarcinoma cells. Double-stranded cloned DNAs containing sequences derived from a chicken actin gene and the 5′ and 3′ coding regions of the 1.9-kb IE HCMV gene inserted into the pUC plasmid, as well as pUC alone, were bound to nitrocellulose filters and hybridized to ^{32}P-nuclear runoff products as described in Materials and Methods. The regions of the 1.9-kb IE gene used in this analysis are mapped at the bottom of the figure. The most significant hybridization is found in the CMV-infected HFF at 5 h postinfection and in the cytomegalovirus-infected differentiated teratocarcinoma cells after 5 h. All hybridization, including that to actin and to the coding regions of the IE gene, was sensitive to concentrations of α-amanitin (HCMV-α) which specifically inhibit polymerase II transcription.

TABLE 1. Transfection of VA-13 and Tera-2 cells with HCMV-neo plasmid

Sample	DNA transfected (μg)	No. of colonies per 5×10^5 cells	
		VA-13[a]	Tera-2
pUC-neo[b]	0.1	1	0
	1.0	0	0
	10.0	1	0
pJTE201[c]	0.1	0	0
	1.0	2	1
	10.0	9	1
$pSV_{tk}neo$[d]	0.1	0	2
	1.0	32	25
	10.0	180	153
pSV-2neo[e]	0.1	25	0
	1.0	136	0
	10.0	702	0
pHCMVneo	0.1	272	0
	1.0		2
	10.0	1,332	4

[a] W138 cells transformed with SV40 virus.
[b] Neomycin gene inserted into the *Bam*HI site of pUC18; no known eucaryotic transcription signals.
[c] Neomycin gene containing the SV40 promoter.
[d] Neomycin gene containing the herpes simplex virus type 1 TK promoter.
[e] Neomycin gene containing the SV40 promoter and enhancer.

the Tera-2 cells are stimulated to differentiate with RA, the production of these transcription factors activates the gene. Alternatively, in the second model negative factors which prevent activation of the enhancer may be present in the undifferentiated but not in the differentiated Tera-2 cells.

Previous studies have shown that during productive infection stable RNA levels of the major IE gene are diminished or not detected late in infection (22). In vitro transcription studies with productively infected HFF and differentiated Tera-2 nuclei indicate that the gene is transcriptionally inactive at 48 h. The residual RNA levels seen at this interval may reflect the stability of the message in the cell. These observations demonstrate a regulation of the gene at the transcriptional level which may have a similar or different mechanism of action than those described above. In one scenario, transcription factors necessary for maintaining the activity of the gene may be depleted during infection, therefore inactivating the gene. A more likely model would involve the production during the virus replication cycle of *trans*-acting factor(s) possibly the gene product itself, which interacts with the promoter-enhancer element and represses activity. An example of this model is seen with a correlate gene in adenovirus in which the adenovirus E1A product represses the activity of adenovirus type 2 E1A, Py, and SV40 enhancers (2, 46).

Regulation of the enhancer may reside in the 5′ upstream region. A comparison of the structural features of the promoter region with the active and inactive IE gene showed the presence of constitutive and inducible DNase I-hypersensitive sites. The majority of the constitutive DNase I sites existed in the area which substitutes for SV40 enhancer function, whereas the inducible DNase I sites (−650, −775, −875, and −950) were located outside this region. Chromatin structure alterations associated with the functional state of a gene have been described in several systems including the chicken lysosome gene (13). Different sets of DNase I sites were found 5′ to the promoter, depending on whether this gene was constitutively expressed in macrophages or hormone induced in chicken oviduct. In the chicken oviduct one of the DNase I sites 5′ to the transcriptional start site of gene reversibly changes with the presence of steroid hormone. A hormone-induced DNase I site was also observed in the vitellogenin gene (4). These sites are thought to be induced by the binding of the hormone-receptor complex. Closer to the situation in the HCMV promoter region is the observation of persistent and inducible changes in the chromatin structure of the mouse mammary tumor virus long terminal repeat which accompanies activation by dexamethasone (52). Two classes of hypersensitive sites were detected within the promoter region. The constitutive hypersensitive sites were present irrespective of the presence of hormone and correlate with the constitutive sites in the HCMV enhancer. The other class was composed of glucocorticoid-induced hypersensitive sites which were similar to the HCMV-inducible sites that appeared only when the gene was transcribed. Unlike the mouse mammary tumor virus long terminal repeats which demonstrate an increased DNase I sensitivity which persists for several cell generations after hormone treatment, the HCMV promoter region shows a shift in the molarity of the DNase I sites proximal and distal to the cap site of the RNA relative to the activity of the gene. This phenomenon may reflect differential bindings of proteins around the enhancer element and sequences 5′ to this region.

Recently, a high-affinity binding site for nuclear factor 1 (NF-1), a DNA-binding protein purified from HeLa cells (27, 28), was identified in the immunoglobulin M gene (21). Although the binding site was located outside the enhancer region, the colocalization of a DNase I-hypersensitive site with the NF-1 binding site in immunoglobulin-producing cells suggested a biological function. By DNase I footprinting of the NF-1 binding site in the immunoglobulin M gene and by comparison of the protected sequence with similar NF-1 binding sites in the adenovirus type 5 terminal repeat (32) and human c-*myc* gene (37), the consensus sequence 5′ . . . . TGGA/CNNNNNGCCAA. . . .3′ was derived. Examination of the HCMV 5′ flanking sequence of the major IE gene revealed the presence of four of these NF-1 binding consensus sequences between bases −619 to −777. Recently, footprinting analysis has demonstrated that these four sites are bound by NF-1 in vitro (L. Hennighausen, personal communication). The coincidence of inducible HCMV DNase I sites near these sites suggests that the NF-1 protein may be involved in expression of the HCMV gene. Although the HCMV enhancer can functionally operate without the presence of the NF-1-binding site, binding of the protein may regulate activity or determine cell specificity of the enhancer. The importance of these distal, inducible DNase I sites and the models proposed are the subjects of current experiments.

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