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Adenoviral Early Region 4 Is Required for Efficient Viral DNA Replication and for Late Gene Expression†

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H2*dl*808 is a defective deletion mutant of human adenovirus 2 lacking most of transcriptional early region 4. Although the mutant can be grown in the complementing cell line W162, it is defective in human cell lines normally used to propagate adenovirus. In such nonpermissive cells, H2*dl*808 exhibits a severe defect in late gene expression, accumulating very small amounts of viral late messages and producing correspondingly small amounts of viral late proteins. H2*dl*808 also exhibits a defect in viral DNA synthesis: 24 h after infection, H2*dl*808-infected nonpermissive cells contain five- to sevenfold less viral DNA than those cells infected with wild-type adenovirus. H2*dl*808-infected nonpermissive cells eventually accumulate a significant amount of viral DNA. However, the rate of synthesis of viral proteins late in mutant infection remains much lower than that observed in wild-type infection at a time when DNA accumulation is comparable. Thus, the mutant's late protein synthesis defect is probably not due solely to its reduced accumulation of viral DNA. Finally, H2*dl*808 is much less efficient than wild-type virus in the inhibition of host cell protein synthesis in infections of nonpermissive cells. These observations imply roles for early region 4 products in several aspects of the viral growth cycle, including DNA replication, late gene expression, and host cell shutoff.

Upon the infection of sensitive cells, human adenoviruses conduct a precisely orchestrated program of gene expression (27, 40). Conventionally, the infectious cycle is divided into two phases, early and late, which are separated by the onset of viral DNA replication, and which are characterized by the predominance of different sets of viral gene products within the infected cell. Studies of viral gene products present during the early phase of adenovirus infection have allowed the assignment of functions in the viral life cycle to some of these products. Early region 1a (E1a) encodes products which are responsible for the activation of all of the other early transcriptional units (4, 23, 30). In addition, E1a products are involved in transformation by adenovirus (16, 21). Early region 1b (E1b) products also participate in transformation, and mutants of E1b have been isolated which degrade viral and cellular DNA, suggesting that E1b may play a role in the protection of DNA sequences during viral infection (7, 18, 36). Some E1b mutants also display defects in late gene expression (2; see below). Early region 2a (E2a) encodes a DNA binding protein which is essential for viral DNA replication (41). This protein may have a regulatory role as well; studies of a mutant which makes a temperature-sensitive protein have suggested that E2a represses transcription from early region 4 (E4) at late times during infection (25). Early region 2b (E2b) encodes two products which are essential for viral DNA replication. The first is a virally encoded DNA polymerase (8). The second is the terminal protein, which is covalently joined to each of the two 5′ ends of the linear viral DNA, and which serves as the primer for viral DNA synthesis (29, 35).

In addition to the transcriptional regions described above, there are three regions expressed early in infection for which no functions are known. Mutants of adenovirus in which early region 3 (E3) sequences have been deleted are viable, demonstrating that this region is dispensible for growth in tissue culture (12, 15). After the onset of DNA replication, the viral major late promoter produces transcripts which code for most of the viral late gene products and cover most of the right 80% of the viral genome. However, this promoter is also active at early times, producing a truncated transcript corresponding roughly to late region 1 (L1) (24, 34). No role for the early products of this region is known. Finally, E4 lies at the extreme right end of the viral genome. S1 nuclease studies and sequence analysis have indicated that E4 may encode up to 24 separate mRNAs, and 16 polypeptides have been identified by in vitro translation of E4 messages (38, 39, 42). Until recently, the only well-characterized E4 mutants were one defective in the production of an 11-kilodalton (kDa) E4 polypeptide, which is phenotypically wild type (33), and another that carries a deletion extending beyond E4 (6). It has not been possible to deduce the contribution of the E4 lesion to the phenotype of the latter mutant; thus, it has not yet been possible to assign functions unambiguously to E4 gene products.

Previously, we reported the construction of a cell line, W162, which contains and expresses E4 sequences (43). This line has allowed us to propagate a defective mutant, H2*dl*808, which carries a large deletion contained entirely within E4 (Fig. 1). Using this mutant, we have begun to examine the role of E4 in the adenovirus life cycle.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were obtained from J. Williams and are maintained in autoclavable Eagle minimal essential medium (Flow Laboratories, Inc.) containing 10% calf serum (MEMC10). Vero cells were obtained from A. M. Lewis and are maintained in MEMC10. W162 cells are a vero cell derivative that contain adenovirus E4 and support the growth of the E4 deletion mutant H2*dl*808 (43). This line was derived by transfection of Vero cells with a plasmid which contained the Adenovirus type 5 (Ad5) *Eco*RI B fragment (map units 83.6 to 100) and the dominant selectable gene *gpt*

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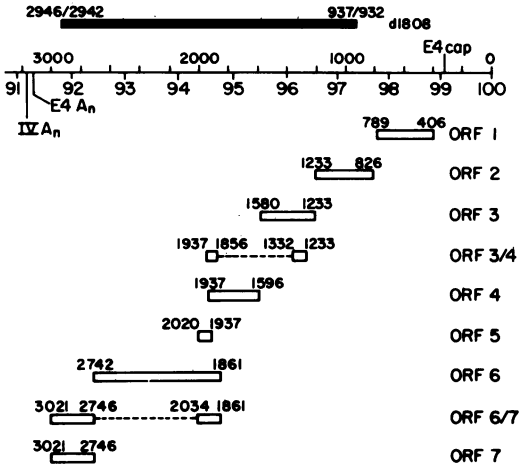

FIG. 1. Deletion 808 and the open reading frames of E4. The extent of the 808 deletion is indicated by the black bar, and the positions of postulated protein-coding regions (open reading frames; ORF) are indicated by open boxes. *dl*808 is a 2,008-base-pair deletion extending from within a run of A · T base pairs at positions 2946 to 2942 on the left to within a second run of A · T base pairs at positions 937 to 932 on the right. Eight A · T base pairs are preserved at the novel joint (E. Bridge and G. Ketner, unpublished data). Open reading frames 1 through 7 are those deduced by Hérrisé et al. (14) from sequence data, with nucleotide positions numbered as described by Gingeras et al. (10). The fused open reading frames, ORF 3/4 and ORF 6/7, were inferred from the nucleotide sequences of cloned E4 cDNAs (9, 42). The positions of the cap and poly(A) addition sites for E4 mRNAs and the poly(A) addition site for fiber (IV) mRNA are also marked. The scale is marked in map units and distance, in nucleotide pairs, from the right genomic end.

(22). The Ad5 *Eco*RI B fragment contains all of E4 and sequences corresponding to the body of the fiber gene. Originally isolated in medium which selected for the expression of the *gpt* gene, W162 cells have been carried in nonselective medium (MEMC10) for over a year with no loss of E4-complementing activity. W162 cells grown in selective medium and in MEMC10 were therefore used interchangeably in these studies.

Wild-type Ad2 was originally from A. M. Lewis. H2*dl*808 is an Ad2 deletion mutant lacking the viral DNA sequences between about 92 and 97.1 units on the standard map (Fig. 1). Its growth on W162 cells has been described (43).

Viral infections. Cell monolayers growing in 24-well tissue culture plates were infected at a multiplicity of infection of 5 to 10 PFU/cell with mutant or wild-type virus suspended in 0.5 ml of MEMC10. The titers of both viruses were determined on W162 cells. After 2 h of incubation at 37°C, the innoculum was removed and replaced with 1 ml of appropriate medium per well.

Labeling of viral proteins. At 24 h after infection, the medium was removed from cell monolayers, and the monolayers were rinsed with 0.2 ml of medium containing 5% of the normal concentration of methionine per well. This was replaced with 0.2 ml of the same medium containing 10 μCi of [^{35}S]methionine (Amersham Corp.) per ml. The cells were incubated at 37°C for 2 h, the labeling medium was replaced with 0.2 ml of MEMC10, and the incubation was continued at 37°C for an additional hour. The medium was removed, and the cells were lysed with a solution containing 10% β-mercaptoethanol, 10% glycerol, 4% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue, and 50 mM Tris hydrochloride (pH 6.8). Samples were stored at −20°C.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed essentially as described by Laemmli (17). Gels were 10% polyacrylamide, with an acrylamide/bisacrylamide ratio of 30:0.45 or 30:0.67. Samples were boiled for 2 min and vortexed vigorously for 1 min before loading. The gels were impregnated with 2,5-diphenyloxazole and dried as described by Bonner and Laskey (5). Autoradiograms were made at −80°C on Kodak XAR-5 film.

Isolation of cytoplasmic RNA and RNA hybridization. Cytoplasmic RNAs were isolated as described by Maniatis et al. (20). Total cytoplasmic RNA (2.5 μg/lane) was electrophoresed in 1% agarose–3% formaldehyde gels (28) at 3 V/cm for 6 h. Gels were soaked for 20 min in 10× SSC (1× SSC is 150 mM NaCl–15 mM sodium citrate, pH 7.0), and the RNA was transferred for 17 h to Biodyne filters which had been presoaked 20 min in 10× SSC (20). Filters were baked for 1 h in vacuo at 80°C and prehybridized for 2 h in 6× SSC–50% formamide–1% SDS–50 μg of denatured salmon sperm DNA per ml–10 mM phosphate buffer (pH 7.0)–0.2% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone at 42°C (1). The prehybridization solution was replaced with fresh prehybridization solution containing 0.02 μg of the appropriate probe (labeled with ^{32}P by nick translation [31] and denatured by boiling for 5 min and chilling on ice) per ml. Hybridizations were carried out for 17 h at 42°C. Filters were washed with 2 liters of 0.1× SSC–0.6% SDS at 65°C over 30 min, air dried, and analyzed by autoradiography on Kodak XAR-5 film at −80°C. Probes for late messages were Ad2 restriction fragments cloned into M13 or pBR322 as follows: late region 1, *Hin*dIII-I (32.2 to 37.9 map units); late region 2, *Hin*dIII-D (41.8 to 51.0 map units); late region 3, an *Hin*dIII-to-*Bam*HI fragment (51.0 to 60.1 map units); late region 4, *Hin*dIII-H (73.4 to 79.7 map units); and late region 5, *Eco*RI-E (83.6 to 89.8 map units).

Preparation and restriction analysis of nuclear DNA. After the removal of the medium from cell monolayers, the cells were lysed with 0.14 M NaCl–1.5 mM $MgCl_2$–0.5% Nonidet P-40–10 mM Tris hydrochloride (pH 7.4). Nuclei were pelleted through a sucrose cushion (0.14 M NaCl, 1.5 mM $MgCl_2$, 1.0% Nonidet P-40, 24% sucrose, 10 mM Tris hydrochloride [pH 7.4]) in 1.5-ml Eppendorf tubes at 7,000 × *g* for 20 min (20). The nuclear pellet was suspended in 0.3 ml of 0.6% SDS–1 mM EDTA–20 mM Tris hydrochloride (pH 7.4)–1 mg of pronase per ml and incubated overnight at 37°C. The DNA was extracted twice with phenol and twice with chloroform and stored at 4°C. DNAs were treated with RNase A (200 μg/ml) for 30 min at 37°C after restriction enzyme digestion performed as recommended by the enzyme supplier. Reactions were ended by the addition of 1/4 volume of loading buffer (1.5% SDS, 10 mM EDTA, 25% glycerol, 0.05% bromophenol blue, 10 mM Tris hydrochloride [pH 8.0]). Digested DNAs were electrophoresed in 1.4% agarose gels in Tris-acetate buffer (40 mM Tris hydrochloride, 5 mM $NaC_2H_3O_2$, 1 mM EDTA) at 7 V/cm for 4 h, stained with ethidium bromide, and visualized by UV illumination.

Dot-blot hybridization. DNAs to be analyzed were treated with RNase A (200 μg/ml) at 37°C for 30 min. After extraction with phenol and chloroform, the DNAs were denatured by boiling for 5 min and quick chilling on ice. The solution was adjusted to a final concentration of 6× SSC and spotted under gentle vacuum through a 96-well manifold onto a nitrocellulose filter presoaked in 6× SSC. Subsequent treat-

ment of the filter and hybridization to radioactively labeled adenovirus DNA was as described previously (43).

RNAs to be analyzed were treated with DNase (Worthington Diagnostics) at 0.1 mg/ml at 37°C for 1 h in the presence of 10 mM vanadyl ribonucleosides (Bethesda Research Laboratories) and then extracted once with phenol and once with chloroform. The solution (50 μl) was made 7% formaldehyde and diluted to 500 μl with 17× SSC–7% formaldehyde. After heating to 65°C for 5 min, the samples were spotted as described above onto a filter presoaked in 20× SSC. Treatment of the filter and RNA-DNA hybridization were as described previously (43).

RESULTS

Late protein synthesis in H2*dl*808-infected cells. Previously, we reported that H2*dl*807, which carries a large deletion that extends into E4, is defective in the production of some, but not all, of the viral late proteins (6). To see whether this was a defect shared by H2*dl*808, we examined late protein synthesis by H2*dl*808 in a nonpermissive cell line (HeLa) and in the permissive, E4-containing cell line W162. Proteins were labeled with [^{35}S]methionine from 24 to 26 h after infection and were analyzed on SDS-polyacrylamide gels. By 24 h after the infection of HeLa cells by wild-type adenovirus, a number of viral late proteins were readily identifiable (Fig. 2). At the same time and multiplicity of infection, only very small amounts of these proteins were present in HeLa cells infected with H2*dl*808. In contrast, in the E4-containing cell line W162, the synthesis of most late proteins by H2*dl*808 was comparable to that directed by wild-type virus. Thus, in nonpermissive cells, H2*dl*808 was profoundly defective in late protein production. E4 seems, therefore, to be required directly or indirectly for efficient adenovirus late gene expression. Two additional lines were tested for their ability to support efficient late gene expression by H2*dl*808: Vero, the parental line from which W162 cells were derived, and 293, a human cell line which expresses E1 sequences (11). The results obtained with these lines were similar to those obtained with HeLa cells.

The synthesis by H2*dl*808 of one viral protein was not restored to the wild-type level in W162 cells. Protein IV (fiber), which is encoded by late region 5 (L5), was underproduced by H2*dl*808 even on W162 cells. L5 is adjacent to E4 and is transcribed in the opposite direction. Since the 808 deletion does not affect sequences found in mature fiber mRNA (43) (Fig. 1), the failure of H2*dl*808 to produce fiber in permissive cells may reflect the existence of downstream sequences, essential for the production of normal quantities of fiber protein, which are removed by the mutation. Small amounts of normal fiber were produced in W162 cells; immune precipitation with fiber-specific antiserum (supplied by C. Anderson) detected a protein of the correct mobility on gels (data not shown).

Wild-type Ad2 effectively shuts off host protein synthesis at late times during infection. H2*dl*808 inhibited host protein synthesis much less effectively, as shown by the persistence of host proteins in mutant-infected HeLa cells labeled 24 h postinfection (Fig. 2). (In some cases, inhibition of host protein synthesis was observed at later times; see Fig. 5b). On W162 cells, the mutant's ability to inhibit host protein synthesis was restored.

Finally, in non-permissive cells, H2*dl*808 substantially overproduced the viral 72-kDa DNA binding protein (Fig. 2) encoded by E2a. On W162 cells, the production of that protein was normal.

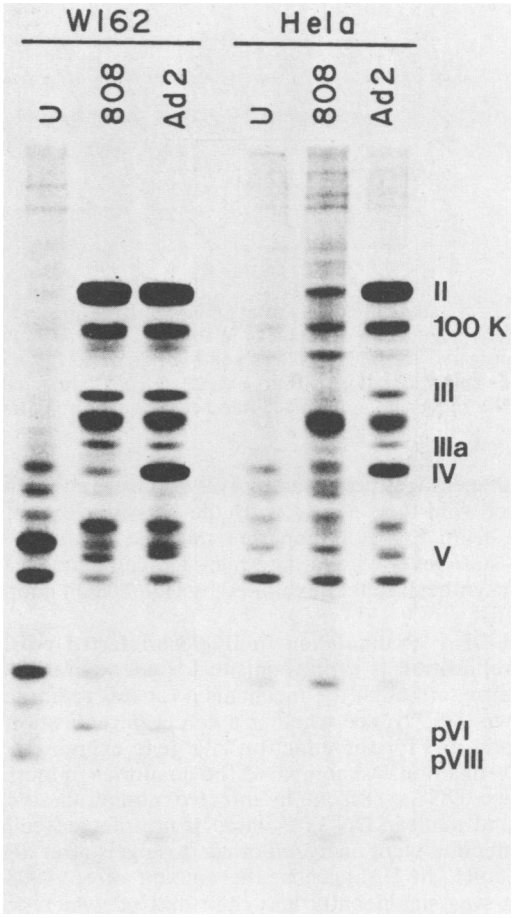

FIG. 2. Proteins produced by Ad2- and H2*dl*808-infected cells. Proteins were labeled with [^{35}S]methionine from 24 to 26 h after the infection of HeLa or W162 cells with wild-type Ad2 or H2*dl*808. Labeled proteins were electrophoresed in a 10% SDS–polyacrylamide gel, and the gel was fluorographed. The positions of viral proteins are indicated on the right.

Steady-state levels of viral late messages in H2*dl*808-infected cells. The adenovirus late proteins are encoded by late messages which fall into five families, as defined by the positions of their 3′ ends (44). These messages are produced by differential processing of a transcript originating from a single promoter at 16.7 map units (3). To see whether the reduction of viral late protein synthesis in H2*dl*808-infected HeLa cells was a reflection of a reduction in the level of the corresponding mRNAs, cytoplasmic RNA was isolated and analyzed. RNAs were electrophoresed in a 1% denaturing agarose gel, transferred to a membrane filter, and hybridized to radioactive adenovirus DNA corresponding to each of the five late gene families. Figure 3a shows an autoradiogram of one set of such filters. RNA isolated from HeLa cells infected with wild-type Ad2 produced a characteristic pattern of intense bands when hybridized to each of the specific probes. In contrast, RNAs extracted from H2*dl*808-infected HeLa cells hybridized very weakly to these probes, indicating that the levels of viral RNAs were strikingly reduced in mutant-infected cells. Upon long exposure, RNAs of the correct mobilty could be identified in autoradiograms of gels displaying RNA extracted from mutant-infected cells (Fig. 3b), indicating that the messages which were produced were not grossly altered in their structure. In W162 cells, the

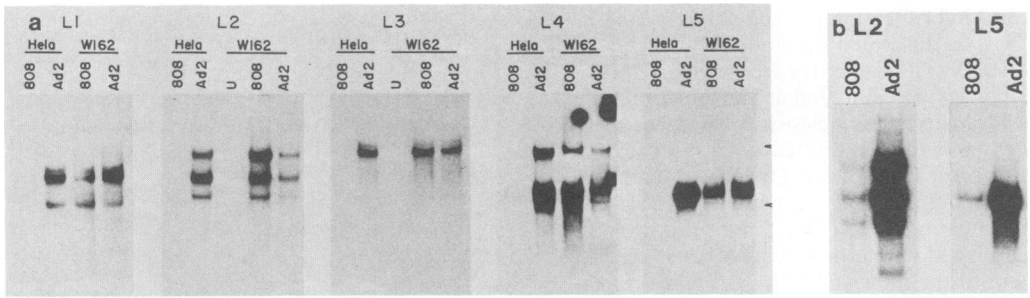

FIG. 3. Steady-state levels of cytoplasmic RNA present in cells infected by Ad2 or H2*dl*808. (a) Total cytoplasmic RNA was isolated at 24 h after the infection of HeLa or W162 cells with Ad2 or H2*dl*808, and electrophoresed in 1% agarose–3% formaldehyde gels. The RNA was transferred to Biodyne filters and hybridized to ^{32}P-labeled probes specific for each of the five viral late regions (L1 through L5; see Materials and Methods). U, RNA extracted from uninfected cells. (b) Long exposures of filters from a, made to detect the presence of rare viral RNA sequences in H2*dl*808-infected HeLa cells. Filters hybridized to the L2 and L5 probes are displayed.

levels of messages produced by H2*dl*808 were comparable to those of wild-type virus, with the possible exception of mRNA from L5. It is apparent that the reduction in the steady-state level of viral late message can account for the protein synthesis defect exhibited by H2*dl*808 on nonpermissive cells.

Viral DNA accumulation in H2*dl*808-infected cells. Viral DNA replication is a prerequisite for adenoviral late gene expression, although the mechanism for this requirement is unknown (37). To see whether a defect in replication might be responsible for the effect on late gene expression of the H2*dl*808 deletion, we compared the quantities of mutant and wild-type DNAs present in infected nonpermissive cells. Total and nuclear DNAs isolated from infected cells 24 h after infection were analyzed on agarose gels after digestion with *Eco*RI. In HeLa cells, the amount of H2*dl*808 DNA present was significantly less than that of wild type DNA (Fig. 4a). Measurements by dot hybridization of viral DNA present in mutant and wild-type infections indicated a five- to sevenfold reduction in accumulation by the mutant (Fig. 4b). On W162 cells, H2*dl*808 and wild-type virus accumulated DNA to similar levels. The rate of adenoviral DNA accumulation in the nuclei of infected nonpermissive cells was also measured by dot hybridization. Typically, the maximal rate of accumulation of hybridizable material in mutant-infected cells was three to five times less than that observed in wild-type infections (Fig. 5a).

Protein synthesis defect of H2*dl*808 and gene dosage. In the experiment presented in Fig. 5a, the level of nuclear adenoviral DNA present in H2*dl*808-infected nonpermissive cells 36 h after infection was comparable to that present 18 h after infection with wild-type virus. If the protein synthesis defect exhibited by H2*dl*808 were due solely to its reduced accumulation of viral DNA, the level of viral proteins produced by these two cell populations should be comparable as well. Figure 5b shows an autoradiogram of ^{35}S-labeled proteins isolated during the course of this experiment and analyzed on a 10% SDS–polyacrylamide gel. The level of viral late protein synthesis observed 36 h after infection with H2*dl*808 was much lower than that observed 18 h after a wild-type infection. Thus, gene dosage is not likely to account entirely for the protein synthesis defect of the mutant.

DISCUSSION

H2*dl*808 is a defective mutant of adenovirus type 2 which has suffered a large deletion of DNA from E4. To study the effect of this deletion on the viral life cycle, and thus to probe the role of E4 in viral growth, we have examined the phenotype of H2*dl*808 in HeLa cells. These cells are permissive for growth by wild-type Ad2, but do not support the growth of the mutant.

In HeLa cells, H2*dl*808 fails to make normal quantities of the viral late proteins. The steady-state levels of messages which encode these proteins are correspondingly reduced, and it is likely that this reduction in message levels accounts for the late protein phenotype. Since DNA replication is a

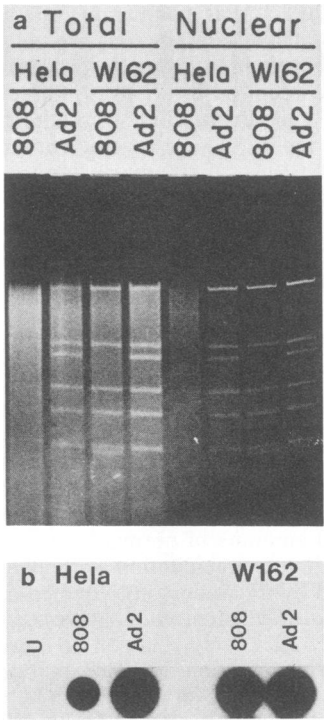

FIG. 4. DNA present in cells infected by Ad2 or H2*dl*808. Total and nuclear DNAs were isolated 24 h after infection of HeLa or W162 cells by Ad2 or H2*dl*808. (a) DNAs were subjected to restriction digestion by *Eco*RI, electrophoresed on a 1.4% agarose gel, and visualized by UV illumination after ethidium bromide staining. (b) Nuclear DNAs were denatured and dotted onto nitrocellulose filters. Viral sequences were detected by hybridization to ^{32}P-labeled adenoviral DNA. An autoradiogram of DNA from one experiment is displayed.

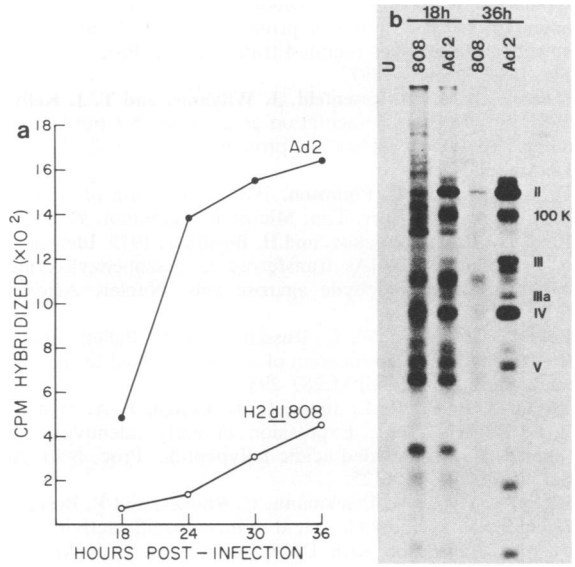

FIG. 5. DNA accumulation and viral protein synthesis in H2*dl*808-infected cells. (a) Viral DNA accumulated in HeLa cells infected either with H2*dl*808 or Ad2, measured by dot-blot hybridization, is plotted versus time after infection. (b) Fluorogram of a 10% SDS–polyacrylamide gel displaying the proteins synthesized in HeLa cells infected with either H2*dl*808 or Ad2 18 and 36 h after infection. The cells were labeled with [^{35}S]methionine for 2 h beginning at the indicated time. U, Uninfected cells. The positions of viral proteins are indicated on the right.

prerequisite for late gene expression by adenovirus, we examined the ability of H2*dl*808 to produce DNA in HeLa cells. Viral DNA accumulates more slowly in mutant than in wild-type infections, and by 24h after infection, wild-type Ad2-infected cells have accumulated approximately five- to sevenfold less nuclear DNA than those infected by H2*dl*808. This reduction probably accounts for a portion of the effect of the mutation on late mRNA levels, but does not seem entirely responsible for the defect in late gene expression displayed by H2*dl*808; mutant-infected cells 36 h after infection produce much lower levels of viral late proteins than do cells 18 h after infection by wild-type virus, although the levels of nuclear viral DNAs in those cells are similar. Although this analysis assumes that nuclear DNA levels measured by hybridization reflect the number of active transcriptional templates present in the nuclei, the data are consistent with independent roles for E4 products in the accumulation of normal quantities of viral DNA and in the efficient expression of the viral late genes.

In addition to its protein synthesis defect, H2*dl*808 exhibits reduced ability to inhibit host cell protein synthesis and overproduces at least one early viral protein (the 72-kDa DNA binding protein). In neither case is the basis for the phenotype understood, although the overproduction of viral early proteins might reflect a failure to turn off their synthesis related to the defect in the inhibition of host cell protein synthesis.

The accumulation of nuclear DNA, the inhibition of host protein synthesis, and the steady-state levels of viral late messages produced by H2*dl*808 approach wild-type levels when the virus is grown in the E4-containing cell line W162. This indicates that these phenotypes are the result of the mutation in E4, and that they are not the result of *cis* effects of the deletion.

Studies of DNA synthesis in vitro have identified three viral gene products necessary for adenoviral DNA replication: a DNA binding protein encoded by E2a, the viral terminal protein encoded by E2b, and a DNA polymerase also encoded by E2b (19, 26, 41). The data presented here suggest that an E4 product is also involved in the production of viral DNA, either directly (perhaps as a component of the replication machinery) or indirectly (for example, via the regulation of E2 expression). Mutants of E1b which degrade DNA and thus accumulate reduced amounts of viral DNA have been described (7, 18, 36). Although the phenotype of H2*dl*808 might also reflect a role for E4 in stabilizing viral DNA after its synthesis, the behavior of the E1b mutants is distinctive and unlike that of H2*dl*808. It is not likely, therefore, that E4 acts to protect DNA in the same way as E1b.

Analysis of E4-encoded RNAs by hybridization and translation in vitro have revealed a multitude of potential E4 products, and several potential protein-coding regions have been identified in E4 on the basis of sequence data (Fig. 1) (14, 38, 39, 41). The 808 deletion destroys all of the postulated coding regions save the one nearest the E4 promoter, and the complex phenotype of the mutant might therefore reflect the disruption of several genetic elements with different functions. Although little is known about any of the E4 products, a protein from this region, (25 kDa), has been shown to associate with a protein (58 kDa) encoded by E1b (32). It is noteworthy that the protein synthesis phenotype of an E1b 58K mutant appears similar to that of H2*dl*808 despite apparently normal DNA replication by the E1b mutant. It is possible that the 25-kDa protein of E4 and the 58-kDa protein of E1b interact to mediate the normal accumulation of viral late messages and the inhibition of host protein synthesis.

We have described the phenotype of a mutant that carries a deletion mutation removing the coding sequences for almost all potential E4 gene products. An analysis of this mutant and of a similar mutant constructed in vitro (13) suggests roles for E4 in viral DNA accumulation, late gene expression, and the inhibition of host cell protein synthesis. The isolation of additional mutants which affect specific gene products within E4 should allow the determination of the role of individual E4 products within the adenovirus life cycle.

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