Thymidine kinase transcription is regulated at G₁/S phase by a complex that contains retinoblastoma-like protein and a cdc2 kinase
(cell cycle/DNA-binding proteins/inducible DNA-binding activity Y1)

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ABSTRACT Transcription of the murine thymidine kinase gene, which is coregulated with the G₁/S phase transition, is activated by changing the binding of protein complexes Y11 and Y12 to three upstream DNA motifs. Y11 is replaced by Y12 shortly before S phase. Y11 contains a protein of 110 kDa that binds to the DNA motif sites and may be an underphosphorylated murine retinoblastoma protein, shown by its molecular mass, timing of its activity, and antibody recognition. An H1 kinase related to cdc2 cofractionates with both complexes. We propose that this kinase phosphorylates the murine retinoblastoma protein, releasing transcriptional inhibitions by Y11 and permitting cell cycle progression. These results provide a cycle-related molecular target for such complexes. They are based on investigations of cycle control in infected cells. The Y1 complexes are similar but not identical to complexes that include a cellular protein, E2F, that was originally found to bind to adenovirus DNA.

Cell proliferation depends upon growth factors that initiate intracellular signals resulting in transcriptional activations (1, 2). Of critical importance are events in G₁ phase that lead to the initiation of S phase, which includes the onset of DNA synthesis. These events require production of proteins such as the enzymes involved in DNA synthesis. Activation of genes is observed and is controlled by transactivating factors (1). One good candidate for a regulatory protein component is pRB, the retinoblastoma protein, which in G₁ phase prevents proliferation (3–6). Another potential G₁/S phase regulator is a cdc2-like kinase that associates with cyclin A or a G₁ cyclin to phosphorylate pRB and permit cell progression through G₁/S phase (7–10). Nevins and coworkers (11) have reported that underphosphorylated pRB and cyclin A are present in two distinct transcription factor E2F complexes in human U937 cells. In synchronized NIH 3T3 cells a G₁-phase-specific E2F complex was replaced by a cyclin A-containing complex near the G₁/S boundary (12). La Thangue and coworkers (13) have identified a complex of DRTF1, a E2F-like transcription factor, which contains both pRB and cyclin A (13). Connection of proteins to the mechanism that activates the onset of S-phase events is, however, not understood. We present experiments that make this connection.

Inductions of S-phase enzymes are tightly coupled with the onset of DNA synthesis (1, 2), suggesting that factors regulating the expression of these enzymes may also control production of transcription factors that initiate DNA synthesis. To investigate these events, we studied the murine thymidine kinase upstream promoter (14). We performed DNase I footprint analysis and identified three DNA motif (MT) sequences (MT1, MT2, and MT3) that bind proteins (15). These MT sequences are similar but not identical to those of E2F/DRTF1 (16–18). To further study MT sequence-binding proteins, we performed band-shift analysis by using synthetic MT oligonucleotides and identified an inducible DNA-binding protein complex, Y1, now called Y12 (15). Y12 was found in S-phase but not in G₀ or G₁-phase nuclear extracts in normal BALB/c 3T3 A31 cells (A31 cells) (15). Y12 was altered in benzo[a]pyrene-transformed A31 cells (BPA31 cells) (19), suggesting a role in normal growth regulation. Indeed, results from mutagenesis of specific MT sequences located either upstream or downstream of the thymidine kinase translation site reveal deregulated expression of a thymidine kinase–reporter gene (J. L. Fridovich-Keil and A.B.P., unpublished data).

In the studies presented here, we have observed a G₁-phase-specific complex Y11 and an S-phase-specific complex Y12, both of which contain a murine pRB (pRBm). We have also found that a cdc2-like protein is present in Y11 and this cdc2-like kinase might regulate the conversion of Y11 to Y12 at G₁/S phase.

MATERIALS AND METHODS

Materials. Oligonucleotides containing the MT3 sequence (15) were synthesized on an Applied Biosystems model 308 synthesizer at the Dana-Farber Oligonucleotide Facility. The annealed oligonucleotides were end-labeled with [α-³²P]dCTP and the Klenow fragment of DNA polymerase I (20) and used for both band-shift and Southwestern assays. Salmon sperm DNA (27–4564–01) and double-stranded poly(dI–dC) (27–7880–01) were purchased from Pharmacia. [γ-³²P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from NEN Research Products. Alkaline phosphatase–agarose (P0762), histone H1 (type III-S, H5505), CAMP-dependent kinase inhibitor peptide (P8140), and all other chemicals were from Sigma. The Immulon-Blot alkaline phosphatase assay kit was from Bio-Rad. Goat anti-mouse IgG, rabbit anti-mouse IgG, and purified mouse anti-human pRB monoclonal antibodies 245, 340, and 349 were from GIBCO/BRL.

Cell Culture, Cell Synchronization, and Nuclear Extract Preparation. A31 cells were grown as monolayer culture (20), and cell synchronization after serum deprivation was achieved as described (14). Entry of cells into S phase was monitored by incorporation of [³H]thymidine (21). Nuclear protein extracts were prepared from the tissue culture cells essentially by the method of Dignam et al. (22).

Band-Shift Analysis. The band-shift assays were performed as described (23). Protein samples were incubated at room temperature for 20 min with a ³²P-labeled MT3 probe (≈30,000 cpm per reaction mixture) and poly(dI–dC) nonspecific DNA competitor (1.25 μg per reaction mixture) in 12.5 μl of binding buffer [10 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/5% (vol/vol) glycerol]. The reaction mixture was then loaded directly onto a 4% polyacrylamide gel.

Abbreviations: pRB, retinoblastoma protein; pRBm, murine pRB.
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For phosphorylation experiments, Y1 binding was assayed in the presence of 2 mM MgCl₂ and 1 mM ATP. For alkaline phosphatase experiments, A31 nuclear extract was incubated with alkaline phosphatase-agarose at 37°C as indicated and then assayed for Y1 binding. For antibody blocking experiments, the nuclear extract was first incubated with an antibody for 5 hr at 4°C and then assayed for Y1 activity.

**Southwestern Blot Analysis.** The Southwestern blot assays were performed essentially as described (24). Protein samples were electrophoresed on an SDS/polyacrylamide gel and then electrophoretically transferred to a nitrocellulose filter. The filter was treated for 3 hr at 4°C with blocking solution [5% (wt/vol) low-fat dry milk, 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1 mM diethiothreitol, and sonicated denatured salmon sperm DNA (20 µg/ml)] and then washed twice with binding buffer (10 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM diethiothreitol/0.25% low-fat dry milk). The washed filter was then incubated for 3 hr at room temperature in binding buffer containing a 32P-labeled MT3 probe (1 × 10⁶ cpm per lane) and poly(dI-dC) nonspecific competitor DNA (2 µg per lane). The labeled filter was washed five times with binding buffer, dried, and autoradiographed.

**Western Blot Analysis.** Immunoblot assays were performed according to Bio-Rad instructions. Electrophoresis and transfer of protein samples were performed as described in the Southwestern blot analysis. The nitrocellulose blot was blocked, washed, and incubated with the first antibody. The filter was washed to remove the unbound first antibody and then incubated with the second antibody, which is a goat anti-rabbit (or -mouse) IgG conjugated to alkaline phosphatase. Finally, the filter was incubated with the color development reagents to visualize protein bands.

**Histone H1 Kinase Assay.** The H1 kinase assay was performed as described (25). A protein sample was incubated in 10 µl of buffer K [cAMP-dependent protein kinase inhibitor peptide (0.1 µg/µl)/H1 histone (0.25 µg/µl)/0.1 mM ATP/5 µCi of [γ-32P]ATP]. After a 20-min incubation at 30°C, the reaction was stopped by adding 10 µl of 2X SDS sample buffer [125 mM Tris·HCl, pH 6.81/4% (wt/vol) SDS/20% glycerol/10% 2-mercaptoethanol/0.01% bromophenol blue] and boiling for 5 min, and the reaction mixture was subjected to gel electrophoresis. The gel was then fixed and washed five times with 5% (wt/vol) trichloroacetic acid containing 1% sodium pyrophosphate (Sigma), dried, and exposed to film at -70°C.

**Heparin-Agarose Chromatography of Y1 Proteins.** In a representative experiment, a crude nuclear extract (140 µg of protein), made from BPA31 cells (24 g) grown by the Massachusetts Institute of Technology Cell Culture Center, was loaded onto a heparin-agarose column, which was precolumned with buffer T (20 mM Tris·HCl, pH 7.5/10% glycerol/1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM diethiothreitol/0.01% Nonidet P-40) containing 0.1 M NaCl. The column was washed with the same buffer and the unbound proteins were collected (flow-through fraction, ~70 mg of protein). The bound proteins were recovered by a 50-ml linear gradient elution from 0.1 to 1 M NaCl in buffer T.

**RESULTS AND DISCUSSION**

**Y1 Is Replaced by Y12 at G₁/S Phase.** The Y11 complex (formerly TKE) appeared only in G₁ phase (6 and 11 hr). The Y12 binding activity dramatically increased after G₁/S (11 hr) (Fig. 1A), as reported (15). Another retardation band labeled p60 is derived from a DNA-binding protein of 60 kDa (see Fig. 3) since a 5000-fold-purified p60 fraction shifted the MT3 oligonucleotide to the same position (unpublished data). Two other weak bands, X and W, were also seen in some experiments. All these bindings are sequence-specific (ref. 15; data not shown).

To confirm the presence of two Y1 complexes, we fractionated nuclear extracts, prepared from exponentially growing BPA31 cells, with a heparin-agarose column and mea-

![Fig. 1. Two DNA-binding Y1 protein complexes. (A) G₁-phase-specific Y11 and S-phase-specific Y12. 32P-labeled (~30,000 cpm per reaction mixture) synthetic oligonucleotides containing the MT3 sequence (15) were used in all the band-shift assays. The MT3 probe was mixed with 10 µg of crude nuclear extract prepared from A31 cells harvested at the indicated times in hours above the lanes after serum stimulation. Free and complexes of Y11, Y12, and p60 are indicated. (B) Y11 and Y12 complexes in fractions eluted from a heparin-agarose column. Each fraction (2 µl, ~3 µg) as indicated above the lanes, was used in the band-shift assay. (C) Elimination of Y11 and Y12 retardations by alkaline phosphatase treatment. A31 nuclear extract (3 µg) was incubated with 10 µg of alkaline phosphatase-agarose (+) or 5 µg of agarose beads alone (-) at 37°C for 15 (lanes 2 and 2) or 30 (lanes 3 and 4) min. The reaction mixtures were centrifuged and the supernatants were recovered for band-shift assays. (D) Enhancement of ATP of Y12 binding activity in heparin column fractions. Band-shift assays were conducted with 2 mM MgCl₂ in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM ATP. Fraction 33 (lanes 1 and 2) or 34 (lanes 3 and 4) (2 µl, ~1 µg), eluted from another heparin-agarose column preparation, was used.](image-url)
sured Yi DNA-binding activity in each fraction. Both Y11 (located in fractions 75–78) and Yi2 (fractions 63–78) were enriched 10- to 20-fold by this column (Fig. 1B).

The Yi1 complex in G1-phase nuclear extract was unstable and changed to Yi2 complex after storage, after being frozen and thawed several times or treated with deoxycholate, or during separation on a Sephacryl S-300 gel filtration column (unpublished data). Treatment with alkaline phosphatase abolished Yi1 binding rapidly and Yi2 binding more slowly (Fig. 1C). Phosphorylation activated the Yi2 complex. A 30-min incubation of 1 mM ATP and the two fractions (equivalent to fraction 81 in Fig. 1B) that eluted just beyond the Yi1 and Yi2 peaks (fractions 33 and 34 from another heparin column preparation) and had low Yi activities increased their Yi2 binding activity 10- to 20-fold (Fig. 1D). Adenosine 5’-[γ-thio]triphosphate, an analog of ATP, had no such effect (data not shown). These data indicate the involvement of a kinase in production of Yi2 at the G1/S boundary.

pRBm Is Present in Both Yi1 and Yi2. Both Yi1 and Yi2 have molecular masses >240 kDa, measured by Sephacryl S-300 gel filtration (unpublished data), suggesting that they are multiprotein complexes. To identify these proteins, we preincubated A31 nuclear extracts with various antibodies against pRB, cdc2, or cyclins and then assayed Yi by gel retardation. Monoclonal antibody 245 against human pRB eliminated both Yi1 and Yi2 retardations; another human pRB monoclonal antibody, 340, and goat anti-mouse IgG controls did not show these effects (Fig. 2A). With Western blot analysis of A31 crude nuclear extracts, antibody 245, but not antibody 340, identified two forms of pRBm, p100 and p110 (Fig. 2B). Other anti-human pRB antibodies, including antibodies 349, 277, 104, and 133, affected Yi complexes less (data not shown). To further determine if Yi2 contains pRBm, we performed band-shift assays with partially purified Yi2 fractions from a heparin column (see Fig. 1B). Only pRB antibody 245, but not the control antibody, eliminated the Yi2 complex (Fig. 2C). Thus, pRBm or a related protein was present in Yi2. These data show specific interactions between antibody 245 and pRBm in both Yi complexes.

A cdc2-Like Protein Is Present in Yi1. Since a cdc2-like kinase might regulate the conversion of Yi complexes, we next examined effects of antibodies to cdc2 (Fig. 2D). An antibody against recombinant human p34cdc2 (lane 5), but not against its C-terminal sequence (lane 6), selectively inhibited Yi1, suggesting that a cdc2-like protein is in the Yi1 complex. The Yi2 complex in a partially purified preparation from a heparin column was not affected by those antibodies against cdc2 protein or cyclins A, B, or CYL1 (Fig. 2C). Thus these proteins were not present or were not recognized in the Yi2 complex by these antibodies. We prefer the latter interpretation since a cdc2-like kinase copurified with the Yi2 protein in a 200-fold enriched fraction eluted from a MT3-binding-sequence-DNA affinity column (unpublished data).

Yi1 and Yi2 Have Different DNA-Binding Components. To study the DNA-binding component of each complex, we performed a Southwestern blot analysis of nuclear extracts prepared from A31 cells in various cycle stages. Two major DNA-binding proteins of 60 (p60) and 110 (p110) kDa were detected by any MT oligonucleotide but not by the AP1-binding site probe (data not shown), indicating relative sequence specificity. p60 binding was constitutive (Fig. 3A). In contrast, p110 activity was cycle-regulated, being very low in early G1 and peaking in mid-G1 phase (4–8 hr).

Both p60 and p110 were found in heparin column fractions 57–84 prepared from BPA31 nuclei (Fig. 3B). In some frac-

![Fig. 2.](image.png)
because Southwestern blot analysis detects protein monomers that bind to DNA whereas a band shift detects binding complexes. (ii) The peaks of both Y1I and p110 were found in G1 phase (Figs. 1A and 3A). (iii) Binding of Y1I and of p110 was unstable with storage at −20°C and abolished by alkaline phosphatase treatment (Fig. 1C; data not shown).

The DNA-Binding Protein p110 May Be Underphosphorylated pRBm. The timing in G1 phase of p110 activity, its molecular mass (∼110 kDa), and the presence of pRBm in Y1 suggest that p110 is an underphosphorylated form of pRBm (3–6). If so, p110 should be phosphorylatable in vitro. In fractions 75–84, which contain both Y1I and p110, several proteins with molecular masses of 87, 95, 110, 120, and 130 kDa were phosphorylated in vitro by endogenous kinase(s). The phosphorylation of these proteins had the same profiles as p110 activity, and roughly the same profile as of Y1I (compare Figs. 3C to 3B and 1B), suggesting that some or all of these proteins may be different forms of pRBm; only one of them had strong DNA-binding activity. However, attempts to stain these proteins with monoclonal antibody 245 against human pRB failed (data not shown).

pRB is a substrate for cdc2 kinase in vitro and probably in vivo (26–28). A purified authentic cdc2 kinase phosphorylated a partially purified p110 fraction in vitro (unpublished data), and antibodies to pRB and cdc2 disrupted Y1I retardation (Fig. 2A and D). High levels of H1 kinase activity were found in p110 fractions. They were in a considerably wider peak than Y1I perhaps because cdc2 is a component of several complexes that bind to various DNA sequences (Fig. 3C). A cdc2-like protein of ∼33 kDa was detected by Western blot analysis in the fractions containing p60, p110, and Y1 with an antisera directed to a PSTAIR amino acid sequence (Fig. 3D) that is conserved in cdc2-like kinases (29–31). Another human recombinant p34 and polyclonal antibody to the C terminus of human cdc2 also both detected a 33-kDa protein in the same fractions (data not shown). Furthermore, after a 5000-fold purification of p60, obtained after five chromatographic steps, we were still able to detect H1 kinase activity. This material contained only a half-dozen polypeptides including p33 and p60 bands, as visualized by silver staining (unpublished data). Our results suggest that activation of Y1I is mediated by a cdc2-like kinase.

We summarize our findings in a minimal model. In G1 phase underphosphorylated pRBm (p110), a cdc2 protein, p60, and other factors form a large inhibitory Y1I complex. At the G1/S boundary, a newly synthesized cyclin associates with and activates the cdc2 kinase, which then phosphorylates pRBm and releases it from binding DNA. Then, p60 binds to DNA in place of pRBm as part of the transcriptionally active Y1I complex, which may contain inactivated pRBm and other proteins, totaling 240 kDa.

Our results support the role of a functional cell-cycle-regulating target for complexes such as E2F and Y1. Further characterization and purification of each component will be needed to establish this model and also to answer remaining questions: What reaction controls the replacement of Y1I by Y1E at the G1/S boundary and how is this process altered in transformed cells?

Note. Since this paper was submitted, further information regarding cell-cycle-dependent transcription complexes has been reported: a G1-phase-specific E2F complex contained pRB whereas an S-phase-specific E2F complex contained cyclin A, cdk2, and p107 (32, 33). Our preliminary data using labeled MT3 and E2F as well as E2F mutant binding sequences and self- and cross-competition in a band-shift assay indicated that our S-phase-specific complex (Y12), which was reported (15), is related to the S-phase-specific E2F complex. But our G1-phase-specific complex (Y1I) is different from the G1-phase-specific E2F complex.

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