Two Homologous Enhancer Elements in the Chicken Vimentin Gene May Bind a Nuclear Factor in Common with a Nearby Silencer Element*

Edward B. Perkins, Janet G. Cunningham, Alma M. Bracete, and Zendra E. Zehner§

From the Department of Biochemistry and Molecular Biophysics and the Massey Cancer Center, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia 23298

Vimentin, a cytoskeletal protein belonging to the intermediate filament protein family, exhibits a complex pattern of expression. In the case of the chicken vimentin gene, several regulatory elements within the 5′ region of the gene have been characterized, including an enhancer activity between –160 and –320, which may contribute to the down-regulation of vimentin expression during myogenesis. In this study, sequences within this region were examined via transient transfections of various deletion constructs, and two distinct enhancer elements were found, one on either side of a previously described silencer element. These two enhancer elements also enhanced transcription when fused separately to the basal promoter region of the chicken vimentin gene. Gel mobility shift assays, UV cross-linking experiments, and DNase I protection studies indicate that these two enhancer elements and the silencer element all contain a common binding site for the previously described 95-kDa silencer element binding protein, suggesting that this regulatory protein can act as both an activator and a repressor.

The initiation of transcription is a key control point for eukaryotic gene expression, and regulation of this critical event is central to producing the correct tissue-specific and temporal pattern of gene expression. Regulation is primarily accomplished by gene-specific DNA-binding transcription factors, which either increase or decrease the rate of transcription initiation (1, 2). The target sequences for these transcription factors can be either upstream or downstream of the transcription start site and are often quite far removed from the start site. Over the last few years, a number of enhancer and repressor elements, along with their corresponding DNA-binding transcription factors, have been well characterized (1). As a result, the distinction between enhancers and repressors has become less definitive. For example, several studies have demonstrated how a single gene, by way of either alternative splicing or the use of alternative translational start sites, can encode for both an enhancer and a repressor protein (3). In other cases particular transcription factors have been shown to either enhance or repress transcription depending on the absence or presence of a metabolic intermediate (4), the concentration of the transcription factor itself (5, 6), or the number of DNA-binding sites present (7).

Vimentin belongs to the intermediate filament protein (IFP) family, a related group of structural proteins which are prominent components of the eukaryotic cytoskeleton (8, 9). IFPs can be subdivided into six distinct types, based on sequence and site of synthesis. These include keratins in epithelial cells, desmin in muscle cells, lamins in the nucleus, neurofilaments in neurons, glial fibrillary acidic protein in glial cells, and vimentin in cells of mesenchymal origin. IFPs are more dynamic structures than previously thought (10), and their importance has been emphasized by the recent discovery that mutations in specific keratin genes can give rise to genetic skin diseases (11–13). Among the different IFP types, vimentin exhibits a complex pattern of expression (14, 15) and is often coexpressed with one of the other IFPs, usually early in development. For example, vimentin is coexpressed with desmin in the early stages of myogenesis but not in the later stages (16). Vimentin is also frequently expressed in cultured cell lines, regardless of origin (17, 18). Regulation of vimentin gene expression is correspondingly complex, involving multiple regulatory elements, including both enhancers and silencers. In the case of the chicken vimentin gene, these regulatory elements are all located upstream of a promoter region that encompasses the first 160 bases upstream of the start site and provides a constitutive level of activity (15, 19). Several regulatory elements upstream of this proximal promoter region, along with associated DNA binding proteins, have been identified and characterized. Three homologous silencer elements (SEs), denoted SE1, SE2, and SE3, and an associated SE binding protein have been identified (20, 21). Approximately 1 kilobase upstream of the most distal silencer element (SE3) is an antisilencer element, which overrides the negative effect of the silencer element but shows no independent enhancer activity (22).

The SE closest to the transcription start site (SE1) lies in a region, between –160 and –320, that we have previously shown contains a tissue-specific enhancer of possible importance for the down-regulation of vimentin during myogenesis (23, 24). In this report, we examine this upstream region in more detail and show that two homologous enhancer elements exist here, one on each side of SE1. Gel mobility shift assays, DNase I protection studies, and UV cross-linking experiments are used to argue that these two enhancer elements bind to the

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† Present address: Children’s Hospital Oakland Research Institute, 747 52nd St., Oakland, CA 94609.

‡ To whom correspondence should be addressed: Tel.: 804-828-8753; Fax: 804-828-1473; E-mail: Zehner@vcuvax (bitnet), Zehner@gems.vcu.edu (internet).

1 The abbreviations used are: IFP, intermediate filament protein; SE, silencer element; GMSA, gel mobility shift assay; PEE, proximal enhancer element; bp, base pair(s); CAT, chloramphenicol acetyltransferase.
previously described SE binding protein, suggesting that this regulatory protein can both activate and repress transcription.

MATERIALS AND METHODS

Oligonucleotide Synthesis, Labeling, and Annealing—The DNA fragments used in the gel mobility shift assays (GMSAs) and the UV cross-linking experiments were made by annealing complementary strands. The following oligonucleotides were synthesized, along with their complementary strands, for the two proximal enhancer elements (PEEs): 5'-CTCGAGGGGGCGGCAC-3' and 5'-CTAAGAGAAGAGGGAGGG-3'. The following strands were synthesized for the three SEs: SE1 (5'-CTCGAGAGGGGGCGGCACACG-3' and 5'-CTCGAGTGGCTCCGAGGCTTGGT-3'), SE2 (5'-CTCGAGATGGGCCCCATAGACCCAG-3' and 5'-CTCGAGCAGGAGCGCTGGCGGAGCTG-3'), SE3 (5'-CTCGAGGAGCGCTGGCGGAGCTG-3' and 5'-CTCGACCTGCAAGGGGCTATGCTTATGCTGCGGACCGCT-3'). The SEs include Sal restriction site sequences at the ends for cloning purposes unrelated to these experiments. Before annealing, each strand was labeled using [γ-32P]ATP and T4 polynucleotide kinase. The complementary strands were annealed in 0.3 M KCl, 10 mM Tris (pH 7.8), and 1 mM EDTA by heating to 95°C and slowly cooling to 25°C. The double-stranded fragments were separated from unincorporated 32P-nucleotides on a 6% polyacrylamide gel, electroeluted, and ethanol-purified.

Plasmids—The p8CAT expression vector was used to test the transcriptional activity of various vimentin 5'-flanking sequences. p8CAT is a pEMBL derivative (25) containing the bacterial chloramphenicol acetyltransferase gene, the adenovirus E1A resistance gene, and the pUCB multicloning site. Several different vimentin 5'-flanking sequences were placed in front of the CAT gene using standard molecular biology techniques (26). Plasmids pcV-160, pcV-320, and pcV-767 have been described previously (20, 24). Plasmids pcV-302 and pcV-283 were created by Bal31 digestion of pcV-320. Naturally occurring EcoRI and BamHI restriction sites were used to generate pcV-200 and pcV-179, respectively. PEE1 and PEE2 were cloned into the KpnI and BamHI sites, respectively, of pcUC18. To accomplish this, the same oligonucleotides described above, representing the complementary strands of PEE1 and PEE2, were synthesized with KpnI and BamHI restriction site sequences, respectively, on the ends. After annealing, these double-stranded fragments were directly ligated into the KpnI and BamHI sites of pcUC18. The following scheme was used to generate a construct containing PEE1 in front of the basal promoter elements contained in pcV-160. The pcV-160 plasmid was cut with EcoRI to create a fragment containing the upstream promoter elements up to ~160, and this fragment was ligated into the EcoRI site of the pcUC18 plasmid, which contained PEE1 in the BamHI site. HindIII digestion of this intermediate pcUC18 construct introduced a fragment containing PEE1 in front of the upstream promoter elements, and this fragment was ligated into the HindIII site of p8CAT to produce the final PEE1/160 reporter plasmid. A plasmid containing PEE2 in front of 160 was constructed similarly.

Cell Culture, Transient Transfections, and CAT Assays—Mouse L-cell fibroblasts were maintained in 90% Dulbecco's modified Eagle's medium, 10% fetal calf serum, 2 mM glutamine, and 0.1 mg/ml gentamycin at 37°C in 5% CO2 as monolayer cultures. Cells were plated at a density of 5 × 10^4 cells/100-mm tissue culture dish 24 h prior to transfection. Transfections and CAT assays were performed as described previously (21). Activity was calculated as pmol of acetylated p-nitrophenol/10^6 cells and is reported as percentage relative to pcV-160 or pcV-320.

Preparation of Nuclear Extracts—Crude nuclear extracts were made from HeLa cell cultures by the method of Dignam (27). Aliquots were stored at −70°C in Dignam buffer D (20 mM HEPES, pH 7.9, 20% [v/v] glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol).

Purification of the SE Binding Protein—A detailed description of the purification and characterization of the SE binding protein will be the subject of a different report.2

GMSA—Oligonucleotides representing each strand of the various DNA fragments were labeled and annealed as described above. The DNA fragments (6 ng) were incubated with 750 ng salmon sperm DNA, 1.25 μg of poly(dI-dC), and 8 μg of crude HeLa nuclear extract in a buffer containing 10 mM HEPES (pH 7.9), 4 mM Tris, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% (v/v) glycerol. The total reaction volume was 20 μl, and binding reactions were incubated on ice for 20 min. Binding reactions with affinity-purified protein fractions were performed in the same way, except that no salmon sperm DNA was included. For competition assays, small nonspecific DNA fragments were produced by blunt cleavage electrophoresis of pUC18 plasmid DNA and isolated by gel electrophoresis.

RESULTS

Two Enhancer Elements Are Present in the Upstream Region—We had previously reported that the upstream region from −160 to −320 in the chicken vimentin gene contained an enhancer potentially important for the down-regulation of vimentin during myogenesis. To examine this region in more detail in a nonmyogenic cell line (mouse L-cells), several other constructs, containing progressively more of the DNA sequence between −160 and −320, were created via Bal31 digestion and transiently transfected into mouse L-cells (Fig. 1A). Two short sequences within this region were found to significantly enhance expression of the CAT reporter gene: the 22-bp sequence between −179 and −200, referred to as proximal enhancer element 1, and the 19-bp sequence between −302 and −320, referred to as proximal enhancer element 2.

Although both PEE1 and PEE2 enhanced transcription, the CAT activity of pcV-320, which contains both enhancers, was only about 2-3 times that of pcV-160. This is likely due to the silencer element that is located between PEE1 and PEE2. We have previously described three homologous silencer elements (SE1, SE2, and SE3), which are important in regulating expression of the vimentin gene (20, 21). Their positions relative to PEE1, PEE2, and the basal promoter region are illustrated in Fig. 1B. One of these silencer elements, SE1, is located between −236 and −253 and has been shown to decrease the transcriptional activity of pcV-160 by about 75% when placed directly upstream of −160 (21). The repressive effect of SE1 was evident in the transfections described here, with construct pcV-283 showing a low level of CAT activity compared with pcV-200 (Fig. 1A). The single SE and the two enhancer elements are all present in construct pcV-320, with the net effect being a 2-3-fold increase over pcV-160.

To confirm the ability of PEE1 and PEE2 to independently enhance transcription, these two elements were separately cloned in front of the basal promoter region contained in pcV-160 and transiently transfected into mouse L-cells (Fig. 2). In these constructs, possible interactions between PEE1 and PEE2 or between SE1 and the enhancer elements were elimin-
When placed directly upstream of -160, both PEE1 and PEE2 continued to show enhancing activity of about 4- and 2-fold, respectively, providing additional evidence for their functional significance. PEE1 also appeared to be a stronger enhancer than PEE2, which correlates with the data shown in Fig. 1A, where PEE1 produced a larger increase in transcription than PEE2.

Both Enhancer Elements Specifically Bind a Nuclear Protein—Since sequence elements that help regulate transcription typically act by binding to trans-acting factors, we carried out GMSAs to look for DNA binding to nuclear proteins. PEE1 and PEE2 were synthesized and used to perform GMSAs as described under "Materials and Methods." Both PEE1 and PEE2 bound nuclear protein(s) to produce complexes of nearly equal mobility (marked L in Fig. 3A). Binding reactions with competitor DNA showed that for both PEE1 and PEE2 an excess of unlabeled PEE1 or PEE2, respectively, effectively eliminated any binding to the labeled fragment (lanes 3 and 8). For band L with both PEE1 and PEE2, an excess of unlabeled DNA from an unrelated sequence did not compete effectively for binding (lanes 6 and 11), indicating that this complex represents sequence-specific binding to PEE1 and PEE2. For band U, an excess of unlabeled nonspecific DNA did reduce the binding to PEE1, probably because the nonspecific DNA, which is a mixture of small fragments resulting from a Hae III digest of pUC plasmid DNA, contained some sequences that were similar to PEE1.

The observation that band L has nearly equal mobility with both PEE1 and PEE2 led us to investigate whether PEE1 and PEE2 were binding the same protein. Competition with the other enhancer element in excess (lanes 4 and 9) did indeed show effective cross-competition for band L, indicating that the same protein, or proteins with similar mobilities and DNA binding specificities, are binding to both PEE1 and PEE2. Band U was not affected by the addition of excess unlabeled PEE2 (lane 9). As an additional control, binding reactions with an excess of unlabeled SE3 were carried out for both PEE2 and PEE1 (lanes 5 and 10). Surprisingly, an excess of unlabeled SE3 abolished all binding to both PEE1 and PEE2, resulting in almost no detectable band L or band U. This suggests that this protein is binding to sequences common to PEE1, PEE2, and SE3.

To further investigate the possibility that these elements are all binding the same protein, another GMSA, comparing all three SEs plus PEE1 and PEE2, was performed. As shown in Fig. 3B, all three SEs bound a protein to produce a band of equal mobility to band L observed with PEE1 and PEE2. We had previously reported that all three SEs bind the 95-kDa SE binding protein in GMSAs or Southern blots (21), suggesting that the SE binding protein is present in band L in Fig. 3A. SE3 also produced a complex with the same mobility as band U observed with PEE1 (Fig. 3B, lane 6), explaining why unla-
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FIG. 3. GMSA analysis. Crude nuclear extract (8 μg) prepared from Hen's cell cultures was incubated with 6 ng of radiolabeled DNA as described under "Materials and Methods." A, nuclear protein binding to both PEE1 (22 bp) and PEE2 (19 bp). Lane 1, control reaction with no protein extract added. Either no competitor (lanes 2 and 7) or the following unlabeled competitor DNA was added: Lanes 3 and 8, PEE2 and PEE1, respectively; lanes 4 and 9, PEE1 and PEE2, respectively; lanes 5 and 10, SE3; lanes 6 and 11, nonspecific DNA. Both PEE1 and PEE2 bind a lower band (marked L), while PEE1 also binds an upper band (marked U). Reactions with unlabeled competitor DNA (lanes 3–6 and 8–11) include a 500-fold molar excess of competitor DNA. B. nuclear protein binding to both enhancer elements and all three SEs. Lane 2 is a control with no protein extract added.

UV Cross-linking of Protein-DNA Complexes—To help confirm whether the same protein is binding PEE1, PEE2, and the three SEs, and to establish whether it is the SE binding protein or some other protein, the GMSA in Fig. 3B was repeated. After electrophoresis, the wet gel was exposed to UV radiation to covalently cross-link the protein-DNA complexes, as described under "Materials and Methods." The cross-linked complexes were separated by SDS-PAGE, and a single predominant band of nearly equal mobility was observed migrating between the 88- and 125-kDa markers (Fig. 4). The slight differences in mobility between the bands are likely attributable to the small differences in the size of the labeled DNA fragments. While PEE1, PEE2, SE1, and SE2 are all between 19 and 32 bases in size, SE3 is 47 bp, and a protein covalently bound to it would be expected to migrate at a slightly slower rate. The result of this experiment strongly suggests that all of these DNA fragments are specifically binding to the 95-kDa SE binding protein.

DISCUSSION

Previous studies of the chicken vimentin gene have demonstrated that several upstream regulatory elements, both positive and negative, function in an integrated but as yet incompletely understood fashion to regulate gene expression (15). In the case of the chicken vimentin gene, positive and negative elements have also been identified that appear to correspond to...
the regulatory elements in the chicken vimentin gene, although not necessarily in the same number or absolute position (28, 29).

We have previously described a region between 160 and 320 bases upstream of the transcription start site in the chicken vimentin gene, which contains a tissue-specific enhancer of possible importance for the down-regulation of vimentin expression during myogenesis (23, 24). Here we have used transient transfection assays with various deletion constructs (Fig. 1A) to show that two separate enhancer elements, PEE1 and PEE2, exist in this region, one on each side of a previously characterized SE (21). Transient transfection assays with each of these enhancer elements fused separately to the basal promoter region (−160) support the functional importance of these two enhancer elements (Fig. 2). When binding to nuclear proteins was tested via GMSA, both PEE1 and PEE2 produced a protein-DNA complex with equal mobility (Fig. 3A, band L), and the addition of both specific and nonspecific competitor DNA confirmed that this complex is the result of a sequence-specific interaction. Unlabeled SE3 was also an effective competitor in this experiment (Fig. 3A, lanes 5 and 10), and a second GMSA confirmed that both enhancer elements and all three silencer elements produce a complex with equal mobility (Fig. 3B). UV cross-linking of these complexes suggests that the 95-kDa SE binding protein is binding to all of these elements (Fig. 4). Efforts to purify the SE binding protein using a SE1/DNA affinity column were under way at the same time as these experiments, 2 and a nearly pure fraction containing the SE binding protein was available for use in DNase I protection studies. This purified fraction contains only two major components when stained with either Coomassie Blue (Fig. 5) or silver (data not shown): the SE binding protein around 95 kDa and another protein around 66 kDa. DNase I protection studies of PEE1, PEE2, SE1, and SE3 confirm that all of these elements have a region or regions protected against DNase I cleavage when incubated with this purified fraction (Fig. 6A). Furthermore, a sequence comparison reveals a homologous eight-base region where all four elements show protein binding (Fig. 6B, box), providing a common basis for DNA-protein interaction. The two SEs show an additional area of protection, and a comparison of these DNase I footprints (underlined bases) with previously described footprints using less purified nuclear extracts (21).
extracts (highlighted bases) shows good correlation for all protected areas (21).

Studies on regulation of the human vimentin gene support the relevance of these results. A computer search of the human vimentin 5' end identified one sequence that exactly matches the eight-base sequence in PEE1 (Fig. 6B). Interestingly, this matching human sequence lies immediately upstream of a 19-bp negative element (29–31), which was clearly protected in DNase I footprints (29). We have previously shown using Southwestern blots and UV cross-linking that this 19-bp negative element binds the same 95-kDa protein as the chicken SEs (21).

These results suggest that the same protein can bind both an enhancer and a silencer element, thereby activating or repressing transcription. In recent years, several instances of such dual regulation by the same transcription factor or related transcription factors, have been demonstrated. In the case of the Wilms' tumor gene product WT1, separate domains of WT1 mediate activation and repression, but the same DNA binding site is utilized in both cases. Whether WT1 activates or represses transcription depends on the number of binding sites and their position within the gene. In cases where related transcription factors with opposite effects have been described, the two proteins can be derived from the same gene, either as a result of alternative splicing (3, 32–34) or multiple translational start sites (35), or from separate genes (36, 37). Although two proteins of noticeably different molecular weights often result, this is not always the case. The gene for the POU domain nuclear protein I-POU, for example, can be alternatively spliced to create twin of I-POU, which activates transcription instead of repressing it like I-POU and differs from I-POU by only two amino acids (34). An example involving two proteins from separate genes is found in the interferon regulatory system, where the regulatory factors IRF-1 and IRF-2 possess similar molecular weights and DNA binding specificities but produce opposite effects on the transcription of the interferon gene and interferon-inducible genes (36, 37). IRF-1 and IRF-2 are derived from separate but related genes.

In our case, the 95-kDa SE binding protein is clearly interacting with both the positive and negative regulatory elements, as demonstrated by GMSAs, UV cross-linking experiments, and DNA footprints. It is unlikely that the opposing effects depend on binding site position or number, as with WT1, since these elements always increase (PEE1 and PEE2) or decrease (SE1, SE2, SE3) transcription when placed independently in front of the CAT reporter gene. A more promising hypothesis is that the SE binding protein is producing opposing effects due to differences in the binding site itself. To test this hypothesis, work is currently under way to construct a series of mutations that can convert the silencer element to an enhancer of transcription and vice versa.

Another possibility is that additional proteins, acting in concert with the SE binding protein, control whether these DNA elements act in a positive or negative manner. The 66-kDa protein, which copurified with the 95-kDa SE binding protein on the DNA affinity column, is one candidate, and preliminary results do indicate that it is essential for binding of the SE binding protein. A GMSA using a partially purified fraction of the SE binding protein that did not contain the 66-kDa protein produced no binding (Fig. 7B, lanes labeled –66). Subsequent addition of the 66-kDa fraction restored binding (lanes labeled Both). Interestingly, the 66-kDa protein was never detected in the UV cross-linking experiments, perhaps because it only contacts the 95-kDa protein and not the DNA itself. It is also possible that the 66-kDa protein and the SE binding protein are binding as a dimer, but the nature of the contacts between the 66-kDa protein and the DNA does not permit UV cross-linking under our conditions. In this case the 66-kDa protein might still be responsible for part of the DNase I protection pattern, and the eventual purification of this protein should allow us to examine more detailed and informative footprints. In addition to the 66-kDa protein, other proteins that play a role in gene regulation could be removed during purification. Since the functional assay used during purification was binding to SE1, proteins that do not directly bind to the DNA, but that nevertheless are important components in vivo, could be missed. The successful purification of the 95-kDa SE binding protein and production of antibodies will eventually allow us to immunoprecipitate intact complexes from labeled extracts. Analysis of these complexes will help us unravel how these regulatory elements and factors interact in vivo to activate or repress transcription of the vimentin gene.

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