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REGULACIÓ DEL PROMOTOR DE *Sp3*

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RESULTATS

1. ARTICLE I:

Characterization of the 5'-flanking region of the human transcription factor *Sp3* gene,

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BBA-Gene Structure and Expression **1730(2)**:126-136 (2005).

Prèviament a la realització d'aquest treball, al nostre grup de recerca s'havia estudiat la regulació del promotor del factor de transcripció Sp1. Els promotors regulats per Sp1 són també regulats per Sp3 i és la relació entre aquestes dues proteïnes la que determina el grau d'activació final dels promotors diana. Per aquesta raó, ens vam proposar estudiar també la regió promotora del factor de transcripció Sp3, donat que en aquell moment no existien dades sobre la regulació de la regió 5'-UTR del gen *Sp3*. Els resultats i conclusions obtingudes van ser els següents:

- Es va procedir al clonatge, a partir de cèl·lules HeLa, de 546 bp de la regió promotora del factor de transcripció Sp3. Mitjançant anàlisi computacional es van identificar seqüències consensus per als factors de transcripció: Elk-1, Myb, NF-1, AP-1, Sp1, NF-Y, AP-1 i USF.
- Utilitzant les tècniques de Primer extension i RNAsa protection, es van determinar múltiples inicis de transcripció per a Sp3 entre -70 i -132 respecte a l'inici de traducció.
- Es va definir la regió mínima promotora com la regió continguda als primers 225 bp respecte l'inici de traducció i es va definir com a promotor proximal la regió situada a -281 bp respecte l'inici de traducció.
- Mitjançant assajos de retardació de la movilitat electroforètica es va demostrar que els factors de transcripció Sp1 i Sp3 eren capaços d'unir-se a la regió proximal del promotor de Sp3.
- Assajos de transfecció transitòria i activitat luciferasa van permetre demostrar que el factor de transcripció Sp3 era capaç de regular positivament el seu propi promotor i que Sp1 també era un activador del promotor de Sp3. La combinació dels dos factors tenia un efecte additiu sobre el promotor proximal.
- La caixa d'unió per a NF-Y era funcional, tal com van demostrar els assajos de retardació de la movilitat electroforètica i els assajos luciferasa. NF-Y era capaç d'unir-se al promotor de Sp3 i activar-lo.

- La combinació dels factors de transcripció Sp1 i Sp3 amb NF-Y va tenir efectes additius sobre el promotor de Sp3.

En conclusió, en aquest treball, s'aïllà la seqüència promotora del gen Sp3 i s'inicià un estudi dels possibles factors de transcripció que podrien estar regulant aquest gen.

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Promoter paper

Characterization of the 5′-flanking region of the human transcription factor *Sp3* gene

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Abstract

A fragment of 1079 bp from the 5′-flanking region of the human *Sp3* gene was isolated and characterized. The *Sp3* promoter is a GC-rich region that contains putative binding sites for Elk-1, c-Myb, NF-1, Ap1, Sp1, NF-Y, Ap2 and USF. Several transcriptional start sites located between 70 and 132 bp upstream of the translational start site were identified. The proximal promoter was contained in the first 281 bp 5′ of the translational start, whereas the region including up to −225 relative to the translational start was referred as the minimal promoter. Transient transfections and luciferase assays revealed activation of the *Sp3* proximal promoter upon overexpression of either Sp1 or Sp3, alone or in combination. Gel-shift and supershift assays demonstrated specific binding of Sp1 and Sp3 proteins to the GC box located in the proximal promoter of *Sp3*. Overexpression of NF-YA had a synergistic effect on Sp1 overexpression and an additive effect on Sp3 overexpression. Additionally, overexpression of NF-YA, Sp1 and Sp3 altogether had a synergistic effect on Sp3 promoter activity. Furthermore, binding of the NF-Y complex to the CCAAT box located in the proximal promoter of *Sp3* was observed in gel-shift assays.

Keywords: Sp3; Promoter; Sp1; NF-Y; Gene regulation; Transcription

1. Introduction

Sp3 is an ubiquitous transcription factor that belongs to the Sp/XKLF (specificity protein/Krüppel-like factor) family of transcription factors, characterized by the presence of three conserved Cys_2His_2 zinc fingers that form their DNA-binding domain [1]. Sp/XKLF members recognize the same GC-(GGGGCGGGG) and GT-(GGTGTGGGG) boxes, which are important for the expression of many different ubiquitous as well as tissue-specific cellular and viral genes [2,3]. The Sp/XKLF family comprises a large number of homologous transcription factors, and Sp3 belongs to a subgroup together with Sp1, Sp2 and Sp4. Sp1–4 contain two major glutamine-rich activation domains that are essential for transcriptional activation. Next to these glutamine-rich domains, serine/threonine-rich stretches are located that may be a target for post-translational modification [2].

Although Sp3 was found to be highly homologous to Sp1, its transcriptional activity is context-dependent and is influenced by both promoter structure and cell type [4]. Studies from several laboratories have shown that Sp3 functions as both transcriptional activator and repressor [5]. Upon cotransfection with Sp1, additive and synergistic effects have been observed. However, it has also been described that Sp3 represses Sp1-activated transcription in the case of promoters containing multiple adjacent binding sites. Interestingly, in the case of the human *Sp1* promoter, Sp3 is able to counteract Sp1-mediated transcription [6]. Additionally, Sp3 can act as an activator or repressor of Sp1-mediated activation depending on the availability of specific co-activators, co-repressors or other transcription factors [3]. The complex activity of Sp3 is due to the glutamine-rich activation domains, similar to those found in Sp1 and Sp4 and, adjacent to these, an inhibitory domain, unique to Sp3, which can be posttranslationally regulated by sumoylation [7,8], acetylation [9,10] and phosphorylation [11,12].

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Knock-out mice lacking Sp3 exhibit defects in bone and tooth development, suggesting a non-redundant role in regulation of genes important for terminal differentiation in these tissues [13,14]. Sp3-deficient mouse embryos develop until birth, but die invariably of respiratory failure immediately after birth [13].

The chromosomal localization of Sp3 is 2q31, spanning more than 55 kb and encompassing seven exons. Additionally, a recent study has shown the existence of a pseudogene located on chromosome 13, spanning approximately 4.0 kb [15]. Transcription factor Sp3, originally also designated as SPR-2, was cloned by recognition site screening using the GT-box motif of the uteroglobin promoter as a probe [1]. Independently, Sp3 was obtained from a human T-cell library by low stringency screening with the zinc finger-encoding region of human Sp1 as a probe [16].

Because of the difficulty in cloning the 5′ end of the mRNA, it was thought for a long time that the full-length Sp3 protein of 110–115 kDa was derived from translational initiation at a non-AUG codon [17], whereas two small Sp3 isoforms of 60–70 kDa were thought to derive from internally initiated translation at AUG start sites [17]. However, in 2002, human genomic DNA sequences were reported that encompassed three exons coding for 85 additional N-terminal amino acids of Sp3 [18]. Moreover, other studies have shown that in vivo four isoforms of Sp3 are expressed, that differ in the extent of the amino terminal part and that are all derived from alternative translational start sites [19]. The two long isoforms of Sp3 can act as transcriptional activators on certain promoter settings, whereas the two small isoforms appear to be always inactive [19]. Furthermore, a recent report describes the complexity of Sp3 transcripts, with the characterization of one transcript of 4.3 kb, encompassing exons 1 to 7, a splice variant lacking exon 3 which initiates transcription within a region just 5′ of exon 2 and does not possess exon 1, a transcribed sequence which appears to initiate within intron 2 and continues to encompass exons 3 to 7 and could be referred as a alternate 5′ UTR, and a putative splice variant lacking exon 4. Transcripts lacking exon 4 would lack the two internal open reading frames that give rise of the two smaller isoforms, which purportedly lack activation domains [15].

In the present work, we started the study of the *Sp3* gene promoter. We cloned a 1079 bp fragment from the 5′ region of the human *Sp3* gene, identified several transcriptional start sites, analyzed the putative binding sites for transcription factors and investigated the role of Sp1, Sp3 and NF-Y in the regulation of the *Sp3* promoter activity.

2. Materials and methods

2.1. Cell culture

HeLa human cervical carcinoma cells, 293T adenovirus-transformed human embryonic kidney cells expressing SV40 large T antigen, HT29 human colon adenocarcinoma cells and MCF-7 human breast adenocarcinoma cells were grown in Ham's F-12 medium supplemented with 7% fetal bovine serum (Gibco). Cultures were maintained at 37 °C in a humidified 5% CO_2-containing atmosphere.

2.2. Isolation of the Sp3 promoter

Contig AC016737, a 196085-bp piece of genomic DNA corresponding to human clone RP11-394I13, was reported by Sulston and Waterston [20].This clone is part of the RPCI-11 human BAC library that was prepared from the blood of one male donor as described by [21] and was obtained from the Roswell Park Cancer Institute (Buffalo, NY). Clone RP11-394I13 was used to isolate by PCR a 1079 bp fragment corresponding to the *Sp3* promoter region, using the following specific primers:

Fwd 5′-CAAGAGGATAAAGAGGCCGGG-3′
Rev 5′-GGTTGCTCTCTCGGCTTTACGTACCGGTCATAGTGTGTTTAGGGC-3′

Additionally, human genomic DNA from HeLa cells was used to isolate by PCR a smaller fragment from the *Sp3* promoter region. The forward primer used to generate this 546-bp fragment, 5′-CCAGCTCAGCTCCCCTACAGC-3′, was also designed from contig AC016737, and the reverse primer was the same used for the 1079-bp fragment. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing kit v.2.0 (Applied Biosystems).

2.3. Database searching and data submission

The 1079 bp sequence corresponding to the 5′ flanking region of the *Sp3* gene was submitted to GenBank (AY251018). For sequence comparison, the BLAST 2.2 program was used [22].

The putative transcription factor binding sites present in the promoter were analyzed using the Match™ software using TRANSFAC 6.0. The presence of putative CpG islands in the 5′-flanking region of the *Sp3* gene was analyzed by the default setting (GC% over 50% and observed CpG/expected CpG ratio over 0.6, for a minimum of 200 bases) of the CpGplot program from the European Molecular Biology Open Software Suite [23].

The alignment of the 5′-UTR sequences from human (AY251018) and mouse (AC124765) *Sp3* genes were carried out using the ClustalW (1.75) algorithm at the European Molecular Biology Network (http://www.es.embnet.org/Doc/phylodendron/clustal-form.html) [24].

2.4. Total RNA and genomic DNA preparation

Total RNA was extracted from HeLa and 293T, HT29 and MCF-7 cells using the Ultraspec™ RNA reagent (Biotecx) in accordance with the manufacturer's instruc-

tions. Genomic DNA was prepared according to standard protocols.

2.5. Primer extension

One hundred ng of Sp3-Rev 45-mer primer with the following sequence: 5′-GGTTGCTCTCTCGGCTTTACG-TACCGGTCATAGTGTGTTTAGGGC-3′ was end-labeled with 20 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) using T4 poynucleotide kinase (New England Biolabs). Then, 30 μg of total RNA either from HeLa or 293T cells was hybridized with 10^5 cpm of the labeled primer in a total volume of 15 μl containing 150 mM KCl, 10 mM EDTA, and 100 mM Tris/HCl, pH 8.3. The same amount of yeast RNA was used as a negative control. The hybridization was performed at 80 °C for 5 min followed by 65 °C for 90 min. After hybridization, the reverse transcription reaction was carried out at 37 °C for 60 min upon the addition of 500 μM dNTPs, 10 mM dithiothreitol (DTT), 20 units of RNAsin (Promega), 10 mM $MgCl_2$ and 200 units of murine leukemia virus reverse transcriptase M-MLV RT (GIBCO) in 50 mM Tris/HCl, pH 8.0. Ten μl of the reaction mixture was run on a 6% polyacrylamide 8 M urea gel in 1× TBE (0.1 M Tris/HCl, 90 mM boric acid, 1 mM EDTA, pH 8.3), dried and visualized by autoradiography.

2.6. Ribonuclease protection assay

For the ribonuclease protection assay, an antisense RNA probe of 464 nt was generated using a construction engineered by unidirectional cloning of PCR fragments from the Sp3 promoter between the *Xho*I and *Hin*dIII sites on the pSP72 vector (Promega). The PCR fragment was generated using a forward primer in the 5′-UTR of human *Sp3* gene (upper case) preceded by an arbitrary sequence (lower case) including a *Hin*dIII restriction site (underlined) (5′-cagtgctgaagcttGGGAGGCGGGCACAGCGGGGT-3′) and a reverse primer located in the first exon of Sp3 (upper case) followed by the same arbitrary sequence (lower case) as the forward primer but including a *Xho*I restriction site (underlined) instead (5′-cagtgctgctcgagCGGTCATAGT-GTGTTTAGGGCACCTCAGGCGGG-3′).

The construct was linearized using the *Bam*HI restriction site present in pSP72, and the antisense RNA probe was synthesized from 1 μg of linearized plasmid using SP6 RNA polymerase in a total volume of 20 μl containing 50 μCi of [α-32P]UTP (3000 Ci/mmol, Amersham Pharmacia Biotech), 500 μM each of ATP, CTP and GTP, 10 μM of UTP, 40 U of RNAsin, and 40 U of SP6 RNA polymerase, in SP6 transcription buffer (Roche) at 37 °C for 1 h.

The probe was gel-purified on a 5% polyacrylamide gel and eluted in 400 μl of Elution Buffer (0.1% SDS, 1 M NH4OAc, 0.1 M EDTA, pH 5). RNase protection assays were performed with 30 μg of total RNA using the RPAII kit (Ambion) with the following modifications: Briefly, total RNA was hybridized to the antisense RNA probe (8×10^4 cpm) in 30 μl of hybridization buffer (80% Formamide, 40 mM PIPES, 400 mM NaCl, and 1 mM EDTA) overnight at 42 °C and subsequently treated with RNase T1 (100 U/ml) for 30 min a 30 °C. Protected fragments were precipitated, electrophoresed in 5% polyacrylamide 8 M urea sequencing gels and visualized by phosphorimaging after the gel was dried.

2.7. Luciferase constructs

Progressive deletion constructs in the *Sp3* promoter region were engineered by unidirectional cloning of PCR fragments from the *Sp3* promoter between the *Nhe*I and *Xho*I sites of the reporter luciferase vector pGL3-basic (Promega). These PCR fragments were generated using a common reverse primer and five different forward primers. The *Sp3*-specific sequences for these primers were taken from contig AC016737 (in uppercase, see below). For the forward primers, these specific sequences were preceded by an arbitrary sequence (in lowercase, see below) including a *Nhe*I restriction site (underlined). The reverse primer followed a similar structure but contained a *Xho*I restriction site (underlined) in the arbitrary sequence.

Fwd 1	5′-tcaagtcaggctagcGGGAGGCGGGCACAGCGGGG-3′
Fwd 2	5′-tcaagtcaggctagcCTGGACCAATGAGCACAGCCG-3′
Fwd 3	5′-tcaagtcaggctagcGAGGGGCGGGAGTTCCGGGCG-3′
Fwd 4	5′-tcaagtcaggctagcGAGTTCCGGGCGGGCTGTCAC-3′
Fwd 5	5′-tcaagtcaggctagcTCACCCTCTTCCCCCCTTTTG-3′
Rev	5′-cagtgctgcctcgagCAGGCGGGGCTCCCCGCCGCC-3′

2.8. Transfections, cotransfections and luciferase assays

HeLa cells were seeded into 6-well plates the day before transfection at a density of 2×10^5 cells/well in Ham's F12 medium containing 5% fetal bovine serum. The medium was renewed before transfection. Transfection was performed using FUGENE™ 6 (Roche Molecular Biochemicals). For each well, 3 μl of FUGENE™ 6 in 100 μl of serum-free F-12 medium was incubated at room temperature for 5 min. The mixture was added to 250 ng of each of the promoter deletion constructs and 250 ng of pCMV SPORT-βgal corresponding to the reporter gene β-galactosidase. In cotransfections, the construct pSp3-FOR4 and the β-gal reporter plasmid were mixed with increasing amounts of Sp1 (CMV-Sp1), Sp3 (CMV-Sp3) or NF-YA expression vectors before the addition of FUGENE™ 6 in serum-free F-12 medium. For co-transfections with the dominant negatives, 250 ng of pCMV-DNSp1 or pCMV-DNSp3 were mixed with construct pSp3-FOR4 and the β-gal reporter plasmid. Luciferase activity was assayed 24 h after transfection. Cell extracts were prepared by lysing the cells with 200 μl of freshly diluted 1× Reporter Lysis Buffer (Promega). The lysate was centrifuged at 13,000 rpm for 2 min to pellet the cell debris and the supernatants were

transferred to a fresh tube. A 10-μl aliquot of the extract was added to 25 μl of the luciferase assay substrate (Promega) and the luminescence of the samples were read immediately on a TD-20/20 Luminometer, in which the light production (relative light units) was measured for 10 s. Each transfection was performed in triplicate.

Transfection efficiency was corrected by β-galactosidase activity. A 10-μl aliquot of the cell extract was mixed with 290 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG) solution (880 μg/ml ONPG, 67 mM Na_3PO_4, 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, pH 7.5). The absorbance of the mixture was determined at 420 nm after 30 min of incubation at 37 °C.

2.9. Electrophoretic mobility shift assays

Nuclear extracts were prepared from exponentially growing HeLa cells as described [25]. The probes were constructed by annealing commercially synthesized complementary single-stranded oligodeoxynucleotides corresponding to the putative Sp1 or NF-Y DNA binding sites present in the *Sp3* promoter (underlined):

Sp1 probe: 5′-AAGAAGAGGGGCGGGAGTTCCGGGC-3′
NF-Y probe: 5′-CGCCTGGACCAATGAGCACA-3′

The ds oligonucleotides were gel-purified, end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech), and used as probes in the gel shift experiments. DNA binding assays were performed as described [26] and they were visualized using a PhosphorImager with ImageQuant software v 5.2 (Molecular Dynamics).

In the supershift experiments, 1 μg of rabbit polyclonal antibody PEP-2 (Santa Cruz) against Sp1, 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 1 h before the addition of the probe.

3. Results

3.1. Cloning of the 5′-flanking region of human transcription factor Sp3

To clone the 5′ region of the *Sp3* gene, we used the sequences AJ310752 [27] and AF494280 [18] as virtual probes to perform a BLAST comparison using the unfinished High Throughput Genomic Sequences database finding contig AC016737, corresponding to clone RP11-349I13, which contained additional sequence in the 5′-region. Then, specific primers described in Materials and methods were used to isolate a 1079 bp fragment by PCR from clone RP11-349I13. This DNA corresponding to the 5′-flanking region of human *Sp3* was sequenced and submitted to GenBank (accession number AY251018).

To confirm that the sequence obtained from clone RP11-349I13 was also present in HeLa cells, we isolated a 546-bp fragment by PCR using genomic DNA from HeLa cells and primers designed from contig AC016737. Sequence analysis was performed to establish the location of the 5′-flanking region AY251018 in relationship with other Sp3 genomic sequences already reported in the literature, mRNA sequences and Sp3-derived ESTs, and the results of such analysis are presented in Fig. 1A.

3.2. Mapping of the putative cis-acting elements in the Sp3 promoter

The human *Sp3* gene 5′-flanking region (546 bp) was analyzed using the Match™ program version 1.0. This software is designed to search potential binding sites for transcription factor nucleotide sequences using the library of mononucleotide weight matrices from TRANSFAC 6.0. The search was performed using 0.95 and 0.90 as cutoffs for core and matrix similarity respectively, and revealed putative binding sites for Elk-1, c-Myb, NF-1, Ap1, Sp1, NF-Y, Ap2 and USF. The *Sp3* promoter sequence was also analyzed using the CpGplot program (www.ebi.ac.ac.uk/emboss/cpgplot). One CpG island of 236 bp was identified (−461 to −200). Comparison between human and mouse *Sp3* promoter regions using the ClustalW alignment software revealed a high degree of homology between the two species (Fig. 1B) and suggests that the putative binding sites located along the conserved regions could have relevance in vivo.

The sequence, the DNA boxes, the CpG island in the human *Sp3* promoter and the alignment between human and mouse promoters are shown in Fig. 1B.

3.3. Transcriptional initiation in the Sp3 promoter

Transcriptional initiation was analyzed by primer extension and by ribonuclease protection assay. The primer extension analysis was carried out with a 45-nt labeled oligonucleotide using RNA preparations from HeLa and 293T cells (Fig. 2A). A common transcriptional start site was observed around position −70 from the translational start. A minor band could also be observed in HeLa cells, corresponding to initiation around position −100. A negative control was performed using yeast RNA.

To confirm the location of *Sp3* transcriptional start, the 5′ ends of Sp3 transcripts were mapped by RNase protection using a probe that spanned 464 nt and RNA from three different human cell lines. As can be seen in Fig. 2B, RNA from HeLa, HT29 and MCF-7 protected several fragments of the probe that migrated, as determined from their relative electrophoretic mobility, at 77, 105, 115 and 139 nt, the sizes expected for transcription initiation at nucleotides −70, −98, −108 and −132 from the translational start,

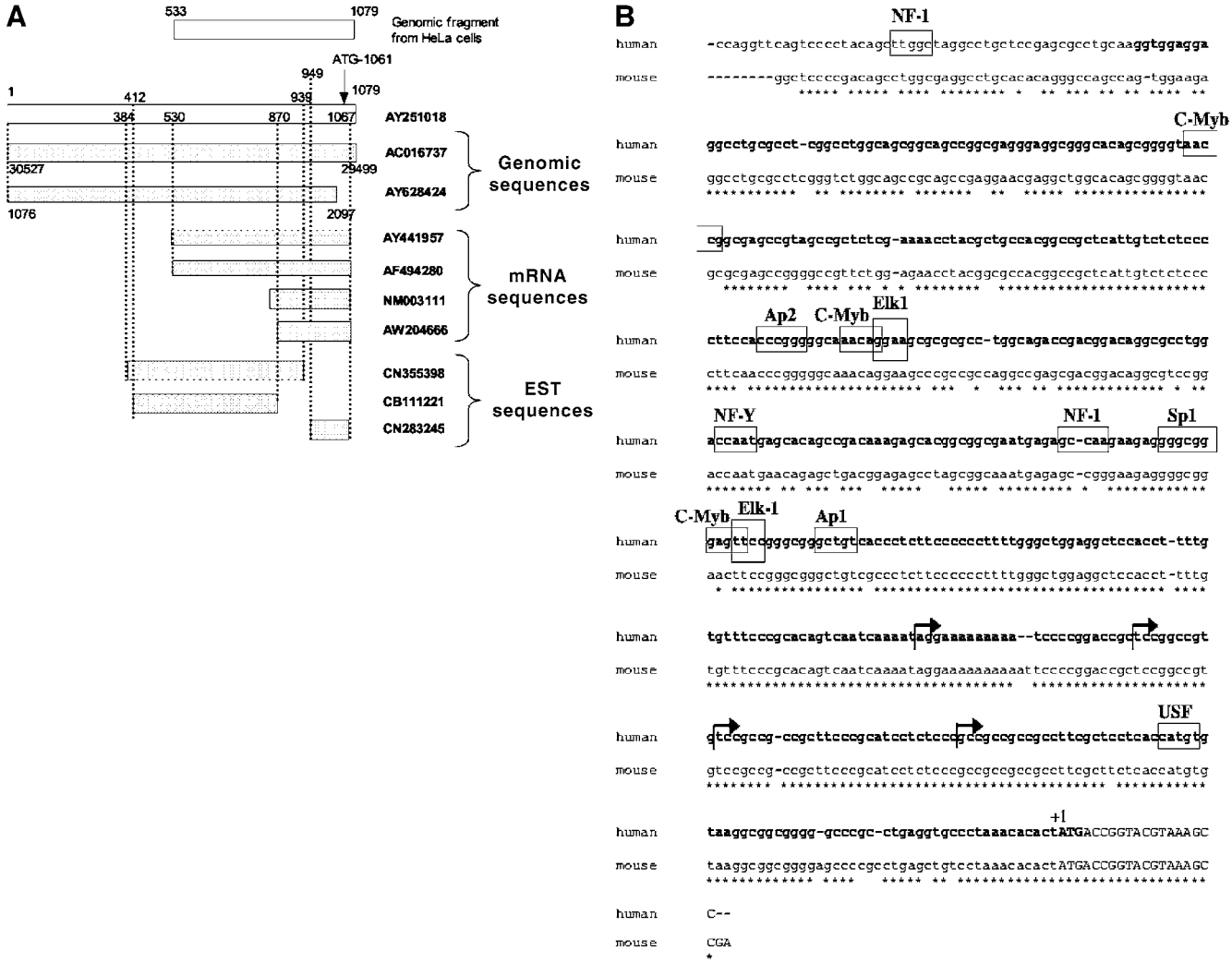

Fig. 1. Structural analysis of the *Sp3* 5′-flanking region. (A) Sequence analysis of the location of the 5′-flanking region AY251018 in relationship with other Sp3 genomic sequences, mRNA sequences and Sp3-derived ESTs. The different sequences used in the analysis are identified by their GenBank accession numbers. Dotted lines demark location of overlap between sequences, and the overlapping regions are indicated by numbers corresponding to specific nucleotide positions in sequence AY251018. (B) Putative binding sites for transcription factors are indicated with boxes and CpG islands are indicated in bold. The different transcription start sites identified by RNAse protection are indicated by arrows. The most N-terminally ATG codon described by Oleksiak and Crawford [18] that initiates translation corresponds to the first three nucleotides of the sequence in capital letters. For comparison between human and mouse *Sp3* promoter regions, the homology between both species is indicated with stars in the figure.

respectively. Additionally, other minor bands could also be observed.

DNA sequencing analysis revealed that the *Sp3* gene lacks basal elements such as a TATA box or an initiator element.

3.4. Transcriptional activity of Sp3 promoter deletions

To localize the DNA elements that are important for basal and stimulated transcription of the *Sp3* promoter, a series of 5′-deletion constructs were generated by PCR and cloned into the promoterless pGL3-basic, a luciferase reporter vector. The resulting constructs, containing 5′-flanking regions from −417 to −198 relative to the translational start codon (ATG) of *Sp3*, were transiently transfected into HeLa cells. Transfection of HeLa cells with pSp3-FOR5, which contained the longest 5′ sequence, yielded a 33.7-fold increase in promoter activity relative to pGL3 alone (Fig. 3). Deletion from nucleotide −417 to nucleotide −281 (pSp3-FOR4) reduced Sp3 promoter activity only by 5%. Further deletion from nucleotide −281 to −225 (pSP3-FOR3) reduced the activity by 70% but it was still 7 times higher than that of the reporter vector alone. However, deletion to nucleotide −213 (pSp3-FOR2) abolished the promoter activity altogether (Fig. 3). These data suggest that the sequence between −281 and −225 contains elements that contribute to the basal transcription activity of the *Sp3* gene. The 5′-flanking region including up to −281 relative to the translational initiation codon could be referred as the proximal promoter, and the 5′-flanking region including up to −225 relative to the translational initiation codon could be referred as the minimal promoter.

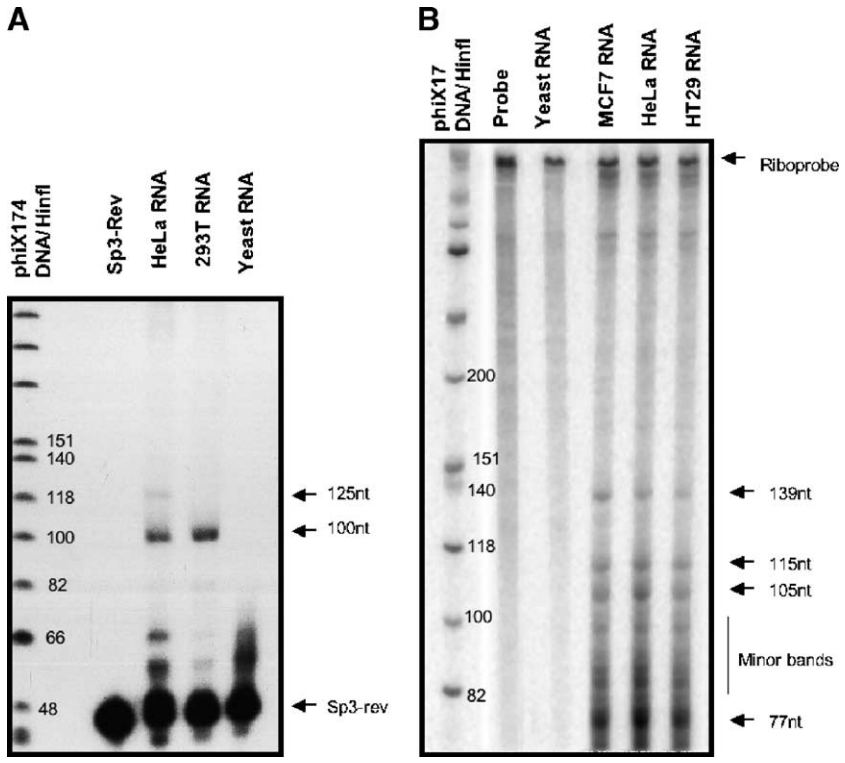

Fig. 2. Determination of the transcriptional start of the human *Sp3* promoter. (A) Primer extension by reverse transcription using a specific 45-mer (Sp3-rev primer) and 30 μg of total RNA from either HeLa or 293T cells is shown. Yeast RNA was used as a negative control. The band of 105 nt reflects a transcription start site around position −70 relative to the translation initiation codon. The band of 125 nt present in HeLa cells reflects an additional transcription start site around position −100. (B) Ribonuclease protection assay was performed with an antisense 464 nt RNA probe and 30 μg of total RNA from HeLa, HT29 and MCF-7 cells using the RPAII kit. Protected fragments were precipitated, electrophoresed in 5% denaturing polyacrylamide sequencing gels and visualized by phosphorimaging after the gel was dried. The size of the protected fragments was determined according to their relative electrophoretic mobility.

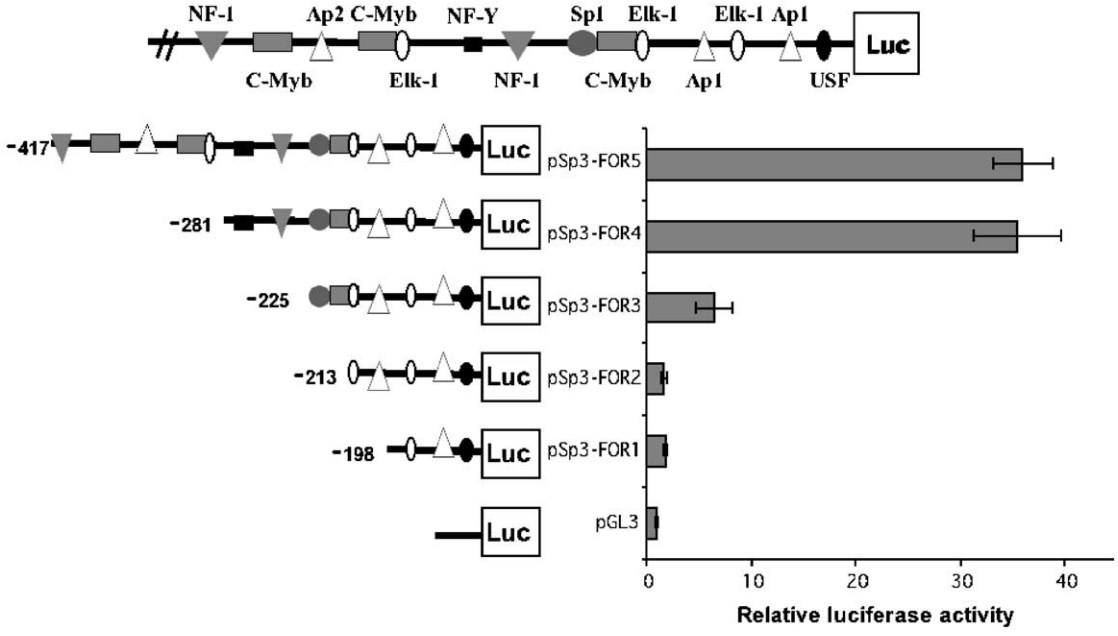

Fig. 3. Deletion analysis of *Sp3* promoter activity in HeLa cells. Depicted on the left side of the figure are the *Sp3* promoter-luciferase deletion constructs. The location of putative nuclear protein binding sites for Elk-1, c-Myb, NF-1, Ap1, Sp1, NF-Y, Ap2 and USF are indicated. The 5′-end point of each deletion mutant is indicated, and all constructs start at position −38 from the ATG. Each construct was transiently transfected into HeLa cells and assayed for luciferase (Luc) activity. Transfections were performed in duplicate, and the results are the mean of three experiments ±S.E. Luciferase activity is normalized to the β-galactosidase activity for each sample and is expressed relative to that of pGL3 basic vector.

3.5. Regulation of Sp3 transcriptional activity by Sp1 and Sp3 proteins

Transient cotransfections in HeLa cells were performed with the proximal promoter pSp3-FOR4 together with expression vectors for Sp1 and/or Sp3. This construct pSp3-FOR4 included the complete Sp1 recognition sequence (consensus and flanking nucleotides) and a NF-Y binding site. As shown in Fig. 4A, cotransfection of pSp3-FOR4 with either Sp1 or Sp3 led to an increase in promoter activity compared to the pSp3-FOR4 construct alone. Moreover, contransfection with both Sp1 and Sp3 increased pSp3FOR4 activity up to 4-fold (Fig. 4A). Cotransfection of pSp3-FOR4 with either Sp1 or Sp3 dominant negatives abolished Sp3 promoter activity (Fig. 4B). These results demonstrated that the *Sp3* promoter was activated by both its gene product and by Sp1.

3.6. Binding analysis to the GC-box in the proximal promoter

Given that the sequence contained in the pSp3-FOR4 construct contained a putative binding site for Sp1 found by computational analysis, we next performed electrophoretic mobility shift assays to characterize and identify the actual DNA-protein binding sites. Using a probe spanning from nucleotides −230 to −206 relative to ATG, which contains the binding site for Sp1, and nuclear extracts from HeLa cells, five major bands could be observed (Fig. 5). The binding pattern obtained in the gel-shift analysis was characterized using antibodies against Sp1 (PEP-2) and Sp3 (D-20). The upper band corresponded to binding of the probe by Sp1 since it was supershifted using the Sp1 antibody. In addition, the lowermost band almost disappeared completely in the presence of the Sp1 antibody. The two intermediate bands corresponded to binding by Sp3. Surprisingly, the intensity of the lowermost band was also decreased in the presence of the Sp3 antibody, suggesting that this band could be due to binding by Sp1 and Sp3 altogether (Fig. 5).

3.7. Regulation of Sp3 transcriptional activity by NF-Y

Since one CCAAT box corresponding to the binding site for the NF-Y complex was identified on the pSP3FOR4 sequence corresponding to the proximal promoter, and that functional synergism between NF-Y and Sp1/Sp3-binding sites has been described for several gene promoters, we sought to examine the role of NF-Y in the regulation of the *Sp3* promoter. To this end, transient cotransfections were performed in HeLa cells using the luciferase construct pSp3FOR4, together with increasing amounts of an expression vector for the NF-YA subunit. As shown in Fig. 6A, the NF-YA expression vector activated the *Sp3* promoter in a dose-dependent manner.

We next cotransfected pSp3FOR4 with expression vectors for NF-YA and Sp1. We observed that overexpression of Sp1 and NF-YA proteins had a synergistic effect on the transcriptional activation of the *Sp3* minimal promoter (Fig. 6B). Additionally, transient cotransfections in HeLa cells were performed with pSp3-FOR4 together with NF-Y and Sp3. As shown in Fig. 6B, cotransfection of pSp3-FOR4 with both NF-Y and Sp3 led to an additive increase in promoter activity compared to the effect produced by Sp3 or NF-Y alone. Finally, when cotransfecting pSp3-FOR4 together with Sp1, Sp3 and NF-Y, a synergistic effect on Sp3 transcriptional activation was observed (Fig. 6B). These results demonstrated that *Sp3* promoter activity might require the concerted action of Sp1, Sp3 and NF-Y.

3.8. Binding analysis to the CCAAT box in the Sp3 proximal promoter

The promoter sequence in the pSp3-FOR4 construct contained a putative binding site for NF-Y in addition to a

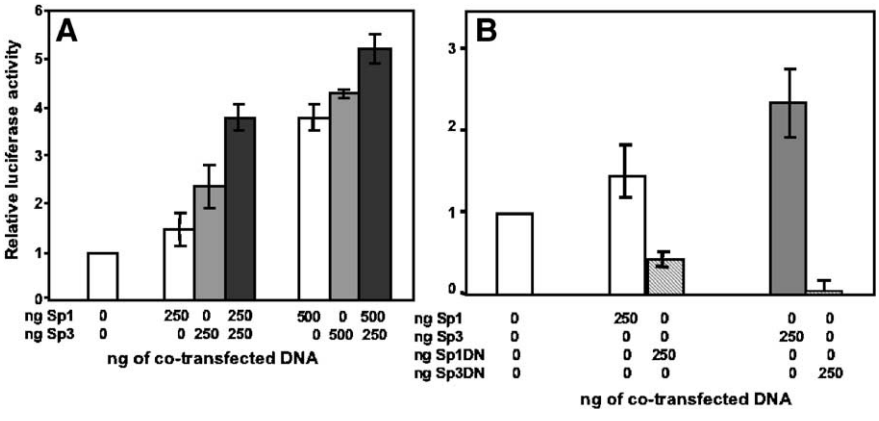

Fig. 4. Effect of Sp1 and Sp3 proteins on *Sp3* promoter activity in HeLa cells. (A) 250 ng of pSp3-FOR4 construct were cotransfected in HeLa cells with expression vectors for either Sp1, Sp3 or both. After 24 h, luciferase activity was determined. Cotransfections were performed in duplicate, and the results are the mean of three experiments ±S.E. Luciferase activity was normalized to β-galactosidase activity for each sample and it is expressed relative to that of pSp3-FOR4 alone. (B) 250 ng of pSp3-FOR4 construct were cotransfected in HeLa cells with expression vectors for either Sp1 or Sp3 dominant negatives. Other conditions as in A.

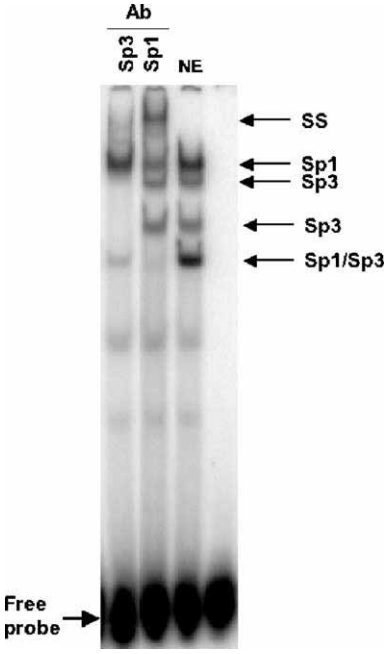

Fig. 5. Characterization of Sp1 and Sp3 binding to *Sp3* proximal promoter. Binding reactions were performed with 20,000 cpm of ds probe, 2 μg of nuclear extracts (NE) from exponentially growing HeLa cells and 1 μg of poly [d(I-C)] as the nonspecific competitor. Supershift mobility assays were performed in the presence of specific antibodies against either Sp1 or Sp3. The shifted and supershifted bands are indicated by arrows.

Sp1/Sp3 binding site. Therefore, we performed electrophoretic mobility shift assays using a probe spanning from nucleotides −262 to −282 relative to ATG, which contains the binding site for NF-Y, and nuclear extracts from HeLa cells (Fig. 7). The binding pattern obtained in the gel-shift analysis was characterized using an antibody against NF-YB (C-20). Two of the bands corresponded to binding of the probe by NF-Y since they were both supershifted in the presence of the NF-YB antibody (Fig. 7).

4. Discussion

In the present study, we isolated and identified the 5′-flanking region of the human *Sp3* gene. This sequence allowed us to characterize the human *Sp3* promoter region and identify *cis*-element regions involved in the regulation of the human *Sp3* gene. Similar to the human *Sp1* gene promoter [25], the 5′-flanking region of the human transcription factor *Sp3* lacks a TATA box [28]. The TATA box is typically located 30 bp upstream from the transcription initiation site and helps to specify the transcription initiation site. There is also no initiator element (TCAGT) in the human *Sp3* promoter that in the absence of a TATA box commonly drives transcriptional initiation [29]. In contrast, this region is GC-rich and contains potential binding sites for several well-characterized transcription factors, located along a conserved region between human and mouse. The absence of TATA box and the presence of several GC boxes have been found primarily in housekeeping genes [30] and it is expected to result in initiation of transcription at several locations [31,32]. Schatt et al. [33] reported that the presence of upstream enhancers in the absence of a TATA box resulted in initiation from sites scattered around the promoter region. Consequently, multiple transcription initiation sites may be a characteristic of genes that lack a TATA box in the promoter. In this direction, the mapping of the transcriptional start using primer extension and ribonuclease protection assays revealed the presence of several transcriptional start sites located around positions −70, −98, −108 and −132 relative to the translation initiation start, respectively, in HeLa, HT29 and MCF-7 cells. The identification of multiple transcription starts sites for the *Sp3* human gene is in accordance with [34] although in our experiments the locations of the different transcriptional starts sites by RNAse protection are further downstream in the 5′ region of the *Sp3* promoter, and they are independent of the cell line analyzed. According to [15], the 5′ end of human Sp3 exon 1 begins at bp 29,998 of the human chromosome 2 genomic sequence (AC016737), indicating that other transcriptional start sites can be found upstream of those reported in the present work.

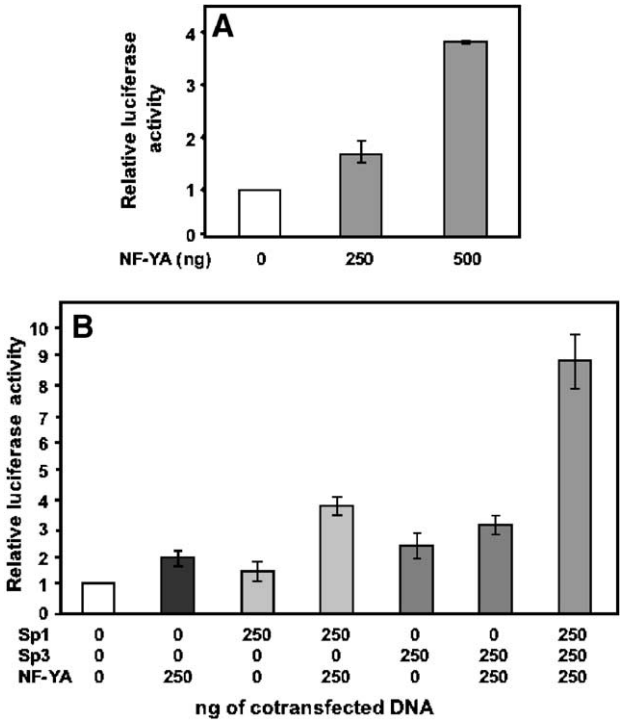

Fig. 6. Effect of overexpression of NF-YA, Sp1 and Sp3 on *Sp3* promoter activity. (A) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 and the indicated amounts of an expression vector encoding NF-YA. After 24 h of culture, cell lysates were assayed for luciferase activity. Luciferase activity was normalized to β-galactosidase activity for each sample. Results are expressed relative to the activity obtained upon transfection with the pSp3-FOR4 construct alone. Data represent the mean ± S.E. from three experiments. (B) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 together with the indicated amounts of expression vectors for NF-YA, Sp1 and/or Sp3. Other conditions as in A.

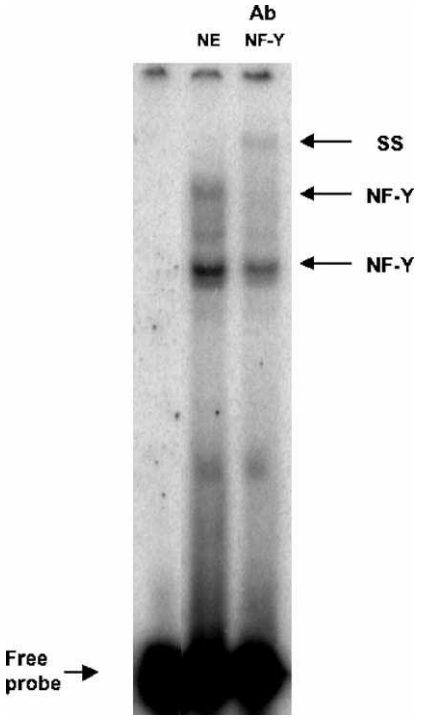

Fig. 7. Characterization of NF-Y binding to *Sp3* proximal promoter. Binding reactions were performed with 20,000 cpm of ds probe, 2 μg of nuclear extracts (NE) from exponentially growing HeLa cells and 1 μg of poly [d(I-C)] as the nonspecific competitor. Supershift mobility assays were performed in the presence of a specific antibody against NF-YB. Shifted and supershifted bands corresponding to NF-Y binding are indicated by arrows.

We found that the 5′-flanking region including up to −281 relative to the translational initiation codon contains all the elements necessary to achieve basal promoter activity. Computer analysis indicates the presence of one putative Sp1-binding site between nucleotides −223 to −215 that matches the structural determinants of Sp1-binding specificity described in [35] and which is consistent with previous studies showing that the transcription of other TATA-less promoters frequently involves the action of a proximal Sp1 site [36]. The computational analysis of this promoter region also revealed the presence of putative binding sites for Elk-1, c-Myb, NF-1, Ap1, Sp1, NF-Y, Ap2 and USF.

A main conclusion of this work is that the *Sp3* gene is positively autoregulated. Indeed, co-transfection experiments of *Sp3* proximal promoter constructs (pSp3-FOR4) and an Sp3 expression vector in HeLa cells demonstrate that Sp3 can activate its own transcription activity. Several studies have shown that Sp3 is a transactivator for promoters containing a single Sp-binding site. However, Sp3 preferentially inhibits Sp1-activated transcription of promoters containing multiple Sp-binding sites. Moreover, Sp3 can act as an activator or repressor of Sp1-mediated activation, depending on the sequence context and the availability of specific co-activators, co-repressors or other transcription factors [3].

The mechanism of positive autoregulation has already been described for other transcription factors such as Sp1 [6,25], NF-κB2 [37], junD [38] and CREB [27]. For all these transcription factors, the actual binding of these proteins to their gene promoters has been shown. We demonstrated that several DNA–protein complexes were produced in HeLa cells with a probe including the putative Sp1-binding site present in the *Sp3* promoter. Supershift experiments in the presence of specific antibodies allowed us to identify Sp1 and Sp3 as the proteins responsible for the shifted bands, thus demonstrating that Sp1 and Sp3 bind to the GC box located in the proximal promoter of *Sp3*.

In addition to Sp1 and Sp3, the proximal *Sp3* promoter also contains a putative binding site for NF-Y. NF-Y is a ubiquitous transcription factor that binds to CAAT elements in the proximal promoter of a wide variety of mammalian genes [39]. NF-Y consists of three subunits, termed A, B and C (with molecular masses of 42 kDa, 36 kDa and 40 kDa, respectively), all of which are essential for DNA binding [40–43]. Whereas NF-Y binding to the CAAT-box is frequently 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 proximal promoter construct pSp3-FOR4, which contains a NF-Y binding site, the cotransfection of NF-Y transactivated the basal promoter, probably recruiting other endogenous transcription factors. When NF-Y was co-transfected with either Sp1 or Sp3, a cooperative activation of promoter activity was observed. Furthermore, cotransfection of all three transcription factors caused a synergistic effect on Sp3 promoter activity. The functional synergism between NF-Y and Sp1 is effective for the *Sp3* promoter, as previously shown for the *Sp1* promoter [6] and other gene promoters as well [44–51]. In addition, our results indicate that NF-Y could enhance transactivation from Sp3. Although the mechanism for a functional interaction between NF-Y and Sp proteins is 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 through direct protein–protein interaction between NF-Y and other factors, or through its association with histone acetylases that 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 [52,53]. It is worth mentioning that Sp3 transcriptional activity can also be regulated by acetylation. Furthermore, by means of gel-shift and supershift analyses, we demonstrated that the NF-Y complex binds to the *Sp3* promoter using a probe including the putative NF-Y binding site present in this promoter.

To summarize, in this work, we defined the proximal promoter of the human *Sp3* gene. Both Sp1 and Sp3 activate

Sp3 promoter transcription as demonstrated by their binding to the recognition sequence present in the *Sp3* proximal promoter and by functional analysis using luciferase assays. In addition, NF-Y enhances transactivation from either Sp1 or Sp3 on *Sp3* transcriptional activity. Thus, our results indicate an important role for Sp1, Sp3 and NF-Y in the transcriptional regulation of the *Sp3* proximal promoter.

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2. ARTICLE II:

Transcriptional regulation of the 5'-flanking region of the human transcription factor *Sp3* gene by NF-1, c-Myb, B-Myb, AP-1 and E2F.

Alicia Tapias, Carlos J.Ciudad and Véronique Noé
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En el treball anterior, s'havia estudiat la regulació del promotor de *Sp3* pels membres de la família Sp, Sp1 i Sp3, i pel factor de transcripció NF-Y. En aquest segon treball, ens vam proposar estudiar més detalladament la identitat dels factors de transcripció responsables de regular el promotor proximal de Sp3. Les conclusions obtingudes van ser les següents:

- Els assajos de retardació de la movilitat electroforètica així com d'immunoprecipitació de la cromatina ens van permetre observar que els factors c-Jun, c-Myb, B-Myb, NF-1C i E2F1 s'unien al promotor de Sp3.
- Als assajos de retardació de la movilitat electroforètica, la utilització d'una sonda per la caixa d'unió de NF-1 va mostrar que NF-Y era capaç de reconéixer aquesta caixa a més de la seva pròpia al promotor de *Sp3*.
- Amb una sonda que contenia les caixes Myb i Sp, que es troben parcialment solapades al promotor de *Sp3*, es va poder establir que les proteïnes Myb no s'unien a la seva caixa d'unió quan ho feien les proteïnes Sp.
- Els factors de transcripció NF-1, c-Jun, c-Fos, c-Myb i B-Myb eren activadors de la transcripció de Sp3, tal com es va demostrar mitjançant assajos luciferasa o amb la determinació dels nivells de mRNA de Sp3 després de sobreexpressar o inhibir aquestes proteïnes.
- El factor de transcripció E2F1/DP1 es comporta com a repressor del promotor de Sp3, la seva sobreexpressió produeix una disminució de l'activitat del promotor i dels nivells de mRNA endogens de *Sp3*. A més, aquest efecte és capaç de contrarestar l'activació produïda per les proteïnes Sp sobre el promotor proximal de *Sp3*.

En aquest treball es demostra com el promotor de *Sp3* està regulat per diferents factors de transcripció: NF-1, AP-1, c-Myb, B-Myb i E2F1/DP1. Alguns d'aquests factors

s'expressen de forma ubicua, però altres són dependents de teixit, com c-Myb que s'expressa en teixits hematopoiètics, i altres, com AP-1, són capaços d'activar-se en front d'estímuls extracel·lulars.

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Transcriptional regulation of the 5′-flanking region of the human transcription factor *Sp3* gene by NF-1, c-Myb, B-Myb, AP-1 and E2F

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ABSTRACT

We analyzed in detail the proximal promoter of transcription factor Sp3, which expands 281 bp from the translational start. This sequence contains putative binding sites for Sp1, NF-Y, NF-1, Myb, AP-1 and E2F transcription factors. In this work, we further explored the role of these boxes on the regulation of the *Sp3* gene. Gel-shift and competition assays showed specific binding of NF-1, Myb, AP-1 and E2F. Furthermore, chromatin immunoprecipitation assays demonstrated that Sp1, Sp3, NF-Y, NF-1, c-Myb, B-Myb, c-Jun and E2F1 actually occupied the *Sp3* promoter in HeLa cells. Transient transfections and luciferase assays revealed activation of the *Sp3* proximal promoter upon overexpression of NF-1, c-Myb, B-Myb, c-Jun and c-Fos, and repression after overexpression of E2F/DP1. Point mutation of the binding sites for NF1, Myb, AP1 and E2F and cell incubation with specific siRNAs further confirmed the role of these transcription factors in the regulation of the *Sp3* promoter. The regulation of the endogenous *Sp3* gene was also observed at the mRNA level when the studied transcription factors were overexpressed or knocked down by siRNA incubation. These results help to explain the complex regulation of the *Sp3* gene, which depends, at least in part, on the relative amount of Sp1, Sp3, NF-Y, NF-1, c-Myb, B-Myb, AP-1, and E2F proteins in the cell.

1. Introduction

The transcription factor specificity protein 3 (Sp3) belongs to the Sp/X Krüppel-like factor (XKLF) family of nuclear proteins to which Sp1 also belongs. The Sp family of transcription factors is characterized by the presence of three zinc fingers that form their DNA-binding domain [1] and allow them to bind the GC and GT boxes for the regulation of basal/constitutive expression of a diverse range of viral and cellular genes both in normal and cancerous tissues [2]. These motifs are also involved in the maintenance of the methylation-free status of the CpG islands [3,4].

Although the expression pattern, the structure, and the DNA-binding capacity of Sp3 are very similar to Sp1, their physiological role appears to be different since experiments performed with cultured cells and Sp3- and Sp1-deficient mice revealed significant differences between Sp1 and Sp3 [5,6]. Both Sp1 and Sp3 are ubiquitously expressed in mammalian cells and can have additive or synergistic effects on gene activation, but Sp3 is also able to repress transcription driven by Sp1 [7,8], by competing for common target sequences with similar binding affinities [1,8–11].

Sp3, unlike Sp1, is inactive or acts only as a weak activator on many reporter constructs containing multiple Sp-binding sites. The molecular basis for these effects has been attributed to its complex structure: two glutamine-rich activation domains, similar to those found in other Sp family members and, adjacent to these, an inhibitory domain, unique to Sp3. Moreover, Sp3 can be posttranslationally regulated by sumoylation [12,13], acetylation [14,15] and phosphorylation [16]. Furthermore, four different isoforms of Sp3 can be found, that differ in the extent of the amino terminal part and that are all derived from alternative translational start sites [17]. The two long isoforms of Sp3 can act as transcriptional activators on certain promoter settings whereas the two small isoforms appear to be always inactive [17].

Our aim was to study the regulation of the *Sp3* gene itself. In a previous article we described the isolation of 1079 bp from the 5′-flanking region of the human *Sp3* gene (accession number AY251018), mapping multiple initiation sites (between −70 and −132 bp upstream of the translational start). The minimal promoter sequence expanded 225 bp and the proximal promoter 281 bp relative to the translational start site. One Sp1-box was found to bind both Sp1 and Sp3 and overexpression of these factors increased luciferase activity from a construction containing the proximal promoter of Sp3 (pSp3FOR4). In addition, we demonstrated that a CCAAT box present in the promoter was bound by NF-Y complex and that NF-Y was activating the *Sp3* promoter. Furthermore, the proximal promoter contains potential binding sites for NF-1, AP-1, Myb, and E2F (Fig. 1).

In the present study, we performed a more detailed analysis of the *Sp3* promoter to ascertain the transcription factors that would actually bind to the proximal promoter sequence. We also tested the functionality of the binding sites (wild type and mutated versions) within the proximal promoter of *Sp3* using luciferase constructs in

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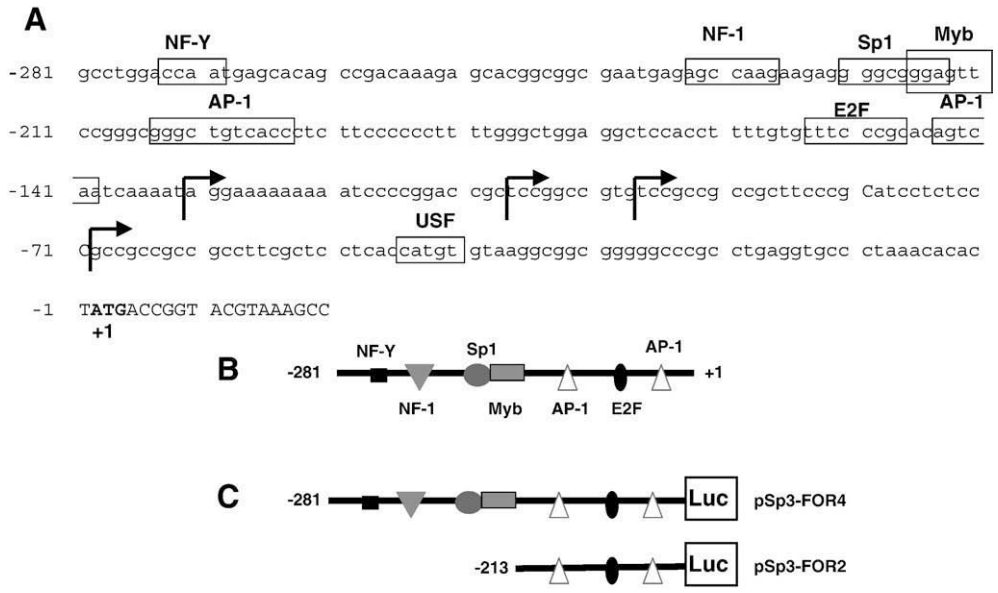

Fig. 1. Structural analysis of the *Sp3* proximal promoter. A) *Sp3* proximal promoter sequence. Putative binding sites for transcription factors are indicated by boxes. Transcriptional start sites are indicated by arrows. The ATG codon that initiates translation is shown in bold. B) Scheme of the *Sp3* proximal promoter. C) Scheme of the pSp3FOR4 and pSp3FOR2 constructs used throughout this study.

cotransfection experiments performed with expression vectors for NF-YA, Sp3, Sp1, NF-1, c-Myb, B-Myb, E2F/DP1 and c-Jun/c-Fos, and luciferase assays conducted after siRNA incubation against NF-1, c-Myb, c-Jun and E2F1. We analyzed the regulation *in situ* of the *Sp3* promoter by chromatin immunoprecipitation using specific antibodies against Sp1, Sp3, NF-Y, NF-1, c-Myb, B-Myb, c-Jun and E2F1. Additionally, we determined the effect of the overexpression or the knock down of these different transcription factors on the mRNA expression of the endogenous *Sp3* gene.

2. Materials and methods

2.1. Cell culture

HeLa human cervical carcinoma, HT29 human colon adenocarcinoma and K562 human erythroleukemia cell lines were grown in Ham's F-12 medium supplemented with 5% fetal bovine serum (Gibco). Cultures were maintained at 37 °C in a humidified 5% CO_2-containing atmosphere.

2.2. Plasmid constructs and siRNA

Constructs pSp3FOR4 and pSp3FOR2, containing different fragments of the *Sp3* promoter, were previously described [18]. A scheme is presented in Fig. 1C.

The pCMV-CTF-IΔUTR (NF-1/CTF) vector was a generous gift from Dr. Nicolas Mermod (Institut de Biotechnologie UNIL-EPFL, Universitée de Lausanne, Lausanne, Switzerland). The expression vectors for Sp1, Sp3 and NF-YA were generously provided by Dr R. Tjian (Department of Molecular and Cell Biology, University of California, Berkeley, CA, U.S.A.), by Dr G. Suske (Molecular Biology Institut, University of Marburg, Germany), and by Dr R. Mantovani (University of Milan, Italy), respectively. c-Myb and B-Myb expression vectors were generous gifts from Dr. G. Raschellà (Ente Nuove Tecnologie Energia Ambiente – ENEA, Rome, Italy). CMV-c-Jun and CMV-c-Fos expression plasmids were generously provided by Dr. Tom Curran (St. Jude Children's research hospital. Memphis. Tennessee). The expression vectors for E2F1 and DP1 were kindly provided by Dr. T. Kouzarides (The Wellcome Trust Cancer Research Institute, Cambridge, U.K.) The expression vector for Sp3 corresponded to the original Sp3 cDNA sequence, a N-terminally truncated version [1], which has been currently used to study Sp3, as stated in Sapetschnig et al [17]. This Sp3 expression construct produces long and short forms of Sp3 as determined by Western blot.

To mutate the different binding sites present in the *Sp3* promoter, the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) was used following the manufacturer's instructions. Briefly, the pSp3FOR4 construct was used as template in a PCR reaction where the two primers bearing the desired mutations hybridized to the same region of the plasmid. Following temperature cycling, the PCR product was treated with DpnI enzyme for 1 h to digest the parental DNA template. The nicked vector DNA bearing the mutation was then transformed into XL1-Blue supercompetent cells. The mutated plasmids were sequenced using the BigDye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems).

The forward primers used for the different constructs were the following (mutations are indicated in bold):

pSp3-NF-Ymut: 5′-GCC TAG CCT GGA CC**C C**TG AGC ACA GCC GAC-3′ to mutate the NF-Y binding site.
pSp3-NF1mut: 5′-CAC GGC GGC GAA TGA GA**T AA**A AGA AGA GGG GCG GGG-3′ to mutate the NF1 binding site.
pSp3-Mybmut: 5′-GGG GCG GGA G**A**T CCG GGC GGG-3′ to mutate the Myb binding site.
pSp3-AP1mut: 5′-CGG GCG GGC TGT **TG**C CCT CTT CCC CC-3′ to mutate the AP1 binding site.
pSp3-E2Fmut: 5′-GCT CCA CCT TTT GTG TTT CC**A T**CA CAG TCA ATC AAA ATA GG-3′ to mutate the E2F binding site.

siRNAs against NF-1, c-Myb, c-Jun and E2F1 were purchased from Santa Cruz. Control siRNAs (Silencer siRNA screening panel) were purchased from Ambion.

2.3. Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared from exponentially growing HeLa, HT29 and K562 cells as described [19]. The probes were constructed by annealing commercially synthesized complementary single-stranded oligodeoxynucleotides corresponding to putative NF-1, Myb, AP-1 or E2F DNA-binding sites (underlined) present in the *Sp3* promoter:

NF-1 probe: 5′-CAC GGC GGC GAA TGA GAG CCA AGA AGA-3′.
Myb probe: 5′-GGG GAG GGA GTT CCG GGC GG-3′.
AP-1 probe: 5′-CGG GCG GGC TGT CAC CCT CTT-3′.
E2F probe: 5′-TTT TGT GTT TCC CGC ACA GTC AAT-3′.

In order to better characterize the binding to the partially overlapped Sp1-Myb boxes, a probe corresponding to a fragment of the *Sp3* promoter from positions −230 to −206 that contains these binding sequences [18] was mutated (mutations are indicated in bold):

Sp1mut probe: 5′-AAG AAG AGG **TT**C GGG AGT TCC GG GC-3′.
Mybmut probe: 5′-AAG AAG AGG GGC GGG AG**A** TCC GG GC-3′.

The ds oligonucleotides were gel-purified, end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (3000 Ci/mmol, Amersham GE Healthcare), and used as probes in the gel-shift experiments. DNA-binding assays were performed as described [20] and visualized using a PhosphorImager with ImageQuant software v 5.2 (Molecular Dynamics).

In the competition experiments, increasing amounts of unlabeled consensus sequences for NF-1 (5′-GTC CCT TGG CAC GTG GCC AAC TGT G-3′), AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′) or E2F (5′-ATT TAA GTT TGC CGC CCT TTC TCA A-3′) were added to the binding reactions with the nuclear extract for 15 min before the addition of the radiolabeled probe which was incubated for an additional period of time of 30 min.

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In the supershift experiments for NF-Y, 1 μg of goat polyclonal antibody against NF-YB (CBF-A, C-20, Santa Cruz) or 1 μg of unspecific IgGs (Sigma) was added to the reaction mixture and incubated on ice for 15 min before the addition of the probe.

2.4. ChIP analysis

Formaldehyde cross-linking and ChIP were performed as described previously [21]. HeLa cells or K562 cells (semiconfluent 100 mm dish) were washed with phosphate-buffered saline and incubated for 10 min with 1% formaldehyde. The reaction was quenched with 0.1 M glycine and cells were sonicated into chromatine fragments showing an average length of 500 to 800 bp. The chromatin solution was precleared by incubation with protein G-Sepharose for 2 h at 4 °C, divided into aliquots, and incubated with the antibodies overnight. Immunoprecipitation was performed with 5 μg of antibodies against Sp1 (PEP 2), Sp3 (D-20), B-Myb (H-115), c-Jun (N), E2F-1 (C-20), NF-YB (C-20), and C-Myb (H-141) (all from Santa Cruz) or unspecific IgGs (Sigma). Immune complexes were precipitated with Protein G-Sepharose (GE Healthcare). Before use, protein G-Sepharose was blocked twice at 4 °C with herring sperm DNA (1 μg/μl) (120–3000 nucleotides length) and BSA (1 μg/μl) for 2 h and overnight. Cross-linking was reversed by heating the sample to 65 °C overnight. DNA was purified, and a specific region from −383 to −154 relative to the translational start site of the *Sp3* promoter was amplified by PCR using the following primers:

Fwd 5′-TAGCCGCTCTCGAAAACCTAC-3′.
Rev 5′-AACACAAAAGGTGGAGCCTCC-3′.

As a negative control, the following two primers were used:

Fwd: 5′-ATGGTTGCCACTGGGGATCT-3′.
Rev: 5′-TGCCAAAGCCTAGGGGAAGA-3′.

These negative control primers flank a region of genomic DNA between the GAPDH gene and the chromosome condensation-related SMC-associated protein (CNAP1) gene.

Amplification was performed by Real Time PCR in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with 1 μl of immunoprecipitated DNA and the primers indicated above using the Fast SYBR Green Master Mix (Applied Biosystems). The reaction was performed following the manufacturer's recommendations. The percent of input was calculated by the standard ΔΔCt method.

2.5. Cotransfections, and luciferase assays

HeLa cells were seeded into 6-well plates the day before transfection at a density of 2×10^5 cells/well in Ham's F12 medium containing 5% fetal bovine serum. The medium was renewed before transfection that was performed using FUGENE™ 6 (Roche Molecular Biochemicals). For each well, 3 μl of FUGENE™ 6 was mixed with 100 μl of serum-free F-12 medium during 5 min and then incubated with the vectors used for 20 min, all at room temperature. In cotransfections, 250 ng of either pSp3-FOR4 or pSp3FOR2 was mixed with the indicated amounts, alone or in combination, of NF-1/CTF, c-Myb, B-Myb, c-Jun, c-Fos, E2F1, DP1, Sp1, Sp3 and/or NF-YA expression vectors before the addition of FUGENE™ 6 in serum-free F-12 medium.

When siRNAs against specific transcription factors were used, HeLa cells were seeded into 6-well plates the day before siRNA treatment at a density of 1×10^5 cells/well in Ham's F12 medium containing 5% fetal bovine serum. siRNA treatment was performed using Metafectene (Biontex). For each well, 2 μl of Metafectene in serum-free F-12 medium and 100 nM of siRNA in 100 μl of serum-free F12 were mixed and incubated for 20 min, all at room temperature. After 24 h, transfection of the pSp3FOR4 construct was performed as described. Results were referred to a control treatment performed using siRNA control 1 from the Silencer siRNA screening panel (Ambion).

Luciferase activity was assayed 30 h after transfection. Cell extracts were prepared by lysing the cells with 200 μl of freshly diluted 1× Reporter Lysis Buffer (Promega). The lysate was centrifuged at 12,000 ×*g* for 2 min to pellet the cell debris. The supernatants were transferred to a fresh tube and the protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's protocol. A 10 μl-aliquot of the extract was added to 25 μl of the luciferase assay substrate (Promega) and the luminescence of the samples were read immediately on a Glomax™ 20/20 Luminometer, in which the light production (relative luminescence units) was measured for 10 s. Each transfection was performed in triplicate.

Transfection efficiency was corrected by β-galactosidase activity upon cotransfection with the pCMV SPORT-βgal vector. A 10 μl-aliquot of the cell extract was mixed with 290 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution (880 μg/ml ONPG, 67 mM Na_3PO_4, 1 mM MgCl2, 45 mM β-mercaptoethanol, pH 7.5). The absorbance of the mixture was determined at 420 nm after 30 min of incubation at 37 °C.

2.6. RT-Real Time PCR

Total RNA was extracted from HeLa cells using the Ultraspec™ RNA reagent (Biotecx) in accordance with the manufacturer's instructions.

Complementary DNA was synthesized in a total volume of 20 μl from RNA samples by mixing 1 μg of total RNA, 125 ng of random hexamers (Roche Molecular Biochemicals), in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 U of RNAsin (Promega), 0.5 mM dNTPs (AppliChem), 200 U of M-MLV reverse transcriptase (Invitrogen) and 50 mM Tris–HCl buffer, pH 8.3. The reaction mixture was incubated at 37 °C for 60 min. The cDNA product was used for subsequent amplification by Real Time PCR.

Sp3 mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 μl of the cDNA mixture and the assays-on-demand Hs01595811_m1 for Sp3 and Hs99999901_s1 for 18S RNA (both from Applied Biosystems). 18S RNA was used as an endogenous control. The reaction was performed following the manufacturer's recommendations. Fold-changes in gene expression were calculated using the standard ΔΔCt method.

2.7. Western blot analysis

Overexpression and knock down of the different transcription factors was performed using the corresponding expression vectors or the specific siRNAs as described. HeLa total extracts were prepared in extraction solution for 1 h at 4 °C (50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% Glicerol (V/V), 1% Triton X-100, Protease inhibitor cocktail). Cell debris was removed by centrifugation (10,000 × *g*, 10 min).

Cells extracts were resolved on SDS-7% or 12% polyacrylamide gels [22], and transferred to a PVDF membrane (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with antibodies against NF1, B-Myb, c-Myb, c-Jun, c-Fos or E2F1 (all from Santa Cruz). Signals were detected by secondary horseradish peroxidase-conjugated antibody (1:2500 dilution) and enhanced chemiluminescence, as recommended by the manufacturer (GE Healthcare).

2.8. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using Student's *t* test and analysis of variance (ANOVA) followed by a Newman multiple comparison test using Prism version 4.00 software for Macintosh (GraphPad Software; San Diego, CA, USA). *p* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Detailed analysis of the Sp3 proximal promoter

In a previous work we described the cloning of the 5′-flanking region of the human *Sp3* gene [18], defined the proximal promoter pSp3FOR4, and demonstrated the binding of Sp1, Sp3 and NF-Y by EMSA to this sequence. Putative binding sites for AP-1, Myb, NF-1 and E2F are also found along this sequence (Fig. 1). In the present study, to ascertain which transcription factors could bind to this sequence, we performed a detailed binding analysis using two approaches: i) gel-shift assays using as probes different regions of the proximal promoter containing the binding sites found by computational analysis (Fig. 2A), and ii) chromatin immunoprecipitation assays, using HeLa or K562 chromatin and antibodies against the transcription factors studied here (Fig. 3).

Four different probes that contained binding sites for NF-1, Myb, AP-1, or E2F were used in binding experiments together with nuclear extracts from HeLa cells.

Using a 27 bp probe spanning from nucleotides −250 to −224, which contains the binding site for NF-1, three major bands could be observed (Fig. 2B). Competition assays using an oligonucleotide containing the consensus sequence for NF-1 [23] only decreased the bottom band, leaving the top bands unaffected, indicating that the bottom band was due to NF-1 binding (Fig. 2B). Since we found by computational analysis that the NF-1 box could also be bound by NF-Y, although with less probability, we performed supershift analysis using an antibody against NF-YB. The two upper bands in Fig. 2C were supershifted, corresponding to binding of the probe by NF-Y. This effect was not observed when unspecific IgGs were used in the supershift assay. Thus, the upper bands observed in gel shifts using the NF-1 probe corresponded to NF-Y binding and were not competed by the addition of the NF-1 consensus sequence. NF-Y probably binds with less affinity to the NF-1 consensus than to the binding site for this transcription factor present in the *Sp3* promoter.

In the case of the probe containing the Myb binding site (20 bp), aside from nuclear extracts from HeLa cells, we also used extracts from K562 and HT29 cells as positive controls; the K562 human erythroleukemia cell line because c-Myb expression is predominant in hematopoietic systems, and the HT29 human colon carcinoma cell line which

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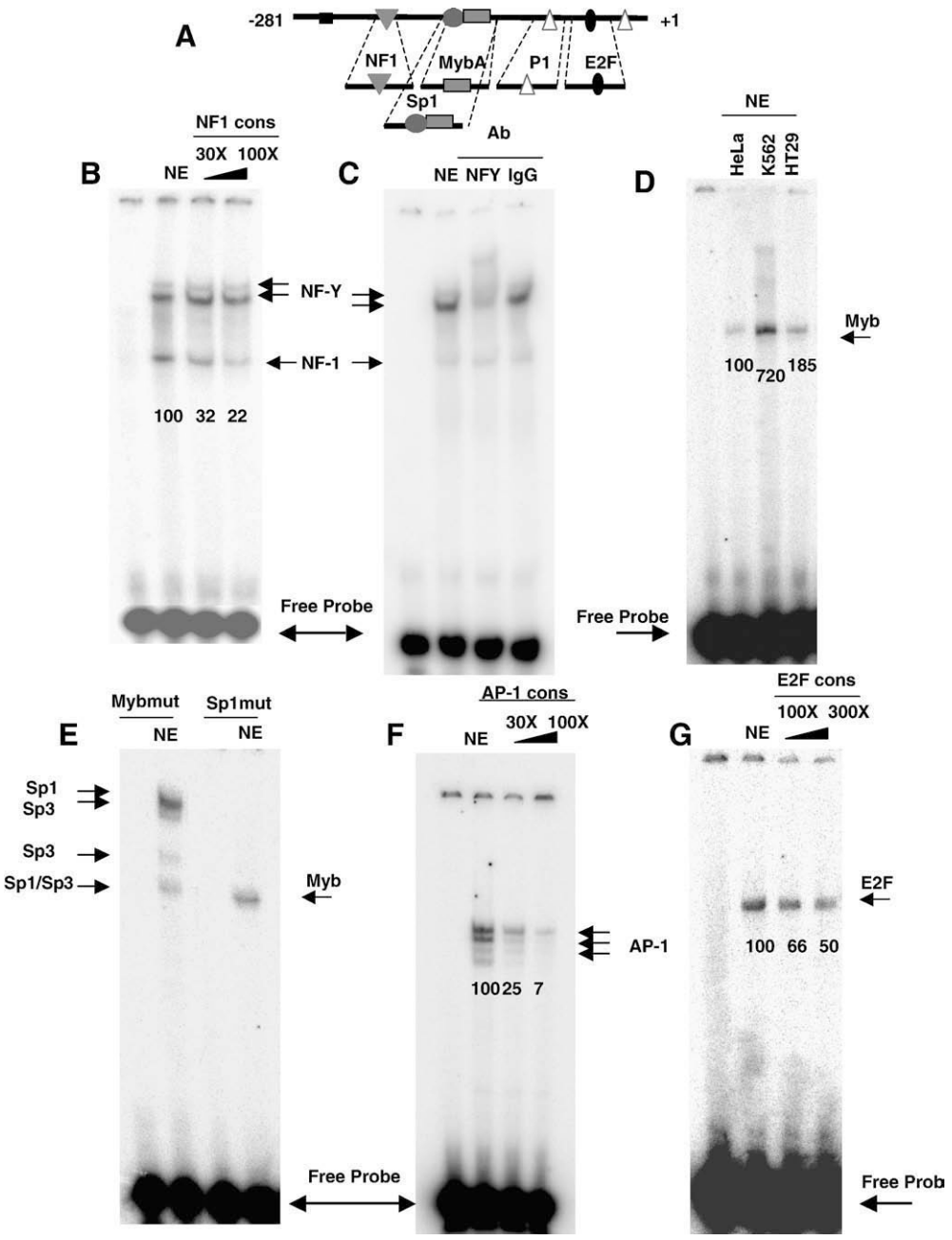

Fig. 2. Binding analysis of the *Sp3* proximal promoter. A) A scheme of the probes used is shown. B–G) Binding reactions were performed with 20,000 cpm of ds probe (B and C, NF-1 probe; D, Myb probe; E, Mybmut and Sp1mut probes; F, AP-1 probe; G, E2F probe), 2 μg of nuclear extracts (NE) from exponentially growing HeLa cells (and K562 and HT29 cells in panel D) and 1 μg of hearring sperm as the nonspecific competitor. Competition assays were performed in the presence of an excess of cold probe containing the respective consensus (cons) binding site. Quantification was performed by phosphorimaging and referred to the binding obtained in the absence of competing DNA. The numbers underneath the bands represent the percentage of remaining binding referred to the control. In panel C supershift was performed by the addition of a specific antibody against NF-YB or unspecific IgGs as a negative control. Bands corresponding to the binding of transcription factors to DNA are indicated by arrows.

overexpressed this factor. As it can be observed in Fig. 2D, a major band is formed in all three cell lines and the binding of this band is increased more than 7-fold in K562 cells compared to HeLa cells. Furthermore, minor bands can also be observed in the K562 cell line probably due to the existence of an alternative translational start for c-Myb [24].

The probe used in these experiments corresponded to the partially overlapping Sp1 and Myb DNA-binding sites present in the *Sp3* promoter. However, only Myb proteins are able to bind to the DNA in these conditions due to the absence of the necessary flanking sequences of the Sp1 box. Starting with a wild type probe corresponding to the *Sp3* promoter sequence from −230 to −206 [18], probes bearing a mutation either on the Sp1 or the Myb binding sites were generated and used in gel-shift assays. The binding pattern of the wild type probe corresponded to Sp1 and Sp3 as previously described [18]. As shown in Fig. 2E, mutation of the Myb site did not cause any effect on the binding pattern of Sp proteins whereas mutation of the Sp1 site abolished Sp1 and Sp3 binding and revealed a new band, with the same mobility as the shifted band observed with the Myb probe used in Fig. 2D. Altogether these experiments demonstrated that Myb binds to the *Sp3* proximal promoter *in vitro* only when Sp1 binding is impaired.

The binding pattern with the 21 bp probe containing the AP-1 box, four bands representing different combinations of members from the AP-1 family, can be observed in Fig. 2F. To confirm the specificity of this binding, we performed competition experiments using increasing amounts of a cold probe containing the consensus sequence for AP-1

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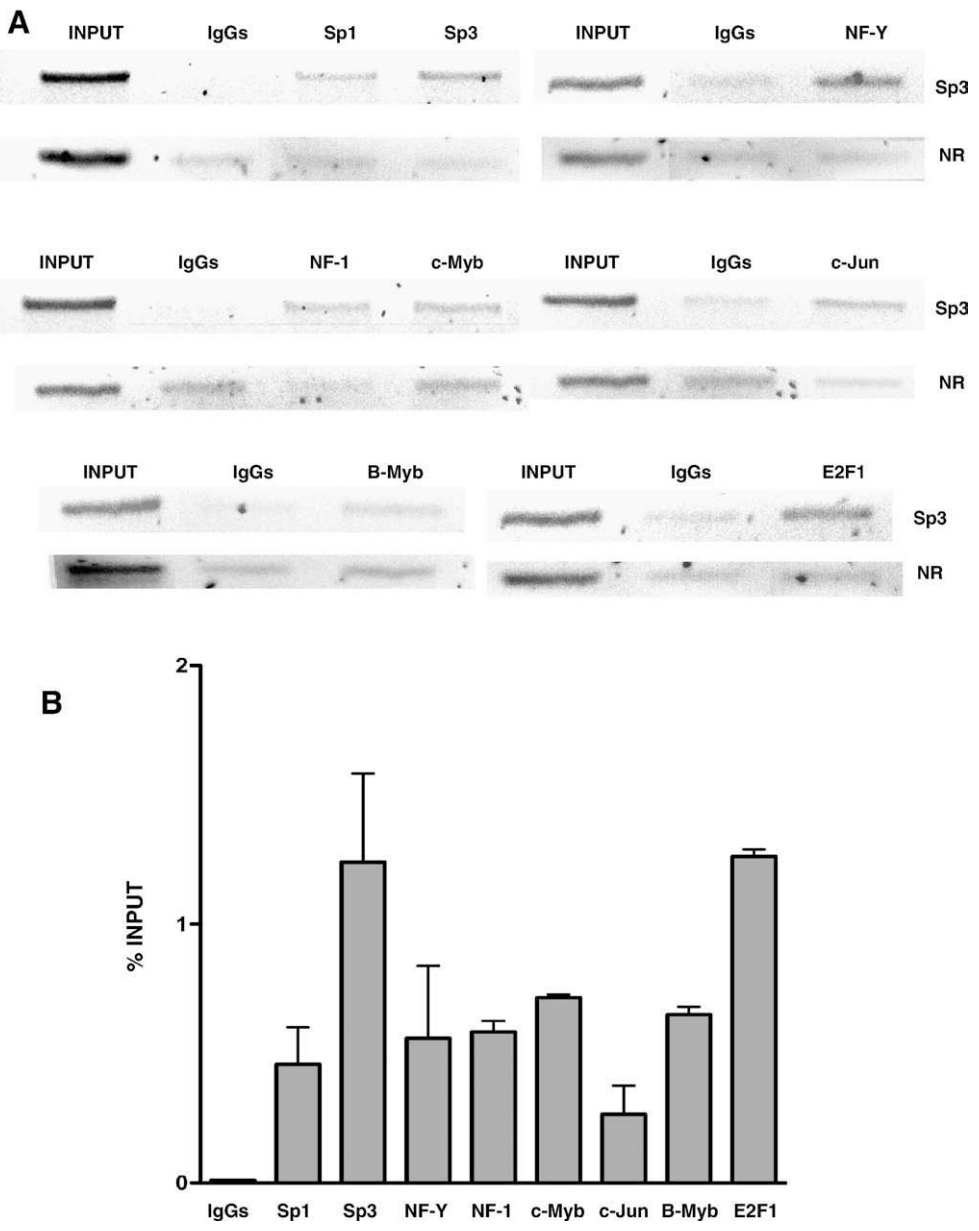

Fig. 3. ChIP analysis of the *Sp3* promoter. ChIP assays for the Sp3 promoter region were performed using HeLa cells for Sp1, Sp3, NF-Y, NF-1, B-myb, c-Jun and E2F1 immunoprecipitation, or K562 cells for C-myb immunoprecipitation. DNA bound to the selected proteins was immunoprecipitated using specific antibodies and amplified by PCR. Rabbit IgG was used as negative control for the immunoprecipitation. A) Representative images of the PCR products corresponding to amplification with specific Sp3 primers (Sp3) and negative control primers flanking a region of genomic DNA (NR) for each antibody are shown. B) Quantification of ChIP analysis was performed by Real Time PCR and results are expressed as percent of the input, taking into account that ΔCt (Sp3)=Ct Sp3 promoter−Ct genomic DNA, ΔΔCt=ΔCt (Sp3)−ΔCt (IgG) and then referred to the input, considering the dilution factor.

(Promega). The four bands observed using HeLa nuclear extracts were all partially competed when using a 100-fold excess of the AP-1 consensus (Fig. 2F). No AP-1 binding was found when using a probe containing the most downstream AP-1 of the *Sp3* promoter in the gel-shift assays (data not shown).

Using a 24 bp fragment containing the E2F binding site present in the ` promoter, one band could be observed. To confirm the identity of this band, increasing amounts of cold probe containing the E2F consensus sequence (Santa Cruz) were used in competition experiments. Addition of the E2F consensus decreased E2F binding (Fig. 2G),

A prerequisite for the activity of a transcription factor on a putative target gene is the finding that it is bound to relevant sequences *in vivo*. We performed the chromatin immunoprecipitation technique (ChIP) to determine whether this was the case for the *Sp3* gene promoter and Sp1, Sp3, NF-YB, E2F1, B-Myb c-Jun, NF1 and c-Myb transcription factors. As can be seen in Fig. 3A, using specific antibodies, a fragment of the *Sp3* promoter was amplified by Real Time PCR in all cases demonstrating the occupancy of the binding sites *in vivo* by the corresponding transcription factors. When unrelated control primers were used, no enrichment referred to the unspecific IgGs was observed, demonstrating the specificity of the amplification of the *Sp3* promoter (Fig. 3A). Results expressed as the percent of input for each transcription factor are shown in Fig. 3B.

3.2. Regulation of Sp3 transcriptional activity by NF-1

Since NF-1 is able to bind the *Sp3* proximal promoter, we examined its role in the regulation of the *Sp3* 5′-flank. To this end, transient cotransfections were performed in HeLa cells using the luciferase construct pSp3FOR4, together with increasing amounts of an expression vector for the NF-1 transcription factor (pCMV-CTF-IΔUTR). The *Sp3* promoter was activated by NF-1 overexpression in a dose-

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dependent manner. The knock down of NF-1 with 100 nM of a specific siRNA (siNF-1) decreased pSp3FOR4 luciferase activity down to 50% referred to the control siRNA (Fig. 4B), thus demonstrating the activating effect of NF-1 on *Sp3* transcription. Overexpression of NF-1 upon transfection with pCMV-CTF-IΔUTR and the decrease in NF-1 protein levels upon incubation with siNF-1, analyzed by Western blot, are shown in Fig. 4A. Incubation with a control siRNA did not cause a significant change in NF-1 protein levels (Fig. 4A). Mutation of the NF-1 binding site in the *Sp3* promoter led to a 2-fold increase in luciferase activity, probably due to a better accessibility of Sp1 to its recognition sequence, and overexpression of NF-1 further increased this activity (Fig. 4C). Altogether these results suggest that the effect of NF-1 on *Sp3* transcription is due to the interaction of NF-1 with other transcription factors that regulate the *Sp3* promoter in addition to its direct binding to specific sequences within the promoter.

To examine the relationship between NF-1 and NF-Y, which can be bound to the same DNA region, transient cotransfections in HeLa cells were performed with pSp3-FOR4 together with NF-1 and NF-YA, either alone or in combination. Cotransfection of pSp3-FOR4 with both NF-YA and NF-1 led to an additive increase in promoter activity compared to the effect produced by NF-1 or NF-YA alone (Fig. 4D).

NF-1 has been described to counteract Sp1 transcriptional activation, so we next cotransfected pSp3FOR4 with expression vectors for NF-1, Sp1 or Sp3 alone or in combination to evaluate their effect on *Sp3* expression. We observed that overexpression of NF-1 combined with Sp3 had an additive effect on the transcriptional activation of the *Sp3* proximal promoter. Moreover, when cotransfection of NF-1 was performed together with Sp1, the effect on *Sp3* transcription was synergistic. Therefore, there is no antagonism between NF-1 and Sp1 or Sp3 on the transcriptional activity of *Sp3* promoter (Fig. 4E).

These results suggest that although NF-1 does not have much effect on *Sp3* transcription by itself, it contributes, when combined with other proteins, to the global transcriptional control of the *Sp3* promoter.

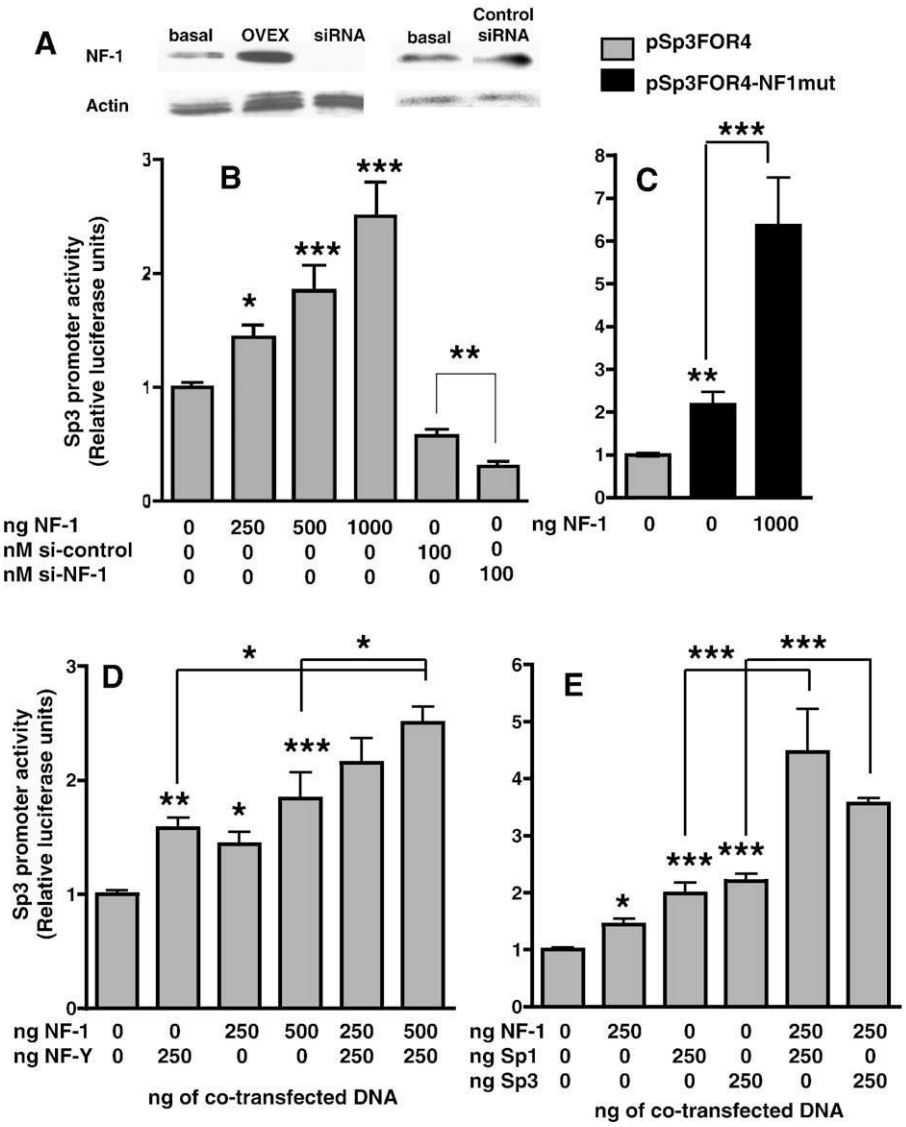

Fig. 4. Effect of NF-1, NF-Y, Sp1 and Sp3 on *Sp3* promoter activity. A) Western blot of the basal protein levels of NF-1 in HeLa cells, after overexpression (OVEX) of 250 ng of expression vector, or incubation with a specific siRNA or a control siRNA. B) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 and the indicated amounts of an expression vector encoding NF-1 or a siRNA against NF1. After 30 h of culture, cell lysates were assayed for luciferase activity. Luciferase activity was normalized to the β-galactosidase activity for each sample. Results are expressed relative to the activity obtained upon transfection with the pSp3-FOR4 construct alone. C) HeLa cells were cotransfected with 250 ng of construct pSp3-NF1mut together with the indicated amounts of expression vectors for NF-1. Other conditions are as in B. D) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 together with the indicated amounts of expression vectors for NF-1 and NF-YA. Other conditions are as in B. E) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 together with the indicated amounts of expression vectors for NF-1, Sp1 and Sp3. Other conditions are as in B. Results represent the mean < 0.05, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the pSp3-FOR4 construct alone.

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3.3.. Synergistic transcriptional activation of Sp3 produced by Myb and Sp proteins

The Myb binding site present in the *Sp3* promoter, between positions −218 and −210, overlaps the GC-box where Sp1 and Sp3 can bind [18] (Fig. 1). Since we previously reported the role of Sp1 and Sp3 on *Sp3* regulation [18], in this study we examined the role of the Myb proteins combined with Sp1 and Sp3 on *Sp3* regulation. In this regard, transient cotransfections were performed in HeLa cells using the luciferase construct pSp3-FOR4, together with increasing amounts of expression vectors for c-Myb and B-Myb, alone or in combination, or in the presence of a siRNA against c-Myb. As shown in Fig. 5B, 250 ng of c-Myb and B-Myb expression vectors increased the promoter activity and the combination of the two plasmids increased the activity in an additive way to 2.5-fold. The overexpression of B-myb and c-Myb upon transfection with the corresponding expression vectors was confirmed by Western blot (Fig. 5A). Treatment of cells with c-Myb siRNA decreased pSp3FOR4 luciferase activity up to 50% the activity determined with a control siRNA (Fig. 5B), in accordance with the decrease in the levels of c-Myb protein, whereas the incubation with a control siRNA did not cause a significant change in c-Myb protein levels (Fig. 5A). However, mutation of the Myb binding site in pSp3FOR4 increased luciferase activity up to 2-fold as compared to the wild type promoter. Given that the Myb binding site in the *Sp3* promoter corresponded in fact to a partially overlapped Myb-Sp1 box, mutation of the Myb site could favor Sp1 and Sp3 binding to their recognition sequence and hence the activation of transcription. Overexpression of c-Myb in combination with the

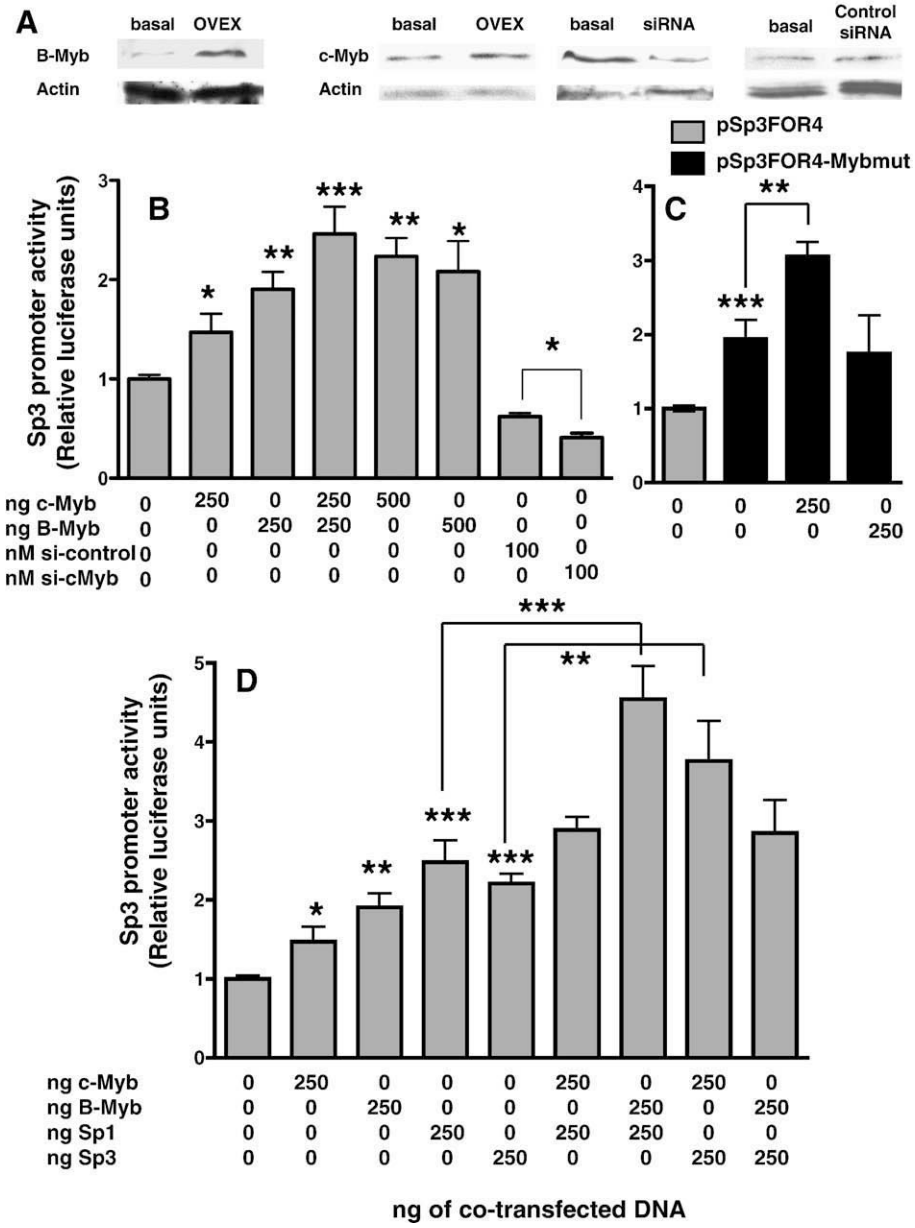

Fig. 5. Effect of c-Myb, B-Myb, Sp1 and Sp3 on *Sp3* promoter activity. A) Western blot of B-Myb basal protein levels in HeLa cells and after overexpression (250 ng of expression vector), and of c-Myb basal protein levels, after overexpression (250 ng of expression vector), or incubation with a specific siRNA or a control siRNA. B) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 and the indicated amounts of expression vectors encoding c-Myb and B-Myb or a siRNA against c-Myb. After 30 h of culture, cell lysates were assayed for luciferase activity. Luciferase activity was normalized to the β-galactosidase activity for each sample. Results are expressed relative to the activity obtained upon transfection with the pSp3-FOR4 construct alone. C) HeLa cells were cotransfected with 250 ng of construct pSp3-Mybmut together with the indicated amounts of expression vectors for c-Myb and B-myb. Other conditions are as in B. D) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 together with the indicated amounts of expression vectors for c-Myb, B-Myb, Sp1 and Sp3. Other conditions are as in B. Results represent the mean<0.05, $^{**}p<0.01$ and $^{***}p<0.001$ compared to the pSp3-FOR4 construct alone.

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mutated promoter increased luciferase activity up to 3-fold whereas the change in luciferase activity upon B-myb overexpression was not significant (Fig. 5C). Altogether these results suggest that the effect of Myb on *Sp3* transcription is mainly due to the interaction of Myb proteins with other transcription factors that regulate the *Sp3* promoter and not to their direct binding to specific sequences within the promoter. In this direction, cotransfection of the luciferase reporter plasmid pSp3-FOR4 plus the expression vectors c-Myb or B-Myb together with the expression vectors for Sp1 or Sp3 led to synergistic or additive effects on transcriptional activation. Specifically, the combination of Sp1 with B-Myb, and of Sp3 with c-Myb caused a modest synergistic increase on the transcriptional activation of the *Sp3* proximal promoter, whereas the combination of Sp1 with c-Myb, and of Sp3 with B-Myb had an additive effect (Fig. 5B). Thus B-Myb and c-Myb proto-oncogene products, which are DNA-binding proteins and transcriptional transactivators, can cooperate with Sp1 and Sp3 in activating the *Sp3* promoter in HeLa cells.

3.4. Regulation of Sp3 transcriptional activity by c-Jun and c-Fos proteins

To determine the role of AP-1 proteins on *Sp3* transcriptional regulation, transient cotransfections in HeLa cells were performed with either 250 ng of the luciferase construct pSp3FOR4, bearing the proximal promoter of *Sp3*, or pSp3FOR2, which contains 213 bp of the *Sp3* promoter (Fig. 1). Each of these basal vectors was cotransfected together with increasing amounts of expression vectors for c-Jun and c-Fos proteins, alone or in combination. As shown in Fig. 6B, cotransfection with c-Jun or c-Fos increased the promoter activity by 2-fold. The pSp3FOR2 vector that contains only the AP-1 and E2F boxes present in the proximal promoter was also activated by c-Jun

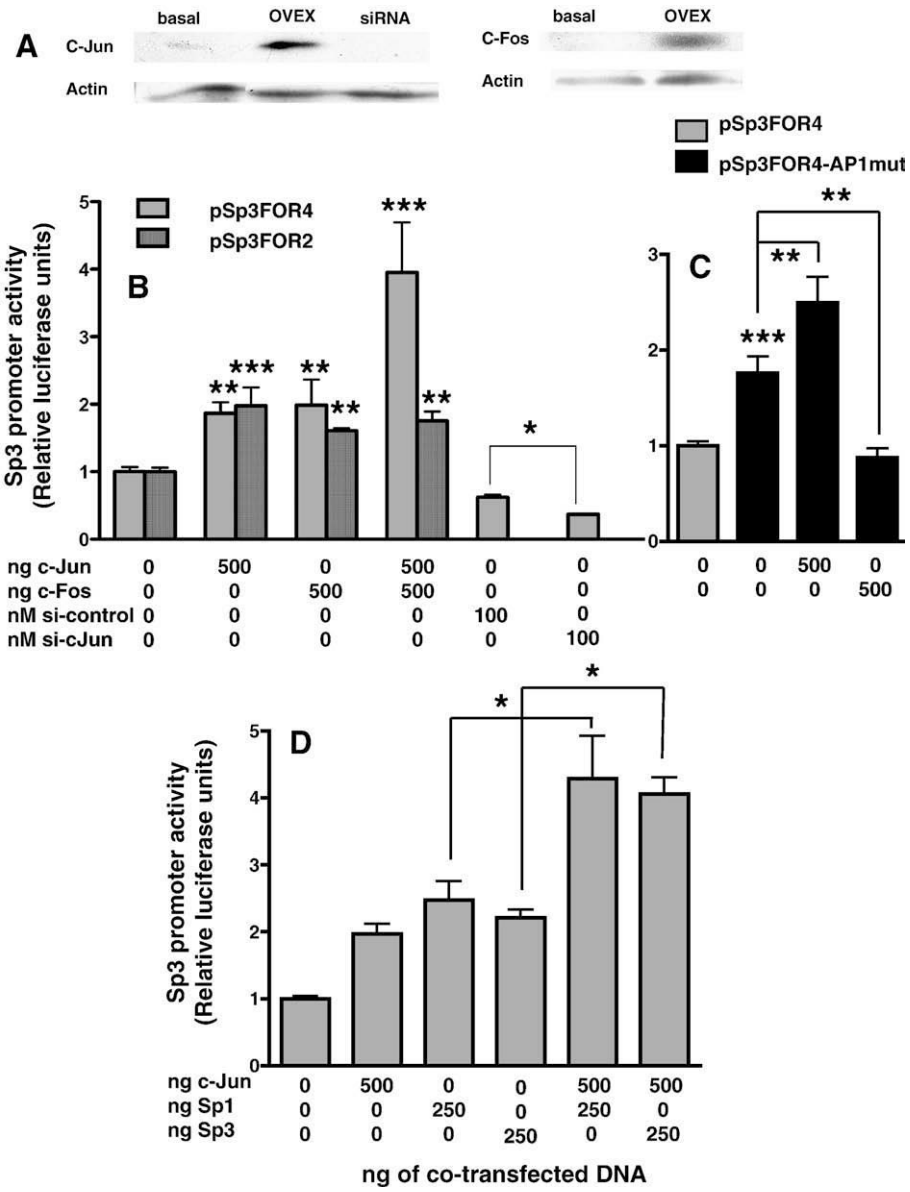

Fig. 6. Effect of Jun/Fos, Sp1 and Sp3 on *Sp3* promoter activity. A) Western blot of the basal protein levels of c-Jun and c-Fos in HeLa cells after overexpression (500 ng of each expression vector) and c-Jun protein levels after siRNA incubation. B) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 or pSp3FOR2 and the indicated amounts of expression vectors encoding Jun and Fos proteins or a siRNA against c-Jun. After 30 h of culture, cell lysates were assayed for luciferase activity. Luciferase activity was normalized to the β-galactosidase activity for each sample. Results are expressed relative to the activity obtained upon transfection with the pSp3-FOR4 or pSp3-FOR2 constructs alone. C) HeLa cells were cotransfected with 250 ng of construct pSp3-AP1mut together with the indicated amounts of expression vectors for c-Jun and c-Fos. Other conditions are as in B. D) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 together with the indicated amounts of expression vectors for Jun, Sp1 and/or Sp3. Other conditions are as in B. Results represent the mean <0.05, $^{**}p<0.01$ and $^{***}p<0.001$ compared to the corresponding control pSp3-FOR4 or pSp3FOR2 alone.

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and c-Fos (Fig. 6B). The overexpression of c-Jun and c-Fos upon transfection with the corresponding expression vectors was confirmed by Western blot (Fig. 6A) Luciferase activity of pSp3FOR4 was also assayed after cell incubation with a siRNA against c-Jun (si-c-Jun). In these conditions, luciferase activity was reduced by 40%, in keeping with the decrease in its protein level upon incubation with si-c-Jun (Fig. 6A) and demonstrating the activator effect of c-Jun on the *Sp3* promoter. Mutation of the distal AP1 binding site in pSp3FOR4 increased luciferase activity less than 2-fold, as it was the case for NF-1 and Myb, overexpression of c-Jun further increased this activity, whereas c-Fos overexpression did not affect luciferase activity of the mutant construct (Fig. 6C).

Given that c-Jun interacts with Sp1 [34–36] and that this interaction seems to be necessary for the activity of certain promoters, we performed cotransfection experiments with pSp3FOR4 using expression vectors for c-Jun and Sp1 or Sp3. As shown in Fig. 6D, the effects of Sp1 and Sp3 on the activation of the *Sp3* promoter were increased in an additive way by the presence of c-Jun.

3.5. Inhibition of the Sp3 promoter by E2F

To study the effect of E2F on *Sp3* promoter activity, transient cotransfections in HeLa cells were performed with 250 ng of the luciferase construct pSp3FOR4, which contains one binding site for E2F, together with increasing amounts of expression vectors for E2F and DP1. The combination of E2F and DP1 expression vectors inhibited the *Sp3* proximal promoter (pSp3FOR4) in a dose-dependent manner down to 50% when 1 μg of E2F and 1 μg of DP1 were combined with the pSP3FOR4 construct (Fig. 7B). Incubation of HeLa cells with 100 nM of a siRNA against E2F1 before pSp3FOR4 transfection led to a significant increase in luciferase activity (Fig. 7B). Overexpression of E2F upon transfection with the corresponding expression vector and

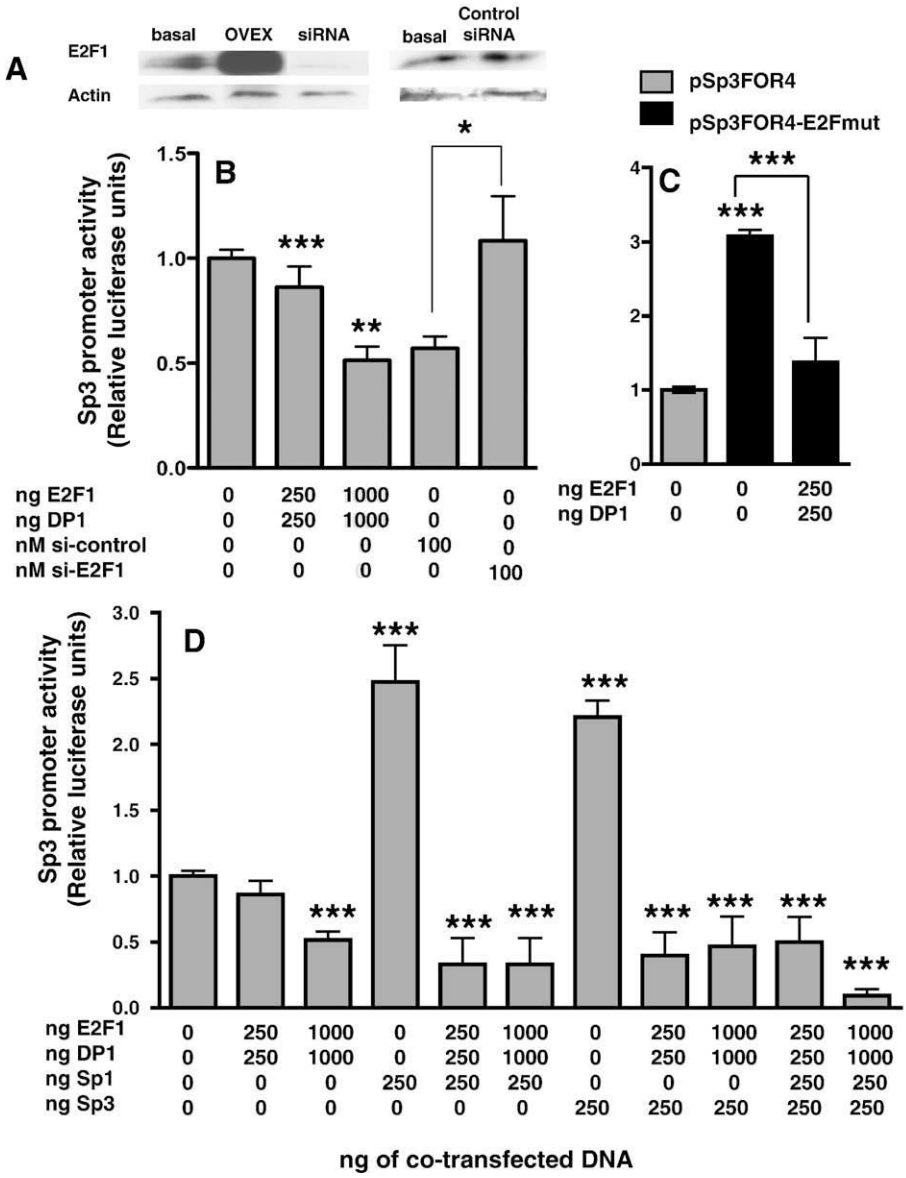

Fig. 7. Effect of E2F, DP1, Sp1 and Sp3 on *Sp3* promoter activity. A) Western blot of the basal protein levels of E2F1 in HeLa cells, after overexpression (250 ng of expression vector), or incubation with a specific siRNA or a control siRNA. B) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 and the indicated amounts of expression vectors encoding E2F and DP1 proteins or a siRNA against E2F1. After 24 h of culture, cell lysates were assayed for luciferase activity. Luciferase activity was normalized to the β-galactosidase activity for each sample. Results are expressed relative to the activity obtained upon transfection with the pSp3-FOR4 construct alone. C) HeLa cells were cotransfected with 250 ng of construct pSp3-E2Fmut together with the indicated amounts of expression vectors for E2F1 and DP1. Other conditions are as in B. D) HeLa cells were cotransfected with 250 ng of construct pSp3-FOR4 together with the indicated amounts of expression vectors for E2F, DP1, Sp1 and/or Sp3. Other conditions are as in B. Results represent the mean<0.05, $^{**}p<0.01$ and $^{***}p<0.001$ compared to the pSp3-FOR4 construct alone.

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the decrease in its protein level upon incubation with the specific siRNA was confirmed by Western blot (Fig. 7A). Incubation with a control siRNA did not cause a significant change in E2F protein levels (Fig. 7A). Mutation of the E2F binding site in pSp3FOR4 increased luciferase activity up to 3-fold as compared to the wild type promoter. However, overexpression of E2F1/DP1 in combination with the mutated promoter decreased luciferase activity down to 50% (Fig. 7C). These results suggest that the effect of E2F on *Sp3* transcription is due to the interaction of E2F with other transcription factors that regulate the *Sp3* promoter in addition to its direct binding to the DNA. Given that E2F interacts with Sp1 [25–27] and that E2F-DP1 behaved as an inhibitor for the *Sp3* promoter, we performed cotransfection experiments with pSp3FOR4 using expression vectors for E2F and DP1 in combination with Sp1 and/or Sp3. The effect of Sp1 and Sp3 on the activation of the *Sp3* promoter was reverted by the presence of E2F-DP1 even well below the basal level (Fig. 7D).

3.5. Regulation of the endogenous Sp3 gene promoter

To study the effect of the different transcription factors on the endogenous levels of Sp3, we performed RT-Real Time PCR to quantify Sp3 mRNA levels in HeLa cells after overexpressing or knocking down these transcription factors.

Overexpression of NF-YA, B-Myb, c-Jun and c-Fos increased Sp3 mRNA levels up to 6-fold, whereas overexpression of Sp1, NF-1 and c-Myb increased Sp3 mRNA levels by 3-fold. Overexpression of E2F1/DP1 decreased Sp3 mRNA levels down to 40% of the control (Fig. 8A). All changes were significant and are in keeping with the changes in the transcriptional activity of the *Sp3* promoter measured in the luciferase assays (Figs. 4–7). Knock down of Sp1, NF-Y, NF1, and c-Myb led to a modest reduction in Sp3 mRNA levels, although significant, whereas in the presence of a siRNA against E2F Sp3 mRNA levels were increased compared to the control. Knock down of Sp3 led to a significant reduction in Sp3 mRNA levels (Fig. 8B)

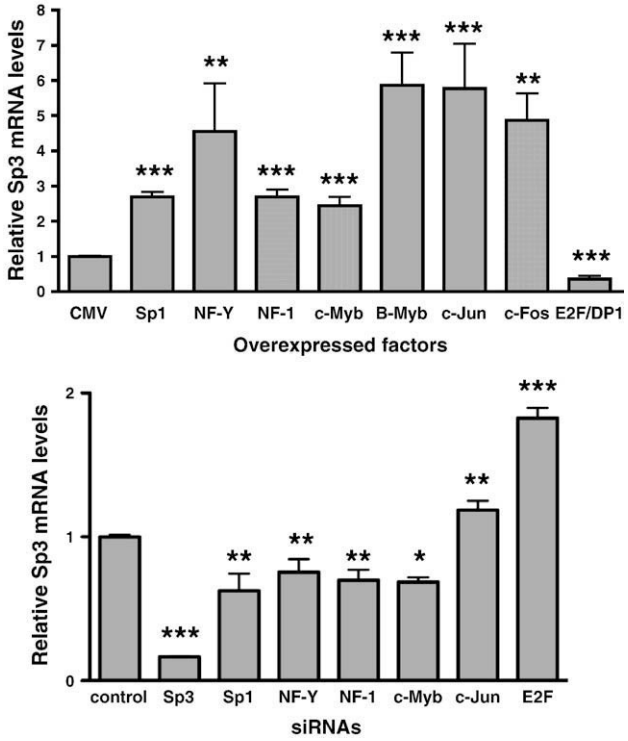

Fig. 8. Effect of Sp1, Sp3, NF-Y, c-Myb, B-Myb, c-Jun, c-Fos and E2F/DP1 on Sp3 endogenous levels. A) HeLa cells were transfected with 5 μg of the indicated expression vectors or 2.5 μg of each E2F and DP1 expression vectors using 6 μl of FUGENE™ 6. After 72 h RNA was extracted and RT-Real Time PCR was performed as described. Results are expressedc relative to the Sp3 mRNA levels obtained upon transfection with the empty vector pCMV. B) HeLa cells were incubated with 100 nM of the indicated siRNAs using Metafectene. After 72 h RNA was extracted and RT-Real Time PCR was performed as described. Results are expressed relative to the Sp3 mRNA levels obtained upon incubation with a control siRNA. Results represent the mean<0.05, $^{**}p<0.01$ and $^{***}p<0.001$ compared to the control.

4. Discussion

Recently, we cloned the 5′-flanking region of the human transcription factor *Sp3* gene [18]. In the present study we further characterize the upstream region of the proximal *Sp3* promoter contained into the pSp3FOR4 construct by means of two strategies: *in vitro*, using gel-shift assays and promoter-luciferase reporter constructs, and *in vivo*, using chromatin immunoprecipitation and determination of the expression of the endogenous *Sp3* gene. The combination of these two strategies allowed us to determine that the putative binding sites for NF-1, Myb, AP-1 and E2F present in the *Sp3* promoter sequence are functional and that Sp3 transcription is regulated by the direct binding of these transcription factors to the DNA as well as through different protein interactions, suggesting the existence of transcriptional complexes.

The NF-1 family of nuclear regulatory factors, can activate [28–30] or repress [31–34] transcription of different genes by binding to DNA as dimers to the symmetric consensus sequence TTGGC(N5)GCCA on a duplex DNA [35–38]. In this study, we show that the NF-1 box present in the *Sp3* promoter is not only bound by NF-1, but also by NF-Y, and that coexpression of NF-1 and NF-Y increases *Sp3* promoter activity in an additive way. NF-1 multiplicity of functions is achieved through the interaction with other nuclear factors, the local chromatin architecture and/or the activation state of intracellular signaling pathways. In this direction, it has been described that NF-1 down-regulates the activation mediated by Sp1 of some genes such as the rat poly-(ADP-ribose)polymerase and the DPGFA-chain [39,40] and that Sp1 down-regulates the activation mediated by NF-1 of the collagen1 promoter [41]. Interestingly, in our studies, NF-1/CTF and Sp1 activate the *Sp3* promoter construct in a synergic way.

Our results indicated that the *Sp3* promoter is regulated by c-Myb, a transcription factor that regulates proliferation, differentiation and apoptosis of immature lymphoid and myeloid cells [23] and plays a critical role in T-cell development and function [24]. c-Myb and B-Myb overexpression increases *Sp3* promoter activity and the endogenous levels of Sp3 mRNA, and the combination of these factors with Sp1 and Sp3 demonstrates a cooperation among all these nuclear proteins to transactivate the *Sp3* promoter. Combination of Sp1 and B-Myb has a synergistic effect on the *Sp3* promoter, possibly because of the ability of B-Myb to act through a binding-independent mechanism in cooperation with the Sp1 factor [42]. In this direction, it is worth mentioning that Myb proteins do not bind to the endogenous *Sp3* promoter unless the Sp1 box is mutated, in which case Myb binds to the overlapped Sp1-Myb recognition site.

Only the distal AP-1 box of the two sequences found in the proximal promoter by computational analysis is actually bound by AP-1 proteins. AP-1 (activating protein 1) is a collective term referring to transcription factors composed of Jun, Fos or ATF (activating transcription factor) subfamilies that bind as dimers to a common DNA site called AP-1 site [43,44]. c-Jun is the most potent transcriptional activator in its group [45] and exists as homo- and heterodimers. However, the Fos proteins, which cannot homodimerize, form stable heterodimers with Jun proteins and thereby enhance their DNA-binding activity [44]. c-Jun and c-Fos overexpression produced the same increase on the pSP3FOR4 luciferase activity; and their combination had an additive effect, demonstrating that the *Sp3* promoter is regulated by AP-1 through Jun/Fos dimers although the contribution of Jun/Jun and Jun/ATF-2 dimers cannot be excluded. Even though this AP-1 box is located in a region where low basal promoter activity remains [18], c-Jun and c-Fos are able to transactivate the *Sp3*

promoter and to increase Sp3 mRNA levels suggesting that this functional AP-1 box might regulate transcription under certain circumstances. According to this hypothesis, AP-1 family members are induced by a wide variety of signals, including, but not limited to, UV and ionizing radiation, oxidative stress, neuronal depolarization, cytokines (tumor necrosis factor alpha, gamma interferon, and interleukin 1), and viral infection [46–53]. In the case of oxidative stress, it is interesting to note that Sp3 is induced significantly in cortical neurons to enhance survival [54]. Moreover, c-Jun has been reported to interact with Sp family members and enhance their transcriptional activity on promoters such as p21 and Smad6 [55,56]. In agreement with these results, here we show that coexpression of c-Jun with Sp1 and Sp3 in HeLa cells further enhances transcriptional activity in an additive way using the pSp3FOR4 construct.

One important finding of this study is that E2F-1/DP1 represses transcription of the human *Sp3* gene and decreases Sp3 mRNA levels. Other studies have also described inhibitory effects for E2F1 [57,58]. Active E2F transcription factor is a heterodimer that contains one member of the E2F family (E2F1-6) and one member of the DP family (DP1 or DP2) [59]. E2F transcription factor can act as a transcriptional activator or repressor, depending on the promoter context. Classically, E2F is considered a transcriptional activator, although, it has also been described that binding of a pocket protein (pRB, p107 or p130) to E2F inhibits its transcriptional activity or converts this factor in an active repressor through recruitment of histone deacetylase and methyl transferase activity. In our experiments, overexpression of E2F1 caused the suppression of Sp-mediated transcriptional activation of the *Sp3* promoter, produced by either Sp1 or Sp3 proteins. This repression could be as a result of the formation of a specific transcriptional complex between E2F and Sp1 and/or Sp3, given that these proteins can physically interact with each other [27], and taking into account that the relative positions of the Sp and E2F binding sites in a promoter could affect their effect on transcription [60–62]. The final complex between E2F and Sp proteins could finally interfere with transcription of the *Sp3* gene.

In summary, our data offer insight into the complex mechanism of Sp3 regulation that involves the existence of protein complexes that include several ubiquitous factors like Sp1, Sp3, NF-Y, NF-1, B-Myb and E2F, as well as inducible factors like Jun and Fos and tissue-specific factors like C-Myb.

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3. ARTICLE III:

Sp1 is regulated by Cell Cycle related proteins.

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Estudis previs al grup van demostrar que el promotor de *Sp1* era regulat positivament pel seu propi producte Sp1. En aquest treball ens vam proposar estudiar en més profunditat la regulació del promotor de *Sp1,* tenint en compte la possible regulació per proteïnes que interaccionessin amb la proteïna Sp1 i poguessin estar modulant la seva activitat. Les conclusions obtingudes van ser les següents:

- Mitjançant un cribatge amb un Array d'Anticossos i la posterior validació dels resultats vam demostrar que les proteïnes CDK4, p21, Rad51, BRCA2 i SKP2 podien interaccionar amb la proteïna Sp1.
- Les proteïnes CDK4, p21, Rad51, BRCA2 i SKP2 eren reclutades al promotor de Sp1 tal com van demostrar els assajos de ChIP, i activaven el promotor de Sp1 en assajos luciferasa.
- La sobreexpressió de E2F1/DP1, Rb, Stat3, NFκB (p65) i p53 sobre el promotor de Sp1 també es va analitzar, donat que existeixen referències a la literatura que descriuen la seva interacció amb Sp1. E2F1/DP1, Rb i Stat3 eren activadors del promotor de Sp1, mentre que NFκB i p53 eren repressors d'aquest promotor.
- Les proteïnes p21, SKP2, E2F1/DP1, Rb, Stat3, NFκB i p53 modulaven l'activitat d'un promotor que contenia només caixes Sp1, suggerint la possibilitat de que els efectes observats puguin constituir un mecanisme general de control de la transcripció dependent de Sp1.
- La interacció Sp1-p21 es va analitzar en cèl·lules que contenien p21 induïble per IPTG. L'expressió de p21 era capaç d'activar el promotor de Sp1 així com augmentar els seus nivells de mRNA i l'activitat del promotor dependent de Sp1. Al mateix temps, l'expressió de p21 va produir una disminució dels nivells de proteïna Sp1 mitjançada per un augment de la seva degradació.

En resum, aquest treball descriu la interacció de Sp1 amb les proteïnes CDK4, p21, SKP2, Rad51 i BRCA2 i l'efecte que té aquesta interacció sobre l'expressió del gen *Sp1*. A més s'analitza l'efecte d'altres reguladors del cicle cel·lular dels que la interacció amb Sp1 ja havia estat descrita anteriorment.

REGULATION OF Sp1 BY CELL CYCLE RELATED PROTEINS

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ABSTRACT

Sp1 transcription factor regulates the expression of multiple gens, including the Sp1 gene itself. We analyzed the ability of different cell cycle regulatory proteins to interact with Sp1 and to affect Sp1 promoter activity. Using an antibody array, we observed that CDK4, SKP2, Rad51, BRCA2 and p21 could interact with Sp1 and we confirmed these interactions by co-immunoprecipitation. CDK4, SKP2, Rad51, BRCA2 and p21 also activated the Sp1 promoter. Among the known Sp1-interacting proteins, E2F-DP1, Cyclin D1, Stat3 and Rb activated the Sp1 promoter, whereas p53 and NFκB inhibited it. The proteins that regulated Sp1 gene expression were shown by positive chromatin immunoprecipitation to be bound to the Sp1 promoter. Moreover, SKP2, BRCA2, p21, E2F-DP1, Stat3, Rb, p53 and NFκB had similar effects on an artificial promoter containing only Sp1 binding sites. Transient transfection of CDK4, Rad51, E2F-DP1, NFκB, p21, p53 and Stat3 also increased mRNA expression from the endogenous *Sp1* gene in HeLa cells. p21 expression from a stably integrated inducible promoter in HT1080 cells activated Sp1 expression at the promoter and mRNA levels, but at the same time it decreased Sp1 protein levels due to the activation of Sp1 degradation. The observed multiple effects of cell cycle regulators on Sp1 suggest that Sp1 may be a key mediator of cell cycle associated changes in gene expression.

INTRODUCTION

Sp1 transcription factor regulates a wide range of cellular processes (Philipsen & Suske, 1999), including cell cycle regulation, hormonal activation, apoptosis and angiogenesis (Safe & Abdelrahim, 2005). It belongs to a family of nuclear proteins, called Sp/KLF (specificity protein/Krüppel-like factor) that recognizes the GC-rich DNA-binding core sequences GC-(GGGGCGGGG) and GT-(GGTGTGGGG) boxes due to the presence of three conserved Cys_2His_2 zinc fingers that form the DNA-binding domain (Bouwman & Philipsen, 2002).

Regulation of Sp1-dependent transcription can be affected by changes in Sp1 abundance, as it occurs during the cell cycle with an increase during G1 phase; DNA binding activity or transactivation activity, and it can involve direct protein-protein interactions with other nuclear factors in which the other factor is not necessarily bound to promoter DNA. Several protein-binding sites have been identified throughout Sp1 (Li et al., 2004). For example, Sp1-interacting proteins include several viral proteins; members of the basal transcription machinery, several transcription activators and cell cycle regulators (Li et al., 2004). In the latter case, the retinoblastoma protein has been described to interact physically with Sp1 in a complex that enhances the transcriptional activation by Sp1 (Noe et al., 1998). In addition, Sp1 can also interact with the E2F-family of transcription factors which is believed to integrate cell-cycle progression with transcription through its cyclical interactions with important cell cycle regulators, such as Rb, cyclins and cyclin-dependent kinases (Slansky & Farnham, 1996). Moreover, Sp factors have been proposed to be essential for the transactivation of the p21Cip1 promoter by members of the p53 family of proteins (Koutsodontis et al., 2005).

Posttranslational modifications also regulate Sp1 activity, including glycosylation, acetylation, phosphorylation and sumoylation (Li et al., 2004; Spengler & Brattain, 2006). Many kinases are able to phosphorylate Sp1 and its phosphorylation state could also be affected by protein phosphatases (Chu & Ferro, 2005). CyclinE-CDK2 and CyclinA-CDK2 complexes which are important regulators of the mammalian cell cycle, phosphorylate Sp1 forming stable complexes with this transcription factor bound to the DNA (Banchio et al., 2004).

We previously described the cloning of the 5'-flanking region of the human Sp1 gene, where a number of putative binding sites for transcription factors were found. Within the minimal promoter sequence, expanding 217 bp from the transcription start site, two Sp1 binding sites were described. Sp1 promoter was mainly regulated by Sp1, Sp3 and NF-Y, although E2F was also found to bind and activate the Sp1 promoter (Nicolas et al., 2003; Nicolas et al., 2001).

In the present study we performed a more detailed analysis of the Sp1 promoter taking into account its regulation by protein-protein interactions. Using a proteomic array

system, we first screened for new Sp1-interacting proteins among cell cycle regulators. We confirmed by coimmunoprecipitation the interaction of Sp1 with Rad51, CDK4, SKP2, BRCA2 and p21 and we studied the effect of the overexpression of these proteins both on the Sp1 promoter and the endogenous Sp1 mRNA level. Other known Sp1-interacting proteins were also tested for its function on Sp1 regulation. We analyzed the regulation of the Sp1 promoter *in situ* by chromatin immunoprecipitation using specific antibodies against the different proteins studied. Additionally, an artificial promoter was used in order to ascertain if the effects observed were unique to the Sp1 promoter or a general mechanism regulating Sp1-dependent transcription. Interaction of Sp1 with p21 was further studied using HT1080 p21-9 cells with inducible p21 expression (Chang et al., 1999). Although p21 activated Sp1 transcription both at the promoter and mRNA levels, it did not increase Sp1 protein levels due to the activation of Sp1 protein degradation.

RESULTS

Sp1 interacts with different cell cycle regulators

To identify proteins that might interact with transcription factor Sp1, whole cell extracts from HeLa cells were screened for Sp1 interactors using an antibody array (Hypromatrix). Among all the Sp1-interacting proteins identified in the Antibody Array, the following were selected for further study: CDK4 and p21, that play a pivotal role in the control of eukaryotic cell cycle; SKP2 that binds to different substrates for ubiquitination by the SCF complex; Rad51 and BRCA2 that participate in DNA-damage responses; and Stat3, p53, NFκB, E2F and Rb for which the interaction with Sp1 had been previously described and that were used as positive controls.

The interactions between Sp1 and several selected proteins identified in the antibody array were confirmed by coimmunoprecipitation. Sp1, CDK4, SKP2, Rad51 and BRCA2 were immunoprecipitated from HeLa whole-cell extracts using specific antibodies and the presence of Sp1 in the immunoprecipitates was determined by western blot analysis. Sp1 was detected in the immunoprecipitates of antibodies against these proteins, demonstrating that all the selected proteins were able to interact with Sp1 (Figure 1A). No signal corresponding to Sp1 was detected when the immunoprecipitation was performed with unspecific IgGs. The interaction between Sp1 and p21 was established by immunoprecipitation using a p21-specific antibody and HeLa cells overexpressing p21 (Fig 1B).

Effects of Sp1-interacting proteins on Sp1-promoter

We used the minimal promoter of the Sp1 gene (pGL3FOR2), which is mainly regulated through two Sp1 sites (Nicolas et al., 2003), to test the effect of the Sp1-interacting proteins. SKP2, Rad51, CDK4, BRCA2 and p21 showed significant transcriptional activation of the Sp1 promoter (Figure 2A). In addition, Rb, Stat3 and CyclinD1 activated the Sp1 promoter, whereas p53 and NFkB decreased Sp1 promoter activity by more than 50%. E2F/DP1 was an activator of Sp1 transcription as previously described (Nicolas et al., 2003).

To better characterize the effect of Sp1-interacting proteins on Sp1-dependent regulation, we used two Sp1 promoter constructs containing different point mutations: pSdNmut, bearing mutations at the NF-Y site and one of the Sp1 boxes, and pSNSmut, with the NF-Y and both Sp1 boxes mutated (scheme in Fig. 2B). All the Sp1-interacting proteins had the same effect on pSdNmut as on the wild type promoter (Figure 2B). In contrast, a decrease in promoter activity was observed when the two Sp1 boxes were mutated, demonstrating the relevance of the Sp1 binding site for the effect of the interacting proteins.

Overexpression of IκB and a dominant negative mutant of p53 caused activation of the Sp1 promoter whereas dominant negatives of Rb and Stat3 decreased the activity of the

construct (data not shown). The combination of BRCA2 and Rad51 produced a discreet increase in SP1 promoter-luciferase activity compared to the effect caused by overexpression of each protein alone (Fig.3A).

Different combinations of cell cycle regulators p53, p21, CDK4, CyclinD1, Rb and E2F1 were also tested for their effect on the Sp1 promoter. As shown in figure 3B, p53 was able to counteract p21-induced activation of Sp1 promoter. Moreover, the combination of p21 and CDK4 activated Sp1 promoter but to a lower extend than p21 alone. The combination of p21 and Rb caused an increase in Sp1 promoter, to a higher level than p21 or Rb alone. Additionally, the combination of Rb and CDK4 also increased Sp1 promoter activity and this effect was additive compared to the activation caused by either Rb or CDK4 alone. Finally, the combination of Rb with E2F activated Sp1 promoter to an intermediated level, higher than E2F alone but lower than Rb alone.

Regulation of the endogenous *Sp1* gene

To study the effect of the interacting proteins on the endogenous levels of Sp1, we performed RT- Real Time PCR to quantify Sp1 mRNA levels in HeLa cells after the overexpression of these proteins. As shown in figure 4, overexpression of p53 and NFκB decreased Sp1 mRNA levels more than 50%, whereas E2F1, p21, Cyclin D1, Stat3, SKP2, Rad51, CDK4, BRCA2 and Rb, increased the Sp1 mRNA levels significantly, in accordance with the results on Sp1 promoter activity. All the changes were in keeping with the changes in the transcriptional activity of the *Sp1* promoter measured in the luciferase assays (Fig. 2 and 3).

Sp1-interacting proteins are bound to the Sp1 promoter *in vivo*.

To examine whether the effects of the exogenous Sp1-interacting proteins on Sp1 transcription reflected the interaction of the corresponding endogenous proteins with the native Sp1 promoter, we performed ChIP analysis of chromatin from HeLa cells using specific antibodies against different proteins. As shown in figure 5, amplification of the Sp1 promoter was observed from chromatin immunoprecipitated using antibodies against Sp1, Stat3, SKP2, NFkB, p21, p53, BRCA2, CDK4 and E2F, demonstrating that both Sp1 and the endogenous Sp1-interactive proteins are bound at the Sp1 promoter containing Sp1-binding sites present in the Sp1 promoter.

Effect of Sp1-interacting proteins using an artificial promoter

To test if the effects observed with the Sp1 gene promoter were a general mechanism that could affect other Sp1-regulated promoters, we used a luciferase construct with a promoter that contained five tandem Sp1 binding sites (pG5-5x(GC)-Luc) (Lee et al., 2005). As shown

in Fig. 6, p53 and NFkB were able to significantly decrease promoter activity whereas Rb, SKP2, E2F1/DP1, Stat3, BRCA2 and p21 increased promoter activity by 2-fold. CDK4, Rad51, CyclinD1 had no effect on the artificial promoter controlled by Sp1.

Effect of p21 on Sp1-dependent transcription

Given that p21 caused the higher effects on the Sp1 promoter and that p21 expression has been shown to result in multiple changes in gene expression in human HT1080 fibrosarcoma and other cell lines (Chang et al., 2000; Roninson, 2003), we further characterized the effect of p21 on Sp1. Transient transfection used in the other experiments leads to very high and often supraphysiological expression levels of the transfected protein, and therefore we used HT1080 p21-9 cells with stably integrated p21 which is inducible by IPTG (isopropyl-β-thio-galactoside) to the levels that are similar to those observed in response to DNA damage or at the onsert of senescence (Chang et al., 2000). First, we transfected HT1080 p21-9 with the Sp1 promoter construct pSdNmut. p21 induction by IPTG increased Sp1 promoter activity in a dose-dependent manner (figure 7A), thus confirming our previous results with transiently transfected p21. Moreover, endogenous Sp1 mRNA levels increased up to 2-fold after 72h of IPTG addition (Fig. 7B). Finally, the artificial promoter controlled exclusively by Sp was activated by 2-fold after IPTG addition (Fig. 7C).

To confirm Sp1 induction by p21 as observed at the RNA level through protein analysis, Sp1 protein levels were analyzed after IPTG addition. Surprisingly, p21 induction caused a decrease rather than an increase in Sp1 protein levels (Fig. 8A). To determine whether this effect on Sp1 protein levels was due to an increase in Sp1 degradation we tested the effect of the calpain inhibitor E64d on p21-induced Sp1 depletion. Incubation of p21-9 induced cells with E64d for 24 hours not only prevented the decrease in the Sp1 protein but also increased its levels up to 190% of the control (Fig 8B). This result is consistent with previously observed induction of Sp1 mRNA by p21 and suggests that Sp1 depletion was due to protein degradation. Incubation of p21-9 cells with E64d for longer periods of time also caused the inhibition of p21 degradation and its accumulation (data not shown).

DISCUSSION

Protein-protein interactions among sequence-specific DNA-binding transcription factors suggest novel modes of regulation. One DNA-bound transcription factor can recruit a second factor to the promoter where binding sites for the latter are absent. The aim of this work was to identify new Sp1-interacting proteins and to study the regulation of the Sp1 promoter by such proteins. Interactions between Sp1 and cell cycle regulators have been well documented and some of the proteins that interact with Sp1 are able to bind among themselves, suggesting that Sp1 could be part of different protein complexes.

We previously described the interaction between Sp1 and the retinoblastoma gene product Rb (Noe et al., 1998). Rb is a target of the cyclin/CDK complexes, mainly CDK4/cyclinD, and its function depends, at least in part, on the interactions with the E2F family of DNA-binding transcription factors (Harbour & Dean, 2000). E2F1 and CDK2 also interact with Sp1 forming stable complexes (Banchio et al., 2004; Noe et al., 1998; Rotheneder et al., 1999). In the present work we demonstrate that Sp1 is able to bind to CDK4 and a CDK inhibitor p21. Overexpression of all these cell cycle regulators (CDK4, Rb, cyclinD1, E2F and p21) increased Sp1 promoter activity and *Sp1* mRNA levels. The activation of the Sp1 promoter by the combination of CDK4 and p21 is in agreement with the fact that p21 plays a permissive role in cell cycle progression as a consequence of its ability to cooperate with CDK4, and to function as an assembly factor for CyclinD-CDK4 complex, as reported in (Chen et al., 1996; Cheng et al., 1999; LaBaer et al., 1997). One might expect that the combination of Rb and CDK4 would lead to a change in the phosphotylation state of Rb. However, since the interaction between Sp1 and Rb is independent of Rb phosphorylation state (Noe et al., 1998), the combination of Rb and CDK4 produces an additive effect in Sp1 transcription. In keeping with this observation and taking into account that p21 activates Rb dephosphorylation (Roninson, 2002), the combination of p21 and Rb caused the activation of the Sp1 promoter. In the case of combining E2F and Rb, the resulting effect on Sp1 transcription is mainly due to Rb, present in its hypophosphorylated form, which is able to repress E2F, either by sequestering this factor or by turning E2F into an active repressor (Harbour & Dean, 2000). E2F and Rb were able to activate the artificial promoter containing only Sp1 sites demonstrating that their effect on the Sp1 gene promoter was mediated directly by Sp1-interaction. On the contrary, overexpression of Cyclin D1 and CDK4 had no effect on this promoter suggesting that they might need the presence of additional factors rather than Sp1 alone to activate Sp1 transcription.

Another complex interacting with Sp1 is SCF^{skp2} that plays an important role in the ubiquitin-mediated degradation of some cell cycle proteins such as p27 (Carrano et al., 1999) and some transcription factors such as E2F1 (Marti et al., 1999). The SCF^{skp2} complex is composed of four subunits: cullin, SKP1, RBX1 and the F-box protein SKP2 (Weissman,

2001). The first three proteins form a common scaffold onto which different F-box proteins can be assembled, conferring specificity to the complex. Cdc34 is also included in this complex and enables transfer of the ubiquitin molecule to specific targets (Patton et al., 1998). Cdc34, p19Skp1, RbX1&2 and p45SKP2 are present in the antibody array and gave positive signals for Sp1 interaction. Given that SKP2 is the F-box of the complex and recognizes the substrate, we validated its interaction with Sp1 by co-immunoprecipitation. Different studies have suggested a close link between the activator function of many transcription factors and their ubiquitylation/degradation (Conaway et al., 2002). Our data demonstrate that SKP2, rather than inhibiting Sp1 promoter, which could be expected as a result of Sp1 degradation, activates the Sp1 promoter and increases Sp1 mRNA levels. This effect has also been observed for the interaction between c-myc and SKP2 (von der Lehr et al., 2003). It is conceivable that Sp1 could be recruiting SCF^{SKP2} to the promoter to degrade negative regulators of transcription at the promoter level. This hypothesis is supported by the results of the ChIP assays that were positive for SKP2 at the Sp1 promoter level and with the result that SKP2 also activates the artificial promoter containing only Sp1 boxes. Another hypothesis could be that ubiquitination of Sp1 and the subsequent cleavage of its N-terminus could degrade a negative regulatory domain thus converting Sp1 into a better transcriptional activator in accordance with (Spengler & Brattain, 2006).

BRCAs are tumor suppressor genes involved in multiple pivotal cellular processes. These proteins contribute to DNA repair, transcriptional regulation in response to DNA damage and maintenance of chromosomal stability, thereby protecting the genome from damage. Many of these functions are mediated by a large number of cellular proteins that interact with BRCAs (Yoshida & Miki, 2004). The interaction between BRCA1 and Sp1 has been described for the regulation of IGF-IR gene expression (Abramovitch et al., 2003). Here we show that Sp1 is able to bind to BRCA2 in addition to BRCA1 and that this interaction increases Sp1 promoter activity and Sp1 mRNA endogenous levels. Moreover, BRCA2 is able to activate a promoter driven only by Sp1 indicating that BRCA2 interacts with Sp1 and that additional factors present in the Sp1 promoter are not necessary for the resulting activation. Rad51 and RBBP are additional components of the repair machinery present in the antibody array that originated positive spots for Sp1 interaction. The interaction of Sp1 with Rad51 was also confirmed by co-immunoprecipitation. Rad51 was able to activate *Sp1* transcription at the promoter and mRNA levels. However, Rad51 did not affect Sp1-dependent activation of the promoter containing only Sp1 binding sites. Combination of BRCA2 and Rad51 increased Sp1 promoter activity to a moderate degree, suggesting that they are forming a part of a complex in which more proteins are needed for the maximum increase in Sp1 transcription. The interaction between BRCA2 and Rad51 has been reported

(Marmorstein et al., 1998) and there is genetic evidence that it is fundamental for the maintenance of cell division and chromosome structure (Yoshida & Miki, 2004).

Sp1 interactions with other proteins also involve several transcription factors: Stat1, Stat3, p53 and NFκB. Tumor suppressor p53 is involved in transcriptional activation of important genes, such as p21, in the regulation of cell cycle and apoptosis (Levine, 1997) and it is involved both in DNA damage and in cell cycle control. In our experiments, p53 overexpression decreased the activity of the Sp1 promoter. Transcriptional repression of other genes by p53 revealed that Sp1 was prevented from binding to the promoter region by a p53-Sp1 protein complex (Borellini & Glazer, 1993; Ohlsson et al., 1998; Yamabe et al., 1998). Our results are in keeping with these observations; p53 counteracted the positive effect of p21 on Sp1 transcription, probably because Sp1 is prevented by p53 from binding to its binding site. NFκB is a nuclear effector in a signaling pathway responsive to a large number of extracellular stimuli in many cells (Gerondakis et al., 2006). NFκB is capable of participating in the transcriptional regulation of target genes, independently of its DNA binding site, through cooperation with other transcription factors. NFκB and Sp1 bind cooperatively and activate transcription of the human immunodeficiency virus synergistically (Perkins et al., 1994). Additionally, NFκB has been described as a transcriptional inhibitor for the gluconeogenic enzyme PEPCK by sequestration of a co-activator protein such as CBP (Xiao, 2004). Accordingly, in our model NFκB behaved as a repressor of Sp1-dependent activation of both the Sp1 promoter and the artificial promoter.

Stats are a family of transcription factors involved in ligand-dependent growth stimulation or differentiation as well as in antiproliferative effects (Darnell, 1997; Schindler, 1998). Both Stat1 and Stat3 have been reported to interact with Sp1 (Loeffler et al., 2005; Look et al., 1995). Stat3 activates transcription of VEGF through its interaction with a Sp1/DNA complex and not by its direct binding to a palindromic enhancer element (Loeffler et al., 2005). In agreement with these observations, in our conditions, Stat3 is an activator of both Sp1 promoter and the artificial promoter, and it binds to the Sp1 promoter.

p21 was studied in further detail as it was able to strongly activate the Sp1 promoter and there are several reports showing that p21 affects transcription of many different genes (Chang et al., 2000; Gregory et al., 2002; Poole et al., 2004). We tested the effects of p21 on Sp1 promoter and Sp1 mRNA levels in HT1080 p21-9 cells, which carry a stably integrated inducible p21 transgene inducible by IPTG addition to physiologically meaningful levels. We confirmed that p21 was able to activate transcription form the Sp1 promoter and to increase Sp1 mRNA expression in this system, as previously observed in HeLa cells transiently transfected with p21. The activation of Sp1 at the promoter and mRNA levels after p21 induction is in accordance with previous observations using CDK inhibitors (Penuelas et al.,

2003). This effect could be caused by dephosphorylation of Sp1 at concrete sites that could increase its transcriptional activity directly or increase the degradation of its inhibitory domain (Spengler & Brattain, 2006). An increase in Sp1 transcriptional activity would increase Sp1 transcription as Sp1 is positively autoregulated.

To further characterize the effects of p21 on Sp1, we analyzed the effect of p21 on Sp1 protein levels by Western blot. Surprisingly, p21 induction did not increase but rather reduced Sp1 protein levels by 80%. The decrease in Sp1 protein levels observed after 24 hours of p21 induction was reverted by the protease inhibitor E64d, indicating that p21 induced Sp1 protein degradation. This effect of p21 was in accordance with Sp1 degradation in aged animal tissues as well as in cells undergoing replicative senescence (Ammendola et al., 1992; Bouwman & Philipsen, 2002; Oh et al., 2007), where p21 induction plays a pivotal role (Roninson, 2002). p21 was previously found to promote the degradation of key cell cycle regulatory proteins, including p53 (Broude et al., Cell Cycle, 2007) and Rb (Broude et al., 2007); the latter protein can be degraded through the same pathway as Sp1 (Nishinaka et al., 1997). Aside from recruiting Sp into this proteolytic pathway, p21, as mentioned above, could potentially cause dephosphorylation of Sp1 increasing the degradation of its inhibitory domain, converting Sp1 into a more unstable protein (Spengler & Brattain, 2006). The dual effect of p21 on Sp is similar to its effect on Rb, where p21 activates Rb by dephosphorylation and concomitantly inactivates it by triggering its degradation (Broude et al., 2007).

In summary, Sp1 function and transcription are regulated by different cell cycle related proteins identified by their interaction with Sp1 protein. Multiple effects of cell cycle regulators on Sp1, observed in the present study, suggest that Sp1 may be a key mediator of cell cycle associated changes in gene expression.

MATERIALS AND METHODS

Plasmid constructs

pGL3FOR2, containing the minimal promoter of Sp1, and pSdNmut and pSNSmut, containing point mutations for the Sp1 and NFY boxes on the Sp1 promoter, have been previously described in detail (Nicolas et al., 2001) and in a scheme presented in Fig. 2. pG5-5x(GC)-Luc contains the luciferase gene controlled by five tandem Sp binding sites and was a generous gift from Dr. Man-Wook Hur.

The expression vectors for the different proteins used in this study were obtained from the following investigators: Sp1 (Dr R. Tjian), E2F1 and DP1 (Dr. T. Kouzarides), pCMV-Cip1 (p21) (Dr. S. Elledge), Stat3 (Dr JC. Lacal), PC53-SN3 (Dr. B.Vogelstein), p45SKP2 (Dr. C. Serra-Pagès), Rad51 (Dr. M. Defais), BRCA2 (Dr J. Bueren), NFkB (Dr. C. Caelles), CyclinD1 (Dr. N. Agell), CDK4 (Dr. M. Barbacid) and Rb (Dr. WG Kaelin).

Cell Culture

HeLa human cervical carcinoma cells were grown in Ham's F-12 medium supplemented with 5% foetal bovine serum (Invitrogen). p21-9 cells were grown in DMEM/GlutaMAX supplemented with 10% foetal calf serum (Invitrogen). Cultures were maintained at 37ºC in a humidified 5% CO_2-containing atmosphere. The derivation, maintenance and p21 induction by IPTG in HT1080 p21-9 cells has been previously described (Chang et al., 1999).

Screening of the AntibodyArray

HeLa total extracts were prepared in extraction solution at 4°C (120 mm NaCl, 25 mm KCl, 2 mm EGTA, 1 mm EDTA, 0.1 mm DTT, 15 mm Tris-HCl, pH 7.5, 0.5% Triton X-100, 10 mg/mL leupeptin, 0.5 mm phenylmethylsulfonyl fluoride). The debris was removed by centrifugation (10 000 g, 15 min).

HeLa total extracts were diluted at a concentration of 2mg/ml in extraction solution containing 1% dry milk. Subsequently, the total extract was incubated with the AntibodyArray™ (Cell Cycle array, Hypromatrix) containing 60 antibodies, as indicated by the manufacturer (see list at http://www.hypromatrix.com). Briefly, the AntibodyArray™ was blocked with 5% dry milk in TBST (150 mm NaCl, 25 mm Tris-HCl, 0.05% Tween-20, pH 7.5) for 1h at room temperature. Then, the membrane was incubated for 2 h at room temperature with the total extract, and washed in TBST (3 x15 min). The membrane was incubated with biotinylated anti-Sp1 antibody (PEP 2, Santa Cruz) and then with streptavidin peroxidase. Binding of the Sp1 antibody was detected by enhanced chemiluminiscence, as recommended by the manufacturer (GE Healthcare).

Co-immunoprecipitations

HeLa total extracts were prepared by incubation in extraction solution for 30 minutes on ice (50 mM Tris-HCl pH8, 50 mM KCl, 5% Glicerol, 0.1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 10 mg/ml Leupeptin). Cell debris was removed by centrifugation (10,000 g, 10 min). In the case of p21 immunoprecipitation, $5x10^5$ HeLa cells were transfected with 5 μg of p21 expression vector using FUGENE™ 6 (Roche Applied Science) 48 h before preparation of the extracts. Total extracts from HeLa cells were immunoprecipiated using specific antibodies (Santa Cruz) for Sp1 (PEP 2), BRCA2 (H-300), SKP2 (H-435), Rad51 (H-92), CDK4 (H-22), p21 (C-19) or unspecific IgGs (Sigma) with the aid of Protein G-Sepharose (GE Healthcare). Then, the pellets were washed three times in extraction solution, resolubilized in loading buffer, boiled for 10 minutes and subjected to Western blot analysis using anti-Sp1 antibody (PEP 2, Santa Cruz) as described in (Noé et al, 1998)

Cotransfections and Luciferase Assays

HeLa cells were seeded into 6-well plates the day before transfection at a density of 2×10^5 cells/well in Ham's F12 medium containing 5% foetal bovine serum. The medium was renewed before transfection, which was performed using FUGENE™ 6 (Roche Applied Science). For each well, 3μl of FUGENE™ 6 in 100 μl of serum-free F-12 medium was incubated at room temperature for 5 min. The mixture was added to the vectors used. In cotransfections, 250ng of the construct pGL3FOR2, pSdNmut or pSNSmut, described in (Nicolas et al., 2001), were mixed with the indicated amounts of Sp1, E2F, DP1, p21, CyclinD1, Stat3, p53, SKP2, NFκB, p21, CDK4, BRCA2 and Rb expression vectors before the addition of FUGENE™ 6 in serum-free F-12 medium.

Luciferase activity was assayed 30 h after transfection. Cell extracts were prepared by lysing the cells with 200 μl of freshly diluted 1x Reporter Lysis Buffer (Promega). The lysate was centrifuged at 12,000 g for 2 min to pellet the cell debris. The supernatants were transferred to a fresh tube and a 30 μl-aliquot of the extract was added to 30 μl of the luciferase assay substrate (Promega). The luminiscence of the samples was read after 10 min on a Glomax™ 20/20 Luminometer, in which the light production (relative luminiscence units) was measured for 10 seconds. Each transfection was performed in triplicate.

Transfection efficiency was corrected by by normalizing the firefly luciferase activity expressed from tested promoter by the control renilla luciferase resulting from cotransfection with the pCMV renilla luciferase vector. 30 μl of the Stop & Glo solution were added to the luciferase mix after reading luciferase activity and were incubated for 10 min before reading the luminescence. Alternatively, when overexpression of the plasmid increased the renilla

activity, luciferase results were corrected by protein concentration using the Bio-Rad protein assay reagent according to the manufacturer's protocol.

For luciferase assays with HT1080 p21-9 cells, cells were seeded into 6-well plates the day before transfection at a density of 2 x 10^5 cells/well in DMEM/Glutamax medium containing 10% foetal calf serum. The medium was renewed before transfection, which was performed using FUGENE™ 6 (Roche Applied Science). For each well, 3 μl of FUGENE™ 6 in 100 μl of serum-free F12 medium was incubated at room temperature for 5 min. The mixture was added to the vectors used. 24 hours after transfection, cells were split and IPTG was added to the medium at the indicated concentrations in one of the two wells corresponding to one transfection. Cells were collected at the indicated times and luciferase activity was measured using the Dual Luciferase Reporter Kit (Promega) according to the manufacturer's instructions. Renilla activity determined for cells in the absence of IPTG was used to normalize the results.

ChIP analysis

Formaldehyde cross-linking and ChIP were performed as described in (Imbriano et al., 2005; Tapias et al., 2008). Briefly, HeLa cells (semiconfluent 100 mm dish) were washed with phosphate-buffered saline and incubated for 10 minutes with 1% formaldehyde. The reaction was quenched with 0.1 M glycine and cells were sonicated to obtain chromatin fragments of an average length of 500 to 800 bp. Immunoprecipitation was performed with protein G-Sepharose (GE Healthcare) and 5 μg of antibodies (Santa Cruz) specific for Sp1 (PEP 2), BRCA2 (H-300), SKP2 (H-435), Rad51 (H-92), CDK4 (H-22), Stat3 (H-190), E2F-1 (C-20), p53 (FL-393) and NFκB p65 (A) or 10μg of antibodies Rb (C-15) and p21 (C-19). Non-specific IgG (Sigma) was used as a negative control. The chromatin solution was pre-cleared by incubation with protein G-sepharose for 2 h at 4ºC, divided into aliquots, and incubated overnight with different antibodies. Before use, protein G-Sepharose was blocked with herring sperm DNA (1 μg/μl) (120-3000 nucleotides length) and BSA (1 μg/μl) for 2 h and then overnight, all at 4ºC.

Sp1 promoter was amplified by PCR using the following specific primers:

Fwd 5'- GCAAGCGAGTCTTGCCATTGG -3'

Rev 5'- CGCTCATGGTGGCAGCTGAGG -3'

PCR was performed in a final volume of 30 μl for at least 28 cycles. Then, 10 μl samples of the PCR products were electrophoresed in a 2% agarose gel and the amplified fragments were visualized after EtBr staining.

For quantification, amplification was performed by Real Time-PCR in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with 2 μl of immunoprecipitated

DNA and the primers indicated above using the Fast SYBR Green Master Mix (Applied Biosystems). The reaction was performed following the manufacturers recommendations. The percent of input was calculated by the standard ΔΔCt method. As a negative control, the following primers were also used:

Fwd: 5´-ATGGTTGCCACTGGGGATCT-3´

Rev: 5´-TGCCAAAGCCTAGGGGAAGA-3´

These negative control primers flank a region of genomic DNA between the GAPDH gene and the chromosome condensation-related SMC-associated protein (CNAP1) gene

Sp1 mRNA endogenous levels

1 µg of expression vectors for E2F, DP1, p21, CyclinD1, Stat3, p53, SKP2, NFκB, p21, CDK4, BRCA2 and Rb was transfected using Fugene 6.

Total RNA was extracted from HeLa cells using the Ultraspec™ RNA reagent (Biotecx) in accordance with the manufacturer's instructions.

cDNA was synthesized in a total volume of 20 µl from RNA samples by mixing 1 µg of total RNA, 125 ng of random hexamers (Roche Molecular Biochemicals), in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 units of RNAsin (Promega), 0.5 mM dNTPs (AppliChem), 200 units of M-MLV reverse transcriptase (Invitrogen) and 50 mM Tris-HCl buffer, pH 8.3. The reaction mixture was incubated at 37ºC for 60 min. The cDNA product was used for subsequent amplification by Real Time-PCR.

Sp1 mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 µl of the cDNA mixture and the Assays-on-demand Hs00412720_m1 for Sp1 and Hs99999901_s1 for 18S RNA (both from Applied Biosystems). 18S RNA was used as an endogenous control. The reaction was performed following the manufacturers recommendations. Fold-changes in gene expression were calculated using the standard ΔΔCt method.

Sp1 protein levels measurement

HT1080 p21-9 total extracts were prepared in extraction solution for 1 hour at 4°C (50 mM Hepes, 0.5M NaCl, 1.5mM MgCl2, 1mM EGTA, 10% Glicerol (V/V), 1% Triton X-100, Protease inhibitor cocktail). Cell debris was removed by centrifugation (10,000 g, 10 min).

Cells extracts were resolved on SDS-7% or 12% polyacrylamide gels (Laemmli, 1970), and transferred to a PVDF membrane (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with antibodies against Sp1, p21 (both from Santa Cruz) or Actin (Sigma). Signals were detected by secondary horseradish peroxidase-conjugated antibody (1:2500 dilution) and enhanced chemiluminiscence, as recommended by the manufacturer

(GE Healthcare). Quantification of the bands was performed using the ImageQuant software (Molecular Dynamics).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using Student's t test using SPSS 13 software for Macintosh. P values of less than 0.05 were considered to be statistically significant.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Coimmunoprecipitation of Sp1 with BRCA2, SKP2, Rad51, CDK4 and p21.

A) Immunoprecipitates were obtained from 1 mg of HeLa total cell extract by incubation with 5 μg of the indicated specific antibodies. After washing, the bound proteins were resolubilized and Sp1 protein was detected by Western analysis. The first lane (TE) shows the signal corresponding to Sp1 from 200μg of HeLa total extract.

B) Coimmunoprecipitation using p21-antibody. 5 μg of p21 expression vector were transfected into HeLa cells using Fugene 6. Total extracts were obtained and 1 mg of each extract was used for coimmunoprecipitation with p21 antibody. Other conditions were as in figure 1A.

Figure 2. Regulation of Sp1 promoter by overexression of Sp1-interacting proteins.

A) HeLa cells were cotransfected with 250 ng of pGL3FOR2 (scheme at upright A) and 500 ng of the indicated expression vectors. After 30 h, cell lysates were assayed for luciferase activity. Firefly luciferase activity was normalized to the renilla luciferase activity for each sample. Results are expressed relative to the activity obtained upon transfection with pGL3FOR2 alone. B) HeLa cells were cotransfected with 250 ng of pSdNmut or pSNSmut (scheme at upright B) together with 500 ng of the indicated expression vectors. Results are expressed relative to the activity obtained upon transfection with pSdNmut alone. Other conditions were as in Fig. 3A.

Results represent the mean ± SE of 3 different experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ Results are expressed relative to the pGL3FOR2 or pSdNmut constructs alone.

Figure 3. Effect of overexpression of different combinations of Sp1-interacting proteins on Sp1 promoter activity.

A) Combination of the DNA-repair proteins Rad51 and BRCA2. Cells were co-transfected with 250 ng of pSdNmut and the indicated amounts of BRCA2 and/or Rad51. After 30 h, cell lysates were assayed for luciferase activity. Other conditions were as in figure 2B.

B) Different combinations of cell cycle regulators p53, p21, CDK4, Rb, E2F and cyclinD1. HeLa cells were co-transfected with 250ng pSdNmut construct and the indicated amounts of the corresponding expression vectors. Other conditions were as in Fig 2B.

Figure 4. ChIP analysis of the Sp1 promoter.

ChIP assays for the Sp1 promoter region were performed using HeLa cells. DNA bound to the immunoprecipitated Sp1, SKP2, Stat3, NFkB, p53, E2F1, Rad51, CDK4, BRCA2, Rb and p21 using specific antibodies, was amplified by PCR. Rabbit IgG was used as negative control (marked as C-). A) Representative images of the PCR products corresponding to the amplification for each antibody using Sp1- specific primers are shown. B) Quantification of ChIP analysis was performed by Real Time PCR and results are expressed as percent of the input, taking into account that ΔCt (Sp1) = Ct Sp1 promoter – Ct genomic DNA, $\Delta\Delta$Ct = ΔCt (Sp1) - ΔCt (IgG) and then referred to the input, considering the dilution factor.

Figure 5. Effect of Sp1-interacting proteins on Sp1 endogenous levels.

HeLa cells were transfected with 1 µg of the indicated expression vectors using 3 µl of FUGENE^{TM} 6. After 72 h RNA was extracted and RT-Real Time PCR was performed as described. Results are expressed relative to the Sp1 mRNA levels obtained upon transfection with the empty vector pCMV.

Results represent the mean ± SE of 3 different experiments and are expressed relative to the control using a CMV vector without insert. *p<0.05, **p<0.01 and ***p<0.001

Figure 6. Effect of Sp1-interacting proteins on an artificial promoter containing only Sp1-binding sites.

HeLa cells were cotransfected with 250 ng of pG5-5x(GC)-Luc and 500 ng of the indicated expression vectors. After 30 h, cell lysates were assayed for luciferase activity. Firefly luciferase activity was normalized to the renilla luciferase activity for each sample. Results are expressed relative to the activity obtained upon transfection with pG5-5x(GC)-Luc and pCMV.

Results represent the mean ± SE of 3 different experiments and are expressed compared to the pG5-5x(GC)-Luc construct cotransfected with 500 ng of an empty CMV vector.

*p<0.05, **p<0.01 and ***p<0.001 Results are expressed relative to the pGL3FOR2 or pSdNmut constructs alone.

Figure 7. Effect of inducible p21 on Sp1-dependent function and expression.

A) Effect of p21 induction on Sp1 promoter. HT1080 p21-9 cells were cotransfected with 500 ng of pSdN construct before induction with increasing amounts of IPTG. At indicated times, cells were assayed for luciferase activity. Firefly luciferase activity was normalized to renilla luciferase activity as described in Materials and Methods. Results are expressed relative to the activity obtained upon transfection with pSdN without IPTG addition.

B) Effect of p21 induction on Sp1 mRNA levels. HT1080 p21-9 cells were treated with the indicated amounts of IPTG. After 72 h RNA was extracted and RT-Real Time PCR was performed as described. Results are expressed relative to the Sp1 mRNA levels obtained without adding IPTG.

C) Effect of p21 induction on a promoter containing only Sp1-binding sites. HT1080 p21-9 cells were transfected with the pG5-5x(GC)-Luc vector and IPTG was added to the medium at the concentrations indicated. Results are expressed relative to the activity obtained upon transfection with pG5-5x(GC)-Luc without IPTG addition.

Results represent the mean ± SE of 3 different experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to the pSp3-FOR4 construct alone.

Figure 8. Effect of p21 induction on Sp1 protein levels.

A) Sp1, p21 and Actin protein levels were determined by Western Blot in HT1080 p21-9 cells after the addition of 50 μM IPTG at different times.

B) Quantification of the results presented in A. Quantification was performed using ImageQuant software (Molecular Dynamics). The representation is in a semilogarithmic scale.

C) Immunoblotting of Sp1, p21 and Actin in HT1080 p21-9 cells treated for 24 hours with 50 μM IPTG and 3 μg/ml of the inhibitor E64d (Sigma).

D) Quantification of the results presented in C.

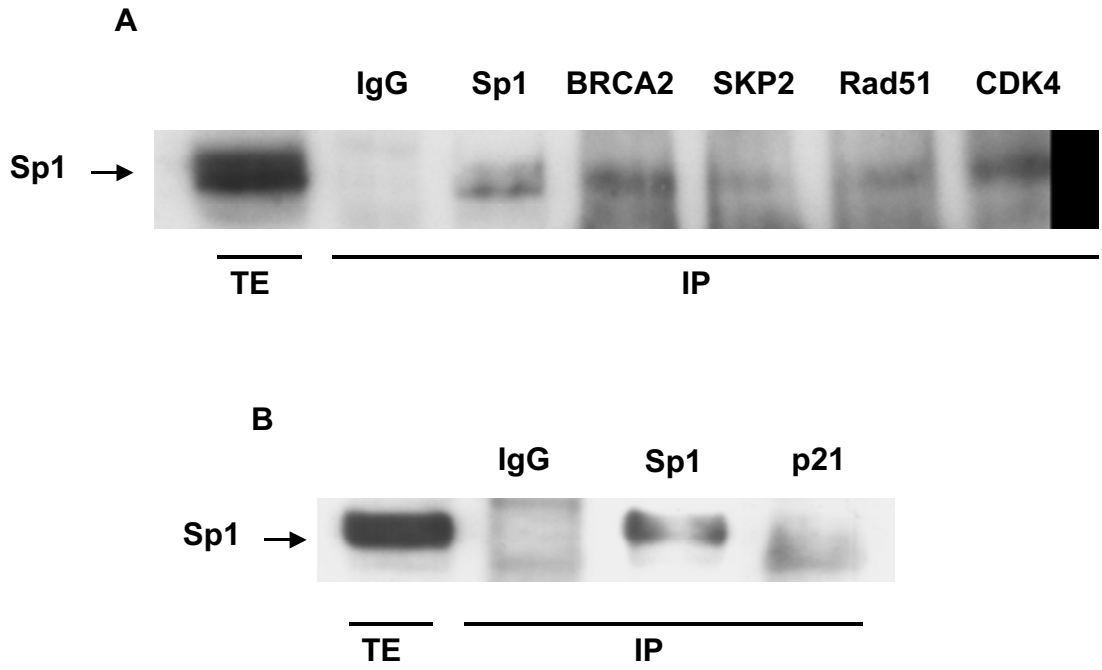
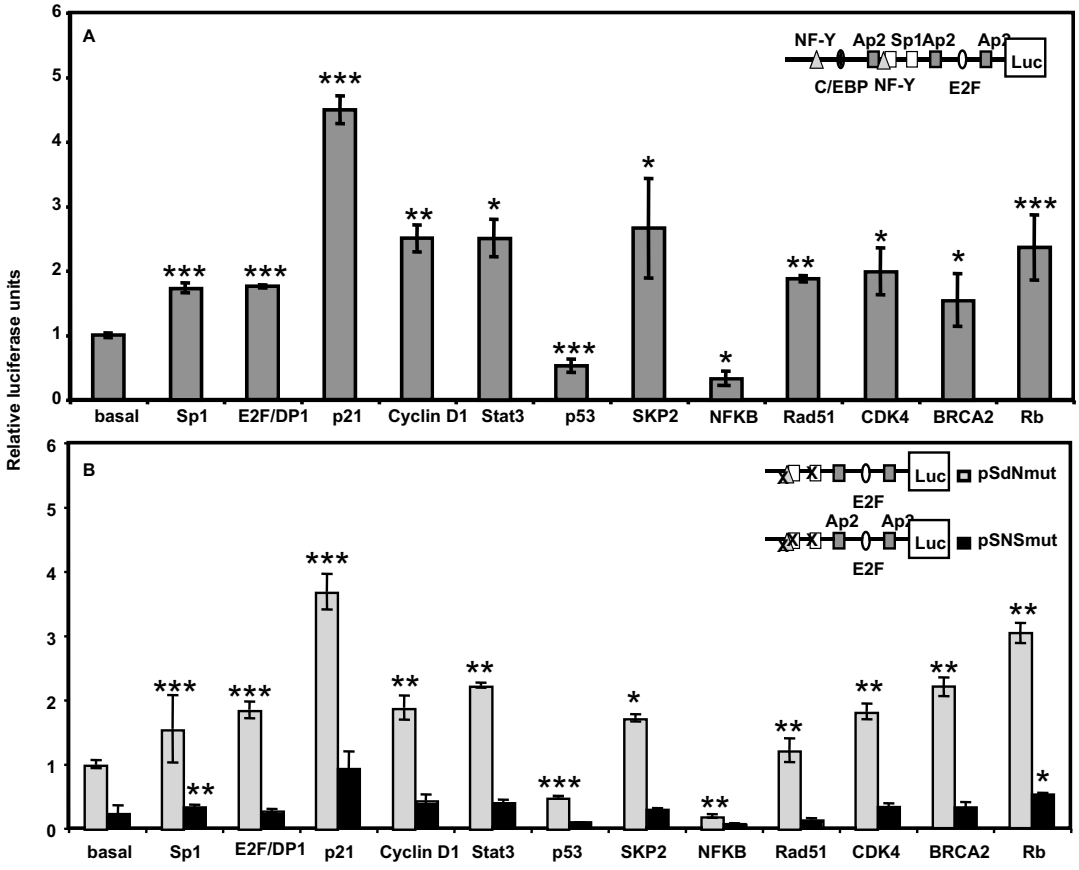


Figure 1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NF-Y
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SKP2
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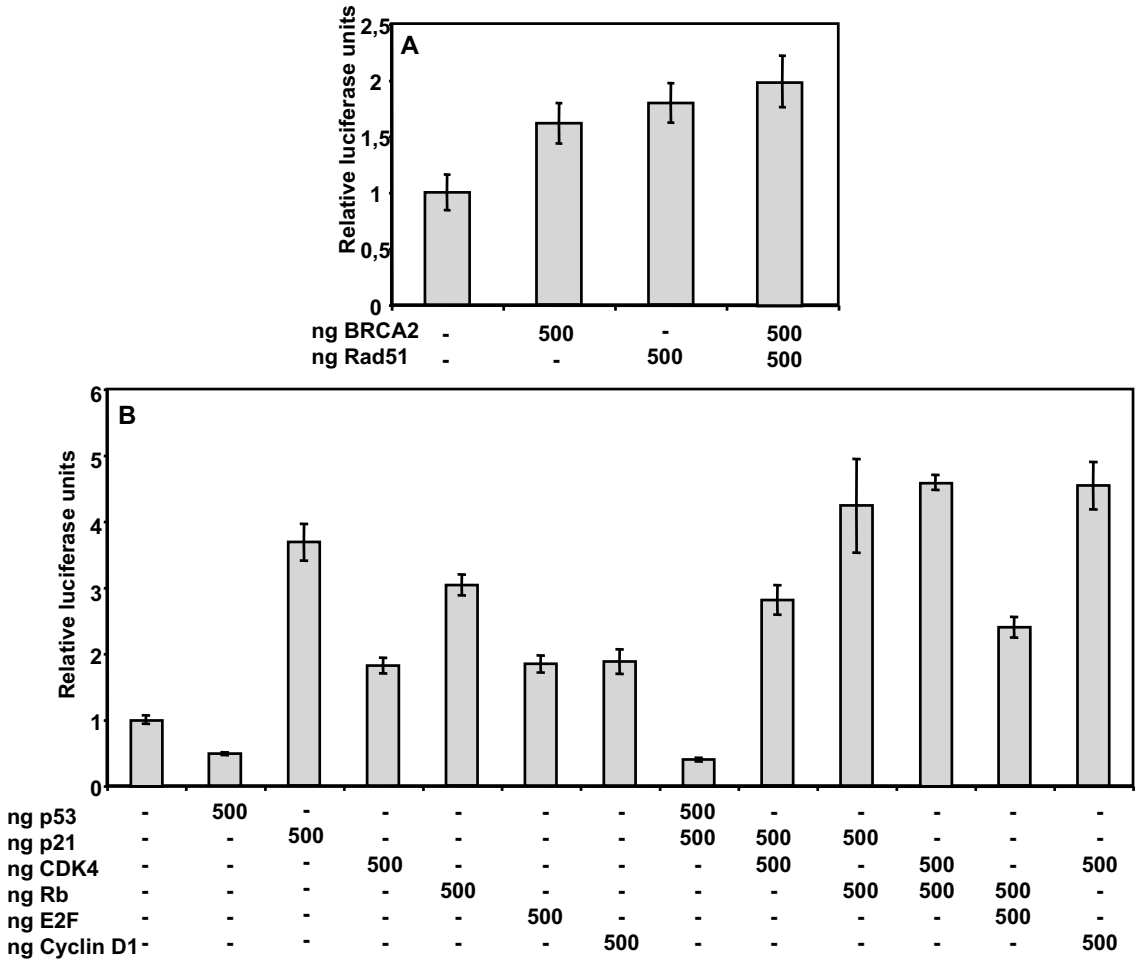
Figure 2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ng Cyclin D1 - - - - - - 500 - - - - - 500

Figure 3

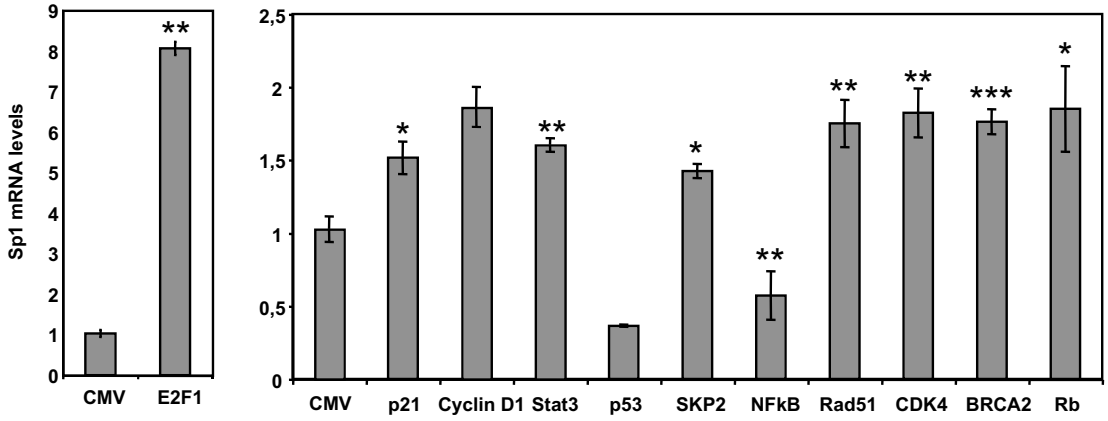
Sp1 mRNA levels
9
8
7
6
5
4
3
2
1
0
**
CMV
E2F1
2,5
2
1,5
1
0,5
0
*
**
*
**
*
**
**
***
*
CMV
p21
Cyclin D1
Stat3
p53
SKP2
NFkB
Rad51
CDK4
BRCA2
Rb

Figure 4

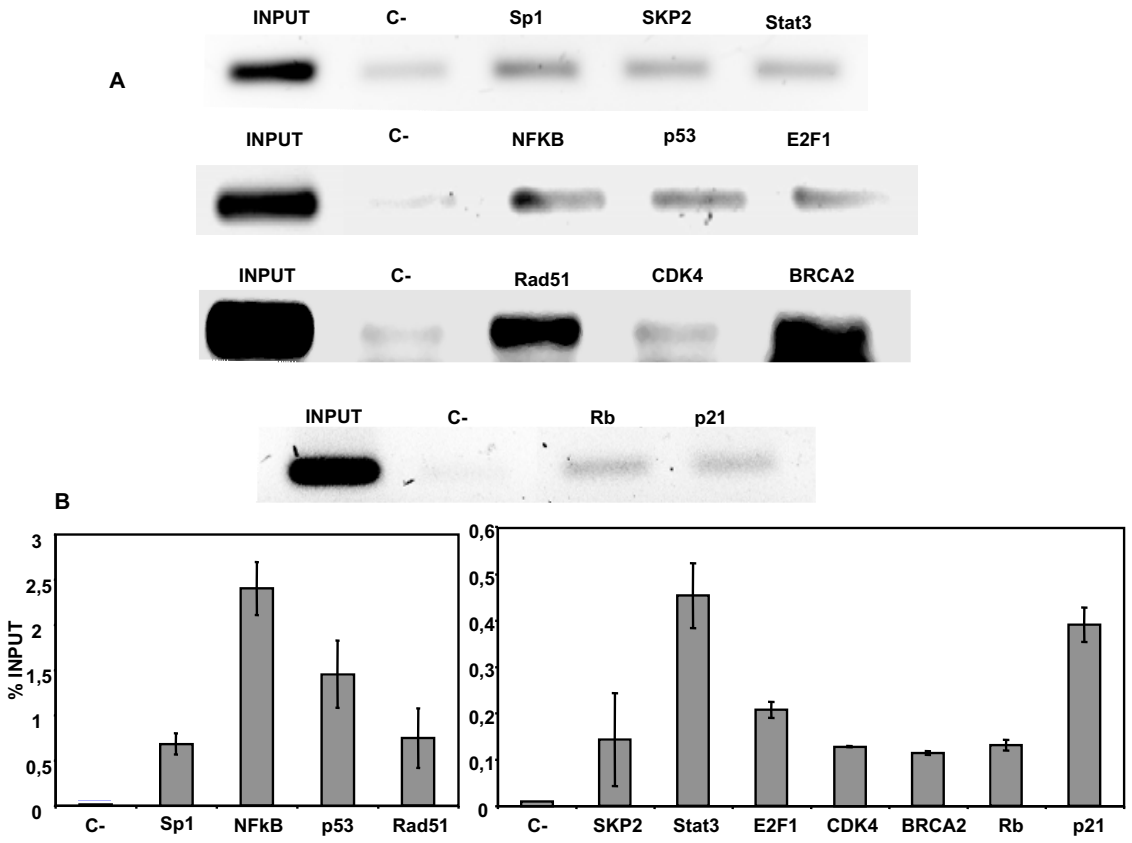
A
INPUT
C-
Sp1
SKP2
Stat3
INPUT
C-
NFKB
p53
E2F1
INPUT
C-
Rad51
CDK4
BRCA2
INPUT
C-
Rb
p21
B
% INPUT
3
2,5
2
1,5
1
0,5
0
C-
Sp1
NFkB
p53
Rad51
0,6
0,5
0,4
0,3
0,2
0,1
0
C-
SKP2
Stat3
E2F1
CDK4
BRCA2
Rb
p21

Figure 5

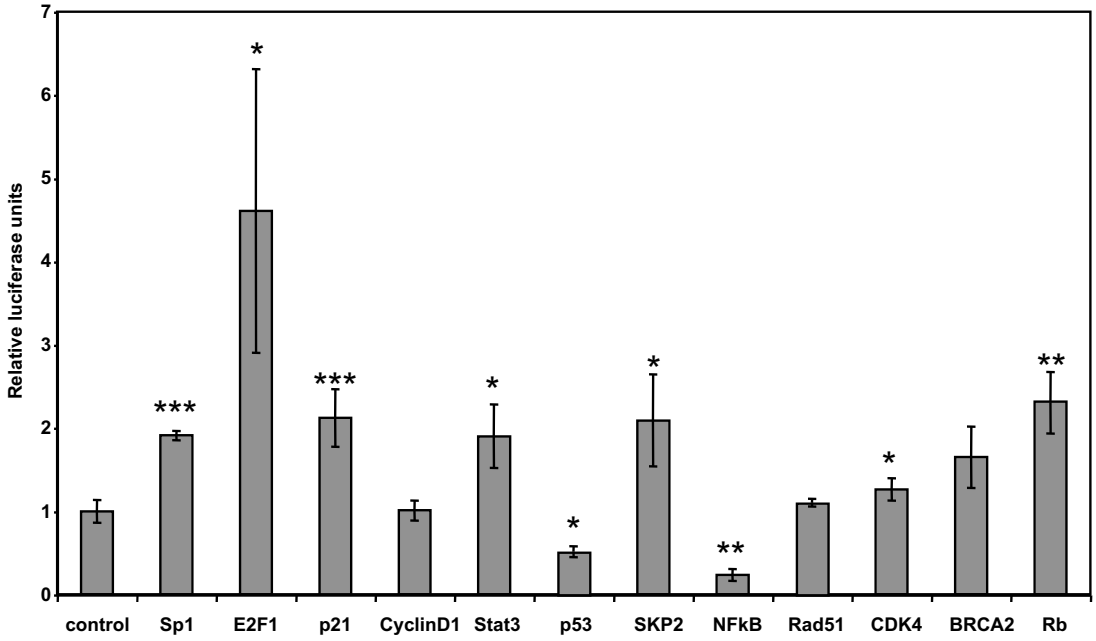
Relative luciferase units
0
1
2
3
4
5
6
7
control
Sp1
E2F1
p21
CyclinD1
Stat3
p53
SKP2
NFkB
Rad51
CDK4
BRCA2
Rb
***
*
***
*
*
*
**
*
**

Figure 6

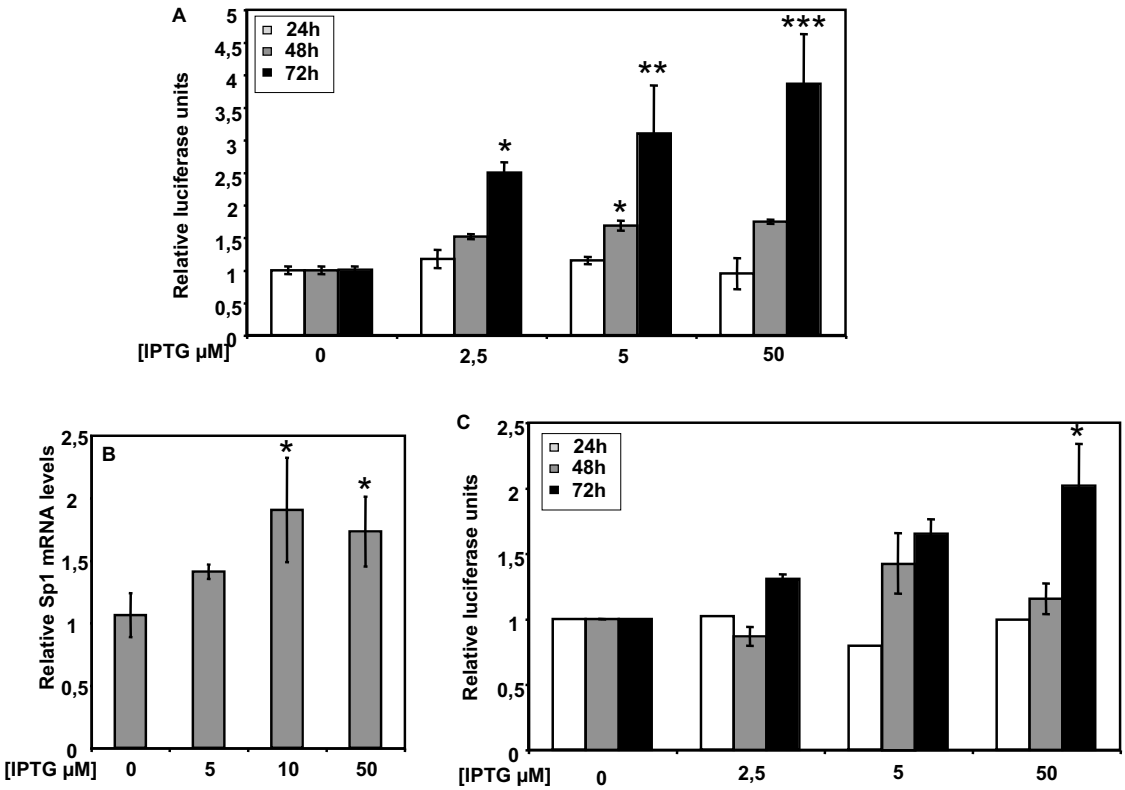
A
24h
48h
72h
Relative luciferase units
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
*
**
***
[IPTG µM]
0
2,5
5
50
B
Relative Sp1 mRNA levels
2,5
2
1,5
1
0,5
0
*
*
[IPTG µM]
0
5
10
50
C
24h
48h
72h
Relative luciferase units
2,5
2
1,5
1
0,5
0
*
[IPTG µM]
0
2,5
5
50

Figure 7

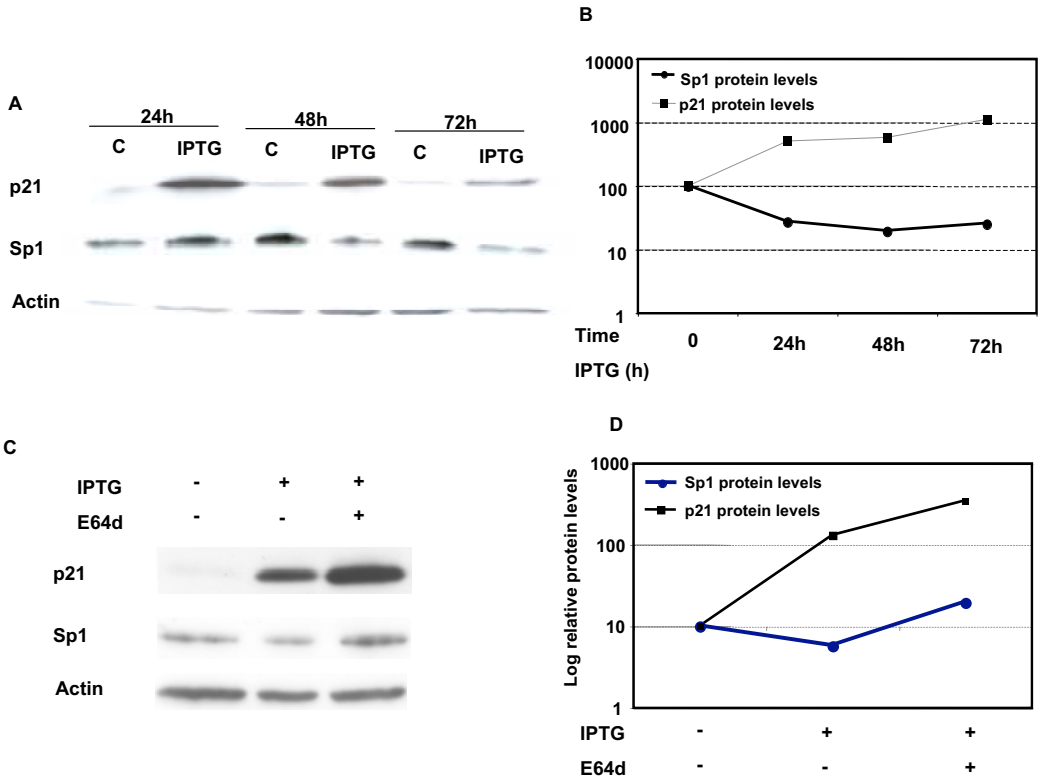
A
24h
48h
72h
C
IPTG
C
IPTG
C
IPTG
p21
Sp1
Actin
B
10000
1000
100
10
1
Sp1 protein levels
p21 protein levels
Time
IPTG (h)
0
24h
48h
72h
C
IPTG
-
+
+
E64d
-
-
+
p21
Sp1
Actin
D
Log relative protein levels
1000
100
10
1
Sp1 protein levels
p21 protein levels
IPTG
-
+
+
E64d
-
-
+

Figure 8