Cell Cycle-coupled Variation in Topoisomerase IIα mRNA Is Regulated by the 3′-Untranslated Region

POSSIBLE ROLE OF REDOX-SENSITIVE PROTEIN BINDING IN mRNA ACCUMULATION

Mammalian topoisomerase IIα (Topo II) is a highly regulated enzyme essential for many cellular processes including the G2 cell cycle checkpoint. Because Topo II gene expression is regulated posttranscriptionally during the cell cycle, we investigated the possible role of the 3′-untranslated region (3′-UTR) in controlling Topo II mRNA accumulation. Reporter assays in stably transfected cells demonstrated that, similar to endogenous Topo II mRNA levels, the mRNA levels of reporter genes containing the Topo II 3′-UTR varied during the cell cycle and were maximal in S and G2/M relative to G1. Topo II 3′-UTR sequence analysis and RNA-protein binding assays identified a 177-nucleotide (base pairs 4772–4949) region containing an AUUUUA motif sufficient for protein binding. Multiple proteins (84, 70, 44, and 37 kDa) bound this region, and the binding of 84- and 37-kDa (tentatively identified as the adenosine- or uridine-rich element-binding factor AUF1) proteins was enhanced in G1, correlating with decreased Topo II mRNA levels. The binding activity was enhanced in cellular extracts or cells treated with thiol-reducing agents, and increased binding correlated with decreased Topo II mRNA levels. These results support the hypothesis that cell cycle-coupled Topo II gene expression is regulated by interaction of the 3′-UTR with redox-sensitive protein complexes.

Mammalian topoisomerase IIα (Topo II) is a multifunctional protein involved in many cellular processes including replication, repair, transcription, recombination, chromosome condensation and segregation, and the G2 cell cycle checkpoint pathway (1–5). Topo II levels (mRNA, protein, and activity) increase in late S phase, peak in G2/M, and rapidly decrease following mitosis (4, 6–8). Consistent with these observations, inhibitors of Topo II have been shown to arrest mammalian cells before mitosis (4, 6–8). Consistent with these observations, inhibitors of Topo II have been shown to arrest mammalian cells before mitosis (4, 6–8).

The regulation of mRNA stability has emerged as an important control mechanism of gene expression. Although the mechanisms that alter mRNA stability of different genes have unique features, in general, sequences located in the 3′-untranslated region (UTR) and their interactions with specific proteins regulate mRNA stability (14–17). The most common 3′-UTR stability determinants are adenosine- or uridine-rich elements (AREs), which include AUUUUA, AUUUA, and AUUUA/AUUUA motifs (18–22). Furthermore, a family of proteins, the AU-binding proteins, including AUFI (23), Hel-N1 (24), AUH (25), HuR (26), and AUBF (27), have the capacity to bind with high affinity to mRNA containing ARE. Binding of AU-binding proteins (e.g. 15-, 18-, and 19-kDa AUBF and 37-, 40-, 42-, and 45-kDa AUFI) to AREs correlates with either mRNA stabilization (27, 28) or destabilization (23, 29, 30). These observations suggest that ARE elements can be both positive and negative regulators of mRNA stability, depending on the sequence context in which the sequence elements are located and the precise protein composition of the RNA-protein complex. In addition, posttranslational modifications involving oxidation/reduction as well as phosphorylation/dephosphorylation of AU-binding proteins have also been shown to affect ARE binding (23, 27, 31). Binding to cytokine and lymphokine mRNAs can be reversibly blocked by the thiol-oxidizing agent diamide but irreversibly inhibited by the thiol-alkylation agent N-ethylmaleimide (NEM) and enhanced by the thiol-reducing agent 2-mercaptoethanol (2-ME) (31, 32). Thus, it is possible that subtle changes in redox potential during cell growth or in response to environmental stimuli may influence ARE-AU-binding protein binding and alter the stability of ARE-containing mRNA. We showed earlier that the cell cycle-coupled accumulation of Topo II mRNA levels is regulated by changes in mRNA stability (6). The present study was designed to test the idea that Topo II gene expression during the cell cycle is regulated by 3′-UTR via interactions with redox-sensitive protein complexes.

MATERIALS AND METHODS

Cell Culture and Extract Preparation—HeLa cell cultures, synchronization by selective detachment of mitotic cells, and flow cytometric analysis of cell cycle positions were performed following previously described procedures (6). Mouse NIH 3T3 fibroblast cells were grown in Dulbecco’s modified Eagle’s medium with 10% calf serum and antibiot-
ics. Exponentially growing cells were synchronized by replacing the growth medium with medium containing 0.5% serum and serum-stimul-
ted to reenter the cell cycle. Cytoplasmic extracts were prepared following the method described by Konarska and Sharp (33). In selected experiments, diithiothreitol (DTT) was omitted during the preparation of cytoplasmic extracts. To determine protein mobility shift assays, RNA-protein binding reactions were treated with RNase T1 (5 units/reaction) and separated by electrophoresis on 4.5% native polyacrylamide gel in 45 mM Tris, 45 mM boric acid, and 1.2 mM EDTA buffer, pH 7.4. The RNA-protein complexes were analyzed by a PhosphorImager (STORM 840, Molecular Dynamics).

Protein Cross-linking—RNA-protein binding reactions as described above were UV-irradiated (2500 J/ml for 3 min) and incubated with RNase A (0.2 µg/µl) and RNase T1 (10 units) at 37 °C for 20 min. Protein samples were analyzed by electrophoresis on 10% SDS-polyacrylamide gel along with prestained protein molecular size markers (Life Technologies) and visualized by exposing the dried gel to a Phos-
phorImager screen.

Northern Blot Analysis—Northern blot analysis was performed according to a previously published protocol (6). Radiolabeled probes, prepared by random priming, were the human Topo II cDNA (35), β-galactosidase (CLONTECH), human p21 (37), human geminin (38), or glyceraldehyde 3-phosphate dehydrogenase (CLONTECH).

Measurement of Glutathione and N-Acetyl-L-cysteine Levels—Intra-
cellular reduced and oxidized glutathione as well as N-acetyl-
L-cysteine (NAC) levels were assessed following previously published protocols (39, 40). Cell pellets were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1.34 mM diethylenetriaminepentaacetic acid. Total glutathione content was determined by the method of Anderson (41). Reduced (GSH) and oxidized (GSSG) glutathione were distin-
guished by the addition of 2 µl of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 µl of sample. NAC levels in cells were measured follow-
ing derivatization with N-(1-pyrenyl)maleimide using a 15-cm C18 Re-
liasil column (Column Engineering, Ontario, CA) coupled with high performance liquid chromatography with fluorescent detection (40). All biochemical determinations were normalized to the protein content of whole cell homogenates using the method of Lowry et al. (42).

Measurement of Intracellular Prooxidant Production during the Cell Cycle—HeLa cells synchronized by mitotic shake-off were replated and harvested at representative times for measurement of oxiproxidant produc-
tion (39). Cells were spun down, and the pellets were resuspended in 1 ml of phosphate-buffered saline supplemented with 5.5 mM glucose at 37 °C. Samples were labeled with 10 µl of 1 mg/ml C-400, an oxidation-
sensitive fluorescent probe (Molecular Probes, Inc., Eugene, OR) and incubated 15 min at 37 °C and then placed on ice immediately. Labeled samples were analyzed by flow cytometry (excitation at 488 nm, emission at 535 ± 10 nm); 20,000 cells from each sample were analyzed to obtain mean fluorescence intensity and corrected for autofluorescence obtained from samples of unlabeled cells. The vari-
ations in mean fluorescence intensity during the cell cycle were calcu-
lated relative to early G1 cells (2 h after mitotic shake-off).

RESULTS

A Regulatory Role for 3′-UTR in the Cell Cycle-coupled Ac-
cumulation of Topo II mRNA Levels—To determine if the 3′-
UTR of Topo II mRNA has a role in cell cycle-specific increase in Topo II mRNA levels, reporter assays in stably transfected 3T3 cells were performed. Stably transfected cells containing HA1Topo2, HßgalTopo, or HßgalsIV40 reporter constructs were synchronized by serum starvation and serum stimulation to reenter the cell cycle. Cells were harvested at various times for analysis of cell cycle position and reporter mRNA levels. Represent-
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H\(\Delta\)Topo2-transfected cell populations was analyzed by Northern blotting, an mRNA band of approximately 2.2 kb, representing the H\(\Delta\)Topo2 reporter construct, was detected with a radiolabeled probe specific for the human Topo II cDNA (Fig. 1A). H\(\Delta\)Topo2 reporter mRNA levels were low in G1 (0–12 h poststimulation) and increased 12–16-fold at mid-S to G2 phases (16–24 h poststimulation). These results were comparable with our previously published results obtained mitotically selected HeLa cells (6) and indicated that the truncated Topo II cDNA reporter construct contains sequence element(s) regulating Topo II mRNA levels. Since the H\(\beta\)galTopo reporter mRNA levels (Fig. 1C) also showed similar cell cycle-coupled variations (as compared with H\(\Delta\)Topo2), we concluded that sequences within the open reading frame of Topo II are not involved in the regulation of mRNA levels. Therefore, the increase in H\(\Delta\)Topo2 and H\(\beta\)galTopo reporter mRNA levels might be regulated either by changes in the Topo II promoter activity or by a combination of promoter activity and posttranscriptional regulation governed by the Topo II 3′-UTR. Results from the H\(\beta\)galSV40 reporter construct (Fig. 1C), which contains the Topo II promoter but not the 3′-UTR, showed a 6–8-fold decrease in H\(\beta\)galSV40 mRNA levels compared with H\(\beta\)galTopo mRNA levels during 20–24 h poststimulation. The low level of H\(\beta\)galSV40 mRNA during 20–24 h poststimulation can be attributed to the 2-fold increase in Topo II transcription that has been reported previously to occur during late S phase by us (6) and other investigators (34, 43). Results from a representative Northern blot measuring the variations in endogenous Topo II mRNA levels are shown in Fig. 1B. The kinetics of endogenous Topo II mRNA accumulation was comparable to reporter mRNA levels during the cell cycle. Overall, these results demonstrate that Topo II 3′-UTR has a regulatory role in accumulation of Topo II mRNA levels during the mammalian cell cycle.

A complete sequence analysis of the Topo II 3′-UTR was performed to determine if sequences known to regulate mRNA levels are present in the 3′-UTR. The hTopo2 plasmid (35) was digested with NdeI and XhoI restriction enzymes and cloned into pBS II SK(+), and both strands were sequenced. Sequence analysis showed that the Topo II mRNA has a long (≈1.1-kb) 3′-UTR with several ARE motifs (four AUUUU, one AUUUA, and one AUUUUA; Fig. 2) present throughout the 3′-UTR with several ARE motifs (four AUUUA, one AUUUUA, and one AUUUUA). To more precisely define the binding site, a riboprobe representing the 177-nt (base pairs 4772–4948; Fig. 2) sequence overlapping the proximal and middle regions of Topo II 3′-UTR RNA was synthesized. RNA-protein complex formation was analyzed by gel mobility shift assay. Results presented in Fig. 3 show that the mobility of all three transcripts was retarded in their migration when compared with the control reaction that did not contain the protein extract (compare lane 1 with lane 2, lane 6 with lane 7, and lane 12 with lane 13). The retardation in mobility was due to an RNA-protein interaction because proteinase K treatment of cellular extracts prior to the binding assay abolished the retardation (lane 11). To analyze the specificity of the RNA-protein complex, competition experiments were performed. The RNA-protein binding assay was carried out in the presence of an unlabeled specific competitor or a nonspecific competitor RNA (\(\beta\)-galactosidase). The addition of a 1-fold excess of specific unlabeled competitor RNA had a minimal effect on RNA-protein complex formation (lanes 3, 8, and 14). However, a 10-fold excess of specific unlabeled proximal and middle transcripts decreased the RNA-protein complex formation by more than 90% (lanes 4 and 9). In contrast, a 10-fold excess of the unlabeled distal transcript did not compete with the labeled distal transcript for complex formation. A 10-fold excess of the nonspecific competitor RNA (\(\beta\)-galactosidase) caused no change in complex formation (lanes 5, 10, and 16). These results demonstrate that protein binding to both the proximal and middle portions of the Topo II 3′-UTR is specific in contrast to nonspecific binding to the distal region. Alternatively, the protein(s) bound to the distal region could be highly abundant and therefore might not be competed away under these conditions.

To further localize the sequence region involved in Topo II 3′-UTR RNA-protein complex formation, cross-competition assays were performed in the presence of unlabeled transcripts from the proximal or the middle region. Cellular proteins bound to labeled proximal transcript could be competed more than 90% with a 10-fold excess of unlabeled proximal competitor RNA (compare lanes 2 and 3, Fig. 4A). Interestingly, a 10-fold excess of unlabeled competitor RNA representing the middle region of Topo II 3′-UTR mRNA also competed (lane 4), while a nonspecific competitor RNA (\(\beta\)-galactosidase, lane 5) did not. Alternatively, the RNA-protein complex formed from the middle region of Topo II 3′-UTR mRNA (Fig. 4B, lane 7) could be competed away with 10-fold excess of unlabeled middle competitor RNA (lane 8) or unlabeled RNA from the proximal region of Topo II 3′-UTR (lane 9). These results indicate that the overlapping sequence spanning the proximal and middle regions of Topo II 3′-UTR may contain the motifs necessary for specific RNA-protein complex formation.

### Table I

| Phase | Cells in each phase of the cell cycle | % | % | % | % |
|-------|-------------------------------------|---|---|---|---|
| G1    | 80                                  | 82 | 35 | 14 | 28 |
| S     | 8                                   | 7  | 58 | 76 | 43 |
| G2 + M| 12                                  | 11 | 7  | 10 | 29 |

**Table I.** Cell cycle phase distribution in H\(\Delta\)Topo2-transfected NIH 3T3 cells synchronized by serum starvation and serum-stimulated to reenter the cell cycle.
FIG. 1. Topo II 3'-UTR has a regulatory role in mRNA accumulation during the cell cycle. Northern blot analysis of reporter mRNA levels in stably transfected NIH 3T3 cells. Schematic diagrams of reporter constructs are shown at the top of each panel. hTop promoter consists of the 650-nt human Topo II promoter (thick line), exon 1, intron, and exon 2 (34). Δ-Topo cDNA represents a truncated form of human Topo II cDNA (base pairs 1–3013 deleted). hTop and SV40 3'-UTR represent 3'-untranslated regions of human Topo II and SV40 DNAs. Stably transfected 3T3 cells containing H4Topo2 (A), HgCuTopo (C), or HgCuSV40 reporter constructs were synchronized by serum starvation and serum-stimulated reentry to the cell cycle. Cells were harvested at the indicated times for RNA isolation and analyzed by Northern blotting. RNAs from H9-UTR cDNA.

FIG. 2. Several AU-rich elements are present in Topo II 3'-UTR. Sequence analysis of Topo II 3'-UTR cDNA. Boldface letters represent AU-rich elements. Translation termination and polyadenylation signals are in boldface type and underlined. Uppercase letters represent coding sequence, and lowercase letters represent 3'-untranslated region. The putative protein-binding region is double underlined.

showed approximately 40% inhibition in complex formation (lane 21, Fig. 4C). The competition with poly(U) at higher concentration could be due to the formation of a double-stranded region with uridylate residues present within the 177-nt sequence. Furthermore, unlabeled c-fos ARE transcript containing the AUUUUUA motif also competed with 177-nt riboprobe and UV-irradiated, and unbound RNA was digested with RNases A and T1. Proteins were separated by SDS-gel electrophoresis and visualized by autoradiography. In the absence of UV irradiation, no cross-linking of labeled probe to protein was observed (Fig. 5, lane 1). In contrast, several polypeptide bands of apparent molecular masses of 84, 70, 44, and 37 kDa (Fig. 5, lanes 2–4) were detected in UV-irradiated reactions. Protein binding was specific and could be competed with 10 ng of poly(U) (lane 5). Furthermore, while the binding of the 70- and 44-kDa polypeptides was detected in extracts from both G1 and S phase cells, binding of the 84- and 37-kDa polypeptides was specific to the G1 phase. These results support the idea that differential binding of proteins to the Topo II 3'-UTR might regulate Topo II mRNA accumulation during the cell cycle.

Since the molecular mass of the 37-kDa polypeptide (Fig. 5A) is similar to that of the ARE-binding protein AUFI (23), and the 177 nt of Topo II 3'-UTR contains an AUUUUA motif, we determined whether AUFI is present in the RNA-protein complex. Protein extract prepared from asynchronously growing HeLa cells was incubated with or without a polyclonal antibody to AUFI, and the immune complex was separated using protein A-Sepharose. Immunoblotting of the supernatants showed that, while AUFI is present in the supernatant from the control reaction, it was not detected in the supernatant from the immunoprecipitation reaction performed with the AUFI polyclonal antibody (data not shown). RNA-protein binding assays were then performed using the control or AUFI-immunodepleted supernatant. While the supernatant from the control reaction showed no change in complex formation, depletion of AUFI from the protein extract inhibited complex formation by more than 90% (compare lanes 1 and 2, Fig. 5B). Binding reactions performed with bovine serum albumin alone (lane 3)
showed no retardation in mobility of the transcript and served as a negative control for the assay. The presence of AUF1 in the Topo II 3′-UTR RNA-protein complex was further verified by comparing binding reactions with c-fos 3′-UTR transcript as a positive control. RNA-protein binding reactions with the 177-nt Topo II 3′-UTR transcript, c-fos 3′-UTR transcript, or a control reaction without any transcript were first resolved by RNA-protein gel shift assay and excised under autoradiographic guidance. In the control lane, an identical region of the gel compared with the radioactive band was also excised. Bound proteins in the excised bands were separated by SDS-gel electrophoresis, transferred to Immobilon-P membrane, and immunoblotted with a polyclonal antibody to AUF1. The control reaction without the transcript did not show any AUF1 immunoreactive polypeptide band (Fig. 5C, lane 1). In contrast, AUF1 was detected in both the 177-nt Topo II and c-fos 3′-UTR RNA-protein complexes (lanes 2 and 3). These results identify AUF1 as one of the proteins present in the Topo II 3′-UTR RNA-protein complex and suggest that the interaction of AUF1 with the AUUUUUA motif in Topo II 3′-UTR may have a regulatory role in determining Topo II mRNA levels during the cell cycle.

Oxidation/reduction Sensitivity of Protein Binding to the Topo II 3′-UTR—Since proteins that bind AREs have been shown previously to demonstrate alterations in binding based on the oxidation/reduction (redox) state of the protein complexes (31, 32), redox sensitivity of protein binding to the 177-nt Topo II 3′-UTR was investigated (Fig. 6). Protein extract was prepared from asynchronously growing HeLa cells using extraction buffer that lacks a reducing agent. Binding reactions were then performed with increasing concentrations of the thiol-reducing agent DTT, or 2-ME, prior to the addition of the 177-nt riboprobe and analyzed by RNA gel shift assay (Fig. 6A, lanes 2–5). Binding of cellular proteins to the Topo II 3′-UTR increased 5–6-fold in protein extracts pretreated with the reducing agents DTT (lanes 3 and 4) or 2-ME (lane 5). The assay was then repeated in extracts supplemented with 2 mM DTT and increasing concentrations of a thiol-oxidizing agent (diamide) or a thiol-alkylating agent, NEM (Fig. 6B). A dose-dependent inhibition in protein binding was observed in extracts that were pretreated with the sulfhydryl-oxidizing agent, diamide (1, 5, 10, and 20 mM; lanes 6–9). While 1 and 5 mM of diamide caused 40 and 90% decreases in protein binding (lanes 6 and 7), both 10 and 20 mM of diamide completely abolished the complex formation (lanes 8 and 9). The inhibition in protein binding in 5 mM diamide-treated extract could be reversed by...
treatment with the thiol-reducing agent, 2-ME (compare lanes 7 and 10). Treatment with NEM also showed a dose-dependent inhibition in protein binding to Topo II 3’-UTR (1 and 10 mM, lanes 11 and 12). Because thiol alkylation is an irreversible reaction, inhibition in RNA-protein binding by NEM could not be reversed by the addition of DTT or 2-ME to the reaction mixture (data not shown). Taken together, these results indicate that reduced thiol residues in Topo II 3’-UTR binding proteins participate in the RNA-protein complex formation and that oxidation of these thiol residues to the disulfide form abolishes binding.

If the above in vitro results are relevant to in vivo regulation, then it is reasonable to postulate that manipulations of intracellular redox status would influence Topo II 3’-UTR RNA-protein binding and mRNA levels. To address this question, asynchronously growing HeLa cells were treated with 20 mM NAC and assayed for NAC uptake, reduced and oxidized glutathione content, RNA-protein binding, and Topo II mRNA levels. By 2 h, intracellular NAC uptake was approximately 4 nmol/mg protein and increased to almost 6 nmol/mg protein by 6 h of treatment (Fig. 7A). Consistent with these results, the ratio of reduced to oxidized glutathione increased approximately 1.5- to 2.0-fold during this time frame, demonstrating that NAC treatment altered the intracellular redox state in favor of a more reducing environment. Topo II 3’-UTR mRNA-protein gel mobility shift assays performed with protein extracts from duplicate samples showed that protein binding increased 2- to 3-fold in NAC-treated cells compared with untreated controls (compare lanes 2–4, Fig. 7B). Results from Fig. 7B (inset) showed that the increase in protein binding was not due to changes in AUF1 protein levels following NAC treatment. Interestingly, Topo II mRNA levels under these conditions decreased dramatically to 10% of control at 2–6 h after NAC treatment (Fig. 7, C and D). These results provide in vivo evidence that a shift to a more reducing environment enhances protein binding to the 177-nt Topo II 3’-UTR, which correlates with a decrease in mRNA levels.

If these apparent redox-sensitive alterations in Topo II 3’-UTR mRNA-protein complex formation are a reflection of events occurring during the cell cycle, then a lower oxidation potential in G1 (relative to S, G2, and M) would favor Topo II 3’-UTR mRNA-protein binding, resulting in decreased mRNA levels. In contrast, a relatively more oxidizing environment in late S, G2, and M would inhibit complex formation, resulting in increased mRNA levels. To determine if variations in redox potential occur during the cell cycle, synchronized HeLa cells collected by mitotic shake-off were replated and harvested at representative times for analysis of protein expression.

Cells were stained with an oxidation-sensitive fluorescent probe (C-400) and analyzed by flow cytometry. Oxidation of the probe 1 was greatest in cells immediately following mitotic shake-off (0 h postmitotic shake-off); 2) was minimal in G1 cells (2–7 h); and 3) increased 3- to 5-fold in late S/G2 cells (14–20 h) relative to G1 (Fig. 8A). An analysis of Topo II mRNA levels at representative time points during the cell cycle showed that the lower oxidation potential during the G1 phase (Fig. 8A) correlated with a decrease in Topo II mRNA levels (Fig. 8B). Similarly, an increase in oxidation potential during S/G2 phase correlated with an increase in Topo II mRNA levels. These observations are consistent with the results from Figs. 6 and 7, suggesting that a relatively reducing environment during G1 favors protein binding to the Topo II 3’-UTR and correlates with a decrease in Topo II mRNA levels. In contrast, a relatively greater oxidation potential during S/G2 phase would be expected to inhibit protein binding to Topo II 3’-UTR, resulting in increased Topo II mRNA levels.

The idea that the 3’-UTR containing the ARE motifs participates in the regulation of mRNA levels during the cell cycle was further investigated by measuring mRNA levels of two other ARE-containing cell cycle genes, p21 (37) and geminin (38). Both mRNAs contain AREs including the AUUUA motif in their 3’-UTRs. HeLa cells synchronized by mitotic shake-off were replated, and total cellular RNA isolated at 4, 12, 18, and 24 h after plating. Cells from duplicate dishes were assayed for cell cycle position by flow cytometric determination of DNA content. Approximately 85% of the cells were in G1 at 4 h after plating, and by 12 h 76% of the cells were in S phase (Fig. 9A). At 18 h after plating, 42% of the cells entered G2, and by 24 h approximately 65% of the cells entered G2 of the next generation. Consistent with previous reports, Northern blot analysis showed that Topo II mRNA levels were low in G1 (lane 1) and increased 15-fold during late S and G2 (lanes 2 and 3, Fig. 9B). Following cell division, Topo II mRNA levels decreased to basal levels (lane 4) in G1 of the next generation. Interestingly, both geminin and p21 mRNA levels were low in G1 (lane 1) and increased approximately 2- (geminin) and 5-fold (p21) during late S and G2 (lanes 2 and 3). The mRNA levels of both geminin and p21 decreased during G1 of the subsequent generation (lane 4). These results support the idea that AREs...
Topo II 3'-UTR and mRNA Accumulation

including the AUUUUUA motif present in the 3'-UTRs of Topo II, geminin, and p21 could possibly contribute to the regulation of mRNA accumulation during the cell cycle.

**DISCUSSION**

We have shown previously that Topo II mRNA levels increase 15–16-fold during late S and G2, compared with the G1 phase of the HeLa cell cycle and that variations in Topo II mRNA levels are associated with significant changes in mRNA stability (6). The present study investigates whether Topo II mRNA accumulation of two other AUUUUUA-containing cell cycle genes (geminin and p21) also varied during the cell cycle, and similar to Topo II their mRNA levels were low in G1. This is intriguing because, in general, the pentameric AUUUA motifs have been shown to regulate mRNA levels (18, 27, 29, 30). Although the role of heptamer ARE motifs in the regulation of mRNA levels has not been reported previously, it is well known that the nonamer ARE motif (UUAUUUAUU) can act as a potent mRNA destabilizer (21). Our results suggest that the potential influence of ARE-mediated mRNA instability may extend beyond the control of cytokine and proto-oncogene expression and include other growth-regulatory genes. Future studies will determine if the AUUUUUA motif by itself or in combination with other AREs or non-AREs confers cell cycle regulation of Topo II, geminin, and p21 gene expression.

Cytokine and proto-oncogene mRNA decay are thought to involve an AU-rich binding protein AUF1, which complexes with heat shock proteins hsc70-hsp70, translation initiation factor eIF4G, and poly(A)-binding protein (44). Multiple proteins with apparent molecular masses of 84, 70, 44, and 37 kDa...
bind to Topo II 3'-UTR and identified AUF1 as one of the proteins present in the RNA-protein complex. Since the 37-kDa protein has a molecular mass similar to AUF1 and its binding was enhanced in G1 (Fig. 5A), the possibility that enhanced binding could be related to changes in absolute quantities of AUF1 during the cell cycle was investigated. An immunoblot analysis of AUF1 in whole HeLa cell extracts prepared from cells in G1 and S showed that the absolute quantities of immunoreactive AUF1 protein did not vary during the cell cycle (data not shown). Thus, the absolute amount of AUF1 protein does not appear to influence Topo II mRNA levels, but posttranslational modifications (e.g. phosphorylation and/or oxidation/reduction) might regulate AUF1 activity during the cell cycle. Since AUF1 consists of four protein isomers, our results do not eliminate the possibility that other isomers of AUF1 may be involved in the cell cycle regulation of Topo II mRNA levels. Nonetheless, these results indicate the potential role of AUF1 in ARE-mediated regulation of cell cycle gene expression.

Our results further indicated that the protein binding to Topo II 3'-UTR is sensitive to redox reactions both in vitro and in vivo. In vitro protein binding to the 177-nt Topo II 3'-UTR was found to be reversibly enhanced by thiol-reducing agents and inhibited by thiol-oxidizing agents. These results support earlier in vitro reports demonstrating redox sensitivity of protein binding to ARE-containing cytokine and lymphokine mRNAs (27). In vivo experiments in which cells were treated with a thiol-reducing agent showed that a shift to a more reducing intracellular environment resulted in increased RNA-protein binding as well as more than 90% reduction in Topo II mRNA levels (Fig. 7). Taken together, these results indicate that thiol residues (presumably cysteine residues) in ARE-binding proteins (e.g. AUF1) participate in the binding to the Topo II 3'-UTR mRNA and that reduction of these thiol residues is required for binding. Since AUF1 has three cysteine residues in its two RNA binding domains (23), it is possible that one or more of these cysteine residues are involved in redox sensitivity of RNA-protein binding during the cell cycle. Thus, subtle changes in redox states during cell growth or in response to environmental stimuli may influence Topo II 3'-UTR mRNA-protein interactions, thereby affecting mRNA levels. Consistent with this assumption, a lower oxidation potential during G1 in synchronized cells was associated with more protein binding to Topo II 3'-UTR, correlating with decreased mRNA levels. Likewise, a relatively more oxidizing environment during S and G2 was associated with less protein binding, correlating with increased Topo II mRNA levels. The variations in intracellular redox states (Fig. 8) are consistent with previous findings (45), suggesting that a relatively high intracellular oxidation potential is associated with progression toward mitosis. Furthermore, our results also support the idea that proteins regulating cell cycle progression are sensitive to the redox potential of the microenvironment (46, 47).

In summary, results from this study support the hypothesis that a posttranscriptional mechanism regulated by the interaction of 3'-UTR mRNA with redox-sensitive protein complexes containing AUF1 may regulate Topo II mRNA accumulation during the cell cycle.

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Cell Cycle-coupled Variation in Topoisomerase IIα mRNA Is Regulated by the 3′-Untranslated Region: POSSIBLE ROLE OF REDOX-SENSITIVE PROTEIN BINDING IN mRNA ACCUMULATION

Prabhat C. Goswami, Jamie Sheren, Lee D. Albee, Azemat Parsian, Julia E. Sim, Lisa A. Ridnour, Ryuji Higashikubo, David Gius, Clayton R. Hunt and Douglas R. Spitz

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