Temperature-Sensitive Formation of the DNA Replication Complex in Adenovirus 31-Infected Cells

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Received for publication 4 September 1973

A temperature-sensitive mutant of adenovirus 31 was defective in formation of the DNA replication complex, suggesting the existence of a virus-coded protein necessary for the complex-formation.

Pearson and Hanawalt (1) reported the formation of the DNA replication complex in adenovirus 2 (Ad2)-infected HeLa cells and Wallace and Kates (6) reported the isolation of viral DNA-protein complexes from nuclei of Ad 2-infected KB cells. The analysis of a temperature-sensitive (*ts*) mutant of Ad 31, *ts13*, suggests that the mutant is defective in the initiation of viral DNA replication at the nonpermissive temperature (3). The present communication describes the failure of the mutant DNA to form the DNA replication complex and suggests the existence of a virus-coded protein necessary for the initiation of adenovirus DNA replication.

Human embryo kidney (HEK) cells, Ad 31 strain 1315 wild type (*w*), and *ts13* were used. The characteristics of these viruses and the routine methods of cell culture and virus assay were described previously (3, 4). The permissive and the nonpermissive temperatures were 33.5 and 39.5 C, respectively.

HEK cells were infected with either *w* or *ts13* at 39.5 C. At 44 h postinfection (PI), the cells were labeled with ³H-thymidine (5 Ci/mmol, 10 μCi/ml) for 30 min. The cells were then washed with isotonic phosphate-buffered saline (PBS) and harvested with EDTA-trypsin. The cells were washed with cold PBS and suspended in PBS at 4 × 10⁶ cells/ml. The cell suspension received Nonidet P-40 (NP-40, Shell Chemical Co., final 0.5%), kept in an ice-bath for 10 min and centrifuged at 1,500 rpm for 10 min to sediment nuclei. The nuclei were suspended in TMK buffer (0.01 M Tris-hydrochloride, pH 7.5, 0.001 M MgCl₂, 0.1 M KCl) at 4 × 10⁶ nuclei/ml. The supernatant was designated as the cytoplasmic fraction. The DNA-nuclear membrane complex was isolated by the "M-band" technique described by Tremblay et al.

(5). The suspension of nuclei (0.5 ml) was gently mixed with an equal volume of 0.2% Sarkosyl and layered onto a two-layered tube of sucrose (4 ml of 15% sucrose on 6 ml of 47% sucrose) in TMK buffer. The tubes were centrifuged at 15,000 rpm for 30 min in an SW-41 rotor. A sharp white band (M-band, Mg²⁺-Sarkosyl crystals) formed at the interface was separated from the fraction remaining on the 15% sucrose (the top fraction). The acid-precipitable radioactivity in each fraction after M-band centrifugation revealed that viral DNA was present only in the M-band fraction in *w*-infected cells. No viral DNA was present in the M-band fraction of *ts13*-infected cells similarly treated (Fig. 1). This observation showed that the viral DNA replication complex was formed in *w*-infected cells in agreement with the report by Pearson and Hanawalt (1) and that *ts13* was defective in the formation of the complex at the nonpermissive temperature.

To analyze the fate of infecting virions, labeled virions were prepared. HEK cells were infected with either *w* or *ts13* at an input multiplicity of 5 to 10 PFU/cell at 35 C (permissive for *ts13*) for 24 h and then received ³H-thymidine (4 μCi/ml). After the cytopathic effect became complete, the cells were harvested. Virions were purified therefrom by isopycnic centrifugation in CsCl gradients as described previously (2). Purified virions were either diluted with or dialyzed against Eagle minimum essential medium and sterilized by filtration through a Millipore filter pretreated with 0.2% polyvinyl-pyrrolidone solution as described by Yoshino and Morishima (7).

HEK cells were infected with labeled virions of either *w* or *ts13*. After adsorption at 35 C for 2 h, the cells were incubated in MEM at 39.5 C. Immediately after adsorption and at 8 and 24 h PI, the cells were harvested and separated into cytoplasm, M band, and top fractions. The acid-precipitable radioactivity in each fraction

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FIG. 1. HEK cells in small bottles were infected with either *w* or *ts13* at 5 PFU/cell and incubated at 39.5 C. At 44 h PI, the cells were labeled with ^3H -thymidine (10 $\mu\text{Ci/ml}$) for 30 min, treated by the M-band technique as described in the text, and separated into 12 fractions from the top. The acid-precipitable radioactivity in each fraction was counted. Symbols: ●, *w*-infected, ○, *ts13*-infected.

was measured (Table 1). The radioactivity associated with the M band increased with the incubation time in *w*-infected cells (19% at 2 h PI, 76% at 24 h PI), whereas it did not in *ts13*-infected cells (24% at 2 h PI, 24% at 24 h PI). However, co-infection with labeled virions of *ts13* and unlabeled virions of *w* resulted in the increase in the acid-precipitable radioactivity associated with the M band after incubation at 39.5 C for 24 h (63%). When *w*-infected cells were incubated at 39.5 C for 24 h in the presence of cycloheximide, the acid-precipitable radioactivity associated in the M band did not increase (23% at 24 h PI). HEK cells infected with *ts13* alone showed an increased radioactivity associated with the M band after incubation at 33.5 C for 44 h (59%). However, the radioactivity in the M band remained constant during additional incubation at 39.5 C for 2 or 4 h, (70 or 61%). The reconstruction experiment, in which labeled viral DNA was mixed with nuclei of either uninfected or *w*-infected cells (24 h PI) and treated similarly, showed that only a small portion of viral DNA was present in the M band (4% in uninfected cells, 7% in infected cells). A similar experiment with heat-denatured viral DNA gave the same result. These controls confirmed that the increase in viral DNA associated with the M band was not an artifact. The

TABLE 1. Localization of infected Ad 31 DNA in cells^a

Virus	Incubation		Localization			Ratio ^b	
	Temp (C)	Time (h)	Cytoplasm (counts/min)	Top (counts/min)	M band (counts/min)	M/input ($\times 10^{-4}$)	M/total (%)
<i>w</i>	35	2	666	158	525	13	19
<i>w</i>	39.5	8	172	624	1,542	39	65
<i>w</i>	39.5	24	118	532	2,172	54	76
<i>w</i> + CH ^c	39.5	24	385	1,273	493	12	23
<i>ts13</i>	35	2	1,336	331	543	14	24
<i>ts13</i>	39.5	8	815	680	610	15	28
<i>ts13</i>	39.5	24	152	1,350	531	13	24
<i>ts13</i> + <i>w</i> ^d	39.5	24	200	315	913	23	63
<i>ts13</i>	33.5	44	198	539	1,074	27	59
<i>ts13</i>	33.5	44 ^e	168	335	1,227	32	70
<i>ts13</i>	39.5	2					
<i>ts13</i>	33.5	44 ^e	224	430	1,024	26	61
<i>ts13</i>	39.5	4					

^a HEK cells in small bottles were infected with labeled virions of either *w* (400,000 counts per min per bottle, 130 PFU/cell) or *ts13* (400,000 counts per min per bottle, 130 PFU/cell). After adsorption at 35 C for 2 h, cells were incubated at the temperature for the time indicated. Cells were then harvested and separated into cytoplasmic, nuclear top, and M band fractions. The acid-precipitable radioactivity in each fraction was determined and expressed in counts per minute per fraction.

^b Ratio of the acid-precipitable radioactivity (counts per minute) in the M band fraction to the input radioactivity (4×10^5 counts/min, M/input) and to the total radioactivity (the sum of counts per minute in three fractions, M/total).

^c After adsorption, the cells were incubated in the presence of cycloheximide (20 $\mu\text{g/ml}$).

^d Co-infection with labeled *ts13* and unlabeled *w* (10 PFU/cell).

^e Infected HEK cells were incubated at 33.5 C for 44 h and at 39.5 C for 2 or 4 h.

following conclusion may be drawn from these observations. (i) The input viral DNA became associated with the M band. (ii) *ts13* was defective in this function at the nonpermissive temperature. (3). This defect of *ts13* was complemented by co-infection with *w*. (iv) Protein synthesis de novo was necessary for the association. (v) *ts13* DNA once associated was not liberated from the M band at the nonpermissive temperature.

These observations suggest that the formation of the parental viral DNA replication complex is an essential step for viral DNA replication and that there is a virus-coded protein necessary for the input viral DNA to form the complex. The protein may be diffusible and modify either the nuclear constituent or the parental viral genome, so that the input viral DNA can form the replication complex. It remains to be determined whether a factor other than the complex-forming protein is also necessary for the initiation of *ts13* DNA replication at the nonpermissive temperature, or the protein of *ts13* is denatured at the nonpermissive temperature to an inactive form.

We are grateful to T. Yamashita for instruction of the M-band technique, to K. Oda for valuable suggestion and discussion, and to M. Matsumoto for review of the manuscript.

This work was supported in part by grants from Ministry of Education of Japan and from the Princess Takamatsu foundation for Cancer Research.

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