Insulin-like Growth Factor-I Regulates Transcription of the Elastin Gene through a Putative Retinoblastoma Control Element

A ROLE FOR Sp3 ACTING AS A REPRESSOR OF ELASTIN GENE TRANSCRIPTION

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Previous studies have demonstrated that insulin-like growth factor-I (IGF-I) increases elastin gene transcription in aortic smooth muscle cells and that this up-regulation is accompanied by a loss of protein binding to the proximal promoter. Sp1 has been identified as one of the factors whose binding is lost, and in the present study we show that Sp3 binding is also abrogated by IGF-I, but in a selected manner. In functional analyses using Drosophila SL-2 cells, Sp1 expression can drive transcription from the elastin proximal promoter, while co-expression of Sp3 results in a repression of Sp1 activity. Footprint and gel shift analyses position the IGF-I responsive sequences to a putative retinoblastoma control element (RCE). Mutation of the putative RCE sequence as assessed by transient transfection of smooth muscle cells results in an increase in reporter activity equal in magnitude to that conferred by IGF-I on the wild type promoter. Together these results support the hypothesis that IGF-I-mediated increase in elastin transcription occurs via a mechanism of derepression involving the abrogation of a repressor that appears to be Sp3 binding to the RCE.

Elastin is a resilient connective tissue protein present abundantly in tissues such as skin and lung, and in the large blood vessels of the cardiovascular system. Aortic elastin fibers, assembled extracellularly from cross-linked tropoelastin monomers and microfibrillar proteins, allow tissues of the cardiovascular system to undergo repeated stretching and recoil, a necessary property for maintaining physiologic pressure gradients. Production of elastin, like other connective tissue proteins, is developmentally regulated. The greatest elastogenic period occurs during late fetal and early neonatal periods (1–4), followed by a drop in elastin production and mRNA steady state levels in adulthood (5–7). However, in response to injury such as in the atherosclerotic lesion, elastin production is up-regulated (8). Lesions are accompanied by a rise in a variety of growth factors, including IGF-I, which is generally not expressed in the adult artery (8). Since there is a significant temporal correlation between the rise of IGF-I levels in blood plasma (4), the initial burst of developmental aortic elastogenesis (1–4), and the production of a connective tissue matrix accompanying the atherosclerotic plaque (8), elucidation of mechanisms whereby IGF-I increases elastogenesis is of great interest.

Recently our laboratory has shown that the increase in elastin gene transcription elicited by IGF-I in aortic SMC is accompanied by a loss of DNA-protein binding to an inhibitor sequence in the proximal promoter (9). In situ footprint analysis of the deprotected region coupled with results from transient transfections performed with deletion constructs suggested that the specific sequence affected is −165 to −137 bp (EFE 5/6) of the promoter. Gel shift analysis of an oligomer representing this sequence with nuclear extract isolated from control SMC showed complex formation with Sp1 and other zinc-dependent transcription factors, whereas nuclear extract from IGF-I-treated cells did not form these complexes even though Sp1 was present. These results led us to hypothesize that IGF-I treatment results in the loss of Sp1 binding by the release of an inhibitor whose release may be mediated by the phosphorylation of Rb (10).

In the present study we have pursued the identification of other zinc-dependent factors capable of complexing with the EFE 5/6 sequence in an attempt to understand why this sequence acts as a repressor in control SMC. We have found that Sp3 is the other zinc-dependent protein capable of binding the EFE 5/6 sequence and that this transcription factor is capable of repressing Sp1-driven elastin promoter CAT activity in a Drosophila melanogaster cell line. Furthermore, through mutational analyses of the proximal promoter, we show that the repressor sequence within the proximal promoter is not the initially described EFE 5/6 but instead is a sequence located just downstream, i.e. −137 to −123 bp, which is very similar to a RCE element. Interestingly, Sp3 and Sp1 binding to this sequence appears to be differentially regulated by IGF-I treatment, thus providing a possible mechanism for derepression.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit polyclonal IgG preparations directed against the carboxyl-terminal 15 amino acids of the human Rb p110 sequence (Rb C-15), amino acid residues 520–538 of human Sp1, and residues 676–695 of the carboxyl terminus of human Sp3 and its corresponding peptide antigens were purchased from Santa Cruz Biotechnology, Inc. Polyclonal IgG directed against bacterially expressed Sp3 (11) was

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generously provided by Guntram Suske, Institut für Molekularbiologie und Medizin, and Dr. Maciej G. Czaja, National Cancer Institute, and double-stranded oligodeoxynucleotide corresponding to the Sp1 consensus sequence ATTCGATGGGGCGGGGGGCACC was purchased from Promega. Single-stranded oligomers and their complementary sequences corresponding to the wild type –165 to –137 bp EFE 5′/6′ element CTAATGGTCCATTTGCCGCCCGGT and mutated EFE 5′/6′ CTAATGGTCCCCGGAAGGCGGCCCGGT, as well as the wild type EFE 4′/5′, was purchased from the elastin promoter TCCCCGGGGCCCCCTCCCCAGGCTCCTCCCCCTCCT, were purchased from the DNA core facility at Boston University Medical Center. Double-stranded oligomers were prepared by heating the complementary strands to 95 °C for 5 min in 10 mM Tris, pH 8.0, 10 mM MgCl₂ and then allowing the mixture to cool to room temperature over a 2-h period. Total DNA from each corresponding experiment was gel purified with and without mutation of the EFE 5′/6′ were generated as described by Wolfe et al. (9). Insulin-like growth factor-I was purchased from New England Biolabs. Chloramphenicol acetyltransferase (CAT) enzyme used for positive controls was purchased from Pharmacia Biotech Inc.

Characterization of DNA Vectors—The –195CAT reporter construct containing –195 to +2 bp of the human elastin promoter was used to replace the SV40 early promoter in a pCAT vector as described previously (9). The mutated EFE 5′/6′ –195 to +2 bp expression construct (M5) was prepared by digesting the –195CAT reporter construct with restriction endonucleases Smal and SacII to remove a 50-bp fragment containing EFE 6 and EFE 5. The digestion products were resolved on a low melting agarose gel, gel purified with the gel purification kit, and the 20 CAT and the remaining elastin promoter (~4.5 kilobase pairs) were eluted from the gel. Two oligonucleotides (sense and antisense) corresponding to bases –195 to –146 bp and –195 to –148 bp, respectively, with mutated sequence from CCCCT to GAG in the region of EFE 5 (~151 to 148) were synthesized. The sequence of the sense oligonucleotide was GGGCCCGGGGACTGTTGACGGCCGGCCCTCTCTGCTTGTGACGGAC, and the antisense oligonucleotide GGGCCGACTCCCGAGAGCCTCGGTGGAGGCTTGTTGAGCCGGGCCCTTTCGTAATCTTGCAGGC. These oligomers were annealed and ligated to the 4.5-kilobase pair DNA was preincubated with nuclear extracts for 30 min at 25 °C before subjecting the sample to electrophoresis. For competition experiments, a 100-fold molar excess of unlabeled competitor DNA was preincubated with nuclear extracts for 30 min at 25 °C before the addition of radiolabeled probe. Western Blot Analysis—Nuclear extracts (50–100 μg) were fractionated on a 6% SDS-polyacrylamide gel according to Laemmli (23). The gel was electrophoretically transferred to nitrocellulose as described by Towbin et al. (24) before being probed with commercially available primary antibodies recognizing Sp1 and Sp3 used at 1:10,000 and 1:3,333 dilution, respectively. Horseradish peroxidase-conjugated Protein A secondary antibody was diluted 1:3000 for blots probed with Sp1 antibody and 1:1000 for blots probed with Sp3 antibody. Immunodetection of proteins was visualized by the ECL method (Amersham) according to the manufacturer's instructions.

DNase Footprint Analysis—Characterization of DNA-protein interactions by DNase footprint analysis was performed essentially as described previously (25). The –195 to +2 bp and –195 to +2 bp CAT reporter constructs were linearized with SacI, ligated with Klenow, and separated from free [32P]dNTP by the spin-column procedure (14). Radiolabeled DNA (0.1–2.5 ng, 20–200 fmol, 20,000–100,000 cpm) was incubated with nuclear extract (5–30 μg) or Sp1 (0.4 footprinting unit) and 10 μg of poly(dI-dC) for 30 min at room temperature. The resultant protein-DNA complexes were resolved on a 4% native polyacrylamide gel and visualized by autoradiography. For supershift experiments, 2 μl of specific antisera was preincubated with nuclear extract 30–60 min at room temperature, prior to the addition of radiolabeled probe. Reactions were allowed to incubate for an additional 30 min at room temperature before subjecting the samples to electrophoresis. For competition experiments, a 100-fold molar excess of unlabeled competitor DNA was preincubated with nuclear extracts for 30 min at 25 °C before the addition of 2 μl of [32P]-labeled DNA.

RESULTS

Sp3 Binds the EFE 5′/6′ Sequence—Previously, we have reported that Sp1 is capable of binding the EFE 5′/6′ sequence of the elastin promoter, the same sequence we have hypothesized is the major response element to IGF-1. In the latter study, we
demonstrated that other zinc-requiring transcription factors bind to this sequence and their binding, in addition to that of Sp1, is abrogated by IGF-I treatment of the SMC. What was somewhat puzzling to us was why the loss of Sp1 binding could result in an activation of elastin gene transcription, since our data suggested that IGF-I increased transcription of the elastin gene through a mechanism of derepression. Since several studies have shown that Sp1 is a member of a family of transcription factors (27, 28) whose individual members are able to confer either activation (Sp1, Sp2, and Sp4) or inhibition (Sp3) of transcription, we tested the possibility that Sp3 may bind the EFE 5/6 sequence.

Fig. 1 shows an autoradiogram of a gel shift analysis generated from the incubation of the EFE 5/6 element with nuclear extract from control SMC in the absence or presence of Sp3 antibody. Incubation of the probe with nuclear extract in the absence of antibodies results in the formation of three major complexes corresponding to complexes I, II, and III as shown previously (10). Interestingly, preincubation of SMC nuclear extract with antibodies to Sp3 results in the abolition of both complexes Ib and II. The majority of supershifted protein-DNA complex remains at the top of the gel, which is consistent with other studies using this antibody preparation (11). Although not shown, we have used a commercially prepared antibody preparation (Santa Cruz Biotechnology, Inc.) with similar reactivity of two closely migrating bands designated Ia and Ib, with Ia exhibiting the slower migration.

![Supershift analysis of the EFE 5/6 using antibodies to Sp3.](image)

**FIG. 1.** Supershift analysis of the EFE 5/6 using antibodies to Sp3. Ten μg of control SMC nuclear protein was incubated with 2 μl of Sp3 antiserum or no antiserum for 1 h on ice with 1 μg of poly(dI-dC). One ng of 32P-labeled EFE 5/6 probe was then added to the mixture, and incubated 30 min at room temperature prior to separation on a 4% equilibrated native polyacrylamide gel. Bands denoted I, II, and III represent three major protein-DNA complexes. Complex I is composed of two closely migrating bands designated Ia and Ib, with Ia exhibiting the slower migration.

Fig. 2 gives the result of a typical transfection experiment in which a constant amount of the −195CAT construct was transfected alone or with increasing amounts of either Sp1 or Sp3 expression plasmid. To reduce variations in transfection efficiency due to differences in DNA concentrations between experimental conditions, the total amount of DNA used was kept constant by supplementing with vector DNA. The results show that in the absence of Sp1 or Sp3, expression of the elastin reporter constructs is undetectable and that co-transfection with Sp1 but not Sp3 is able to activate expression of the reporter gene in a dose-dependent manner. Western blot analysis of nuclear extract prepared from parallel cultures transfected with either Sp1 or Sp3 vectors alone confirmed the expression of these factors (data not shown), eliminating the possibility that the lack of Sp3 transcriptional activation is due to the absence of protein. Additionally, these results demonstrate that the elastin promoter is capable of being driven by Sp1, which is consistent with its constitutive-like features (34).

In order to determine if Sp3 could inhibit Sp1 activation of the elastin gene promoter, co-transfection experiments were performed where −195CAT and Sp1 expression vector DNA amounts were kept constant and increasing amounts of Sp3 expression vector were co-transfected. The data obtained (Fig. 3) demonstrate that co-transfection of 1–8-fold excess of Sp3 to Sp1 expression vector DNA results in a dose-dependent, ~50% diminution of Sp1-driven activity as determined by densitometric analysis of the acetylated products. Although not shown, increasing the amount of Sp3 vector beyond a 8-fold excess to
40-fold did not significantly affect promoter activity. The fact that Sp3 does not completely abrogate Sp1 activation suggests that within a cellular environment the two transcription factors may bind differently.

**Mutation of the EFE 5/6 Sequence Fails to Mimic the IGF-I Effect on Elastin Promoter Activity**—Our previous study (10) coupled with the data described above showed that both Sp1 and Sp3 are capable of binding the EFE 5/6 sequence and that Sp1 activation of the elastin gene promoter is partially inhibited by Sp3 in functional analyses. Since our initial hypothesis was that IGF-I treatment of SMC results in an increase of elastin transcription through derepression, we examined the functional consequences of mutating the EFE 5/6 sequence to inhibit Sp1/Sp3 binding. The mutation introduced involved the change of CCC at residues −151 to −148 bp of the elastin promoter to GAGA. This mutation results in a EFE 5/6 oligomer that no longer is capable of binding proteins that are lost by IGF-I treatment of SMC (35). In order to test the effectiveness of the mutation on the binding of factors identified previously (10), the mutated versions of the EFE 5/6 oligomer and the larger 59-bp fragment (−195 to −136 bp) were incubated with either recombinant Sp1 or nuclear extract isolated from control SMC. The autoradiograms shown in Fig. 4 (A and B) demonstrate that the introduction of the mutation results in the loss of complexes affected by IGF-I (9, 10) and in the loss of ability of Sp1 to bind significantly.

Since the mutation of the EFE 5/6 sequence was able to mimic the IGF-I response with respect to the loss of DNA-protein complexes by gel shift analysis, this mutation was introduced into the −195CAT construct and analyzed in transient transfection experiments to determine the functional consequence on the promoter activity in both control and IGF-I-treated SMC cells. Fig. 5 provides the results of three separate transfection assays and includes an inset showing the actual autoradiogram of one typical transfection analysis. The data obtained demonstrate that the mutation introduced into the EFE 5/6 sequence causes a decrease in CAT activity in the control SMC and that IGF-I is able to up-regulate CAT activity in a manner comparable with the wild type promoter sequence.

Both of the results are contrary to our predictions, since we had anticipated that the inhibition of Sp1/Sp3 binding in the control SMC would functionally confer the same increase in CAT activity elicited by IGF-I and abolish any IGF-I up-regulation. These results clearly demonstrate that the EFE 5/6 element is not the repressor sequence responsible for the derepression postulated for IGF-I treatment and appears instead to bind positive mediators of transcription as mutation in this area decrease activity to ~65% of the wild type promoter.

**A RCE Sequence Positioned at −137 to −123 bp Binds Sp1/Sp3, and IGF-I Treatment Appears to Selectively Affect Sp3 Binding**—The results described above led us to re-examine the data that resulted in our initial targeting of the EFE 5/6 sequence as the IGF-I responsive sequence. This designation was based first on transient transfections of a series of elastin gene promoter constructs, which showed that a repressor sequence was present between −195 and −136 bp. The promoter frag-
ment spanning −136 to +2 bp had basal activity equal in magnitude to that conferred on the −195 to +2 by IGF-I and did not exhibit any significant responsiveness to IGF-I. This result, coupled with gel shift analysis of the promoter fragments showing that IGF-I treatment resulted in a loss of DNA-protein complexes within the −195 to −136 bp region, led to the hypothesis that IGF-I caused a derepression of elastin gene expression by abrogating binding to a repressor protein(s) (9). We further confirmed the ability of the specific sequence within the −195 to −136 bp fragment, i.e. the EFE 5/6 sequence, to exhibit a loss of DNA-protein binding complexes within nuclear extracts obtained from IGF-I-treated SMC (10).

Based on the above observations, we considered the possibility that the restriction cleavage generated by EcoNI 3′ of −136 bp to generate the −136 to +2 promoter/CAT reporter construct may have disrupted a critical repressor sequence. In examining the sequence surrounding the cleavage site, we have found a putative RCE sequence at −137 to −123 bp, CCCCCAGGCCTCCCC, which has 73% identity with −102 to −88 bp of the RCE identified in the promoter of the c-fos proto-oncogene CCCCAGGCCTCCCC (core element underlined) (36). The RCE of the c-fos gene has recently been shown to bind both Sp1 and Sp3 (37, 38). These latter investigations showed that in transient co-transfection assays in SL-2 cells both Sp1 and Sp3 can positively influence transcription through the RCE element and that both can be superactivated by co-transfection with Rb. This is the first demonstration that we are aware of which suggests that Sp3 may also act as a positive effector of transcription; the significance of this observation has yet to be determined. It is interesting to note that our laboratory has found that this area of the elastin proximal promoter is protected in DNase footprint analysis using nuclear extract isolated from control SMC and has been designated EFE 4 spanning −137 to −125 bp (see Ref. 9).2

In order to test whether the sequence spanning the EcoNI

Fig. 5. Transient transfection of SMC with wild type and mutated EFE 5/6 promoter constructs. SMC cells were transfected with 15 μg of either 195CAT reporter (WT) or the mutated 195CAT reporter (M5), followed by treatment with 50 ng/ml IGF-I (I) or control (C). Cell lysates were assayed for CAT activity, and the acetylated products were separated by thin layer chromatography and visualized by autoradiography (insert). Quantification was performed by exposure of the plate to a phosphorimager, and relative CAT activity was expressed as percent of the control (C) wild type −195CAT activity.

restriction site might be important in influencing elastin gene promoter activity in control and IGF-I-treated SMC, several different experimental approaches were used. First DNase footprint analysis of the wild type and mutated −195 to +2 fragments was performed with nuclear extracts isolated from control SMC to determine if the area surrounding the putative RCE element was protected. The footprint analysis given in Fig. 6 shows that the EFE 4 plus sequences immediately 5′ of this sequence are protected by DNA binding factors and that the footprint patterns in this region are not affected by the mutation introduced into the EFE 5/6 sequence.

Gel shift analyses were then performed using an oligomer encompassing −151 to −117 bp of the promoter and representing a continuum between the EFE 4 and 5 sequences. Fig. 7 shows a gel shift and supershift analysis using the radiolabeled EFE 4/5 probe incubated with nuclear extract from control SMC in the absence or presence of antibodies to Sp1, Sp3, and Rb. The probe forms three specific complexes with control nuclear extract that can be specifically competed with unlabeled probe (data not shown). Complex I′ appears as a broad band that is composed of three complexes, two of which can be individually supershifted with either Sp1 or Sp3 antibody or both abrogated by Rb antibody. The remaining component of complex I′, which is neither Sp1 nor Sp3, is not a zinc finger protein as titration with EDTA or 1,10-phenanthroline did not abrogate the binding of this protein (data not shown). Complex II′ is lost with the addition of either Sp3 or Rb antibody, showing that this complex is the result of Sp3 binding. Complex III′ exhibits a very broad band and may comprise multiple complexes formed by unidentified nuclear factors similar to those we have found binding to the EFE 5/6 sequence (10).

After determining that both Sp1 and Sp3 were capable of binding to the EFE 4/5 sequence in control cells, we next wished to determine if treatment with IGF-I would affect the binding of these factors. The EFE 4/5 probe was incubated with nuclear proteins from both control and IGF-I-treated cells in the presence and absence of excess unlabeled Sp1 consensus sequence (Fig. 8). When compared with the three major protein complexes formed from control nuclear proteins, nuclear ex-

FIG. 5. Transient transfection of SMC with wild type and mutated EFE 5/6 promoter fragments.Wild type (195) and mutated (M5) promoter fragments were subjected to DNase digestion after incubation of 32P-labeled DNA with control (C) nuclear extract or nuclear extract from SMC treated with IGF-I (I) as specified in the text. The free (F) lane represents digestion of the M5 32P-labeled DNA in the absence of nuclear proteins. The putative RCE is represented by the thicker bar, and the site of the mutation introduced into the EFE 5 sequence is represented by the thinner bar.

2 J. A. Foster and C. B. Rich, unpublished data.
Fig. 7. Gel shift analysis of the EFE 4/5 sequence. Ten μg of control SMC nuclear protein was incubated with 2 μl of either Sp1, Sp3, or Rb antiserum or no antiserum for 1 h at room temperature with 10 μg of poly(dI-dC). One ng of 32P-labeled EFE 4/5 probe was then added to the mixture and incubated 30 min at room temperature prior to separation on a 4% equilibrated native polyacrylamide gel. Bands denoted I′, II′, and III′ represent three major protein-DNA complexes.

Fig. 8. Gel shift analysis of the EFE 4/5 sequence with nuclear proteins from control and IGF-I-treated cells with and without competition with Sp1 consensus oligomer. Ten μg of poly(dI-dC) was incubated with 10 μg of nuclear proteins from either control (C) or IGF-I-treated (I) cells with (+) or without (−) preincubation with a 100-fold molar excess of Sp1 consensus sequence 30 min at 25 °C prior to the addition of 1 ng of 32P-labeled EFE 4/5 oligomer. The resultant protein-DNA complexes were separated by electrophoresis and visualized by autoradiography. Bands denoted I′, II′, and III′ represent three major protein-DNA complexes.

IGF-I Regulates Transcription of Elastin through Putative RCE Element

Previous studies from our laboratory have focused on elucidating the mechanism whereby IGF-I increases levels of aortic elastogenesis. Using neonatal SMC as a model system, we have shown that IGF-I is capable of increasing levels of elastin gene transcription in cells rendered quiescent. In addition, we have shown by transient transfections and gel shift analyses that the up-regulation of elastin promoter activity resides within an element residing in the −195 to −2 bp area of the promoter and that the overall effect appears to be the release of an inhibitor of transcription, as evidenced by the loss of protein-DNA complexes within the responsive area of the proximal promoter (9). Further investigation demonstrated that several zinc-requir-
IGF-I Regulates Transcription of Elastin through Putative RCE

Although not initially targeted as the IGF-I-responsive sequence, the EFE 4/5 area appears to be a potential site for a repressor sequence whose binding may be affected by IGF-I treatment. In order to test directly the significance of the putative RCE within this area, a mutation was introduced to this sequence and the consequences examined by transient transfections of SMC. The results obtained demonstrated that mutation of the RCE sequence results in a 3-fold increase in promoter activity in control SMC, which is consistent with the RCE binding a repressor. Within this study we have achieved our initial objective, which was to identify a DNA binding factor that could repress elastin gene transcription. Our results have also brought forth several new questions: specifically, how Sp3 can selectively bind to the RCE sequence over Sp1 in vivo and how IGF-I might preferentially affect Sp3 and not Sp1 binding to this sequence as determined in gel shift assays. In regard to the first question of Sp3/Sp1 selectivity in binding, Hagen et al. (27) have shown by structural analysis of cDNAs encoding Sp1, Sp3, and Sp4 that all three deduced protein possess a high degree of structural conservation, suggesting that they act through binding to the same DNA elements. Gel shift analysis using several different GT and GC boxes (11) as well as promoter fragments from different gene promoters (29–31) reveal that Sp1 and Sp3 can bind to the same DNA fragment. We have previously shown that Sp1 can compete effectively with Sp3 binding to the EFE 5/6 sequence, suggesting that the two factors share identical or overlapping sites (10). On the other hand, functional studies have suggested that the actual binding or the functionality imparted by the binding of Sp1 and Sp3 to specific sites within an active promoter fragment are not comparable. Using Drosophila SL-2 cells, Birnbaum et al. (30) examined the ability of Sp3 to repress Sp1-driven transcription from several eukaryotic promoters that contain G/C boxes. These investigators found that Sp3 repressed Sp1 activation with the dihydrofolate reductase promoter, which contains multiple functional G/C boxes. In contrast, Sp3 was not capable of affecting Sp1 activation with the histone H4 or the thymidine kinase promoters, both cell cycle-controlled promoters containing single G/C boxes. The data provided in the present study show that Sp3 can effectively inhibit only 50% of Sp1 activation, suggesting that the in vivo binding of the two factors is not equivalent. In composite these studies suggest that Sp3 abrogation of Sp1-driven transcription is promoter-specific. The specificity could depend upon the context and/or number of functional Sp1 binding sites, possibly by disrupting the ability of Sp1 to act synergistically or could reflect local changes in DNA structure, which result in the preferential binding of Sp3 over Sp1. It is interesting to point out in regard to this latter possibility that there is evidence suggesting that Sp1 binding to GC regions is influenced by DNA structure, specifically the formation of a non-B helix involving a single-stranded nature (39). In this regard, the elastin proximal promoter exhibits several hypersensitive DNase areas surrounding both the EFE 5/6 and EFE 4/5 sequences (see Fig. 6). This general area of the elastin gene sequence also contains several CT boxes that have been implicated in the regulation of the c-Myc promoter by imparting unusual DNA conformations, including single-stranded and triplex structures (40). We have further found that the EFE 5/6 sequence exhibits single-stranded DNA binding, as evidenced by competition with single-stranded oligomers and Southwestern analysis using these oligomers (35). Although we have not yet examined the ability of the EFE 4/5 sequence to confer single-stranded DNA binding, it may be possible that differential binding of Sp1/Sp3 is a result of DNA structural changes conferred by CT box binding factors such as heteronuclear ribonucleoprotein K and cellular nucleic acid-binding protein (41), which may be present in Complex III (see Fig. 7).

The second question raised by this study is how IGF-I treatment might selectively alter the ability of Sp3 and Sp1 to bind DNA. Our previous report showed IGF-I treatment results in an abrogation of Sp1 binding to the EFE 5/6 sequence (10). In the present study we have shown that Sp3 is a component of the complexes lost within EFE 5/6 sequence. We also show that binding to the EFE 4/5 oligomer exhibits a selective loss of Sp3 binding, suggesting that IGF-I might result in a cascade of events that result in differential effects in transcription factor binding. A potential mechanism for regulating the binding of Sp1 has been presented by Chen et al. (42). These investigators partially purified a protein (Sp11) from CV-1 cells of approximately 20 kDa, which, when released from hyperphosphoryl-
IGF-I treatment. Our future studies are focused on the structural and functional analysis where the repressor identified, i.e. p107 and p130 (44), for dictating control of numerous positive and negative transcription factors, the possibility exists that IGF-I treatment of quiescent SMC results in selective phosphorylation of Rb or one of its cohorts dependent upon activation of a specific cyclin. It is interesting in this regard to note that we have found that co-transfection of the −195CAT promoter with cyclin A, E, and D1 expression vectors resulted in activation of the elastin promoter with cyclin E, whereas cyclins A and D1 had no significant effect. These results suggest that the possibility that within quiescent SMC, IGF-I treatment, which does not cause the cells to enter S phase (9), results in activation of a specific cyclin activity whose function is to phosphorylate Rb or one of its family members resulting in the differential release of an inhibitor affecting primarily Sp3 or initiating a post-translational mechanism targeting Sp3.

In conclusion, the results from our study raise more questions than we originally asked. We began with a simple question, which sought to identify a putative repressor of elastin gene transcription whose binding was specifically affected by IGF-I treatment. We have uncovered a fairly complicated situation where the repressor identified, i.e. Sp3, appears to bind a sequence not previously targeted although not inconsistent with our previous data. Furthermore, we are now faced with different questions relating to the mechanisms dictating both preferential binding of Sp3 to the elastin promoter within the RCE sequence as well as selective abrogation of that binding by IGF-I treatment. Our future studies are focused on the structure of DNA sequences surrounding the RCE and on the impact of IGF-I treatment on specific events known to accompany cell cycle progression.

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