MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 1202–1211
0270-7306/93/021202-10$02.00/0

Vol. 13, No. 2

Control of Simian Virus 40 DNA Replication by the HeLa Cell Nuclear Kinase Casein Kinase I

ALEKSANDRA CEGIELSKA[1,2] AND DAVID M. VIRSHUP[1,2,3]*

Program in Human Molecular Biology and Genetics,[1] Department of Cellular, Viral and Molecular Biology,[2] and Department of Pediatrics,[3] University of Utah, Salt Lake City, Utah 84112

Received 21 August 1992/Returned for modification 6 November 1992/Accepted 12 November 1992

The initiation of simian virus 40 (SV40) DNA replication is regulated by the phosphorylation state of the viral initiator protein, large T antigen. We describe the purification from HeLa cell nuclei of a 35-kDa serine/threonine protein kinase that phosphorylates T antigen at sites that are phosphorylated in vivo and thereby inhibits its ability to initiate SV40 DNA replication. The inhibition of both origin unwinding and DNA replication by the kinase is reversed by protein phosphatase 2A. As determined by molecular weight, substrate specificity, autophosphorylation, immunoreactivity, and limited sequence analysis, this kinase appears to be identical to casein kinase I, a ubiquitous serine/threonine protein kinase that is closely related to a yeast kinase involved in DNA metabolism. The HeLa cell phosphorylation cycle that controls the initiation of SV40 DNA replication may also play a role in cellular DNA metabolism.

Protein phosphorylation has emerged as an important regulatory mechanism in nuclear as well as cytoplasmic processes. Cellular protein kinases and phosphatases participate in, among other events, the regulation of differentiation, transcription, DNA repair, and mitosis. The transition from the G_1 to the S phase of the cell cycle is also dependent on the function of phosphorylation cascades. In *Saccharomyces cerevisiae*, the functions of the Cdc28 and Cdc7 protein kinases are required to initiate DNA synthesis. In higher eukaryotes, $p34^{cdc2}$ and other kinases have also been implicated in controlling the cell's commitment to begin chromosomal replication. However, the downstream events which couple Cdc28 and Cdc2 activation to the actual initiation of DNA synthesis remain unknown.

The study of the replication of the genome of the papovavirus simian virus 40 (SV40) has advanced our understanding of mammalian chromosomal replication because, except for the SV40 large T antigen, all of the factors required for control and execution of replication are present in the host cell. In the initial steps in SV40 DNA replication, T antigen binds as a double hexamer to the viral 64-bp minimal origin of replication and unwinds the duplex DNA, thereby providing a template for new DNA synthesis (3, 5, 13, 20, 21, 40, 47). This origin-unwinding activity of T antigen is controlled by its phosphorylation state (reviewed in references 13 and 29). Unphosphorylated T antigen expressed in *Escherichia coli* is inactive in replication until phosphorylated on threonine 124 by $p34^{cdc2}$ kinase (25). T antigen purified from mammalian cells is phosphorylated on up to seven serines and two threonines clustered at the amino- and carboxy-terminal regions (36). This heavily phosphorylated T antigen is not able to efficiently unwind the origin of replication and initiate SV40 DNA replication in vitro. The mammalian cellular kinase that inactivates T-antigen function has not yet been identified. A number of kinases have been shown to phosphorylate T antigen in vitro, several on sites which are not phosphorylated in vivo (17). The DNA-activated protein kinase phosphorylates T antigen on serine 120 and several other sites (6), and rabbit reticulocyte casein kinase I phosphorylates T antigen on serine 120 and/or 123, as well as several carboxy-terminal sites (17, 42); no functional effects of these kinases on T-antigen activity have been demonstrated.

In vitro studies have also shown that T antigen is activated by site-specific dephosphorylation. Initial studies demonstrated that underphosphorylated T antigen bound more tightly to origin DNA and was more active in the initiation of replication than heavily phosphorylated T antigen (reviewed in references 13 and 29). Subsequently, a cellular factor which markedly stimulated both the origin-unwinding activity of T antigen and in vitro SV40 DNA replication was found to be the catalytic subunit of protein phosphatase 2A ($PP2A_c$) (43, 44). $PP2A_c$ removes phosphoryl groups from one or more of serines 120, 123, 677, and 679 (37). These sites have been found to have a rapid phosphate turnover in infected cells (34). Phosphorylated T antigen is able to bind to the origin as a double hexamer; however, the complexes dissociate rapidly and do not unwind the duplex DNA. In contrast, $PP2A_c$-treated T antigen forms double hexamers on the SV40 origin in a highly cooperative manner; these dephosphorylated double hexamers have a slow off-rate and efficiently unwind the origin region (45). These data suggest that specific interactions between the hexamers are critical for origin-unwinding activity and that the interactions are regulated by the phosphorylation state of T antigen. While single T-antigen tetramers or hexamers may be active as nonspecific DNA helicases (39), the formation of specifically dephosphorylated double hexamers appears to be required for SV40 origin unwinding. The recent demonstration by electron microscopy that the T-antigen double hexamer can remain intact during the origin-unwinding process suggests that the double hexamer may also be the active species in the replication complex (47).

The factors that regulate the initiation of SV40 DNA replication may be involved in the regulation of cellular DNA replication as well. Both in vivo and in vitro, the initiation of viral replication depends on the host cell's position in its replication cycle. In vivo, SV40 DNA replication begins when the host cell enters the S phase, regardless of when during the cell cycle the cell was infected (27). In vitro, S-phase cell extracts are 10-fold more efficient than

* Corresponding author.

G_1-phase cell extracts at supporting both origin-specific unwinding and complete replication of SV40 DNA (30). Subsequent studies have demonstrated that addition of either $PP2A_c$ (43) or $p34^{cdc2}$ kinase activity (11, 12) to G_1-phase cell extracts markedly stimulates their replication activity. $PP2A_c$ directly stimulates the initiation of replication by dephosphorylating T antigen, while stimulation of replication by $p34^{cdc2}$ is indirect and requires an additional, as yet unidentified factor(s) present in unfractionated cellular extract. Neither the quantity nor the activity of protein phosphatase 2A (PP2A) has been found to vary throughout the cell cycle (33, 43). One possible explanation of the data is that the kinase that phosphorylates target sites for $PP2A_c$ may be more active in G_1-phase extracts. These findings suggest that a dynamic balance between cellular kinases and phosphatases can control the initiation of SV40 DNA replication; these factors may also influence the initiation of cellular DNA replication.

We undertook to identify the cellular kinase that inhibits the activity of T antigen in the initiation of DNA replication. We postulated that (i) the kinase would phosphorylate T antigen on serines, on sites that were at least in part accessible to $PP2A_c$, (ii) phosphorylation would occur on physiologic sites, (iii) phosphorylation would inhibit both T-antigen-catalyzed origin unwinding and in vitro SV40 DNA replication, and (iv) this inhibition would be reversible by $PP2A_c$. A 35-kDa protein kinase was purified from HeLa cell nuclear extracts that met these criteria. It appears to be an isoform of casein kinase I. This kinase is highly homologous to an *S. cerevisiae* protein kinase that is involved in DNA metabolism, supporting a role for the mammalian nuclear casein kinase I in DNA metabolism as well.

MATERIALS AND METHODS

The following reagents were obtained from commercial suppliers: DEAE-cellulose (DE-52) was from Whatman; Mono-S 5/5, heparin-Sepharose, and Superdex-75 10/30 columns were from Pharmacia; Affi-Gel Blue chromatography medium was from Bio-Rad; and hydroxylapatite was from Calbiochem. *E. coli* single-stranded DNA-binding protein (SSB) was purified from *E. coli* RLM727 (obtained from M. Wold, University of Iowa) by the method of LeBowitz (22). $PP2A_c$ was purified from bovine heart by a modification of published methods (9). Protein concentrations were determined by the Bradford dye-binding assay with a commercial reagent (Bio-Rad) and bovine serum albumin as a standard.

Preparation of HeLa cell nuclear extract. A total of 5.5×10^{10} HeLa cells were used in the purification. All steps were carried out at 0 to 4°C. Log-phase cells were lysed in hypotonic buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5], 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 5 mM KCl) as described before (49). The nuclei were removed from the lysates by centrifugation at 1,700 × *g* for 10 min, resuspended in hypotonic buffer, and frozen in liquid nitrogen for storage. To prepare the nuclear extract, nuclear pellets were thawed on ice and washed once with hypotonic buffer, once with hypotonic buffer containing 0.1% Nonidet P-40, and once again with hypotonic buffer. The nuclei were then resuspended (3 ml per 10^9 nuclei) in buffer B (20 mM HEPES [pH 7.5], 1 mM DTT, 1 mM EDTA, 10% sucrose, 0.02% Nonidet P-40) containing 400 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg each of leupeptin and pepstatin per ml and incubated on ice for 45 min, with occasional gentle mixing. The residual nuclei and cellular debris were removed by centrifugation at 25,000 × *g* for 30 min, and the supernatant was used directly for purification.

T-antigen kinase assay. The standard kinase reaction mixes contained dialyzed column fractions incubated with 1 μg of T antigen for 15 min at 37°C with 100 μM ATP–5 μCi of [γ-^{32}P]ATP–30 mM HEPES (pH 7.5)–7 mM $MgCl_2$–0.5 mM DTT, in a final volume of 20 μl. Reactions were stopped by the addition of 10 μl of electrophoresis sample buffer (625 mM Tris HCl, pH 6.8, 10% SDS, 50% sucrose, 10 mM DTT). The reaction mixes were then heated to 65°C for 10 min and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel. T antigen was visualized by staining with Coomassie brilliant blue R250; then the gel was dried and autoradiographed, the T-antigen bands were cut out, and incorporated radioactivity was determined by Cerenkov counting. Kinase activity was determined as the number of picomoles of P_i transferred to 1 pmol of T antigen per minute.

T-antigen-dependent origin-specific DNA-unwinding assay. The T-antigen-catalyzed origin DNA-unwinding assay was modified from published methods (45). The unwinding substrate contains two ^{32}P-labeled duplex DNA fragments, a 280-bp fragment with 65 bp of SV40 sequence encompassing the minimal origin of DNA replication, and a 220-bp fragment derived from the vector that serves as an internal control for nonspecific helicase activity. The DNA fragments are generated by a *Pvu*II plus *Asp* 718 digest of pDV.XH and labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. pDV.XH contains the SV40 minimal origin of replication (nucleotides 5211 to 32), with no additional SV40 sequence, cloned between the *Xho*I and *Hin*dIII sites of pBluescript KS(−) (Stratagene) (45).

The unwinding reactions were performed essentially as described before (45) except that 0.5 μg of T antigen was incubated with dialyzed column fractions for 15 min at 37°C in 30 mM HEPES (pH 7.5)–7 mM $MgCl_2$–2 mM DTT–100 μg of bovine serum albumin per ml–4 mM ATP before the remainder of the components were added. The final reaction mix also contained 10 ng of unwinding substrate, 100 ng of sheared calf thymus DNA, 25 ng of *E. coli* SSB, and 40 mM creatine phosphate. After a further 30 min of incubation at 37°C, the reactions were stopped by the addition of SDS, proteinase K, and EDTA, and the reaction mixes were analyzed on an 8% polyacrylamide gel in 90 mM Tris-borate (pH 8.0)–2 mM EDTA as described before (43, 44). The reaction mixes were visualized by autoradiography and quantitated by a Phosphorimager (Molecular Dynamics). Under the conditions of the assay, no unwinding of the nonspecific 220-bp fragment was seen.

In vitro SV40 DNA replication was assayed as described before (49) except that 0.6 μg of T antigen was first incubated with 0.4 U of kinase and/or 400 ng of $PP2A_c$ for 15 min at 37°C as described above. Each reaction mix contained 100 ng of pUC.HSO and 100 μg of HeLa cytoplasmic extract. Okadaic acid (2 μM), a potent inhibitor of PP2A, was added after the preincubation step to prevent further dephosphorylation of T antigen by PP2A present in the cytoplasmic extract.

ATP affinity labeling. ATP affinity labeling was performed by a modification of the method of Clertant and Cuzin (7). [α-^{32}P]ATP (1.55 μM, 1.93×10^7 cpm/pmol) was oxidized for 20 min at room temperature with 0.5 mM sodium metaperiodate (Sigma) and 0.5 mM HCl and then quenched by the addition of glycerol to 8.3%. Then, 12.5 μl of oxidized ATP (final ATP concentration, 0.78 μM) was incubated with

5.1 μg of the Superdex column load (with or without 4 mM unlabeled, unoxidized ATP) or with 10 μl of even-numbered Superdex fractions in a final volume of 25 μl in the presence of 7.7 mM sodium cyanoborohydride. After overnight incubation on ice, the reaction mixes were fractionated by SDS-PAGE on a 10% acrylamide gel, silver stained, dried, and autoradiographed.

For immunoblot analysis, proteins were separated by SDS-PAGE on a 10% gel and then electrophoretically transferred to a polyvinylidene fluoride (Immobilon-P; Millipore) membrane in 12.5 mM Tris-HCl–86 mM glycine (pH 8.3)–20% methanol. The membrane was blocked with 3% nonfat dry milk in 20 mM Tris-HCl (pH 7.5)–500 mM NaCl–0.05% Tween 20 and then probed overnight at 4°C with a 1:500 dilution of a rabbit antiserum raised against a peptide derived from the casein kinase I sequence (32) in the absence or presence of 250 ng of the immunogenic peptide (provided by M. Cobb, University of Texas Southwestern). Immunoreactive proteins were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) and the chemiluminescence substrate AMPPD (Tropix) per the manufacturers' instructions.

RESULTS

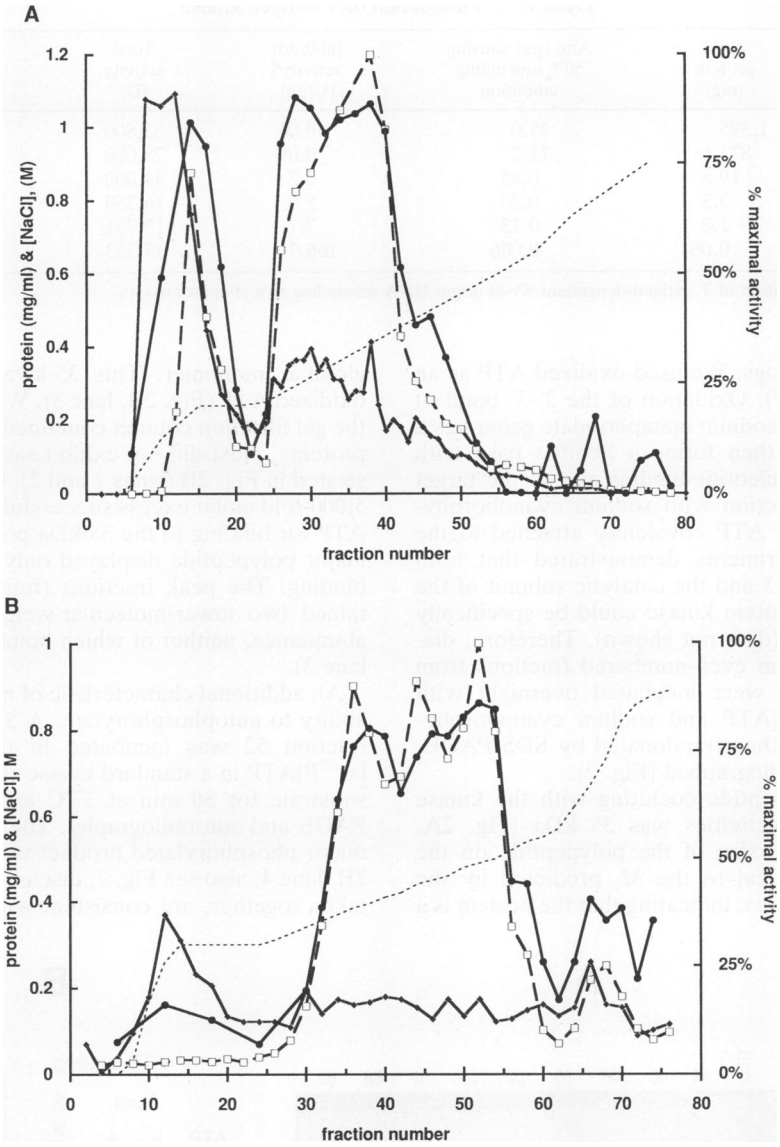
The observation that the site-specific dephosphorylation of T antigen stimulates its activity in the initiation of DNA replication led us to purify the cellular kinase that phosphorylates T antigen and inhibits its activity. A large number of cellular kinases have been shown to phosphorylate T antigen in vitro, frequently on nonphysiologic sites (6, 17, 23). Preliminary studies demonstrated that incubation of T antigen with a number of these kinases (cyclic AMP-dependent protein kinase, casein kinase II, DNA-activated protein kinase, and glycogen synthase kinase-3) had no effect on its activity (unpublished results). Therefore, identification of a kinase that phosphorylates T antigen was necessary but not sufficient. Since the putative inhibitory kinase (operationally referred to as the T-antigen kinase) was expected to block the function of T antigen in the initiation of replication, we also measured the ability of protein fractions to inhibit the T-antigen-catalyzed origin-specific unwinding of duplex DNA (the unwinding assay) (8, 44, 45, 48). All assays were performed with T antigen that had been immunoaffinity purified from recombinant baculovirus-infected Sf9 cells (26, 49). We used insect cell-produced T antigen because it is phosphorylated quantitatively on threonine 124 but is significantly underphosphorylated on the inhibitory serine residues compared with the protein produced in mammalian cells (6, 19). Preliminary studies have shown that baculovirus-produced T antigen is active in origin unwinding without initial dephosphorylation, unlike T antigen produced in mammalian cells (data not shown).

Copurification from nuclear extracts of a T-antigen kinase and origin-specific unwinding inhibitor. Cytosolic and nuclear extracts from log-phase HeLa cells were assayed for both T-antigen kinase activity and inhibition of origin unwinding. A 400 mM NaCl extract of HeLa nuclei (see Materials and Methods) was found to contain both a kinase that phosphorylated T antigen and an activity that inhibited the ability of T antigen to unwind the SV40 origin of replication. The nuclear extracts were depleted of free DNA by passage over DEAE-cellulose in buffer B (Materials and Methods) with 300 mM NaCl, with quantitative recovery of both kinase and unwinding inhibitor activity in the flowthrough. The DEAE-cellulose flowthrough was dialyzed overnight into buffer B, clarified by centrifugation at 25,000 × *g* for 20 min, and loaded onto a 100-ml hydroxylapatite column. Both the T-antigen kinase and the unwinding-inhibitory activities adsorbed to hydroxylapatite and were coeluted with 950 ml of buffer B containing 400 mM NaCl at 35 ml/h directly onto a coupled 10-ml Affi-Gel Blue column. Both the T-antigen kinase and the unwinding-inhibitor activity adsorbed to the Affi-Gel Blue and were coeluted with 100 ml of 400 mM potassium thiocyanate in buffer B. The eluate from Affi-Gel Blue was dialyzed into buffer B and applied to a Mono-S 5/5 fast protein liquid chromatography (FPLC) column. Protein was eluted from the Mono-S column with a 20-ml 0 to 1,000 mM NaCl gradient in buffer B at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The salt concentration was held at 250 mM for 10 fractions to elute a single-strand nuclease activity that occasionally interfered with the origin-unwinding assay. The fractions were assayed for protein concentration, T-antigen kinase activity, and unwinding inhibition (Fig. 1A). Peak activities coeluted between 250 and 400 mM NaCl, suggesting that the protein is heterogeneous and basic. We have not further characterized the T-antigen kinase/unwinding inhibitor peak that eluted at 250 mM NaCl; most likely it represents the leading edge of the kinase/inhibitor activity.

Peak fractions from the Mono-S column were pooled and dialyzed into buffer B. Dialyzed proteins were applied to a 1-ml heparin-Sepharose column, which was then washed with 5 ml of buffer B and eluted with a 20-ml 0 to 1,000 mM NaCl gradient at a flow rate of 0.25 ml/min, and 0.5-ml fractions were collected (Fig. 1B). Kinase and unwinding-inhibitor assays again demonstrated cofractionation of the two activities, which eluted in a broad peak between 300 and 600 mM NaCl. After dialysis into buffer B, peak fractions (a total of 5 ml) were pooled and concentrated to 350 μl with a Centricon-10 microconcentrator (Amicon).

One half of the concentrated heparin-Sepharose peak material was further fractionated by gel filtration (Fig. 1C). The sample was applied to a Superdex 75 10/30 column equilibrated in buffer B with 200 mM NaCl and eluted at a flow rate of 0.5 ml/min, and 200-μl fractions were collected. Even-numbered fractions were dialyzed into buffer B and then assayed both for the ability to phosphorylate T antigen and the ability to inhibit the T-antigen-catalyzed origin-specific unwinding of linear duplex DNA. As in each previous step, the two activities cochromatographed, supporting the hypothesis that the kinase is also the inhibitor of the unwinding reaction. The size of the inhibitory kinase, estimated from its behavior during gel filtration, is 35 kDa. The overall yield of the purification, measured as recovery of unwinding-inhibitor activity, was 24%, with a 4,000-fold purification (Table 1). We speculate that the 140% recovery of the inhibitor in the DE-52 flowthrough is due to the removal of kinase-binding factors, specifically double-stranded DNA (unpublished data), on the anion-exchange matrix. We initially attempted to calculate the purification from the recovery of the T-antigen kinase activity. We found, however, that in the initial chromatographic steps, large amounts of kinase were lost, while there was good recovery of the inhibitor. We attribute this result to the presence of additional nuclear kinases which can phosphorylate T antigen but do not inhibit its activity in the unwinding of the origin of replication.

ATP binding and autophosphorylation of the T-antigen kinase. To further identify the kinase in the gel filtration fractions, we took advantage of the fact that a number of ATP-binding proteins can be covalently labeled with radio-



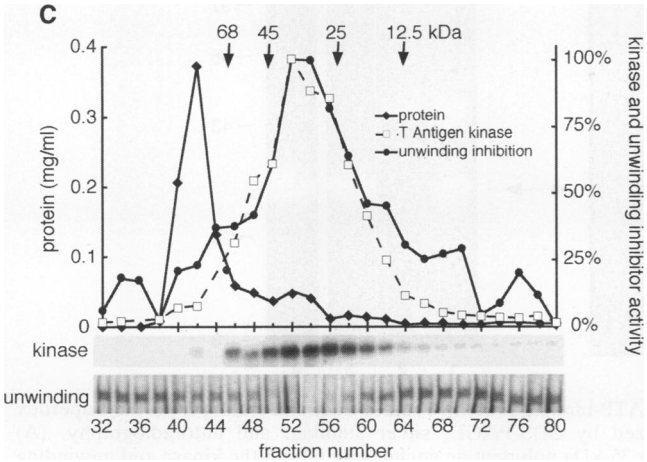


FIG. 1. T-antigen kinase and an inhibitor of T-antigen-catalyzed origin unwinding copurify from HeLa cell nuclear extracts. A 400 mM NaCl extract from HeLa nuclei was fractionated by step elutions from DEAE-cellulose, hydroxylapatite, and Affi-Gel Blue (Materials and Methods). The fractions containing the T-antigen kinase/unwinding inhibitor were further fractionated by cation-exchange (Mono-S, panel A), heparin-Sepharose (panel B), and gel filtration (Superdex 75 10/30, panel C) chromatography. Panel C also shows autoradiographs of T-antigen phosphorylation and origin-specific unwinding assayed in the presence of the indicated fractions from the Superdex column. ◆, protein concentration; □, kinase activity with T antigen as the substrate; ●, inhibition of T-antigen-catalyzed origin unwinding; – – –, NaCl concentration. The molecular size markers used were bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (12.5 kDa).

TABLE 1. Purification of T antigen kinase

Step	Total protein (mg)	Amt (μg) causing 50% unwinding inhibition	Inhibitor activity[a] (U/μg)	Total activity (U)	Yield (%)	Purification (fold)
Nuclear extract	1,395	25.0	0.04	55,800		
DE-52 flowthrough	873.6	11.2	0.09	78,000	140	2.2
Affi-Gel Blue eluate	19.8	0.45	2.2	44,000	79	56
Mono-S peak	3.3	0.20	5.0	16,250	29	125
Heparin-Sepharose	1.8	0.13	7.7	13,731	25	192
Superdex 75	0.08	0.006	166.7	13,333	24	4,167

[a] One unit produces 50% inhibition of T-antigen-dependent SV40 origin DNA unwinding in a standard assay.

active ATP and ATP analogs. We used oxidized ATP as an affinity-labeling reagent (7). Oxidation of the 2′-3′ bond of ribose in [α-^{32}P]ATP with sodium metaperiodate generates a reactive dialdehyde that then forms a Schiff's base with primary amines in the nucleotide-binding site of the target protein. Subsequent reduction with sodium cyanoborohydride leaves the oxidized ATP covalently attached to the kinase. Preliminary experiments demonstrated that both glycogen synthase kinase-3 and the catalytic subunit of the cyclic AMP-dependent protein kinase could be specifically labeled with this reagent (data not shown). Therefore, dialyzed samples (10 μl) from even-numbered fractions from the gel filtration column were incubated overnight with periodate-oxidized [α-^{32}P]ATP and sodium cyanoborohydride. The samples were then fractionated by SDS-PAGE, silver stained, and autoradiographed (Fig. 2).

The predominant polypeptide coeluting with the kinase and unwinding-inhibitor activities was 35 kDa (Fig. 2A, fractions 52 and 54). The size of the polypeptide on the silver-stained gel is identical to the M_r predicted by the Stokes radius of the activities, indicating that the protein is a globular monomer. This 35-kDa polypeptide also bound oxidized ATP (Fig. 2B, lane 3). While the material loaded on the gel filtration column contained a number of ATP-binding proteins, most did not exhibit saturable binding, as demonstrated in Fig. 2B (lanes 1 and 2). Unlabeled ATP added at a 5,000-fold molar excess successfully competed with oxidized ATP for binding to the 35-kDa polypeptide, while the other major polypeptide displayed only a small decrease in ATP binding. The peak fractions (fractions 52 to 54) also contained two lower-molecular-weight polypeptides in lesser abundance, neither of which bound oxidized ATP (Fig. 2B, lane 3).

An additional characteristic of many protein kinases is the ability to autophosphorylate. A 5-μl aliquot from Superose fraction 52 was incubated in the presence of 100 μM [γ-^{32}P]ATP in a standard kinase reaction mix without added substrate for 30 min at 37°C and then analyzed by SDS-PAGE and autoradiography. The 35-kDa polypeptide is the major phosphorylated product under these conditions (Fig. 2B, lane 4; also see Fig. 7, discussed below). These findings, taken together, are consistent with the hypothesis that the

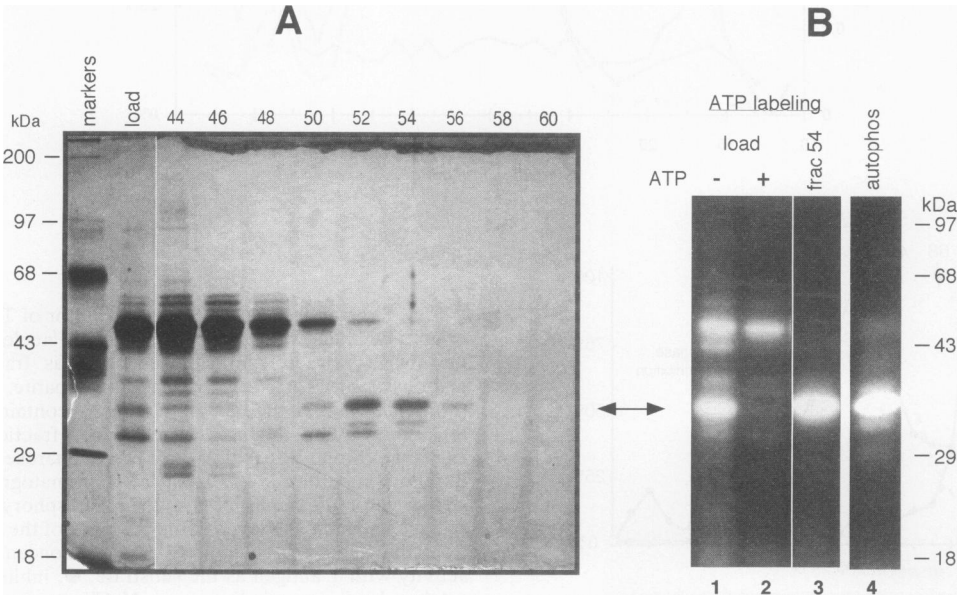


FIG. 2. T-antigen kinase/unwinding inhibitor copurifies with a 35-kDa ATP-binding protein. Protein fractions eluting from the Superdex 75 column were incubated with oxidized [α-^{32}P]ATP and then analyzed by SDS-PAGE, silver staining, and autoradiography. (A) Silver-stained gel of Superdex column load and peak fractions (44 to 60). A 35-kDa polypeptide coelutes with both the kinase and unwinding inhibitor activities. (B) Autoradiograph of [α-^{32}P]ATP-labeled proteins (lanes 1 to 3). Lanes 1 and 2, Superdex column load incubated without (lane 1, −) or with (lane 2, +) 4 mM unlabeled ATP. Oxidized [α-^{32}P]ATP binding to the 35-kDa protein was specifically displaced by excess unlabeled ATP. Lane 3, autoradiograph of Superdex fraction 54 after incubation with oxidized ATP. Lane 4, autophosphorylation of Superdex fraction 52. Double-headed arrow indicates the 35-kDa ATP-binding protein.

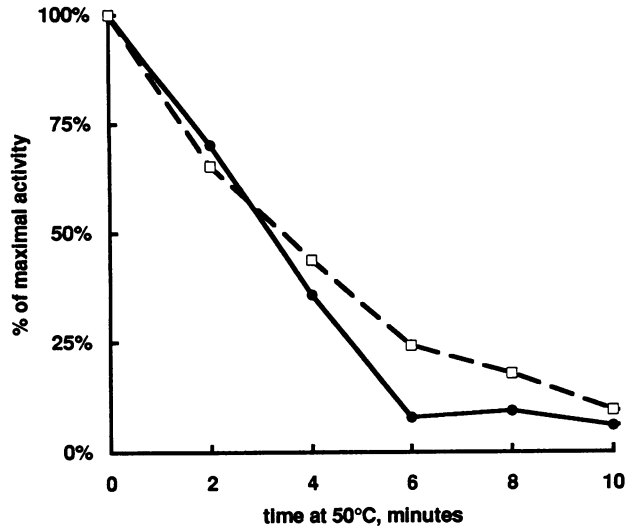


FIG. 3. Time course of heat inactivation of the T-antigen kinase and unwinding inhibitor. A highly purified kinase/inhibitor fraction was incubated at 50°C for the indicated times and chilled on ice, and then aliquots were assayed for both T-antigen kinase activity (□) and the ability to inhibit T-antigen-dependent origin unwinding (●). The results are plotted as the percentage of activity remaining compared with that in a nonheated control.

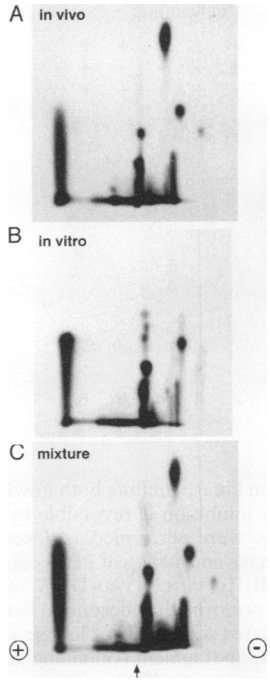


FIG. 4. The kinase phosphorylates T antigen on sites that are phosphorylated in vivo. Two-dimensional phosphopeptide maps of ^{32}P-labeled T antigen were prepared as described previously (37, 43). (A) In vivo-labeled T antigen. (B) In vitro-phosphorylated T antigen. (C) Mixture of equal quantities (by radioactivity) of metabolically labeled and in vitro-labeled T-antigen phosphopeptides. T antigen was metabolically labeled in vivo with $^{32}P_i$ in recombinant-adenovirus-infected 293 cells and immunoaffinity purified or phosphorylated in vitro with [γ-^{32}P]ATP by purified kinase. Radiolabeled T antigen was isolated by SDS-PAGE and then sequentially digested with trypsin and pronase. Electrophoresis was performed in the horizontal direction, followed by ascending chromatography. The origin is indicated by the arrow.

protein that inhibits the activity of T antigen in the initiation of SV40 DNA replication is a 35-kDa monomeric T-antigen kinase.

Thermal inactivation of both kinase and inhibitor activities. The association between the kinase and the unwinding inhibitor was further explored by determining the thermostability of the two activities. Peak inhibitory fractions from the heparin-Sepharose column were incubated for various times at 50°C and then transferred to ice. Aliquots were taken at each time point and assayed for both T-antigen kinase activity and inhibition of T-antigen-dependent origin unwinding. Both activities displayed similar lability at 50°C, with 50% loss of activity after 3 min (Fig. 3). Thus, the cofractionation of the two activities through six chromatographic steps, including separations based on charge, ligand affinity, and size, and their identical rates of thermal denaturation strongly support the conclusion that the kinase and inhibitory activities reside in the same 35-kDa protein.

T-antigen kinase phosphorylates in vivo sites. It was important to determine whether the kinase phosphorylates T antigen on sites that are phosphorylated in vivo in virus-infected cells. Previous studies have established that T antigen produced in mammalian cells is phosphorylated on up to nine sites, two threonines and seven serines (35, 36). Activation of T antigen by PP2A$_c$ is achieved by removal of phosphoryl groups from specific serine residues (37). T antigen phosphorylated with the T-antigen kinase incorporated up to 4 mol of P_i per mol of T antigen, approximately 90% on serines (data not shown). We compared two-dimensional phosphopeptide maps of T antigen metabolically labeled in vivo with $^{32}P_i$ and T antigen phosphorylated in vitro by purified kinase (Fig. 4). Sequential trypsin and pronase digests of in vivo-phosphorylated T antigen generated approximately nine major phosphopeptides (Fig. 4A). Identical proteolysis of in vitro-phosphorylated T antigen generated a smaller set of phosphopeptides, most of which were identical to those found in in vivo-phosphorylated T antigen (Fig. 4B). Three apparently novel peptides appeared to be phosphorylated by the kinase as well, although at much lower efficiencies. A two-dimensional phosphopeptide map containing equal amounts (by counts per minute) of in vivo- and in vitro-labeled T antigen confirmed that the purified T-antigen kinase phosphorylates T antigen on a subset of peptides that are physiologically phosphorylated in infected cells (Fig. 4C). We are unable to determine the specific sites phosphorylated on T antigen from these peptide maps.

Inhibition of unwinding and SV40 DNA replication reversed by PP2A$_c$. Two major predictions of the hypothesis that the site-specific phosphorylation of T antigen is responsible for blocking the initiation of SV40 DNA replication are that (i) phosphorylation of T antigen by the kinase should similarly inhibit its ability to support in vitro SV40 DNA replication and (ii) the inhibition of T-antigen activity by the kinase should be reversible by dephosphorylation with PP2A$_c$. To test these predictions, T antigen was incubated with and without purified kinase and then added to origin-unwinding and in vitro SV40 DNA replication reaction mixes (49) (Fig. 5). In the unwinding assay, phosphorylation of T antigen inhibited origin-specific unwinding by approximately 91% (Fig. 5A, lane 3 compared with lane 5; see also Fig. 1C). Phosphorylation of T antigen likewise inhibited in vitro SV40 DNA replication by up to 89% (Fig. 5B, lane 2 compared with lane 4). Concurrent addition of PP2A$_c$ to the preincu-

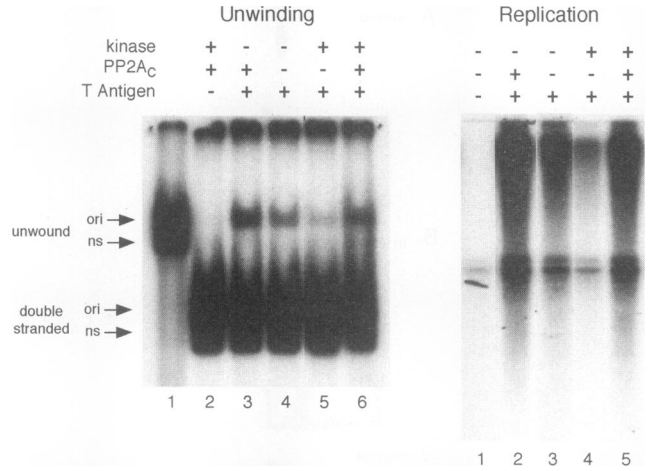


FIG. 5. T-antigen kinase inhibits both unwinding and SV40 DNA replication, and the inhibition is reversible by $PP2A_c$. (A) Unwinding inhibition assays were performed as described in the text, with 0.4 U of purified kinase and 400 ng of $PP2A_c$ in the preincubation mix where indicated. (B) In vitro SV40 DNA replication reactions in HeLa extract were performed as described before (49) except that T antigen was first treated with 0.4 U of kinase and/or 400 ng of $PP2A_c$ as indicated. ori, 280-bp fragment containing the SV40 minimal origin of replication; ns, nonspecific 220-bp fragment from the vector.

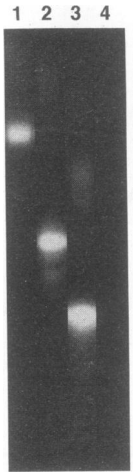


FIG. 6. T-antigen kinase is a casein kinase, not a histone kinase. Autoradiograph of ^{32}P-labeled kinase substrates. Heparin-Sepharose-purified kinase was incubated with 1 μg of T antigen (lane 1), phosvitin (lane 2), casein (lane 3), or histone H1 (lane 4) in the presence of 100 μM [γ-^{32}P]ATP for 10 min at 37°C and then analyzed by SDS-PAGE and autoradiography.

bation reaction mix completely reversed the inhibition of both origin unwinding and DNA replication. Addition of $PP2A_c$ to both reaction mixes in the absence of kinase also stimulated the T-antigen activity, most likely by removing residual inhibitory phosphoryl groups from the baculovirus-produced T antigen (Fig. 5A, lane 3, and Fig. 5B, lane 2) (6, 19).

Inhibition of the complete replication reaction could potentially be due to phosphorylation of proteins present in the replication extract rather than to phosphorylation of T antigen. However, the only protein required in the origin-unwinding reaction besides T antigen is *E. coli* SSB, which is not a substrate for the T-antigen kinase (data not shown). $PP2A_c$-mediated reversal of inhibition was not due to direct inactivation of the kinase, since $PP2A_c$ added after phosphorylation of T antigen similarly reversed the inhibition (data not shown). These data are most consistent with the hypothesis that the kinase inhibits T-antigen function by phosphorylating physiologic, $PP2A_c$-accessible sites on T antigen.

Identification of the inhibitory kinase as casein kinase I. We performed a number of studies to further characterize the kinase. T antigen and the acidic substrates phosvitin and casein were all phosphorylated at a similar rate, while the basic substrate histone H1 was not phosphorylated (Fig. 6). ATP was utilized as a phosphate donor about 10-fold more efficiently than GTP (data not shown). To determine whether the 35-kDa kinase was autophosphorylated in an intra- or intermolecular reaction, equal amounts of kinase were added to increasing volumes of reaction mixture (approximate kinase concentration, 85 to 280 nM) and allowed to phosphorylate for 5 min before the reaction was stopped by the addition of SDS-PAGE sample buffer. The degree of phosphorylation of the 35-kDa protein was the same regardless of its concentration in the reaction mix, demonstrating that the autophosphorylation reaction is intramolecular (Fig. 7). When the autophosphorylation reaction was allowed to continue to completion, up to approximately 6 mol of phosphate per mol of kinase were added, 90% on threonine residues and 10% on serine residues, as determined by phosphoamino acid analysis (data not shown). Autophosphorylation of the kinase had no detectable effect on its activity.

The properties of this kinase are similar to those described for casein kinase I, a basic, low-molecular-weight monomeric messenger-independent protein kinase that preferentially phosphorylates acidic substrates (41). To determine whether the T-antigen kinase was related to casein kinase I, we compared equal amounts (determined by kinase activity on T antigen) of highly purified casein kinase I (from bovine thymus) and the T-antigen kinase (from HeLa cell nuclei) by

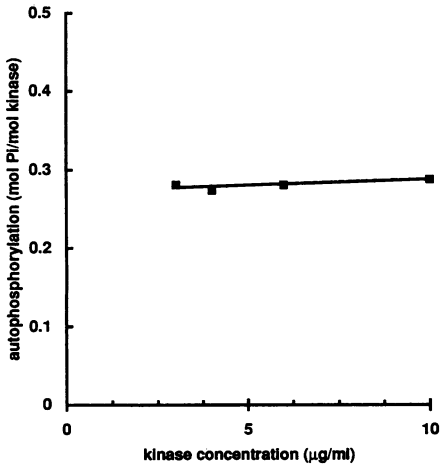


FIG. 7. Kinase autophosphorylation reaction is intramolecular. Purified kinase (0.3 μg) was incubated in a volume of 10 to 100 μl for 5 min at 37°C in the presence of 100 μM [γ-^{32}P]ATP and then isolated by SDS-PAGE. Kinase autophosphorylation was determined by Cerenkov counting of individual 35-kDa kinase bands excised from the gel, and the results are expressed as moles of P_i incorporated per mole of kinase.

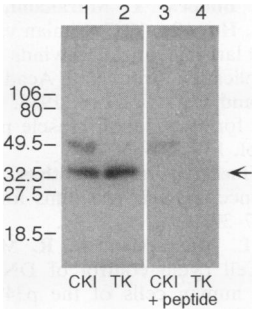


FIG. 8. T-antigen kinase is recognized by anti-casein kinase I peptide antibody. Equal quantities (by T-antigen kinase activity) of bovine thymus casein kinase I (CKI, lanes 1 and 3) and heparin-Sepharose-purified T-antigen kinase (TK, lanes 2 and 4) were probed with a 1:500 dilution of a rabbit antiserum raised against a peptide derived from the casein kinase I sequence in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 250 ng of the immunogenic peptide (32). Sizes are shown in kilodaltons. Arrow indicates 35-kDa kinase.

immunoblotting with a rabbit antiserum raised against a synthetic peptide derived from the bovine casein kinase sequence (32) (Fig. 8). Casein kinase I and the T-antigen kinase appear to be closely related, as determined both by size on SDS-PAGE (35 kDa) and by immunoreactivity. The kinases reacted equally well with the antiserum, and the immunoreactivity was specifically blocked by the inclusion of the antigenic peptide. This antiserum may potentially recognize any of the known isoforms of casein kinase I (32).

To further identify which isoform of casein kinase I we had purified, the amino acid sequence of one tryptic peptide was obtained by the method of Aebersold et al. (1). The sequence, FEEAPDYMY, is identical to amino acids 268 to 276 in the bovine casein kinase Iα isoform, which is also 35 kDa. This peptide sequence is not present in bovine casein kinase Iγ and differs from the bovine casein kinase Iβ amino acid sequence in two potential tryptic cleavage sites. The complete sequence of mammalian casein kinase Iδ is not known, although it is reportedly 50 kDa. Thus, the HeLa nuclear T-antigen kinase is apparently the α isoform of casein kinase I.

DISCUSSION

Alterations in the phosphorylation state of SV40 large T antigen regulate its activity in the initiation of SV40 DNA replication. Using a functional assay, the inhibition of T-antigen-catalyzed SV40 origin unwinding, we have purified a 35-kDa serine/threonine protein kinase. This kinase, which appears to be a nuclear form of casein kinase I, phosphorylates T antigen on physiologic sites and blocks its activity in both origin unwinding and in vitro SV40 DNA replication. The use of a functional assay was critical to the purification, since any number of cellular kinases may phosphorylate T antigen on sites that do not affect its activity.

How does the phosphorylation of T antigen affect the initiation of SV40 DNA replication? In the initial steps of SV40 DNA replication, T antigen forms hexamers in an ATP-dependent manner, two of which then bind to the SV40 origin of replication (24, 28, 47). In the absence of specific inhibitory phosphoryl groups on T antigen, the two hexamers interact to form a stable complex that then unwinds the origin region to provide a template for DNA synthesis (45). Phosphorylated T antigen also binds to the origin as a double hexamer, but the complex is significantly less stable and is unable to unwind the origin duplex DNA. The results presented here demonstrate that PP2A and a nuclear form of casein kinase I reciprocally control the ability of T antigen to initiate SV40 DNA replication in vitro, presumably by controlling the ability of T-antigen hexamers to cooperatively interact while bound to the origin of replication.

What sites on T antigen does this kinase phosphorylate? Phosphopeptide mapping studies have shown that $PP2A_c$ removes phosphoryl groups from serines 120 and 123 and, to a lesser extent, serines 677 and 679 of T antigen purified from SV40-infected cells (37). Since the inhibition of origin unwinding and replication by casein kinase I is reversed by $PP2A_c$, it seems likely that casein kinase I is phosphorylating one or more of these serine residues. Additionally, casein kinase I from rabbit reticulocytes has been shown to phosphorylate T antigen on serines 120 and/or 123 and serines 677 and/or 679; serine 639 may also have been a target (17). Casein kinase I is a serine/threonine kinase which recognizes sites that have several acidic residues amino-terminal to the target residue; for example, serine in the peptide DDDDVASLPGLRRR (2, 16). Significantly, phosphoserine in the sequence S(P)XXS [where S(P) is phosphoserine and X is any amino acid] also creates a casein kinase I recognition site, indicating that casein kinase I activity on certain substrates can be regulated by the phosphorylation state of the substrate (14, 15, 42). Casein kinase I recognition sites in T antigen identified by inspection of the sequence include serine 639 in the sequence DDDDEDS(639) and serine 679 in the sequence SSQS(679). The sequence SQHS(123), encompassing amino acids 120 to 123 in T antigen, could potentially be sequentially phosphorylated on serine 120 (e.g., by the DNA-activated protein kinase [6]) and then on serine 123 by the HeLa nuclear casein kinase I (42). It remains to be determined whether phosphorylation of one or several of these sites is responsible for the inhibition of T-antigen activity or whether additional sites are involved. The simplest prediction is that mutation of the critical serine residue(s) to a nonphosphorylatable amino acid would produce a constitutively active T antigen. Mutant T antigen in which alanine is substituted for serine at position 679 indeed exhibits enhanced binding to the SV40 origin in vitro and increased viral replication in vivo (38), suggesting that the in vitro effects of casein kinase I may be due at least in part to phosphorylation of serine 679. Mutants in which alanine is substituted for serine at either position 120 or 123, while active in in vitro assays (38, 44a), are defective in viral replication in vivo (35, 38). SV40 DNA replication in vivo and in vitro normally begins only when the cell enters the S phase (27, 30, 43). Mutations of serines 120 and 123 may be lethal in vivo because they disrupt the ability of T antigen to delay the initiation of viral replication until the cell enters the appropriate phase of the cell cycle.

Casein kinase I is highly homologous to a yeast protein kinase involved in DNA repair. The kinase that we isolated from HeLa nuclei as the inhibitor of in vitro DNA unwinding is a 35-kDa protein that appears to be identical to bovine casein kinase Iα. Casein kinase I is a basic, messenger-independent protein kinase that has been isolated from membranes, mitochondria, cytosol, and nuclei as a monomer of between 25 and 55 kDa (41). A large number of nuclear, cytosolic, and membrane-bound proteins have been identified as substrates for casein kinase I, but the effect of phosphorylation on their function is not known (41). A cytoplasmic form of casein kinase I phosphorylates glycogen synthase on serine 10, resulting in its inactivation, but only

after the casein kinase I recognition site has been formed by phosphorylation of serine 7 by the cyclic AMP-dependent protein kinase (15). The control of the nuclear casein kinase I is not understood, although an erythrocyte membrane-associated form appears to be regulated by phosphatidylinositol 4,5-bisphosphate (4). A pair of essential but functionally redundant casein kinase I-related genes (*YCK1* and *YCK2*) have been cloned from *S. cerevisiae*, but their biologic function remains unknown (31). The protein most closely related to the bovine casein kinase Iα sequence identified through a search of the GenBank and EMBL libraries (10) is HRR25 (18), an essential *S. cerevisiae* protein kinase. HRR25 is 62% identical and 78% similar to the predicted bovine casein kinase Iα amino acid sequence (32). Yeast cells with mutations in HRR25 are sensitive to DNA damage resulting from endonuclease cleavage, DNA-alkylating agents, and X irradiation and have defects in nuclear segregation and meiotic cell division, suggesting an essential role for this kinase in DNA repair (18). The cellular substrates for HRR25 are not known. Since deletion of HRR25 leads to the accumulation of cells in the G_2 phase of the cell cycle, perhaps HRR25 normally functions in a pathway that suppresses replication until damaged DNA is repaired.

The initiation of SV40 DNA replication has previously been demonstrated to be controlled by cellular events. We have here identified a nuclear form of casein kinase I that directly controls the ability of T antigen to unwind the SV40 origin of replication. This kinase, in conjunction with PP2A, is part of a molecular switch which can turn SV40 DNA replication off and on. A closely related yeast protein kinase appears to play an essential role in DNA metabolism. The nuclear form of casein kinase I may play a role in mammalian DNA replication as well (46).

ACKNOWLEDGMENTS

We thank Alicia Russo and Tom Kelly for assistance with the HeLa nuclear extract; Melanie Cobb for the generous gift of bovine casein kinase I protein, peptide, and antibodies; Bob Schackmann for peptide sequencing; and Scott Shaffer for technical assistance.

This work was supported by NIH grant AI 31657 and grants from the American Cancer Society (IN-178) and the Rocky Mountain Center for the Biology of Development (RFA 90-HD-03) to D.M.V.

REFERENCES

1. **Aebersold, R. H., J. Leavitt, R. A. Saavedra, L. E. Hood, and S. B. Kent.** 1987. Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after in situ protease digestion on nitrocellulose. Proc. Natl. Acad. Sci. USA **84:**6970–6974.
2. **Agostinis, P., L. A. Pinna, F. Meggio, O. Marin, J. Goris, J. R. Vandenheede, and W. Merlevede.** 1989. A synthetic peptide substrate specific for casein kinase I. FEBS Lett. **259:**75–78.
3. **Borowiec, J. A., F. B. Dean, P. A. Bullock, and J. Hurwitz.** 1990. Binding and unwinding—how T antigen engages the SV40 origin of DNA replication. Cell **60:**181–184.
4. **Brockman, J. L., and R. A. Anderson.** 1991. Casein kinase I is regulated by phosphatidylinositol 4,5-bisphosphate in native membranes. J. Biol. Chem. **266:**2508–2512.
5. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58:**671–717.
6. **Chen, Y. R., M. S. P. Lees, P. Tegtmeyer, and C. W. Anderson.** 1991. The human DNA-activated protein kinase phosphorylates simian virus 40 T antigen at amino- and carboxy-terminal sites. J. Virol. **65:**5131–5140.
7. **Clertant, P., and F. Cuzin.** 1982. Covalent affinity labeling by periodate-oxidized [α-^{32}P]-ATP of the large T proteins of polyoma and SV40 viruses. J. Biol. Chem. **257:**6300–6305.
8. **Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz.** 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84:**16–20.
9. **DeGuzman, A., and E. Y. C. Lee.** 1988. Preparation of low-molecular-weight forms of rabbit muscle protein phosphatase. Methods Enzymol. **159:**356–368.
10. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
11. **D'Urso, G., R. L. Marraccino, D. R. Marshak, and J. M. Roberts.** 1990. Cell cycle control of DNA replication by a homologue from human cells of the p34^{cdc2} protein kinase. Science **250:**786–791.
12. **Dutta, A., and B. Stillman.** 1992. *cdc2* family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. EMBO J. **11:**2189–2199.
13. **Fanning, E., and R. Knippers.** 1992. Structure and function of simian virus 40 large tumor antigen. Annu. Rev. Biochem. **61:**55–85.
14. **Flotow, H., P. R. Graves, A. Q. Wang, C. J. Fiol, R. W. Roeske, and P. J. Roach.** 1990. Phosphate groups as substrate determinants for casein kinase I action. J. Biol. Chem. **265:**14264–14269.
15. **Flotow, H., and P. J. Roach.** 1989. Synergistic phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase and casein kinase I. J. Biol. Chem. **264:**9126–9128.
16. **Flotow, H., and P. J. Roach.** 1991. Role of acidic residues as substrate determinants for casein kinase I. J. Biol. Chem. **266:**3724–3727.
17. **Grässer, F. A., K. H. Scheidtmann, P. T. Tuazon, J. A. Traugh, and G. Walter.** 1988. In vitro phosphorylation of SV40 large T antigen. Virology **165:**13–22.
18. **Hoekstra, M. F., R. M. Liskay, A. C. Ou, A. J. DeMaggio, D. G. Burbee, and F. Heffron.** 1991. HRR25, a putative protein kinase from budding yeast: association with repair of damaged DNA. Science **253:**1031–1034.
19. **Höss, A., I. Moarefi, K.-H. Scheidtmann, L. J. Cisek, J. L. Corden, I. Dornreiter, A. K. Arthur, and E. Fanning.** 1990. Altered phosphorylation pattern of simian virus 40 T antigen expressed in insect cells by using a baculovirus vector. J. Virol. **64:**4799–4807.
20. **Hurwitz, J., F. B. Dean, A. D. Kwong, and S. H. Lee.** 1990. The in vitro replication of DNA containing the SV40 origin. J. Biol. Chem. **265:**18043–18046.
21. **Kornberg, A., and T. Baker.** 1992. DNA replication. W. H. Freeman, New York.
22. **LeBowitz, J.** 1985. Biochemical mechanisms of strand initiation in bacteriophage lambda DNA replication. Ph.D. thesis, The Johns Hopkins University, Baltimore, Md.
23. **Lees-Miller, S. P., Y. R. Chen, and C. W. Anderson.** 1990. Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. Mol. Cell. Biol. **10:**6472–6481.
24. **Mastrangelo, I. A., P. V. Hough, J. S. Wall, M. Dodson, F. B. Dean, and J. Hurwitz.** 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature (London) **338:**658–662.
25. **McVey, D., L. Brizuela, I. Mohr, D. R. Marshak, Y. Gluzman, and D. Beach.** 1989. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. Nature (London) **341:**503–507.
26. **O'Reilly, D. R., and L. K. Miller.** 1988. Expression and complex formation of simian virus 40 large T antigen and mouse p53 in insect cells. J. Virol. **62:**3109–3119.
27. **Pages, J., S. Manteuil, D. Stehelin, M. Fiszman, M. Marx, and M. Girard.** 1973. Relationship between replication of simian virus 40 DNA and specific events of the host cell cycle. J. Virol. **12:**99–107.
28. **Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeyer.** 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the

replication origin. J. Virol. **65:**2798–2806.
29. **Prives, C.** 1990. The replication functions of SV40 T antigen are regulated by phosphorylation. Cell **61:**735–738.
30. **Roberts, J. M., and G. D'Urso.** 1988. An origin unwinding activity regulates initiation of DNA replication during the mammalian cell cycle. Science **241:**1486–1489.
31. **Robinson, L. C., E. J. Hubbard, P. R. Graves, et al.** 1992. Yeast casein kinase I homologues: an essential gene pair. Proc. Natl. Acad. Sci. USA **89:**28–32.
32. **Rowles, J., C. Slaughter, C. Moomaw, J. Hsu, and M. H. Cobb.** 1991. Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes. Proc. Natl. Acad. Sci. USA **88:**9548–9552.
33. **Ruediger, R., J. E. V. W. Hood, M. Mumby, and G. Walter.** 1991. Constant expression and activity of protein phosphatase 2A in synchronized cells. Mol. Cell. Biol. **11:**4282–4285.
34. **Scheidtmann, K. H.** 1986. Phosphorylation of simian virus 40 large T antigen: cytoplasmic and nuclear phosphorylation sites differ in their metabolic stability. Virology **150:**85–95.
35. **Scheidtmann, K. H., M. Buck, J. Schneider, D. Kalderon, E. Fanning, and A. E. Smith.** 1991. Biochemical characterization of phosphorylation site mutants of simian virus 40 large T antigen: evidence for interaction between amino- and carboxy-terminal domains. J. Virol. **65:**1479–1490.
36. **Scheidtmann, K. H., B. Echle, and G. Walter.** 1982. Simian virus 40 large T antigen is phosphorylated at multiple sites clustered in two separate regions. J. Virol. **44:**116–133.
37. **Scheidtmann, K. H., D. M. Virshup, and T. J. Kelly.** 1991. Protein phosphatase 2A dephosphorylates simian virus 40 large T antigen specifically at residues involved in regulation of the DNA-binding activity. J. Virol. **65:**2098–2101.
38. **Schneider, J., and E. Fanning.** 1988. Mutations in the phosphorylation sites of simian virus 40 (SV40) T antigen alter its origin DNA-binding specificity for sites I or II and affect SV40 DNA replication activity. J. Virol. **62:**1598–1605.
39. **Stahl, H., P. Droge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5:**1939–1944.
40. **Stillman, B.** 1989. Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. **5:**197–245.
41. **Tuazon, P. T., and J. A. Traugh.** 1991. Casein kinase I and II—multipotential serine protein kinases: structure, function, and regulation, p. 123–164. *In* P. Greengard and G. A. Robinson (ed.), Advances in second messenger phosphoprotein research, vol. 23. Raven Press, New York.
42. **Umphress, J. L., P. T. Tuazon, C. J. Chen, and J. A. Traugh.** 1992. Determinants on simian virus 40 large T antigen are important for recognition and phosphorylation by casein kinase I. Eur. J. Biochem. **203:**239–243.
43. **Virshup, D. M., M. G. Kauffman, and T. J. Kelly.** 1989. Activation of SV40 DNA replication in vitro by cellular protein phosphatase 2A. EMBO J. **8:**3891–3898.
44. **Virshup, D. M., and T. J. Kelly.** 1989. Purification of replication protein C, a cellular protein involved in the initial stages of SV40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA **86:**3584–3588.
44a. **Virshup, D. M., I. Moarefi, and E. Fanning.** Unpublished data.
45. **Virshup, D. M., A. Russo, and T. J. Kelly.** 1992. Mechanism of activation of simian virus 40 DNA replication by protein phosphatase 2A. Mol. Cell. Biol. **12:**4883–4895.
46. **Virshup, D. M., M. S. Wold, D. H. Weinberg, M. G. Kauffman, and T. J. Kelly.** 1990. Cellular proteins involved in SV40 DNA replication in vitro, p. 303–314. *In* C. C. Richardson and I. R. Lehman (ed.), Molecular mechanisms in DNA replication and recombination, vol. 127. Alan R. Liss, Inc., New York.
47. **Wessel, R., J. Schweizer, and H. Stahl.** 1992. Simian virus 40 T antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. J. Virol. **66:**804–815.
48. **Wold, M. S., and T. J. Kelly.** 1988. Purification and characterization of replication protein A, a cellular protein required for in vitro replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. USA **85:**2523–2527.
49. **Wold, M. S., D. W. Weinberg, D. M. Virshup, J. J. Li, and T. J. Kelly.** 1989. Identification of cellular proteins required for SV40 DNA replication. J. Biol. Chem. **264:**2801–2809.