Modulation of DNA topoisomerase II α promoter activity by members of the Sp (specificity protein) and NF-Y (nuclear factor Y) families of transcription factors

Natisha MAGAN*, Agnieszka P. SZREMSKA*, Richard J. ISAACS† and Kathryn M. STOWELL*¹

*Institute of Molecular BioSciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand, and †Regional Cancer Treatment Service, Palmerston North Public Hospital, Ruahine Street, Palmerston North, New Zealand

topo II α (topoisomerase II α) is a major target of several commonly used anticancer drugs and is subject to down-regulation at the transcriptional level in some drug-resistant cell lines and tumours in response to chemotherapy. Clinical resistance to such drugs has been correlated with down-regulation of topo II α at transcription in some drug-resistant cell lines and tumours. Putative binding sites for a variety of transcription factors, including Sp1 (specificity protein 1) and NF-Y (nuclear factor Y) have previously been identified in the topo II α promoter, but their functional significance and interactions have not been described following exposure to anti-cancer drugs. The binding of these factors to specific putative regulatory elements in the topo II α promoter was studied using electrophoretic-mobility-shift assays. Sp1 was found to bind strongly to both distal and proximal GC-rich

elements and NF-Y to ICB1 (the first inverted CCAAT box). The functional significance of transcription-factor binding was studied using transient transfection of HeLa cells using a luciferase reporter driven by a 617-bp minimal promoter containing point mutations in putative regulatory elements. Sp1 and NF-Y were both found to be transcriptional modulators with activator or repressor functions depending on protein/DNA context. Moreover, a functional interaction between Sp1 and NF-Y bound at proximal elements was observed.

Key words: cancer, chemotherapy, drug resistance, topoisomerase II α , nuclear factor Y (NF-Y), specificity factors 1 and 3 (Sp1 and Sp3).

INTRODUCTION

topo II (topoisomerase II) is an essential nuclear enzyme that is required in all organisms for various features of DNA metabolism (reviewed in [1–3]). topo II is also the primary cellular target of a number of widely used anticancer drugs, including doxorubicin, mitozantrone and etoposide (reviewed in [4,5]).

Human cells express two isoforms of topo II, designated α (M_r 170 000) and β (M_r 180 000). The expression of the two isoforms varies in different physiological states, with topo II α levels fluctuating during the cell-cycle and with proliferation state, while topo II β levels appear to be more ubiquitously expressed (reviewed in [1]). Expression of both isoforms at both the protein and mRNA level has been correlated with response to chemotherapy in cell lines and tumour tissues, with high levels of expression often conferring increased sensitivity to such therapies and low levels conferring resistance (reviewed in [4–6]).

In different physiological states, expression of human topo II α mRNA is primarily regulated by activity of the promoter [7–9]. Maximal activity of the promoter appears to be regulated in the first 617 bp [10], within which lie a number of putative *cis*-acting elements including two Sp (specificity protein) consensus sites (GC1 and GC2) and four inverted CCAAT boxes (ICB1–ICB4).

We have previously shown that the reduction of topo II α mRNA levels induced by confluence arrest is mediated by the binding of the transcription factor NF-Y (nuclear factor Y) to a specific ICB (ICB2 from position –109 to –105) [7]. Others have suggested that the proximal ICBs are critical in regulating the transcriptional response to cell-cycle phase [11,12], and to heat shock [13]. Recently the Sp1 transcription factor has been implicated in the cell-proliferation-dependent regulation of topo

II α gene expression in the rat by binding to a separate Sp consensus site [14]. In terms of chemotherapy drug resistance, the transcription factor Sp3 has been indirectly implicated in the evolution of acquired chemotherapy resistance to teniposide [15], while others have shown a reduced level of NF-Y in doxorubicin-resistant cells [16].

There are significant differences in the DNA sequence and the position of *cis*-acting elements in different species (reviewed in [1]), indicating that methods of regulation for one species may not directly apply to others. Furthermore, there has been little published data to explain the mechanisms of basal transcription in the human topo II α promoter.

In the present study we have investigated the basal regulation of the human topo II α promoter by exploring the binding of transcription factors to the Sp and one NF-Y consensus sites in the first 617 bp. We used promoter constructs containing single or multiple point mutations with transient transfections and EMSAs (electrophoretic-mobility-shift assays) to demonstrate not only binding to the particular *cis*-acting elements, but also the effects of such binding on the expression of topo II α . We also explore interactions between the different *trans*-acting factors, providing evidence that such interactions are critical in regulating basal gene expression.

EXPERIMENTAL

Reporter gene constructs

The WT (wild-type) reporter construct was prepared by excising the 617 bp topo II α promoter region from plasmid pHGH (a plasmid that contains an 1.8 kbp cDNA for human growth

Abbreviations used: EMSA, electrophoretic-mobility-shift assay; ICB, inverted CCAAT box; NF-Y, nuclear factor Y; Sp1, specificity protein 1; topo II, topoisomerase II; WT, wild-type.

¹ To whom correspondence should be sent (e-mail k.m.stowell@massey.ac.nz).

Table 1 Oligonucleotides used for introducing mutations into topo II α promoter-reporter gene constructs and for mutated competitors in EMSAs

Oligonucleotide	DNA sequence (5'–3')
GC1mtF	GCTTCGTGCGTGCTAAAGGAAG
GC1mtR	CTTCCTTTAGCAGCACGAAGC
ICB1mtF	CGAGTCAGGGATTCCCTGGTCTGC
ICB1mtR	GCAGACCAGGGAATCCCTGACTCG
GC2mtF	AGGGTAAAGGTGGCGTGGTTGAGGCA
GC2mtR	TGCCTCAACCACGCCACCTTACCC

hormone as a reporter gene) [7] using the restriction endonucleases *Xba*I and *Bam*HI and subcloning into the luciferase reporter vector pGL3 Basic (Promega, Madison, WI, U.S.A.) using the *Nhe*I and *Bgl*II sites respectively. Point mutations were introduced into the GC2, GC1 and ICB1 elements sequentially by PCR mutagenesis [17] using *Pwo* (*Pyrococcus woesei*) polymerase (Roche Diagnostics New Zealand Limited, Auckland, New Zealand) under the conditions described by the manufacturer with the flanking primers RV3 and GL2 (Promega) and the mutagenic primers listed in Table 1.

Single mutant constructs with mutations in regions –546 to –566, –57 to –80 and –37 to –59 were named GC2[–], GC1[–] and ICB1[–] respectively. A double mutant construct was named GC1[–]/ICB1[–]. PCR products were digested with *Kpn*I and *Nco*I, purified using phenol/chloroform extraction, followed by ethanol precipitation and ligated in the pGL3 Basic vector that had been prepared in the same manner. Double and triple mutant constructs containing the mutated GC2 element were prepared by excising a *Hind*III fragment ($\approx$ 400 bp) containing the mutated GC2 region from the GC2[–] mutant and subcloning this into each of the other constructs after removing the corresponding *Hind*III fragment. These constructs were named GC2[–]/GC1[–], GC2[–]/ICB1[–] and GC2[–]/GC1[–]/ICB1[–]. All clones were sequenced using BigDye terminators and analysed on an ABI Prism 377–36 automated sequencer to confirm orientation and the presence of the appropriate mutations.

Transient transfections and reporter gene assays

HeLa cells were maintained on minimum essential medium supplemented with non-essential amino acids (Sigma–Aldrich Pty. Ltd., Castle Hill, NSW, Australia), penicillin/streptomycin (Gibco BRL/Invitrogen Corporation) and 10% (v/v) fetal-calf serum (Gibco BRL/Invitrogen Corporation). Transient transfections were carried out in 12-well tissue-culture plates with cells at $\approx$ 60% confluence using 1 μ g of reporter vector, 1 μ g of the pSV β -gal control vector (Promega) and plus or minus various amounts of Sp1 co-expression vector (EF1 α /Sp1; gift from Dr Merlin Crossley, Department of Biochemistry, University of Sydney, Sydney, Australia) using FuGENE 6TM (Roche Diagnostics) transfection reagent according to the manufacturers' instructions. After 48 h the cells were harvested and assayed for luciferase activity using a Luciferase Assay Kit (Promega) and FLUOStar galaxy detection (BMG LabTechnologies PTY, Melbourne, Australia) according to the manufacturers' instructions. β -Galactosidase activity was measured using a spectrophotometric assay [18]. Relative luciferase activity was calculated from the ratio between relative light units produced from luciferase assays and the absorbance at 420 nm for the β -galactosidase assays. All values were normalized to the WT construct that was arbitrarily set at 100%.

Table 2 Oligonucleotides used for probes and competitors in EMSAs

Oligonucleotide	DNA sequence (5'–3')
GC2wtF	AGGGTAAAGGGGCGGGGTTGAGGCA
GC2wtR	TGCCTCAACCCGCCCTTTACCC
GC1wtF	GCTTCGGGCGGGCTAAAGGAAG
GC1wtR	CTTCCTTTAGCCGCCCGGAAGC
ICB1wtF	GAGTCAGGGATTGGCTGGTCTGC
ICB1wtR	GCAGACCAGCAATCCCTGACTC
ICB1wt/GC1wtF	CGAGTCAGGGATTGGCTGGTCTCTTCGGGCGGGCTAAAGGAAG
ICB1wt/GC1wtR	CTTCCTTTAGCCGCCCGGAAGCAGACCAGCAATCCCTGACTCG
ICB1mt/GC1wtF	CGAGTCAGGGATTCCCTGGTCTGCTTCGGGCGGGCTAAAGGAAG
ICB1mt/GC1wtR	CTTCCTTTAGCCGCCCGGAAGCAGACCAGGGAATCCCTGACTCG
ICB1wt/GC1mtF	CGAGTCAGGGATTGGCTGGTCTGCTTCGTGCGTGCTAAAGGAAG
ICB1wt/GC1mtR	CTTCCTTTAGCACGCACGTAAGCAGACCAGCAATCCCTGACTCG
ICB1mt/GC1mtF	CGAGTCAGGGATTCCCTGGTCTGCTTCGTGCGTGCTAAAGGAAG
ICB1mt/GC1mtR	CTTCCTTTAGCACGCACGAAGCAGACCAGGGAATCCCTGACTCG

EMSAs

EMSAs were carried out using ³²P-labelled double-stranded oligonucleotides representing GC2, GC1, ICB1 or a composite ICB1/GC1 element. Oligonucleotides used as probes are listed in Table 2. Unlabelled competitor oligonucleotides were either homologous with the probe oligonucleotides or mutated sequences as used for the PCR mutagenesis. Mutated ICB1/GC1 sequences are listed in Table 2. Mutant single element oligonucleotides were as listed in Table 1. All oligonucleotides were obtained from SigmaGenosys (Sigma–Aldrich). F and R refer to forward and reverse strands respectively for each complementary pair. One oligonucleotide of the complementary pair was end-labelled using γ -riboATP and T4 polynucleotide kinase. The unlabelled complementary oligonucleotide was then annealed to the labelled oligonucleotide in the presence of 50 mM KCl, and the double-stranded product purified using 10% PAGE in 1 $\times$ TBE buffer (0.089 M Tris/0.089 M boric acid/2 mM EDTA). The position of the labelled double-stranded oligonucleotide was determined by autoradiography for 1 min and the oligonucleotide eluted from the gel slice in 50 mM KCl overnight at 37 °C. Approx. 0.5 ng (10000–20000 c.p.m.) of double-stranded oligonucleotide was used in each assay with approx. 10 μ g of cell extract as the protein source. Each assay contained 1 μ g of poly (dI-dC) (Amersham Biosciences NZ Ltd., Auckland, New Zealand) as non-specific competitor, 20 mM Tris, pH 7.6, 8% ficoll, 50 mM KCl, 0.2 mM EDTA and 0.5 mM dithiothreitol in a total volume of 20 μ l. Labelled probes were added last after a 15 min pre-incubation on ice. For supershift assays, antibodies (diluted as recommended by the manufacturer) were added at the start of the pre-incubation period. Free probe and bound complexes were separated by 4% PAGE at 200 V for approx. 2 h. Gels were dried under vacuum on to DE-81 paper and exposed to X-ray film for several hours or overnight. Antibodies against Sp1 and Sp3 were obtained from Santa Cruz Biotechnology Inc. and those against NF-Y subunits from Rockland Immunochemicals, Gilbertsville, PA, U.S.A.

Preparation of cell extracts for EMSA

HeLa cells were grown to confluence in 14 cm-diameter culture dishes and whole-cell extracts prepared [19]. Protein concentrations were determined using the Bio-Rad protein assay reagent according to the manufacturers' instructions.

Figure 1 Sp1 and Sp3 can bind specifically to the topo II α promoter

(A) EMSA of the GC1 element with HeLa-cell extracts. Lane 1, free probe; lanes 2–5 contain probe plus HeLa cell extract; lane 3, supershift with anti-NF-YB antibodies; lane 4, supershift with anti-Sp1 antibodies; lane 5, anti-Sp3 antibodies do not supershift any complexes. (B) EMSA of the GC2 element with extracts from HeLa-cell extracts. Lane 1, free probe; lanes 2–5 contain probe plus cell extract; lane 3, supershift with anti-Sp1 antibodies; lane 4, supershift with anti-Sp3 antibodies; lane 5, anti-NF-YB antibodies do not supershift any complexes. (C) EMSA of the ICB1 element with HeLa-cell extracts. Lane 1, free probe; lanes 2–5 contain probe plus cell extract; lane 3, supershift with anti-NF-YB antibodies; lane 4, supershift with anti-Sp1 antibodies; lane 5, anti-Sp3 antibodies do not supershift any complexes. NS refers to non-specific DNA–protein complexes.

RESULTS

As both Sp1 and Sp3 have been implicated in the transcriptional regulation of topo II α , we sought to determine whether either or both could bind to, and modulate, transcription from the consensus sequences GC1 (–37 to –59) and GC2 (–546 to –566) of the topo II α promoter.

DNA binding assays

A series of *in vitro* EMSAs was carried out with individual double-stranded oligonucleotides and HeLa-cell extracts. Specific interactions between both GC1 and GC2 elements of the human topo II α promoter and Sp1 were observed using both competitor oligonucleotides at a 100-fold excess (results not shown) and a specific antibody against Sp1 (Figure 1A, lane 4, and Figure 1B, lane 3, respectively). In addition, Sp3 was shown to interact specifically with the GC2 element (Figure 1B, lane 4), but not with GC1. In a similar set of binding assays originally designed to assess the importance of the ICB1 element in interacting with the transcription factor NF-Y, we observed a supershift not only with antibodies against NF-Y (Figure 1C, lane 3), but also with antibodies against Sp1 (Figure 1C, lane 4). A specific interaction between NF-Y and GC1 was also observed (Figure 1A, lane 3). These observations suggest that Sp1 can be recruited to the ICB1 element in the absence of its cognate DNA binding site and that NF-Y can be recruited to the GC1 element in the absence of

an ICB via protein–protein interactions between Sp1 and NF-Y. Functional interactions between Sp1 and NF-Y have been demonstrated on other promoters [20–23], but not on the topo II α promoter, which led us to study in more depth a possible interaction between NF-Y and Sp1 at the region containing ICB1 and GC1 elements. For these experiments, a longer double-stranded oligonucleotide was used, which included both ICB1 and GC1 as well as the intervening sequence (–37 to –80). EMSA clearly show that four proteins are capable of binding specifically to this sequence (labelled I–IV in Figures 2 and 3). In an attempt to determine whether the complete DNA sequence was required for protein binding, we carried out EMSA using double-element ICB1/GC1 competitor oligonucleotides carrying mutations in either or both ICB1 or GC1 (Figure 2). Competition with the homologous WT oligonucleotide (Figure 2, lanes 3–5) demonstrated that all four bands were specific DNA–protein complexes. Only complex II could not be competed for with an oligonucleotide mutated in ICB1 (ICB1mtGC1wt; Figure 2, lanes 7–9). Strong competition for complex II and weak competition for complexes III and IV was observed with an oligonucleotide mutated in GC1 (ICB1wt/GC1mt; Figure 2, lanes 11–13). No significant competition was observed for any of the complexes when the competitor oligonucleotide contained mutations in both ICB1 and GC1 (ICB1mt/GC1mt; Figure 2, lanes 15–17). These results suggested that the proteins were able to bind ICB1 or GC1 in a specific manner, with complex II

Figure 2 A composite ICB1/GC1 element representing the topo II α promoter binds four proteins

Lane 1 contains free probe, while lanes 2–17 contain probe plus HeLa-cell extract. Lanes 3–5 show competition with an oligonucleotide homologous with the labelled probe; lanes 7–9 show competition with a composite ICB1/GC1 oligonucleotide containing a mutation in the ICB1 site; lanes 11–13 show competition with a composite ICB1/GC1 oligonucleotide containing a mutation in the GC1 site; lanes 15–17 show competition with a composite ICB1/GC1 oligonucleotide containing a mutation in the both ICB1 and GC1 sites. The positions of the four specific DNA–protein complexes are indicated by roman numerals. NS refers to non-specific DNA–protein complexes. Competitors were added at 50-, 100- and 200-fold molar excess over probe.

Figure 3 Single-element competitors show less affinity for DNA–protein complexes formed on the composite ICB1/GC1 element

Lane 1 contains free probe, while lanes 2–17 contain probe plus HeLa-cell extract. Lanes 3–5 show competition with an oligonucleotide corresponding to the ICB1 element; lanes 7–9 show competition with a mutated ICB1 element; lanes 11–13 show competition with an oligonucleotide corresponding to the GC1 element; lanes 15–17 show competition with a mutated GC1 element. The positions of the four specific DNA–protein complexes are indicated by roman numerals. NS refers to non-specific DNA–protein complexes. Competitors were added at 50-, 100- and 200-fold molar excess over probe.

formation being more dependent on ICB1 and complexes I, III and IV being more dependent on GC1.

Potential interactions between proteins bound at ICB1 and GC1 were investigated using single-element competitors; either ICB1 or GC1 with or without mutations (Figure 3). Complexes I, II and IV showed significant competition with WT ICB1

Figure 4 Composite ICB1/GC1 element representing the topo II α promoter binds Sp1, Sp3, NF-Y and an uncharacterized protein

(A) Lanes 1–4 contain probe plus HeLa-cell extract. Lanes 2–4 show supershifts with antibodies against Sp1, NF-YB and NF-YA respectively (NF-Y is a trimeric protein consisting of three subunits, A, B, and C). (B) Lanes 1 and 2 contain probe plus HeLa-cell extract. Lane 2 shows a supershift with antibodies against Sp3. NS refers to non-specific DNA–protein complexes.

(Figure 3, lanes 3–5). Using a WT GC1 competitor, significant competition for complex I only was observed (Figure 3, lanes 11–13). No significant competition was observed with mutated ICB1 (Figure 3, lanes 7–9) or mutated GC1 (Figure 3, lanes 15–17). Taken together, these results suggest that the proteins forming complexes I, II and IV at the composite ICB1/GC1 element, have a strong affinity for the single ICB1 element alone. The single GC1 element appears to compete only for complex I, suggesting that the proteins in complexes II, III and IV have a higher affinity for the ICB1/GC1 composite element than they do for GC1. Using antibodies, proteins in these complexes were identified as NF-Y (complex II), Sp1 (complex I), Sp3 (complex IV) and an unidentified protein (complex III) (Figure 4). The unidentified protein complex showed weak competition for the ICB1 element, but no competition for the GC1 element (Figure 3, lanes 3–5). This suggests that while the unidentified protein complex requires the composite element for formation, it has some affinity for the ICB1 element alone. Collectively these assays demonstrated that ICB1 is the dominant element of the two and strongly suggest an interaction between Sp1/3 and NF-Y is capable of occurring at the composite consensus site. Of the three proteins identified in the complexes, the weakest interaction appears to be between the composite element and Sp3; therefore we sought to determine whether Sp1 could modulate transcriptional activity of the topo II α promoter.

Functional assays

HeLa cells were transiently transfected with the luciferase reporter vector pGL3 Basic harbouring the –617 promoter regions of

Figure 5 Sp1 activates topo II α

Relative luciferase activity is plotted against amount of Sp1 expression vector in each transfection. All transfections were carried with 1 μ g of the -617 WT topo II α . Results are means $\pm$ S.E.M. ($n = 9$). * $P < 0.001$ (obtained by comparing relative luciferase activities plus and minus Sp1).

human topo II α [7,10]. This region contains both the distal GC2 and proximal GC1 element as well as all five ICB elements and the transcription start site. Each assay contained the pSV β Gal vector used as a control for transfection efficiency. The effect of Sp1 on transcription was tested by co-transfecting increasing amounts of an Sp1 expression vector. A 2–6-fold activation of transcription was observed (Figure 5), clearly demonstrating that Sp1 is a transcriptional activator of human topo II α . There was no effect of Sp1 on the vector pGL3 Basic, suggesting this was a specific effect of Sp1 on the topo II α promoter. A series of functional assays was then carried out using constructs carrying mutations in either

ICB1, GC1 or GC2 as well as combinations of these mutations in the presence and absence of the co-expression vector for Sp1. Each assay was normalized against the transcriptional activity of the WT -617 topo II α promoter in the absence of Sp1 (Figure 6). In this set of assays expression from the WT human topo II α promoter was increased 2–3-fold by the addition of 0.25 μ g of Sp1 co-expression vector. It is important to note that HeLa cells express significant amounts of both Sp1 and Sp3, as shown in Figures 1–4, so the effect of Sp1 on the WT promoter is likely to be masked by the endogenous Sp1 and/or Sp3. Lower, but significant, activation of expression due to Sp1 was also observed for each of the mutant promoter constructs, with the exception of constructs containing mutations in both GC1 and GC2. This was expected, as Sp1 is thought to interact via GC elements. These data clearly show that Sp1 is a transcriptional activator of human topo II α gene expression. A mutation in ICB1 resulted in a dramatic reduction in basal transcription, but mutations in either GC1 or GC2 did not change expression markedly from that of the WT. However, the activation caused by Sp1 remained in the presence of the ICB1 mutation as long as either the GC1 or GC2 element was intact. Interestingly, the presence of mutations in both GC1 and GC2 did not decrease expression in the absence of Sp1. The residual transcriptional activity from the triple mutant construct is likely to be due to other functional interactions between NF-Y and the other three ICBs, ICB2, 3 and 4, within the -617 region of the topo II α promoter.

DISCUSSION

The EMSAs clearly show that four specific protein complexes can form with the composite ICB1/GC1 element of the topo II α promoter. Antibody supershift assays identified proteins within these complexes as Sp1, Sp3, NF-Y and an as yet uncharacterized protein. Competitor assays with mutated oligonucleotides suggested that Sp1/3 could be recruited to the composite element via putative interactions with NF-Y and vice versa. The uncharacterized protein appears to bind to the GC1 element (Figure 2;

Figure 6 Reporter gene assays

Relative luciferase activity is plotted against plasmid construct. The black and grey bars represent the relative luciferase activity in the absence and presence of 0.25 μ g of the Sp1 expression vector respectively. Results are means $\pm$ S.E.M. ($n = 9$). ** $P < 0.01$ and * $P < 0.05$ (obtained by comparing relative luciferase activities plus and minus Sp1).

compare lanes 7–9 with lanes 11–13), but also appears to be recruited in an NF-Y-dependent manner (Figure 3; compare lanes 3–5 and 11–13).

Sp1 is well known as a transcriptional activator responsible for recruiting the RNA polymerase to promoters, and NF-Y has been suggested to be an architectural protein responsible for bending DNA and recruiting other activators to promoter regions [24–28]. Moreover, functional interactions between Sp1 and NF-Y have been shown previously in a number of different promoter contexts, including that of the human *cdc25C* gene [20], the rat fatty acid synthase gene [21,22] and the p27(Kip1) gene [23]. To our knowledge this is the first demonstration that Sp1 can interact with, and regulate, the human topo II α promoter and that the interaction may be mediated by NF-Y.

Transient co-transfection of an Sp1 expression vector with the WT topo II α reporter construct has demonstrated that Sp1 is a transcriptional activator of topo II α (Figure 5). A series of transient transfection assays have confirmed a role for NF-Y and Sp1 at the ICB1, GC1 and GC2 elements respectively of the human topo II α promoter. NF-Y acting at ICB1 appears to be an activator of basal transcription and Sp1 activates transcription at GC1 through cross-talk with NF-Y bound at ICB1. Sp1 acting at the GC2 element appears to repress basal transcription via a putative interaction at the ICB1 and GC1 elements, possibly mediated by NF-Y, and an uncharacterized protein. It is possible that residual binding of Sp1 (or the uncharacterized protein) in this region via the ICB1 element is sufficient for basal transcription from the human topo II α promoter. In contrast, transcription from the promoter bearing mutations in all three elements showed the lowest activity and activation by Sp1 was not observed. The promoter construct bearing a mutation in ICB1 and GC2 showed approximately WT activity in the absence of Sp1 and a significant increase in activity in the presence of Sp1. This observation, together with the slight increase in expression in the absence of Sp1 observed for the mutant GC2 construct, suggests that proteins bound to the GC2 element may act as repressors of transcription. Collectively these assays demonstrated that the ICB1 element is the most important of those tested for basal regulation of transcription from the human topo II α promoter. But more importantly, some type of cross-talk is likely to exist between Sp1 (and/or Sp3) bound at the GC1 and GC2 elements, and this cross-talk is likely to be mediated via the interaction of NF-Y at ICB1. Our experiments do not distinguish between Sp1 and Sp3, as both proteins are expressed endogenously in HeLa cells. There may also be a role for the uncharacterized protein in both recruiting a multiprotein complex and in basal transcription from the topo II α promoter. Confirmation of these roles awaits the identification of this protein and further functional analysis. A model for this putative interaction is suggested in Figure 7. NF-Y bound at ICB2 is shown as a possible architectural protein responsible for bending DNA [24]. NF-Y bound at ICB2 has previously been shown to be responsible for the confluence-induced down-regulation of the topo II α promoter [7]. Those authors showed that a mutation in ICB2 was responsible for an up-regulation of transcription at confluence arrest. Our model supports this functional role for NF-Y, as it would be able to bend the DNA to accommodate the proposed interaction between GC2 and GC1 (Figure 7A). An inability for GC2 to bind Sp1/3 would result in an opening of the promoter structure such that a functional repressive interaction between Sp1/3 bound at GC2 and GC1 would not occur (Figure 7B). This would result in an increase in transcription and could be a possible scenario in the situation where low levels of Sp1/3 are observed. If NF-Y cannot bind at ICB1, less Sp1/3 and/or the uncharacterized protein would be recruited to the promoter, resulting in a decrease in transcription

Figure 7 Putative interactions of NF-Y and Sp1/3 with the human topo II α promoter

(A) Basal transcriptional regulation of human topo II α . NF-Y is shown as bending the promoter at ICB2, and binding at ICB1 such that it could contact Sp1/3 bound at GC1. The uncharacterized protein is depicted as being able to contact both NF-Y and Sp1/3 and may mediate the interaction between NF-Y and Sp1/3 bound at GC2. (B) Loss of the repressive effect of Sp1/3 at GC2. With low levels of Sp1/3 the GC2 would be unoccupied, so that NF-Y bound at ICB1 and Sp1/3 bound at GC1 together with the uncharacterized protein could act as positive regulators of topo II α transcription. When Sp1/3 levels are limiting, preferential binding would occur at the ICB1/GC1 elements because of the physical interaction between the two transcription factors. (C) Loss of proteins binding at ICB1 results in repression of transcription. (D) High levels of Sp1/3 would result in activation of transcription mediated by interactions between GC1 and GC2.

(Figure 7C). When all proteins can bind at ICB1, GC1 and GC2 an increase in transcription would occur (Figure 7D). This could be the scenario when high levels of Sp1 are present. DNA looping and self-association of Sp1 into multimeric complexes to create transcriptional activator function have been reported previously [29,30]. In summary, this model predicts that Sp1 would be a transcriptional activator of topo II α that is recruited to the proximal promoter via an interaction with NF-Y acting also as a transcriptional activator. The strength of this functional interaction would also depend on the presence of the uncharacterized protein and the amount of Sp1/3 present in the cell. Our data from both EMSA and transient transfection assays provide strong evidence of a functional interaction between Sp1/3 and NF-Y. This interaction could be direct or it could be mediated by the uncharacterized protein in Complex III or other protein components of the four complexes identified by EMSA. Sp1 has been shown to be a transcriptional activator of the rat topo II α promoter acting via the GC2 element [14] which is equivalent to the human GC1 element. In some promoter contexts both Sp1 and Sp3 have been shown to be transcriptional activators [31–33], while in another study Sp3 has been shown to be an Sp1-dependent transcriptional repressor [34]. More importantly, the functional interactions between NF-Y and Sp1/3 have implications for the response of tumours to chemotherapy. Sp3 RNA levels were decreased in merberone-resistant CEM cells and this correlated with a down-regulation of topo II α

[35]. Those authors also showed that co-transfection of Sp3 induced topo II α activity in drug-sensitive CEM cells in a dose-dependent manner. Conversely Sp3 levels were shown to be elevated in etoposide/teniposide-resistant KB cells, and this elevation correlated with a decrease in topo II α levels [15]. The differing responses of topo II α expression to Sp3 could either be due to the different drugs used to generate the resistant cells or to a cell-specific response. Decreased levels of NF-Y correlating with a down-regulation of topo II α have been reported in doxorubicin-resistant multiple myeloma cells [16]. NF-Y appears to act as a repressor at the IBC2 element of human topo II α in confluence-arrested Swiss 3T3 cells [7], and as an activator or a repressor of basal transcription at the ICB1 element in HeLa cells (the present study) depending on the ability of Sp1/3 to bind at the GC2 element. It is clear that the DNA-protein and protein-protein interactions occurring at the topo II α promoter are complex and depend on the stage of the cell cycle as well as cell type. Both NF-Y bound at ICB1 and Sp1/3 bound at GC1 and GC2 have essential functional roles in the regulation of topo II α gene expression. It will be important to identify the upstream signalling pathways controlling the binding, activity and interactions between these transcription factors. If there are differences between malignant and normal cells in the activity or regulation of these pathways, this would present an opportunity to not only increase tumour-cell response to chemotherapy, but it may enable us to minimize toxicity to normal cells from drugs which interact with the topo II α promoter. To this end we are currently working towards characterizing the fourth protein contributing to the proposed multiprotein complex regulating topo II α promoter activity.

This work was supported by the Palmerston North Medical Research Foundation and The Cancer Society of New Zealand.

REFERENCES

- Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J. and Hickson, I. D. (1998) Physiological regulation of eukaryotic topoisomerase II. *Biochim. Biophys. Acta* **1400**, 121–137
- Wang, Q. (1996) DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635–692
- Watt, P. M. and Hickson, I. D. (1994) Structure and function of Type II DNA topoisomerases. *Biochem. J.* **303**, 681–695
- Froelich-Ammon, S. J. and Osheroff, N. (1995) Topoisomerase poisons – harnessing the dark side of enzyme mechanism. *J. Biol. Chem.* **270**, 21429–21432
- Pommier, Y. (1993) DNA topoisomerase I and II in cancer chemotherapy: update and perspectives. *Cancer Chemother. Pharmacol.* **32**, 103–108
- Beck, W. T., Danks, M. K., Wolvert, J. S., Kim, R. and Chen, M. (1993) Drug resistance associated with altered DNA topoisomerase II. *Adv. Enzyme Regul.* **33**, 113–127
- Isaacs, R. J., Harris, A. L. and Hickson, I. D. (1996) Regulation of the Human topoisomerase II α gene promoter in confluence-arrested cells. *J. Biol. Chem.* **271**, 16741–16747
- Ng, S. W., Eder, J. P., Schnipper, L. E. and Chan, V. T. (1995) Molecular cloning and characterization of the promoter for the Chinese hamster DNA topoisomerase II α gene. *J. Biol. Chem.* **270**, 25850–25858
- Sandri, M. I., Hochhauser, D., Ayton, P., Camplejohn, R. C., Whitehouse, R., Turley, H., Gatter, K., Hickson, I. D. and Harris, A. L. (1996) Differential expression of the topoisomerase IIa and b genes in human breast cancers. *Br. J. Cancer* **73**, 1518–1524
- Hochhauser, D., Stanway, C. A., Harris, A. L. and Hickson, I. D. (1992) Cloning and characterization of the 5'-flanking region of the human topoisomerase II α gene. *J. Biol. Chem.* **267**, 18961–18965
- Falck, J., Jensen, P. B. and Sehested, M. (1999) Evidence for repressional role of an inverted CCAAT box in cell cycle-dependent transcription of the human DNA topoisomerase II α gene. *J. Biol. Chem.* **274**, 18753–18758
- Adachi, N., Nomoto, M., Kohno, K. and Koyama, H. (2000) Cell-cycle regulation of the DNA topoisomerase II α promoter is mediated by proximal CCAAT boxes: possible involvement of acetylation. *Gene* **245**, 49–57
- Furukawa, M., Uchiyama, T., Nomoto, M., Takano, H., Morimoto, R. I., Naito, S., Kuwano, M. and Kohno, K. (1998) The role of an inverted CCAAT element in transcriptional activation of the human DNA topoisomerase II α gene by heat shock. *J. Biol. Chem.* **273**, 10550–10555
- Yoon, J. H., Kim, J. K., Rha, G. B., Oh, M., Park, S. H., Seong, R. H., Hong, S. H. and Park, S. D. (1999) Sp1 mediates cell proliferation-dependent regulation of rat DNA topoisomerase II α gene promoter. *Biochem. J.* **344**, 367–374
- Kubo, T., Kohno, K., Ohga, T., Taniguchi, K., Kawanami, K., Wada, M. and Kuwano, M. (1995) DNA topoisomerase II α gene expression under transcriptional control in etoposide/teniposide-resistant human cancer cells. *Cancer Res.* **55**, 3860–3864
- Wang, Q., Zambetti, G. P. and Suttle, D. P. (1997) Inhibition of DNA topoisomerase II α gene expression by the p53 tumor suppressor. *Mol. Cell. Biol.* **17**, 389–397
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59
- Herbomel, P., Bourachot, B. and Yaniv, M. (1984) Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell (Cambridge, Mass.)* **39**, 653–662
- Ladias, J. A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V. and Cladaras, C. (1992) Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3. *J. Biol. Chem.* **267**, 15849–15860
- Zwicker, J., Gross, C., Lucibello, F. C., Truss, M., Ehler, F., Engeland, K. and Muller, R. (1995) Cell cycle regulation of cdc25C transcription is mediated by the periodic repression of the glutamine-rich activators NF-Y and Sp1. *Nucleic Acids Res.* **23**, 3822–3830
- Roder, K., Wolf, S. S., Beck, K. F. and Schweizer, M. (1997) Cooperative binding of NF-Y and Sp1 at the DNase I-hypersensitive site, fatty acid synthase insulin-responsive element 1, located at –500 in the rat fatty acid synthase promoter. *J. Biol. Chem.* **272**, 21616–21624
- Roder, K. H., Wolf, S. S. and Schweizer, M. (1997) Interaction of Sp1 and NF-Y in the diet-induced regulation of the rat fatty acid synthase (FAS) gene. *Biochem. Soc. Trans.* **25**, 72S
- Inoue, T., Kamiyama, J. and Sakai, T. (1999) Sp1 and NF-Y synergistically mediate the effect of vitamin D(3) in the p27(Kip1) gene promoter that lacks vitamin D response elements. *J. Biol. Chem.* **274**, 32309–32317
- Ronchi, A., Bellorini, M., Mongelli, N. and Mantovani, R. (1995) CCAAT-box binding protein NF-Y (CBF, CP1) recognizes the minor groove and distorts DNA. *Nucleic Acids Res.* **23**, 4565–4572
- Framson, P. and Bornstein, P. (1993) A serum response element and a binding site for NF-Y mediate the serum response of the human thrombospondin 1 gene. *J. Biol. Chem.* **268**, 4989–4996
- Reith, W., Siegrist, C. A., Durand, B., Barras, E. and Mach, B. (1994) Function of major histocompatibility complex class II promoters requires cooperative binding between factors RFX and NF-Y. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 554–558
- Hasan, S., Koda, T. and Kakinuma, M. (1994) An upstream NF-Y-binding site is required for transcriptional activation from the hst promoter in F9 embryonal carcinoma cells. *J. Biol. Chem.* **269**, 25042–25048
- Mantovani, R. (1999) The molecular biology of the CCAAT-binding factor NF-Y. *Gene* **239**, 15–27
- Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P. and Hough, P. V. (1991) DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5670–5674
- Su, W., Jackson, S., Tjian, R. and Echols, H. (1991) DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. *Genes Dev.* **5**, 820–826
- Li, J. M., Nichols, M. A., Chandrasekharan, S., Xiong, Y. and Wang, X. F. (1995) Transforming growth factor β activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site. *J. Biol. Chem.* **270**, 26750–26753
- Biggs, J. R., Kudlow, J. E. and Kraft, A. S. (1996) The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemic cells. *J. Biol. Chem.* **271**, 901–906
- McEwen, D. G. and Ornitz, D. M. (1998) Regulation of the fibroblast growth factor receptor 3 promoter and intron 1 enhancer by Sp1 family transcription factors. *J. Biol. Chem.* **273**, 5349–5357
- Hagen, G., Muller, S., Beato, M. and Suske, G. (1994) Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO J.* **13**, 3843–3851
- Mo, Y. Y., Wang, Q. and Beck, W. T. (1997) Down-regulation of topoisomerase II α in CEM cells selected for merbarone resistance is associated with reduced expression of Sp3. *Cancer Res.* **57**, 5004–5008