Independent Mutations in Ad2ts111 Cause Degradation of Cellular DNA and Defective Viral DNA Replication

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An adenovirus mutant, Ad2ts111, has previously been shown to be temperature sensitive for viral DNA replication in vivo and also to induce degradation of cellular DNA. Soluble nuclear extracts prepared from Ad2ts111-infected HeLa cells grown at either the permissive (32°C) or the nonpermissive (39.5°C) temperature are thermolabile for elongation but not for initiation of DNA replication in vitro. Adenovirus single-stranded-DNA-binding protein purified from wild-type-infected cells can complement these extracts at the restrictive temperature in vitro. The DNA-binding protein synthesized in Ad2ts111-infected cells is stable at the nonpermissive temperature and is phosphorylated, as is the wild-type protein. In contrast, the mutant DNA-binding protein synthesized in Ad5ts125-infected cells is unstable. Ad2ts111 and Ad5ts125 do not complement each other for virus growth in vivo. These results suggest that Ad2ts111 contains a mutation in the DNA-binding protein that affects viral DNA synthesis. Finally, we demonstrated that, unlike viral DNA synthesis, the induction of cellular DNA degradation in Ad2ts111-infected cells is not temperature sensitive and that this phenotype is a result of a mutation in early region 1 on the virus genome. Thus, the two phenotypes displayed in Ad2ts111-infected cells, namely, the temperature-sensitive replication of viral DNA and the degradation of cell DNA, are the result of two separate mutations.

Characterization of cell extracts capable of initiation and elongation of adenovirus DNA replication in vitro has led to the identification of five proteins that are required to complete one round of DNA synthesis (4, 29, 35). Three of these proteins, encoded by the virus genome, are the adenovirus single-stranded-DNA-binding protein (DBP), the terminal protein precursor (pTP), and the adenovirus DNA polymerase (Adpol), whereas the other two are cellular proteins. The identification and characterization of these virus-encoded proteins has been greatly facilitated by the use of adenovirus mutants that contain alterations in replication functions.

Recently, by using complementation of defective Ad5ts36- or Ad5ts149-infected cell extracts, the Adpol was purified and shown to contain a DNA polymerase activity that is required for both initiation and elongation of DNA synthesis in vitro (10, 24, 30, 37). Similarly, defective extracts have been prepared from cells infected with another mutant, Ad5ts125, and in this case the defect was complemented in vitro by DBP purified from wild-type-infected cells (18, 30). Furthermore, extracts prepared from Ad5ts125-infected cells grown at the permissive temperature could be inactivated by extensive incubation at 37°C in vitro, and these inactive extracts could also be complemented by wild-type DBP (18, 41). To date, mutations affecting the pTP have not been reported.

Martin et al. (28) isolated a temperature-sensitive mutant of adenovirus type 2 (Ad2), Ad2ts111, that was defective for viral DNA replication and also had the unusual phenotype of inducing degradation of cell chromosomal DNA (6). Wild-type adenovirus normally does not induce degradation of cell DNA. This mutant was reported to complement both Ad5ts36 (in the early region E2B Adpol gene) and Ad5ts125 (in the early region E2A DBP gene), and thus the Ad2ts111 mutant represented a third, unidentified complementation group of mutants that affect DNA replication. In addition,

because this mutant also induced degradation of cell DNA, it suggested that the biochemical defect in DNA replication and cell DNA degradation may be caused by the same mutation.

By utilizing a combination of genetic studies in vivo and biochemical studies in vitro, we showed that the DNA replication defect in Ad2ts111 is due to an altered single-stranded-DNA-binding protein and that the degradation of cellular DNA induced by this mutant is due to a different mutation in early region 1 on the virus genome. Initial characterization of the DNA degradation phenotype is also presented. A preliminary observation related to these results was reported previously (36).

MATERIALS AND METHODS

Cells and viruses. All viruses were propagated in suspension cultures of HeLa cells, and plaque titers were determined on monolayer cultures of HeLa or 293 cells. Wild-type Ad2 was propagated at 37°C; Ad5ts125 (11) was obtained from H. S. Ginsberg and C. S. H. Young (Columbia University, New York, N.Y.) and was grown at 32.5°C (permissive temperature); Ad2ts111 (28) was obtained from J. Sussenbach (State University of Utrecht, The Netherlands) and was also grown in HeLa cells at 32.5°C. Both temperature-sensitive mutants gave at least 100-fold more plaques at 32.5°C than at 38.5°C (the nonpermissive temperature from plaque assays). Viruses were purified as described previously (26).

DNA replication in vitro. Nuclear and cytoplasmic extracts from virus-infected HeLa cells were prepared as described by Challberg and Kelly (3), except that cells were infected at a multiplicity of infection of 50 PFU per cell. Extracts from Ad5ts125- and Ad2ts111-infected HeLa cells were prepared in a similar manner after infection of cells at 32.5°C for 40 h or at 39.5°C for 21 h. DNA replication with Ad2 DNA-protein complex as template DNA was performed as described previously (37). Reaction mixtures were set up on ice and then placed directly at either 37 or 30°C and incubated for 1 h. The formation and detection of the pTP-dCMP

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initiation complex by using crude nuclear extracts prepared from virus infected cells has also been described previously (40).

Enzymes and proteins. The adenovirus single-stranded-DNA-binding protein was purified from wild-type-infected HeLa cells by the procedure described by Schechter et al. (34). Samples of the purified preparation appeared as a single band as determined by Coomassie brilliant blue staining after electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (21). Restriction enzymes were purchased from New England Biolabs, Inc., Boston, Mass.

Immunoprecipitations. Virus-infected HeLa monolayer cells were labeled with either [35 S]methionine or 32 P at various times after infection and at various temperatures. The cells were scraped from the dish, washed with phosphate-buffered saline, and then resuspended in lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride; 0.5 ml per 10^6 cells). The cell suspension was then sonicated twice for 10 s and centrifuged in an Eppendorf centrifuge for 10 min at 4°C. The supernatant was used for immunoprecipitations with a monoclonal antibody (B6-6) made against the adenovirus 5 (Ad5) DBP (31). Immunoprecipitations with 100 μ l of each extract and 100 μ l of antibody were performed as described by Ross et al. (32) with slight modifications. The immunoprecipitates were analyzed by electrophoresis on 15% SDS-polyacrylamide gels (21) and autoradiography.

Immunofluorescence. HeLa cells growing in monolayer culture on glass cover slips at 50% confluency were infected with each virus at a multiplicity of infection of 10 PFU per cell and incubated at 32.5°C for 24 h. The cells were then incubated for a further 4 h at either 32.5 or 39.5°C and then washed with phosphate-buffered saline and fixed in 3.7% formaldehyde for 3 min followed by acetone-methanol (1:1 [vol/vol]) for 5 min at -20°C. The cells were then air dried, rehydrated in phosphate-buffered saline, and incubated with the monoclonal antibody against Ad5 DBP (B6-6) for 30 min at room temperature. This antibody was washed from the cells, and a second, fluorescein-conjugated goat anti-mouse antibody (diluted 1 in 20; Antibodies Inc., Davis, Calif.) was added for 30 min at room temperature. The second antibody was washed from the cells with phosphate-buffered saline, and the cells were placed under a cover slip in mounting medium containing 0.1% *p*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.).

Extraction of cell DNA. DNA from infected cells was extracted by the method of Hirt (13) with slight modifications. The "Hirt" supernatant was extracted with phenol and chloroform-isoamyl alcohol (24:1 [vol/vol]) followed by ethanol precipitation. Before gel electrophoresis, samples were digested with pancreatic RNase A (20 μ g/ml).

RESULTS

Replication in vitro with Ad2ts111-infected cell nuclear extracts. To investigate whether the temperature-sensitive defect in Ad2ts111-infected cells could be reproduced in vitro, soluble nuclear extracts were prepared from both wild-type-infected HeLa cells grown at 37°C and mutant-infected HeLa cells grown at 39.5°C for 21 h in the presence of hydroxyurea. The template DNA for replication was restriction enzyme-digested Ad2 DNA-protein complex, and specific replication of viral DNA was measured by the preferential replication of the origin-containing terminal fragments (*Eco*RI A and C fragments and *Hind*III G and K fragments) (15). Figure 1a demonstrates that wild-type nuclear extracts supported adenovirus-specific DNA synthesis, whereas

FIG. 1. DNA replication in extracts prepared from wild-type- and Ad2ts111-infected cells. Nuclear extracts prepared from cells infected with Ad2 at 37°C or Ad2ts111 at 39.5°C were incubated with restriction enzyme-digested Ad2 DNA-protein complex as template DNA at 37°C in vitro. The reaction products were incubated with RNase (20 μ g/ml) for 15 min at 37°C followed by incubation with pronase (1 mg/ml) and SDS (0.5%) for 1 h and then subjected to electrophoresis in a 1% agarose gel. (a) Gel stained with ethidium bromide. (b) Autoradiogram of the dried gel. Lanes 1, *Eco*RI-digested DNA-protein complex; lanes 2, *Hind*III-digested DNA-protein complex. The respective fragments are indicated at the side of each panel.

Ad2ts111 (39.5°C) nuclear extracts did not. In the ethidium bromide-stained gel (Fig. 1a), the smear of DNA over the Ad2 template DNA in the lanes containing Ad2ts111 nuclear extract is degraded cellular DNA induced in Ad2ts111-infected cells (see below).

The DNA replication in Fig. 1 was performed at 37°C in vitro, which is close to the in vivo nonpermissive temperature of 39°C. It was possible that the DNA synthesis in Ad2ts111 nuclear extracts was thermolabile in vitro, and Fig. 2a shows that this was indeed the case. Nuclear extracts prepared from Ad2ts111-infected cells grown at either the permissive (32.5°C) or the nonpermissive (39.5°C) temperature were used for DNA replication in vitro with *Hind*III-digested template DNA. Both extracts replicated DNA at 30°C, but neither replicated DNA at 37°C, demonstrating that DNA synthesis with Ad2ts111 nuclear extracts was thermolabile, irrespective of the temperature of virus infection in vivo.

The thermolabile defect in Ad2ts111 nuclear extracts could have been at the stage of initiation of DNA synthesis or at a subsequent stage in chain elongation. Initiation of adenovirus DNA synthesis is measured by the formation of a

FIG. 2. Thermolabile elongation of DNA synthesis in vitro. (a) Nuclear extracts from Ad2ts111-infected cells grown at 32.5 or 39.5°C were incubated with *Hind*III-digested Ad2 DNA-protein complex at 30 or 37°C in vitro. The reaction products were subjected to agarose gel electrophoresis and autoradiography as described in the legend to Fig. 1. G_s refers to the single-stranded *Hind*III G fragment produced by multiple rounds of initiation (15). (b) Nuclear extracts prepared from Ad2-infected cells grown at 37°C or Ad2ts111-infected cells grown at 39.5°C were incubated with Ad2 DNA-protein complex in the presence of [α -³²P]dCTP, and the reaction products were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Reactions were at 30 or 37°C as indicated. The molecular weight markers were phosphorylase B (94,000), bovine serum albumin (68,000), and ovalbumin (43,000) as indicated on the right of the panel.

covalent complex between the pTP and dCMP, which then acts as a primer for DNA synthesis (5, 25, 35, 40). This reaction is dependent upon pTP, Adpol, and a host protein (factor I) (35). Figure 2b demonstrates that the formation of the pTP-dCMP complex was not thermolabile in vitro in Ad2ts111 nuclear extracts and suggests that the virus-coded pTP and Adpol are not defective, at least for initiation of DNA synthesis.

Complementation of the Ad2ts111 defect with DBP. A third virus-encoded protein that is required for DNA replication is DBP. This protein, present in both nuclear and cytoplasmic extracts prepared from adenovirus-infected cells, is defective in extracts prepared from the early region E2A mutant Ad5ts125 (18, 30). Thus, nuclear extracts prepared from

HeLa cells infected with Ad5ts125 at the nonpermissive temperature (39.5°C) do not support adenovirus DNA replication at any temperature in vitro. Figure 3 demonstrates that a cytoplasmic extract prepared from Ad2ts111-infected cells grown at either the permissive (32.5°C) or nonpermissive (39.5°C) temperature was able to complement, at 30°C in vitro, the defective Ad5ts125 nuclear extracts. The complementing activity present in Ad2ts111 cytoplasmic extracts was thermolabile in vitro, as evidenced by the absence of DNA replication at 37°C, whereas complementation of Ad5ts125 nuclear extracts by DBP purified from wild-type Ad2-infected cells was not thermolabile. These results implicated DBP as being responsible for the thermolabile defect in Ad2ts111 extracts.

DBP was purified from the nuclei of Ad2 wild-type-infected HeLa cells and used in complementation experiments with nuclear extracts prepared from wild-type Ad2-, Ad2ts111-, and Ad5ts125-infected cells (Fig. 4). In these experiments (Fig. 4b, c, and d), the nuclear extracts were

FIG. 3. Complementation of Ad5ts125 nuclear extracts in vitro. Nuclear extracts were prepared from Ad5ts125-infected cells grown at 39.5°C and cytoplasmic extracts from cells infected with Ad2ts111-infected cells grown at 32.5 or 39.5°C as indicated. All reactions contained *Hind*III-digested template DNA, Ad5ts125 nuclear extract, and 10 μ l of Ad2ts111 cytoplasmic extract (32.5 or 39.5°C) or 0.5 μ g of Ad2 DBP as indicated and were incubated at 30 or 37°C for 1 h. The reaction products were subjected to agarose gel electrophoresis and autoradiography as described in the legend to Fig. 1.

FIG. 4. Complementation of nuclear extracts with purified DBP. (a-d) Nuclear extracts were prepared from Ad2-, *Ad2ts111*-, and *Ad5ts125*-infected cells grown at 37, 39.5, and 39.5°C, respectively, and fractionated over DEAE-cellulose as described previously (37). An unfractionated wild-type Ad2 extract is shown in panel a. (b-d) Replication reactions contained nuclear extracts, *Hind*III-digested Ad2 DNA-protein complex in the presence (+) or absence (-) of purified DBP, and were incubated at 37°C for 1 h before agarose gel electrophoresis and autoradiography. The lanes in d are not aligned with the lanes in a through c. (e) Incorporation of [α - 32 P]dCMP into DNA with increasing amounts of purified DBP added to *Ad2ts111* or *Ad5ts125* nuclear extracts prepared from cells grown at 39.5°C and incubated for 1 h at 37°C in vitro.

passed through a DEAE-cellulose column to remove the nucleic acid present (especially in the *Ad2ts111* nuclear extracts), thus eliminating the possibility that the high amount of degraded DNA in *Ad2ts111* nuclear extracts was inhibiting DNA synthesis at 37°C. A control containing unfractionated crude extract from Ad2-infected cells (Fig. 4a) is shown as a comparison for the amount of replication observed with the Ad2 DEAE extract (Fig. 4b). Both *Ad5ts125* nuclear extracts (Fig. 4d) and *Ad2ts111* nuclear extracts (Fig. 4c) were complemented for DNA synthesis in vitro by the addition of purified DBP at 37°C, and the amount of complementation was dependent on the concentration of DBP for both *Ad5ts125* and *Ad2ts111* nuclear extracts (Fig. 4e). In each case, especially with the *Ad5ts125* nuclear extract but even with the Ad2 nuclear extract, the addition of DBP decreased the background of nonspecific DNA replication. The reason for this is not known, but it may be due to DBP binding to gaps in the template DNA. These experiments clearly demonstrate that the defect in *Ad2ts111* DNA replication in vitro is due to an alteration in the virus-coded single-stranded-DNA-binding protein, which is a product of early region E2A.

Stability of DBP produced in *Ad2ts111*-infected cells. The inability of *Ad2ts111* to replicate viral DNA in vivo could be due to either an unstable DBP or an alteration in the intracellular localization of DBP. Figure 5a and b show by

immunoprecipitation analysis that the DBP was synthesized in *Ad2ts111*-infected HeLa cells at the nonpermissive temperature and was produced at levels similar to that of DBP synthesized in wild-type-infected cells grown in the presence of hydroxyurea, which mimics the DNA replication block in *Ad2ts111*. Furthermore, DBP, which is normally phosphorylated in wild-type-infected cells (16, 23, 33), was also phosphorylated in *Ad2ts111*-infected cells (Fig. 5c). Figure 5c also demonstrates that the phosphorylated form of DBP in both wild-type- and *Ad2ts111*-infected cells was stable after a 4-h shift to the nonpermissive temperature, suggesting that the replication defect was not due to degradation or dephosphorylation of DBP.

Figure 6 shows an immunofluorescence analysis of HeLa cells infected with Ad2 wild type, *Ad2ts111*, and *Ad5ts125*. Cells were infected at the permissive temperature (32.5°C), and at 24 h after infection were either left at 32.5°C or shifted to the nonpermissive temperature (39.5°C) for a further 4 h. The cells were then fixed and stained by using a monoclonal antibody against Ad5 DBP and a fluorescein-labeled second antibody. Both Ad2 wild type and *Ad2ts111* show intense nuclear staining at both temperatures, whereas *Ad5ts125* shows intense fluorescence only at 32.5°C and weak diffuse fluorescence at 39.5°C, which was due to the degradation of the unstable *Ad5ts125* DBP (12, 23). In the *Ad2ts111* 39.5°C nuclei, the staining pattern was slightly different from that at

FIG. 5. Immunoprecipitation analysis of DBP. Cells were infected with either Ad2 or Ad2ts111 at a multiplicity of infection of 20 PFU per cell and were labeled with [^{35}S]methionine or ^{32}P as shown and then harvested. The DBP was immunoprecipitated from cell extracts by using a monoclonal antibody against Ad5 DBP. In each case, the left lane shows the first immunoprecipitate and the right lane a second immunoprecipitate from the supernatant that remained after precipitation of the first antibody with Staph A. (a) Cells were incubated at 39.5°C for 18 h and labeled with [^{35}S]methionine for 4 h. (b) Cells were incubated at 39.5°C for 17 h and the Ad2 wild-type-infected cells also contained 10 mM hydroxyurea. The cells were labeled for 3 h. (c) Cells were incubated at 32.5°C for 24 h and then labeled with ^{32}P for 1 h at 32.5°C. The ^{32}P was then removed and cells were incubated at either 32.5 or 39.5°C for a further 4 h. All immunoprecipitates were subjected to electrophoresis on 15% SDS-polyacrylamide gels and autoradiographed.

32.5°C in that the globular pattern of staining was not as pronounced. However, the DBP in Ad2ts111-infected cells grown at 39.5°C remained in the nucleus and was stable for at least 4 h.

Two mutations in the Ad2ts111 genome. It has been reported previously that Ad2ts111 can be complemented for the replication defect by coinfection with Ad5ts125 and that the Ad2ts111 mutation mapped within the left 30 map units (percent) of the genome (7, 28). These results would eliminate a primary defect in the DBP gene (62 to 67 map units on the genome). We have reexamined the map location of the Ad2ts111 mutation, particularly to determine whether the DNA replication defect and the cellular DNA degradation defect were caused by the same mutation.

Table 1 shows that Ad2ts111 could complement mutant Ad5ts149 (mutation located at 18 to 22.5 map units) for lytic growth at the nonpermissive temperature but could not complement the Ad5ts125 mutation. This result indicates that the DBP in Ad2ts111-infected cells could not complement the defective DBP from Ad5ts125 and suggested that the primary defect for temperature-sensitive growth in Ad2ts111 was in the E2A DBP gene. Furthermore, we have been able to isolate temperature-independent recombinants between Ad2ts111 and Ad5, and these recombinants synthesize normal amounts of viral DNA in vivo at 39.5°C (unpublished data), but, surprisingly, these ts^+ recombinants retained the second phenotype of Ad2ts111, the ability to induce degradation of cell DNA.

To confirm the possibility that the cell DNA degradation defect might be unrelated to the temperature-sensitive defect in DBP, we constructed recombinants between Ad5d/309 DNA and Ad2ts111 DNA and screened recombinants for the DNA degradation phenotype. Ad5d/309 is a nondefective virus containing a single *Xba* site at 3.8% (17). Six of 15

recombinants formed when the Ad2ts111 *Bgl*III E fragment (0 to 9.1 map units) and the Ad5d/309 *Xba* A fragment (3.8 to 100 map units) were cotransfected into 293 cells had the DNA degradation phenotype, suggesting that the lesion causing this phenotype maps between 3.8 and 9.1 map units. Four of these six recombinants which had the DNA degradation phenotype also contained the Ad2ts111 *Xmn*I restriction enzyme site at 6.1 map units (Ad5 DNA does not contain this site). Furthermore, these recombinants were not temperature sensitive for growth. These results suggest that the Ad2ts111 genome contains two mutations; one is in the DBP gene, conferring a temperature-sensitive phenotype for viral DNA replication and lytic growth, and the second causes degradation of cell DNA and maps in early region 1 between 3.8 and 9.1 map units. A prediction from these results is that the DNA degradation phenotype should be observed in the absence of viral DNA replication.

Degradation of cell DNA in Ad2ts111-infected cells. It was noted above (Fig. 1) that the nuclear replication extracts prepared from Ad2ts111-infected cells contained a smear of DNA that was visualized by ethidium bromide staining of the DNA after electrophoresis through an agarose gel. Nuclei were prepared from either wild-type-, Ad2ts111-, or Ad5ts125-infected HeLa cells that had been grown in the presence of hydroxyurea, which inhibits both virus and cell DNA replication. The nuclei were successively incubated in buffers containing 0.1 M NaCl, 0.35 M NaCl, and finally 2 M NaCl for 0.5 h on ice. After each incubation, the nuclei were centrifuged and resuspended in buffer containing increasing amounts of NaCl as indicated, and each supernatant was treated with RNase followed by pronase and then subjected to agarose gel electrophoresis (Fig. 7). DNA was released from Ad2ts111-infected cell nuclei at 0.1 M and 0.35 M NaCl but was not detected in similar elutions from either wild-

FIG. 6. Immunofluorescence analysis of DBP. Cells were infected with either Ad2, Ad2ts111, or Ad5ts125 at 32.5°C for 24 h and then incubated at either 32.5 or 39.5°C for a further 4 h. The cells were then stained with a monoclonal antibody directed against the Ad5 DBP and with a fluorescein-labeled second antibody.

type- or Ad5ts125-infected cell nuclei. This result suggests that the degraded DNA is not tightly associated with the nuclear structure. Since the infected cells were grown in the presence of hydroxyurea, which inhibits viral DNA replication and hence late protein synthesis, this result demonstrates that the degraded DNA is of cellular origin and that the altered protein causing degradation is an early gene function, which is consistent with the map location of the mutation.

Figure 8 demonstrates that the degraded DNA could also be extracted by the method of Hirt (13) and that the degradation was specific for Ad2ts111-infected cells (Fig. 8a). Furthermore, the DNA degradation phenotype was not temperature sensitive (Fig. 8b) and increased with increasing multiplicity of infection (Fig. 8c). The fact that the degradation was not temperature sensitive confirmed the genetic separation of the two mutations in the Ad2ts111 genome.

DISCUSSION

The adenovirus DBP is a multifunctional protein, participating in viral DNA replication (11, 14, 18, 42), autoregulation of its synthesis, and the expression of other early genes

(1, 2, 32) as well as in the regulation of late gene expression in nonpermissive cells (19). The Ad2ts111 mutant contains a defective DBP that results in thermolability of DNA replication in vivo and in vitro. The mutation functionally inactivates the DBP but does not alter its stability or its ability to be phosphorylated at the nonpermissive temperature, nor does it alter the intracellular localization of DBP. This is in contrast to the altered DBP present in Ad5ts125-infected cells, which is not stable at the nonpermissive temperature

TABLE 1. Complementation of Ad2ts111 by DNA-negative *ts* mutants of type 5 virus at 38.5°C

Type 5 virus	Expt	Single infection yield (S)		Double infection yield (D)	Ratio (D/S)
		Type 5 virus	Ad2ts111		
Ad5ts125	1	9×10^7	3×10^7	2×10^8	2.2
	2	6×10^7	9×10^7	1×10^8	1.1
Ad5ts149	1	6×10^7	8×10^6	1×10^9	16.6
	2	2×10^7	2×10^7	6×10^8	30

and is rapidly dephosphorylated (23). Furthermore, the DBP present in nuclear extracts prepared from Ad5ts125-infected cells grown at the permissive temperature requires extensive incubation at 37°C to inactivate the protein in vitro, whereas the DBP present in the Ad2ts111 nuclear extracts is inactivated immediately upon incubation at 37°C in vitro. It is likely, therefore, that the mutation affecting the Ad2ts111 DBP is different from the mutation affecting the Ad5ts125 DBP. Recently, the Ad5ts125 mutation has been localized to the carboxy-terminal half of DBP (20). This is the region of the protein required for complementation for DNA synthesis of defective Ad5ts125 nuclear extracts in vitro (9, 41). D'Halluin et al. (7) have suggested that the mutation causing temperature-sensitive growth of Ad2ts111 maps within the left 30 map units on the virus genome, but our data are not consistent with this conclusion. We are currently sequencing the Ad2ts111 DBP gene to determine the location of the mutation within the protein.

Another phenotype of Ad2ts111 is the degradation of cell DNA upon infection of HeLa cells, which is the result of an early gene function. We have shown that this defect is a result of an early region 1 mutation and unlike the DNA replication defect is not temperature sensitive. D'Halluin et al. (6) have also observed this phenotype in Ad2ts111-infected cells, but they have attributed this defect to the same mutation that affects DNA synthesis (7, 28). It is not

FIG. 7. Detection of degraded DNA in infected cell nuclei. HeLa cells infected with Ad2, Ad2ts111, or Ad5ts125 were grown in the presence of hydroxyurea for 21 h at 37, 39.5, and 39.5°C, respectively. Nuclei were isolated (3), and NaCl was added to a final concentration of 0.1 M for 0.5 h on ice. After centrifugation (15,000 $\times$ g, 20 min, 4°C), the nuclei were resuspended in buffer C (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH, pH 7.5, 10% [wt/vol] sucrose) containing 0.35 M NaCl for 0.5 h on ice. The nuclei were again collected by centrifugation and resuspended in buffer C containing 2 M NaCl for 0.5 h on ice. The nuclei were then collected by centrifugation, and all supernatants were digested with RNase followed by pronase in the presence of 0.5% SDS, and a sample was subjected to electrophoresis through a 1% agarose gel. The marker was Ad2 DNA digested with endonuclease *Xba*I.

FIG. 8. Analysis of Hirt supernatant DNAs. HeLa cells were infected with virus and incubated at various temperatures for 41 h, and DNA was isolated by the method of Hirt (13) and subjected to electrophoresis through a 1% agarose gel. (a) Cells were mock infected or infected at a multiplicity of infection of 10 PFU per cell with Ad2, Ad5, or Ad2ts111 and then incubated at 37°C. (b) Cells were mock infected or infected with Ad2ts111 at a multiplicity of infection of 10 PFU per cell and then incubated at the temperature shown. (c) Cells were mock infected or infected with various multiplicities of infection with Ad2ts111 and then incubated at 37°C. In each panel, the marker DNA is *Hind*III-digested Ad2 DNA.

clear why our results differ from those previously reported, but it is not likely to be due to different assays for DNA degradation. Recombinants that we have constructed which contain only the left 9.1% of the Ad2ts111 genome degrade cell DNA and are not temperature sensitive for viral DNA replication; thus, we conclude that two mutations in the Ad2ts111 genome are responsible for the two phenotypes. We are currently investigating the biochemical defect in Ad2ts111-infected cells that causes degradation of cell DNA and are mapping the mutation more precisely within early region 1.

A number of human adenovirus type 12 mutants (*cyr*) have been isolated (38) and shown to induce degradation of cell DNA and pronounced cytopathic effects in human KB cells (8). These mutants are defective for oncogenic transformation and map to early region E1b on the virus genome (22, 27, 38, 39). It is likely that these mutants contain mutations similar to that of the mutation affecting DNA degradation in Ad2ts111.

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