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Characterization of a glucocorticoid responsive element and identification of an AT-rich element that regulate the link protein gene

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ABSTRACT

The cartilage matrix is composed of characteristic components including type II collagen, aggrecan and link protein. In this paper, we report two DNA elements that regulate the link protein gene. Using transient transfection assays with link protein gene constructs in chondrocytes, chloramphenicol acetyl transferase (CAT) assays were used to measure the transcriptional activity of the link protein gene. Previously, we identified an enhancer-like activity within the first intron of the gene. In this paper, we report an active 34 bp (+1390 to +1424) fragment within this region that contains a glucocorticoid-like response element (GRE). Both deletion of, and site-specific mutations within this sequence motif reduced the dexamethasone-inducible activity. The GRE-like sequence from the rat link protein gene, or the homologous sequence from the human link protein gene were included in vectors containing the thymidine kinase promoter linked to the CAT gene (tkCAT). Both human and rat elements transferred the ability to respond to dexamethasone and hydrocortisone with a >10-fold induction. Deletions through the promoter from –923 to –900 identified a second site required for both glucocorticoid and serum responsiveness. A four base substitution at this site resulted in a loss of serum responsiveness. This region contains an AT-rich element, similar to the AT-rich elements involved in homeotic protein regulation of the growth hormone gene and the muscle creatine kinase gene. Southwestern analysis using oligonucleotides containing the AT-rich element from the link protein gene or the muscle creatine kinase gene, identified a 32 kDa protein band from nuclear extracts of chick chondrocytes. Using these AT-rich oligonucleotides in band-shift analyses, nuclear extracts of chick sternal muscle, rat chondrosarcoma and chick sternal chondrocytes each showed formation of different complexes suggesting cell specificity. AT-rich elements have been identified as binding sites for homeodomain-containing proteins and can contribute to gene regulation by serum response factors. The identification of an AT-rich element in the link protein gene suggests similar functions for this element.

INTRODUCTION

Chondrocytes produce a unique extracellular matrix in which type II collagen fibers are embedded in a large aggregate structure of aggrecan (proteoglycan core), link protein and hyaluronic acid (for review see 1–3). The interactions between aggregate and collagens are responsible for the mechanical properties of cartilage, including stiffness, resistance to shearing and compression, and elasticity. The synthesis of components of the cartilage matrix is regulated by vitamins, hormones and growth factors (4–11). The effects of growth factors are dependent on the developmental stage of the cartilage (6,10,11). Aggrecan and link protein are also expressed in some tissues other than cartilage though at much reduced amounts (12,13).

Glucocorticoids affect gene expression in a number of cell types and tissues. A direct affect on transcription is mediated through the glucocorticoid response element (GRE; refs 14–16). Glucocorticoids, such as dexamethasone, affect the cartilage matrix, but their actions are controversial. Dexamethasone can inhibit proteoglycan synthesis *in vivo* (17,18) and *in vitro* (19,20). However, some reports show that glucocorticoid treatment stimulates proteoglycans synthesis *in vitro* (21,22). These results may be related to differences in experimental protocols, animal models, the type of cartilage and the concentrations of glucocorticoids utilized (23–25).

Glucocorticoid treatment can also induce changes in chondrocyte morphology, differentiation and viability. Morphological changes include the induction of a pericellular refractile area, an increase in the number of club-shaped membrane protrusions (26) and the loss of endoplasmic reticulum. At high dexamethasone concentrations, cell death results (27). Glucocorticoids are required for the maintenance of cartilage explants in organ culture, and for the continued growth of prechondrocytic mesenchymal cells under low serum conditions in cell culture (28). In addition, treatment with glucocorticoids can induce the differentiation of chondrocytes from a progenitor cell line, RCJ3.1 (28). Glucocorticoid receptors have been identified in cartilaginous tissues including adult articular cartilage (29).

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Glucocorticoid responsiveness can be modulated by other factors. For example, additional factors such as growth hormone, insulin-like growth factor (IGF-I) and insulin may act synergistically to regulate cartilage matrix synthesis. Dexamethasone and growth hormone synergize to stimulate the formation of a cartilaginous matrix by the Swarm rat chondrosarcoma (30). Treatment of rabbit costal chondrocytes with a combination of insulin and dexamethasone stimulates the formation of link protein- and aggrecan protein-rich fractions (22). Similarly, treatment of rabbit costal chondrocytes with dexamethasone, followed by IGF-I treatment, increases the incorporation of $^{35}SO_4$ into proteoglycans several-fold (31).

Glucocorticoids have also been shown to induce the synthesis of cartilage link protein and its mRNA. Dexamethasone treatment induces link protein mRNA in the rat calvaria-derived cell line, CFK2 (32), and induces a link protein-rich fraction in rabbit costal chondrocytes (22). The inductive affects of dexamethasone are most pronounced as these cultures of chondrocytes reach confluence (22,32).

The 5′ region of the link protein gene has been isolated from several species including rat (33), chicken (34) and human (33,35). Our initial studies indicated that the 5′-flanking region of the rat gene contains elements that enhance transcription (33). Continuing with these studies, we report here two elements that can regulate link protein gene transcription. One is a glucocorticoid-like response element (GRE; refs 14–16), the second is an AT-rich element similar to regulatory elements identified in the rat growth hormone gene (36,37), and the muscle creatine kinase gene (38). AT-rich elements can be required for serum responsiveness (39,40) and cell-type specific gene expression (36–38,40). Our experiments are particularly interesting since it is the first reported identification of an AT-rich element required for transcriptional activity in a cartilage gene, and suggests a widespread distribution of AT-rich regulatory elements.

MATERIALS AND METHODS

Cell culture

Chick embryo chondrocytes (CEC) were obtained from the sterna of day 15 or 16 embryos (Truslow Farms, Chestertown, MD). Digestion of sterna with collagenase B (0.6%, Boehringer Mannheim) yielded isolated chondrocytes as described (41). Typically, preparations from 36 sterna yielded ~10^8 cells. Cells were maintained in Ham's F12 medium (Gibco), containing 2.0% fetal calf serum or medium containing serum depleted of growth factors. Depleted serum was prepared by mixing 56 g of BioRad resin (AG 1-X) and 500 ml of fetal calf serum for 24 h at 4°C. The serum was decanted and 56 g of fresh resin was added and mixed for another 24 h at 4°C. The serum was decanted, filtered through a 0.45 μm filter, and then incubated with 50 g of acid-washed activated charcoal for 24 h at 4°C. To remove the charcoal, the serum was centrifuged and filtered through a 0.45 μm filter and then filter sterilized through a 0.22 μm filter. The depleted serum was stored in 50 ml aliquots at –20°C.

Construction of site-specific mutations

Oligonucleotides corresponding to sequences within the first intron of the rat gene, containing one or two altered nucleotide residues, were phosphorylated with polynucleotide kinase. Oligonucleotides with appropriate mutations were annealed to a single-stranded M13 template containing the complementary DNA to the rat first intron. Double strand DNA synthesis was completed with the Klenow fragment of DNA polymerase. The wild-type strands were removed using an oligonucleotide-directed mutagenesis kit, version 2 (Amersham), as directed by the manufacturer. Mutations were identified by plaque hybridization using labeled oligonucleotides as probes. The mutagenized fragments were isolated from *Bam*HI-digested, replicative-form phage DNA. Mutagenized fragments were subcloned into CAT reporter plasmids as described below. These site-specific mutations were confirmed by dideoxy-sequencing using a Sequenase kit according to the manufacturer's protocols (US Biochemicals).

Construction of recombinant CAT plasmids

tkCAT derivatives were constructed by subcloning double-stranded oligonucleotides from the link protein gene into the *Bam*HI site of the tkCAT plasmid; a site which is adjacent but upstream to the thymidine kinase promoter (described as LS-37tkcat; ref. 42). Oligonucleotide inserts were confirmed by dideoxy DNA sequencing. CAT reporter plasmids (43) containing various fragments of the promoter and the first intron of the link protein gene were prepared using Bal-31 digestion as described previously (33), or by polymerase chain reaction (PCR; ref. 44) using sequence-specific primers. Inserts were confirmed by DNA sequencing. Plasmid constructs for transfection were prepared using plasmid DNA from twice-banded CsCl–EtBr gradient equilibrium centrifugation.

CAT assays

CECs were transfected with 15 μg of DNA of each plasmid construct, using the calcium phosphate precipitation method (45) as described previously (33). The CAT enzyme assay was performed by phase extraction of cell lysates (46). A total of 6 μg of the CMV-luciferase construct (47,48) was added to 10 ml of $CaCl_2$ (200 mM) and aliquoted into 1 ml samples. To each aliquot, 60 μg of one of the CAT reporter chimeras was added and this solution added to 1 ml HEPES-buffered saline (HBS). After 20–30 min at room temperature, 0.5 ml of the calcium phosphate precipitate (150 ng of the CMV-luciferase construct and 15 μg of each CAT reporter construct) was added to a 60 mm^2 plate of CEC (~3×10^6 cells). After a 3 h incubation at 37°C, the cells were washed once with PBS, and 3 ml of new media added. When indicated, glucocorticoids, either dexamethasone or hydrocortisone (10^{-7} and 10^{-6} M, respectively), were added and incubation continued for an additional 36 h at 37°C.

Determination of transfection efficiency

Transfection efficiency was confirmed by co-transfection of a CMV-luciferase reporter plasmid as described above (48). Cell lysates were prepared and the amount of luciferase in each cell lysate was determined in duplicate using a luminometer (Moonlight 2010, Applied Bioluminescence). For each transfection, an efficiency factor was determined from the luciferase data and used to normalize each CAT assay.

Oligonucleotides

Oligonucleotides were synthesized on an Applied Biosytems 380B automated DNA synthesizer. The 26-residue oligonucleotide, 5′-gtggaggggTcatggtgAtcttatga-3′, used for preparation of the

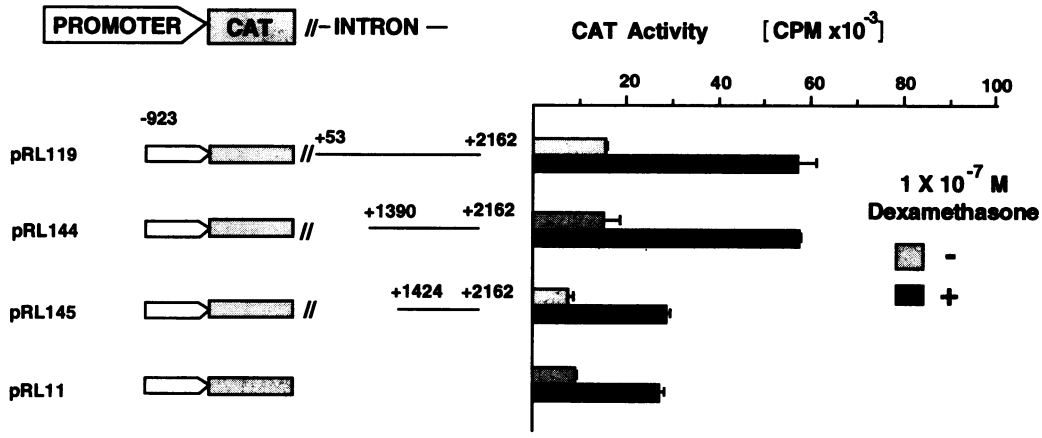

Figure 1. Diagrammatic representation of constructs containing the rat link protein gene 5′ region controlling the CAT reporter expression. DNA sequence of the rat and human link protein genes were previously reported. The rat gene for link protein has GenBank accession number X55057, while the human gene has accession number X55056. Calcium phosphate transfections were performed using chondrocytes of day 15 chick sterna, and CAT expression was assayed by phase extraction. Transfection efficiency was determined by co-transfection with CMV-luciferase, in serum-depleted conditions. Intron fragments analyzed were +1390 to +2162, +1424 to +2162 or +53 to +2162 inserted into PSVOCAT. Each bar is the mean and standard error of two different plates of cells.

double-site mutation was purified by electrophoretic fractionation on a 15% polyacrylamide gel, buffered with 7 M urea–TBE. The oligonucleotides were eluted from the excised gel slice overnight and purified on a Water's C18 Seppack column as suggested (Applied Biosystems). The 17 residue oligonucleotide, 5′-agggg-acCtggtgttct-3′, used to introduce the single-site mutation, was synthesized and deprotected and then used without additional purification. The 35 residue oligonucleotide, 5′-ctcccctcgagt-tatCGCGaaatgtctatttgttcag-3′, was used to introduce a 4 bp substitution mutation at the AT-rich element using PCR.

The single-stranded DNA oligonucleotides (250 ng) used in band-shift and Southwestern analyses were radiolabeled with [^{32}P]ATP using T4 polynucleotide kinase (PNK). The oligonucleotides were heat treated at 65°C for 20 min, and annealed at 37°C for 2 h. Oligonucleotides used in Southwesterns were incubated for an additional 30 min with PNK and 1 mM ATP before heat treatment at 65°C. After annealing, the oligonucleotides were ligated in a final concentration of 15% polyethylene glycol to form concatomers. The formation of multimeric forms of the DNA was confirmed by electrophoresis through 10% polyacrylamide (TBE).

Nuclear extracts

Chondrocytes from chick sterna were prepared as described above, and cultured for 4 h before nuclear extracts were prepared. Cells from a rat chondrosarcoma were a gift from Dr T. Calabro (Cleveland Clinic), prepared as described (49). Muscle cells and adipocytes were isolated by a single rapid homogenization of chick day 14 breast muscle (pectoralis) or overlying adipose tissue, respectively. Debris was removed using a nylon filter. Nuclear proteins were extracted as described (50). The integrity of cells and nuclei were monitored using trypan blue staining and microscopy.

Southwestern analysis

For Southwestern analysis, 10 µg of nuclear extract was precipitated on ice for 10 min with an equal volume of acetone and collected by centrifugation. The precipitate was resuspended in SDS-sample buffer, heated at 100°C for 5 min, and electrophoresed on 13% polyacrylamide gels (SDS–PAGE). Proteins were electrophoretically transferred to nitrocellulose filters. The blot was blocked by incubating in binding buffer (30% glycerol, 50 mM KCl, 0.1% NP-40, 2 mM DTT, 20 mM Tris–HCl pH 7.8 and 0.05 µg/ml dIdC) and 2.5% powdered milk for 1 h. The blocking solution was aspirated and the blot was incubated with concatenated [^{32}P]ATP labeled double-stranded oligonucleotide (~40 ng) in binding buffer for 2 h at 4°C. The filter was then washed with three changes of binding buffer (without glycerol) for 5 min each and exposed to X-ray film.

Band mobility shift assays

Nuclear extracts were incubated with 20 000 c.p.m. of the appropriate ^{32}P-labeled oligonucleotide probe at 4°C for 30 min in nuclear binding buffer (20 mM Tris–HCl pH 7.8, 100 mM KCl and 2 mM DTT) and fractionated by electrophoresis on a non-denaturing 8% polyacrylamide gel (40 mM Tris and 300 mM glycine).

RESULTS

Identification of a glucocorticoid responsive element (GRE) in the first intron of the rat link protein gene

Previously, we described a 2.2 kb fragment within the first intron of the rat link protein gene which activated transcription of the gene (33). The most transcriptionally active region within the intron was found between +750 and +2162 bp. Two additional deletions, one to +1390 bp (pRL144) and one to +1424 (pRL145), showed substantial differences in CAT assays (Fig. 1). These differences were particularly evident in serum-containing conditions. For example, in one CAT assay 62 000 c.p.m. were produced from the pRL144 construct (+1390 to +2162) versus 30 000 c.p.m. from the pRL145 construct (+1424 to +2162). Since DNA sequence analysis of this region (+1390 to +1424) revealed a sequence homologous to a GRE, these constructs were further assayed in depleted serum conditions with and without dexamethasone. In these experiments the pRL144 construct showed similar gluco-

corticoid-induced activity to that of the original 2.2 kb intron fragment, pRL119. In contrast, a deletion to +1424 bp (pRL145) resulted in significant loss of enhancer activity, and was similar to the promoter without the link protein intron, pRL11. In general, assays performed with the plasmids containing this GRE region (+1390 to +2162), revealed dexamethasone-inducible activities that were ≥2-fold greater than plasmids which did not contain the GRE (+1424 to +2162 bp). Examination of Figure 1 also indicates that the 923 bp promoter construct, pRL11, contains sequences that respond to dexamethasone. However, sequences homologous to a complete GRE have not been identified in this 923 bp promoter region.

Conservation of the GRE in the human and rat link protein genes

Previously, we reported a comparison between the 5′ regions of the human and rat link protein genes (33). Although the region of the human link protein gene corresponding to the rat +1400 region was not determined, there appeared to be a divergence between the two genes at +1100. To identify the corresponding region in the human link protein gene, a region of the rat gene containing the GRE (+1395 to +1502 bp) was used as a probe for Southern hybridization with the human clone. A DNA fragment from the human gene was found to hybridize to the rat probe, and the fragment was characterized by DNA sequencing.

The sequences of the rat GRE and the corresponding putative human GRE were compared with GREs characterized in other genes (Fig. 2). The rat GRE sequence is nearly identical with the GRE consensus motif (14), matching in all positions but one, while the corresponding sequence of the human gene was slightly less conserved.

		654321-		+123456
Consensus	GRE	ggtaca	nnn	tgttct
Human	+1649	ggggAAaca	tta	tgtActtat
Rat	+1389	aggggAaca	tgg	tgttcttat
MMTV	-187	atggTtaca	aac	tgttcttaa
MMTV	-134	tttggtaTC	aaa	tgttctgat
TAT	-2515	tgcTgtaca	gga	tgttctagc

Figure 2. Comparison of rat and human link protein gene GREs with several additional GREs. Nucleotide sequences are from GREs identified in the mouse mammary tumor virus (MMTV; ref. 53) and the tyrosine amino transferase gene (TAT; refs 51,52). The DNA sequence numbering was presented elsewhere (14). Nucleotides differing from the consensus sequences are underlined and capitalized.

Activity of the link protein GRE with the heterologous tk promoter

The glucocorticoid-inducible activities of both the human and the rat GRE elements were tested using a heterologous promoter, the thymidine kinase promoter, linked to the CAT reporter gene (LS-37tkCAT; ref. 42). Synthetic oligonucleotides corresponding to these GRE elements were placed upstream of the tk promoter in the LS-37tkCAT construct and then the plasmids were transfected into chondrocytes (Fig. 3). The presence of the rat or human GRE elements caused an increase in CAT activity in response to hydrocortisone or dexamethasone treatment. Control oligonucleotides, including an SP1 consensus sequence and a GRE-like half-site found in the link protein promoter,

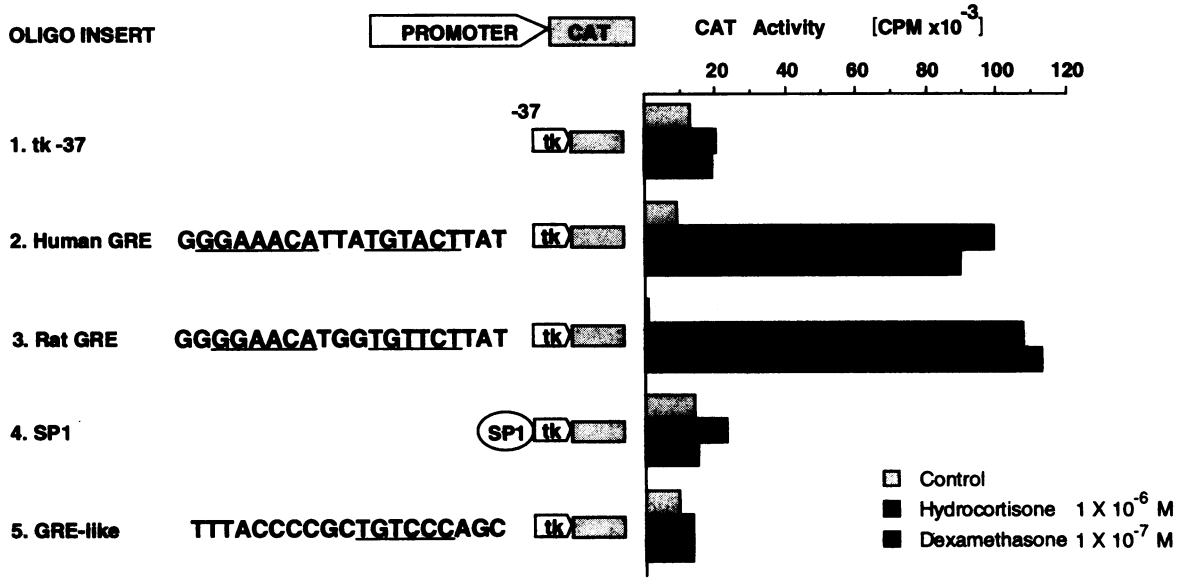

Figure 3. Glucocorticoid-mediated induction of a heterologous promoter by the rat and human link protein gene GREs. The minimal promoter for tkCAT (LS -37; ref. 42) was modified and in one case an oligonucleotide containing an SP1-site was inserted (E. Pascal and R. Tjian, unpublished). The tkCAT reporter was used as the cloning vector for the rat GRE, human GRE or GRE-like elements of the rat promoter. CAT assays including either dexamethasone (10^{-7} M) or hydrocortisone (10^{-6} M) were performed in 5% depleted serum. Dexamethasone induction was repeated in three independent experiments. The hydrocortisone effect has been tested twice. Data from a representative experiment is shown.

A.

	654321-		+123456
Consensus GRE	ggtaca	nnn	tgttct
pR119	aggggAaca	tgg	tgttcttat
pRL119m1	aggggAacC	tgg	tgttcttat
pRL119m2	aggggATca	tgg	tgAtcttat

B.

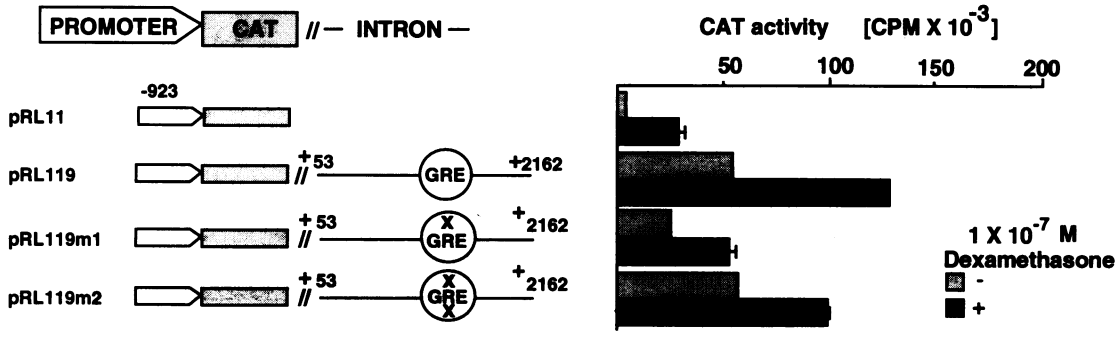

Figure 4. The effect of site-specific GRE mutations on transcriptional activity of the rat link protein gene first intron. The site-specific mutations shown were introduced into the GRE and then analyzed by transfection into chick sternal chondrocytes. pRL119m1 and pRL119m2 are identical to pRL119 except for the point mutations shown. Assays were performed in duplicate and repeated in three experiments using p119, p119m1 and p119m2. The effects of mutations corresponding to the single- and double-site mutations analyzed here, were previously reported (52,53).

5′-TGTCCC-3′ (–865 to –870), did not induce any transcriptional activity (Fig. 3). This indicated that both the rat and human GREs can mediate dexamethasone and hydrocortisone-induced transcriptional activity.

Site-specific mutations in the GRE

To evaluate the importance of the GRE in the 2.2 kb first intron fragment, site-specific mutations were generated within the GRE and their effects assayed (Fig. 4). A double-site mutation (A→T at –2, and T→A at +2; refs 51,52) and a single-site mutation (A→C at –1; ref. 53) were introduced into the link protein promoter–CAT plasmid pRL119 (Fig. 4A). These base changes are inhibitors of glucocorticoid induction in the tyrosine aminotransferase gene (51,52) and mammary tumor virus promoter (53) . The single-site mutation (pRL119m1) and the double-site mutation (pRL119m2) reduced the dexamethasone-inducible GRE activity by ~70 and 20%, respectively. The inability of these mutations to completely inhibit inducible transcriptional activity suggests that sequences surrounding the GRE, and promoter sequences, also contribute to glucocorticoid induction.

A promoter region required for dexamethasone and serum responsiveness

A series of promoter deletion constructs were prepared in order to delineate which region of the promoter was required for glucocorticoid responsiveness. A deletion of 135 bp between –923 and –788 (pRL121) resulted in a greatly reduced capacity for glucocorticoid induction (Fig. 5A). In addition, serum responsiveness was reduced by this 135 bp promoter deletion (Fig. 5B). Deletions from –788 to –120 showed no further effects. In the presence of 2% serum (that was not depleted of growth factors), dexamethasone treatment increased CAT activity ~4-fold, while deletion from –923 to –788 eliminated this increase in CAT activity (Fig. 5B). The induction of CAT activity in the presence of 2% non-depleted serum was greater than the induction in the presence of depleted serum, suggesting that serum factors may play a role in glucocorticoid responsiveness of the promoter element. To further map this responsiveness, smaller deletions within the –923 and –788 region of the promoter were tested. Results showed that dexamethasone induction of CAT activity was lowered by a 9 bp deletion from –923 to –914, and further reduced by deletion to –900 (Fig. 6A). Further deletions to –786 and –620 did not have any additional large effects on the induction of CAT activity (Fig. 6A). Serum responsiveness (without added dexamethasone) was also reduced by deletions of the promoter from –923 to –900, as well as by a 4 bp substitution within this site (Fig. 6B). Examination of this DNA sequence indicated an AT-rich sequence that is conserved in both rat and human genes. This AT-rich DNA is similar in sequence to an AT-rich element within the rat growth hormone gene and an AT-rich element in the muscle creatine kinase gene (Fig. 6C).

Southwestern analysis

Southwestern analysis was performed to address the presence and molecular size of proteins factors which bind to the AT-rich element. Double-stranded ^{32}P-radiolabeled oligonucleotides corresponding to the link protein promoter AT-rich element, or to the AT-rich element of the muscle creatine kinase (MCK) gene, were used as probes in Southwestern blot analysis. The link protein promoter AT-rich element oligonucleotide (LP-AT-rich)

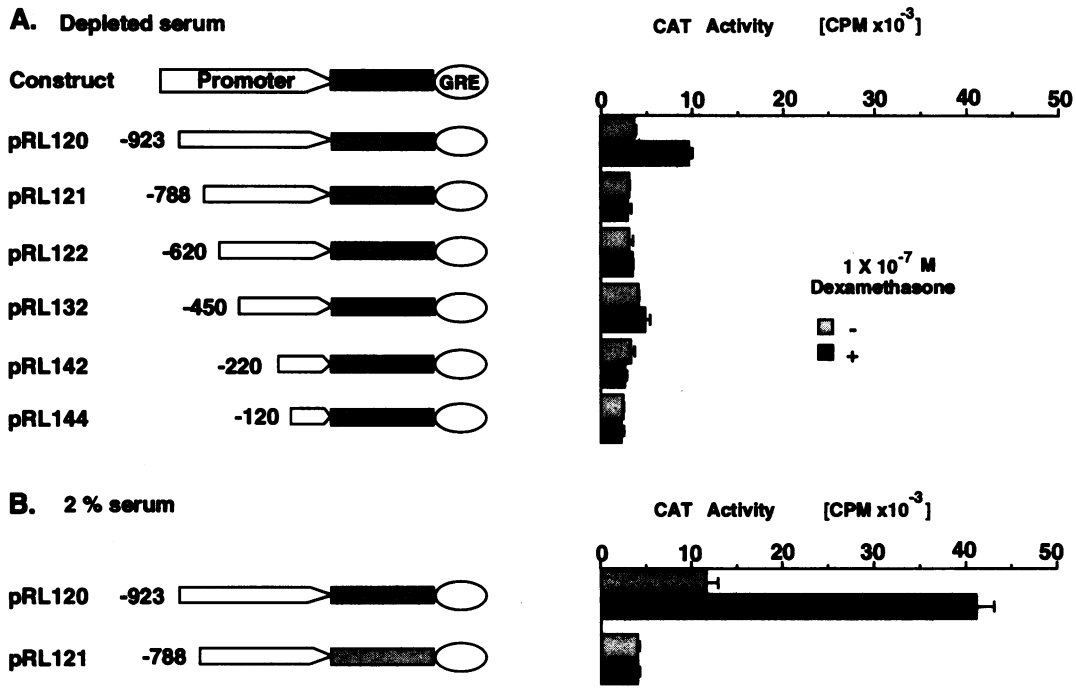

Figure 5. CAT assay analysis of promoter deletions and glucocorticoid responsiveness. **(A)** Transfections and dexamethasone inductions were performed in depleted serum as described previously. Promoter deletions were inserted upstream of the CAT promoter while a single GRE was inserted into the *Bam*HI site of pSVOCAT. **(B)** Glucocorticoid inductions were performed as above, but the cells were grown in 2% serum.

bound to a 32 kDa protein(s) in nuclear extracts isolated from chondrocytes, skeletal muscle cells (pectoralis) and adipocytes (Fig. 7). The MCK AT-rich element also bound to a 32 kDa protein(s) in nuclear extracts isolated from chondrocytes (Fig. 7). To confirm the specificity of oligonucleotide/protein binding, unlabeled oligonucleotides corresponding to the MCK sequence (0.1 μg/ml) or the link protein AT-rich element (0.1 μg/ml) were used to compete with the binding of the labeled link protein probe or the MCK probe, respectively. In each case, inclusion of the unlabeled probe blocked binding of the labeled probe to the 32 kDa protein(s) from chondrocytes (data not shown).

Band mobility shift analysis

Band mobility shift assays were performed to reveal native complexes formed by the interaction of nuclear proteins with AT-rich elements (Fig. 8). The link protein AT-rich element formed three distinct complexes when incubated with nuclear extracts from chondrocytes. The two largest complexes bound the greatest proportion of the shifted oligonucleotide, suggesting that they had the highest affinity for the AT-rich element. The link protein AT-rich element formed three complexes when incubated with extract from rat chondrosarcoma cells. These three complexes were distinct is size from those formed with extracts from chondrocytes. Three major complexes were formed between the link protein AT-rich element (LP-AT-rich) and chick muscle nuclear extract. The two smallest complexes formed were the same size as the two smallest complexes formed with the chick chondrocytes, while the largest complex was unique in size. The mutated link protein AT-rich element (LP-AT-rich-mu), which resulted in a loss of both basal and serum responsive activity, was also examined in band mobility shift assays. The mutated element formed two complexes in addition with those formed with the wild-type LP-AT-rich element when incubated with chondrocyte extracts. One complex was larger than all of the wild-type complexes and the second was larger than the smallest wild-type complex. The mutated oligonucleotide also bound with reduced affinity to the complexes indicated by the arrow. LP-AT-rich-mu formed complexes with the rat chondrosarcoma cell extract similar to those formed with the wild-type element, although a larger proportion of the oligonucleotide bound to the two smaller complexes and the smallest complex may be unique. The mutated element, LP-AT-rich-mu, formed one small complex with muscle extract in addition to those complexes formed with the wild-type LP-AT-rich element. In addition, the two small complexes bound a greater proportion of the LP-AT-rich-mu oligonucleotide in the muscle extract. The MCK AT-rich element formed a similar pattern of complexes as did the LP-AT-rich element with extracts from both chondrocytes and muscle cells, although the two larger complexes bound less oligonucleotide. The MCK-AT-rich element and rat chondrosarcoma extract formed a distinct smaller complex and resulted in changes in affinity of the oligonucleotides for the complexes. In general, the complexes formed by LP-AT-rich and MCK-AT-rich elements were similar and the complexes formed was dependent on the cell-type examined.

DISCUSSION

The identification of GREs in the link protein gene was complicated by the existence of at least two distinct regulatory sites. We have characterized two of these elements, one in the promoter region and another in the first intron. The responsive

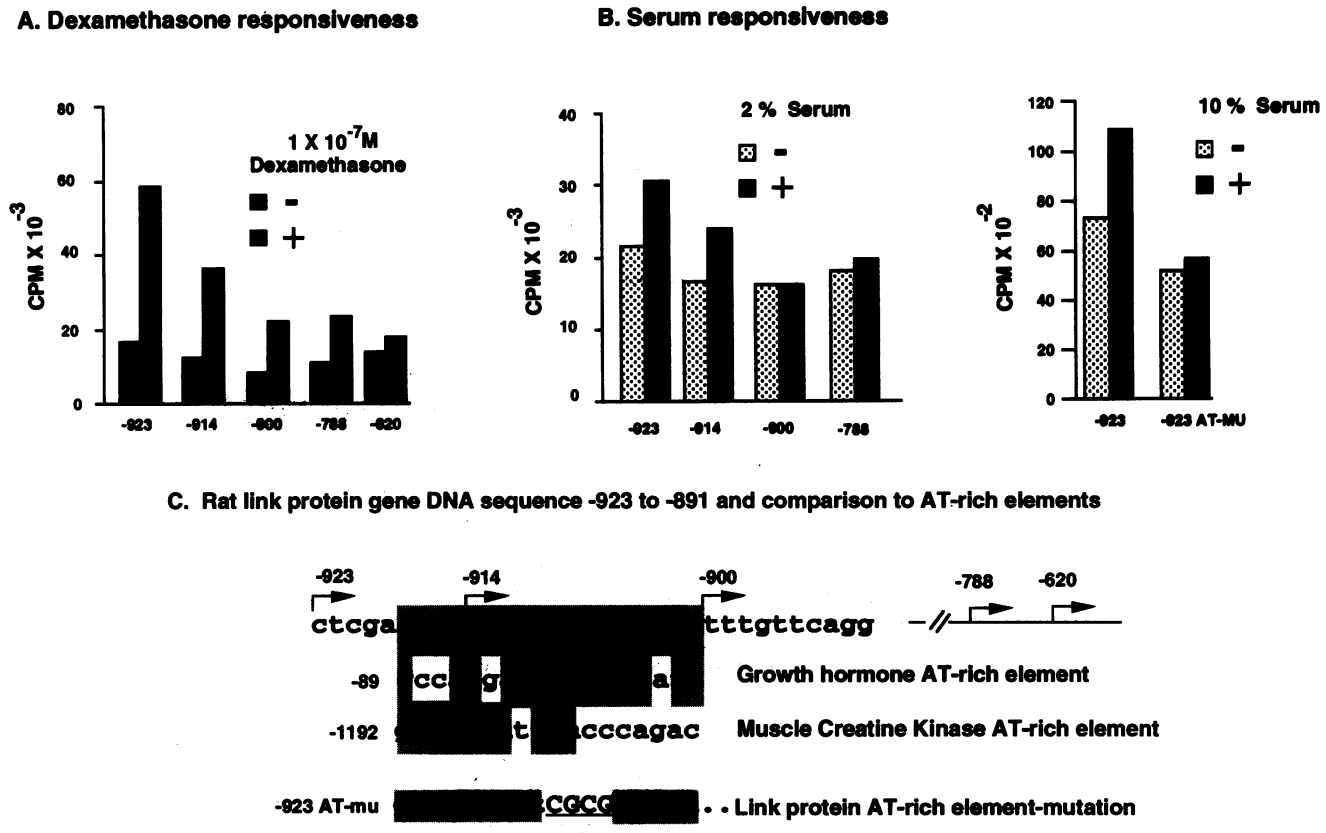

Figure 6. Fine mapping of elements required for serum and glucocorticoid responsiveness in the rat link gene promoter. Short promoter deletions were constructed each containing a GRE inserted into the *Bam*HI site of pSVOCAT, and each tested for serum and glucocorticoid responsiveness. The DNA sequence from –923 to –891 of the gene for rat link protein is shown. Each arrow identifies the start nucleotide of a deletions analyzed in the panels above. Each panel is from one representative experiment. (**A**) Dexamethasone responsiveness requires DNA sequences –923 to –901 of the rat link protein gene. (**B**) CAT activity was compared in depleted or 2% serum. A reduction in serum responsiveness is identified as sequences from –923 to –901 are deleted. (**C**) The transcriptionally active AT-rich sequence aligned with AT-rich sequences in the growth hormone gene (36,37) and the muscle creatine kinase gene (38). The lower DNA sequence diagrams the 4 bp mutation construct, replaced bases are in capital letters and underlined.

element in the first intron of the rat link protein gene matched the sequence of a consensus GRE at 11 of 12 nucleotide positions (14–16,51,53,54). The responsive element identified within the promoter region does not share sequence homology with a GRE, but resembles previously identified AT-rich elements.

Several experiments support a role for the GRE element in the rat first intron in regulating link protein gene expression. First, deletion of DNA sequences from +1390 to +1424 containing this GRE resulted in a significant loss of dexamethasone-inducible activity. Secondly, when two site-specific DNA mutations, which disrupt the function of other GREs, were introduced at this element a reduction in responsiveness was also observed. Activity of the first intron GRE enhancer was confirmed by testing constructs containing sequences corresponding to the rat or the human element in conjunction with the thymidine kinase promoter (LS-37tkCAT; ref. 42). Sequences corresponding to both the human and the rat elements conferred glucocorticoid responsiveness on the tk promoter, inducing CAT activity by >10-fold.

These experiments may also indicate additional regulatory elements. For example, the failure of the site-specific mutations to completely inactivate the activity of the first intron suggests the existence of additional regulatory elements. Further, deletion through the GRE results in a substantial loss of basal activity in addition to the inhibition of dexamethasone-inducible activity, suggesting DNA elements required for basal transcriptional activity may overlap with the region of the GRE.

Initial analyses of the promoter showed that a 135 bp sequence (–923 to –788 bp) was required for full responsiveness to glucocorticoid treatment by the link protein gene. However, there were no consensus GRE sites in this region. Additional promoter deletions identified a 23 bp sequence (–923 to –900) required for responsiveness to both serum and glucocorticoid treatment. This AT-rich region did not confer dexamethasone responsiveness comparable to the intron GRE-tkCAT constructs (data not shown). This sequence contains a motif similar to the well-characterized AT-rich elements in the growth hormone gene and the muscle creatine kinase gene promoter (36–38). The link protein promoter AT-rich element was further analyzed by Southwestern analysis and band-shift assays using the AT-rich region from MCK for comparison. Southwestern analysis indicated that this site bound with high specificity to a 32 kDa protein(s) in nuclear extracts isolated from chondrocytes, muscle or adipose tissues. The AT-rich element from the muscle creatine kinase also bound to a 32 kDa protein(s) in nuclear extracts isolated from chondrocytes. The molecular mass of 32 kDa is similar to homeodomain-containing proteins. Further, the link protein AT-rich element can bind recombinant Pit-1 and Hox 4.7

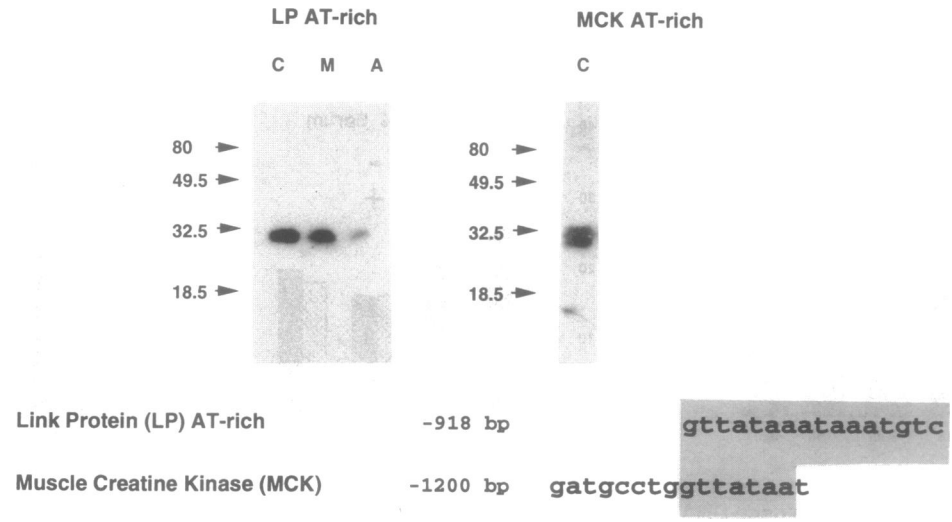

Figure 7. Southwestern analysis of the link protein promoter AT-rich element and the muscle creatine kinase AT-rich element. Nuclear proteins from chondrocytes (lane C), muscle cells (lane M) or adipocytes (lane A) were fractionated by electrophoresis through a 13% polyacrylamide gel (SDS–PAGE) and blotted onto a nitrocellulose filter. The first panel was probed with the link protein gene AT-rich element. The link protein gene AT-rich probe was prepared from [^{32}P]ATP labeled oligonucleotides, 5′-tcgagttataaataaatgtcg-3′ and 5′-tcgacagacatttatttataac-3′. Labeled oligonucleotides were annealed and ligated into concatomers. The second panel was probed with the muscle creatine kinase element (MCK). The MCK probe was prepared using [^{32}P]ATP labeled oligonnucleotides, 5′-gatcgatgcctggttataat-3′ and 5′-gatcattataaccaggcatc-3′, in concatomeric form (second panel, lane C).

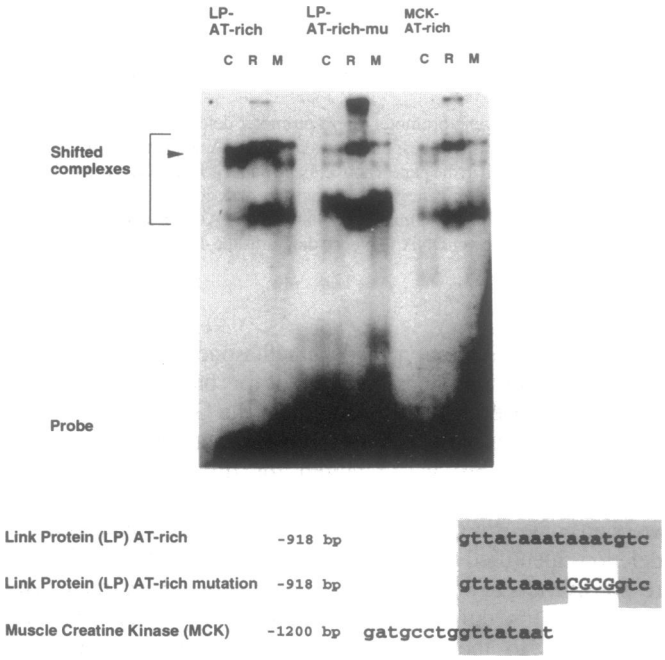

Figure 8. Electrophoretic mobility shift assay of AT-rich elements. Double-stranded oligonucleotide was radiolabeled with [^{32}P]ATP using PNK, incubated with nuclear extract at 4°C and fractionated by electrophoresis through a non-denaturing 8% polyacrylamide gel. AT-rich elements analyzed include: the link protein AT-rich element (LP AT-rich), the muscle creatine kinase element (MCK AT-rich) or a 4 bp substitution in the link protein AT-rich element (LP AT-rich Mu). Nuclear extracts in lanes C, M, R were from chick sternal chondrocytes, muscle cells, or rat chondrosarcoma, respectively.

using Southwestern analysis (data not shown). Thus, it is likely that the band detected with the link protein promoter element represents either a single homeodomain protein or mixture of homeodomain proteins. Band-shift assays revealed that the link protein AT-rich element forms cell-type-specific DNA–protein complexes. The appearance of cell-type-specific complexes in native conditions may indicate the presence of multiple factors, and interactions that are dependent on the cell-type. Thus the proteins binding at this site could be different in each tissue, but have similar molecular sizes.

AT-rich elements are required for several functions including tissue-specificity, serum and hormone responsiveness and developmental regulation of gene regulation. The muscle creatine kinase gene AT-rich region is required for responses to myogenic factors during muscle differentiation (37). The AT-rich element of the growth hormone gene promoter is the recognition site for the Pou-containing homeodomain protein, Pit-1. Pit-1 is required for cell type-specific gene expression (36,37,56,57) and directs responsiveness to hormones (58–60). An AT-rich element in the c-*fos* gene is required for serum responsiveness and directs the interaction between two families of transcriptional factors; the homeodomain-containing proteins, and the MADS box-containing family of proteins (the term MADS box is derived from the first letter of each protein member MCM1, agamous, deficiens and serum response factor; ref. 39). Interactions between these two families of transcriptional factors are thought to be a conserved mechanism for determining cellular identity in yeast, plants and animals (39, and references therein). Since AT-rich elements can bind to distinct families of homeodomain-containing proteins, such as the Pou-containing homeodomain Pit-1 or a paired rule-like homeodomain-containing protein such as Phox (39) or Mhox (38), additional experiments will be required to characterize binding and activation through this element. Further examination of the link protein gene 5′ region has revealed additional AT-rich elements within regions of high DNA sequence conservation between the rat and human genes. Additional experiments will be required to address the significance of these elements, their binding characteristics, and

functional activity. Our present study presents evidence that the –900 to –923 AT-rich region in the link protein promoter is a candidate site required for interactions with both homeodomain-containing proteins, and serum response factors, and may direct hormone responsiveness.

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