Regulation of the Human Topoisomerase IIα Gene Promoter in Confluence-arrested Cells*

(Received for publication, February 29, 1996, and in revised form, April 24, 1996)

Richard J. Isaacs†, Adrian L. Harris, and Ian D. Hickson§

From the Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Expression of DNA topoisomerase IIα mRNA and protein reflects the proliferative state of mammalian cell lines and tissues with high levels in actively cycling cells and marked down-regulation during serum deprivation or cell density-induced growth arrest. Using stably integrated gene fusions comprising the human topoisomerase IIα promoter with a growth hormone reporter gene, we have localized elements required for the differential activity of the topoisomerase IIα promoter in proliferating and confluence-arrested cells. Deletion analysis localized the region of the promoter that responded to changes in the cellular growth state to between −101 and −144 base pairs. Mutation analysis identified an inverted CCAAT box (ICB) located at −108 to −104 as necessary for promoter down-regulation in confluence-arrested cells, while several other potential cis-acting elements, including four additional ICBs, were shown to be required. The critical element was recognized in vitro by the CCAAT box binding factor, NF-Y, with levels of binding activity higher in extracts from proliferating cells than from confluence-arrested cells. We conclude that the differential regulation of topoisomerase IIα gene expression in cycling and confluence-arrested cells is mediated, at least in part, through proliferation-specific binding of factors to an ICB element in the gene promoter.

Topoisomerase II is a homodimeric nuclear enzyme required for many different aspects of DNA metabolism. Mutations in the genes encoding budding and fission yeast topoisomerase II enzymes cause a loss of cell viability due to a failure to segregate newly replicated chromosomes at mitosis (1–3). Many of the other roles performed by topoisomerase II are not essential for the maintenance of cell viability, probably because they are shared with topoisomerase I. Included in this latter category are roles as a replication "swivelase" and as a suppressor of chromosome instability in regions of the genome containing repetitive DNA elements (reviewed in Refs. 5–8).

Many of the key functional analyses on topoisomerase II have been performed in yeast both because of the availability of temperature-sensitive top2 mutants in these organisms and because yeast cells express only one topoisomerase II protein. In contrast, human cells express two closely related but genetically distinct isozymes of topoisomerase II designated topoisomerase IIα and -β (9–14). The respective roles of the two human isozymes are unknown, although both can functionally substitute for their yeast counterparts (15).

In mammalian cells, topoisomerase IIα and/or -β are among the most important cellular targets for a range of anticancer drugs (reviewed in Refs. 17–20). High levels of topoisomerase II gene expression correlate with relative sensitivity of cells to these agents, while low levels confer relative drug resistance. For this reason, considerable effort has been devoted to understanding the mechanisms by which topoisomerase II genes are regulated in mammalian cells. Identification of the mechanisms that regulate topoisomerase IIα gene expression in vivo may permit specific modulation of tumor cell chemosensitivity. In addition, the two topoisomerase II genes appear to be differentially regulated both in vivo and in cultured cell lines during changes in cellular growth. The β isozyme of topoisomerase II is expressed ubiquitously in vivo and is present in both proliferating and quiescent cell populations in vitro. In contrast, the α isozyme is one of the best available markers of the proliferative state of cells in vitro and in vivo (21–24).

Topoisomerase IIα protein is virtually absent from cells induced to undergo growth arrest by serum withdrawal. Moreover, after the release of cells from a serum-deprived state, topoisomerase IIα protein appears to accumulate to a significant level only in the G2 and M phases of the subsequent cell cycle (22). Consistent with this, immunohistochemical studies have shown that topoisomerase IIα protein is undetectable in quiescent or differentiated cell populations in vivo but is present in the proliferative cell fraction of all tissues (23, 24).

We have previously described the structure of the human topoisomerase IIα gene promoter and have defined the minimal unit necessary for full promoter activity (26). We have now extended that work to an analysis of the elements in the promoter that are necessary for the differential regulation of the topoisomerase IIα gene in proliferating and confluence-arrested cell populations. We show that a CCAAT box, present in the inverted orientation (inverted CCAAT box (ICB)), is essential for the down-regulation of promoter activity in confluence-arrested cells and that this ICB is recognized by the ubiquitous transcription factor NF-Y (also called CBF, ACF, and CP1; Refs. 27–29).

MATERIALS AND METHODS

Cell Culture—Human MCF-7 breast carcinoma cells and mouse fibroblasts derived from Swiss 3T3 cells were grown in Dulbecco's mod-

* This work was supported in part by the Imperial Cancer Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by an Overseas Research Student Award from the Universities of the United Kingdom.

§ To whom correspondence should be addressed: Tel.: 44-1865-222417; Fax: 44-1865-222431; E-mail: hickson@icrf.icnet.uk.

1 J. Jensen, S., Redwood, C. S., Jenkins, J. R., Andersen, A. H., and Hickson, I. D. (1996) Mol. & Gen. Genet., in press.

2 H. Turley, M. Comley, S. Houlbrook, I. D. Hickson, K. Gatter, and A. L. Harris, submitted for publication.

3 The abbreviations used are: ICB, inverted CCAAT box; EMSA, electrophoretic mobility shift assay; HGH, human growth hormone; bp, base pair(s); PCR, polymerase chain reaction; ATF, activating transcription factor.
Regulation of Human Topoisomerase IIα Gene Promoter

Topoisomerase IIα from pSVGH (30), which was a generous gift from Dr. J. Firth, Oxford, and human growth hormone (HGH) as a reporter gene. pHGH was derived from waspGEM-7Zf(9). The pHGH derivative containing the 5′-117 promoter fragment was used to generate single-stranded DNA templates. Primers used for mutating the ICB1, ICB2, and first nucleotide sequences of all promoter fragments were confirmed using DNA sequencing. This was performed using the Muta-gene Site-directed Mutagenesis system (Promega). Antisense oligonucleotides were purified by electrophoresis on a 20% polyacrylamide gel. 5′-End labeling of the oligonucleotides was performed using phage T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol). Labeled oligonucleotides were purified through a Sephadex G-50 column and then annealed to a 4-fold molar excess of the unlabeled complementary strand. Unlabeled oligonucleotides used as competitors were annealed in a 1:1 equivalent quantity. All EMSA reactions were performed essentially as described by Firth et al. (35). Briefly, reaction mixtures (20 μl) contained 50 μM KCl, 1 μM MgCl2, 0.5 mM EDTA, 5 μM dithiothreitol, 5% (v/v) glycerol, and 1 μg of poly(dI-dC). This mixture was incubated with nuclear extract (5 μg) for 5 min at 20°C before radiolabeled oligonucleotide (approximately 0.1 ng) and specific competitor DNA were added. Incubation was continued for an additional 10 min at 20°C. A total of 10 μg of nuclear extract was used on a 5% polyacrylamide gel in 0.5 × TBE (50 mM Tris, 50 mM boric acid, 0.1 mM EDTA, pH 7.9) at 4°C.

**RESULTS**

Use of Human Growth Hormone mRNA and Protein as Reporters of Topoisomerase IIα Gene Promoter Activity—In order to study the activity of the human topoisomerase IIα gene promoter in different cell lines, we sought a reliable reporter system that permitted quantification of both the mRNA and protein products of the reporter gene. We have previously described fusions of the topoisomerase IIα gene promoter with the bacterial chloramphenicol acetyltransferase gene (26). However, while analysis of chloramphenicol acetyltransferase protein is straightforward, the chloramphenicol acetyltransferase mRNA is generally of very low abundance due to its short half-life. Moreover, since chloramphenicol acetyltransferase is an intracellular protein, quantification of its activity requires that the experiment be terminated and the cells lysed. In contrast, HGH mRNA is readily detectable using the RNase protection assay (30), and the HGH protein is secreted into the culture medium permitting regular sampling of the medium over time from the same cell population for quantification of protein levels via an immunoassay. For these reasons, a series of plasmids was constructed in which various regions of the topoisomerase IIα promoter were cloned upstream of a promoterless growth hormone protein coding region in pBGH (32). These constructs were then used to study promoter activity in cells that were either proliferating or arrested by growth to confluence.

Fig. 1a shows the sequence of the region of the topoisomerase IIα promoter previously demonstrated to exhibit maximal promoter activity in transient transfection experiments (26). Within the promoter sequence are a number of DNA elements that closely match the consensus for transcription factor binding sites, including two GC boxes (potential Sp1 factor binding

Fig. 1. a, sequence of the human topoisomerase IIα promoter. Bases are numbered (as indicated on the left) with respect to the major transcription start site, which is designated +1 and marked with an arrow. Putative cis-acting elements, underlined and annotated above, include GC boxes (Sp1 sites) at positions -562 and -51, ICBs at positions -385, -259, -175, -108, and -68, and an ATF site at -226. The ATG start codon is overlined. b, schematic representation of putative transcription factor binding motifs and the truncated promoter derivatives constructed. The 5' limit of each promoter fragment is indicated on the left. The major transcription start site is marked with an arrow. The positions of the GC boxes (GC), ICBs (C), ATF site (ATF), and growth hormone reporter gene (HGH) are indicated.

Expression of the Topoisomerase IIα Gene Promoter Is Down-regulated in Confluence-arrested Human and Mouse Cell Lines—Initially, we tested whether the expression of the topoisomerase IIα mRNA was regulated by cellular growth state. Fig. 2, a and b, shows that the level of mRNA for the endogenous topoisomerase IIα gene was down-regulated approximately 4-fold in confluence-arrested human MCF-7 cells compared with exponentially growing cells. Fig. 2, a and b, also indicates that the HGH mRNA expressed from the full-length 1964-bp topoisomerase IIα promoter fragment was similarly down-regulated in the same population of confluence-arrested MCF-7 cells. In parallel, the level of HGH protein secreted into the medium was monitored over time as the cells grew to confluence and were held in a growth-arrested state for 48 h. Fig. 2c shows that HGH protein levels increased linearly with time during exponential cell growth, but this rise in expression virtually ceased as cells attained full confluence. The data in Fig. 2, a–c, demonstrate that expression of the HGH mRNA and protein reporters accurately reflects the expression of the endogenous topoisomerase IIα gene and suggest that a change in promoter activity is the primary determinant of the differential expression of topoisomerase IIα mRNA in proliferating and confluence-arrested cells.

Although MCF-7 and other human cell lines of epithelial origin are useful for demonstrating growth state regulation of genes, 3T3 mouse fibroblasts are significantly more attractive model systems (36). This is because 3T3 cells are known to show strong contact inhibition of growth and can be held in a confluence-arrested state for many days without loss of viability. For this reason, we analyzed the growth state regulation of the endogenous topoisomerase IIα gene in Swiss 3T3 cells. Fig. 3, a–c, shows that expression of the mouse topoisomerase IIα gene was virtually ablated in confluence-arrested Swiss 3T3 cells. We conclude, therefore, that topoisomerase IIα gene expression is down-regulated in both human and mouse cell lines in response to cell cycle arrest by growth to confluence. Because of their ability to achieve such tight regulation of topoisomer-
ase II α gene expression by alterations in growth state, we used Swiss-3T3 cells to study the activity of various deletion or mutated constructs of the topoisomerase II α gene promoter. For each of the analyses described below, a minimum of two cell clones was tested for each construct stably transfected into the Swiss 3T3 host cell line with experiments performed in triplicate.

Identification of a Region of the Topoisomerase II α Promoter Required for Mediating Down-regulation of Activity in Confluence-arrested Cells—The expression of HGH protein was monitored in Swiss 3T3-derived cell lines stably transfected with constructs containing various different fragments of the human topoisomerase II α promoter (see Fig. 1b). Fig. 4, a–d, shows that HGH expression from the −2617-bp and −2144-bp promoter fragments was switched off as cells attained a confluence-arrested state (indeed, a small decline in protein level was observed, presumably reflecting the protein half-life in medium). However, those transfectants containing the −2101-bp or −256-bp fragments of the promoter showed continued expression of HGH for at least 48 h after confluence-induced arrest was attained. These data indicate that all of the essential elements for transcriptional repression in contact-inhibited cells are present within the −2144-bp region of the topoisomerase II α promoter and, furthermore, that elements essential for this regulation are located in the region between −2144 and −2101 bp of the promoter.

An Inverted CCAAT Box in the Topoisomerase II α Gene Promoter mRNA expression was detectable in the loading control and to define the position of the α1-globin-specific protected fragment; lanes 3–5, RNA from Swiss 3T3 cells in exponential phase (lane 3), at 90% confluence (lane 4), and 24 h after full confluence was attained (lane 5). b, quantification of the mRNA levels from samples in lanes 3–5 of panel a. Values were normalized to the α1-globin signal. c, flow cytometric analysis of cells from which RNA was extracted for analysis in lanes 3 (proliferating) and 5 (fully confluent) of panel a. The percentage of cells in the G0/G1 phase in each cell population is indicated.

FIG. 3. Regulation of topoisomerase II α mRNA in confluence-arrested Swiss 3T3 cells. a, ribonuclease protection assay on RNA extracted from exponentially growing or confluent Swiss 3T3 cells. All samples contained both the α1-globin (133-bp protected fragment) and topoisomerase (Topo II α) probes (208-bp protected fragment). Lane 1, Escherichia coli tRNA alone (negative control for nonspecific hybridization); lane 2, K562 RNA, a control to confirm that no topoisomerase II α mRNA expression was detectable in the loading control and to define the position of the α1-globin-specific protected fragment; lanes 3–5, RNA from Swiss 3T3 cells in exponential phase (lane 3), at 90% confluence (lane 4), and 24 h after full confluence was attained (lane 5). b, quantification of the mRNA levels from samples in lanes 3–5 of panel a. Values were normalized to the α1-globin signal. c, flow cytometric analysis of cells from which RNA was extracted for analysis in lanes 3 (proliferating) and 5 (fully confluent) of panel a. The percentage of cells in the G0/G1 phase in each cell population is indicated.

FIG. 4. Deletion analysis of the topoisomerase II α promoter in stably transfected Swiss 3T3 cells. Levels of growth hormone protein secreted over time from cells transfected with the −617-bp (panel a), −144-bp (panel b), −101-bp (panel c), and −56-bp (panel D) promoter constructs are shown. Cells in each case were grown to full confluence and then incubated for an additional 48 h without a change of medium. mu, milliunits.
moter Is Essential for Down-regulation of Promoter Activity in Confluence-arrested Cells—The short region of the promoter between −2101 and −144 bp that effects growth state regulation of promoter activity contains only one clearly discernible transcription factor binding site, an ICB (ICB2; see Fig. 1) at bp −2108 to −2104. This putative binding site was, therefore, destroyed by site-directed mutagenesis converting the ICB motif from ATTGG to ATTCC in the −2617-bp promoter fragment. The level of expression from this mutated promoter was then quantified in stably transfected 3T3 cells under proliferating and confluence-arrested culture conditions. Fig. 5a shows that mutation of ICB2 completely abrogated the down-regulation of HGH expression normally seen in confluence-arrested cells.

We considered the possibility that elements present in the −2101-bp fragment, which although not necessary for negative regulation during confluence arrest, might, nevertheless, cooperate with the critical ICB2 element to achieve complete growth state regulation of the topoisomerase IIα gene promoter. In addition to ICB1, the −101-bp fragment contains a consensus GC box that is a potential binding site for the transcription factor Sp1. Fig. 5, b and c, shows that mutation of these two sites did not significantly influence the down-regulation of HGH expression that was seen as cells attained a confluence-arrested state.

To confirm that ICB2 was the critical element in the 144-bp promoter fragment, we carried out ribonuclease protection assays on RNA from the transfectants expressing HGH from the mutated ICB2 promoter construct (and from the ICB1 mutant promoter as a control). Fig. 6, a–c, shows that while the level of human growth hormone mRNA expressed from the promoter fragment carrying a mutated ICB1 element was down-regulated in confluence-arrested cells, no such down-regulation was seen in cells expressing growth hormone from the mutated ICB2 promoter construct. The endogenous mouse topoisomerase IIα gene was down-regulated as normal in both of these transfected cell populations (Fig. 6, a and b). These results demonstrate that mutation of the ICB2 promoter element causes the regulation of the HGH reporter gene to be discordant with that of the endogenous topoisomerase IIα gene in the same cell population. Moreover, the ICB1 and ICB2 elements clearly are functionally distinct in their effects on confluence-induced regulation of the topoisomerase IIα promoter.
Identification of Protein Factors That Bind to ICB2 in the Topoisomerase IIa Gene Promoter—To identify and characterize protein factors that could bind to the ICB2 element in the topoisomerase IIa promoter, EMSAs were performed using a radiolabeled 30-bp double-stranded oligonucleotide spanning ICB2. Nuclear extracts from Swiss 3T3 cells contained a factor that bound specifically to this oligonucleotide (Fig. 7, lane 1). This binding was eliminated by the addition of a 100-fold excess of unlabeled competitor DNA of identical sequence (Fig. 7, lane 2) but not by the addition of a similar excess of oligonucleotide containing a mutated ICB2 (ICB2-M; Fig. 7, lane 3). Consistent with the ICB being the only protein recognition element in this oligonucleotide, no protein-DNA complex was obtained when the mutated ICB-M oligonucleotide alone was used in the EMSA (Fig. 7, lane 4). Antibody “supershift” analyses revealed that the ICB binding factor contained two subunits of the NF-Y protein complex (NF-YA and NF-YB; lanes 5 and 6, respectively) but not CTF/NF-1 (lane 7), a second previously identified CCAAT box binding factor. Lanes 8–14 of Fig. 7 show a set of EMSAs equivalent to those in lanes 1–7, with the exception that the extracts were prepared from confluence-arrested Swiss 3T3 cells. Although the result is qualitatively similar to that obtained with an extract from proliferating cells, the level of ICB binding activity was considerably lower (approximately 4-fold) in the extract from the arrested cells. Taken together, these results indicate that ICB2 of the topoisomerase IIa gene promoter is recognized by a protein complex that contains the NF-YA and NF-YB subunits and that this complex is present at a reduced level in confluence-arrested cells.

DISCUSSION

We have shown that the activity of the human topoisomerase IIa gene promoter is strongly down-regulated in cells undergoing confluence-induced growth arrest. We have localized the region of the promoter that responds to the negative growth signals invoked by cell confluence to a short stretch within 144 bp of the major CAP site and have shown that an ICB element within this region is necessary for the observed down-regulation. Furthermore, we have identified the NF-Y transcription factor as a component of a proliferation-induced complex that can bind in vitro to the critical ICB2 element in the topoisomerase IIa gene promoter.

The 144-bp fragment of the topoisomerase IIa promoter that is necessary and sufficient for responding to proliferation-specific signals contains only two proximal ICBs out of the five present in the full promoter together with a putative GC box. However, this GC box is an imperfect match to the consensus Sp1 factor binding site due to having only three guanine residues on either side of the central cytosine residue instead of the usual four. The ICB2 located at –108 to –104 clearly plays a pivotal role in the negative regulation of topoisomerase IIa mRNA expression. In contrast, ICB1 is neither necessary nor sufficient for growth state regulation. The features that distinguish the critical ICB2 from ICB1 (and indeed the other three ICBs within the promoter) were not identified in the current study, but they could include either the spacing of the CCAAT motif from other promoter elements or differences within the specific sequences flanking the different ICBs within the promoter. However, we have obtained no clear evidence that the different CCAAT boxes are recognized differentially by protein factors in cell extracts.4

Ng et al. (37) have analyzed the role of ICBs in the regulation of basal expression of the Chinese hamster topoisomerase IIa gene. Their data indicate that an approximately 290-bp region of the 5′-flanking region spanning the CAP site of the gene, which is highly conserved in the human gene, contains maximal promoter activity. The three ICBs within this region (equivalent to ICB1, -2, and -3 of the human gene) were each shown to contribute to basal promoter activity, although ICB1 and -3 were relatively more important for this effect than was ICB2. It would appear, therefore, that the ICBs in the topoisomerase IIa promoter play an important role both in regulating basal gene expression and, at least in the case of ICB2, in mediating down-regulation of transcription in response to negative growth signals.

We have shown that the CCAAT box-binding protein, NF-Y, is a component of a proliferation-specific complex that bind to ICB2. NF-Y is a heteromeric transcription factor comprising at least three subunits named NF-YA, NF-YB, and NF-YC (28). All three of these subunits appear to be necessary for sequence-specific DNA binding activity (38). The NF-Y factor has been implicated in controlling the activity of numerous other promoters, including those required for the transcription of the major histocompatibility complex class II gene (39) and the thymidine kinase gene (40). Moreover, NF-Y has been shown to bind to the CCAAT sequence in the promoters of several genes expressed in a tissue-specific manner (16, 25, 27). Recently, Ronchi et al. (16) showed that NF-Y can induce bends of 62–82° at the site of a CCAAT box in DNA and that the degree of bending is influenced by the flanking sequence. Hence, a possible explanation for our findings is that changes in NF-Y binding to ICB2, either qualitative or quantitative, induce a critical conformational change in the promoter that in turn alters the affinity of cis-acting elements within the minimal promoter for their trans-acting factors.

Our data are also consistent with the idea that ICB2 acts as a negative regulator of the topoisomerase IIa promoter in con-

4 R. J. Isaacs, A. L. Harris, and I. D. Hickson, unpublished data.
fluence-arrested cells and that this repression may be relieved in proliferating cells by the binding of NF-Y to this element. Nevertheless, it seems likely that this is only one facet of the growth state regulation of topoisomerase IIα gene expression since NF-Y was still detectable in extracts from cells in confluence-arrested cells. This regulation cannot simply be a consequence of a lack of binding of a positive regulatory factor to ICB2 since elimination of ICB2 prevents down-regulation of promoter activity in confluent cells. It remains to be determined if modification of NF-Y subunits, such as by phosphorylation, reinforces the effects brought about by alterations in NF-Y levels seen in proliferating and growth-arrested cell population.

The growth of cells to confluence could, in principle, induce an arrest via many pathways, including nutrient deprivation and contact inhibition. Nutrient deprivation appeared to play only a very minor role, if at all, in negatively regulating topoisomerase IIα gene expression since actively cycling cells fed conditioned medium taken from cultures held at confluence for 48 h continued to express HGH at nearly normal rates. Moreover, medium taken from confluence-arrested cells did not show a significant change in pH compared with fresh growth medium. Further work is required to define the signaling pathway leading to a down-regulation of the topoisomerase IIα promoter in confluent cells.

It is clear from previous studies that transcription factors other than CCAAT box-binding proteins can mediate down-regulation of some genes during cell cycle arrest brought about by growth to confluence. For example, the ATF binding site in the cyclin A promoter is recognized by both the ATF-1 and cAMP response element-binding protein. Our data indicate that the putative ATF site at −227 bp is not necessary for confluence-induced down-regulation of the topoisomerase IIα promoter.

In summary, we have shown that the activity of the topoisomerase IIα gene promoter is regulated by the growth state of human and mouse cell lines and that an ICB element plays a critical role in this regulation. The challenge now is to delineate the biochemical changes to transcription factors, such as NF-Y, that occur in confluence-arrested cells to bring about this negative regulation.

Acknowledgments—We thank Dr. R. Mantovani for anti-NF-YA and NF-YB antibodies, Dr. J. Firth for pHGH, Drs. Chris Norbury and B. Edgar for the mouse cDNA library, and E. Clemson for typing the manuscript. We also thank Dr. C. Norbury for reading the manuscript and G. Campbell for performing the HGH assays.

5 R. J. Isaacs, A. L. Harris, and I. D. Hickson, unpublished observations.