MOLECULAR AND CELLULAR BIOLOGY, Feb. 1994, p. 934–943
0270-7306/94/$04.00+0

Vol. 14, No. 2

Multiple Silencer Elements Are Involved in Regulating the Chicken Vimentin Gene

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Received 12 July 1993/Returned for modification 2 September 1993/Accepted 22 October 1993

Vimentin, a member of the intermediate filament protein family, exhibits tissue- as well as development-specific expression. Transcription factors that are involved in expression of the chicken vimentin gene have been described and include a *cis*-acting silencer element (SE3) that is involved in the down-regulation of this gene (F. X. Farrell, C. M. Sax, and Z. E. Zehner, Mol. Cell. Biol. 10:2349–2358, 1990). In this study, we report the identification of two additional silencer elements (SE1 and SE2). We show by transfection analysis that all three silencer elements are functionally active and that optimal silencing occurs when multiple (at least two) silencer elements are present. In addition, the previously identified SE3 can be divided into three subregions, each of which is moderately active alone. By gel mobility shift assays, all three silencer elements plus SE3 subregions bind a protein which by Southwestern (DNA-protein) blot analysis is identical in molecular mass (approximately 95 kDa). DNase I footprinting experiments indicate that this protein binds to purine-rich sites. Therefore, multiple elements appear to be involved in the negative regulation of the chicken vimentin gene, which may be important in the regulation of other genes as well.

Vimentin is a member of the family of intermediate filament proteins (IFPs). These structural proteins are so named because they are intermediate in size (11 nm) to the other principal cytoskeletal structural proteins: the microfilaments (5 nm; actin) and the microtubules (25 nm; tubulin). Proteins that make up the IFP family can be divided into several distinct types: acidic and neutral-basic keratins (types I and II, respectively), desmin, vimentin, glial fibrillary acidic protein (GFAP), peripherin (type III), neurofilaments (type IV), lamins (type V), and nestins (type VI) (29, 49).

Although a specific function has yet to be determined, IFPs are thought to play a major role in the composition of the cellular infrastructure. For example, immunofluorescence studies with vimentin antibodies show a complex network of scaffolding surrounding the nucleus and extending to the cell periphery (6). In addition, it appears that vimentin actually contacts both the nuclear surface (at its carboxy terminus) as well as the plasma membrane (at its amino terminus), suggesting that vimentin is important not only in cytoplasmic organization but also in cellular organization, communication, and perhaps information transport into and out of the nucleus as well (48, 49). However, unequivocal evidence has still not been demonstrated (6).

In general, IFPs are expressed in a cell-specific manner and as such are useful determinants for the embryonic origins of cells (48, 49). For example, desmin is found in muscle cells, the keratins (acidic and basic) are found in epithelial cells, GFAPs are found in astrocytes and glial cells, nestins are found in neuroepithelial stem cells, and vimentin is found in cells of mesenchymal origin. Vimentin is unique among the IFPs in two additional ways: it is found in almost all cells grown in culture regardless of origin, and whenever two IFPs are coexpressed in the same cell, one of these is always vimentin (27).

During development, the IFP genes are differentially expressed. For example, switches from vimentin to GFAP in glial cells and from vimentin to desmin in muscle cells have been observed (27). This differential expression of IFPs in specific lineages, as well as during development, suggests that a dynamic control mechanism must be at play.

Current models of IFP gene regulation, as well as that of eucaryotic genes in general, invoke numerous *cis*-acting elements interacting with *trans*-acting factors to produce variable expression patterns. To date, elements that enhance transcriptional activity have proven more amenable to analysis, and several have been isolated and characterized (4, 22, 28, 39). For example, induction of vimentin expression has been linked to serum, platelet-derived growth factor, and phorbol esters (23, 38, 39), in addition to more general transcription factors such as SP-1 (8). In the chicken vimentin gene, differential vimentin expression has been shown to involve several *cis* elements and *trans* factors (43–45, 50, 53, 54). However, one of the more interesting aspects of vimentin gene regulation is that it is also down-regulated during development. The primary locale of this negative regulation was determined to be a 40-bp silencer element (SE3) located at −607 to −567 from the start site of transcription (14, 44). This element was shown to bind a factor of 95 kDa (14), the silencer element protein (SEP). Upon further analysis, SE3 was observed to contain some sequence similarity to the octamer-binding sequence (4) which has been observed in a negative element in the human c-*myc* gene (51). However, the data suggest that SEP is a different factor.

One important conclusion from this study was that while SE3 could act in a position- and orientation-independent manner, a single copy yielded only a partial reduction in transcription within the native context of the gene and 36% compared with that of the heterologous herpes simplex virus (HSV) thymidine kinase (tk) promoter (ptkCAT) (14). In fact, multiple SE copies (more than two) were required to completely silence the ptkCAT gene. Preliminary data sug-

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gested that there were probably additional SEs within the vimentin gene as well (14). Here, we show by transfection analyses that there are at least three SEs active within the chicken vimentin gene. Moreover, all three elements appear to bind the same silencer protein (95 kDa) as measured by gel mobility shift assays (GMSAs), UV cross-linking experiments, and Southwestern (DNA-protein) blots. Transfection data suggest that for complete repression of transcription, multiple (at least two) SE copies are required.

The presence of multiple negative elements is not unique to the chicken vimentin gene. Since our initial report, multiple negative elements have also been found in the chicken lysozyme (2, 3), rat glutathione *P*-transferase (19), human ε-globin (9), interleukin-4 (30), collagen II (42), and hamster (52) and human (41) vimentin genes. Possible mechanistic models are presented to account for the need for multiple SEs.

MATERIALS AND METHODS

Plasmids. Standard molecular biology techniques (1) were used to construct vimentin-chloramphenicol acetyltransferase (CAT) reporter gene plasmids. p8CAT, a pEMBL derivative (10) from which the F1 origin of replication has been removed and the bacterial CAT gene inserted, was used as a reporter gene (43). Various vimentin 5′-flanking sequences were placed in front of the CAT gene (pcV-160, pcV-320, pcV-567, and pcV-607) as described previously (14). Plasmids pcV-302 and pcV-283 were constructed by BAL 31 digestion of pcV-320 followed by filling in with Klenow and ligation. Three fragments, SE3 (−607 to −567), AGGAGCGCTGTGCCCGAAGCAAAGCGATGCCCCT CCTGCAG; SE2 (−486 to −462), GAGCGCGCTGAGCCC ATGAGCACAG; and SE1 (−253 to −236), AGGAGCGCT GGCGGAGCA, plus complementary strands were synthesized and subsequently cloned in front of pcV-160 and pcV-320 in unique sites left over from cloning the −160 and −320 fragments in the multicloning site (MCS) of pUC18. SE1 was cloned into a *Kpn*I site, SE2 was cloned into an *Xba*I site, and SE3 was cloned into a *Bam*HI site. Additionally, SE3 subfragments were synthesized and cloned 5′ to the pCV-160 and pCV-320 vectors as follows: SE3/A (−607 to −591) was cloned into a *Xba*I site, SE3/A+B (−607 to −582) was cloned into a *Bam*HI site, SE3/B (−590 to −582) was cloned into a *Sal*I site, and SE3/C (−581 to −568) was cloned into a *Kpn*I site. The human 19-bp negative element was synthesized as 5′-TCGACTGGCGTGGTGCCACCGG ACG-3′ plus the complementary strand and cloned into the *Sal*I site of pUC18 (41).

The plasmid ptkCAT was also used. This plasmid consists of the HSV tk promoter in front of the CAT gene (35). SE3, SE3/A, SE3/A+B, SE3/B, and SE3/C were fused upstream of the ptkCAT promoter at a unique *Bam*HI site.

Cell culture, DNA transfections, and CAT assays. Mouse L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 10% fetal calf serum (FCS), and 0.1 mg of gentamicin per ml. Twenty-four hours prior to transfection, the cells were seeded at 7×10^5 cells per 100-mm tissue culture dish in DMEM plus 10% FCS. The cells were transfected with 15 μg of plasmid DNA by the calcium phosphate coprecipitation technique. The media were removed from the dishes, and the DNA-calcium phosphate precipitate was gently added. The DNA was allowed to sit on the cells at 25°C with gentle agitation every 5 min. After 20 min, 6 ml of medium (DMEM plus 10% FCS) was carefully added, and the cells were incubated for 12 h, after which the medium was removed and 8 ml of fresh medium was added. The cells were harvested 24 h later, washed in phosphate-buffered saline, pelleted, and resuspended in 100 μl of 0.25 M Tris (pH 7.5). Protein was extracted by repeated freeze-thaw in 250 mM Tris (pH 7.6). The protein suspension was clarified by centrifugation at 15,000 × *g* for 5 min at 4°C, and the supernatant was collected. Protein concentrations were determined by the method of Lowry (31), and the protein was stored at −20°C. CAT activity was determined as previously described (17). Experimental results were quantitated by excising the radioactive spots from silica plates and determining their ^{14}C content by liquid scintillation counting. Each set of DNA transfections included p8CAT as a control. CAT activity was reported as the percent activity versus pcV-160, pcV-320, or ptkCAT activity, depending on the experiment. All values reported are the average of usually six to eight separate transfections. Error bars were calculated by using the standard error of the mean (SEM).

Protein extraction. HeLa cells were grown as 3-liter suspension cultures in spinner flasks to a density of 5×10^5 cells per ml. Crude nuclear extract was obtained as previously described (1, 13). Further enrichment was obtained by ammonium sulfate precipitation to 50% saturation and then by dialysis in Dignam buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT] [both PMSF and DTT were added fresh]). Fractions were collected and stored at −70°C for up to 6 months. Further fractionation for GMSAs was performed on some preparations by dialysis in buffer D minus KCl, followed by separation on a 10-ml phosphocellulose column (P11 cellulose phosphate; Whatman Inc.) equilibrated in buffer D minus KCl. The column was washed with 3 column volumes of buffer D minus KCl. Protein was eluted off of the column with 3 column volumes of a step gradient consisting of buffer D with 0.1, 0.2, 0.3, or 1.0 M KCl. The eluted fractions were adjusted to 0.1 M KCl, aliquoted, and stored at −70°C for up to 6 months. For DNA footprinting experiments, crude, nuclear extracts were size fractionated on Sephacryl S300 gel filtration columns in TM buffer (50 mM Tris-HCl [pH 7.9], 0.1 M KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 10% [vol/vol] glycerol, 1 mM DTT, 0.1 mM PMSF, 1 mM sodium metabisulfite, 1 mM benzamidine-HCl [all added fresh prior to use]) (8). Fractions containing SEP binding activity were determined by DNA footprinting or GMSAs and pooled accordingly.

GMSAs. Radiolabeled fragments corresponding to SE1, SE2, or SE3 were analyzed by GMSAs as described previously (14). ^{32}P-labeled fragments (1 to 2 ng) were incubated with 8 to 10 μg of partially purified protein as described above and analyzed on a 4% nondenaturing gel.

Footprint analysis. The SEs cloned into the MCS of pUC18 were digested with *Eco*RI for SE1 and SE3 or *Hin*dIII for SE2, labeled with [α-^{32}P]dATP and Klenow fragment, extracted with phenol-chloroform, and concentrated by ethanol precipitation. A second digest was then performed with either *Hin*dIII for SE1 and SE3 or *Eco*RI for SE2, and end-labeled fragments were separated on a gel, excised, eluted, and reprecipitated with ethanol. For each reaction mixture, the ^{32}P-labeled DNA (15 to 25 fmol) was combined with 10 μl of 10% (wt/vol) polyvinyl alcohol, 1 μl of 1-mg/ml calf thymus DNA, and water to a 25-μl final volume and then was thoroughly vortexed. This mixture is referred to as the DNA mix. Increasing amounts of partially purified protein

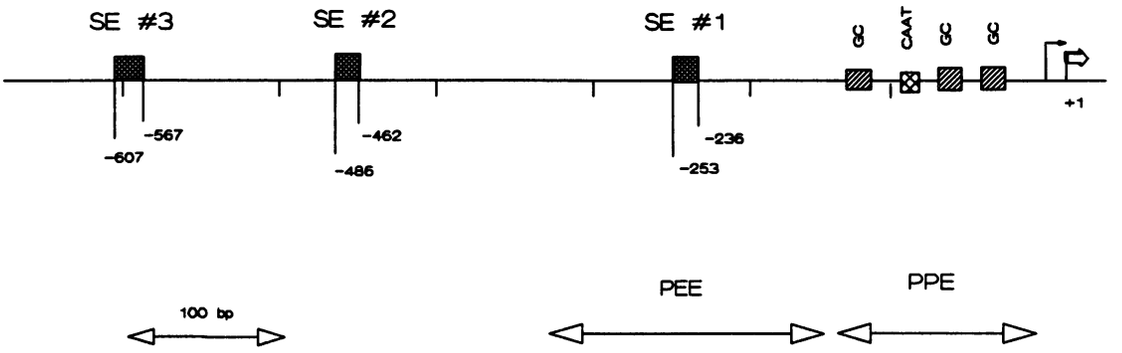


FIG. 1. Relative locations of the chicken vimentin SEs. The location and composition of the PPE (−160 to +1) and PEE (−320 to −160) are shown. The two transcriptional start sites are noted by arrows, with the larger arrow depicting the major start site (43). Positions of the three SEs are determined relative to this site.

(S300 pool described above) were mixed on ice with buffer Z (25 mM HEPES [pH 7.6], 12.5 mM $MgCl_2$, 1 mM DTT [freshly added], 10 μM $ZnSO_4$, 10% [vol/vol] glycerol, 0.1% [vol/vol] Nonidet P-40), to yield a final volume of 25 μl. The KCl concentration was adjusted to 0.1 M KCl, as necessary. Twenty-five microliters of the DNA mix was added to increasing amounts of protein-buffer Z solution to yield a final volume of 50 μl. The DNA-protein mixture was mixed by flicking the tubes, incubated on ice for 15 min, and then incubated at 25°C for 1 min. Fifty microliters of a 5 mM $CaCl_2$-10 mM $MgCl_2$ solution was then added. After 1 min, the reaction was initiated by the addition of 2 μl of DNase I (2.5 mg/ml; Sigma), freshly diluted to 1:800, and the reaction was stopped after 1 min by the addition of 90 μl of stop solution (20 mM EDTA [pH 8.0], 1% [wt/vol] sodium dodecyl sulfate [SDS], 0.2 M NaCl, 250 μg of glycogen per ml). Hydroxyradical footprints with 1,10-phenanthroline–copper ion were performed as previously described (26). Following incubation of either footprinting reaction mixture, the DNA was treated with phenol-chloroform, ethanol precipitated, and analyzed on a 8% polyacrylamide–8 M urea sequencing gel. The dried gel was placed on XAR film with an enhancer screen for visualization.

Southwestern blot. Southwestern blots were performed as previously described (14, 43, 50). Briefly, 100 μg of crude HeLa nuclear extract was separated on a denaturing SDS-polyacrylamide gel (6.5% stacking and 10% separating). Following a 30-min soak, the gel was electroblotted onto nitrocellulose for 3 h at 0.7 A (constant current) in gel transfer buffer. The membrane was blocked for 1 h with a solution of 5% Carnation nonfat dry milk in TNED (50 mM Tris [pH 7.6], 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT), washed for 2 to 5 min in TNED, and probed with ^{32}P-labeled DNA (of equal radiospecific activity, 0.65×10^6 to 2×10^6 cpm/ml) in 25 ml of TNED for 1 h with gentle agitation. Following hybridization, the membrane was washed with 50 ml of TNED two to three times (10 min each) with gentle agitation, air dried, and exposed to XAR film for 18 to 24 h with an intensifying screen.

UV cross-linking experiments. Radiolabeled fragments corresponding to SE1 and the human 19-bp negative element (41) were analyzed by GMSAs as described previously (14). Each strand of the 19-bp element or SE1 was ^{32}P labeled with [γ-^{32}P]ATP and polynucleotide kinase to an equal radiospecific activity, annealed, and gel purified. SE1 and Δ19 were incubated with 40 μg of crude HeLa nuclear extract, and protein-DNA complexes were separated by 4% nondenaturing polyacrylamide gel electrophoresis. The wet gel was wrapped in Saran Wrap, exposed to UV light at 254 nm from a UVGL-25 UV lamp (UVP, Inc.) from a distance of 4 cm for 10 min and placed on XAR film for 2 h at 4°C. The retarded protein-DNA complexes were cut out, soaked in SDS-sample buffer for 10 min at 25°C, and then placed in the bottom of the wells of a denaturing SDS-polyacrylamide gel (3.9% stacking and 8% separating). The gel was electrophoresed for 1.5 h at 175 V, dried, and then placed on XAR film with an enhancer screen and exposed for 36 h at −70°C.

RESULTS

Previously, we have shown that expression of the chicken vimentin gene is down-regulated in part because of the presence of a 40-bp *cis*-acting negative element (SE3) located at −607 to −567 from the transcriptional start site (14). Moreover, it was noted that a single copy of this element fused to either the vimentin or the HSV tk promoter was unable to completely inhibit transcription. Multiple (more than two) copies were required to repress ptkCAT transcription by 90%. At the time, it was pointed out that there might be another SE within vimentin's 5′ end (−566 to −321), because expression of the pcV-567 construct was considerably less than that of pcV-320.

Transient transfections of SE constructs. Computer-assisted analysis (12) of vimentin's 5′-flanking region revealed two additional negative-element homologies, SE1 (−236 to −253) and SE2 (−486 to −462), compared with the original SE3 (Fig. 1 [see Fig. 8]). Interestingly, SE1 was located within a larger region known to contain enhancer activity (43, 44). In order to determine whether these two additional negative elements were functionally relevant, various vimentin 5′-flanking regions containing these regions were fused to the CAT gene (p8CAT) and transfected into mouse L929 cells, which are known to express vimentin (14, 43, 50) (Fig. 2). A negative element between −283 and −160 which repressed transcriptional activity from vimentin's proximal promoter element (PPE; pcV-160) by 90% was discovered. As more 5′-flanking sequence was added, this decrease in transcription was overcome in pcV-320 (proximal enhancer element [PEE] plus PPE), which essentially restored full transcriptional activity. Extending the 5′-flanking region out to −567 nucleotides revealed yet another negative element, resulting in an 87% decrease in transcriptional activity from that of the pcV-160 construct. Continuing to −607 nucleotides further reduced gene expression, yielding a total repression of 98% over that exhibited by the promoter alone (pcV-160). These results confirmed our suspicion that there

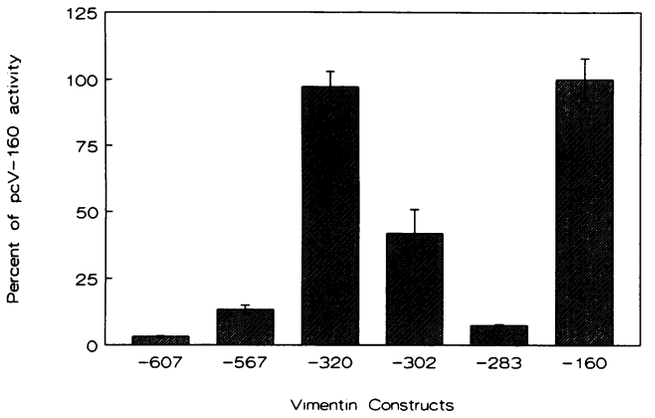


FIG. 2. Transient transfections of various chicken 5'-flanking chimeric constructs in mouse L929 cells. Each construct is identified by the length of 5'-flanking DNA that it contains from the transcription start site at +1 to −160, for example, pcV-160. CAT activity is expressed as a percentage relative to that of the PPE pcV-160, which is set as 100% activity. Results are an average from six to eight separate transfections, and error bars represent the SEM.

were indeed two additional negative elements involved in the regulation of the chicken vimentin gene. Moreover, the aforementioned computer-generated homologies (SE1 and SE2) were located within these functionally defined silencer regions.

To confirm that these decreases in transcription were due only to the three SEs described in Fig. 1 and 2, these elements were individually synthesized and fused to pcV-160 and pcV-320 as described in Materials and Methods. Each construct was transiently transfected into mouse L929 cells (Fig. 3). All three SEs markedly inhibited transcription to various degrees. Both SE3 and SE2 decrease transcriptional activity by approximately 50%. When two tandem copies of SE3 were present [$(SE3)^2$], an additional 25% decrease was noted, for a total repression of approximately 75%. This was similar to earlier observations with ptkCAT (14), for which we noted additive silencing as the SE3 copy number was increased from one to four, i.e., one copy yielded a 36% decrease in transcriptional activity, two copies yielded 68%, and four copies yielded 90%. However, SE1 fused to pcV-160 yielded even stronger repression, a 75% reduction in transcription, similar to that seen when two copies of SE3 were present.

When either SE2 or SE3 was cloned in front of pcV-320 (the PEE plus PPE), complete silencing (98%) was observed (Fig. 3, inset). It should be noted that these constructs already contain SE1 in its proper context within the 5'-flanking region of the vimentin gene. This in effect creates a construct with two copies of the negative, *cis*-acting elements contributing to the overall level of transcription.

Transient transfections of SE3 subregion constructs. When we originally examined SE3, we noted that this element could be conveniently divided into three subregions: region A, 5'-CCAGGAGCGCTGTGCCCG-3' (−607 to −591); region B, 5'-AAGCAAAGC-3' (−590 to −582); and region C, 5'-GATGCCCCTCCTGCAG-3' (−581 to −568) (see Fig. 8). These subdivisions were chosen because region B exhibits partial similarity (six of eight match) to the octamer-binding site ATGCAAAT (4). Subsequently, we showed that the protein (OBP100) that normally binds to this sequence does not bind to SE3 (14). Nevertheless, it gave us a useful means to further delineate SE3. Regions A+B, A, B, and C were subsequently synthesized, fused to pcV-320, and transfected into mouse L929 cells (Fig. 4). All constructs showed a

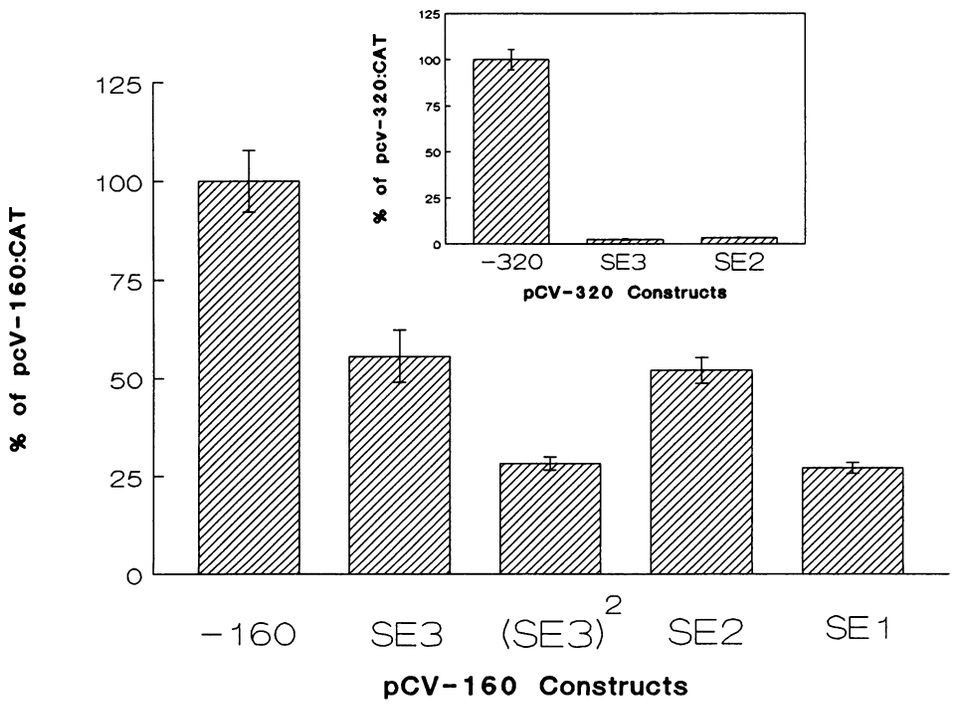


FIG. 3. In vivo effects of each SE on vimentin's promoter, pcV-160 (PPE), or PEE plus PPE, pcV-320 (inset). CAT activity is expressed as a percentage for each construct relative to that of pcV-160 or pcV-320 (inset). Results are an average of six to eight separate transfections, and error bars represent the SEM.

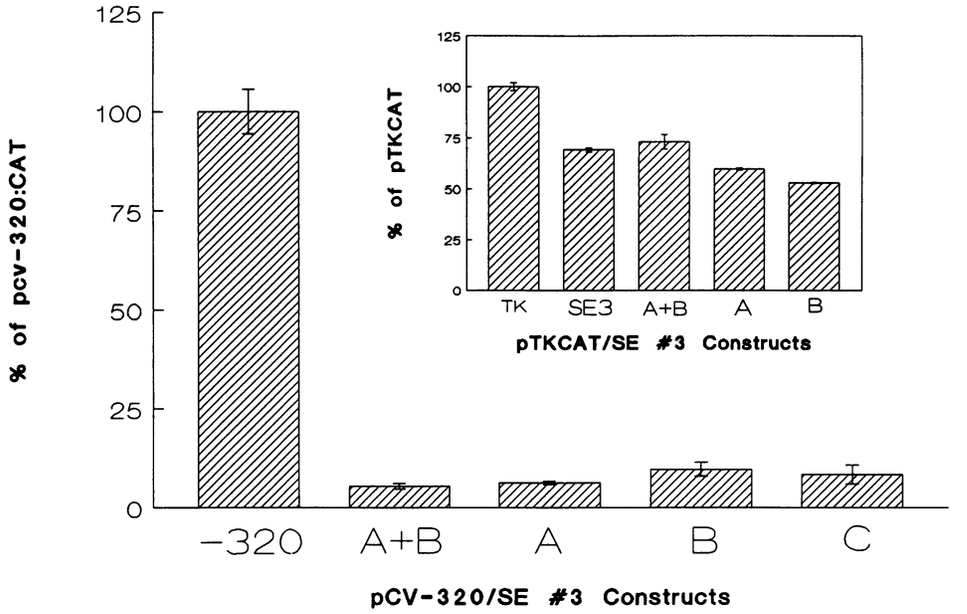


FIG. 4. In vivo effects of SE3 or its subregions on vimentin, pcV-320, or the heterologous tk promoter activity (inset). A+B, A, B, and C represents SE3 subregions versus the entire 40-bp sequence. CAT activity is expressed as a percentage relative to that of pcV-320 or ptkCAT (inset). Error bars represent the SEM.

relatively high degree of transcriptional silencing. Region A+B decreased transcription 95%, whereas region A, B, or C alone had slightly less effect, an average of 92%.

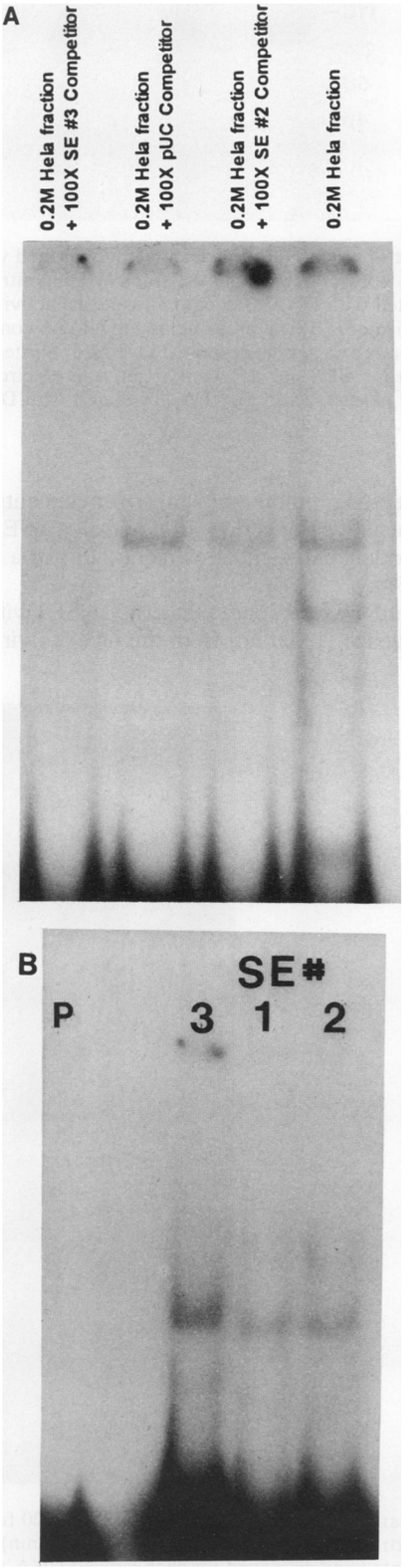
Since Farrell et al. reported that SE3 could also repress transcription (36%) of the heterologous tk promoter (ptk-CAT) (14), we decided to assess the silencing ability of the SE3 subregions on the tk promoter as well (Fig. 4, inset). Comparable silencing activity was seen with either the intact SE3 (31%) or its various subregions A+B, A, and B at 27, 40, and 47%, respectively. Curiously, region B exhibited the best repression, although this small difference may not be significant. In conclusion, it would appear that the subregions of SE3 are all equally efficient at silencing transcription of either the homologous vimentin or the heterologous HSV tk promoter.

All three SEs plus SE3 subregions bind a protein of the same size. Transient-transfection data suggest that repression of vimentin gene expression is due to the presence of three SEs. Initial data suggested that SE3 was able to bind a 95-kDa protein (14). We therefore initiated studies to determine whether SE1 and SE2 bound the same or a different protein.

GMSAs were performed with each SE as described in Materials and Methods. Nuclear extracts from HeLa cells were used for these assays for four reasons: (i) previous results suggested that SE3 conferred the same relative repression whether the cell source was mouse L929 or HeLa cells, (ii) SE3 was shown to bind a 95-kDa protein in mouse L929 cell extracts as well as in HeLa cell extracts, (iii) HeLa cells are easy to grow in sufficient quantity for binding studies, and (iv) binding was shown to be more abundant in HeLa than mouse L929 extracts (14). Figure 5A shows that SE3 exhibits two shifted bands with partially purified HeLa extract eluted off a phosphocellulose column with 0.2 M KCl. Competition with a 100X excess of specific competitor, SE3, or nonspecific competitor (the MCS from pUC) shows that the top band represents specific binding. Binding exhibited by the bottom band is nonspecific because it can be inhibited with any DNA fragment. On the other hand, SE2 does significantly compete with SE3 for binding, although it does not completely prevent protein binding. This could suggest a relatively higher binding affinity of SEP for SE3 versus SE2. Similar specific competition can also be seen with SE1 (data not shown). In Fig. 5B, each SE exhibits a similar bandshift pattern. No binding to the MCS of pUC18 (lane P) was noted. Since the relative mobilities of these bands are due primarily to the size of the protein bound rather than DNA length and DNA length is roughly equivalent, we can conclude that these elements may be binding a protein of similar molecular mass.

In summary, the GMSA data suggest that all three SEs bind a protein of approximately the same molecular mass and, at least with respect to SE3 versus SE2 or SE1, this protein binds at least in vitro with slightly different affinities. To ensure that the same protein was binding each element, Southwestern blots were performed. Previously, we had shown that SE3 bound a protein of 95 kDa (14). Equal concentrations of crude HeLa nuclear extract were subject to denaturing electrophoresis on a 10% polyacrylamide gel (Fig. 6A). The fractionated proteins were blotted onto nitrocellulose and probed with equal radiospecific activity probes of SE3 (lane 1), SE2 (lane 2), and SE1 (lane 3). All three chicken vimentin SEs bound proteins of equal molecular mass (approximately 95 kDa). Binding was specific, as we previously showed that no binding occurs to a DNA fragment containing the GC box consensus sequence (14) or the MCS of pUC18 (data not shown).

Analysis of the 5′ end of the human vimentin gene revealed a negative region from −529 to −277 (38, 39) with additional silencing activity located further upstream, 5′ to −795 (39, 41). Originally, by sequence homology we found a potential match to SE3 at −475 to −464; however, functional corroboration was lacking. Recently, by functional CAT assays, the sequence of this negative element was defined to a 19-bp element located at −338 to −319 with additional partial matches to other regions within the 5′ end of the human vimentin gene (41). Because this region was thought



to exhibit little homology to our SE3, it was assumed to be a different element and factor. However, further examination of this region does suggest a partial homology (6 of 10) to that subregion of SE3 or SE1 (region A [see Fig. 8]) which is important for protein binding, as measured by DNase I or chemical footprinting methods. Therefore, we synthesized the functional 19-bp SE from the human gene and determined whether it could bind the same 95-kDa protein as SE1, SE2, and SE3. Figure 6A (lane 4) clearly shows that the human 19-bp element binds a protein with a molecular mass similar to that of the protein bound by the three chicken vimentin SEs.

In order to confirm the molecular mass of this protein by an alternative method, radiolabeled DNA was UV cross-linked to protein in shifted bands from GMSAs similar to those displayed in Fig. 5B. The bands were eluted and the size of the cross-linked ^{32}P-labeled protein was determined on SDS–10% polyacrylamide gels (Fig. 6B). In this case, both SE1 and the 19-bp element from the human gene bound a protein of the same molecular mass, approximately 95 kDa, in agreement with results obtained by Southwestern blots. The free DNA band bound no radiolabeled protein, and both SE2 and SE3 gave the same results (data not shown).

Surprisingly, all three subregions of SE3, i.e., A, B, and C, were capable of repressing transcription when fused to pcV-320 or ptkCAT (Fig. 4). In order to confirm that they were also able to bind the same 95-kDa protein, we repeated the Southwestern blot analysis with each subregion cloned in pUC8 as a probe (Fig. 6C). As expected, all three subregions of SE3 as well as the entire SE were able to bind the 95-kDa protein, although binding to subregion C was less evident.

Identification of binding sites in the three SEs by DNA footprinting. To assess where the actual contact points are located within the three SEs, we performed DNase I footprinting experiments. ^{32}P-labeled DNA fragments containing each SE were incubated with crude HeLa nuclear extracts as described in Materials and Methods. For each SE, specific protection was seen (Fig. 7, good [*] versus moderate [·] protection is denoted). Increasing protein concentration seems to protect two AG-rich regions in SE3 and SE1. In both cases, these two regions are roughly one turn of the helix apart. In addition, SE3 shows an additional region of protection at the extreme 3′ end (region C) of the element, about 1.5 turns away from the second protected site. SE2, however, shows a slightly different pattern with a central region of protection containing three nucleotides which show no evidence of protein binding. In addition, hypersensitive sites can be seen for all three elements (Fig. 7 and 8, ^). These results are summarized in Fig. 8.

To further confirm DNA-protein contact sites by an alternative method, we performed an additional footprint on SE3 with the nuclease activity of 1,10-phenanthroline–copper ion (26). These results (data not shown) are included in Fig. 8 as open circles below SE3. Note the comparable protection of the same bases (in AG-rich regions) in SE3 both by enzymatic digestion and chemical footprinting methods.

FIG. 5. GMSAs of HeLa nuclear protein binding to the three SEs. (A) ^{32}P-labeled SE3 DNA (1 to 2 ng) was incubated with 10 μg of a 0.2 M KCl fraction of HeLa nuclear extract off a phosphocellulose column as described in Materials and Methods. The effects of a 100-fold excess of nonradiolabeled SE3, SE2, or the pUC MCS are shown as indicated. (B) ^{32}P-labeled SE3 (40 bp) (lane 3), SE2 (30 bp) (lane 2), or SE1 (18 bp) (lane 1) was incubated with 8 to 10 μg of the 0.2 M KCl fraction described above. Lane P, ^{32}P-labeled MCS (51 bp) from pUC18.

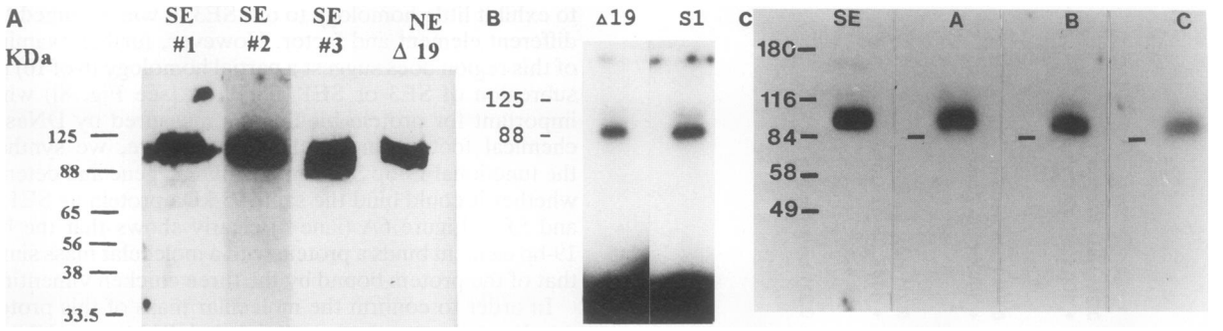


FIG. 6. Assessment of protein binding to the various SEs. (A) Southwestern blot of crude HeLa nuclear extracts probed with each SE DNA. Crude HeLa nuclear extract (100 μg) was separated on a denaturing SDS–10% polyacrylamide gel and transferred to nitrocellulose as described previously (14, 50) and in Materials and Methods. The filters were incubated with DNA of equal radiospecific activity, i.e., SE3, SE2, SE1, and the 19-bp negative element from the human vimentin gene (41) as indicated. (B) UV cross-linking of GMSA complexes. SE1 (S1) or Δ19 (1 pmol) was incubated with 40 μg of crude HeLa nuclear extract and separated on gels as described for Fig. 5. Shifted bands were cut out, soaked in SDS-containing sample buffer, placed in the wells of a denaturing SDS-polyacrylamide gel, and electrophoresed as described in Materials and Methods. (C) Southwestern blot of crude HeLa nuclear extracts as in panel A, incubated with DNA of equal radiospecific activity for the entire SE versus subregion A, B, or C cloned in pUC8.

DISCUSSION

Studies from several laboratories (14, 37, 39, 41, 45, 50, 52) clearly indicate that multiple elements, both positive and negative, are active in maintaining vimentin's pattern of gene expression. Previously, we (43, 44) as well as others (38, 39, 41) have shown that the vimentin gene not only is controlled by the expected promoter and enhancer elements but also is under the negative transcriptional control of a SE (14) as well as an overriding antisilencer element (50) (for a review, see reference 53).

In this study, we compared the original SE3 with the entire 5′-end sequence (1,700 bp) from the chicken vimentin gene

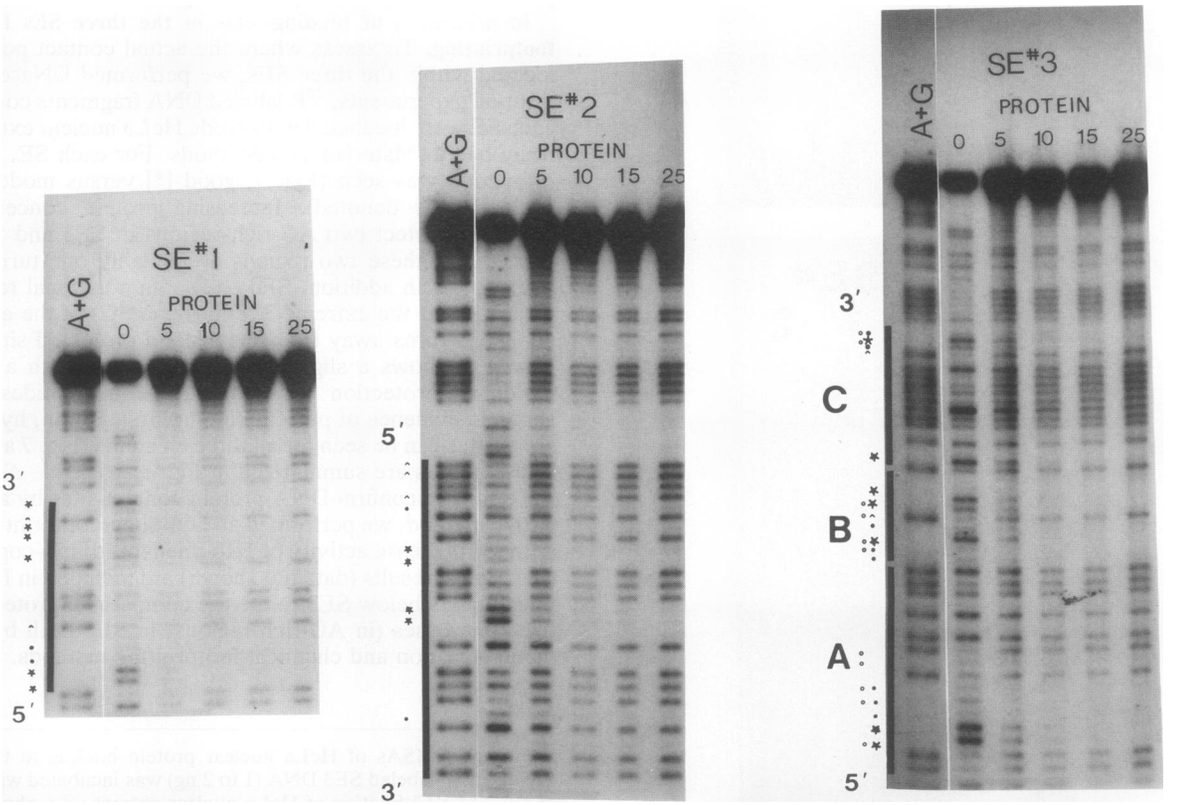


FIG. 7. DNase I footprints of the various SEs with partially purified HeLa nuclear extracts. Various ^{32}P-labeled SEs (20 fmol) as noted were incubated with increasing amounts (in micrograms) of partially purified HeLa extract (pool from a Sephacryl S300 column) and digested as described in Materials and Methods. Maxam and Gilbert A+G ladders were generated and displayed for each labeled DNA. Bars indicate locations of each SE within the pUC vector; SE3 shows the three subregions as A, B, and C. Asterisks denote positions of good protection, dots indicate positions of moderate protection, and carets indicate positions of hypersensitive sites. Open dots for SE3 indicate positions that were protected in the copper-phenanthroline footprinting method (data not shown).

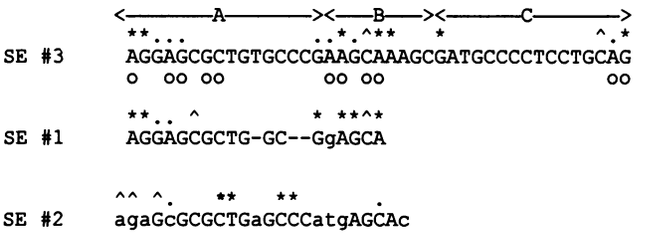


FIG. 8. Comparison of the DNA sequences of the three chicken vimentin SEs. SE1 and SE2 are aligned against SE3. Nucleotide matches are indicated by capital letters; mismatches are shown by lowercase letters. Gaps are indicated by hyphens. *, very good protection; ·, moderately good protection; ^, hypersensitive sites; ○, sites protected in SE3 by the copper-phenanthroline footprinting method.

and discovered two additional putative silencer elements (SE1 and SE2) (Fig. 1 and 8). Here, we functionally show that SE1 and SE2 both work within the context of the vimentin gene (Fig. 2), as well as when fused to either the pcV-160 or pcV-320 expression vectors (Fig. 3). Moreover, SE1 appears to be more active than either SE2 or SE3, both within the context of the vimentin gene, i.e., 93% repression (Fig. 2), or when fused to pcV-160, i.e., 75% repression (Fig. 3). However, SE2 and SE3 approach the silencing activity of SE1 when they are present in more than one copy, either by themselves, as in the pcV-160 constructs, or when they are joined to SE1, as in the pcV-320 constructs (Fig. 3). A further dissection of SE3 reveals three subregions, all of which are active in silencing transcription, especially in conjunction with SE1, which is naturally present in pcV-320 (Fig. 4).

In vitro analysis of the SEP by GMSAs (Fig. 5), Southwestern blots (Fig. 6A), and UV cross-linking experiments (Fig. 6B) suggests that the same factor which interacts with SE3 (14) or its subregions A, B, and C also interacts with SE1 and SE2. Moreover, all three SEs plus the corresponding 19-bp element from the human gene (41) specifically bind a protein of the same molecular mass (approximately 95 kDa) as measured by Southwestern blots and UV cross-linking data. Therefore, we conclude that these SEs and their *trans*-acting factor are identical in both the chicken and the human vimentin genes. However, in both cases there are additional upstream negative elements, the relatedness of which is unknown (38, 39, 41). Since the chicken vimentin gene appears to contain multiple copies of the same element binding an identical 95-kDa factor, it will be interesting to see whether the same arrangement exists for the human vimentin gene. The human gene is known to contain additional negative elements, but the nature of these sequences and binding factors is uncharacterized at present (38, 39, 41). Perhaps multiple copies of the same element and factor are required there as well.

Because the sequences of these elements are only partially identical, we were curious as to how one factor could specifically bind all three versions. To address this question, we performed DNase I footprinting experiments on all three SEs (Fig. 7). Each element exhibited protection of specific bands (Fig. 8), with the pattern of binding for SE1 and SE3 being the most similar. For both of these elements, two separate regions of protection which are about one turn of the helix apart and are AG rich are seen. Additionally, SE3 has a third region of protection at the extreme 3′ end which is about 1.5 turns of the helix from the previous DNase I-protected region. This could explain how subregions of SE3 (A, B, or C) are active in repressing transcription (Fig. 4) and can all bind the same 95-kDa protein, albeit to various degrees (Fig. 6C). On the other hand, SE2 exhibited only a single region of protection roughly in the center of the element which on this strand is pyrimidine rich. Previously, we have shown that SE3 is equally active in either orientation (14), and therefore, we conclude that probably all three elements exhibit no restriction for orientation. In addition, that portion of the human 19-bp negative element which exhibits the greatest homology to SE3 or SE1 is G rich. Therefore, we conclude that a purine-rich sequence on one strand is important for protein binding to all of these SEs, and mutagenesis studies to confirm which bases are required for functional binding are under way. It is likely that considerable sequence divergence may be tolerable, since SE2 (as well as subregions of SE3) appears to work as well as SE3 in functional assays (Fig. 3 and 4).

Recently, van de Klundert et al. (52) identified two functional SEs in the hamster vimentin gene, Sil1 (−741 to −712) and Sil2 (−655 to −635). Sil2 has a limited sequence similarity to SE3 (8 of 21) and human vimentin (7 of 16) at −811 to −796, which is not within the functional 19-bp negative element delineated by Salvetti et al. (41). Moreover, Southwestern blot data with Sil2 indicate that this element binds a 200-kDa factor. Southwestern blots with Sil1 have not been reported. The fact that 200 kDa is far larger than the protein that we detect here suggests that either hamster has unique SEs and/or factors or Sil2 represents the distal silencer activity which both the chicken (53a) and human (39, 41) vimentin genes contain but about which nothing is known. We feel confident about the 95-kDa size of SEP, because we have determined this molecular mass by two independent methods, i.e., Southwestern blots and UV cross-linking experiments from GMSAs, with nuclear extracts from a variety of tissues and cells, and in different species such as chickens, mice, and humans.

All these SEs, plus subregions of SE3, appear to act by repressing vimentin's active promoter elements, which include multiple GC boxes, a CCAAT, and a TATA box. Indeed, SE3 as well as its subregions are capable of silencing the heterologous but related HSV tk promoter (Fig. 4, inset) which also contains multiple GC boxes, a CAAT, and a TATA box (22). This suggests that vimentin's SEs are repressing common promoter elements and/or their associated factors or more general transcription factors such as TFIIA to -J, as shared between the tk and vimentin promoters. Therefore, they are not likely to be unique elements which only pertain to vimentin gene expression.

From our in vitro binding studies, it is likely that SEP binds DNA as a monomer or homodimer. If DNA binding is due to a heterodimer, the subunits would have to be of the same molecular weight, because only a single band is observed under the denaturing gel conditions of the Southwestern blot (14) or in the UV cross-linking experiments. It is possible that in vivo the factor is multimeric, but this could occur only if the 95-kDa monomer is capable of DNA binding or the heterodimer subunits are of approximately the same size, thus appearing as a single band in the Southwestern blot and UV cross-linking gels. A resolution of this question will have to await SEP purification and/or production of a SEP antibody which can be used to purify SEP complexes from cellular extracts and then a rigorous examination of all associated components. Experiments to purify SEP with a DNA affinity column are in progress.

Footprinting experiments revealed a noticeable difference

between the single, central binding site for SE2 versus the two binding regions observed for SE1 and SE3. This could be due to a protein having two contact sites on the DNA, either within a single SE (such as for SE1 or SE3) or between two different SEs, i.e., one binding site in SE2 with a secondary binding site in SE3 or SE1. From functional studies with either ptkCAT or pcV-320 versus the pcV-160 constructs, it is apparent that additive silencing occurs with the addition of extra SE copies. This suggests that some kind of stabilizing interaction is occurring across multiple SE copies in order to attain higher levels (90%) of transcriptional repression. A requirement for the presence of multiple SEs may prove to be a general rule in genes utilizing this type of negative transcriptional control. For example, the chicken lysozyme gene as well as others has been shown to contain multiple negative elements (2, 3).

In vitro footprinting experiments reveal distinct hypersensitive sites suggestive of protein-induced DNA bending (carets in Fig. 7 and 8). That the three SEs within the chicken vimentin gene are spaced 100 to 200 nucleotides apart would permit DNA bending. Such bending could be induced or stabilized in vivo by SEP binding, which could lead to transcriptionally inactive loops, preventing the formation of an active preinitiation complex. In support of this theory is that significant repression (greater than 90%) requires at least two SEs. Currently, experiments to determine whether SEP binding induces DNA bending in vitro and whether this could be fundamental to repression as has been suggested for some DNA-binding proteins are under way (18, 24, 46, 55).

On the other hand, these SEs might act directly to block transcription initiation by interfering with the binding or association of one or more of the general transcription factors TFIIA to -J. In this scenario, SEP might be involved in the formation of a tertiary structure which is inhibitory to transcription directly through protein-protein interactions with the preinitiation complex, through DNA looping effects, or through any combination thereof. Repressors that interfere with the assembly of a functional preinitiation complex have been described (20, 21, 32–34). However, in these cases the repressor doesn't bind a specific DNA sequence. Recently, it has been suggested that acidic activators positively influence transcription by interacting with TFIIB (40), thereby enhancing the rate-limiting step to transcription initiation. In our case, perhaps these silencers block such functional interactions and therefore repress transcription. If this is the case, then our SEs should be able to repress a variety of promoter elements. Presently, we are testing this hypothesis by fusing SE1 to a variety of promoter elements from various genes.

A good example of a defined system requiring multiple SEs consists of the yeast silent mating type loci. Brand et al. (7) showed that, as with the chicken vimentin gene, the *HMR* silencer actually consists of three subregions, listed in order, 5′ to 3′: A, E, and B. The E region consisted of a binding site for a protein called RAP-1, and complete silencing occurred only when the E element was examined in conjunction with either A or B (for a review, see reference 15). Within region A is a consensus ARS sequence, and within region B is a domain that contributes to, but is not sufficient, for ARS function. It is thought that in this system DNA replication is required for silencing to occur, implying that correct chromatin structure is required (36). The fact that our DNase I footprints produce hypersensitive sites suggests that the configuration of the DNA is important to the chicken vimentin gene as well; however, in vimentin, the change in structure of the DNA may be a consequence of the protein-DNA interaction rather than a prerequisite for binding to occur.

The type of silencing activity exhibited by the chicken vimentin gene is in contrast to the negative regulation seen, for example, by the protein Id (5) in genes activated by MyoD, E12, and E47, or by the protein IκBβ (25) in genes activated by Rel and NF-κB. In these cases, an inhibitory protein binds an activator protein in the cytosol and prevents DNA binding. Another type of negative regulation that seems unlikely here is when positive DNA elements are partially overlapped by negative regulatory domains (16) or when a single DNA domain may bind two different factors with opposite and mutually exclusive effects (11, 21).

In summary, several genes are known to contain negative elements (2, 3, 7, 9, 19, 30, 41, 42, 52). However, the chicken vimentin gene contains multiple copies of the same SE which are required for total silencing of gene transcription. Probably this is also the case for the human gene, as additional homologies to the 19-bp negative element were mentioned (41). It is likely that multiple SEs may be a common feature of some gene families. That multiple elements are required at all may be due to the requirement that these genes be carefully regulated during development and cell cycle. Further experiments will be required to determine how these multiple elements cooperate in repressing gene transcription.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant HL45422 to Z.E.Z.

We acknowledge the contribution of Brent Perkins to this work and thank Indira Carey and Alma Bracete for their critical reviews of the manuscript.

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