BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 105–109 (1997)
ARTICLE NO. RC965893

Cell Cycle-Dependent Regulation of the Mouse DNA Topoisomerase IIα Gene Promoter

Noritaka Adachi, Masao Kobayashi, and Hideki Koyama[1]
Kihara Institute for Biological Research, Yokohama City University, Maioka-cho 641-12, Totsuka-ku, Yokohama 244, Japan

Received November 26, 1996

Expression of DNA topoisomerase (topo) IIα varies through the cell cycle with its peak in G_2/M. To investigate the mechanism controlling the topo IIα gene expression, we cloned the 5′ upstream region of the mouse topo IIα gene. Although there was no TATA-like sequence, two GC and seven CCAAT boxes were found in the upstream region 5′ distal to the major transcription start sites, which were located 137, 124, and 105 bp upstream from the ATG start codon. Luciferase vectors with the upstream sequences were constructed and transfected into HeLa cells, followed by cell cycle arrest either in G_1 by treatment with mimosine, in S with thymidine, or in G_2/M with colcemid. We found that the topo IIα gene promoter has the cell cycle-dependent activity, which is low in G_1, rises in S, and peaks in G_2/M. We suggest that the level of topo IIα mRNA is determined by the cell cycle-regulated promoter.

Eukaryotic DNA topoisomerase II (topo II) is a ubiquitous nuclear enzyme that can alter the topology of DNA, by transiently breaking both strands of DNA helix, passing another segment of DNA duplex through the break, and religating the two strands (reviewed in Ref. 1). The enzyme has been implicated in many cellular processes, such as replication, transcription, recombination, or chromosome condensation and segregation (2-9).

Yeast cells express only one topo II protein that is shown to be essential for cell viability, being required for chromosome segregation at the time of cell division (2-4). In contrast, mammalian cells express two closely related but genetically distinct isoforms, topo IIα and topo IIβ (10-14), and their respective roles remain unclear. Recent studies have shown that expression of topo IIα protein varies through the cell cycle with its peak in G_2/M and lowest level in G_1 (15-17), whereas topo IIβ is expressed constantly irrespective of the cell cycle position. These findings raise the possibility that topo IIα is required mainly for chromosome condensation and/or segregation.

More recently, Goswami *et al.* (18) have reported that the level of the topo IIα mRNA during the cell cycle parallels that of the protein, and that the mRNA level is determined through changes in mRNA stability with a half-life of 30 min in G_1 and >4 hr in late S. The 5′ upstream sequences of the human, hamster, and rat topo IIα genes have recently been isolated, and the promoter regions conferring a basal activity characterized (19-21). However, it remains unexplored whether the topo IIα gene promoter contributes to the cell cycle-regulated expression of this gene. In this paper, we describe the isolation and characterization of the 5′ upstream region of the mouse topo IIα gene. Using transient expression assays with the firefly luciferase reporter gene, we have demonstrated that the topo IIα promoter possesses the cell cycle-dependent activity that is maximal in G_2/M.

MATERIALS AND METHODS

Isolation of the 5′ upstream region of the mouse topo IIα gene. By systematic Southern blot analyses of genomic DNA from mouse ES cells, we found that the 5′ upstream region of the topo IIα gene resides in a 9 kb *Hin*dIII fragment, together with the exons which correspond to nucleotides 1 to 573 of the topo IIα cDNA (data not shown). Hence, ES genomic DNA was digested with *Hin*dIII, religated to allow self-circularization, and used as a template for an inverse polymerase chain reaction with the following primers: pR5, 5′-CTGGGCGGAGCAGTATATGTTCC-3′ (139-117 of the cDNA); and p550, 5′-GCGTACAAGAAAATGTTCAAACAG-3′ (550-573 of the cDNA). The amplified 2.4 kb fragment was digested with *Hin*dIII and *Bam*HI, and the resulting 1.1 kb fragment was subcloned into pUC119, followed by sequencing using the dideoxy nucleotide chain termination method (22).

Plasmid construction. The 982 bp *Sac*I-*Nco*I fragment or the 479 bp *Bgl*II-*Nco*I fragment was subcloned into the pGL3-Promoter vector (Promega), upstream of the firefly luciferase gene at the corresponding restriction sites, generating pSac and pBgl, respectively (see Fig. 3). In each case, translation of the luciferase gene starts

[1] To whom correspondence should be addressed. Fax: +81-45-820-1901. E-mail: koyama@yokohama-cu.ac.jp.

0006-291X/97 $25.00

from the authentic ATG start codon at the *Nco*I site for both the luciferase and mouse topo IIα gene, thereby preventing artificial results that could arise from chimeric constructs.

Primer extension. Total cellular RNA was isolated from mouse ES cells using ISOGEN (Nippon Gene). An oligonucleotide, 5′-TCC-TGAAGAGGCTCGAGAATCCGG-3′, corresponding to positions −32 to −9 from the ATG start codon was used as a primer. Labelled primer (50 pmol) and the total RNA (100 μg) were denatured for 10 min at 70°C, and then incubated at 37°C for 30 min. Primer extension was carried out in a total volume of 50 μl containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM dNTPs, 2 U RNase inhibitor, and 300 U SUPERSCRIPT II reverse transcriptase (Gibco BRL). After incubation for 2 hr at 42°C, the reaction was stopped with 4 μl of 0.5 M EDTA and 1 μl of 5 μg/ml RNase. Following extraction with phenol/chloroform, the products were analyzed on a 4% polyacrylamide gel containing 7 M urea. The sizes were accurately determined with reference to a sequencing reaction in which the same primer was used.

Cell culture, transfection, and luciferase assays. HeLa cells were grown in ES medium (Nissui) supplemented with 5% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were plated into a 12-well cluster dish (Costar), grown for about 20 hr, and transfected using the lipofection method as described previously (23). Briefly, 0.4 ml of serum-free ES medium containing 300 ng of plasmid DNA and 2 μl of LipofectAMINE (Gibco BRL) was added to the cell layer ($\sim 4 \times 10^5$ cells). After 5-hr incubation, the medium was replaced with growth medium and the cells were grown for further 18 hr, followed by treatment with one of the following: 0.4 mM mimosine (Sigma) for 24 hr, 2 mM thymidine (Wako) for 16 hr, or 25 ng/ml colcemid (Wako) for 24 hr. Cells were then harvested with Reporter Lysis Buffer (Promega). For luciferase assays, cell lysates were mixed with Luciferase Assay Reagent (Promega) and light intensity was measured for 30 sec on a luminometer (BLR-301, Aloka). Results were expressed as relative light units (RLUs).

Flow cytometry. Synchronized cells were collected, fixed with ethanol, treated with RNase, stained with propidium iodide, and analyzed on a Coulter EPICS XL flow cytometer (24).

RESULTS

Sequence Analysis of the 5′ Upstream Region of the Mouse topo IIα Gene

We isolated a genomic DNA containing the 5′ upstream region of the mouse topo IIα gene and determined its nucleotide sequence (Fig. 1). To identify the site(s) of transcription initiation, the 5′ end of topo IIα mRNA was determined by primer extension analysis (Fig. 2). This experiment led to the identification of three major start sites located 137, 124, and 105 bp upstream from the ATG start codon. The distal start site is designated hereafter as +1 unless otherwise stated. Inspection of the sequence 5′ distal to the start sites showed no TATA-like sequence, indicating that the mouse topo IIα gene is a TATA-less gene. Instead, several consensus sequences characteristic of eukaryotic promoter elements are present in this immediate upstream region (Fig. 1). There are two GC boxes, potential binding sites for Sp1 transcription factors, one of which is located in the inverted orientation. There are also seven CCAAT boxes, five of which are present in the inverted orientation.

Promoter Activity of the 5′ Upstream Region of the Mouse topo IIα Gene

In order to test the upstream sequences for promoter activity, the 5′ upstream region −845/+137 or −342/+137 of the mouse topo IIα gene was linked to the firefly luciferase reporter gene in a vector devoid of any promoter/enhancer sequences, generating pSac and pBgl, respectively (Fig. 3A). Each construct was then transfected into HeLa cells using the lipofection method. Prior to luciferase assay, the transfected cells were treated with drugs to arrest them in different phases of the cell cycle; mimosine to block in late G_1, thymidine in S, or colcemid in G_2/M. Fig. 3B shows representative flow cytometry profiles of cell cycle position as determined by DNA content after treatment with these drugs. After a 24-hr treatment with mimosine, a plant amino acid capable of arresting cells in late G_1(25, 26), 87% of the cells were blocked in G_1. Thymidine blocked 65% of the cells in S after a 16-hr treatment, and colcemid blocked 95% of the cells at the G_2/M boundary after a 24-hr treatment. Following such cell cycle arrests for 16 or 24 hr, the cells were harvested and assayed for their luciferase activity. As shown in Fig. 3C, the pBgl-transfected cells arrested in G_2/M had 4∼5-fold greater luciferase activity than the cells arrested in late G_1. The cells arrested in S had an intermediate level. These results indicate that the upstream region −342/+137 of the mouse topo IIα gene has promoter activity, which is low in G_1, rises in S, and peaks in G_2/M.

Very similar results were obtained when the pSac construct was transfected (Fig. 3C), suggesting that the region −845/−343 plays no noticeable role in the efficiency or regulation of transcription. A luciferase vector with the SV40 early promoter sequence (pGL3-Promoter) was similarly transfected into HeLa cells, which were then blocked in different phases of the cell cycle and subjected to luciferase assay. This promoter showed approximately 2.5-fold greater activity in S than in G_1, while its activity in G_2/M was lower (70%) than that in S (Fig. 3C). The activity of the pBgl or pSac construct in colcemid-arrested cells was comparable to, or rather higher than, that of the pGL3-Promoter vector, suggesting that the topo IIα promoter activity is considerably high in G_2/M.

Taken together, our data indicate that the upstream region of the mouse topo IIα gene has the promoter activity that is strongly cell cycle-regulated, peaking in G_2/M.

DISCUSSION

In this study, we have cloned and characterized the 5′ upstream region of the mouse topo IIα gene (Figs. 1 and 2), and, using transient expression assays with the luciferase reporter gene, we have demonstrated that

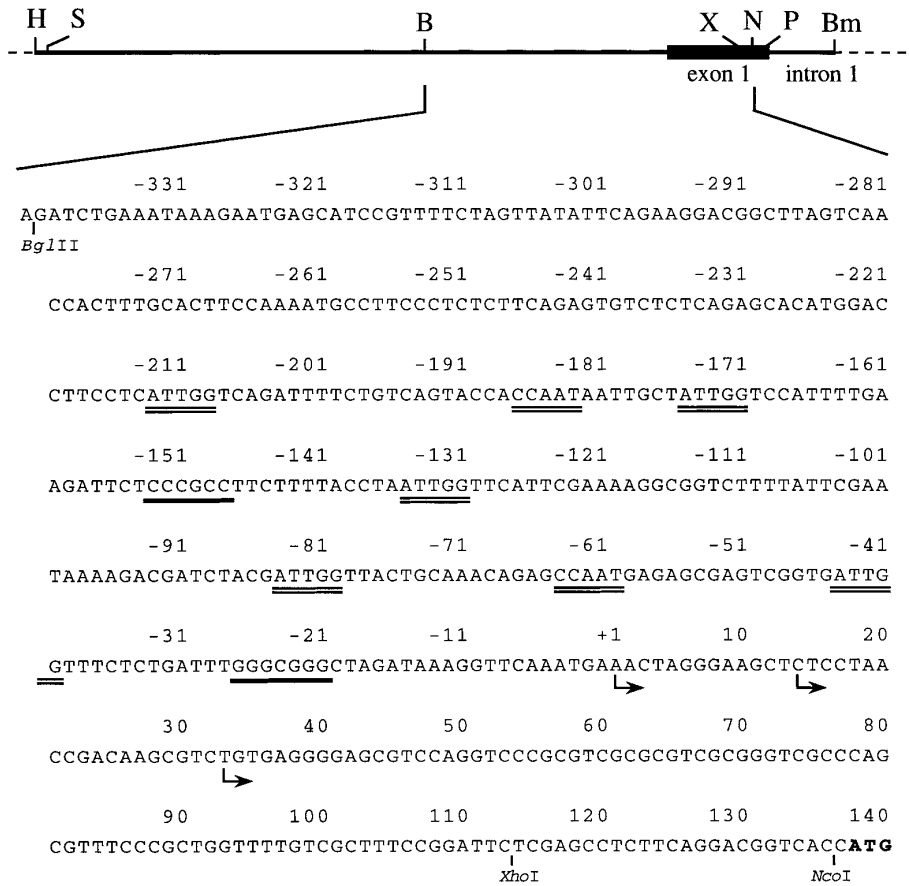

FIG. 1. Sequence of the 5′ upstream region of the mouse topo IIα gene. (Upper) Restriction map of the upstream region. The first exon is indicated as a closed rectangle. Selected restriction sites are indicated: B, *Bgl*II; Bm, *Bam*HI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; S, *Sac*I; X, *Xho*I. (Lower) The nucleotide sequence of the 482 bp fragment located immediately upstream of the ATG start codon (boldface). The three major transcription start sites are indicated by arrows. Bases are numbered with respect to the distal transcription start site designated as +1. The GC boxes are underlined, and the CCAAT boxes are doubly underlined, five of which are in the inverted orientation. The nucleotide sequence will appear in the DDBJ/EMBL/GenBank DNA databases with the Accession Number D88147.

the topo IIα gene promoter is strongly cell cycle-regulated in HeLa cells: the promoter activity is low in G_1, rises in S, and reaches a peak in G_2/M (Fig. 3). In addition, similar results have been obtained in mouse NIH 3T3 cells (Adachi *et al.*, unpublished results). This promoter activity parallels the pattern of topo IIα mRNA levels (18). We suggest, therefore, that the cell cycle-dependent promoter activity determines the level of topo IIα mRNA. Consistent with this idea, the topo IIα gene promoter shows little activity in G_0, in which topo IIα mRNA is undetectable (Adachi *et al.*, unpublished results). However, Goswami *et al.* (18) have reported that changes in topo IIα mRNA stability during the cell cycle contribute to the fluctuation of topo IIα mRNA levels, since the half-life of the mRNA is 30 min in G_1 and $>$4 hr in late S. Therefore, it is probable that, in addition to changes in the rate of transcription, changes in mRNA stability are responsible for the cell cycle-regulated expression of this gene.

The mouse topo IIα gene promoter has a moderately high GC content, no TATA-like sequence (Fig. 1), and the transcriptional start sites are scattered in several positions (Fig. 2). These are the characteristics of promoters of housekeeping genes (27). Comparison of the 5′ upstream sequences of the human, hamster, and rat topo IIα genes with the mouse gene revealed that the promoters, as well as the noncoding regions, of these genes share a high degree of homology (Adachi *et al.*, unpublished results). This suggests that mammalian topo IIα genes share the same machinery for transcriptional regulation. The mouse topo IIα gene promoter contains two GC and seven CCAAT boxes, five of which are located in the inverted orientation (Fig. 1). The two GC and all the inverted CCAAT boxes are well conserved (Adachi *et al.*, unpublished results), raising the possibility that these sequences play a pivotal role in the regulation of topo IIα promoter activity. In contrast, two direct CCAAT boxes are not found in the other topo IIα promoters, suggesting that these sequences in the mouse promoter are regulatory ele-

ments specific for the mouse gene; otherwise, they might be nonfunctional.

Inverted CCAAT boxes are known to be important for the cell cycle-dependent transcriptional regulation of the human thymidine kinase gene (28, 29) and for the serum-inducible transcription of the human DNA polymerase α gene (30). Ng *et al.* (20) have shown the protein binding at and around the inverted CCAAT boxes present in the hamster topo IIα gene and their significance for basal promoter activity. Therefore, it is likely that the inverted CCAAT boxes found in the mouse gene are also involved in the transcriptional regulation. It remains to be elucidated, however, whether these sequences contribute to the cell cycle-dependent promoter activity. Further study is required to characterize the elements which may control the basal and/or cell cycle-regulated expression of the topo IIα gene.

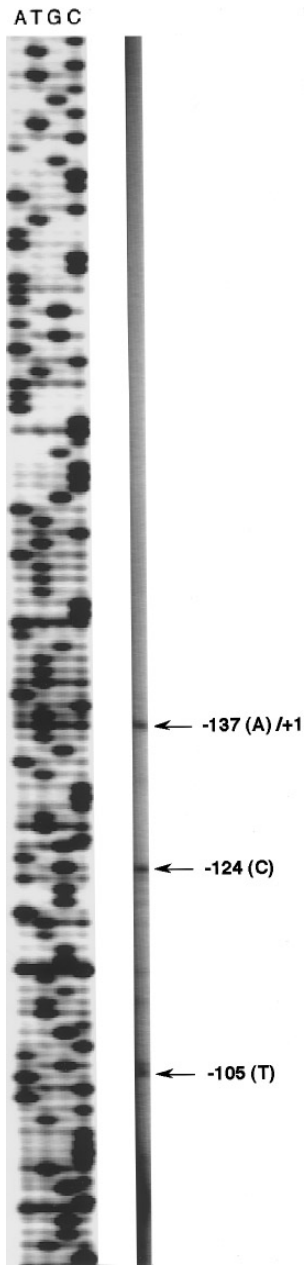

FIG. 2. Determination of the transcription start site of the mouse topo IIα gene by primer extension. The right-most lane shows the products of a primer extension reaction. The three major transcription start sites are indicated by arrows. A sequencing reaction was carried out using the same primer, allowing accurate determination of the sizes.

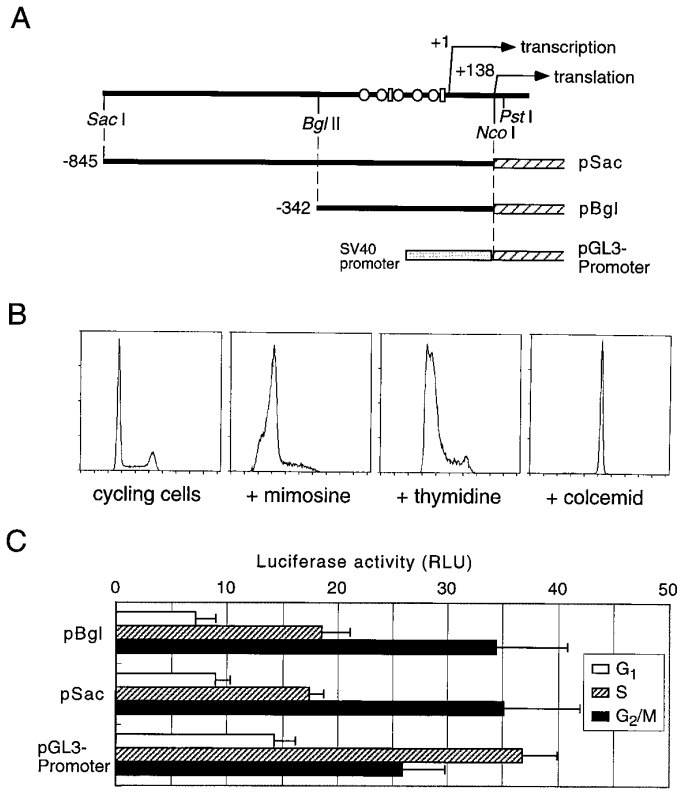

FIG. 3. Promoter activity of the 5′ upstream region of the mouse topo IIα gene. (A) Structure of the reporter constructs. Thick lines indicate the topo IIα gene 5′ upstream region. Nucleotides −845 to +137 or −342 to +137 of the mouse topo IIα gene were linked to the firefly luciferase gene (hatched region), generating pSac and pBgl, respectively. The GC and inverted CCAAT boxes are shown by open rectangles and circles, respectively. (B) Cell cycle position after treatment with drugs. Representative flow cytometry profiles of HeLa cells are shown. Cells were treated with one of the following: no drug (cycling cells), 0.4 mM mimosine for 24 hr, 2 mM thymidine for 16 hr, or 25 ng/ml colcemid for 24 hr. (C) Promoter activity in HeLa cells. HeLa cells were transfected, using the lipofection method, with either the pSac, pBgl, or pGL3-Promoter vector. Following cell cycle arrest, the cells were harvested and assayed for their luciferase activity. Results were expressed as relative light units (RLUs). Error bars represent the standard deviation of three separate experiments.

ACKNOWLEDGMENTS

We thank Drs. A. Kikuchi and H. Ikeda for supports and helpful suggestions. This work was supported by grants from Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences and

the Ministry of Education, Science, Sports and Culture, Japan. The first two authors made an equal contribution to the work.

REFERENCES

1. Wang, J. C. (1985) *Annu. Rev. Biochem.* **54,** 665–697.
2. DiNardo, S., Voelkel-Meiman, K., and Sternglanz, R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2616–2620.
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. Brill, S. J., and Sternglanz, R. (1988) *Cell* **54,** 403–411.
6. Christman, M. F., Dietrich, F. S., and Fink, G. R. (1988) *Cell* **55,** 413–425.
7. Kim, R. A., and Wang, J. C. (1989) *J. Mol. Biol.* **208,** 257–267.
8. Kim, R. A., and Wang, J. C. (1989) *Cell* **57,** 975–985.
9. Adachi, Y., Luke, M., and Laemmli, U. K. (1991) *Cell* **64,** 137–148.
10. Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. (1989) *Biochemistry* **28,** 8154–8160.
11. Tsai-Pflugfelder, M., Liu, L. F., Liu, A. A., Tewey, K. M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., and Wang, J. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7177–7181.
12. Chung, T. D. Y., Drake, F. H., Tan, K. B., Per, S. R., Crooke, S. T., and Mirabelli, C. K. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9431–9435.
13. Adachi, N., Miyaike, M., Ikeda, H., and Kikuchi, A. (1992) *Nucl. Acids Res.* **20,** 5297–5303.
14. Jenkins, J. R., Aytn, P., Jones, T., Davies, S. L., Simmons, D. L., Harris, A. L., Sheer, D., and Hickson, I. D. (1992) *Nucl. Acids Res.* **20,** 5587–5592.
15. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth Diff.* **2,** 209–214.
16. Taagepera, S., Rao, P. N., Drake, F. H., and Gorbsky, G. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8407–8411.
17. Kimura, K., Saijo, M., Ui, M., and Enomoto, T. (1994) *J. Biol. Chem.* **269,** 1173–1176.
18. Goswami, P. C., Roti Roti, J. L., and Hunt, C. R. (1996) *Mol. Cell. Biol.* **16,** 1500–1508.
19. Hochhauser, D., Stanway, C. A., Harris, A. L., and Hickson, I. D. (1992) *J. Biol. Chem.* **267,** 18961–18965.
20. Ng, S. W., Paul Eder, J., Schnipper, L. E., and Chan, V. T. W. (1995) *J. Biol. Chem.* **270,** 25850–25858.
21. Park, S. H., Yoon, J. H., Cho, H. A., Kwon, Y. D., Seong, R. H., Hong, S. H., and Park, S. D. (1995) *J. Biochem.* **118,** 725–733.
22. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
23. Fujimaki, K., Aratani, Y., Fujisawa, S., Motomura, S., Okubo, T., and Koyama, H. (1996) *Somat. Cell Mol. Genet.*, in press.
24. Krishan, A. J. (1975) *J. Cell Biol.* **66,** 188–193.
25. Lalande, M. (1990) *J. Biol. Chem.* **186,** 332–339.
26. Gilbert, D. M., Neilson, A., Miyazawa, H., DePamphilis, M. L., and Burhans, W. C. (1995) *J. Biol. Chem.* **270,** 9597–9606.
27. Sehgal, A., Patil, N., and Chao, M. (1988) *Mol. Cell. Biol.* **8,** 3160–3167.
28. Knight, G. B., Gudas, J. M., and Pardee, A. B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8350–8354.
29. Kim, Y. K., and Lee, A. S. (1991) *Mol. Cell. Biol.* **11,** 2296–2302.
30. Pearson, B. E., Nasheuer, H. P., and Wang, T. S. F. (1991) *Mol. Cell. Biol.* **11,** 2081–2095.