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Deletion of the E4 region of the genome produces adenovirus DNA concatemers

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ABSTRACT **Two mutants containing large deletions in the E4 region of the adenovirus genome H5dl366 (91.9–98.3 map units) and H2dl808 (93.0–97.1 map units) were used to investigate the role of E4 genes in adenovirus DNA synthesis. Infection of KB human epidermoid carcinoma cells with either mutant resulted in production of large concatemers of viral DNA. Only monomer viral genome forms were produced, however, when mutants infected W162 cells, a monkey kidney cell line transformed with and expressing the E4 genes. Diffusible E4 gene products, therefore, complement the E4 mutant phenotype. The viral DNA concatemers produced in dl366- and dl808-infected KB cells did not have any specific orientation of monomer joining: the junctions consisted of head-to-head, head-to-tail, and tail-to-tail joints. The junctions were covalently linked molecules, but molecules were not precisely joined, and restriction enzyme maps revealed a heterogeneous size distribution of junction fragments. A series of mutants that disrupted single E4 open reading frames (ORFs) was also studied: none showed phenotypes similar to that of dl366 or dl808. Mutants containing defects in both ORF3 and ORF6, however, manifested the concatemer phenotype, indicating redundancy in genes preventing concatemer formation. These data suggest that the E4 ORFs 3 and 6 express functions critical for regulation of viral DNA replication and that concatemer intermediates may exist during adenovirus DNA synthesis.**

Adenovirus DNA replication has been extensively studied (reviewed in refs. 1 and 2). Electron microscopy demonstrated that replication initiates at the ends of the linear genome and proceeds via strand displacement, producing a semiconservatively replicated daughter genome and a single-stranded (type II) molecule which may form a duplex panhandle by virtue of inverted terminal repeats (ITRs). This type II molecule then is the substrate for another round of DNA synthesis producing a second daughter genome (3).

Insight into the mechanism of the initiation of replication has been gained from *in vitro* reconstitution of DNA synthesis using isolated viral DNA and plasmid constructs. Genetic data and an *in vitro* model of DNA replication defined the role of three essential viral proteins: a single-stranded-DNA-binding protein, a DNA polymerase, and a terminal protein that is covalently linked to the 3′ terminal deoxycytosine of the genome and is critical for the initiation of DNA synthesis (4–6). Four host proteins have also been defined as important for viral DNA replication: nuclear factor I and nuclear factor III/Oct-1, which are transcription factors (ref. 7 and references therein); nuclear factor II, a type I topoisomerase (8); and pL, a 5′-to-3′ exonuclease (9).

An *in vivo* model for viral DNA synthesis used plasmid constructs to investigate the sequence requirements for replication. The ITR is essential, and a plasmid containing a single complete copy of the repeat can be rescued by duplicative transfer of one end of the genome to the other (10) or by replication-induced recombination (11). The last 45 bp of the genome contain the necessary sequence information for replication, and any deletion of the terminal cytosine abolishes DNA synthesis (12, 13).

Other viral gene products may be involved in viral DNA synthesis, and other mechanisms not yet elucidated may be required for completion of viral DNA replication. Some phenomena not accounted for in the current model include the following: circular, supercoiled, and multimeric viral DNA molecules, which were identified in electron microscopic studies (14); the pL 5′-to-3′ exonuclease required in the *in vitro* model (9) produces defective strands that need additional processing for maturation; a large proportion of viral particles produced during infection carry partially replicated single-stranded DNA (15); and concatemers of input DNA are formed in the *in vivo* plasmid model of viral DNA synthesis (10–13). Topoisomerase, which is required in the *in vitro* model, should not be needed to relieve tortional stress in the linear molecules predicted by the current model. In addition, more than the three viral genes defined by the current model are needed for viral DNA replication, since mutations in the E4 region have a significant effect on viral DNA synthesis (16–18).

To gain a better understanding of the functions of the E4 genes in adenovirus DNA synthesis, the DNA replication phenotypes of E4 mutants were investigated. These experiments revealed the formation of viral DNA concatemers, which suggests that multimeric genomes are a consequence of disruption of a normal viral gene function and that the mechanism of adenovirus DNA synthesis may differ from the presently accepted model.

MATERIALS AND METHODS

Cell Culture and Viruses. Monolayer cultures of KB cells (human epidermoid carcinoma cell line) and W162 cells [a Vero monkey kidney cell line which contains an integrated copy of the adenovirus type 5 (Ad5) E4 region was supplied by G. Kettner, The Johns Hopkins University] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. E4 mutants H5dl366, in351, in352, dl355, dl356, dl358, and dl359 were supplied by T. Shenk (17); H2dl808 was supplied by G. Kettner (16); and E4 inORF3, E4 inORF3/inORF6, E4 inORF3/dl355, and dl366+ORF3 were supplied by P. Hearing (18). See Fig. 1 for location of mutations.

Purified virions, prepared by CsCl equilibrium density centrifugation, were used in all infections. Material isolated from the gradient was disrupted with 0.1% SDS and A_{260} was measured. An A_{260} of 1 indicates 10^{12} particles per ml (17). When purified Ad5 was titrated on KB cells, a 10:1 ratio of total particles to plaque-forming units was present. In all

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Abbreviations: Ad*n*, adenovirus type *n*; ITR, inverted terminal repeat; ORF, open reading frame; PFGE, pulsed-field gel electrophoresis.

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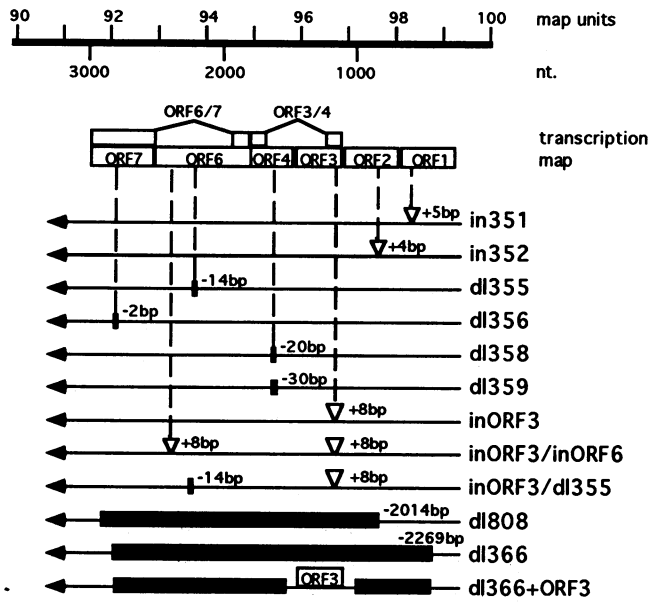

FIG. 1. E4 transcription map and mutant viruses. The physical map of the E4 region is shown both in map units (above the line) and in nucleotides (below the line). The translational open reading frames (ORFs) are shown as boxes correctly oriented with respect to the physical map with the ORF number either within the box or above the box, in the case of alternative splicing. The E4 mutants described in the text are labeled to the right. The vertical dotted lines locate the mutation on the transcription map. Solid bars correspond to deletion mutations. Triangles above the lines correspond to insertions. The size of each mutation is noted.

experiments 200 particles per cell (20 plaque-forming units for Ad5 wild type) were employed.

Pulsed-Field Gel Electrophoresis (PFGE). PFGE gels were run as described (19). In brief, 10^6 infected cells were removed from culture plates with trypsin and resuspended in 0.25 ml of phosphate-buffered saline, which was immediately mixed with 0.25 ml of liquid 1% low-melting-point agarose. This solution was cast into blocks and, after solidification, incubated at 55°C for 24 hr in 1% SDS/250 mM EDTA with proteinase K at 5 mg/ml. For restriction endonuclease digestion, proteinase K was inactivated, and 100-fold excess restriction enzyme was added and incubated for 12 hr. All gels were electrophoresed at a pulse frequency of 20 sec, 300 V, for 24 hr. The gel was washed for 20 min in 0.2 M HCl before Southern transfer.

Two-Dimensional Gel. The first dimension was run in a 0.7% agarose gel buffered with Tris/borate/EDTA. A lane was cut from this gel and placed in a long slot of a 1% agarose gel buffered with 50 mM NaOH and 1 mM EDTA. The second dimension was run at 0.5 V/cm at 4°C and blotted directly onto membranes.

Southern Transfer. Hybond-N membranes (Amersham) were used. All washes were done at 65°C with 45 mM NaCl/4.5 mM sodium citrate, pH 7. The E1A probe was a PCR product from 550 bp to 1000 bp, and the E4 probe was the terminal *Kpn* I fragment of Ad5. Radioactivity from [α-^{32}P]dNTPs was incorporated into the probes by random priming and Klenow DNA polymerase incubation.

DNA Library. DNA was obtained from agarose blocks used in PFGE by phenol extraction at 65°C and digested with *Hin*dIII. A 1:1 weight ratio of adenovirus DNA was ligated to *Hin*dIII-digested, dephosphorylated pUC18. The pUC18 library was transfected into *Escherichia coli* HB101 and plated onto Hybond-N. Two replicate filters of the original were made for these experiments. Only those colonies that hybridized in both filters were scored as positive.

RESULTS

Viral DNA Replication Phenotype of E4 Mutants. The studies described below used a series of twelve E4-region mutants (16–18). The designation of the mutant, type, and location of the mutations are shown in Fig. 1. In summary, dl808 is an Ad2 derivative with most of the E4 region deleted, and dl366 is an Ad5 derivative in which deletions affect all of the E4 ORFs. Each ORF that has been identified in the E4 region has been individually mutated in the Ad5 derivatives in351, in352, dl355, dl356, dl358, dl359, and inORF3. The Ad5 derivatives inORF3/inORF6 and inORF3/dl355 each contain double mutations located in ORF3 and ORF6. The Ad5 derivative dl366+ORF3 has an intact ORF3 with appropriate splicing signals reinserted into the large E4 deletion of dl366.

As previously described, dl366 and dl808 do not produce infectious virus in KB cells (16, 17), although viral DNA synthesis does proceed and there is some multiplicity leakiness in the replication-deficient phenotype (18). Large E4 deletions do multiply in W162 cells, a Vero cell line that expresses the functional E4 region (16, 17). Fig. 2*A* presents a Southern blot of uncut DNAs that were isolated from KB cells 4, 24, or 48 hr after infection with Ad5 wild type, dl366, or dl808. PFGE was used to separate these high molecular weight DNAs, and all Southern blots in Fig. 2 were hybridized with Ad5 E1A DNA, which does not cross hybridize with KB cellular DNA. At 24 and 48 hr after infection, both E4 deletion mutants produced a ladder of bands with equal distance between the bands (evenly spaced, individual bands were apparent in the 48-hr lanes of dl366 and dl808 on ethidium bromide staining of the pulsed-field gel; data not shown). Migration of viral DNA under the conditions of this PFGE is linearly related to the size of the DNA, and the splaying of the lanes is an expected consequence of the conditions used. The 190-kb marker was located between the fifth and sixth bands of dl808, which indicates that the size difference separating each band was between 31 and 38 kb.

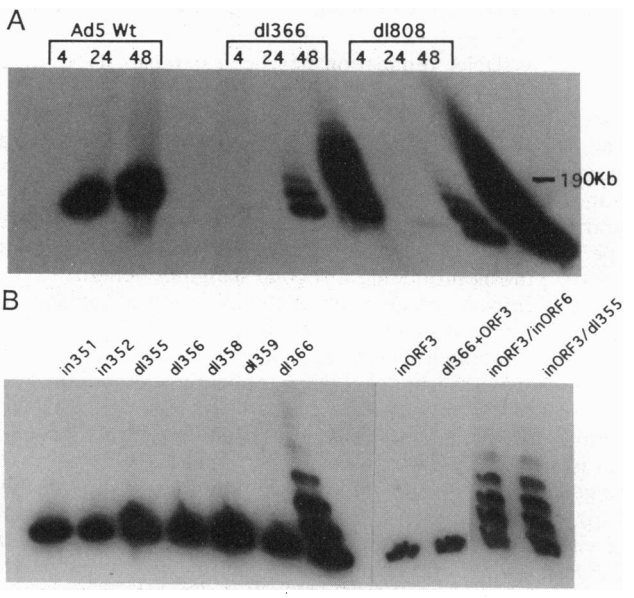

FIG. 2. Southern blots of pulsed-field gels hybridized with E1A DNA. All lanes contained uncut DNA derived from infected KB cells. (*A*) Time course of infection. DNA was isolated 4, 24, and 48 hr after infection. DNAs from the first set of three lanes were from cells infected with Ad5 wild type (Wt), the second set of three lanes contained DNA from cells infected with dl366, and the third set of three lanes contained DNA from cells infected with dl808. Size standard on the right is *Trypanosoma brucei* 118 D (20). (*B*) Two separate pulsed-field gels. The first seven lanes are from one gel and the last four lanes are from another. The mutant used for infection is labeled above each lane.

The deletion in dl808 produces a genome that is 33 kb long (16). The even spacing and molecular size of each band are compatible with concatemer formation in which unit genomes are added to one another. As the infection progressed, the number of bands increased, and the proportion of DNA in the highest molecular weight DNA increased. These data suggest that the lower molecular weight products are precursors to the higher molecular weight DNA.

Effect of Mutation of Genes in the E4 Region. To define which ORF in E4 was responsible for production of concatemers, a series of mutants were used in which each E4 ORF was individually mutated. The in351, in352, dl355, dl356, dl358, dl359, and inORF3 mutants produced only single bands (Fig. 2*B*; see Fig. 1 for the ORF affected by each of these mutations). E4 ORF3 and ORF6 are redundant regulators of viral DNA synthesis (18). Therefore, a series of double mutants in ORF3 and ORF6 were tested for the concatemer phenotype. Individual mutations in dl355 (an ORF6 mutation) and in inORF3 (an ORF3 mutation) synthesized only monomer genomes (Fig. 2*B*). When the double mutants inORF3/dl355 and inORF3/inORF6 were used, a ladder of bands similar to that seen with the large E4 mutations was produced (Fig. 2*B*). Finally, when only ORF3 was added back to the large E4 deletion of dl366, no concatemers were formed (Fig. 2*B*, dl366+ORF3). These data demonstrate that suppression of concatemers is a combined effect of ORF3 and ORF6 of E4. Only monomer genomes were produced in any mutant in which either ORF3 or ORF6 remained intact.

Structure of the DNA Junctions. The finding that multimers were synthesized during viral DNA synthesis of E4 mutants made defining the orientation of the genomes at the concatemer joints important for understanding the mechanism of concatemer formation. A restriction map of the junction molecules in the multimers of dl366 and dl808 was attempted using the restriction enzyme *Hin*dIII. This experiment gave a surprising result. Instead of producing one or more new restriction fragments that would define the orientation of the junction (see below), a heterogeneous distribution of fragments was generated ranging in size from 2 kb to 10 kb [Fig. 3*A*, lane 5 (dl366) and lane 8 (dl808)]. The size of the E1A terminal *Hin*dIII fragment from a monomer genome was 2.8 kb in Ad5, dl366, and dl808 (Fig. 3*A*, lanes 1, 4, and 7). The E4 terminal *Hin*dIII DNA fragments were 1.1, 1.7, and 1.8 kb, respectively (data not shown). The largest junction fragment predicted from ligation of the E1 end to itself is 5.6 kb, but when *Hin*dIII digests of multimeric genomes were probed with E1A DNA the smear extended beyond 10 kb, implying that a process other than simple ligation of one genome to another was operating. Also, fragments smaller than the starting 2.8 kb were produced, demonstrating that either a gain of new *Hin*dIII sites or a loss of genetic information occurred in some of the molecules generated during concatemer formation.

One possible explanation of the smear of junction fragments is that the DNA molecules in the smear are highly structured and therefore migrate aberrantly in agarose. Another possible explanation is that the terminal fragments have long single-stranded components that hybridize to one another. To test these possibilities, DNA from dl366-infected cells was used in two-dimensional gel electrophoresis (Fig. 3*B*). The first dimension used a nondenaturing agarose gel. The second dimension employed a denaturing alkali gel electrophoresed with 50 mM NaOH. If the smear was due either to an aberrant DNA structure or to annealing of one fragment to another, the molecules present in the smear should migrate at different molecular sizes in the first and second dimensions. If, however, a majority of the junction molecules were linear and covalently intact, then a diagonal should be formed in which the relative migration under nondenaturing and denaturing conditions was the same. The two-dimensional gel in Fig. 3*B* demonstrates a diagonal in which the apparent molecular sizes under nondenaturing and denaturing conditions were the same. These findings indicate that the smear of the junction fragments was predominantly composed of linear, covalently linked molecules.

Orientation of Concatemer Junctions. Given the imprecise joints demonstrated in the *Hin*dIII map, another restriction enzyme, which produces larger fragments, may be more useful to map the orientation of the junction between two genomes in the concatemer. *Bam*HI cuts once in Ad5 wild type and dl366, producing two fragments of 21.5 and 14.5 kb in wild type (Fig. 4*A*, lane 1) and two fragments of 21.5 and 10 kb in dl366 (Fig. 4*A*, lane 4). The E1A probe hybridizes to the 21.5-kb fragment of Ad5 wild type and dl366 (Fig. 4*B*, lanes 1 and 4), which will be called the head of the genome. The E4 probe hybridizes to the 14.5-kb fragment of Ad5 wild type (Fig. 4*C*, lane 1) and to the 10 kb fragment of dl366 (Fig. 4*C*, lane 4); this E4 fragment will be termed the genome tail.

To determine whether the concatemers consisted of head-to-head and tail-to-tail genomes (i.e., E1 joined to E1, and E4 joined to E4) or head-to-tail genomes (i.e., E1 joined to E4) *Bam*HI restriction maps of the junction fragments were prepared. If the genomes of dl366 were joined head-to-head

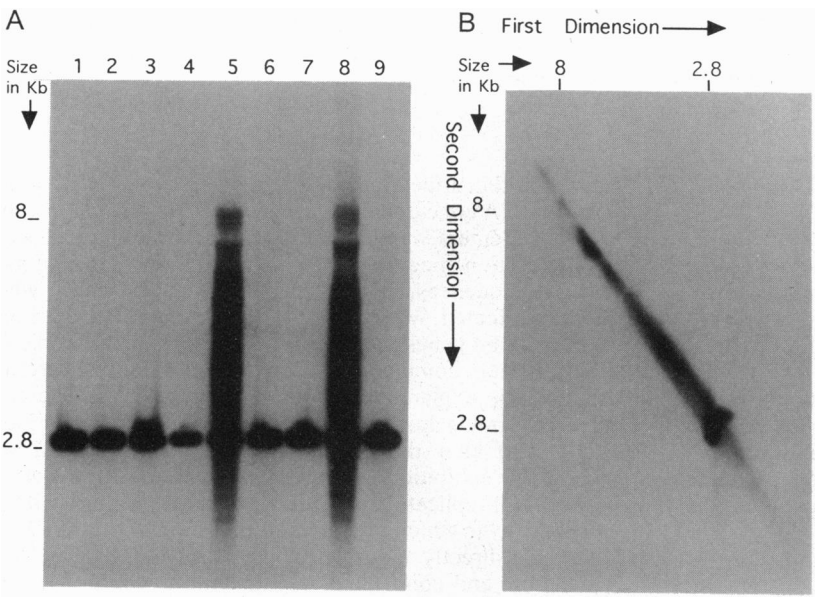

FIG. 3. Southern blot analysis of agarose gel electrophoresis of *Hin*dIII-digested DNA probed with E1A. (*A*) Lanes 1–3, Ad5 wild type; lanes 4–6, dl366; lanes 7–9, dl808; lanes 1, 4, and 7, cesium chloride gradient-purified virus; lanes 2, 5, and 8, total DNA obtained from KB cells 48 hr after infection; lanes 3, 6, and 9, total DNA obtained from W162 cells 48 hr after infection. (*B*) Two-dimensional agarose electrophoresis of DNA obtained from KB cells 48 hr after infection with dl366. The first dimension was run under nondenaturing conditions. The second dimension was run under denaturing conditions.

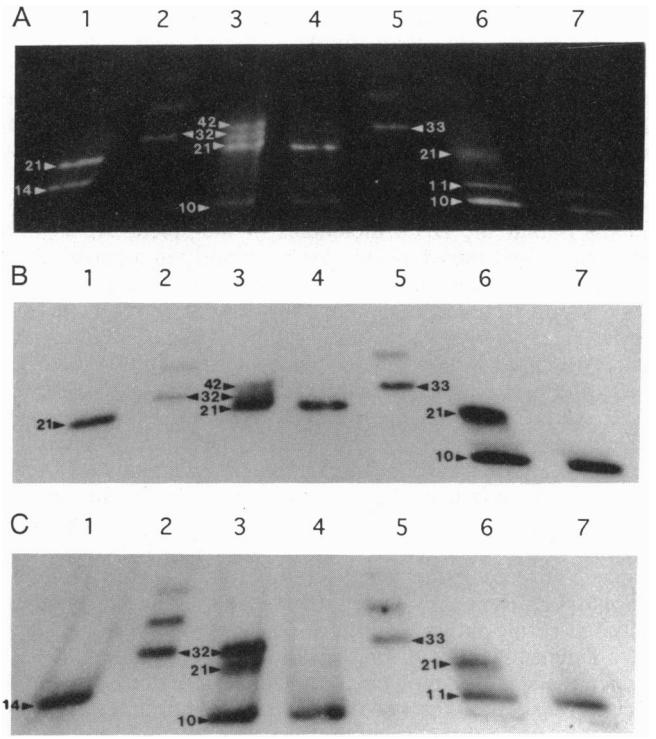

FIG. 4. PFGE of adenovirus DNA. Numbers with arrowheads indicate predicted sizes of bands in kilobases. (*A*) Ethidium bromide stain. Lane 1, *Bam*HI-digested Ad5 wild type; lane 2, undigested dl366 from infected KB cells; lane 3, *Bam*HI-digested dl366 from KB cells; lane 4, *Bam*HI-digested dl366 from W162 cells; lane 5, undigested dl808 from KB cells; lane 6, *Bam*HI digested dl808 from KB cells; lane 7, *Bam*HI digested dl808 from W162 cells. (*B*) Southern blot of the same gel shown in *A* probed with E1A DNA. (*C*) Southern blot of the same gel probed with E4 DNA.

in the concatemers, the junction created by multimerization should be ≈42 kb and would hybridize only with an E1A probe. If the joints were head-to-tail, then the newly created fragment would be 32 kb and would hybridize with both E1A and E4 DNAs. If the joints were tail-to-tail, then the newly created fragment would be 20 kb and would hybridize with only E4 DNA.

When *Bam*HI-digested dl366 concatemers were examined, three new bands were formed. Two additional bands of 32 and 42 kb were seen in the ethidium bromide-stained gel after PFGE (Fig. 4*A*, lane 3), and a third new band, which was not resolved from the 21.5-kb head fragment on ethidium bromide staining, was apparent at 21 kb when a Southern blot of the pulsed-field gel was probed with E4 DNA (Fig. 4*C*, lane 3). The new 42-kb band hybridized only with E1A DNA (Fig. 4*B*, lane 3) and therefore represents a head-to-head junction molecule. The new 32-kb band comigrated with the monomer band of uncut dl366 (compare the bands marked 32 in Fig. 4, lanes 2 and 3) which hybridized with both E1A DNA (Fig. 4*B*, lane 3) and E4 DNA (Fig. 4*C*, lane 3). This monomer-length band, therefore, represents a head-to-tail junction molecule. The predicted size of the tail-to-tail junction is 20 kb. No additional band was apparent at this size in the ethidium bromide-stained gel, but a new band was seen with the E4 probe (Fig. 4*C*, lane 3, marked 21). Therefore, the tail-to-tail junction fragment existed but was not resolved from the head flanking fragment in this gel system. These data indicate that all possible junction molecules existed in dl366 concatemers and that the joining reaction was not orientation-specific.

The interpretation of the dl808 PFGE banding pattern was more difficult, since *Bam*HI cuts this Ad2 derivative at two sites (the site at base pair 15403 is missing; L. H. Epstein and C. H. Young, personal communication), yielding two 10-kb bands and one 11-kb band. Therefore, the three new junction molecules would range in size from 20 to 22 kb. Although a smear of new fragments representing junction molecules was seen around 21 kb when concatemers of dl808 were digested with *Bam*HI (Fig. 4, lane 6), the individual forms were not separated from each other by PFGE.

Therefore, a cloning approach was undertaken to determine the types of junction molecules present in this mutant. Two DNA libraries were made from *Hin*dIII-digested dl808 DNA and Ad5 wild-type DNA. The viruses were isolated from infected KB cells, and the digested DNA was inserted into the *Hin*dIII site of pUC18. When the Ad5 wild-type library was probed, no E1A-hybridizing colonies were obtained. Terminal *Hin*dIII restriction fragments of Ad5 wild type should not be represented in this library, since one end of the terminal fragment is blunt. The DNA library derived from dl808 had 16 colonies that hybridized with E1A and/or E4 DNA. Four colonies hybridized only with E1A DNA and, therefore, represented head-to-head junctions; 8 colonies hybridized only with E4 DNA and thus represented tail-to-tail junctions; and 4 colonies hybridized to both E1A and E4 probes and represented head-to-tail junctions. Therefore, all possible joint orientations existed in the viral concatemers of dl808-infected as well as dl366-infected KB cells.

Complementation of E4 Deletion Mutants. The concatemers that were produced with the E4 deletion mutants could be due to loss of gene function encoded in the E4 region or the result of some structural feature of the mutated viral DNA that allowed annealing of one genome to another. The presence of a copy of the E4 region incorporated into the chromosome of the W162 cell line allowed testing of this question. When DNA was isolated from dl366- or dl808-infected W162 cells, only monomer viral DNA was detectable (data not shown). This demonstrated complementation of the E4 deletion phenotype by E4 gene expression. Data supporting this conclusion are shown in Figs. 3 and 4. When dl366 or dl808 was propagated in KB cells and the DNA was digested with *Bam*HI (Fig. 4*A*, lanes 3 and 6) or *Hin*dIII (Fig. 3*A*, lanes 5 and 8), bands not predicted from the restriction map of the monomer were present. These new bands represent junction molecules (see above). When these mutants were used to infect W162 cells, the only bands that appeared were predicted from the monomer restriction map (Fig. 4*A*, lanes 4 and 7; Fig. 3*A*, lanes 6 and 9). This finding demonstrates that the mutant phenotype could be complemented by expression of E4 genes that were not physically linked to the viral genome. These data indicate that the products of the E4 ORF3 and ORF6 are essential for normal viral DNA synthesis and, hence, responsible for prevention of concatemer accumulation.

DISCUSSION

Mutations that affect both ORF3 and ORF6 of E4 were found to form DNA concatemers during viral synthesis in KB cells. The concatemers were formed by covalent joints that were not orientation-specific and frequently involved loss or gain of DNA sequences. Concatemers were not observed when mutants infected W162 cells, a line transformed with and expressing E4 genes, which demonstrated that diffusible E4 gene products complemented the E4 mutations. There are two possible explanations of this observation. (*i*) The concatemer-forming mutants have lost gene functions (ORF3 and ORF6) that alter the activity of mammalian factors, and without the inhibition or stimulation of these host factors, normal viral replication intermediates are drawn into a dead-end pathway in which concatemers are formed. (*ii*) ORF3 and ORF6 are directly involved in the pathway of viral DNA replication, and concatemer accumulation occurs when the

pathway is blocked. These two sets of possibilities are not mutually exclusive.

Host-mediated ligation has been invoked to explain the appearance of concatemers of input plasmids containing adenovirus origins of replication that was observed in the *in vivo* model of viral DNA replication (10, 12, 13). This explanation of plasmid concatemer formation is an example of a dead-end pathway in which concatemers are formed because the host DNA ligase produces multimeric genomes. Activity of a mammalian ligase is not, however, sufficient to explain concatemer formation of test plasmids, because replicating constructs in the *in vivo* model have covalently attached terminal protein (10) and, therefore, would not be substrates for ligase until the serine adduct had been removed from the 5′-terminal phosphate. At least one other enzymatic activity, such as a cellular endonuclease or exonuclease, would be required to remove the protein adduct. Alternatively, a viral molecule could be produced by a cellular DNA primase–polymerase that could initiate viral DNA replication without a terminal protein. An exonuclease, endonuclease, or DNA primase–polymerase would create a viral genome that had lost DNA sequences and was a substrate for DNA ligase. Another possibility is that small regions of homology present in direct and inverted subrepeats contained in the ITR of the adenovirus genome could be a substrate for a host recombination pathway. Recombination in a palindrome within an ITR of two different viral genomes would produce multimers directly. If recombination occurred at a low fidelity with internal subrepeats in the ITR, DNA sequences could be lost. Any of these four enzyme activities could lead to junctions with imprecise joints and no orientation specificity, which were observed in dl808 and dl366.

The mechanism by which the E4 mutants could cause persistence of host factors responsible for concatemer formation may be through loss of the host shutoff phenotype. In normal viral infection, host DNA synthesis and protein synthesis are shut off (17). With mutants in which E4 ORF6 is the only E4 gene expressed, host proteins are efficiently shut off, and viral replication is normal (21). But after infection with mutants in ORF6, host DNA and protein synthesis persist 24 hr into infection (16, 18). ORF6 mutants would be expected to have higher levels of host enzyme activity than would be found in wild-type adenovirus infection. The abnormal persistence of host enzymatic activities such as an endonuclease, recombinase, or DNA polymerase is not sufficient to explain the concatemer phenotype, since mutants such as dl355, an ORF6 mutant that does not suppress host DNA synthesis, form only monomers.

Alternatively, concatemers could be normal intermediates of viral DNA synthesis, and ORF3 and ORF6 could be involved in the pathway that converts multimers to monomers. Evidence that multimers exist in adenovirus replication comes from electron microscopy (14). When protease is not used in viral DNA isolation, multimers and circular molecules are frequently found; protease treatment resolves these molecules to unit genomes (14). The electron microscopic study that defined the currently held model for viral DNA replication did use protease for viral DNA preparation (3). Other data that support the possibility that multimers are formed during replication come from the *in vivo* model of adenovirus DNA replication in which multimers are formed (10–13).

A rolling circle model found in bacteriophage ϕX174 or a rolling hairpin model found in adeno-associated virus would produce concatemers (22), but the former produces only head-to-tail molecules and the latter produces only head-to-head molecules that progress from one to two to four to eight genome lengths. The finding of head-to-head and head-to-tail junctions with size increments of one genome length in the E4 mutants suggests that both models of adenovirus DNA synthesis are unlikely.

One model that could account for the current observations is a replication-associated overlap recombination in which the leading-strand synthesis is protein-primed from both ends of the genome and produces single-stranded molecules that are available for duplex formation and completion of the genome. Concatemers would be formed after priming of a palindrome of one ITR with the reiterated sequences of another ITR. The occurrence of the palindrome within the ITR would allow head-to-head and head-to-tail molecules to form. Such overlap recombination was observed between two plasmid constructs in which each carried a single copy of the adenovirus ITR (11). The T7 bacteriophage presents another model in which linear DNA molecules that carry terminal redundancies replicate through concatemer intermediates (22). The 5′-to-3′ exonuclease needed in the *in vitro* model of adenovirus DNA replication (9) would produce molecules similar to those of T7. Neither of these models predicts the loss of DNA sequences at the junctions as observed with H5dl366 and H2dl808 DNA synthesis. If a multimer is in the pathway of viral DNA replication, then ORF3 and ORF6 would be involved both in the fidelity of the reaction and in resolving higher-order forms to monomers.

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