Volume 2 · Number 2 · 1992
GENE EXPRESSION

The SV40 early transcriptional regulatory element is unable to direct gene expression in pituitary GH-3 cells

Timothy A. Coleman, Yong-Tai Hou, and John J. Kopchick

Department of Zoological and Biomedical Sciences, Molecular and Cellular Biology Program and Edison Animal Biotechnology Center, Ohio University, Athens, Ohio

The SV40 early (SV40E) transcriptional regulatory element (TRE) is able to direct heterologous gene expression in a variety of eukaryotic cell lines. This ability is conferred, in part, by the presence of several cis-elements. Transfection studies, mutational analyses, and in vitro DNA binding assays have demonstrated that the SV40E TRE is capable of interacting with several cellular transcription (trans) factors. In the present study, we have investigated the inability of the SV40E TRE to direct gene expression in cultured rat anterior pituitary GH-3 cells. Gel shift analysis demonstrated that nuclear factors within these cells can recognize and specifically bind to DNA containing SV40 enhancer sequences. Surprisingly, we have found that both HeLa and GH-3 cells possess relatively equal quantities of Sp1-specific RNA; however, a dramatic decrease in Sp1 protein was seen in GH-3 cells. Transfection studies utilizing CAT reporter plasmids revealed that the intact SV40E TRE is inactive in these cells, and that subsequent deletion of a region(s) where nuclear factor binding occurs does not result in detectable levels of gene expression. Thus, removal of cis-sites potentially involved in repressor binding does not result in activation of the SV40E TRE in these cells. Subcloning an SV40 enhancer fragment upstream of a heterologous TK promoter yielded chimeric TREs that could direct high levels of gene expression in HeLa but not GH-3 cells. Therefore, the prototypic SV40 enhancer, in the context of GH-3 cells, cannot enhance gene expression.

Transcription of eukaryotic DNA is a complex process that can be regulated developmentally, tissue-specifically, or in response to extracellular signals (Darnell, 1982; Maniatas et al., 1987; Serfling et al., 1985). This process involves interaction of DNA sequences with cellular transcription (trans) factors (Dynan and Tjian, 1985; Ptashne, 1988; Mitchell and Tjian, 1989; Johnson and McKnight, 1989). Early studies identified two distinct types of cis-acting regulatory sequences required for Pol II-directed RNA polymerase: promoters, which are located close to the initiation site and act in a position-dependent manner; and enhancers, which can be located far from the initiation site and act in a position- and orientation-independent manner (Khoury and Gruss, 1983; Gluzman, 1985; Serfling et al., 1985; Guarente, 1988).

In the region of the SV40 viral DNA that includes the origin of replication, a prototypic transcriptional regulatory element (TRE) has been demonstrated. Deletion mapping and mutagenesis studies have delineated a 418 bp region (position 5171 to 346 of the viral genome) that is important for viral replication, as well as expression of both early and late genes

Received October 24, 1991; revision accepted February 11, 1992.

Correspondence: John J. Kopchick, Edison Animal Biotechnology Center, 201 Wilson Hall, Ohio University, Athens, OH 45701
Tel (614) 593-4534 Fax (614) 593-4795.

 1052-2166/92/0202/175-15$2.00

(Fig. 1A; Gruss et al., 1981; Moreau et al., 1981; Gidoni et al., 1985). The SV40 early (SV40E) TRE has been shown to contain several modules which by themselves have no or weak enhancing activity, but which can act synergistically to yield high levels of activity (Herr and Clark, 1986; Ondek et al., 1988; Schirm et al., 1987; Fromental et al., 1988). Subsequent analysis has demonstrated that these modules contain binding sites for a variety of cellular transcription factors including TFIID, Sp1, AP-1, AP-2, AP-3, AP-4, AP-5, Oct-1, and NF-KB (Fig. 1B; reviewed by Jones et al., 1988). The ability of the SV40E TRE to direct heterologous gene expression in different cell lines must be due, in part, to the array of cis-elements present within this region (Nomiyama et al., 1987; Ondek et al., 1987; Rosales et al., 1987; Schirm et al., 1987).

We have previously reported on the inability of the SV40E TRE to direct heterologous gene expression after transfection into cultured rat anterior pituitary (GH-3 and P3) cells (Coleman et al., 1991a). This is not due to an inability of these cells to take up SV40-CAT plasmid DNA, nor is it a result of cellular transformation, as non-transformed pituitary cells (P3) demonstrate essentially the same profile of heterologous gene expression. Schirm et al. (1987) had previously shown that synthetic multimers of SV40 modules linked to a β-globin test gene could yield enhancer activity in 9 of 10 cell lines tested. None of these synthetic SV40 motifs was capable of directing β-globin transcription in GH-3 cells, as determined by quantitative S1 nuclease mapping. These data suggest a possible transcriptional down-regulation of the SV40E TRE in pituitary GH-3 cells, but they do not rule out such posttranscriptional effects as mRNA processing, transport, or stability (Darnell, 1982). If this effect was due to decreased transcriptional initiation, however, then plausible explanations include the lack of a trans-activator(s) or the presence of a trans-repressor(s).

In the present study we set out to determine whether GH-3 cells contained proteins that could interact with the SV40 enhancer. In vitro gel shift assays demonstrated the presence of factor(s) that could specifically bind SV40 enhancer sequences. Utilizing fragments of the SV40 enhancer, or synthetic double-stranded oligonucleotides, we were able to localize factor(s) binding to an upstream region (between position 205–279) of the viral TRE (Tooze, 1981). Curiously, we also observed little specific DNA binding of GH-3 cell nuclear proteins to an SV40 fragment containing multiple Sp1 binding sites (Dynan and Tjian, 1983). Subsequent studies demonstrated low levels of immuno-reactive Sp1 in GH-3 cell extracts.

Based on the possibility that the binding of GH-3 cell nuclear proteins might represent a repressor protein, a series of 5′ deletion mutants were created to remove these upstream binding sites. Removal of upstream TRE sequences that include negative regulatory elements (NREs) have previously been shown to result in increased levels of gene expression (Goodbourn et al., 1986; Baniahmad et al., 1987; Gaub et al., 1987; Imler et al., 1987; DeSimone and Cortese, 1989; Herbst et al., 1990).

All of these 5′ truncated SV40E TRE deletion mutants were inactive in directing reporter gene expression in GH-3 cells. Finally, we wished to determine whether a fragment from the SV40E TRE, which contains a number of the previously mentioned cis-elements, could enhance gene expression in either HeLa or GH-3 cells when fused to a heterologous promoter. SV40:thymidine kinase (TK) chimeric TREs were all active in directing CAT gene expression in HeLa cells, but were found to yield only slightly elevated levels in GH-3 cells. The relative inactivity of the SV40E TRE in GH-3 cells, therefore, might be due to both the lack (or low level) of a specific trans-activator such as Sp1, and the presence of cis-elements that silence SV40E-directed gene expression within the context of these cells.

Materials and methods

Cell culture

Human cervical carcinoma (HeLa; ATCC CCL2) and rat anterior pituitary (GH-3; ATCC CCL 82.1) cell lines were maintained in culture medium consisting of Dulbecco's Modified Eagle's Media (DMEM) containing 10% Nu-serum (Collaborative Research Inc.), 2 mM L-glutamine, and 50 ug/ml gentamicin (Gibco).

Transient transfection and CAT assays

Transfections were carried out in the presence of DEAE-dextran. Approximately 5×10^5 cells were plated onto 22-mm tissue culture plates. After 24-hour incubation in culture medium,

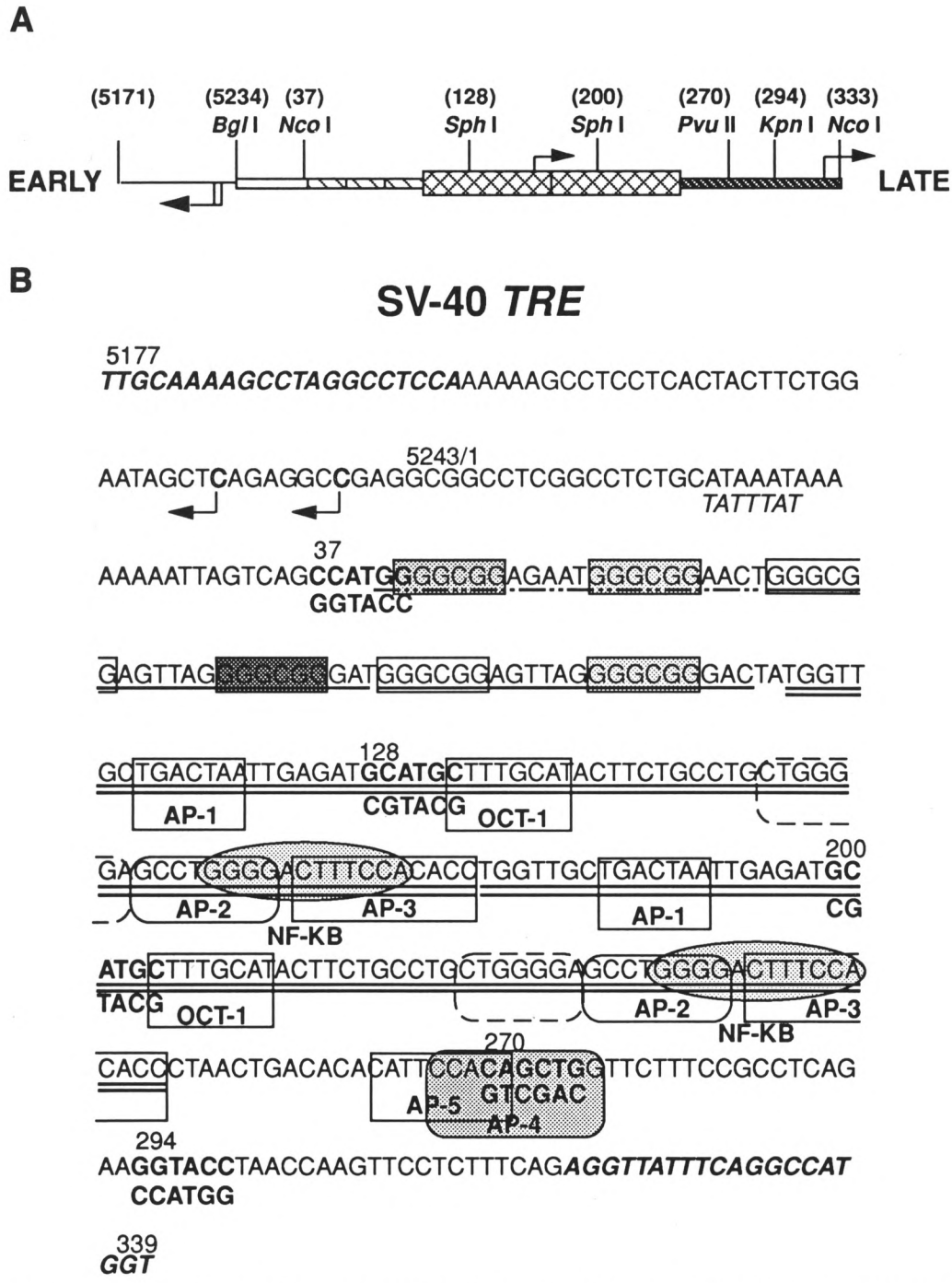

Figure 1. Complexity of the SV40E TRE. **A.** The region between positions 5171 and 333 of the SV40 viral genome is depicted. This region includes the viral origin of replication, as well as TREs for both early (to the left) and late (to the right) viral gene expression. Regions involved in early gene expression include a CAP site (arrows), TATA box (open box), three 21-bp repeats (light hatching), two 72-bp direct repeats (cross hatching) and unique upstream sequences (dark hatching). **B.** SV40 nucleotide sequence cloned into pBLCAT-3 to yield pSVE405 (Coleman et al., 1991b). Sequence in italics at position 5177–5197 and 320–339 correspond to oligonucleotides used in the PCR amplification of the SV40E TRE. Promoter elements include two major early CAP sites (arrows) and a TATA box (ATAAATA). Cis-sites for transcription factors Sp1 (GGCGGG), AP-1 (TGACTAA), OCT-1 (CTTTGCAT), AP-2 (GCCTGGGG), NF-KB (GGGGACTTTCCA), AP-3 (CTTTCCACACC), AP-5 (CATTCCACA) and AP-4 (CCACAGCTGG) are also indicated (reviewed by Jones et al., 1988). Restriction sites used to generate deletion mutants include Nco I (37), Sph I (128 and 200), Pvu II (270), and Kpn I (294).

the cells were rinsed twice with serum-free DMEM. Cells were transfected with 1 μg of plasmid DNA diluted in 0.5 ml of serum-free DMEM containing 0.1 mg of DEAE-dextran. For HeLa cells, the DNA:DEAE-dextran solution was incubated on the cells for 1 hour, after which the solution was removed and the cells shocked for 90 seconds with 1.0 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffered saline (21 mM HEPES pH 7.15; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM Glucose) containing 5% dimethyl sulfoxide (DMSO), as described previously (Lopata et al., 1984). For GH-3 cells, the incubation time was 30 minutes, and the DMSO shock was omitted (Pasleau et al., 1985; Coleman et al., 1991a). After the appropriate incubation, the cells were washed sequentially with phosphate-buffered saline (PBS) and serum-free DMEM, then cultured in 1.0 ml of complete medium.

CAT assays were performed as described (Neumann et al., 1987) with the following changes (Coleman et al., 1991b). Three days after transfection, cells were rinsed with 1.0 ml of PBS/EDTA (PBS containing 1 mM EDTA) and lysed by addition of 100 μl of 100 mM Tris-HCl pH 7.5, followed by 100 μl of the same solution containing 0.2% Trition X-100 (Nachtigal et al., 1989). The cell suspension was transferred to a 1.5 ml Eppendorf tube, centrifuged at 1300 × *g*, and heat-inactivated at 68°C for 10 minutes; insoluble material was removed by centrifugation at 16,000 × *g* for 10 minutes. Cell extract (150 μl) was added to 7 ml scintillation vials, followed by addition of a reaction mix (100 μl) containing 2 μl [^{3}H]-acetyl-CoA (0.5 mCi/ml; 200 mCl/mmol; DuPont-NEN), 50 μl of 5 mM chloramphenicol (in water), 15 μl of 1.0 M Tris-HCl pH 8.0, and 33 μl of water. The reactions were then overlaid with 5 ml of scintillation fluid (Econo-fluor, DuPont-NEN), and radioactivity was determined after 1, 2, and 4 hours incubation at room temperature. The total amount of protein used per assay was determined using a BioRad protein assay, and CAT activity was expressed as counts per minute/per hour of incubation/per μg of cell protein (cpm/hr/μg protein).

Plasmid constructs

DNA manipulations were carried out using standard cloning techniques (Maniatas et al., 1982). The plasmid pBLCAT-3, which contains the *cat* coding sequence fused to the SV40 t-intron and polyadenylation signal, was linearized at a unique Bgl II site located upstream of the gene (Luckow and Schutz, 1987). Treatment with Klenow polymerase and dNTPs yielded a 4344 bp linear fragment that served as vector for all subsequent manipulations. Inclusion of an ~1200 bp DNA fragment containing the human cytomegalovirus immediate-early (CMV) TRE resulted in the plasmid pCMV-CAT (Coleman et al., 1991a). Ligation into this vector of a PCR amplified 405 bp fragment containing the SV40E TRE resulted in the plasmid pSVE405 (Coleman et al., 1991b).

The 5′ deletion mutants of the SV40E TRE were constructed as follows. After polymerase chain reaction (PCR) amplification as previously described, a 405 bp fragment containing the SV40E TRE was isolated by 1% agarose electrophoresis, elution, and subsequent purification on Elu-tip D column (Schleicher & Schuell). This material was digested with Kpn I, Pvu II, Sph I, and Nco I, and the fragments were cloned into the linear pBLCAT-3 vector as described above. The clones obtained are depicted in Figure 6. All inserts are flanked by unique BamH I and Xho I restriction sites at the 5′ and 3′ junctions, respectively.

Chimeric TREs containing an SV40 enhancer fragment fused to a minimal herpes TK promoter (Fig. 7) were constructed by insertion of an Sph I-Sph I fragment from pSVE-338 (Fig. 6) into the unique Sph I site of pBLCAT-2 (Luckow and Schutz, 1987). This fragment contains SV40 enhancer sequences located between positions 200 and 273 of the viral genome and includes the following cis-sites: Oct-1, AP-2, AP-3, NF-KB, and AP-5. Twenty-nine base pairs of flanking sequence from the multiple cloning site of pBLCAT-3 are also present in this fragment. Recombinants containing single and triple inserts in either orientation were identified by restriction enzyme cleavage analyses. All recombinants were verified by nucleotide sequencing.

RNA isolation and analysis

Total cellular RNA was extracted from both HeLa and GH-3 cells using RNAzol™ (Cinna/Biotecx) as suggested by the manufacturer; 5, 10, and 20 μg of each sample was denatured and fractionated by 1.5% Formaldehyde agarose gel electrophoresis as described (Maniatas et al.,

1982). After overnight transfer to Nytran (Schleicher and Schuell), the filters were hybridized to either a 28S rRNA probe (Barbu and Dautry, 1988) or an Sp1 cDNA probe (Kadonaga et al., 1987).

Cell extracts and Western blots

Total cell extract was prepared according to S. P. Jackson, University of California, Berkeley (personal communication). Sub-confluent 100 mm dishes of HeLa and GH-3 cells were rinsed 1× with PBS and harvested in 1 ml PBS/EDTA. The cells were collected by centrifugation at 1300 × *g* for 10 minutes and resuspended in 100 µl PBS/EDTA, after which cell volume was estimated. Cells were pelleted as described above and resuspended with 5 volumes of dH_2O per volume of cell pellet. Finally, the cell suspension was lysed by addition of SDS sample buffer, and the viscosity was reduced by repeated passage through a 22 g hypodermic needle.

Equal volumes of cell extracts were resolved on a 7.5% SDS-PAGE, and protein bands were visualized by Coomasie staining. Equivalent protein profiles were observed from both HeLa and GH-3 cell extracts (data not shown). To determine the relative amounts of Sp1 present in these cells, Western blot analysis using a polyclonal rabbit anti-Sp1 Ab (a gift from S. P. Jackson) were performed as described (Jackson and Tjian, 1988).

Nuclear extracts and heparin agarose fractionation

Nuclear extracts from sub-confluent HeLa and GH-3 cells were prepared as described by Dignam et al. (1983). All steps were performed at 4°C. To enrich the extracts for DNA-binding proteins, nuclear extracts were adsorbed onto Heparin-agarose as described (Briggs et al., 1986). After washing with buffer containing 100 mM KCl, bound proteins were eluted by stepwise salt elutions containing 400 mM and 1.0 M KCl. Proteins eluted with 400 mM KCl (HA-400) were pooled and dialyzed against two changes of Buffer D (20 mM HEPES pH 7.9; 20% v/v glycerol; 100 mM KCl; 0.2 mM EDTA; 0.5 mM PMSF; 0.5 mM DTT). Protein concentrations were determined using a BioRad assay and were found to average between 5–10 µg of protein per µl for both nuclear extracts and HA-400 pooled fractions.

Gel shift assay

Double-stranded DNA fragments obtained by digestion of plasmid DNA with restriction enzymes were radiolabeled with Klenow polymerase and [α^{32}P]dNTP and used for the binding assays (Fried and Crothers, 1981). Also, double-stranded synthetic oligonucleotides with staggered ends were prepared and labeled as described above. DNA fragments, 0.5 µg of poly (dI-dC) as a nonspecific competitor, and 5–20 µg of protein extracts were incubated in a total volume of 20 µl containing 10 mM Tris-HCl (pH 7.5); 1 mM EDTA; 1 mM DTT; 50 mM NaCl; 5% glycerol at room temperature for 60 minutes and separated by 8% polyacrylamide gel electrophoresis in a Tris-borate buffer.

Results

CAT activity in HeLa versus GH-3 cells

HeLa and GH-3 cells were transiently transfected with plasmid DNA in which the CMV or SV40E TREs directed expression of the *cat* reporter gene (pCMV-CAT and pSVE405, respectively). High levels of CAT activity were obtained from HeLa cell extracts after transfection with either plasmid DNA. In GH-3 cells, the CMV TRE is very active, and the SV40E TRE is inactive at directing detectable levels of *cat* gene expression (Fig. 2).

DNA binding by GH-3 nuclear proteins

Nuclear extracts from HeLa and GH-3 cells were assayed for their ability to recognize and bind different fragments of the SV40 enhancer. HeLa cell extracts served as a positive control because all the trans-factors that bind the corresponding cis-sites of the SV40E TRE (described in Fig. 1B) have been shown to be present in these cells (Briggs et al., 1986; Lee et al., 1987; Macchi et al., 1989; Mercurio and Karin, 1989; Mermod et al., 1988; Mitchell et al., 1987, Sturm et al., 1987). To determine whether any SV40 enhancer-specific trans-factors were present in GH-3 cells, a 296 bp Nco I-Nco I fragment (Fig. 1A) containing the three 21 bp repeats, two 72 bp repeats, and unique upstream enhancer sequence was utilized. Significant shifting of this fragment was observed with increasing amounts of either the HeLa or GH-3 nuclear extracts (Fig. 3A, lanes B–D and H–J). Moreover,

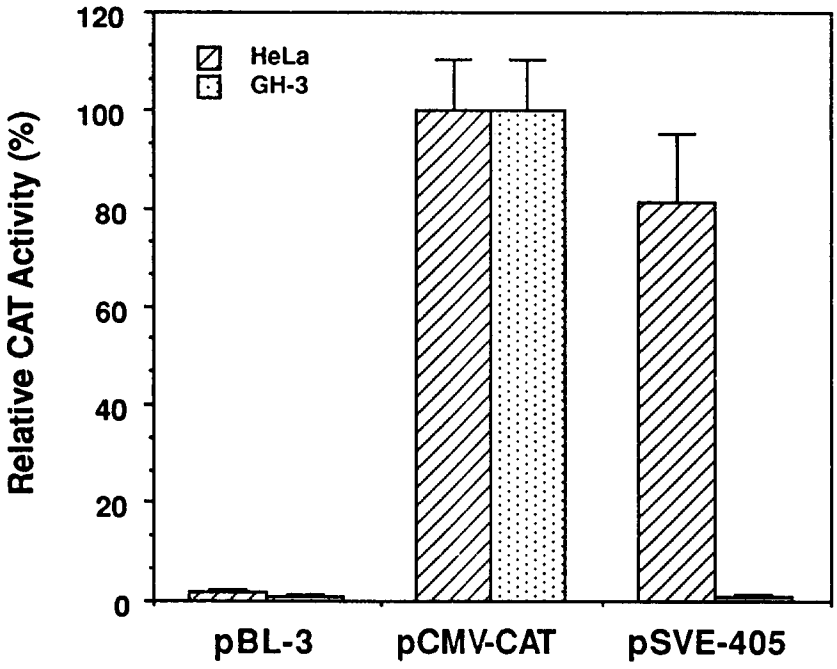

Figure 2. Relative CAT activity obtained after transient transfection of pCMV-CAT and pSVE405 into HeLa and GH-3 cells. Cells were transfected with 1.0 μg of each plasmid DNA as described. The CAT activity is expressed as cpm/hr incubation/μg total protein and is expressed relative to pCMV-CAT which represents 100%. Values represent the mean ± SD of two independent transfections (n = 4).

this binding was specific, since excess cold fragment competed this binding (Fig. 3A, lanes E and F, K and L). It should be noted that a difference in the absolute amount of binding was observed. With 10 μg of HeLa cell nuclear extract, quantitative shifting of the labeled DNA fragment was observed (Fig. 3A, lane C). Furthermore, a 20-fold excess of cold competitor was required to abolish this binding. Ten μg of GH-3 cell nuclear extract could not quantitatively shift this fragment, while a 10-fold molar excess of cold competitor abolished this DNA binding (Fig. 3A, lanes I and K, respectively). Taken together, these data suggest a difference in the quantity of SV40-specific trans-factors present within these two cell lines.

In order to establish the location within the SV40 enhancer at which binding occurred, the above mentioned 296 bp fragment was labeled and subsequently digested with Sph I. Two DNA fragments were obtained: a 91 bp Nco I-Sph I portion which contains the six Sp1 binding sites and an AP-1 site, and a 133 bp Sph I-Nco I fragment which contains the cis-sites for Oct-1, AP-2, AP-3, NF-KB, AP-5, and AP-4. Gel shift analysis using the 91 bp fragment and HeLa nuclear extract revealed significant binding by protein(s) that should represent the ubiquitous factor Sp1 (Fig. 3B, lanes A–D). We did not observe significant binding of the GH-3 cell nuclear extract to this fragment (Fig. 3B, lanes E and F). Three separate nuclear extract preparations demonstrated the same lack of binding (data not shown).

Significant DNA binding to the 133 bp fragment was observed using both HeLa and GH-3 cell nuclear extracts (Fig. 3B, lanes G–J). This fragment contains several binding sites for cellular transcription factors. Significantly, different gel shift patterns were observed with either HeLa or GH-3 nuclear extracts, suggesting that differences existed in the compliment of trans-factors present within these cell lines.

Based on these data and the fact that several previously identified cis-sites are present within this region (Fig. 1B), two double-stranded oligonucleotides were prepared. They spanned regions from position 205–237 (oligonucleotide #1) and 238–279 (oligonucleotide #2) of the SV40E TRE and were used in gel shift analyses (Fig. 4). For oligonucleotide #1, which contains Oct-1 and AP-2 sites, similar profiles of DNA binding were observed with both HeLa and GH-3 cell nuclear extracts or HA-400 fraction (Fig. 4A, lanes A–E and H–L). This binding could be competed by addition of excess cold oligonucleotide #1 (Fig. 4A, lanes F and G, M and N). The DNA-binding pattern to oligonucleotide #2 seemed similar when either HeLa or GH-3 nuclear extracts were utilized (Fig. 4B, lanes A and B, H and I). With the pooled HA-400 fraction, a change in the binding pattern was ob-

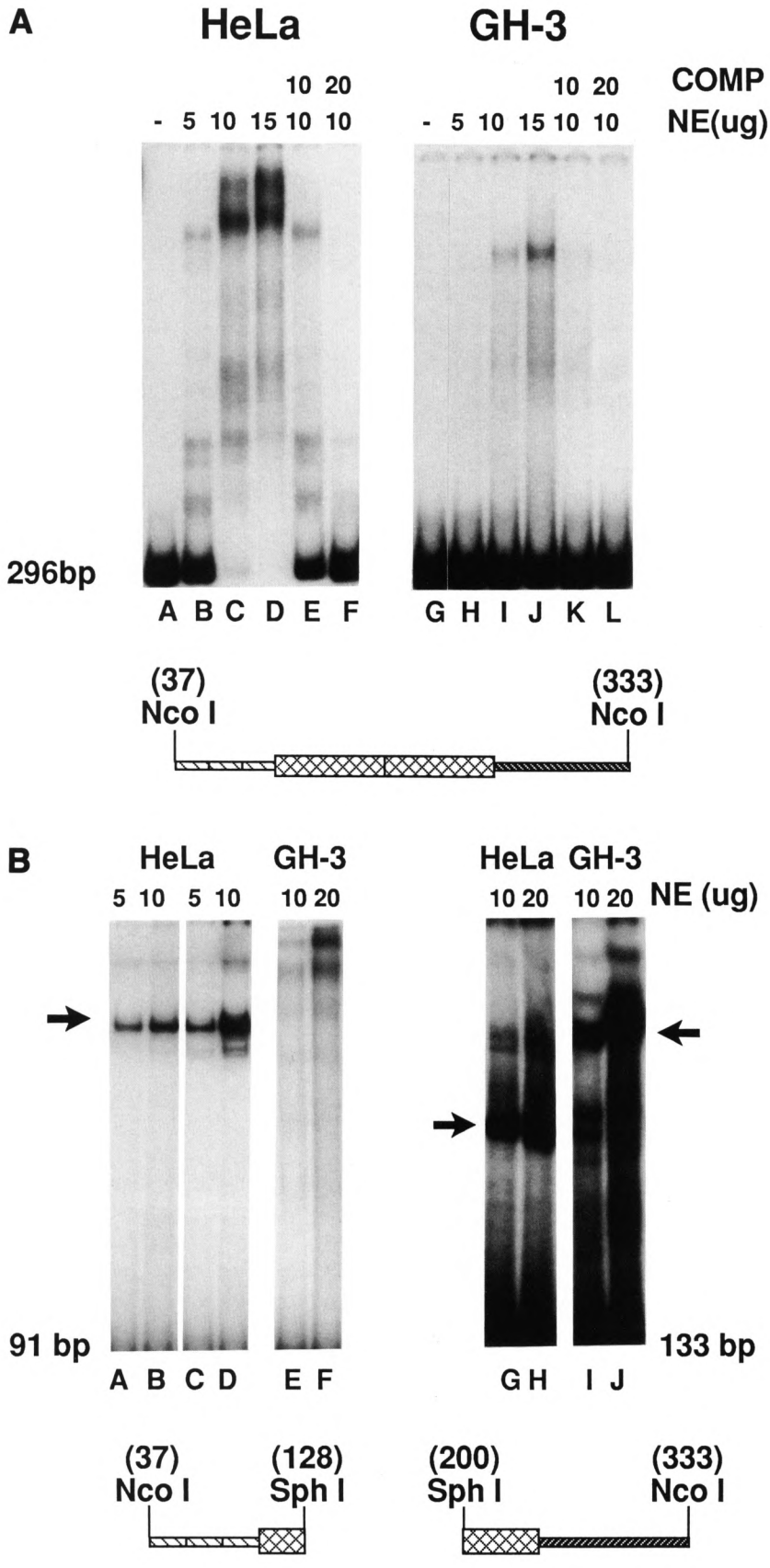

Figure 3. Binding of nuclear factors from HeLa and GH-3 cells to SV40 enhancer DNA fragments. **A.** A 296 bp Nco I-Nco I fragment which contains the three 21-bp repeats (light hatching), two 72-bp direct repeats (cross hatching) and unique upstream sequence (dark hatching) was labeled and used in gel shift assays. Shifted bands were observed with increasing amounts of nuclear extracts (NE) from both HeLa (lanes B–D) and GH-3 (lanes H–J) cells. In the presence of 10 μg of each extract, 10 and 20-fold molar excess of cold competitor (COMP) could specifically compete this binding (lanes E–F and K–L). **B.** The 296 bp Nco I-Nco I fragment was labeled and subsequently digested with Sph I to yield a 91 bp Nco I-Sph I fragment which contains cis-sites for Sp1 and AP-1, and a 133 bp Sph I-Nco I fragment which contains cis-sites for Oct-1, AP-2, NF-KB, AP-3, AP-5 and AP-4. Gel shift analyses were performed using the 91 bp (lanes A–F) or 133 bp (lanes G–J) fragments and HeLa cell NE (lanes A and B, G and H) or a commercially available extract enriched for Sp1 (lanes C–D; Stratagene #203002) or GH-3 cell NE (lanes E and F, I and J). Binding that should represent Sp1 is indicated by the arrow on the left panel. Positions of different complexes formed using the 133 bp fragment and HeLa or GH-3 NE are indicated by arrows on the right panel.

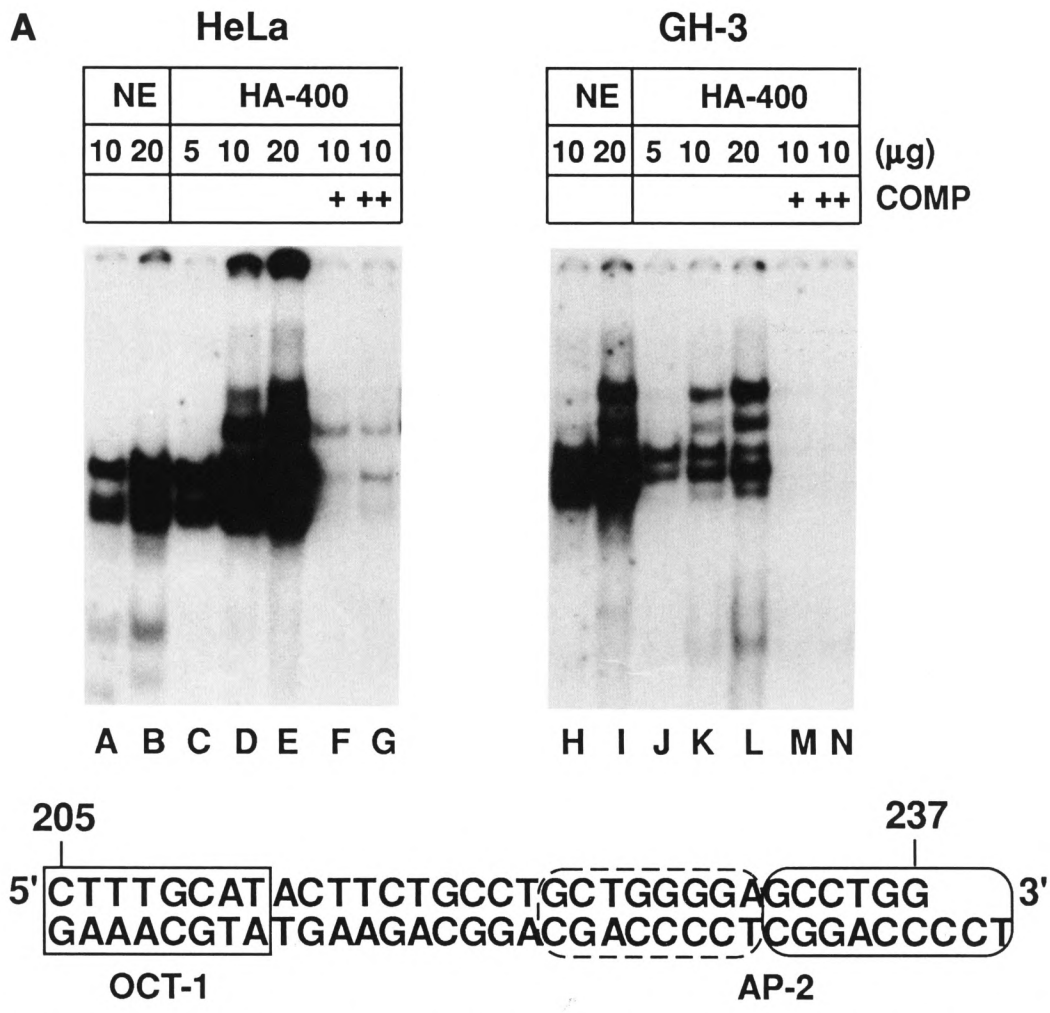

Figure 4. Binding of nuclear factors from HeLa and GH-3 cells to double-stranded oligonucleotides corresponding to SV40 enhancer fragments. **A.** Increasing amounts of nuclear extracts from HeLa (lanes A and B) and GH-3 (lanes H and I) cells or heparin-agarose pooled fractions (HA-400) from HeLa (lanes C–E) and GH-3 (lanes J–L) cells were used with a double-stranded oligonucleotide containing Oct-1 and AP-2 sites. This sequence is identical to that found in SV40 between positions 205–237 (oligonucleotide #1). Specific binding could be competed with 50 (+) and 100-fold (++) molar excess cold oligonucleotide (COMP; lanes F and G, M and N).

served in comparing HeLa and GH-3 cell preparations (Fig. 4B, lanes C–E versus J–L). This DNA binding could be specifically competed with excess cold oligonucleotide #2 (Fig. 4B, lanes F and G, M and N).

Activity of deletion mutants in HeLa and GH-3 cells

Based on the possibility that binding of nuclear proteins from GH-3 cells might serve to repress transcription from the SV40E TRE, a series of 5′ and internal deletion mutants were constructed and assayed for their ability to direct *cat* gene expression after transient transfection into both HeLa and GH-3 cells. The results are shown in Figure 5. Truncation of the SV40E TRE yielded concomitant reduction in SV40E-directed gene expression after transfection into HeLa cells. The profile is similar to previously reported results (Gorman, 1982b; Zenke et al., 1986). In GH-3 cells, all SV40E deletion mutants were unable to direct *cat* gene expression.

Activity of SV40E enhancer:TK promoter chimeric plasmids

Chimeric TREs were constructed by insertion of an SV40 enhancer fragment upstream of a minimal TK promoter (Fig. 6A). This SV40 fragment contains enhancer sequences located be-

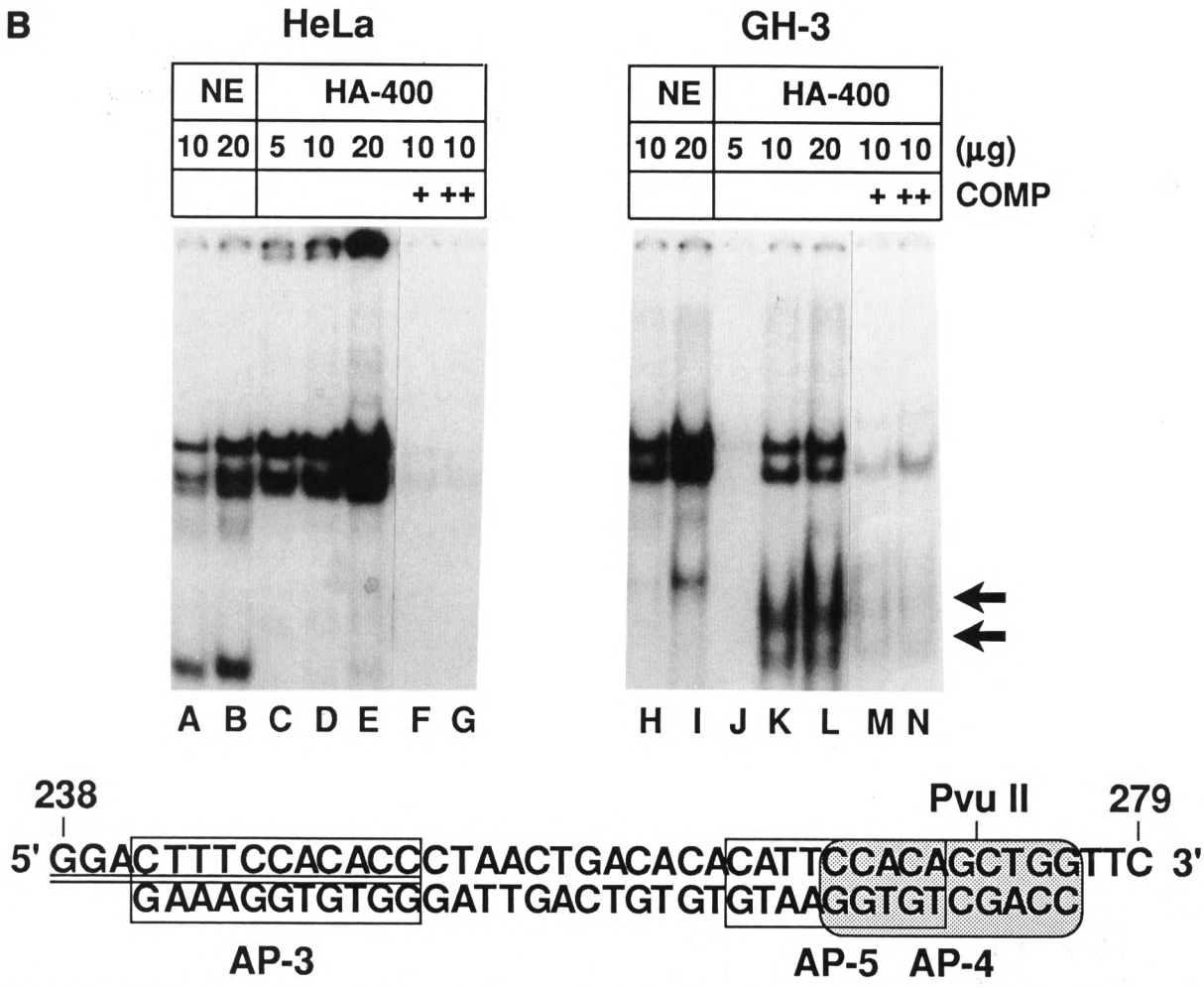

Figure 4 (continued). **B.** Extracts and competition are as described in **A** except that oligonucleotide #2, corresponding to SV40 sequences between 238–279, was used. This oligonucleotide contains binding sites for AP-3, AP-5, and AP-4. A difference in binding pattern between HeLa and GH-3 cells was observed as indicated by the arrow.

tween positions 200 and 273 (Fig. 1B) and has been shown to bind nuclear proteins from both HeLa and GH-3 cells (Fig. 4). Clones were identified that contained single or triple SV40 inserts in either early or late transcriptional orientation. Transfection of these chimeric plasmids into HeLa cells and subsequent activity profiles demonstrated that this fragment could indeed enhance gene expression (25- to 94-fold relative to TK alone). An increase in CAT activity was observed when the SV40 DNA fragment was inserted upstream of the TK promoter in either transcriptional orientation and multiple inserts resulted in higher activity than single copies suggesting additive effects (Fig. 6, hatched bars). These plasmids, when transfected into GH-3 cells had a small stimulatory effect (1.8- to 2.4-fold relative to TK alone) on *cat* gene expression (Fig. 6, stippled bars). Though these values are significantly different from pBLCAT-2-directed (TK) gene expression, they represent a dramatically reduced ability (13- to 40-fold) of this SV40 fragment to enhance gene expression in GH-3 cells. Thus, neither the intact SV40E TRE, nor the chimeric SV40 enhancer:TK promoter clones, could direct high levels of *cat* gene expression in pituitary GH-3 cells.

Analysis of Sp1 levels in GH-3 cells

Because Sp1 has been shown to be required for efficient SV40E-directed gene expression (Dynan and Tjian, 1983; Gidoni et al., 1984 and 1985), and due to the apparent lack of Sp1-like DNA binding activity in GH-3 cell nuclear ex-

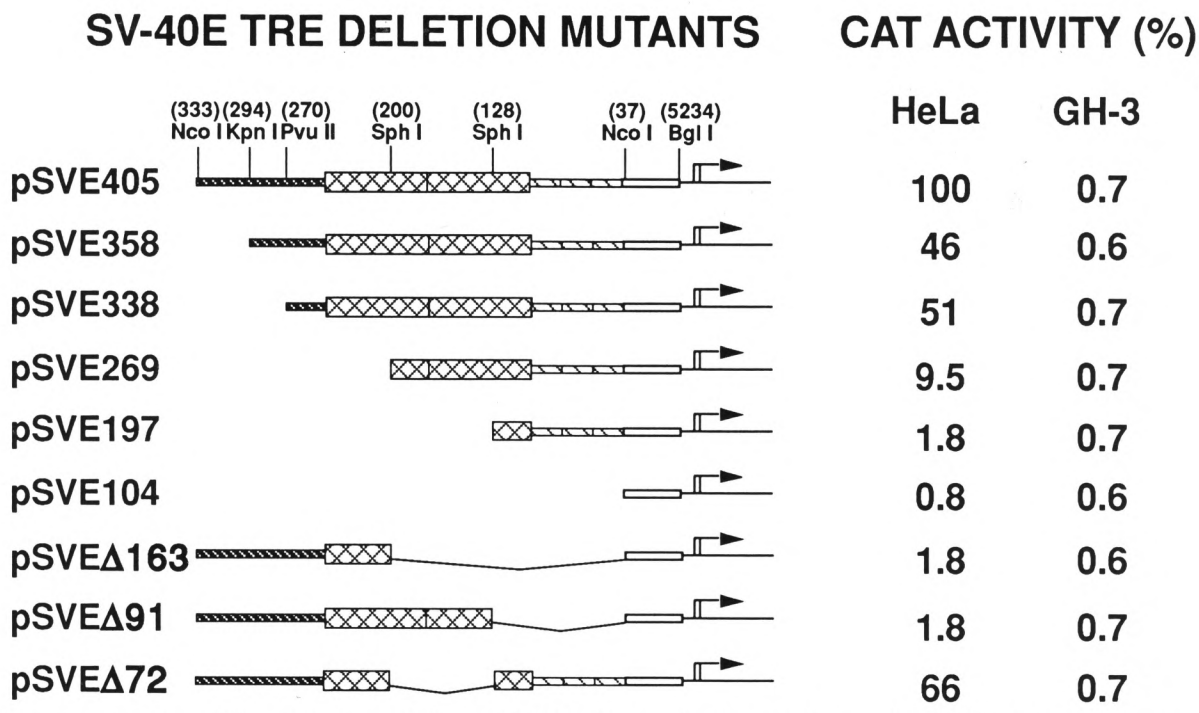

Figure 5. Relative CAT activity from HeLa and GH-3 cells transiently transfected with SV40E deletion mutations. Plasmid designations appear at the left. For ease of presentation, the polarity of the TRE has been switched such that early gene expression is now to the right. Activity from HeLa cells is expressed relative to pSVE405. In GH-3 cells, pCMV-CAT serves as 100%. Conserved elements and restriction sites are as indicated in Figure 1A. The level of CAT activity directed by pSVE104, which includes the TATA and CAP sites, is equal to that of the promoterless plasmid, pBLCAT-3 (data not shown). Values represent the mean values of three separate transfections (n = 6).

tracts (Fig. 3B, lanes E and F), we determined the level of Sp1 in GH-3 versus HeLa cells. Northern blot analysis revealed the presence of equivalent levels of Sp1-specific RNA in both HeLa and GH-3 cells (Fig. 7A). To assess the level of protein, Western blots using an Sp1-specific polyclonal Ab and total cell extracts were performed. Though Sp1-specific RNA was present at approximately the same level in both cell lines, the amount of Sp1 protein was approximately 20-fold lower in GH-3 cells versus HeLa cells (Fig. 7B). While Sp1 is thought to be ubiquitously expressed, recent studies which quantified the levels of Sp1 (mRNA and protein) in different mouse tissues and at different stages of development demonstrated dramatic differences of up to 100-fold in the level of Sp1 present in a variety of cell types (Saffer et al., 1991).

Discussion

The SV40E TRE can direct heterologous gene expression after transient transfection into a wide variety of cultured cell lines (Gorman et al., 1982a; Nomiyama et al., 1987; Ondek et al., 1987; Rosales et al., 1987; Schirm et al., 1987). This prototypic TRE has been shown to possess a promoter that includes a CAP site and TATA box (Benoist and Chambon, 1981; Mathis and Chambon, 1981), as well as a very potent enhancer (Banerji et al., 1981; Gruss et al., 1981; Moreau et al., 1981). The enhancer contains different domains and is subdivided into three imperfect 21-bp repeats, two 72-bp direct repeats and a unique upstream sequence that is required for maximal SV40E-directed gene expression. Housed within these domains are cis-sites, or consensus binding sites, for several cellular trans-factors (Briggs et al., 1986; Lee et al., 1987; Macchi et al., 1989; Mercurio and Karin, 1989; Mermod et al., 1988; Mitchell et al., 1987; Sturm et al., 1987). The activity of the SV40E TRE, therefore, is dependent on the compliment of trans-factors available within a given cell.

Based on earlier results we and others have concluded that the SV40E TRE is incapable of directing detectable levels of heterologous gene expression in transformed rat anterior pituitary

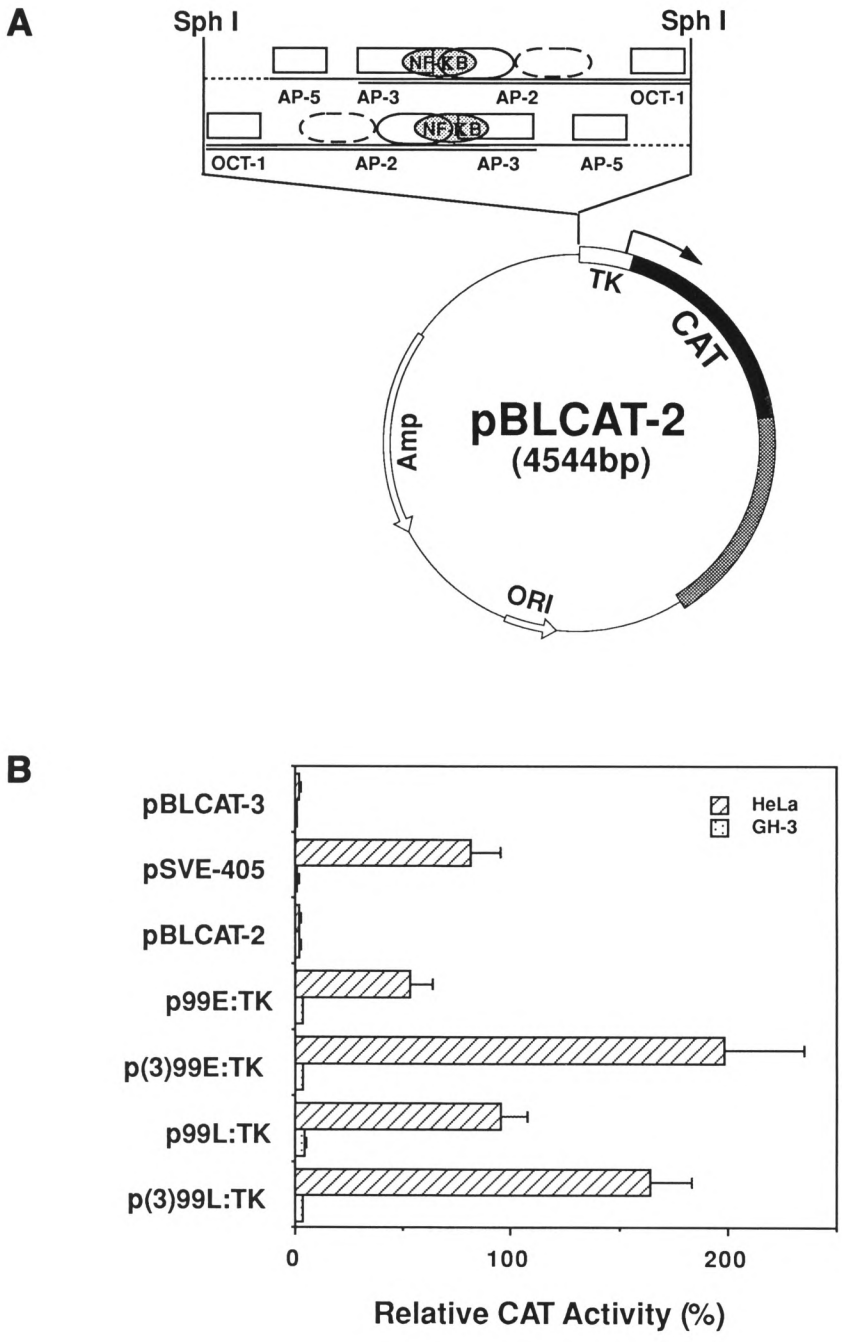

Figure 6. Construction and transfection results with chimeric SV40 enhancer:TK promoter TREs. **A.** A 99 bp Sph I-Sph I fragment from pSVE338 containing cis-sites for Oct-1, AP-2, NF-KB, AP-3, and AP-5, as well as 29 bp of the multiple cloning site from pBLCAT-3 (dotted lines) was ligated into the unique Sph I site in the multiple cloning region of pBLCAT-2. pBLCAT-2 contains a minimal TK promoter (TK) with a CAP site (solid arrow), TATA box, two Sp1 binding sites and a CAAT box. Single and triple inserts in both SV40 transcriptional orientations were identified and used for transfection into HeLa and GH-3 cells. **B.** Relative CAT activity from HeLa (hatched bars) and GH-3 (shaded bars) cells transiently transfected with SV40 enhancer:TK promoter chimeric TREs. Plasmid pCMV-CAT served as a positive control in GH-3 cells (data not shown). p99E:TK contains a single SV40 insert in the early (E) orientation and p(3)99E:TK contains three repeated inserts all in the early orientation. Clones containing single or triple SV40 inserts in the late orientation (L) are also shown. All chimeric TREs were active in directing CAT gene expression in HeLa cells, while little CAT activity was obtained from transfected GH-3 cells using these plasmids. Values represent the mean ± SD from three separate transfections (n = 6).

GH-3 cells (Camper et al., 1985; Schirm et al., 1987; Coleman et al., 1991a). This does not seem to be an artifact of cellular transformation as non-transformed rat pituitary cells are also incapable of supporting SV40E-directed gene expression, nor is it due to an inability of these cells to take up SV40-CAT plasmid DNA (Coleman et al., 1991a). We believe that the inability of the SV40E TRE to direct gene expression must be due, in part, to low or repressed levels of reporter gene transcription.

In an attempt to define the process which results in the lack of activity, we first determined whether nuclear factors from GH-3 cells could recognize and bind the SV40 enhancer. Utilizing a 296 bp fragment containing the intact enhancer, we demonstrated that factors are present within these cells that can interact with the

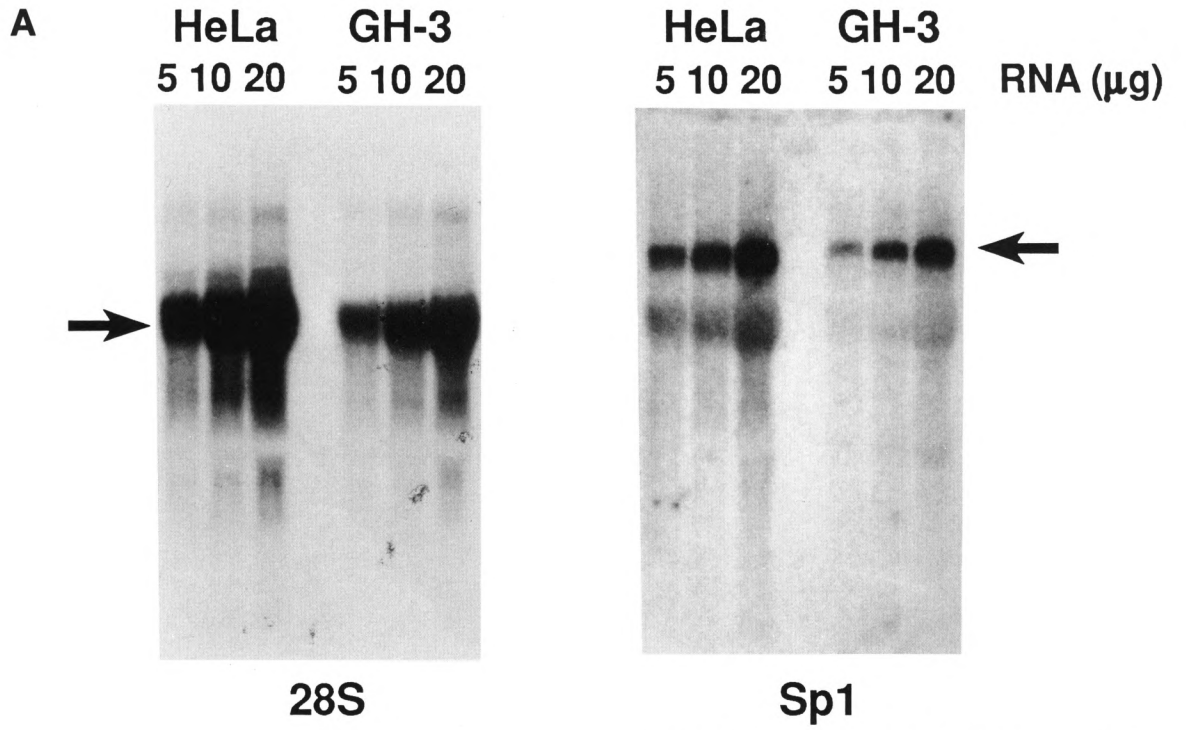

Figure 7. Analysis of Sp1 mRNA and protein in HeLa versus GH-3 cells. **A.** Northern blot analysis was performed and the relative levels of Sp1 mRNA in the two cell lines determined. Equivalent blots with increasing amounts of total RNA were hybridized with either a 28S rRNA-specific oligonucleotide probe to control for the amount of RNA blotted onto the membrane, or an Sp1-specific cDNA probe. Sp1-specific RNA of the correct molecular mass (~8.2 Kb) was found in both HeLa and GH-3 cells at equivalent levels. **B.** Western blot analysis using total cell extracts from HeLa and GH-3 cells and a polyclonal Sp1 antibody. 1, 3, and 9 µl of total cell extracts and 1, 2, and 4 ng of pure Sp1 (Promega) were separated by 7.5% SDS-PAGE and transferred to nitrocellulose. The membrane was exposed to Sp1 Ab followed by goat anti-rabbit alkaline phosphatase Ab. A doublet of 95–105 Kd comigrating with purified Sp1 could be seen in HeLa cell extracts, whereas a significantly reduced signal was observed using the GH-3 cell extract.

SV40 enhancer. Gel shift analyses revealed quantitative differences between HeLa and GH-3 cell nuclear extracts. In particular, less shifting was observed when the GH-3 cell nuclear extracts were used (Fig. 3A).

Using enhancer fragments, differences in binding patterns became more apparent. Curiously, no shifting of a 91 bp fragment which contains several Sp1 binding sites was observed using GH-3 cell nuclear extract (Fig. 3B, lanes E–F), while a prominent shifted band was seen when a control HeLa cell extract was used. Also, different gel mobility shift profiles were observed using the 133 bp Sph I-Nco I fragment (Fig. 3B, lanes G–J), which might be indicative of differences in the array of trans-factors within these two cell lines.

To further localize the cis-sites involved in nuclear extract binding to the 133 bp fragment, synthetic double-stranded oligonucleotides were used. Oligonucleotide #1 contained sequences between 205 and 237 of the viral genome and

included cis-elements for the trans-factors, Oct-1 and AP-2. It is similar in sequence to the coreSPHI oligonucleotide which was shown to demonstrate lymphoid cell specific activity (Schirm et al., 1987). Oligonucleotide #2 contained sequences between 238 and 279 of the viral genome and included cis-elements for AP-3, AP-5 and AP-4. This oligonucleotide is equivalent to the sum of the coreC and corePvuII oligonucleotides described by Schirm et al. (1987). The coreC oligonucleotide was shown to be transcriptionally active in 9 of 10 cell lines tested, being found inactive only in GH-3 cells. Gel shift studies demonstrated the presence of factors within GH-3 cells that could recognize and bind both of these oligonucleotides, yet fail to yield transcriptional activation of the SV40E TRE. In order to determine whether binding of these factors represented binding of a 'repressor' protein, 5′ deletion mutants were constructed which removed these upstream sequences. Though a gradual loss of SV40E-directed *cat* gene expression was observed after transfection into HeLa cells, none of these mutations resulted in detectable levels of SV40E-directed gene expression in GH-3 cells (Fig. 5). Finally, to determine whether the SV40 enhancer could function in these cells when fused to a heterologous promoter, several chimeric plasmids were constructed in which an enhancer fragment from SV40 was ligated upstream of a minimal TK promoter. This fragment contained sequences recognized by GH-3 cell nuclear proteins. The TK promoter alone is not capable of directing *cat* gene expression in GH-3 cells; however, it can be made functional when fused to the pituitary specific prolactin enhancer (Iverson et al., 1990). Again, the chimeric SV40:TK TREs were all capable of directing high levels of *cat* gene expression after transient transfection into HeLa cells. These same chimeric plasmids directed only slightly elevated levels of *cat* gene expression in GH-3 cells.

We have also performed experiments in which other SP-1 cis elements containing TREs have been attached to CAT and assayed for their ability to direct gene expression in GH-3 cells. These TREs include the Herpes Simplex Virus (HSV) TK, HSV-VP16, HSV-IE3, and SV40L elements. All were inactive in GH-3 cells (data not shown).

Another possible explanation for the inactivity of the SV40E TRE in GH-3 cells might be a limiting amount of Sp1. Sp1-specific RNA of the correct molecular mass is present in GH-3 cells as determined by Northern blot analysis (Fig. 7A); however, the amount of protein, as determined either by gel shift with a fragment that contains several Sp1 binding sites (Fig. 3B, lanes E–F) or by Western blot analysis (Fig. 7B, lanes G–I), is dramatically lower than in HeLa cells. The reason for this difference in the ratio of Sp1 RNA:protein between the two cell types, and the overall contribution of this difference to the inactivity of the SV40E TRE in GH-3 cells, remain to be determined.

The SV40E TRE has been used to drive heterologous gene expression in a number of different cell lines (Gorman et al., 1982a; Nomiyama et al., 1987; Ondek et al., 1987; Rosales et al., 1987; Schirm et al., 1987). Few examples exist of cell lines where the SV40E TRE is incapable of directing heterologous gene expression. One such model of transcriptional repression demonstrated that the adenovirus E1A gene product could repress SV40E-directed gene expression in HeLa cells (Borrelli et al., 1984). Subsequent studies in non-differentiated embryonal carcinoma (EC) cells demonstrated that the enhancers from SV40 and Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) were repressed in these cells (Gorman et al., 1985; Sleigh and Lockett, 1985). It has since been shown that undifferentiated EC cells contain an E1A-like activity which disappears upon differentiation (reviewed by Nevins, 1987). Loss of this cellular E1A-like activity results in the ability of SV40 to direct gene expression in differentiated EC cells. Together, several points can be realized from these data: (1) GH-3 cells represent terminally differentiated cells and therefore are not analogous to undifferentiated EC cells. (2) To our knowledge, this cell line was not transformed via adenovirus infection. Additionally, we have previously reported the inability of the SV40E TRE to direct gene expression in non-transformed rat anterior pituitary P3 cells (Coleman et al., 1991a). (3) Finally, we have found the MoMLV LTR to be very active in directing heterologous *cat* gene expression in GH-3 cells (T. Coleman and J. Kopchick, unpublished results). This suggests that E1A, or a cellular E1A-like activity, is not involved in repression of the SV40E TRE in pituitary GH-3 cells.

Thus, the prototypic SV40E TRE must be

viewed as an enhancer which is dependent on the nuclear trans-factors present within a given cell. In rat anterior pituitary (GH-3 and P3) cells, the SV40E TRE is inactive. Our data suggest that this inactivity could be due, in part, to low levels of a trans-activator, i.e., Sp1. This does not exclude the possibility that a cellular factor(s) could repress SV40E-directed gene expression in these pituitary cells. Though significantly more documentation of trans-activators currently exists, the involvement of tissue- or cell-specific trans-repressors in modulating gene expression cannot be overlooked (Levine and Manley, 1989). Ongoing studies in our laboratory are aimed at identifying the exact mechanism of SV40E TRE silencing in GH-3 cells.

Acknowledgments

We thank Drs. W. Bergman, W. Blue, and C. James for reviewing the manuscript and Mr. P. Wiehl for synthesis of oligonucleotides. J. J. Kopchick is supported in part by the State of Ohio's Eminent Scholar Program, which includes a grant from Milton and Lawrence Goll.

The costs of publishing this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC Section 1734 solely to indicate this fact.

T. A. Coleman is currently in the Department of Gene Regulation, Roche Institute of Molecular Biology, Nutley, NJ 07110.

References

J. Banerji, S. Rusconi, and W. Schaffner (1981), Cell 27, 299–308.

A. Baniahmad, M. Muller, C. Steiner, and R. Renkawitz (1987), EMBO J 6, 2297–2303.

V. Barbu and F. Dautry (1989), Nucl Acids Res 17, 7115.

C. Benoist and P. Chambon (1981), Nature 290, 304–310.

E. Borrelli, R. Hen, and P. Chambon (1984), Nature 312, 608–612.

M. R. Briggs, J. T. Kadonaga, S. P. Bell, and R. Tjian (1986), Science 234, 47–52.

S. A. Camper, Y. A. S. Yao, and F. M. Rottman (1985), Journ Biol Chem 260, 12246–12251.

T. A. Coleman, P. Chomczynski, L. A. Frohman, and J. J. Kopchick (1991a), Mol Cell Endocrinol 75, 91–100.

T. A. Coleman, Y. T. Hou, and J. J. Kopchick (1991b), Gene 106, 243–248.

J. E. Darnell (1982), Nature 297, 365–371.

V. DeSimone and R. Cortese (1989), Nucl Acids Res 17, 9407–9415.

J. D. Dignam, R. M. Lebovitz, and R. G. Roeder (1983), Nucl Acids Res 11, 1475–1489.

W. S. Dynan and R. Tjian (1983), Cell 35, 79–87.

W. S. Dynan and R. Tjian (1985), Nature 316, 774–777.

M. Fried and D. M. Crothers (1981), Nucl Acids Res 9, 6505–6525.

C. Fromental, M. Kanno, H. Nomiyama, and P. Chambon (1988), Cell 54, 943–953.

M-P. Gaub, A. Dierich, D. Astinotti, I. Touitou, and P. Chambon (1987), EMBO J 6, 2313–2320.

D. Gidoni, W. S. Dynan, and R. Tjian (1984), Nature 312, 409–413.

D. Gidoni, J. T. Kadonaga, H. Barrera-Saldana, K. Takahashi, P. Chambon, and R. Tjian (1985), Science 230, 511–517.

Y. Gluzman (1985), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

S. Goodburn, H. Burstein, and T. Maniatas (1986), Cell 45, 601–610.

C. M. Gorman, G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard (1982a), Proc Natl Acad Sci USA 79, 6777–6781.

C. M. Gorman, L. F. Moffat, and B. H. Howard (1982b), Mol Cell Biol 2, 1044–1051.

C. M. Gorman, P. W. J. Rigby, and D. P. Lane (1985), Cell 42, 519–526.

P. Gruss, R. Dhar, and G. Khoury (1981), Proc Natl Acad Sci USA 78, 943–947.

L. Guarente (1988), Cell 52, 303–305.

R. S. Herbst, E. M. Boczko, J. E. Darnell, and L. E. Babiss (1990), Mol Cell Biol 10, 3896–3905.

W. Herr and J. Clarke (1986), Cell 45, 461–470.

J-L. Imler, C. Lemaire, C. Wasylyk, and B. Wasylyk (1987), Mol Cell Biol 5, 2558–2567.

R. A. Iverson, K. H. Day, M. d'Emden, R. N. Day, and R. A. Maurer (1990), Mol Endo 4, 1564–1571.

S. P. Jackson and R. Tjian (1988), Cell 55, 125–133.

P. F. Johnson and S. L. McKnight (1989), Annu Rev Biochem 58, 799–839.

N. C. Jones, P. W. J. Rigby, and E. B. Ziff (1988), Genes Devel 2, 267–281.

Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian (1987), Cell 51, 1079–1090.

G. Khoury and P. Gruss (1983), Cell 33, 313–314.

W. L. Lee, P. Mitchell, and R. Tjian (1987), Cell 49, 741–752.

M. Levine and J. L. Manley (1989), Cell 59, 405–408.

M. A. Lopata, D. W. Cleveland, and B. Sollner-Webb (1984), Nucl Acids Res 12, 5207–5717.

B. Luckow and G. Shutz (1987), Nucl Acids Res 15, 5490.

M. Macchi, J-M. Bornert, I. Davidson, M. Kanno, R. Rosales, M. Vigneron, J-H. Xiao, C. Fromental, and P. Chambon (1989), EMBO J 8, 4215–4227.

T. Maniatas, E. F. Fritsch, and J. Sambrook (1982), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

T. Maniatas, S. Goodburn, and J. A. Fischer (1987), Science 236, 1237–1245.

D. J. Mathis and P. Chambon (1981), Nature 290, 310–315.
F. Mercurio and M. Karin (1989), EMBO J 8, 1455–1460.
N. Mermod, T. J. Williams, and R. Tjian (1988), Nature 332, 557–561.
P. J. Mitchell, C. Wang, and R. Tjian (1987), Cell 50, 847–861.
P. J. Mitchell and R. Tjian (1989), Science 245, 371–378.
P. Moreau, R. Hen, B. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon (1981), Nucl Acids Res 9, 6047–6088.
M. W. Nachtigal, B. E. Nickel, M. E. Klassen, W. Zhang, N. L. Eberhardt, and P. A. Cattini (1989), Nucl Acids Res 17, 4327–4337.
J. R. Neumann, C. A. Morency, and K. O. Russian (1987), Biotech 5, 444–447.
J. R. Nevins (1987), Microbiol Rev 51, 419–430.
H. Nomiyama, C. Fromental, J. H. Xiao, and P. Chambon (1987), Proc Natl Acad Sci USA 84, 7881–7885.
B. Ondek, A. Shepard, and W. Herr (1987), EMBO J 6, 1017–1025.
B. Ondek, L. Gloss, and W. Herr (1988), Nature 333, 40–45.
F. Pasleau, M. J. Tocci, F. Leung, and J. J. Kopchick (1985), Gene 38, 227–232.
M. Ptashne (1988), Nature 335, 683–689.
R. Rosales, M. Vigneron, M. Macchi, I. Davidson, J. H. Xiao, and P. Chambon (1987), EMBO J 6, 3015–3025.
J. D. Saffer, S. P. Jackson, and M. B. Annarella (1991), Mol Cell Biol 11, 2189–2199.
S. Schirm, J. Jiricny, and W. Schaffner (1987), Genes Devel 1, 65–74.
E. Serfling, M. Jasin, and W. Schaffner (1985), Trends Genet 1, 224–230.
M. J. Sleigh and T. J. Lockett (1985), EMBO J 4, 3831–3837.
R. A. Sturm, T. Baumruker, B. R. Franza, and W. Herr (1987), Genes Dev 1, 1147–1160.
J. Tooze (1981), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
M. Zenke, T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon (1986), EMBO J 5, 387–397.