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The Palindromic Series I Repeats in the Simian Cytomegalovirus Major Immediate-Early Promoter Behave as Both Strong Basal Enhancers and Cyclic AMP Response Elements

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A 600-base-pair (bp) enhancer region upstream from the major IE94 gene of simian cytomegalovirus (SCMV) produces very strong basal expression of associated gene products. This domain consists of multiple sets of interspersed repetitive elements, including 11 copies of a conserved 16-bp palindromic sequence with the consensus CCATTGACGTCAATGG. These series I repeats contain an 8-bp core TGACGTCA that resembles the cyclic AMP (cAMP) response element (CRE) of cellular genes. In transient chloramphenicol acetyltransferase assays in K562 human erythroleukemia cells, a set of deleted variants of the IE94 promoter all responded up to 15-fold to induction by cAMP. However, successive removal of most of the SCMV 16-bp motifs reduced basal expression over 20-fold. The cAMP stimulation was also manifested at the steady-state RNA level after SCMV infection of K562 cells and was detectable within 1.5 h after treatment of DNA-transfected cells. Addition of a single 30-bp oligonucleotide encompassing the 16-bp palindrome conveyed up to 10-fold cAMP responsiveness onto a heterologous weak promoter but had no effect on basal expression. In contrast, two or more adjacent copies produced 20- to 40-fold increases in basal expression and provided greater than 200-fold activation in the presence of cAMP. Similar effects were obtained when the oligonucleotides were placed in a downstream location relative to the reporter gene. Studies with mutant oligonucleotides revealed that both the core CRE and the flanking sequence portions of the 16-bp elements were essential for enhancer function. Both components were also important for maximum cAMP responsiveness. Band shift assays with fractionated nuclear extracts from Raji lymphocytes revealed multiple competable complexes with cellular DNA-binding factors that recognized the series I elements. Three distinct CREB-like factors were detected that required only the core 8-bp elements for binding. We conclude that the 16-bp series I repeats provide a major contribution to the constitutive enhancer properties of the IE94 promoter and also act as functional CREs. The cAMP response properties appear likely to play a key role in reactivation of the virus from a latent state in appropriately differentiating cell types.

Both simian and human cytomegaloviruses (SCMV and HCMV) produce lytic infection in cultured permissive human diploid fibroblast cells. However, only a single family of major viral gene transcripts is expressed under conditions of cycloheximide block to new protein synthesis. These are defined as the immediate-early (IE) mRNAs. The corresponding major IE1 polypeptides of either 94 (SCMV) or 68 (HCMV) kilodaltons (kDa) are also overexpressed in conditions of cycloheximide reversal in the presence of actinomycin D (30, 57). Furthermore, in nonpermissive rodent fibroblasts, these IE1 species are the only significant CMV proteins produced at any stage after infection, even in the absence of inhibitors (28, 33). Part of the explanation for this very limited pattern of gene expression during the initial stages of CMV infection lies in the unique properties of the 5′ upstream region of the CMV major IE gene, which represents one of the strongest known RNA polymerase II promoters that are active in mammalian cells. We demonstrated originally that the intact isolated IE94 gene itself and IE94 promoter-driven chloramphenicol acetyltransferase (CAT) reporter genes are expressed abundantly and constitutively in stable DNA-transfected lines of mouse L-cell and Vero cell origin (29, 42, 43). The isolated IE94 promoter-enhancer region from SCMV(Colburn) also functions three- to fourfold more efficiently than the intact classical 2 × 72-base-pair (bp) enhancer region from the simian virus 40 (SV40) early gene promoter in transient assays in Vero cells and up to 200-fold better than the IE175 promoter from herpes simplex virus (HSV) in microinjected *Xenopus* oocytes (31, 48; K.-T. Jeang, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1984). Similarly, both the HCMV and murine CMV (MCMV) major IE promoters efficiently substitute for the SV40 2 × 72-bp repeats in an SV40 enhancer trap assay (4, 13), and the HCMV IE68 protein can be expressed constitutively in permanent cell lines established from DNA-transfected Vero cells (35a).

Despite the strong constitutive basal properties of the IE promoter, many transformed human and mammalian cell lines fail to express any HCMV viral gene products, and transcription of the IE promoter is repressed (34, 35). However, induction of some of these cell types to differentiate, for example, after retinoic acid treatment of human NTera2 teratocarcinoma stem cells, activates both IE transcription and subsequent stages in the viral lytic cycle (18, 34, 35, 45). Similarly, although SCMV IE94 mRNA is expressed constitutively in human teratocarcinoma cells, it

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is inducible in mouse F9 teratocarcinoma cells (34, 35). Since latent MCMV can be reactivated from monocytes in vivo after differentiation into macrophages, the differential control of IE gene expression may be important in latency. Therefore, the nature of the elements that contribute to both the constitutive enhancerlike features and the cell-type-specific repression and induction properties of the SCMV IE94 promoter are of considerable interest.

Both the SCMV IE94 and HCMV IE68 genes have large and complex upstream regulatory domains that include at least six sets of repetitive elements. In the IE94 promoter, the enhancerlike A domain located between −630 and −50 is structurally distinguishable from two further far-upstream domains. The B domain from −1300 to −630 consists of tandemly repeated nuclear factor 1 (NF1)-binding sites (31; Jeang, Ph.D. thesis, 1984), and the C domain from −2300 to −1300 contains an A+T-rich bent-DNA feature (Y.-N. Chang and G. S. Hayward, manuscript in preparation). However, these far-upstream domains appear to be absent in IE68 and are replaced instead with a negative-acting element between −900 and −550 (45). Within the IE94 600-bp enhancer region we recognize several distinct sets of multicopy repetitive sequences (referred to as the series I, II, III, IV, and V repeats). These include 11 16-bp series I palindromic repeats (31; Jeang, Ph.D. thesis, 1984) as well as three nuclear factor NFκB-like consensus elements (series III). The overall structure and arrangement of consensus elements in the 5′ upstream enhancer regions of IE94 and IE68 are summarized in Fig. 1A and B. Most of these consensus elements are conserved in IE68 but have been scrambled in their arrangement relative to one another (4, 56, 58). One major difference is that in IE94, but not in IE68, the NFκB-like sites are clustered between positions −560 to −440 and overlap with consensus sites for serum response factor (SRF) (Chang and Hayward, in preparation). Preliminary footprinting studies have demonstrated that several cellular factors bind to repetitive elements within the enhancer domain of IE68 (16), but there was no detailed information available about the role of individual repetitive elements or binding factors in the constitutive enhancers of either promoter.

This work was stimulated by recognition that the consensus sequence for the 11 copies of interspersed series I repeats found in IE94 (Table 1) closely resembles a duplicated portion of the upstream sequences in a domain of the human somatostatin gene promoter that contains signals for response to cyclic AMP (cAMP) in transient assays (39, 40). Many subsequent studies have identified similar sequences in other cAMP-responsive cellular genes, such as alpha gonadotropin, phosphoenolpyruvate carboxykinase, and c-*fos*, and narrowed the consensus cAMP response element (CRE) motif to an 8-bp element, 5′-TGACGTCA-3′ (1, 26, 52, 53). This sequence appears to represent two overlapping inverted copies of the pentamer TGACG, which alone is capable of binding to at least one major 45-kDa phosphorylated cellular transcription factor referred to as CREB or CREF (59). Related sequences (TGACG or TGACGT) have also been identified as functionally important components of adenovirus and human T-cell leukemia virus type I (HTLV-I) promoters. In the E4 and other adenovirus early promoters, they represent binding sites for a cellular factor called ATF, which appears to mediate responses to E1A (36–38). In HTLV-I, they represent critical elements within the 3 × 21-bp repeats of the long terminal repeat (LTR) promoter that mediate responses to *trans*-activation by the Tax protein (27). Here we demonstrate that the 16-bp series I palindrome repeats from the SCMV major IE promoter exhibit properties similar to those of active CREs from several cellular genes. In addition, they represent functionally important components of the constitutive IE94 enhancer domain.

MATERIALS AND METHODS

Cells, viruses, and reagents. Stocks of the African green monkey cytomegalovirus SCMV(Colburn) were passaged in human foreskin fibroblast cells as described previously (28). Mouse F9 stem teratocarcinoma cells and rat NG108 neuroblastoma-glioma cells were obtained from J. Shaper and R. Schnaar, Johns Hopkins School of Medicine. All monolayer cell cultures were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum. Human K562 erythroleukemia cells were obtained from the American Type Culture Collection (Rockville, Md.) and grown in suspension cultures in RPMI 1640 medium plus 10% fetal calf serum. Infection of K562 and Vero cells was carried out with supernatant virus at a multiplicity of infection of 10 PFU per cell. 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; B7880; Sigma Chemical Co., St. Louis, Mo.) was added to the culture medium at a final concentration of 1 mM, isobutyl methylxanthine (IMX; Sigma) was added at 0.5 mM, and retinoic acid (Sigma) was added at 1 μM.

Plasmids and reporter gene constructions. The full-length promoter construction IE94(−990/+30)-CAT in plasmid pTJ278 was described previously (31). Selected 5′-deletion variants were constructed by BAL 31 digestion from the upstream *Sal*I linker site, and new *Sal*I linkers were added. The 5′ boundaries of pDG7, pDG5, pTJ279, pTJ280, pDG3, pDG2, and pDG1 were determined by dideoxynucleotide sequencing with M13 derivatives and are summarized in Fig. 1. The construction referred to as ENH/A10-CAT contains the IE94 enhancer region sequences from −405 to −67 placed at the *Bgl*II site upstream from the SV40 enhancer-minus early promoter reporter gene (A10-CAT) in plasmid pYNC24a (Fig. 1C). The IE94(−91/+30)-CAT reporter gene in plasmid pYNC45 was constructed by inserting a 28-bp synthetic oligonucleotide pair containing a *Bgl*II site plus sequences corresponding to the series I repeat element at positions −91 to −73 into the 5′ *Sal*I linker site at −69 in pDG2. Subsequent insertion of tandem repeats of a 30-mer oligonucleotide pair (LGH45/46) containing the wild-type consensus series I repeat bounded by *Bam*HI and *Bgl*II sites gave pYNC50 (two copies) and pYNC51 (four copies). Similarly, pYNC47 contains an insertion of the 28-mer sequence at a *Sal*I site 3′ of the IE94(−69/+30)-CAT reporter gene in pDG2. Addition of the 30-mer LGH45/46 oligonucleotide pair at the 3′ *Bgl*II site in pYNC47 produced pYNC52 (single copy) and pYNC53 (four tandem repeats).

Multiple tandemly repeated copies of the wild-type LGH45/46 oligonucleotide pair were also inserted either at the 5′ *Bgl*II or 3′ *Bam*HI site in pA10-CAT to create pYNC54 (5′, single copy, forward orientation), pYNC55a (5′, two copies, forward orientation), pYNC55b (5′, two copies, backward orientation), pYNC56 (5′, three copies, forward orientation), pYNC57 (5′, four copies, forward orientation), pYNC58 (3′, single copy, backward orientation), and pYNC82 (3′, three copies, forward orientation). The mutant oligonucleotides shown in Fig. 2 were also inserted at the 5′ upstream *Bgl*II site in pA10-CAT to produce pYNC64 and pYNC70 (monomer and dimer copies of LGH127/128 in the forward orientation), pYNC66 (dimer copy of LGH125/126), and pYNC67 (dimer copy of LGH133/

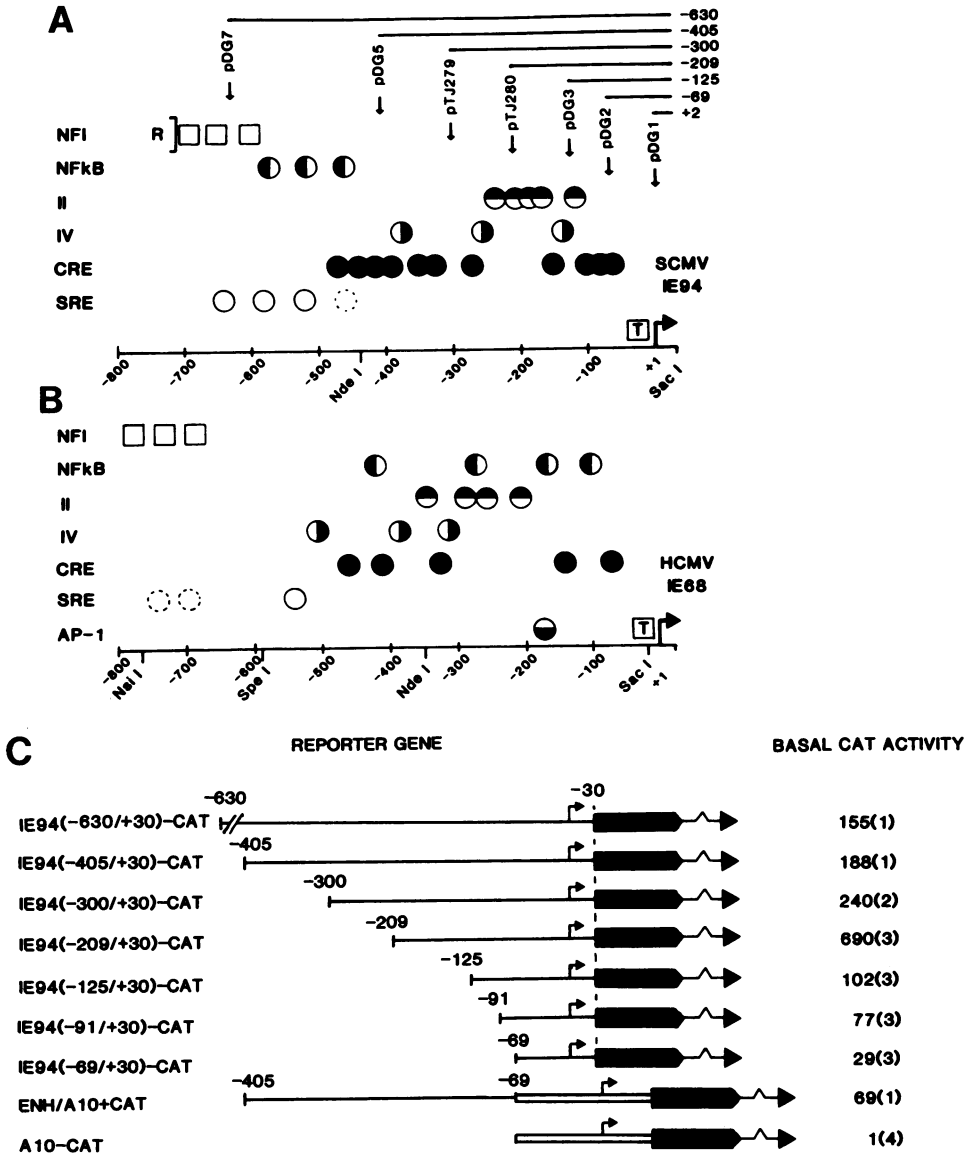

FIG. 1. Organization and enhancerlike properties of the proximal 5′ upstream domain of the major IE promoter from SCMV(Colburn). (A and B) Comparison of interspersed repeat consensus elements within the enhancer region of the SCMV and HCMV major IE promoters. (A) Proximal 800 bp of the SCMV IE94 promoter, based on sequence data from Jeang (Ph.D. thesis, 1984), Jeang et al. (31), and Y.-N. Chang et al. (manuscript in preparation). The endpoints for a set of 5′-deletion mutations derived from a plasmid containing the IE94(−990/+30)-CAT gene (pTJ278) are also indicated. (B) Arrangement of equivalent repeated elements in the HCMV IE68 promoter (4, 58). Individual consensus elements are represented as follows: ●, series I elements (referred to as 19-bp elements in HCMV), which we show here to be both CREs and recognition sites for CREB (or ATF) factors; ◓, series II elements (referred to as 21-bp elements in HCMV); ◐, series III elements (referred to as 18-bp elements in HCMV), which resemble binding sites for the cellular NFκB and HT2F1 factors (3, 44); ◑, series IV elements (referred to as 16-bp elements in HCMV); □, series V elements, which are palindromic NF1-binding sites (20, 31); ○, series VI elements, which resemble core SREs and bind to the cellular serum response factor (Chang et al., in preparation); ◌, diverged variants of the SRE sites. T, TATATAA box; R, beginning of the more distal cluster of 20 × 30-bp tandemly repeated NF1-binding sites in IE94 (31). (C) Basal expression data for 5′-deleted IE94-CAT reporter gene constructions in DNA-transfected K562 human erythroleukemia cells. Cultures were transfected by the DEAE-dextran procedure with 2 μg of each plasmid DNA sample. The results are presented as average levels of [^{14}C]chloramphenicol acetylation activity relative to a basal A10-CAT activity of 1.0. The number of determinations for each sample is indicated in parentheses. Arrowed solid bars represent the CAT-coding cassette with SV40 early gene-derived poly(A) and splicing signals; open bars indicate minimal SV40 early gene promoter and leader region sequences in A10-CAT (SV40 positions −110 to +58); lines represent SCMV IE94 sequences, with 5′ and 3′ endpoints indicated.

134). The validity of each construction was confirmed by dideoxynucleotide-sequencing analysis on alkali-denatured plasmid DNA, using a CAT gene leader sequence primer.

RNA extraction, DNA transfection, and CAT assays. Total-cell RNA was isolated from infected or DNA-transfected K562 cells by the guanidinium isothiocyanate procedure followed by sedimentation through a cushion of CsCl and then was suspended in buffer, extracted with phenol-isoamyl alcohol-chloroform, and ethanol precipitated. Introduction of 10 μg of plasmid DNA into 2 × 10^7 K562 cells in

TABLE 1. Comparison of SCMV series I repeats with consensus CREs and CREB factor- or ATF-binding sites

Sequence			Description	
	CCATTGACGTCAATGG		SCMV consensus 16-bp palindrome	
−481	CCATTGACGTCAATGG[a]	−466	SCMV (Colburn) IE94 series I repeats	
−446	CTATTGACGTCATATGG	−430		
−420	CTATTGACGTATATGG	−405		
−401	CCATTGACGTCAATTA	−386		
−355	CCATTGACGTCAATAGG	−340		
−331	CCATTGACGTCAATGG[a]	−316		
−277	CTATTGACGTCAATGA	−262		
−164	CCATTGACGTCAATGG[a]	−149		
−112	CCATTGACGTCAATGG[a]	−97		
−90	GCAATGACGCAAATGG	−75		
−69	CCATTGACGTAAATGG	−54		
−466	CCGTTGACGTCAATAG	−451	HCMV (Towne) IE68 series I repeats	
−413	CCATTGACGTCAATGG[a]	−398		
−330	CTATTGACGTCAATGA	−315		
−144	CCATTGACGTCAATGG[a]	−129		
−70	CCGTTGACGCAAATGG	−55		
−128	AAATTGACGTCATGGT	−113	Chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone	α-Subunit, 2 × 18-bp repeats
−146	AAATTGACGTCATGGT	−131		
−53	TGGCTGACGTCAGAGA	−37	Rat somatostatin	
−79	GGAGTGACGTCATCT.	−64	Parathyroid hormone	
−48	..CTTGACGTCAGCC.	−34	Thyroid hormone	
−80	.CTGTGACGTGCTTC.	−65	Vasoactive intestinal polypeptide	
−80	..TCTGACGTAAGGGG	−95	Phosphoenolpyruvate carboxykinase	
−82	..GCTGACGCAGGCC.	−97	Proenkephalin	
−70	CCAGTGACGTAGGA..	−55	c-*fos*	
−101	GCGTTGACGACAACCC	−86	HTLV-I LTR (3 × 21-bp repeats)	
−200	GCCCTGACGTGTCCCC	−185		
−249	GCTCTGACGTCTCCCC	−234		
−331	.AAGTGACGTTTTTG.	−317	Ad2[b] E1A	
−405	.GGGTGACGTAGTAG.	−392		
−437	.TTGTGACGTGGCGC.	−424		
−80	GAGATGACGTAGT...	−65	Ad2 E2A	
−50	CCTGTGACGAAAGC..	−65	Ad2 E3	
−40	.AAATGACGTAACGG.	−55	Ad2 E4	
−148	.AAGTGACGATTTGA.	−133		
−172	.AAGTGACGTAACGT.	−157		
−238	.GGGTGACGTAGGTT.	−223		
−270	.TTGTGACGTGGCGC.	−255		

[a] Perfect match to consensus 16-bp element.
[b] Ad2, Adenovirus type 2.

suspension culture was performed by the DEAE-dextran (250 μg/ml)-plus-chloroquin (50 μg/ml) procedure described by Jeang et al. (27). F9 and NG108 cells were transfected by the short-term calcium phosphate-plus-glycerol procedure (46). For CAT assays, transfected cells were harvested after 48 h; where appropriate, cAMP and IMX were added to the culture medium 20 h before harvesting. Preparation of extracts for assay of CAT activity by conversion of [^{14}C]chloramphenicol to its 1′- and 3′-acetylated derivatives was carried out as described by O'Hare and Hayward (46, 47).

Labeled synthetic oligonucleotides. Single-stranded oligonucleotides were synthesized by Scott Marrow, Johns Hopkins School of Hygiene, purified by high-pressure liquid chromatography procedures, and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The complementary strands of each pair were heated to 65°C in 150 mM NaCl, annealed by slow cooling, and separated from unincorporated nucleotides on Sephadex G-25 columns. The sequences of the oligonucleotides used here are listed in Fig. 2.

Nuclear extracts and DNA-binding assays. Raji cells are an Epstein-Barr virus (EBV)-immortalized human B-lymphocyte line derived from an African Burkitt's lymphoma patient. Nuclear extracts were prepared by a modified high-salt lysis procedure (12), bound to a heparin-agarose column, and then eluted in a gradient of 0.3 to 0.75 M NaCl as described by Jones et al. (32). For some experiments, the fractions containing CRE-binding activity were pooled and concentrated. Gel mobility retardation assays (5) were carried out essentially as described by O'Neill and Kelly (50) and apRhys et al. (2), using 2% agarose gels in 0.1× Tris-acetate-EDTA buffer (pH 7.5). DNA-protein binding reactions were carried out at 23°C for 30 min in 50 mM NaCl plus binding buffer with 0.01% Nonidet P-40, 40 μg of poly(dI-dC) per ml, and 5 fmol of ^{32}P-end-labeled oligonucleotide probe DNA. Bovine serum albumin (1 mg/ml) was added when purified column fractions were used.

RESULTS

Basal enhancer properties of the SCMV(Colburn) IE94 upstream region in K562 cells. In earlier transient assays in

FIG. 2. Sequences of the wild-type and various point mutant series I 30-mer oligonucleotide pairs. The shaded nucleotides on the top strand in each pair represent the consensus perfect 16-bp palindrome, which includes an *Aat*II site. The group 2 oligonucleotides differ from those in group 1 only at positions 21, 22, and 23 to allow the introduction of a *Kpn*I tag as well as the *Pvu*II site. FCCF indicates a wild-type series I consensus 16-bp palindrome, where C represents the TGACG core sequence and F represents the flanking sequence.

Vero cells, measurements of basal CAT enzyme expression from a series of 5′ deletions in the IE94(−990/+30)-CAT reporter gene construction (pTJ278) revealed an additive effect of the presence of upstream sequences. Deletion to −405, −209, −125, and −69 retained 80, 15, 3, and 1%, respectively, of the maximal activity (not shown). However, since Vero cells did not prove useful for cAMP induction studies, we switched to the human K562 erythroleukemia cell line and used the DEAE-dextran rather than calcium phosphate coprecipitation procedure for DNA transfection. This cell line in suspension culture was used successfully previously by Jeang et al. (27) to study CREs in the LTR promoter-enhancer region of HTLV-I. In general, so much CAT enzyme was produced in this system that our assays had to be carried out with 10-fold (and sometimes 100-fold) dilutions of the extracts to maintain linear kinetics over the 1-h incubation period. The overall pattern of higher basal expression when more of the upstream IE94 sequences were present persisted in the K562 cells (Fig. 3C) except that the highest constitutive activity occurred with the −209 deletion mutant. Retention of farther-upstream sequences produced a small decrease in basal levels, whereas IE94(−125/+30)-CAT (pDG3) and IE94(−69/+30)-CAT (pDG2) both displayed a fivefold greater residual activity compared with that of the intact promoter than was anticipated from the Vero cell data. In addition, the IE94 upstream sequence from −405 to −69 (without its associated TATAAA box element) was placed upstream from the minimal SV40 early promoter in A10-CAT (pYNC24A) and yielded a nearly 70-fold increase in basal CAT expression. Thus, these results confirmed that the IE94 5′ upstream region displays the properties of a movable constitutive enhancer in K562 system also, although the relative contributions of various portions of the sequence appear to vary somewhat among cell types.

The IE94 promoter-enhancer region responds to cAMP induction in transient assays. Several IE94-CAT 5′-deletion plasmids were transfected into K562 cells, and the levels of CAT enzyme were examined both with and without the addition of 8-Br-cAMP to the growth medium. The treatment was initiated 28 h after DNA transfection, and the cultures were harvested 20 h later. The relative levels of CAT activity obtained in a representative experiment are shown in Fig. 3A. Importantly, all IE94-CAT targets retaining 5′ upstream sequences as far as positions −91 or beyond gave between 8- and 14-fold stimulation after cAMP induction, compared with 12-fold stimulation for the HTLV-I LTR-CAT positive control DNA. The minimal IE94(−69/+30)-CAT target (pDG2) containing only one series I element gave fivefold stimulation, whereas control CAT gene constructions containing the intact IE175 and IE110 promoters from HSV and the intact or minimal SV40 early promoter in SV2-CAT or A10-CAT all failed to respond significantly.

To determine whether other, more indirect inducers of cAMP levels were also effective, we compared the results of addition to K562 cells of 8-Br-cAMP alone, IMX (an inhibitor of cAMP phosphodiesterase) alone, forskolin (an inducer of adenylate cyclase) alone, or combinations of these agents (Fig. 3B). The target gene in this case was the IE94(−125/+30)-CAT construction with three series I repeats in plasmid pDG3. Compared with mock treatment with the same levels of ethanol or dimethyl sulfoxide solvents, treatment with all of these agents proved to be effective and resulted in four- to sixfold induction of expression, with the mixtures of cAMP and IMX being slightly superior at eightfold. These findings support the conclusion that the phenomenon is indeed related to cAMP levels. The cAMP-plus-IMX regimen was used in all subsequent experiments in K562 cells.

Examination of other cAMP-inducible cell types. Since only minimal cAMP responses could be measured in Vero cells,

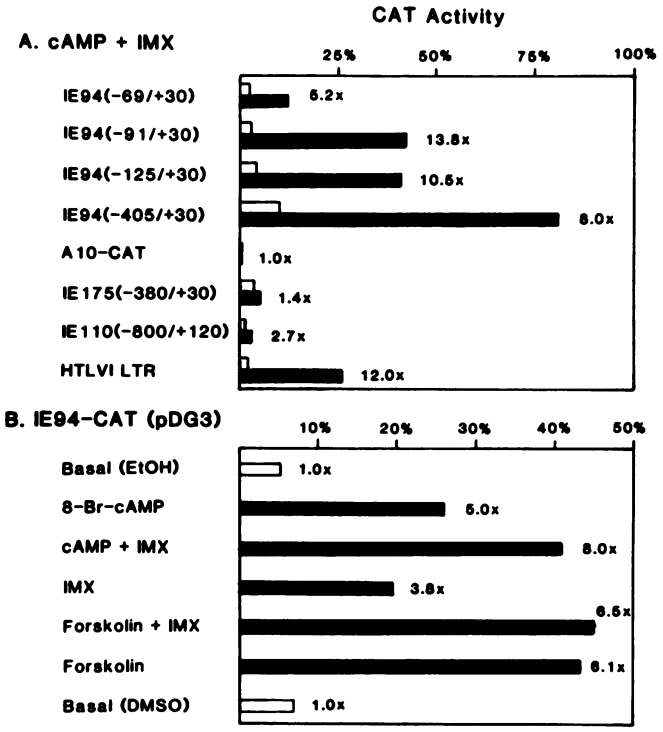

FIG. 3. Demonstration that the IE94 promoter responds to cAMP induction in K562 human erythroleukemia cells. Shown are results of typical transient expression assays with various target CAT reporter gene constructions (2 μg of DNA in each sample). Symbols: □, levels of CAT enzyme activity (percent conversion) in the absence of cAMP treatment; ■, levels obtained after treatment with 8-Br-cAMP plus IMX. The fold induction value is indicated for each pair of samples. (A) Target gene specificity and the effect of 5′ deletions. (B) Effects of different cAMP induction regimens on levels of CAT activity for transfected IE94(−125/+30)-CAT DNA (plasmid pDG3).

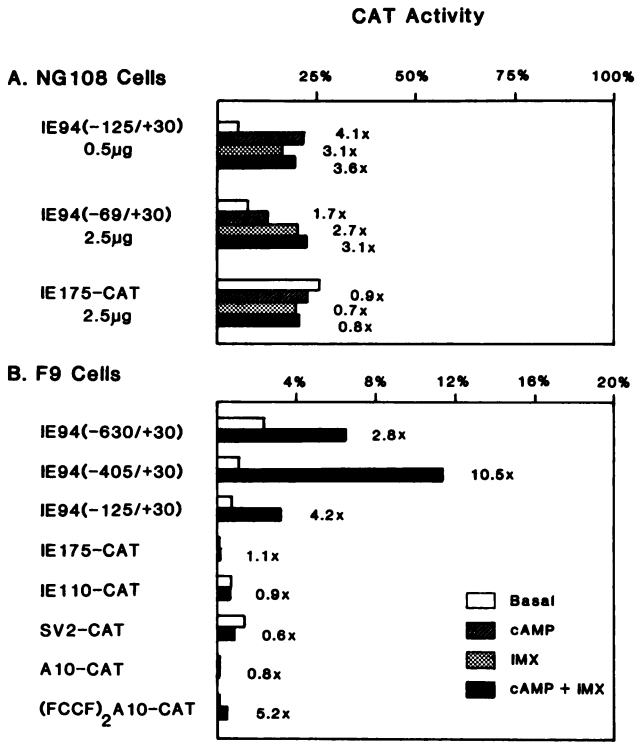

FIG. 4. cAMP induction of IE94-CAT expression in neuroblastoma and teratocarcinoma cell lines. The histograms illustrate the results of transient CAT expression assays with various target reporter genes (input amounts of plasmid DNA are indicated). Fold induction values relative to basal activity of 1.0 are indicated. (A) Rat NG108 cells. Symbols are as shown in panel B. (B) Mouse F9 teratocarcinoma stem cells. Symbols: □, basal levels after treatment with retinoic acid only; ■, treatment with retinoic acid plus cAMP. Each sample received 2 μg of target plasmid DNA. $(FCCF)_2$A10-CAT contains two inserted wild-type series I consensus oligomers (pYNC55a).

which presumably either are not sensitive to cAMP induction or already contain maximal levels of cAMP, we also examined two other cell types: the rat NG108 neuroblastoma cell line, which can be induced to partially differentiate after cAMP or nerve growth factor treatment, and mouse F9 teratocarcinoma cells, which differentiate best after treatment with retinoic acid plus dibutyryl cAMP. The F9 cells were of particular interest because expression of the IE94 mRNA after SCMV(Colburn) infection does not occur until after retinoic acid induction (35). The results of cAMP treatment on expression of transfected IE94(−125/+30)-CAT target DNA in NG108 cells were very similar to those in K562 cells. Maximal stimulation of fourfold occurred with 0.5 μg of IE94(−125/+30)-CAT target DNA, using either 8-Br-cAMP alone, IMX alone, or the combination of the two (Fig. 4A). In this experiment, 2.5 μg of input IE94(−125/+30)-CAT DNA gave basal and induced levels beyond the linear range of the assay, but a two- to threefold stimulation was observed with 2.5 μg of IE94(−69/+30)-CAT DNA. In comparison, 2.5 μg of the HSV IE175-CAT gene DNA gave threefold higher basal activity than did IE94(−69/+30)-CAT, but the cAMP treatment yielded a slight reduction in activity.

In the F9 stem cells, IE94(−125/+30)-CAT DNA responded 4-fold to cAMP activation, and IE94(−405/+30)-CAT gave a 10-fold stimulation (Fig. 4b). Basal activity with IE94(−630/+30)-CAT DNA was 4.5-fold higher than for IE94(−125/+30)-CAT in this experiment, but the induction was greatly reduced. All of the heterologous control plasmids, including the complete SV40 early promoter-enhancer construction, gave lower basal activity than did IE94(−123/+30)-CAT, and none were inducible by cAMP treatment. Retinoic acid alone had no significant induction effects even on the IE94-CAT target DNAs over the short period of time involved in this experiment (12 h, compared with 3 to 4 days for differentiation effects). Therefore, although lower levels of expression in general in the NG108 and F9 cells made it more difficult to document, the cAMP response of the IE94 promoter is clearly not confined to K562 cells and should be evident in all cell types in which cAMP levels can be manipulated.

cAMP induction occurs at the RNA level in both transient assays and infected cells. To determine whether the CAT assay measurements reflected accurate initiation events at the RNA level, we also used S1 nuclease analysis of whole-cell mRNA isolated at various times from K562 cell cultures transfected with IE94-CAT DNA. The results with an IE94-CAT-derived ^{32}P-labeled riboprobe revealed that a 295-bp protected RNA species appeared within 1.5 h in cAMP-treated cells (Fig. 5a). This same species was not detectable until 6 h in uninduced cells. At both time points, the induced mRNA was at least 5- to 10-fold more abundant than the uninduced species. Furthermore, the start site for these

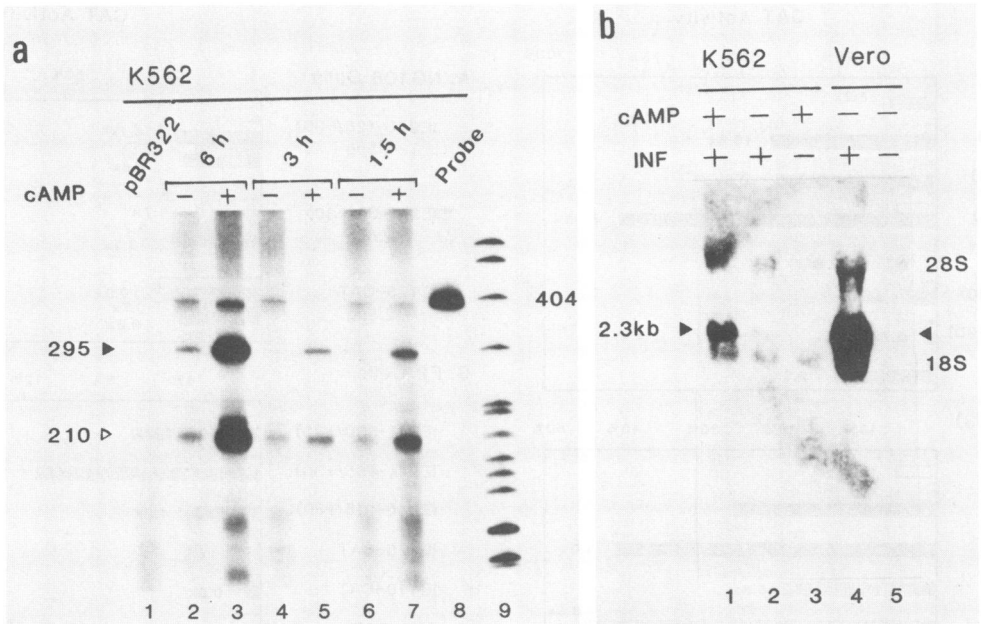

FIG. 5. Stimulation of correctly initiated IE94 transcripts in transfected and infected cells. (a) S1 Analysis of IE94-CAT mRNA synthesized in DNA-transfected K562 cells in the presence (+) or absence (−) of 8-Br-cAMP. Total RNA samples were harvested at 48 h after transfection with pDG3 DNA and 1.5, 3, or 6 h after addition of 8-Br-cAMP plus IMX and then annealed with a 404-nucleotide uniformly ^{32}P labeled pGEM complementary-strand riboprobe (Promega Biotec, Madison, Wis.) that included sequences from −69 to +298 in the IE94-CAT reporter gene (pYNC71). Digestion with S1 nuclease and fractionation on a 6% polyacrylamide–urea gel revealed protection of an approximately 295-nucleotide region, which corresponds to the known viral transcription start site 30 bp upstream from the *Sac*I site. The second band at 210 nucleotides apparently reflects partial S1 cleavage at a site near the initiation codon of the CAT-coding sequences. Control samples represent RNA from pBR322 DNA-transfected cells (lane 1) and undigested probe DNA (lane 8). (b) Effect of cAMP on the levels of IE94-specific transcripts in SCMV(Colburn)-infected K562 cells. 8-Br-cAMP plus IMX was added 20 h before infection in lanes 1 and 3. Total-cell RNA was harvested at 18 h after infection (lanes 1 and 2) or mock infection (lane 3) of K562 cells and subjected to Northern blot hybridization with a ^{32}P-labeled plasmid DNA probe containing IE94 exon 4 sequences labeled by the random-priming procedure (15). Control mRNA samples from infected and mock-infected Vero cells are shown in lanes 4 and 5. Label trapped with the 18S and 28S rRNA is visible in all lanes. The 2.3-kilobase-pair mRNA is visible only in those K562 cells that were pretreated with cAMP and corresponds to the major IE94 species produced in Vero cells.

reporter gene mRNAs was estimated to lie 30 bp upstream from the *Sac*I site and therefore corresponds exactly to that for the major intact viral IE94 mRNA species observed in SCMV(Colburn)-infected Vero cells (Jeang, Ph.D. thesis, 1984).

It was also necessary to demonstrate that the effects of cAMP on IE94-CAT gene expression in transient assays could be detected with the intact viral gene in SCMV-infected cells. Although transformed human cell lines are known to be very poor hosts for HCMV infection and fail to express IE68 mRNA, SCMV-infected Vero and human teratocarcinoma stem cells do synthesize IE94 mRNA and protein (34, 35). Therefore, although we did not anticipate much IE94 expression from SCMV(Colburn)-infected K562 cells, we asked whether cAMP treatment could increase the levels of IE94 viral mRNA. The results with cultures that had been pretreated for 18 h with cAMP showed that a 2.3-kilobase-pair mRNA species containing IE94 exon 4 mRNA sequences was indeed detectable by Northern (RNA) blot hybridization analysis at 12 h after infection in the presence of cycloheximide (Fig. 4b). In contrast, this mRNA was not detectable in K562 cells infected in the absence of cAMP. Note that the amount of IE94 mRNA was still an order of magnitude lower than that in a parallel sample from Vero cells infected in the absence of cAMP.

Addition of extra 16-bp repeats to the IE94 minimal promoter increases basal expression. Although farther-upstream sequences were clearly important for the highest levels of basal and induced CAT enzyme activity, the smallest deleted version of the promoter that we tested responded fivefold to cAMP induction. This IE94(−69/+30)-CAT target gene (pDG2) also still gave 30-fold-higher basal activity than did the minimal SV40 early promoter in A10-CAT. Interestingly, this minimal IE94 promoter retained one imperfect copy of the series I repeats but had lost all of the other recognized repetitive elements (Fig. 1). To attempt to address whether the higher basal expression obtained with additional upstream sequences might be accounted for primarily by loss of the other series I elements, rather than by loss of the NFκB-like, serum response element (SRE), series II, or series IV repeats, for example, we prepared a 28-bp synthetic oligonucleotide pair that restored the second series I repeat sequence in its correct location when inserted at the *Sal*I linker site in pDG2. This new construction [the IE94(−91/+30)-CAT gene in plasmid pYNC45] yielded 2-fold higher basal expression than did the minimal promoter and exhibited between 9- and 14-fold activation after cAMP treatment (Fig. 3B and 6b). The *Bgl*II site introduced at position −94 also allowed insertion of additional head-to-tail tandem repeats of a consensus 30-bp series I oligonucleotide pair bounded by *Bam*HI and *Bgl*II linker sites. Target reporter plasmids containing a total of either three or five tandem repeats of the consensus 30-mer added 5′ to the minimal promoter gave five- to ninefold increases in basal expression (Fig. 6b, pYNC50 and pYNC51). Similarly, addition of either two or five head-to-tail tandem repeats at the

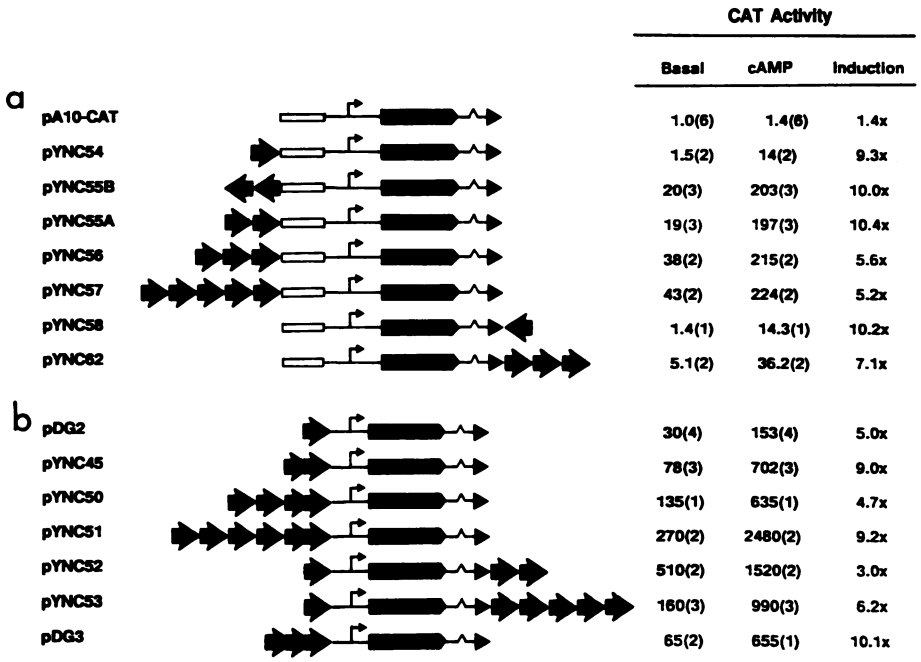

FIG. 6. Summary diagram showing that addition of consensus wild-type 30-bp series I repeats confers both cAMP-induced responses and enhancer properties. Shown are the structures of test gene constructions and the measured basal and cAMP-induced levels of CAT enzyme activity for each plasmid relative to a basal A10-CAT level of 1.0. The number of determinations for each test plasmid is indicated in parentheses. Also shown is fold induction in the presence of cAMP compared with that in the absence of cAMP. Symbols: ■, CAT gene coding sequences; □, six adjacent Sp1 factor-binding sites from the minimal SV40 early promoter in A10-CAT. Arrows illustrate the location, spacing, and orientation of all natural and synthetic series I 16-bp repeats. (a) Set of eight series I insertion constructions with consensus 30-mer oligonucleotides placed either 5′ or 3′ from the parent A10-CAT reporter gene; (b) set of seven series I insertion constructions based on the minimal IE94 promoter in IE94(−69/+30)-CAT (pDG2) and the reconstructed parent IE94(−91/+30)-CAT gene (pYNC45) and including the IE94(−125/+30)-CAT (pDG3) deletion control.

*Bam*HI site 3′ to the IE94(−91/+30)-CAT gene again boosted basal expression between 6- and 17-fold (Fig. 6b, pYNC52 and pYNC53). The high basal CAT activity of these constructions approached that of the fully intact natural promoter-enhancer region in the HCMV IE68(−760/+10)-CAT reporter gene (i.e., up to 500-fold higher than basal A10-CAT activity) (Fig. 7). The structures of all of these test genes and the averaged results from a number of independent assays are summarized in Fig. 6b. The data for both basal and cAMP levels are given relative to the basal A10-CAT activity, which was set at an arbitrary value of 1.0. Thus, in the presence of cAMP, the construction with five added 5′ tandemly repeated series I elements (pYNC51) gave an overall 80-fold increase in the presence of cAMP over the basal levels for the minimal IE94(−69/+30)-CAT gene in pDG2. This value is 1,750-fold higher than that for the minimal SV40 promoter in pA10-CAT. Note that the multicopy constructions gave no significant increases in cAMP response over the 10-fold effect produced by the two-copy version (pYNC45).

The series I repeat elements convey both high basal expression properties and cAMP responsiveness to a heterologous promoter. The results described above indicated that the 16-bp repeats probably play a major role in the high basal activity of the IE94 promoter and that they may represent both orientation- and distance-independent enhancer elements. To confirm these properties, we asked whether the series I repeat oligonucleotides could also transfer the cAMP response or basal enhancer properties to a weak nonresponsive heterologous promoter. Different numbers of tandemly repeated copies of the 30-bp consensus oligonucleotide were inserted either upstream or downstream from the minimal promoter elements of the SV40 early-gene control region in plasmid A10-CAT. Sample results with some of these reporter gene plasmids are shown in Fig. 8 (lanes 1 to 10 and 21 to 26), and all of the quantitative data that were obtained in an extensive series of similar experiments are summarized and compared in Fig. 6a. Four separate 5′-multicopy constructions were examined, two with two tandem copies each of the inserted consensus element arranged in opposite orientations (pYNC55A and pYNC55B), plus one with three copies (pYNC56) and one with four copies (pYNC57). With both of the dimers, the basal activity of A10-CAT in K562 cells was stimulated 20-fold. In addition, the level of induction by cAMP treatment was increased from 1.4-fold for A10-CAT to approximately 10-fold in both orientations. The three- and four-copy constructions both gave 40-fold increased basal activity and 5-fold cAMP responses. Overall, the levels of CAT activity in all four of these constructions in the presence of cAMP were boosted an average of 200-fold compared with basal A10-CAT activity. Thus, although the absolute activity levels achieved in the pA10-CAT background were only approximately 10% of those obtained in the IE94 minimal promoter background (Fig. 7), the maximum basal activation achieved with 5′ inserts was several-fold greater in pA10-CAT.

In contrast to the multiple-copy constructions, the addition of a single 5′ copy of the series I oligonucleotide into A10-CAT (pYNC54) failed to influence basal expression at all but still conferred nearly full cAMP responsiveness (9.3-fold). Similarly, a single copy placed at the *Bam*HI site at the 3′ end of the A10-CAT gene (pYNC58) conveyed

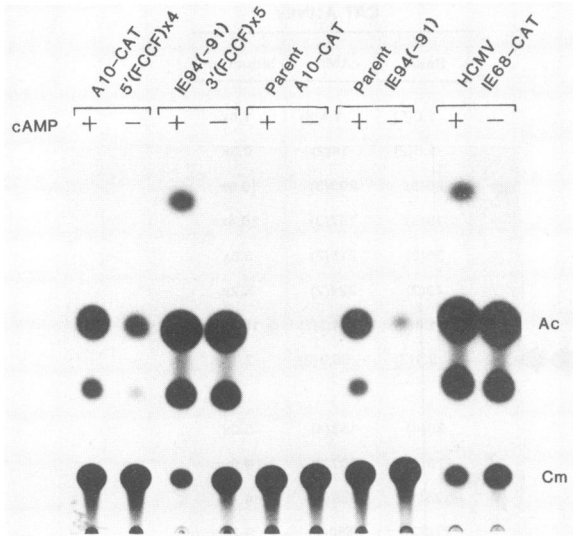

FIG. 7. Effect of addition of multiple copies of the consensus 30-bp series I oligonucleotides to the minimal IE94 and SV40 early gene promoter-driven reporter genes. The autoradiographs show the results of transient CAT expression assays after addition of 2 μg of each plasmid DNA sample to K562 cells in the presence (+) or absence (−) of cAMP plus IMX. The target reporter gene used for each pair of samples is indicated. These are contained within the following plasmids: lanes 1 and 2, pYNC57 (A10-CAT plus 4 × wild-type 30-mer placed upstream); 3 and 4, pYNC53 [IE94(−91/+30)-CAT plus 5 × wild-type 30-mer placed downstream]; 5 and 6, parent pA10-CAT; 7 and 8, parent pYNC45 [IE94(−91/+30)-CAT]; 9 and 10, pCATwt760 [IE68(−760/+10)-CAT]. Cm, Substrate [^{14}C] chloramphenicol spot; Ac, 3′-acetyl and 1′,3′-acetyl [^{14}C]chloramphenicol products. Quantitation of these data are included in Fig. 6.

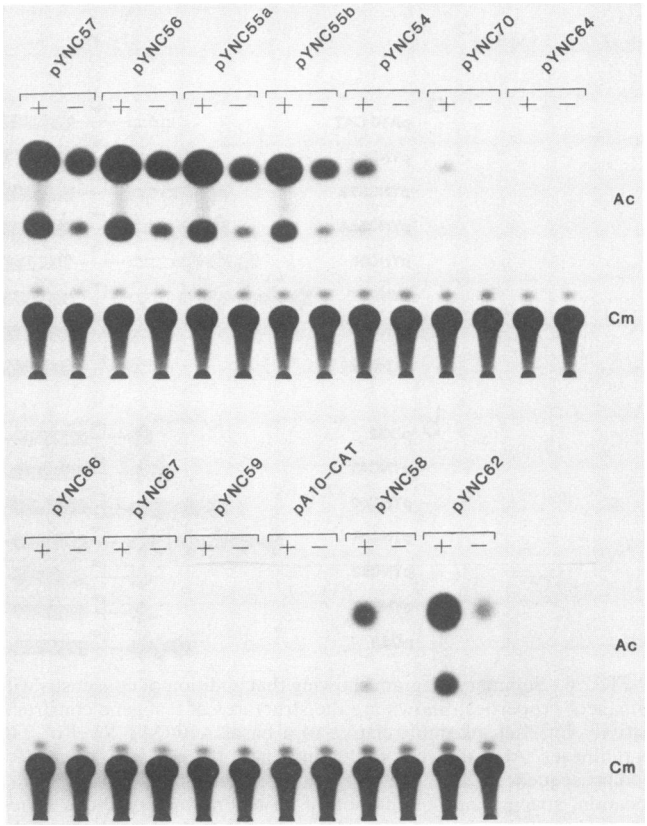

FIG. 8. Comparison of the effects of different copy number, position, and sequence of synthetic series I oligonucleotides on levels of CAT expression from the minimal SV40 early gene promoter. The autoradiographs show the results of a typical set of parallel transient CAT expression assays in DNA-transfected K562 cells, using equivalent amounts of extracts prepared from cells grown in the presence (+) or absence (−) of cAMP plus IMX. A summary of the structure of each target reporter gene plasmid used and of quantitative measurements from this and other similar experiments are given in Fig. 6 and 9.

equally strong cAMP responses (10.2-fold) without influencing basal levels. Three tandemly repeated copies placed in a 3′ position behind the A10-CAT gene (pYNC62) gave only a modest gain in basal levels (5.1-fold) but still yielded 35-fold-increased expression overall in the presence of cAMP. These properties are totally consistent with those of classical distance- and orientation-independent basal enhancer elements.

Evidence that sequences flanking the SCMV CRE consensus are critical for basal enhancer properties. Additional modified A10-CAT target gene constructions similar to those described above were prepared by using mutated 30-bp consensus series I oligonucleotides. In these constructions, either the core 8-bp CRE site only was retained or one or both half-TGACG sites were mutated and the flanking sequences were retained (Fig. 2). The wild-type and mutant oligonucleotides were each examined as either monomers or tandem dimers placed upstream of A10-CAT (Fig. 8, lanes 5 to 22). Summary quantitative data for all of the relevant constructions tested are given in Fig. 9. As a positive control, the monomer form of the intact wild-type 16-bp palindrome (pYNC54) conferred a 9.3-fold cAMP response but gave an insignificant increase in basal expression (1.6-fold). Insertion of this oligonucleotide as a tandemly repeated dimer (pYNC55a) resulted in a large increase in basal expression (27-fold) that was further induced an average of 7.3-fold by cAMP. In dramatic contrast, the use of any of the three mutated oligonucleotides as either a monomer (pYNC64 and pYNC59) or dimer (pYNC70, pYNC66, and pYNC67) yielded slight decreases in basal expression compared with the A10-CAT level. Surprisingly, even a tandemly repeated dimer of the mutated sequence that retained the intact 8-bp CRE but lacked the normal palindromic flanking sequences (pYNC70) responded only partially to cAMP treatment (3.8-fold). Furthermore, the dimer insert with only the 5-bp half-CRE sequence intact (pYNC66) gave an even lower response (1.9-fold). In summary, mutations in either the core CRE consensus element or the flanking sequences of the 16-bp series I repeat all reduced both the basal expression and the cAMP responses of dimer constructions to minimal levels.

Detection of cellular nuclear proteins that bind to the 16-bp elements. Many studies with CREs from cellular genes have reported the detection of one or more cellular factors that bind to sequences containing either the full consensus 8-bp motif TGACGTCA or the 5-bp half-site TGACG (1, 39, 59). However, not all promoters with these same consensus sequences either respond to cAMP or bind to these factors (10, 11). We were interested in knowing whether cellular transcription factors from human lymphoid cells might bind to the SCMV series I 16-bp repeat oligonucleotides. We chose to use nuclear extracts from the Raji strain of EBV-transformed B cells, partly because we had purified a 45-kDa CREB-like protein from this source and shown that it

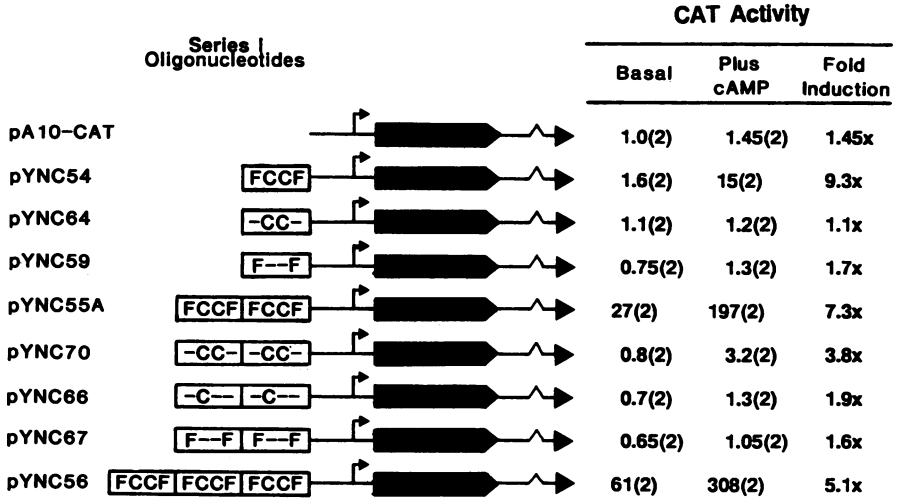

FIG. 9. Analysis of copy number and DNA sequence requirements for both basal enhancer activity and cAMP responses after insertion of wild-type or mutated 30-bp oligonucleotides into an A10-CAT background. FCCF indicates a wild-type series I consensus 16-bp palindrome, where C represents the TGACG core sequence and F represents the flanking sequence. Dashes indicate mutated subelements. The number of transient CAT assay determinations performed for each test plasmid is given in parentheses.

footprinted over a consensus 8-bp CRE sequence motif in EBV DNA (D. Rawlins, unpublished data).

Using an unfractionated nuclear extract or the partially purified CREB protein (eluting at 0.55 to 0.6 KCl from a heparin-agarose column), we carried out gel shift-up assays with the consensus IE94 series I 16-bp repeat oligonucleotides. The ^{32}P-labeled wild-type 30-mer plus mutations that either lacked the flanking sequences or disrupted one or both of the overlapping TGACG core motifs were each used as probes. Both the wild-type (FCCF) and core (-CC-) oligonucleotides produced a doublet and occasionally triplet pattern of DNA-protein complexes (C1, C2, and C3) in the presence of 40 μg of poly(dI-dC) competitor per ml (Fig. 10a, lanes 1, 2, 5, and 6). Even a probe retaining only the 5-bp TGACG portion of the 16-bp palindrome (-C--) gave a single complex similar in abundance and mobility to the C2 species obtained with the 8-bp-core-only (-CC-) probe (Fig. 10b, lane 6). In comparison, the flanking-sequence-only probe (F--F) yielded none of these gel shift complexes and instead gave only a minor and much faster-migrating species (labeled F in Fig. 10a, lane 4). An unrelated 30-mer-NFκB oligonucleotide probe containing known NFκB protein-binding sites from the human immunodeficiency virus (HIV) LTR promoter-enhancer region was used as a positive control and formed a single DNA-protein complex, which migrated with mobility similar to that of the C2 complex (Fig. 10a, lane 8). The specificity of CREB binding to the core (-CC-) probe was confirmed by a competition experiment (Fig. 10c). The unlabeled homologous core (-CC-) oligonucleotide proved to compete slightly with the labeled (-CC-) probe at a 500-fold excess and very efficiently at a 5,000-fold excess (lane 7). Similar concentrations of the heterologous NFκB oligonucleotide failed to affect binding of the C2 complex (lane 12) but, as expected, did compete with the B complex formed by the HIV LTR probe.

We also scanned the entire Raji cell heparin-agarose gradient with the wild-type (FCCF), core (-CC-), and flanking sequence (F--F) 30-mer probes. The NFκB 30-mer was again used for comparison. Four distinct major shift-up complexes (C1, C2, C3, and NS [nonspecific]) plus several minor species were detected with the wild-type probe (Fig. 11a), and all of them were also detected with the probe containing only the central 8 bp of the core palindrome (not shown). Note that the strong binding species (NS) formed in fractions 1 to 3 represents a relatively nonspecific activity that bound to all of these probes and also to several other unrelated oligonucleotides that we tested. The probe retaining only the flanking regions of the palindrome (F--F) detected just a single potential minor unique species (F) in addition to the nonspecific complex (Fig. 11b, lane 4). Finally, NFκB-binding activity eluted much earlier off the column (predominantly in fractions 4 to 6) and was therefore clearly resolved from the C2 species (Fig. 11c). On the basis of the binding patterns obtained with all four probes, we conclude that there are at least three distinct proteins that form complexes specifically with the 8-bp palindrome sequences. One major binding species (C2) was concentrated in fractions 10 and 11 and corresponded to the activity in the partially purified CREB fraction used in the previous experiment (Fig. 11a, lane 19). The C1 and C3 species eluted at lower salt concentrations than did C2 (fractions 6 to 8 and 8 to 10) and yielded slightly faster and slightly more slowly migrating complexes, respectively. None of these proteins bound to the flanking sequences probe. At higher input levels of protein extract, additional slower-migrating complexes were sometimes seen with both the FCCF and -CC- probes but never with the pentamer consensus (-C--) probe (not shown). These forms may correspond to the dimeric or higher-oligomer forms of CREB that were proposed by Yamamoto et al. (59).

DISCUSSION

The results presented here argue strongly for a model in which the series I 16-bp repeats provide the bulk of the constitutive enhancer properties of the SCMV IE94 promoter, at least in certain cell types. In their natural promoter context, the first three copies of the 16-bp elements lie directly adjacent to one another and are spaced 21 and 22 bp apart on the same face of the helix between positions −111 and −53 (Fig. 1A). Only these three series I elements (plus one series II element) remain within the IE94(−125/+30)-

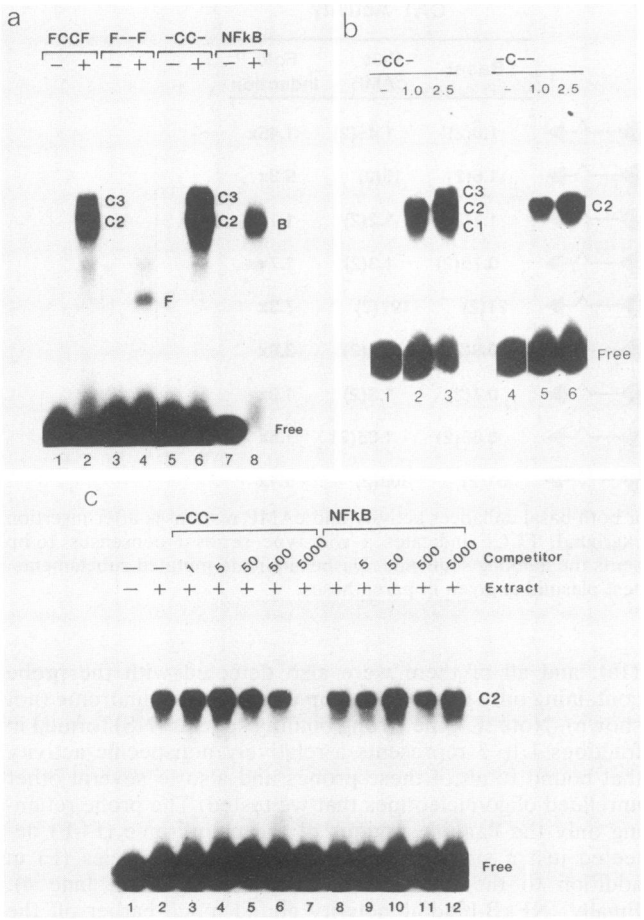

FIG. 10. Detection of CREB-like cellular DNA-binding factors that recognize the IE94 series I repeats in gel mobility retardation assays. (a) Comparison of complexes formed with ^{32}P-end-labeled 30-mer oligonucleotide probes containing intact (FCCF), core-sequence-only (-CC-), or flanking-sequence-only (F--F) consensus series I sequences. An HIV LTR NFκB probe was used as a nonrelevant positive control for binding specificity. +, Addition of 2.5 μl of unfractionated Raji cell nuclear extract; −, absence of extract. (b) Different binding patterns of 8-bp (-CC-) compared with 5-bp (-C--) core CRE probes. The volume of unfractionated Raji cell extract added used is given in microliters. (c) Specific competition for binding to the labeled 8-bp (-CC-) core consensus oligonucleotide probe with unlabeled series I 8-bp (-CC-) core CRE oligonucleotides but not with the unlabeled NFκB oligonucleotide probe. The fold excesses of unlabeled competitor oligonucleotides used in lanes 3 to 7 (-CC-) and 8 to 12 (NFκB) are indicated. Free, Position of free unbound oligonucleotide probes; B, NFκB complex; C1, C2, and C3, core CRE-binding activities; F, possible flanking-sequence binding activity.

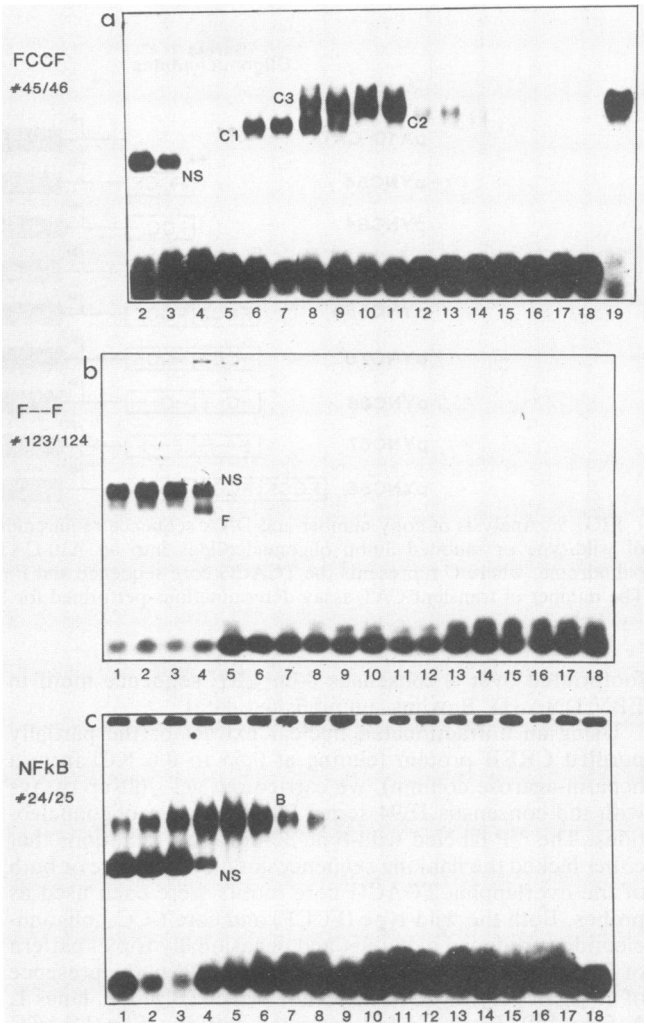

FIG. 11. Demonstration of three distinct Raji cell binding activities that recognize the IE94 CRE core consensus sequences. The autoradiographs show gel mobility retardation assays with labeled oligonucleotide probes to heparin-agarose column fractions of the Raji cell nuclear extracts. B, NFκB complex; C1, C2, and C3, core CRE-like binding activities; F, possible flanking-sequence-specific binding activity; NS, nonspecific binding activity. Fractions: 1 to 16, successive pooled samples from the 0.3 to 0.75 M NaCl elution gradient; 17 and 18, 0.75 and 1.25 M NaCl washes; 19, sample of partially purified Raji cell CREB-binding activity (C2) used in the experiment shown in Fig. 10c and in renaturation studies (not shown).

CAT 5′-deletion construction, which retains 15% of the maximal basal promoter activity in both K562 and F9 cells. On the basis of the dramatically increased basal expression obtained after addition of multiple 30-mers of the series I repeats into either the IE94(−69/+30)-CAT or the A10-CAT background, we anticipate that the other eight farther-upstream series I repeats in IE94 may also contribute to the overall basal enhancer activity, although their spacing is less regular, with center-to-center distances of 25, 24, 28, and 35 bp occurring among the six adjacent sites between positions −479 and −346. It is not clear whether these additional elements simply represent redundancy or whether they provide other response specificities in a variety of cell types in conjunction with different combinations of nearby elements. We also do not know as yet what significance to place on the fact that the SCMV IE94 promoter-enhancer region contains 12 copies of the series I repeats, whereas IE68 contains 5 and the MCMV major IE promoter retains only 1 intact 16-bp repeat (plus a number of half-sites). Similarly, there are sequence variations among the IE94 series I repeats, with only 8 of the 12 (4 in HCMV) retaining a perfect TGACGTCA core CRE motif with its central *Aat*II restriction enzyme cleavage site. It remains to be determined whether these sequence variations have some functional significance or represent residual effects of an apparently rather dynamic evolutionary divergence process that involved multiple amplification and rearrangement events.

Considering the 20-fold effects on basal expression of intact dimers compared with monomers of the series I repeats in A10-CAT, the relatively high basal activity of the minimal IE94(−69/+30)-CAT construction that contains the single most proximal series I element is somewhat puzzling. No other obvious recognizable motifs except for the TATAAA box are present within this region, yet it gives up to 30-fold-higher basal expression than does unmodified A10-CAT in K562 cells. There are no consensus Sp1 sites anywhere in the IE94 promoter, and therefore the context is very different from that of A10-CAT, with its cluster of six adjacent Sp1 sites. Perhaps another, unidentified control element or the relative proximity to the TATAAA box are critical factors here. Alternatively, there may be differences in the relative binding or functional affinity of the somewhat imperfect proximal series I element compared with the fully palindromic consensus elements that we synthesized.

Our results show clearly that the intact consensus 16-bp elements found in the SCMV IE promoter behave similarly to the cellular 8-bp palindromic CREs. Furthermore, they can also function in a 3′ position and contribute significantly as tandem repeats to basal expression, i.e., they behave as classical (although inducible) enhancers. Nevertheless, our mutational analysis shows that the 8-bp CREs alone, and therefore presumably also the CREB-like factors that bind to them, are not sufficient for this effect. Interestingly, an initial analysis with synthetic somatostatin consensus CREs failed to detect increased basal expression, using tandem repeats placed upstream of the A10-CAT promoter (40). However, a more recent and very thorough analysis with tandemly repeated copies of the complex 18-bp elements from the chorionic gonadotropin gene revealed basal enhancement results very similar to ours (10, 11). In addition, these authors showed that in certain sequence contexts, such as in the parathyroid hormone and glucagon promoters, the standard consensus 8-bp CRE is both insufficient to give basal enhancement and unable to bind CREB or to compete for binding to active CRE sequences. Similarly, certain alterations in the spacing or sequence of the paired 18-bp elements totally abolished even the cAMP responses without changing the consensus 8-bp core TGACGTCA sequence. The data currently available do not provide any obvious rules about allowable CRE contexts that produce functional enhancerlike elements. The flanking sequences in the SCMV and HCMV 16-bp elements are conserved but differ significantly from all of the other active cellular gene contexts that have been described to date.

Our analysis revealed three distinct CRE-binding factors in the Raji cell extracts, each of which eluted at a different salt concentration from a heparin-agarose column. Species C2, which was the only one of the three that bound to a single TGAGC half-site probe, has been shown by renaturation analysis to be a protein of 45 kDa (Rawlins, unpublished data) and therefore closely resembles the CREB or ATF species described by others. The slower-migrating complexes may potentially involve higher-molecular-weight CRE-binding proteins similar to these detected with HTLV-I 21-bp repeat probes (27). Although c-Jun/c-Fos heterodimers have some affinity for CREs (K.-T. Jeang, unpublished data), we have shown elsewhere that uninduced Raji cells are relatively devoid of AP-1-binding activities (P. M. Lieberman et al., submitted for publication). Hoeffler et al. (22) and Gonzalez et al. (19) have recently cloned and sequenced genes encoding human CREB proteins and shown that they contain the conserved adjacent basic and leucine zipper DNA-binding domains typical of the closely related *jun/fos*/CEBP/GCN4 family of cellular transcription factors.

We were unable to find convincing evidence in Raji cell extracts for factors that specifically recognized the flanking portions of the 16-bp palindromes. Perhaps the palindromic nature of the flanking sequences alone promotes cooperative interactions between pairs of adjacent 8-bp elements. However, additional studies will also be necessary directly within K562 cell extracts (prepared before and after cAMP treatment) to seek appropriate DNA-binding activities that might be specific for the series I flanking sequences. It is noteworthy that synergistic effects of other factor-binding sites lying adjacent to CREs have been described recently in the adenovirus, gonadotropin, and proenkephalin systems (7, 9, 14, 25) and that the appropriate juxtaposition of two different elements in a duplicated arrangement is a central theme in the enhanson model proposed by Ondek and colleagues for the SV40 enhancer (49).

The factors and events that initiate and control the transcription and expression of the IE genes of CMV are likely to be of major importance for determining the outcome of the infection process (i.e., lytic, latent, reactivated, or abortive). Both the HCMV and SCMV major IE gene promoter-enhancer regions drive expression of alternatively spliced IE1 and IE2 gene products, both in infected permissive human foreskin fibroblast cells (55) and in transient DNA transfection systems (51). The IE2 genes encode nuclear phosphorylated proteins that behave as powerful nonspecific *trans*-activators of gene expression in transient cotransfection assays (8, 21, 41, 51) and also negatively autoregulate transcription from their own promoters (51). The functions of the more abundant IE1 proteins of HCMV and SCMV are not yet known, but they are highly acidic phosphorylated nuclear proteins (54; Jeang, Ph.D. thesis, 1984) and appear to associate tightly with condensed chromosomes in mitotic cells (35a).

The immediate biological relevance of the presence of CREs in the major IE promoters of SCMV and HCMV is somewhat difficult to assess at present. Synthesis of the CMV IE2 *trans*-activator protein presumably leads to activation of the full lytic cycle pathway. Therefore, it seems likely that IE2 gene expression must be either shut down or repressed during latent infection in monocytes or other lymphoid cell types. The CREs might function to detect and react to changes in the differentiated state of latently infected cells, with high levels of cAMP perhaps leading to lytic cycle reactivation. On the other hand, there is reason to suspect that the IE1 protein is selectively expressed during latent infection (23; LaFemina et al., in press) and that an excess of IE2 would specifically down-regulate expression from the major IE promoter (51). Since the series III and V consensus repeat elements in the IE94 promoter also bind to NFκB, H2TF1, and SRE factors present in Raji cell extracts (Y.-N. Chang et al., unpublished data), the possibilities for inducible responses to other stimuli that influence lymphocyte activation appear to be complex and varied. Furthermore, the probability that the CREs act as ATF-binding sites leads one to expect that the CMV IE promoters may respond to transactivation by the E1A protein of adenovirus. Unfortunately, a critical unknown in attempts to rationalize the modulation of expression of the CMV major IE promoters is the functional role of the IE1 nuclear antigen during lytic compared with latent infections.

Recent analyses from other laboratories have indicated that both the series I (CRE)- and series III (NFκB)-equivalent elements in the HCMV major IE68 promoter contribute

to basal expression (17) and that the NFκB-like repeat elements mediate transcriptional stimulation in response to T-cell activation (6, 24).

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