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Tissue specific activity of the proximal human calcitonin receptor promoter is mediated by Sp1 and an epigenetic phenomenon

M.D. Pondel*, G.A. Partington, R. Mould

Department of Cellular and Molecular Medicine, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK

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Abstract To identify *cis*-acting sequences transcriptionally regulating the human calcitonin receptor (hCTR) gene, hCTR promoter/luciferase gene constructs were transiently or stably transfected into hCTR-positive and -negative cell lines. Luciferase assays demonstrated that the proximal hCTR promoter (hCTRP1) was transcriptionally active in all cell lines tested. High-level hCTRP1 activity depended on an 11 bp Sp1/Sp3 binding site. Electrophoretic mobility shift assay showed that this region bound the transcription factors Sp1 and Sp3. We further showed that hCTRP1 was strongly activated by the 11 bp Sp1/Sp3 binding site in hCTRP1/luciferase-, Sp1-transfected *Drosophila* S2 cells. Bisulphite-mediated sequencing of genomic DNA from hCTR-expressing and -non-expressing cell lines demonstrated that the endogenous hCTRP1 was hypomethylated in all cell lines tested. These results suggest that the hCTRP1 is activated by the tissue-ubiquitous transcription factor Sp1 and that an epigenetic process unrelated to CpG methylation represses its activity in hCTR-negative tissues.

Key words: Calcitonin receptor; Transcriptional regulation; Sp1; Sp3

1. Introduction

The calcitonin receptor (CTR) is a member of the seven trans-membrane domain G protein-coupled receptor superfamily [1,2]. Its binding to calcitonin (CT), a 32 amino acid peptide hormone, stimulates activation of the adenylate cyclase/cAMP protein kinase A pathway [3], the phosphoinositide-dependent phospholipase C pathway and the protein kinase C pathway [4,5]. CT inhibits osteoclast-mediated bone resorption and increases Ca^{2+} excretion by the kidney [6–8]. High-affinity receptors for CT have been identified in a variety of tissues such as brain [9,10], testis [11], spermatozoa [12], kidney [13,14], osteoclasts [15–17], skeletal muscle [18], breast cancer cell lines and human primary breast cancer [19–21]. Recently, we identified the presence of CTRs in the chronic myelogenous leukaemia cell line K562 [22].

Both human and mouse CTR (hCTR and mCTR) genes have been shown to be regulated by multiple promoters. The mCTR gene contains three promoters: P1, P2 and P3. Data suggest that the P3 promoter is only transcriptionally active in osteoclasts [23]. At least two promoters, hCTRP1 (proximal) and hCTRP2 (distal), have been identified and shown to regulate hCTR gene expression [24]. Transcriptional analysis of a 4.9 kb DNA fragment (4.5 kb 5′-flanking region + 441 bp 3′-flanking region) containing hCTRP1 and hCTRP2 in transgenic mice suggest its transcriptional activity is restricted to CTR-expressing tissues [25]. Deletion mapping of this fragment in transfected hCTR-positive T47D breast cancer cells demonstrated that only 97 bp of hCTRP1 5′-flanking region (cap = 0) contains at least 70% of the transcriptional activity of the 4.9 kb hCTR promoter fragment [24]. However, transcriptional regulatory elements within this region and the transcription factors they interact with have not yet been identified.

In this paper, we employed hCTRP1 deletional analysis and electrophoretic mobility shift assays (EMSA) to identify DNA sequences and the transcription factors they interact with that regulate hCTRP1 transcriptional activity. The role Sp1/Sp3 transcription factors play in hCTRP1 transcriptional activity was examined and bisulphite-mediated sequencing of hCTRP1 was carried out to determine if CpG methylation of hCTRP1 was associated with repression of its transcriptional activity in non-hCTR-expressing cell lines.

2. Materials and methods

2.1. Cell lines and culture

hCTR-positive human breast cancer cell lines T47D and MCF-7 and the hCTR-negative human foreskin cell line HS27 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 100 μg/ml penicillin and 100 U/ml streptomycin. The hCTR-negative embryonic kidney cell line HEK293 was maintained as above except Earle's modified essential medium (MEM) supplemented with MEM non-essential amino acids was employed. *Drosophila* Schneider 2 (S2) cells were maintained in Schneider's *Drosophila* medium (Gibco) supplemented with 10% heat-inactivated foetal calf serum, 100 μg/ml penicillin and 100 U/ml streptomycin.

2.2. DNA transfections

For transient transfections in mammalian cell lines, cells were transfected with 1–2 μg of specific hCTR/luciferase construct, 0.2 μg of a co-transfection control plasmid pRLTK (Promega) and Fugene 6 (Roche) according to the manufacturers' instructions. Luciferase assays were performed employing a dual luciferase receptor assay system (Promega) and a Turner Design TD-20/20 luminometer. For stable transfection assays, T47D and HEK293 cells were transfected with 2 μg of specific plasmid as above. Pools of G418 (1.2 mg/ml)-resistant clones for each construct were harvested and luciferase assays performed as above on 20 μg of lysed cell extract. For transient transfections into Schneider cells, 0.5 μg of various hCTR constructs were co-transfected with varying amounts of pPacSp1 and/or pPacSp3 and 1 μg of a β-galactosidase co-transfection control plasmid p97b (kind

*Corresponding author. Fax: (44)-20-8725 0064.
E-mail address: mpondel@sghms.ac.uk (M.D. Pondel).

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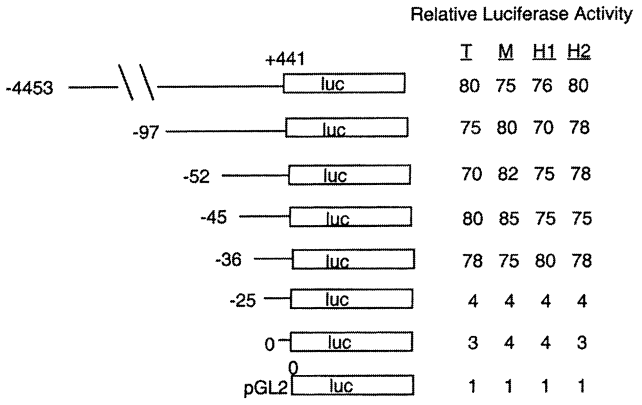

Fig. 1. hCTR promoter/luciferase deletion analysis in transiently transfected cell lines. Amount of 5′-flanking region relative to hCTRP1's most 5′ transcriptional start site (cap = 0) is indicated. 441 bp of hCTR 3′-flanking region are present in all constructs except pGL2-basic. Relative luciferase activities were determined by comparing luciferase activity of cells transfected with hCTR/luciferase constructs to cells transfected with the promoter-less pGL2-basic luciferase plasmid. Background luciferase levels = 1. Cell lines employed were: T47D (T); MCF-7 (M); HS27 (H1); HEK293 (H2). These data represent averages of three independent sets of transfections per construct all giving similar (< 10% variation) results.

gifts of Prof. G. Suske). Transfections and luciferase assays were performed as above.

2.3. Generation of hCTR/luciferase constructs

An hCTR/luciferase construct containing 4.5 kb of 5′-flanking region (relative to hCTRP1's most 5′ start site of transcription) and 441 bp of 3′-flanking region [24] was cut with *Acc*65I/*Afl*II and purified. Sense and antisense oligos corresponding to sequences −1 to −52, −1 to −45, −1 to −36, −1 to −25 (also relative to hCTRP1's most 5′ start site of transcription) and each containing a 5′ *Acc*65I and 3′ *Afl*II site were annealed and ligated into the *Acc*65I/*Afl*II-cut vector. To generate an hCTR construct with 0 bp of 5′-flanking region, the *Acc*65I/*Afl*II-cut vector was purified, filled in with Klenow and blunt-end ligated. For stable transfections, luciferase construct pGL2-basic (Promega) and pGL2-basic containing 4.5 kb or 97 bp of the hCTR 5′ region were cut with *Sca*I and a blunt-ended SV40 promoter/neo gene fragment ligated in.

2.4. Nuclear extracts and EMSA

Preparation of nuclear extracts from all cell lines was carried out as previously described [26]. Double-stranded oligonucleotides C1 5′-CCGCCTGTCGGGAGGCGGGCGGGGGTGGGGCGGGAGCGCAGGC-3′; C2 5′-CGGGCGGGGGTGGGGCGGGAGCGCAGGC-3′; C3 5′-AGGCGGGAGGCGGGCGGGGGTGGGGCGGGA-3′; C4 5′-CGGGGGTGGGGCGGGAGCGCAGGC-3′; C5 5′-AAGGGGGGCTGGCTCTCATCAATTCTGCTGCCACCTCCTCTGC-3′; C6 5′-CGGGAGCGCAGGCTAGGATTGAGACTCTT-3′; C7 5′-CCGCCTGTCGGGAGGCGGAGGTGGGTGGGGCGGGAGCGCAGGC-3′; and C8 5′-CCGCCTGTCGGGAGGCGGGCGGGGGTGAGGTGGGAGCGCAGGC-3′ were employed for EMSA as previously described [26]. Each double-stranded oligonucleotide has three unpaired bases at its immediate 5′ and 3′ ends. A variant Sp1 binding site [27] present in oligos C1–C4 has been underlined. Sp1 goat polyclonal antibody (sc-59X) was purchased from Santa Cruz. Sp3 antibody was a kind gift from Prof. G. Suske.

2.5. Methylation analysis of hCTRP1

A modified bisulphite-mediated genomic sequencing protocol was employed [28]. Bisulphite-treated DNA was subjected to nested polymerase chain reaction (PCR) utilising two sets of primers. Set 1: (sense) 5′-GAGGGTGTTAGGGGGAAAGAAGAGGAGT-3′; (antisense) 5′-AATCCTCCCTCTCCGCCCCTCTCCT-3′; Set 2: (sense) 5′-TGGGATAAGGTTGTTGTGGAAA-3′; (antisense) 5′-CCCCTAACTTACTTTCTACCTCCCC-3′. Qiagen Taq polymerase was employed for PCR according to the manufacturer's instructions. PCR products were cloned into the vector pGEM T Easy (Promega) and DNA from 8–10 clones representing each cell line was subjected to DNA sequence analysis.

3. Results

3.1. hCTRP1 deletional analysis in hCTR-positive/negative cell lines

To identify *cis*-acting sequences regulating hCTRP1 activity, seven hCTR/luciferase constructs containing 4.5 kb, 97, 52, 45, 36, 25, and 0 bp of 5′-flanking region relative to hCTRP1's most 5′ transcriptional start site were transiently transfected into hCTR-positive T47D and MCF-7 cells. Luciferase assays demonstrated that a construct with only 36 bp of 5′-flanking region was as transcriptionally active as the 4.5 kb hCTR promoter. However, the deletion of an additional 11 bp reduced expression levels to that of a construct containing no hCTR 5′-flanking region (Fig. 1). This 11 bp region is highly GC-rich (> 90%), contains a variant Sp1/Sp3 binding site (GGGGTGGGGC) [27] and has potential Sp1/Sp3 binding sites at its 5′/3′ junctions (see Section 2).

To assess the tissue specificity of hCTRP1, we transiently transfected the above constructs into the hCTR-negative cell lines HEK293 and HS27. Luciferase activity from all hCTRP1 constructs was identical in both hCTR-expressing and -non-expressing cell lines. Even the construct containing 4.5 kb of hCTR promoter and shown to contain sufficient 5′-flanking region for CTR tissue-specific expression in transgenic mice [25] was transcriptionally active in hCTR-negative cells (Fig. 1). Similar results were obtained in stably transfected T47D and HEK293 cells (data not shown). These results demonstrate that hCTRP1 is transcriptionally promiscuous in transiently or stably transfected cell lines.

3.2. EMSA analysis of the hCTRP1 activation region

To examine the interaction of the 11 bp hCTRP1 activation region and its immediate 5′ and 3′ junctions with nuclear regulatory transcription factors, three oligo probes (C2–C4) (see Fig. 2 and Section 2) overlapping this region were synthesised and EMSA carried out employing nuclear extracts from T47D cells (Fig. 3). ^{32}P end-labelled C2–C4 probes detected three complexes (A, C and D) while probes C2 and C3 detected an additional complex (B) (see lanes 1, 6 and 11). It should be noted that complex A is non-uniform and consists of multiple bands. Unlabelled oligonucleotides C2 and C3 effectively competed for binding of transcription factors in all complexes (see lanes 3, 4, 8, 9, 13 and 14). Unlabelled

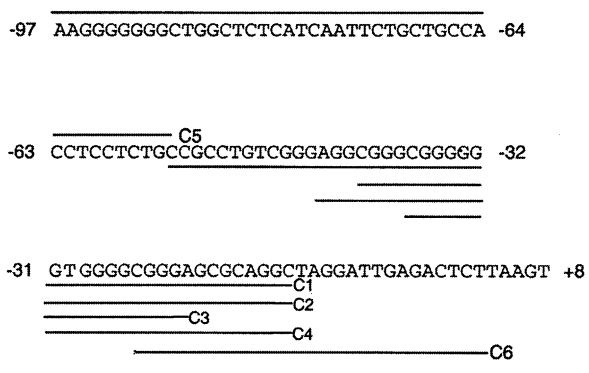

Fig. 2. Positions of double-stranded oligonucleotides employed for gel shift analysis of the hCTRP1 promoter (see Figs. 3 and 4).

probe C4 competed for transcription factor binding in all complexes except factor present in complex B (lanes 5 and 10). These results demonstrate that the transcription factor in complex B binds immediately 5′ to the 11 bp hCTR activation region and no factors detectably bind immediately 3′ to this region. Site-directed mutagenesis of the region just 5′ to the 11 bp hCTR activation region (see oligo C7 in Section 2) eliminated complex B while mutagenesis of the 11 bp activation region (see oligo C8 in Section 2) eliminated complex B but not A and C (data not shown). We hypothesise that transcription factors present in complex A and C, normally binding to the 11 bp activation region, are able to bind 5′ to this region when their preferred binding site(s) are mutated. Subsequent displacement of transcription factor in complex B then occurs. Probe C1 (see Fig. 2 and Section 2), which overlaps region C2–C4, also effectively competed for the binding of transcription factors in all complexes (lanes 2, 7 and 12). No complexes were detected when oligos C5 and C6 (see Fig. 2 and Section 2) were employed (data not shown). Together, these results suggest that only the C2–C4 region of 97 bp of hCTRP1 5′-flanking region detectably binds transcription factors. Furthermore, the hCTRP1 11 bp core activation region binds factors in three complexes (A, C and D) while an additional factor present in complex B binds immediately 5′ to this region.

The 11 bp hCTR activation region contains a variant core (GGGGTGGGGC) Sp1/Sp3 binding site. To determine if any of the DNA/protein complexes in this region were due to the presence of Sp1/Sp3, EMSA on C2–C4 double-stranded oligonucleotides was carried out with Sp1/Sp3 antibodies (Fig. 4). At least one band comprising part of complex A on oligos C2–C4 super-shifted with Sp1 antibodies (lanes 3, 7 and 11)

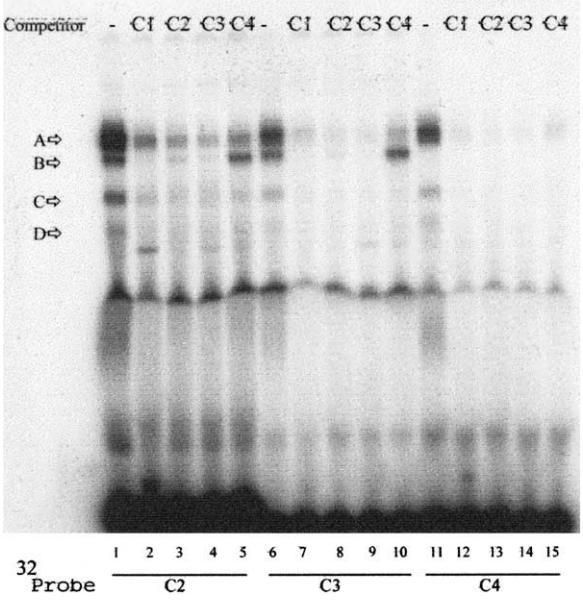

Fig. 3. EMSA of nuclear regulatory factors from T47D breast cancer cells binding to ^{32}P end-labelled oligos C1–C4. Each oligo was also employed as an unlabelled competitor. The ^{32}P end-labelled oligonucleotide probes were used at a concentration of 40 fmol/assay and unlabelled competitor oligonucleotides were used at a concentration of 2 pmol/lane. Non-specific competitor poly-dIdC (0.5 μg/lane) was present in all lanes. Complexes A–D are indicated. This experiment was performed at least three times, each giving similar results.

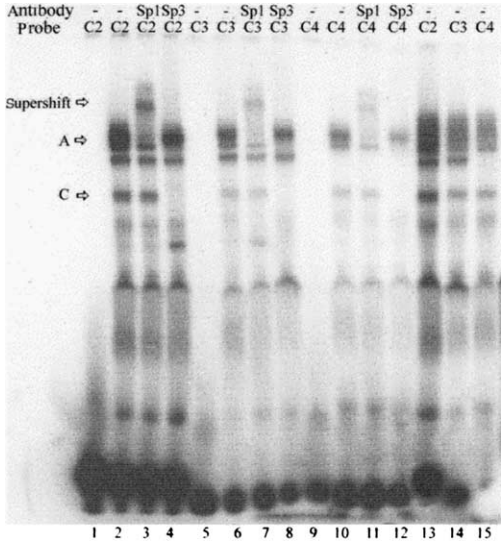

Fig. 4. EMSA with antibodies to factors binding to ^{32}P end-labelled oligos C2–C4. Antibody to Sp1 or Sp3 (0.2 μg/lane) was included in lanes 3, 4, 7, 8, 11, 12. Nuclear extracts from T47D were employed for lanes 2–4, 6–8, 10–12, and HEK293 for lanes 13–15. Lanes 1, 5 and 9 contain no nuclear extract. ^{32}P end-labelled probes were used at a concentration of 40 fmol/assay. This experiment was performed at least three times, each giving similar results.

while anti-Sp3 caused complex C to disappear (lanes 4, 8 and 12). Lanes 13–15 suggested that extract from the non-hCTR-expressing cell line HEK293 contained identical Sp1/Sp3 complexes. Subsequent experiments employing HEK293 nuclear extract and Sp1/Sp3 antibodies detected the presence of Sp1/Sp3-containing complexes as above (data not shown). Overall, the results suggest that the tissue-ubiquitous transcription factors Sp1 and Sp3 interact with the 11 bp hCTRP1 activation region.

3.3. *Activation of hCTRP1 in* Drosophila *Schneider cells*

To further assess the role Sp1/Sp3 plays in hCTRP1 activity, we transfected luciferase constructs containing 97, 36, 25, and 0 bp of hCTR 5′-flanking into *Drosophila* S2 cells which are well known to lack endogenous Sp proteins. The expression levels of these constructs were examined in the presence or absence of 5 ng of co-transfected Sp1 or Sp3 expression constructs (Fig. 5). Luciferase activity from constructs containing 97 and 36 bp of 5′-flanking region was 40-fold above pGL2-basic background levels when co-transfected with the Sp1 expression plasmid pPacSp1. However, deletion of the 11 bp hCTRP1 activation region caused up to a 93% drop in hCTRP1 activity similar to expression from a construct containing no hCTR 5′-flanking region. In the absence of co-transfected pPacSp1, all constructs expressed at background levels. Luciferase activity from constructs containing 97 and 36 bp of hCTRP1 flanking region was only two- to three-fold above background levels when co-transfected with 5 ng of pPacSp3. Co-transfected pPacSp3 had no detectable effect on expression from hCTR constructs containing 25 and 0 bp of 5′-flanking region. Results identical to the above were also achieved with 2–20 ng of co-transfected pPacSp1 or pPacSp3. However, at levels above 50 ng of pPacSp1/pPacSp3, squelching of hCTRP1 transcriptional activity was evident (data not shown). The results of these experiments demonstrate that Sp1 transcriptionally activates hCTRP1 pri-

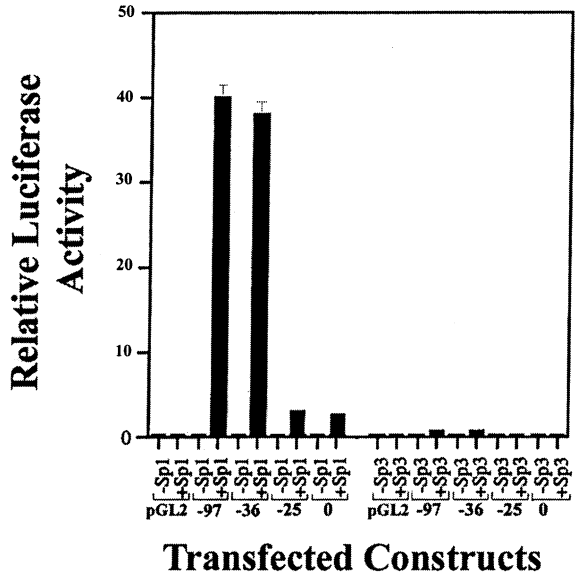

Fig. 5. hCTR promoter/luciferase deletion assays in *Drosophila* S2 cells co-transfected with or without pPacSp1/pPacSp3. Amount of hCTR 5′-flanking region for each construct is indicated. 0.5 μg of each hCTR construct was co-transfected with/without 5 ng of pPacSp1 or pPacSp3. Relative luciferase activity was compared between cells transfected with hCTR constructs (with/without co-transfected pPacSp1/pPacSp1) and the promoter-less pGL2-basic plasmid (relative luciferase activity = 1). The data are the average of three independent experiments each giving similar results.

marily by its interaction with the 11 bp hCTR activation region. Transactivation by Sp1 is also mediated, although to a much lesser extent, by sequences 3′ to hCTRP1's most 5′ start site of transcription.

To determine if Sp3 can inhibit hCTRP1 activation by Sp1 in S2 cells, varying pPacSp1 and pPacSp3 concentration ratios

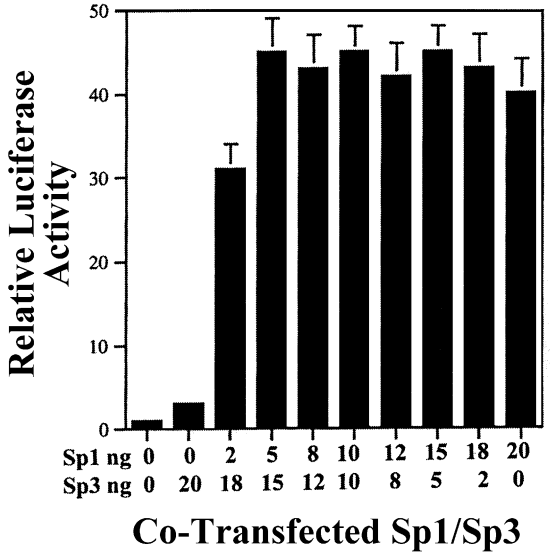

Fig. 6. −97 hCTR/luciferase activity in transiently transfected *Drosophila* S2 cells. Varying ratios of pPacSp1 and pPacSp3 were co-transfected with 0.5 μg of −97 hCTR/luciferase construct. Relative luciferase activity was compared to cells transfected with −97 hCTR/luciferase alone (relative luciferase activity = 1). The data are the average of three independent experiments each giving similar results.

were co-transfected with 0.5 μg of −97 hCTR/luciferase construct into S2 cells (see Fig. 6). The data demonstrate that when a 3:1 ratio of pPacSp3 to pPacSp1 is transfected in Schneider cells, hCTRP1 activity is still increased up to 45-fold. Only when nine times more pPacSp3 is present than pPacSp1 (9:1) is a modest but consistent decrease (25%) in hCTRP1 activity observed.

3.4. Methylation mapping of hCTRP1

hCTRP1 contains a highly GC-rich region with multiple CpG dinucleotides (see Fig. 7A). The 11 bp hCTRP1 activation region is present within this region. One possible mechanism by which hCTR gene transcription could be repressed in specific tissues in vivo is by CpG methylation of this region. However, such a process is unlikely to be recapitulated in transiently or stably transfected cell lines resulting in hCTRP1 transcriptional promiscuity. We therefore employed bisulphite-mediated sequencing of genomic DNA from hCTR-expressing (MCF-7) and -non-expressing cells (HS27 and HEK293) to examine the methylation state of the endogenous hCTRP1. Sequence analysis on cloned bisulphite-treated genomic DNA from all the above cell lines demonstrated that

A

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-425 ACTCACCTCCCTCCGGAATTTGCATTCTCCGGGGAGGGGACAGG
                  30              29
-381 GCTCCCAAGAAAGCTGTCTCCCAGTCCAGACTGTCGCCCCCCTC
                                  28
-337 TCCCTCCCTACTCAAGGTCTAACTCGGGTCCCTCGCCTGCTTCC
                              27      26
-293 TGTGTTTACGCGGCGCTTTAGTCTCCCGGACTCGCAGGGTGAGC
           2524 23            22    21
-249 CCCAGCCCTGACTGGAGCGAGACAGCAGCCGCGAGCGCAGCCCC
                     20            1918  17
-205 ACTCGCGGGCCGGGGCGACTGGGGCTGGCGCGAGGCTCACGGAG
       1615   14   13              12 11       10
-161 CTCACCAGCTCGCCCCTCCCTCTCCTGGGACAGGAGGGGGCTGA
                9
-117 CTGGGGTGGCGGGGTCCGGGAAGGGGGGCTGGCTCTCATCAATT
                8       7
 -73 CTGCTGCCACCTCCTCTGCCGCCTGTCGGGAGGCGGGCGGGGGT
                           6     5     4    3
 -29 GGGGCGGGAGCGCAGGCTAGGATTGAGAC-1
        2     1
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B

Clone	Cell Line	CpG methylated
1,3,6-10	MCF-7	0
2	MCF-7	5,30
4	MCF-7	9
5	MCF-7	4
1,6	HEK293	0
2	HEK293	22
3	HEK293	7,14
4	HEK293	5,6
5	HEK293	10,12,13,29
6	HEK293	0
7	HEK293	5,20
8	HEK293	0
1,2,4	HS-27	0
3	HS-27	10,13,14
5	HS-27	26
6	HS-27	0
7	HS-27	29
8	HS-27	0
9	HS-27	5
10	HS-27	4,5

Fig. 7. Bisulphite-mediated sequencing of the hCTRP1 promoter in MCF-7, HS27 and HEK293 cells. A: Sequence analysis of 425 bp of hCTRP1 5′-flanking region. Each CpG dinucleotide is numbered and underlined. B: Methylation state of hCTRP1 CpG dinucleotides in MCF-7, HS27 and HEK293 cells. Genomic DNA from each cell line was bisulphite-treated and restriction enzyme-cut followed by PCR amplification of CpG-rich region. PCR products were subcloned and 8–10 clones (representing each cell type) subjected to DNA sequence analysis.

the entire GC-rich region of hCTRP1 is relatively methylation-free (hypomethylated) (see Fig. 7B). This suggests that a DNA methylation-mediated mechanism is not responsible for the repression of hCTRP1 activity in non-hCTR-expressing cells.

4. Discussion

Although both the human and mouse CTR promoters have been cloned and sequenced, specific DNA sequences that activate their transcription have not previously been described. In this paper we defined an 11 bp region of hCTRP1 that is necessary for its high-level activity in a variety of cell types. This region contains a variant Sp1/Sp3 binding site [27] along with consensus Sp1/Sp3 binding sites at its 5′ and 3′ junction. EMSAs with antibodies to Sp1 and Sp3 demonstrate these factors bind to this activation region. Although our analysis detected the presence of other unidentified factors (present in complexes B and D) binding on or near this region, the well-known ability of Sp1 to activate gene transcription combined with our results demonstrating that Sp1 and its interaction with the 11 bp hCTR activation region can significantly transcriptionally activate hCTRP1 in *Drosophila* S2 cells suggests that Sp1 plays a major role in the regulation of hCTRP1 activity. Our results showing that Sp3 only marginally increases hCTRP1 activity is consistent with a variety of studies showing Sp3 is either inactive or a weak activator of transcription [29–31]. Since Sp1 and Sp3 bind to the same recognition sequence, it is reasonable to hypothesise that the ratio of Sp3 and Sp1 in different cell types can determine the level to which the hCTRP1 promoter can be activated. However, we found that even a 9:1 ratio of Sp3 to Sp1 caused only a modest decrease (25%) in hCTRP1 activity. This contrasts with Kumar and Butler [31] who demonstrated that a 3:1 ratio of Sp3 to Sp1 reduced ornithine decarboxylase promoter activity in Schneider cells up to 97%. As the 11 bp hCTR activation region contains a variant Sp1 binding site [27] it is possible that Sp1 may have a greater binding affinity for this site than does Sp3. This hypothesis is currently being tested.

Previous work with transgenic mice suggested that a DNA fragment containing 4.5 kb of hCTR 5′-flanking region directed expression of a linked *lacZ* gene specifically to CTR-expressing tissues in vivo [25]. The observation that the same fragment is transcriptionally active in transiently and stably transfected hCTR-negative cells lines suggests that sequences which may act to repress hCTRP1 promoter activity in specific tissues in vivo are not functional in stably and transiently transfected cells. Such a lack of regulatory function in transfected cells versus transgenic mice has been previously demonstrated by Stanworth et al. [32]. They showed that regulatory elements directing human ζ-globin gene expression specifically to embryonic erythroid cells in transgenic mice failed to suppress ζ-globin gene expression in adult transfected erythrocytes. We hypothesise that an epigenetic process takes place early in mouse development that acts to repress hCTR gene expression in specific tissues. Such a process does not appear to be recapitulated in transiently or stably transfected cell lines.

One epigenetic process believed to play a major role in the control of tissue-specific gene regulation in vivo is cytosine methylation. Heritable patterns of CpG methylation have been shown to repress transcription by blocking the access of transcription factors and inducing the formation of inactive chromatin [33]. Recently, we demonstrated that in vitro methylation of the hCTR promoter represses its transcriptional activity in transfected cells (unpublished results). However, our results showing that the hCTRP1 promoter is hypomethylated in both hCTR-expressing and -non-expressing cell lines suggest that CpG methylation is not the key epigenetic mechanism that represses hCTR gene expression in non-hCTR-expressing tissues. Additional studies examining patterns of histone acetylation and chromatin structure of the hCTR gene are under way to elucidate the mechanism(s) that restrict the expression of the hCTR gene in vivo.

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