Identification of Adenovirus 2 Early Genes Required for Induction of Cellular DNA Synthesis in Resting Hamster Cells

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Received 6 January 1981/Accepted 16 March 1981

tsAF8 cells are temperature-sensitive (*ts*) mutants of BHK-21 cells that arrest at the nonpermissive temperature in the G₁ phase of the cell cycle. When made quiescent by serum restriction, they can be stimulated to enter the S phase by 10% serum at 34°C, but not at 40.6°C. Infection by adenovirus type 2 or type 5 stimulates cellular DNA synthesis in *tsAF8* cells at both 34 and 40.6°C. Infection of these cells with deletion Ad5*dl*312, Ad5*dl*313, Ad2Δp305, and Ad2+D1 and temperature-sensitive (H5*ts*125, H5*ts*36) mutants of adenovirus indicates that the expression of both early regions 1A and 2 is needed to induce quiescent *tsAF8* cells to enter the S phase at the permissive temperature. This finding has been confirmed by microinjection of selected adenovirus DNA fragments into the nucleus of *tsAF8* cells. In addition, we have shown that additional viral functions encoded by early regions 1B and 5 are required for the induction of cellular DNA synthesis at the nonpermissive temperature.

Small DNA viruses, such as simian virus 40, polyoma viruses, and adenoviruses, can induce resting (G₀) cells to enter the S phase (5, 24, 25, 28, 30). The identification of the virus-coded protein(s) responsible for the induction of host DNA replication is of interest in terms of viral gene expression, virus-cell interaction and regulation of the G₀-G₁-S transition of the cell cycle.

We have shown (23) that adenovirus 2 (Ad2) is able to induce cellular DNA synthesis in a G₁-specific, temperature-sensitive (*ts*) mutant (*tsAF8* cells) derived from BHK-21 cells (3, 17). Cell DNA synthesis is induced by Ad2 infection at both the permissive and nonpermissive temperatures, even though the temperature-sensitive mutation prevents serum-stimulated cells from entering the S phase (21).

In this paper we describe experiments to identify the regions of the adenovirus genome involved in the induction of cellular DNA synthesis both by the use of deletion and temperature-sensitive mutants of the virus and by microinjection of adenovirus DNA restriction fragments.

MATERIALS AND METHODS

Cell culture. BHK-21 and *tsAF8* cells were grown and made quiescent as previously described (21).

Viruses. Ad2, Ad2 Δp305 (isolated by N. Newell),

Ad5, H5*ts*125 (6), and H5*ts*36 (29) were grown in Hela cell culture maintained in Dulbecco modified Eagle medium supplemented with 5% calf serum. Ad5*dl*312 and Ad5*dl*313 (11) were grown in 293 cell culture maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. Ad2+D1 (13) was grown in monkey CV1 cells in Dulbecco modified Eagle medium in the presence of 5% calf serum. Viruses were purified as described by Philipson et al. (20) using two cycles of CsCl density gradient centrifugation.

Infection. *tsAF8* and BHK-21 cells were made quiescent by a 48-h incubation in medium containing 0.5% serum. The medium from quiescent cells was collected and saved. The viruses were diluted in Hanks balanced salt solution containing 20 mM MgCl₂ and were allowed to absorb for 1 h at 37°C (multiplicity of infection, 500 to 1,000 PFU/ml). The cultures were then refed with the serum-depleted medium, with the exception of *tsAF8* cells to be incubated at 40.6°C to which was added 10% serum medium.

Preparation of Ad2 DNA fragments. Ad2 DNA was purified as described by Pettersson and Sambrook (19). The purified Ad2 DNA was digested to completion with restriction endonuclease *Bam*HI or *Hpa*I under conditions suggested by the supplier (Bethesda Research Laboratories, Bethesda, Md.). After digestion the mixture was deproteinized with chloroform-isoamyl alcohol. The fragments were resolved by electrophoresis in 0.7 or 1% agarose and visualized by long-wavelength UV-induced fluorescence of ethidium bromide-stained gels, and DNA was isolated from gel slices by electroelution (16). pBR730, provided by D. Klessig, is a recombinant pBR322 plasmid that contains Ad5 sequences from 59.5 to 75.9 map units inserted at the pBR322 *Bam*HI-*Eco*RI sites. pHEB4,

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obtained from D. Solnick, is a recombinant pBR322 plasmid that includes Ad2 sequences from map units 0.8 to 4.5 inserted at the pBR322 *HindIII-EcoRI* sites. Plasmid DNA were prepared by the procedure of Tanaka and Weisblum (26) and were linearized by digestion with *Bam*HI restriction endonuclease.

Microinjection. *tsAF8* cells were plated on 22- by 22-mm glass slides and made quiescent by incubation at 34°C in medium containing 1% serum for 6 days. About 10⁻¹¹ ml of purified, intact Ad2 DNA and Ad2 DNA fragments at a concentration of 0.5 to 1.0 mg/ml in 10 mM Tris-hydrochloride (pH 7.2) was injected into the nuclei of *tsAF8* cells by the glass capillary microinjection technique of Graessmann and Graessmann (9). After microinjection, the cells were incubated at 34°C in the same medium for 40 h. For the last 24 h of incubation 1 µCi of [³H]thymidine per ml was present in the medium.

Immunofluorescence. *tsAF8* cells were fixed for 15 min with methanol at -20°C. Ad2-specific 72K DNA-binding protein was visualized in microinjected cells by indirect immunofluorescence using hamster anti-72K serum (C783, provided by A. Lewis) as the first antibody and fluorescein isothiocyanate-labeled goat anti-hamster globulin as the second antibody.

Autoradiography. Incorporation of [³H]thymidine into *tsAF8* cells was determined essentially as described by Baserga and Malamud (1). A minor modification of the procedure was used for autoradiography of microinjected cells (A. Graessman, personal communication). Briefly, methanol-fixed *tsAF8* cells were first stained by the immunofluorescence method for the Ad2 72K protein (see above) and then coated with Kodak Nuclear Track Emulsion diluted 1:1 with water. The slides were developed after 3 or 4 days of exposure and mounted in Aqua-Mount (a nonfluorescing medium from Lerner Laboratories, Stamford, Conn.).

RESULTS

Effect of infection with adenovirus deletion or temperature-sensitive mutants on

the induction of cellular DNA synthesis in quiescent *tsAF8* and BHK-21 cells. *tsAF8* and BHK-21 cells infected with wild-type Ad2 or Ad5 or with deletion or temperature-sensitive mutants were examined for DNA synthesis at 34 and 40.6°C by autoradiography, as described above.

Table 1 shows the proportion of cells synthesizing DNA. As already reported (21), 10% serum stimulates quiescent *tsAF8* cells to enter the S phase at the permissive temperature, but fails to do so at the nonpermissive temperature. In contrast 10% serum stimulates DNA synthesis at both temperatures in BHK-21 cells, the parent line from which *tsAF8* cells were derived.

tsAF8 cells infected with the wild-type Ad5 synthesized DNA at both the permissive and nonpermissive temperatures, as already reported for Ad2 infection (23). The requirement of different early regions for the induction of cellular DNA synthesis in *tsAF8* at 34 and at 40.6°C as well as in BHK-21 cells, as control, was tested by infecting the cultures with different mutant viruses. The mutation of the viruses used in these experiments has been mapped within five different early regions (E1A, E1B, E2, E3, E5) of the adenovirus genome (Table 1).

(i) **Region E1A.** The virus used to analyze the effect of early region 1A, Ad5d1312, has a deletion between 1.5 and 4.5 map units (11). Ad5d1312 failed to stimulate cell DNA synthesis both at the permissive and nonpermissive temperatures in either *tsAF8* cells or BHK-21 cells.

(ii) **Region E1B.** Ad5d1313, with a deletion between 3.5 and 10.5 map units (11), lacks the carboxy-terminal protein of the early region 1A and essentially all of early region 1B. Infection of *tsAF8* cells with Ad5d1313 resulted in induction of cell DNA synthesis at the permissive

TABLE 1. DNA synthesis in *tsAF8* and BHK-21 cells after infection with adenovirus deletion and temperature-sensitive mutants^a

Treatment	Mutated region	Map coordinates of mutation	% Labeled cells			
			<i>tsAF8</i>		BHK-21	
			34°C	40.6°C	34°C	40.6°C
Mock infection			15.0		14.0	17.5
Serum stimulation (10%)			89.0	18.2	87.2	89.0
Wild-type Ad2 infection			73.3	69.5	72.1	74.3
Wild-type Ad5 infection			72.0	78.3	69.5	73.5
Ad5d1312 infection	E1A	1.5-4.5	18.2	8.5	14.0	10.5
Ad5d1313 infection	E1B	3.5-10.5	76.0	10.2	67.5	71.0
Ad2+D1 infection	E2	64.0-74.0	25.0	22.1	28.0	23.5
H5ts125 infection	E2	61.3-62.9	71.0	12.0	65.3	15.7
Ad2Δp305 infection	E3	83.0-85.0	73.5	77.2	77.0	79.8
H5ts36 infection	E5	18.5-22.0	80.0	11.0	78.5	81.2

^a In all the experiments the cells were continuously labeled with [³H]thymidine (0.05 µl/ml), and the percentage of labeled nuclei was determined at 40 h after serum stimulation or infection.

temperature, but not at the nonpermissive temperature, whereas infection of BHK-21 cells with the same virus induced cell DNA synthesis at both 34 and 40.6°C.

(iii) **Region E2.** To test the requirement of early region 2, which encodes the single-stranded DNA-binding protein, for the initiation of cell DNA replication in *tsAF8* and BHK-21 cells, we used two different viruses, Ad2+D1 and H5ts125. Ad2+D1 is a defective Ad2-simian virus 40 hybrid virus (13) which contains the entire early region of the simian virus 40 genome. The simian virus insertion replaces the segment of the adenoviral DNA mapping between 64 and 74 map units, i.e., much of early region 2. The mutation of H5ts125 has been physically located in the early region 2 (7, 10) between 61.3 and 62.9 (R. Galos and J. Williams, unpublished results) and results in the production of thermolabile 72K DNA-binding protein (27) that is unable to promote viral DNA replication (14). Ad2+D1 failed to induce cell DNA synthesis in *tsAF8* and BHK-21 cells at either 34 or 40.6°C, whereas H5ts125 failed to stimulate cell DNA synthesis at 40.6°C (Table 1). These results indicate the requirement of early region 2 for the initiation of cell DNA synthesis. It is unclear why the simian virus 40 A gene inserted in the Ad2+D1 hybrid virus DNA did not have effect on the stimulation of cellular DNA synthesis. Current investigations seem to suggest that Ad2 has an inhibitory effect on this function of the Simian virus 40 A gene.

(iv) **Region E3.** The early region 3 has been located on the Ad2 genome between coordinates 76.6 and 86.1 (4). The deletion mutant $\Delta p305$ of Ad2 is a viable virus with a small deletion in this region of the Ad2 DNA between map units 83 and 85 (T. Kelly, personal communication). From our results (Table 1) the deleted region seems to be nonessential to the ability of Ad2 to stimulate host DNA replication: $\Delta p305$ was capable of stimulating both *tsAF8* and BHK-21 cells to enter the S phase at 34 and 40.6°C.

(v) **Region E4.** The requirement of early region 4 for the induction of cell DNA synthesis in *tsAF8* and BHK-21 cells remains unclear for lack of viruses with mutations in early region 4. However, we can exclude its participation in the stimulation of DNA synthesis at least in *tsAF8* cells at the permissive temperature based on our results from microinjection experiments (see below). At present, we cannot establish its role on the initiation of DNA synthesis at 40.6°C.

(vi) **Region E5.** The mutation in H5ts36 has been located by marker rescue analysis to be between 18.5 and 22 map units (8). An early single-stranded RNA has been mapped in this

region, which is designated region 5 (8). However, the relationship between the *ts* mutation and the single-stranded RNA is not clear, due to the presence of several proteins encoded in that region (15).

H5ts36 failed to induce cell DNA synthesis at 40.6°C only when the cells infected were *tsAF8* cells, but not in BHK-21 cells, a behavior similar to the early region 1B mutant virus, Ad5dl313 (Table 1).

Expression of adenovirus genome and stimulation of cellular DNA synthesis in quiescent *tsAF8* cells after microinjection of selected adenovirus DNA fragments. The nuclei of quiescent *tsAF8* cells were microinjected either with intact Ad2 DNA, with A (59.5 to 100 map units) and B (0 to 29 map units) fragments from a *Bam*HI restriction digest, with *Bam*HI fragment B alone, with a DNA segment from 59.5 and 75.9 map units or from 0.8 to 4.5 map units (each cloned in a pBR322 plasmid), or with mixtures of them, as indicated in Table 2. After microinjection, cells were incubated at 34°C for 40 h (3 H]thymidine was included for the last 24 h) and then fixed, stained for the viral 72K protein, and autoradiographed by the techniques described above.

The cells were evaluated for immunofluorescent staining and autoradiographic grains by a Zeiss Microscope by using, alternatively, fluorescence excitation or transmitted light. (Table 2). Cells injected either with intact Ad2 DNA or with *Bam*HI A and B fragments that include all

TABLE 2. DNA synthesis in *tsAF8* cells after microinjection of adenovirus DNA fragments^a

Injection ^b	Staining for Ad2 72K protein	% Labeled <i>tsAF8</i> cells at 34°C
None	—	21.3
Intact Ad2 DNA	+	87.0
Ad2 DNA A and B fragments of <i>Bam</i> HI digest (59.5–100 and 0–29)	+	85.7
Ad2 DNA B fragment of <i>Bam</i> HI digest (0–29)	—	22.1 ^c
pHEB4 (0.8–4.5)	—	20.5 ^c
pBR730 (59.5–75.9)	—	18.3 ^c
pBR730 (59.5–75.9) and B fragment of <i>Bam</i> HI digest (0–29)	+	73.2
pBR730 (59.5–75.9) and C fragment of <i>Hpa</i> I digest (4.5–25.5)	—	17.5 ^c
pBR730 (59.5–75.9) and pHEB4 (0.8–4.5)	+	75.0

^a In all experiments 3 H]thymidine (1 μ Ci/ml) was added to the medium 16 h after injection. Cells were examined for immunofluorescence, and the labeling index was calculated on the number of positive cells observed 40 h after injection.

^b Numbers within parentheses indicate the map coordinates of the fragments.

^c The labeling index was calculated on the number of labeled cells in the total microinjected area.

of the early regions of the Ad2 genome were positive for the presence of the Ad2 72K protein. In addition, 85 to 87% of the 72K protein-positive cells were also synthesizing DNA as indicated by the autoradiography with [3 H]thymidine.

The fragment B of *Bam*HI digest (0 to 29 map units), as well as the pHEB4 plasmid, including only the early region 1A (0.8 to 4.5 map units) were not able to initiate DNA synthesis when microinjected in *tsAF8* cells.

Microinjection of the plasmid pBR730 including the entire early region 2 (59.5 to 75.9 map units) resulted in neither expression of the 72K protein nor a significant increase in the percentage of cells synthesizing DNA. On the contrary, when microinjection into the nucleus of quiescent *tsAF8* cells was performed with a mixture of pBR730 and the fragment B of *Bam*HI digest or with a mixture of pBR730 and pHEB4 plasmids, the expression of the 72K protein was detected, and 73.5 and 75% of the positive cells, respectively, were labeled with [3 H]thymidine. The fragment C of an *Hpa*I digest (4.5 to 25 map units) failed to stimulate the production of the DNA binding protein when injected together with pBR730 plasmid, and the mixture had no effect on the initiation of DNA synthesis in microinjected *tsAF8* cells, as shown in Table 2.

DISCUSSION

We have previously reported the ability of Ad2 to stimulate cellular DNA synthesis in *tsAF8* cells, even in a condition restrictive for serum stimulation such as the nonpermissive temperature of 40.6°C (23).

The question about the identity of viral genes involved in the initiation of cellular DNA synthesis and in the regulation of the cell cycle flow has been partially answered in this paper. The results obtained after infection of *tsAF8* and BHK-21 cells with viruses having a deletion or a mutation in the early regions 1A (Ad5d1312) and 2 (Ad2+D1 and H5ts125) indicate that at least these two early regions are needed for the initiation of cellular DNA replication.

The use of mutant viruses for this type of analysis has certain limitations. For instance, in our experiment it was not possible to investigate the role of the early region 4 due to a lack of mutants, and the interpretation was based on negative results. A more detailed investigation was made possible by the use of the manual microinjection technique. The introduction of viable, selected adenovirus DNA fragments into the nucleus of *tsAF8* cells not only confirmed the results obtained by infection with the mutant viruses, but also provided additional infor-

mation. We have clearly shown that the expression of early region 2 in resting cells is regulated by the presence of early region 1A. In addition, our results also indicated that fragments of adenovirus DNA, including only the early regions 1A and 2, are sufficient to initiate cell DNA synthesis in quiescent *tsAF8* cells incubated at the permissive temperature. Because studies on transcription have revealed that the region 1A plays a regulatory role on the transcription of the other early regions (2, 12), a question arises: is the 72K protein, as a product of early region 2, the only direct requirement for the induction of cell DNA synthesis, or are the translation product(s) of region 1A also directly involved beyond their requirement for the expression of early region 2?

The results obtained after infection of *tsAF8* cells at the nonpermissive temperature are complex and may require further investigation. The block in the G₁ phase of the cell cycle of *tsAF8* cells at the nonpermissive temperature is due to a defective RNA polymerase II, as indicated by biochemical and genetic analysis (21, 22, 25a). Although this conclusion is supported also by the observation that at the nonpermissive temperature the adenovirus DNA is not replicated in *tsAF8* cells (18, 23), it is somewhat surprising in view of the fact that early viral genes are transcribed. Apparently, this transcription, whose nature and extent have not yet been fully characterized, is not sufficient to cause viral DNA replication to occur. Our data indicate that the stimulation of cellular DNA synthesis by Ad2 observed at the nonpermissive temperature requires additional viral function. The expression of early regions 1B and 5 is needed, in addition to the early regions 1A and 2, as indicated by the data obtained after infection with Ad5d1313, deleted in region 1B (11), and with H5ts36, in which the mutation is in region 5 (8). Both viruses failed to induce DNA synthesis in *tsAF8* at the nonpermissive temperature, but they were able to do so in BHK-21 cells from which *tsAF8* cells derived.

At present we have not yet analyzed this feature by microinjection of selected fragments. This is in part due to the lack of information on the identity of the product(s) and coding sequences from region 5. Considering that the results obtained by infection at the permissive temperature have been confirmed by microinjection, it seems reasonable to assume that at least early regions 1B and 5 are involved in the induction of cell DNA synthesis in *tsAF8* cells at the non-permissive temperature, although we cannot exclude the participation of early region 4.

Finally, our findings suggest that different viral functions may act at different points in the G₁ phase of the cell cycle, ruling out, in this way, the possibility that adenoviruses act directly on the cell DNA machinery.

ACKNOWLEDGMENTS

We thank T. Kelly, T. Shenk, A. Lewis, Jr., D. Klessig, and D. Solnick for supplying viruses, serum, and plasmids. We are grateful to J. Sambrook for helpful discussions and to L. Chow and T. Broker for careful reading of this manuscript.

This work was supported by Public Health Service research grant GM22359 to R.B. from the National Cancer Institute and research grant CD54 to M.R. from the American Cancer Society; G.J.J. was a trainee under Public Health Service grant CA 09214 from the National Cancer Institute.

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