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Adenovirus Type 2 Coded Single-Stranded DNA Binding Protein: In Vivo Phosphorylation and Modification

YUN-HUA JENG,* WILLIAM S. M. WOLD, KENJI SUGAWARA,[1] ZVEE GILEAD, AND MAURICE GREEN

Institute for Molecular Virology, Saint Louis University School of Medicine, St. Louis, Missouri 63110

Received for publication 30 November 1976

The adenovirus type 2-coded single-stranded DNA binding protein (DBP) was shown to be a phosphoprotein and to exist in at least two forms that differ in mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After a 30-min pulse with [^{35}S]methionine or $^{32}PO_4$, ^{35}S- or ^{32}P-labeled DBP had a nominal molecular weight of 74,000, whereas after a 30-min label followed by a 20-h chase, ^{35}S- and ^{32}P-labeled DBP had a nominal molecular weight of 77,000. Both large and small forms of ^{35}S- and ^{32}P-labeled DBP bound to single-stranded DNA-cellulose columns and were eluted by 0.4 to 0.6 M NaCl; both forms also were immunoprecipitated by antiserum against adenovirus type 1-simian virus 40-induced tumor cells (this antiserum contains antibodies against DBP) and by monospecific antiserum against 95 to 99% purified DBP. With highly purified ^{32}P-DBP labeled 7 to 10 h postinfection, it was shown that the ^{32}P radioactivity was firmly associated with protein material (i.e., not contaminating nucleic acids or phospholipids) and had properties expected of a phosphoester of an amino acid; paper electrophoresis of acid hydrolysates of this preparation identified phosphoserine but not phosphothreonine. Phosphoserine but not phosphothreonine was also identified in acid hydrolysates of another preparation of ^{32}P-DBP labeled for 30 min, chased for 20 h, and then immunoprecipitated by adenovirus type 1-simian virus 40 antiserum.

Human adenovirus 2 (Ad2) is an oncogenic DNA virus that replicates in the nucleus of permissive human cells (reviewed in reference 18 and in W. S. M. Wold, M. Green, and W. Büttner, *In* D. P. Nayak, ed., *Molecular Biology of Animal Viruses*, in press). The productive infection proceeds in an "early" stage, when 23 to 32% of the asymmetric genome is expressed as mRNA, and in a "late" stage, which follows the initiation of viral DNA replication at 6 to 7 h postinfection (p.i.). Virus-coded or -induced proteins synthesized early after infection probably are involved in viral DNA replication, regulation of viral gene expression, and transformation of nonpermissive cells. A protein of 75,000 (75K) daltons, which binds to single-stranded DNA but not to native DNA, is produced in large quantities in early stages of Ad2 infection (24, 27). An analogous Ad5 DNA binding protein (DBP) of 72K was isolated from abortively infected monkey cells (28). Recently an Ad12 DBP of 58K to 60K has been described (29). Ad2 and Ad5 DBPs of 41K to 45K are also synthesized, but peptide analyses (20), genetic studies (29), and immunoprecipitation studies (27a) indicate that these are derived from the larger DBP (possibly degradation products). Ad2 DBP has been shown to be viral coded by cell-free translation of hybridization-purified Ad2 early mRNA into a 72K polypeptide with a peptide map similar to that of DBP isolated from infected cells, and the DBP gene has been mapped in Ad2 *Eco*RI-B fragment (Ad2 map position, 0.59 to 0.71) (16). Recently, Ad2 DBP has been purified to 95 to 99% homogeneity; sucrose gradient and gel filtration studies indicate that it does not have a typical globular protein structure (27).

DBP is probably involved in viral DNA replication, since it is found in both nuclear membrane and soluble Ad2 DNA replication complexes (24, 33, 34; M. Arens, T. Yamashita, R. Padmanabhan, T. Tsuruo, and M. Green, manuscript in preparation). Genetic studies indicate that DBP functions in the initiation of viral DNA replication (30). DBP is synthesized in large quantities, which suggests that its role is stoichiometric rather than catalytic (28), as in the case of T4 gene 32 protein (1). Many Ad2-

[1] Present address: Department of Microbiology, Faculty of Medicine, Kyoto University, Konoe-cho, Yoshida, Sakyo-hu, Kyoto, Japan 606.

transformed cells neither contain the DBP gene (23) nor synthesize DBP (15, 27a; P. H. Gallimore, personal communication), indicating that DBP does not function to maintain cell transformation.

Many cellular processes are controlled by modifications, such as adenylation (10), methylation (4), and phosphorylation (22), of key regulatory proteins. We, therefore, investigated whether Ad2 DBP undergoes posttranslational modification. In this communication, we demonstrate that Ad2 DBP is phosphorylated at serine residues, and that at least two forms of DBP exist that differ in mobility by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Chemicals. [^{35}S]methionine (Met) (400 Ci/mmol), [^{3}H]leucine (40 Ci/mmol), and inorganic $^{32}PO_4$ (carrier-free) were obtained from New England Nuclear Corp.; 1-β-D-arabinofuranosylcytosine (ara-C), phosphoserine, phosphothreonine, pancreatic DNase (EC 3.1.4.5), and Pronase were secured from Sigma Chemical Co.; acrylamide came from Eastman Kodak Co.; *N,N*-methylenebisacrylamide, *N,N,N',N'*-tetramethyladenediamine (TEMED), and ammonium persulfate were procured from Bio-Rad Laboratories; SDS was bought from BDH Chemical, Ltd.; pancreatic RNase A (EC 2.7.7.16) and *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) were both obtained from Worthington Biochemical Corp.; and pancreatic RNase T_1 was acquired from Calbiochem.

Preparation of labeled protein extracts. Human KB cells (clonal line) were grown at 37°C in Eagle minimal essential medium (MEM) containing 5% horse serum. Cells were infected at 6×10^6 cells/ml with Ad2 (strain 38-2) at 100 PFU/cell in MEM without horse serum. The purification of Ad2 has been described (9). After 1 h of adsorption, cells were suspended at 3.5×10^5 cells/ml in Met-free or phosphate-free MEM with 5% horse serum. At 4 h p.i., ara-C was added to a concentration of 20 μg/ml. At 0.5 h prior to labeling, cells were centrifuged and resuspended at 6.5×10^5 cells/ml in warm Met-free or phosphate-free MEM with 5% horse serum and 20 μg of ara-C per ml. Cells were pulse-labeled from 7 to 7.5 h p.i. with 25 μCi of [^{35}S]Met or 0.5 mCi of $^{32}PO_4$ per ml. Chased samples were prepared by washing cells twice in warm, complete MEM containing 5% horse serum and 20 μg of ara-C per ml, at the end of the labeling period, and then incubating cells in fresh wash medium for 20 h. Harvested cell pellets were washed twice with cold phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}. Cells were then suspended at 10^7 cells/ml in reticulocyte standard buffer (10 mM Tris-hydrochloride[pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$), placed in an ice bath for 15 min, and sonically disrupted. Cell extracts were digested with 25 μg of DNase per ml at 37°C for 1 h in 5 mM $MgCl_2$. EDTA was added to 10 mM, and extracts were further digested with 20 μg each of RNase A and T_1 per ml at 37°C for 30 min. Extracts were then dialyzed for 16 h at 4°C against 10 mM Tris-hydrochloride (pH 7.5) and 1 mM 2-mercaptoethanol, and the samples were either analyzed directly or stored at −70°C. Mock-infected protein extracts were similarly prepared, except that virus was not added.

Analytical and preparative SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel experiments were prepared as described (11, 14, 26). Gradient gels of 8 to 21% polyacrylamide with 0.1% SDS were formed as slabs between glass plates; slabs were 1.5 mm thick and 100 or 270 mm long. A stacking gel of 5% polyacrylamide and 0.13% bisacrylamide (analytical gels contained up to 20 sample wells) was cast above the separating gel. Electrophoresis was performed at 7°C with a constant current of 20 mA. After electrophoresis, analytical gels were dried under vacuum and autoradiographed with Kodak single-coated panoramic dental X-ray film (DF-85). Apparent molecular weights of proteins were estimated by comparison of their migration rate in SDS-polyacrylamide gels with known molecular weight standards. Cylindrical gels (0.6 by 10 cm) containing 6% polyacrylamide were electrophoresed at 2 mA/gel for 3 h. Gels were fractionated into 2-mm slices with a Gilson gel fractionator and counted in 5 ml of Aquasol in a liquid scintillation counter.

To localize protein bands in preparative gels, a lightly loaded analytical gel with labeled protein markers was run in parallel with each batch of preparative gels. The gels were kept wet during autoradiography, and the localized phosphoprotein bands were cut out and eluted from gels with agitation at 37°C in 10 volumes of 5 mM $NaHCO_3$ containing 0.05% SDS. After 16 h, acrylamide fragments were removed by centrifugation, and the supernatant was dried. A small portion of protein from preparative gels was checked for purity by analytical SDS-polyacrylamide gel electrophoresis.

Immunoprecipitation of DBP from crude extracts and DNA-cellulose purified materials. Indirect radioimmunoprecipitation was carried out as described (7, 8) with minor modifications. Immunoglobulin G (IgG) was isolated from an antiserum (a gift from R. Gilden) against extracts of Ad1-simian virus 40 (SV40)-induced hamster tumor cells (6); this antiserum contains antibodies to DBP (7, 8). Samples from infected and mock-infected extracts were mixed with 20 μg of Ad1-SV40 IgG or nonimmune IgG in 240-μl reaction mixtures and incubated for 18 h at 4°C. Then 45 μl of goat serum anti-hamster IgG were added and incubation continued for 2 h at 37°C. Immunoprecipitates were collected by centrifugation for 1.5 min at 10,000 × *g* in a Beckman 152 microfuge, washed three times with 150 μl of phosphate-buffered saline containing 0.5 M urea, 0.5% Nonidet P-40, and 1% deoxycholate, suspended in an equal volume of gel electrophoresis sample buffer (11), and electrophoresed on SDS-polyacrylamide gels.

Some immunoprecipitations were conducted with guinea pig IgG against 95 to 99% purified DBP (anti-DBP) (27a); these utilized a modified radioimmunoprecipitation buffer (10 mM sodium phosphate [pH

7.2], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) for both incubation and washing (5). Portions of DBP-containing eluates from DNA-cellulose were adjusted to 1× radioimmunoprecipitation buffer and mixed with 60 μg of anti-DBP guinea pig IgG or nonimmune guinea pig IgG; the reactions were then incubated for 18 h at 4°C. Then 60 μl of goat serum anti-guinea pig IgG were added to form the immunoprecipitates.

Purification of ^{32}P-labeled DBP. A detailed description of the DBP purification method is presented elsewhere (27). Briefly, infected cells were simultaneously labeled with 2 μCi of [^{3}H]leucine and 0.5 mCi of $^{32}PO_4$ per ml from 7 to 10 h p.i. in the presence of ara-C. Cytoplasmic extracts were prepared and chromatographed on a single-stranded DNA-cellulose column (24). Bound proteins were released from the column by stepwise elution with buffer A (20 mM Tris-hydrochloride [pH 8.1], 50 mM NaCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, and 10% glycerol) containing 0.05 M, 0.2 M, 0.4 M, 0.6 M, and 2.0 M NaCl, respectively. Fractions eluted with 0.6 M NaCl were pooled, treated with 1% sodium deoxycholate for 1 h at 4°C, and dialyzed against buffer B (10 mM Tris-hydrochloride [pH 7.5], 5 mM NaCl, 1 mM 2-mercaptoethanol, 0.02% Nonidet P-40, 0.4 M urea, and 10% glycerol). The dialyzed material was then applied to a DEAE-Sephadex column equilibrated with buffer B, and proteins were eluted with a linear gradient of buffer B and 0.3 M NaCl in buffer B. The major eluted radioactive peak containing DBP was pooled and analyzed by SDS-polyacrylamide gel electrophoresis.

Identification of phosphorylated amino acids. ^{32}P-labeled DBP purified by DNA-cellulose and DEAE-Sephadex chromatography was further purified by electrophoresis in preparative SDS-polyacrylamide gels as described above. The ^{32}P-labeled DBP band was eluted from the gel and dried, and the dried residue was hydrolyzed in 1 ml of 6 N HCl at 110°C for 2 h. A 2-h period was selected as the hydrolysis time to maximize hydrolysis of the peptide bond while limiting hydrolysis of the phosphorylated amino acid. Under the conditions of partial acid hydrolysis used, about 20% of the liberated phosphoserine would be hydrolyzed (12). The hydrolysate was dried and dissolved in 16 μl of water containing 40 μg of unlabeled phosphoserine and phosphothreonine each and subjected to paper electrophoresis (2). Electrophoresis was carried out on Whatman no. 1 filter paper with a formic acid-acetic acid-water (30:90:880) mixture (pH 1.9) at 600 V for 16 h at 4°C. The paper was dried, and unlabeled phosphoamino acid markers developed with ninhydrin reagent (Sigma). The position of radioactivity was determined with a Packard radiochromatogram scanner. Rechromatography was performed by eluting the radioactive spots from the paper chromatogram with water and subjecting the dried material to paper electrophoresis as described above.

RESULTS

Pulse-chase experiments showing that ^{35}S- and ^{32}P-labeled DBP decrease in mobility in SDS-polyacrylamide gel electrophoresis during chase periods. Ad2-infected and mock-infected KB cells were labeled with [^{35}S]Met or $^{32}PO_4$ for 30 min, or for 30 min followed by a 20-h chase. To prevent the transition to late stages of infection, ara-C was added 4 h p.i., and cells were labeled in the presence of ara-C. Whole cell extracts were prepared, and polypeptides were resolved by SDS-polyacrylamide gradient slab gel electrophoresis. Figure 1 shows an autoradiogram of one such experiment. After a 30-min pulse, ^{35}S-labeled DBP migrated with an apparent molecular weight of 74K (lane A), but after a 20-h chase the apparent molecular weight of DBP had increased to 77K (lane C). The ^{35}S-labeled DBP band was not present in lanes representing pulse (lane B)- or pulse-chase (lane D)-labeled, mock-infected cell extracts. Analyses of proteins labeled with $^{32}PO_4$ indicated that DBP, and no other Ad2-induced polypeptide, was associated with the phosphate label (in Fig. 1 cf. lane E with F, and lane G with H). The ^{32}P-labeled DBP behaved like ^{35}S-labeled DBP in pulse-chase experiments: ^{32}P-labeled DBP had a nominal molecular weight of 74K after a 30-min pulse (lane E) and 77K after a 20-h chase (lane G). In Fig. 1, there are no apparent virus-specific ^{32}P-labeled polypeptides of 41K to 45K daltons, i.e., representing the DBP subspecies of these apparent molecular weights (20). Apparently the ^{32}P-labeled DBP subspecies were present in insufficient amounts to be observed in the autoradiogram, because in other experiments these polypeptides were shown to be phosphorylated (data not shown).

Immunoprecipitation by Ad1-SV40 IgG of pulse-labeled and pulse-chase-labeled ^{35}S- and ^{32}P-DBP. Antisera against cell extracts of Ad1-SV40- and Ad2-SV40-induced hamster tumor cells contain antibodies to Ad2 DBP (7, 8). As shown in Fig. 2, IgG from Ad1-SV40 antiserum precipitated ^{35}S-labeled DBP from cells labeled for 30 min (lane F), or for 30 min followed by a 20-h chase (lane J). The immunoprecipitated DBPs showed the same apparent molecular weight change as untreated samples (lanes A and C, pulse-labeled and pulse-chase-labeled infected cells); i.e., the chased DBP had a slightly reduced mobility in the gels. In control experiments, IgG from nonimmune hamsters did not immunoprecipitate ^{35}S-DBP from pulse-labeled (lane E) or pulse-chase-labeled (lane I) infected cell extracts; also, no ^{35}S-labeled polypeptides were immunoprecipitated from mock-infected cell extracts by Ad1-SV40 IgG (pulse – lane H, chase – lane L) or normal hamster IgG

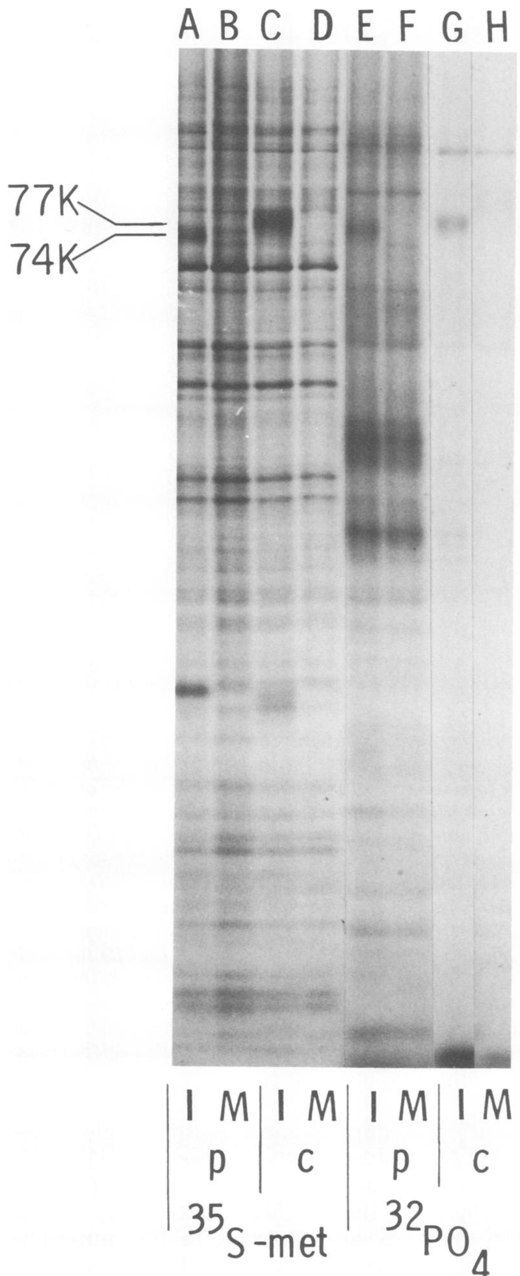

FIG. 1. *Autoradiogram of SDS-polyacrylamide gel electropherogram showing two electrophoretic forms (an apparent molecular weight of 74K, after a 30-min pulse, and 77K, after a 30-min pulse followed by a 20-h chase) of ^{35}S- and ^{32}P-labeled DBP. Ad2-infected (I) and mock-infected (M) KB cells were pulse-labeled with [^{35}S]Met or $^{32}PO_4$ 7 to 7.5 h p.i. (p), or 7 to 7.5 h p.i. followed by a 20-h chase (c) in the presence of ara-C. Total cell protein extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. (A,B) ^{35}S pulse; (C,D) ^{35}S pulse-chase; (E,F) ^{32}P pulse; (G,H) ^{32}P pulse-chase.*

(pulse—lane G, chase—lane K). Ad1-SV40 IgG also precipitated ^{32}P-DBP from both pulse (lane R)- and pulse-chase-labeled (lane V) infected cell extracts. Again, the chased ^{32}P-labeled DBP had a slightly reduced mobility. As with ^{35}S-labeled DBPs, the ^{32}P-labeled DBPs immunoprecipitated from pulsed and pulse-chased infected cell extracts had the same apparent molecular weight as ^{32}P-labeled DBP in samples not immunoprecipitated (lanes M and O). Control immunoprecipitation experiments performed with ^{32}P-labeled infected and mock-infected cell extracts revealed no detectable ^{32}P-labeled DBP (lanes Q, S, T, U, W, and X). Thus DBP-specific antisera immunoprecipitated 74K and 77K forms of both ^{35}S- and ^{32}P-labeled DBP. High-titer monospecific guinea pig antiserum against 95 to 99% purified DBP also precipitated ^{35}S- and ^{32}P-labeled DBP (data not shown).

Pulse-labeled and pulse-chase-labeled ^{32}S- and ^{32}P-DBPs that bind to single-stranded DNA-cellulose. Ad2 DBP binds to single-stranded DNA and can be eluted from single-stranded DNA-cellulose with 0.6 M NaCl (24). To test whether both 74K and 77K forms of ^{35}S- and ^{32}P-labeled DBP have a similar affinity for DNA, infected and mock-infected KB cells were labeled with [^{35}S]Met or $^{32}PO_4$ for 30 min (or for 30 min followed by a 20-h chase), and cytoplasmic extracts were chromatographed on single-stranded DNA-cellulose columns. About 90 to 95% of the labeled proteins passed through with the void volume in 50 mM NaCl. The column was washed with buffers of increasing ionic strength (see above). Proteins eluted with 0.6 M NaCl were electrophoresed on SDS-polyacrylamide gels. As shown in Fig. 3, pulse-labeled ^{35}S-DBP (lane A) and ^{32}P-DBP (lane E), as well as pulse-chase-labeled ^{35}S-DBP (lane C) and ^{32}P-DBP (lane G), bound to DNA-cellulose and were eluted by 0.6 M NaCl. Both pulse-chased ^{35}S- and ^{32}P-labeled DBPs had lower mobility on the gel. Thus both 74K and 77K forms of ^{35}S- and ^{32}P-labeled DBPs bind to single-stranded DNA and are eluted from the column at the same NaCl concentration.

Purification of ^{32}P-labeled DBP by DNA-cellulose and DEAE-Sephadex chromatography and preparative SDS-polyacrylamide gel electrophoresis. The experiments described above demonstrate that ^{32}P label is apparently firmly associated with DBP (i.e., not removed by SDS and by chromatography on DNA-cellulose). For further studies on the phosphorylated component, ^{32}P-labeled DBP was purified by sequential DNA-cellulose and DEAE-Sephadex chromatography (which yields DBP of 95 to 99% purity) (27), followed by preparative SDS-gel

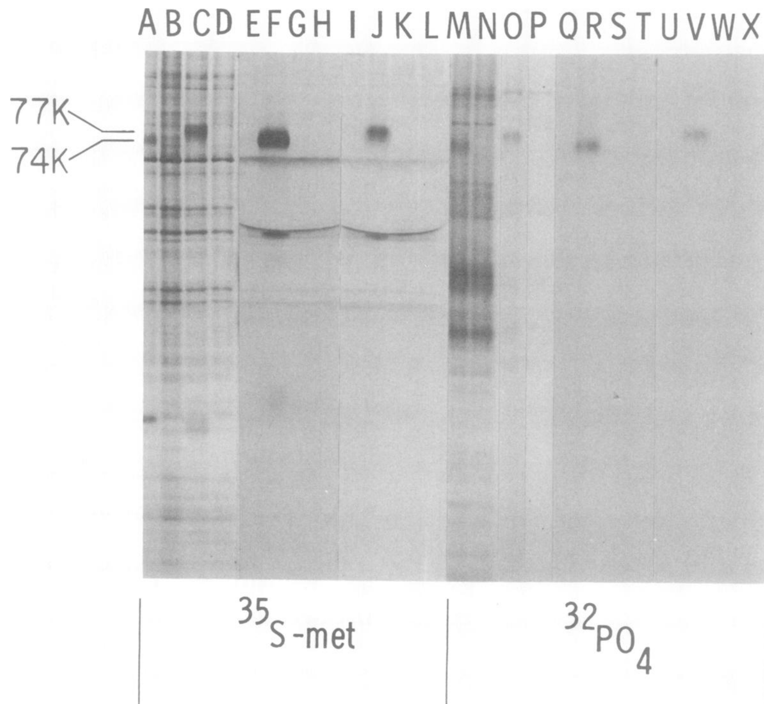

FIG. 2. *Autoradiogram of SDS-polyacrylamide gel electropherogram showing that Ad1-SV40 IgG immunoprecipitates both 74K (seen after 30-min pulse) and 77K (seen after 30-min pulse followed by 20-h chase) forms of ^{35}S- and ^{32}P-labeled DBP. Ad2-infected and mock-infected cells were labeled with [^{35}S]Met or $^{32}PO_4$ (from 7 to 7.5 h p.i., or 7 to 7.5 h followed by a 20-h chase), total cell extracts were prepared, and immunoprecipitation experiments were performed with Ad1-SV40 and nonimmune hamster IgGs. A through D and M through P, respectively, show ^{35}S- and ^{32}P-labeled extracts electrophoresed before immunoprecipitation: pulse-labeled extracts are in A (Ad2 infected), B (mock infected), M (Ad2 infected), and N (mock infected); pulse-chase-labeled extracts are in C (Ad2 infected), D (mock infected), O (Ad2 infected), and P (mock infected). E through L (^{35}S-labeled extracts) and Q through X (^{32}P-labeled extracts) show the results of the immunoprecipitation experiments. (E and Q) Infected pulse-labeled cells versus nonimmune hamster IgG; (F and R) infected pulse-labeled cells versus Ad1-SV40 IgG; (G and S) mock-infected, pulse-labeled cells versus nonimmune IgG; (H and T) mock-infected, pulse-labeled cells versus Ad1-SV40 IgG; (I and U) infected chase-labeled cells versus nonimmune IgG; (J and V) infected chase-labeled cells versus Ad1-SV40 IgG; (K and W) mock-infected, chase-labeled cells versus nonimmune IgG; and (L and X) mock-infected, chase-labeled cells versus Ad1-SV40 IgG.*

electrophoresis. Infected cell proteins were labeled lightly with [^{3}H]leucine and copurified with ^{32}P-labeled proteins. Each stage of purification was monitored by gel electrophoresis. A labeling time of 7 to 10 h p.i. was chosen to obtain maximal synthesis of DBP, as indicated by preliminary experiments. The cytoplasmic extract was adsorbed to a single-stranded DNA-cellulose column, and bound proteins were eluted with buffers of increasing ionic strength. ^{32}P-labeled polypeptides eluted with each NaCl concentration were analyzed by gel electrophoresis, and the results are shown in Fig. 4. Lanes A and B, respectively, show marker ^{35}S-labeled polypeptides from infected and mock-infected cell extracts; these extracts were prepared by the "cycloheximide enhancement" procedure to increase the levels of labeled Ad2-induced early polypeptides (11). Ad2-induced polypeptides of 74K (pulse form of DBP), 21K, 19K, 17.5K (faint), 15K, 11.5K, 11K, and 8K are apparent in this autoradiogram. Figure 4 shows the ^{32}P-labeled polypeptides present in total cell (lane C) and cytoplasmic (lane D) extracts, and the following NaCl eluates from DNA-cellulose: 0.05 M (lane E), 0.2 M (lane F), 0.4 M (lane G), 0.6 M (lane H), and 2.0 M NaCl (lane I). ^{32}P-labeled DBP was eluted mainly with 0.6 M NaCl (lane H) and to a lesser extent with 0.4 M NaCl (lane G). Thus ^{32}P-labeled DBP is eluted from DNA-cellulose at the same NaCl concentrations as ^{3}H-labeled DBP reported previously (24, 28). ^{32}P-labeled DBP is not apparent in lanes C and D, repre-

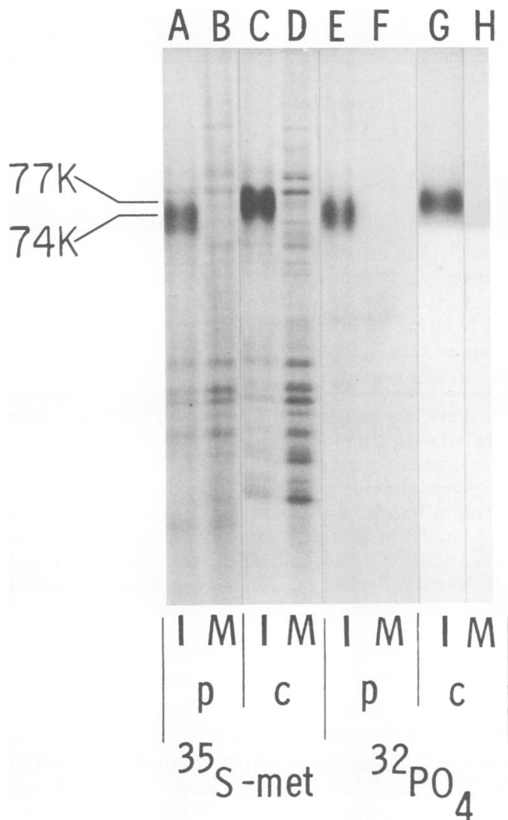

FIG. 3. *Autoradiogram of SDS-polyacrylamide gel electropherogram showing that both 74K (pulse) and 77K (chase) forms of ³⁵S- and ³²P-labeled DBP bind to single-stranded DNA-cellulose columns and are eluted by 0.6 M NaCl. Ad2-infected (I) mock-infected (M) cells were pulse-labeled with [³⁵S]Met or ³²PO₄ 7 to 7.5 h p.i. (p), or the same period followed by a 20-h chase (c). Cytoplasmic extracts were prepared and chromatographed on single-stranded DNA-cellulose columns. Bound proteins were eluted with 0.05 M, 0.4 M, and 0.6 M NaCl. The proteins eluted by 0.6 M NaCl were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. (A,B) ³⁵S pulse; (C,D), ³⁵S pulse-chase; (E,F) ³²P pulse; (G,H) ³²P pulse-chase.*

senting total cell and cytoplasmic extracts (which contain DBP), because equal radioactivity was applied to each lane, and ^{32}P-labeled DBP was enriched in the 0.4 and 0.6 M NaCl eluates from DNA-cellulose. The 74K ^{32}P-labeled polypeptide eluted from DNA-cellulose was shown to be DBP by immunoprecipitation of both the 0.4 and 0.6 M NaCl eluates with monospecific IgG against purified DBP (data not shown).

The 0.6 M NaCl eluate from DNA-cellulose was further purified by chromatography on DEAE-Sephadex. ^{32}P-labeled proteins co-chromatographed with the ^{3}H-labeled proteins that were used to monitor the purification. The peak fractions were pooled and analyzed by electrophoresis on cylindrical gels. The ^{3}H- and ^{32}P-labeled DBP comigrated in the cylindrical gel (data not shown), suggesting the covalent association of ^{32}P label with purified DBP. The remaining material was electrophoresed on a preparative slab gel; the band corresponding to ^{32}P-labeled DBP was cut from the gel, and the ^{32}P-labeled DBP was eluted and chemically analyzed as described below.

DBP contains phosphoserine but not phosphothreonine. The phosphate linkage properties of the ^{32}P-labeled DBP band isolated by preparative gel electrophoresis, as described

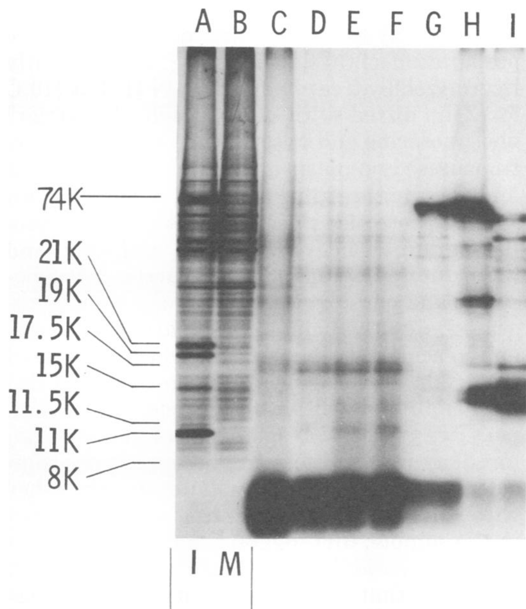

FIG. 4. *Autoradiogram of SDS-polyacrylamide gel electropherogram showing the ³²P-labeled polypeptides eluted from a single-stranded DNA-cellulose column by various NaCl concentrations. Ad2-infected cells were labeled with ³²PO₄ 7 to 10 h p.i., and the cytoplasmic extract was chromatographed on a single-stranded DNA-cellulose column. The bound proteins were eluted with increasing NaCl concentrations. (A) Ad2 early infected KB cells extracts labeled with [³⁵S]Met 7 to 8 h p.i. in the presence of ara-C after cycloheximide pretreatment (i.e., Ad2 early protein markers); (B) mock-infected, ³⁵S-labeled cell extracts treated the same as infected cells. (C through I) show ³²P-labeled polypeptides as follows: (C) total cell extracts; (D) cytoplasmic extract before DNA-cellulose; (E) DNA-cellulose flow-through and 0.05 M NaCl wash; (F) 0.2 M NaCl eluate; (G) 0.4 M NaCl eluate; (H) 0.6 M NaCl eluate; and (I) 2.0 M NaCl eluate.*

above, were analyzed. As shown in Table 1, treatment with hot trichloroacetic acid or organic solvents did not solubilize significant ^{32}P counts, suggesting that the ^{32}P label was associated with protein and not with RNA or DNA or phospholipids contaminating the DBP preparation. Pronase treatment completely solubilized the ^{32}P counts, which establishes that the radioactivity was associated with DBP. The phosphate linkage was at most 20% resistant to treatment with alkali, and it was 90% resistant to acid, as expected for a phosphoester of serine or threonine; this excluded phosphohistidine as a major component of DBP, because phosphoimide linkages are stable to alkalai and sensitive to acid (10, 19). Consistent with this, alkaline phosphatase partially solubilized ^{32}P counts, indicating a phosphomonoester bond (17).

To identify the phosphoamino acid, the same preparation of ^{32}P-labeled DBP was partially hydrolyzed by treatment with 6 N HCl at 110°C for 2 h, mixed with 40 μg each of "carrier" phosphoserine and phosphothreonine, and electrophoresed on paper at pH 1.9. Phosphoserine and phosphothreonine markers, not mixed with sample, were also electrophoresed. The electropherogram was developed with ninhydrin and then autoradiographed. The carrier phosphoserine and phosphothreonine in the sample migrated slightly behind the marker phosphoamino acids, possibly because trace amounts of materials (e.g., oligoacrylamide) had retarded the mobility of phosphoamino acids in the sample. Two radioactive spots appeared on the autoradiogram of this electropherogram: one coincided with inorganic phosphate and the other with the carrier phosphoserine ninhydrin spot in the sample; this suggests that serine but not threonine residues of DBP are phosphorylated. To verify that the radioactivity was in phosphoserine, the carrier phosphoserine spot was eluted, evaporated repeatedly, and reelectrophoresed on paper. As shown in Fig. 5, the radioactivity again coincided with phosphoserine and inorganic phosphate. In the reelectrophoresis experiment, the mobility of the carrier phosphoserine in the sample coincided exactly with the marker phosphoserine. These results indicate that serine but not threonine residues of DBP are phosphorylated. The $^{32}PO_4$ spot on the paper electropherograms presumably arose by hydrolysis of ^{32}P-labeled phosphoserine residues of DBP, because the first electrophoresis step should have eliminated inorganic phosphate from the carrier phosphoserine.

In the above experiment, DBP was labeled from 7 to 10 h p.i., so that it is not clear whether both the pulse and pulse-chase forms of DBP contain phosphoserine. To demonstrate that the chased form contains phosphoserine, Ad2-infected cells were labeled with $^{32}PO_4$, from 7 to 7.5 h p.i., then chased with normal MEM for 20 h. Total cellular proteins were extracted and immunoprecipitated by Ad1-SV40 IgG; then the immunoprecipitates were electrophoresed on SDS gels. As described above, the ^{32}P-labeled DBP band was cut from the gel, the DBP was eluted and was acid hydrolyzed, and the hydrolysate was subjected to paper electrophoresis. As shown in Fig. 6, the ^{32}P counts coincided with phosphoserine and inorganic phosphate. Thus the chased form of DBP contains phosphoserine but not phosphothreonine.

TABLE 1. *Properties of the phosphate linkage associated with highly purified ^{32}P-labeled DBP*[a]

Treatment	Trichloroacetic acid-insoluble ^{32}P counts (%)
Control	100
10% Trichloroacetic acid, 20 min at 90°C	100
10% Trichloroacetic acid, 20 min at 90°C plus ethanol-ether (3:1) extraction, 30 min at 60°C	100
0.1 N HCl, 15 min at 100°C	90
0.1 N KOH, 15 min at 100°C	25
Pronase, 1 h at 37°C	0
Alkaline phosphatase, 1 h at 37°C	30

[a] Portions corresponding to about 500 cpm were treated with trichloroacetic acid, trichloroacetic acid plus organic solvents, HCl, KOH, Pronase, or alkaline phosphatase (19). One hundred μg of bovine serum albumin was added at the end of the incubation, and the proteins were precipitated with cold 10% trichloroacetic acid. The acid-insoluble materials were collected on glass-fiber filters, washed, dried, and counted for radioactivity.

DISCUSSION

We have shown that Ad2 DBP is phosphorylated as indicated by (i) identification of ^{32}P-labeled DBP in infected cell extracts, (ii) binding and elution of ^{32}P-labeled DBP from single-stranded DNA-cellulose, (iii) immunoprecipitation of ^{32}P-labeled DBP by Ad1-SV40 IgG and monospecific anti-DBP IgG, and (iv) identification of ^{32}P-labeled serine in purified and immunoprecipitated DBP. Pulse-chase experiments also indicated that DBP exists in at least two forms that differ in mobility by SDS-polyacrylamide gel electrophoresis. After a short (30 min) pulse, DBP had an apparent molecular weight of 74K, but, after a pulse followed by a 20-h chase, the apparent molecular weight of DBP increased to 77K, and the 74K form was no

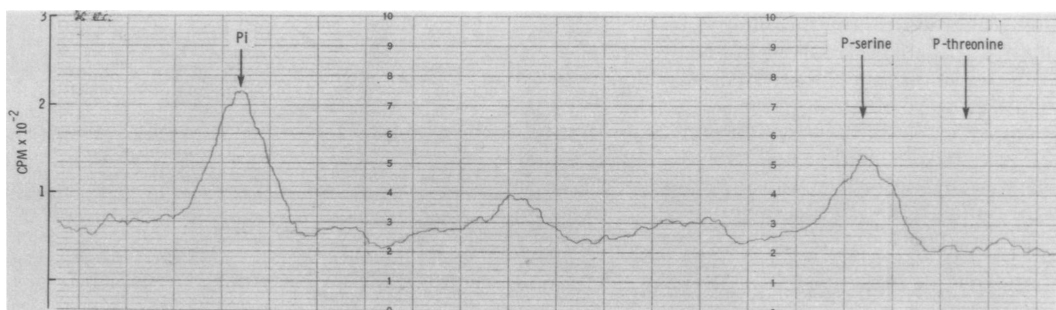

FIG. 5. *Identification of ^{32}P-labeled phosphoserine in highly purified ^{32}P-labeled DBP. Ad2-infected cells were labeled with $^{32}PO_4$ from 7 to 10 h p.i., and ^{32}P-labeled DBP was purified from cytoplasmic extracts by sequential chromatography on DNA-cellulose and DEAE-Sephadex (27), followed by preparative SDS-polyacrylamide gel electrophoresis. The ^{32}P-labeled DBP band was eluted from the gel, partially hydrolyzed in HCl, mixed with unlabeled "carrier" phosphoserine and phosphothreonine, and electrophoresed on paper as described in the text. Phosphoserine and phosphothreonine "markers" (i.e., not mixed with the ^{32}P-labeled DBP sample) were also electrophoresed. The "carrier" phosphoserine and phosphothreonine in the sample migrated slightly behind the marker phosphoserine and phosphothreonine. The radioactivity coincided with phosphoserine in the sample and with inorganic phosphate. The phosphoserine radioactive spot was eluted from the paper chromatogram and electrophoresed a second time. The results of the second electrophoresis are shown in this figure. Ninhydrin spray was used to locate the position of unlabeled amino acids, and a Packard radiochromatogram scanner was used to determine the position of radioactivity. The origin is located to the extreme right of the paper scan.*

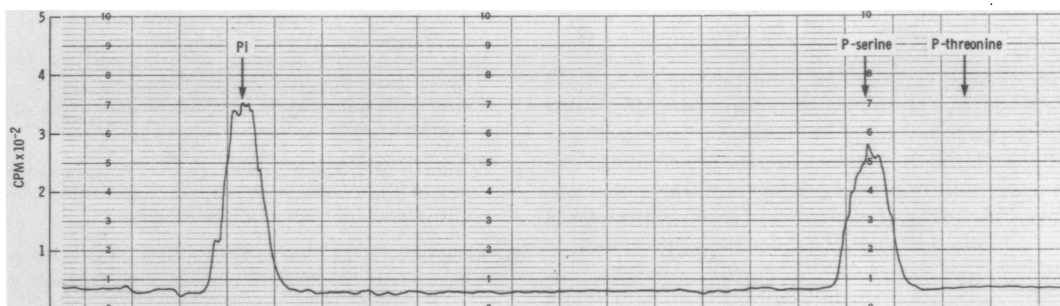

FIG. 6. *Identification of ^{32}P-phosphoserine in pulse-chase labeled ^{32}P-DBP after immunoprecipitation by Ad1-SV40 IgG and preparative SDS-polyacrylamide gel electrophoresis. Ad2-infected cells were labeled with $^{32}PO_4$, from 7 to 7.5 h p.i., then chased in normal MEM for 20 h. ^{32}P-labeled DBP was immunoprecipitated from cytoplasmic extracts by Ad1-SV40 IgG, then electrophoresed on SDS-polyacrylamide gels. ^{32}P-labeled DBP was eluted from the gel, acid hydrolyzed, mixed with carrier phosphoserine and phosphothreonine, and subjected to paper electrophoresis. The figure shows the correspondence between sample radioactivity and carrier phosphoserine present in the sample. "Marker" phosphothreonine and phosphoserine (i.e., not mixed with the sample) had the same electrophoretic mobilities as "carrier" phosphoserine and phosphothreonine in the sample.*

longer observed. Both 74K and 77K forms of ^{35}S- and ^{32}P-labeled DBP bound to single-stranded DNA and were immunoprecipitated by Ad1-SV40 IgG.

Other workers have commented on alterations of Ad2 DBP mobility on SDS-polyacrylamide gels. Anderson et al. (3) reported that late after infection Ad2 DBP appeared as a diffuse band, and occasionally as a doublet. In studies of viral-specific proteins during late stages of infection, by Walter and Martin (31) with Ad2-SV40 hybrids, and Weber (32) with an Ad2 late polypeptide-processing mutant, a similar reduction in the mobility of this polypeptide (presumably) was observed. Lewis et al. (16) found that DBP synthesized by cell-free translation of early Ad2 mRNA occasionally appeared as a doublet. Both forms were identical by peptide analysis and were very similar to DBP isolated from infected cells. The results of our pulse-chase experiments, together with data of other workers, may indicate that 74K DBP is the primary translation product that is then phosphorylated and further modified in

some unknown manner that affects the DBP mobility in gel electrophoresis. Both 74K and 77K forms of ^{35}S- and ^{32}P-DBP could be detected when cells were pulse- or pulse-chase-labeled in the presence of ara-C, which indicates that DBP can be phosphorylated and converted to the apparent larger form in the absence of viral DNA replication. Highly purified DBP has a frictional ratio of 1.86 and tends to aggregate unless treated with detergent, which suggest that DBP does not have typical globular protein structure and has hydrophobic surface regions (27). Possibly phosphorylation and other post-translational modifications contribute to these physical properties of DBP.

The roles of phosphorylation and other possible posttranslational modifications in DBP function are unknown. Both 74K and 77K forms of ^{35}S- and ^{32}P-labeled DBP bind to single-stranded DNA, but we have no accurate estimate of the affinity of each form to DNA. Also, it is not known whether nonphosphorylated DBP will bind to DNA. Protein phosphorylation is believed to play a key regulatory role in eukaryotic cell metabolism. The role of phosphoproteins (modulated by adenosine 3′,5′-cyclic monophosphate) in intermediary metabolism is well established, and protein phosphorylation may be involved in regulation of membrane function (22). Particularly germane to Ad2 DBP is that phosphorylation of histones and especially acidic DNA binding proteins may function in regulation of gene expression (13, 22, 25). The SV40 T-antigen is phosphorylated (R. Carroll, D. Livingstone, P. Tegtmeyer, personal communication), and this protein may have many biological functions, including initiation of viral DNA replication and expression of late genes, as well as maintenance of the transformed phenotype of SV40-transformed cells. Ad2 DBP seems to be an excellent model system to study the general role that phosphorylation plays in the biochemistry of eukaryotic DNA binding proteins.

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