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Adenovirus Early Function Required for Protection of Viral and Cellular DNA

JEAN-CLAUDE D'HALLUIN,* CHANTAL ALLART, CHANTAL COUSIN, PIERRE A. BOULANGER, AND GUY R. MARTIN

Laboratoire de Virologie Moléculaire, Institut National de la Santé et de la Recherche Médicale, 59045 Lille, France

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Studies were done to characterize a DNA-negative temperature-sensitive (*ts*) mutant of human adenovirus type 2, H2 *ts*111. The temperature-sensitive defect, which was reversible on shift-down in the absence of protein synthesis, was expressed as early as 2 h postinfection, and the results of density-labeling experiments are in agreement with at least a DNA replication initiation block. On shift-up, after allowing viral DNA synthesis at permissive temperatures, the newly synthesized viral DNA and the mature viral DNA were cleaved into fragments which sedimented as a broad peak with a mean coefficient of 10-12S. This cleavage was more marked in the presence of hydroxyurea as the DNA synthesis inhibitor. Parental DNA in infected cells was degraded to a much lesser extent regardless of the incubation temperature. In contrast, the parental DNA was strongly degraded when early gene expression was permitted at 33°C before shift-up to 39.5°C. Furthermore, cellular DNA was also degraded at 39.5°C in *ts*111-infected cells, the rate of cleavage being related to the multiplicity of infection. This cleavage effect, which did not seem to be related to penton base-associated endonuclease activity, was also enhanced when early gene expression was allowed at 33°C before shift-up. The *ts*111 defect, which was related to an initiation block and endonucleolytic cleavage of viral and cellular DNA, seemed to correspond to a single mutation. The implication of the *ts*111 gene product in protection of viral and cellular DNA by way of a DNase-inhibitory function is discussed.

Thermosensitive (*ts*) mutants which cannot replicate DNA at nonpermissive temperatures have been isolated from human adenovirus type 2 (Ad2) (10, 12), Ad5 (5, 30), Ad12 (11, 23), and Ad31 (25, 26). Partial characterization of these mutants suggests that adenovirus replication needs at least three virus-coded early gene products (8, 10, 23), each probably being required for a step in chain initiation. Moreover, a very early function has been detected which does not seem to be directly involved in DNA synthesis (10). To our knowledge, no other DNA synthesis-related early function associated with adenovirus DNA-negative phenotypes has been previously reported.

In the present paper, an early function which is altered in cells infected at the nonpermissive temperature with the H2 *ts*111 DNA-negative mutant (12) is reported. Data presented show that viral as well as cellular DNAs are degraded throughout the replicative cycle. The results suggest that an early viral function prevents both viral and cellular DNAs from endonuclease-like cleavage.

MATERIALS AND METHODS

Cells and virus. KB cells were cultured in suspension in Joklik-modified F13 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% horse serum. HeLa cells were grown as monolayers in Eagle minimum essential medium containing 10% calf serum.

The wild-type (WT) Ad2 was originally supplied by J. F. Williams (Carnegie-Mellon Institute, Pittsburgh, Pa.). The Ad2 thermosensitive mutant *ts*111 has undergone preliminary characterization (12). Virus particles were produced and purified as previously described (4). Infectivity was measured by the fluorescent focus assay technique (16) or by the plaque method on HeLa cells. The infectivity titers were expressed in fluorescent focus units or PFU. Cells were infected at a multiplicity of infection (MOI) of 10 to 100 PFU/cell as indicated in each experiment.

Radioisotopes and counting method. [^{3}H]thymidine (27 Ci/mmol) and [^{14}C]thymidine (53.6 mCi/mmol) were purchased from the Commissariat à l'Energie Atomique (Saclay, France). The radioactive samples were precipitated with cold 10% trichloroacetic acid and filtered on Whatman GF-C filters. The filters were washed successively with 2% trichloroacetic acid and cold ethanol, air dried, and counted in a

toluene-based scintillation fluid in an Intertechnique liquid spectrometer.

Labeling conditions. Infected cells were labeled with [^{3}H]thymidine (5 μCi/ml). For pulse-chase experiments, 5×10^{-5} M nonradioactive thymidine (Sigma Chemical Co., St. Louis, Mo.) was added. Density labeling of DNA was accomplished by adding 10^{-4} M 5-bromo-2'-deoxyuridine (Sigma). Noninfected HeLa cells were labeled with [^{14}C]thymidine (1 μCi/ml). WT and *ts*111-purified, DNA-labeled virus particles were prepared from infected cells maintained at 33°C and labeled with [^{3}H]thymidine (1 μCi/ml) from 24 to 96 h. Temperature shifts were made on HeLa cell monolayers by replacing the medium with a medium prewarmed at the required temperature.

Analysis of DNA by zonal sedimentation. (i) Alkaline sucrose gradient centrifugation. The method used for alkaline sucrose gradient centrifugation was that described by Doerfler (5). A total of 5×10^5 to 1×10^6 cells suspended in 100 μl of Tris-saline (0.15 M NaCl + 0.01 M Tris-hydrochloride, pH 7.5) were loaded on top of a 5 to 20% alkaline sucrose gradient (0.3 M NaOH + 0.7 M NaCl + 0.01 M EDTA) and overlaid with 0.4 ml of lysis buffer (1 M NaOH + 0.05 M EDTA). After standing for 16 h at 4°C, the gradients were centrifuged at 4°C in an SW 41 rotor at 35,000 rpm for 5 (viral DNA) or 3 (cellular DNA) h.

(ii) Centrifugation in neutral condition. A total of 10^6 cells were suspended in 0.01 M Tris + 0.001 M EDTA + 0.3 M NaCl + 0.03 M trisodium citrate (pH 7.4) containing 0.5% Sarkosyl (Ciba-Geigy) and 1 mg of pronase (Boehringer; predigested at 37°C for 3 h and heated for 2 min at 85°C before use) per ml and lysed for 3 h at 37°C. Lysates were gently pipetted on top of a 5 to 20% neutral sucrose gradient (1 M NaCl + 0.01 M EDTA + 0.05 M Tris + 0.1% Sarkosyl, pH 7.4) and centrifuged for 5 h at 35,000 rpm at 4°C in an SW 41 rotor. 5-Bromo-2'-deoxyuridine density-labeled DNA was analyzed by centrifugation on a self-generating CsCl gradient (1.7 g/ml) for 72 h at 35,000 rpm and 20°C in an R 50Ti rotor. The gradients were collected dropwise from the bottom of the tubes, and the fractions were assayed for acid-precipitable radioactivity.

Assay for penton base-associated endonuclease activity. The assay for penton base-associated endonuclease activity was carried out as described by R. G. Marusyk (personal communication).

Virus particles (WT and *ts*111) were purified by two cycles of equilibrium centrifugation in CsCl. The virion preparations were diluted to the same optical density (at 260 nm) per milliliter. Virion-derived pentons were obtained by dialysis of virions versus distilled water for 2 h, followed by dialysis against 0.005 M Tris-maleate buffer, pH 6.2, for 16 h at 20°C. The dialysate was centrifuged at 35,000 rpm for 2 h at 20°C in an SW 50.1 rotor. The supernatant, dialyzed for 2 h against 0.01 M citrate, pH 4.5, was used as a source of virion-derived pentons. Adenovirus DNA was extracted from purified WT adenovirus. The incubation mixture consisted of 0.01 M NaCl, 0.01 M citrate, 0.002 M $MgCl_2$, pH 4.5, and 1.5 μg of Ad2 DNA plus an aliquot of the virion-derived penton in a total volume of 20 μl. The reaction was carried out at 39.5°C for 10 min and was stopped by adding 0.01 M EDTA. The size of the DNA was determined by electrophoresis in agarose gel.

Inhibitors. Cycloheximide (Boehringer-Mannheim) was used at a concentration of 20 μg/ml, and hydroxyurea (Serva) was used at a final concentration of 0.01 M.

RESULTS

Viral DNA synthesis after temperature shift-up and shift-down. The efficiency of viral DNA synthesis shutoff after shift-up to the nonpermissive temperature was studied. KB cells infected with *ts*111 were incubated at 33°C for 40 h and subsequently shifted to 39.5°C or kept at 33°C. The infected cells were then labeled with [^{3}H]thymidine from 40 to 42 h postinfection (p.i.). Analysis of labeled DNA at 42 h p.i. on alkaline sucrose gradients showed that there was almost no viral DNA synthesis after shift-up (Fig. 1). Shift-down experiments were performed to determine if the *ts*111 DNA block was reversible. KB cells infected with *ts*111 were maintained at 39.5°C for 17 h and shifted to 33°C or kept at 39.5°C. [^{3}H]thymidine was then added from 17 to 19 h p.i. Viral DNA synthesis immediately resumed after the shift-down even when protein synthesis was inhibited by cycloheximide (Fig. 2).

These data indicate that after shift-up to 39.5°C the capacity to synthesize *ts*111 DNA slows down rapidly and that inactivation of the thermosensitive *ts*111 function is readily reversible, even in the absence of protein synthesis. The existence of fragments lighter than 34S, noticed particularly in cycloheximide-treated cells, suggested the possibility of viral DNA degradation or lack of ligation of Okazaki-like fragments.

Reversibility of the *ts*111 defect by temperature shift-down: infectious virus and viral DNA. To establish the time at which the *ts*111 altered function was expressed in the infectious cycle, *ts*111-infected cells were incubated for varying periods at 39.5°C and shifted down to 33°C. The reversibility of the mutation was determined by titrating the infectious *ts*111 virus produced and by quantitating the synthesized 34S DNA.

HeLa cells infected with *ts*111 were incubated for varying periods of time at 39.5°C and shifted down to 33°C until 98 h p.i. The infectious virus present in crude cell lysates was titrated by the fluorescent focus assay. Figure 3b shows the curve obtained by plotting the values of final virus yields versus time. As early as 2 h p.i., a strong effect was observed at the nonpermissive temperature. It can be concluded, therefore, that the function altered in *ts*111 infection is essential very early in the productive cycle. Analysis of

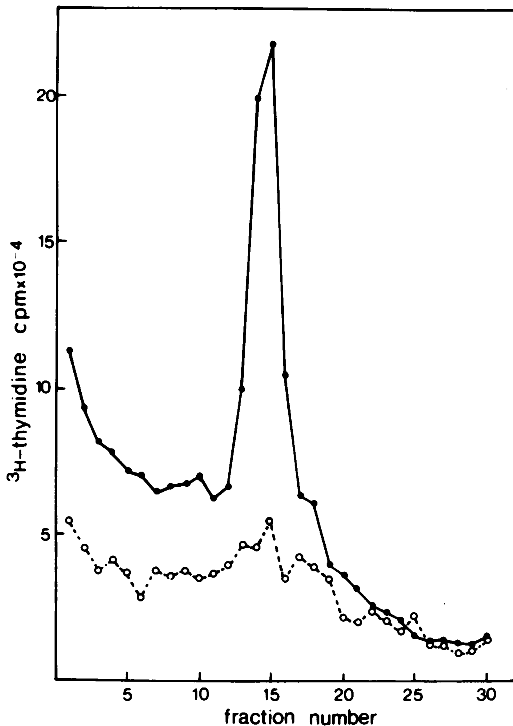

FIG. 1. *Analysis in alkaline sucrose gradients of the DNA synthesized on shift-up to 39.5°C in KB cells infected with mutant ts111. KB cell suspensions inoculated with H2 ts111 at an MOI of 25 PFU/cell were incubated at 33°C for 40 h and subsequently shifted to 39.5°C (○) or kept at 33°C (●). The infected cells were labeled with [^{3}H]thymidine (10 μCi/ml) from 40 to 42 h p.i. At the end of the labeling period, the cells were washed, and aliquots of 5 × 10^5 cells were lysed in alkali on top of alkaline sucrose gradients. The conditions of centrifugation have been described. The direction of sedimentation is to the left.*

the viral DNA synthesis capacity was carried out by infecting KB cells with *ts*111 and incubating the infected cells at 39.5°C for 10, 16, 20, 24, and 32 h before shift-down to 33°C until 44 h p.i. The infected cells were then labeled with [^{3}H]thymidine between 40 and 44 h p.i. Alkaline sucrose gradient analysis revealed that extracted DNA sedimented almost exclusively at 34S. The amount of viral DNA synthesized was determined from the radioactivity in the 34S peak, and the results were expressed as a percentage of the amount of labeled 34S viral DNA synthesized in *ts*111-infected cells at the permissive temperature (Fig. 3a). It was noticed that DNA synthesis was much less rapidly depressed than expected from the previous results. This suggests that a fraction of the viral DNA which appeared on shift-down was somehow unable to evolve into infectious virions.

Viral DNA replication and *ts*111 function. The possible implication of the altered *ts*111 function in the viral DNA replication process was determined as follows. KB cells infected with *ts*111 at 33°C and labeled with [^{3}H]thymidine from 40 to 42 h p.i. were chased for 4 h at 33 or 39.5°C in the presence of 5-bromo-2'-deoxyuridine (10^{-4} M). After Sarkosyl-pronase treatment, the infected cell lysates were subjected to equilibrium centrifugation in cesium chloride. Controls were performed with WT virus (not shown). 5-Bromo-2'-deoxyuridine was not incorporated into viral DNA in the shift-up experiment, whereas a peak was observed at the heavy-

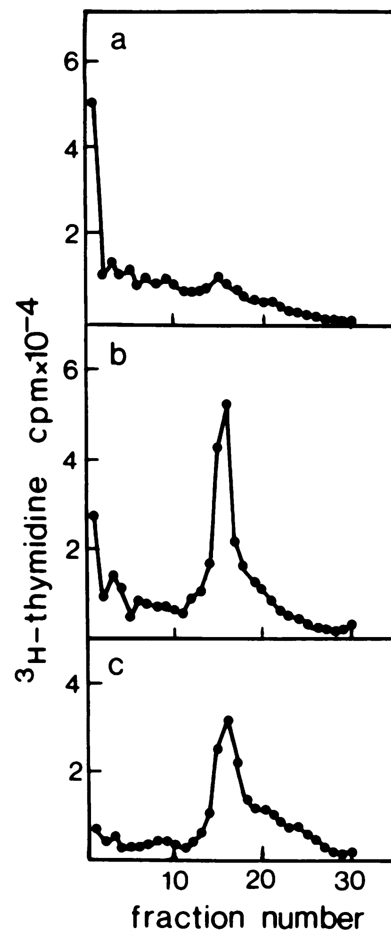

FIG. 2. *Analysis in alkaline sucrose gradients of the DNA synthesized on shift-down to 33°C in KB cells infected with ts111. KB cells inoculated with H2 ts111 at an MOI of 25 PFU/cell were incubated at 39.5°C for 17 h and kept at 39.5°C (a) or shifted to 33°C (b) in the presence of 20 μg of cycloheximide per ml (c). The cells were labeled from 17 to 19 h p.i. with [^{3}H]thymidine (10 μCi/ml). At the end of the labeling period, the cells were treated as described in the legend to Fig. 1.*

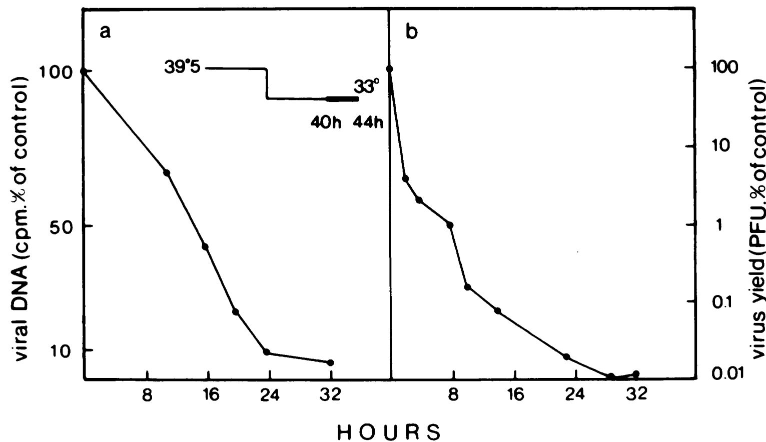

FIG. 3. *Time dependence of the ts111 defect reversibility. (a) Viral DNA synthesis: 5 × 10^{5} KB cells infected with ts111 (MOI = 25) were incubated at 39.5°C for 0, 10, 16, 20, 24, and 32 h before shift-down to 33°C until 44 h p.i. The cells were labeled from 40 to 44 h p.i. with [³H]thymidine (10 μCi/ml). At the end of the labeling period, the cells were treated as described in the legend to Fig. 1. The amounts of synthesized viral size DNA were determined from the radioactivity in the 34S peak. The results are expressed as a percentage of the quantity of labeled viral DNA in ts111-infected cells incubated at 33°C until 44 h p.i. (b) Virus yields: 5 × 10^{6} HeLa cells infected with ts111 (MOI = 25) were incubated at 39.5°C for different lengths of time before shift-down to 33°C until 98 h p.i. Infectious virus production was monitored by the fluorescent focus method, and the results are expressed as a percentage of the virus yield at 98 h p.i. at 33°C. The x axis indicates the time p.i. at which cultures were shifted down to 33°C.*

light density position with extracts from cells maintained at 33°C (Fig. 4). These results indicate that observable reinitiation and elongation do not take place at the nonpermissive temperature.

Fate of newly synthesized viral DNA. To determine whether the viral DNA synthesized at the permissive temperature was affected when the mutation was expressed, the following experiment was carried out. KB cells infected with *ts*111 were incubated at 33°C and labeled with [³H]thymidine between 40 and 42 h p.i. The infected cells were separated into three aliquots; one was stored at +4°C before analysis, and cold thymidine was added to the two others before incubation at 39.5 or 33°C until 46 h p.i. Labeled DNA was analyzed in alkaline sucrose gradients (Fig. 5). A predominant 34S peak was observed just after labeling, together with 40–100S intermediate-size DNA and heavy cellular DNA (Fig. 5a) as described previously (2, 7, 20). After a 4-h chase at 33°C, the 34S peak diminished and slower-sedimenting fragments appeared (Fig. 5b). When the 4-h chase was performed at 39.5°C, the radioactivity was found at sedimentation rates between 7 and 34S (Fig. 5c). Trichloroacetic acid-precipitable counts remained constant during the chases. The *ts*111 lesion, which appeared fully expressed at 39.5°C, was therefore also observed at 33°C, although to a much lesser degree. Under the same conditions, the WT DNA appeared stable (12). These data indicate that the function modified by the *ts*111 mutation is essential to the maintenance of normal-length viral DNA.

The low-molecular-weight DNA material was of viral origin since it hybridized at a rate of >90% with Ad2 WT DNA and at <2% with control KB cell DNA. When analyzed in agarose gel electrophoresis, the cleaved DNA showed a polydisperse pattern, with a band smearing between the position of *Eco*RI fragments A and C of Ad2 DNA used as markers, suggesting a low degree, if any, of cleavage specificity (not shown).

Effect of DNA synthesis inhibition on viral DNA degradation. To determine whether the *ts*111 lesion affects the DNA molecules during their replication cycle or their stability independently of the replication process, hydroxyurea was used as a DNA synthesis inhibitor (24, 28). *ts*111-infected HeLa cells were incubated at 33°C for 42 h and labeled with [³H]thymidine between 40 and 42 h p.i. The infected cells were divided into three equal parts; one was removed immediately after labeling and stored at +4°C until analyzed, and cold thymidine was added to the two others, which were immediately shifted to 39.5°C and maintained at this temperature for 4 h. Hydroxyurea was added to one aliquot at the time of shift-up. DNA was analyzed in alkaline and neutral su-

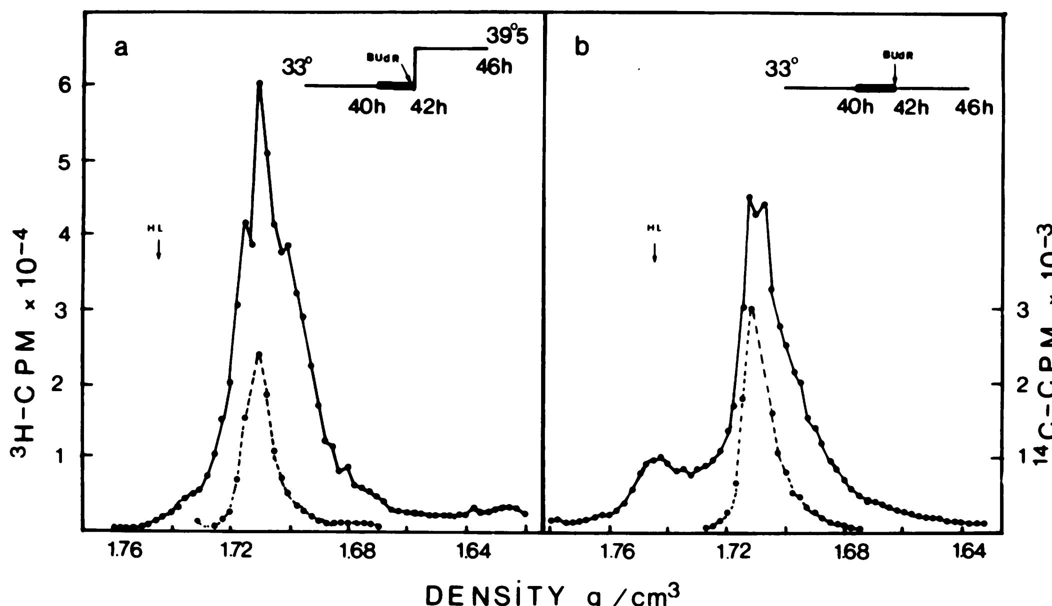

FIG. 4. *CsCl density gradient centrifugation of H2 ts111 DNA labeled with 5-bromo-2'-deoxyuridine (BUdR) at 39.5°C. KB cells infected with H2 ts111 (MOI = 15) were incubated for 42 h at 33°C and labeled with [^{3}H]thymidine (10 μCi/ml) from 40 to 42 h. BUdR (10^{-4} M) and nonradioactive thymidine (5×10^{-5} M) were added at 42 h p.i. Cells were harvested after a further 4 h of incubation at (a) 39.5 or (b) 33°C and treated with Sarkosyl and pronase as described in the text. Viral DNA was analyzed by centrifugation on a self-generating CsCl gradient (1.7 g/ml) in the presence of ^{14}C-labeled Ad2 marker DNA. HL indicates the heavy-light density position. Symbols: ●——●, ^{3}H; ●- - -●, ^{14}C.*

crose gradients (Fig. 6). Immediately after labeling, single-stranded DNA was found at 34S and at lower S values, confirming the occurrence of partial degradation even at the permissive temperature (Fig. 6a). After the chase at 39.5°C, the DNA peak was observed at a sedimentation rate always lighter than 34S (Fig. 6b). When hydroxyurea was added at the time of shift-up, the amount of light DNA fragments strongly increased (Fig. 6c). These results suggest that the main altered function in *ts*111-infected cells is not related to the viral DNA replication process, since DNA breakdown still occurred at a higher rate in the absence of DNA replication.

Analysis on neutral sucrose gradients showed that viral DNA sedimented between 31S and 10S (Fig. 6d), or in some experiments only at 31S. Radiolysis did not account for these results since the sedimentation pattern was not modified regardless of the total incorporated radioactivity. Therefore, single-strand "nicks," starting points of the degradation process, may explain these data.

Pulse-chase of viral DNA. It was shown above that newly synthesized viral DNA was cleaved into small fragments at 39.5°C. Since mature viral DNA might be protected against degradation, it was of interest to study the fate of labeled viral DNA at 39.5°C after different periods of chase at 33°C. KB cells infected with *ts*111 at 33°C and labeled with [^{3}H]thymidine from 40 to 42 h p.i. were shifted up to 39.5°C for 4 h at 42, 46, 50, and 64 h p.i. Hydroxyurea was added at the time of shift-up to prevent viral DNA from being repaired during the chase period at 39.5°C.

The amount of label observed in the alkaline sucrose gradient 34S peak after the 2-h pulse decreased slightly after an 8-h chase at 33°C, and only a few lighter fragments appeared. In contrast, whenever the chase period at 33°C was followed by a shift-up to 39.5°C, marked DNA fragmentation occurred (not shown). These data suggest that the function altered in *ts*111 infection is essential to the integrity of viral DNA regardless of its time of synthesis.

Fate of parental DNA. Since synthesized viral DNA was broken down at 39.5°C, an experiment on the fate of parental DNA at the nonpermissive temperature was performed. KB cells were infected with [^{3}H]thymidine-labeled *ts*111 virions, and the parental viral DNA was examined in alkaline sucrose gradients under the following conditions of incubation: (i) 39.5°C for 16 h (Fig. 7a); (ii) 33°C for 48 h (Fig. 7b); (iii) 33°C for 24 h and shift-up for 16 h (Fig. 7c); (iv)

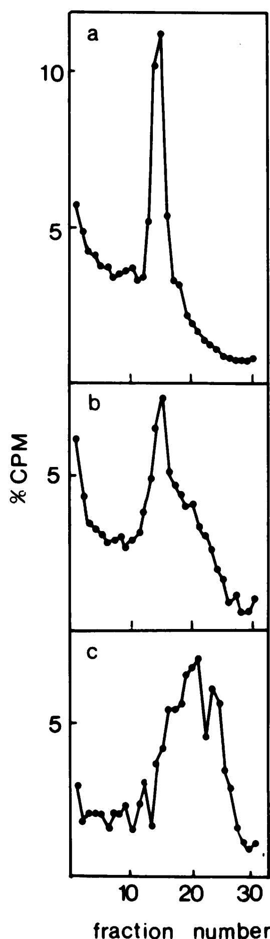

FIG. 5. *Analysis of newly synthesized DNA in KB cells infected with ts111. KB cell suspensions were infected with ts111 (MOI = 25), incubated at 33°C for 42 h, and labeled from 40 to 42 h p.i. with [^{3}H]-thymidine (10 μCi/ml). (a) Cells harvested at 42 h p.i. (b) Cells incubated and radioactivity chased at 33°C until 46 h p.i. (c) Cells incubated at 39.5°C and chase performed until 46 h p.i. Analysis was performed on alkaline sucrose gradients as indicated in the legend to Fig. 1. The results are expressed as a percentage of counts per minute (CPM) in the gradient.*

33°C for 48 h and shift-up for 16 h (Fig. 7d); (v) addition of hydroxyurea and then incubation at 33°C for 48 h and shift-up for 16 h (Fig. 7e). A control experiment was carried out by coinfecting KB cells with DNA-labeled *ts*111 and unlabeled WT for 18 h at 39.5°C (Fig. 7f). The *ts*111 parental DNA was slightly degraded at 39.5°C (Fig. 7a) and 33°C (Fig. 7b). In contrast, incubation at 33°C for 24 (Fig. 7c) and 48 (Fig. 7d) h followed by a shift-up at 39.5°C for 16 h provoked a strong degradation of the parental DNA. This degradation was much more marked when hydroxyurea was added (Fig. 7e). The data presented indicate that parental DNA is only slightly degraded when infection takes place at the nonpermissive temperature, whereas it is almost completely broken down when early genes are expressed for 24 and 48 h at 33°C before incubation at 39.5°C.

Fate of prelabeled cellular DNA. Since the above results suggested that the *ts*111 altered function might be implicated in the protection of viral DNA, it was of interest to establish whether this function was specific for protection of viral DNA or could also affect the fate of cellular DNA. HeLa cell monolayers were labeled at 37°C for 24 h with [^{14}C]thymidine before infection with *ts*111 at 39.5°C. Similar experiments were performed with WT-infected cells or cells doubly infected with *ts*111 and *ts*114. *ts*114 is a DNA-negative mutant which complements *ts*111 (12). The cellular DNA was analyzed in alkaline sucrose gradients at 40 h p.i. When cells were infected with *ts*111, a large fraction (46%) of the cellular DNA sedimented between 20 and 50S (Fig. 8a), whereas single infection with WT (not shown) or double infection with *ts*111 and *ts*114 (Fig. 8b) resulted in reduced degradation (20 and 18%). These data show that the normal function of the product modified in *ts*111 infection may be inhibition of nonspecific cellular nucleases.

Influence of MOI on cellular DNA breakdown. During previous experiments, it was noticed that the *ts*111 mutant could not be used at a high MOI as high levels of late cytotoxicity were produced (unpublished data). It was of interest, therefore, to look at the fate of cellular DNA when cells were infected at several different MOIs. HeLa cells in monolayer were labeled at 37°C for 48 h with [^{14}C]thymidine and chased for 24 h. The cells were infected at 39.5°C with *ts*111 at MOIs of 5, 25, 50, 100, and 200 PFU/cell. The following controls were carried out at the same temperature: mock-infected cells, WT-infected cells (MOI = 100), and WT-*ts*111 double-infected cells (MOI = 5 or 50 each). DNA was analyzed on alkaline sucrose gradients at 24 h p.i. Results are summarized in Table 1. Whereas DNA breakdown in control experiments was 24 and 27%, it increased with *ts*111 infection from 34% at an MOI of 5 to 63% at an MOI of 200. The MOI does, then, affect the rate of DNA degradation. These data may be explained in at least two ways: (i) *ts*111 infectious virus may contain a modified structural protein with enhanced endonucleolytic activity; (ii) the amount of the putative altered protein could be a function of the number of expressed genomes. The data presented above do not allow a clear choice between the two explanations.

Penton base-associated endonuclease

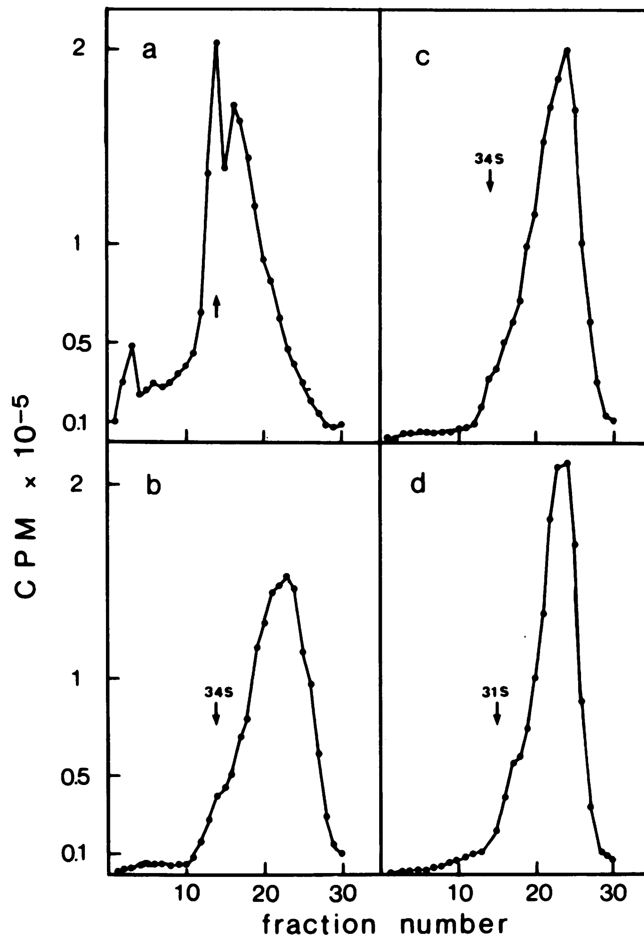

FIG. 6. *Fate of the newly synthesized viral DNA on shift-up to 39.5°C in the presence of hydroxyurea. KB cells infected with ts111 (MOI = 25) were incubated at 33°C until 42 h p.i. and labeled with [^{3}H]thymidine (10 μCi/ml) from 40 to 42 h. The added radioactivity was chased with 5 × 10^{-5} M nonradioactive thymidine. Analysis in alkaline sucrose gradients was performed as indicated in the legend to Fig. 1. (a) Cells at 42 h p.i. (b) Cells after 4-h chase at 39.5°C. (c) Cells after 4-h chase at 39.5°C in the presence of hydroxyurea (10 mM). Analysis in neutral sucrose gradients was performed as described in the text. (d) Cells after 4-h chase at 39.5°C in the presence of hydroxyurea (10 mM). The arrow indicates the position of the Ad2 marker DNA.*

activity. Endonuclease activity has been found in association with the penton subunit of adenovirus (3, 13). This activity has also been described and characterized in extracts from uninfected and adenovirus-infected cells (17, 18, 29).

An experiment was carried out to establish if the penton base-associated endonuclease activity was modified in the *ts*111 virion. Virion-derived pentons (WT and *ts*111) and Ad2 DNA were prepared, and the assays were performed as described in Materials and Methods. Three concentrations (1×, 5×, 10×) of WT and *ts*111 virion-derived pentons were studied. The degree of cleavage was almost identical for the WT and *ts*111 penton preparations at each concentration (not shown). This result shows that the *ts*111 mutation apparently does not affect the penton-associated endonuclease activity. This is in agreement with the finding that this activity is of cellular origin (L. Tsang and R. G. Marusyk, personal communication).

Relationship between synthesis of early proteins and cellular DNA breakdown. The possibility that an increase in cellular DNA breakdown was related to the enhanced synthesis of *ts*111-induced early proteins was studied by infecting cells with *ts*111 at the permissive temperature for a convenient period before shift-up to 39.5°C, thus allowing expression of the mutation. HeLa cells were labeled for 72 h with [^{14}C]thymidine. The cells were infected with

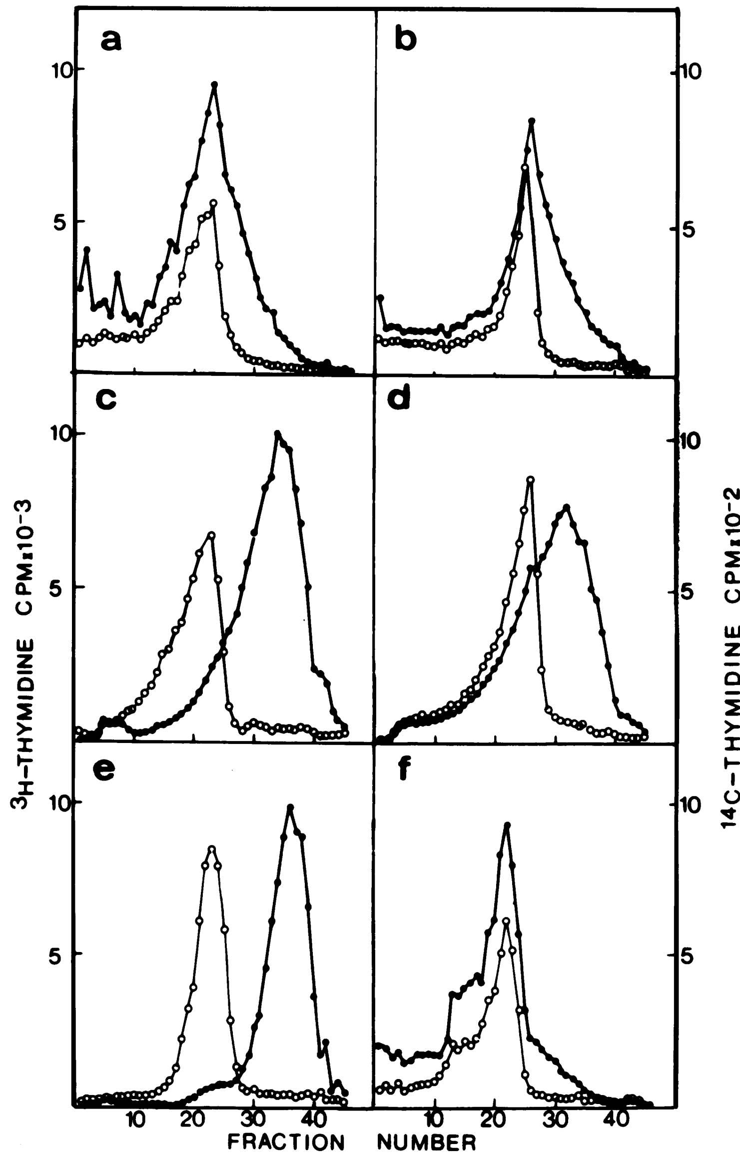

FIG. 7. *Size distribution of ^{3}H-labeled Ad2 DNA after various conditions of incubation of KB cells infected with ^{3}H-labeled ts111 virus. KB cell suspensions were infected with ^{3}H-labeled ts111 virus (2.5 × 10^{-4} cpm/PFU) at an MOI of 100. After incubation under several conditions, the DNA was analyzed by zonal sedimentation in alkaline sucrose density gradients as described (●). ^{14}C-labeled Ad2 marker DNA was added on top of the gradient (○). (a) Incubation at 39.5°C for 16 h. (b) Incubation at 33°C for 48 h. (c) Incubation at 33°C for 24 h followed by 16 h of incubation at 39.5°C. (d) Incubation at 33°C for 48 h followed by 16 h of incubation at 39.5°C. (e) Incubation at 33°C for 48 h followed by 16 h of incubation at 39.5°C in the presence of hydroxyurea (10 mM). (f) Incubation at 39.5°C for 16 h after coinfection with WT (MOI = 100) and ^{3}H-labeled ts111 (MOI = 100).*

*ts*111 at 33°C for 24 h in the presence of hydroxyurea to maintain them in the early phase of the virus cycle. Infected cells were then shifted up to 39.5°C for 24 h with or without hydroxyurea. Controls were performed with WT. The results summarized in Table 2 show that preincubation at 33°C before shift-up greatly increased cellular DNA breakdown. These data suggest that a modified *ts*111 early protein, functional at 33°C, must lose its DNA-protective function at 39.5°C.

DISCUSSION

The temperature-sensitive mutant *ts*111 of human Ad2 is a phenotypical DNA-negative

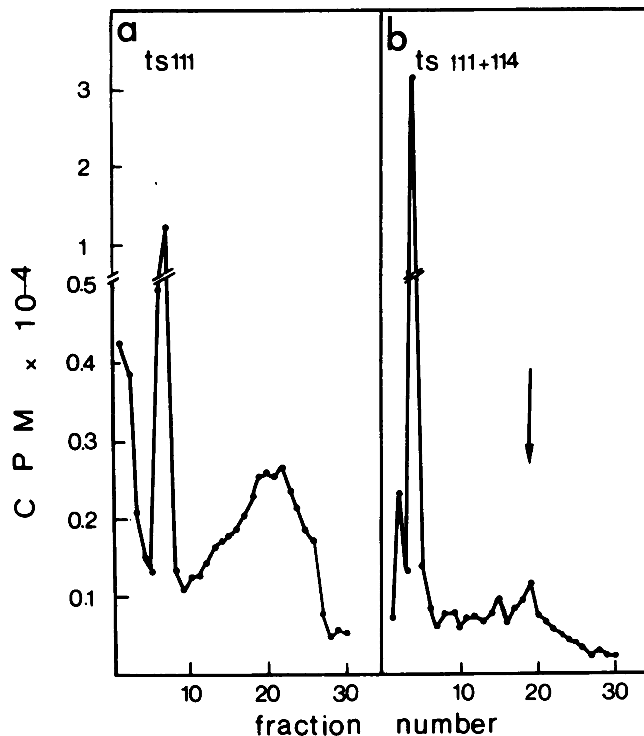

FIG. 8. *Size distribution of ^{14}C-labeled cellular DNA after ts111 infection at 39.5°C. HeLa cell monolayers were labeled with [^{14}C]thymidine (1 µCi/ml) for 24 h at 37°C. After being washed, cells were infected with (a) ts111 (MOI = 25) or (b) ts111 and ts114 (MOI = 25 each) and incubated at 39.5°C for 40 h. Labeled cellular DNA was analyzed in alkaline sucrose gradients as described in the text. The arrow indicates the position of the Ad2 marker DNA.*

mutant belonging to a unique complementation group differing from the H5 *ts*125, which is altered in the DNA-binding 72K protein function. It also complements the H5 *ts*36 by a factor of about 400 (12). H2 *ts*111 elicits a defect which provokes viral DNA synthesis shutoff on shift-up to nonpermissive temperature (Fig. 1). On shift-down, viral DNA synthesis immediately resumes, even in the presence of protein synthesis inhibitors (Fig. 2). Therefore, the altered gene product which is not degraded at 39.5°C becomes functional on shift-down to 33°C. The implicated function is essential from the beginning of the infectious cycle. However, the effect of the *ts*111 mutation is much more pronounced on the virus yield than on the amount of viral DNA synthesized (Fig. 3). These data may be explained by the fact that the production of infectious virions is the result of multiple steps, only one of which is viral DNA synthesis. Like all adenovirus DNA-negative mutants described thus far, H2 *ts*111 appears to be defective in DNA initiation at 39.5°C, as revealed by density labeling and temperature shift-up experiments. The elongation process is also affected (Fig. 4). The most important feature of the H2 *ts*111 alteration is seen when viral DNA synthesis is allowed at 33°C before shift-up to 39.5°C. The 34S DNA synthesized at the permissive temperature is rapidly degraded into small fragments at 39.5°C, with the cleavage being strongly enhanced in the presence of hydroxyurea, a DNA synthesis inhibitor (Fig. 5). These results indi-

TABLE 1. *Influence of MOI on cellular DNA degradation*[a]

Virus	MOI (PFU/cell)	% Cellular DNA at S values < 30
H2 *ts*111	5	34
H2 *ts*111	25	44
H2 *ts*111	50	59
H2 *ts*111	100	63
H2 *ts*111	200	63
WT	100	27
H2 *ts*111 + WT	2 × 5	24
H2 *ts*111 + WT	2 × 50	24
Mock-infected		25

[a] HeLa cell monolayers were labeled at 37°C for 48 h with [^{14}C]thymidine (1 µCi/ml) and chased for 24 h. After infection, cells were incubated at 39.5°C for 24 h. Cellular DNA was analyzed in alkaline sucrose gradients as described in the text.

TABLE 2. *Cellular DNA degradation after infection with WT and H2 ts111 under various conditions of incubation*[a]

Expt	Conditions of incubation	% Cellular DNA at S values < 30	
		WT	H2 *ts*111
I	33°C/48 h	6.7	36.4
	33°C/24 h + 39.5°C/24 h	11.6	86.7
	33°C/24 h + 39.5°C/24 h(+HU[b])	11.6	87.3
	39.5°C/24 h	3.3	12.0
II	33°C/24 h(+HU)	ND[c]	11.8
	33°C/48 h(+HU)	8.3	20.1
	33°C/24 h(+HU) + 33°C/24 h 33°C/24 h	7.6	23.3
	33°C/24 h(+HU) + 39.5°C/24 h	11.9	69.1
	33°C/24 h(+HU) + 39.5°C/24 h(+HU)	7.0	71.5
	39.5°C/24 h	8.0	ND
	39.5°C/24 h(+HU)	10.7	19.9

[a] HeLa cell monolayers were labeled at 37°C for 72 h with [^{14}C]thymidine (1 μCi/ml). After being washed, cells were infected with WT or *ts*111 (MOI = 10) under different conditions. Cellular DNA was analyzed in alkaline sucrose gradients as described in the text.

[b] Hydroxyurea (HU; 10 mM) was added at the beginning of the indicated incubation period.

[c] ND, Not done.

cate that protection of newly synthesized viral DNA requires an active *ts*111 gene product and suggest that the main altered function is related to an endonucleolytic-like degradation which likely starts by single-strand nicks.

When parental DNA is examined late in *ts*111 infection at 39.5 or 33°C, a fraction of the label sediments at a rate lower than 34S (Fig. 7a and b), whereas the bulk of the label cosediments with intact WT DNA marker (Fig. 7f), as previously observed with DNA-labeled WT virions (9). In contrast, extensive parental DNA degradation is observed on shift-up to 39.5°C for 16 h after 24 or 48 h of incubation at 33°C (Fig. 7c and d), particularly under conditions of DNA synthesis inhibition (Fig. 7e). According to these data, the parental DNA appears to be partially protected even in the absence of active *ts*111 gene product, which is consistent with the finding that stretches of parental DNA are found in nucleosome-like structure (22). At permissive temperature, the parental DNA enters into replication intermediates and protection may be assumed to occur by binding of the 72K protein (14) and the involvement of the *ts*111 gene product. Inactivation of this gene product on shift-up to 39.5°C would allow endonuclease cleavage.

Inhibitory activity towards cellular DNases has been demonstrated in phage-bacteria systems (1, 15, 19, 27) and during infection of KB cells by Ad5 (14). Similarly, H2 *ts*111 might be altered genetically in a DNase-inhibitory function. This hypothesis is supported by the fact that cellular DNA is markedly degraded on infection with *ts*111 at 39.5°C. This cleavage is related to MOI (Table 1) but does not seem to be due to a modification of the penton base-associated endonuclease activity (3, 13). The functional role of the observed endonucleolytic DNA cleavage is unknown. Available data do not suggest a restriction-like process. However, integration of viral DNA fragments into cellular DNA (2, 7, 20) might be one of the consequences of this activity. In this hypothesis, the enhancement of cell DNA cleavage observed when *ts*111 expression is allowed at 33°C before shift-up (Table 2) might be the result of integration of viral DNA units into cell DNA, a phenomenon which could have occurred at a normal rate at the permissive temperature. Viral DNA sequences have been found in high-molecular-weight, newly synthesized DNA from cells infected at 39.5 or 33°C with *ts*111 (data not shown) or with other Ad2 temperature-sensitive mutants (21).

From the results reported here, it appears that H2 *ts*111 presents two phenotypical characteristics: (i) absence of DNA initiation and elongation; (ii) endonucleolytic cleavage of viral and cellular DNA. As *ts*111 might be a double mutant, four revertants were isolated by culture at nonpermissive temperature and cloned. They were tested for virus yield at permissive and nonpermissive temperatures, soluble antigen production, and cellular DNA degradation. The four revertants had a WT serological phenotype and the same effect on cell DNA as WT, strongly suggesting that *ts*111 is a single mutant. Attempts to detect the implicated virus-coded protein failed: the sodium dodecyl sulfate-polyacrylamide gel pattern of the *ts*111 early proteins was similar to that of WT (not shown). Further experiments are required to determine how the *ts*111 gene product is implicated in DNA initiation and elongation and in cellular DNase inhibition.

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