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A DNA-Activated Protein Kinase from HeLa Cell Nuclei

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A DNA-activated protein kinase (DNA-PK) was purified from nuclei of HeLa cells. Activity was associated with a single high-molecular-mass (approximately-300,000 Da) polypeptide when analyzed by gel filtration, denaturing polyacrylamide gel electrophoresis, and Western immunoblotting using a monoclonal antibody that also inhibits enzyme activity. Nuclear localization was indicated by subcellular fractionation and confirmed by immunofluorescence on whole cells. Double-stranded DNA stimulated phosphorylation of the 300-kDa polypeptide in purified preparations as well as phosphorylation of the exogenous substrates α-casein, simian virus 40 large T antigen, and the human heat shock protein hsp90. Autophosphorylation led to inactivation of the enzyme. The phosphorylation of casein was stimulated over 30-fold by DNA and was specific for serine and threonine residues. Bovine serum albumin and histone H1 were poor substrates for DNA-PK, and no phosphorylation of immunoglobulin G or histones other than H1 was observed. Supercoiled or heat-denatured DNA and synthetic double-stranded RNA or RNA-DNA copolymers did not stimulate casein phosphorylation by DNA-PK. Interaction of the enzyme with DNA in the absence of exogenous substrates was demonstrated by thermal inactivation and gel mobility shifts. These characteristics identify DNA-PK as distinct from other protein kinases described in the literature and suggest that activation by DNA is an important feature of the enzyme's in vivo function.

Protein kinases regulate cellular activities at multiple levels (8, 14, 15), including the mediation of responses to specific external stimuli. Many growth factor receptors (44), oncogene products (15), and cell cycle regulatory factors (13) possess intrinsic protein kinase activity and furthermore are themselves often regulated by phosphorylation. Signaling pathways affecting gene activity and cell division may include protein kinases within the cell nucleus, functioning either as targets of second messengers or as distal components of protein kinase cascades (22, 23).

Among the protein kinases that have been purified from or localized to the nuclei of eucaryotic cells, the two best-studied enzymes, referred to as NI (6, 39, 41) and NII (6, 11, 31, 40) (also called nuclear casein kinases [42]), have been isolated from a variety of species and tissues, including HeLa cells (45). A histone H1 kinase, which plays a key role in mitotic regulation (2, 13), phosphorylates a number of nuclear substrates in vivo (21). Other, less well characterized protein kinases may be localized in the nucleus, including the product of the proto-oncogene *mos* (43); a chromatin-associated kinase which phosphorylates DNA-bound histones, especially histone H3 (36); a cyclic GMP (cGMP)-dependent kinase which also phosphorylates histones preferentially (12); and two salt-sensitive activities from HeLa cells (10, 30). An enzyme related to protein kinase C has been shown to contain a putative nuclear localization signal (25), and under certain conditions protein kinase C translocates from cytoplasm to the nuclear membrane (17). The cAMP-dependent protein kinase has also been found to accumulate in nuclei in response to cAMP stimulation (24, 35). Ohtsuki et al. (28) identified a cyclic nucleotide-independent enzyme from mouse spleen nuclei that was distinct from casein kinase II and phosphorylated histones as well as several nonhistone chromosomal proteins.

Relatively few protein kinases have been shown to be activated or stimulated by nucleic acids. One kinase that is activated by double-stranded RNA (33) specifically phosphorylates the translation factor EIF-2a (9). The enzyme isolated from mouse spleen nuclei by Ohtsuki et al. was stimulated up to fivefold by DNA specifically for phosphorylation of two small nonhistone proteins (26–28), and histone phosphorylation by the chromatin-associated cGMP-dependent kinase is also stimulated by DNA (12); in both of these cases, however, the stimulation results from interaction of DNA with the substrate rather than with the enzyme (12, 26).

Although no nuclear protein kinase has yet been shown to require DNA as a general cofactor, DNA-dependent protein phosphorylation was observed in crude cytoplasmic extracts from HeLa cells and *Xenopus* oocytes (42) and in HeLa nuclear extracts (4). Here we report the purification of a protein kinase from HeLa cell nuclei with the unique characteristic that it is activated specifically by double-stranded DNA (dsDNA) for the phosphorylation of a variety of substrates. Lees-Miller and colleagues have been studying an enzyme that is probably identical to the one described here and describe its partial purification and characterization in the accompanying report (19). This enzyme, which we call DNA-PK, is the first example of a protein kinase with a generalized functional requirement for DNA.

MATERIALS AND METHODS

Cells. HeLa cells (line S3; ATCC CCL 2.2) were grown at 37°C in suspension (4) or in monolayer (5). For enzyme purification, batches of 3×10^9 cells (approximately 6 g) were harvested from 6-liter suspension cultures by centrifugation at 4°C and 800 × *g* for 20 min, washed once in 600 ml of ice-cold phosphate-buffered saline (0.2 g of KH_2PO_4, 0.2 g of KCl, 8 g of NaCl, 1.15 g of Na_2HPO_4 per liter), recentrifuged, suspended in 30 ml of ice-cold 50 mM Tris hydrochloride (pH 7.9)–0.1 mM EDTA–10 mM α-monothioglycerol–0.1 mM dithiothreitol (DTT)–30% (vol/vol) glycerol), frozen in a dry ice-ethanol bath, and stored at −100°C.

Purification of DNA-dependent kinase. All steps were carried out at 0 to 4°C. Nuclei were isolated and extracted by

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the method of Dignam et al. (7) in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μg each of leupeptin, pepstatin A, and aprotinin per ml). The 0.4 M KCl nuclear extract from 50 g of HeLa cells was concentrated by $(NH_4)_2SO_4$ precipitation (7). The precipitate was dissolved in 10 ml of buffer B (20 mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% [vol/vol] glycerol, 0.02% polyethylene sorbitan monolaurate [Tween 20]) containing 0.1 M KCl and dialyzed against 250 ml of the same buffer for a total of 18 h, with one buffer change. The resulting precipitate was removed by centrifugation at 15,000 × *g* for 20 min, and the supernatant solution was either stored at −100°C or used directly for purification. For all subsequent steps, buffer B with the stated additions was used. The concentrated nuclear extract was adjusted to 40 mM KCl by addition of buffer B, adsorbed to a column (2.5 by 19 cm) of DEAE-Sephacel equilibrated with buffer B plus 40 mM KCl, and eluted with a 500-ml linear 0.04 to 0.5 M gradient of KCl in buffer B at 1 to 2 ml/min. Fractions containing DNA-stimulated casein phosphorylation activity (assayed as described below) were pooled and adsorbed to a phosphocellulose column (2.5 by 4.0 cm). After a wash with 50 ml of buffer B plus 0.15 M KCl, the column was eluted with a 250-ml linear 0.04 to 1.0 M gradient of KCl in buffer B. Active fractions were dialyzed against buffer B plus 0.3 M KCl and 15% glycerol, concentrated by ultrafiltration in a Minicon-B15 device (Amicon; molecular weight cutoff, 15,000), layered onto a column (0.75 by 80 cm) of Sephacryl S-300HR equilibrated with buffer B plus 0.3 M KCl and 10% glycerol, and eluted with the same buffer. Fractions containing peak activity were pooled, dialyzed against buffer B plus 0.05 M KCl, and adsorbed to a column (1 by 5 cm) of reactive red 120 agarose 300CL (Sigma). The column was washed with 10 ml of dialysis buffer, and enzyme activity was eluted with a linear gradient of 0.05 to 0.5 M KCl in buffer B. Peak fractions (eluting at approximately 0.2 M KCl) were pooled, dialyzed against buffer B plus 0.1 M KCl, and stored in small aliquots at −100°C. At this stage, DNA-PK activity was nearly pure, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and silver staining; this preparation was used for some biochemical characterization experiments. Further purification to constant specific activity was accomplished by step elution from dsDNA-cellulose prepared as described by Potuzak and Wintersberger (29), loading in buffer B plus 0.05 M KCl, and elution with buffer B plus 0.5 M KCl, followed by dialysis against buffer B plus 0.03 M NaCl and fast protein liquid chromatography (FPLC), using a Mono Q HR5/5 column (Pharmacia) and a linear gradient of 0.03 to 0.5 M NaCl (see Fig. 2). Enzyme activity from any of the final three steps was stable for 72 h in buffer B plus 0.1 M KCl and for at least 2 months when stored at −100°C in the same buffer.

Protein kinase assay. The standard assay mixture contained 0.5 to 12 μl of extract or enzyme and 20 μg of enzymatically dephosphorylated α-casein that had been heated at pH 9.0 and then neutralized prior to use (39), with or without 1 μg of sonicated salmon DNA (average size of approximately 300 nucleotides, determined by agarose gel electrophoresis and ethidium bromide staining) in a final volume of 20 μl containing 0.6× buffer B. Reactions were started by addition of [γ-^{32}P]ATP (1 to 3 μCi/nmol) and $MgCl_2$ to give final concentrations of 100 μM and 7.5 mM, respectively, incubated at 37°C for 5 or 10 min, and stopped with 5 μl of 0.2 M EDTA. Radioactivity bound to DEAE-paper was determined as described previously (4). Incorporation of isotope by the most highly purified preparations was linear for at least 20 min.

Monoclonal antibodies. Monoclonal antibodies were produced in collaboration with Thomas Shenk, Department of Molecular Biology, Princeton University. Two 8- to 12-week-old female BALB/c mice were each injected intraperitoneally with 30 to 50 μg of highly purified enzyme protein (from the Mono Q step) and RIBI adjuvant. Injections were repeated after 2 and 5 weeks. Anti-DNA-PK titer 6 days after the second booster immunization was determined by dot Western immunoblot. The spleen from one seropositive animal was removed, washed three times in warm RPMI medium, and crushed, and the cells were collected from the supernatant. Fusions with SP2/0 nonproducer variant myeloma cells (selected from a hybridoma between MOPC-21 and BALB/c spleen cells) at a 10:1 ratio were accomplished by incubation with polyethylene glycol in HEPES-buffered RPMI at 37°C for 60 min. Fusion mixtures were then plated in RPMI plus 20% fetal calf serum under selective conditions onto a feeder layer of thymocytes in 96-well plates such that each well contained one to five hybridomas. When colonies were 1 mm in diameter, tissue culture supernatants from these pools were screened by dot blots and in some cases by Western analysis and/or inhibition and immunoprecipitation of DNA-PK activity. Positive pools were subcloned at limiting dilution, and 40 to 50 individual colonies from each pool rescreened by the appropriate assay. The immunoglobulin type and subclass were determined by a commercial hemagglutination assay (Serotek), and immunoglobulin Gs (IgGs) were then purified from the tissue culture supernatants by chromatography on protein G-Sepharose (Genex). Two monoclonal antibodies, MAb18-2 and MAb42-26, used in this study were both of the IgG1 subclass, and a third, MAb25-4, was IgG2a.

Western blots. Five percent denaturing SDS-polyacrylamide gels were run as described previously (4), and the polypeptides were transferred at constant voltage (to give an initial current of 2.5 mA/cm^2) for 1.5 h at room temperature with 25 mM Tris chloride (pH 10.4)–20% methanol as the anode buffer and 25 mM Tris (pH 9.4)–40 mM 6-aminohexanoic acid–20% methanol as the cathode buffer in a Polyblot (ABN, Inc.) semidry transfer apparatus. More than 90% of the 300-kDa polypeptide was transferred under these conditions, as determined by inclusion of ^{32}P-labeled autophosphorylated enzyme as a marker. The filters were rinsed to remove gel debris in TBST (TBS plus 0.05% Tween-20; TBS is 10 mM Tris hydrochloride [pH 7.5], 0.14 M NaCl, 1.5 mM $MgCl_2$), blocked in TBSTM (TBST plus 5% nonfat dry milk) at 4°C overnight with shaking, and incubated with a monoclonal antibody (2 ml of high-titer tissue culture supernatant) for 2 h at room temperature in TBSTM. After four washes with TBSTM, the membrane was incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma) in TBSTM for 30 min and then extensively washed with TBSTM, TBST, and finally TBS. Color development was with Protoblot reagent (Promega Biotec). Dot blots for screening monoclonal supernatants were done in the same way as Western blots except that aliquots of enzyme containing 50 ng of protein were immobilized on nitrocellulose sheets by vacuum filtration.

Indirect immunofluorescence. Monolayer cells growing on coverslips were fixed by immersion in acetone-methanol (70:30) for 5 min at −20°C, incubated with primary antibody (mouse monoclonal IgG) for 30 min at 37°C, stained with fluorescein-labeled goat anti-mouse IgG, and photographed with the aid of a Zeiss epifluorescence microscope.

TABLE 1. Purification of DNA-PK

Purification step	Total activity[a] (kU)	Stimulation by DNA[b]	Total protein (mg)	Sp act (kU/mg)
Whole-cell homogenate[c]	294.3	1.5	2,257.50	0.39
Cytoplasmic S-100	943.1	6.7	524.28	2.16
Nuclear extract	1,046.0	3.8	932.20	1.52
DEAE-Sephacel	1,602.0	46.5	136.00	12.04
Phosphocellulose	620.1	20.4	12.31	59.97
Sephacryl S-300HR	150.5	23.6	1.80	87.30
Reactive red agarose	104.6	11.6	1.07	107.35
DNA-cellulose	68.6	19.8	0.66	109.48
Mono Q	41.5	33.9	0.35	122.92

[a] Activity with sonicated salmon DNA (50 μg/ml) minus activity without added DNA. In each case, a time course was done and activity was determined from the initial slope. One unit = 1 pmol of ^{32}P transferred from [γ-^{32}P]ATP to casein at 37°C in 1 min; the concentration of ATP was 0.5 mM.
[b] Activity in the presence of DNA divided by activity without DNA.
[c] Prepared by high-salt extraction of a hypotonic lysate (4, 7).

DNA electrophoretic mobility shift assay. Complementary synthetic single-stranded oligonucleotides were end labeled with ^{32}P by using T4 polynucleotide kinase (20), annealed by heating to 70°C in 0.1 M NaCl and slow cooling, phenol extracted, and separated from unincorporated ATP by gel filtration. Binding reaction mixtures contained 0.5 ng of labeled oligonucleotide (approximately 40,000 cpm), 30 ng of purified enzyme (DNA-cellulose fraction) unless otherwise noted, 10 mM Tris chloride (pH 7.5), 1 mM DTT, 1 mM EDTA, 50 to 150 mM NaCl, and 5% glycerol in a total volume of 10 μl. After 30 min at 30°C, the mixture was chilled on ice and electrophoresed through a 4% polyacrylamide gel (29:1 acrylamide-bisacrylamide) in Tris-glycine buffer at 5 to 10°C as described by Singh et al. (37).

Determination of phosphoamino acids. The chromatographic method of Schaffer (32), as modified by Rose et al. (31), was used. Enzymatically dephosphorylated α-casein (30 μg) was phosphorylated by purified DNA-PK (0.2 μg) for 30 min under standard conditions (see above) in the presence of 50 μg of double-stranded salmon DNA per ml in a 100-μl reaction volume. The protein was precipitated by cold trichloroacetic acid, dissolved in 400 μl of 2 N HCl, and incubated for 18 h at 100°C in a sealed glass ampoule. The entire sample was then diluted with H_2O to 0.5 M HCl, loaded onto a Dowex 50W-X4 column, and eluted with 0.05 N HCl. The order of elution of phosphoamino acid standards as determined by A_{214} and A_{280} was *O*-phosphothreonine, *O*-phosphoserine, *O*-phosphotyrosine.

Reagents. Chromatography resins were obtained from Pharmacia-LKB or Whatman (phosphocellulose P-11); salmon sperm DNA, enzymatically dephosphorylated α-casein, phosvitin, Tween 20, and phosphoamino acids were from Sigma; DNase I (RNase free), calf intestine alkaline phosphatase, T4 polynucleotide kinase, and purified histone fractions were from Boehringer Mannheim; and restriction endonucleases were from International Biochemicals and Bethesda Research Laboratories. Synthetic nucleic acid heteropolymers were from Boehringer Mannheim and Pharmacia-LKB. Synthetic oligonucleotides corresponding to the Ap1-binding site at −70 of the human collagenase gene (1), the NFκB-binding site of the mouse κ light-chain immunoglobulin gene enhancer (34), and the H2TF1-binding site from the mouse *H-2K*^{b} class I major histocompatibility gene enhancer (3) were gifts from T. Shenk and U. Müller (Princeton University), and oligonucleotides containing the consensus Sp1- and Ap1-binding sites were purchased from Stratagene. Bacterial plasmids were a gift from L. E. Babiss (Columbia University) and were propagated and purified by standard methods (20). Baculovirus-cloned simian virus 40 (SV40) T antigen was a gift from E. Fanning (University of Munich), and purified, kinase-free human hsp90 was a gift from C. Anderson and S. Lees-Miller (Brookhaven National Laboratories).

RESULTS

Purification of DNA-PK. Table 1 summarizes the data from a representative purification. Nuclear and soluble cytoplasmic fractions obtained after hypotonic swelling and mechanical shearing contained approximately equal amounts of DNA-stimulated protein kinase activity. However, DEAE-Sephacel chromatography of the nuclear extract yielded a 60% increase in DNA-dependent phosphorylation, indicating that inhibitors or phosphatases in crude extracts probably led to underestimation of activity. In addition, it is likely that traces of DNA present in some nuclear extracts would have the same effect, because enzyme activity was determined by subtracting the amount of casein phosphorylation in the absence of DNA from that observed after addition of DNA. Chromatography over phosphocellulose without the intervening DEAE step also resulted in increased DNA-dependent activity (Table 2). Several peaks from the DEAE column had cyclic nucleotide-independent protein kinase activity, but only one (eluting between 0.15 and 0.25 M KCl) contained DNA-stimulated activity. Fractionation of this peak by chromatography on phosphocellulose resolved two casein-phosphorylating activities. The major component was a single peak that eluted between 0.25 and 0.35 M KCl and was stimulated by DNA; a heterogeneous minor component, which eluted at a higher salt concentration, was not affected by DNA. Further fractionation by gel filtration and successive affinity columns containing the Reactive Red 120 agar-

TABLE 2. Specific activities of DNA-PK and marker enzymes in subcellular fractions

Fraction	Casein phosphorylation[a]						Activity[b]		
	Before PC			After PC			Glucose-6-phosphate dehydrogenase	Malate dehydrogenase	Glutamate dehydrogenase
	+DNA	−DNA	Net	+DNA	−DNA	Net			
Nuclear	2,238	1,968	270	1,236	155	1,081	<80	<13	128
100,000 × *g* supernatant	367	223	144	166	33	130	4,495	97	78
100,000 × *g* pellet	998	618	380	327	80	247	2,000	389	370

[a] Expressed as picomoles of ^{32}P transferred to casein per minute per milligram at 30°C. The concentration of ATP was 300 μM. Salmon DNA (100 μg/ml) was added to reaction mixtures as indicated, and net DNA-dependent phosphorylation was calculated by subtraction. For phosphocellulose (PC) chromatography of the crude subcellular fractions, 0.2-ml samples were adjusted to 0.1 M KCl and applied to 0.6-ml column of phosphocellulose; after a wash with 6 ml of buffer B plus 0.1 M KCl, enzyme was eluted with 1 ml of buffer B plus 0.5 M KCl (see Materials and Methods).
[b] Determined spectrophotometrically by NADP reduction (38); expressed as picomoles of NADPH produced per second per milligram.

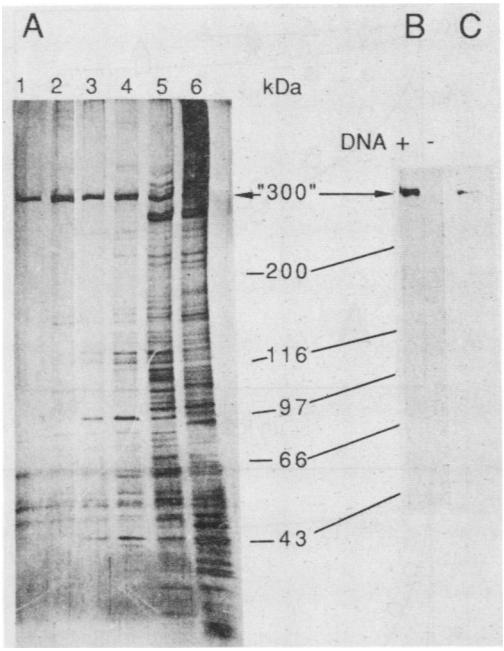

FIG. 1. Denaturing PAGE of fractions from DNA-PK purification. (A) The indicated amounts of protein from samples containing peak DNA-PK activity from various purification steps were separated by 5 to 12.5% gradient SDS-PAGE and silver stained. Lanes: 1, Mono Q, 1.5 μg; 2, dsDNA-cellulose, 2.5 μg; 3, Sephacryl S-300HR, 2.5 μg; 4, phosphocellulose, 5 μg; 5, DEAE-Sephacel, 10 μg; 6, nuclear extract, 10 μg. The positions of size markers are indicated. (B) A 1.5-μg sample of purified enzyme was incubated with [γ-^{32}P]ATP with or without DNA under standard conditions and separated by 7.5% SDS-PAGE, and the gel was dried and fluorographed. (C) Western blot of purified DNA-PK (50 ng) probed with MAb18-2.

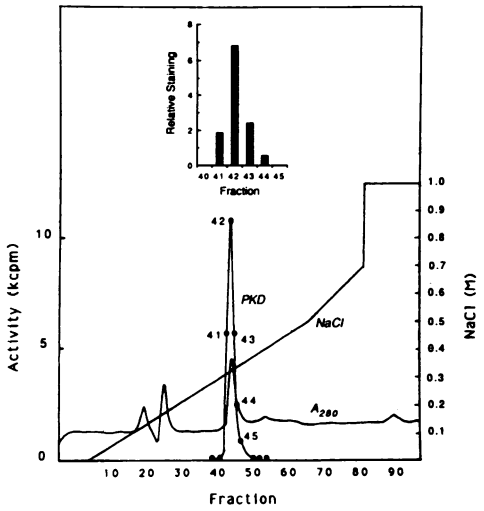

FIG. 2. FPLC of purified DNA-PK on a Mono Q HR5/5 column. Enzyme purified by DNA-cellulose chromatography was processed as described in Materials and Methods. (Insert) Indicated fractions were analyzed by 7.5% SDS-PAGE and stained with Coomassie brilliant blue, and the visible band at approximately 300 kDa was scanned with an ISCO recording densitometer. Staining intensity was determined by integration of the peak areas.

ose and dsDNA-cellulose and by ion-exchange FPLC on Mono Q resin resulted in an essentially homogeneous preparation, with little increase in specific activity in the final steps. The apparent purification was about 100-fold, and the overall recovery was 4%, as judged from the activity measured in nuclear extracts. (Both figures are subject to the quantitative uncertainty in determining total kinase activity in the crude cell fractions.) The low yield probably resulted from inactivation during purification, because only one peak of protein kinase activity was observed in all purification steps after the phosphocellulose column. Attempts to reconstitute activity by addition of each Sephacryl fraction to the fraction containing peak DNA-stimulated activity failed to detect additional forms of the enzyme that might have required a polypeptide present in the peak fraction to activate the enzyme. Thus, the actual degree of purification based on recovery of protein, rather than on specific activity, is likely to have been greater than 100-fold. Estimation of the amount and recovery of DNA-PK by Western blotting using monoclonal antibodies (see below) suggested that the 300-kDa polypeptide associated with DNA-PK activity (Fig. 1A) may comprise as much as 1% of the total nuclear protein solubilized in 0.4 M KCl, which would be equivalent to 0.2% of the proteins on a molar basis, assuming an average molecular mass for nuclear proteins of 60,000 Da. If the ratio of activity to immunoreactive protein in nuclear extracts is assumed to be 1:1, it can be estimated from Western blots of purified enzyme that as much as 80% of the enzyme may have been inactivated during purification (data not shown).

In all purified enzyme fractions, the ratio of casein phosphorylation in the presence and absence of DNA was markedly increased over that in crude extracts, with purified preparations typically containing only 2 to 5% DNA-independent kinase activity. Occasionally, as in the red agarose fraction shown in Table 1, the extent of DNA-independent activity was somewhat greater. DNA-independent phosphorylation could be due to traces of other contaminating protein kinases, to a basal DNA-independent activity of DNA-PK, or to fragments of cellular DNA that might have remained bound to the enzyme or copurified with it. Effects of salt, temperature, pH, and inhibitors of DNA-activated casein phosphorylation were similar for DNA-stimulated and DNA-independent activities (although this was often difficult to measure for the low DNA-independent activity), suggesting that DNA-independent activity was not due to contaminating kinases (data not shown).

DNA-PK activity is associated with a 300-kDa polypeptide. Analysis of the polypeptide composition of pooled peak fractions by SDS-PAGE and silver staining demonstrated that activity copurified with a high-M_r polypeptide (Fig. 1A). No other polypeptides were detected in the peak fractions from the final chromatographic step; the diffusely staining bands migrating at 40 to 60 kDa in all lanes (including the marker lane) were probably contaminating keratins. DNA-PK activity eluted from the Mono Q column as a symmetrical peak at approximately 0.25 M KCl (Fig. 2); upon analysis by SDS-PAGE and staining with Coomassie brilliant blue, densitometry showed that the staining intensity of the 300-kDa polypeptide corresponded closely to the amount of enzyme activity in each fraction (Fig. 2, inset). None of the minor peaks of 280-nm absorbance contained any polypeptide detectable on silver-stained gels. After Mono Q chromatography of some purified enzyme preparations, silver staining often revealed a faint set of additional bands below the 300-kDa band in each fraction that contained the 300-kDa polypeptide. These minor bands were recognized by monoclonal antibodies (see below) that re-

acted with the major 300-kDa polypeptide and were therefore likely to be partial degradation products (T. H. Carter, I. Sun, and I. Vančurová, unpublished data). Thus, the enzyme activity is associated with the large polypeptide of approximate M_r 300,000, which probably exists in its active, undenatured form as a monomer, because molecular exclusion chromatography on Sephacryl S-300HR indicated a Stokes radius for the native enzyme equivalent to that of a 280,000-Da globular protein (data not shown).

Of several monoclonal antibodies specific for the 300-kDa polypeptide, one (MAb18-2) detected the polypeptide on Western blots (Fig. 1C) and also inhibited DNA-dependent casein phosphorylation in a dose-dependent manner (Fig. 3A). A purified preparation of DNA-PK (through the Reactive Red agarose step) was incubated with indicated amounts of purified IgG for 2 h at 0°C in buffer B with 0.1 M KCl but without DTT. In some reactions, enzyme was incubated for 30 min with sonicated salmon DNA (50 μg/ml) before the addition of antibody. DNA-dependent kinase activity was then assayed as described in Materials and Methods. MAb18-2 reproducibly inhibited kinase activity by 50%, whereas neither another monoclonal antibody of the same IgG subclass (MAb42-26) that also reacted with the 300-kDa polypeptide on Western blots (not shown) nor purified mouse IgGs inhibited activity (Fig. 3A). Interestingly, preincubation of the enzyme with DNA protected DNA-PK against inactivation by MAb18-2, suggesting that the portion of the native DNA-PK molecule comprising the epitope recognized by this antibody is conformationally altered or partially occluded by DNA binding. An alternative explanation for these data could be that MAb18-2 binds to DNA or to a DNA-enzyme complex. The Western blot data make this is unlikely, however, as do the results of immunoprecipitation experiments (see below).

Partial inhibition of kinase activity could have been due to the presence of another, non-cross-reacting DNA-activated kinase, to a weak interaction between MAb18-2 and DNA-PK, or to antibody binding in such a way as to interfere with, but not abolish, kinase activity. To distinguish between these possibilities, the experiment shown in Fig. 3A was repeated, except that immune complexes were removed by binding to anti-mouse immunoglobulins immobilized on polyacrylamide beads, and the supernatant solution was then assayed for DNA-PK (Fig. 3B). Both MAb18-2 and MAb42-26 immunoprecipitated ≥95% of the DNA-PK. However, over five times as much MAb18-2 as MAb42-26 was required for 50% removal of DNA-PK, suggesting that partial inhibition of DNA-PK activity in solution by MAb18-2 may have been due to a relatively weak antibody-antigen interaction. Since no DNA was added before kinase activity was measured, it is unlikely that all of the DNA-PK activity could have been immunoprecipitated by MAb18-2 if this antibody recognized either DNA or an epitope composed of a DNA-enzyme complex.

Subcellular localization of DNA-PK. Stimulation of activity by dsDNA is consistent with nuclear localization of DNA-PK. If this were the case, the dsDNA-stimulated casein phosphorylating activity in the cytoplasmic S-100 fraction could have resulted from leakage of the enzyme from nuclei during their isolation. However, the nuclear isolation procedure used for DNA-PK purification was relatively simple, and it is possible that the activity associated with the nuclear pellet resulted from contamination with cytoplasmic constituents. We therefore examined the intracellular localization of DNA-PK by using a more rigorous method to isolate nuclei and also by indirect immunofluorescence on whole cells.

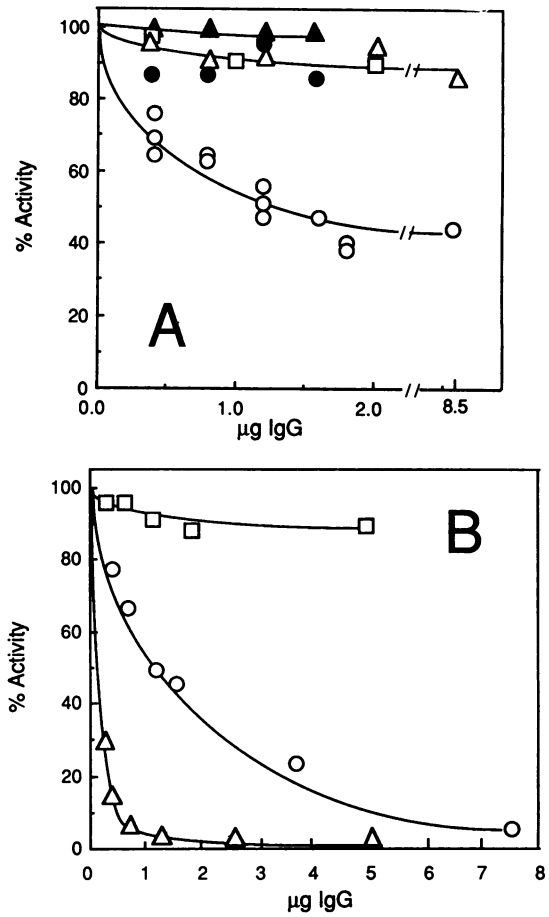

FIG. 3. Effect of monoclonal antibodies on DNA-PK activity. (A) Indicated amounts of monoclonal antibodies (mouse, IgG subclass) specific for the 300-kDa polypeptide in purified DNA-PK preparations or of a mixture of nonspecific mouse IgGs were incubated with 100 ng of purified enzyme in a total volume of 40 μl for 2 h at 0°C prior to assay of enzyme activity. Symbols: ○, MAb18-2; △, MAb42-26; □, mixed IgGs. In some cases, enzyme was preincubated with sonicated native salmon DNA for 30 min before addition of antibody (filled symbols). Data are expressed as percentages of the enzyme activity without antibody. Each symbol represents the average of two determinations. (B) Enzyme was incubated with IgGs as for panel A; after 2 h, 20 μl of a 20% suspension of Immunobeads (Bio-Rad) containing immobilized rabbit anti-mouse immunoglobulin in buffer B plus 0.1 M KCl was added to each tube, and incubation continued for 1 h at 0°C before centrifugation at 12,000 × *g* for 10 s. The supernatant solution was readsorbed to a second aliquot of Immunobeads and then assayed for DNA-PK activity.

For cell fractionation, HeLa cells grown in suspension were disrupted by mechanical shearing in isotonic buffer, and nuclei were isolated by repeated centrifugation through hypertonic sucrose as described by Kornberg et al. (16). The washed nuclei were then extracted in 0.6 M KCl as described by Dignam et al. (7) to solubilize the DNA-PK activity. The cytoplasmic supernatant obtained after low-speed centrifugation of the original cell lysate was further separated into soluble and particulate fractions by high-speed centrifugation. Each fraction was then assayed for DNA-dependent phosphorylation of casein before and after chromatography

on phosphocellulose and was also assayed directly for the cytosolic enzymes glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and malate dehydrogenase (EC 1.1.1.37) and for the mitochondrial enzyme glutamate dehydrogenase (EC 1.4.1.2). The specific activity of DNA-PK after phosphocellulose chromatography was eight times greater in purified nuclei than in the soluble cytoplasmic fraction (Table 2). Before chromatographic separation, the calculated specific activity in nuclei was considerably lower than this because of a high level of DNA-independent casein phosphorylation. Since our assay conditions would detect other casein kinases, and contaminating DNA would also lead to enhanced DNA-independent casein kinase activity, the phosphocellulose step probably yields a more accurate figure for the relative amounts of DNA-PK in the various subcellular fractions than does direct assay of the fractions. No cytosolic marker enzyme activity was detected in the nuclei. Although the highly purified nuclei were still contaminated with mitochondria, as judged by glutamate dehydrogenase activity, the specific activity of DNA-PK in these nuclei was four times that in the cytoplasmic particulate fraction, which contained the bulk of the mitochondria. These results are consistent with a nuclear localization for the majority of active DNA-PK molecules in growing HeLa cells. Results of indirect immunofluorescence also suggested that most DNA-PK molecules are nuclear (Fig. 4). The monoclonal antibody MAb18-2 stained most nuclei of HeLa cells growing in monolayer as well as the nuclei of cells of the human epidermoid carcinoma line A431.

Autophosphorylation. Autophosphorylation of DNA-PK was tested by incubating purified enzyme with [γ-^{32}P]ATP under standard reaction conditions with and without dsDNA. When the trichloroacetic acid-precipitated proteins were analyzed by SDS-PAGE and autoradiography, no phosphorylated polypeptides were observed in the absence of DNA, whereas a 300-kDa polypeptide was the predominant radiolabeled species observed when the enzyme was incubated with DNA alone (Fig. 1B). Since it was often observed that concentrated preparations of enzyme did not continue to phosphorylate casein for as long a period as did more dilute enzyme, the possibility that autophosphorylation inactivated the enzyme was tested by incubating purified DNA-PK with ATP and Mg^{2+} in the absence of added substrate and then analyzing its ability to phosphorylate casein, while also measuring the incorporation of ^{32}P into the 300-kDa polypeptide during the first incubation in a parallel experiment (Fig. 5). The kinetics of inactivation closely followed the labeling of the 300-kDa polypeptide. After 30 min of incubation in the presence of Mg^{2+} and ATP, 95% of the kinase activity was lost; during the same period, an average of 0.87 mol of phosphate was transferred per mol of the 300-kDa polypeptide in two experiments, consistent with inactivation of DNA-PK by a single autophosphorylation event. Inactivation of the kinase was not an indirect effect of ATP depletion during the preincubation, because the same result was obtained when additional ATP was added to the casein phosphorylation reactions (data not shown).

Protein substrate specificity. A number of different proteins were tested for the ability to act as phosphate acceptors. Phosvitin, α-casein, serum albumin, mouse IgGs, mixed histones H2a, H2b, H3, and H4 (without H1), purified histone H1, the human heat shock protein hsp90, and SV40 large T antigen were all used as substrates (Fig. 6). Little or no phosphorylation of any substrate was observed in the absence of DNA, even at relatively high protein concentrations. T antigen and denatured α-casein were the best substrates on a phosphorylation-per-unit-mass basis; hsp90 was somewhat less well phosphorylated, and denatured bovine serum albumin was weakly phosphorylated (Fig. 6A). A small amount of DNA-stimulated phosphorylation of histone H1 was observed after a long exposure of the autoradiogram, but phosphorylation of immunoglobulins, phosvitin, and histones other than H1 was not detected (Fig. 6B). Because casein was the best routinely available substrate for DNA-PK in the presence of DNA, characterization of the enzyme used phosphorylation of this substrate as the standard assay.

The phosphoamino acids produced in α-casein by the DNA-activated kinase were determined after phosphorylation by highly purified enzyme under standard conditions, trichloroacetic acid precipitation, acid hydrolysis, and fractionation by ion-exchange chromatography. All of the radioactivity was recovered in peaks that comigrated with P_i (55%), *O*-phospho-L-serine (39%), and *O*-phospho-L-threonine (6%) (Fig. 7).

Effects of polynucleotides. A number of natural and synthetic polynucleotides were tested for their effects on casein phosphorylation (Table 3). Only double-stranded polydeoxyribonucleotides were effective at stimulating kinase activity, whereas a synthetic DNA-RNA heteroduplex and poly(rI) · poly(rC), an activator of the double-stranded RNA-activated kinase, were inhibitory. In several cases, polynucleotides were digested with a restriction endonuclease and/or S1 endonuclease and repurified by phenol extraction before incubation in protein kinase reactions (Table 4). Activation required linear, double-stranded polydeoxyribonucleotides, regardless of the source. S1 endonuclease treatment did not prevent various DNA preparations from activating DNA-PK (data for one preparation are shown). Although supercoiled plasmid DNA did not activate the enzyme, saturating concentrations of plasmid that had been repeatedly frozen and thawed to generate nicked circular molecules were able to activate the kinase nearly as well as was the same preparation that had been completely linearized by digestion with *Eco*RI. The fact that about five times more nicked DNA than linearized DNA was required for half-maximal activation probably reflects the fact that more than half of the former preparation remained supercoiled, as judged by gel electrophoresis and ethidium bromide staining (data not shown).

To compare the abilities of different DNAs to activate the enzyme, the apparent K_a and V_{max} values for activation were determined by using highly purified enzyme from the DNA-cellulose step (Table 5). Although the range of ability to stimulate casein phosphorylation (V_{max}) varied by only a factor of 5, the relative activating affinities of each polynucleotide for DNA-PK (i.e., the concentration required for half-maximal stimulation of casein phosphorylation) varied over 2 orders of magnitude. Synthetic double-stranded oligonucleotides from 22 to 35 bp in length were better activators of DNA-PK at low concentrations than was either salmon DNA or poly(dA-dT) but only slightly better than linearized plasmid DNA or poly(dI-dC). For comparative purposes, activation affinities are expressed as nanograms per milliliter rather than molarity because DNA molecules 300 to 3,000 nucleotides in length could be expected to bind multiple molecules of enzyme, whereas it is possible that oligonucleotides of 20 to 30 bp bind only a single molecule of DNA-PK. Although an oligonucleotide with a binding sequence for the transcription factor Ap1 had the highest affinity constant for DNA-PK activation, a functional Ap1-binding site was not required for activation, because a larger

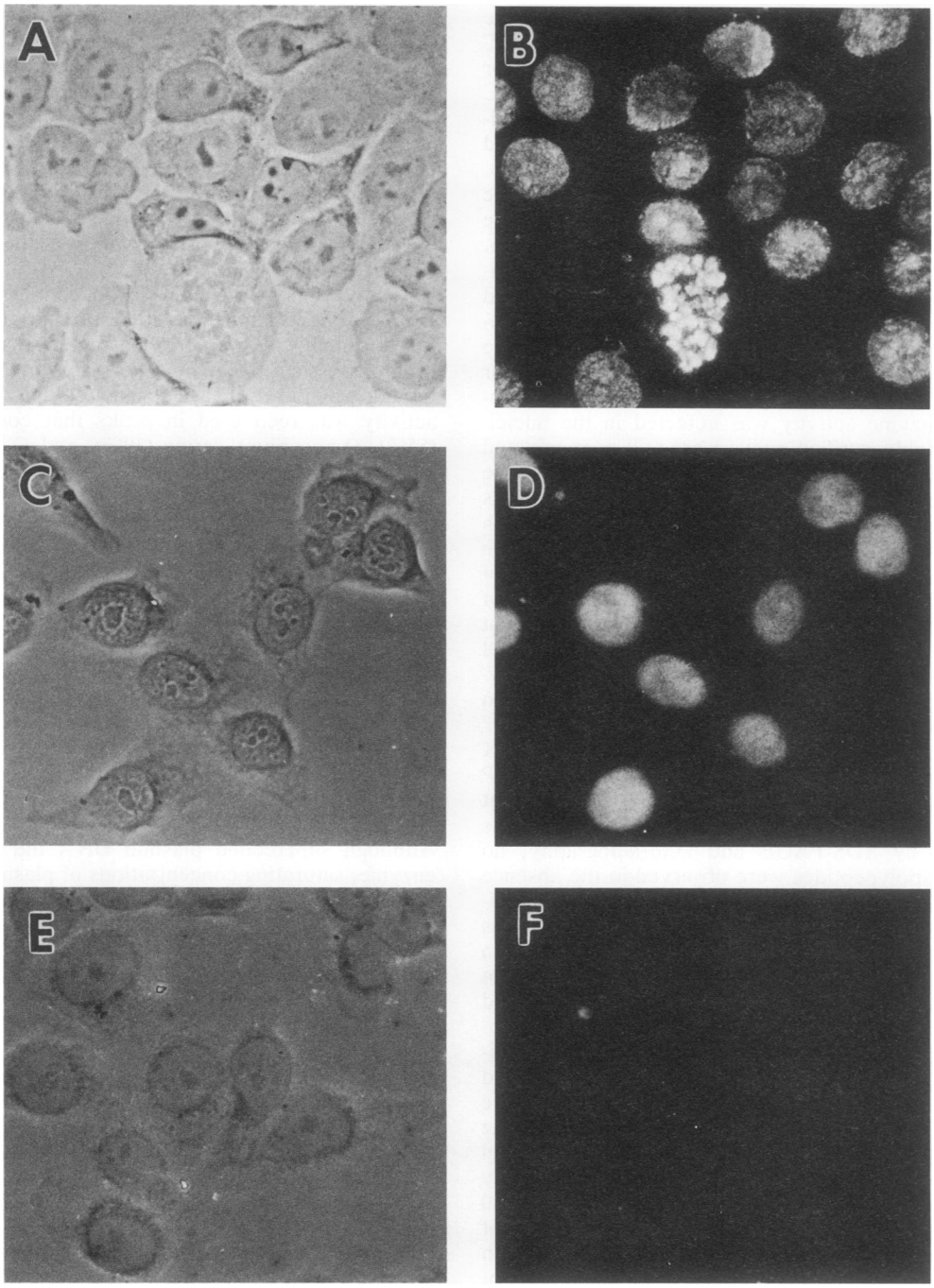

FIG. 4. Localization of DNA-PK by indirect immunofluorescence. HeLa cells growing on glass slides were fixed and stained by indirect immunofluorescence. (A, C, and E) Phase-contrast photomicrographs of the HeLa cell monolayer (A and E) and A431 cells (C). (B and D) Epifluorescence using MAb18-2 as the primary antibody; same fields as in panels A and C, respectively. (F) Epifluorescence using mixed mouse IgGs as the primary antibody.

oligonucleotide with a single base change that disrupts Ap1 binding activated DNA-PK with the same K_a and V_{max} as its cognate unaltered oligonucleotide.

Interaction with dsDNA. Considering the fact that phosphorylation of casein, a substrate not normally associated with DNA in vivo, was nevertheless stimulated specifically by dsDNA, several experiments were undertaken to test for physical interaction of the enzyme with DNA under standard reaction conditions in the absence of exogenous substrate. Attempts to observe cosedimentation of purified enzyme and DNA on glycerol gradients were frustrated by an inability to recover enzyme activity, which resulted from irreversible inactivation of DNA-PK at high glycerol concentrations. Therefore, thermal inactivation studies were done in the presence and absence of dsDNA. Exposure of DNA-PK to DNA strikingly reduced the stability of the enzyme at 50°C (Fig. 8). Loss of activity did not result from loss of the ability of heated DNA to stimulate the enzyme, because unheated

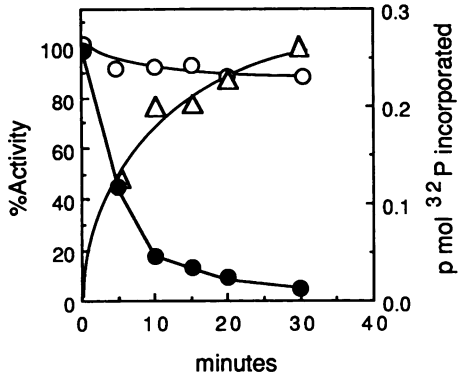

FIG. 5. Inhibition of DNA-PK activity by autophosphorylation. A 375-ng sample of enzyme from the DNA-cellulose step was incubated at 37°C under standard conditions in 50-μl reaction volumes in the presence of 50 μg of salmon DNA per ml and 7.5 mM $MgCl_2$ with (●) or without (○) 100 μM ATP. At the indicated times, duplicate samples containing 30 ng of enzyme were withdrawn from each tube and assayed for kinase activity by addition of casein to 1 mg/ml, DNA to 50 μg/ml, and [γ-^{32}P]ATP. In a third tube, enzyme was incubated exactly as described above in the presence of DNA except that [γ-^{32}P]ATP (10 μCi/nmol) was also added. At the indicated times, 10-μl samples were withdrawn, mixed with 2× sample buffer, and subjected to SDS-PAGE on a 7.5% gel. After drying and fluorography, radioactivity incorporated into the 300-kDa polypeptide was determined by cutting the bands from the gel and scintillation counting (△).

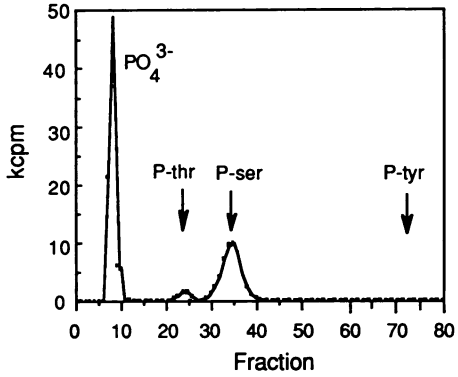

FIG. 7. Phosphoamino acid analysis. Casein was phosphorylated by using [γ-^{32}P]ATP, acid hydrolyzed, and fractionated on a Dowex 50W-X4 column, and the fractions were analyzed for radioactivity. The elution positions of *O*-phosphoamino acid standards are shown by arrows.

enzyme was fully activated by heat-treated preparations of either DNA alone or DNA that had been heated in the presence of enzyme (data not shown).

Binding of purified DNA-PK to dsDNA was also tested by its ability to shift the electrophoretic mobility of a radiolabeled oligonucleotide. 5′-End-labeled DNA was incubated with purified enzyme or nuclear extract in the presence or absence of various unlabeled oligo- and polynucleotides or monoclonal antibodies and then analyzed by 4% PAGE and autoradiography (Fig. 9A). DNA-PK retarded the electrophoretic mobility of the same Ap1-binding 35-bp oligonucleotide that was also a good activator of casein phosphorylation (Table 5), and increasing concentrations of a purified enzyme preparation gave rise to an increasing number of protein-DNA complexes, with a low-mobility complex appearing at the highest protein/DNA ratios (Fig. 9A, lanes 1 to 7). The same pattern of protein-DNA complexes was observed with a nuclear extract in place of purified DNA-PK (lane 8), and as with the purified enzyme, this pattern was seen only in the absence of nonspecific DNA competitor (data not shown). The low-mobility complex in both nuclear

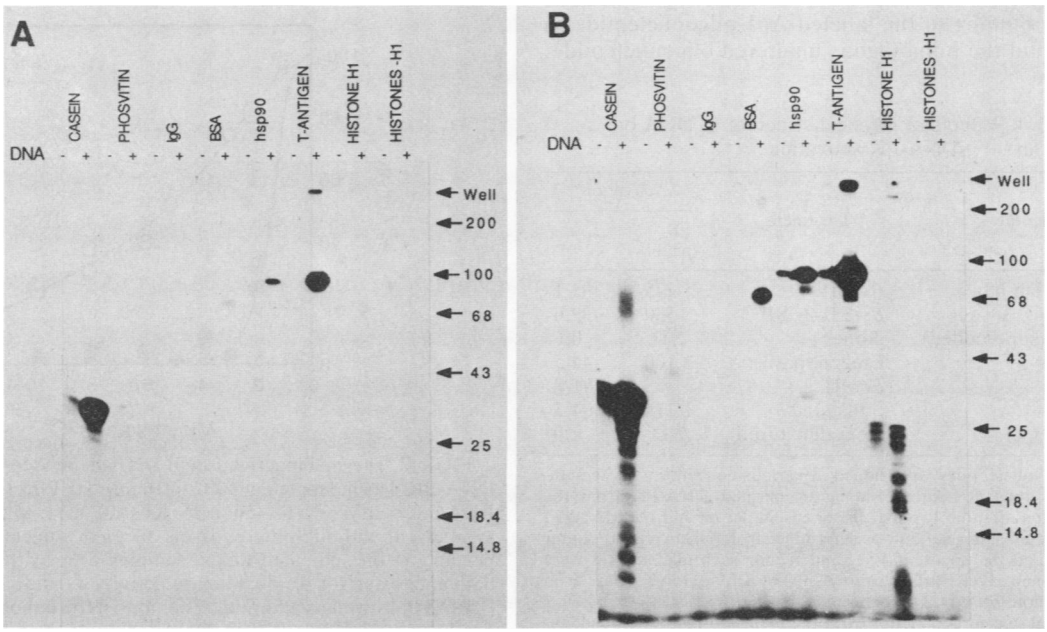

FIG. 6. Phosphorylation of purified proteins by DNA-PK. Substrate proteins were added at 250 μg/ml to a standard phosphorylation reaction with and without sonicated salmon DNA. After 10 min, reactions were stopped with electrophoresis sample buffer and separated by 12.5% SDS-PAGE. The dried gel was fluorographed. The positions of size markers were determined by Coomassie staining and are indicated in kilodaltons. Gels were exposed for 6 h (A) and 24 h (B). BSA, Bovine serum albumin.

TABLE 3. Effects of different polynucleotides on stimulation of casein phosphorylation

Polynucleotide[a]	Activity[b]	Stimulation[c]
None	2.4	1.0
Sonicated salmon DNA	70.5	29.4
Poly(dI-dC)	38.8	16.2
Poly(dA-dT)	31.2	13.0
Poly(dG) · poly(dC)	16.1	6.7
Poly(rI) · poly(rC)	1.5	0.6
Poly(rG) · poly(dC)	0.0	

[a] Present at 100 μg/ml.
[b] Expressed as nanomoles of ^{32}P transferred to α-casein per minute per milligram at 37°C. Each reaction contained 100 ng of protein from the DNA-cellulose fraction. Values are means of three experiments, with standard deviations within ±10%.
[c] Ratio of casein phosphorylation with and without added polynucleotide.

extracts and purified enzyme could be supershifted by incubation with a mixture of monoclonal antibodies specific for the 300-kDa polypeptide (lanes 9 and 15) but not by a monoclonal antibody against SV40 T antigen (lane 11) or a mixture of mouse IgGs (lane 10). The supershift was also specific for anti-300-kDa monoclonal antibodies in purified enzyme preparations, and antibodies alone did not alter the mobility of the labeled probe (data not shown). Addition of 100 μg of poly(dI-dC) per ml completely inhibited formation of the two complexes of lowest mobility and substantially reduced the amount of the third complex (lanes 12 and 14). Considering the differences in the relative K_as of different types of DNA, however, competition by lower amounts of DNA was used to determine whether some sequence specificity of DNA-PK binding could be discerned (Fig. 9B). In this experiment, using a different enzyme preparation of roughly equivalent purity to that used in Fig. 9A, only the low- and highest-mobility complexes were observed. As with activation of casein phosphorylation, binding of DNA-PK to the oligonucleotide did not require a functional Ap1-binding site, since the mutant Ap1 oligonucleotide competed for binding to the labeled Ap1 oligonucleotide as efficiently as did the homologous unaltered oligonucleotide.

TABLE 4. Effects of physical structure of DNA on DNA-PK activation

Polynucleotide	Treatment	Activation[a]	
		K_a (ng/ml)	V_{max}
Bacteriophage lambda	*Bst*EII[b]	275	92.1
	*Bst*EII + S1[c]	530	99.6
pUC18 (highly supercoiled)	None	ND	0.5[d]
pUC18 (nicked)	Freeze-thaw	2,100	44.5
	*Eco*RI	375	141.8
Salmon sperm	Sonicated	1,114	94.5
	Heat denatured	ND	0.0[d]

[a] Oligo- or polynucleotides were added at various concentrations to standard kinase reactions containing 100 ng of purified DNA-PK from the DNA-cellulose step in Table 1, plus 1 mg of casein per ml and 100 μM ATP. The rate of casein phosphorylation was determined in duplicate reactions after 10 min at 37°C, and the apparent V_{max} and K_a for each nucleic acid were determined by Lineweaver-Burk analysis. Units of activity for V_{max} are as in Table 3 (see footnote *b*). ND, Not determined.
[b] DNA was digested to completion with the indicated restriction endonuclease.
[c] DNA was digested with S1 endonuclease after the restriction endonuclease.
[d] Activity was determined from triplicate reactions containing the polynucleotide at 100 μg/ml.

TABLE 5. Effect of DNA sequence on DNA-PK activation

Oligo- or polynucleotide[a]	Size (bp)	Apparent K_a (ng/ml)[b]	Relative activation[b,c]
Ap1	35	191	1.25
Ap1M	35	199	1.12
Ap1#2	22	28	1.73
Sp1	22	70	1.31
NFκB#1	22	95	2.13
NFκB#2	24	107	2.42
pUC18 (*Eco*RI digested)	2,686	375	1.50
Poly(dA-dT)	~1,000	2,924	0.45
Poly(dI-dC)	~1,000	442	0.53
Sonicated salmon DNA	~300	1,114	1.0

[a] Synthetic double-stranded oligonucleotides were as follows. Ap1#1 is the transcription factor Ap1-binding site from the human collagenase gene enhancer, GGATGTTATAAAGCATGAGTCAGACACCTCTGGCT. The Ap1-binding sequence is underlined. Ap1M is the same sequence with ATG at the beginning of the Ap1-binding site changed to AGG. AP1#2 is the consensus Ap1-binding site (from Stratagene, Inc.), CTAGTGATGAGTCAGCCGG ATC. Sp1 is the transcription factor Sp1-binding site (Stratagene, Inc.), GATCGATCGGGGCGGGGCGATC. NFκB#1 is the transcription factor NFκB-binding site from the mouse immunoglobulin light-chain enhancer, CAGAGGGGACTTTCCGAGAGGC. NFκB#2 is the NFκB-binding site from the human interleukin-2 enhancer, CAAAGAGGGATTTCACCTCAC-TCC.
[b] Determined by Lineweaver-Burk analysis as described in Table 4, footnote *a*.
[c] V_{max} relative to the V_{max} for salmon DNA, which was 94.5 nmol of ^{32}P transferred to casein per min per mg. The activity without added DNA was 3.4 nmol/min/mg.

However, two G+C-rich oligonucleotides containing binding sites for the transcription factors NFκB (34) and H2TF1 (3) competed about 10-fold less well than did the two Ap1-related oligonucleotides. In these experiments, the two complexes of different mobilities were each competed for with equivalent kinetics by the unlabeled oligonucleotides. Although salmon DNA and poly(dI-dC) were approximately as good competitors of the high-mobility complex as were

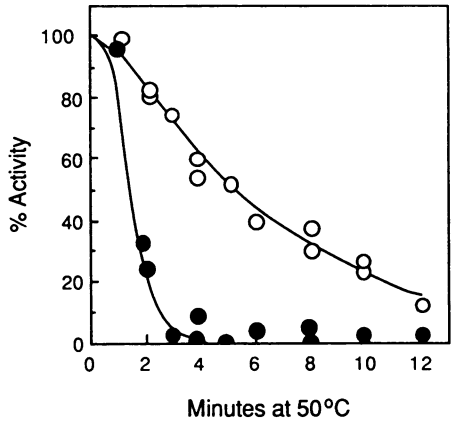

FIG. 8. Thermal inactivation of DNA-dependent kinase activity. DNA-PK was incubated at 50°C in 16 mM HEPES (pH 7.9)–0.8 mM EDTA–0.8 mM EGTA–50 mM KCl–10 mM $MgCl_2$ with (filled symbols) or without (open symbols) 67 μg of sonicated salmon DNA per ml. At the indicated times, samples were withdrawn to an ice bath and tested for residual kinase activity by addition of casein and [^{32}P]ATP, as well as addition of salmon DNA to those tubes that had not been preincubated with DNA. Activity was measured under standard conditions at a final DNA concentration of 50 μg/ml. Each datum point is the average of duplicate reactions; results of two separate experiments are shown. Results are expressed as percentage of activity of unheated samples.

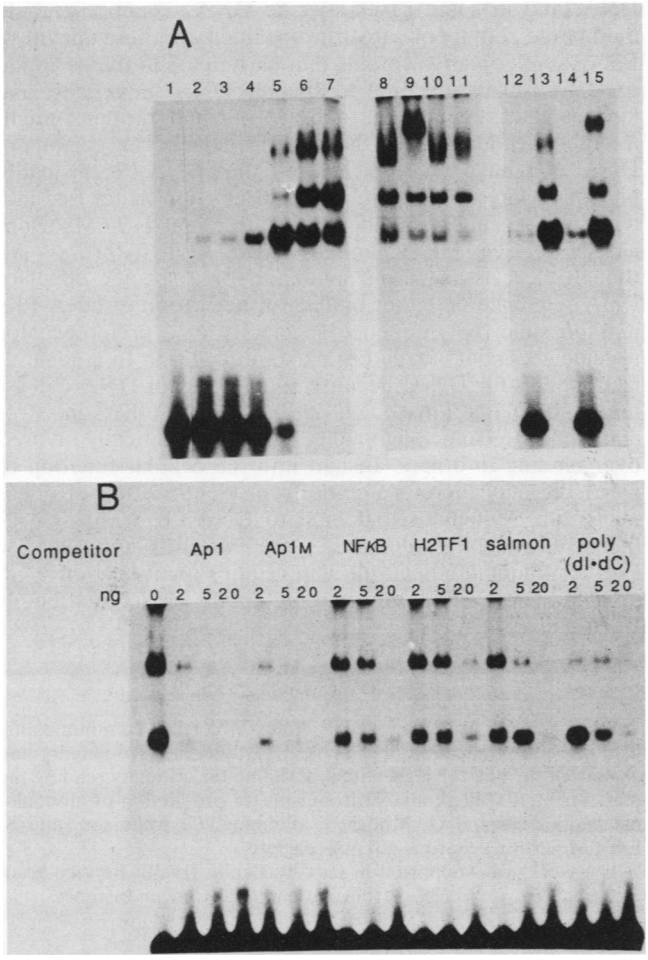

FIG. 9. Effect of DNA-PK on electrophoretic mobility of DNA. A 0.5-ng sample of ^{32}P-labeled Ap1 oligonucleotide (Ap1#1; see footnote *a* of Table 5) was incubated with purified enzyme or nuclear extract as indicated, and the complexes were resolved by electrophoresis as described in Materials and Methods. (A) In lanes 1 to 7, 0, 0.5, 1.0, 2.5, 10, 20, and 30 ng, respectively, of DNA-PK protein from the DNA-cellulose step were included in the reactions, incubated at 60 mM NaCl. Other lanes: 8 to 11, 1.7 μg of nuclear extract plus no addition (lane 8), 1 μl of a mixture of MAb18-2, -42-26, and -25-4 (0.33 μg each) in buffer B plus 0.1 M KCl (lane 9), 1 μl (1 μg) of MAbM37 purified control antibody against SV40 T antigen (from E. Harlow) (lane 10), and 1 μl of mixed mouse immunoglobulin G (lane 11); 12 and 13, 30 ng of purified enzyme without antibodies and with (lane 12) or without (lane 13) 100 μg of poly(dI-dC) per ml; 14 and 15, 30 ng of purified enzyme with 1 μl of the same monoclonal antibody mixture as in lane 9, with (lane 14) or without (lane 15) poly(dI-dC), incubated at 120 mM NaCl. (B) A 30-ng sample of purified DNA-PK was incubated with labeled DNA at 150 mM NaCl in the presence of the indicated amounts of unlabeled competitor oligo- or polynucleotides (see footnote *a* of Table 5 for a description of the oligonucleotides).

the G+C-rich oligonucleotides, poly(dI-dC) was almost as efficient a competitor for the low-mobility complex as was the Ap1 oligonucleotide itself.

DISCUSSION

DNA-PK appears to be a novel type of protein kinase. If the 300-kDa polypeptide is indeed the enzyme, its combination of large size and monomeric structure would be unique among serine-threonine protein kinases: NI was reported to exist as a monomer or homodimer (40) of 25-kDa subunits, and the mouse spleen enzyme (28) was reported to exist as a monomer with molecular mass of 45 kDa, whereas NII is a multisubunit enzyme composed of polypeptides in the range of 20 to 60 kDa (9, 31, 38, 46). Several receptors for polypeptide growth hormones possess protein kinase activity and have molecular masses approaching 300 kDa (44), but all are tyrosine specific, and none has been found in association with cell nuclei. We cannot at present rule out the possibility that DNA-PK is another protein that strongly associates with the 300-kDa polypeptide and requires this association for its stability in vitro but is present in such small amounts that it is not easily detected by silver staining. If this were the case, its specific activity would have to be at least an order of magnitude greater than that reported for typical preparations of, for example, the NII casein kinase. It would also be necessary to postulate that MAb18-2 interfered with this putative enzyme's association with the 300-kDa polypeptide. Thus, the simplest explanation of our data is that the 300-kDa polypeptide and DNA-PK are one and the same.

Two published reports describe protein kinase activities that have similarities to DNA-PK. An activity that copurified with a polypeptide of approximately 300 kDa was partially purified by Friedrich and Ingram (10), although no effect of DNA was reported, and a DNA-stimulated protein kinase activity has recently been found to phosphorylate unique sites on hsp90 (18). The partial purification and characterization of this latter enzyme are described in the accompanying paper (19). Direct biochemical comparison of this activity to DNA-PK, including the phosphorylation sites on hsp90 and partial peptide mapping of the autophosphorylated 300-kDa polypeptide from each preparation, suggest that these two enzymes are either identical or very closely related (T. H. Carter, S. P. Lees-Miller, and C. W. Anderson, J. Cell Biol. **109**:217a, 1989).

The requirement for dsDNA is the most unusual characteristic of DNA-PK. Highly purified enzyme was stimulated as much as 60-fold by exogenous DNA for phosphorylation of α-casein (Table 5). Earlier studies from our laboratory and others also demonstrated that certain polypeptides in cell extracts were phosphorylated only in the presence of dsDNA (4, 41). The dependence of DNA-PK on dsDNA for phosphorylation of casein and other substrates distinguishes this enzyme from the mouse spleen kinase described by Ohtsuki et al. (26–28).

The physical form of the DNA appears to be important for stimulation of casein phosphorylation. The inability of most supercoiled DNA preparations to activate DNA-PK efficiently was consistent both in purified enzyme preparations (Table 4) and in crude cell extracts (4). The possible importance of the structure of free ends of dsDNA for DNA-PK activation was assessed by using several different restriction endonucleases, as well as sonication, to generate DNA fragments, and the resultant DNA preparations were also treated with a single-strand-specific nuclease to generate flush-ended DNA molecules. None of these treatments substantially changed the ability of each DNA preparation to activate the enzyme as long as the DNA was not supercoiled, indicating that neither short terminal regions of single-stranded DNA nor specific terminal nucleotide sequences are required for activation. Thus, if DNA-PK is activated by binding to free ends of dsDNA, this interaction must be tolerant of a wide range of molecular structures. The fact that a plasmid preparation containing an increased

amount of nicked, closed circular DNA as a result of freezing and thawing was found to activate the enzyme (Table 4) is consistent with the requirement of DNA-PK for nonsupercoiled dsDNA, as opposed to linear DNA molecules containing free ends.

The increased rate of thermal inactivation of DNA-PK in the presence of DNA shows direct protein-nucleic acid interaction, as does the ability of purified DNA-PK to retard the electrophoretic mobility of an oligonucleotide. It is possible that enhanced thermal inactivation resulted from partial denaturation of the DNA double helix in the vicinity of DNA-PK binding, because denatured DNA can inhibit DNA-dependent casein phosphorylation of DNA-PK under certain conditions (4; Table 4), but a direct helicase assay failed to show such activity for the purified enzyme (I. Vančurová and T. H. Carter, unpublished). A comparison of the kinetic data for activation of DNA-PK in Table 5 with the gel shift competition data in Fig. 9B suggests that DNA activates the kinase activity of DNA-PK by binding to the enzyme rather than to its substrates. The K_a for kinase activation by the Ap1 and NFκB oligonucleotides was between 5 and 10 times lower than the K_a for salmon DNA but only 2 to 4 times lower than the K_a for poly(dI-dC). The fact that competition in the gel retardation assay reflected approximately the same relative range of affinities argues for direct activation of DNA-PK by binding to dsDNA.

The formation of several species of protein-DNA complex with different electrophoretic mobilities, at least two of which were competed for with similar kinetics by oligonucleotides and sheared salmon DNA, is consistent either with more than one DNA-binding site on each DNA-PK molecule or the binding of more than one enzyme molecule to each oligonucleotide. Either of these models would in turn be consistent with the observation that the lowest-mobility complex was present only at high protein/DNA ratios. However, the inability of monoclonal antibodies against DNA-PK to alter the mobility of the two fastest-running complexes and the fact that Western blots of native gels show that the bulk of the 300-kDa polypeptide migrates at the position of the low-mobility complex in the presence or absence of DNA (data not shown) suggest that other explanations are more likely. One is that the enzyme preparation, which had been purified over a DNA-cellulose column, contained other, unrelated DNA-binding proteins that gave rise to the two high-mobility complexes. Alternatively, it is possible that the additional DNA-binding proteins which copurify with DNA-PK are proteolytic fragments of the enzyme that retain DNA-binding ability but have lost the epitopes recognized by the monoclonal antibodies. Further studies using more highly purified enzyme, and use of additional monoclonal antibodies in supershift experiments, may resolve this question.

Although binding of DNA-PK to the Ap1 oligonucleotide was more efficiently competed for by the homologous unlabeled oligonucleotide than by either of two G+C-rich oligonucleotides or by sonicated salmon DNA, the fact that a 40-fold excess of all competitors substantially reduced the binding of DNA-PK to the labeled oligonucleotide demonstrates a generalized affinity of DNA-PK for DNA, regardless of sequence. Whether this general affinity for DNA is reflected in the biological activity of DNA-PK or is an artifact of in vitro assay conditions remains to be determined. The biological function of DNA-PK is not known. However, the in vivo substrates for nuclear protein kinases are likely to include chromatin or regulatory and structural proteins associated with it. It is therefore reasonable that DNA, and in some cases specific DNA sequences, could modify the activity or substrate specificity of these enzymes. For example, many proteins that participate in transcription or DNA replication bind to specific DNA sequences, and kinases that regulate the activity of such proteins might interact specifically with the same or neighboring regions of DNA. We have recently learned that DNA-PK probably phosphorylates the generalized transcription factor Sp1 in a template-dependent manner (S. P. Jackson, J. J. MacDonald, S. P. Lees-Miller, and R. Tjian, Cell, in press). Although there is as yet no evidence for a specific effect of DNA sequence on either binding or activation of DNA-PK, it is possible that sequence specificity or a preference for specific secondary structure exists in addition to a generalized affinity for DNA, because some types of DNA consistently stimulated kinase activity better than others in vitro, and binding to some oligonucleotides was kinetically favored over binding to others. Insight into the cellular function of DNA-PK may therefore come from identification of oligonucleotide sequences that bind to DNA-PK with high affinity, and these experiments are currently in progress. Further understanding will also require identification of in vivo substrates and information about the regulation of DNA-PK activity.

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