Evidence for a cell-specific negative regulatory element in the first intron of the gene for bovine elastin

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A cell-specific negative regulatory element has been identified in the first intron of the gene for elastin in a region between 442 and 464 bp from the translational start site. This regulatory element functions both when it is located 5' of the promoter and 3' of the chloramphenicol acetyltransferase (*CAT*) gene. The inhibition is observed both with the homologous elastin promoter and the heterologous SV1 promoter in transient expression experiments using rat aortic smooth-muscle cells. No inhibition was observed

with NIH 3T3, Hep G2 and little, if any, with HeLa cells. Cell specificity was further confirmed by DNA mobility shift assays and the position of the negative regulatory element was localized with the use of synthetic duplex oligomers. It is proposed that this negative element plays a significant role in the modulation of the expression of the gene for elastin in the smooth-muscle cells of the aorta during development.

INTRODUCTION

Elastin is a major structural protein of tissues, such as aorta, lung and skin, that require extensibility and recoil for their proper function (Anwar, 1990). The production of elastin, the principal precursor of elastic fibres, occurs during a fairly defined period during development (Davidson and Giro, 1986). Although there are characteristic differences in the developmental regulation of elastin synthesis in the various elastogenic tissue types, the bulk of elastogenesis occurs during late fetal and early neonatal periods and is essentially fully repressed by adulthood. Recently, various factors such as 1,25-dihydroxyvitamin D₃ (Pierce et al., 1992), 12-*O*-tetradecanoylphorbol 13-acetate (Parks et al., 1992), tumour necrosis factor- α (Kahari et al., 1992), transforming growth factor- β (Marigo et al., 1993), insulin-like growth factor-I (Wolfe et al., 1993) and interleukin-1 β (Mauviel et al., 1993) have been shown to modulate the level of expression of the gene for elastin. The complex regulation of elastin production provides a challenging model to study the molecular signals involved in developmentally regulated gene expression.

The recent isolation and characterization of genomic clones of human and bovine elastin, including the 5'-flanking region of these genes, have made it possible to study these genes at a molecular level (Yeh et al., 1989; Bashir et al., 1989; Kahari et al., 1990; Manohar et al., 1991). Deletion analyses of the 5'-flanking region of the gene for human elastin revealed the presence of several putative regulatory elements, but no cell-specific *cis*-element was detected in this region (Kahari et al., 1990). The promoter-chloramphenicol acetyltransferase gene (*CAT*) constructs used in these studies showed similar activities in elastogenic and non-elastogenic cells.

With the use of transient transfection and DNA mobility shift analyses we have identified the presence of a cell-specific negative regulatory element in the first intron of the gene for bovine elastin. The results of this study are described in this paper.

MATERIALS AND METHODS

Construction of *CAT* reporter clones

The 416-bp elastin promoter (–1 to –416) (Figure 1) was

constructed in several cloning steps as shown in Figure 2. First, an *Ava*I/*Ava*I restriction fragment (protruding ends were filled using Klenow fragment; Sambrook et al., 1989) of the elastin gene promoter (–1 to –135) was ligated into the *Sma*I site of pT7T3 19U (Pharmacia Biotechnology, Baie d'Urfe, Quebec, Canada). The orientation and possible recombination errors were screened for in each of the cloning steps by restriction mapping and/or DNA sequencing. The G at position –1 was contributed by the *Sma*I site (5'-CCCGGG-3') from the polylinker of pT7T3 19U, as confirmed by sequence data. Adjacent to the 3' end of the cloned 135-bp fragment is a *Bam*HI site in the pT7T3 19U polylinker and overlapping the *Ava*I site, at the 5' end of this fragment, is a *Stu*I site (–127 to –131). Further upstream of this *Stu*I site, in the elastin promoter, is a *Pst*I site (–411 to –416). The *Bam*HI/*Stu*I fragment from the previous clone was mixed with the *Pst*I/*Stu*I fragment from the elastin promoter and ligated into the *Bam*HI/*Pst*I sites of pT7T3 19U to yield the clone (P1) containing the 416-bp promoter.

The *CAT* gene was isolated from pSV2-CAT (Gorman et al., 1982) and ligated into the *Bam*HI/*Hind*III sites of pBluescript II KS[–] (pBS) (Stratagene, La Jolla, CA, U.S.A.) to produce the promoterless construct, pBS-CAT. The promoters were inserted 5' of the *CAT* gene using the available pBS polylinker sites.

The *Hind*III site marked the 5' end of the *CAT* gene in pBS-CAT. However, the available *Hind*III site in the P1 clone was on the 5' end of the 416-bp promoter. In order to reverse its orientation, P1 was digested with *Bam*HI and the protruding nucleotides were deleted by the exonuclease activity of bacteriophage T4 DNA polymerase (T4 pol) (Sambrook et al., 1989); after the T4 pol was removed by phenol/chloroform extraction, the 416-bp fragment was released using *Pst*I. The pT7T3 19U vector was first digested with *Sph*I and also treated with T4 pol, followed by digestion with *Pst*I. The *Pst*I/'blunt' fragment was then ligated into the *Pst*I/'blunt' vector. This resulted in the –1 position of the 416 bp promoter being adjacent to the *Hind*III site. This clone (P2) was then digested with *Hind*III and *Kpn*I to release the 416-bp fragment which was ligated into the *Hind*III and *Kpn*I sites of pBS-CAT to produce the 416-bp elastin promoter-CAT construct (pEL416-CAT) (Figure 3). To construct the 129-bp elastin promoter construct

Smooth-muscle-cell nuclear extracts were prepared from 8-day-old cultures. Extracts were prepared from smooth-muscle and NIH 3T3 cells using a protocol described by Chodosh (1988) and stored at -80°C . Briefly, cells were lysed by incubation in a hypotonic buffer [10 mM Hepes, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.2 mM phenylmethanesulphonyl fluoride (PMSF) and 0.5 mM dithiothreitol (DTT)] for 10 min on ice, followed by 10 strokes in a Dounce homogenizer. The nuclei were packed by centrifugation at 3300 *g* and resuspended in half volume of a low-salt buffer [20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl_2 , 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT], and then half volume of a high salt-buffer [20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl_2 , 1.4 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT] was added in a dropwise fashion while stirring. This mixture was gently stirred

Figure 2 Schematic illustration depicting the construction of the various CAT-plasmids

The *Aval/Aval* (−1 to −135) and the *StuI/StuI* (−130 to −416) fragments are from the elastin promoter, and the i136 *BamHI/PstI* fragment (382 to 517) is from the first intron (Figure 1). Experimental details are given in the text. The chimeric constructs, pEL129-CAT, pEL416-CAT, pSV1-CAT, pEL129-CAT-i136, pEL416-CAT-i136, pSV1-CAT-i136 and pi136-EL416-CAT, are described in more detail in Figure 3.

for 30 min to extract the nuclear proteins. The mixture was then centrifuged for 30 min at 15000 *g* in a SS34 rotor at 4 °C, and the supernatant containing the nuclear extract was removed and dialysed against 100 vol of buffer [20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT]. Protein concentration was determined by the Bio-Rad protein assay method (Bio-Rad Laboratories, Mississauga, Ont., Canada) according to the manufacturer's instructions.

DNA mobility shift assay

The DNA probe (i136-bp) was purified by gel electrophoresis using Biogel agarose and other reagents in the MERmaid kit as described by the manufacturer (BIO 101, La Jolla, CA, U.S.A.). The fragment (approx. 200 fmol) was radiolabelled with [α - 32 P]dXTP (> 3000 Ci/mmol) using Klenow fragment (Sambrook et al., 1989). Free radiolabelled nucleotides were removed using the Bio-Spin 6 column according to the manufacturer's instruc-

Figure 3 Schematic representation of the chimeric *CAT* constructs

The construct pEL416-CAT represents the *CAT* gene driven by the elastin promoter (–1 to –416); pEL416-CAT-i136 is the *CAT* construct driven by the 416-bp elastin promoter containing the 136-bp intronic fragment located 3' of the *CAT* gene (the orientation of the intronic fragment is in the 5' to 3' orientation in all constructs). The pi136-EL416-CAT construct contains the *CAT* gene driven by the 416-bp elastin promoter with the intronic fragment located 5' of the promoter; pEL129-CAT is the *CAT* gene driven by the –1 to –129 elastin promoter; pEL129-CAT-i136 is the *CAT* gene driven by the 129-bp basic elastin promoter and the intronic fragment placed 3' of the *CAT* gene; pSV1-CAT is the *CAT* gene driven by the SV1 promoter; pSV1-CAT-i136 is the *CAT* gene driven by the SV1 promoter and the intronic fragment is placed 3' of the *CAT* gene. The vector used to construct these chimeric constructs was pBS II KS⁺.

tions (Bio-Rad Laboratories). Nuclear extract (20 µg of protein) was preincubated for 10 min with 4 µg of poly(dIdC) in a buffer containing 70 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT, 0.1 % Triton X-100, 5 % (v/v) glycerol and 10 mM Tris, pH 7.5, in a final volume of 20 µl. The ³²P-labelled DNA probe was then added and incubated for 15 min. All incubations were carried out at ambient temperature. The incubation mixtures were resolved by electrophoresis through a 5 % non-denaturing polyacrylamide gel in 45 mM Tris/borate and 1.0 mM EDTA buffer, pH 8.0. Electrophoresis was performed at 25 °C at 100 V (12.5 V/cm). The gels were dried and analysed by autoradiography. In assays involving competitor and non-competitor DNA, a 10–250 molar excess of the respective DNA was added to the preincubation mixture.

Two single-stranded DNA oligomers and their complementary strands (there is a 3-base overhang on the 5' ends of each strand) were synthesized and purified by the HSC/Pharmacia Biotechnology Service Center (Toronto, Ont., Canada) using Pharmacia's Gene Assembler system. The first set of complementary DNA oligomers (C1) was 5'-TTGCACACCTGTGGACCCAG-3' and 5'-CCACTGGGTCCACAGGTGTG-3', and the second set (C2) was 5'-AGGGACAGGGGACCTGGGCT-3' and 5'-TCCAGCCCAGGTCCCCTGTC-3'. Duplex oligomers (C1 and C2) were prepared by heating complementary strands in 10 mM Tris, pH 8.0, 1.0 mM EDTA to 95 °C for 5 min and then slowly cooling to room temperature. The 5' overhangs of the oligomers were used for labelling with [α -³²P]dXTP or filled with unlabelled dXTP using Klenow fragment.

RESULTS AND DISCUSSION

A part of the nucleotide sequence of the gene for bovine elastin containing –1 to –416 of the 5'-flanking region, the first exon and 435-bp of the first intron is shown in Figure 1. The sequences relevant to this study and those which were used in the *CAT* constructs are identified. The intronic fragment (382 to 517) was

chosen because this fragment contains two regions that are highly conserved between the genes for human and bovine elastin (Manohar et al., 1991). The sequence 442 to 462 also contains a consensus enhancer core sequence (TGTGGA; Sassone-Corsi and Borrelli, 1986). The various *CAT* constructs (Figure 3) were transfected into cultured neonatal rat aortic smooth-muscle, NIH 3T3, Hep G2 and HeLa cells and the transient expression of the *CAT* gene constructs was measured by *CAT* assays. Neonatal rat smooth-muscle cells are known to synthesize elastin and transcribe the gene for elastin in culture, while the other cells are non-elastogenic (Oakes et al., 1982; Barone et al., 1988; Kahari et al., 1990; Wolfe et al., 1993).

Figure 4 shows examples of the results of representative *CAT* assays after transient expression of the *CAT* gene constructs in smooth-muscle cells. The results of all the transient transfection experiments are presented in Table 1. The *CAT* gene driven by elastin gene promoters (pEL416-CAT and pEL129-CAT) was expressed in all four cell types used. This observation is consistent with previously published reports that the 5'-flanking region of the gene for human elastin lacks cell-specificity elements (Fazio et al., 1990; Kahari et al., 1990). However, the presence of the intronic fragment (i136) from the gene for bovine elastin inhibited the transient expression of the *CAT* gene in all constructs, including pSV1-CAT-i136, in smooth-muscle cells by about 50–60 %. The degree of inhibition was somewhat less when the fragment was present 5' of the promoter (pi136-EL416-CAT; 48 %) than that observed when the fragment was placed 3' of the *CAT* gene (pEL416-CAT-i136; 65 %). Also, there was approx. 50 % inhibition when the basic elastin promoter was used (pEL129-CAT-i136), and with the SV1 promoter the inhibition was about 60 % (pSV1-CAT-i136). There was no inhibition in NIH 3T3 and Hep G2 cells and marginal, if any, in HeLa cells. These results clearly suggest that this intronic fragment contains a cell-specific negative regulatory element. It may be mentioned that the presence of regulatory elements in the intronic sequences of several genes such as those for different collagen types (Ramirez and Di Liberto, 1990; Vuorio and de Crombrughe, 1990) and

Figure 4 Representative *CAT* assays of rat aortic smooth-muscle cells transfected with the *CAT* gene constructs

Cells were transiently transfected with 20 µg of *CAT* plasmid and 10 µg of β -galactosidase plasmid (PCH110) using the calcium phosphate co-precipitation method and harvested 48 h after transfection. Results from the β -galactosidase assay were used to normalize the amount of cell lysates used for the *CAT* assay. The acetylated and non-acetylated [14 C]chloramphenicol were separated by t.l.c.

Table 1 CAT activity obtained from transient expression of the chimeric CAT constructs in rat aorta smooth-muscle, NIH 3T3, Hep G2 and HeLa cells

Cells were transfected with 20 μ g of CAT plasmid and 10 μ g of β -galactosidase plasmid (PCH110). The results from the β -galactosidase assays were used to normalize the amount of cell lysates used for the CAT assays. The values are given as percentage conversion of [14 C]chloramphenicol to its acetylated derivatives, as described in the Materials and methods section. The percentage conversion was set at 100% for pEL416-CAT, pEL129-CAT and pSV1-CAT. The activities of the corresponding constructs containing the i136 fragment are indicated as percentage $\pm$ S.D. Each data point represents the results from a minimum of three independent experiments.

Constructs	CAT activity (%)			
	Smooth muscle	NIH 3T3	Hep G2	HeLa
pEL416-CAT	100	100	100	100
pEL416-CAT-i136	35 $\pm$ 3.2	107 $\pm$ 3.5	90 $\pm$ 4.6	80 $\pm$ 7.8
pi136-EL416-CAT	52 $\pm$ 4.2	98 $\pm$ 5.9	99 $\pm$ 13.4	91 $\pm$ 18.2
pEL129-CAT	100	100	100	100
pEL129-CAT-i136	55 $\pm$ 8.0	91 $\pm$ 1.2	115 $\pm$ 15.1	94 $\pm$ 21.1
pSV1-CAT	100	100	100	100
pSV1-CAT-i136	37 $\pm$ 6.8	104 $\pm$ 4.6	103 $\pm$ 3.1	91 $\pm$ 6.2

immunoglobulins (Banerji et al., 1983; Gillies et al., 1983) has been well established.

The intronic fragment was also subjected to DNA mobility shift assays using nuclear proteins from smooth-muscle and NIH 3T3 cells. As shown in Figure 5(a) (lane 10), two discrete retarded bands (indicated by the arrow) above the free probe were observed in the presence of nuclear extract from smooth-muscle cells. There is also a diffuse band above these bands. The mobility shift of the lower of the two bands indicated by the arrow was essentially abolished by 60-fold excess and higher of unlabelled probe (Figure 5, lanes 3–5). The control competitor DNA is a portion of the elastin gene promoter sequence (–1 to –137) of similar length to the intronic fragment. No change in the intensity of these bands was seen with the control DNA, even at the highest molar concentration used (Figure 5a, lanes 6–9). Competition similar to the unlabelled fragment was also observed (Figure 5a, lanes 12 and 13 and 16–17; the lower band was more efficiently competed out) when the synthetic duplex oligomer C1, corresponding to region 442 to 464 in the intronic fragment, was used as the competitor. However, no change in the intensity of the retarded bands was observed with equal concentrations of the duplex oligomer C2, corresponding to region 482 to 504 of the intronic fragment (lanes 14 and 15). Furthermore, when the duplex oligomer C1 was used as a probe, two discrete retarded bands were also observed in the presence of nuclear extract of smooth-muscle cells (Figure 5b, lane 1). In this case, these bands are well separated from the free probe, apparently due to the small size of the probe (23-bp versus 136-bp from the intronic fragment, Figure 5a). Again the lower band was more efficiently abolished by the unlabelled C1 as compared with the upper band (Figure 5b, lanes 2 and 3), but the duplex oligomer C2 at equal concentration had no noticeable effect (Figure 5b, lane 4). Figure 5(c) shows the results of a DNA mobility shift assay with nuclear extract from NIH 3T3 cells. The shifted bands are apparently due to non-specific binding and the profile is very different from the assay in which smooth-muscle-cell nuclear extract was used. No change in the intensity of the retarded bands was observed, even when 250 molar excess of the duplex oligomer C1 (Figure 5c, lane 3) or C2 (lane 4) was used as the competitor. This

Figure 5 DNA mobility shift assays

(a) The DNA-binding assay was carried out using nuclear proteins from smooth-muscle cells and 32 P-labelled intronic fragment (136-bp). Competition was carried out with unlabelled intronic fragment, duplex oligomers C1 and C2 (C1 and C2 corresponds to the sequence 442 to 464 and 482 to 504 respectively; Figure 1) and an unrelated DNA fragment of comparable size (control). The amount (molar excess) of unlabelled DNA added was as follows: lanes 1–5, 10 $\times$, 30 $\times$, 60 $\times$, 100 $\times$ and 150 $\times$ of unlabelled i136 respectively; lanes 6–9 and 11, 30 $\times$, 60 $\times$, 100 $\times$, 150 $\times$ and 250 $\times$ of control DNA respectively; lane 12, 150 $\times$ and lane 13, 250 $\times$ of duplex oligomer C1; lane 14, 150 $\times$ and lane 15, 250 $\times$ of C2; lane 16, 150 $\times$ of each of C1 and C2; lane 17, 250 $\times$ of each of C1 and C2. Lane 10 contains the radiolabelled fragment and nuclear extract only, and lane 18 contains the radiolabelled fragment without addition of nuclear proteins (free probe). The arrow indicates the two discrete retarded bands. (b) The assay was carried out using nuclear extract from smooth-muscle cells and the probe was 32 P-labelled duplex oligomer C1. Lane 1 contains only nuclear proteins and the radiolabelled probe; lane 2, 150 $\times$ and lane 3, 250 $\times$ molar excess of unlabelled C1; lane 4, 250 $\times$ molar excess of C2; and lane 5 contains the radiolabelled probe (free probe). The arrow indicates the two discrete retarded bands. (c) In this binding assay nuclear extract from NIH 3T3 cells was incubated with the 32 P-labelled 136-bp intronic fragment. Lane 1 contains only nuclear proteins and radiolabelled probe; lane 2, 150 $\times$ and lane 3, 250 $\times$ molar excess of C1; lane 4, 250 $\times$ molar excess of C2; lane 5, 250 $\times$ molar excess of each of C1 and C2. Lane 6 is the radiolabelled fragment only (free probe).

suggests that the cognate *trans*-factor was absent in NIH 3T3 cells.

These results are consistent with, and provide additional support for, the cell-specific nature of the *cis*-acting element present in the intronic fragment. The competition experiments in the mobility shift assay with the synthetic oligonucleotides (Figure 5a, lanes 12–17) localized the *cis*-element to nucleotides 442 to 464 in the first intron of the gene for bovine elastin. As this sequence is highly conserved between the genes for human and bovine elastin, it is reasonable to suggest that this sequence plays a similar role in the gene for human elastin. A search of the GenBank Data Bank revealed the presence of a similar sequence in the fifth intron of the human troponin C gene, but to our knowledge the role of this sequence in that gene has not been examined (Gahlmann and Kedes, 1990). It has been reported that there is a phenotypic variation within smooth-muscle cells of aorta as a function of distance from the heart, and that there is a decreasing gradient of relative elastin synthesis and of relative abundance of mRNA for elastin down the length of the aorta (Davidson et al., 1985, 1986; Selmin et al., 1991). Therefore, it is

reasonable to suggest that the cell-specific negative regulatory element of the first intron reported here may play a role in this type of phenotypic variation within the smooth-muscle cells of aorta.

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