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# Isolation and Characterization of a Viable Adenovirus Mutant Defective in Nuclear Transport of the DNA-Binding Protein

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**The isolation and characterization of an adenovirus mutant, Ad5*dl*802r1, containing two independent deletions in the 72-kilodalton (kDa) DNA-binding protein (DBP) gene is described. The two deletions remove amino acids 23 through 105 of DBP, resulting in the production of a 50-kDa product. Expression of this truncated DBP was delayed 12 to 24 h compared with that of the 72-kDa protein produced by wild-type adenovirus type 5. The DBP was located primarily in the cytoplasm of infected cells, whereas the wild-type product was predominantly nuclear. Therefore, DBP appears to contain a nuclear localization signal within the deleted region. Ad5*dl*802r1 DNA synthesis, viral late gene expression, and virus production were all delayed 12 to 24 h and were approximately 10-fold lower than with wild-type adenovirus type 5. These phenotypic properties can be accounted for by the delay in synthesis and the inefficient accumulation of the 50-kDa DBP within the nucleus of infected cells. The truncated DBP also lacks the majority of amino acids which are phosphorylated in the normal protein. The loss of these phosphorylation sites does not appear to seriously impair the ability of the protein to carry out its functions.**

The multifunctional DNA-binding protein (DBP) of human adenovirus types 2 and 5 (Ad2 and Ad5, respectively) binds single-stranded DNA (64), the ends of double-stranded DNA (20, 55), and RNA (1, 15). It is encoded by early region 2A (E2A) (40) and is expressed at substantial levels both early and late during the infectious cycle (19, 39).

The protein is composed of 529 amino acids and has a molecular weight of 59,042 (36, 37); however, on sodium dodecyl sulfate (SDS)-polyacrylamide gels it has an apparent size of 72 kilodaltons (kDa). DBP is extensively phosphorylated at a number of serine and threonine residues (7, 29, 41, 42), and 11 of these sites have been tentatively mapped (3; R. S. Mann, Ph.D. thesis, New York University, New York, 1987). Although no function has yet been ascribed to this posttranslational modification, it may be required for DBP to perform its diverse roles. DBP accumulates predominantly in the nuclei of infected cells (59, 68), though late in infection a cytoplasmic subclass has been detected (16). Given its large size, DBP probably contains a nuclear localization (NL) signal, as has been shown for other proteins found in this intracellular compartment (for a review, see reference 17).

DBP is anisometric. Mild treatment with several proteases, including chymotrypsin, cleaves the molecule into two fragments: a carboxy-terminal (C-t) ~44-kDa fragment and a ~26-kDa amino-terminal (N-t) fragment (29, 55). The 44-kDa C-t fragment retains the ability to bind DNA and RNA (15, 29) and is able to complement an in vitro adenovirus DNA replication system which requires the addition of exogenous DBP (6, 61). The 26-kDa N-t portion of DBP contains all, or nearly all, of the protein phosphorylation sites (3, 29; Mann, Ph.D. thesis). Two stretches of basic amino acids resembling the NL signal of the simian virus 40 (SV40) large T antigen are also located in this portion of the molecule (37, 69). In addition, 21 of the first 101 amino acids of Ad2 DBP are proline, which may account for the anomalous migration of the protein on SDS-polyacrylamide gels.

DBP appears to directly or indirectly play several important roles during the viral infectious cycle. These include an essential role in the elongation and efficient initiation of viral DNA synthesis (21, 24, 65, 66; for a review, see reference 58), the regulation of viral early (8, 12, 13, 23, 43, 52, 53) and late (31) gene expression, and virion assembly (44). In addition, DBP has been implicated in cellular transformation (49) and adeno-associated virus replication (25, 50).

Most of the functions attributed to DBP are based on studies of viral mutants containing lesions in the E2A gene. These mutants can be grouped into four general classes (70). The first class consists of the temperature-sensitive (*ts*) mutants located in the C-t. The prototype mutant of this class, Ad5*ts*125 (*ts*125), produces an altered DBP which is thermolabile for binding to DNA and at nonpermissive temperatures (i.e., 39°C) is defective for viral DNA replication both in vivo (18) and in vitro (24). In addition, infections with *ts*125 at nonpermissive temperatures result in the overaccumulation of early adenovirus mRNAs (8, 12) and overproduction of DBP (13). Since overexpression of early genes compared with wild-type (WT) Ad5 occurs even in the presence of inhibition of DNA replication (12), the results imply that DBP has a second function, namely regulation of early viral genes, including autoregulation of its own gene.

The second class of mutants are revertants of *ts*125 and Ad5*ts*107 (*ts*107; an independently derived mutant containing the identical lesion found in *ts*125; 35). These revertants contain second-site mutations in the C-t portion of DBP which allow the virus to grow well on HeLa cells at 39°C. The role of DBP in virion assembly is suggested by the phenotype of one such revertant, R(*ts*107)202. This mutant produces apparently normal amounts of late viral structural proteins but is deficient at assembling virions in human 293 cells at nonpermissive temperatures (44).

The third class of E2A mutants, typified by Ad2hr400, produce altered DBPs which allow the adenovirus to grow productively in semipermissive monkey cells. The block to

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WT adenovirus growth in these cells is complex and includes a reduction in late viral gene transcription (26), aberrant processing of at least one late mRNA—that encoding the capsid fiber protein (5, 30)—and poor utilization of this mRNA in vivo (4). Ad2hr400 is able to overcome these blocks and multiplies as efficiently in monkey cells as in human cells. This class of mutants contain alterations in the N-t region of DBP (2, 10, 33, 36).

The fourth class consists of a series of E2A deletion mutants which are unable to grow on HeLa cells and are absolutely defective for viral DNA replication (48). One of these mutants, Ad5*dl*802 (*dl*802) contains a 242-base-pair (bp) deletion near the 5′ end of the DBP gene which causes a frameshift. The predicted E2A translation product encoded by *dl*802 should contain only the first 22 amino acids of DBP which are fused to 31 amino acids originating from the alternate reading frame. However, no detectable DBP-related protein is expressed during infections with this virus. While the steady-state levels of most early adenovirus mRNAs in *dl*802 infections were similar to those of WT Ad5, messages originating from the E2A region were significantly reduced. This result calls into question the presumptive role of DBP in repressing early gene expression and suggests that the protein may be a positive *trans*-acting regulator of its own gene.

In this report, we describe the isolation and partial characterization of a revertant of *dl*802 which is able to grow on HeLa cells. This revertant, Ad5*dl*802r1 (r1), restores the correct reading frame of the E2A gene of *dl*802 and represents a new class of viable DBP deletion mutants.

## 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, and 100 μg of penicillin per ml.

Ad5 was originally obtained from J. F. Williams. The construction of *dl*802, which contains a deleted *Kpn*E fragment from Ad2 within an Ad5 background, has been described elsewhere (48). However, the 242-bp deletion in *dl*802 which was previously reported (48) to fuse bp 64 to bp 307 of the DBP gene (the A of the initiating ATG is defined as bp 1) is reported here to fuse bp 66 to bp 309 of the gene. Both are equivalent at the nucleotide level; however, our current designation reflects that the predicted translation product maintains the WT amino acid (Arg) at position 22. Virus infections were performed at multiplicities of between 10 and 20 PFU per cell. After adsorption for 70 min at 37°C in phosphate-buffered saline, the cells were washed with phosphate-buffered saline and incubated in culture medium at 37°C.

**DNA sequence analysis.** DNA sequence analysis was performed by the dideoxy-chain termination method of Sanger et al. (54) with modifications described in the Sequenase booklet "Step-by-step protocols for DNA sequencing with Sequenase" (United States Biochemical Corporation). The *Bam*HI (Ad5 map unit 59.5)-to-*Eco*RI (Ad2 map unit 70.7) fragments of *dl*802 and r1 were cloned into pUC119 and pUC120 (67). Synthetic primers were used to sequence both strands of an approximate 300-nucleotide region around the site of the original deletion in *dl*802.

**In situ analysis of DBP and viral DNA.** In situ fractionation of cell monolayers, immunofluorescent microscopy studies using a monoclonal antibody (38-2) directed against the C-t of DBP, and in situ hybridization by using an Ad2 Patho-Gene identification kit (Enzo Biochem, Inc.) were carried out as described previously (68).

**Analyses of viral DNA synthesis.** Virus-infected HeLa cell monolayers were labeled for 4 h at 37°C with 50 μCi of [$^3$H]thymidine per 60-mm tissue culture dish at various times postinfection. Cells were washed once with phosphate-buffered saline and scraped in 0.2 ml of 10 mM Tris (pH 7.5)–1 mM EDTA. The suspensions were lysed on top of preformed 5 to 20% alkaline sucrose gradients as described elsewhere (11) with the exception that no CsCl cushion was used. After 16 h at 4°C, the gradients were centrifuged for 5 h at 150,000 × *g* and 4°C in a Beckman SW41 rotor. Aliquots (~0.5 ml) of the respective gradients were collected and subjected to trichloroacetic acid precipitation. Acid-precipitable counts were collected on glass fiber filters, dried, and analyzed by scintillation counting.

Analysis of adenovirus DNA synthesis by using a modified Hirt procedure to isolate low-molecular-weight DNA from infected cells has been described previously (47).

**Protein analyses.** [$^{35}$S]methionine labeling of proteins synthesized in vivo, immunoprecipitations, and immunoblotting of proteins were carried out as described previously (4, 32), with the substitution of 5% nonfat dry milk (Carnation) for 5% bovine serum albumin as a blocking agent in the immunoblot procedure. The molecular size of the r1 DBP was estimated by comparison with a set of $^{14}$C-labeled molecular weight markers (Amersham Corp.).

## RESULTS

**Isolation of *dl*802 revertants.** The E2A deletion mutant *dl*802 is completely defective for growth and plaque formation on HeLa cells (48). However, passage of *dl*802 on human cells resulted in the production (selection) of revertant viruses which had reacquired the ability to form plaques on HeLa cell monolayers. Plaques appeared 1 to 2 days later than those produced by WT Ad5 virus. Although they were initially very small (i.e. <1 mm), by 8 to 10 days postinfection the plaques were similar in size to those of the WT (~3 to 5 mm). Several of these revertants were plaque purified twice on HeLa cells and grown to high-titer stocks ($2 \times 10^9$ to $5 \times 10^9$ PFU/ml) for analysis. The initial characterization of one revertant, r1, is described below.

**r1 produces a 50-kDa DBP-related protein.** The 242-bp deletion in *dl*802 is located near the 5′ end of the DBP gene which not only removes ~80 amino acids from the N-t region of the protein but also causes a frameshift mutation. *dl*802-infected HeLa cells do not produce a detectable DBP-related protein (48; Fig. 1). To determine whether the ability of r1 to grow on HeLa cells was due to restoration of the correct reading frame, r1-infected cells were examined for the presence of a DBP-related protein. HeLa cells were infected with Ad5, *dl*802, or r1 virus and [$^{35}$S]methionine-labeled proteins synthesized at various times postinfection were analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A new DBP-related protein of ~50 kDa was present in r1 infections but absent in mock, *dl*802, and Ad5 infections (Fig. 1). That this 50-kDa product was indeed related to DBP but lacks amino acids in the N-t region was supported by the observation that it could be immunoprecipitated with monoclonal antibodies (37-3, 38-2) directed against the C-t portion but not with monoclonal antibodies (16-5, 18-9) directed against the N-t portion of the WT protein (data not shown).

The kinetics of synthesis of the r1 50-kDa DBP was strikingly different from that of the WT protein (Fig. 1). The

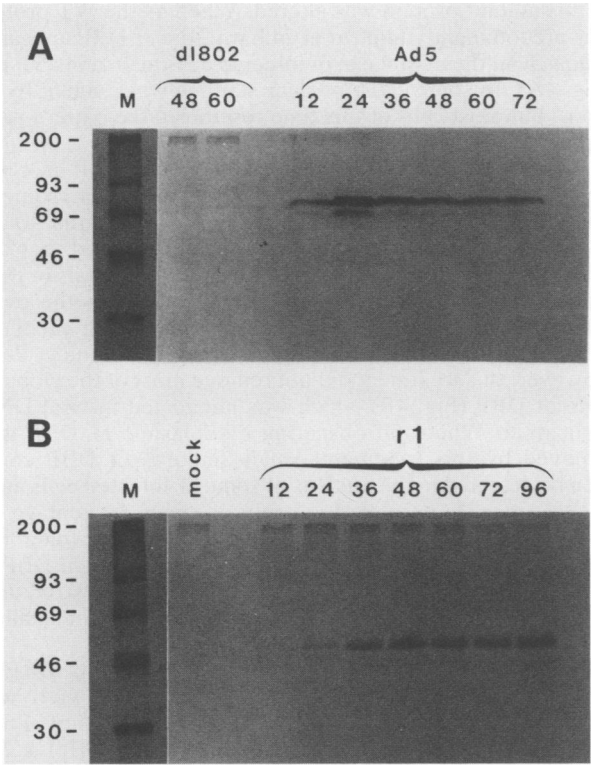


FIG. 1. Synthesis of Ad5 and r1 DBPs in infected HeLa cells. Confluent monolayers were infected with Ad5, *dl*802 (A), or r1 (B). Synthesis of DBP at various times p.i. was determined by labeling with 100 μCi of [$^{35}$S]methionine per 60-mm tissue culture dish for 2 h. Aliquots of each labeled cell lysate were immunoprecipitated with polyclonal anti-DBP serum and fractionated by using SDS-PAGE (15% polyacrylamide gel). Times after infection (in hours) are shown above each lane. Mock-infected cells were included as a control. The sizes (in kilodaltons) of $^{14}$C-molecular size standards (labeled M) run on these same gels are indicated to the left of each panel.

WT protein could be seen as early as 6 to 9 h postinfection (p.i.) (68), and in the experiment shown the Ad5 72-kDa DBP was clearly discernible at 12 h p.i. Synthesis then peaked at 24 h p.i., after which DBP production declined slightly. In contrast, synthesis of the r1 DBP was first detected at 24 h p.i. and reached a plateau by 36 h p.i. Although DBP synthesis in r1 infections was initially delayed compared with that in Ad5 infections, by ~36 h p.i. it was approximately equal to that in Ad5 infections.

Accumulation of the DBPs produced by r1 and Ad5 were compared by Western blot (immunoblot) analysis (Fig. 2). The WT protein was detectable at 12 h p.i., and its accumulation reached a plateau by 24 h p.i. The accumulation of the r1 DBP was delayed 12 to 24 h. Trace amounts of r1 DBP were first detected at 24 h p.i. (not readily seen in Fig. 2), after which its level increased up to 48 h p.i. The amount of Ad5 and r1 DBP which had accumulated by 48 h p.i. did not change significantly at later times (i.e., even after 72 h p.i.; data not shown). Peak accumulation of r1 DBP was reduced ~5- to 10-fold compared with the WT protein maximum level.

The apparent differences in kinetics and rates of 50-kDa protein synthesis and accumulation compared to WT DBP could be due to differences in the ability of the polyclonal anti-DBP serum to recognize the intact, WT DBP versus the deleted, 50-kDa DBP-related protein. To address this, DBP synthesis and accumulation experiments were repeated with monoclonal antibodies, 37-3 and 38-2, which are directed against the C-t half of the DBP; this C-t domain is shared with the 50-kDa protein. Identical results were obtained with the monoclonal and polyclonal antibodies (data not shown). These results are consistent with the conclusion that both the synthesis and accumulation of the r1 50-kDa DBP were delayed 12 to 24 h compared with that of the Ad5 WT protein and that the 50-kDa DBP failed to reach the high levels found in WT-infected cells.

**DNA sequence of r1.** The 242-bp deletion in *dl*802 fuses bp 66 to bp 309 of the WT DBP gene (the A of the initiating ATG is defined as bp 1). To examine the DBP gene of the r1 virus in greater detail, the *Bam*HI-*Eco*RI fragment (59.5 to 70.7 WT map units) was isolated from purified viral DNA, cloned into pUC vectors, and partially sequenced. *dl*802 was treated in a similar manner both for comparative reasons and because the reported sequence for this mutant was determined from a plasmid construct rather than from the intact virus. While both viruses contained the 242-bp deletion previously reported for *dl*802, the r1 DNA was found to

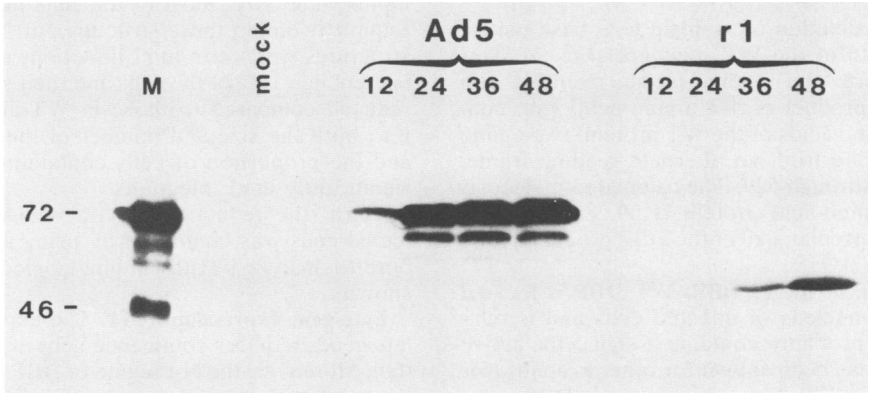


FIG. 2. Accumulation of Ad5 and r1 DBPs in infected HeLa cells. Accumulation of DBP at various times p.i. was determined by immunoprecipitating equal fractions of infected cell lysates with anti-DBP serum, fractionating the products on an SDS-10% polyacrylamide gel, and subjecting this gel to Western blot analysis. Times after infection (in hours) are shown above each lane, and the infecting virus is indicated. Purified DBP containing a number of proteolytic subspecies was run in lane M as a standard and the size (in kilodaltons) of the intact protein as well as its major breakdown product are indicated to the left of the blot.

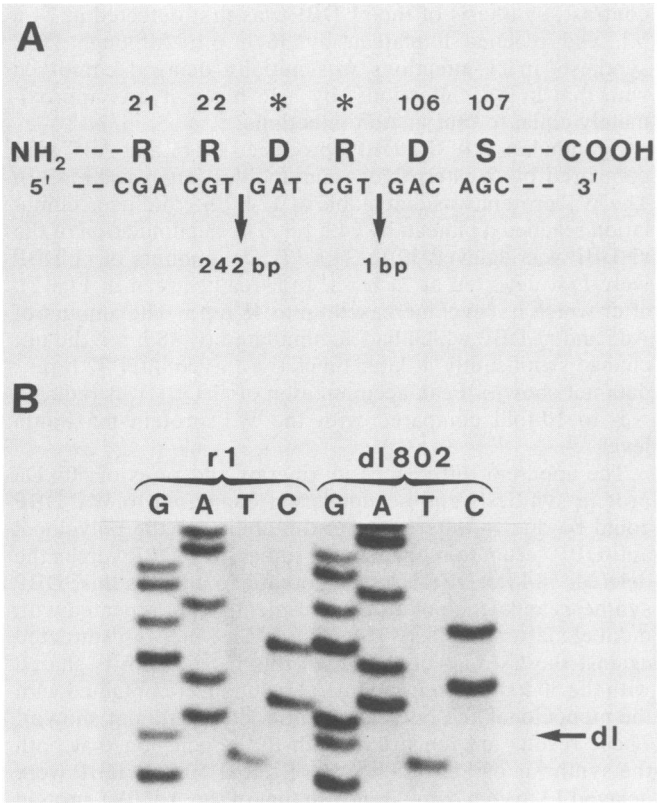


FIG. 3. DNA and amino acid sequence of r1. (A) Representation of the DNA sequence and predicted translation product of the region surrounding the sites of the deletions in the DBP gene of r1. The top line indicates the amino acid residue number of the WT DBP. *, Two amino acids encoded by r1 because of the use of an alternate reading frame. The second line is the single-letter code for the actual amino acids encoded by the mutant virus. The third line is the nucleotide sequence of the DNA-coding strand which we determined. The location of the 242-bp deletion found in r1 and its parent virus *dl*802, as well as the 1-bp deletion found only in the revertant, is indicated by arrows. (B) Sequencing gel comparing r1 with its parent *dl*802; the gel is read 5′ (bottom) to 3′ (top). The dideoxynucleotide added to the respective sequencing reactions are indicated above each lane. The position of the G residue which has been deleted (dl) from the revertant and which restores the correct reading frame of the gene is indicated by an arrow.

contain an additional deletion of a single G-C base pair at nucleotide position 315 of the WT sequence (Fig. 3). This second deletion restores the reading frame, resulting in a predicted translation product of 448 amino acids that consists of the first 22 amino acids of the WT protein, two amino acid residues originating from an alternate reading frame, and WT residues 106 through 529. The calculated molecular weight of this 448-amino-acid protein is 50,280, which is consistent with the molecular size of the DBP produced in r1 infections (Fig. 1 and 2).

**Intracellular location of the r1 DBP.** WT DBP is located predominantly in the nucleus of infected cells and is relatively large. Thus, it probably contains a signal for active nuclear transport, as has been shown for other proteins (for a review, see reference 17). Two regions in the DBP, near amino acids 43 and 85 (37, 69), resemble the NL signal identified in the SV40 T antigen (27, 38). Since both of these potential NL signals are deleted in r1, immunofluorescence (IF) was used to determine whether the intracellular location of this mutant protein was altered. Whereas the WT protein was predominantly nuclear at 24 h p.i., the r1 DBP appeared primarily in the cytoplasm of infected cells at 36 and 48 h p.i. (Fig. 4A). In some cells, nuclear staining was evident by 48 h p.i., but most cells at this time resembled the pattern at 36 h p.i.

Adenovirus DNA is replicated in the nuclei of cells. Because DBP is essential for viral DNA replication, some r1 DBP must enter this compartment for the virus to be viable. Voelkerding and Klessig (68) demonstrated that different subclasses of DBP can be isolated on the basis of their different extractabilities in situ. DBP present in the cytoplasm of cells, as well as a diffuse-staining nuclear subclass, could be extracted with 150 mM NaCl and 1% Nonidet P-40. However, this treatment did not remove most of the globular form of DBP (Fig. 4B) which was implicated in viral DNA replication. When the obscuring cytoplasmic r1 DBP was removed by this treatment, small amounts of DBP could then be detected in the nucleus of some r1-infected cells at 48 h (Fig. 4B). (At 36 h p.i., only a small percentage of r1-infected cells exhibited this nuclear staining [data not shown].) In r1-infected cells, the extraction-resistant DBP in the nucleus was not found in the large globular structures evident in WT infections. Rather, it was present in smaller, less regularly shaped structures.

**Replication of r1 DNA.** The ability of the mutant DBP to participate in adenovirus DNA replication in vivo was demonstrated by three criteria. First, the synthesis of r1 and Ad5 DNA was compared by labeling infected HeLa cell monolayers with [$^3$H]thymidine at various times p.i. and analyzing the products on alkaline sucrose gradients. Detectable r1 DNA synthesis was delayed approximately 24 h (cf. Fig. 5A and 5B) and never reached the high levels seen with WT Ad5 (Fig. 5C). Even at late times (48 to 72 h p.i.) viral DNA replication in r1-infected cells was depressed (7- to 20-fold reduced) compared with that of WT-infected cells. As controls, cells infected with *dl*802 (Fig. 5D) or mock infected (data not shown) were analyzed in parallel. A small peak of labeled r1 DNA was observed above these control backgrounds.

Second, viral DNA replication in r1-infected cells was studied by using in situ hybridization (Fig. 6). This allows individual cells to be analyzed. The presence of viral DNA in globular structures (Fig. 6), similar to those observed for DBP by IF (Fig. 4A), was readily apparent in Ad5 infections at 24 and 48 h p.i. Although there was variation in the number and size, most of the cells infected with Ad5 were found to contain these structures at 24 h p.i. While similar structures were seen in r1 infections at 24 h p.i., they were present in <10% of the cells and their size and numbers were reduced compared to those in WT-infected cells. By 48 h p.i., both the size and number of these globular structures and the proportion of cells containing them had increased significantly in r1 infections.

Third, the reduction in viral DNA replication in r1-infected cells was confirmed by using a modified Hirt procedure to analyze [$^3$H]thymidine-labeled viral DNA (data not shown).

**Late gene expression by r1.** The expression of adenovirus late genes requires commencement of viral DNA replication (60). Moreover, the N-t region of DBP has been implicated in the regulation of late-gene expression (4, 5, 10, 26, 31). Since the r1 DBP is missing a significant portion of this region of the protein, the expression of viral late proteins by the mutant was compared with that of Ad5.

Infected cells were labeled with [$^{35}$S]methionine at various

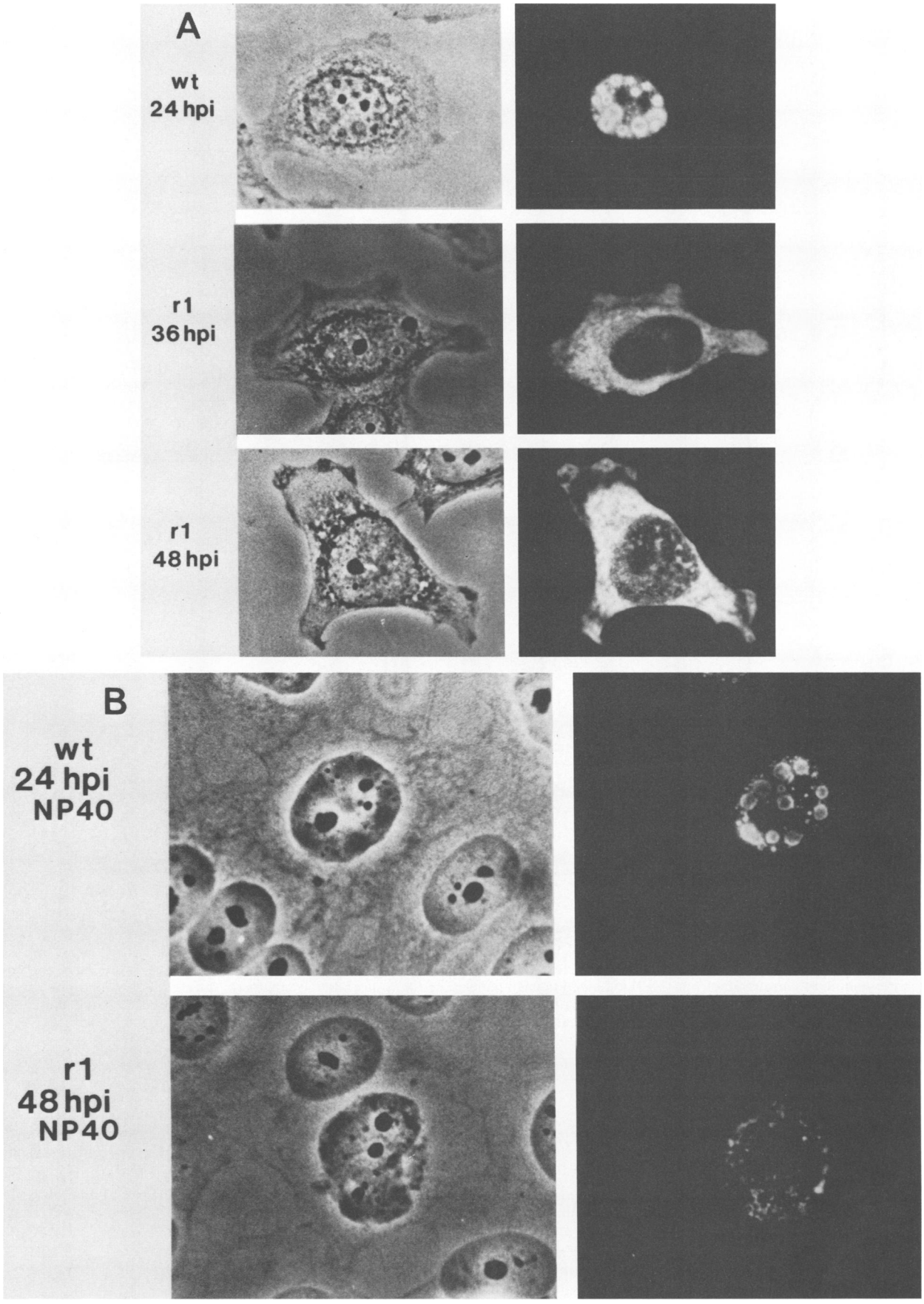


FIG. 4. Cellular distribution of DBP in Ad5 and r1 infections of HeLa cells. Infected cells grown on glass cover slips were fixed at the time shown and sequentially reacted with a monoclonal antibody (38-2) directed against the C-t of DBP, followed by fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G. The left panels in A and B are phase images of infected cells, and the corresponding right panels are these same cells visualized by IF showing the location of DBP. Panel A, Infections with Ad5 (wt) or r1; panel B, phase images and IF analyses of DBP in Ad5 (wt) and r1 infections after in situ extraction with 1% Nonidet P-40–150 mM NaCl. Magnification, ×400.

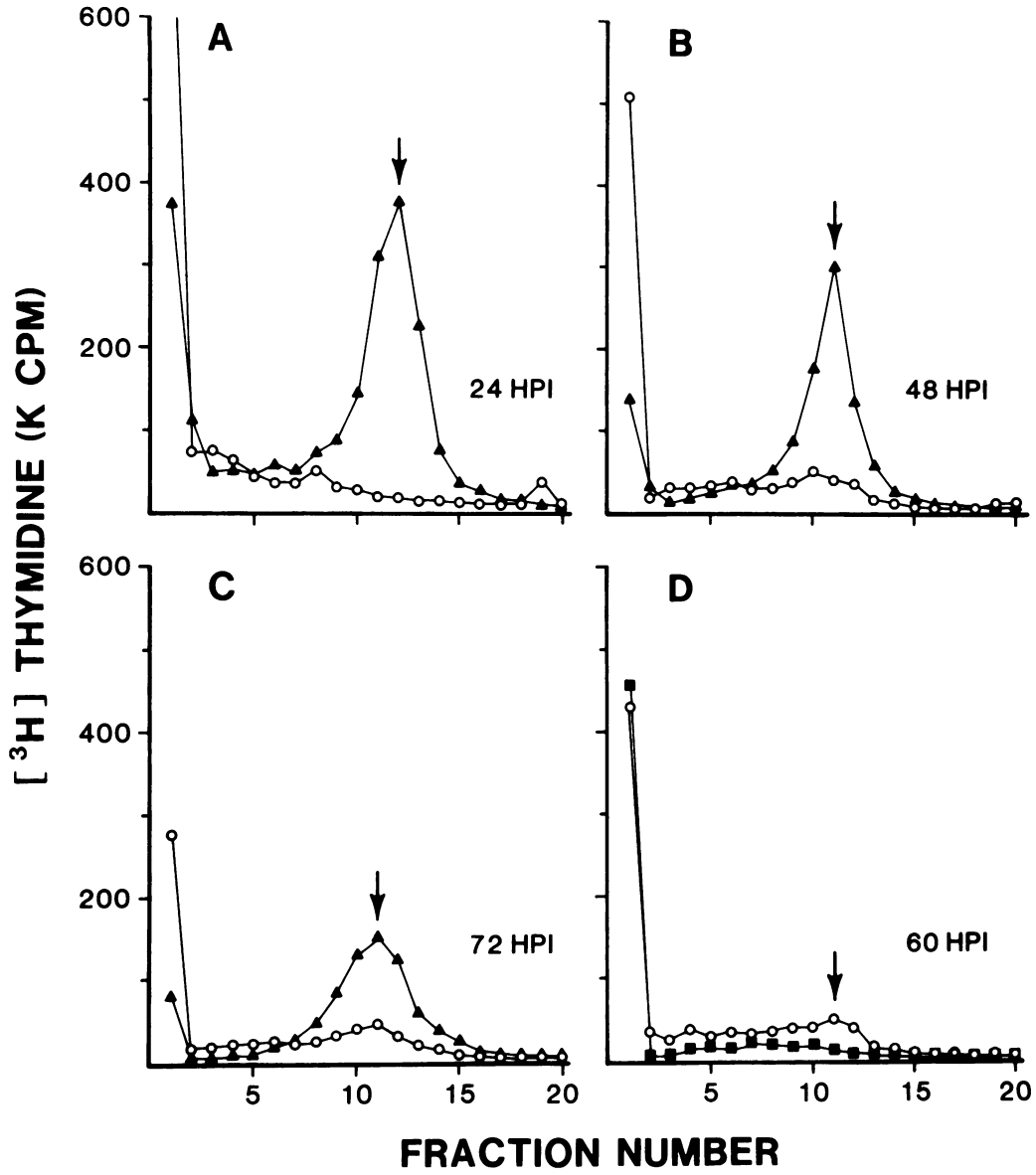


FIG. 5. DNA synthesis in Ad5 and r1 infections of HeLa cells. Confluent monolayers were infected with the respective virus and labeled with [$^3$H]thymidine at various times p.i. (HPI) indicated in each panel. The times indicated are midpoint during the label. Labeled products were then analyzed by alkaline-sucrose gradient centrifugation. Trichloroacetic acid-precipitable counts from fractionated gradients are shown. The bottom of the gradients are to the left of each graph. The position of viral DNA is indicated by an arrow in Ad5 (▲)-, r1 (○)-, or *dl*802 (■)-infected cells. Panel D includes as a control a gradient profile from *dl*802-infected cells.

times p.i., and the newly synthesized proteins were analyzed by SDS-PAGE (Fig. 7A). Ad5-infected cells produced large amounts of late viral proteins by 24 to 72 h p.i. In contrast, only low levels of late-gene expression was observed in r1-infected cells at 48 h p.i., after which the level of synthesis of late viral proteins increased steadily up to 96 h p.i. (the last time point at which a measurement was taken). One of the late proteins, fiber, was immunoprecipitated from these same labeled cell lysates and subjected to SDS-PAGE (Fig. 7B). The expression of fiber in r1 infections could not be detected at 24 h p.i. (data not shown) and was reduced ~10- and 5-fold at 48 and 72 h p.i., respectively, compared with levels in Ad5 infections at these same times (Fig. 7B). The synthesis of this protein in r1-infected cells at 96 h p.i. was comparable to that seen in Ad5 infections at 24 h p.i.

**Production of infectious r1 virus.** To determine whether the reduction or delay in r1 DNA replication and late-gene expression affected virus production, the growth curves of Ad5 and r1 were compared. The appearance of infectious r1 progeny was delayed ~24 h compared with that of Ad5 (Fig. 8). The rate of accumulation of r1 virus was also slower and the final yield (burst size) was 7- to 15-fold lower than for Ad5.

## DISCUSSION

In this report, we have described the isolation and partial characterization of an adenovirus mutant, r1, which contains two independent deletions of 1 and 242 bp within the E2A gene of the virus (Fig. 3). The larger deletion, which removes

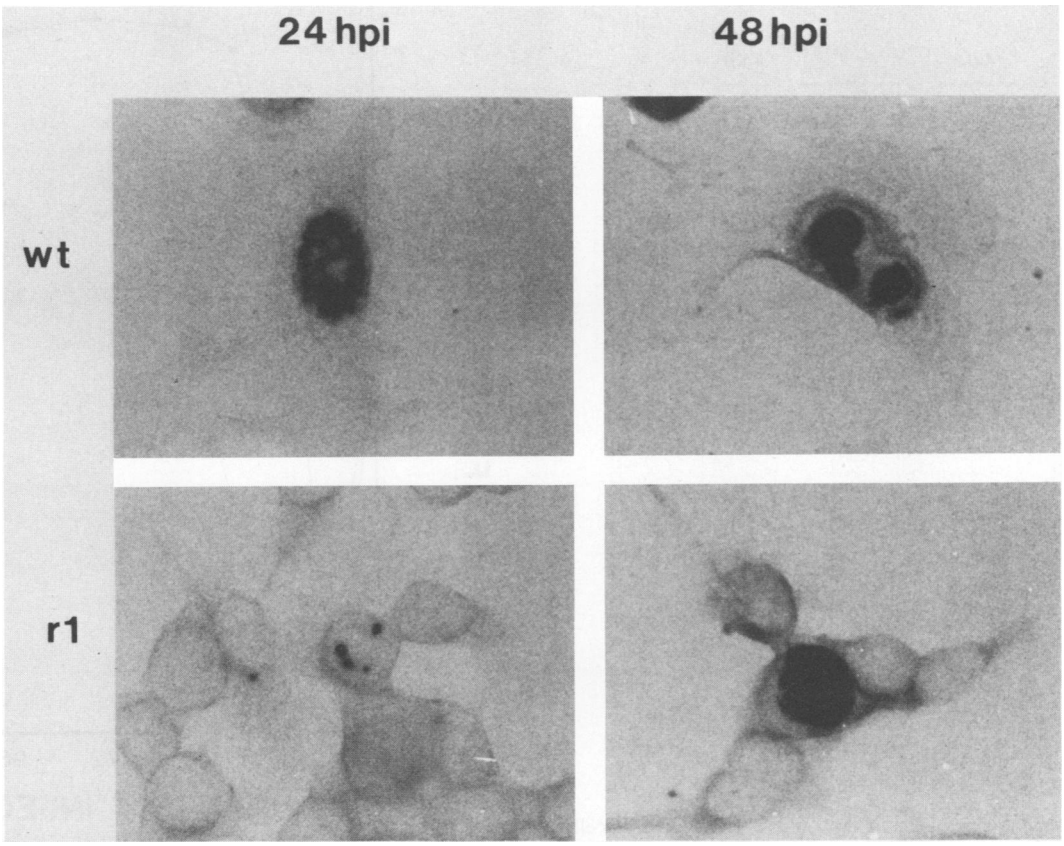


FIG. 6. Localization of viral DNA during Ad5 (wt) or r1 infections of HeLa cells. Infected cells grown on glass cover slips were analyzed by in situ hybridization using a biotinylated Ad2 probe. The reaction product, a dark brown precipitate, denotes the hybridization signal. Magnification, ×200.

bp 67 through 308 of the gene and causes a frameshift mutation, was generated in vitro by using the exonuclease Bal 31 to construct the virus *dl*802 (48). *dl*802 produces no detectable DBP-related protein and is therefore unable to replicate its DNA or to grow on HeLa cells. The second deletion of 1 bp (bp 315 of the WT gene) occurred spontaneously during passage of *dl*802 on HeLa cells and (i) restores the correct reading frame of the E2A gene, (ii) leads to the expression of a DBP-related protein having an apparent size of ~50 kDa (Fig. 1B), and (iii) restores the ability of the virus (r1) to grow on HeLa cells (Fig. 8). The net effect of these two deletions is to remove the WT DBP amino acids 23 through 105 (Fig. 3).

The loss of amino acids 23 to 105 from WT DBP appears to have a dramatic effect on the structure of this molecule. The molecular weight of the WT protein predicted from its sequence is 59,042, (36, 37) but its molecular size on SDS-polyacrylamide gels is 72 kDa. Phosphorylation increases the molecular size from 70 to 72 kDa, as shown by pulse-chase experiments and in vitro dephosphorylation of purified protein (29, 42). A likely explanation for the unusual gel mobility of the WT protein is its high proline content, which could result in a coiled, nonglobular structure. The N-t region of the WT protein is especially rich in this amino acid; 21 of the first 101 residues of Ad2 DBP are proline. The r1 DBP lacks 20 of these proline residues and has a molecular size (~50 kDa) similar to the size predicted by its sequence (50,280).

The deletion in r1 removes 9 of the 11 tentatively mapped phosphorylation sites of the DBP (3; Mann, Ph.D. thesis). Nonetheless, the r1 DBP was phosphorylated (data not shown). The significance of DBP phosphorylation, however, is difficult to address, as no function has yet been attributed to this modification. Studies of other phosphoproteins, for example the large T antigen of SV40 (56, 57) and the L polypeptide of the vesicular stomatitis virus (14), have demonstrated that certain phosphorylated amino acids are essential for these proteins to fulfill their respective roles, whereas other sites appear to be dispensible. Whether or not DBP also contains essential phosphorylation sites cannot be determined from our study, but it would appear that those presumptive phosphorylation sites which are deleted from the r1 DBP are not critical for its functions in cell culture.

WT DBP was found predominantly in the nuclei of infected cells, while the r1 protein was primarily cytoplasmic (Fig. 4). This strongly suggests that the WT molecule contains a NL signal. This signal is presumably located between amino acids 23 to 105 of the WT protein, since this is the region which is missing from the r1 DBP.

NL signals have been defined for a number of polypeptides (for a review, see reference 17). They generally consist of a short stretch of basic amino acids. Two regions of DBP located near amino acid residues 43 and 85 of the WT protein have this property and might serve as NL signals. The DBP sequence beginning at amino acid 85 of the WT Ad2 protein is Lys-Lys-Lys-Lys-Lys-Arg-Pro. This sequence closely resembles the NL signal identified in the SV40 T antigen, Pro-Lys-Lys-Lys-Arg-Lys-Val (27, 38). In addition, this region is highly conserved in DBPs produced by all adenovirus serotypes sequenced to date (for a review, see refer-

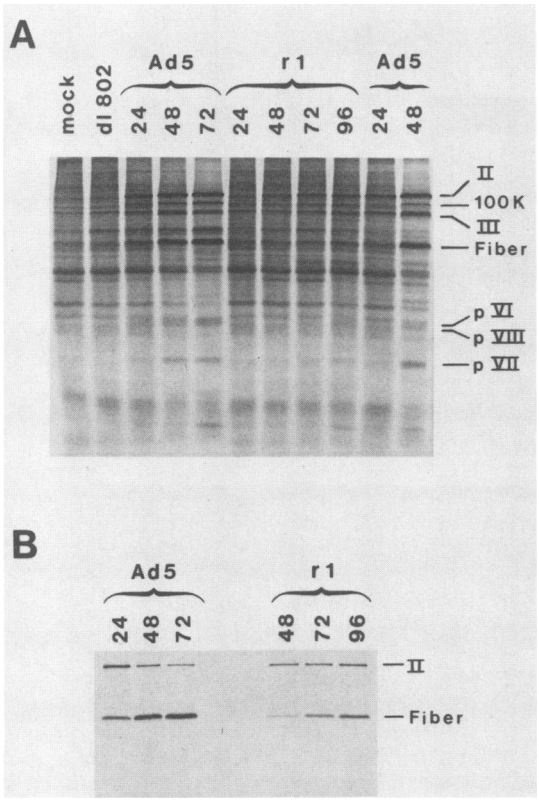


FIG. 7. Late gene expression in Ad5- and r1-infected HeLa cells. Confluent monolayers were infected with Ad5, *dl*802, or r1. At various times p.i., cells were labeled with 100 μCi of [$^{35}$S]methionine per 60-mm culture dish for 2 h. Labeled proteins were extracted and analyzed directly by SDS-PAGE (15% polyacrylamide gel) (A) or were subjected to immunoprecipitation using polyclonal anti-fiber serum prior to gel electrophoresis (B). The time p.i. (in hours) when label was added and the infecting virus are indicated above each lane. The position of several late viral proteins are shown to the right of the autoradiogram. The anti-fiber serum also reacted with hexon (II). In contrast with that of fiber, the immunoprecipitation of hexon was not quantitative and thus can not be used as an indicator of viral late gene expression.

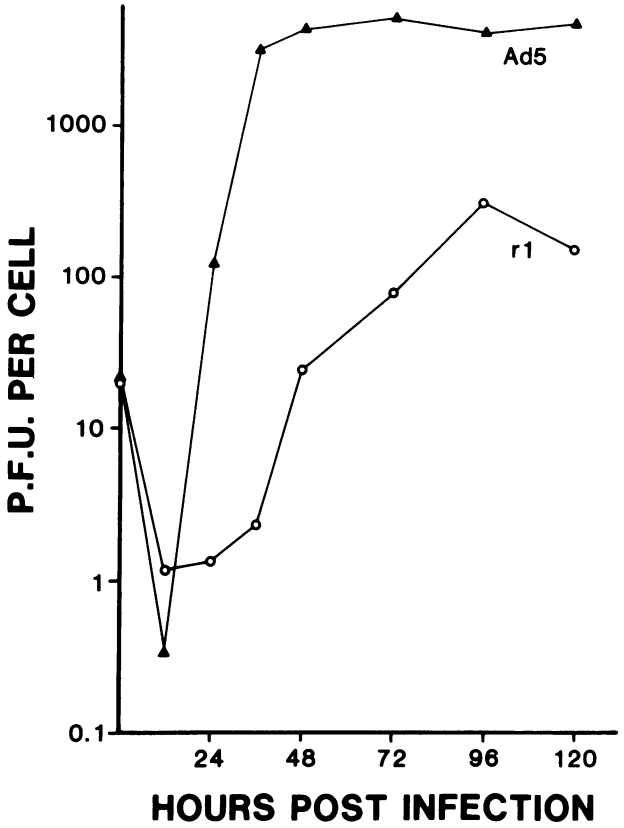


FIG. 8. Growth curves of Ad5 (▲) and r1 (○) on HeLa monolayer cells. Infected cells were harvested at the various times shown. Virus yield was determined by titration of the cell lysates on HeLa monolayer cells.

ence 69). The first three lysines (amino acids 85 to 87) are completely conserved, and five out of seven amino acids in this region are always the basic residues lysine or arginine. In contrast, the majority of the N-t portion of DBP is highly divergent between serotypes (69), suggesting that this conserved sequence plays an important function, perhaps in nuclear transport. More importantly, we have found that alterations of this sequence affect nuclear localization (N. Morin and D. F. Klessig, unpublished data). A second potential DBP NL signal, Pro-Lys-Lys-Arg-Leu-Arg-Arg-Arg, is located between amino acids 43 and 50. This region is not highly conserved in different adenovirus serotypes but shows some resemblance to the SV40 T antigen signal and contains a high proportion of basic residues. Several other proteins, including the polyomavirus large T antigen, have been shown to contain two NL signals (22, 34, 45, 51). Our results are consistent with one or both of these regions being involved in active transport of DBP into the nucleus.

Despite the obvious defect in r1 DBP nuclear localization, some of the protein was able to enter this compartment (Fig. 4B). Globular proteins having a molecular size less than 60 kDa apparently are able to passively diffuse through pores into the nucleus at rates proportionate to their size (9; for a review, see reference 17). The r1 DBP has a molecular size of ~50 kDa and therefore may be able to passively enter the nucleus of an infected cell (albeit at a slow rate). A less likely possibility is that the r1 DBP still retains a weak NL signal which allows for inefficient active transport of the mutant protein.

The large quantity of DBP produced during WT adenovirus infections and the amount of DBP molecules bound in vitro to single-stranded DNA (1 per 7 to 20 bp; 55, 62, 63) suggest that the protein is required in stoichiometric amounts to fulfill its role in DNA replication. Thus, it was surprising that the small amount of r1 DBP in the nucleus was sufficient to allow moderate levels of DNA synthesis. The accumulation of r1 DBP is reduced 5- to 10-fold compared with that of the WT protein. While this reduction is similar to the decrease in viral DNA replication, late-gene expression, and virus yield, most of the r1 DBP is located in the cytoplasm. By IF, it appears that, at most, only 10% of r1 DBP is found in the nucleus. Thus, the reduction of nuclear DBP may be 50-fold or more compared with that of WT infections. Since DNA synthesis was only reduced 5- to 10-fold, this implies that DBP in WT infections is produced in excess.

The phenotype of the r1 virus can be explained in large part by the inability of its 50-kDa DBP to efficiently localize to the nucleus. The slow entry of this protein into the nucleus, coupled with its lowered level of synthesis and accumulation, could both delay the onset and reduce the amount of viral DNA replication (Fig. 5). Since adenovirus

late-gene expression requires viral DNA replication (60), this would also be delayed (Fig. 7). Additionally, the lower number of viral DNA templates should lead to less late viral mRNA. The delay and reduction in the synthesis of capsid proteins (products of the late viral mRNAs), together with the depression in viral genomes, would then lead to a similar delay and reduction in the production of infectious virus (Fig. 8). In general, our results are consistent with this model. Viral DNA replication, late-gene expression, and virus yield were all delayed approximately to the same extent, and the final levels of each of these virus products were similarly reduced.

However, one aspect of the r1 infectious cycle which is difficult to explain by this model was the delay in expression of the viral DBP (Fig. 1). This delay is not easily accounted for by the retardation of viral DNA replication, since DBP is expressed prior to replication. We reported previously that the steady-state E2A mRNA levels in *dl*802 were reduced approximately fivefold compared with that of the WT virus (48). Although we have not confirmed that the delay in r1 DBP expression is due to a reduction in steady-state mRNA levels, it is conceivable that poor early expression of the E2A gene in both viral mutants is caused by a similar mechanism. This defect may be due to the 242-bp deletion within this gene which acts in *cis* to either destabilize the message or disrupt efficient transcription of the E2A region. However, in light of recent results with a second revertant, Ad5*dl*802r2 (r2), this explanation for poor early expression of the DBP gene in *dl*802 and r1 is unlikely. r2 contains the same 242-bp deletion found in r1 but restores the correct reading frame of the DBP gene via a more complex mutation (unpublished results). The mutation in r2 appears to have fortuitously regenerated a NL signal in the protein, enabling it to enter the nucleus more efficiently than the r1 product. If the retardation in r1 DBP synthesis was due to a *cis*-acting defect in the E2A gene or its mRNA, then both *dl*802 revertants should exhibit a similar delay. In contrast, the kinetics of expression of the r2 DBP was more similar to that of WT Ad5 than to that of r1.

A second explanation for the reduced E2A message level of *dl*802 was that DBP *trans* activates its own gene (48). If, as expected, *trans* activation occurs in the nucleus, then the failure of r1 to efficiently express its DBP early would correlate with the proteins slow entry into this compartment. Experiments are in progress to directly address this possibility.

What is the function of the N-t domain of DBP? Genetic analysis suggests that it is necessary for proper expression of the late viral genes, at least in monkey cells (4, 5, 10, 26, 31). However, loss of approximately half of this domain in r1 (amino acids 23 to 105) does not appear to seriously impair this function in HeLa cells. The ~10-fold reduction in late-gene expression can be accounted for by the depression in r1 DNA replication, presumably resulting from the delayed synthesis and failure of the r1 DBP to efficiently nuclearly localize. This region also does not appear to be essential for viral DNA replication in vivo, consistent with previous in vitro studies (6, 61). Depression of DNA synthesis can again be readily explained by r1 DBP retarded synthesis and transport to the nucleus. The small amount of mutant DBP which enters the nucleus appears to efficiently participate in viral DNA replication.

In addition to the NL signal(s) contained in the N-t domain and presumably located in the deleted region of r1 (amino acids 23 to 105), other segments of this domain are likely to be important for viral growth on HeLa cells. The very N-t portion of the molecule may be such an area. Both of the *dl*802 revertants (r1 and r2) restore the correct DBP reading frame via a second deletion 3′ to the original 242-bp deletion, thus leaving the first 22 amino acids of the WT protein intact. Attempts to construct a DBP mutant missing amino acids 2 to 40 of the WT protein on human 293 cells have thus far been unsuccessful (H. L. Vos, D. Brough, D. F. Klessig, and J. S. Sussenbach, personal communication). We also have been unable to isolate viable revertants of the completely defective mutant Ad5*dl*801 (unpublished results). This mutant is similar to *dl*802 except that it contains a larger deletion of 313 bp which fuses bp 22 of the DBP coding region to bp 336 (48) and, like *dl*802, also causes a frameshift mutation. This deletion effectively removes the WT amino acids 8 through 112 of the protein. Therefore, the genetic information needed to encode amino acids 8 to 22 and 106 to 112 are absent from *dl*801 but are present in r1. Both of these regions contain amino acids which are conserved among DBPs from various adenovirus serotypes (28, 46, 69). The revertant r2, however, does not restore the correct DBP reading frame until amino acid 131 (WT protein residue number). These observations suggest that the first 22 amino acids of DBP may be required in some way for the protein to perform its essential roles.

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