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The Complete Sequence of the Host Cell Factor 1 (HCFC1) Gene and Its Promoter: A Role for YY1 Transcription Factor in the Regulation of Its Expression

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We report here the complete sequence of the Host Cell Factor (HCFC1) gene, including two kilobases of the 5′-flanking region and 5.9 kb of the first intron. The upstream and 5′-untranslated regions contain several putative transcriptional factor binding sites and a 17-nt-long repeated element (SiSa element) present in six regularly spaced copies, of which five are perfectly identical, while the sixth has a transition substitution (CT for TC) at nucleotides 13 and 14. Four copies are contained in the flanking region, the fifth is at the beginning of the mRNA (position +9), and the sixth is at position 195 of the mRNA. This 17-bp element contains at its 5′ side an octamer sequence known to bind the Yin/Yang 1 (YY1) transcription factor; another YY1 binding octamer is present at the end of the first intron. The promoter also contains several Sp1 binding sites, some of which are located very close to SiSa elements. We demonstrate that YY1 binds to the 5′ half of the SiSa element, whose 3′ region binds in gel shift experiments an additional, as yet unidentified nuclear factor. Therefore the YY1 binding site in HCFC1 overlaps the site of a second factor, as has been described in several YY1-site-containing promoters. This suggests that HCFC1 expression might be regulated by the reciprocal interaction of several transcription factors.

INTRODUCTION

The Host Cell Factor (also called C1, VCAF, and CFF) was originally defined as a cellular component involved in the action of the Herpes Simplex transactivator protein VP16 (Wilson *et al.*, 1993). VP16 is known to be one of the strongest transcriptional transactivators, exerting its function by binding to DNA in concert with the POU homeodomain-containing protein Oct-1. However, the presence of cellular extract enhances the transcriptional activity of VP16/Oct-1 complex by one or two orders of magnitude (Kristie and Sharp, 1993). This enhancement is due to the Host Cell Factor, which consists of a family of polypeptides (with molecular mass ranging from 110 to 300 kDa) encoded by a single gene, whose cDNA has been cloned (Wilson *et al.*, 1993). It has been assumed that all the polypeptides derive from posttranslational processing of a polypeptide encoded by a single transcript of 8 kb (Frattini *et al.*, 1994; Wilson *et al.*, 1995). However, the possibility that alternative RNA processing could contribute in part to the diversity of HCFC1 polypeptides in a subset of tissues has not yet been completely ruled out (Frattini *et al.*, 1994). The role of this gene in the physiology of the cell is completely unknown. In addition, nothing is known about its regulation, other than the finding that it is ubiquitously expressed. Our previous studies have established that the HCFC1 mRNA is assembled from 26 exons spread over a region of approximately 24 kb on the Xq28 chromosomal region, close to the Renin Binding Protein gene, in a 180-kb contig that also includes the V2R and L1CAM genes (Faranda *et al.*, 1995; Frattini *et al.*, 1994).

In the present paper we report the complete sequence of the gene, including 2146 bp upstream of the mRNA start site and the whole 5.9-kb first intron, where regulatory elements are usually found. In addition to the detection of many putative binding sites for known DNA binding proteins, a highly conserved 17-bp sequence was found to be present six times at regular intervals in the 5′ region of the gene. This motif is capable of binding the transcription factor Yin/Yang 1 (YY1) as well as another unidentified factor, suggesting that HCFC1 expression is regulated by the interaction of these factors.

MATERIALS AND METHODS

Genomic clone, subcloning, DNA sequencing, and sequence analysis. The genomic clone for sequencing was obtained from a cosmid library made in pWE15 vector from human/hamster hybrid X3000.11 as previously described (Frattini *et al.*, 1994; Villa *et al.*, 1992). Cosmid fragments were subcloned into pBluescript II KS(+) vector (Stratagene). For sequencing, DNA templates were prepared using the Wizard kit (Promega) and sequenced by the dideoxynucleotide chain

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termination method using the Sequenase kit (USB). The strategy was based on the sequencing of restriction fragments subcloned in pBluescript using vector primers and on internal primers designed to cover any gaps. The whole genomic locus, including 2146 bp upstream from the reported cDNA clone and 0.5 kb from the 3′ end of the transcript, was sequenced with a more than fivefold redundancy, with every region sequenced on both strands. The exon boundaries were determined by comparing the cDNA to the genomic sequence. Computer analysis of nucleotide sequences was performed with the GCG software package on a VAX 3600 computer. Homology searches were performed in GenBank (Release 88, 3/95) and EMBL (Release 42, 3/95). The Accession No. L20010 refers to the sequence of the cDNA as determined by Wilson and co-workers (Wilson *et al.*, 1993). The Accession No. of the genomic sequence is X79198, and for the promoter region and the first intron the Accession No. is X84221.

Cell lines. A panel of established human cell lines was used for the electrophoretic mobility shift assay (EMSA). HeLa and Chang cells were grown in DMEM (Gibco) supplemented with 10% bovine serum; Jurkat and Ramos cells were grown in RPMI supplemented with 10% fetal bovine serum and 50 μM β-mercaptoethanol for Ramos; 293 cells were grown in DMEM supplemented with 10% fetal bovine serum; SK-N-SH cells were grown in DMEM supplemented with 15% fetal bovine serum; and T84 cells were grown in HAM12 medium supplemented with 10% bovine serum.

Electrophoretic mobility shift assay. Nuclear extracts (NE) were prepared from 10^7 cells following the method by Andrews and Faller (1991) with minor modifications. In brief, scraped cells were washed once in PBS, pelletted, and frozen on dry ice. The pellet was then thawed on ice, resuspended in 400 μl of cold buffer A (10 mM Hepes; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT); 0.2 mM PMSF), and left on ice 10 min; after a brief spin, the nuclear pellet was resuspended in 50 μl of ice-cold buffer C (20 mM Hepes; 25% glycerol; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT) and incubated on ice for 20 min before spinning in a microfuge for 10 min at 4°C. Protein concentration of the crude nuclear extract was measured with the Bio-Rad protein assay reagent. The following oligonucleotides were synthesized: SiSa, TAGTCTCAAGATGGCGGCTCCCAGGGCGGCGGCCTCTAT; SiSa 5′, TAGTCTCAAGATGGCGGCCTCATA; SiSa 3′, TAGTCTCCCAGGGCGGCCTCATA; wt YY1, CCCGCTTCAAAATGGAGACCCTCGGCCTCATA; mutant YY1, CCCGCTTCAAAATTTAGACCCTCGGCCTCATA; and oligo 10, TATGAGGCCG.

Double-stranded oligonucleotides were produced by Klenow polymerase extension of oligo 10 annealed to each of the other oligos and labeled by end phosphorylation (using NEB T4 polynucleotide kinase) in the presence of [γ-^{32}P]ATP. In the competition experiments, 100-fold molar excess of unlabeled probe was added during the first incubation step.

Binding reactions were carried out in two steps (10 min preincubation before adding the probe and second incubation) either on ice or at room temperature as indicated, in 10 μl containing 7.5 μg NE and 5×10^5 cpm of ^{32}P end-labeled probe in buffer B (75 mM NaCl; 15 mM Tris–HCl, pH 7.5; 1.5 mM EDTA; 0.2 or 1.2 mM DTT, see Results; 5% glycerol; 20 μg/ml BSA). The resulting complexes were resolved on a 4% native polyacrylamide gel, which was then dried and exposed to X-ray film.

RESULTS

Sequence Analysis

The whole HCFC1 gene is contained in cosmid 430. We determined the complete sequence of the locus, including 2146 bp upstream of the transcription start site as well as 0.5 kb downstream from the 3′ end of the transcript.

From an analysis of the whole sequence, it appears that most introns are small, with the exception of the first one, which spans 5918 nt. No significant homology was detected in databases, with the exception of a single *Alu* sequence (nucleotides 4944–5174, in the 3′ region of the first intron), in the reverse orientation to HCFC1 transcription. In addition, we identified a polypyrimidine stretch at the beginning of the first intron, starting at nt 566 and ending at nt 760 (there are only nine purines in 194 bases).

Regulatory Elements in the Upstream Region, the 5′ Untranslated Region, and the First Intron

We analyzed the first 8601 bp of the sequence containing 2146 bp of 5′-flanking region, the first exon, and the first intron, whose sequence is shown in part in Fig. 1. The promoter region appears to be very G + C rich, with 66% G + C content compared to 54% in the first exon and 57% in the first intron. There is neither a TATA nor a CAAT box in the promoter region, as usually happens with housekeping genes. Twenty-three Sp1 sites (GGGCGG) were found (see Fig. 1): 15 in the promoter (12 in the forward and 3 in the reverse orientation), 2 in the untranslated region (UTR) of the first exon, and 6 in intron 1. A short 5′-GAGGAAGGGAGGAGAGAGAGG-3′ purine stretch (designated "short GAGA" motif) surrounds the transcription start site. Two additional GAGAGAG motifs, identical to the (CT)n element found in the *Drosophila* hsp26 gene (Lu *et al.*, 1993), are present in the middle of intron 1.

Among several other possible binding sites for known transcription factors, we identified a conserved 17mer element (CAAGATGGCGGCTCCCA, arbitrarily named SiSa) that is repeated six times (SiSa 1–6 in Fig. 1), regularly spaced at about 0.2-kb intervals, with SiSa5 falling at the very beginning of the mRNA and SiSa6 being in the 5′ UTR. In two cases, Sp1 and SiSa elements are closely associated; SiSa1 and SiSa5 lie very close to Sp1.f10 and Sp1.r4, respectively. In addition, Sp1.f12 is 16 nt upstream of SiSa4. Finally, SiSa5 is closely associated to the short GAGA motif.

The SiSa element contains an 8-bp sequence (AAGATGGC) identified in the regulatory elements of several other genes, among them the heavy chain immunoglobulin gene (Lenardo *et al.*, 1987; Park and Atchison, 1991), the rpL30 and rpL32 ribosomal proteins (Hariharan *et al.*, 1991), and the polyoma enhancer (Caruso *et al.*, 1990). In the HCFC1 gene one additional AAGATGGC motif is found at position 6300, in the first intron.

A search of databases for the SiSa element revealed its presence in two instances: in the human prealbumin gene (Accession No. M11518), where a SiSa motif in the reverse orientation encompasses the transcriptional start site, and in the mouse E46 mRNA for an E46 protein of unknown function (Accession No. X61506). In this case the context of this SiSa motif is identical, for the extent of 8 bp 5′ and 1 bp 3′, to that of SiSa1 in the HCFC1 promoter.

Interestingly, however, sequences closely resembling SiSa are present at the very beginning of the transcripts of four other genes: the BFT3 transcription fac-

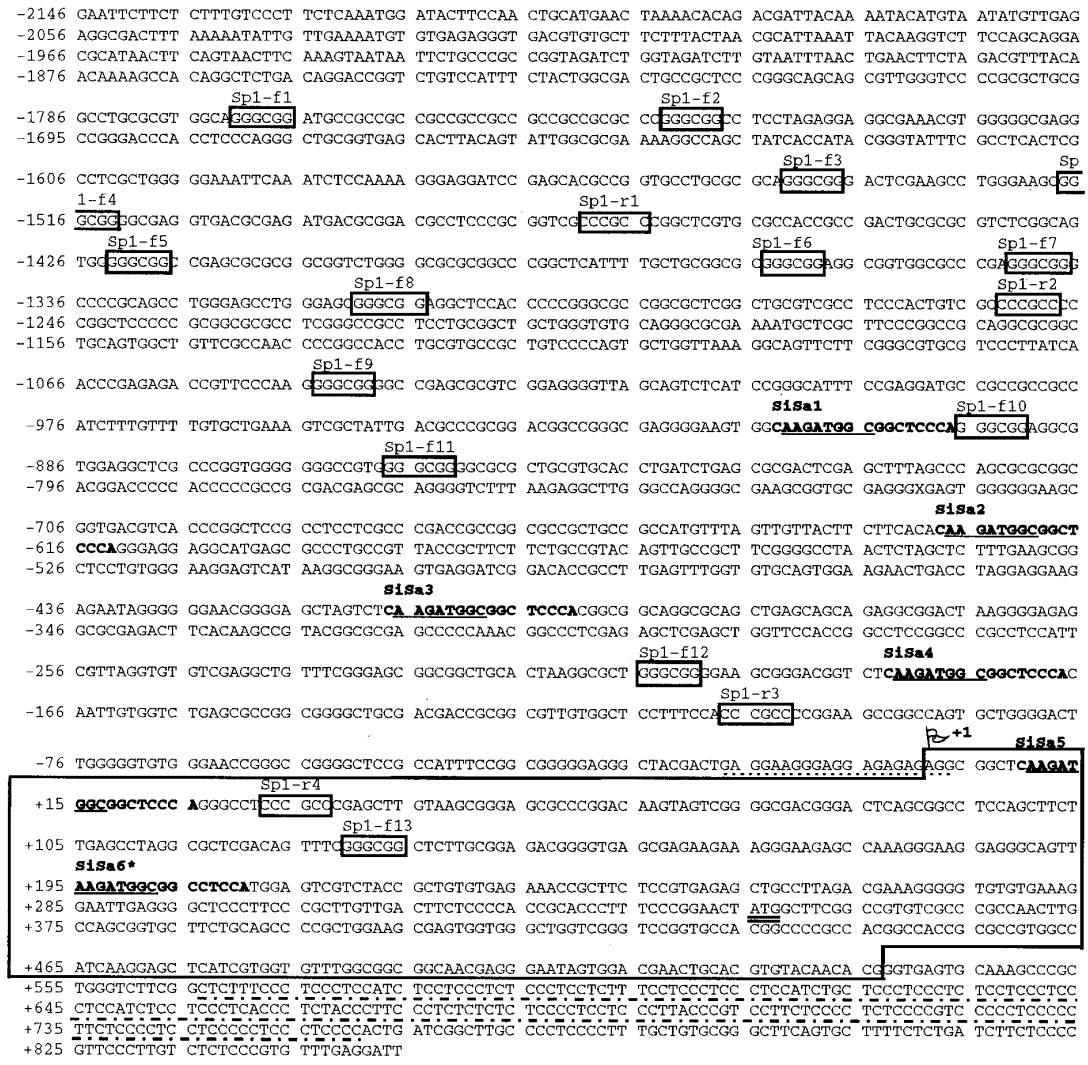

FIG. 1. Nucleotide sequence of 3000 bp of the human HCFC1 gene, including the promoter, the first exon, and part of the first intron. Sequence is numbered relative to position +1 (small flag), representing the first nucleotide of the published cDNA sequence. Sp1 sites are boxed and numbered with orientation indicated by f (forward) and r (reverse). SiSa elements are shown in boldface, with the YY1 elements underlined. The first exon is enclosed in the large box. The short GAGA motif at the transcription initiation site is underlined with dots, the start ATG is double underlined, and the polypyrimidine stretch is underlined with dashes and dots.

tor homologue (Accession No. M90355), the B-raf protein (Accession No. X67052), the NADH dehydrogenase (Accession No. X63215), and a cDNA with unknown function (Accession No. D21064).

All Cells Contain an Activity Capable of Binding the SiSa Element

NE from a panel of established human cell lines of various origins exhibit a binding activity when incubated with a double-stranded oligonucleotide representing the SiSa element, as shown in Fig. 2. Although the same amount of extract was used for all cells, the relative amount of binding activity seems to vary among them. However, this finding may be due to the instability of the binding activity when only PMSF is present as protease inhibitor. We found that the best results, under these conditions, are obtained when the two incubation steps (see Materials and Methods) are carried out on ice and that a brief incubation at higher temperature (2 min at 37°C or 10 min at 25°C) is sufficient to abolish all binding (data not shown).

The SiSa Element Contains Two Distinct Binding Sites

To gain further insight into the binding activity, we synthesized half site oligos (containing the 5′ and 3′ ends, respectively; see Materials and Methods) to be used in EMSA experiments. Figure 3a shows the result of a binding and competition experiment performed with Jurkat cell NE (a similar binding pattern is obtained with both 293 and HeLa NE; data not shown). When the full-length motif is used as probe (lanes 1–5), binding can be competed efficiently by a 100-fold molar excess of unlabeled oligo of the same species (lane 3) or containing the 5′ 10 nt of the element (oligo S5′; lane 4), but not by oligo S3′ (containing 8 nt at the 3′ end; lane 5); this suggests that

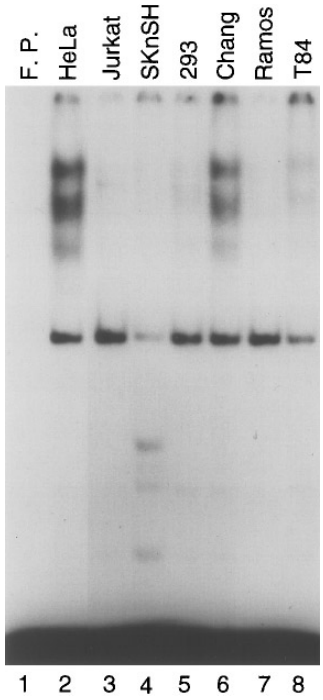

FIG. 2. Nuclear extract from all cells examined can bind to the SiSa element. EMSA obtained with full-length SiSa oligo alone (lane 1) or with nuclear extracts from epithelial carcinoma cells (HeLa, lane 2), T-cell leukemia (Jurkat, lane 3), neuroblastoma (SK-N-SH, lane 4), embryonic kidney transformed (293, lane 5), liver immortalized (Chang, lane 6), B-cell lymphoma (Ramos, lane 7) and colon carcinoma (T84, lane 8).

all of the observed binding capacity is included in the first 10 nt of the motif.

Lanes 6–10, obtained with labeled probe S5′, show the same pattern of competition, consistent with the above interpretation. Accordingly no factor, under these conditions, binds to the 3′ half site of the SiSa element (lanes 11–15). Interestingly, when the reaction conditions are slightly altered, namely when DTT concentration is lowered from 1.2 to 0.2 m*M*, specific binding can be observed with labeled oligo S3′. Figure 3b shows an assay performed in 0.2 m*M* DTT with the 293 cell line NE (the same result is obtained with HeLa cell NE; data not shown) in which labeled full-length probe was used: two distinct DNA/protein complexes can be seen (lane 1, CI and CII), both specific for the full SiSa element (absent in lane 2, where they are competed by excess of unlabeled oligo). When the binding reaction is performed in the presence of 100-fold excess oligo S5′, only CI is detected, and CII is not visible (lane 3). Conversely, complex II is visible only when excess unlabeled oligo S3′ is added to the reaction, indicating that CI and CII are formed on the 3′ and 5′ halves of the element, respectively. As the two complexes are also clearly defined when only the full-length labeled SiSa element is present in the reaction mixture and no higher (slower) complex is visible, it appears that the binding of the two complexes is mutually exclusive; i.e., factor(s) I can bind to SiSa only if factor(s) II does not, and vice versa. A similar behavior has been observed with the transcription factor YY1 in many regulatory regions, including the AAV P5 promoter (Shi *et al.*, 1991), where it was demonstrated that the sites to which either factor binds overlap by a few nucleotides, explaining the mechanics of the alternative binding. We are currently investigating the nature of complex I, its binding properties, and the details of the alternative biological conditions that favor the binding of one complex vs the other.

Complex II Is Produced by YY1 Binding

The AAGATGGC octamer is known to bind the transcription factor YY1. To investigate if the factor binding SiSa is YY1, we synthesized *bona fide* YY1 oligos, as well as a mutant oligo known not to bind (Shi *et al.*, 1991). Figure 4a shows that both the complete SiSa element and the 5′ part (containing the YY1 binding site) can efficiently compete for the factor that binds to the *bona fide* YY1 oligo. Conversely, when excess *bona fide* YY1 oligo is present in the reaction, no binding is observed on the SiSa element (not shown).

To confirm further that the nuclear factor that binds SiSa is indeed YY1, we used monoclonal antibodies against YY1 (a generous gift of T. Shenk, Princeton University) in the EMSA reaction. This monoclonal antibody was previously shown to inhibit YY1/DNA complex formation (Shi *et al.*, 1991); Fig. 4b demonstrates that the characteristic band does not appear when the antibody is included in the reaction (compare lanes 1 and 2, and lanes 9 and 10). This effect is not obtained with an unrelated monoclonal antibody (against LDL receptor; lanes 3, 7, and 11), nor is any effect seen when the antibody is present, but not the nuclear extract (lanes 4, 8, and 12).

Finally, to prove the identity of YY1 as the protein that binds SiSa, we carried out partial proteolytic clipping of the complexes by using low concentrations of proteinase K (Lee and Schwartz, 1992). DNA/protein complexes were produced by incubating labeled YY1, SiSa, or S5′ oligos with HeLa cell NE for 20 min at room temperature; proteinase K was added at the concentration indicated in Fig. 4c for 5 min before samples were loaded on the gel. The similarity of the digestion pattern (compare lanes 1–6, 7–12, and 13–18) is further evidence that the three oligos bind to the same protein(s).

DISCUSSION

We have previously reported that the HCFC1 gene is assembled from 26 exons spanning a region of approximately 24 kb in Xq28 (Frattini *et al.*, 1994). We report here the whole 26-kb sequence of the gene including the 5′-flanking region and the first intron, where regulatory elements are usually contained. We found a 194-bp polypyrimidine stretch and a short putative GAGA motif surrounding exon 1, as well as two additional GAGAGAG in intron 1: these sequences might represent binding sites for GAGA factor, which

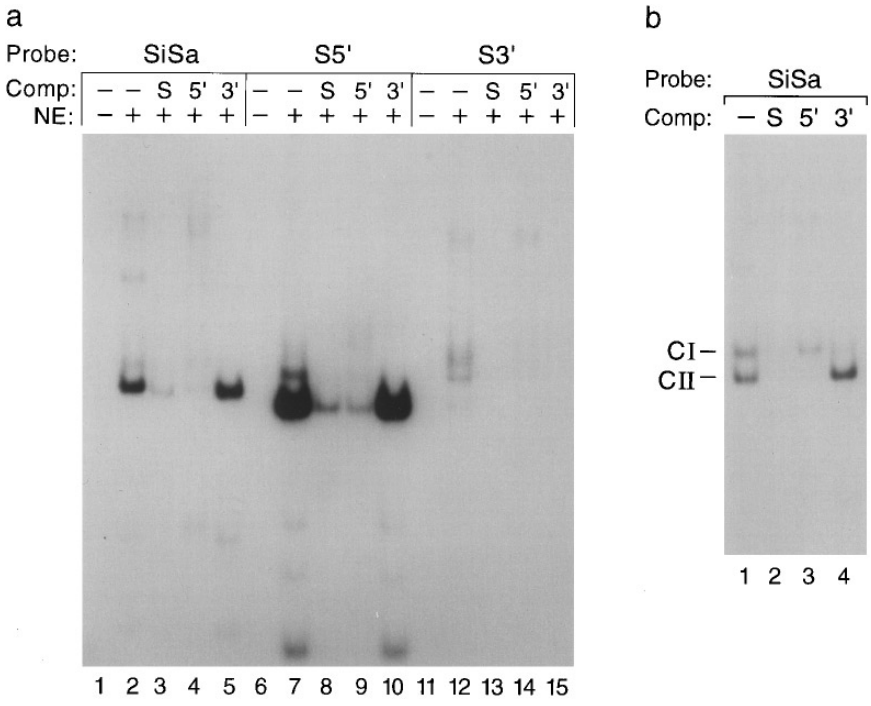

FIG. 3. SiSa contains two functional sites. (a) Binding and competition experiments performed with Jurkat cell NE. 7.5 mg of NE was used for each reaction (where indicated), with full-length SiSa probe (lanes 1–5), the 5′ 10 nt (S5′, lanes 6–10), or the 3′ 7 nt (S3′, lanes 11–15). Competitor at 100 times molar excess was added prior to the probe as indicated at the top of each lane. (b) Binding and competition assays were performed in 0.2 m*M* DTT using full SiSa as probe and competitors are as indicated.

in some promoters has a role in nucleosome disruption and rearrangement (Tsukiyama *et al.*, 1994), modulating expression of the gene (Lu *et al.*, 1993). The possible role of GAGA motifs in the HCFC1 gene has not been investigated here.

In agreement with its apparent housekeeping role (Frattini *et al.*, 1994; Wilson *et al.*, 1995), HCFC1 does not contain either a TATA or a CAAT box in its promoter but contains a number of putative binding motifs, some of which are shown in Fig. 1. We also have identified a repeated element, named SiSa, present in six copies in the promoter and 5′ UTR and in a shorter version in the first intron. The repetition of this sequence together with the fact that it contains a motif present in one or more copies in other regulatory elements strongly suggests that it plays a role in the *in vivo* regulation of the HCFC1 gene. We demonstrate that the SiSa element is able to form at least two different complexes *in vitro.* One of these involves the transcription factor YY1, which binds the first 10 nucleotides of SiSa. The identity and properties of the second factor(s), which binds to the 3′ portion, are currently under investigation. The overlapping of YY1 binding sites with sites for other transcription factors is a frequent occurrence (see, for example, Vincent *et al.*, 1993; Lee *et al.*, 1992; Ye *et al.*, 1994; Raich *et al.*, 1995).

YY1 can also act as an initiator in TATA-less promoters (Seto *et al.*, 1991; Usheva and Shenk, 1994), and Sp1 sites in the vicinity can enhance this function *in vivo* (Lee *et al.*, 1993; Seto *et al.*, 1993). In this regard it is noteworthy that SiSa5 is located across the HCFC1 transcription start and that two of the Sp1 sites in the promoter map very close to SiSa5 and SiSa6.

The versatility of YY1 is intriguing as it can act as an initiator and as a transcriptional repressor or activator (Shrivastava and Calame, 1994). This dual YY1 function is probably dependent on many factors. First, the orientation of the binding sequence with respect to the direction of the transcription could be involved, as in the c-fos promoter (Natesan and Gilman, 1993). In this respect, though, Ye *et al.* (1994) found that the enhancer effect of YY1 sequence in the GM-CSF gene is independent of the orientation, as expected for a true enhancer element.

Second, YY1 function is clearly affected by the interaction with other proteins. In many cases of negatively regulated elements, the YY1 site overlaps with different motifs, such as the CArG (Gualberto *et al.*, 1992), the mammary gland factor binding site (Meier and Groner, 1994; Raught *et al.*, 1994) or the κB site (Lu *et al.*, 1994), suggesting that negative regulation is caused by the invasion by YY1 of sequence motifs partially shared with other (positive) transcription factors.

Another form of interaction seems to occur at the adeno-associated virus promoter, where repression produced by YY1 binding can be relieved by the addition to the complex of the adenoviral protein E1A, which does not bind DNA per se (Lewis *et al.*, 1995; Shi *et al.*, 1991). Curiously, a complementary situation exists in the α-1 acid glycoprotein gene, where it is YY1 that, upon binding to another specific factor, relieves the transcriptional repression exerted by this other

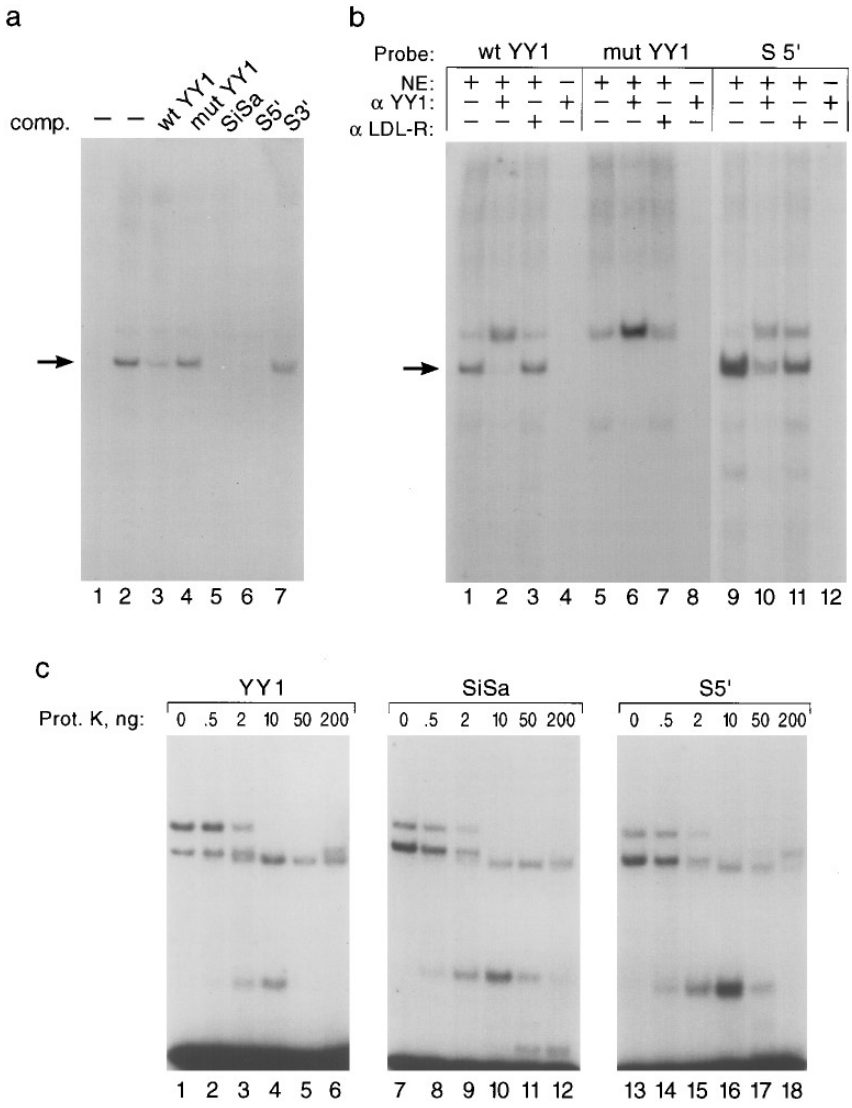

FIG. 4. S5′ binds to YY1. (a) Binding and competition analysis performed with HeLa cell NE, YY1 oligo as probe, and 100-fold molar excess competitors as indicated at the top of each lane. Lane 1 shows free probe only. (b) Anti-YY1 monoclonal antibodies prevent the formation of the DNA/protein complex both with YY1 probe (lane 2) and with S5′ probe (lane 10). NE was incubated with α-YY1 (lanes 2, 6, and 10) or unrelated monoclonal antibody as control (lanes 3, 7, and 11) prior to probe addition (wt YY1, lanes 1–4; mutant YY1, lanes 5–8; or S5′, lanes 9–12). (c) Partial proteolytic cleavage of the DNA/protein complexes obtained with YY1, SiSa, or S5′ and increasing amounts of proteinase K, as indicated.

protein (Lee and Lee, 1994). An additional factor influencing the activity of YY1 could be the exact sequence to which it binds: it has been noted that the consensus sequence for YY1 as an activator is more strict than the consensus for YY1 as a repressor (Shrivastava and Calame, 1994).

Finally, other factors like the number of YY1 sites, the position with respect to the nucleosomes, and the presence or absence of a TATA box could also modulate YY1 function.

In conclusion, we have determined the whole sequence of the HCFC1 gene and its promoter region. This allowed us to identify a 17-bp element repeated a total of six times in the promoter and 5′ UTR, which contains two binding motifs: one is for the YY1 transcription factor, probably acting in the HCFC1 context as a positive regulator, while the other has not yet been characterized. This gene is unique among known promoters in containing six copies of this binding motif plus one 8-bp YY1 binding motif in the first intron, and further studies are needed to establish the way in which YY1 and the other transcription factor(s) act in this specific promoter.

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