Sp1 mediates cell proliferation-dependent regulation of rat DNA topoisomerase IIα gene promoter

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DNA topoisomerase IIα (topo IIα) is an essential nuclear enzyme required for chromosome segregation during mitosis. Consistent with its critical role in cell division is the fact that the expression of the gene for topo IIα is strongly regulated by the proliferation state of cells. Using a transient expression system, we determined the contribution of putative *cis*-acting elements in its promoter region to its basal level and cell proliferation-dependent transcription. Experiments with 5′ and/or 3′ serial deletion and site-directed mutation revealed that (1) maximal promoter activity resides in the fragment extending to position −663 bp from the ATG initiation codon, (2) minimal promoter activity is harboured at −195 bp, (3) the defined minimal promoter contains only two putative elements, inverted CCAAT box 4 (ICB4) (−166 to −162 bp) and the most proximal GC-rich box in the promoter (GC2) (−149 to −143 bp), and (4) ICB4 is most important in the basal-level transcription of the gene for rat topo IIα. The luciferase activities of the mutated reporter plasmids in G_0-arrested and exponentially growing cells showed that proliferation-specific regulation is controlled mainly by GC2. Electrophoretic mobility-shift assays indicated that Sp1 binds specifically to the GC2 site. The extent of DNA–protein complex formation increases after the stimulation of cells to proliferate. These results indicate that the increased binding activity of Sp1 to GC2 is important in the up-regulation of the gene for topo IIα in growing cells.

Key words: *cis*-acting elements, basal-level transcription, up-regulation.

INTRODUCTION

Eukaryotic DNA topoisomerase II (topo II) enzymes have similar structures and biochemical properties [1,2]. The homodimer consisting of 150–180 kDa subunits changes the topology of DNA by generating double-strand breaks and transporting another duplex DNA. Yeast and *Drosophila melanogaster* express only one type of topo II protein that is essential for cell viability, required for chromosome segregation at the time of cell division [3,4]. In avian and mammalian species, topo II exists in two isoforms that are closely related but genetically distinct [5–7]: the 170 kDa topo IIα and the 180 kDa topo IIβ. Despite their high degree of similarity, the two isoforms are differentially regulated and have distinct biochemical properties and probably different functions [7–10]. However, their respective roles are unclear at present. The cellular concentration of topo IIα, but not that of topo IIβ, is generally correlated with the cell proliferation state [11–13]. The two isoforms are differentially expressed during the cell cycle: the expression level of topo IIα protein is shown to be maximal in G_2/M and minimal in G_1, whereas topo IIβ is expressed at a constant level [11–13].

The 5′-upstream sequences of the human, hamster, mouse and rat genes for topo IIα have been isolated and analysed for basal promoter activity [14–17]. The overall nucleotide sequences of these promoters are similar: they are moderately rich in GC and have no TATA box. These characteristics have commonly been found in the promoters of housekeeping genes [18]. In addition, multiple CCAAT boxes with inverted orientation and GC boxes were located within the region near –700 bp. Inverted CCAAT boxes (ICBs) are important for basal-level transcription and one ICB has been found to mediate down-regulation during contact inhibition [19,20]. NF-Y, a CCAAT-box-binding protein, recognizes these ICBs. Adachi et al. [17] showed that the 5′-upstream region of the mouse gene for topo IIα spanning − 850 bp has cell-cycle-dependent promoter activity. However, Goswami et al. [21] have reported that the level of topo IIα mRNA during the cell cycle parallels that of the protein and that the mRNA level is regulated by changes in mRNA stability with a half-life of 30 min in G_1 and over 4 h in late S-phase. It is possible that changes in both the rate of transcription and the stability of mRNA are responsible for cell-cycle-regulated topo IIα expression.

Genetic and biochemical approaches have been successful in determining functional roles for topo II enzymes. However, the mechanisms of its transcriptional regulation at the promoter level are just beginning to be elucidated. In this study, the cell-proliferation-dependent transcriptional regulation of the gene for rat topo IIα was analysed at the molecular level to understand the mechanism of expression of the mammalian gene during cell division.

Abbreviations used: AP1, activator protein 1; ATF, activating transcription factor; C/EBP, CCAAT-enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; GC2, the most proximal GC-rich box in the promoter; ICB, inverted CCAAT box; topo II, DNA topoisomerase II.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Z29676.

We investigated the *cis*-acting elements and their cognate binding factors involved in the basal level and the growth-state-dependent regulation of the expression of the gene for topo IIα. To elucidate the mechanisms, we transfected a series of reporter plasmids containing various additions or site-specific mutations in the promoter region and the luciferase gene into NIH 3T3 cells. We found that ICB4 is critical for basal-level transcription and that the most proximal GC-rich box in the promoter 2 (GC2), has a role in cell-proliferation-dependent expression of the gene for topo IIα. With the use of an electrophoretic mobility-shift assay (EMSA), we show that Sp1 binds specifically to GC2 and that its binding activity increases after growth stimulation.

MATERIALS AND METHODS

Construction of luciferase reporter plasmids

To generate reporter plasmids containing 5′-serially deleted promoter sequences, blunt-ended PCR was performed with appropriate upstream primers. Each amplified DNA was phosphorylated and inserted into the *Sma*I site of the promoterless luciferase reporter plasmid pGL3-BASIC (Promega Corp., Madison, WI, U.S.A.). Plasmids with 3′ deletion were also generated by the PCR-based cloning method. For example, the −663/−133 plasmid was prepared as follows with the −663/−11 plasmid as a template, and PCR was performed with RV3 and Rpro133 primers. The PCR product was digested with *Kpn*I and subcloned into pGL3-BASIC predigested with *Kpn*I and *Sma*I. Mutations were introduced into the promoter region of the gene for rat topo IIα, as described by Ho et al. [22]. The first PCR was performed with a combination of RV3/MR-series primers or MF-series primers/GL2. After elution, the first PCR product was used as a template DNA for the second PCR with RV3 and GL2 primers. The second PCR product, digested with *Kpn*I and *Bgl*II, was cloned into pGL3-BASIC.

Table 1 Oligonucleotides used for the construction of luciferase reporter plasmids and EMSA

Mutated sequences are in bold and underlined.

Name	Sequence (5′ → 3′)	Corresponding region
Fpro2765	CTCGAGGATCTGAGTTCA	−2765 to −2748
Fpro1713	CAGGACAGAGGACAGGAG	−1713 to −1697
Fpro1163	GTGGTGTCGACACACTAGC	−1163 to −1146
Fpro663	GCCGTGACGAATTGTAGC	−663 to −647
Fpro417	AGTTTAGTCGACCGCTTTGC	−417 to −398
Fpro236	AGACCTTTGTGACGTAAAAGACCGT	−236 to −212
Fpro195	GATTGCTGTCGACAGAGCAA	−195 to −176
Fpro138	CGGGCTAGTCGACTGTTCAA	−138 to −119
Rpro11	CTGAAGGGGCTCGAGAATCC	−11 to −30
Rpro170	CGACTCGCTCTCATTTGCTCTGTT	−170 to −193
Rpro152	ATCAGAGGAACCAATCACCGACT	−152 to −174
Rpro133	ATTCATCTAGCCCGCCCAAATCA	−133 to −155
RV 3	CTAGCAAAATAGGCTGTCCC	pGL vector upstream
GL 2	CTTTATGTTTTTGGCGTCTTCCA	pGL vector downstream
WF/ICB4	GAGCGAGTCGGTGATTGGTTCCTCTGATTT	−179 to −150
WR/ICB4	CAAATCAGAGGAACCAATCACCGACTCGCT	−151 to −178
MF/ICB4	AGTCGGTGATT**CC**TTCCTCTGAT	−174 to −152
MR/ICB4	AATCAGAGGAA**GG**AATCACCGAC	−151 to −173
WF/CEBP	AGACCGTCTGCGATTGATTGCTGTAAACA	−218 to −188
WR/CEBP	TCTGTTTACAGCAATCAATCGCAGACGGT	−186 to −216
MF/CEBP	CGTCTGCGATT**CC**TTGCTGGTAA	−214 to −191
MR/CEBP	TTTACCAGCAA**GG**AATCGCAGAC	−190 to −213
WF/GC2	TCTGATTTGGGCGGGCTAGATGA	−157 to −135
WR/GC2	ATTCATCTAGCCCGCCCAAATCA	−133 to −155
MF/GC2	CCTCTGATTTG**T**GCG**T**GCTAGATGAA	−159 to −134
MR/GC2	ATTCATCTAGC**A**CGC**A**CAAATCAGAG	−133 to −158
WF/ICB3	CCTTTACCTAATTGGTTCATTCGAA	−281 to −257
WR/ICB3	TTTTCGAATGAACCAATTAGGTAAA	−255 to −279
MF/ATF	AGACCTTTGTG**C**C**CC**AAAAGACCGT	−236 to −212
MR/ATF	GACGGTCTTTT**GG**G**G**CACAAAGGTC	−211 to −235

Cell culture, transfection and luciferase assay

Mouse fibroblast NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were plated at a density of 2×10^6 cells in a 60 mm dish and incubated for 24 h at 37 °C under humidified air/CO_2 (19:1). NIH 3T3 cells were transfected with 5 μg of luciferase reporter construct by the calcium phosphate method, as described previously [23]. To correct for variability in transfection efficiency, 1 μg of pCMV-β-GAL (in which CMV and β-GAL stand for cytomegalovirus and β-galactosidase respectively) (Stratagene, La Jolla, CA, U.S.A.) was co-transfected in all experiments. Each construct was transfected at least three times; each transfection was done in triplicate. Cell extracts were prepared by harvesting cells with Reporter Lysis Buffer (Promega). For luciferase assays, cell lysates were mixed with Luciferase Assay Reagent (Promega) and the light intensity was measured for 30 s with a luminometer (Lumat LB9501; Berthold, Oak Ridge, TN, U.S.A.). Measurements of β-galactosidase activity were performed in accordance with the instructions provided by Stratagene. Standardization with β-galactosidase activity was performed as described by Sambrook et al. [24].

For measuring basal promoter activity, the transfected cells were incubated for 20 h. After two washes in PBS, the medium was replaced with the complete medium and cells were harvested by gentle scraping after 24 h. In experiments with serum-stimulated cell proliferation, transfected cells were incubated for 20 h and then incubated in the starving medium containing 0.5% FBS for 40–48 h before cell-cycle progression with 15% FBS for 18 h.

Statistical significance was assessed by Student's t test on data pooled from three or more separate transfections. Differences were considered significant at $P < 0.05$. Values are given as means ± S.E.M.

Preparation of synthetic deoxyoligonucleotides

Synthetic deoxyoligonucleotides (listed in Table 1) were provided by Genosys Biotechnology (Woodland, TX, U.S.A.).

Flow cytometry analysis

Cells were collected, fixed with 70% (v/v) ethanol and treated with 1 mg/ml RNase A. They were stained with 50 μg/ml propidium iodide and the DNA content of individual cells was analysed with a flow cytofluorometer (FACStar^{PLUS}; Becton Dickinson, Franklin Lakes, NJ, U.S.A.). The percentage of cells in each phase of the cell cycle was calculated with a computer program supplied by the manufacturer.

EMSA

A probe was made from double-stranded oligonucleotides containing the putative *cis*-acting element. The probe was radio-

labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Unincorporated isotope was removed with a G50 spin column (Amersham Pharmacia Biotech, Uppsala, Sweden). A typical reaction mixture contained 2–3 fmol of labelled DNA probe (20000 c.p.m.), 1 μg of poly(dI-dC)·poly(dI-dC), 20 mM Hepes pH 7.9, 60 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol, 1 mM PMSF and 5 μg of crude nuclear extract in the final volume of 30 μl. Crude nuclear extracts were prepared from NIH 3T3 cells as described by Andrew and Faller [25]. The binding reaction was performed at room temperature for 15 min. Subsequently, each sample was subjected to electrophoresis through 5% non-denaturing polyacrylamide gel in 0.5 × TBE buffer (44 mM Tris base/44 mM boric acid/1 mM EDTA). Before electrophoresis, polymerized EMSA gel was pre-run at room temperature for 1 h. Gel electrophoresis was performed, at room temperature, at 15 V/cm for 2 h without buffer recirculation. The gel was dried and analysed by autoradiography.

For competition experiments, the reaction mixture was incubated with unlabelled oligonucleotides for 15 min before the addition of radiolabelled probe. In the supershift assay, goat polyclonal IgG against Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the reaction mixture after the addition of the probe.

RESULTS

Sequence analysis of the promoter region of the gene for topo IIα

Previously, we reported the promoter activity and sequence of the −663 bp region (from the ATG translation initiation codon) of the gene for rat topo IIα [16]. Because other upstream *cis*-elements could be involved in the transcriptional regulation of this gene, we isolated a genomic clone containing the 2.7 kb 5′-upstream region and determined its complete nucleotide sequence. A partial nucleotide sequence of the topo IIα promoter is shown in Figure 1. Within the −665 bp region there are two GC boxes at −289 and −149 bp (putative Sp1-binding site), one of which is in the reverse orientation. Four copies of a CCAAT motif (at −348, −311, −271 and −166 bp), all of which are in the reverse orientation (ICB), and activator protein 1 (AP1) (at −244 bp), activating transcription factor (ATF) (at −227 bp) and a CCAAT-enhancer-binding protein (C/EBP)-binding site (at −207 bp) were found in succession. Several potential *cis*-acting elements were also found in the region between −2765 and −665 bp.

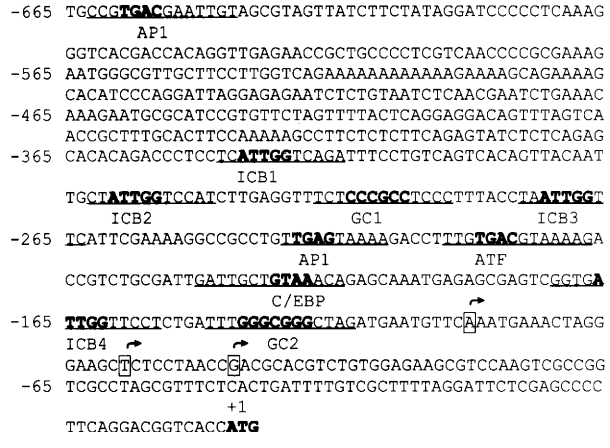

Figure 1 Partial nucleotide sequence of 5′-upstream DNA of the gene for rat topo IIα

Bases are numbered at the left with respect to the translation initiation codon ATG, which was designated +1. Putative regulatory elements, GC boxes, ICBs, ATF and AP1 factor-binding sites are underlined and annotated below; core sequences for binding are shown in bold. Three major transcription start sites are boxed and indicated by arrows.

Localization of important elements for basal-level transcription

To delineate DNA sequences that were important for basal-level promoter activity, NIH 3T3 cells were transfected with a series of luciferase reporter plasmids containing various lengths of the rat topo IIα 5′-flanking sequences (Figure 2). The basal expression of luciferase was essentially unchanged with deletions between −2765 and −1713 bp. There was a 30% decrease in basal promoter activity with deletion to −1163 bp. The −663/−11 construct directed the highest level of luciferase activity. These observations suggested the presence of negative elements between −1163 and −663 bp. Plasmid −195/−11, lacking three of the four ICBs, the C/EBP, ATF and AP1 sites, retained 60% of promoter activity relative to the activities of plasmids −2765/−11 and −1713/−11. Although there was a gradual decrease in basal activity with deletion between −663 and −195 bp, a minimal promoter region can be defined as the sequences up to −195 bp. Further deletion to 138 bp (plasmid −138/−11) resulted in a substantial loss of basal expression. Hence the most important positive element was found to reside within −195 to −138 bp. Because this region contained two potent *cis*-acting elements (ICB4 and GC2), three plasmids with the same 5′ end and different 3′ ends (−663/−133, −663/−152, and −663/−170) were tested to assess in detail the functions of each element.

When ICB4, residing between −166 and −162 bp, was deleted (plasmid −663/−170), approx. 50% of the promoter activity was lost. In contrast, plasmid −663/−152, lacking GC2 (−149 to −143 bp), did not show decreased activity. Two other plasmids with 5′ and 3′ deletions, −195/−134 and −195/−150, were prepared and analysed further. Plasmid −195/−134 had approx. 80% luciferase activity compared with that of the −195/−11 construct. In contrast, the −195/−150 construct directed very low promoter activity, suggesting that ICB4 alone was not sufficient for promoter activity and that additional elements were required. Taken together, these results indicate the presence of positive regulatory elements in the regions −663 to −417 bp (containing AP1), −417 to −195 bp (containing ICB1–3, GC1, ATF, C/EBP and AP1) and −195 to −152 bp (containing ICB4). The elements within all three areas seemed to be necessary for maximal topo IIα promoter activity for basal-level transcription; however, the ICB4 element might be the most important because deletion of this region caused the most marked decrease in promoter activity.

ICB4 is a major regulatory element for basal-level transcription

To show this critical function of ICB4 further, ICB4 and other elements within −663 to −11 bp were mutated in the plasmid −663/−11 to generate plasmids mutATF, mutC/EBP, mutICB4, mutGC2 and mutICB4/GC2 by PCR-based site-directed mutagenesis. Figure 3 shows that the transcription of the reporter gene with mutated ICB4 was decreased to 55% compared with the wild-type −663/−11. No significant decrease in transcription was observed when ATF or C/EBP was mutated. Plasmid mutICB4GC2, with mutated ICB4 and GC2, showed similar activity to that of mutICB4. This finding suggests that

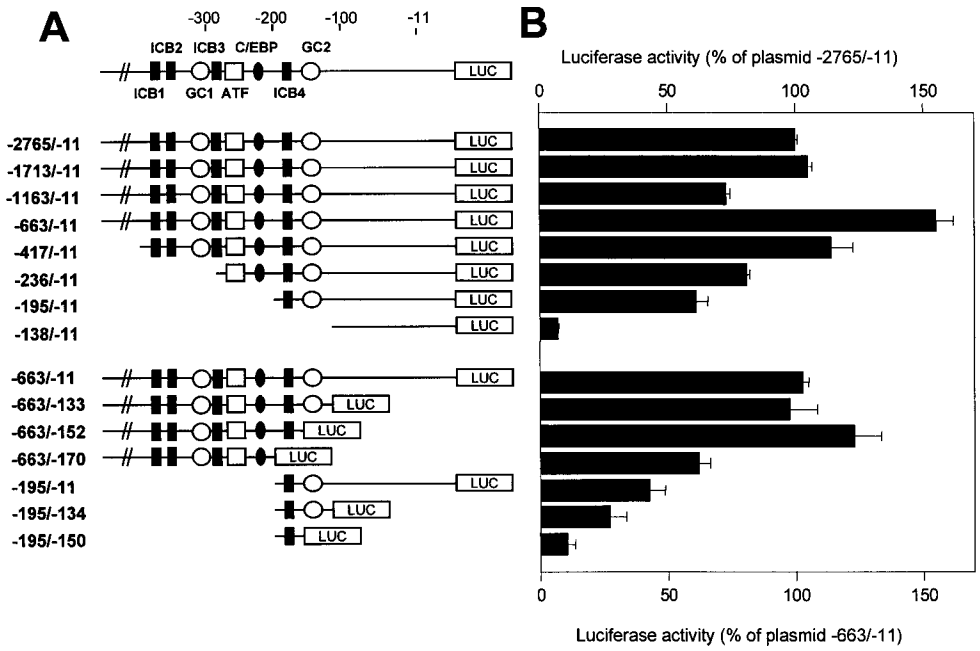

Figure 2 Basal promoter activities of 5′ and/or 3′ serially deleted reporter constructs

(**A**) Plasmids containing various lengths of the 5′-flanking sequence of the gene for topo IIα and the luciferase (LUC) reporter gene. The *cis*-acting elements are indicated by boxes and circles. Each plasmid (5 μg per 60 mm dish) was transfected into NIH 3T3 by the calcium phosphate method. For each construct, the plasmid pCMV-βGAL was co-transfected to correct for differences in transfection efficiency. Luciferase and β-galactosidase activities were measured and the relative activities of luciferase were calculated. (**B**) The luciferase activity (mean ± S.E.M.) of each plasmid expressed as a percentage of the positive control (plasmid −2765/−11 or plasmid −663/−11).

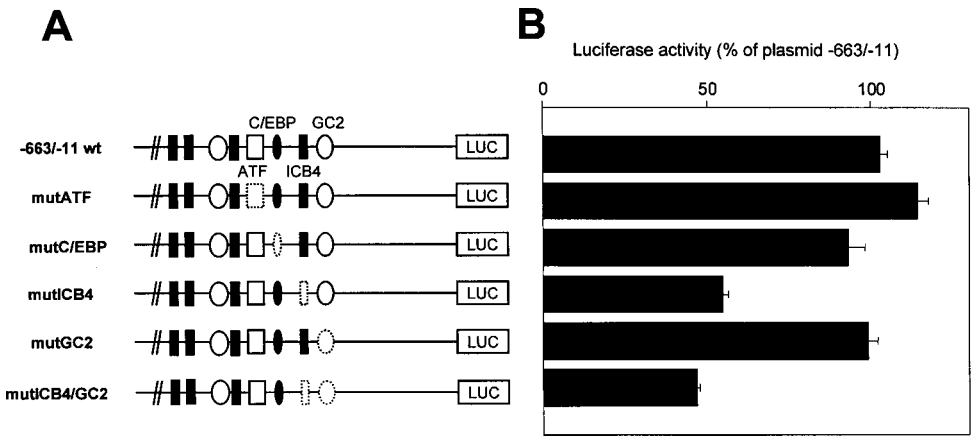

Figure 3 Mutation analysis of putative *cis*-acting elements in promoter activities of the gene for rat topo IIα in basal-level transcription

(**A**) Plasmids containing various mutations in *cis*-acting elements of gene for the promoter of the rat topo IIα gene and the luciferase (LUC) reporter gene. The elements drawn in dotted lines represent inactivation. (**B**) The activity of each mutated plasmid as a percentage of that of the wild-type plasmid −663/−11.

ICB4 is the critical positive element for the basal-level transcription of the gene for rat topo IIα.

Cell proliferation-dependent expression of the gene for topo IIα

Although the expression of the gene for topo IIα seems to be closely related to growth states and the cell cycle, the identity of the elements mediating this regulation is not known. To this end, a series of luciferase reporter constructs with 5′ and/or 3′ truncations or mutations were tested in a transient expression assay in both G_0-arrested and growing cells.

The synchronization of NIH 3T3 cells at G_0 was achieved by serum withdrawal for 40 h; cell arrest was released by including 15% (v/v) FBS in the medium. The change in cell cycle distribution during serum stimulation is shown in Figure 4A. The percentage of cells in G_0/G_1 was not altered until 12 h after the serum replenishment. The number of cells in $S/G_2/M$ were markedly increased at 14 h and reached a maximum at 18 h. The cell population in $S/G_2/M$ was decreased after 18 h. At 24 h after treatment, flow cytometry showed the ordinary cell cycle pattern described by Okazaki et al. [26]. A time course of topo IIα promoter activity corresponded well to these results (Figure

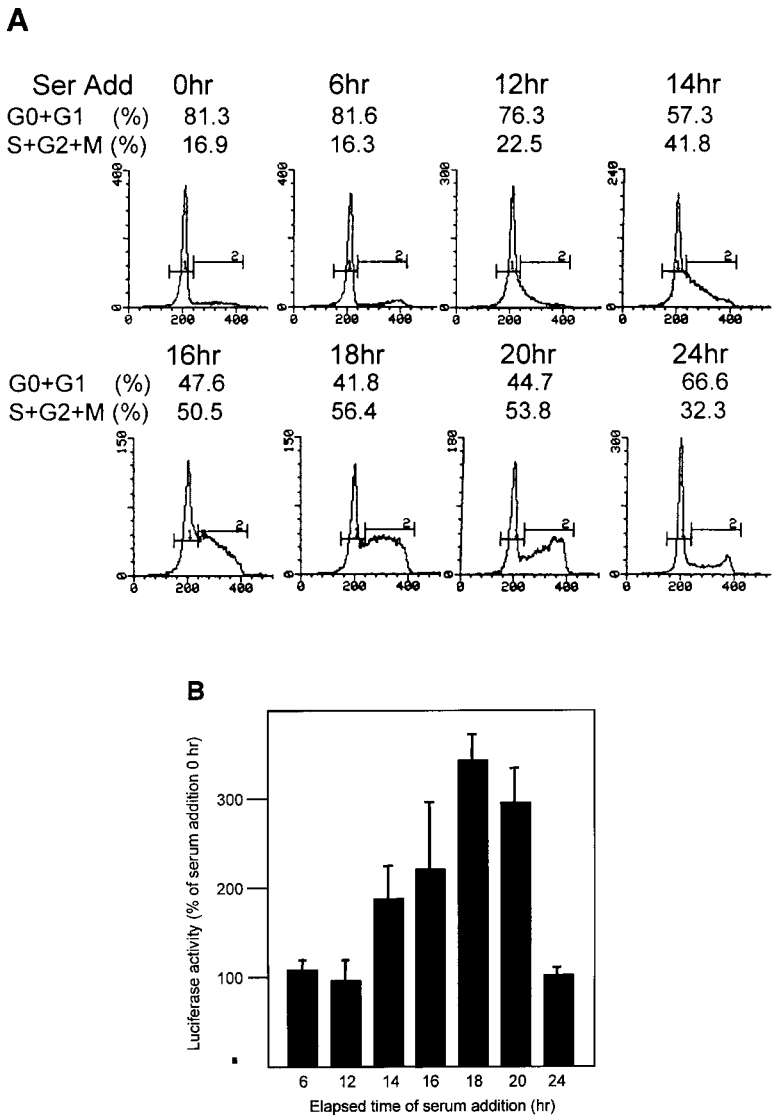

Figure 4 Cell cycle analysis and time course of expression of the gene for topo IIα after serum replenishment

(**A**) Flow cytometry analysis of NIH 3T3 cells. At each time point, cells were collected and their DNA content was analysed by flow cytometry. The percentage of cells in different phases of the cell cycle are indicated. (**B**) NIH 3T3 cells were transfected with −2765/−11 plasmid. After 20 h, cells were incubated with starvation medium for 40 h followed by a new medium containing 15% serum for various durations. The relative ratio was calculated from the luciferase activity in growing cells with respect to that in starved cells.

4B). Expression of the reporter gene was maximal at 18 h after serum replenishment and decreased at 24 h. These observations indicated that this experimental system served a good model to investigate the cell-proliferation-dependent regulation of expression of the gene for topo IIα.

DNA region spanning −152 to −138 bp mediates up-regulation in growing cells

To determine whether the positive elements in the −663/−417, −417/−195 and −195/−152 sequences were involved in cell-proliferation-dependent regulation, a series of truncated luciferase reporter plasmids were transfected into NIH 3T3 cells and analysed. For each construct the ratio of luciferase activity in growing cells to that in G_0-arrested cells was calculated (Figure 5). Results for the 5′-deleted constructs showed that the luciferase activity in growing cells was higher (approx. 3-fold) for all plasmids containing the rat topo IIα promoter except for plasmid −138/−11. This indicated that the sequence within −195 to −138 bp, containing ICB4 and GC2, could control the expression of the gene for topo IIα in a proliferation-specific manner. This finding was similar to the results of basal-level transcription. Plasmid −663/−152, lacking only GC2, showed significantly decreased induction. In addition, plasmid −663/−170 showed a similar degree of induction, although luciferase activities in both growing and resting cells were 50% of that of −663/−11. These results suggested that GC2 is important for the up-regulation of topo IIα in growing cells.

GC2 is a critical element for up-regulation in growing cells

To confirm the above result, plasmids with mutated sequences were tested (Figure 6). The five plasmids with mutations were transfected; cells were then harvested as described above. The

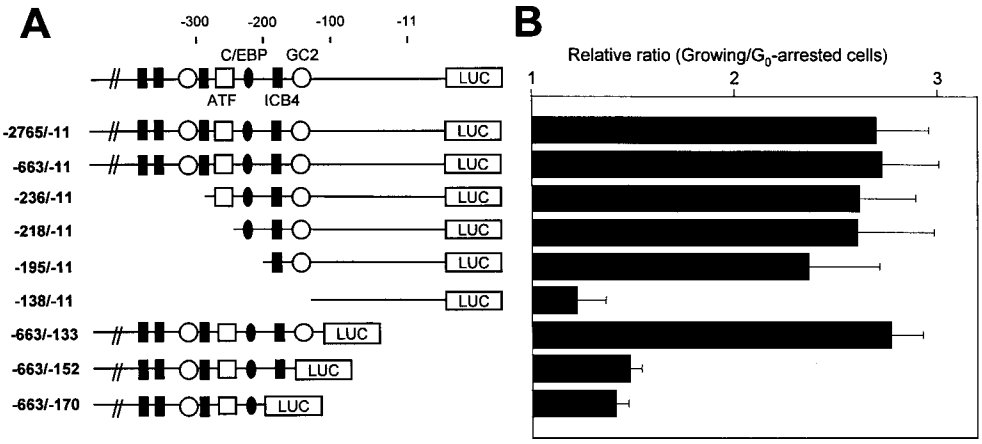

Figure 5 Cell proliferation-dependent regulation of rat topo IIα promoter

At 20 h after transfection, the medium was replaced with starvation medium containing 0.5% (v/v) FBS to arrest at G_0. After 40–48 h, the medium was replaced with medium containing 15% (v/v) FBS to stimulate proliferation. The cells were harvested after 18 h. (**A**) Schematic representation of structures of reporter plasmids. (**B**) Ratio of the luciferase activity in growing cells to that in G_0-arrested cells.

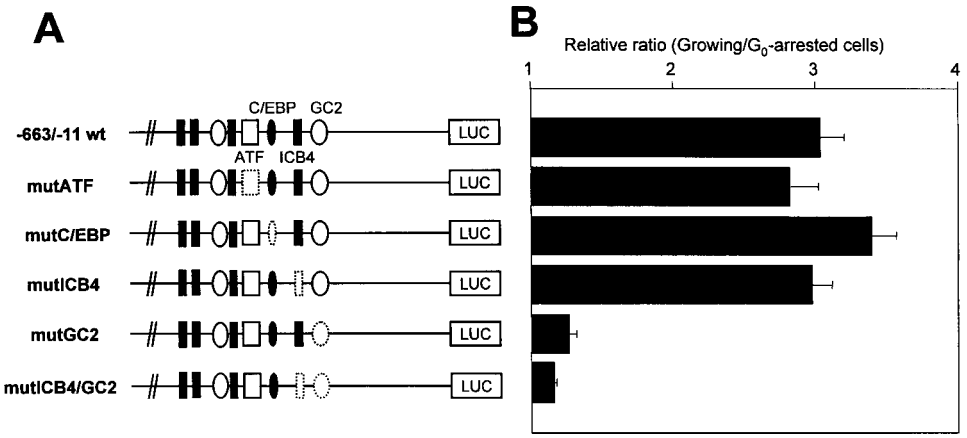

Figure 6 Mutation analysis of putative *cis*-acting elements of the promoter for the gene for rat topo IIα in cell-proliferation-dependent expression

(**A**) Plasmid containing various mutations of *cis*-acting elements of the promoter for the gene for rat topo IIα and the luciferase (LUC) reporter gene. The elements drawn in dotted lines represent inactivation. (**B**) Ratio of the luciferase activity in growing cells to that in G_0-arrested cells.

luciferase activities of mutGC2 and mutICB4/GC2 were found not to be increased in growing cells. In addition, the luciferase activities of other mutated constructs in growing cells were not significantly different from those in G_0-arrested cells. Thus the GC2 sequence was a major activation element in growing cells. ICB4 seemed to function as a basal enhancer and facilitated maximal up-regulation. For the full activation of expression of the gene for rat topo IIα in growing cells, both GC2 and ICB4 were required.

Sp1 progressively binds to the GC2 element after stimulation of cell growth

Because GC2 was shown to be involved in the activation of transcription of the gene for topo IIα in growing cells and that GC2 had a putative Sp1-binding site, EMSA was performed with a radiolabelled double-stranded oligonucleotide (−157 to −135 bp) spanning GC2. Nuclear extracts from NIH 3T3 cells at 18 h after serum addition contained a factor that bound to this oligonucleotide (Figure 7A, lane 2, labelled 'w/o'). To investigate the binding specificity of nuclear protein to the GC2 oligonucleotide, competition assays were also performed. Adding unlabelled GC2 (Figure 7A, lanes 3–6), but not the oligonucleotide containing mutated GC2 (Figure 7A, lanes 7–10), efficiently eliminated this binding. The Sp1 oligonucleotide harbouring the consensus sequence for the Sp1-binding site effectively displaced the labelled probe, although it was more efficient as a competitor (Figure 7A, lanes 11–13).

Supershift analyses with anti-Sp1 antibody indicated that the nuclear protein binding to GC2 is a ubiquitous transcription factor, Sp1 (Figure 7B). To determine whether the binding of Sp1 could be growth-regulated, nuclear extracts were prepared from NIH 3T3 cells at various time points during growth stimulation and EMSA was performed. Nuclear extracts from resting cells produced weak Sp1 binding. The binding increased as growth-stimulated cells entered the cell cycle, reaching 2- and 3-fold enhancement at 14 and 18 h respectively.

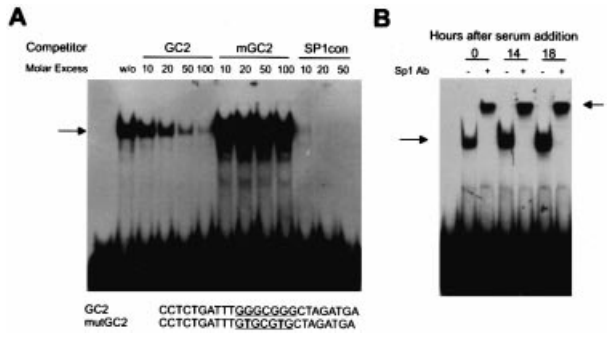

Figure 7 Formation of DNA–protein complex by using an oligonucleotide containing a GC2 site

(**A**) Competitive EMSA with GC2 oligomer with the use of an Sp1 consensus oligomer as a competitor. Nuclear extracts were prepared from NIH 3T3 cells 18 h after serum addition. Labelled double-stranded oligonucleotide containing GC2 (−149 to −143 bp) was mixed with the nuclear extracts (approx. 5 μg), which had been preincubated with the indicated molar excess of self (GC2), mutated GC2 (mGC2) or Sp1 consensus oligomer (SP1CON, Stratagene). Competitors were used in 10-fold, 20-fold, 50-fold and 100-fold molar excesses as indicated above. The DNA–protein complex and the free DNA probe were separated on a 5% (w/v) non-denaturing polyacrylamide gel. Lane 1 contained no extract. The DNA–protein complex is indicated by the arrow. The nucleotide sequences of GC2 and mutGCS2 are shown at the bottom. Binding core sequences are underlined and the mutated bases are shown in bold. (**B**) EMSA with GC2 by using nuclear extracts from cells at different time points after serum addition. Supershifted bands obtained with nuclear extracts as indicated were preincubated with Sp1 polyclonal antibody. The DNA–protein complex is indicated by the arrow. Lane 1 contained no extract.

DISCUSSION

The control of transcription of the gene for topo IIα has a pivotal role in cancer chemotherapy and cell cycle progression. Here we show that the transcription rate of topo IIα in growing cells is higher than that in non-growing cells (Figure 4). This observation is consistent with earlier reports that the levels of topo IIα mRNA reflected the cell's growth state and were related to the cell cycle [17,20,21]. However, the exact sequences of positive regulatory elements and their cognate DNA-binding proteins were not delineated.

A number of potential transcription-factor-binding sites exist in the 630 bp region upstream of the translation start codon, including two GC boxes (putative Sp1-binding site). One GC box (−149 to −143 bp) is close to the transcription initiation site within the defined minimal promoter (−195 bp; Figure 2) and the other is located between −289 and −284 bp in an inverted orientation. It is relatively common for at least one GC box to be located within the promoter of a housekeeping gene [18] and GC boxes are functional in the inverted orientation [27–29]. There are slightly more CpG than GpC dinucleotides in the region containing the minimal promoter and the 5′-untranslated sequences of the gene for topo IIα, whereas the CpG dinucleotide is greatly under-represented in genomic sequences lacking a regulatory function [30]. The gene for rat topo IIα, like that for human topo IIα, has several ICBs in an inverted orientation, one of which is within the minimal promoter region.

Luciferase reporter gene transfection experiments identified positive regulatory elements within the promoter that were important for basal topo IIα transcription at −663 to −417 bp (containing AP1), −417 to −195 bp (containing ICB1–3, GC1, ATF, C/EBP and AP1), and −195 to −138 bp (containing ICB4 and GC2) (Figure 2). The minimal promoter was mapped to within the region near −195 bp. The fragment extending to −663 bp consistently directed higher luciferase activity, suggesting that the sequences between −2765 and −663 bp influence the promoter activity in a negative fashion. In the gene for human topo IIα, the maximum promoter activity was observed in a region including −557 bp from the transcription start site; the minimal promoter region was defined as the sequence extending 90 bp upstream of the major transcription start site [14]. In this paper, both the minimal and maximal promoters of rat topo IIα were mapped to similar regions to those in human topo IIα.

Deletion and mutation analyses indicated that ICB4, within −166 to −162 bp, was the most important element for basal-level transcription (Figures 2 and 3). A comparison of the rat and human promoter sequences revealed that ICB4 corresponded to ICB1 of the human promoter. Ng et al. [15] and Isaacs et al. [20] have shown that ICBs are functionally important for transcriptional regulation in Chinese hamster ovary cells and Swiss 3T3 cells respectively. Therefore ICB in the mammalian topo IIα promoter seems to act as a major regulatory element in basal-level transcription.

ICB is present in many cellular housekeeping and tissue-specific promoters [31–40]. The CCAAT sequence is positioned at approx. 60–80 bp upstream of the transcription start site in the above genes and has been shown to be important in their transcription. More than half a dozen different factors that recognize this CCAAT motif have been described, including C/EBP [41], NF1/CTF [42], NF-Y (also known as CBF, CP1 and YEBP [43,44]) and ACF [45]. NF1 binds as a homodimer and C/EBP can bind both as a homodimer and as a heterodimer [46]. NF-Y is clearly distinct from the other known CCAAT-box-binding proteins in that it is composed of A, B and C subunits of 42, 36 and 40 kDa respectively [47,48]. The ICBs of the mouse topo IIα promoter are recognized by NF-Y [19,20]. EMSA with ICBs used in this study confirmed that NF-Y binds to ICB (results not shown). This strongly indicates that NF-Y is a key *trans*-acting factor in controlling the basal-level transcription of the mammalian gene for topo IIα.

Isaacs et al. [20] have demonstrated that ICB2 (−108 to −104 bp) has important roles in the down-regulation of the human topo IIα promoter during contact inhibition and that NF-Y mediates this regulation through DNA–protein complex formation. In contrast, the present study revealed that a GC box (GC2), but not ICB, mediates the regulation of topo IIα in a cell-growth-specific manner (Figures 5 and 6) and that ICB4 might simply amplify the Sp1 action on the gene for topo IIα. In addition, the binding activity of Sp1 to GC2 increases during growth stimulation (Figure 7B). Changes in NF-Y binding to ICB4 in growing and arrested cells were not detected (results not shown). Several lines of evidence show that Sp1 is important in late S/G_2-specific transcription of some genes, supporting our results [38,39,48–50]. Because the mouse gene for topo IIα promoter has a cell-cycle-dependent activity (low in G_1, rises in S, and peaks in G_2/M), it is likely that Sp1 is involved in the growth-state-dependent or cell-cycle-dependent regulation of the gene for topo IIα.

The discrepancy between our results and those of Isaacs et al. [20] might be due to the use of different cell lines, reporter systems or experimental systems for growth arrest and/or species-specific regulation mechanisms. The different growth arrest methods might have been an important factor. Cell cycle arrest achieved by contact inhibition or serum starvation might cause different cellular events. Because ICB4 is also required for the maximal induction of the gene for topo IIα in growing cells, it is likely that multiple elements including Sp1 and NF-Y contribute to its cell-proliferation-dependent expression.

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REFERENCES

1 Roca, J. (1995) Trends Biochem. Sci. **20**, 156–160
2 Wang, J. C. (1996) Annu. Rev. Biochem. **65**, 635–692
3 Holm, C., Goto, T., Wang, J. C. and Botstein, D. (1985) Cell **41**, 553–563
4 Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K. and Yanagida, M. (1987) Cell **50**, 917–925
5 Tsai-Pflugfelder, M., Liu, L. F., Liu, A. A., Tewey, K. M., Whang-Peng, J., Knutsen, T., Hueben, K., Crose, C. M. and Wang, J. C. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7177–7181
6 Jenkins, J. R., Ayton, P., Jones, T., Davies, S. L., Simmins, D. L., Harris, A. L., Sheer, D. and Hickson, I. D. (1992) Nucleic Acids Res. **20**, 5587–5592
7 Tsutsui, K., Tsutsui, K., Okada, S., Watanabe, M., Shohmor, T., Seki, S. and Inoue, Y. (1993) J. Biol. Chem. **268**, 19076–19083
8 Chung, T. D. Y., Drake, F. H., Atan, K. B., Per, S. R., Crooke, S. T. and Mirabelli, C. K. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9431–9435
9 Drake, F. H., Hoffmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T. and Mirabelli, C. K. (1989) Biochemistry **28**, 8154–8160
10 Negri, C., Chiesa, R., Cerino, A., Bestagno, M., Sala, C., Zini, N., Maraldi, N. M. and Astaldi Ricotti, G. C. B. (1992) Exp. Cell Res. **200**, 452–459
11 Wossener, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K. and Drake, F. H. (1991) Cell Growth Differ. **2**, 209–214
12 Kimura, K., Saijo, M., Ui, M. and Enomoto, T. (1994) J. Biol. Chem. **269**, 1173–1176
13 Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J. and Hickson, I. D. (1998) Biochim. Biophys. Acta **1400**, 121–137
14 Hochhauser, D., Stanway, C. A., Harris, A. L. and Hickson, I. D. (1992) J. Biol. Chem. **267**, 18961–18965
15 Ng, S. W., Eder, J. P., Schnipper, L. E. and Chan, V. T. W. (1995) J. Biol. Chem. **270**, 25850–25858
16 Park, S. H., Yoon, J. H., Cho, H., Kwon, Y. D., Seong, R. H., Hong, S. H. and Park, S. D. (1995) J. Biochem. (Tokyo) **118**, 725–733
17 Adachi, N., Kobayashi, M. and Koyama, H. (1997) Biochem. Biophys. Res. Commun. **230**, 105–109
18 Dynan, S. W. (1986) Trends Genet. **2**, 196–197
19 Herzog, C. E. and Zwelling, L. A. (1997) Biochem. Biophys. Res. Commun. **232**, 608–612
20 Isaacs, R. J., Harris, A. L. and Hickson, I. D. (1996) J. Biol. Chem. **271**, 16741–16747
21 Goswami, P. C., Roti, J. L. R. and Hunt, C. R. (1996) Mol. Cell. Biol. **16**, 1500–1508
22 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Gene **77**, 51–59
23 Kim, D. S., Ahn, S. K., Yoon, J. H., Hong, S. H., Kim, K. E., Maurer, R. A. and Park, S. D. (1994) Mol. Endocrinol. **8**, 528–536
24 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25 Andrew, N. C. and Faller, D. V. (1991) Nucleic Acids Res. **19**, 2499
26 Okazaki, K., Nishizawa, M., Furono, N., Yasuda, H. and Sagata, N. (1992) EMBO J. **11**, 2447–2456
27 Everett, R. D., Baty, D. and Chambon, P. (1983) Nucleic Acids Res. **11**, 2447–2464
28 Gidoni, D., Kadonaga, J. T., Barrera-Saldana, H., Takahashi, K., Chambon, P. and Tjian, R. (1985) Science **230**, 511–517
29 Mcknight, S. and Tjian, R. (1986) Cell **46**, 795–805
30 Bird, A., Taggart, M., Frommer, M., Miller, O. J. and Macleod, D. (1985) Cell **40**, 91–99
31 Papazafiri, P., Ogami, K., Ramji, D. P., Nicosia, A., Monaci, P., Cladaras, C. and Zannis, V. (1991) J. Biol. Chem. **266**, 5790–5797
32 Raymondjean, M., Pichard, A.-L., Gregori, C., Ginot, F. and Kahn, A. (1991) Nucleic Acids Res. **19**, 6145–6153
33 Tronche, F., Rollier, A., Sourdive, D., Cereghini, S. and Yaniv, M. (1991) J. Mol. Biol. **221**, 31–43
34 Garlatti, M., Tchennokov, V., Daheshia, M., Feilleux-Duche, S., Hanoune, J., Aggerbeck, M. and Barouki, R. (1993) J. Biol. Chem. **268**, 6567–6574
35 Filatov, D. and Thelander, L. (1995) J. Biol. Chem. **270**, 25239–25243
36 Ronchi, A. E., Bottardi, S., Mazzucchelli, C., Ottolenghi, S. and Santoro, C. (1995) J. Biol. Chem. **270**, 21934–21941
37 Wright, K. L., Moore, T. L., Vilen, B. J., Brown, A. M. and Ting, P.-Y. (1995) J. Biol. Chem. **270**, 20978–20986
38 Zwicker, J., Gross, C., Lucibello, F. C., Truss, M., Ehlert, F., Engeland, K. and Muller, R. (1995) Nucleic Acids Res. **23**, 3822–3830
39 Zwicker, J., Lucibello, F. C., Wolfman, L. A., Gross, C., Truss, M., Engeland, K. and Muller, R. (1995) EMBO J. **14**, 4514–4522
40 Linhoff, M. W., Wright, K. L. and Ting, J. P.-Y. (1997) Mol. Cell. Biol. **17**, 4589–4596
41 Graves, B. J., Johnson, P. F. and McKnight, S. L. (1986) Cell **44**, 565–576
42 Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J. and Tjian, R. (1987) Cell **48**, 79–89
43 Dorn, A., Bollekens, J., Staub, A., Benoist, C. and Mathis, D. (1987) Cell **50**, 863–872
44 Hatamochi, A., Golumbek, P. T., Van Schaftingen, E. and de Crombrugghe, B. (1988) J. Biol. Chem. **263**, 5940–5947
45 Raymondjean, M., Cereghini, S. and Yaniv, M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 757–761
46 Roman, C., Platero, J. S., Shuman, J. and Calame, K. (1990) Genes Dev. **4**, 1404–1405
47 Maity, S. N., Sinha, S., Rutehouser, E. C. and de Crombrugghe, B. (1992) J. Biol. Chem. **267**, 16574–16580
48 Dalton, S. (1992) EMBO J. **11**, 1797–1804
49 Sinha, S., Maity, S. N., Lu, J. and de Crombrugghe, B. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 1624–1628
50 Henglein, B., Chenivesse, X., Wang, J., Eick, D. and Brechot, C. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 5490–5494

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