Cloning and initial characterization of the promoter region of the rat cysteine-rich intestinal protein gene

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Cysteine-rich intestinal protein (CRIP) is an intestinal Zn^{2+} -binding protein containing a single copy of the double Zn^{2+} -finger arrangement known as the LIM motif. CRIP is developmentally regulated and can be induced by glucocorticoid hormones during the early suckling period. In this report we show that CRIP mRNA levels are induced by dexamethasone in cultured rat intestinal epithelial cells (IEC-6). Analysis of the 2644 bp of the 5'-flanking region of the CRIP gene revealed that the CRIP promoter lacks classical CAAT and TATA boxes but contains GC-rich regions in the proximal end of the promoter that probably function in transcription initiation. In addition to

binding sites for transcription factors such as Sp-1, AP-2, OCT and GATA-2, there are multiple glucocorticoid-response elements. CRIP promoter constructs fused to the chloramphenicol acetyltransferase reporter gene and transfected into IEC-6 cells confirmed glucocorticoid responsiveness and the presence of negative acting elements. Mobility-shift assays revealed the presence of nuclear factors that bind to the CRIP promoter as a result of dexamethasone treatment. These experiments provide the initial data required to explore further the regulation of this tissue-specific developmentally regulated Zn^{2+} -finger protein.

INTRODUCTION

Cysteine-rich intestinal protein (CRIP) is a tissue-specific developmentally regulated protein [1,2]. It has been shown to bind Zn^{2+} in the intestine of the neonatal [2] and adult [3] rat. Analysis of its sequence revealed that CRIP is a member of a family of proteins with a cysteine-rich domain known as the LIM motif [1]. This motif, Cys-Xaa₂-Cys-Xaa₁₇₋₁₉-His-Xaa₂-Cys-Xaa₂-Cys-Xaa₂-Cys-Xaa₇₋₁₁-(Cys)-Xaa₈-Cys, has been postulated to form a double Zn^{2+} -finger arrangement [4], and has been shown to be a component of several transcription factors [5]. Thus it has been hypothesized that CRIP is a transcription factor [6]. In contrast with the known LIM-containing transcription factors, CRIP has only a single copy of the LIM motif and lacks a homeodomain [4]. It has been proposed that 'LIM only' proteins, such as CRIP, may be involved in protein-protein interactions with transcription factors [7] or other proteins [8]. In addition, CRIP has been shown to bind Zn^{2+} during the absorptive process [3] and may play a role in cellular Zn^{2+} movement [3,9], protein-protein interactions or function in a Zn^{2+} -dependent developmental/differentiation process. CRIP also has many features in common with the cryptidins and other cysteine-rich proteins [10].

CRIP gene expression is highly regulated both spatially and temporally. Expression occurs predominantly in the cells of the mid-villar region of the small intestine and increases dramatically during the first three postnatal weeks [1,2]. During this time period, CRIP expression is susceptible to induction by dexamethasone (Dex; a glucocorticoid hormone) [2,6]. To our knowledge this is the first example of a hormonally regulated LIM-motif-containing protein. In adult animals, inducibility by injected Dex is suppressed [2]. We have cloned and sequenced the

5'-flanking region of the rat CRIP gene to examine regulation of this intestinal protein. Here we report this sequence and use transient-transfection studies and a gel mobility-shift assay to begin to explore the characteristics of the CRIP promoter that may explain the developmental and hormonal regulation of the CRIP gene and eventually give insight into its function.

EXPERIMENTAL

Animals

Pregnant Sprague-Dawley rats (Harlan-Sprague-Dawley, Indianapolis, IN, U.S.A.) were received on day 14 of gestation. A commercial diet (Rodent Laboratory Chow no. 5001, Ralston-Purina, St. Louis, MO, U.S.A.) and water were provided *ad libitum*. Neonatal pups were a random mixture of males and females. On postnatal day 5, 14 pups were injected subcutaneously with 1 mg/kg Dex. On day 6, nuclear extracts were prepared from small intestines of Dex-treated and untreated control pups. In addition, intestines from pups from each group were used to prepare total RNA for Northern-blot analysis. Animal experiments were approved by the institutional animal care committee.

Cultured intestinal cells

Cultured IEC-6 cells, a normal adult rat epithelial cell line derived from the crypt region of the small intestine, were obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 100 units/l insulin and 5% bovine serum supplemented with penicillin, streptomycin and glutamine [11]. Cells were treated with 5 μ M Dex for up to 30 h and harvested for Northern-blot analysis.

Abbreviations used: CRIP, cysteine-rich intestinal protein; GRE, glucocorticoid-response element; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; NRE, negative response element; Dex, dexamethasone.

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The nucleotide sequence reported in this paper will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under accession number L25263.

Northern-blot analysis

Total cellular RNA from IEC-6 cells or rat intestine was prepared using the guanidinium isothiocyanate method [12]. RNA samples (15 µg) were subjected to agarose-gel electrophoresis (1%, w/v) containing 0.66 M formaldehyde and stained with ethidium bromide (added before electrophoresis) to verify RNA integrity. Capillary blotting was used to transfer RNA to Nylon membranes (Genescreen; NEN, Boston, MA, U.S.A.). After u.v. cross-linking, blots were probed with random-primed ³²P-labelled rat CRIP, cryptdin or metallothionein cDNA [2]. Loading of lanes was confirmed by ethidium bromide staining and hybridization of ³²P-labelled rat β-actin cDNA. Filters were autoradiographed at -70 °C. Densitometry of the autoradiographs provided relative quantification of hybridization.

Preparation of nuclear extracts

Nuclear extracts were prepared from small intestines using modifications [13] of the methods of Gorski et al. [14] and Shapiro et al. [15]. Briefly, tissues were homogenized in homogenization buffer (10 mM Hepes, pH 7.6, 0.5 mM spermidine, 0.15 mM spermine, 25 mM KCl, 2 M sucrose, 10% glycerol, 1 mM EDTA). The homogenate was layered over this buffer and centrifuged at 18000 g (*r*_{av}, 7.0 cm) for 30 min. Nuclear pellets were resuspended in homogenization buffer with glycerol (9:1, v/v) and rehomogenized. The homogenate was again layered over the buffer and centrifuged at 18000 g (*r*_{av}, 7.0 cm) for 30 min. Pelleted nuclei were resuspended in 20 mM Hepes, pH 7.9, containing 0.75 mM spermidine, 0.15 mM spermine, 2 mM dithiothreitol (DTT) and 25% glycerol and mixed 9:1 (v/v) with satd. (NH₄)₂SO₄ and extracted for 30 min at 4 °C. Samples were then centrifuged at 2 °C for 90 min at 150000 g (*r*_{av}, 5.9 cm). The supernatant was removed and precipitated by the addition of 0.33 g/ml solid (NH₄)₂SO₄ and centrifugation at 85000 g (*r*_{av}, 5.9 cm) for 20 min. The pellet was dissolved in 0.5 ml of buffer (20 mM Hepes, pH 7.9, containing 20% glycerol, 100 mM KCl and 2 mM DTT) and dialysed against this buffer. Extracts were stored at -70 °C and used within 5 days of preparation.

Genomic library screening, mapping, subcloning and sequencing

The 5'-flanking region of the rat CRIP gene was isolated by screening a rat genomic library in the Lambda Dash II vector (Stratagene, La Jolla, CA, U.S.A.) as described [16]. Briefly, recombinant phages were plated on 150 mm × 15 mm plates and incubated at 39 °C for 12–16 h. Duplicate lifts were made from chilled plates using Nylon membranes (Hybond-N, Amersham International, Amersham, Bucks., U.K.). Bound DNA was then denatured in 1.5 M NaCl/0.5 M NaOH for 2 min and neutralized for 5 min in 1.5 M NaCl/0.5 M Tris/HCl, pH 8.0. Filters were then washed in 2 × SSPE (1 × SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.7), cross-linked by u.v. irradiation, prewashed, hybridized [16] to a random-primed ³²P-labelled CRIP cDNA probe and autoradiographed.

Positive clones were characterized by digestion with restriction endonucleases and agarose-gel electrophoresis and Southern-blot analysis [17] using CRIP cDNA and 5'- and 3'-oligonucleotides [2] as probes. A single 2.8 kb fragment hybridized to the CRIP cDNA and the 5'-oligonucleotide probe, but not to the 3'-oligonucleotide probe. Subsequent mapping showed that this fragment, containing approx. 3 kb of sequence 5' to the CRIP coding sequence, was cut on the 5' end with *Eco*-

Figure 1 CRIP promoter constructs

The CRIP promoter was subjected to exonuclease III digestion to produce constructs of various lengths. The constructs shown were fused to the bacterial CAT gene and transiently transfected into cultured IEC-6 cells.

RI and on the 3' end with *Xba*I. This 2.8 kb fragment was subcloned into pGEM-7Zf(+) (Promega, Madison, WI, U.S.A.).

Sequencing of both strands of DNA was performed using the *Taq* DyeDeoxy Terminator Cycle Sequencing provided by the DNA Sequencing Core Facility at the University of Florida after exonuclease III digestion [16]. For regions not sequenced from nested deletions, sequences were generated by synthesis of oligonucleotide primers.

Construction of chloramphenicol acetyltransferase (CAT) plasmids

Promoter constructs were generated by digestion of the 2.8 kb promoter fragment in pGEM-7Z with *Kpn*I and *Apa*I. This produced a 1000 bp fragment that was determined by Southern-blot analysis and sequencing to be immediately upstream from the CRIP genomic coding sequence. This 1 kb promoter fragment was subsequently blunt-end cloned into the *Xho*I site of pGEM-7Z and subjected to exonuclease III digestion. Promoter constructs starting at positions -782, -471, -451, -235, -163 and -10 to position +55 were cloned into the *Hind*III and *Xba*I sites of a plasmid containing the bacterial gene for CAT (pCAT-Enhancer; Promega). Construct lengths were determined by sequencing. In addition, the full-length promoter fragment, starting at position -2744, was cloned into the pCAT vector (Figure 1).

Transfection of CRIP promoter constructs

IEC-6 cells were transiently transfected with CRIP promoter constructs (*n* = 4–6 cultures in at least two separate experiments) by incubation for 12 h with 4 µg of plasmid DNA/dish and 2 µl of dioctadecylamidoglycylspermidine/µg of plasmid DNA (Transfectam; Promega) in serum-free DMEM supplemented with insulin, glutamine and antibiotics. Cells were co-transfected with 3 µg of pSV-β-galactosidase control plasmid (Promega) to normalize for transfection efficiency.

Assays of CAT and β-galactosidase

Transient expression of CAT in transfected cells was assayed by a method based on liquid-scintillation counting of CAT reaction products [18]. After transfection, washed cells were incubated for 5 min in 1 ml of TEN buffer (40 mM Tris/HCl, pH 7.5, 1 mM EDTA, 15 mM NaCl). Cells were then scraped from the plate, and centrifuged at 13000 g (*r*_{av}, 5.5 cm) for 10 min at 4 °C. The

Figure 2 Time course of Dex-mediated increase in CRIP mRNA in IEC-6 cells

IEC-6 cells were cultured in DMEM with 10% fetal calf serum and treated with 5 μ M Dex for up to 30 h. At each time point, cells were harvested and total RNA was isolated. Total RNA (15 μ g/lane) was separated by agarose-gel electrophoresis, transferred to a Nylon filter, and hybridized with 32 P-labelled CRIP and β -actin cDNA probes.

supernatant was removed and the pellet resuspended in 90 μ l of 0.25 M Tris/HCl, pH 8.0. Cells were subjected to three freeze-thaw cycles. The extract was incubated for 10 min at 60 $^{\circ}$ C and

stored at -70° C. The assay of CAT activity was performed by adding 50 μ l of cell extract to 0.25 M Tris/HCl, pH 8.0, containing 2 μ l of [14 C]chloramphenicol (0.025 mCi/ml) and 5 μ g of *n*-butyryl-CoA in a final volume of 125 μ l. Samples were incubated overnight at 37 $^{\circ}$ C. The reactions were terminated by the addition of 300 μ l of mixed xylenes and vortexing for 30 s. The upper phase was removed and back-extracted by the addition of 100 μ l of 0.25 M Tris/HCl, pH 8.0. The xylene phase (200 μ l) was removed for liquid-scintillation counting. Samples were counted for 10 min at 1% error. β -Galactosidase activity was measured in cell extracts by monitoring the transformation of *O*-nitrophenyl β -D-galactoside to galactose and *O*-nitrophenol [19].

Gel electrophoretic mobility-shift assay

Promoter fragments starting at positions -782 and -471 to position $+55$ were end-labelled with 32 P using polynucleotide kinase. The labelled DNA (0.5 ng) was incubated at room temperature with 1.5 μ g of poly(dI-dC) for 30 min. Nuclear extract (5 μ g of protein) from control or Dex-treated animals was added and incubated for 30 min in a final volume of 25 μ l of

ATGCATCCAACGCGTTGGGAGCTCTCCGGATCCAAGCTTATCGATTTCGAACCCGGGTACCGAATTCAGGAGTGGGAGAAGGAGCTATCTCTG
 -2550 GGGTGGGGCAGTCGAGAGAAGAAACACAGACTGGGTATTGACCCACGTGCCTTAGCATCGCGTCTCTGGGTAGGAGGTAGGAAGTCTGTCAAGAAGTC
 -2450 CCTGGGTGTTAGATAGATAGTGGTGGCATACAATTTTAAATCCACGCGCTTAGGAATGAGAGGCAGGAAGATCTCTGAGTTTGAGGCCAGCCTGGTCTACA
 -2350 TAGTGAGTTCAGGATAGCTAGGGCTACACAGTGAAACCTTTTTCAGGAACAAAAACAAAAACAAAAACAAAAACGAGGTCCCTAAGTGCAGATAGAA
 -2250 ACGTAGCTATTTGCTCTGAGAGAGACCACATAGACCGGAGAGCCACCGCAAAGGTGATTTGCCCCACAGGTATGCCTACCAGCCCCAGCACATGTATG
 -2150 TGCATTTAAGCCTTTAGCTTCTGACTCATACACCATCAGAGGGAAGGCAGGCTGCCAGGTGCCTTATGCCTCCGCCCAGTGCACACCCCTGGCAGGCAGT
 -2050 TGTTGGCTTCTCCCATGGACAGTGAGGTAGTGTGCTTTGTGTGTGGCCAGTCCCCAGGGATCTATGGTGGTAGAGACACCACCCCTCCACATGGCTTCTC
 -1950 CTTATATTACCAAGAGCTTGGCTCAGATCTCCCTTCCAGAACCTCTGCCCATAGGATAAGCCTCCCATTCCTCTCTCCTTTGTAGACTAAGTCAGGAG
 -1850 CTATAGAAATTTGCTCTTTTAAACCAAGGATTGAGAGGCTACAAGAAATGGCTGGGGAGGAGTGGAGAGATGGCTCAGTGGTTAAGAGCAGACT
 -1750 GTTCTTCCAGAGGTCTGAGTTCAATTCCAGCAACCATGTTGGCTCTACAACCATCTGTGATGGGATCCGATGCCTCTCTGTGTGCCCCAAGAC
 -1650 AGCACATGGTGTAATCACATACATAAAATAAATAAATCTTAAAAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAATGGCTGGGGG
 -1550 CCAGAGGGTGGGGTGGGGTGGGGCAGGCTGGTGGCTTAGGTCCAGAAACCACTGGACCTAAGGGGTGCCAAACAGGCCTTTAGGTGGGTCTTAGGTAA
 -1450 TGGTAGGCTAGAAGGTGGGANTCTGTCTCTGTAGGTTTCAACCCCTGTTCTTGGAGCTCTCAAGGCTTCAGAAGGTAGAGCTGGGTAATTGTGGAGCTCCAGT
 -1350 TTGGGACAGAAGTACAGAACCTCAAGTTAGGGTTCTGGGAGGAAGGTAGTGTTCGGGGGGGTACATTTGCAACCCCTGGGTAGTCCCTGATCCATGATC
 -1250 CATCACTAATCCCATCTTGAGAGTACTGACTCTCACTTCACCACTGAGGAGGAGTGGAGGTACCCCTGAGCCTTGACAGAGCCAGGCCAGGGGGTAG
 -1150 ATAAAGGGTAGGTGTGAACGGGAGGCTCAGTCAGGGCTGAACAGTGAGAGCTGAATGTCCAGCTATCAGCAAGGGCACCATCAGCTTGTAGGCAACAGT
 -1050 CAGAGTCCCAATCCCCATCGACTCTACAACTACTCTGAACTCAGGGCAGTAGAAGCCCCACCAGCCCTCCATATTTACTGTATGTAGGCTTAGTCTAG
 -950 TCTCTACCTGGTACCAACCAGACTCAGCCTCCTCTTAGCTGGCGGTGGACTTGGACCGCTGAGCTCTGGGCGCTTCTCCAGAGATAAGAACCACTC
 -850 TTCAATCAATGTGCTCAAGGCATCAGGGAAGCGGCTGGGCTGAGCTTCACTCTGTTAGGTGCTCAAGCTTAGATTCTGTGGTTCCTGGATGGGAGTC
 -750 CCCTCTTCTGATTTCCCTGTGAAGTTGTGGCTGCAGTCCAGTCTGTGGCAAAGGTCAGGGTGTTCATGCGGCTGGCCAGGGTGAACCTCCTGGTGT
 -650 CTGTGTGGGTAGATCTCCAAAGGCTGGGTGGGAAGGGTTGGGTTACAAGGCTGAGACTATGAATGTCAAGTCTCAACTTTGAGAAGCGTTAAATATG
 -550 TGCAGACTCCTTGATGCTAATTGTCATCTCTGTGCTTTATTTGAGAGAGAGAGAGAGAGTGTGTTGAAGCAGGGCTTTCTCACCCCTGGAAACCAA
 -450 ATTGACCTCACCAGTTTTATTAGGACCGCAATCACACCACATCCGGCTTATCTGCACTTCAGAAGTTTCTGTGCCCAGCCGCTCCAGCTAGCCCT
 -350 CGCAGGTGCAAGAGGCTAGGTTGGGCGCTGCGATCTTCAGGGGAGGACCAACGTCTCTATTAGGACCTGAGGCTAGCCCTGCCCTAGGCAGGGGCG
 -250 GGGCCTGGGTCTGCAGGAGCACCCGGAATCTTAGAGGGTCCCCGAAGACTCAGCACTCTGCATGGCACTTGGCCAGTTTATCTTCCGGAGGCGAGC
 -150 ATGGACCAGATGACCAATCTTGGCCGGTCCGCCTAGATCAGAGTTCACAGGGGTCCAAGTCCCGAACTGAGGGGGTGGGGCGGGGGCGGGGCTTAAAGG
 -50 GCAGGGCCACGGGTCCCTGAAAAGCGCGGACAGGGCGGCGCCACCCAGTccagagcctacaacactacttcttctagctgcagccacttgtgcaggac

Figure 3 Sequence of CRIP genomic promoter with putative response elements

Both strands of the CRIP genomic promoter were sequenced by *Taq* DyeDeoxy Terminator Cycle Sequencing after exonuclease III digestion. The sequence represents 2644 bp upstream from the start site. Regions with high sequence similarity to known response elements are underlined and identified (GRE, glucocorticoid-response element; Sp-1; NF-1; GATA-2; AP-2; OCT and CACCC boxes).

100 mM Hepes (pH 7.9) containing 10% glycerol, 50 mM KCl and 1 mM DTT. The entire volume was then loaded on to a 5% polyacrylamide gel for electrophoresis (30 mA for 90 min). After the gels were dried, bands were detected by autoradiography.

RESULTS

CRIP induction by Dex in IEC-6 cells

The time course of Dex induction of CRIP mRNA was determined by Northern-blot analysis. This information was used to determine the maximal Dex induction in transiently transfected IEC-6 cells. Maximal CRIP mRNA levels (approx. 2-fold induction) were reached 12 h after treatment with 5 μ M Dex. As a control, metallothionein mRNA was also examined in these cells and found to be increased 10-fold by 12 h (Figure 2). The possible effects of Dex on differentiation of IEC-6 cells was also explored by treatment of cells with Dex for up to 30 h and measuring cryptdin mRNA levels by Northern-blot analysis. No effect on cryptdin mRNA was found at any time point (results not shown).

Figure 4 CAT activity in transiently transfected IEC-6 cells

Promoter constructs of decreasing length were fused with the CAT gene and transfected into IEC-6 cells in serum-free DMEM. Constructs were tested in the presence (▨) or absence (□) of 5 μ M Dex. Normalized data (c.p.m.) are reported as means $\pm$ S.E.M. ($n = 4-6$) from at least two separate experiments.

Figure 5 Effect of Dex on CRIP mRNA in 6-day-old rats

The Dex-induced increase in rat intestinal CRIP mRNA is shown by Northern-blot analysis of total RNA from Dex-treated (+Dex) and control (-Dex) pups. Pups were treated with Dex (1 mg/kg) on postnatal day 5 and intestines harvested on day 6. Total RNA (15 μ g/lane) was separated by agarose-gel electrophoresis, transferred to a Nylon filter and hybridized with ³²P-labelled β -actin and CRIP cDNA probes.

Analysis of promoter sequence

The CRIP gene promoter was sequenced, and regions within it with high sequence similarity to known response elements were identified. The sequence determined was for 2744 bp upstream from the start site identified for the murine CRIP gene [1]. The complete sequence is shown in Figure 3. Putative response elements and their positions are marked. The CRIP promoter lacks both classical CAAT and TATA boxes. The first 100 bp (from the start site to position -100) is a GC-rich region containing 74% GC residues. Putative hormone response elements for binding of the glucocorticoid and retinoic acid receptors are also present. Three regions with high sequence similarity to the known GRE consensus sequence (GCTACAnnnTGTTCT) were initially identified starting at positions -1750, -1416 and -697. An additional putative GRE, having complete sequence identity with part of a GRE found in the rat α 1-acid glycoprotein gene promoter, was identified [20]. Like several other known GREs, it does not contain the classical GRE consensus TGTCT [21,22]. The CRIP promoter contains multiple regions that may serve as binding sites for transcription factors. Immediately upstream from the GRE at -697 is a potential binding site for the transcription factor NF-1. A sequence corresponding to an octamer motif (ATTGTCAT) is found beginning at position -523. There are also six CACCC boxes found throughout the promoter, and binding sites for the transcription factors Sp-1, AP-2 and GATA-2 (Figure 3).

Transient-transfection analysis in IEC-6 cells

Transfection studies were conducted to identify regions in the genomic promoter responsible for basal activity, glucocorticoid induction and transcriptional repression during development. Using the reporter gene for CAT, the CRIP promoter constructs were tested for basal and Dex-induced activity in transiently transfected IEC-6 cells. Basal and Dex-induced CAT activities (c.p.m.) for each construct are shown in Figure 4. Constructs p2644CAT and p782CAT both had low basal activities. Deletion of the promoter to position -471 (p471CAT) resulted in removal of the octamer motif and a 6.6-fold increase in basal CAT activity. When the CRIP promoter was shortened to position -451 (p451CAT), deleting the CACCC box at position -465, an additional increase in basal activity from 6.6-fold to 8-fold was observed (Figure 4), suggesting that the CACCC box may play a role in the transcriptional repression of the CRIP gene. Treatment with Dex increased CAT activity 2-3-fold over basal activity in all but the shortest construct, p10CAT (Figure 4).

Mobility-shift assay

Gel mobility-shift assays were performed to detect the presence of nuclear proteins responsive to glucocorticoid treatment. We reasoned that if CRIP expression responds to Dex in neonatal rats, a glucocorticoid-induced nuclear protein may be involved in the observed up-regulation during the late suckling period. First, intestinal RNA from Dex-treated and control 6-day-old pups was analysed by Northern-blot analysis to confirm Dex induction of CRIP mRNA in animals used for the preparation of nuclear extracts. Dex treatment resulted in a 2-fold increase in CRIP mRNA levels in these animals (Figure 5). Promoter fragments were then incubated with nuclear extracts prepared from intestines of control and Dex-treated 6-day-old rats. Mobility-shift assay of these complexes showed that Dex treatment resulted in a single shifted band in both promoter fragments tested (Figure 6). Lanes 2 and 4, representing promoter fragments

Figure 6 Gel mobility-shift analysis of CRIP promoter fragments

Two CRIP promoter fragments starting at positions -782 (a) and -471 (b) were labelled with ^{32}P and used in gel mobility-shift analysis with $5\text{ }\mu\text{g}$ of nuclear extract prepared from intestine of Dex-treated (+Dex) and control (–Dex) 6-day-old rats.

hybridized with nuclear extracts from intestines of Dex-treated pups, clearly showed band retardation compared with lanes 1 and 3, which represented promoter fragments incubated with nuclear extracts from control pups (Figure 6).

DISCUSSION

The transcription factor Sp-1 may play a role in the initiation of CRIP transcription. Binding of Sp-1 appears to be essential for transcription initiation from a number of TATA-less promoters [23,24]. This may involve the interaction of bound Sp-1 at the initiation site and the transcription factor TFIID [25]. Zhu et al. [26] have proposed a regulatory role for overlapping Sp-1 sites such as those found between positions -74 and -58 of the CRIP promoter.

The longest CRIP promoter constructs (p2644CAT and p782CAT) had low basal activities. Deletion of the promoter to position -451 (p451CAT) resulted in a significant increase in basal CAT activity. This suggests that between positions -782 and -451 there are negative response elements (NREs). Similar NREs have been identified in other genes. Transfection of HepG2 cells with 5' deletions of the caeruloplasmin promoter revealed several NREs between positions -5200 and -732 [27]. Negative acting elements that maintain low basal expression have also been identified [28,29]. Repressor proteins that bind to the CRIP promoter between positions -782 and -451 could explain the tissue specificity of the CRIP expression. In this model, tissues showing little or no expression of CRIP mRNA, such as liver or kidney, would have high levels of synthesis of repressor protein.

A repressor protein could also help explain the developmental increase in intestinal CRIP mRNA. A developmental decrease in expression of repressor protein could result in the observed increase in intestinal CRIP mRNA levels with age. The exact binding site for the hypothesized repressor protein has not been identified. As discussed, the NRE in the CRIP promoter appears to be between positions -782 and -451 . The octamer motif identified in this region has been shown in other genes to act as an NRE [30]. Also CACCC boxes, such as those found at position -465 in the CRIP gene, have been shown to down-regulate the monoamine oxidase B gene [26].

CRIP mRNA is clearly regulated by glucocorticoid hormones in the neonatal intestine and cultured IEC-6 cells. It is possible that the observed increase in CRIP mRNA is due to generalized maturational effects on the neonatal small intestine or induction of differentiation in IEC-6 cells. However, the results reported here show that the increase in mRNA is, at least in part, due to the interaction of Dex-induced nuclear factors with the CRIP gene promoter. Evidence for direct glucocorticoid action was obtained by transient-transfection analysis and mobility-shift assay. The fact that a shift occurred in the major band with nuclear extracts prepared from intestines of Dex-treated 6-day-old animals suggests the presence of a nuclear factor that is induced by glucocorticoid hormones and binds to the CRIP promoter.

GREs identified in promoters are either simple or composite. Simple GREs in a promoter result in glucocorticoid-receptor-enhanced transcription without the requirement for additional transcription factors or protein-protein interactions [31]. In contrast, hormonal regulation from composite GREs requires both receptor and non-receptor factors [32], and is probably the major mode of glucocorticoid regulation [33].

Comparison of the CRIP genomic promoter sequence with the known GRE consensus sequences revealed that there are four promoter regions with high sequence similarity to known GREs. However, CAT analysis of the 5' deletion constructs made from the CRIP promoter suggests that these GREs are not fully functional in this system, as deletion of the promoter to position -163 did not result in a significant reduction in Dex-induced CAT activity. There are a number of possible explanations for these results. First, CRIP is a developmentally regulated protein, increasing during the first 3 weeks of life. During this period CRIP mRNA levels are most susceptible to induction by glucocorticoids. It is possible that the distal GREs are only functional during the developmental period. This effect may not have been evident in our transient-transfection studies as IEC-6 cells are derived from adult rat intestinal epithelial cells. Unfortunately, our attempts to induce CRIP mRNA with Dex in cultured IRD-98 cells, which are derived from fetal rat intestine, were not successful. Secondly, although CRIP mRNA can be induced by Dex in IEC-6 cells, these cells are derived from the crypt region of the rat intestine. We have previously shown that CRIP mRNA is most strongly expressed in the mid-villar region of the intestine. This is confirmed by the low basal levels of CRIP mRNA in IEC-6 cells. Thus it is possible that IEC-6 cells may not produce nuclear factors required for induction from composite GREs. Other regions with high sequence similarity to known GREs, but apparently not active in this transient-transfection system, may be active in other tissues or at other developmental periods.

We have shown that transcription of the CRIP gene is probably under the control of multiple elements in the promoter region which may confer the observed glucocorticoid responsiveness, tissue specificity and developmental regulation of the CRIP gene. These experiments provide the initial data required to explore

further the unique regulation and function of this Zn²⁺-binding LIM-motif-containing protein.

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