Transcriptional regulation of the human *Sp1* gene promoter by the specificity protein (Sp) family members nuclear factor Y (NF-Y) and E2F

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We analysed in detail the minimal promoter of transcription factor Sp1, which extends 217 bp from the initiation of transcription. Within this sequence we identified putative binding sites for Sp1, nuclear factor Y (NF-Y), activator protein 2 ('AP-2'), CCAAT/enhancer-binding protein ('C/EBP') and E2F transcription factors. In one case, the boxes for Sp1 and NF-Y are overlapping. Gel-shift and supershift assays demonstrated specific binding of Sp1, Sp3 and NF-Y proteins. Transient transfections and luciferase assays revealed activation of the Sp1 minimal promoter upon overexpression of Sp1 itself, NF-Y and E2F. Whereas overexpression of NF-Y or E2F had an additive effect on Sp1 overexpression, the activation of Sp1 transcription

due to Sp1 was counteracted by Sp3 overexpression. Mutagenesis analysis of the NFY/Sp1-overlapping box revealed that both factors compete for this box, and that when the NF-Y site of this overlapping box is specifically mutated there is an increase in Sp1 binding, thus increasing transcriptional activity. These results help to explain the complex regulation of the *Sp1* gene, which depends on the relative amounts of Sp1, Sp3, E2F and NF-Y proteins in the cell.

Key words: E2F, gene promoter, gene regulation, nuclear factor Y (NF-Y), Sp1, Sp3.

INTRODUCTION

Sp1 is a ubiquitously expressed transcription factor that plays a major role in the transcription of a number of gene promoters [1]. In addition to Sp1, other members of the specificity protein (Sp) family [2], i.e. Sp2 [3], Sp3 [3,4] and Sp4 [4], have also been cloned. Sp2 does not recognize the same sequences as Sp1, and Sp4 expression is restricted to brain tissue [5]. Both Sp1 and Sp3 are ubiquitously expressed in mammalian cells, competing for common target sequences with similar binding affinities, including the GC and GT boxes [1,3,4,6,7]. Although Sp1 is a transcriptional activator [1], Sp3 can act either as a transcriptional activator or as a repressor [5,6]. The bifunctionality of Sp3 is dependent upon the promoter context as well as the cellular background [8].

Nuclear factor-Y (NF-Y), also known as CBF (CCAAT-binding factor), CP1 or YEBP, was originally identified through its binding to the conserved Y-box element in the human or mouse major histocompatibility complex class II E α promoter [9–11]. This Y-box contains an inverted CCAAT box (5'-ATTGG-3'), and is involved in a number of systems transactivating transcription [12,13]. NF-Y, which is highly conserved among species, is ubiquitously expressed and is composed of three subunits, NF-YA, NF-YB and NF-YC, all of which are necessary for DNA binding [12,14]. NF-YB and NF-YC interact closely with one another, and their association precedes further association with NF-YA and the binding of the whole complex to the cognate DNA sequences [15].

Both Sp1 and NF-Y are ubiquitously expressed transcription factors with a major role in the transcription of many genes [1,12,13]. Moreover, there are abundant examples of promoters that require the concerted action of Sp1 and NF-Y [16–22], and in some cases a physical interaction between these factors has been demonstrated [21,23,24].

Although the activity of Sp1 is believed to be constitutive, it has recently been shown to participate in several cases of regulated gene transcription. There is evidence that its activity can be modulated, as in the case of differentiation [25,26], the cell cycle [27,28], development [29] and enhancement of drug resistance [30]. Therefore our aim was to study the regulation of the *Sp1* gene itself. In a previous paper we described the cloning of the 5'-flanking region of the human *Sp1* gene, and a number of putative binding sites for transcription factors were found [31]. Within the minimal promoter sequence, expanding 217 bp relative to the transcription start site, two Sp1 binding sites were described: (i) a perfectly characterized downstream site, where both Sp1 and Sp3 bind; and (ii) a GC box located further upstream that can be bound by purified recombinant Sp1 protein, although most of the binding was produced by a different transcription factor [31]. In addition, the minimal promoter contained potential binding sites for CCAAT/enhancer-binding protein (C/EBP), activator protein 2 (AP-2), NF-Y and E2F. There are two NF-Y binding sites, one of them overlapping with the upstream Sp1 site. The putative E2F box is at the level of the transcriptional start site.

In the present study, we performed a more detailed binding analysis of the Sp1 promoter in order to clarify which transcription factors would actually bind to the minimal promoter sequence. We also tested the functionality of the binding sites within the minimal promoter using luciferase constructs in co-transfection experiments performed in combination with expression vectors for NF-Y, Sp3, Sp1 and E2F.

MATERIALS AND METHODS

Cell culture

HeLa human cervical carcinoma cell line was grown in Ham's F12 medium supplemented with 5% (v/v) fetal bovine serum

Abbreviations used: AP, activator protein; CBF, CCAAT-binding factor; C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; NF-Y, nuclear factor Y; Sp, specificity protein; TNT, transcribed and translated.

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Table 1 Oligonucleotide probes used to generate mutations in the D-FOR2U probe

The sequences of the oligonucleotides used in the reverse primers of the PCR mutagenesis are indicated. Underlined nucleotides correspond to the mutations introduced. Wt, wild-type.

Oligonucleotide	Sequence
Wt (reverse)	5'-TGCTGCCCCGCCCAATGAGGG-3'
Smut	5'-TGCTGCCCC <u>G</u> GCCCAATGAGGG-3'
Nmut	5'-TGCTGCCCCGCC <u>C</u> ATGAGGG-3'
SNmut	5'-TGCTGCCCC <u>G</u> <u>G</u> <u>C</u> <u>T</u> ATGAGGG-3'

(Gibco). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Transcription and translation *in vitro*

cDNAs for the human Sp1 and NF-YA, -YB and -YC transcription factors were transcribed and translated (TNT) in one step using rabbit reticulocyte lysates from 1 µg of the corresponding plasmid with the aid of a commercial kit (Promega), according to the manufacturer's instructions.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared from exponentially growing HeLa cells as described previously [32] with the following modification: the amount of Triton X-100 used for cell lysis was 0.05%.

DNA binding assays were performed as described previously [27], but using herring sperm as the non-specific competitor. The FOR2U probe was prepared as described in [31]. FOR2U (113 bp) was used as the template for obtaining the probes U-FOR2U (61 bp) and D-FOR2U (52 bp). These probes were prepared by PCR using the following primers: (i) for probe U-FOR2U, the forward primer was 5'-CGCAACTTAGTCTCA-CACGCC-3' and the reverse primer was 5'-CGGTGGAATTA-TCCAATGGCAAG-3'; (ii) for probe D-FOR2U, the forward primer was 5'-TCTTTCTTCTGCAAGTCCCTC-3' and the reverse primer was 5'-TGCTGCCCCGCCCAATGAGGG-3'. In order to better characterize the NF-Y/Sp1 box, the D-FOR2U probe was mutated by PCR, introducing mutations in the reverse primers to generate Smut, Nmut and SNmut probes (Table 1).

The PCR fragments were gel-purified, end-labelled with T4 polynucleotide kinase (New England Biolabs) using [γ -³²P]ATP (3000 Ci/mmol; Amersham Biosciences), and used as probes in the gel-shift experiments.

In the competition experiments, increasing amounts of unlabelled consensus sequences for C/EBP [5'-TGCAGATTGC-

GCAATCTGCA-3'; (Santa Cruz)], AP-2 [5'-GATCGAACT-GACCGCCCGCGGCGCGT-3'; (Promega)] or Sp1 [5'-ATT-CGATCGGGGCGGGGCGAGC-3'; (Promega)] were added to the binding reactions with the nuclear extract for 15 min before the addition of the D-FOR2U radiolabelled probe.

In the supershift experiments, 0.5 µg of rabbit polyclonal antibody PEP-2 (Santa Cruz) against Sp1, which does not cross-react with Sp2, Sp3 or Sp4, 1 µg of rabbit polyclonal antibody D-20 (Santa Cruz) against Sp3, or 1 µg of goat polyclonal antibody C-20 (Santa Cruz) against NF-YB (CBF-A) were added to the reaction mixture and incubated on ice for 15 min, before the addition of each probe.

When performing EMSA experiments with TNT proteins, 2 µl of Sp1 was pre-incubated in the absence or in the presence of increasing amounts of NF-YA, -YB and -YC. The mixture was placed on ice for 15 min before the addition of the diluted D-FOR2U probe (4000 c.p.m.), and then incubated for a further 30 min before electrophoresis.

Luciferase mutated constructs

Luciferase construct pGL3FOR15, a new deletion of the Sp1 promoter region engineered by unidirectional cloning, was created using the same methodology as that described by Nicolás et al. [31]. This construct contains 129 bp of the 5'-flanking region of the human transcription factor Sp1 relative to the transcriptional start site. Point mutations of this construct, pNmut, pSmut, pSSmut, pSNmut and pSNSmut, were generated using pGL3FOR2 as the template, and pSdmut and pSdNmut were created using pSSmut as the template. The primers used for these constructs are shown in Table 2.

Co-transfections and luciferase assays

HeLa cells were plated in 35-mm dishes the day before transfection, at a density of 2.25×10^5 cells/well, in Ham's F-12 medium containing 5% fetal bovine serum. The medium was renewed 2 h before transfection. For each dish, 3 µl of FuGENE™ 6 (Boehringer Mannheim) in 100 µl of serum-free Ham's F-12 medium was incubated at room temperature for 5 min, and then added to the DNA mix. In co-transfection experiments, 250 ng of the corresponding luciferase construct plus 250, 500 or 750 ng of each co-expression vector (Sp1, Sp3, NF-YA or E2F) was used. The DNA/lipid mixture was incubated at room temperature for 15 min and then added to the cells for 28 h.

Luciferase activity was assayed after the expression time. Cell extracts were prepared by lysing the cells with 200 µl of freshly diluted 1X Reporter Lysis Buffer (Promega). The lysate was centrifuged at 13000 rev./min (9500 g) for 2 min in order to pellet the cell debris. The supernatants were transferred to a fresh tube, and their protein concentration was determined using the

Table 2 Oligonucleotides used to generate mutations in the pGL3FOR15 construct

Mutations included in the forward (Fwd) primers are shown in bold and are underlined. The sequence that is in lower-case lettering and which is underlined in the forward primers represents the *NheI* restriction endonuclease site, and *XhoI* in the reverse primer. WT, wild-type; Rev, reverse.

Oligonucleotide	Sequence	Construct
Fwd15WT	5'-tcaagtcaggctagcCCTCCCTCATTGGGCGGGG-3'	pFOR15 pSdmut
FwdNmut	5'-tcaagtcaggctagcCCTCCCTCATGGGCGGGG-3'	pNmut pSdNmut
FwdSmut	5'-tcaagtcaggctagcCCTCCCTATTGGGCGGGG-3'	pSmut
FwdSNmut	5'-tcaagtcaggctagcCCTCCCTCATACGGGCGGGG-3'	pSNmut
FwdSSmut	5'-tcaagtcaggctagcCCTCCCTCATTGGGCGGGGAGCAGAGAGGGGCGGGG-3'	pSSmut
Rev	5'-cagtgcgtcctcagGCTCAAGGGGGTCTGTCCGG-3'	

BioRad protein assay reagent according to the manufacturer's protocol. A 10 μ l aliquot of the extract was added to 25 μ l of luciferase assay substrate (Promega), and the luminescence of the samples was read immediately on a TD-20/20 luminometer, in which light production (relative light units) was measured for 10 s. Each transfection was performed in duplicate. Luciferase activity was normalized to cellular protein concentration.

RESULTS

Detailed analysis of the Sp1 minimal promoter

In a previous paper we described the cloning of the 5'-flanking region of the human *Sp1* gene [31], defined the minimal promoter FOR2, and performed binding analysis using this sequence (FOR2 probe), shown schematically in Figure 1(A). We reported, within this 276 bp sequence, two Sp1 binding sites, one of which (located the furthest downstream) was fully characterized. However, the identity of the proteins that bind to the more upstream GC box in the FOR2 probe was not clear. Although the FOR2U probe could be bound by purified recombinant Sp1 protein, the

gel-shift pattern with nuclear extracts seemed to be due to the additional contribution of another transcription factor. Sp1 binding accounted for only 5% of the total binding, as determined by phosphorimaging in supershift experiments using Sp1 antibody (results not shown).

In the present work, use of the Transfac database revealed an NF-Y box overlapping the upstream Sp1 box (Figure 1A). Therefore we performed a detailed binding analysis using different regions of the FOR2U probe in order to ascertain which transcription factor could bind to this sequence (Figure 1A). The FOR2U probe was divided into two new probes of similar length, U-FOR2U (61 bp) and D-FOR2U (52 bp). The U-FOR2U probe contains one putative NF-Y-binding site. D-FOR2U contains putative binding sites for C/EBP, AP-2, NF-Y and Sp1, the last two sites overlapping (Figure 1A). The binding pattern with all three probes was very similar; two shifted bands were observed with the FOR2U and U-FOR2U probes, and three with the D-FOR2U probe (Figure 1B).

We then characterized the binding patterns obtained in the gel-shift analysis using the D-FOR2U probe with antibodies against Sp1 (PEP-2) and Sp3 (D-20) (Figure 2B, lanes 2 and 3; the structures of the probes U-FOR2U and D-FOR2U are shown in Figure 2A). The upper band in Figure 2(B) (labelled 'a') corresponded to Sp1 binding, since it disappeared almost completely in the presence of the Sp1 antibody (Figure 2B, lane 2). In contrast, Sp3 was not bound to the D-FOR2U probe, since no changes in the binding pattern in either the absence or presence of an Sp3 antibody were observed (Figure 2B, lane 3). Competition analysis using increasing amounts of the consensus sequence for Sp1 also showed that the upper band was due to Sp1 binding (Figure 2B, lanes 4 and 5). As a further control, we used human recombinant Sp1, which produced a shifted band with the same mobility as the upper band seen in the gel-shift with the nuclear extracts (Figure 2B, lane 7). The putative binding sites for AP-2 and C/EBP included in the D-FOR2U probe were analysed by competition analysis with increasing amounts of their consensus sequences. The binding generated was competed (Figure 2B, lanes 9–12), thus indicating that AP-2 and C/EBP did not bind to the D-FOR2U probe. The possible binding of transcription factor NF-Y was also tested using the D-FOR2U probe. Supershift experiments using an antibody against NF-YB (C-20) (Figure 2B, lane 3) showed that the binding (bands labelled 'b' and 'c') disappeared almost completely.

Probe U-FOR2U contains only one putative NF-Y-binding site, and the binding pattern obtained with nuclear extracts (Figure 2C, lane 5) was similar to that observed with the FOR2U probe. Supershift experiments using an antibody against NF-YB caused the disappearance of the binding produced by U-FOR2U, giving rise to a supershifted band at the top of the gel (Figure 2C, lane 6). In a similar fashion, the NF-YB antibody also led to the complete disappearance of the binding when performing supershift analysis with the FOR2U probe (Figure 2C, lane 9).

Regulation of the Sp1-mediated transcription by NF-Y

In the FOR2 sequence corresponding to the minimal promoter we defined two CCAAT boxes, putative binding sites for the NF-Y complex. Since the binding of NF-Y to its consensus sequence in the FOR2 probe was confirmed by supershift analysis (Figure 2B), we also wanted to examine the role of the NF-Y complex in the regulation of the Sp1 promoter. To this end, transient co-transfections were performed in HeLa cells using the luciferase construct pGL3FOR2, together with increasing amounts of an expression vector for the NF-YA subunit (NF-YA13; [33]). As

Figure 1 Binding analysis of the FOR2U sequence

(A) Diagram summarizing the sequences used as probes in the gel-shift assays; the putative binding sites for transcription factors are depicted. (B) Binding reactions were performed with 20 000 c.p.m. of each probe ([FOR2U, D-FOR2U (D) and U-FOR2U (U)], 2 μ g of nuclear extracts (NE) from exponentially growing HeLa cells, and 2 μ g of herring sperm DNA as the non-specific competitor.

Figure 2 Binding analysis of the D-FOR2U probe

(A) The structures of the probes U-FOR2U and D-FOR2U are shown, showing the positions of the respective transcription-factor-binding sites. The nucleotide sequence (attgg) of the NF-Y-binding site that overlaps with that of Sp1 is highlighted in bold for both probes. (B) Binding reactions were performed with 20 000 c.p.m. of the D-FOR2U probe, 2 μ g of nuclear extracts (NE) from exponentially growing HeLa cells and 2 μ g of herring sperm DNA as the non-specific competitor (lanes 1, 6 and 8). Supershift-mobility assay with the D-FOR2U probe in the presence of specific antibodies against Sp1 or Sp3 (Ab Sp1 and Ab Sp3; lanes 2 and 3 respectively). Recombinant purified Sp1 protein (rSp1; Promega) (25 ng) was used as a control (lane 7). Competition analysis of the D-FOR2U probe with increasing amounts (20- and 200-fold excess) of consensus sequence for Sp1, AP-2 or C/EBP (Sp1cs, Ap2cs and C/EBPcs; lanes 4 and 5, 9 and 10, and 11 and 12 respectively). (C) Supershift mobility assay was performed with the NF-YB antibody (Ab) in the presence of D-FOR2U, U-FOR2U and FOR2U probes (lanes 3, 6 and 9 respectively).

shown in Figure 3(A), the NF-YA expression vector activates the Sp1 promoter in a dose-dependent manner. In contrast, when a dominant-negative mutant of NF-YA (NF-YAm29; [33]) was used in the co-transfection experiments, no significant change in Sp1 promoter activity could be observed (Figure 3A).

There are many examples of promoters that require the concerted action of Sp1 and NF-Y [16–20,22], and it has been shown that the Sp1 protein interacts physically with the NF-Y complex [21,23,24]. Therefore we co-transfected the luciferase reporter plasmid pGL3FOR2 plus the expression vector NF-YA or the equivalent dominant-negative plasmid, NF-YAm29, together with the expression vector for Sp1. We observed that overexpression of Sp1 and NF-YA proteins had an additive effect on the transcriptional activation of the Sp1 minimal promoter (Figure 3B). In contrast, the same amount (250 ng) of NF-YAm29 did not modify the activation produced by overexpression of Sp1, although higher amounts of this dominant-negative plasmid (500 ng) partially reversed the activation produced by Sp1 overexpression (Figure 3B).

Mutational analysis of the overlapping NF-Y/Sp1 box

To characterize the binding properties of the NF-Y/Sp1 overlapped sequence, mutants in the NF-Y and/or Sp1 boxes

corresponding to the D-FOR2U probe were constructed (Figure 4A). Subsequently, gel-shift analyses with nuclear extracts from HeLa cells and these four probes were performed. Three retarded bands were observed (Figure 4B, lane 2, bands labelled 'a', 'b' and 'c') when using the wild-type D-FOR2U probe. The upper band (a) was supershifted when incubated with the Sp1 antibody, and the other two bands (b and c) were decreased when incubated with the NF-YB antibody. In contrast, when the probe carrying the Sp1 mutation was used (Smut), the upper band (a) disappeared (Figure 4B, lane 6) and the NF-YB antibody was able to supershift bands b and c. When the probe carrying the NF-Y mutation was used (Nmut) the formation of the two lower complexes (b and c) was abolished (Figure 4B, lane 10), and the Sp1 antibody was able to supershift the binding of the upper complex (a) (Figure 4B, lane 11). Incubation with the NF-YB antibody did not affect the binding pattern of the Nmut probe. Finally, when both Sp1 and NF-Y boxes were mutated within the same probe (SNmut), no binding was observed upon incubation of this probe with the nuclear extract (Figure 4A, lane 14). Therefore point mutation in either Sp1 or NF-Y boxes abolished the binding of the respective transcription factors.

To complement the characterization of these two boxes, we performed competition experiments using the wild-type sequence as the probe and the mutant sequences as competitors (Figure

Figure 3 Effect of overexpression of NF-YA and Sp1 on Sp1 promoter activity in HeLa cells

(A) Cells were co-transfected with 250 ng of the basal reporter gene construct (pGL3FOR2) and the indicated amounts of an expression vector encoding NF-YA or the corresponding dominant-negative NF-YAm29 vector. (B) Cells were co-transfected with 250 ng of the basal reporter gene construct (pGL3FOR2) and the indicated combinations of expression vectors for NF-YA, NF-YAm29 and Sp1. After 28 h of culture, cell lysates were assayed for luciferase activity. Data represent the means $\pm$ S.E.M. for three experiments. Luciferase activity was normalized to mg/protein for each sample. Promoter activity was expressed as a percentage of values obtained upon transfection with the pGL3FOR2 construct.

4C). Competition with SNmut dsDNA did not significantly decrease the binding (Figure 4C, lanes 10–12). Moreover, when competitions were performed with Nmut dsDNA, the binding hardly changed (Figure 4C, lanes 7–9).

Additionally, EMSA experiments with a probe containing the NF-Y/Sp1-overlapping box and Sp1 and NF-Y TNTs were performed. When D-FOR2U probe was incubated with 2 μ l of Sp1 TNT in the absence or presence of increasing amounts of NF-YA, B and C TNTs, the binding corresponding to Sp1 progressively disappeared as quantified by phosphorimaging (Figure 5A). Given the competition between Sp1 and NF-Y for the overlapping box, we performed EMSA analyses using nuclear extracts from exponentially growing HeLa cells with D-FOR2U, Smut or Nmut probes to determine the influence of each mutation on the binding to the other site (Figure 5B). The shifted band ('a') caused by Sp1 protein increased 3.5-fold in intensity, as determined by phosphorimaging, when the NF-Y box was mutated. However, the binding of NF-Y was not significantly changed when mutating the Sp1 site.

To study the effect of these point mutations on the regulation of the Sp1 promoter, transient co-transfections in HeLa cells were performed. In these series of experiments, the wild-type luciferase construct was pGL3FOR15, which includes the overlapping NF-Y/Sp1 box. The same point mutations introduced in the NF-Y/Sp1 box for the gel-shift analyses were present in the luciferase mutants: pNmut, pSmut, pSSmut, pSNmut and pSNSmut. Moreover, we constructed pSdmut and pSdNmut, containing the Sp1 downstream box mutated instead of the Sp1 upstream box (Figure 6). All the basal constructions containing an Sp1-mutated site resulted in a decreased luciferase activity.

However, mutation of the NF-Y box increased Sp1 promoter activity in HeLa cells. In addition, each basal construct was co-transfected with 500 ng of the expression vector for Sp1, NF-YA, NF-YAm29 or E2F. All these overexpressed proteins increased the corresponding basal activity, except in the case of plasmid encoding NF-YAm29. Sp1 overexpression increased the activity of each mutant, even when the Sp1 boxes were mutated. However, the degree of activation when any Sp1 box was mutated was always lower than that obtained with the wild-type. NF-YA overexpression showed a similar behaviour to Sp1 overexpression, but to a lesser extent.

Regulation of Sp1-mediated transcription by Sp1 family members

In a previous report [31], we demonstrated that Sp1 could activate its own minimal promoter using the pGL3FOR2 luciferase construct in 293T cells. To examine further the role of the Sp family members in the regulation of the Sp1 promoter, transient co-transfections in HeLa cells were performed with pGL3FOR2 and increasing amounts of an expression vector for Sp1 or Sp3, as well as combinations of both. As shown in Figure 7, increasing amounts of the Sp1 expression vector (250–750 ng) alone activate, in a dose-dependent manner, the Sp1 promoter, achieving a 25-fold increase compared with pGL3FOR2 alone. In contrast, increasing amounts of the expression vector Sp3, co-transfected with the luciferase construct pGL3FOR2, did not significantly activate the promoter.

We then co-transfected Sp1 and Sp3 expression plasmids together with pGL3FOR2. Co-transfections with constant

Figure 4 Effect of point mutations in the overlapping NF-Y/Sp1 box on binding to the D-FOR2U probe

(A) Scheme of the different probes used in the binding experiments. (B) Binding reactions were performed with 20000 c.p.m. of the D-FOR2U (lanes 1–4), Smut (lanes 5–8), Nmut (lanes 9–12) or SNmut (lanes 13–14) probes, 2 μ g of nuclear extracts (NE) from exponentially growing HeLa cells, and 2 μ g of herring sperm DNA as the non-specific competitor. A supershift mobility assay is shown, with each probe indicated in the presence of specific antibodies against Sp1 or NF-YB (Ab Sp1 and Ab NF-YB respectively; lanes 3–12). (C) Competition experiments were performed with 20000 c.p.m. of the D-FOR2U probe, 2 μ g of nuclear extracts (NE) from exponentially growing HeLa cells and 2 μ g of herring sperm DNA as the non-specific competitor, in the presence of a 10-, 50- or 200-fold molar excess of each competitor double-stranded DNA probe (lanes 2–12).

amounts of Sp3 (250 ng) and increasing amounts of Sp1 (250–750 ng) counteracted the activation of Sp1 transcription by Sp1 (Figure 7).

Regulation of Sp1 transcription by E2F

The Sp1 promoter cloned in our previous work [31] contained other putative binding sites in addition to Sp1 and NF-Y (e.g. E2F). Since this transcription factor is involved in cell cycle regulation, we analysed the contribution of the E2F box located

in the minimal promoter of Sp1 at the level of the transcriptional start site [31]. Transient co-transfections in HeLa cells were performed with either 250 ng of the luciferase construct pGL3FOR2, which contains the minimal promoter of Sp1, pGL3FOR1, which contains 146 bp of the Sp1 promoter, or pGL3, which lacks the promoter sequence (Figure 8A). Each of these basal vectors was co-transfected together with increasing amounts of an expression vector for E2F. As shown in Figure 8(B), the E2F expression vector activated the Sp1 minimal promoter (pGL3FOR2) in a dose-dependent manner. The pGL3FOR1 vector was also activated in a dose-dependent

Figure 5 Analysis of the competition between Sp1 and NF-Y proteins for the overlapping NF-Y/Sp1 box

(A) EMSA of the D-FOR2U probe. The labelled D-FOR2U probe (4000 c.p.m.) was incubated with *in vitro*-synthesized 1, 1.5 or 2 μ l of TNT NF-Y protein (lanes 1–3 respectively), or with 2 μ l of TNT Sp1 protein (lane 4). Lanes 5–7 are identical with lanes 1–3, except for the presence of 2 μ l of *in vitro*-synthesized Sp1. The quantification by phosphorimaging of the competition is shown in the associated histogram on the right of the gel. (B) Binding reactions were performed with 20000 c.p.m. of the labelled D-FOR2U, Smut or Nmut probes, 2 μ g of nuclear extract from exponentially growing HeLa cells and 2 μ g of herring sperm DNA as the non-specific competitor. The quantification by phosphorimaging of the increase in Sp1 binding is shown to the right of the gel.

manner by E2F. As a control, we checked that the pGL3 vector was not activated by E2F. Given that E2F interacts with Sp1 [34–36] and that this interaction seems to be necessary for the activity of certain promoters, we performed co-transfection experiments with pGL3FOR2 using expression vectors for E2F and Sp1. As shown in Figure 8(C), the effect of Sp1 on the activation of the Sp1 promoter was increased by the presence of E2F.

DISCUSSION

In the present study, we have proceeded with the further characterization of the 5'-flanking region of the human transcription factor *Sp1* gene, which we have cloned recently [31].

We analysed in detail the upstream region of the minimal promoter Sp1 (FOR2U sequence). This sequence was divided into two probes, U-FOR2U (61 bp), which contains a putative binding site for NF-Y, and D-FOR2U (52 bp), presenting putative binding sites for AP-2, C/EBP and Sp1, the latter overlapping with an NF-Y-binding site. By means of gel-shift, supershift and competition analyses, we demonstrated that the

NF-Y complex binds to both probes, whereas the transcription factor Sp1 only binds to the D-FOR2U probe. AP-2 and C/EBP did not bind to this sequence. It is worth noting that the overlapping NF-Y/Sp1 box can bind both factors, although NF-Y produced the majority of the observable binding (Figure 2).

NF-Y is a ubiquitous transcription factor that binds to CCAAT elements in the proximal promoter of a wide variety of mammalian genes [13]. NF-Y consists of three subunits, termed A, B and C (with molecular masses of 42, 36 and 40 kDa respectively), all of which are essential for DNA binding [14,37–39]. Whereas NF-Y binding to the CCAAT box is often essential for gene transcription, particularly for TATA-less genes, NF-Y by itself is largely unable to activate transcription. Rather, its transcriptional role relates to its capacity to enhance transactivation from nearby activating elements and/or to facilitate the positioning of transcriptional factors at the transcriptional start site. In our transfection analyses using the minimal promoter construct pGL3FOR2, which contains two NF-Y-binding sites, the co-transfection of NF-Y transactivated the basal promoter, probably recruiting other endogenous transcription factors, while co-transfection with the dominant-negative mutant of NF-Y did

Figure 6 Effect of point mutations of the overlapping NF-Y/Sp1 boxes on Sp1 promoter activity in HeLa cells

Cells were co-transfected with 250 ng of the basal reporter gene construct pFOR15, pNmut, pSmut, pSSmut, pSNmut, pSNSmut, pSdmut or pSdNmut (indicated by the uppermost of the five bars for each construct in the right-hand panel) and 500 ng of an expression vector encoding Sp1, NF-YA, NF-YAm29 or E2F proteins (indicated, in descending order respectively, by the lower four bars for each construct). After 28 h of expression, cell lysates were prepared and assayed for luciferase activity. Data represent the means $\pm$ S.E.M. for four experiments. Luciferase activity was normalized to mg/protein for each sample. Promoter activity was cross-referenced with values obtained with the pFOR15 construct.

not increase luciferase activity. When NF-Y was co-transfected with Sp1, an additive activation of promoter activity was observed (Figure 3), which was not seen if the dominant negative of NF-Y was used. There is ample precedence for a functional synergism between NF-Y and Sp1, already described for rat pyruvate kinase M [24], fatty acid synthase [23,40], MHC class II-associated invariant chain [41], p27kip1 [20], human tissue inhibitor of metalloproteinases-2 ('TIMP-2') [42], extracellular-signal-regulated protein kinase 2 [43], Na/K-ATPase α 3 subunit [44], E2F [45], the vitamin D₃-induced human p27 (*Kip2*) gene [20], type-A natriuretic peptide receptor [21], the UV-induced human multidrug-resistance-1 gene [18], the cystathionine β -synthase-1b gene [46] and the nicotinic receptor β 4 subunit [16], among others. Although the mechanism(s) is/are not entirely understood, it may be that NF-Y binding introduces distortions in the DNA structure essential to the recruitment of critical transcription factors. This might be via direct protein-protein interaction between NF-Y and other factors, or via its association with histone acetylases, which maintains chromatin in an open configuration, thus activating transcription. Alternatively, NF-Y subunits B and C contain histone-like motifs that could be targets for acetylation/deacetylation by the histone-modifying enzymes [47,48]. In at least one report [41] it has been demonstrated that the half-life of either NF-Y or Sp1 binding is dramatically increased when both transcription factors are bound to the proximal promoter of the MHC class II-associated invariant chain (*Ii*) gene.

In the present study, we analysed carefully the NF-Y/Sp1-overlapping box in order to elucidate the contribution of each transcription factor to the binding, and the resulting transcriptional activity. Point-mutation analyses of Sp1 and/or NF-Y boxes within the D-FOR2U probe confirm that the binding in this overlapping box is mainly due to NF-Y, which is able to shift two complexes ('b' and 'c'), whereas Sp1 produces only one faint band ('a') (Figure 4B). Competition experiments using these mutants as competitors corroborate the nature of this binding (Figure 4C). Moreover, NF-Y competes with Sp1 binding to the overlapping box, as shown in the TNT experiments (Figure 5A). In keeping with this, when the NF-Y site in this overlapping box is mutated, there is a substantial increase (more than 3-fold) in Sp1 binding (Figure 5B). Since NF-Y binds most of the molecules of the wild-type probe, it is not surprising that mutation of the Sp1 site did not significantly alter NF-Y binding.

It is interesting to note that the mutation in the Nmut probe makes the Sp1 upstream box the same as the Sp1 downstream box. However, in spite of having the same sequence, the binding of the Sp1 upstream box is much weaker than that of the Sp1 downstream box, as shown in Nicolás et al. [31]. This differential binding ability of Sp1 to both probes could be caused by a dissimilar DNA secondary structure.

Nonetheless, each of the two Sp1 boxes are functional, and their mutation (pSmut or pSdmut) leads a 60% decrease in luciferase activity compared with the wild-type construction (pFOR15) (Figure 6). The mutation of both Sp1 boxes, to render

Figure 7 Effect of overexpressing Sp1 and/or Sp3 on Sp1 promoter activity in HeLa cells

Cells were co-transfected with 250 ng of pGL3FOR2 and increasing amounts of expression vectors for Sp1 and/or Sp3. After 28 h of expression, cell lysates were assayed for luciferase activity. Data represent the means $\pm$ S.E.M. for three experiments. Luciferase activity was normalized to mg/protein for each sample.

the double mutant pSSmut, does not produce an additional reduction in the activity of the reporter gene. In contrast, mutation of the NF-Y site at the NF-Y/Sp1-overlapping box (pNmut) causes an increase in luciferase activity. This result suggests that NF-Y could be acting as a repressor through the NF-Y/Sp1-overlapping box. However, overexpression of NF-YA increased basal luciferase activity in all constructs, including the wild-type (Figure 6). The increase in basal activity observed with the pNmut construct could be explained by the results gathered from the binding experiments. There is competition between NF-Y and Sp1 for the overlapping box, although NF-Y is bound preferentially. In the case of the Nmut probe, the binding of Sp1 is increased appreciably, and, given that in our model Sp1 is a stronger activator than NF-Y, mutation of this NF-Y site is translated into an increase in Sp1-promoter activity.

The same NF-Y/Sp1-overlapping boxes were observed by Valor et al. [16] in the nicotinic receptor gene. These researchers performed NF-Y mutation analysis in three different cell lines and, in two of them (C2C12 and COS cell lines), found the same increase in transcriptional activity. Consequently, it was suggested that complex mechanisms are behind the differential effect of the NF-Y mutation in the cell types, perhaps involving different protein modifications, protein-protein interactions, and/or a distinct balance between NF-Y and Sp1 in the three cell lines analysed [16].

We have reported previously that the *Sp1* gene is positively autoregulated [31]. Sp3 is another transcription factor that could also bind to the same sequence and, via supershift experiments, we demonstrated that Sp3 was able to bind to the Sp1 promoter. Sp3 can act as an activator or repressor of Sp1-mediated

activation, depending on the sequence context, the number of Sp1-binding sites and the availability of specific co-activators, co-repressors or other transcription factors [49]. In our model, we found no significant effect when transfecting HeLa cells with an expression vector for Sp3 alone. In contrast, when co-transfecting HeLa cells with a constant amount of Sp3 and increasing amounts of Sp1, we found that Sp3 counteracted Sp1-mediated transactivation. The action of Sp3 on the Sp1 promoter is not surprising, since it has been reported in other systems that Sp3 overexpression suppresses the Sp1-mediated activation of transcription [8,50–52].

Synergistic activation by E2F-1 and Sp1 has been reported for the dihydrofolate reductase promoter in SL2 cells [35], and for the murine thymidine kinase promoter in 3T6 cells [36]; in the latter case, its interaction with Sp1 enables E2F-1 to displace the Sp1 that is bound to histone deacetylase-1, a negative regulator of transcription [53]. We have demonstrated that the minimal promoter of Sp1, which contains a putative E2F-binding site, could be activated in a dose-dependent manner by E2F, and with more potency in the presence of an Sp1-binding site. Furthermore, an additive activation was observed when co-transfecting E2F together with an Sp1 expression vector in HeLa cells. This additive effect could be explained by E2F/Sp1 binding and activation of the Sp1 promoter through the E2F box, or E2F and Sp1 binding to each corresponding DNA box, since there are consensus sequences for both transcription factors in the minimal promoter of Sp1.

Moreover, in the co-transfection experiments using the luciferase mutants, the overexpression of Sp1, NF-YA or E2F increases luciferase activity, even when the Sp1 or NF-Y boxes

Figure 8 Effect of overexpression of E2F on Sp1 promoter activity in HeLa cells

(A) Scheme of the luciferase construction used in the co-transfection experiments. (B) Cells were co-transfected with 250 ng of the basal reporter gene construct pGL3, pGL3FOR1 or pGL3FOR2 (as demonstrated by the key in A), and 250 or 750 ng of an expression vector for E2F. (C) Cells were co-transfected with 250 ng of the basal reporter gene construct pGL3FOR2, with the indicated combinations of the expression vectors for E2F and Sp1. After 28 h of expression, cell lysates were assayed for luciferase activity. Data represent the means $\pm$ S.E.M. for three experiments. Luciferase activity was normalized to mg/protein for each sample, and promoter activity of each co-transfection was cross-referenced with the control value of each construct.

are mutated. This effect could be explained by the formation of a general complex that governs the transcriptional activity. In spite of the direct binding to DNA, proteins could mediate their activation effect through the whole complex. Additionally, we cannot rule out that a small proportion of either the Sp1 or NF-Y proteins still interacts with the DNA mutants.

In a previous paper [31], we concluded that the *Sp1* gene is positively autoregulated. An interesting conclusion of the present work is that the *Sp1* gene promoter is regulated by a number of different transcription factors, including Sp1, Sp3, NF-Y and E2F, all of which are ubiquitously expressed factors. The abundance of these four factors is essential for the final activity of the Sp1 promoter, because Sp3 is compensating for the activation produced by Sp1, and also because NF-Y is a minor activator transcription factor. Thus the relationship between the amounts of Sp1, Sp3, NF-Y and E2F proteins in the cell could be crucial in terms of the final promoter activity.

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