Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8343–8347, August 1994
Biochemistry

Phosphorylation of the p34 subunit of human single-stranded-DNA-binding protein in cyclin A-activated G_1 extracts is catalyzed by cdk–cyclin A complex and DNA-dependent protein kinase

(DNA replication/cell cycle regulation/DNA transactions)

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ABSTRACT **The human single-stranded-DNA-binding protein (HSSB, also called RP-A) is a trimeric complex (p70, p34, and p14) required for multiple functions in DNA transactions. We report here that the p34 subunit of HSSB was hyperphosphorylated by kinase activities present in G_1 extract (obtained from HeLa cells in G_1 phase) preincubated with human cyclin A. This hyperphosphorylated HSSB product included at least four species of p34 that migrated more slowly through denaturing polyacrylamide gels than the hypophosphorylated form. Fractionation of cyclin A-activated G_1 extract identified two kinases involved in the hyperphosphorylation of HSSB p34: cdk–cyclin A complex and DNA-dependent p350 protein kinase (DNA-PK). Kinetic analysis revealed that in cyclin A-activated G_1 extract, p34 was first phosphorylated by cdk–cyclin A prior to the action of DNA-PK. Addition of $p21^{cip1}$, a specific inhibitor of cdk–cyclin A but not DNA-PK, nearly abolished the hyperphosphorylation of HSSB p34 in G_1 extract preincubated with cyclin A. This suggests a requirement of the cdk–cyclin A activity for the phosphorylation of p34 by DNA-PK in G_1 extract.**

The human single-stranded-DNA (ssDNA)-binding protein (HSSB, also called RP-A), a complex containing polypeptides of 70, 34, and 14 kDa (p70, p34, and p14), was originally identified as a HeLa cell protein required for the *in vitro* replication of simian virus 40 (SV40) DNA (see refs. 1–3 for reviews). HSSB plays multiple roles in this replication reaction. In the initiation reaction, it stabilizes the ssDNA regions resulting from T antigen-mediated unwinding and assembles an initiation complex with T antigen as well as the DNA polymerase α–primase complex essential for primer synthesis. HSSB is also involved in the elongation stage of DNA replication. It activates DNA polymerases δ and ε to catalyze DNA chain elongation of primed templates in reactions dependent on both proliferating-cell nuclear antigen and activator 1 complex (also called RF-C) (4).

In addition to its role in replication, HSSB has been shown to function in other cellular activities. It is essential for DNA excision repair in a cell-free system (5). It stimulates the rate of homologous DNA pairing reaction catalyzed by the human homologous pairing protein 70-fold (6). More recently, several groups have shown that HSSB binds the acidic domain of transcription factors VP16 and p53 (7–9). Luche *et al.* (10) have recently found that *Saccharomyces cerevisiae* SSB binds to sequence elements that are involved in regulating transcription.

Each of the three SSB subunits of *S. cerevisiae* is encoded by a single essential gene (11). While the p70 subunit of HSSB binds to ssDNA (12), the biological functions of the p34 and p14 subunits are not known. Monoclonal antibodies against p34 block SV40 DNA replication, suggesting a direct role of p34 in this reaction (12).

Din *et al.* (13) have shown that the p34 subunit of HSSB is phosphorylated in a cell cycle-dependent manner. It is underphosphorylated in the G_1 phase of the cell cycle and becomes extensively phosphorylated during the $G_1 \rightarrow$ S-phase transition. The p34 subunit remains phosphorylated until M phase, when dephosphorylation occurs. Subsequent studies have demonstrated that both p34 and p70 are phosphorylated *in vitro* by the cdc2–cyclin B kinase complex (14, 15). Fotedar and Roberts (16) have reported a protein kinase activity that phosphorylates HSSB p34 in a ssDNA-dependent reaction.

We report that HSSB p34 was hyperphosphorylated in G_1 extract preincubated with human cyclin A and that this was due to the combined actions of the cdk–cyclin A complex and the p350 DNA-dependent protein kinase (DNA-PK).

MATERIALS AND METHODS

Protein Reagents. HSSB, human cyclin A, and $p21^{cip1}$ were purified as described (12, 17, 18), and cdc2–cyclin B from sea star and protein phosphatase 2Ac were purchased from Upstate Biotechnology (Lake Placid, NY).

Preparation of G_1 Extract. Twenty liters of HeLa cells, grown to $5–6 \times 10^5$ cells per ml at 37°C in Joklik's medium with 5% bovine calf serum, were treated with nocodazole (40 ng/ml) for 12 hr to arrest cells at the G_2/M boundary. The cells were centrifuged at $1300 \times g$ for 5 min at 22°C, washed free of drug by gentle resuspension in 4 liters of phosphate-buffered saline, and centrifuged again. Pelleted cells were gently suspended in 16 liters of Joklik's medium with 10% bovine calf serum and then allowed to recover for 2 hr at 37°C. Hexamethylenebisacetamide (HMBA; ref. 19) was added to 4 mM and the mixture was incubated for 6 hr to arrest cells in G_1. Flow cytometry of the HMBA-arrested cells, carried out with the Becton Dickinson Immunocytometry system, showed that ≈90% of cells were in G_1 phase. Cytosolic extract of G_1 cells was prepared as described (12).

Immunoblot Assays. Monoclonal antibodies (mAbs) against HSSB p34 (12) were used for immunoblot analysis (20).

Kinase Assays. For the phosphorylation of HSSB with G_1 extract, reaction mixtures (10 μl) containing 40 mM creatine phosphate (pH 7.7), creatine kinase at 25 μg/ml, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, G_1 extracts, and cyclin A were incubated at 37°C as indicated.

For the phosphorylation of HSSB p34 by purified kinases, reaction mixtures (10 μl) containing 40 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM ATP and kinase as indicated were incubated at 37°C for 45 min. After sepa-

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Abbreviations: ssDNA, single-stranded DNA; HSSB, human ssDNA-binding protein; DNA-PK, DNA-dependent protein kinase; HMBA, hexamethylenebisacetamide; mAb, monoclonal antibody; SV40, simian virus 40.

ration of products by SDS/PAGE, the ^{32}P forms of p34 were visualized with Coomassie brilliant blue or silver staining and excised for liquid scintillation counting.

Conditions for the phosphorylation of histone H1 and quantitation of H1 kinase activity were as reported (15).

Purification of cdk–Cyclin A and DNA-PK. G_1 extract (18.7 mg) was incubated with human cyclin A (0.18 mg) for 20 min at 30°C in the presence of 40 mM creatine phosphate (pH 7.7), creatine kinase at 25 μg/ml, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 4 mM ATP. The reaction mixture was then loaded onto 25–40% glycerol gradients (6 × 5 ml) in buffer A [25 mM Tris·HCl, pH 7.5/1 mM EDTA/0.01% Nonidet P-40/0.1 mM phenylmethanesulfonyl fluoride with antipain (0.2 μg/ml) and leupeptin (0.1 μg/ml)] plus 0.2 M NaCl and centrifuged at 190,000 × *g* for 18 hr. Two peaks of kinase activity that phosphorylated HSSB p34, with *s* values of 15 S and 5 S, respectively, were separately pooled and dialyzed against 2 liters of buffer B (buffer A/10% glycerol)/0.05 M NaCl for 3 hr at 4°C. The 15S fraction (0.6 mg of protein) was loaded onto a double-stranded DNA-cellulose column (Sigma, 0.7 ml, 0.7 cm × 1.8 cm) equilibrated with buffer B/0.05 M NaCl. After the column was washed with equilibration buffer (7 ml), bound material was eluted with 4 ml of buffer B/0.5 M NaCl. The eluate was then concentrated by centrifugation using a Centriflow-25 cone (Amicon). The resulting fraction (0.5 ml, protein at 0.15 mg/ml) was enriched in p350 DNA-PK as well as both the p70 and p80 subunits of the Ku antigen, as determined by immunoblot analysis (data not shown).

The 5S fraction (13 mg of protein) was mixed with glutathione *S*-transferase (GST)–$p13^{suc1}$ beads (Upstate Biotechnology, 0.4 ml, 2.5 mg of GST–p13/ml of beads), equilibrated with buffer B/0.05 M NaCl, and rocked for 2 hr at 4°C. The mixture was then packed into a small column (0.4 ml, 0.7 cm × 1 cm), successively washed with buffer B/0.5 M NaCl (4 ml) and 0.05 M NaCl (2 ml), and eluted with buffer B/0.05 M NaCl/5 mM glutathione (4 ml). After dialysis against 2 liters of buffer B/0.05 M NaCl for 2 hr at 0°C, the fraction was concentrated 16-fold with a Centriflow-25 cone. The resulting fraction (0.13 unit/μl) contained cyclin A, cdk2, and cdc2, as determined by immunoblot analysis (data not shown). One unit is defined as 1 nmol of phosphate incorporated into histone H1 (1 μg) after a 30-min incubation at 30°C.

Isolation of Phosphorylated HSSB. Phosphorylated HSSB was isolated from reaction mixtures by adsorption to ssDNA cellulose columns in the presence of protein phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, and 100 nM okadaic acid), followed by elution with 2 M NaCl/45% (vol/vol) ethylene glycol.

RESULTS

Cyclin A-Dependent Phosphorylation of HSSB p34 in G_1 Extract. To examine the effect of human cyclin A on the phosphorylation of HSSB by G_1 extract, cyclin A was preincubated with G_1 extract to allow formation of the cdk–cyclin A kinase complex (21). This procedure activated the histone H1 kinase activity of G_1 extract 4- to 5-fold (data not shown). Following preincubation of G_1 extracts with cyclin A, exogenous underphosphorylated HSSB (1.5 μg) was added and the mixture was further incubated as indicated (Fig. 1*A*). The phosphorylation of HSSB was then analyzed by examining electrophoretic mobility changes of HSSB p34, using mAbs against p34 for immunoblot detection. In the absence of cyclin A, only limited amounts of p34 were converted into slow-migrating forms (Fig. 1*A*, lane 2). In contrast, after a 30-min incubation with cyclin A, nearly all of the p34 subunit was converted into multiple slow-migrating forms designated b–e (lane 5). Form b appeared after a 5-min incubation and then decreased after 30 min (lanes 3–6). In contrast, form e was detected after a 15-min lag and its level continued to increase up to 60 min. Forms c and d reached steady levels after 15 min.

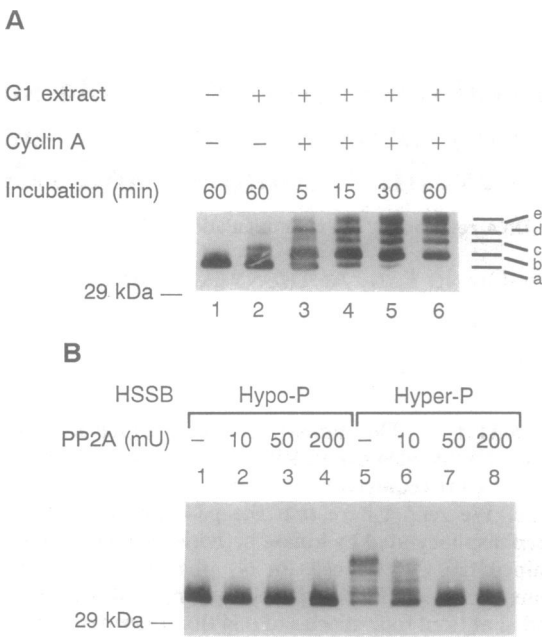

FIG. 1. (*A*) Cyclin A-dependent phosphorylation of HSSB p34. G_1 extracts (52 μg) were preincubated with cyclin A (0.5 μg) at 30°C for 20 min and incubated with HSSB (1.5 μg) for various periods as indicated. Aliquots (0.3 μl) were subjected to SDS/12.5% PAGE followed by immunoblot analysis with mAbs against the p34 subunit of HSSB. (*B*) Protein phosphatase 2Ac dephosphorylates hyperphosphorylated HSSB p34. Purified hypophosphorylated HSSB (lanes 1–4) or hyperphosphorylated (lanes 5–8) HSSB (0.6 μg), isolated after incubation with cyclin A-activated G_1 extracts for 60 min, was incubated with various amounts (millilunits, mU) of protein phosphatase 2Ac (PP2A), as indicated, for 30 min at 30°C in reaction mixture (5 μl) containing 20 mM Hepes (pH 7.5), 7 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 M NaCl. Aliquots (0.5 μl) were analyzed by immunoblot using p34 mAb.

HSSB containing multiple slow-migrating forms of p34 was isolated and treated with protein phosphatase 2Ac. The phosphatase converted the slow-migrating forms of p34 (Fig. 1*B*, lane 5) into a single fast-migrating, hypophosphorylated form (lanes 6–8) but had no effect on underphosphorylated p34 (lanes 1–4). This indicates that the altered mobility of p34 observed following incubation with cyclin A-activated G_1 extracts is due to extensive phosphorylation.

Identification of Kinases Involved in the Hyperphosphorylation of p34. Fractionation of cyclin A-activated G_1 extract identified cdk–cyclin A and DNA-PK as the enzymes involved in hyperphosphorylation of HSSB p34.

The extent of phosphorylation of HSSB p34 by partially purified cdk–cyclin A was similar to that caused by purified cdc2–cyclin B (purchased from UBI) (see below). Phosphorylation of p34 with purified preparations of cdc2–cyclin B converted 50% of the substrate into a slow-migrating product (Fig. 2*A Upper*, lane 3, form b1). Only form b1 of p34, but not the fast-migrating form a1, was labeled with ^{32}P (Fig. 2*A Lower*, lane 3), incorporating approximately one phosphate residue per molecule.

We then analyzed the phosphorylation of HSSB p34 by DNA-PK. In the presence of single-stranded poly(dT) (174 nt), DNA-PK converted 50% of p34 into three slow-migrating forms—b2, c2, and d2 (Fig. 2*B Upper*, lane 4). Forms b2, c2, and d2, but not a2, were labeled with ^{32}P (Fig. 2*B Lower*, lane 4). However, in the absence of added DNA, only low levels of p34 phosphorylation by DNA-PK were observed (lane 3).

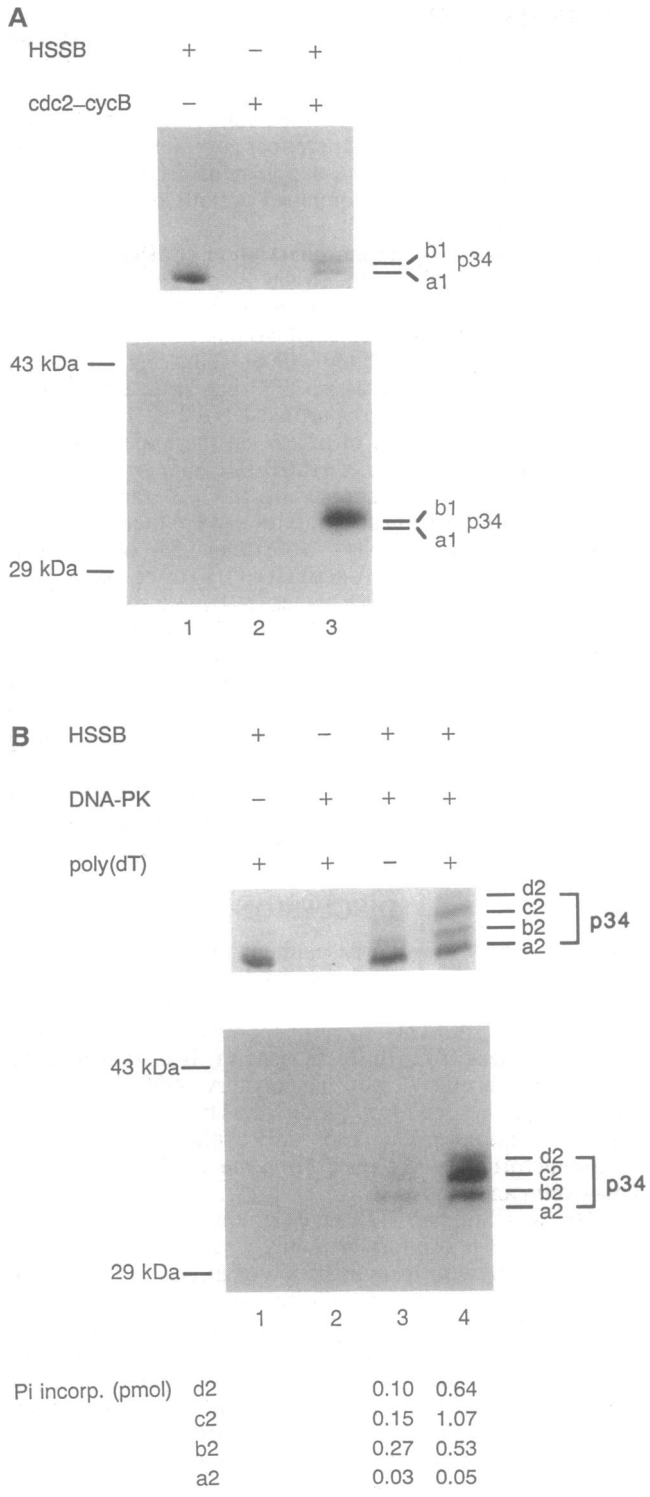

FIG. 2. (*A*) Phosphorylation of HSSB p34 by cdc2–cyclin B. HSSB (0.8 μg) was incubated with cdc2–cyclin B (cdc2–cycB, 0.18 unit) in the absence (*Upper*) or presence (*Lower*) of [γ-^{32}P]ATP (5 μCi; 1 μCi = 37 kBq). Reaction mixtures were then subjected to SDS/12.5% PAGE followed by staining with 0.1% Coomassie brilliant blue (*Upper*) or autoradiography (*Lower*). Incorporation of ^{32}P into the b1 and a1 forms of p34 by cdc2–cyclin B was 0.79 and 0.04 pmol, respectively. (*B*) Phosphorylation of HSSB p34 by DNA-PK. HSSB (0.8 μg) was incubated with the DNA-PK fraction (0.15 μg) in the absence (lane 3) or presence (lane 4) of poly(dT) (174 nt long, 0.16 μg). The reaction shown in *Lower* was carried out in the presence of [γ-^{32}P]ATP (5 μCi). Reaction mixtures were analyzed by SDS/12.5% PAGE followed by staining with silver (*Upper*) or autoradiography (*Lower*). Quantitation of phosphate incorporated into the four forms of p34 is shown below the autoradiograph.

While the addition of DNA stimulated the formation of the b2 species 2-fold, the production of c2 and d2 required the presence of DNA. We estimated that b2 and c2 contained approximately two and four phosphate residues per molecule of p34, respectively. Incubation of HSSB with highly purified preparations of DNA-PK and the Ku antigens (kindly provided by W. Dynan, ref. 22) resulted in a pattern of p34 phosphorylation similar to that observed with our preparations (data not shown).

Reconstitution of HSSB p34 Hyperphosphorylation with Partially Purified Kinases. Phosphorylated forms b1 and c1 of p34 resulting from incubation with either cdc2–cyclin B (Fig. 3*A*, lane 3) or cdk–cyclin A (lane 4) preparations migrated faster than forms b2, c2, and d2 that were generated after phosphorylation with DNA-PK (lane 5). This suggested that DNA-PK phosphorylated the p34 subunit of HSSB at different sites than the other two kinases. The electrophoretic mobilities of forms b1 and c1 were similar to those of products b and c formed following incubation with cyclin A-activated G_1 extract (lanes 2 and 6). However, none of the b2–d2 derivatives corresponded to p34 species that migrated between b and e. This suggests that form b, produced after 5 min of incubation with cyclin A-activated G_1 extract, is likely to be the product arising from the action of cdk–cyclin A kinase. The phosphorylation products formed by DNA-PK alone

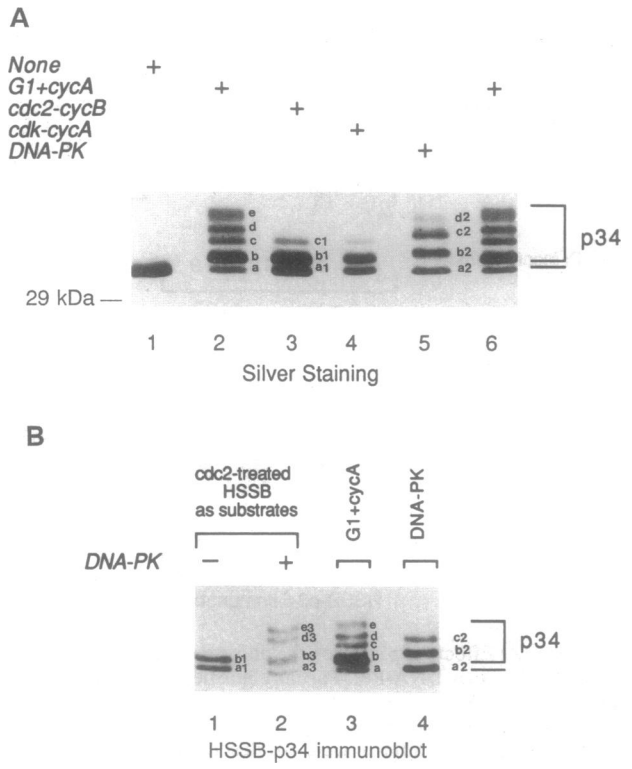

FIG. 3. (*A*) Comparison of the different phosphorylated p34 subunit products formed by different kinases. HSSB, isolated as the hypophosphorylated form (0.15 μg, lane 1) or after incubation with cyclin A-activated G_1 extracts (0.25 μg, lanes 2 and 6), with cdc2–cyclin B (0.6 μg, lane 3), cdk–cyclin A (0.3 μg, lane 4), or DNA-PK (0.2 μg, lane 5), was separated by SDS/12.5% PAGE followed by silver staining. (*B*) Combined phosphorylation of HSSB p34 by cdc2–cyclin B and DNA-PK. Phosphorylated HSSB (0.3 μg), isolated after incubation with cdc2–cyclin B, was incubated with (lane 2) or without (lane 1) DNA-PK (0.15 μg) and poly(dT) (0.16 μg) at 37°C for 45 min. Aliquots (0.8 μl) were subjected to SDS/12.5% PAGE analysis along with hyperphosphorylated HSSB isolated from cyclin A-activated G_1 extracts (25 ng, lane 3) or DNA-PK treatment (50 ng, lane 4). The separated material was then analyzed by immunoblot with p34 mAb.

(lane 5) differed from those formed by G_1 extract supplemented with cyclin A (lanes 2 and 6).

We then examined the phosphorylation of HSSB p34 resulting from the combined actions of cdc2–cyclin B and DNA-PK. Coincubation of cdk–cyclin A and DNA-PK with HSSB resulted in low levels of p34 phosphorylation (data not shown). The reason for this is unknown. However, when HSSB was initially phosphorylated by cdc2–cyclin B and reisolated prior to phosphorylation with DNA-PK, hyperphosphorylated p34 forms d3 and e3 were observed (Fig. 3*B*, lane 2). These forms migrated almost identically to forms d and e (lane 3). The absence of form c in this reconstitution reaction may be due to the low yield of form c1 following incubation with cdc2–cyclin B (lane 1). Similar results were obtained with the partially purified cdk–cyclin A preparation (data not shown). Thus, hyperphosphorylated p34 species d and e, formed with cyclin A-supplemented G_1 extract, most likely resulted from the combined actions of cdk–cyclin A and DNA-PK.

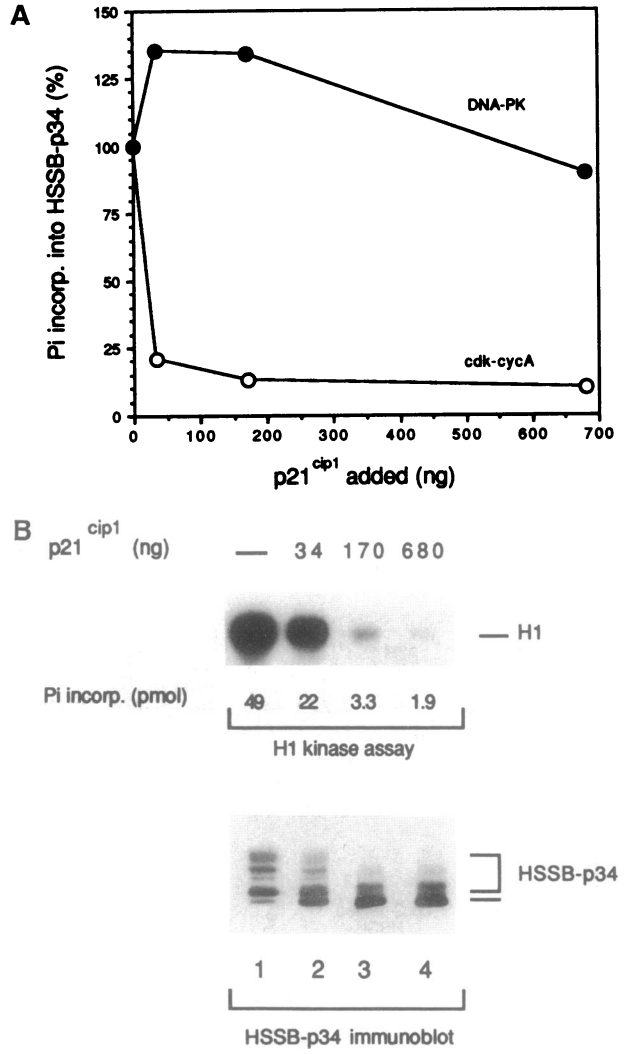

FIG. 4. (*A*) Effects of $p21^{cip1}$ on the phosphorylation of HSSB p34 by cdk–cyclin A or DNA-PK. Partially purified cdk–cyclin A (0.13 unit), or DNA-PK fraction (0.15 μg) along with poly(dT) (0.16 μg), was incubated with HSSB (0.8 μg) in the presence or absence of $p21^{cip1}$. The other additions were as described in *Materials and Methods*, including [γ-^{32}P]ATP (5 μCi). The phosphorylated p34, separated by SDS/12.5% PAGE, was quantitated as described in *Materials and Methods*. The activity is presented as percent of phosphate incorporated. In the absence of $p21^{cip1}$, 0.63 and 2.56 pmol of ^{32}P were incorporated into p34 by cdk–cyclin A and DNA-PK, respectively. These values represent 100% activity. Since $p21^{cip1}$ did not affect formation of the phosphorylated forms b2–d2 catalyzed by DNA-PK, the amounts of these three forms produced were added together. (*B*) Effect of $p21^{cip1}$ on the phosphorylation of HSSB p34 in G_1 extract preincubated with cyclin A. G_1 extract (52 μg) was preincubated with cyclin A (0.5 μg) in the absence (lane 1) or presence of $p21^{cip1}$, as indicated (lanes 2–4), for 20 min at 30°C. (*Upper*) Aliquots (0.5 μl) were assayed for histone H1 kinase activity. (*Lower*) The preincubated reaction mixtures were further incubated with HSSB (1 μg) for 60 min at 37°C. Aliquots (0.25 μl) were then subjected to immunoblot analysis using p34 mAb.

$p21^{cip1}$ Inhibits Hyperphosphorylation of HSSB p34 in G_1 Extract Preincubated with Cyclin A. $p21^{cip1}$ is a specific inhibitor of cyclin-dependent kinases (ref. 18; see ref. 23 for a review). Phosphorylation of HSSB p34 by partially purified cdk–cyclin A was inhibited >90% following addition of purified $p21^{cip1}$; in contrast, $p21^{cip1}$ had little effect on the phosphorylation of p34 by purified DNA-PK (Fig. 4*A*).

To examine the effect of $p21^{cip1}$ on the hyperphosphorylation of the p34 subunit of HSSB in cyclin A-activated G_1 extract, $p21^{cip1}$ was preincubated with G_1 extract along with cyclin A. Aliquots from such mixtures were then assayed for histone H1 kinase activity. More than 95% of H1 kinase activity present in cyclin A-activated G_1 extract was inhibited by $p21^{cip1}$ (Fig. 4*B Upper*, lanes 2–4). As shown by p34 immunoblot analysis (Fig. 4*B Lower*), in the absence of $p21^{cip1}$, p34 was hyperphosphorylated to form multiple slow-migrating forms (lane 1), similar to those observed above. However, the addition of $p21^{cip1}$ significantly inhibited formation of the slow-migrating forms of p34, resulting in the accumulation of the fast-migrating, hypophosphorylated form (lanes 2–4). Thus, $p21^{cip1}$, presumably due to its inhibition of the cdk–cyclin A activity, nearly abolished the hyperphosphorylation of HSSB p34.

DISCUSSION

Here we report that the p34 subunit of HSSB was hyperphosphorylated by the cdk–cyclin A and the DNA-PK activities present in G_1 extract following preincubation with human cyclin A. This hyperphosphorylated p34 product included at least four different species, b–e, that migrated more slowly through denaturing polyacrylamide gels than the hypophosphorylated form. While forms b and c of p34 are the phosphorylation products of cdk–cyclin A, the slower-migrating forms d and e result from the combined actions of cdk–cyclin and DNA-PK.

Dutta and Stillman (14) have demonstrated that purified cdc2–cyclin B phosphorylates only a subset of the peptides derived by chymotrypsin digestion of *in vivo* labeled HSSB p34. This suggests that another kinase(s) phosphorylates the p34 subunit, and our results indicate that DNA-PK is a likely candidate. The phosphorylation of HSSB p34 by DNA-PK is consistent with the observations of Fotedar and Roberts (16), who reported that a kinase present in cytosolic extracts phosphorylated p34 in a ssDNA-dependent manner.

In cyclin A-activated G_1 extract, the phosphorylated p34 form b formed by cdk–cyclin A appeared rapidly, after a 5-min incubation. In contrast, phosphorylated form e, which resulted from the combined actions of cdk–cyclin A and DNA-PK, accumulated following a lag period of 15 min. This suggests that cdk–cyclin A catalyzes the phosphorylation of HSSB p34 prior to phosphorylation by DNA-PK.

$p21^{cip1}$, a specific inhibitor of cdk–cyclin A but not DNA-PK, nearly abolished the hyperphosphorylation of HSSB p34 in G_1 extract preincubated with cyclin A. These observations suggest a requirement of the cdk–cyclin A activity for the phosphorylation of p34 by DNA-PK in G_1 extract. We propose (Fig. 5) that during $G_1 \rightarrow$ S-phase progression, the expression of cyclin A leads to the formation of active cdk–cyclin A kinase complexes that initiate efficient phosphorylation of p34 allowing further phosphorylation by

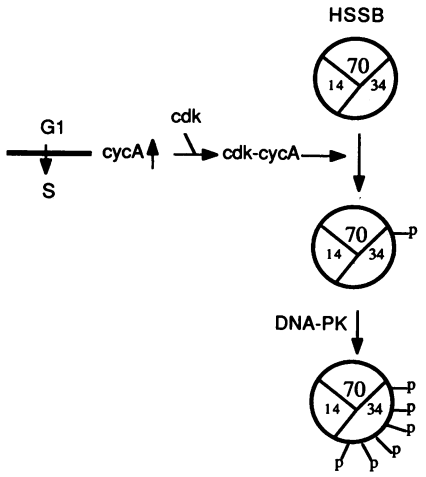

FIG. 5. A working model for a two-step cyclin A-dependent hyperphosphorylation of HSSB p34 during the cell cycle $G_1 \rightarrow$ S-phase transition. Based on results presented in Fig. 2*A*, the product of HSSB p34 phosphorylation by cdk–cyclin A contained one phosphate residue per molecule. The amount of phosphate incorporated into p34 by the combined actions of cdk–cyclin A and DNA-PK has not been quantitated. As shown in Fig. 3*A*, both of the phosphorylated p34 forms d and e produced by the action of two kinases migrated more slowly through SDS/polyacrylamide gels than form c2, which contained four phosphate residues per molecule (see Fig. 2*B*). For this reason, we believe that form e should contain at least six phosphate residues.

DNA-PK that converts HSSB into a hyperphosphorylated form.

Partially purified DNA-PK, derived from cyclin A-activated G_1 extract, efficiently phosphorylated HSSB p34 and yielded three phosphorylated forms of p34 with mobilities distinct from those formed by phosphorylation with cdk–cyclin A or cdc2–cyclin B. However, in the absence of cyclin A, or when cdk–cyclin A activity was inhibited by $p21^{cip1}$, DNA-PK present in G_1 extract did not phosphorylate p34 efficiently. It is unclear how the cdk–cyclin A activity controlled the action of DNA-PK in the phosphorylation of p34 in G_1 extract. There are at least three possibilities to explain this finding. (*i*) It is possible that cdk-catalyzed phosphorylation of p34 is a prerequisite for the action by DNA-PK. (*ii*) cdk–cyclin A may activate the Ku antigens in G_1 extract that are required for DNA-PK activity (ref. 22; see ref. 24 for a review). (*iii*) cdk–cyclin A may directly activate the phosphorylation of p34 by DNA-PK during the $G_1 \rightarrow$ S transition. Dutta and Stillman (14) have shown that a mutant p34 protein in which two serine residues from the putative cdc2-cyclin B phosphorylation sites were replaced with alanine residues was not phosphorylated *in vivo*. This observation seems to support the first possibility.

Carty *et al.* (25) have recently observed that extract prepared from HeLa cells following ultraviolet irradiation supported SV40 DNA replication poorly, and the p34 subunit of HSSB isolated from this extract was hyperphosphorylated. The phosphorylated p34 products are similar to those that we have observed with extract prepared from cells arrested in G_1 with HMBA. The mechanism of HMBA-mediated prolongation of murine erythroleukemia cells in the G_1 phase has been linked to its effects in suppressing cyclin A mRNA and protein levels and cdc2-catalyzed histone H1 kinase activity (26), as well as resulting in the accumulation of underphosphorylated retinoblastoma protein (27). It remains to be determined whether the hyperphosphorylation of p34 affects the biological functions of HSSB in DNA transactions.

We thank Drs. J. W. Harper and S. J. Elledge for providing purified $p21^{cip1}$, Dr. W. Dynan for purified DNA-PK and Ku antigens, Dr. T. Carter for mAb against p350 DNA-PK (28), and Dr. R. Burgess for mAbs against the p70 and p80 subunits of Ku antigen (29). We thank Drs. F. Dean and F. Muller for critical reading of the manuscript and Mrs. C. Holo and B. Philips for expert technical assistance. Z.-Q.P. is supported by a fellowship from the Leukemia Society, and J.H. is a professor of the American Cancer Society. This work is supported by Grant GM38559 from the National Institutes of Health.

1. Challberg, M. & Kelly, T. (1989) *Annu. Rev. Biochem.* **58**, 671–717.
2. Stillman, B. (1989) *Annu. Rev. Cell Biol.* **5**, 197–245.
3. Hurwitz, J., Dean, F. B., Kwong, A. D. & Lee, S.-H. (1990) *J. Biol. Chem.* **265**, 18043–18046.
4. Lee, S.-H., Pan, Z.-Q., Kwong, A. D., Burgers, P. M. J. & Hurwitz, J. (1991) *J. Biol. Chem.* **266**, 22707–22717.
5. Coverley, D., Kenny, M. K., Munn, M., Rupp, W. D., Lane, D. P. & Wood, R. D. (1991) *Nature (London)* **349**, 538–541.
6. Moore, S. P., Erdile, L., Kelly, T. & Fishel, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9067–9071.
7. Li, R. & Botchan, M. R. (1993) *Cell* **73**, 1207–1221.
8. He, Z., Brinton, B. T., Greenblatt, J., Hassell, J. A. & Ingles, C. J. (1993) *Cell* **73**, 1223–1232.
9. Dutta, A., Ruppert, J. M., Aster, J. C. & Winchester, E. (1993) *Nature (London)* **365**, 79–82.
10. Luche, R. M., Smart, W. C., Marion, T., Tillman, M., Sumrada, R. A. & Cooper, T. G. (1993) *Mol. Cell. Biol.* **13**, 5749–5761.
11. Brill, S. J. & Stillman, B. (1991) *Genes Dev.* **5**, 1589–1600.
12. Kenny, M., Schlegel, U., Furneaux, H. & Hurwitz, J. (1990) *J. Biol. Chem.* **265**, 7693–7700.
13. Din, S.-U., Brill, S. J., Fairman, M. P. & Stillman, B. (1990) *Genes Dev.* **4**, 968–977.
14. Dutta, A. & Stillman, B. (1992) *EMBO J.* **11**, 2189–2199.
15. Pan, Z.-Q. & Hurwitz, J. (1993) *J. Biol. Chem.* **268**, 20433–20442.
16. Fotedar, R. & Roberts, J. M. (1992) *EMBO J.* **11**, 2177–2187.
17. Connell-Crowley, L., Solomon, M. J., Wei, N. & Harper, J. W. (1993) *Mol. Biol. Cell* **4**, 79–92.
18. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993) *Cell* **75**, 805–816.
19. Gambari, R., Marks, P. A. & Rifkind, R. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4511–4515.
20. Pan, Z.-Q., Chen, M. & Hurwitz, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6–10.
21. Pan, Z.-Q., Amin, A. & Hurwitz, J. (1993) *J. Biol. Chem.* **268**, 20443–20451.
22. Dvir, A., Stein, L. Y., Calore, B. L. & Dynan, W. S. (1993) *J. Biol. Chem.* **268**, 10440–10447.
23. Pines, J. (1994) *Trends Biochem. Sci.* **19**, 143–145.
24. Anderson, C. W. (1993) *Trends Biochem. Sci.* **18**, 433–437.
25. Carty, M. P., Zernik-Kobak, M., McGrath, S. & Dixon, K. (1994) *EMBO J.* **13**, 2114–2123.
26. Kiyokawa, H., Ngo, L., Kurosaki, T., Rifkind, R. A. & Marks, P. A. (1992) *Cell Growth Differen.* **3**, 377–383.
27. Richon, V. M., Rifkind, R. A. & Marks, P. A. (1992) *Cell Growth Differen.* **3**, 413–420.
28. Carter, T., Vancurova, I., Sun, I., Lou, W. & DeLeon, S. (1990) *Mol. Cell. Biol.* **10**, 6460–6471.
29. Knuth, M. W., Gunderson, S. I., Thomson, N. E., Strasheim, L. A. & Burgess, R. R. (1990) *J. Biol. Chem.* **265**, 17911–17920.