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Identification of Two Nuclear Subclasses of the Adenovirus Type 5-Encoded DNA-Binding Protein

KARL VOELKERDING† AND DANIEL F. KLESSIG†*

Department of Cellular, Viral, and Molecular Biology, University of Utah School of Medicine, Salt Lake City, Utah 84132

Received 26 December 1985/Accepted 3 July 1986

The synthesis, accumulation, and subcellular distribution of the adenovirus serotype 5 DNA-binding protein (DBP) has been examined during the infectious cycle in HeLa cells. With the onset of viral DNA replication and entry into the late phase, two nuclear subclasses of DBP are distinguishable by immunofluorescence microscopy and can be separately isolated by in situ cell fractionation. The first subclass, represented by diffuse-staining DBP, is released by the addition of 1% Nonidet P-40–150 mM NaCl. The second subclass of DBP, which is sequestered into intranuclear globular structures, requires a high ionic strength (2 M NaCl) for extraction and appears to be associated with centers of active viral DNA replication. This association is based on the observations that (i) DBP within the globules and viral DNA, as detected by in situ hybridization, form identical structures that colocalize within the nuclei of infected cells, (ii) the formation of DBP globular structures requires the onset and continuation of viral DNA replication, and (iii) once formed, the globular structures can be perturbed by modulating viral DNA synthesis.

The DNA-binding protein (DBP) encoded by early region 2A (E2A) of the human adenovirus genome is a 72-kilodalton (kDa) phosphoprotein (21, 29) which is synthesized both early and late during the infectious cycle (5, 30). A large number of functions have been ascribed to this protein which are based primarily on analyses of DBP mutants; these functions include roles in viral DNA replication, viral early and late gene expression, and virion assembly.

The role of the DBP in viral DNA replication is firmly established. The prototype DBP temperature-sensitive (*ts*) mutant, Ad5ts125, provided the first genetic evidence for this role. At the nonpermissive temperature, Ad5ts125 is defective for viral DNA synthesis (14). With the advent of the in vitro DNA replication systems for adenovirus, this function was directly demonstrated (19). DBP is required for the strand elongation reaction of viral DNA synthesis (12, 15) and may play a role in the initiation reaction as well (32, 48).

Evidence for involvement of the DBP in viral early gene expression comes from several studies. Microinjection of adenovirus genes or mRNAs into cells suggests that DBP may stimulate both early region 1B (E1B) (40) and early region 4 (E4) gene expression (39). Studies with *ts*, mutants such as Ad5ts125 or Ad2ts400 indicate that at intermediate and later times in infection DBP may negatively regulate expression of early genes. Infection with these mutants is characterized by higher levels of accumulation of several early mRNAs in *ts* mutant- versus wild type (wt)-infected cells (9, 10). The negative regulation attributed to wt DBP may be mediated by effects on the cytoplasmic stabilities of early region 1A (E1A) and E1B mRNAs (6) and the transcription rate of the E4 gene family (33). Furthermore, DBP has been shown to repress E4 transcription in vitro (18). In contrast to the results obtained with the *ts* mutants and by microinjection studies, Rice and Klessig (38) have shown that the E2A deletion mutant Ad5dl802, which produces neither intact DBP nor DBP-related polypeptides, exhibits a pattern of early gene expression very similar to that of *wt* virus. This work thus questions the role of DBP as a regulator of early gene expression.

Further support for multifunctional nature of DBP comes from studies of abortive infections by human adenovirus in monkey cells. wt adenovirus fails to multiply in monkey cells due to a complex block to viral late gene expression. This block includes a reduction in the rate of transcription of late genes (20), alterations in the pattern of mRNA splicing for the fiber polypeptide (3, 23), and poor utilization of this mRNA in vivo (2). Host range mutants of adenovirus which overcome these blocks and thus are capable of productive growth in monkey cells contain alterations in the DBP gene (1, 8, 24, 27). These results suggest that DBP may interact with the RNA transcription apparatus, with RNA itself (perhaps at the level of splicing), or directly or indirectly with the host cell translational machinery.

Finally, evidence for the role DBP in virion assembly comes from studies done with a revertant of one of the *ts*, DNA-replication-negative mutants, Ad5ts107. The revertant, R(ts107)202, produces apparently normal amounts of late viral structural proteins but is deficient at assembling virions in human 293 cells at the nonpermissive temperature (34).

Physical mapping of the two general classes of DBP mutations suggests that this polypeptide contains at least two functionally distinct domains (1, 8, 25–28). Whereas *ts* mutants reside in the 3′ two-thirds of the gene, the host range mutants are located in the 5′ portion of the gene. Moreover, digestion of purified DBP with a variety of proteases generates a carboxyl-terminal fragment of approximately 44 kDa and an amino-terminal fragment of approximately 26 kDa (21, 41). The 44-kDa carboxyl-terminal fragment can bind single-stranded DNA (21) and can substitute for intact DBP in an in vitro DNA replication system (4). The 26-kDa amino-terminal fragment, which contains most of the protein's phosphorylation sites (21), retains its activity(ies) required for normal late gene expression even when the DBP DNA replication function is perturbed by *ts* mutations in the

* Corresponding author.
† Present address: Waksman Institute, Rutgers-The State University of New Jersey, Piscataway, NJ 08854.

carboxyl terminus (37). Together these results suggest that DBP contains at least two functionally and physically separable domains.

The pleiotropic nature of DBP implies that there may be several subclasses of the protein which carry out different functions. Delineating such potential subclasses of DBP might be achieved by demonstrating that they associate with different macromolecules and structures in the cell. This approach has been successfully used to establish subclasses of simian virus 40 large T antigen (42) and of myc proteins (13). Once isolated, different populations of DBP could be separately analyzed for their biochemical characteristics. Such a system to define and isolate these subpopulations would also provide a framework upon which to compare and contrast the distribution and interactions of mutant DBP proteins.

In this study we examined the synthesis, accumulation, and subcellular distribution of DBP during adenovirus type 5 (Ad5) infection of HeLa cells. With the onset of viral DNA replication and entry into the late phase, two nuclear subclasses of DBP could be distinguished. These two subclasses could be biochemically separated by in situ cell fractionation. The involvement of one of these subclasses in viral DNA replication or late gene expression was examined.

MATERIALS AND METHODS

Cells, viruses, and infections. HeLa cells, originally obtained from J. F. Williams, were cultivated as monolayers in Dulbecco modified Eagle medium (Flow Laboratories, Inc.) supplemented with 10% calf serum (Irvine Scientific), 100 μg of streptomycin per ml, 100 μg of penicillin per ml, and 2 mM L-glutamine. For immunofluorescence (IF) microscopy and in situ hybridization studies, cells were grown on glass cover slips (Corning Glass Works) precleaned by boiling for 5 min in 0.1 N HCl and followed by extensive rinses in distilled H_2O and subsequent heat sterilization.

Ad5 and Ad5ts149 were originally obtained from J. F. Williams. Virus infections were performed at multiplicities of between 10 and 30 PFU per cell. After adsorption for 60 min at 37°C in phosphate-buffered saline (PBS), the cells were washed with PBS and incubated in culture medium at the appropriate temperature.

IF microscopy. For IF microscopy studies, mock- or virus-infected cells grown on cover slips were washed briefly in PBS, fixed for 20 min in −20°C methanol, and then rehydrated in PBS. Primary antibody incubations were at 37°C for 30 min with either a mouse anti-DBP monoclonal antibody or a polyclonal rabbit antisera raised against purified DBP. After three 5-min washes in PBS, secondary antibody reactions with either fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin G (Sigma Chemical Co.) or FITC-conjugated goat anti-rabbit immunoglobulin G (Meloy Laboratories) were performed at 37°C for 30 min. Cover slips were then washed three times in PBS and once in distilled H_2O and then mounted on glass slides with 90% glycerol–10% PBS. Slides were viewed on a Zeiss microscope equipped with epifluorescence and recorded on Kodak Plus-X-pan film.

In situ hybridization. In situ hybridization was performed using a biotinylated Ad2 or Ad5 probe supplied in an Ad2 Patho-Gene identification kit (Enzo Biochem, Inc.). Conditions for hybridization, binding of streptavidin-horseradish peroxidase complex to the hybridized probe, and subsequent enzymatic detection with the chromogen diaminobenzidine tetrahydrochloride in the presence of hydrogen peroxide were as outlined in *Bio-Note 101* (Enzo Biochem). Slides were viewed on a Nikon Optiphot microscope and recorded on Polaroid type 55 land film.

Colocalization by in situ hybridization and immunofluorescence. Mock- or virus-infected cells grown on glass slides were fixed for 20 min in −20°C acetone and then air dried. In situ hybridization using a biotinylated Ad5 probe with subsequent detection using FITC-conjugated avidin utilized reagents and followed conditions recommended by Enzo Biochem. Briefly, the Ad5 probe in a 50% formamide hybridization solution was applied to slides and covered with a glass cover slip. The slides were placed on a 95°C heating block for 1 min for denaturation, which was followed by hybridization at room temperature for 15 min. After several washes with PBS, FITC-conjugated avidin diluted in PBS was applied for 15 min at room temperature and then the slides were washed in PBS. Subsequent immunodetection of DBP was performed using either a polyclonal rabbit antisera or a mouse anti-DBP monoclonal antibody as a primary antibody, followed by rhodamine-conjugated secondary antibodies (Boehringer Mannheim Biochemicals). Antibodies were diluted in PBS, and incubations were carried out for 15 min at 37°C.

Cell fractionation. In situ fractionation of cell monolayers or cover slip cultures was as described by Staufenbiel and Deppert (43) with the following modifications. Cells were washed briefly, first with cold PBS and then with cold 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.2 (MES)–1.5 mM $MgCl_2$. Fraction 1 consisted of pooled sequential extractions for 5 and then 10 min on ice in 1% Nonidet P-40 (NP-40)–150 mM NaCl–1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–5 mM dithiothreitol (DTT)–1.5 mM $MgCl_2$–10 mM MES. Fraction 2 consisted of a 5-min extraction on ice using the fraction 1 buffer but with the omission of NP-40. Fraction 3 consisted of two successive 5-min extractions on ice in 2 M NaCl–1 mM EGTA–5 mM DTT–1.5 mM $MgCl_2$–10 mM MES. Solubilization of residual cell structures was achieved by incubation for 30 min on ice with 1% Empigen BB (Albright and Wilson, Inc.)–25 mM KCl–1 mM EGTA–5 mM DTT–40 mM Tris (pH 9.0). After extraction all fractions were supplemented with phenylmethylsulfonyl fluoride to a final concentration of 100 μg/ml and immediately frozen at −20°C to prevent proteolysis. For IF microscopy analysis, extracted cell structures were briefly rinsed in cold 10 mM MES–1.5 mM $MgCl_2$, fixed for 20 min in −20°C methanol, rehydrated in PBS, and incubated with primary and secondary antibodies as described above.

Protein analyses. Labeling of proteins synthesized in vivo, immunoprecipitations, and immunoblotting of proteins were carried out as described previously (22), with the substitution of 5% nonfat dry milk (Carnation) for 5% bovine serum albumin as a blocking agent in the immunoblot procedure. Autoradiographs for quantitation were obtained without intensifying screens so that band intensity could be used as a linear measure of the amount of labeled protein. Densitometer tracings were performed with a Joyce-Loebel model 3Cs microdensitometer, and areas under the peaks were determined with a Numonic Corp. model 1224 electronic graphics calculator.

RESULTS

Synthesis and accumulation of DBP. At various times after infection of HeLa monolayers with Ad5, proteins were labeled with [^{35}S]methionine and immunoprecipitated with

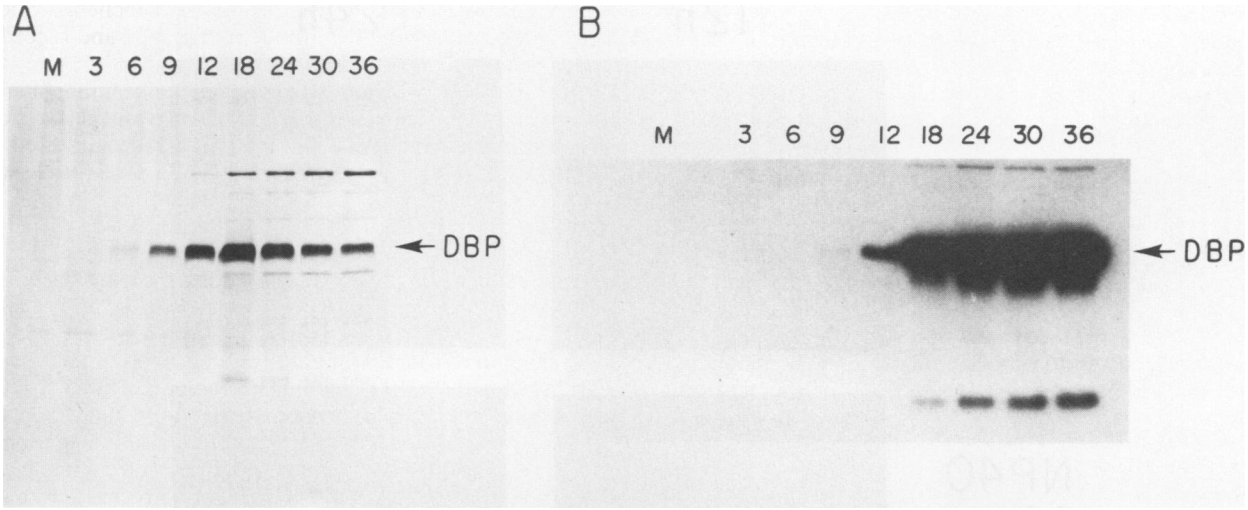

FIG. 1. Synthesis and accumulation of DBP during Ad5 infection. Confluent monolayers of HeLa were infected with 20 PFU of Ad5 per cell and incubated at 37°C. (A) Synthesis of DBP at various times after infection, as determined by labeling with 75 μCi of [^{35}S]methionine per ml for 1 h at 37°C. Equal fractions of each labeled cell lysate were immunoprecipitated with polyclonal rabbit anti-DBP serum and fractionated on a 12.5% SDS-polyacrylamide gel and subjected to autoradiography. Times after infection (in hours) are shown above each lane. (B) Accumulation of DBP. Equal fractions of each labeled cell lysate were fractionated on a 10% gel, transferred to nitrocellulose, and incubated first with polyclonal rabbit anti-DBP serum and then with ^{125}I-labeled protein A. The ^{35}S emission was blocked by an extra sheet of film placed between the immunoblot and a second film which recorded the ^{125}I emission. M, Mock-infected cells.

anti-DBP serum. DBP synthesis was first detected at 6 h postinfection (p.i.), increased dramatically during the next 12 h, peaked at approximately 18 h p.i., and then gradually declined (Fig. 1A). Accumulation of DBP was examined in the same experiment by immunoblot analysis (Fig. 1B). DBP was first detected at 9 h p.i., increased most rapidly during the next 9 h, and then increased only moderately (2×) during the next 18 h.

Cellular distribution of DBP. To determine the intracellular location of DBP during Ad5 infection of HeLa cells, IF microscopy was utilized (Fig. 2). In pilot experiments, different methods of fixation were employed, including ad-

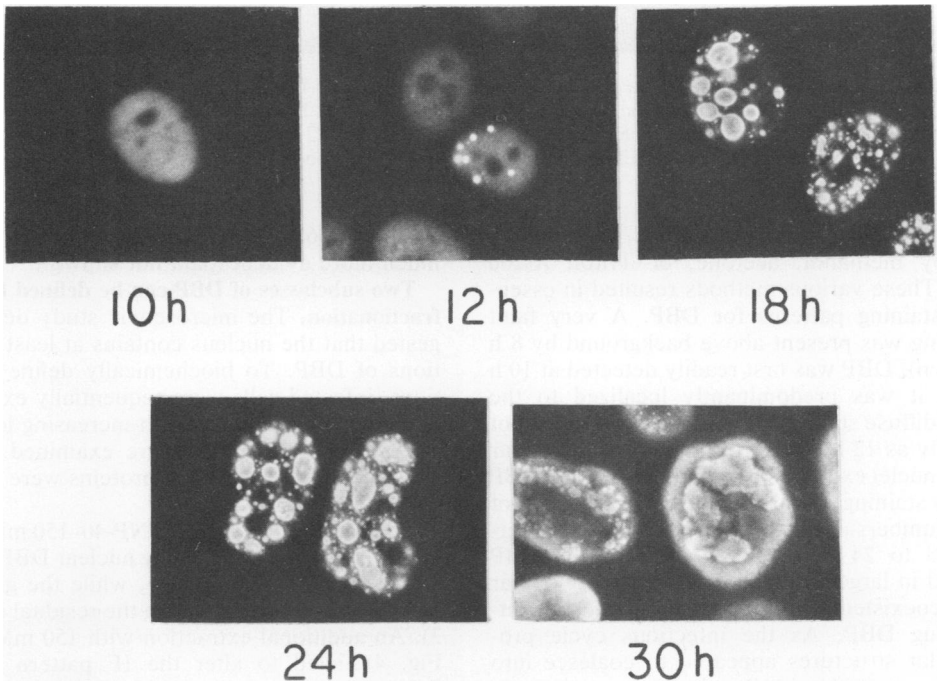

FIG. 2. Cellular distribution of DBP during Ad5 infection of HeLa cells. Infected cells grown on glass cover slips were fixed at the time shown in −20°C methanol for 20 min and sequentially reacted with a mouse anti-DBP monoclonal antibody, followed by FITC-conjugated sheep anti-mouse immunoglobulin G. Only nuclear fluorescence is seen. A rabbit anti-DBP polyclonal antibody gave an identical IF pattern. Magnification, ×600.

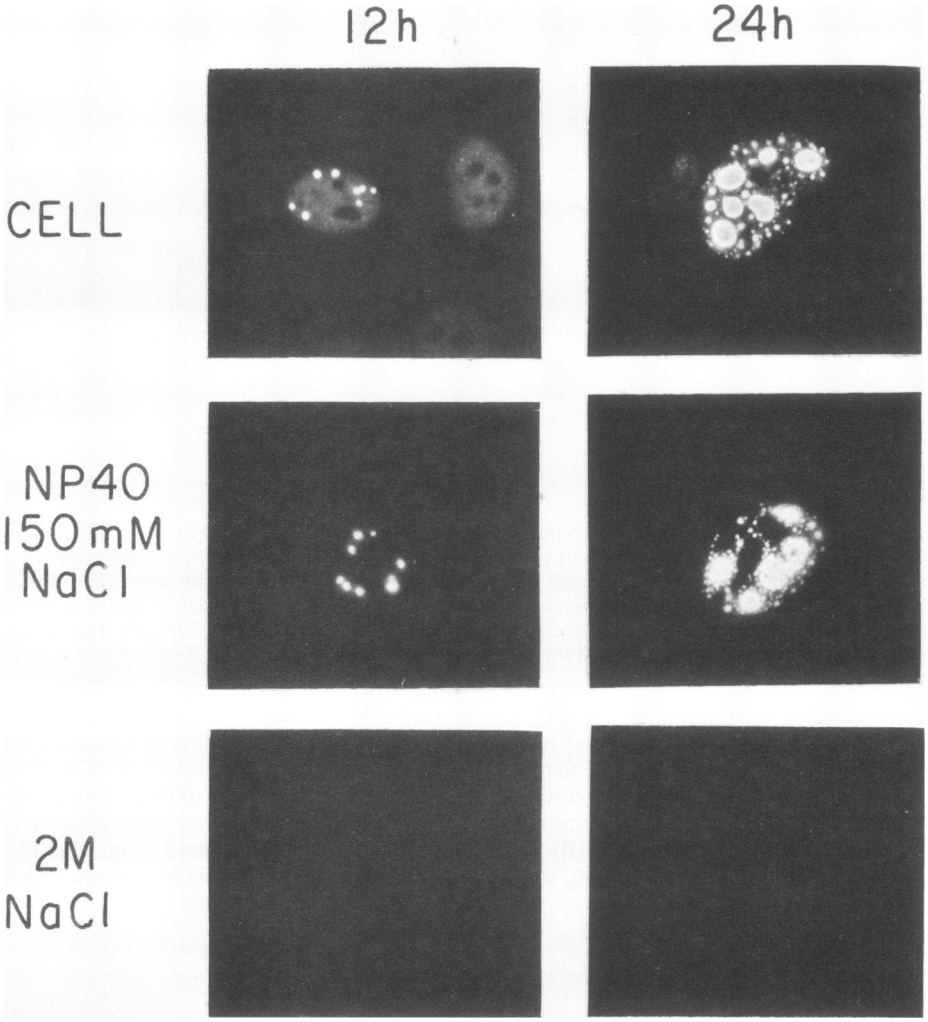

FIG. 3. IF analyses of DBP in Ad5-infected HeLa cells after in situ extraction. Ad5-infected HeLa cells (12 and 24 h p.i.) on parallel cover slips were analyzed by IF directly or after extraction in situ with 1% NP-40–150 mM NaCl or with 1% NP-40–150 mM NaCl followed by 2 M NaCl. Control (unextracted) cells and extracted cell structures were fixed in −20°C methanol for 20 min and incubated with an anti-DBP monoclonal antibody followed by reaction with an FITC-conjugated secondary antibody.

dition of methanol, acetone, methanol-acetone, or formaldehyde, followed by methanol, acetone, or Triton X-100 permeabilization. These various methods resulted in essentially similar IF staining patterns for DBP. A very faint cytoplasmic staining was present above background by 8 h p.i. (data not shown). DBP was first readily detected at 10 h p.i. At this time it was predominantly localized to the nucleus and had a diffuse staining pattern. Nucleoli were not stained. By as early as 12 h p.i. a transition began to occur wherein some cell nuclei exhibited both diffuse-staining DBP and more intensely staining, small globular concentrations of DBP. Increasing numbers of nuclei exhibited this sequestering process by 18 to 24 h p.i., with most of the DBP becoming localized in larger, randomly distributed globular structures which coexisted with a small proportion of diffuse-nuclear-staining DBP. As the infectious cycle proceeded, the globular structures appeared to coalesce into large, amorphous forms (30 h p.i.). Starting at approximately 24 h p.i. a faint, cytoplasmic staining again became evident. Photographic exposures, which were taken to most faithfully reproduce the nuclear DBP staining pattern (24 h p.i.; Fig. 2), do not reveal this faint cytoplasmic DBP. Very late in infection (36 to 40 h p.i.), the cytoplasmic staining became much more evident (data not shown).

Two subclasses of DBP can be defined by successive in situ fractionation. The microscopic study described above suggested that the nucleus contains at least two major populations of DBP. To biochemically define these two populations, infected cells were sequentially extracted in situ with nonionic detergent and with increasing ionic strengths. The residual cell structures were examined by IF microscopy (Fig. 3), and the extracted proteins were analyzed by immunoblotting (Fig. 4).

After extraction with 1% NP-40–150 mM NaCl (fraction 1, Fig. 4), the diffusely staining nuclear DBP could no longer be detected by IF microscopy, while the globular DBP structures remained intact within the residual cell structures (Fig. 3). An additional extraction with 150 mM NaCl (fraction 2, Fig. 4) failed to alter the IF pattern (data not shown). Subsequent extraction with 2 M NaCl (fraction 3, Fig. 4) resulted in the loss of the globular structures. The nuclear matrix, which is resistant to this extraction procedure, contained only trace amounts of DBP (Fig. 3). The faint, cytoplasmic DBP seen at 24 h p.i. was removed by extrac-

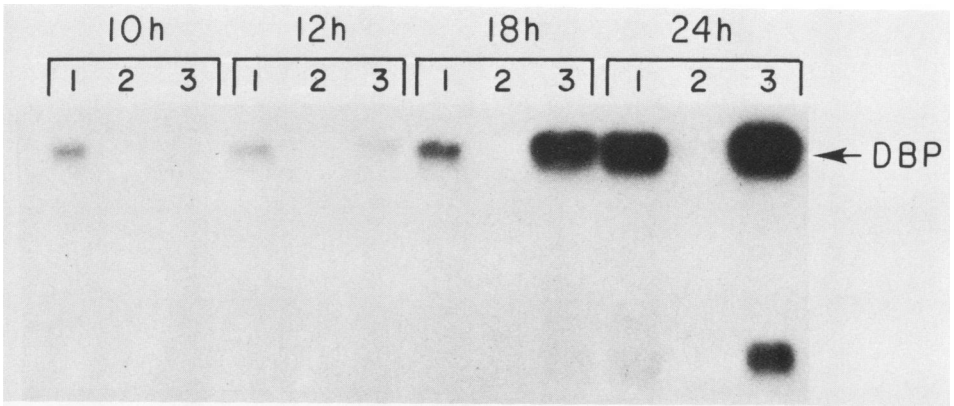

FIG. 4. Two populations of DBP defined by successive in situ fractionation. Ad5-infected HeLa monolayers at various times (in hours) after infection were sequentially extracted with a buffer containing 1% NP-40–150 mM NaCl–1 mM EGTA–5 mM DTT–1.5 mM $MgCl_2$–10 mM MES (lanes 1), with the same buffer minus NP-40 (lanes 2), and with the same buffer minus NP-40 but containing 2 M NaCl (lanes 3). Equal fractions of each extract were immunoprecipitated with polyclonal rabbit anti-DBP serum, fractionated by 10% SDS-PAGE, and subjected to immunoblot analysis. To obtain detectable levels of DBP at 10 h, 10 times more extract was immunoprecipitated than at other time points.

tion with 1% NP-40–150 mM NaCl. This was also observed for the more predominant cytoplasmic DBP seen very late in the infectious cycle (data not shown). In other experiments extraction with intermediate salt concentrations of 0.5 and 1.0 M NaCl, subsequent to an initial 1% NP-40–150 mM NaCl fractionation step, resulted in the globular structures taking on an increasingly ragged appearance, presumably due to partial extraction of DBP.

The proteins released during this in situ fractionation were analyzed by using immunoblots (Fig. 4). At 10 h p.i., when DBP is predominantly located in the nucleus and exhibits a diffuse staining pattern, essentially all DBP was released with 1% NP-40–150 mM NaCl. DBP was found by 12 h p.i. both in the 1% NP-40–150 mM NaCl fraction and in the 2 M NaCl fraction. By 18 to 24 h p.i., when DBP is predominantly in globular structures, most of the DBP was released by 2 M NaCl. When intermediate NaCl concentrations (0.3 to 1.0 M) were applied subsequent to extraction with 1% NP-40–150 mM NaCl, partial release of DBP occurred (data not shown). After extraction with 2 M NaCl the residual cytoplasmic structure and nuclear matrix could be solubilized by extraction with the dipolar ionic detergent Empigen BB. Only trace amounts of DBP could be detected in this fraction (data not shown).

Newly synthesized DBP is rapidly partitioned. By 24 h p.i. approximately 75% of the DBP is associated with the 2 M NaCl releasable fraction. To examine the kinetics of DBP association with this fraction, Ad5-infected HeLa cells at 24 h p.i. were pulse-labeled with [^{35}S]methionine, chased for various times, and fractionated in situ. The extracted proteins were immunoprecipitated with anti-DBP serum and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 5). Immediately after pulse-labeling for 4 min, slightly greater than 75% of newly synthesized DBP was found in the 1% NP-40–150 mM NaCl releasable fraction, with the remaining DBP already associated with the 2 M NaCl releasable fraction. With a 5-min chase the ratio of DBP distribution was reversed. By 20 min approximately 80% of DBP was in the 2 M NaCl releasable fraction, and this percentage was unchanged at 120 min.

Association of one subclass of DBP with centers of active viral DNA replication. It is likely that DBP observed in the globular structures within the nucleus by IF microscopy and releasable by 2 M NaCl represents a population of the protein associated with centers of active viral replication for

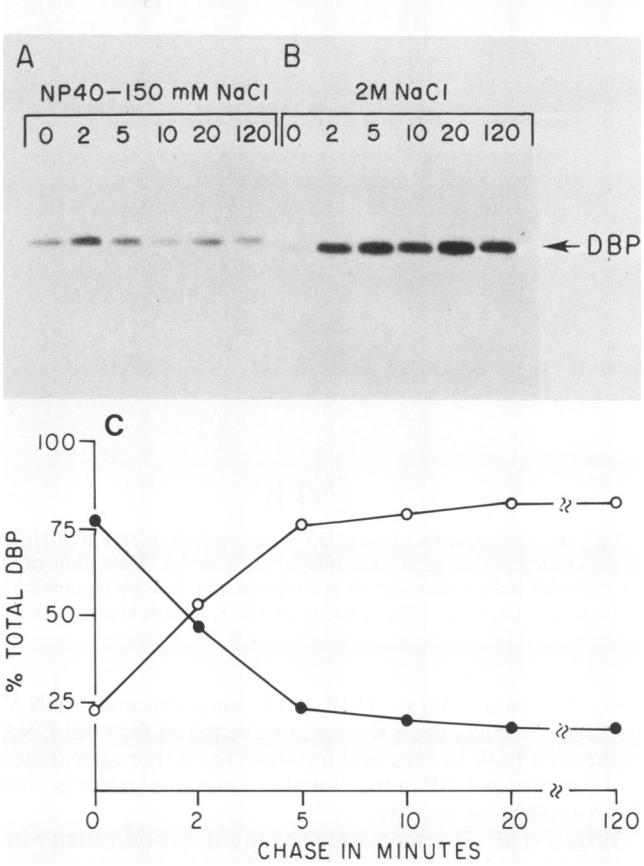

FIG. 5. Kinetics of partitioning of newly synthesized DBP. Ad5-infected HeLa monolayers at 24 h p.i. were pulse-labeled for 4 min with 125 μCi of [^{35}S]methionine per ml and chased with a 2,000-fold molar excess of unlabeled methionine for the times (in minutes) indicated. The monolayers were then fractionated in situ by sequential extraction with buffer containing 1% NP-40–150 mM NaCl–1 mM EGTA–5 mM DTT–1.5 mM $MgCl_2$–10 mM MES (A) and with the same buffer minus NP-40 but containing 2 M NaCl (B). Equal fractions of each labeled cell extract were immunoprecipitated with polyclonal rabbit anti-DBP serum and fractionated on a 12.5% SDS-polyacrylamide gel. The kinetics of partitioning are graphically displayed in panel C (●, NP-40–150 mM NaCl-extracted DBP; ○, DBP released with 2 M NaCl).

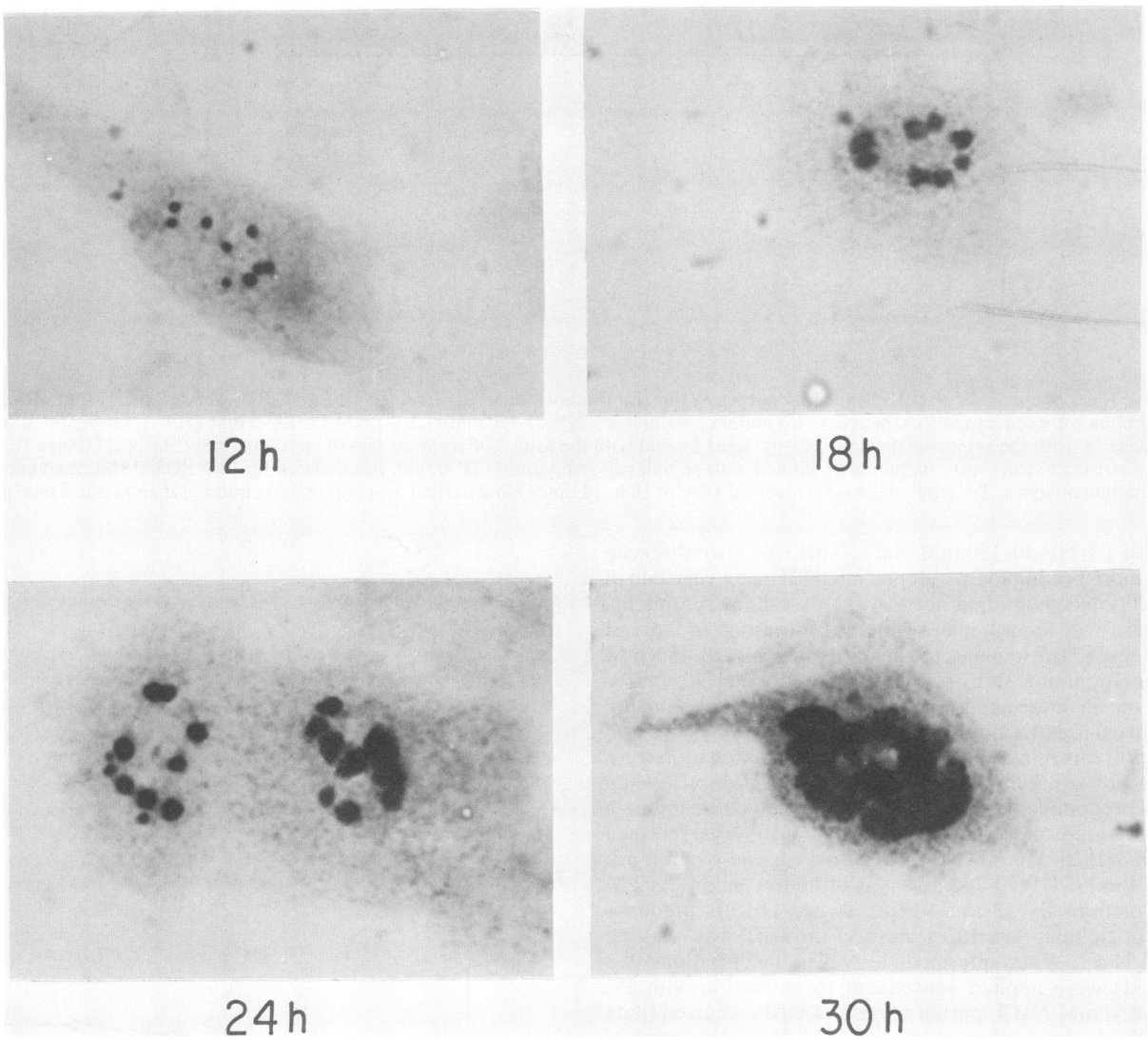

FIG. 6. Localization of viral DNA during Ad5 infection of HeLa cells. Infected cells grown on glass cover slips were analyzed by in situ hybridization by using a biotinylated Ad2 probe. Detection of the hybridized probe was accomplished by reacting the cells first with streptavidin-linked horseradish peroxidase and then by incubation with the chromagen diaminobenzidine tetrahydrachloride in the presence of hydrogen peroxide. The reaction product, a dark brown precipitate, denotes the hybridization signal. Magnification, ×400.

several reasons. First, DBP binds single-stranded DNA. Second, DBP has been shown to be required for viral DNA replication both in vivo and in vitro. Third, the appearance and quantity of DBP in the globular structures parallels viral DNA replication activity.

Brigati et al. (7) demonstrated by in situ hybridization with biotinylated adenovirus probes that during adenovirus infection viral genomes were localized as discrete foci within the nuclei. Since these foci of viral DNA appeared to be similar to the DBP globular structures, experiments were performed to compare the sizes, numbers, and locations of DBP globular structures with the distribution of viral genomes during the infectious cycle as detected by in situ hybridization (Fig. 6). At 12 h p.i. when DBP was first seen in globular structures, small focal concentrations or centers of viral DNA were first evident. As infection proceeded, DBP was increasingly sequestered into the more prominent globular structures with a corresponding enlargement of the viral DNA centers. Thus, there is a temporal correlation between the appearance and distribution of globular structures containing DBP and the centers of adenovirus DNA.

To ensure that the biotinylated probe was specifically hybridizing to adenovirus DNA rather than simply being bound by the adenovirus single-stranded-DNA-binding protein during the hybridization reaction, a heterologous biotinylated probe against herpes simplex virus DNA was employed in parallel experiments. This heterologous probe, while detecting viral DNA in herpes simplex virus-infected cells, did not react with the Ad5-infected cells (data not shown).

To determine whether the DBP globular structures colocalized with viral DNA centers, the same infected cells were subjected to both in situ hybridization with the biotinylated adenovirus DNA probe and immunodetection with anti-DBP serum. Pilot experiments showed that under the conditions of denaturation and hybridization used in the in situ procedure, DBP antigenicity was preserved. Thus, at various times after infection, the cell monolayers were first

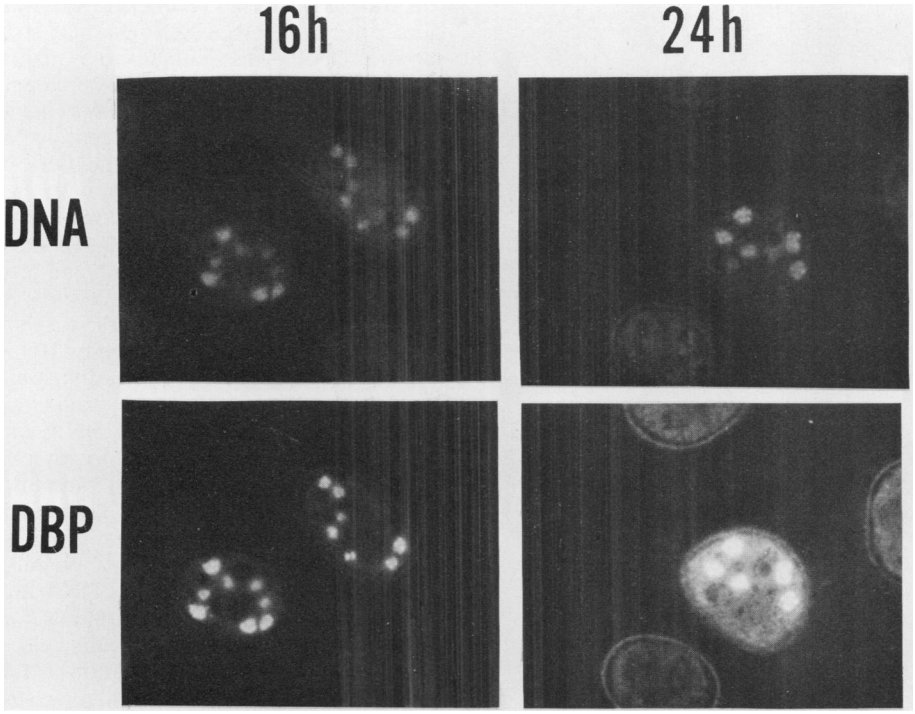

FIG. 7. Colocalization of DBP globular structures with centers of adenovirus DNA. Infected cells grown on glass slides were analyzed by sequential in situ hybridization and immunodetection as described in Materials and Methods. Top row, DNA hybridization with a biotinylated Ad5 probe detected by FITC-conjugated avidin; bottom row, immunolocalization of DBP in the same cell(s) with a rabbit polyclonal antisera and a rhodamine-conjugated secondary antibody. Preimmune serum controls showed no positive staining (data not shown). Magnification, ×600.

hybridized with the viral DNA probe and then reacted with anti-DBP serum. The results of these experiments indicate that the DBP globular structures colocalized with centers of viral DNA (Fig. 7). Moreover, these globular structures and centers exhibited similar fine structures. The similarity in fine structures was particularly evident when either higher magnification was used or exposure times were adjusted to give equal fluorescent signals from the two probes (data not shown).

If the globular structures represent a direct interaction of DBP with centers of active viral DNA replication, this association might be affected by perturbing viral DNA replication. Several approaches were taken to test this. First, viral DNA replication was inhibited with 10 mM hydroxyurea. This prevented the formation of viral DNA centers and the transition of DBP to the globular structures normally seen between 12 and 24 h p.i. (data not shown). Second, viral DNA replication was modulated using the *ts* mutant Ad5ts149. Ad5ts149 contains a mutation in the region coding for the viral DNA polymerase which prevents viral DNA replication at 39.5°C but not at 33°C (11, 16, 44). At the nonpermissive temperature only a diffuse nuclear staining pattern was observed in most of the infected cells even at 24 h p.i. (Fig. 8A). A few cells had minute focal concentrations of DBP, but well-defined globular structures were not seen. However, within 4 h of shifting from the nonpermissive to the permissive temperature, DBP was sequestered into prominent globular structures (Fig. 8A).

To determine whether the association of DBP with globular structures was reversible, Ad5ts149-infected cells cultured at 33°C were shifted to 39.5°C during the late phase of infection. By 3 h postshift approximately 30% of the cells exhibited a partial breakdown of globular structures concurrent with an increase in diffuse-staining nuclear DBP (Fig. 8B), which then became extractable with 1% NP-40–150 mM NaCl (data not shown). With extended incubation at the nonpermissive temperature (6 h), approximately 60% of the infected cells showed partial breakdown of the globular structures. Some of these cells also exhibited prominent cytoplasmic DBP staining not seen in control (unshifted) cells. Furthermore, although approximately 40% of the cells did not show a perturbation of the preexisting DBP globules by 6 h after the temperature shift, the increase in both the size and the number of globular structures that normally occurs (at 33°C) in the absence of the shift was inhibited (data not shown). The DBP globular structures were unaffected by shifting from 33 to 39.5°C in control experiments with Ad5-infected cells (data not shown).

DISCUSSION

The human adenovirus-encoded DBP is a multifunctional polypeptide with a diverse set of roles in the infectious cycle that may include viral DNA replication, viral early and late gene expression, and virion assembly. The performance of such different functions may require unique subclasses of the protein. IF localization of DBP, in both our study and that of Sugawara et al. (46), reveals that the cellular location of DBP changes during the infectious cycle. These distributional alterations may reflect changes in the interactions of the protein not only with other viral products but also with host factors and structures. Thus, intracellular partitioning of functionally distinct subclasses of DBP may occur throughout an infection. By utilizing an in situ cell fractionation procedure developed by Staufenbiel and Deppert (43), we have defined two major nuclear subclasses of DBP which are present in HeLa cells during infection with Ad5.

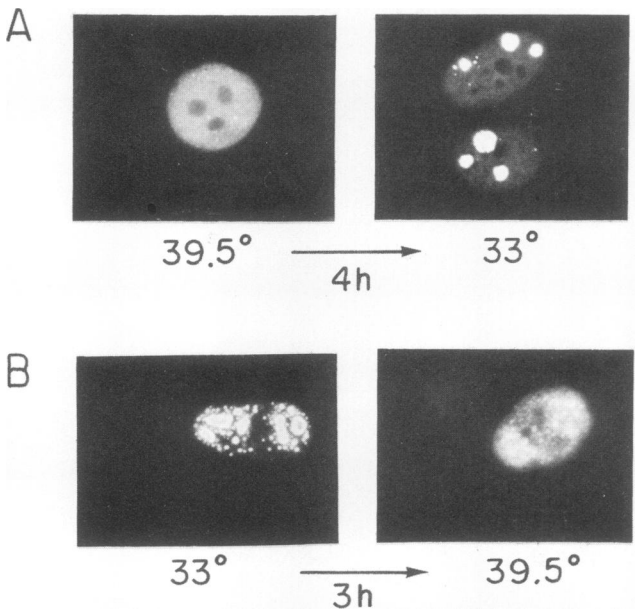

FIG. 8. Effects of temperature shifts on DBP distribution in HeLa cells infected with the *ts* viral DNA polymerase mutant Ad5ts149. (A) HeLa cells infected with Ad5ts149 were maintained at 39.5°C until 24 h p.i. and then shifted to 33°C for 4 h. (B) HeLa cells were infected with Ad5ts149 and maintained at 33°C until 44 h p.i. and then shifted to 39.5°C for 3 h. The distribution of DBP was analyzed by IF before (left side of panel) and after (right side of panel) the temperature shift. DBP distribution was also determined in control (nonshifted) cells at the end of the normal shift period (data not shown).

One nuclear subclass has a diffuse staining pattern and can be released during in situ fractionation with 1% NP-40–150 mM NaCl. Initially seen during the early phase of infection, it subsequently becomes less prominent as infection proceeds to the intermediate and late phases. The function of this subpopulation is unclear at present.

The second nuclear subclass of DBP is sequestered into globular structures that first appear at around 12 h p.i. as small, brightly staining foci. As the infection proceeds, the number and size of these globular structures increase. The globules are resistant to 1% NP-40–150 mM NaCl but can be extracted at a high ionic strength (2 M NaCl). Sequestering of DBP into globular structures could simply result from extensive self-association of the protein as has been shown to occur in vitro (41, 47). The strongest argument against this possibility is provided by studies with DBP deletion mutants such as Ad5dl802 (38). These viruses are totally defective for viral DNA synthesis in HeLa cells but can replicate their DNA in cell lines (e.g., gmDBP2) which produce DBP from an integrated copy of the E2A gene (22). The endogenous DBP levels in these cells are approximately 2 to 5% that seen during the late phases of infection of HeLa cells with wt virus. Similarly, viral DNA replication and viral production with DBP deletion mutants in these cells are a small percentage of that seen with wt virus (38). This suggests that the majority of DBP synthesized during a normal infection with wt virus is needed for viral DNA replication or for other functions required to produce progeny virus and thus cannot simply be sequestered away in nonfunctional globules. Moreover, the observation that the DBP globular structures break down when viral DNA replication is inhibited by shifting Ad5ts149-infected cells to the nonpermissive temperature is not easily reconciled with this aggregation model. The colocalization of DBP globular structures with centers of viral DNA is also difficult to explain by this model.

Sugawara et al. (46) and Ginsberg et al. (17) suggested that the DBP globular structures may represent sites of adenovirus DNA replication. More recently, Puvion-Dutilleul et al. (35) provided evidence that the DBP globules seen by IF light microscopy may correlate with the clear fibrillar inclusions seen by electron microscopy and thought to be sites of adenovirus DNA replication and transcription (50). Our observations are consistent with and provide further support for an assignment of the DBP globular structures as sites of adenovirus DNA replication. First, the temporal appearance of DBP globular structures seen by IF microscopy correlates well with the intranuclear distribution of adenovirus genomes detected by in situ hybridization. Second, the DBP globules and centers of viral DNA can be colocalized by sequential in situ hybridization with a biotinylated adenovirus DNA probe followed by immunodetection of DBP. Third, the formation of DBP globules is dependent on the onset of viral DNA replication. If replication is blocked by hydroxyurea, the formation of globular structures is prevented and no centers of viral DNA are detected by in situ hybridization. Moreover, if viral DNA synthesis is inhibited at the restrictive temperature in cells infected by a virus (Ad5ts149) carrying a *ts* mutation in the gene encoding the viral DNA polymerase, the globular structures do not form (Fig. 8A) (31). When the block to viral DNA replication is removed by shifting to the permissive temperature, DBP globules rapidly appear. Conversely, when Ad5ts149-infected cells are shifted from the permissive to the nonpermissive temperature late in the infectious cycle, preexisting globular structures are perturbed. Similar observations have been made with adenoviruses carrying *ts* lesions in DBP (17, 36, 45). These data provide evidence that this subclass of DBP is actively participating in viral DNA replication rather than only associating with sites of viral DNA accumulation.

Though a significant proportion of DBP associated with these globular structures may be involved in viral DNA replication, it would be premature to designate this as its sole function. First, studies suggest that transcription of late genes and DNA replication can occur simultaneously on the same viral template (50). Second, analysis of the blocks to late gene expression in wt-adenovirus-infected monkey cells—blocks which can be overcome by host range mutations in the DBP gene—suggests that this protein may directly or indirectly be involved in viral RNA transcription or processing (3, 20, 23, 24). Third, we have recently shown that DBP can bind RNA both in vivo and in vitro (V. Cleghon and D. F. Klessig, unpublished data). Finally, our preliminary studies with actinomycin D, an inhibitor primarily of RNA transcription, indicate that the DBP globular structures undergo partial breakdown upon addition of this drug during the late phase of infection (K. Voelkerding and D. F. Klessig, unpublished results). Thus, the globular structures may also serve as sites of late viral gene expression. Moreover, recent work has shown that a proportion of actively replicating viral DNA is associated with late-phase structural proteins, suggesting that it is simultaneously being packaged (49). If the DBP globular structures represent adenovirus replication complexes, such structures might then also be potential sites for virion maturation.

Our studies have focused on a portion of the infectious cycle during which DBP is localized predominantly to the nucleus as determined by IF microscopy and can be delin-

eated into two distinct subclasses by in situ cell fractionation. However, at other times cytoplasmic DBP staining is evident, particularly very late in the infection (beginning 36 to 40 h p.i.) (46; Wolfgang Deppert, personal communication, and our own work). This represents a potential third DBP population. Each of these major subclasses of DBP may ultimately prove to be heterogeneous since 10 or more species of DBP are readily distinguishable by two-dimensional gel analysis and probably represent different phosphorylated forms of the protein (21, 29). Furthermore, the partial release of DBP by intermediate NaCl concentrations (0.3 to 1.0 M) is consistent with either the protein or its association in the globular structures being complex in nature.

The ability to define and isolate two major nuclear subclasses of DBP will allow an examination of their biochemical properties. In addition, a framework has been established from which to begin a comparative analysis of the cellular locations and biochemical interactions of DBPs encoded by E2A mutants. Together these biochemical, cellular, and genetic approaches should provide a better understanding of how this protein performs its myriad of functions.

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