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Species Differences in cis-Elements of the Proα1(I) Procollagen Promoter and Their Binding Proteins

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Abstract Previous studies suggest that there may be species differences in the utilization of cis-elements of the type I collagen genes. The present study was designed to examine this possibility by focusing on two regions of the proα1(I) collagen promoter. One is the GC-rich A1 region (−194/168) that modulates transcriptional activity of the mouse promoter. The other contains a glucocorticoid response element (GRE) implicated in negative glucocorticoid regulation of the rat promoter. Unlike mouse A1 probes, probes representing the analogous human (−195/−168) and rat (−193/−179) regions failed to bind nuclear proteins in gel shift assays. Binding assays with mouse A1 probes containing base substitutions indicated that this behavior could be ascribed to five bases in the human, and two in the rat sequences. In addition, the pattern of expression of c-Krox, a protein that alters transcriptional activity via the mouse A1 element, differed in mouse and human tissues. Computer analysis revealed differences in the arrangement of GRE half-sites in human and rat proα1(I) collagen promoters. In a region of the human promoter (−700/673) analogous to the rat (−672/−633), there are three half-sites, each separated by two nucleotides, that cooperate in binding of glucocorticoid receptor. There also is a proximal half-site at position −335 of the human promoter that binds glucocorticoid receptor, but it is not present in the rat promoter. This study has defined several species-specific differences in the sequences and nuclear protein binding activity of regions involved in transcriptional activity of the proα1(I) collagen promoter. The results suggest that the A1 regions of the human and rat promoters examined here are unlikely to function as regulatory cis-elements, and they provide a framework for investigating the role of GREs in transcriptional regulation. They also suggest that species differences in cis-elements and transcription factors should be taken into consideration when using heterologous systems to study collagen gene regulation. J. Cell. Biochem. 73:408–422, 1999. Published 1999 Wiley-Liss, Inc.†

Key words: type I collagen; promoter elements; DNA binding; species specificity; glucocorticoid response element

Type I collagen, the most abundant type in vertebrates, belongs to the fibrillar class of collagens [van der Rest and Garrone, 1991]. Its triple helical structure consists of two subunits that are synthesized as the precursor chains proα1(I) and proα2(I). The subunits are encoded by separate genes that are expressed at high levels in bone, tendon, and skin. Transcription of the genes is regulated by several growth factors, hormones, cytokines, and nutritional factors [Slack et al., 1990], and it is stimulated in fibrotic diseases [Chojkier, 1993]. Appropriate transcription of the type I collagen genes is important for angiogenesis [Fouser et al., 1991], and wound healing [Clark, 1988]. Although type I collagen plays a major structural role, it also can influence the expression of other genes important in cell differentiation through interaction with integrins [Lin and Bissell, 1992]. Thus, the analysis of the factors involved in the regulation of the type I collagen genes is of great interest.

Minimal sequences required for the transcriptional activity of the proα1(I) promoter have been identified in several species. The CCAAT and TATA elements found within 100 bp upstream of the transcription start site are highly conserved [Slack et al., 1990]. In the mouse, a region between −222 and +116 of the proα1(I) promoter contains sufficient information to direct transcription in reporter gene constructs, and two elements (A1 and B1) within the −190 to −133 region play a role in modulating transcription [Karsenty and de Crombrugghe, 1990; Rhodes et al., 1994]. A factor in NIH-3T3 cell

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nuclear extracts inhibits transcription mediated through the A1 and B1 elements, and nuclear proteins from NIH-3T3 cells bind to these regions [Karsenty and de Crombrugghe, 1990, 1991]. Recombinant mouse c-Krox, a zinc finger protein, also binds to these regions [Galéra et al., 1994, 1996]. The −222/+113 region in the rat proα1(I) promoter directs transcription in transfection experiments, and regions analogous to the mouse A1 and B1 elements bind nuclear proteins that have not been characterized [Kovacs et al., 1996].

There is disagreement as to the minimal size of the human proα1(I) promoter required for transcriptional activity. Transfection and transgenic mouse experiments indicate that a 440 bp region of the human proα1(I) proximal promoter is sufficient in some cells [Houglum et al., 1995; Slack et al., 1990], and tissue specific expression in vivo has been obtained with a 476 bp promoter [Sokolov et al., 1995]. It also has been reported, however, that a 174 bp minimal promoter is sufficient for optimal transcription [Jimenez et al., 1994], which would suggest that the −190/−170 sequence analogous to the mouse A1 region, may not be involved in human promoter activity. Regions further upstream also modulate transcriptional activity of, or confer tissue specific expression to, the human [Slack et al., 1990], mouse [Rossert et al., 1995], and rat promoters [Dhalla et al., 1997; Dodig et al., 1996; Meisler et al., 1995].

Although there is a high degree of conservation between the type I collagen promoters in different species, there are some reported differences. In the mouse and human proα2(I) procollagen promoters the CCAAT box sequence is highly conserved, but only the human promoter contains a flanking sequence, the collagen modulating element, that participates with CCAAT binding factor (CBF) in regulating transcription [Collins et al., 1997]. TGF-β activates transcription of the proα2(I) promoter in mouse through an NF-1 related binding site [Rossi et al., 1988], while in the human, AP-1 [Chung et al., 1996] or Sp1 [Greenwel et al., 1997] sites are involved. Similarly, TGF-β activation of the human proα1(I) promoter utilizes an Sp1 binding sequence [Jimenez et al., 1994], while in the rat, a different site is involved [Ritzenthaler et al., 1993].

Species-specific regulation also could result from differences in the expression or properties of transcription factors. Human c-Krox contains an additional 117 amino acids at its N-terminal compared to the mouse protein, and it binds poorly to the region of the human proα1(I) promoter corresponding to the mouse A1 element [Widom et al., 1997]. The mouse protein is expressed mainly in skin [Galéra et al., 1994], but recent reports indicate that the human protein also is expressed in bone cells and lung fibroblasts [Heegard et al., 1997]. Overexpression of the human protein in mouse NIH-3T3 cells represses transcription of co-transfected rat, human, and mouse proα1(I) promoter constructs [Widom et al., 1997], but the mouse protein activates transcription [Galéra et al., 1994].

Many studies on the regulation of procollagen gene transcription are carried out with heterologous systems. For example, human or rat promoter activity has been analyzed in transgenic mice [Dodig et al., 1996; Houglum et al., 1995; Slack et al., 1990; Sokolov et al., 1995], or in transfected cells from different species [Jimenez et al., 1994; Ritzenthaler et al., 1993]. Therefore, it is important to elucidate species differences in the functioning of cis-elements of the proα1(I) procollagen promoter, and in the expression and properties of proteins that interact with them. Based on the suggestive evidence from previous studies [Heegard et al., 1997; Jimenez et al., 1994], we investigated the possibility that the A1 region in the human proα1(I) collagen promoter functions differently than in the mouse promoter, and also examined the expression of c-Krox in mouse and human cells and tissues. We carried out a computer analysis of the A1 region of the proα1(I) promoter, which predicted that there were no binding sites for known transcription factors in this region of the human promoter. That prediction was confirmed by DNA binding studies with probes containing mouse and human A1 sequences, and nuclear proteins from mouse and human cells. Therefore, an analysis of the sequence differences responsible for loss of binding to the human A1 probe was undertaken. A similar analysis was undertaken for the rat A1 region, which also differs from the mouse. Other regions in the proα1(I) promoter also were analyzed to determine if there were additional species differences in transcription factor binding sites. The analysis revealed that the upstream region containing a glucocorticoid response element (GRE) at positions −655/−650 in the rat gene [Meisler et al., 1995],

differed significantly in the human promoter. Therefore, a detailed study of the binding of glucocorticoid receptor (GR) and nuclear proteins to this region of the human and rat promoters also was carried out.

MATERIALS AND METHODS

Nuclear Extracts

HeLa cell nuclear extracts were purchased from Promega (Madison, WI), and NIH-3T3 nuclear extracts were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Balb 3T3-714 cells, a subclone selected for stringent contact inhibition [Hata and Peterkofsky, 1977], were grown in MEM-5-PIE medium [Peterkofsky and Prather, 1986]. Cultures were initiated at 5–7 $\times$ 10^5 cells per 100 mm dish in 15 ml medium, and were refed on day 3. Cells were harvested on day 4, and nuclear extracts were prepared as described previously [Dignam et al., 1983], except that Tris-HCl buffer replaced Hepes buffer in solutions, phenylmethyl sulfonyl fluoride (0.5 mM), and leupeptin (1 μg/ml) were present in hypotonic buffer A, and 0.5 M KCl replaced NaCl in the extraction buffer. One ml of hypotonic buffer was added to approximately 15–20 $\times$ 10^6 cells, and 2 μl of extraction buffer was added to nuclei from 10^5 cells. Protein concentrations of extracts were measured with BCA reagent (Pierce, Rockford, IL), and they ranged from 5–6 mg/ml. Fetal human skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository, and they were grown and passaged as described previously [Takeda et al., 1992], and were used at passages 10 and 13. Cells were seeded and harvested, and nuclear extracts were prepared, as described for Balb-3T3 cells.

Gel Shift Assay

Complementary oligonucleotides (6 μM) were annealed in 10 mM Tris-HCl, pH 8, and 10 mM $MgCl_2$ by sequential incubation at 95°C for 5 min, 50°C for 30 min, and 25°C for 1 h, and the products were analyzed on 4% agarose gels. Double stranded probes were end labeled with [γ-^{32}P]ATP, and free nucleotide was separated on Select-D columns (5 Prime-3 Prime, Boulder, CO). Nuclear extract (1 μl) was incubated for 10 min at 25°C in a total volume of 8 μl containing gel shift binding buffer (Promega, Madison, WI), and 0.05 mM $ZnClZ_2$, with or without nonradioactive oligonucleotides. Then 2 μl (0.08 pmoles) of ^{32}P-labeled oligonucleotide probe was added and incubation was continued for 20 min. Two μl of gel loading buffer was added, and samples were electrophoresed on 6% non-denaturing polyacrylamide gels (Novex, San Diego, CA). In most cases, the portion of the gel below the tracking dye was sliced off to prevent excessive diffusion of radioactivity from the free probe. The gels were dried and exposed to X-ray film at −80°C. Sequences of oligonucleotide probes and competitors are shown in the figures. Recombinant human glucocorticoid and RXRβ receptors were purchased from Affinity Bioreagents, Inc. (Golden, CO). Recombinant human RARγ receptor, and antibodies against Sp1 and Sp3 for supershift assays, were purchased from Santa Cruz Biotechnology, Inc.

Northern Blots and cDNA Probes

Human and mouse c-Krox cDNAs were prepared by reverse transcription and the polymerase chain reaction (RT-PCR) using mRNA from fetal human skin fibroblasts and Balb-3T3 cells, as described previously [Gosiewska and Peterkofsky, 1995], except that 200 units of SuperScript II RNaseH-free reverse transcriptase (Gibco BRL, Gaithersburg, MD), and 1.6 units/mL of RNasin (Promega) were used. The 18 bp oligonucleotide primers used were based on the mouse sequences at positions 1770 and 1940 [Galéra et al., 1994]. The sequences analogous to the primers in human cDNA are identical, except that the T in position 1944 of the mouse is a C in the human [Widom et al., 1997]. The RT-PCR products were 184 bp and 187 bp for the human and mouse, respectively, because of a three base pair difference in the sequences. This region was selected to ensure specificity, since it is outside the zinc finger domain. The PCR fragments were inserted into pGEM-T plasmid (Promega), and cloning was performed according to the manufacturer's instructions. The identity of the human probe was confirmed by sequencing, and the identity of the mouse clone was confirmed by comparison of HaeIII restriction enzyme digests of the human and mouse fragments. Only the human sequence has a HaeIII site because there is a G in position 1565, which in the mouse sequence is a T. Total RNA was extracted from fetal human skin fibroblasts, and mouse Balb-3T3-714 cells as described previously [Takeda et al., 1992]. Multitissue blots containing poly(A)$^+$ RNA from tissues of 9–11-week-old mice and adult hu-

mans, and poly(A)$^+$ RNA isolated from yeast and drosophila, were purchased from Clontech (Palo Alto, CA). cDNA probes were labeled with [^{32}P-α]dCTP by nick translation, and a 30 base oligonucleotide probe for 28S rRNA [Takeda et al., 1992] was end-labeled with [^{32}P-α]dATP and terminal deoxynucleotidyl transferase. Hybridization was carried out with ExpressHyb hybridization solution (Clontech) according to the manufacturer's instructions.

Western Blotting

A peptide corresponding to amino acid residues 222-234 in mouse c-Krox, and residues 335-347 in human c-Krox, was used to produce antiserum (no. 42075) in rabbits by Research Genetics Inc. (Huntsville, AL). c-Krox antiserum from the third bleed, or prebleed serum, were used at 1:1,000 dilutions. Antibody to the N-terminal region of human GR (SC-1003) was purchased from Santa Cruz Biotechnology, and was used at a 1:2,000 dilution. A control without antibody was processed in parallel. Nuclear proteins were separated by SDS-PAGE, and transferred to Immobilon-P membranes. After incubation of blots with antiserum or controls, immunoreactive protein was detected with biotinylated second antibody, and peroxidase substrate, according to the protocol provided with the Vectastain ABC reagents (Vector Laboratories, Burlingame, CA).

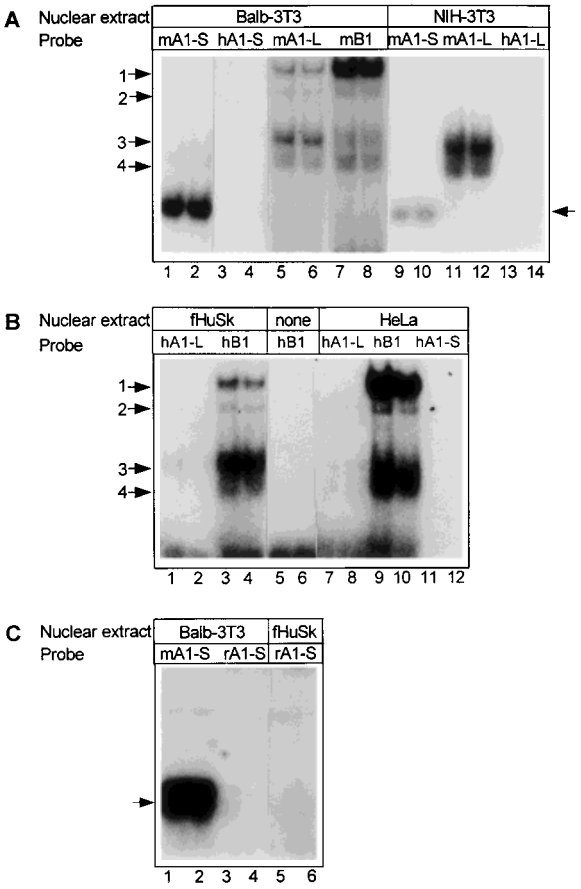
Computer Analysis of Promoter Sequences

The Transcription Element Search System (TESS) was used to analyze promoter sequences. The program can be accessed on the web at www.cbil.upenn.edu/tess/index.html.

RESULTS

Comparison of Human, Rat, and Mouse A1 Regions

Gel shift assays were carried out to determine if human and rat A1 regions displayed differences in binding of nuclear proteins compared to the well-defined mouse element. The sequences of the probes used are shown in Figure 1. Two different mouse probes were used. The mA1-L is identical to one used previously, and slightly longer than the footprints reported (underlined) at nucleotides −190/−170 [Karsenty and de Crombrugghe, 1990] or −190/−172 [Rhodes et al., 1994]. The shorter probe (mA1-S) represents a core region



PROBES		6 8 1213 1516 1819	
mA1-L	-194	TTGCGGGAGGGGGGGCGCTGGGTGGAC	-168
mA1-S	-189	GGAGGGGGGGCGCTG	-175
rA1-S	-193	GG**G**GGG**A**GGG**G**GCTG	-179
hA1-S	-189	**A**GAGGGG**A**G**AT**G**TG**G	-175
hA1-L	-195	CTGCGA**A**GAGGGG**A**G**AT**G**TG**GGGTGGAC	-168
hB1	-140	ATGGAGAGGGGGAGGAGGAGGGAAGGGA	-167
mB1	-133	CGAAGAGGGGGGAGGAGGGAAAGGAAGG	-160

Fig. 1. Failure of human and rat A1 probes to bind to mouse or human nuclear proteins. Gel shift assays were carried out on with the ^{32}P-labeled probes indicated above the lanes. The sequences of probes are shown at the bottom of the figure. The mA1-L and mA1-S probes represent longer and shorter sequences within the mouse (m) A1 region. The complementary sequence of the mB1 probe is shown. The reported mouse A1 and B1 footprints [Karsenty and de Crombrugghe, 1990; Rhodes et al., 1994] are underlined. The hA1-L, hA1-S, hB1, and rA1-S probes represent the analogous human (h) and rat (r) sequences. Differences in the rat and human A1 core regions at the numbered positions are shown in bold type. **A**: Nuclear protein from Balb-3T3 (**lanes 1–8**, 5 μg) or NIH-3T3 (**lanes 9–14**, 5 μg) cells. **B**: Nuclear protein from fetal human skin fibroblasts (fHuSk) at passage 13 (**lanes 1–4**, 5 μg), HeLa cells (**lanes 7–12**, 9 μg), or no extract (**lanes 5 and 6**). **C**: Nuclear protein from Balb-3T3 (**lanes 1–4**, 5 μg) or fetal human skin fibroblasts (fHuSk) at passage 10 (**lanes 5 and 6**, 5 μg). Four complexes formed with mA1-L (A) or hB1 (B) probes are indicated by arrows on the left, and the complex formed with the mA1-S probe is indicated on the right in A and on the left in C.

that contains almost all of the differences in the human and rat sequences (bold type) relative to mouse (numbered), as well as three positions (12–14) that were shown by G→A mutations to be required for binding and transcriptional activity of the A1 region [Karsenty and de Crombrugghe, 1990], and binding of c-Krox [Galéra et al., 1994]. The mB1 probe, shown as the complementary strand, represents the B1 region, which also modulates transcriptional activity of the mouse proα1(I) promoter. The other probes represent analogous regions in the human (hA1-L, hA1-S, hB1), and rat (rA1-S) promoters.

The mA1-L probe formed four complexes with Balb-3T3 nuclear proteins (Fig. 1A, lanes 5 and 6). Complexes 1 and 2 contained proteins related to Sp1, as determined by competition assays (data not shown). They are not observed with nuclear extracts from mouse NIH-3T3 cells (Fig. 1A, lanes 11 and 12), because of the low level of Sp1 binding activity in these cells (data not shown). Complexes 3 and 4 are formed with NIH-3T3 nuclear proteins (Fig. 1A, lanes 11 and 12), results similar to those described previously for binding of A1 probes and NIH-3T3 nuclear extracts [Karsenty and de Crombrugghe, 1990; Rhodes et al., 1994]. The mA1-S probe usually formed a single complex with both 3T3 extracts (Fig. 1A, lanes 1, 2, 9, and 10), although in some assays the complex was resolved as a doublet. This complex also was observed with nuclear extracts from HeLa cells, a human epitheloid carcinoma cell line, and fetal human skin fibroblasts (data not shown). Based on UV crosslinking and SDS-PAGE results, the proteins in mA1-L complexes 3 and 4 differ from the protein in the mA1-S complex (data not shown). The mB1 probe also formed four complexes with mouse Balb-3T3 nuclear extract (Fig. 1A, lanes 7 and 8). Binding with all three probes was specific since it was competed out by the unlabeled forms of the probes (data not shown).

Probes consisting of the human sequences corresponding to either the short (Fig. 1A, lanes 3 and 4) or long (Fig. 1A, lanes 13 and 14) mouse A1 probes did not bind 3T3 nuclear proteins. Failure of the human probes to bind was not due to the use of mouse nuclear extract. Neither the human A1-L (Fig. 1B, lanes 1, 2, 7, and 8) nor the human A1-S (Fig 1B, lanes 11 and 12) probes bound to nuclear proteins from fetal human skin fibroblasts or HeLa cells. The faint bands observed with the human A1-L probe represent nonspecific binding (data not shown). In contrast, the probe corresponding to the human B1 element specifically bound nuclear proteins from both cell types (Fig. 1B, lanes 3, 4, 9, and 10). Supershift assays identified the proteins in complexes 1 and 2 as Sp1 and Sp3, respectively (data not shown). There was no shift in the mobility of the hB1 probe in the absence of nuclear extract (Fig. 1B, lanes 5 and 6), and this was true for all of the probes used in this study (data not shown). A probe (rA1-S) for the rat sequence analogous to mA1-S, also failed to bind nuclear proteins from either mouse (Fig. 1C, lanes 3 and 4) or human (Fig. 1C, lanes 5 and 6) cells. Additional evidence for species differences was obtained by comparing mouse, rat, and human A1-S oligonucleotides as competitors (Fig. 2, lanes 3–10) against the mA1-S probe (Fig. 2, lanes 1 and 2). The human oligonucleotide did not compete even at a 75-times molar excess. The rat oligonucleotide was a slightly better competitor, but nevertheless a 75-times excess was required to achieve the same extent of competition achieved with the mouse oligonucleotide at a 10-times excess.

Mutants of Mouse A1 Probes With Human or Rat Base Substitutions

To investigate the basis for the species-specific loss of binding to the A1 region, we prepared oligonucleotides with individual substitutions in the mA1-S and mA1-L sequences that corresponded to the bases in the human or rat sequences, as indicated in Figure 3. We compared these to previously tested G→A mutations in the mouse A1 region at positions 12–14 (underlined). Mutations at positions 12–14 inclusively, and at position 14 alone, eliminate binding of NIH-3T3 nuclear proteins [Karsenty and de Crombrugghe, 1990], and the 12–14 mutant also does not bind recombinant c-Krox [Galéra et al., 1994]. The G at position 14 is conserved, but positions 12 and 13 differ in rat and human, respectively.

Binding of the wild-type mA1-S probe to nuclear proteins (Fig. 3A, lanes 1, 8, and 9) was eliminated by mutations at positions 12–14 (Fig. 3A, lanes 4 and 5), and 14 (Fig. 3A, lanes 6 and 7). Binding of mutant 12A was greatly reduced (Fig. 3A, lanes 10 and 11), but mutation at position 13 had no effect (Fig. 3A, lanes 12 and 13). The greater labeling of the complex with the 13A mutant was due to the higher specific

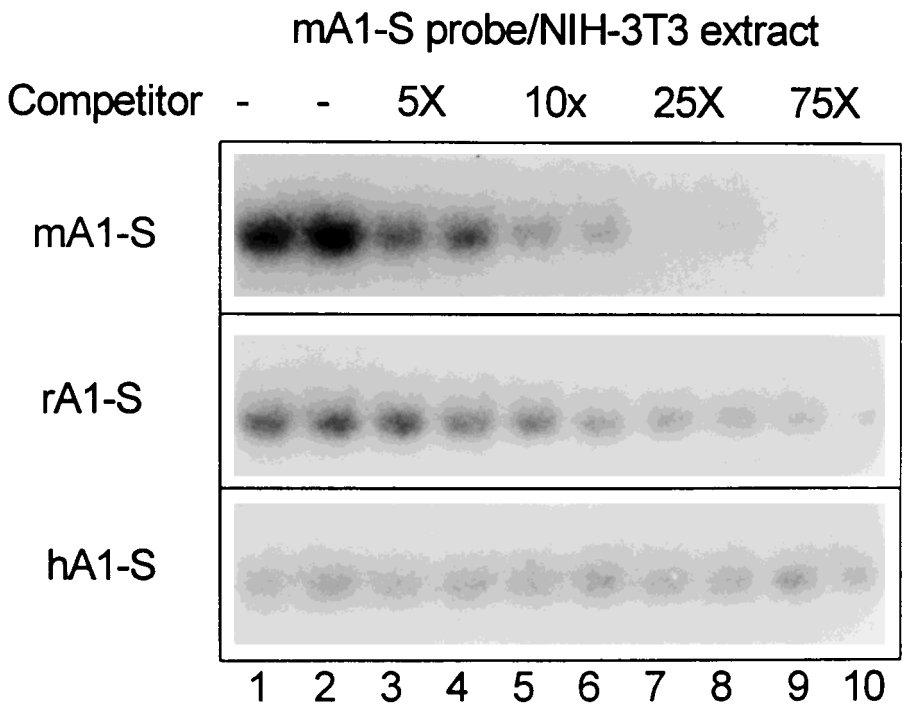


Fig. 2. Competition of mA1-S binding by mouse, rat, and human A1-S oligonucleotides. Gel shift assays were carried out with labeled mA1-S probe as described in the legend to Figure 1, except that either no competitors (**lanes 1 and 2**), or the unlabeled competitors indicated at the left of the panels, were added prior to the probe at 5, 10, 25, and 75-times molar excesses (**lanes 3–10**).

activity of the probe. Mutations in the A1-L probe at positions 14 (Fig. 3B, lanes 9 and 10) and 12-14 (Fig. 3B, lanes 7 and 8) essentially eliminated formation of complexes 3 and 4 compared to the wild-type probe (Fig. 3B, lanes 1 and 2). Complexes 1 and 2 were not observed since NIH-3T3 extract was used. The mA1-L8G mutant did not form complex 3, but labeling of complex 4 increased (Fig. 3B, lanes 3 and 4). With mutant 13A (Fig. 3B, lanes 5 and 6), complex 4 was not labeled, but a new, rapidly-migrating band appeared. Thus, these two mutations appear to affect binding of the A1 region to different proteins. Assays with Balb-3T3 also showed that the Sp1-related complexes were not affected by the 13A mutation (data not shown). A summary of binding experiments with mA1-S mutants and 3T3 nuclear extracts is shown in Table I. The positions at which the rat and human sequences differ and where mutations were made, are shown above the table. At position 16, only the T substitution found in the human sequence was tested, while a G is present in the rat sequence. All substitutions, except for 13A, reduced or eliminated binding. Although 8G was not tested for binding, it was a poor competitor (data not shown), suggesting that it would not bind avidly. Competition assays revealed that the affinities of all mutants, except for 13A, were lower than that of the wild-type, in agreement with the binding results (data not shown).

c-Krox and Collagen Gene Expression in Mouse and Human Cells and Tissues

Previous reports suggested that there may be species differences in c-Krox expression. Mouse c-Krox is expressed almost exclusively in skin, but not in bone [Galéra et al., 1994, 1996]. In contrast, human c-Krox is expressed in bone cells and lung fibroblasts [Heegard et al., 1997], as well as in skin fibroblasts [Widom et al., 1997]. Since such differences could lead to species-specific regulation of the proα1(I) collagen gene, we measured the gene expression of c-Krox relative to proα1(I) collagen in mouse and human cells and tissues. RNA from mouse and human cells was analyzed on Northern blots (Fig. 4). As controls, poly(A)$^+$ RNA from yeast (Fig. 4A, lane 5) and drosophila (Fig. 4A, lanes 6 and 7) was analyzed, since these organisms do not express type I collagen. c-Krox mRNA was expressed in HeLa and 3T3 cells and fetal human skin fibroblasts (Fig. 4A, top panel, lanes

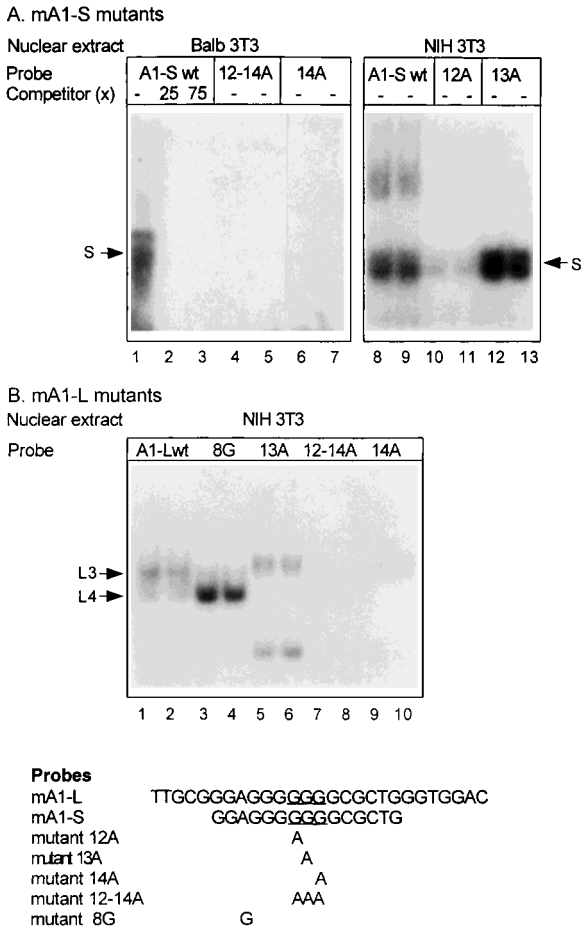


Fig. 3. Effect of substitution mutations on binding of mA1-S and mA1-L probes to mouse nuclear proteins in gel shift assays. The nuclear extracts used are indicated at the tops of the panels. Sequences of the probes are shown at the bottom of the figure with the positions of mutations indicated. **A**: mA1-S wild-type (wt) probe alone (**lanes 1, 8,** and **9**), and with unlabeled mA1-S wt oligonucleotide as competitor (**lanes 2** and **3**); mA1-S mutant probes (**lanes 4–7**, and **10–13**), as indicated at the top of the lanes. **B**: mA1-L wt and mutant probes, as indicated. The specific activities of probes were similar, except for A1-S14A (A, **lanes 6** and **7**) and A1-S13A (A, lanes **12** and **13**) which were 2.5 times that of the wild-type. Positions of major complexes are indicated at the sides of the figure.

1–4). The transcript size (3.9 kb) was in the range previously reported [Galéra et al., 1994; Heegard et al., 1997], although the transcript in HeLa cells was slightly smaller than in the other cells. HeLa cells contained at least three times more c-Krox mRNA than the other cell types, but they did not express proα1(I) collagen mRNA (Fig. 4A, top and middle panels, lanes 1 and 2 compared to lanes 3 and 4), while the level of proα1(I) collagen mRNA was highest in human skin fibroblasts (Fig. 4A, middle panel, lane 4). There was intense hybridization of the c-Krox probe with a shorter transcript in yeast. A BLAST search of the yeast database detected several short sequences that were identical to c-Krox at the nucleic acid level, but the transcripts do not code for other proteins.

Expression of c-Krox protein in cells was measured by Western blotting of nuclear extracts with a specific antibody. Specificity was established by using prebleed serum (Fig. 4B, lanes 1–6). The major specific band (arrow) corresponded to a protein of approximately 47 kDa for mouse (Fig. 4B, lanes 11 and 12), and the human protein was only slightly larger (Fig. 4B, lanes 7–10), which agrees with the theoretical molecular weights of the proteins. In agreement with the results of Northern blotting, the concentration of c-Krox protein in HeLa cells (Fig. 4B, lanes 9 and 10) was more than twice that in human skin fibroblasts (Fig. 4B, lanes 7 and 8), but about the same as in 3T3 cells (Fig. 4B, lanes 11 and 12). The results of Northern and Western blotting appeared to suggest that expression of c-Krox varied inversely with type I collagen expression. Further analysis, however, indicated this was not a general phenomenon.

Analysis of Northern blots containing poly(A)$^+$ RNA from either mouse or human tissues, also detected an approximately 3.9 kb c-Krox transcript (Fig. 5, top panels). c-Krox was expressed in a variety of tissues in both species, but the pattern of expression differed. Overall, c-Krox expression was lower in human tissues (Fig. 5, top right panel) than in mouse (Fig. 5, top left panel), but in both species there was no consistent correlation between these levels, and the concentration of proα1(I) collagen mRNA (Fig. 5, bottom panels). For example, in the mouse the c-Krox mRNA level was highest in kidney, while human kidney had one of the lowest levels (Fig. 5, top panels, lane 7), but both tissues contained essentially no proα1(I) collagen mRNA (Fig. 5, bottom panels, lane 7).

Glucocorticoid Response Elements in Proα1(I) Collagen Promoters

A region of the rat proα1(I) collagen promoter beginning at −900 bp before the transcription start site is required for glucocorticoids to suppress the transcriptional activity of the promoter, and a highly conserved TGTTCT GRE half-site at positions −655/−650 (Fig. 6A, bold underline) is implicated in the process [Meisler

TABLE I. Binding of mA1-S Mutants Containing Human and Rat Substitutions to NIH-3T3 Nuclear Proteins

		6 8 1213 1516 1819	
mA1-L	−194	TTGCGGGAGGGGGGGCGCTGGGTGGAC	−168
mA1-S	−189	GGAGGGGGGGCGCTG	−175
mutants		A G AA AT TG	

Site[a]	Occurrence	Binding[b]
wt		+
6A	human	−
8G	rat	nd
12A	rat	±
13A	human	+
15A	human	−
16T	human(rat)[c]	−
18T	human	−
19G	human	−

[a]Mutations are numbered according to positions indicated above mA1-L.
[b]Measured in gel shift assays; nd, not determined.
[c]G in the rat sequence.

et al., 1995]. A computer analysis was carried out to compare the rat and human sequences in this region of the promoter, which are shown in Figure 6A. GRE half-sites are indicated by inclusion of the complementary sequences and underlining, and the conserved sequence in the rat is underlined in bold. Another sequence at position −669 of the rat promoter (Fig. 6A, dashed underline) was not predicted by the computer program, but it is a functional GRE in the RUGα2μ-globulin gene [Chan et al., 1991]. The rat probes used in gel shift assays contained either both of these sites (rGRE), or only the highly conserved site (rGRE-S), and they are represented by bars above the rat sequence. The sequence of the rGRE-S probe is identical to the mouse sequence in this region. The analogous region of the human promoter (−704/−659) has only a 40% similarity to the rat sequence as determined by a BLAST sequence alignment. TESS analysis predicted a highly conserved GRE half-site at position −697 (no. 1, underlined), and a slightly divergent one at position −681 (no. 3, underlined). There also is a divergent sequence at position −689 (no. 2, dashed underline) that was not predicted by the program, but which is identical to a GRE in the RUGα2μ-globulin gene [Chan et al., 1991]. Probes containing all three sites (hGRE123), or only site 1 (hGRE1) or site 3 (hGRE1), were used in gel shift assays, and these regions are represented by bars above the human sequence. No other transcription factor binding sites were detected in the hGRE123 probe sequence, but a PEA3 site (ACATCCT) was detected at position −671 that is identical to one involved in phorbol ester regulation of the human collagenase gene. Computer analysis of other regions of the promoter also predicted a proximal GRE half-site at position −335 of the human promoter (Fig. 6B, underlined). This sequence is identical to the distal hGRE3, and it is part of an activating GRE in the rat c-H-RAS gene [Strawhecker et al., 1989]. The 33 bp sequence shown was used as a probe. A CBF binding site (GATTGG) also was predicted at position −328. No GRE sequences were detected in an analogous region of the rat promoter, but a divergent GRE half-site [Strawhecker et al., 1989] was predicted at position −337 of the mouse promoter.

Binding of Rat GRE Probes to GR and Nuclear Proteins

A probe containing the conserved GRE half-site at position −655 of the rat promoter binds to a nuclear protein from rat fibroblasts [Meisler et al., 1995], but it was not tested with isolated GR. Therefore, we used gel shift assays to determine whether the 40 bp rGRE probe bound to recombinant human GR, and for comparison also tested nuclear proteins from rat and human cells. The rat probe formed a single complex with GR (Fig. 7A, lane 3). With nuclear extract from rat KNRK fibroblasts, a complex was observed (Fig. 7A, lane 4, marker at right) that migrated slightly faster than the GR complex, and there also was a diffuse band below it. The binding pattern with HeLa extract was

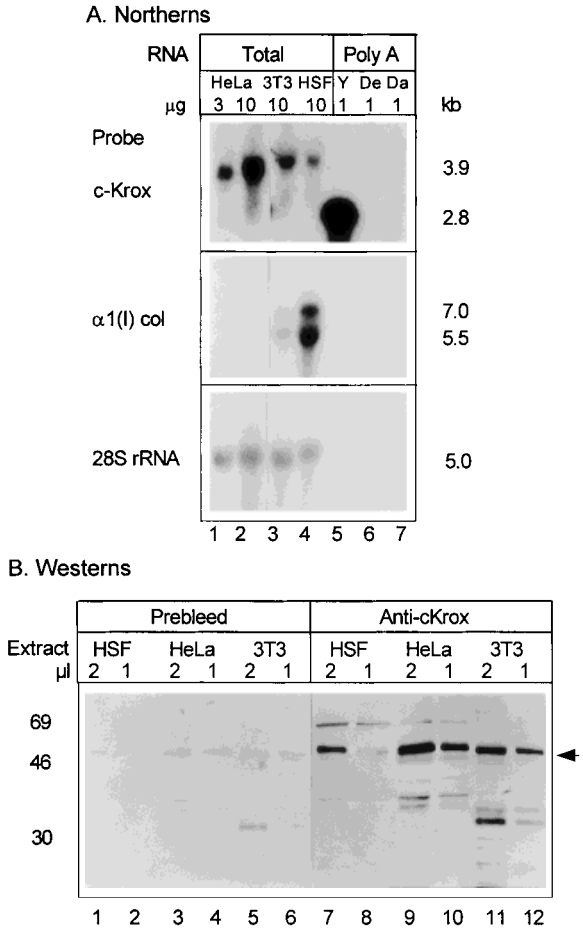


Fig. 4. c-Krox and collagen mRNA levels in human and mouse cells. **A**: Northern blot analysis was carried out with total RNA from HeLa and Balb-3T3 cells, and fetal human skin fibroblasts at passage 10 (HSF), or poly(A)$^+$ RNA from yeast (Y), and embryonic (De) and adult (Da) drosophila. Blots were hybridized sequentially with probes for c-Krox and proα1(I) collagen mRNAs, and 28S rRNA. Positions of RNA standards are indicated at the right. **B**: Western blot analysis of nuclear extracts from HeLa (9 µg/µl), fetal human skin fibroblasts at P-13 (5 µg/µl), and Balb-3T3 cells (6 µg/µl) with either prebleed or c-Krox antiserum at 1:1,000 dilutions, as indicated. The major specific, immunoreactive band of approximately 47 kDa is indicated by an arrow. Positions of molecular weight markers are shown at the left.

similar except that there also was a band at approximately the same position as the GR complex (Fig. 7A, lane 2, markers at left). These bands did not form in the absence of protein (Fig. 7A, lane 1).

Unlabeled oligonucleotides at 25-times molar excesses competed for binding of the rGRE probe to rat (Fig. 7B, lane 3) and human (Fig. 7C, lane 3) nuclear proteins, indicative of specific binding, but there was relatively little competition of the more rapidly migrating, diffuse band. Neither a consensus palindromic GRE (Fig. 7C, lanes 5 and 6) nor the hGRE123 (Fig. 7C, lanes 7 and 8) oligonucleotides competed against the rat probe for binding to HeLa nuclear proteins, even at a 75-times excess. With the rGRE-S probe containing only the conserved site, essentially no binding to nuclear proteins was observed when autoradiograms were exposed for the same time periods used for the longer probe (data not shown).

Binding of Human GRE Probes to GR and Nuclear Proteins

The ability of human GRE probes to bind recombinant GR and nuclear proteins also was examined in gel shift assays. Probes containing either GRE1 or GRE3 separately, along with partial GRE2 sequence, did not exhibit any binding to HeLa nuclear protein when gels were exposed to X-rays for the short time periods used to obtain the autoradiograms described below (data not shown). The hGRE123 and proximal hGRE formed a single complex with recombinant GR (Fig. 8, lanes 3 and 8) that migrated identically, while with the consensus palindromic GRE very little of this complex was formed, and the major complex migrated near the top of the gel (Fig. 8, lane 13). There was no significant binding of any of these probes to RAR or RXR (Fig. 8, lanes 4, 5, 9, 10, 14, and 15), which were tested because several of the hGREs are divergent, and retinoic acid receptor binding sites are related to GREs [Wahli and Martinez, 1991]. There was no binding in the absence of protein (Fig. 8, lanes 1, 6, and 11).

The hGRE123, hGRE$_{prox}$, and consensus GRE probes displayed similar binding to HeLa nuclear proteins, with three complexes formed, as indicated by the arrows (Fig. 8, lanes 2, 7, and 12). The same binding patterns were observed with nuclear extracts from fetal human skin fibroblasts (data not shown). GR is present in HeLa nuclear extracts as determined by Western blotting with an antibody to human GR, and it migrated identically to recombinant human GR at 96 kDa, which corresponds to the monomer form (data not shown). Coupled with the observed binding of the probes to recombinant GR, the results strongly suggest that the human GRE probes are binding to GR in nuclear extracts. An analysis of GR complexes with a negative GRE by electrophoresis in TBE gels demonstrated the formation of three complexes whose proportions varied with the concentra-

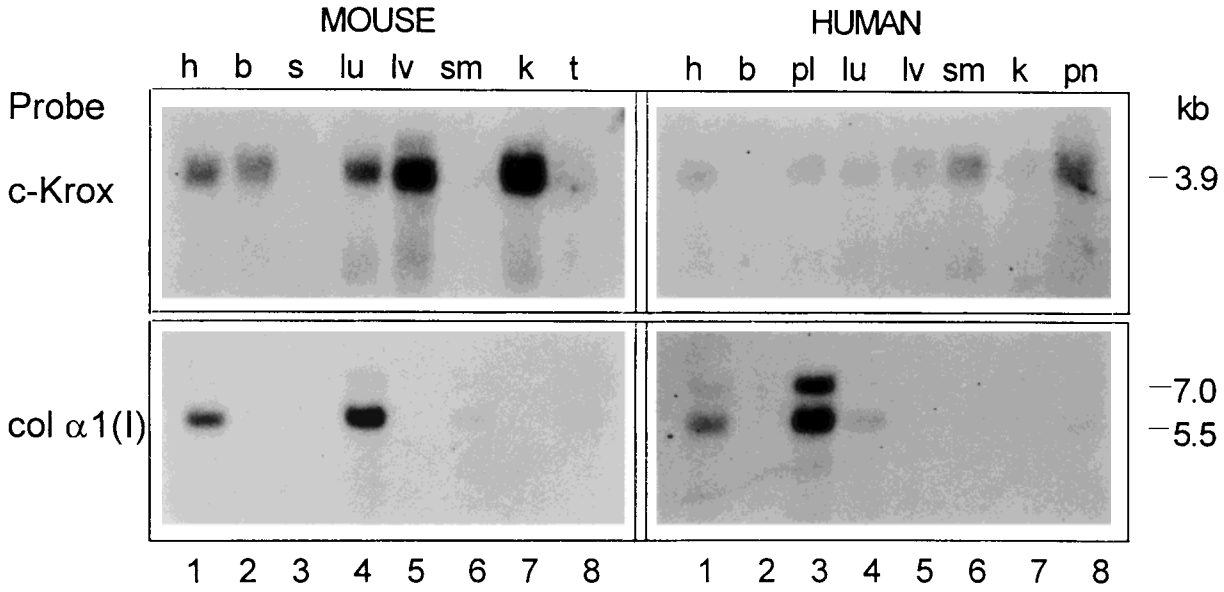


Fig. 5. c-Krox and collagen mRNA levels in human and mouse tissues. Multitissue blots contained 2 μg of poly(A)$^+$ RNA from heart (h), brain (b), spleen (s), lung (lu), liver (lv), skeletal muscle (sm), kidney (k), testes (t), placenta (pl), and pancreas (pn). Blots were hybridized first with the mouse (top left panel) or human (top right panel) c-Krox probes, and after stripping, with the proα1(I) collagen probe (bottom panels). The amount of radioactivity in the mouse c-Krox probe was approximately eight times greater than for the human c-Krox probe, but the human blot was exposed for a longer time to compensate for the difference.

tion of GR [Drouin et al., 1993]. Based on that analysis, the complexes formed between the human and consensus GRE probes with HeLa nuclear proteins appear to correspond to the monomer (complex 3), dimer (complex 2), and trimer (complex 1) forms of GR. From Western blots results, it was estimated that the amount of HeLa extract used in gel shift assays contained about 20% the amount of recombinant GR used, so the formation of multiple complexes with GR in nuclear extracts could result from this difference.

Competition experiments were carried out to determine if binding by the human GREs to nuclear proteins was specific and related to GR. Binding of the human and consensus GREs was specific, since labeling was competed out by the unlabeled forms (Fig. 9A–C, lanes 1–4). Both hGRE123 (Fig. 9A, lanes 5 and 6), and hGREprox (Fig. 9A, lanes 7 and 8) competed well against the consensus GRE, and the consensus GRE also competed against hGREprox (Fig. 9C, lane 6), providing further evidence that the probes bound to GR in nuclear extracts. Unlabeled hGRE123 and hGREprox competed well against each other (Fig. 9B, lanes 9 and 10 and 9C, lanes 7 and 8), but neither hGRE1 nor hGRE3 separately competed against hGRE123 (Fig. 9B, lanes 5–8). These results suggested that GRE1 and GRE3, and perhaps GRE2, cooperate in binding GR. Although TESS predicted a CBF binding site at position −328, an oligonucleotide with a functional conserved CBF site (TGATTGGCT) from the proα1(I) collagen promoter, as well as consensus NF-1 and C/EBP oligonucleotides, did not compete for binding by the hGREprox probe (data not shown).

DISCUSSION

The results of previous studies on factors involved in the regulation of the type I collagen genes suggested that there may be species differences in the utilization of cis-elements. The present study was designed to examine this possibility by focusing on two regions of the proα1(I) collagen promoter. One was the GC-rich A1 region that modulates the transcriptional activity of the mouse promoter [Karsenty and de Crombrugghe, 1990; Galéra et al., 1994], and the other was a region of the rat proα1(I) collagen promoter containing a GRE that has been implicated in the glucocorticoid suppression of promoter activity [Meisler et al., 1997]. A major observation was that, unlike mouse A1 probes, probes representing the analogous human and rat sequences failed to bind nuclear proteins in gel shift assays, and the basis for

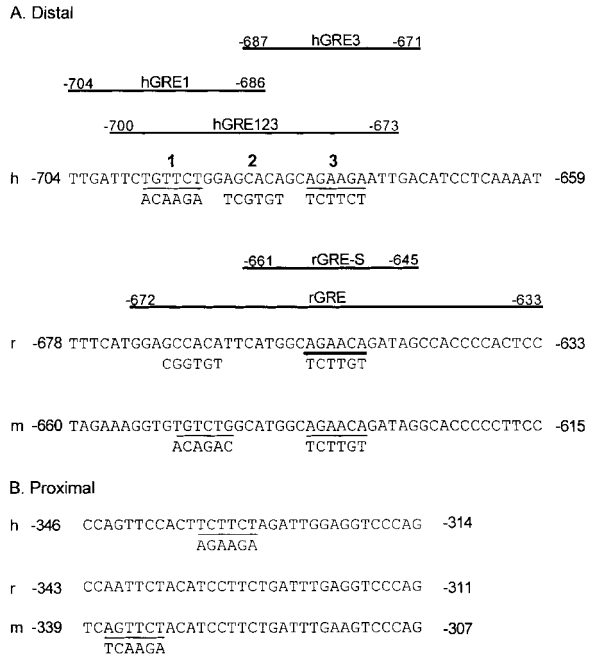


Fig. 6. Sequences of potential GREs in the human, rat and mouse proα1(I) collagen promoters and the oligonucleotides used as probes. GREs are shown with complementary sequences. Those predicted by the TESS program are underlined by a solid line. Those not predicted, but which are found as GREs in other genes as described in the text, are underlined with a dashed line. **A**: Distal GREs. Oligonucleotide probes are indicated as bars above the sequences they represent. A conserved GRE sequence in the rat proα1(I) collagen promoter predicted by computer analysis and previously reported [Meisler et al., 1995], is indicated by a bold underline. Two probes containing this sequence are designated as rGRE, and rGRE-S (short). The sequence of rGRE-S is identical in the mouse promoter. A region in the human promoter analogous to the rGRE sequence contains a highly conserved GRE half-site at −697/−692 (no. 1), and less conserved GREs at −689/−684 (no. 2), and at −681/676 (no. 3). The human (h) probes are designated according to which of these three sites are present. **B**: Proximal GREs. A region of the human promoter containing a predicted GRE at −335/−330 was used as a probe. No GREs were predicted in the analogous rat region, and one divergent sequence was predicted in the mouse.

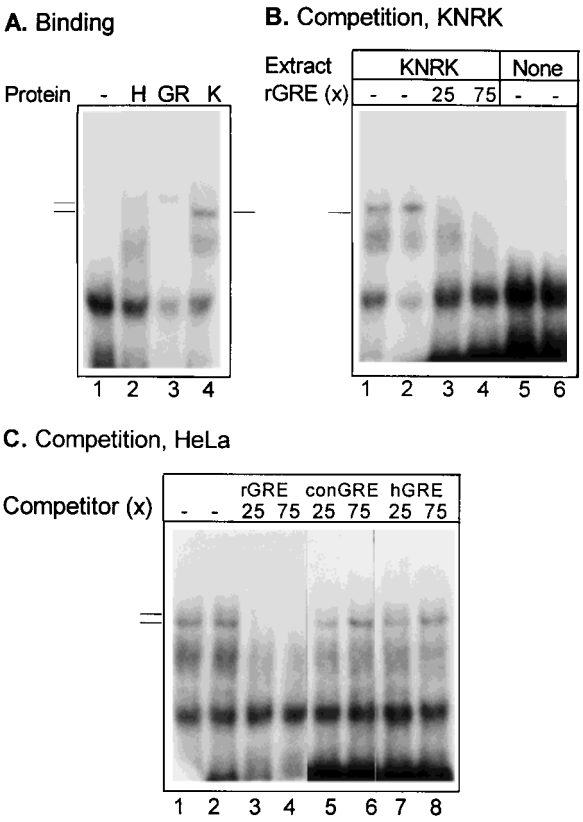


Fig. 7. Binding of the rat GRE probe to human and rat nuclear proteins and recombinant GR. Gel shift assays were carried out with labeled rGRE. The sequences of probes and competitors are described in Figure 6. **A**: No protein (−), HeLa (H) or rat KNRK (K) nuclear proteins, and recombinant human GR. **B**: No protein (none) or KNRK nuclear extract, without (−) or with unlabeled rGRE at 25 and 75 times molar excesses. **C**: HeLa nuclear extract without (−) or with the competitors rGRE, consensus (con) GRE, and hGRE123. The consensus GRE probe, AGAGGATCAAGAACAGGATGTTCTAGAT, contained a TGTTCT palindrome (underlined).

these differences was determined. In addition, the pattern of expression of c-Krox, a nuclear protein that binds to the mouse A1 element, differed in mouse and human tissues. With regard to the region containing the GRE, computer analysis revealed that the arrangement and location of GRE half-sites differs considerably between the human and rat proα1(I) collagen promoters. Probes containing human or rat GREs bound to recombinant GR and GR in nuclear extracts, but there were differences in their binding properties that may be related to their spatial arrangement.

As predicted by computer analysis, the human A1 element did not display any specific binding to human or mouse nuclear proteins in gel shift assays, and it did not compete against mouse probes for binding. In contrast, the mouse core region (−189/−175), formed a single complex with mouse and human nuclear proteins, and a slightly longer probe formed four complexes. The human A1 core sequence differs from the mouse sequence at six positions. Substitution of a human base at five of these positions individually in the mouse A1-S probe, eliminated binding by reducing the affinity of the probe. Failure of the human A1 sequence to bind to nuclear proteins can be explained by the cumulative effect of the five base differences, and the results suggest that the A1 region is

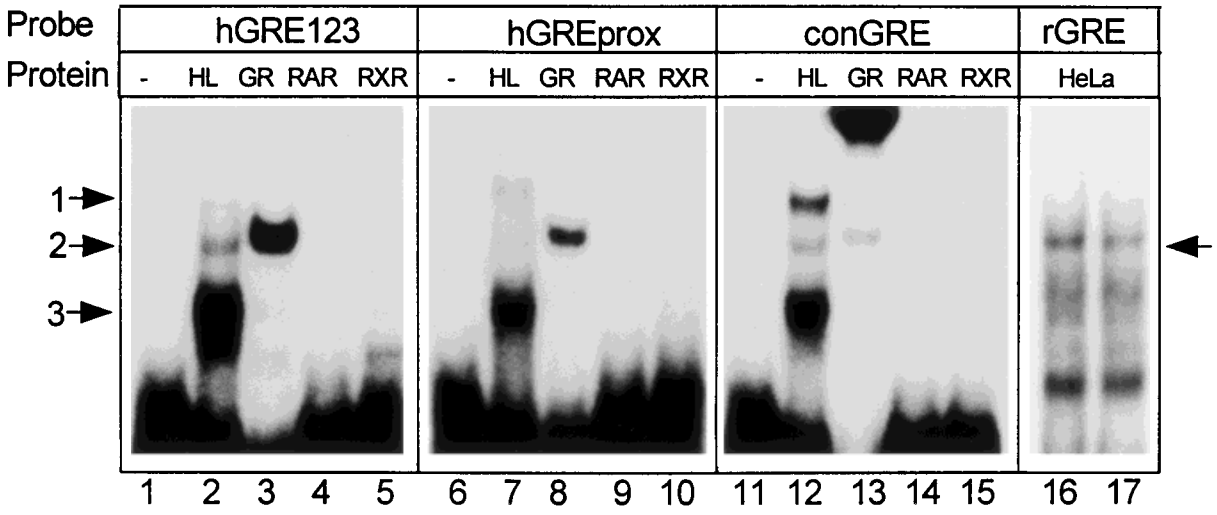


Fig. 8. Binding of GREs to recombinant human nuclear receptors and HeLa nuclear proteins. Gel shift assays were carried out with the hGRE123, proximal (prox) hGRE, and rGRE probes described in Figure 6, and without protein (−) or with HeLa (HL) extract (9 μg), GR (0.5 ng), RARγ (0.2 μg), and RXRβ (2 μg). The position of the specific complex formed with the rGRE probe is indicated by an arrow.

unlikely to play a role in determining the transcriptional activity of the human promoter. On the other hand, a probe containing the human sequence analogous to the mouse B1 region bound to several nuclear proteins, which suggests that it may be important in regulation. These results could explain previous observations that a segment of the proα1(I) collagen promoter starting at position −174, which does not contain the A1 region but does contain the B1 region, is sufficient for optimal transcriptional activity [Jimenez et al., 1994], and that human c-Krox fails to bind to the human A1 region [Widom et al., 1997]. The rat core region also did not bind to nuclear proteins, although it competed weakly against the mouse probe. This result may be explained by the fact that, compared to the human, the rat A1 sequence has fewer differences from the mouse. Substitution at position 12 in the mouse A1 sequence with a base that occurs in the rat, greatly reduced binding, but did not eliminate it. Although the C→G rat substitution at position 16 was not tested, a C→T human substitution eliminated binding, so this position is critical for binding. These data predict that the core A1 region (−193/−179) of the rat promoter does not have a regulatory role. Adjacent sequences, however, may be important since a probe with six additional base pairs at the 5′ end and two at the 3′ end formed a specific complex with rat fibroblast nuclear proteins [Kovacs et al., 1996].

Although previous reports indicated that mouse c-Krox is expressed mainly in skin [Galéra et al., 1994], our study found relatively high concentrations of its mRNA in a number of other tissues. The pattern of expression in mouse and human tissues differed considerably, and overall expression was lower in human tissues. In the mouse, the highest level of expression was in kidney and liver, while expression was relatively low in the corresponding human tissues. In addition, there was no correlation between levels of c-Krox and proα1(I) collagen mRNAs in mouse and human tissues. Thus, while c-Krox affects proα1(I) collagen transcription in isolated systems [Galéra et al., 1994; Widom et al., 1997], it appears to be subordinate to other factors in vivo.

Type I procollagen gene expression is downregulated by glucocorticoid treatment, although there is some disagreement as to how this is achieved. Several studies suggest that proα1(I) collagen mRNA stability is decreased by glucocorticoid treatment [Mahonen et al., 1998; Raghow et al., 1986], while others indicate that regulation is at the transcriptional level [Cockayne et al., 1988; Meisler et al., 1997]. Glucocorticoids regulate transcription positively by binding of GR to a palindromic sequence of two hexamers separated by three nucleotides, or negatively by two different mechanisms [Karin, 1998]. GR can interfere with other transcription factors such as AP-1 by direct interaction

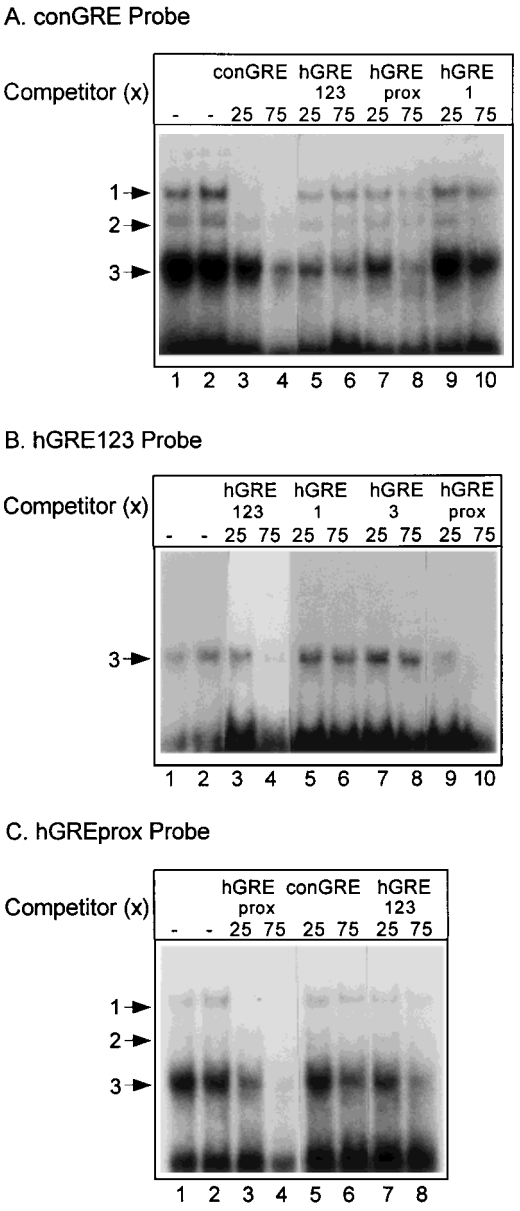


Fig. 9. Binding and competition of human and consensus GRE probes with HeLa nuclear proteins. Gel shift assays were carried out as described in the legend to Figure 8. The competitors used are indicated at the top of the lanes. The three complexes formed with the consensus (con) GRE and the proximal (prox) hGRE are indicated by arrows. For hGRE123, only complex 3 is visible, but longer exposures revealed complexes 1 and 2.

without binding to DNA, or by binding to a negative GRE that may be quite divergent from the prototypical sequence, and may be either a bivalent hexamer [Drouin et al., 1993] or a half-site [Subramanian et al., 1997].

Negative regulation of the rat proα1(I) collagen promoter by dexamethasone is attributed to a conserved GRE half-site at −655/−650 [Meisler et al., 1997]. Mutation of this site, however, does not eliminate the response to glucocorticoid, which may be due to the involvement of the TGF-β response element [Meisler et al., 1997]. In addition to the previously described conserved AGAACA half-site in the rat promoter at position −655, a divergent GRE half-site (TGTCTG) that had not been reported previously, was detected six nucleotides upstream. The rat probe containing both GREs bound to GR, but a shortened probe lacking the divergent GRE did not. This finding suggests that the divergent GRE or another flanking sequence is required for binding. In the analogous distal region of the human promoter, there are three half-sites separated from each other by two nucleotides. The three distal elements appear to cooperate in binding of GR, based on the observation that probes containing either GRE1 or GRE3, and only partial GRE2 sequence, did not bind GR, while a probe containing all three sites (hGRE123) did bind. This conclusion is supported by the finding that the separate hGRE1 and hGRE3 oligonucleotides did not compete against binding of hGRE123. Another species difference was that a divergent GRE half-site was predicted by computer analysis in a proximal region of the human promoter at position −335, but none was detected in the analogous region of the rat promoter. The proximal human GRE also bound recombinant GR and GR in nuclear extracts, which potentially could mean that glucocorticoid regulates the human promoter at both distal and proximal sites, but only at one site in the rat.

The gene for osteocalcin, a bone-specific extracellular protein, also is negatively regulated by glucocorticoid. The species-specific location of GREs in the proα1(I) collagen promoter resembles the arrangement of GREs in the rat and human osteocalcin promoters. In the rat osteocalcin promoter, there is a proximal region with two GRE half-sites separated by four nucleotides located downstream of the TATAA box; a half-site within the OC box adjacent to the CCAAT box; and two half-sites separated by three nucleotides in a distal location [Aslam et al., 1995]. All of these GREs contribute to negative regulation of rat osteocalcin by glucocorticoid in cells [Aslam et al., 1995]. In the human osteocalcin promoter, there is a GRE overlapping the TATAA box, and GR competes for binding of TATA binding factor, resulting in down-regulation of transcriptional activity [Meyer et

al., 1997]. The human promoter does not contain a distal GRE, or the half-site in the OC box [Aslam et al., 1995].

In summary, this study has defined several species-specific differences in the sequences and nuclear protein binding activities of regions known to affect the transcriptional activity of the mouse proα1(I) collagen promoter, and in the expression of c-Krox which regulates mouse promoter activity through the A1 region. The results strongly suggest that the A1 region of the human promoter, and a core A1 region of the rat promoter, are unlikely to act as regulatory cis-elements. The identification of several GREs in the proα1(I) collagen promoter that are functional with respect to binding of GR provides a framework for investigating whether they also function in regulating transcription. The results suggest that the different arrangement and location of these GREs may lead to different mechanisms of regulation in the rat and human proα1(I) collagen promoters, similar to the species differences observed for regulation of the osteocalcin promoter. The species differences reported here also could potentially affect the outcome of studies using heterologous systems to study the regulation of procollagen gene transcription.

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