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Adenovirus E4-Dependent Activation of the Early E2 Promoter Is Insufficient To Promote the Early-to-Late-Phase Transition

CATHARINA HEMSTRÖM,[1] ANDERS VIRTANEN,[1] EILEEN BRIDGE,[1] GARY KETNER,[2] AND ULF PETTERSSON[1]*

Department of Medical Genetics, University of Uppsala, Biomedical Center, S-751 23 Uppsala, Sweden,[1] and Department of Immunology and Infectious Diseases, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205[2]

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The adenovirus E4 ORF6/7 protein has been shown to activate the cellular transcription factor E2F. E2F activation leads to activation of the adenovirus early E2 promoter which controls the production of viral DNA replication proteins. In the present study an adenovirus type 5 cDNA mutant, H5*il*E4L, was constructed. This mutant is capable of making the ORF6/7 polypeptide but lacks the coding sequences for all other E4 products. H5*il*E4L *trans* activates the early E2 promoter to wild-type levels, but still it is defective for viral DNA replication. A mutant expressing ORF6 in addition to ORF6/7, H5*il*E4I, is normal for viral DNA replication. This indicates that activation of the early E2 promoter is insufficient to promote efficient viral DNA replication and that another E4-encoded function is necessary. The ORF6 protein seems to provide this function. We suggest that ORF6/7-induced activation of E2F is not necessary for adenovirus growth in HeLa cells. Rather, this activation might be of importance in the normal, growth-arrested host cell, since E2F has been shown to bind to the promoter regions of a number of immediate-early genes involved in regulation of cell proliferation (M. Mudryj, S. W. Hiebert, and J. R. Nevins, EMBO J. 9:2179–2184, 1990).

Early region 4 (E4) of adenovirus type 5 (Ad5) is located between map units 91 and 100 on the viral genome and is transcribed leftward (33, 44). Genetic analyses have demonstrated a requirement for E4 products during adenovirus infection. Mutants with large E4 deletions, such as H2*dl*808 (52) and H5*dl*366 (13), display complex phenotypes including defects in viral DNA replication, late gene expression, and shutoff of host cell macromolecular synthesis. These mutants can only be propagated on the complementing W162 cell line (51).

A complex set of differentially spliced mRNAs is generated from region E4 during viral infection (10, 48, 49), and the nucleotide sequence reveals seven open translational reading frames (ORFs) (11, 18). Attempts to define roles for specific E4 products have identified the products of ORFs 6 and 3 as individually capable of rescuing viral growth to normal or near-normal levels, respectively (4, 17, 20).

So far only three E4-encoded polypeptides have been identified in infected cells. A 14-kDa polypeptide is the product of ORF3 (9, 43). This protein is associated with the nuclear matrix. ORF6 encodes a 34-kDa nuclear protein (42) which is physically associated with the 55-kDa product of early region 1b (E1b) (42). This complex is thought to function as a unit, since E4 34-kDa mutants and E1b 55-kDa mutants show similar phenotypes (5, 8, 35). Finally, a fusion between ORFs 6 and 7, created by mRNA splicing (ORF6/7), encodes a 19.5-kDa polypeptide (8). This protein, which is found in the nuclei of infected cells, is responsible for the *trans* activation of the early region 2 (E2) promoter (21, 27, 31, 36). The E2 transcription unit, which is responsible for the production of viral DNA replication proteins, is activated during the early phase of infection as a result of an activation of the cellular transcription factor E2F. This activation has been reported to require the products of regions E1a and E4 (1, 14, 36, 38). E2F binds to two adjacent sites in the early E2 promoter, and these sites have been shown to be important elements for basal and E1a-induced expression from this promoter (22, 24, 26, 30, 55). During infection, E2F is modified to a form which binds cooperatively to the early E2 promoter region (14, 15, 53). The E4 19.5-kDa-mediated activation of E2F has been shown to involve a direct physical interaction between these two proteins. This interaction probably results in the formation of a stable complex involving two adjacent E2F factors, thereby enhancing the cooperative interaction of the E2F molecules with the early E2 promoter binding sites (21, 27, 31, 36).

We reported previously on a mutant, H5*il*E4I, encoding mRNAs corresponding to ORF6 and ORF6/7 proteins (17). This mutant multiplied normally in noncomplementing HeLa cells and lacked most of the phenotypic properties associated with H2*dl*808. We have now constructed a mutant, H5*il*E4L, in which region E4 is substituted for by a cDNA only capable of encoding the ORF6/7 mRNA. This mutant shows growth defects similar to those of H2*dl*808, including a severe defect in viral DNA replication. Interestingly, mutant H5*il*E4L *trans* activates the early E2 promoter to wild-type levels. This indicates that normal activation of the promoter which controls the production of viral DNA replication products is not sufficient to promote efficient viral DNA replication. Thus, an additional function is required to regulate viral DNA replication. Since addition of the ORF6 product results in normal viral growth, this protein is likely to be involved in this process.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of W162 and HeLa cells were maintained in Dulbecco modified medium containing 10% newborn calf serum or 10% fetal calf serum,

* Corresponding author.

respectively. Suspension cultures of HeLa cells were grown by the method of Halbert et al. (13).

The structures of mutant viruses are shown in Fig. 1. H2*dl*808 (*dl*808) and H5*il*E4I (*il*E4I) are previously described E4 mutants (17, 52). Plasmid pilE4L contains a reconstructed *Eco*RI-B fragment of Ad5, in which the *Taq*I fragment of region E4 was replaced by a cDNA representing mRNA L of Ad2 (clone 101 [49]). To create a mutant that expressed only this E4 product, pilE4L was introduced into Ad5 by $CaPO_4$ transfection (12) of W162 cells as described by Bridge and Ketner (4). Triply plaque-purified stocks of mutant H5*il*E4L (*il*E4L) were established. The structure of the virus was verified by restriction enzyme digestion of purified mutant DNA.

Ad5 and *il*E4I virus were grown in HeLa cell suspension cultures, and *dl*808 and *il*E4L were grown in W162 cells. Viruses were purified as described before (17). Viral titers expressed as fluorescent focus units (FFU) were determined on monolayer cultures of HeLa and W162 cells as described by Philipson (34).

Viral infections. Monolayer cultures of HeLa cells or W162 cells were infected with wild-type or mutant viruses at a multiplicity of 5 FFU per cell based on titers determined on W162 cells.

DNA replication. Infected HeLa monolayer cells were pulse-labeled with [^{3}H]thymidine (1.0 mCi/ml; 80.7 Ci/mmol) 1 h before the cells were harvested. Viral DNA was extracted at various times after infection, using the protocol of Hirt (19). Purified DNA was digested with *Eco*RI and analyzed by gel electrophoresis followed by ethidium bromide staining and subsequent fluorography.

Protein preparation and analysis. Infected HeLa monolayer cells were pulse-labeled with [^{35}S]methionine (9.63 mCi/ml; 1,169 Ci/mmol) 30 min before harvest as described before (17). Protein extracts were prepared by IsoB-Nonidet P-40 extraction and analyzed by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (17). Extracts used for immunoprecipitation of the E2A-encoded 72-kDa DNA-binding protein (DBP) were prepared as described by Sarnow et al. (42). Immunoprecipitation reactions were performed by the method of Persson et al. (32), using a polyclonal antibody directed against the DBP (25). Western blot (immunoblot) analysis was performed as described by Harlow and Lane (16), using a polyclonal antibody which detects the adenovirus-specific DNA polymerase. Immunofluorescence (IF) microscopy was carried out at 24 h postinfection (p.i.) as described by Morin et al. (28), using the DBP antibody described above, or at 48 h p.i., using the FFU assay described by Philipson (34) for hexon detection.

Preparation and analysis of cytoplasmic RNA. Cytoplasmic RNA was prepared from infected HeLa cells by the method of Brawerman et al. (3). Portions, 10 μg, of total cytoplasmic RNA were subjected to S1 nuclease analysis by the protocol of Berk and Sharp (2). A synthetic oligonucleotide, spanning the cap site of the early E2 promoter (58 nucleotides [nt], starting with the A at nt 27052 and ending with the G at nt 27109, giving a protected fragment of 40 to 41 nt), was 5′-end labeled and used as probe. The resulting S1-resistant material was separated on a 10% polyacrylamide gel containing 7 M urea (41) and analyzed by fluorography. RNase protection analyses were performed by the method of Sambrook et al. (40), using 50-μg portions of total cytoplasmic RNA. A template for in vitro transcription was generated through polymerase chain reaction amplification of Ad5 DNA (46), using synthetic oligonucleotides as primers (nt 14164 to 14184 with the T7 promoter 5′ of the adenovirus sequence and nt 14382 to 14402 of the Ad2 sequence [39]). The T7 transcripts generated from the amplified DNA cover the splice acceptor site of the second leader sequence of the E2B messages (45). The transcripts were gel purified and used for RNase protection experiments, using RNases A and T_1. The RNase-resistant material was separated on a 5% polyacrylamide gel containing 7 M urea (41) and analyzed by fluorography.

RNA blot analyses. Total cytoplasmic RNA (5 μg) was fractionated by gel electrophoresis in 1% agarose–2% formaldehyde gels, and separated RNA was transferred to nitrocellulose filters (40). Hybridizations were carried out at 42°C overnight as described by Svensson and Akusjärvi (47). Rehybridization to filters with an actin probe was carried out after removal of hybridized probe in 1% glycerol at 100°C for 10 min. Probes for early messages were labeled with ^{32}P by random priming (40) and were as follows (nucleotides correspond to Ad2 sequences): E1a nt 0 to 1336 except nt 1111 to 1226 (13S intron); E1b nt 3322 to 3931; E2 nt 22912 to 23303; E3, for Ad2 mRNA, nt 27372 to 30046, and for Ad5, mRNA, the *Xba*I-D fragment corresponding to map units 78.5 to 84.3. To ensure that equal amounts of RNA were present in each lane, the filters were rehybridized against human actin sequences.

RESULTS

Construction and propagation of *il*E4L virus. The E4 mutant *il*E4L was generated by transfection of W162 cells with ligated Ad5 DNA fragments. Three viral restriction fragments were ligated in vitro, together generating the Ad5 genome with the E4 region substituted for by the cDNA sequence of pilE4L (encoding only mRNA L of Ad2), and mutant virus was identified by restriction enzyme analysis of DNA prepared from the resulting plaques. Mutant virus was plaque purified three times and propagated in W162 cells. The only E4 protein expected to be expressed from this mutant is an ORF6/7 fusion polypeptide (Fig. 1).

Mutant *il*E4L does not multiply in HeLa cells. We previously characterized a mutant, *il*E4I, which encoded the E4 ORFs 6 and 6/7. This virus replicated in HeLa cells as efficiently as wild-type virus. The growth properties of mutant *il*E4L, only encoding ORF6/7, were compared with those of *il*E4I, *dl*808, and Ad5. Monolayer cultures of HeLa and W162 cells were infected, and viral stocks were recovered by freeze-thawing of cells and medium at 6, 24, 48, and 72 h p.i. Titers of recovered stocks were determined on W162 cells. The rate of accumulation of newly formed viruses was approximately the same for all four viruses in W162 cells (Fig. 2A), as expected since these cells express endogenous E4 products. In HeLa monolayer cells, mutants *dl*808 and *il*E4L did not multiply, unlike mutant *il*E4I and Ad5, which accumulated high yields of virus (Fig. 2B). The results obtained for *dl*808 and *il*E4I are consistent with those reported earlier (17, 52). Thus, mutant *il*E4L showed a severe growth defect in nonpermissive HeLa cells with final virus yields that were similar to those obtained for *dl*808.

Mutant *il*E4L is defective for viral DNA replication and late protein synthesis. Large E4 deletion mutants, like *dl*808 and H5*dl*366 (*dl*366), accumulate reduced levels of viral DNA and late viral proteins (13, 52). Mutant *il*E4I, on the other hand, encoding only two E4 proteins, is nondefective both for viral DNA replication and in the production of late gene products (17). To examine DNA replication properties and late gene expression of *il*E4L, HeLa monolayer cells were

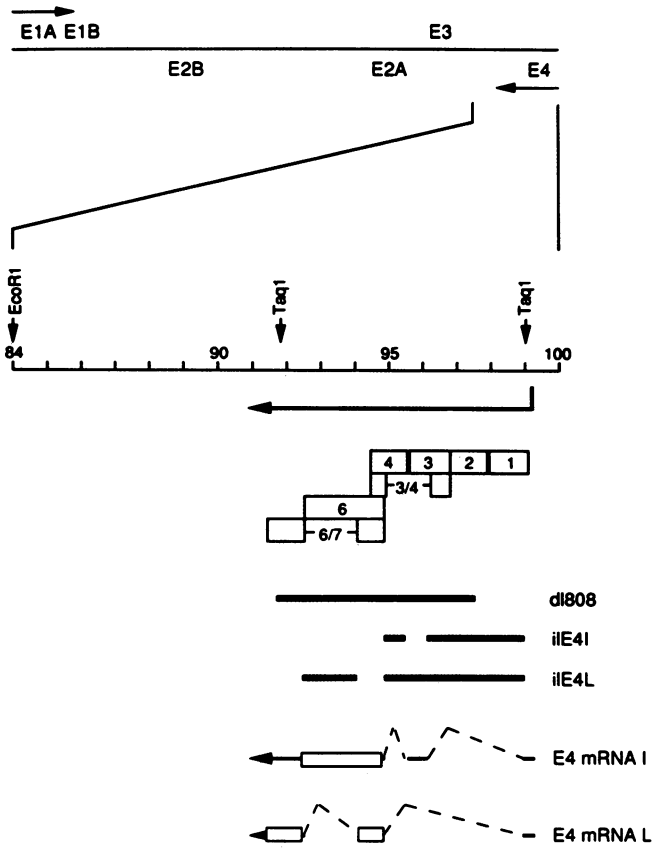

FIG. 1. Physical map of the adenovirus genome and region E4. (Top) Location of the early transcription units. (Bottom) Expansion of the terminal DNA fragment generated by *Eco*RI digestion of the Ad5 genome. The location of the E4 transcription unit is indicated by an arrow. ORFs of region E4 are shown by open boxes, numbered 1 through 7. Sequences absent in mutant viruses *dl*808, *il*E4I, and *il*E4L are indicated by solid bars. The structures of E4 mRNAs I and L are indicated at the bottom. The positions of restriction endonuclease cleavage sites used in the construction of mutant *il*E4L are shown.

infected with mutant or wild-type virus. At different times after infection, cells were pulse-labeled with either [^{3}H]thymidine or [^{35}S]methionine. DNA or protein extracts were prepared and analyzed as described in Materials and Methods. Figures 3 and 4 show that *il*E4L was clearly defective for both DNA replication and late protein synthesis, although it was not as defective as *dl*808. It has previously been reported by us and others (17, 20, 54) that E4 mutants defective for growth accumulate almost wild-type levels of viral DNA by 48 h after infection. This was not seen in these experiments (data not shown). This difference in accumulation of DNA might be an effect of the low multiplicity used in these experiments, since many E4 mutants have been reported to show a multiplicity-dependent phenotype (20).

***il*E4L, but not *dl*808, is normal for early gene expression.** To examine early gene expression in *il*E4L-infected cells, Northern (RNA) blot analyses were performed. HeLa monolayer cells were infected with mutants *il*E4L, *il*E4I, and *dl*808 and with wild-type Ad5. Cytoplasmic RNAs were prepared at 7 h p.i. and Northern blot analyses were performed as described in Materials and Methods. Early mRNAs were identified by hybridization with DNA sequences from regions E1a, E1b, E2, and E3 as probes (Fig. 5A). The resulting fluorograms showed that both *il*E4L and *il*E4I produced normal amounts of mRNA from all four early regions. *dl*808, on the other hand, showed a conspicuous defect in the production of E2 mRNA, while the production of the other early messages was normal or only modestly affected (Fig. 5A). The difference in hybridization pattern obtained with the E3 probe for Ad5, *il*E4I, and *il*E4L compared with *dl*808 can be explained by the difference in splicing patterns for Ad5 and Ad2 mRNAs. Analysis of RNA from Ad2-infected cells gave the same result as obtained with *dl*808 RNA (data not shown). Thus, *il*E4L and *il*E4I were normal for early gene expression while *dl*808 showed a drastically reduced production of E2 mRNAs at 7 h p.i.

Mutant *il*E4L *trans* activates the early E2 promoter to wild-type levels. The 19.5-kDa product of ORF6/7 has been shown to be responsible for the modification of transcription factor E2F, which in turn leads to *trans* activation of the early E2 promoter (21, 27, 31, 36). To examine whether mutant *il*E4L, encoding the ORF6/7 protein, was able to *trans* activate this E2 promoter, cytoplasmic RNAs were prepared from mutant and wild-type virus-infected HeLa cells at 7 h p.i. E2 messages were investigated by the S1 nuclease technique. The results (Fig. 5B) showed that the activity of the early E2 promoter in *il*E4L-infected cells was the same as in Ad5-infected cells. *il*E4I, expressing ORFs 6 and 6/7, also *trans* activated the early E2 promoter to at least normal levels. *dl*808, on the other hand, which does not encode the ORF6/7 polypeptide, was defective for early E2 *trans* activation.

The results described above thus show that mutant *il*E4L fails to replicate DNA, although the E2 promoter clearly is activated at 7 h p.i. Although unlikely, it could be argued that there still is a defect in the expression of the E2B products which like the 72-kDa DBP are controlled by the E2 promoter. The E2B products include the adenovirus-specific DNA polymerase and the terminal protein which are both necessary for viral DNA replication. These proteins are produced in very small amounts during the course of a normal infection, making them very difficult to study. The structures of these mRNAs have only been delineated in cells in which the proteins are overexpressed (45). Northern blot analysis was attempted (data not shown) but no E2B mRNAs were detected even when probes of very high specific activity were used. Instead, an RNase protection experiment was performed by using in vitro transcribed RNA as a probe. The RNA template covered the splice site junction between the first and the second leaders in the E2B mRNAs. E2B mRNAs are expected to protect a fragment of 128 nt from this template. The results (Fig. 5C) show that two closely migrating fragments with estimated lengths of 128 and 116 nt were seen when RNA from wild-type infected cells were used for the analysis. These fragments were absent when RNA from *dl*808- or mock-infected cells were tested (Fig. 5C). It is not known why two bands were seen; perhaps they result from alternative splicing of the leaders. RNA from cells infected with mutants *il*E4I and *il*E4L yielded similar sets of fragments as RNA from wild-type infected cells. In *il*E4I-infected cells there seems to be some overproduction of E2B mRNA, as is the case for E2A mRNA.

We thus conclude that the production of both the E2A and E2B products is normal early after infection with mutant *il*E4L, although it fails to replicate its DNA efficiently.

***il*E4L produces normal amounts of E2 polypeptides.** The

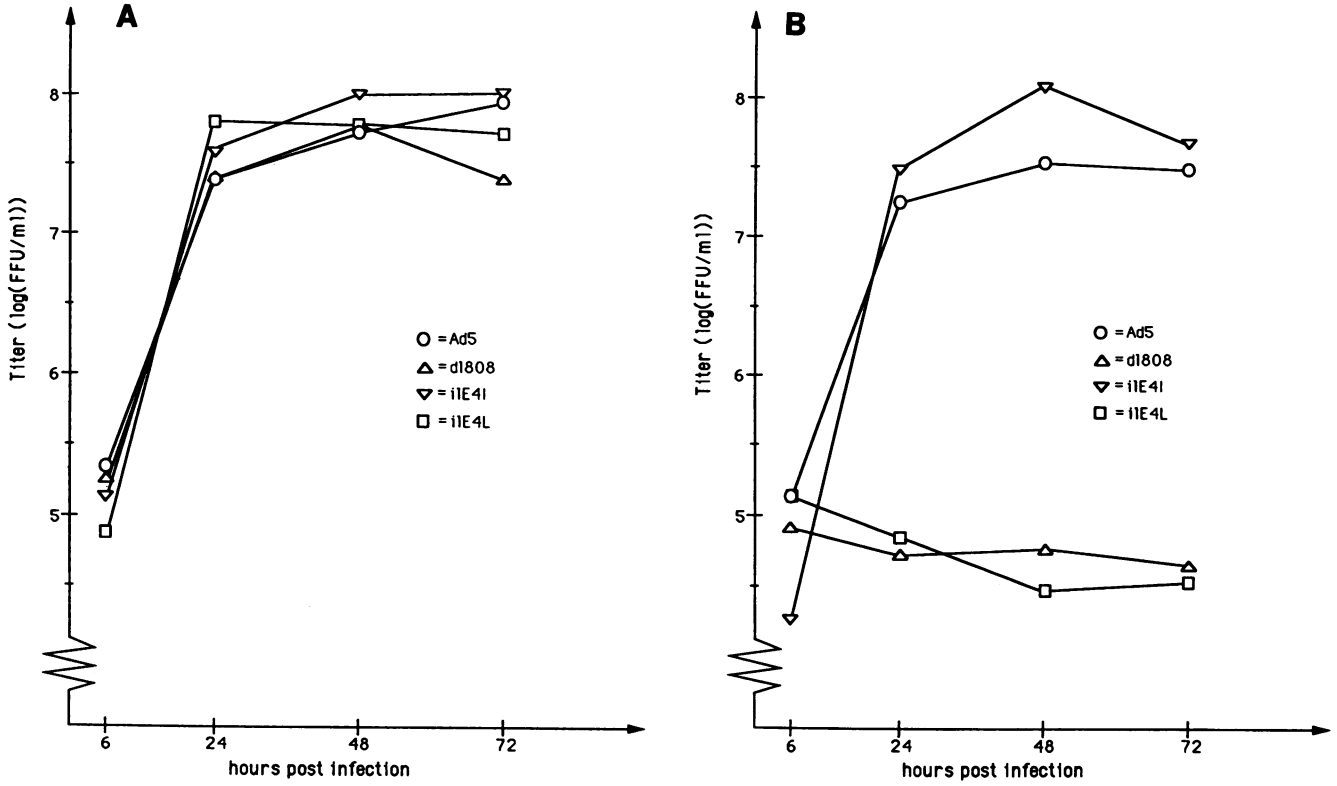

FIG. 2. Growth kinetics of mutant and wild-type viruses. W162 (A) and HeLa (B) cells were infected at a multiplicity of 5 FFU per cell, and virus yields were determined on W162 cells at the times indicated.

data presented above indicated that in *il*E4L-infected cells the promoter responsible for the production of gene products necessary for viral DNA replication is fully active. Still, this virus showed a severe DNA replication defect. To rule out the possibility that this defect was due to inefficient translation of E2 mRNAs, protein lysates were prepared at 7 and 24 h p.i. and subjected to immunoprecipitation, using a polyclonal antiserum directed against the DBP. The production of DBP was normal in *il*E4L-infected cells at 7 h p.i., (Fig. 6) as was the case in *il*E4I-infected cells. *dl*808 showed a decreased production of DBP compared with wild type, as expected. At 24 h p.i., in contrast, all mutants produced normal amounts of DBP. In fact, Ad5 and *il*E4I showed a slightly decreased synthesis compared with mutants *il*E4L and *dl*808. These data indicate the presence of at least one alternative way to activate the E2 promoter, in addition to ORF6/7-induced activation of E2F. The small decrease in DBP production seen for Ad5 and *il*E4I at 24 h p.i. might be explained by the fact that both of these viruses have entered the late phase of infection at this time. At late times, the activity of the E2 promoter decreases and the synthesis of cellular proteins is shut off, which was also seen in Ad5- and *il*E4I-infected cells but not in *il*E4L- and *dl*808-infected cells.

The production of adenovirus-specific DNA polymerase was investigated by Western blot analysis of protein extracts prepared at 7 and 24 h p.i. from mutant- or wild-type infected HeLa cells. At 7 h p.i., the protein was not detectable in any extract, while at 24 h p.i. all four viruses produced detectable amounts of adenovirus-specific DNA polymerase (data not shown).

Intracellular distribution of DBP produced by *il*E4L. Finally, we determined the intracellular distribution of the DBP in *il*E4L-infected cells. HeLa cells were infected with wild-type or mutant viruses. At 24 h p.i., the cells were analyzed by IF microscopy. In Ad5- and *il*E4I-infected cells, the DBP was present in high quantities in almost every cell, associated in globular structures located in the nucleus (Fig. 7). These structures have been identified as sites of viral DNA replication and late gene expression (50). When *dl*808- or *il*E4L-infected cells were examined, similar structures were found, but only in a minority of the cells (Fig. 7). These data, together with the immunoprecipitation data, suggest that the DBP in *il*E4L-infected cells was synthesized normally and that it could be organized into nuclear structures typical for the late phase. These structures were, however, only found in a few cells. The same observation was made for *dl*808-infected cells. This might be a consequence of the DNA replication defect exhibited by these viruses. Only a few cells infected by *dl*808 or *il*E4L appear to enter the late phase. These cells might contain sufficient levels of critical viral products compared with the rest of the cells, possibly due to a multiplicity effect. Even though the average multiplicity was 5 FFU/cell, individual cells might be infected by more than five virus particles. IF microscopy titration experiments with mutant *dl*808 showed that the number of cells expressing hexon protein increased drastically at a critical multiplicity, while upon wild-type infection the increase was linear and foci could be detected at a much lower concentration of virus (data not shown). This suggests that the presence of a threshold number of virus particles within the cell allows the infection to enter the late phase, even in the absence of E4 products.

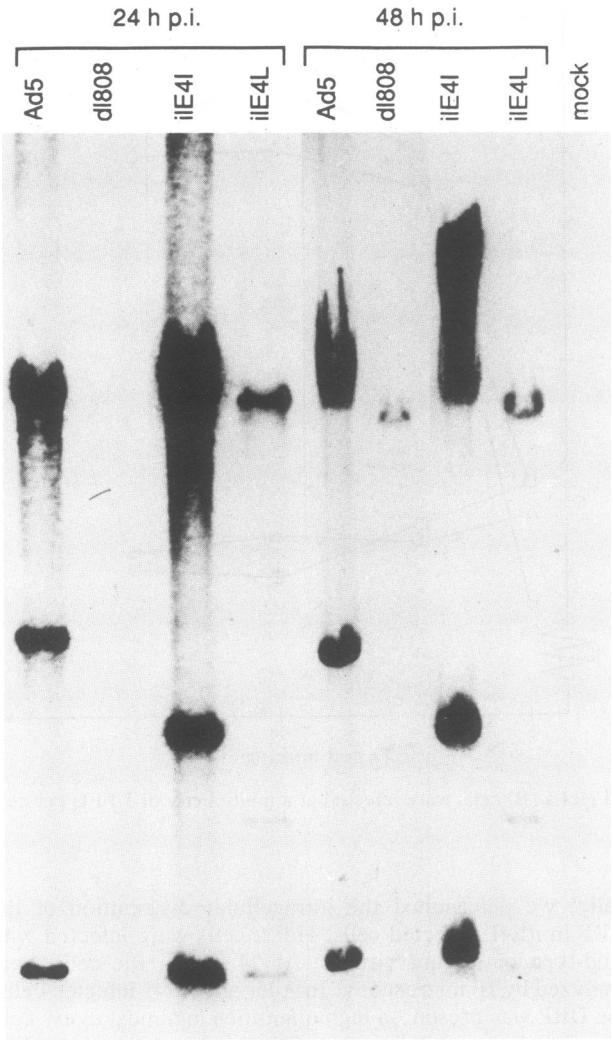

FIG. 3. DNA replication rates determined by electrophoretic analysis of labeled DNAs prepared from mutant or wild-type virus-infected HeLa cells. Infected cells were pulse-labeled for 1 h with [^{3}H]thymidine. At 24 or 48 h p.i., viral DNA was extracted by the protocol of Hirt (19), digested with *Eco*RI, and subjected to electrophoresis. Radioactive DNA fragments were visualized by fluorography.

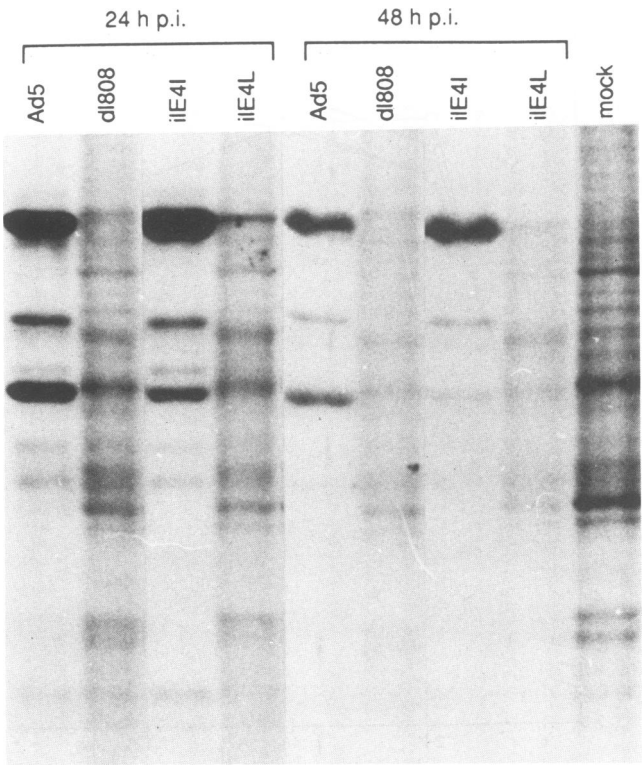

FIG. 4. Protein synthesis in HeLa cells infected with mutant or wild-type virus. Proteins were pulse-labeled for 30 min with [^{35}S]methionine, and the cells were harvested at 24 or 48 h p.i. as described in Materials and Methods. Protein extracts were prepared and subjected to electrophoresis in a 10% polyacrylamide gel containing SDS. Labeled proteins were visualized by fluorography.

DISCUSSION

Adenovirus E4 deletion mutants, such as *dl*808, show very complex phenotypes, including defects in viral DNA replication, late gene expression, and shutoff of host cell protein synthesis (52). To study the properties of region E4 in more detail, we have constructed adenovirus mutants in which region E4 was replaced by cloned cDNAs. We have previously described a mutant, *il*E4I, only capable of producing mRNAs corresponding to ORFs 6 and 6/7. This mutant multiplied in HeLa cells as efficiently as Ad5 and did not show the phenotypes associated with a *dl*808 infection (17). We report here on a mutant, *il*E4L, only encoding the ORF6/7 product. Its phenotype was compared with those of *il*E4I and *dl*808. *il*E4L was defective for growth in HeLa cells (Fig. 2B) and, like *dl*808, showed clear defects in viral DNA replication, late protein synthesis, and host cell shutoff (Fig. 3 and 4).

Examination of early gene expression by Northern blot analyses showed that *il*E4L synthesized the same amounts of early messages as the nondefective viruses Ad5 and *il*E4I. *dl*808, on the other hand, showed clear defects in the production of E2 mRNAs (Fig. 5A), while the levels of E1a, E1b, and E3 messages were normal or only slightly reduced. The reduction in E2 message level was probably due to lack of induction of transcription factor E2F. This cellular protein is modified during adenovirus infection to a form which binds cooperatively to the early E2 promoter (14, 15, 27, 53). This modification has been reported to require the ORF6/7 product of E4 (21, 27, 31, 36) and the absence of region E4 has been shown to lead to a 10-fold reduction in the activity of the E2 promoter (38).

The results presented above showed clear differences between *dl*808 and *il*E4L in the expression of E2 messages. Since the E2 region encodes the proteins required for viral DNA replication, we investigated the synthesis of E2 products in these cells in more detail. In the Northern blot analysis, we only investigated messages corresponding to the 72-kDa DBP, since the other E2 products are produced in such small quantities that they are very difficult to detect by this technique. The E2 promoter produces two other replication proteins, the terminal protein precursor (pTP) and the adenovirus-specific DNA polymerase (for a review, see reference 6). We used the S1 nuclease technique to detect the overall activity of the early E2 promoter. The results (Fig. 5B) were the same as those obtained in the Northern blot analysis, the early E2 promoter was equally

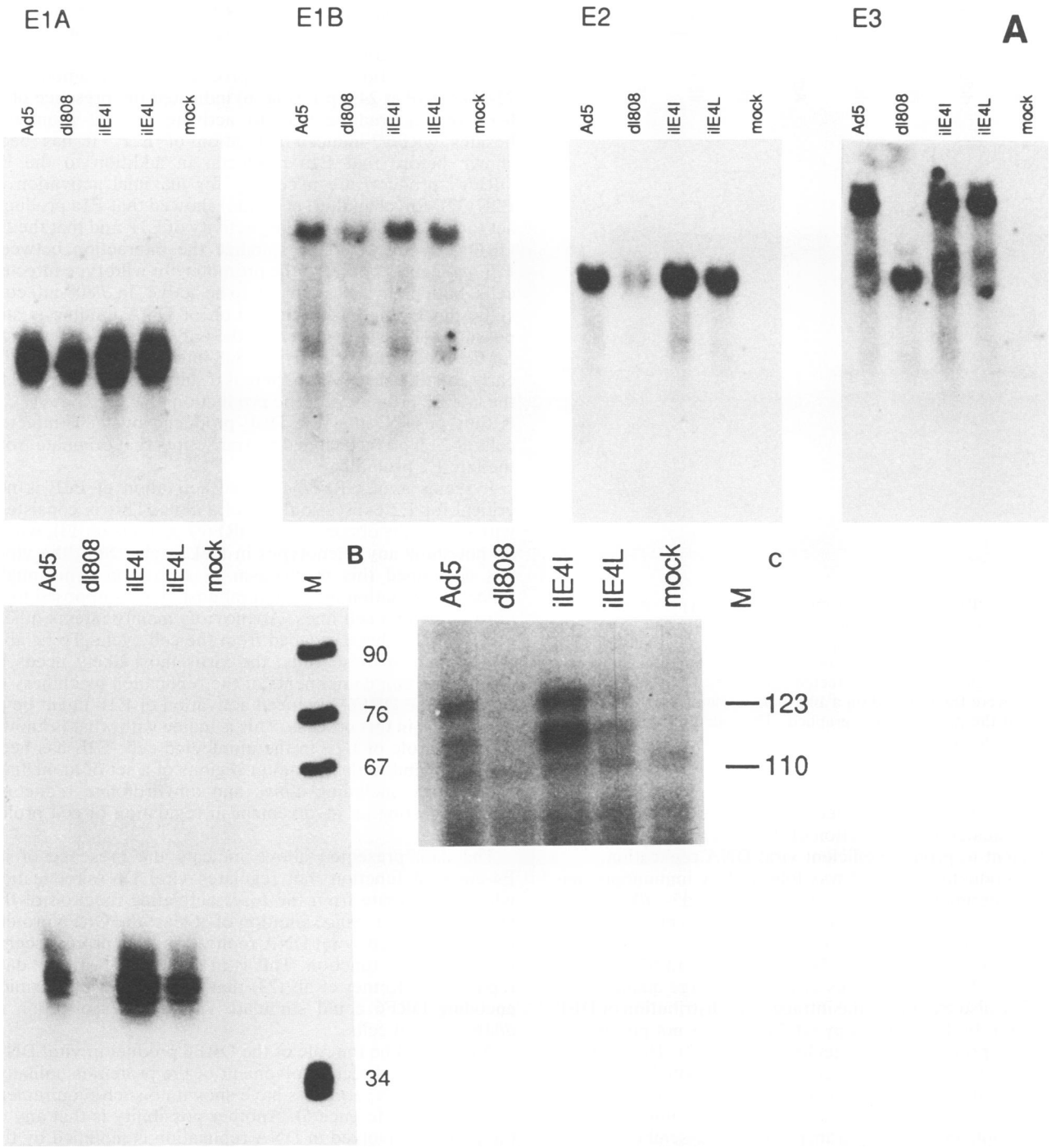

FIG. 5. Analysis of early mRNAs. Total cytoplasmic RNAs were extracted from mutant or wild-type virus-infected HeLa cells at 7 h p.i. (A) Northern blot analysis of early mRNAs: 5-μg portions were subjected to electrophoresis, transferred to nitrocellulose filters, and hybridized to region-specific probe DNAs. (B) S1 nuclease analysis of E2 messages: 10-μg portions of RNA were analyzed. A 5′-end-labeled synthetic oligonucleotide spanning the E2 early cap site was used as probe. Lane M, *Hpa*II-digested pBR322 as size marker. Sizes are given in nucleotides. (C) RNase protection analysis of E2B messages: 50-μg portions of RNA were analyzed, using a probe covering the splice acceptor site of the second E2B leader sequence. Lane M, As in panel B.

active in *il*E4L-, *il*E4I-, and Ad5-infected cells. In contrast, early E2 promoter activity was substantially reduced in *dl*808-infected cells. We also investigated the production of E2B messages by RNase protection (Fig. 5C). The results showed that the E2B mRNAs were produced in *il*E4L-infected HeLa cells, while they were undetectable in *dl*808-infected cells, thus showing that both the E2A and E2B mRNAs are produced at normal levels in *il*E4L-infected

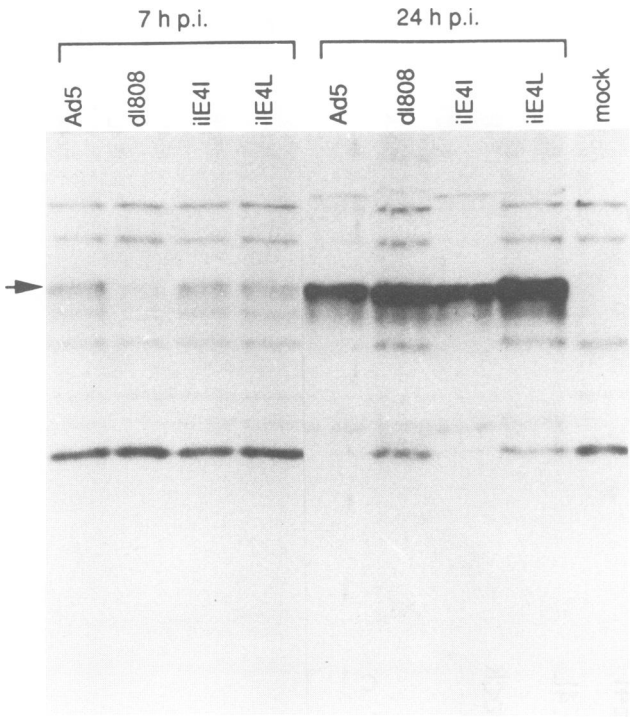

FIG. 6. DBP production in mutant or wild-type virus-infected cells. Proteins were labeled for 1 h before harvest with [^{35}S]methionine, and cell extracts were prepared at 7 or 24 h p.i. as described in Materials and Methods. Equal portions were immunoprecipitated by using a polyclonal antibody directed against the DBP. Immunoprecipitates were fractionated on a 10% polyacrylamide gel containing SDS, and the gel was fluorographed. The location of the DBP is shown by an arrow.

cells. Still, the virus was defective for DNA replication. This result indicated that activation of the early E2 promoter was insufficient to promote efficient viral DNA replication.

The production of DBP was followed by immunoprecipitation experiments (Fig. 6). At 7 h p.i., Ad5-, *il*E4I-, and *il*E4L-infected cells produced similar amounts of DBP, while *dl*808-infected cells showed reduced synthesis. These results were in agreement with the Northern blot and S1 nuclease data. At 24 h p.i., all viruses synthesized large quantities of DBP. We also examined the intracellular distribution of DBP at 24 h p.i. by IF microscopy (at 7 h p.i. it is not possible to detect the protein with this technique) (Fig. 7). The DBPs of wild-type and *il*E4I-infected cells were associated in globular structures in the nucleus. *dl*808- and *il*E4L-infected cells showed the same kind of structures, indicating that the protein could be correctly transported and assembled. However, only a small fraction of the infected cells contained these structures, although both *dl*808 and *il*E4L synthesized large amounts of DBP at this time (Fig. 6). That DBP is not seen in all cells is explained by the difficulty in detecting the protein unless it is assembled in the structures (24a). The low amount of correct structures seen in *dl*808- and *il*E4L-infected cells probably reflects the replication-defective phenotypes of these viruses. E4 products, other than the ORF6/7 product, might be required for the proper formation of these structures. In the absence of the necessary products, the nuclear structures would rarely form, leading to inefficient DNA replication. Alternatively, association of DBP into globular structures might not be a prerequisite for, but rather a consequence of, DNA replication. In this case, E4 products would act to stimulate DNA replication as such, in an as yet unidentified way.

The observation that *dl*808 produced normal amounts of 72-kDa DBP at 24 h p.i. (Fig. 6) indicated the presence of at least one alternative way to activate the E2 promoter, besides ORF6/7-induced activation of E2F. It has been shown before that E1a products, in addition to the E4 ORF6/7 product, are necessary for maximal activation of E2F (37). Raychaudhuri et al. (36) showed that E1a products act to stimulate DNA-binding activity of E2F and that the E4 ORF6/7 product acts to stabilize the interaction between E2F molecules bound to the promoter. In wild-type infected cells, both pathways are likely to be active. In *dl*808-infected cells, the E1a-induced stimulation of DNA binding is still possible. This is, however, not the only possible explanation for the production of DBP in *dl*808-infected cells. During the early-to-late phase switch there is a shift from the early E2 to the late E2 promoter for the production of E2 mRNAs (7). It is thus possible that the DBP produced by *dl*808-infected cells at 24 h p.i. is encoded by transcripts that originate from the late E2 promoter.

It seems as if ORF6/7-induced activation of E2F is not critical for E2 expression in HeLa cells. This is consistent with the results obtained with ORF6/7 mutants (4, 13), which do not show any phenotypes in HeLa cells. Still, the virus has developed this mechanism of activation. This might reflect the situation in the natural host cell, as opposed to in vitro cultivated cell lines. Adenovirus mainly infects quiescent cells that have escaped from the cell cycle. To be able to replicate in these cells, the virus most likely needs to activate certain components of the replication machinery of its host. The ORF6/7-induced activation of E2F might be of importance in this process. This is in line with what is known about the role of E2F in the uninfected cell. E2F has been shown to bind to the promoter regions of a set of immediate early genes, including c-*myc* and dihydrofolate reductase (29), suggesting an involvement in regulation of cell proliferation.

The data presented above indicate the presence of an E4-encoded function that regulates viral DNA replication which is separate from the *trans* activating function of the ORF6/7 product. Since addition of at least the ORF6 protein results in efficient viral DNA replication, this protein seems to provide that function. This is in agreement with the data reported by Ketner et al. (23) that transfection of plasmids encoding ORF6 could stimulate viral DNA replication in *dl*808-infected cells.

What could be the role of the ORF6 product in viral DNA replication? A direct involvement of the protein is unlikely, since in vitro experiments have shown no such requirement (reviewed in reference 6). Another possibility is that any of the proteins involved in DNA replication is modified by the ORF6 product. No such modification has been shown so far. Besides the E2 products, there might exist other virus-encoded proteins which participate in DNA replication in an as yet unidentified way. Any of these proteins could be the target for ORF6-induced activation. Replication-defective viruses also show defects in late protein synthesis. A possible explanation for the reduction in DNA replication is that one or more of the late proteins are, in some way, involved in maintaining efficient DNA replication. This seems unlikely, though, since adenovirus DNA replication in vitro does not require any late gene products. In addition, some E4 mutants are defective for late protein synthesis even though they accumulate normal levels of viral DNA (4). Yet

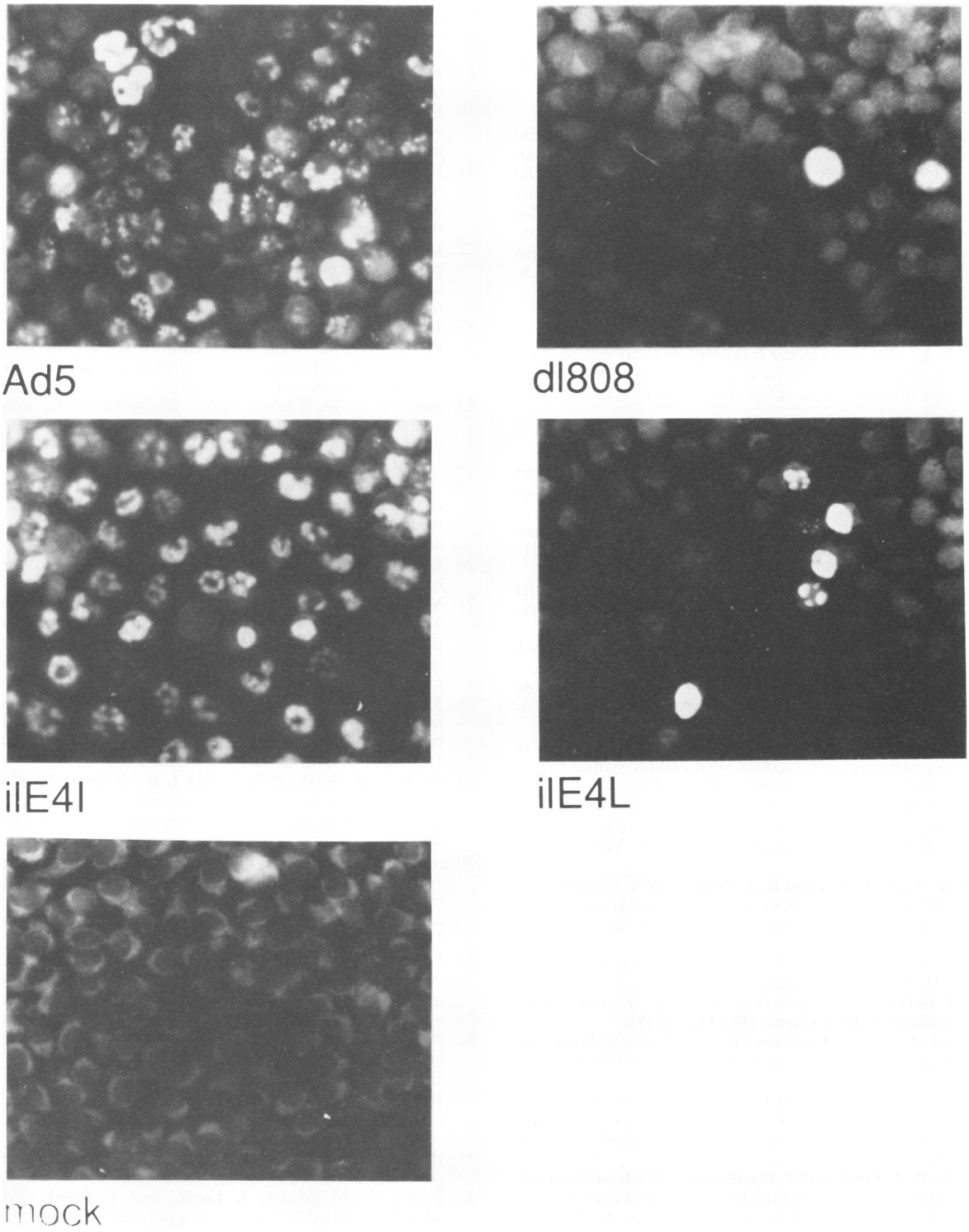

FIG. 7. Intracellular location of DBP in mutant or wild-type virus-infected HeLa cells. Cells were analyzed by IF microscopy at 24 h p.i., using a polyclonal antibody directed against the DBP.

another possibility is that the ORF6 protein is required for the proper assembly of nuclear structures necessary during the replication process, for example, the DBP-containing globular structures. The role of E4 products in the formation of these structures will be further investigated.

In addition to virus-encoded proteins, adenovirus DNA replication requires the participation of several host cell-encoded proteins, including cellular transcription factors and topoisomerase activities (reviewed in reference 6). Whether or not these proteins are regulated or modified during

infection is unknown, but an E4-dependent modification of any of these proteins could also explain the activity of E4 products in regulation of the onset of viral DNA replication.

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