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Identification of a Large Bent DNA Domain and Binding Sites for Serum Response Factor Adjacent to the NFI Repeat Cluster and Enhancer Region in the Major IE94 Promoter from Simian Cytomegalovirus

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The major immediate-early (MIE) transactivator proteins of cytomegaloviruses (CMV) play a pivotal role in the initiation of virus-host cell interactions. Therefore, *cis*- and *trans*-acting factors influencing the expression of these proteins through their upstream promoter-enhancer regions are important determinants of the outcome of virus infection. S1 nuclease analysis and in vitro transcription assays with the MIE (or IE94) transcription unit of simian CMV (SCMV)(Colburn) revealed a single prominent mRNA start site associated with a canonical TATATAA motif. This initiator region lies adjacent to a 2,400-bp 5′-upstream noncoding sequence that encompasses a newly identified 1,000-bp (A+T)-rich segment containing intrinsically bent DNA (domain C), together with the previously described proximal cyclic AMP response element locus (domain A) and a tandemly repeated nuclear factor I binding site cluster (domain B). Deleted MIE reporter gene constructions containing domain A sequences only yielded up to 4-fold stronger basal expression in Vero cells than the intact simian virus 40 promoter-enhancer region, and sequences from position −405 to −69 (ENH-A1) added to a minimal heterologous promoter produced a 50-fold increase of basal expression in an enhancer assay. In contrast, neither the nuclear factor I cluster nor the bent DNA region possessed basal enhancer properties and neither significantly modulated the basal activity of the ENH-A1 segment. A second segment of domain A from position −580 to −450 was also found to possess basal enhancer activity in various cell types. This ENH-A2 region contains three copies of a repeated element that includes the 10-bp palindromic sequence CCATATATGG, which resembles the core motif of serum response elements and proved to bind specifically to the cellular nuclear protein serum response transcription factor. Reporter gene constructions containing four tandem copies of these elements displayed up to 13-fold increased basal enhancer activity and 18-fold tetradecanoyl phorbol acetate responsiveness in U937 cells, but an ENH-A2 DNA segment encompassing two of the core serum response transcription factor binding sites failed to respond to serum induction in NIH 3T3 cells. Although there are overall similarities in the organizations of both the MIE enhancers and MIE transcription units among human CMV, SCMV, and murine CMV, the specific arrangements of repetitive motifs are quite different, and the bent DNA and ENH-A2 domains appear to be unique to SCMV.

The predominant major immediate-early (MIE) genes encoding the IE1 and IE2 nuclear regulatory proteins of cytomegalovirus (CMV) are usually transcribed at high levels of abundance both in cells infected in the presence of cycloheximide and in cells transfected with the isolated cloned genes (21, 23–25, 30, 32, 55, 57, 62, 65). Their phosphorylated protein products have transcriptional transactivation and autoregulation properties (18, 45, 46) and are likely to play key roles in initiating or maintaining gene regulation pathways in both lytic and latent infections (19, 32). Therefore, the structural and functional characteristics of the MIE upstream promoter-regulatory regions are of great interest. Both the IE94 promoter of simian cytomegalovirus (SCMV) and the IE68 promoter of human cytomegalovirus (HCMV) have been described as being among the most constitutively powerful eukaryotic promoters known (6, 22, 43). Nevertheless, in infected cells, transcription from these promoters is dependent on both host cell and input virion factors (53, 56) and can be repressed in stem teratocarcinoma cells, for example, but induced in the same cultures after differentiation with retinoic acid. The properties of the human and simian MIE genes differ in this regard, with the SCMV IE94 gene being expressed after infection of many established or transformed human cell lines that are nonpermissive for expression of HCMV IE68 (30, 31). In addition, expression of the HCMV IE68(IE1) protein is induced after retinoic acid treatment of human NTera-2 teratocarcinoma stem cells (14, 30, 40, 52) but not in the mouse F9 cell line, whereas SCMV IE94 responds to retinoic acid induction in F9 cells but is constitutively expressed in infected NTera-2 cells (30, 31).

The HCMV IE68 promoter contains two well-defined domains, a 500-bp proximal enhancer region (6, 56, 60), which encompasses binding sites for two known cellular factors, cyclic AMP (cAMP) response element binding protein (CREB) and NF-κB (20, 50, 54), as well as a number of additional as yet poorly defined cellular factors (11), and a farther upstream modulatory region that appears to play

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some role in the repression in NTera-2 cells (40, 52) and includes several DNase I hypersensitive sites (39). The SCMV IE94 promoter contains a structurally similar proximal 580-bp enhancer-like domain containing a different arrangement of the same four multicopy interspersed repeat elements, referred to as series I, II, III, and IV elements (22), but the farther-upstream region consists of an expanded 700-bp block of 23 diverged tandemly repeated 30-bp elements that contain binding sites for the cellular nuclear factor I (NFI) and CAAT transcription factor (CTF) DNA binding proteins (26). We have shown that the IE94 proximal promoter region can be activated up to 10-fold by cAMP treatment in human K562 erythroleukemia cells and that the 11 series I consensus repeats represent unusual 16-bp palindromic CREs that bind to the cellular cAMP response factor (7). In addition, these motifs act as strong basal enhancer elements when arranged as multiple tandem repeats.

To further understand the nature of the complex CMV MIE promoter-regulatory regions and to attempt to explain the different biological properties of the human and simian versions, we have made a more detailed characterization of the SCMV IE94 upstream sequences. In particular, we describe the identification and definition of the predominant MIE mRNA start site, a far-upstream "bent DNA" domain, and a second subdomain in the enhancer region that contains an additional set of repeated consensus elements (series V), which bind to the cellular serum response transcription factor (SRF) and contribute to tetradecanoyl phorbol acetate (TPA) responsiveness.

MATERIALS AND METHODS

Cells and virus. Diploid human fibroblast cells were derived from trypsinized primary cultures of foreskin fibroblasts and grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (FCS). Monolayers of Vero and NIH 3T3 cells were grown in DMEM with 5% FCS. SCMV(Colburn) virus is a human isolate, but from DNA hybridization and restriction enzyme analysis, it is clearly of African green monkey origin. The virus was plaque-purified from an original sample obtained from Milan Fiala (Harbor General Hospital, Los Angeles, Calif.), and stocks were routinely passaged at dilutions of 10^{-6} as described previously (23).

Plasmid clones, deletions, and nucleotide sequencing. Unfractionated restriction enzyme cleavage digests from SCMV(Colburn cl-2) virion DNA were cloned into pBR322 plasmid vectors by standard procedures and selected by antibiotic selection followed by colony hybridization with isolated fragment DNA probes (22). Three overlapping fragments, *Hin*dIII-H(pTJ148), *Eco*RI-G(pTJ97), and *Bam*HI-O(pTJ55), that encompassed the MIE gene complex at map coordinates 0.70 to 0.75 were used in these studies. Initial DNA sequence analysis was carried out by the Maxam and Gilbert procedure on selected fragments from a series of 5′ and 3′ *Bal* 31-deleted variants of pTJ148. Additional sequence analysis was performed on a set of single-stranded M13mp10 or M13mp11 subclones by using the Sanger dideoxynucleotide chain termination procedure. The construction of the prototype SCMV hybrid reporter plasmid pTJ278, containing the IE94(−990/+30)-chloramphenicol acetyltransferase (CAT) gene, was described previously (26), together with two 5′-deleted variants referred to as pTJ279 [containing the IE94(−300/+30)-CAT gene] and pTJ280 [containing the IE94(−209/+30)-CAT gene] that were generated by *Bal* 31 nuclease digestion. An additional series of 5′-deleted SCMV IE94-CAT reporter gene plasmids, pDG1, pDG2, pDG3, pDG5, and pDG7, represent further *Bal* 31 derivatives of the IE94 promoter in pTJ278 with added *Sal*I linkers that were placed in front of the CAT coding region cassette in pCATC′ (7).

Plasmid reporter gene constructions for enhancer assays. A series of 18 plasmids containing CAT reporter genes for testing the *cis*-enhancer properties of upstream domain A, B, and C sequences were constructed as follows. For a domain A enhancer subfragment, the IE94(−405/+30)-CAT gene in plasmid pDG5 was cleaved at position +30 with *Sac*I and incubated with *Bal* 31 to create a nested set of 3′ deletions to which *Sal*I linkers were added. Six of these 3′ deletions were sequenced, and a 336-bp *Sal*I fragment from position −405 to −69 (ENH-A1) was chosen for cloning into a modified pUC18 vector (pGH56) containing an inserted *Hin*dIII-*Bgl*II-*Hin*dIII adapter. This sequence in plasmid pYNC20 was subsequently isolated as a *Bam*HI-*Bgl*II fragment and placed in either orientation at the upstream *Bgl*II site in the minimal simian virus 40 (SV40) early promoter in pA10-CAT to create plasmids pYNC24A and pYNC24B containing series I, II, and IV elements only. The same fragment was also placed in either orientation into the 3′ *Bam*HI site in pA10-CAT to create plasmids pYNC30A and pYNC30B.

A representative domain B NFI sequence contained within the 240-bp *Nco*I-*Acc*I fragment (position −1022 to −785) was isolated from pTJ148, and *Sal*I linkers were added after the ends were blunted with Klenow DNA polymerase. This fragment was first cloned at the *Sal*I site in pGH56 as plasmid pYNC3 and was then inserted in either orientation as a *Bam*HI-*Bgl*II fragment into the *Bgl*II site in pA10-CAT to create pYNC26A and pYNC26B.

For domain C test DNA sequences, the *Stu*I-*Nco*I fragment from position −2255 to −1017 was first cloned into pGH56 after the addition of *Sal*I linkers (pYNC9). *Bal* 31 deletion to position −1439 from the *Nco*I side and cleavage with *Acc*I at position −2024 to remove all NFI sites generated a 585-bp *Acc*I-*Eco*RI fragment that was cloned with *Sal*I linkers into pGH56 (pYNC12). This domain C bent DNA fragment bounded by *Bam*HI and *Bgl*II sites was inserted in both orientations into the *Bgl*II site in pA10-CAT to produce plasmids pYNC27A and pYNC27B. The 1,240-bp *Stu*I-*Nco*I fragment containing domain BC bent-plus-NFI sequences was also placed into the 5′ *Bgl*II site of pA10-CAT as pYNC28A and pYNC28B.

A second series of *cis*-enhancer test reporter genes with various combinations of the isolated domain A, B, and C sequences were constructed by inserting additional *Bam*HI-*Bgl*II-bounded NFI, bent, or bent-NFI fragments in both orientations at the *Bgl*II sites in the ENH-A1/A10-CAT constructions pYNC24A and pYNC24B. This created plasmids pYNC31A,B (NFI/ENH/A10-CAT); pYNC32A,B (ENH/NFI/A10-CAT); pYNC35A,B (bent/ENH/A10-CAT); pYNC 36A,B (ENH/bent/A10-CAT); pYNC33A(bent-NFI/ENH/A10-CAT); pYNC33B(NFI-bent/ENH/A10-CAT); pYNC34A (ENH/bent-NFI/A10-CAT), and pYNC34B(ENH/NFI-bent/A10-CAT), respectively.

An additional *cis*-enhancer reporter gene construction encompassing two pairs of overlapping series V and III elements plus one NFI site and a diverged series I element from between positions −601 and −482 was prepared by polymerase chain reaction procedures using synthetic oligonucleotide primers containing *Bgl*II and *Sal*I linkers on the 5′ side and *Sal*I and *Bam*HI linkers on the 3′ side. This ENH-A2 domain sequence from the IE94 gene was inserted in both orientations at the upstream *Bgl*II site in pA10-CAT

to produce plasmids pGH206A and pGH206B and at the *Sal*I site at position −69 in pDG2 (minimal IE94-CAT) to produce pGH208A and pGH208B. The positive control reporter gene in plasmid p268-CAT/Bg5, which contains a functional serum response element (SRE) sequence within a 2.8-kb promoter fragment from the mouse *zif268* gene, was obtained from Barbara Christy and Dan Nathans (9). The TPA responsiveness of the SCMV MIE element from position −585 to −561 encompassing a consensus series V motif was examined with reporter gene constructions containing multiple inserts of a 30-mer double-stranded synthetic oligonucleotide placed into a minimal IE94(−69/+30)-CAT gene background. Plasmid pDG2 was first modified by the addition of an *Sal*I-*Bgl*II-*Sal*I adapter at the *Sal*I site at position −69 (pCJC81), and plasmids containing the oligonucleotide pair LGH218 and LGH219 in one (pCJC111), two (pCJC112), or four (pCJC114) tandemly repeated copies at the *Bgl*II site were selected for use in the assay.

Preparation of IE RNA. Infections were carried out with supernatant virus at a multiplicity of infection of 5 to 30 PFU/ml in cell cultures at 90% confluence. Cycloheximide (final concentration, 50 μg/ml; Sigma) and actinomycin D (final concentration, 10 μg/ml; Sigma) were both prepared fresh and were routinely filtered before use. Removal of the inhibitors ("reversal") was accomplished by rinsing the cell monolayers sequentially three times with PBS. Immediate-early (IE) RNA was extracted from cells that had been infected in the presence of cycloheximide. Pelleted cells were treated with a solution of 4 M guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol (8). Total cell RNA was then separated away from bulk protein and DNA by sedimentation through a CsCl cushion (13). The RNA pellet was resuspended in 0.5 M NaCl–0.5% sodium dodecyl sulfate (SDS)–100 mM Tris (pH 7.4), and residual protein was removed by extraction with a phenol-chloroform-isoamyl alcohol mixture (25:24:1). The RNA sample was then ethanol precipitated and the poly(A)-containing fraction was selected by using an oligo(dT) column (4).

In vitro transcription, S1 nuclease mapping, and RNA gel electrophoresis. Analysis of promoter sites in vitro was conducted with a Manley HeLa cell lysate system (34) purchased from Bethesda Research Laboratories. For each reaction, 2.5 μg of linear DNA template was added to 30 μl of HeLa cell lysate in the presence of [^{32}P]UTP or [^{32}P]riboGTP. Runoff transcripts were analyzed by glyoxal-agarose gel electrophoresis (59) and autoradiography.

For the S1 hybrid protection assay, poly(A)-selected total IE-infected cell RNA and the ^{32}P-labelled DNA probe were pelleted together in ethanol and resuspended in 10 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–400 mM NaCl–1 mM EDTA in 80% formamide. The dissolved sample was then heated to 80°C for 15 min and quickly shifted to a 58°C water bath and incubated for 3 h to allow hybrid formation. S1 digest buffer (200 μl), which contained 280 mM NaCl, 30 mM Na acetate, and 4.5 mM Zn acetate (pH 4.5), was then added together with 500 U of S1 enzyme. Digestion was allowed to proceed at 37°C for 30 min. Carrier tRNA (5 μg) was added, and the entire sample was extracted with phenol-chloroform and precipitated with ethanol. Size analysis of protected DNA was carried out by denaturing urea polyacrylamide gel electrophoresis (5).

DNA transfection and CAT assays. The procedures for introducing plasmid DNA into Vero and NIH 3T3 cells by the calcium phosphate-HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) plus glycerol shock method for transient expression assays and for measuring CAT enzyme activity in extracts from these cells were described in detail previously (42). For U937 cells, transfection was carried out by a DEAE-dextran procedure used previously for B lymphocytes (58). When appropriate, fresh medium containing TPA at 50 ng/ml was added at 24 h, and the cells were harvested at 42 h.

Nuclear protein extracts, oligonucleotides, and electrophoretic mobility shift assay. Nuclear extracts, prepared from Raji cells by a modified high-salt lysis procedure followed by heparin agarose chromatography (27), were generously supplied by Dan Rawlins, Emory University. Fractions containing binding activity for the c-*fos* SRE were pooled and concentrated. Electrophoretic mobility shift assays were carried out through 2% agarose gels in 12 mM Tris-acetate (pH 7.5)–1 mM EDTA–0.05% Nonidet P-40 buffer. Protein-DNA binding reactions were carried out at 23°C for 30 min in 50 mM NaCl binding buffer (3), 1 mg of bovine serum albumin per ml, and 40 μg of poly(dI-dC) with 5 fmol of ^{32}P-labelled oligonucleotide probe DNA. The wild-type (SRF) and point mutant (ΔSRF) SCMV series V consensus site probes were prepared by annealing the complementary 30-mer oligonucleotide pairs LGH218 (5′-GATCCAGTAT TCCATATATGGGTTTTCCTA-3′) and LGH219 (3′-GTCA TAAGGTATATACCCAAAAGGATCTAG-5′) or LGH220 (5′-GATCCAGTATTGGATATATCCGTTTTCCTA-3′) and LGH221 (3′-GTCATAACCTATATAGGCAAAAGGATCT AG-5′). Labelling was accomplished by 3′ extension of the recessed 3′ ends with Klenow DNA polymerase in the presence of [α-^{32}P]dCTP.

Serum response assays. For serum stimulation assays, NIH 3T3 cells were grown in DMEM containing 0.5% FCS for 48 h and were then transfected with target plasmid DNA by the calcium phosphate-HEPES plus glycerol shock procedure. After 48 h, the cells were transferred into fresh DMEM containing 10% FCS and samples were taken for determination of CAT enzyme levels at 2 h intervals (9).

Nucleotide sequence accession number. The entire 3,023-bp MIE regulatory sequence of SCMV(Colburn) determined here is available from GenBank under accession no. L06819.

RESULTS

Characterization of the IE94 promoter and mRNA start sites. To confirm that the 5′-upstream region for the SCMV (Colburn) IE94 gene is associated with only a single unidirectional transcription unit within the 10.8-kb *Hin*dIII-H region, we carried out in vitro runoff transcription analysis as described by Manley et al. (34). In our experience, this assay works efficiently for all known herpesvirus IE genes but often works only poorly for delayed-early (DE) and late genes (41a). In vitro transcription reactions using cloned plasmid DNAs containing the overlapping *Hin*dIII-H(pTJ148), *Eco*RI-G(pTJ97), or *Bam*HI-O(pTJ55) fragments from approximately 0.7 map units in the SCMV(Colburn) genome (29) after cleavage with a variety of restriction enzymes are presented in Fig. 1a. Both the *Hin*dIII-H and *Eco*RI-G fragments (but not *Bam*HI-O) generated abundant transcripts, which in both cases proceeded leftwards, initiating from a single start site mapping close to a *Sac*I site located 1,650 bp to the right of the *Bam*HI O to H boundary and 3,650 bp to the right of the *Eco*RI N to G boundary (Fig. 1b). No other significant mRNA start site was detected on the entire 14-kb region encompassing *Hin*dIII-H, *Eco*RI-G, and *Bam*HI-O (the few minor bands observed represent aggregation with the 28S and 18S rRNA). This corresponds to the expected start site for the major spliced 2.3-kb

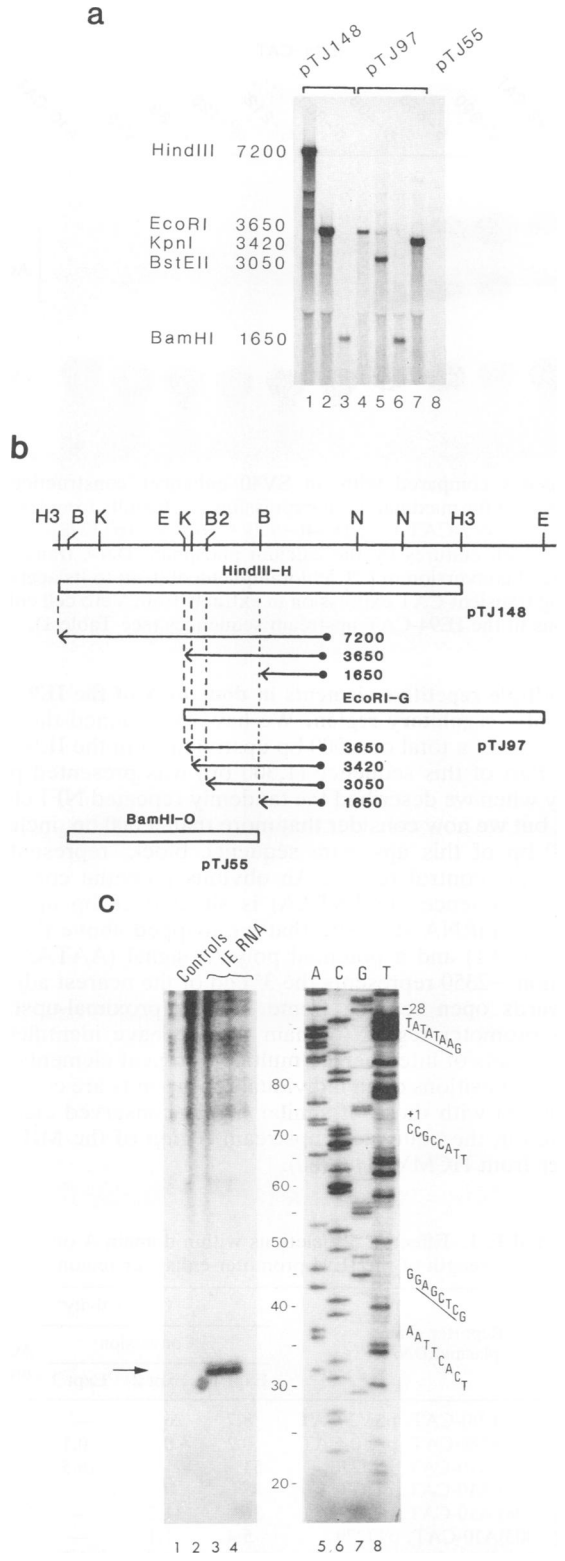

FIG. 1. Detection of a single strong leftwards IE94 transcription start site in the *Hin*dIII-H fragment of SCMV(Colburn). (a) Autoradiograph showing the results of in vitro runoff transcription experiments with uninfected HeLa cell extracts as described by Manley et al. (34). Linear cleaved plasmid DNA samples were incubated with extract in the presence of [α-^{32}P]UTP. After phenol-chloroform extraction, the newly synthesized RNA was fractionated according to size by electrophoresis through a glyoxal-agarose gel. Approximate RNA sizes were estimated relative to single-stranded DNA fragment standards run in parallel lanes. Lanes 1 to 7 show RNA products from incubations with linearized plasmid DNA containing either the 10.8-kb *Hin*dIII-H(pTJ148) fragment or the 9.4-kb *Eco*RI-G(pTJ97) fragment as follows: 1, pTJ148, *Hin*dIII; 2, pTJ148, *Hin*dIII plus *Eco*RI; 3, pTJ148, *Hin*dIII plus *Bam*HI; 4, pTJ97, *Eco*RI; 5, pTJ97, *Eco*RI plus *Bst*EII; 6, pTJ97, *Bam*HI; and 7, pTJ97, *Eco*RI plus *Kpn*I. Lane 8 shows the negative control with the internal 5.2-kb *Bam*HI-O fragment from the left-hand side of *Hin*dIII-H (pTJ55, *Bam*HI). (b) Summary and map of the in vitro runoff transcription results leading to the conclusion that a single strong SCMV MIE mRNA start site occurs near the right-hand *Sac*I site in pTJ148 and pTJ97. (c) Determination of the 5′ end of IE94 mRNA obtained from SCMV(Colburn)-infected Vero cells. The autoradiograph shows S1 nuclease protection of a portion of a uniformly ^{32}P-labelled M13 single-stranded *Sac*I-*Sal*I DNA fragment probe (representing the coding strand from position +32 to −610) after electrophoresis through a urea-polyacrylamide gel. The probe was annealed in the absence of RNA (lane 1), with yeast RNA (lane 2), or with two samples of poly(A)-selected RNA from cells infected with SCMV(Colburn) in the presence of cycloheximide (lanes 3 and 4). Parallel dideoxynucleotide sequencing reactions run on the same gel with the same M13 clone DNA by using the standard 17-mer M13 universal primer are shown in lanes 5 to 8 as size markers. The sequence of parts of the complementary, noncoding strand is given to illustrate that the RNA start site (position +1) lies 32 bp upstream from the *Sac*I site and 28 bp downstream from the TATATAA box sequence.

leftwards-IE mRNA that was mapped within the *Hin*dIII-H fragment in cycloheximide-treated SCMV-infected cells (22, 24).

To definitively locate the initiation site for the MIE species mRNA, we also carried out an S1 nuclease protection experiment with poly(A)-selected RNA from cycloheximide-blocked SCMV(Colburn)-infected cells. A uniformly labelled complementary strand probe was synthesized from an M13 recombinant clone containing a 640-bp viral fragment insert from the region immediately upstream of the *Sac*I site. After denaturation, hybridization with IE RNA, S1 nuclease treatment, and resolution in a urea-polyacrylamide gel, a single abundant 5′-protected fragment of 33 bp was obtained (Fig. 1c, lanes 3 and 4). Control S1 experiments with the same probe hybridized either in the absence of RNA or to carrier yeast RNA only (Fig. 1c, lanes 1 and 2) or with the complementary strand probe (not shown) gave no protected bands in these gels. Parallel chain termination sequencing tracts across the *Sac*I site of the same M13 clone were used both as size markers on the gel and to illustrate the sequence around the cap site (Fig. 1c, lanes 5 to 8).

Constitutive expression of IE94-CAT hybrid genes in DNA transfection assays. To measure the strength of the IE94 promoter in direct comparison to the strong enhancer-containing early promoter from SV40, we used two hybrid reporter gene constructions containing either 990 or 3,500 bp of 5′-upstream IE94 sequences fused to the CAT coding cassette at the *Sac*I site described above: i.e., predicted to be at position +30 in the IE94 leader sequence. Basal levels of CAT enzyme activity produced by the IE94-CAT DNA after transfection into Vero cells were compared with those from pSV2-CAT and pA10-CAT DNA, which contain either the complete −340/+58 SV40 early T-antigen promoter or a −150/+58 derivative lacking most of the 2- by 72-bp enhancer region (33). Initially, yields of [^{14}C]-3-acetyl chloramphenicol with two- and fourfold dilutions of the IE94(−990/+30)-CAT, SV2-CAT, and control A10-CAT ex-

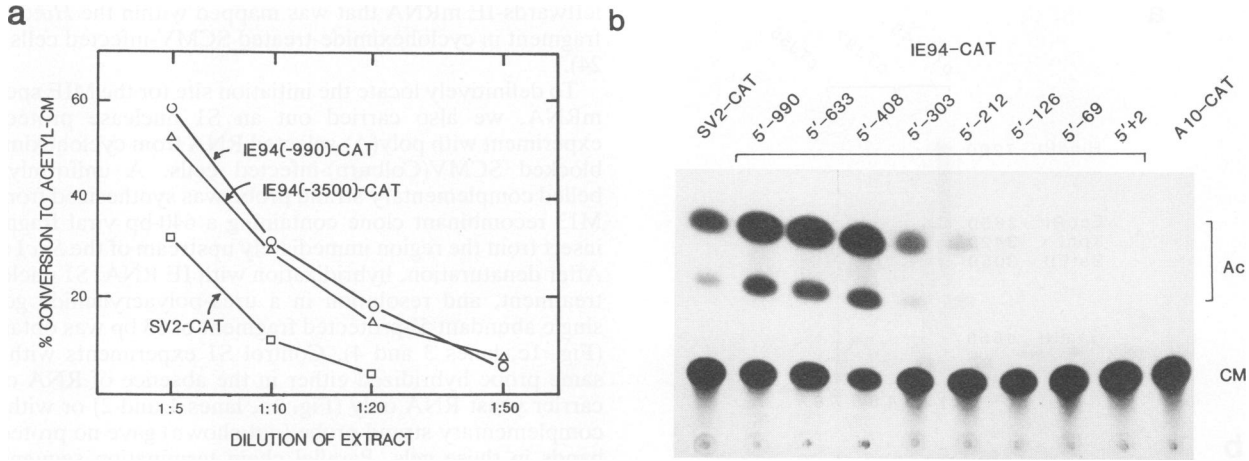

FIG. 2. Strong constitutive expression of SCMV IE94-CAT reporter genes compared with an SV40 enhancer construction and requirement for distal parts of domain A1 from the IE94 promoter-regulatory region for maximal basal expression. (a) Results from transient expression assays with a series of increasingly diluted samples of extracts from SV2-CAT (□), IE94(−990/+30)-CAT (pTJ278) (○), and IE94(−3500/+30)-CAT (pJM50) (△). Plasmid DNA was introduced into Vero cell cultures by the calcium phosphate DNA transfection procedure. After 48 h, extracts of the cultures were assayed for the percentage of conversion of [^{14}C]chloramphenicol (Cm) to its acetylated derivatives (Ac). (b) Autoradiographs of the results of an experiment measuring transient CAT expression in extracts from Vero cell cultures transfected with 2 μg each of a series of plasmid DNAs containing 5′ deletions in the IE94-CAT upstream sequences (see Table 1).

tracts were determined. However, since the percent conversions for the enhancer containing samples were beyond the range giving linear enzyme kinetics, quantitation of the results with a 5- to 50-fold series of dilutions of the IE94(−990/+30)-CAT, IE94(−3500/+30)-CAT, and SV2-CAT extracts were carried out (Fig. 2a). In these experiments, both of the IE94-CAT plasmids gave 3.5-fold greater CAT activity than equivalent amounts of SV2-CAT DNA and at least 200-fold greater activity than equivalent amounts of A10-CAT DNA.

Contribution of domain A sequences to high levels of IE94-CAT basal activity. Evidence that sequences driving high-level basal expression of the IE94-CAT gene are located within the 5′-proximal-upstream regions came from measurements of CAT activity produced in transient assays by a series of 5′-deletion constructions. *Bal* 31 nuclease-generated deletions (with added *Sal*I linkers) that retained sequences out to positions −633, −408, −303, −212, −126, −69, and +2 relative to the mRNA start site were selected, and their *Sal*I-*Sac*I regions were transferred into a CAT coding region vector in a manner equivalent to that used to construct the IE94(−990/+30)-CAT reporter gene. An autoradiograph from one experiment with this series of plasmids in short-term DNA transfections in Vero cells is shown in Fig. 2b, and quantitative data averaging the results from several experiments are summarized in Table 1. The construction containing the minimal IE94 promoter region IE94(−69/+30)-CAT gave low yields of CAT activity equivalent to those obtained with the minimal SV40 early promoter sequences in A10-CAT or with an IE94-CAT construction deleted to position +2 that had no known remaining promoter elements. The basal activity then increased 3.3-fold, with 126 bp of 5′-upstream sequence present and approximately 7-fold more with addition of 5′ sequences out to position −212 or −303. Inclusion of SCMV sequences up to position −408 gave another eightfold increase in activity, but little further increase occurred with either 633 or 990 bp of upstream sequence included. Overall, the removal of sequences from between positions −633 and −69 reduced basal expression almost 200-fold.

Multiple repetitive elements in domain A of the IE94 gene promoter-regulatory region. We have determined the DNA sequence of a total of 4,000 bp upstream from the IE94 start site. Part of this sequence (1,300 bp) was presented previously when we described the tandemly repeated NFI cluster (26), but we now consider that more than 3,000 bp, including 2,400 bp of this upstream sequence block, represents an intergenic control region. An obvious potential consensus TATA sequence (TATATAA) is situated 28 bp upstream from the mRNA start site that we mapped above (labelled position +1) and a potential poly(A) signal (AATAAA) at position −2350 represents the 3′ end of the nearest adjacent leftwards open reading frame. In the proximal-upstream IE94 promoter region (domain A), we have identified six distinct sets of interspersed multicopy repeat elements (Fig. 3a). The positions of all individual repeat units are compared in Fig. 3a with those of similar highly conserved elements located in the equivalent upstream region of the MIE promoter from HCMV (1, 6, 60).

TABLE 1. Effect of 5′ deletions within domain A on basal strength of the IE94 promoter-enhancer region

Reporter gene, plasmid DNA	CAT activity[a]			
	% Conversion			Avg fold increase
	Expt 1	Expt 2	Expt 3	
SV2(−380/+58)-CAT, pSV2-CAT	8.7	26	—[b]	43
SV2(−110/+58)-CAT, pA10-CAT	0.2	0.06	0.1	1.0
IE94(−990)/A10-CAT, pTJ278	54	—	16.5	217
IE94(−633)/A10-CAT, pDG7	45	9.6	—	192
IE94(−408)/A10-CAT, pDG5	36	11.2	—	183
IE94(−303)A10-CAT, pTJ279	5.4	1.1	—	21
IE94(−212)/A10-CAT, pTJ280	1.7	1.5	3.3	21
IE94(−126)/A10-CAT, pDG3	0.5	0.25	—	3.3
IE94(−69)/A10-CAT, pDG2	0.2	0.08	—	1.1
IE94(+2)/A10-CAT, pDG1	0.15	0.06	—	0.9

[a] Transient assays in Vero cells using the calcium phosphate-HEPES-glycerol system.
[b] —, not done.

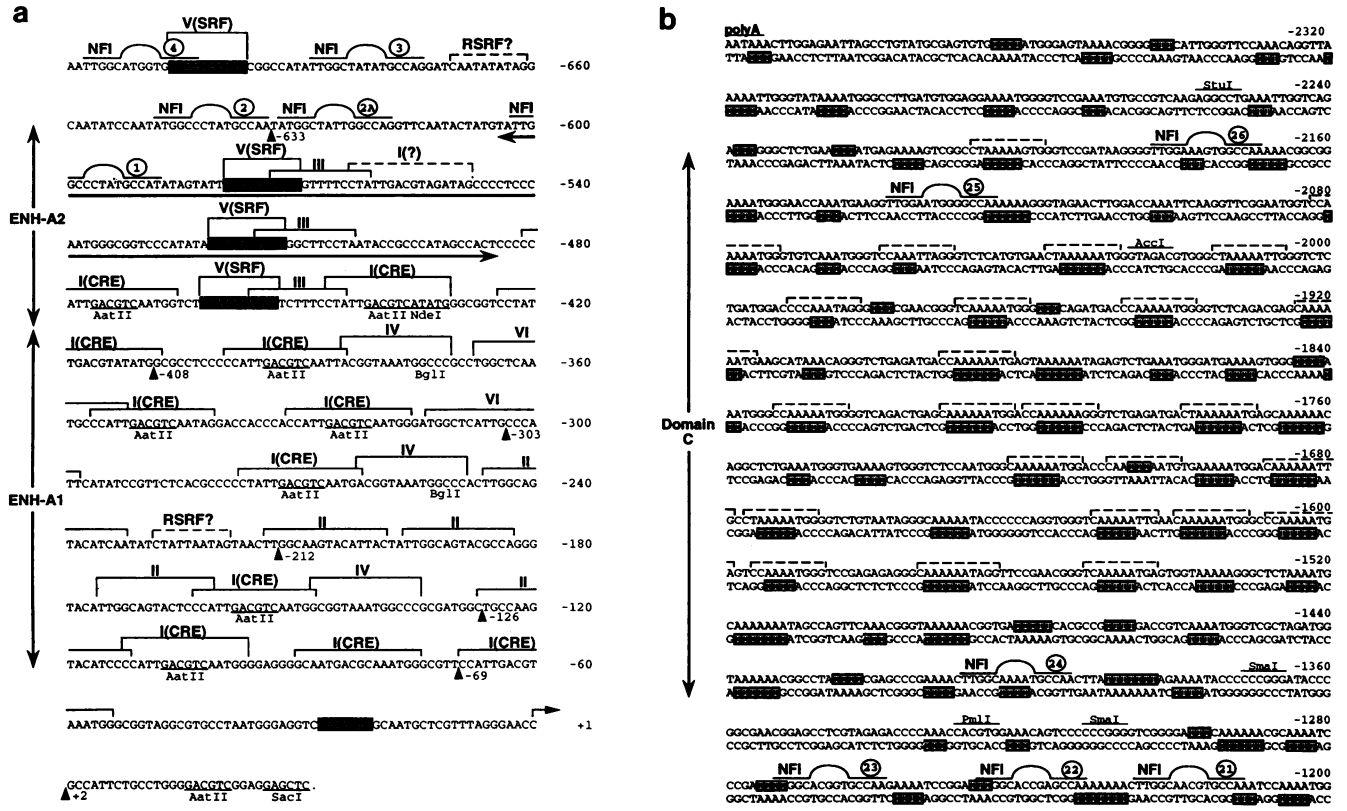

FIG. 3. DNA sequence of the enhancer region and bent-DNA domain within the 5′-upstream promoter region of SCMV IE94. (a) Domain A1 and A2 enhancer region. The TATAAA element, key restriction enzyme cleavage sites, and 5′-deletion boundaries are indicated, together with the locations and identification of proposed series I, II, III, IV, V, and VI repetitive elements and the most proximal NFI sites. The three consensus core SRF motifs (series V) are shown in reverse typescript. The outside boundary positions of the 5′-deletion constructions used here are shown (▲) and the underlined arrowed region (position −601 to −482) represents the sequence used for ENH-A2 functional assays. (b) Double-stranded sequence of 1,200 bp of the far-upstream region encompassing the bent DNA in domain C. Runs of adjacent T4, T5, T6, and T7 motifs that are spaced mostly on the same face of the DNA helix (11 or 22 bp apart on the same DNA strand) are likely to be involved in the bent DNA feature. All poly(T) runs of three or greater are presented in reverse typescript in the sequence. Flanking consensus palindromic NFI sites, key restriction enzyme cleavage sites, and the poly(A) signal at position −2400 associated with the adjacent leftwards open reading frame are also indicated. Close matches to potential SRF and RSRF consensus sequences of CC(A/T)6GG or C(A/T)8G are denoted by overhead broken-line brackets.

Twelve copies of the first set of 16-bp palindromic repeats (series I), which have a consensus sequence of CCATT GACGTCAATGG, including a central *Aat*II site, lie between positions −482 and −54. We have shown previously that monomers of these elements function as CREs and that as dimers and higher oligomers they also exhibit basal enhancer properties in DNA-transfected human K562 erythroleukemia cells (7). The HCMV MIE promoter contains five of these series I (or 19-bp) repeat elements. The second set of repeats (series II) includes five diverged copies of a sequence with the consensus sequence TTGGCAGTACAT CAA. All have the same orientation, and four lie within a 90-bp region between positions −250 and −160. Versions of this series II consensus sequence occur at positions −246 to −232, −214 to −199, −196 to −182, −175 to −161, and −127 to −122 in IE94 and at positions −370 to −356, −258 to −244, and −207 to −193 in IE68, and a plausible additional copy (CTGGACGTACATGAG) appears at position +253 to +267 within intron-1 of IE94 (see Fig. 4). These sites do not obviously resemble known transcription factor consensus elements and there is little data available about whether or not they represent functionally significant elements or binding sites.

Two other sets of repeats referred to as series III (consensus sequence ATGGGTTTCCTA) and series IV (consensus sequence ACGGTAAATGGCCC) each appear three times within domain A in IE94 (Fig. 3a). The SCMV series III repeats resemble but differ from the AGGGACTTTCCA (18-bp) repeats in HCMV IE68, which have been shown to represent consensus NF-κB binding sites (50). The three SCMV series IV repeats, namely ACGGTAAATGGCCC at position −384 to −371, ACGGTAAATGGCCC at position −262 to −249, and GCGGTAAATGGCCC at position −148 to −135, match closely the three repeat elements found in HCMV, ACGGTAAATGGCCC at position −509 to −496, ACGGTAAACTGCCC at position −387 to −374, and ACG GTAAATGGCCC at position −315 to −302, and include (in inverted orientation) the proposed consensus motif for the cellular YY1 DNA binding factor (5′-GCCATNT-3′).

A fifth set of multicopy repeats within domain A have the consensus sequence CCATATATGG (Fig. 3a). This series V motif occurs three times between positions −579 and −453 in SCMV IE94 and once at position −538 to −529 in HCMV IE68. Plausible additional copies of this series V motif are found at position −706 to −697 (CCAATAATGG) in IE94 and at positions −734 to −725 and −687 to −678 (both

TCAATATTGG) in IE68. These motifs all closely resemble the 10-bp core binding site of CCATATTAGG for SRF within the palindromic SREs in the c-*fos*, β-actin, and *zif268* promoters (36, 41, 48). Another potential consensus repeat element, TGGCN4TGCCCA (series VI), is present in two copies at positions −367 to −354 and −313 to −298 and is also conserved as a single copy element at position −297 to −282 in HCMV. Note that the two potential AP-1 sites, TGACTAA at position −239 to −231 and TGACTCA at position −174 to −166 in HCMV, do not appear to be conserved in SCMV IE94. Overall, these six sets of interspersed repeats that we have described together account for nearly 80% of the nucleotides between positions −580 and −54 in the IE94 5′-upstream promoter-regulatory region.

Features of the DNA sequence of the far-upstream domain B and C regions. We refer to the previously described tandemly repeated NFI binding site cluster between positions −1280 and −590 in the SCMV IE94 promoter-regulatory region as domain B (22, 26). This region includes diverged tandem repeats (19 by 30 bp) plus several adjacent shorter repeats, creating a total of 23 consensus palindromic elements of the type TTGG(C/A)N5GCCAA. Most of these repeats represent high-affinity binding sites for purified cellular NFI protein (26), and many of the half sites also include standard CAAT consensus motifs of the form TGCCAAT or AGCCAAT, which would be expected to act as binding sites for additional cellular transcription factors of the CTF type. A further series of core SRE-like sequences, including TCAATATGG, CCAATATGG, TCAAAATGG, CCATAT TGA, and TATATAGG, also occur within almost every copy of the 19- by 30-bp tandem repeats in domain B.

Much of the next 1,000 bp of upstream sequence (designated domain C) that lie beyond the tandemly repeated NFI cluster are characterized primarily by numerous short runs of consecutive thymidines (Fig. 3b). There are 80 occurrences of T3, T4, T5, T6, or T7 within the entire 1,200-bp region shown, with 65 of them being on the same DNA strand. Most remarkably, the central 730-bp segment between positions −2217 and −1488 contains 45 successive poly(T) runs that are all spaced roughly either 11 or 22 bp apart (one or two turns of the double helix) on the same DNA strand. Furthermore, nearly half (8 of 18) of the 22-bp spans have internally located T2 motifs. Only three copies of T3 runs and one T4 run occur over this same region of the opposite (top) strand. Curiously, the whole of domain C is again bounded by consensus palindromic NFI binding sites, i.e., close matches to TTGG(C/A)AN5GCCAA, one at position −1415 to −1400 and two more at positions −2143 to −2128 and −2188 to −2173. Therefore, taking into consideration the five consensus NFI sites between positions +470 and +573 within intron-1 (26), the entire 3,000-bp IE94 control region contains at least 31 high-affinity palindromic NFI binding sites (summarized in Fig. 4). Finally, many of the poly(T) runs are bracketed by A and G or C such that on the opposite strand they again resemble diverged potential core SRE-like elements of the types CCAAAAATGG, TCAAAAATGG, CCAAAAAAGG, and CCAAATTAGG, including six that match the proposed CC(A/T)6GG SRE core site and many others that are nominally either one base diverged from this pattern or fit a consensus sequence of CC(A/T)5GG or CC(A/T)7GG. As many as seven more elements of this diverged core SRE type also occur between positions +100 and +550 in intron-1, including a near-consensus SRF motif of CTATATATGG at position +201 to +210.

Anomalous gel electrophoretic mobility of domain C sequences. The runs of consecutive thymidines spaced at intervals of approximately 11 and 22 bp apart on the same face of the helix resemble the classical arrangement reported for bent DNA within the minicircle DNA of trypanosomes (35) and within the origin of DNA replication in phage lambda (67). However, in those cases, the bent DNA domains cover no more than four to six poly(T) runs over a 150- to 200-bp region. In IE94 domain C, the approriate sequence features could be considered to span a contiguous block from position −2320 to −1377. Several linear DNA fragments from IE94 domain C containing portions of the proposed bent DNA region displayed a slower than expected mobility in polyacrylamide gel electrophoresis. Most dramatically, a 1.2-kb *Stu*I-*Nco*I fragment covering virtually the entire region proved to migrate as a 1.2-kb double-stranded DNA fragment in agarose gels but exhibited aberrant properties in a gradient polyacrylamide gel such that it crossed over the positions of admixed phage λ DNA size markers, and its mobility was reduced to being equivalent to that of a 3.5-kb species in 12.5% polyacrylamide (Fig. 5). This result is entirely consistent with the notion that domain C does indeed adopt the characteristic properties of intrinsically bent DNA. The anomalous mobility of several smaller fragments tested was not as pronounced as that of the *Stu*I-*Nco*I fragment (position −2256 to −1022), and we tentatively conclude that the extraordinary degree of bending shown by this fragment represents a summation of effects from across the entire region rather than being caused by a single localized feature.

Neither domain B nor domain C displays transcriptional enhancer properties or modifies the activity of domain A in transient-expression assays. The proximal 500 bp from the upstream regulatory regions of HCMV IE68 and murine CMV (MCMV) IE97 were originally identified as having standard enhancer properties in an SV40 enhancer trap assay (6, 10). Because of the extremely high-level basal expression properties of IE94-IFN reporter gene constructions in microinjected *Xenopus* oocytes (43), we have presumed that this would also be the case for SCMV, although the only formal proof has come from the use of multicopy consensus synthetic series I CRE sequences placed into A10-CAT or IE94(−69/+30)-CAT reporter genes in K562 cells (7).

To address this question more directly for domains A, B, and C, we isolated DNA fragments representing subsegments from each individual domain, together with an overlapping domain BC fragment, and placed them into the minimal SV40 early promoter reporter gene in plasmid pA10-CAT, either as single fragments or in combinations. The basal expression levels produced by these *cis*-enhancer reporter gene constructions were then compared directly in transient DNA-transfection assays in Vero cells. The structures of 18 of these hybrid test genes and the results of two sets of basal expression assays are summarized in Fig. 6. In the first experiment, only the domain A sequences produced significantly increased CAT expression over that of the A10-CAT gene. The 5′-upstream NFI, bent, and combined NFI-bent DNA segments all failed to increase basal pA10-CAT activity. An inserted forwards-oriented form of the sequence from positions −405 to −69, referred to as the ENH-A1/A10-CAT hybrid gene, gave almost 4-fold stronger basal activity than that of the backwards-oriented ENH-A1/A10-CAT construction, and the enhancement effect was 10-fold greater in a 5′ position compared with enhancement when placed at a 3′ position. In fact, the added SCMV domain A sequence was virtually inactive as a 3′ enhancer,

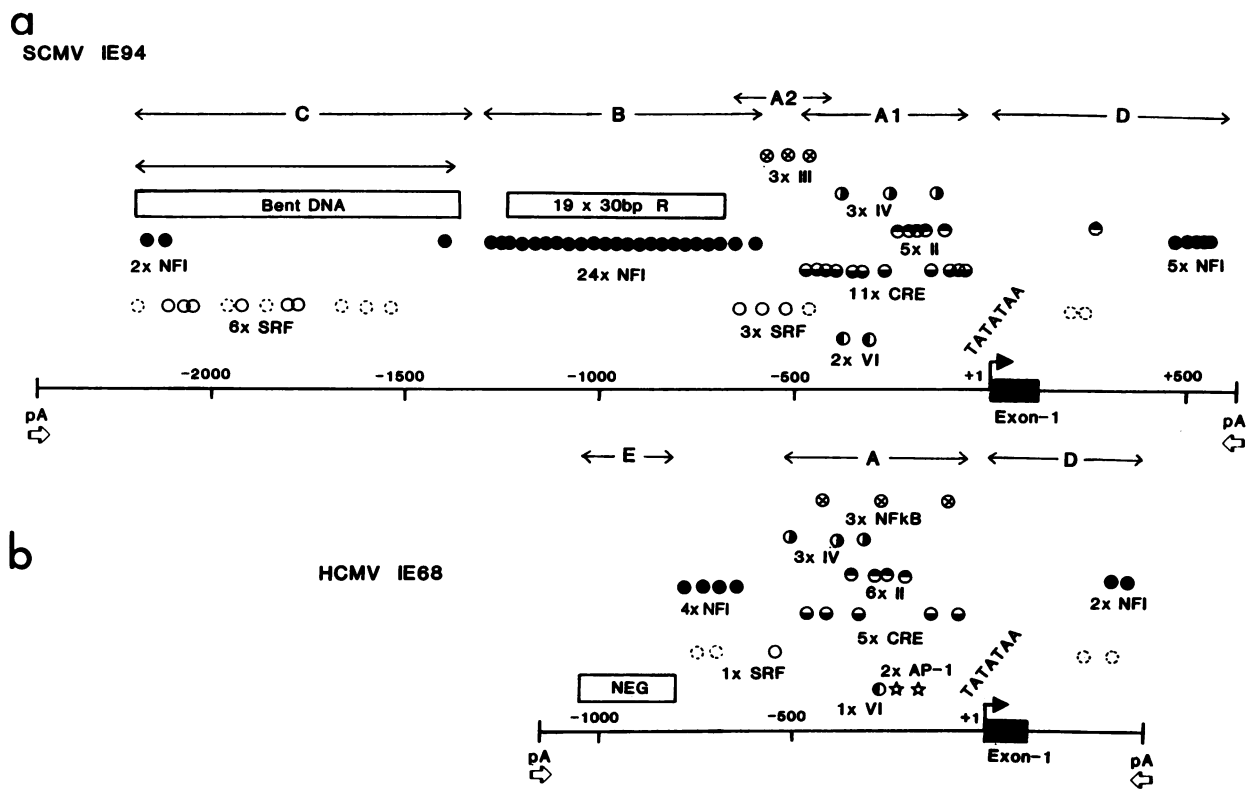

FIG. 4. Arrangement of consensus multicopy repetitive elements and conserved potential transcription factor binding sites within the control regions of the SCMV IE94 and the HCMV IE68 promoters. (a) Summary diagram showing the structural organization of domains A, B, C, and D within the upstream 5′ promoter-regulatory region and first intron of the SCMV IE94 gene. The positions of the TATAA consensus sequence and the positions of individual members of each of the six recognized sets of multicopy repeat elements are indicated (by series) as follows: I, CRE (◒); II, ◓; III, NF-κB-like (⊗); IV, ◑; V, SRF (○); and VI, ◐. ●, NFI. Dotted circles denote some of the near-consensus SRF and RSRF motifs. The numbering system used is based on the first nucleotide in the mRNA molecule representing position +1. Evidence that the domain B tandem repeats represent binding sites for cellular NFI and that the 16-bp series I palindromes represent CREs has been presented previously (7, 26). (b) Comparison with the equivalent region of the HCMV IE68 MIE promoter. The additional (☆) symbols represent potential consensus AP-1 sites. Domain E represents the negative modulator region (39).

and even dimerization of the 3′ ENH-A1 element failed to improve its activity (not shown). Note that the maximum activity of the forwards ENH-A1 segment relative to SV2-CAT was only 20% of that of an IE94-CAT 5′-deletion construction that retained essentially the same domain A sequences out to position −405 within their natural minimal SCMV promoter context.

In a second set of experiments, representative domain B and C segments were added either upstream or downstream of the ENH-A1 segment in the 5′-ENH/A10-CAT constructions. However, their presence gave virtually no significant change in activity in our standard transient Vero cell transfection assays. In all cases, the forwards-oriented 5′-ENH-A1 segment gave 30- to 50-fold stimulation of basal A10-CAT activity (compared with 40-fold for SV2-CAT), and this level of activity was not significantly altered when the NFI or bent DNA sequences were added upstream in either orientation. Similarly, the backwards-oriented 5′-ENH-A1 segment gave 10-fold stimulation of A10-CAT activity on its own, and this value was reduced slightly (but no more than 2-fold) by the interposed NFI, bent, and bent-NFI segments.

The series V motifs bind to SRF. To evaluate whether the series V core SRE-like motifs are functionally significant, we first tested for cellular DNA binding activities that might recognize these motifs (36, 47, 61). The nuclear extracts from human Raji B-lymphoblast cells used in these experiments have proved to be a rich source of various DNA binding activities in both previous (7) and ongoing studies in this laboratory. Two pairs of 30-mer oligonucleotides encompassing nucleotides from position −585 to −561 in the IE94 enhancer region were synthesized, annealed, and labelled and then were used as probes to screen fractions from across a heparin agarose elution profile of a nuclear extract from Raji cells. The first pair of nucleotides (SRF) represented the wild-type sequence, and the second pair (ΔSRF) contained mutations at four critical positions within the potential core SRF binding motif: viz, CCATATATGG changed to GGATATATCC.

The results of electrophoretic mobility shift assays with these two consensus oligonucleotide probes in the presence of poly(dI-dC) competitor DNA are shown in Fig. 7a. Both probes detected an early eluting nonspecific binding activity that was also encountered previously (7), but only the wild-type SRF probe and not the ΔSRF probe produced a more slowly migrating complex that was eluted in three adjacent fractions near the center of the fractionation profile from the heparin agarose KCl gradient. This same binding activity was efficiently competed for with 100-fold excess of the unlabelled double-stranded SRF oligonucleotide pair but

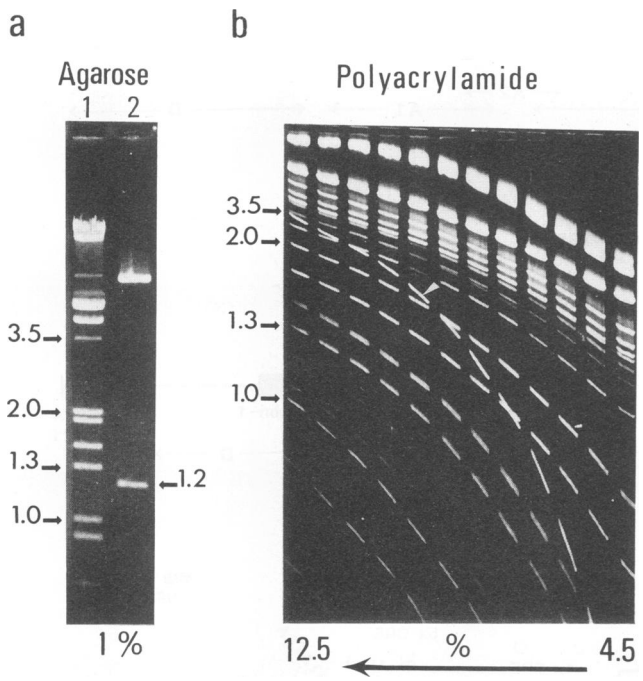

FIG. 5. Anomalous gel electrophoretic mobility of the IE94 domain C bent DNA region. (a) Mobility of the *Stu*I-*Nco*I fragment from position −2260 to −1022 in a 1% agarose gel corresponds to that expected for a 1.2-kb linear DNA fragment. Lane 1 shows phage λ *Hin*dIII-*Eco*RI double-cut markers; lane 2 shows *Sal*I cleavage of plasmid pYNC9 DNA. DNA was detected by ethidium bromide staining and UV fluorescence photography. (b) Altered relative electrophoretic mobility of the same 1,200-bp *Stu*I-*Nco*I fragment depending on the pore size of a polyacrylamide gel matrix. A linear vertical gradient gel of 4.5 to 12.5% polyacrylamide was prepared in a slab gel apparatus. The gel was then turned 90° for new agarose sample slots to be added horizontally across a gradient gel whose concentration increased from right to left. Plasmid pYNC9 DNA was cleaved with *Sal*I and admixed with (an incomplete digest of) *Hin*dIII-*Eco*RI cleaved phage λ DNA, and then aliquots were loaded into each of 12 sample wells; this was followed by electrophoresis and ethidium bromide staining. The apparent molecular weight position of the relevant *Stu*I-*Nco*I fragment (white arrowhead) ranges from 1.2 kb up to 3.5 kb at the highest gel concentration.

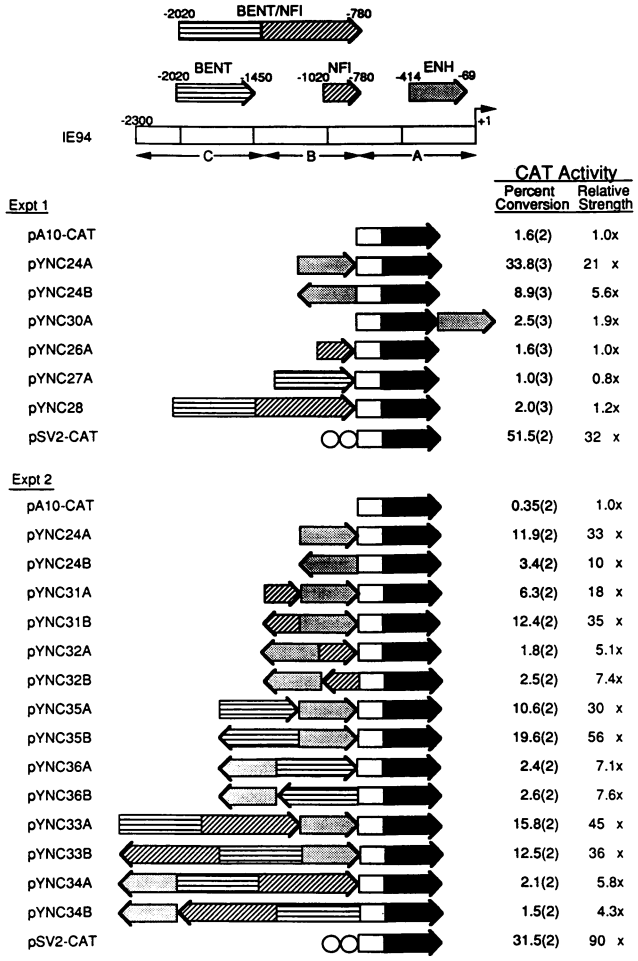

FIG. 6. Domain A but not B or C sequences produce high levels of basal expression and enhancer activity. The figure shows a summary of the results of experiments measuring transient CAT expression in extracts from Vero cell cultures transfected with 2 μg each of a series of A10-CAT-based plasmid DNAs containing various arrangements of added 5′ segments of the enhancer (ENH-A1 domain), NFI cluster (domain B), and bent DNA (domain C) from within the IE94 upstream control region. The data represent averages from either two or three separate measurements as indicated. Solid arrows represent CAT coding sequences. The top portion of the diagram illustrates the relative sizes and locations within the intact IE94 control regions of the four test fragments used in these studies.

was not affected by a 5,000-fold excess of the ΔSRF oligonucleotide pair (Fig. 7b). Therefore, the binding activity is specific and shows the same characteristics expected for the classical 67-kDa SRF protein found to bind to the related SRE motif in the c-*fos*, β-actin, and *zif268* gene promoters (9, 61). In other experiments, we have found that an oligonucleotide representing the c-*fos* SRE itself produces complexes of the same mobility in the same fractions of our Raji cell extract and that it efficiently competes for the binding of this factor to our SCMV series V oligonucleotide probe (not shown).

A DNA segment containing the core SRF binding sites has enhancer properties but does not respond to serum induction. The usual system for examining induction of the c-*fos* and other related SRE-like elements involves the addition of fresh serum or appropriate growth factors to starved growth-arrested mouse NIH 3T3 cells (9, 61). To ask whether the upstream segment of IE94 domain A carrying the SRF motifs is functionally responsive to serum induction, a 120-bp DNA sequence from position −601 to −482 encompassing two series V SRF elements (together with two series III and one possible series I CRE element) was placed in a 5′-upstream test location in the A10-CAT reporter gene vector. This construction (referred to as ENH-A2/A10-CAT) was then transfected into a culture of NIH 3T3 cells that had been maintained in 0.5% serum. After 48 h, the cells were fed with fresh 10% serum and samples were taken for determination of CAT expression levels at 2-h intervals (Table 2). A positive control CAT reporter plasmid containing a known functional SRE from the inducible cellular IE *zif268* gene (9) gave a low level of basal expression and 18-fold induction within 2 h after serum stimulation. In contrast, the results revealed that the SCMV ENH-A2/A10-CAT construction gave a 15-fold enhancement of basal expression in the

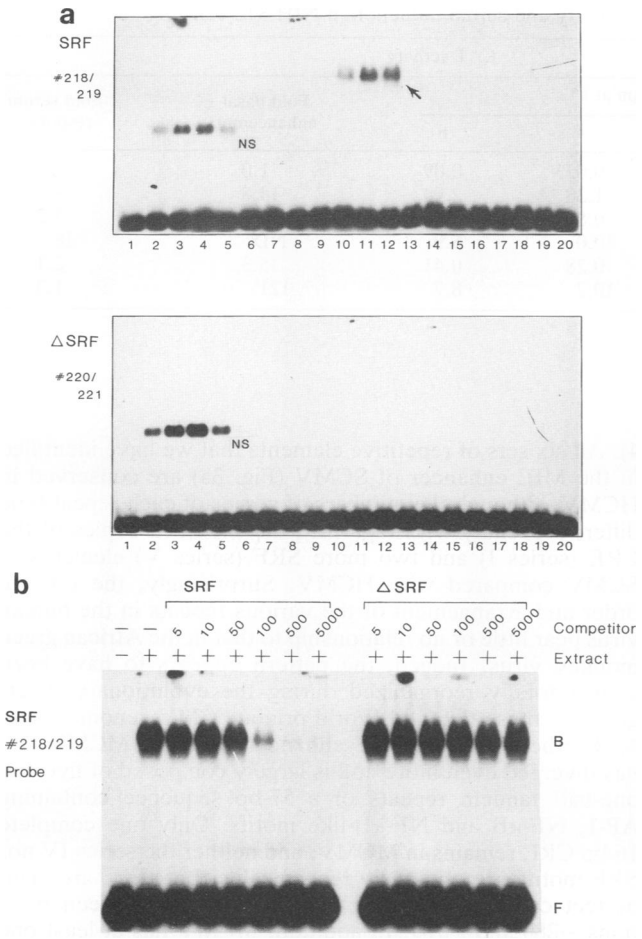

FIG. 7. Series V consensus motifs in the IE94 ENH-A2 region are binding sites for cellular core SRE binding factor (SRF). (a) Detection of series V DNA binding activity in Raji cell nuclear extracts. Electrophoretic mobility shift assay with successive fractions from across a KCl (0.2 to 0.6 M) gradient elution profile from a heparin agarose column containing bound Raji cell proteins. Upper panel, ^{32}P-labelled wild-type series V oligonucleotide probe SRF (#218/219). Lower panel, ^{32}P-labelled mutant series V oligonucleotide probe ΔSRF (#220/221). NS, nonspecific binding activity. Arrow, core SRF binding activity. (b) Competition gel electrophoretic mobility shift assay. Binding of partially purified Raji cell SRF (lane 11 from heparin agarose column above) to 10 ng of ^{32}P-labelled wild-type 30-mer series V oligonucleotide probe DNA (#218/219) in the presence of progressively increasing amounts (5- to 5,000-fold excess) of unlabelled wild-type (SRF) or mutant (ΔSRF) competitor oligonucleotides. Control lanes on the left side of the autoradiograph received either no protein sample and no competitor DNA (first lane) or added protein sample without competitor DNA (second lane). B, bound; F, free probe.

forwards orientation (although only 4.2-fold enhancement in the backwards orientation) and was induced no more than 3-fold by serum treatment.

A second construction containing the ENH-A2 domain placed back into the minimal IE94(−69/+30)-CAT reporter gene was also tested in NIH 3T3 cells. The minimal IE94 promoter itself gave 5-fold greater basal activity than A10-CAT, and this was boosted to 120-fold overall after insertion of the ENH-A2 segment (Table 2). Again there was no significant response to serum induction. Therefore, this upstream ENH-A2 segment of the IE94 promoter-regulatory region contains a second independent basal enhancer activity that is distinct from that of the ENH-A1 segment. However, despite the presence of core SRF binding sites, the ENH-A2 segment appears not to respond to serum induction under standard assay conditions.

Evidence that the series V SRF binding sites contribute to both basal enhancer activity and TPA responsiveness. The same wild-type 30-mer oligonucleotide pair that was used in the SRF DNA binding assays described above was inserted upstream at position −69 into a minimal IE94(−69/+30)-CAT reporter gene as either a monomer or as two and four tandem repeats. These four plasmid constructions were then examined for both basal and phorbol ester (TPA)-induced activity after transfection into human U937 histiocytic leukemia cells. To obtain linear response values, CAT enzyme levels were measured in samples diluted 1- to 5- and 1- to 25-fold as well as in undiluted samples of each extract. Some of the results are presented in the histogram shown in Fig. 8. Overall CAT activity in the presence of TPA increased 2.6-, 4.3-, and 9.3-fold over that of the parent minimal construction with the addition of one, two, and four copies, respectively, of the series V oligonucleotides. This stimulation could be resolved into both a boost in basal expression levels and a separate induction effect, which differed somewhat depending on the number of copies of the inserted element. In the absence of TPA, the basal effect was insignificant with one copy but reached 3.3-fold with two copies and 13-fold with four copies. Direct responsiveness to TPA measured independently of the basal effect reached 4.2-, 37-, and 18-fold for the one-, two-, and four-copy versions, respectively, compared with 1.6-fold for the parent minimal promoter. In undiluted samples, the measured TPA responsiveness of the single-copy construction increased to 5.8-fold. Therefore, only the multiple-copy forms of the element produced basal enhancement, and this increased synergistically with four copies compared to two copies, whereas the direct responses to TPA occurred with just a single copy of the element and were greatest with two copies. Further analysis of the precise sequence requirements for these activities is in progress (7a).

DISCUSSION

The overall structural arrangement of repeated elements in the SCMV MIE promoter-enhancer region differs markedly from that in the herpes simplex virus IE175 and IE110 promoters, as well as from that in either the SV40 enhancer or the long terminal repeat promoter of human immunodeficiency virus. The major novel features are the 12 dispersed copies of the 16-bp palindromic series I CRE consensus sequence CCATTGACGTCAATGG (7) and the presence of at least three SRF binding sites, CCATATATGG, which have not to our knowledge been found previously in any other virus promoter. The SCMV IE94 upstream regulatory region also differs from other viral IE promoter-enhancer regions by the relative paucity of consensus Sp-1 factor binding sites and the lack of any obvious consensus AP-1 sites. However, among the other sets of consensus repeat motifs, the SCMV series III elements have some resemblance to the core NF-κB enhancer sequences.

Comparison of the 5′-upstream promoter-regulatory region from SCMV IE94 with the equivalent sequence in the MIE gene of HCMV (1, 6, 12, 60) reveals an extraordinary pattern of conservation of consensus repeat elements combined with extensive reorganization of their locations (Fig.

TABLE 2. Effects of domain A2 sequences on basal activity and serum induction in NIH 3T3 cells

Test gene, plasmid name	CAT activity					
	% Conversion at h[a]				Fold basal enhancemnt	Fold serum response
	0	2	4	6		
A10-CAT	0.066	0.09	0.053	0.09	1.0	1.5
ENH-A2/A10-CAT, pGH206A	0.98	2.56	1.38	2.94	14.8	3.0
ENH-A2(I)[b]/A10-CAT pGH206B	0.28	0.59	0.80	0.90	4.2	3.2
268(SRE)-CAT	0.92	16.7	10.0	5.9	ND[c]	18
IE94(−69)-CAT, pDG2	0.35	0.74	0.28	0.41	5.3	2.1
ENH-A2/IE94(−69)-CAT, pGH208A	8.0	9.3	10.2	8.7	121	1.3

[a] Time after addition of medium containing serum.
[b] I, Inverted orientation.
[c] ND, not determined.

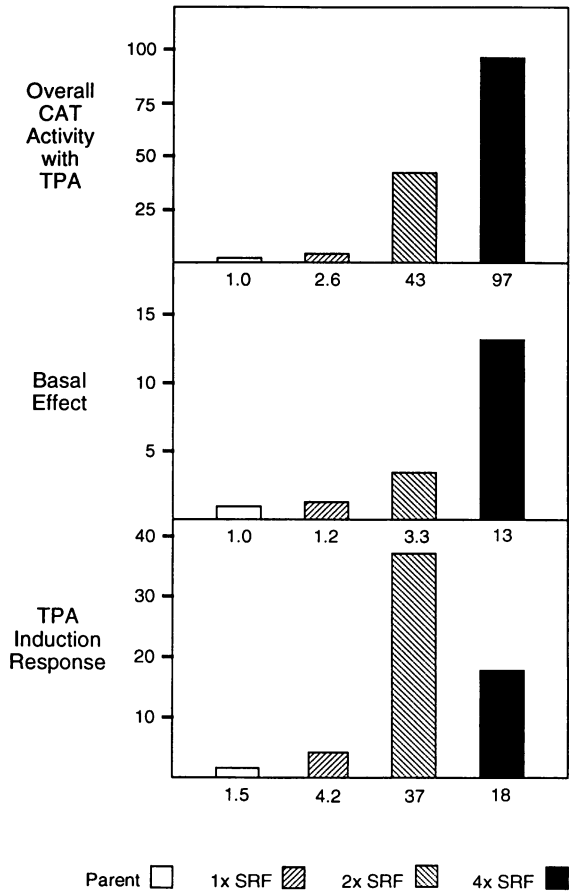

FIG. 8. Basal enhancement and TPA responsiveness of series V elements in U937 cells. Plasmids containing the parent minimal IE94(−69/+30)-CAT reporter gene (pCJC81) and versions with insertions of one (pCJC111), two (pCJC112), and four (pCJC114) tandem copies of the 30-mer series V wild-type oligomers were transfected into U937 cells. Parallel cultures were grown in the presence or absence of TPA and harvested for CAT assays. Relative values on the basis of percent conversion measurements are shown for overall CAT activity in the presence of TPA (at 1:25 dilution), basal activity in the absence of TPA (at 1:5 dilution), and fold stimulation for each construction in the presence of TPA (TPA induction response) compared with basal levels in the absence of TPA (at 1:25 dilution).

4). All six sets of repetitive elements that we have identified in the MIE enhancer of SCMV (Fig. 3a) are conserved in HCMV, although the numbers of copies of each repeat type differ, especially with regard to the six extra copies of the CRE (series I) and two more SRF (series V) elements in SCMV compared with HCMV. Surprisingly, the relative order and arrangement of the various repeats in the human virus bear little or no relationship to that in the African green monkey virus. Indeed, the pattern appears to have been almost totally reorganized during the evolutionary divergence of these two Old World primate CMV genomes (Fig. 4). Furthermore, the MIE enhancer region in MCMV (10) has diverged even more and is largely composed of five and one-half tandem repeats of a 57-bp sequence containing AP-1, NF-κB and NF-κB-like motifs. Only one complete 16-bp CRE remains in MCMV, and neither the series IV nor SRF motifs are obviously recognizable, but there are eight perfect consensus AP-1 sites (TGAGTCA) between positions −280 and −709. In addition, MCMV has at least one close homolog of the SCMV series II repeats (TTGGCACG TACATAAG) at position −316 to −299 and two series of four or five copies each of the related sequences TTGC CCAGTACATAAG and TTGGAGCCAAGTACACTGC, which all include the core AGTACA or CGTACA motif of the series II elements.

The three series III elements in SCMV do not match the classical NF-κB sites nearly as closely as do related sites in the HCMV IE68 enhancer. Indeed, three of the four HCMV 18-bp elements (1, 6, 60), together with five similar motifs in the HCMV US3 gene enhancer (64) and eight in the MCMV MIE promoter (10), have the consensus sequence AGGGACTTTCCA, which includes a perfect match to the standard NF-κB binding elements found within immunoglobulin enhancers (44, 66), in the MSV long terminal repeat and SV40 early promoter core enhancer regions (33, 63) and in the long terminal repeat promoter of human immunodeficiency virus (38). However, because they lack the defining -AC- central dinucleotide that is characteristic of strong NF-κB binding sites, we consider that all three SCMV series III elements and five additional NF-κB-like motifs in MCMV represent distinct motifs relative to the perfect consensus NF-κB sites. The 18-bp elements containing consensus NF-κB sites in HCMV have been shown to bind to cellular NF-κB proteins and to mediate TPA plus phytohemagglutinin responses in human T cells (50). A full analysis of cellular binding factors and functional properties associated with the SCMV series III NF-κB-like elements will be presented elsewhere (7a). Although both the series II and series VI motifs could be considered to be diverged NFI sites, the

entire enhancer region containing these sites failed to bind to affinity-purified NFI in our earlier filter binding assays (26). We have also detected a cellular factor that binds to the SCMV series IV elements (6a), and Kothari et al. (28) have suggested that a factor binding to these sites in HCMV may play a role in repression of IE68 in human teratocarcinoma stem cells.

The contributions to high-level basal expression of different regions within the SCMV enhancer domain appear to vary in different cell types. For example, the patterns of expression for the series of IE94 5'-deletion constructions shown here in Vero cells differ markedly from that in K562 cells, where the minimal region between positions −69 and +30 was of much greater importance and inclusion of sequences beyond position −209 appeared to have negative effects (7). Furthermore, the ENH-A2 segment that was shown here to act as an independent basal enhancer element in NIH 3T3 and U937 cells has a composition (mostly series V and III motifs) quite distinct from that of the ENH-A1 segment (mostly series I, II, and IV motifs).

Despite our demonstration that the core SRF motifs in the SCMV ENH-A2 region represent high-affinity binding sites for the cellular SRF protein, they appear not to act as functional SREs within the context of the 120-bp ENH-A2 fragment that we tested. In the case of the c-*fos* palindromic SRE site, maximal serum response is thought to require adjacent flanking DNA sequences that bind to one or more 62-kDa cellular factors in addition to the core 10-bp binding site for the 67-kDa SRF protein (15). Therefore, in SCMV, either these ancillary factor sites are absent or the high level of basal activity of the region masks or precludes response to serum induction. As judged from the properties of the series V oligonucleotides that we tested, the core SRF motifs in SCMV do contribute to both the high-level basal enhancer properties of the ENH-A2 region and to the strong TPA responsiveness displayed by this region in several T-cell and monocyte-macrophage cell types (7a). However, there are many other consensus core SRF-like and related SRF (RSRF)-like motifs within the IE94 control region that presumably bind to either SRF or SRF-related cellular proteins (47), and yet these other regions do not display either the basal enhancer or TPA response characteristics. The c-*fos* SRE sequence itself also responds to protein kinase C induction by phorbol esters (12), but again both the core and flanking motifs are required for this effect (15). Therefore, it seems likely that other sequences in addition to the series V motifs that are present within our 30-bp oligonucleotides must be involved. Indeed, in subsequent mutagenesis analyses, we have found that both the core SRF binding sites and the adjacent series III motifs are necessary functional components of the SCMV TPA response elements (7a).

The cluster of 23 tandemly repeated palindromic NFI sites that occurs further upstream between positions −1300 and −780 in domain B (22, 26) of the SCMV MIE promoter-regulatory region does not contribute significantly to the high-level basal transcriptional properties, which reside primarily in domain A. An equivalent tandemly repeated NFI cluster is not present in either HCMV or MCMV, although HCMV does have four closely spaced NFI sites at a similar location (17, 26). The role of the large intrinsically bent DNA in domain C is quite enigmatic at present. The combination of bent DNA together with the adjacent NFI cluster and the close relationships between replication origins in IE promoter control elements in SV40, adenovirus, and herpes simplex virus obviously suggest a potential role in DNA replication. Furthermore, the existence of two clusters of NFI binding sites, one as a series of 30-bp tandem repeats, has obvious parallels to the family of 30-bp tandem repeats and dyad symmetry region containing 20 and 4 EBNA-1 binding sites, respectively, within the Epstein-Barr virus latency origin *ori*-P (49). However, the predominant lytic cycle replication origins in SCMV and HCMV map elsewhere in the unique long segment of these genomes (2, 16), and the absence of these features in HCMV and MCMV would be difficult to reconcile with a latency origin function. We are left at present with the suggestion that both regions may play a role in the much wider host range for active MIE gene expression with SCMV than with HCMV (30, 31), perhaps by contributing to opening up of the chromatin structure to permit access of cellular transcription factors to the enhancer region. In support of this notion, preliminary evidence suggests that the inclusion of the NFI region prevents down-regulation of an IE94-CAT reporter gene by superinfection with SCMV in human NTera-2 teratocarcinoma stem cells (10a).

Despite the presence of the bent DNA and the tandem-repeat NFI cluster in SCMV but not in HCMV, the overall organization of the MIE upstream regulatory region of SCMV resembles much more closely that of HCMV than that of MCMV. In particular, a third MCMV MIE region gene, which is transcribed from the opposite side of the divergent 500-bp MCMV enhancer region and has some amino acid homology to the HCMV UL22/TRS-1 gene family, is absent from this location in both HCMV and SCMV.

Finally, we have shown here that IE94-CAT hybrid genes containing domain A sequences from the SCMV MIE promoter express nearly four times more CAT enzyme activity than does the SV40 enhancer from SV2-CAT in transient DNA transfection assays in Vero cells. It is also 40- to 100-fold stronger than the herpes simplex virus type 1 IE175 promoter in microinjected *Xenopus* oocytes (37). Furthermore, the IE94(−990/+30)-CAT reporter gene gives a more than 50-fold higher level of basal CAT activity than does SV2-CAT in transient assays in both human diploid fibroblasts and mouse NG108 neuroblastoma cell lines when the calcium precipitation DNA transfection procedure is used, and it is also expressed abundantly in human Raji, U937, HL60, and Jurkat cell lines after introduction by the DEAE-dextran procedure (33a). SCMV(Colburn) has a much broader host range in cultured human and monkey transformed cell lines than does HCMV(Towne), as demonstrated by constitutive synthesis of the SCMV IE94(IE1) mRNA and protein after infection of these cells, whereas HCMV IE68(IE1) is usually not expressed (30, 31). These differences appear to be related to a combination of both the absence of the HCMV upstream negative modulatory region and the presence of the expanded NFI and bent DNA domains in SCMV. Overall, our results suggest that the complete MIE far-upstream plus enhancer region of SCMV may be an even more useful and powerful reagent than the isolated enhancer from HCMV for directing long-term high-level expression of foreign hybrid genes when introduced into mammalian cells or transgenic animals (51).

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