Involvement of NF-Y and Sp1 binding sequences in basal transcription of the human telomerase RNA gene

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Abstract The proximal promoter of the telomerase RNA gene, hTR, contains four Sp1 sites and one CCAAT box. We have carried out a functional analysis of the role of these sequence elements. Two Sp1 sites downstream of the CCAAT box mediated negative regulation, while the other two Sp1 sites were positive regulators with the strongest effect mediated by the negative regulatory Sp1 site closely flanking the CCAAT box. Basal transcriptional activity is maintained via the CCAAT box even when all four Sp1 sites are mutated, suggesting nuclear factor-Y (NF-Y) is a fundamental regulator of hTR promoter function. Chromatin immunoprecipitation revealed binding of NF-Y, Sp1 and TFIIB to the promoter in vivo. Thus the interaction of NF-Y at the CCAAT box is pivotal to hTR gene transcription and surrounding sequence elements may provide an environment for the regulation of activity through recruitment of additional protein complexes.
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Key words: Telomerase; hTR; Gene promoter; Gene transcription; Chromatin immunoprecipitation; NF-Y; SPI

1. Introduction

The human telomerase core enzyme consists of an essential RNA molecule, hTR, with a template domain for telomeric DNA synthesis and of a catalytic protein, hTERT, with reverse transcriptase activity. Telomerase activity is undetectable in many normal somatic cell types but is re-activated in the majority of cancer cell lines and human tumours where it counteracts cell division-associated telomere attrition. Cell strains with undetectable telomerase activity often spontaneously senesce in vitro and experimental inhibition of activity in cancer cell lines leads to telomere shortening and apoptosis. Therefore, understanding the mechanisms governing expression of telomerase activity is of substantial interest. Cellular control of telomerase expression is complex and reconstitution of telomerase activity was shown to require expression of both hTR and hTERT in vitro [1,2]. Transcriptional regulation is the primary step in eukaryotic gene expression, and appears to constitute the major mechanism for differential telomerase gene regulation. It has been reported that up-regulating hTERT transcription can induce telomerase activity de novo and extend the lifespan of several normal human cell strains [3]. Moreover, activation of endogenous hTERT in some transformed cell lines occurs with concomitant elevation of hTR transcriptional activity that may suggest partially overlapping regulatory mechanisms [4]. In our laboratory we have previously isolated and characterised the human telomerase RNA gene promoter and studies of hTR promoter regulation demonstrated that multiple factors modulate hTR gene expression [5,6]. However, the functional contribution to transcription of individual sequence elements in the core hTR promoter has not been determined yet.

Nuclear factor-Y (NF-Y) plays a central role in hTR transcription [6]. NF-Y is a heterotrimeric transcriptional activator composed of three subunits (NF-YA, B, and C), which complexes with CCAAT box sequences [7,8]. NF-Y subunit sequences are highly conserved among eukaryotes and both NF-YB and NF-YC contain conserved putative histone fold motifs (HFM) [9,10], showing most similarity to histones H2B and H2A. Thus NF-Y subunits are capable of participating in formation of the histone octamer [11]. Studies from several laboratories have suggested that NF-Y functionally and physically interacts with other transcription factors or nuclear proteins both in vitro and in vivo [12,13]. NF-YB and NF-YC have been demonstrated to interact with TATA binding protein (TBP) in vitro [14] and NF-Y may therefore serve a structural role by recruiting TBP and/or TAFIIs to connect upstream activators with the general polymerase II transcription machinery [15,16]. The interaction between NF-Y and GCN5 results in the modulation of NF-Y transactivation potential by aiding the disruption of local chromatin structure [17]. A recent study by Park et al. described induction of TGF-β type II receptor gene transcription involving recruitment of the P/CAF protein to the NF-Y–CCAAT complex after HDAC inhibitor treatment [18]. Chromatin structure plays a vital role in transcriptional regulation by restricting the access of transcription-associated proteins to promoters and it is likely that the interaction of NF-Y with histones and with other co-regulators of transcription performs a critical and central function in the organisation of core promoter activation.

The core promoter regions of genes orchestrate diverse functions including polymerase recruitment, promoter activity, and response to regulatory input [19,20]. The aim of the present study was to define the functional *cis*-acting DNA elements responsible for basal activity of the hTR core pro-

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Abbreviations: EMSA, electrophoretic mobility shift assay; NF-Y, nuclear factor-Y; hTR, human telomerase RNA gene; ChIP, chromatin immunoprecipitation

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moter in human cancer cells and thereby provide a basic model to aid future studies of hTR promoter regulation. Disruption of the hTR CCAAT box is known to abrogate basal activity [6]. In the current study we have analysed the sequences contributing to basal activity of the hTR promoter in the hTR- and telomerase-positive 5637 bladder carcinoma cell line that has previously been included in a comprehensive study of hTR expression in normal, cancer and telomerase-negative immortal cell lines [21]. We identified a strong repressive activity acting through a low affinity Sp1 site closely flanking the CCAAT box. Promoter activity was maintained through the wild-type CCAAT box after mutation of all four surrounding Sp1 sites, suggesting that the active core promoter is organised by NF-Y. This is further supported by the observation that cotransfection of the three subunits of NF-Y significantly enhanced the activity of reporters lacking all four functional Sp1 sites. Finally, we observed recruitment of NF-Y, Sp1 and TFIIB to the hTR promoter in vivo using chromatin immunoprecipitation (ChIP). Previous structural observations from the hTR gene have suggested that, in contrast to lower eukaryotes which utilise Pol III for transcription of the telomerase RNA gene [22], hTR is likely to be transcribed by Pol II. This study provides the first direct evidence that a component of the Pol II transcriptional machinery is specifically recruited to the hTR promoter in vivo and provides the first evidence of a basal transcriptional unit in the hTR promoter through which hTR transcription in cancer cells is likely to be organised predominantly through the activity of NF-Y.

2. Materials and methods

2.1. Materials and cell culture

Antibodies to Sp1 and Sp3 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibody directed against NF-YA, B and C was obtained from R. Mantovani (University of Milan, Milan, Italy). The 5637 cell line, originally established from the primary bladder carcinoma of a 68 years old man in 1974, purchased from DSMZ (No: ACC 35). 5637 cells were maintained at 37°C in 5% CO_2 in 1640 medium supplemented with 10% foetal bovine serum, penicillin, and streptomycin.

2.2. Construction of reporter plasmids and mutagenesis

Primers carrying restriction sites were used for polymerase chain reaction (PCR) with hProm867 [5] as the template to generate a series of 5′ and 3′ terminal deletions with compatible ends for cloning as *Xho*I/*Hin*dIII fragments into the multiple cloning region of the promoter-less luciferase vector pGL3-basic (Promega, Madison, WI, USA). A two-step cloning strategy was used for site-directed mutagenesis to prevent unexpected mutations in luciferase reporter vectors: (i) An hTR 176 bp fragment (2923wt, spanning from −107 to +69 bp) was cloned into the *Xho*I/*Hin*dIII sites in pCR-Script™ plasmid vector (Stratagene, La Jolla, CA, USA), which was used as template for PCR using a QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. (ii) All mutation fragments were reconstructed into the *Xho*I/*Hin*dIII sites of pGL3-basic vectors and verified by DNA sequencing.

Primers carrying the mutations (Table 1) and a second set of primers for subcloning were designed. The PCR reaction was performed with these primers and 2923wt or 2925wt as template to create single site mutation constructs shown in Fig. 2B. The double site mutation construct, mSp1(2), or multiple sites mutation constructs, mSp1(3) and mSp1(4), were generated in several separate PCR reactions using mSp1.1, mSp1(2), and mSp1(3) constructs as templates.

The following oligonucleotides were used as PCR primers: hTR23(+69 to +46) *Hin*dIII, 5′-cgcaagcttTACGCCCTTCTCAGTTAGGGTTAG-3′; hTR25(+10 to −12) *Hin*dIII, 5′-cgcaagctTCCGCAACCCGGTGCGCTGCCG-3′; hTR29(−107 to −88) *Xho*I, 5′-gcgctcgAGCCCGCCCGAGAGAGTGAC-3′; mCCAAT(−74 to −45), 5′-GCGAGAGTCAGCTTGGagtcTCCGTGCGG-3′; conSp1(−49 to −20), 5′-GCGGTCGGCccCCGCcCCCTTTATAAGCCG-3′; mSp1.4(+15 to +36), 5′-GGGCCTGGGtaaGGTaaTGGCC-3′.

Nucleotides corresponding to promoter sequences are given in uppercase letters from 5′ end to 3′ end. Lowercase letters indicate mutated nucleotides or clamps for introduction of underlined restriction enzyme sites.

2.3. Transfection and dual luciferase reporter assay

3.0 μg of hTR promoter plasmids containing firefly luciferase reporters were cotransfected into tumour cells with an internal *Renilla* luciferase control, pRL-SV40 (Promega) using Superfect transfection reagent (Qiagen) as previously described [5,6]. For cotransfection, 5637 cells were cotransfected with 0.5 μg of expression vectors encoding wild-type NF-YA, B and C (kindly donated by Dr R. Mantovani), 1–3 μg of the plasmids containing the luciferase reporter gene and 0.5 μg of pRL-SV40 plasmid for control of transfection efficiency. The total amount of DNA was kept constant at 10 μg with salmon sperm DNA. The activity of both firefly and *Renilla* luciferase was determined 48 h later using the dual luciferase assay kit (Promega). A minimum of three independent transfections were performed in duplicate and specific hTR promoter activity was normalised to protein as described elsewhere [5,6].

2.4. Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts from cultured 5637 cells were made according to our previous study [6]. EMSAs were performed using the EMSA kit (Promega, E3300). 5.0 μg of nuclear extract proteins were incubated in 15 ul of reaction containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM ethylenediamine tetraacetic acid (EDTA), 50 mM NaCl, 10 mM Tris–HCl, pH 7.5 and 2.0 μg poly (dI-dC) with or without 100-fold molar excess of unlabelled DNA competitors on ice for 15 min, followed by addition of the radiolabelled probe. For supershift assays, antibodies against Sp1, Sp3, NF-YA, NF-YB or NF-YC, were added to the reaction mixture 25 min prior to the addition of the probe. All DNA–protein complexes were resolved by electrophoresis on 5% native polyacrylamide. The following double-stranded oligonucleotides were used in EMSAs as probes and/or competitors: consensus Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′, (Promega, E323A); TFIID, 5′-GCAGAGCATATAAGGTGAGGTAGGA-3′ (Promega, E322B). The other oligonucleotides are shown in Table 1.

2.5. ChIP assays

Formaldehyde cross-linking and ChIP were performed as described previously [23]. In brief, 5637 cell cultures were treated with formaldehyde for 10 min followed by the addition of glycine to a final concentration of 0.125 M. Cells were then washed twice with cold phosphate-buffered saline (PBS) and were resuspended in lysis buffer (1% sodium dodecyl sulphate (SDS), 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) with a proteinase inhibitor. After brief sonication to yield an average DNA fragment size of 500 bp, the DNA fragments cross-linked to the proteins were enriched by immunoprecipitation with specific antibodies. A 'no antibody' sample was included as a negative control for the immunoprecipitation step. After reversal of the cross-links and DNA purification, the extent of enrichment was monitored by PCR amplification of promoters using forward and reverse primers to the hTR (5′-TACGCCCTTCTCAGTTAGGGTTAG-3′ and 5′-AGCCCGCCCGAGAGAGTGAC-3′) and hsp70 (5′-CCTCCAGTGAATCCCAGAAGACTCT-3′ and 5′-TGGGACAACGGGAGTCACTCTC-3′) gene promoter fragments [24] and to the GAPDH coding region as a negative control (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′). The PCR product was separated by agarose gel electrophoresis. The input sample (the supernatant of the 'no antibody' sample) was processed with the rest of the samples from the point at which the cross-links were reversed.

3. Results

3.1. Mutation of Sp1 sites inhibits DNA binding complexes

Our previous study [6] showed that Sp1/Sp3 factors activate and repress hTR promoter activity and four Sp1 sites (termed Sp1.1, Sp1.2, Sp1.3 and Sp1.4) have been detected in the hTR proximal promoter from −107 to +69 bp (Fig. 2A). In that

Table 1
List of oligonucleotides used in EMSA

Oligonucleotide		Sequence (5′→3′)
h9(Sp1.1)	−44	CGGCGGCCGCTCCCTTTATAAGCCGACT −17
mut11		CGGCGGCC**ATAG**CCTTTATAAGCCGACT
mut12		CGGCGGCCGCTC**ATGC**TATAAGCCGACT
mut13		CGGCGGCCGCTCCCTT**CGAC**AGCCGACT
h4(Sp1.2)	−110	ACCAGCCCGCCCGAGAGAGT −91
mut2		ACCAGCCCG**AA**CGAGAGAGT
h112(Sp1.3)	−2	CCGGGTTGCGGAGGGTGGGCCTGGG +23
mut3		CCGGGTTGCGGA**AAA**TGGGCCTGGG
h113(Sp1.4)	+17	GCCTGGGAGGGGTGGTGGCC +36
mut4		GCCTGGG**TAA**GGTGGTGGCC
h111(Sp1.3/4)	−2	CCGGGTTGCGGAGGGTGGGCCTGGGAGGGGTGGTGGCC +36

Bold bases represent mutations with respect to the wild-type sequences. Regulatory motifs underlined have previously been reported [35,36].

study, we used EMSA to examine complex formation in 5637 nuclear extracts using the oligonucleotides h9, h4 and h111, which correspond to individual Sp1/Sp3 sites within the hTR proximal promoter (Table 1). Supershift analysis previously identified specific Sp1 and Sp3 binding complexes, labelled in Fig. 1 [6]. To clarify the molecular basis for the response to Sp1/Sp3 protein binding, we first introduced specific mutations at each of these sites (for sequences see Table 1). As shown in Fig. 1, ^{32}P-labelled probes h4, h9 and h111 form DNA–protein binding complexes (lanes 1, 6 and 10 respectively), formation of which is inhibited by the respective unlabelled oligonucleotide (lanes 2, 8 and 11). Oligonucleotides harbouring mutations in Sp1 binding sites did not compete with the labelled DNA for protein complex formation (lanes 3, 4, 9, 12 and 13). Oligonucleotide h9 contains a potential TATA box, however mutant mut13 (from TATA to CGAC) retained the ability to displace nuclear protein binding (lane 5). Additionally, a consensus TFIID oligonucleotide used in EMSA did not compete for h9 binding (data not shown). To confirm that the sequence change did not introduce a new protein binding site in mut12 and to assess the possibility of proteins binding at the TATA box after destruction of the Sp1 site, EMSA was performed using labelled mut12 oligonucleotide as a probe. No specific DNA–protein complex formation was detected within this oligonucleotide (data not shown). These results demonstrated that the hTR TATA box (−28/−25) is not critical for DNA–protein interaction.

3.2. *Refining the functional region in hTR proximal promoter*

We previously reported that NF-Y binding at the hTR CCAAT box plays a central role in the maintenance of transcription from the hTR promoter. To further characterise the functional significance of the CCAAT box and surrounding sequence elements, we performed a series of deletion and mutagenesis experiments on a 176 bp fragment of the hTR proximal promoter (2923wt) (Fig. 3). In initial experiments, deletion of the sequence downstream of the transcriptional start site containing Sp1 sites 1.3 and 1.4 (construct 2925) (Fig. 3A) resulted in a minor increase in promoter activity while mutation of site Sp1.1 in addition to the downstream deletion resulted in a significant enhancement of activity. Thus sequences surrounding the CCAAT box may play a role in the modulation of hTR core promoter activity with upstream elements contributing to transactivation, while the downstream sequence elements have a general repressive role.

To characterise the functional relevance of each Sp1 site in regulation of the hTR promoter, mutant sequences detailed in Fig. 2B were constructed into hTR promoter reporters (Fig. 3A and B) by site-directed mutagenesis, plasmid constructs were transiently transfected into 5637 cells and the promoter activities were monitored. As shown in Fig. 3A, the single site mutation analysis indicated that: (i) Mutation of the TATA box from TATA to CGAC in construct mTATA had no effect on reporter gene expression, consistent with redundancy of this site. (ii) Mutations of Sp1 sites led to activation or sup-

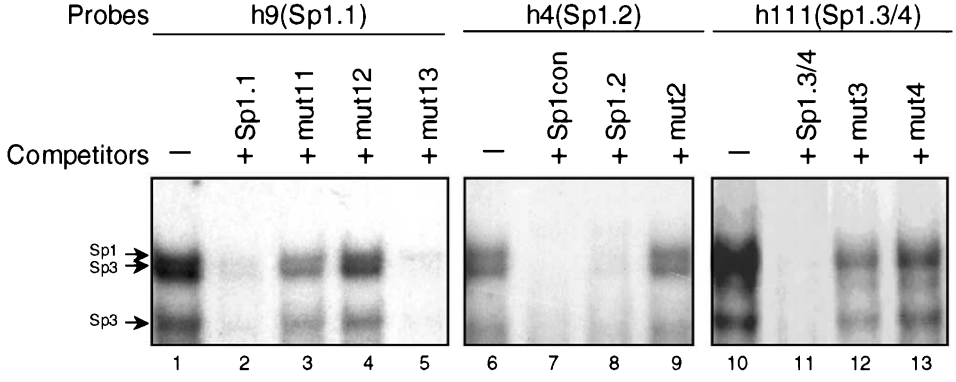


Fig. 1. Disruption of Sp1/Sp3 binding to hTR promoter Sp1 sites. Nuclear extract was mixed with radiolabelled oligonucleotide probes as indicated at the top of each panel and analysed by EMSA. Competition experiments were performed in the presence of 100-fold molar excess of cold competitors. Specific complexes binding Sp1/Sp3 are indicated on the left by arrows.

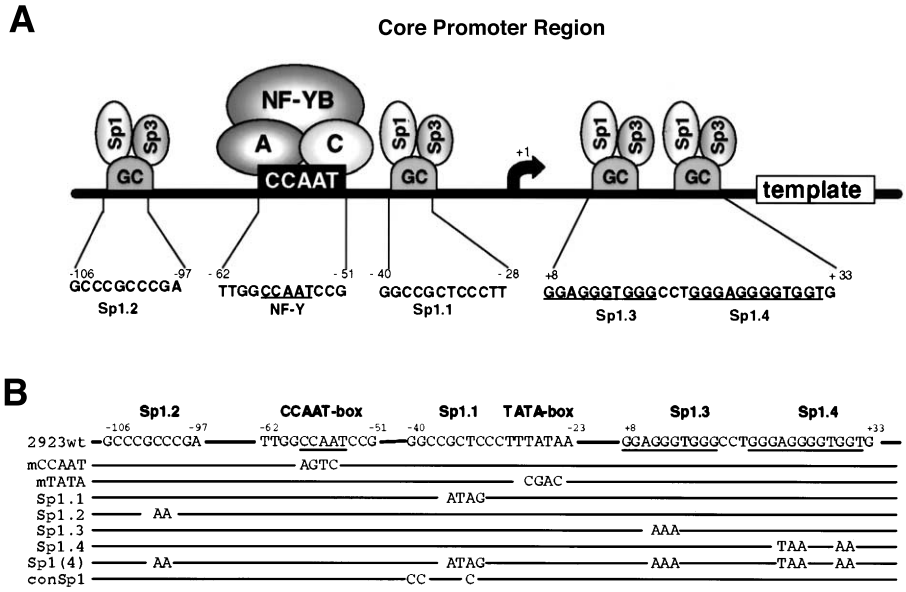


Fig. 2. hTR core promoter and mutation constructs (−107/+69). A: Schematic illustration of the hTR gene promoter. Regulatory sequences (GC box and CCAAT box sites) and their cognate binding factors are shown. The Sp1 and NF-Y binding nucleotide sequences are indicated below. Core promoter regulatory domains identified in this study are illustrated. B: The sequence of the wild-type hTR core promoter (−107/+69) is shown at the top. The name of each mutant construct is indicated on the left hand side. The number on either side of the sequence is related to the transcriptional start site. Dashes indicate an identical sequence to wild type. Mutated nucleotides are shown below the wild-type sequence. The hTR template region is indicated in bold.

pression of promoter activity. Mutation of the Sp1.2 or Sp1.3 sites alone resulted in a minor decrease in promoter activity relative to wild type. Mutation of site Sp1.1, which flanks the hTR CCAAT box, led to the most significant effect on promoter activity (an increase of about four-fold), and mutation of site Sp1.4 also resulted in a small increase in transcriptional activity of 1.5-fold relative to wild type. These data indicate that Sp1.2 and Sp1.3 elements are the sites of action for an activator of hTR promoter activity, and Sp1.1 and Sp1.4 elements are the sites of action for a repressor of hTR promoter activity with Sp1.1 apparently mediating the most dominant effects. Mutation of the CCAAT box (construct mCCAAT) resulted in abrogation of basal activity. Since Sp1.1 flanks the CCAAT box and mediates a strong negative effect, there are at least two interpretations of these data: In one scenario, NF-Y is a vital basal regulator of the hTR promoter whose effects are critical for maintenance of basal activity. An alternative explanation is that the CCAAT box mediates a positive effect, but it is the strong repressive nature of the intact Sp1.1 site in the single mutant construct that is responsible for repression of activity in the absence of an intact CCAAT box. To clarify this issue, we performed a cotransfection analysis using the wild-type 2923 and mCCAAT constructs cotransfected with Sp1 or Sp3 expression constructs. We previously showed that transient overexpression of Sp1 or Sp3 protein can, respectively, up- or down-regulate the proximal hTR promoter activity. We now demonstrate that the stimulatory effect of Sp1 and the repressive effect of Sp3 cotransfections are lost in the context of a construct lacking the wild-type CCAAT box (Fig. 3C), strongly suggesting that the regulatory roles of the Sp1/Sp3 binding sites in the hTR promoter are also dependent on the presence of an NF-Y binding element.

To further determine how the factors binding to Sp1 elements interact and coordinate hTR transcription, double and multiple site-directed mutant constructs were created. As shown in Fig. 3B, mutations of Sp1.2 and Sp1.1 sites together in construct mSp1(2) had a similar effect to mutation of Sp1.1 alone. Since deletion analysis indicates that upstream sequence contributes to transactivation, it is likely that positive regulation can be maintained in the absence of activation by Sp1.2 by factors interacting with Sp1.3. In keeping with this hypothesis, mutation of three Sp1 sites in construct mSp1(3) resulted in a large decrease in promoter activity relative to the double mutant. These data suggest that transactivation unmasked by ablation of the strong repressive effect of Sp1.1 can be mediated either through Sp1.2 or Sp1.3 with neither site showing dominance. The activity of construct mSp1(3) was only slightly greater than the wild type and interestingly, the activity of a construct with combined mutation of all four Sp1 sites within the core promoter (construct mSp1(4)) was similar to the wild-type activity. These data suggest that the major basal activator of the hTR promoter is NF-Y binding at the CCAAT box and that the effect of NF-Y mediated transactivation of the core promoter may be modulated by the presence of a strong repressive element at Sp1.1.

3.3. *A critical Sp1 site in the hTR core promoter represses NF-Y-organised transactivation*

The presence of multiple Sp1 sites exhibiting functional divergence within the hTR promoter, suggests that hTR transcriptional regulation may involve complex interactions between Sp1 and Sp3. It has been demonstrated that the repression of gene promoter activity in general by Sp3 is strictly dependent on the promoter context of the DNA binding sites [25]. Sp1.1 localises to a region in the hTR promoter between the CCAAT box and the transcriptional start site, and is adjacent to the redundant TATA box and may thus be opti-

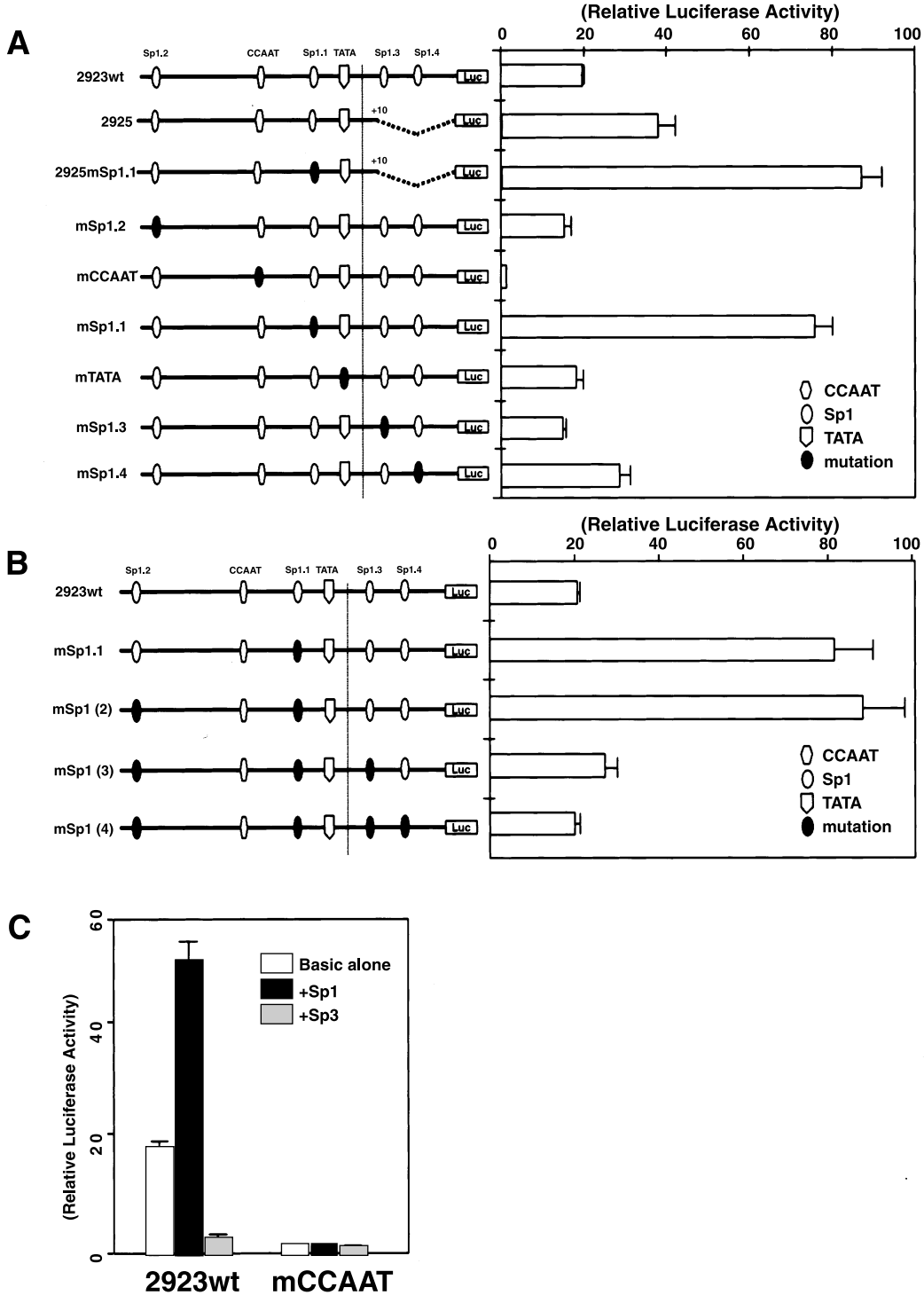


Fig. 3. Scanning mutational analysis of the hTR core promoter. A: Promoter activities of deletions and site-replaced mutant constructs: The various symbols or circles represent the different transcription factor binding sites indicated at the top. Transcriptional start site indicated as broken line. The constructs are shown with a black ellipsoid shape indicating a site-replaced mutation in one or more positions and open ellipsoids representing unmodified sites. The promoter activity is shown on the right hand side. 3 μg of each plasmid were used for transient transfection analysis in 5637 cells. Promoter activities of the mutant constructs were assayed by transfection and compared to the wild-type promoter. The pRL-SV40 vector was used as an internal control to normalise the transfection efficiency. For each transfection the mean and standard deviation of data from three experiments are shown. B: Double and multiple mutation analysis. C: Promoter activity of wild-type and CCAAT mutant reporters cotransfected with Sp1 or Sp3.

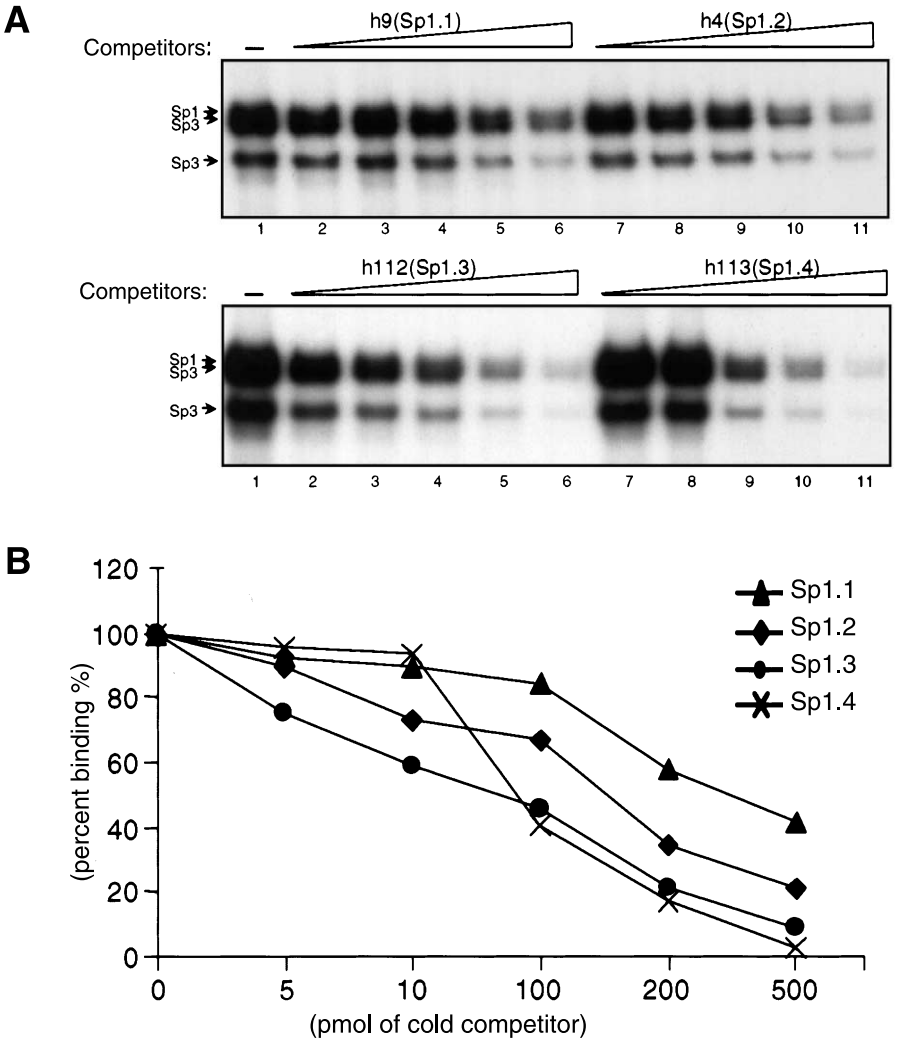
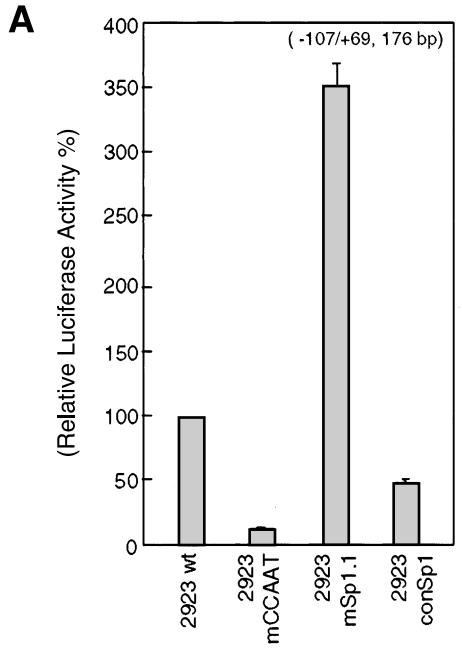


Fig. 4. Differential affinities of the hTR core promoter Sp1 sites. A: Consensus Sp1 oligonucleotide was radiolabelled by kinase treatment using [γ-^{32}P]dATP. Nuclear extract protein was incubated with increasing concentrations of each unlabelled oligonucleotide for 10 min followed by incubation with the Sp1 probe and electrophoresed on a 5% polyacrylamide gel. Quantitative analysis of the dried gel was performed using both a computing phosphorimager with ImageQuant software (Molecular Dynamics) and autoradiography on Kodak XAR-5 films. Three major DNA–protein complexes are indicated with arrows, and lane 1 without competitor was used as a control for quantitative analysis. B: Quantification of the data. Molar excess (from 5×, 10×, 100×, 200× to 500× fold molar present in lanes 2–6 or lanes 7–11) of unlabelled oligonucleotides (h4, h9, h112 and h113) are indicated. Sequences of the oligonucleotide used in competition are shown in Table 1.

mally placed to generate a maximal repressive effect. It is unknown to what extent the affinity of Sp1/Sp3 binding at the non-consensus Sp1.1 site mediates the repressive effect, and to what extent those effects are mediated by its proximity to the CCAAT box. To address this issue, we investigated the relative affinities of each of the four Sp1 sites for Sp1/Sp3 binding in parallel competition experiments utilising a consensus Sp1 oligonucleotide as the probe and competing cold hTR Sp1 oligonucleotides in molar excess. As shown in Fig. 4A, cold oligonucleotides corresponding to sites Sp1.3 and Sp1.4 were able to efficiently compete with binding to labelled consensus Sp1 oligonucleotides, whereas inhibition of complex formation by Sp1.1 and Sp1.2 was less efficient. The relative affinities of the four Sp1 sites in the hTR core promoter are Sp1.4 > Sp1.3 > Sp1.2 > Sp1.1 (Fig. 4B). Therefore, the Sp1.1 site showed the lowest affinity for Sp1/Sp3 binding.

Since mutation of the Sp1.1 site results in the most significant effect on promoter activity and since this site has the lowest affinity for Sp1/Sp3 binding, it seems likely that relatively weak binding to Sp1.1 can greatly inhibit hTR core promoter activity, suggesting the likelihood of a positional importance associated with the site and the possibility of functional interplay with NF-Y. We speculated that increasing the affinity of the Sp1.1 site for Sp1/Sp3 binding should enhance the inhibitory effect of this element. To test this model and to extend the understanding of the role of NF-Y in the regulation of hTR transcription we generated several mutant constructs from the proximal promoter (2923wt; −107/+69). Consistent with the hypothesis that the positioning of Sp1.1 mediates a significant repressive effect on hTR even with relatively low affinity binding, replacement of this sequence with a consensus Sp1 binding site enhanced the repressive effect unmasked in Sp1.1 mutants (Fig. 5A), while mutation of the CCAAT box severely retarded the basal activity. These data



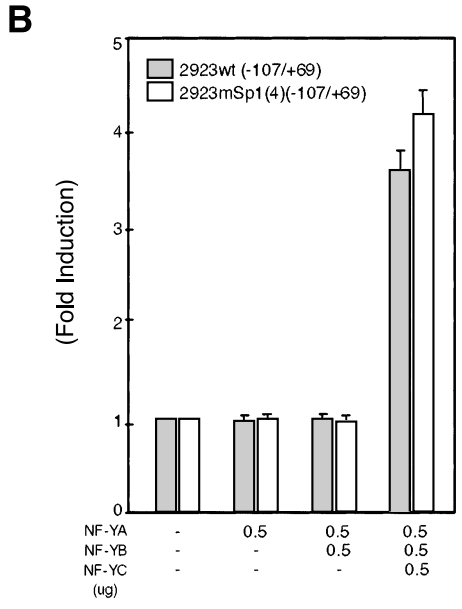


Fig. 5. Functional analysis of hTR gene transcription regulation by the CCAAT box and Sp1.1. A: Comparison of the activity of core promoter regions containing the wild-type, mutant or consensus Sp1 sites or mutant CCAAT box. Promoter activities were assayed by transient transfection and results are expressed as the percent of luciferase activity normalised by *Renilla* and compared to the wild-type promoter. For each transfection the mean and standard deviation of data from three experiments are shown. B: Effects of overexpression of NF-Y on hTR core promoter activity. 5637 cells were cotransfected with 3.0 μg of 2923wt (−107/+69) or 2923mSp1(4) luciferase reporter constructs together with 0.5 μg of expression vectors encoding wild-type NF-YA, B and C. Total input DNA amount for transfection was adjusted with salmon sperm DNA to ensure a constant amount in all transfections. After 48 h of culture, cells were harvested, and the cell lysate was assayed for *Renilla* and luciferase. Data presented are the means of the three independent experiments performed in duplicate.

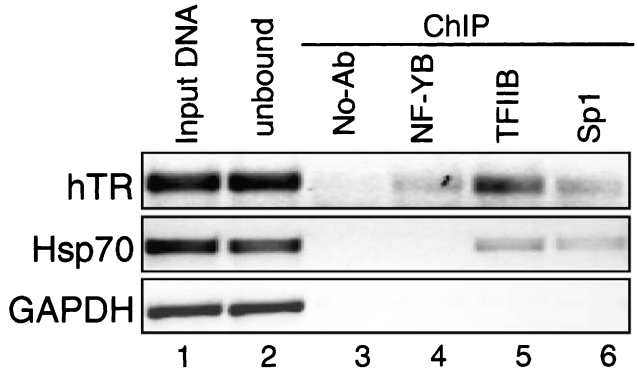


Fig. 6. Immunoprecipitation of the hTR promoter from 5637 cells. Formaldehyde cross-linked chromatin was prepared from 5637 cells and immunoprecipitated with antibodies to NF-YB (lane 4), TFIIB (lane 5) and Sp1 (lane 6), or in the absence of antibody (lane 3). PCR detection of DNA sequences immunoprecipitated with each antibody is shown in these lanes. Lane 2 shows the supernatant of the 'no antibody' sample. PCR was performed with specific primers for the hTR promoter or for the Hsp70 promoter and GAPDH coding region as positive and negative controls. A sample representative of the total input chromatin (input DNA, lane 1) was included in the PCR analysis.

underscore the importance of NF-Y mediated transcriptional control of hTR and suggest that inhibition by factors interacting with the Sp1.1 site is mediated primarily in cooperation with NF-Y.

To reassess the importance of the hTR CCAAT box and address whether NF-Y can directly activate transcription, we performed a transient cotransfection experiment to reconstitute the three subunits of NF-Y (NF-YA, B and C) in 5637 cells. In this experiment, two promoter contexts were used to investigate NF-Y transactivation; a core promoter construct, 2923wt and the core promoter with all four Sp1 sites mutated, 2923mSp1(4). As shown in Fig. 5B, transient expression of NF-YA alone or with NF-YB had no effect on promoter activities, but co-expression of all three subunits together dramatically induced the activity of all promoter constructs, even those lacking all four Sp1 binding elements (from 2.5- to 4.5-fold induction). These results demonstrated that NF-Y is a strong activator and that NF-Y alone can activate hTR transactivation without Sp1 binding.

3.4. NF-Y interacts with the hTR promoter in vivo

The complete set of general transcription factors are thought to form the transcription initiation apparatus at most promoters, but a direct test of this model in hTR has not been carried out in vivo. Moreover, while the telomerase RNA genes of lower eukaryotes are transcribed by Pol III dependent mechanisms, the structure of the hTR gene and promoter indicates that it may be regulated by Pol II [5,6,22], suggesting that the mechanisms regulating telomerase RNA expression may be evolutionarily divergent. To determine whether NF-Y, Sp1 and TFIIB, a component of the general Pol II transcriptional machinery, become associated with the active hTR promoter, we performed a ChIP assay in 5637 cells. 5637 cells were treated with formaldehyde to cross-link proteins to DNA. After sonication, the cross-linked chromatin was immunoprecipitated using anti-NF-YB, Sp1 and TFIIB antibodies. After immunoprecipitation, enrichment of the endogenous hTR promoter fragment in each sam-

ple was monitored by PCR amplification using primers amplifying the hTR promoter region from −107 to +69 bp. To confirm the specificity of the DNA binding activity of factors at the hTR promoter, PCR amplifications of the Hsp70 promoter which binds Sp1 and the general transcription factor TFIIB [24], and the GAPDH coding sequence, which has no binding sites for any of the regulators tested, were included in parallel experiments as positive and negative controls, respectively (Fig. 6). The results of ChIP experiments performed with these antibodies indicate that hTR transcription in living cells involves recruitment of NF-YB, Sp1 and TFIIB factors to the hTR promoter. This is the first evidence of a basal transcriptional mechanism for regulation of the hTR promoter and, taken together, the data presented in this report give a strong indication that NF-Y is an essential component involved in the organisation of the hTR basal transcriptional machinery.

4. Discussion

It is likely that multiple mechanisms regulate the hTR promoter in vivo. We previously observed that NF-Y and Sp1 family transcription factors bound to the hTR promoter to regulate hTR expression. Additionally, we previously described silencing of the hTR promoter in telomerase-negative immortal cells (ALT cells) by methylation of the CpG island in which the proximal promoter is located. The silent (methylated) hTR promoter can be re-activated in some ALT cell lines by combined treatment with the demethylating agent 5-AzaC and the histone deacetylase inhibitor TSA [21]. These studies suggest that regulation of the proximal hTR promoter may be important for the differential regulation of hTR gene expression in normal and cancer cells. In this manuscript we analyse the hTR core promoter and demonstrate the coordinate function of Sp1 and NF-Y binding sequence elements in the basal activity of the hTR promoter in cancer cells. These data provide the first demonstration that the functional hTR core promoter is composed of four Sp1 sites and a CCAAT box. In this core promoter: (i) NF-Y binding at the CCAAT box is crucial for hTR promoter activation. (ii) The TATA box adjacent to Sp1.1 has no function. (iii) Factors binding to Sp1 sites nearby the CCAAT box coordinate with NF-Y to activate or repress hTR transcription. In this model, the transcription factors targeting downstream of the CCAAT box represent an overall negative effect, although the downstream region also contains a sequence (Sp1.3) that can act as positive regulator in the absence of upstream elements that contribute to transactivation. It is possible that differential binding between normal and cancer cells of factors acting on these and other promoter elements may partly underlie the cell selectivity of hTR expression. Indeed, we have recently demonstrated that hTR promoter gene therapy constructs delivered by adenovirus to a range of human cell lines are differentially active between normal cells and cancer cells [26]. Therefore, the present study delineates the fundamental elements of a core promoter structure that will be helpful for future studies that may increase our understanding of the differential regulation of hTR expression.

NF-Y–CCAAT complex formation underlies hTR transcriptional regulation and is likely to be influenced by surrounding modulators. Single and multiple site-directed mutagenesis experiments identified both negative (Sp1.1 and Sp1.4) and weak positive regulatory elements (Sp1.2 or Sp1.3) (Fig. 3), of which the CCAAT flanking Sp1.1 site appeared to be the most highly functional. Disruption of Sp1.1 facilitated transactivation, presumably by factors binding either to the Sp1.2 or Sp1.3 sites since de-repression of activity was only observed in constructs with at least one of these sites intact. These results indicated that hTR transcription can be up- or down-regulated by surrounding Sp1 sites. However, regulation of the core promoter by Sp1 or Sp3 cotransfection required an intact CCAAT box and basal promoter activity was unchanged relative to wild type in a construct lacking all four Sp1 sites. Interestingly, the latter construct could also be transactivated by cotransfection of all three NF-Y subunits. These data indicate that the hTR core promoter can drive reporter gene expression without Sp1/Sp3 directly binding to DNA and suggest that the primary basal activator of hTR transcription is NF-Y while the Sp1/Sp3 binding sites are more likely to perform regulatory roles.

The sequences controlling basal transcription are common to many genes and include the TATA box, CCAAT box, Sp1 site and initiator (Inr) sequence elements. Transcription factors Sp1 and NF-Y have been shown to coordinately regulate many gene promoters and to physically interact with each other and with TBP and general transcription factors [14,16,27]. Moreover, Sp1 sites in TATA-less proximal promoters can regulate start site utilisation [28–30], while the vast majority of CCAAT boxes have been shown to significantly contribute to overall promoter strength and to be strictly required for gene transcriptional activity [8]. Therefore we suggest that coordination of the factors binding to Sp1 sites and the CCAAT box constitute the hTR core transcription machinery in human cancer cells which may mediate the recruitment of components of the Pol II transcriptional apparatus to the hTR promoter in vivo (Fig. 6). NF-Y binding to the CCAAT box is a good candidate to organise promoter architecture and chromatin assembly thereby controlling core hTR transcription by influencing the transcription initiation complex formation. In this speculative model, factors binding to the region downstream of the CCAAT box in the hTR core promoter might directly or indirectly influence initiator binding activity leading to transcriptional repression. Other factors binding to the distal promoter might enhance NF-Ys ability to recruit general transcription factors in the formation of an active transcriptional complex.

In the present study, we identified an NF-Y-organised hTR core promoter and propose a basal transcriptional mechanism. There are elements in the upstream hTR promoter that may potentially allow interaction with other co-regulators for modulation of signals between transcription factors and the core transcriptional machinery. Combinations of ubiquitous transcription factors with cell type-specific factors might thereby regulate hTR gene expression in the cell type-specific manner that has been observed. Studies of the transcriptional regulation of hTR and hTERT promoters will be essential for the rational development of telomerase-directed therapeutics [8,21,31–34]. It is possible that a small number of key transcription factors might be responsible for a complex set of expression patterns in diverse conditions. Studies of the functional motif combinations in the hTR promoter may give more information about molecular mechanisms controlling differential hTR expression patterns in normal and cancer cells.

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