Polynucleotide Ligase Activity in Adenovirus-Infected Human Embryonic Kidney Cell Cultures[1] (34549)

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Polynucleotide ligase, an enzyme discovered recently in normal and bacteriophage-infected *Escherichia coli* (1–6), and in mammalian cells (7) as well as in polyoma virus-infected mouse embryo cultures (8), catalyzes the repair of single-strand interruptions in a DNA duplex by the formation of phosphodiester bonds. Although the *in vivo* role of the ligase has not been elucidated, it has been implicated in various processes such as the repair of DNA lesions (9) and is believed to play an essential role in DNA replication (10). The suggestion has been made that *in vivo* DNA replication involves the synthesis of short single-stranded segments of both DNA strands and the subsequent joining of the DNA fragments by the ligase to form long intact strands (11). Support for this possibility has been obtained by the finding that infection of *E. coli* with ligase mutants of T4 phage resulted in inhibition of phage DNA synthesis (12–14), and the accumulation of newly synthesized short DNA chains (15–19).

We have examined the effect of adenovirus infection on the activity of polynucleotide ligase in human embryonic kidney (HEK) cell cultures. It was previously reported that one of the relatively early events of adenovirus infection in HEK cells is an inhibition of cellular DNA synthesis (20). The enzyme activity was assayed by testing for repair of single-strand breaks produced in HEK cell DNA by the action of pancreatic deoxyribonuclease. This was measured by looking for an increase in the single-strand molecular weight after zone sedimentation in alkaline sucrose-density gradients.

Materials and Methods. Cells and virus. Cultivation of primary cultures of HEK cells, and the preparation and assay of stocks of nononcogenic type 2 and oncogenic type 12 human adenoviruses have been described (21).

Infection of cells. Confluent or nearly confluent monolayer HEK cell cultures, containing about 2×10^6 cells in a 60-mm petri dish, were washed twice with Tris-buffered saline (22), and 0.2 ml of virus suspension was added to the cell layer. The multiplicity of infection was approximately 20–30, a multiplicity high enough to infect all or virtually all cells (21). After adsorption for 1 hr at 37°, the cells were washed twice, covered with 3 ml of medium consisting of Eagle's minimal essential medium containing 4% fetal calf serum and 1 m*M* arginine, and incubated at 37° in a humidified 5% CO_2–air mixture. Control cultures were treated under the same conditions, but no virus was added. Ten sets of infected and control cultures were collected for preparation of the enzyme extract at stated times.

Preparation of HEK cell DNA with single-strand breaks. Cell DNA was labeled by growing HEK cell cultures in the presence of thymidine-^3H (5 μCi/ml, 10 Ci/mmole) for 2 days, and the DNA was subsequently extracted as described previously (20). DNA containing single-strand breaks was prepared by incubating HEK cell DNA with electrophoretically purified deoxyribonuclease (DNase) (Worthington) using essentially the method of Weiss, Live, and Richardson (23). The reaction mixture contained in 5.0

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ml: 67 mM Tris HCl buffer, pH 8.0, 5 mM $MgCl_2$, 0.5 unit DNase, and about 200 μg HEK cell DNA (approximately 10^6 counts/-min). This material was incubated at 20° for 20 min, and then 0.5 M sodium EDTA, pH 7.5, was added to a final concentration of 16 mM. The final mixture was dialyzed for 8 hr at 4° against a solution containing 20 mM NaCl and 10 mM Tris–HCl buffer, pH 8.0, and stored at 0°.

Polynucleotide ligase assay. The assay procedure was partly based on the method of Becker, Lyn, Gefter, and Hurwitz (1). The reaction mixture contained in 0.23 ml: 20 μM Tris–HCl buffer, pH 7.5, 2 μM $MgCl_2$, 5 mμM ATP, 0.05 μM dithiothreitol, approximately 4 μg DNA (approximately 2×10^4 cpm), and crude enzyme extract, containing 120 μg protein, which was prepared as previously described (21). This mixture was incubated for 15 min at 25°, and then immediately layered on a linear 5–20% alkaline sucrose gradient.

Zonal centrifugation. Alkaline sucrose gradients were prepared by layering 0.1–0.2 ml of the enzyme reaction mixture and 0.05 ml of ^{14}C-labeled adenovirus 2 DNA marker (0.5 μg DNA) on top of a 4.5-ml linear gradient of 5–20% sucrose in a solution containing 0.9 M NaCl, 1 mM EDTA, and 0.1 M NaOH. Centrifugation and collection of fractions were carried out as described elsewhere (20). Zone sedimentation using neutral sucrose gradients has been described (20).

Calculation of DNA molecular weight. The average molecular weight was calculated from the equations of Burgi and Hershey (24) and Abelson and Thomas (25):

$$\overline{M}_w \cong \sum_i \frac{\text{counts (i)}}{\text{total counts}} \cdot K \cdot (\text{fraction number (i)})^a$$

where $K = M_{std}/(\text{fraction number}_{std})^a$; $a = 2.63$ (denatured DNA); M_{std} of adenovirus 2 DNA marker $= 21 \times 10^6$ (obtained from Dr. W. Doerfler).

Results and Discussion. Before treatment with DNase, the DNA isolated from HEK cultures was analyzed by zone sedimentation through alkaline sucrose-density gradients (Fig. 1A). The DNA had a single-strand average molecular weight ($\overline{M}_w$) of 26.5×10^6. Limited hydrolysis of the HEK cell DNA catalyzed by pancreatic DNase decreased the $\overline{M}_w$ to 15.0×10^6 (Fig. 1B). Sedimentation analysis of the DNase-treated DNA in neutral sucrose-density gradients showed that most of the DNA had the sedimentation properties of untreated DNA, indicating that the molecular weight of the duplex was essentially unchanged.

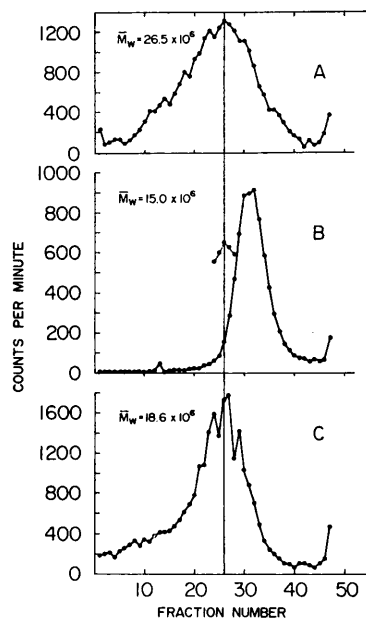

FIG. 1. Alkaline sucrose gradients of digested HEK cell DNA. (A) No treatment; (B) 20-min DNase; (C) 15-min ligase repair using crude enzyme extract prepared from HEK cultures infected for 46 hr with adenovirus 2; ^{14}C Adenovirus 2 DNA marker (. . .).

Incubation of the DNase-treated HEK cell DNA with crude cell-free extracts prepared from noninfected HEK cultures (controls for cultures infected for 2 hr) increased the $\overline{M}_w$ from 15.0×10^6 to 21.8×10^6. This represents 59.1% repair of single-strand nicks in the substrate DNA, assuming that a shift to a $\overline{M}_w$ of 26.5×10^6 corresponds to 100% repair. Enzyme extracts prepared from HEK cultures infected for 46 hr with adenovirus 2 increased the $\overline{M}_w$ of the degraded

TABLE I. Polynucleotide Ligase Activity Found in Noninfected and Adenovirus 2- or 12-Infected HEK Cultures.

Cell extracts	Single-strand average molecular weight ($\bar{M}_w$) $\times 10^6$ [a]			
	22 hr	46 hr	59 hr	77 hr
Noninfected	21.0 (52.1%) [b]	19.5 (39.1%)	18.7 (32.2%)	16.9 (16.5%)
Adeno 2-infected	19.4 (38.2%)	18.6 (31.3%)	17.0 (17.4%)	15.8 (6.9%)
Adeno 12-infected	20.6 (48.7%)	17.3 (19.9%)	16.2 (10.4%)	14.2 (—6.9%)

[a] $\bar{M}_w$ of nicked HEK cell DNA after repair with ligase.

[b] Figures in parentheses indicate % repair of DNase-treated HEK cell DNA with a $\bar{M}_w$ of 15.0×10^6, assuming that a shift to a $\bar{M}_w$ of 26.5×10^6 of the untreated DNA represents 100% repair.

DNA to 18.6×10^6, corresponding to approximately 30% repair (Fig. 1C and Table I).

The ligase activity was measured at various times after infection of HEK cultures with nononcogenic adenovirus 2 or oncogenic adenovirus 12, and compared with that found in control noninfected cultures. Enzyme activity in crude extracts prepared from control cells progressively declined during a period of 77 hr (Table I). At this time, the ligase activity was approximately 30% of that found at 2 hr (Fig. 2). During this period, the rate of DNA synthesis in noninfected cultures also slowly declined. In another study, at 62 hr, the rate of incorporation per cell of thymidine-^{3}H into the DNA of noninfected cultures was approximately 30% of the initial rate (21). The observed decline in DNA synthesis is presumably related to the fact that the dense HEK cultures employed were approaching, or were in the stationary phase of growth. It is striking that activities of the several enzymes tested, namely, thymidine kinase, deoxycytidylic deaminase, and DNA polymerase, all of which catalyze steps in the terminal pathway of thymidine metabolism and DNA synthesis, did not decrease appreciably during the same period (26).

Polynucleotide ligase activity found in adenovirus 2- or 12-infected HEK cell extracts apparently declined at a somewhat faster rate than that of the noninfected cell extracts (Table I, Fig. 2). At 77 hr after infection, ligase activity in adenovirus 2- or 12-infected cultures was only 12%, and 0%, respectively, of that found at 2 hr (Fig. 2).

Only partial repair of single-strand breaks in the substrate DNA was possible under the conditions used (Table I, Fig. 2). Incompleteness or loss of repair could be due to the presence in the crude enzyme extracts of nucleases with endo- or exonucleolytic properties, and these enzymes might produce double- or single-strand breaks in the DNA. The extracts contained some detectable DNase (single-stranded DNA) activity when assayed by the release of acid-soluble radioactivity from labeled DNA (20). The DNase activity, however, was approximately the same in noninfected and adenovirus 2- or 12-infected extracts at various times after infection.

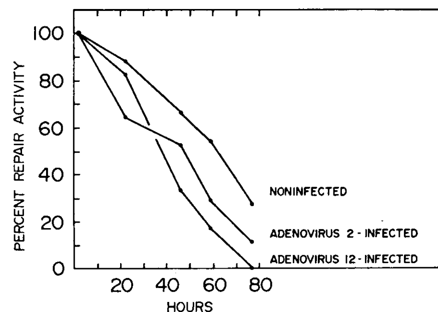

FIG. 2. Repair activity with ligase from control noninfected and adenovirus 2- or 12-infected HEK cultures. The 2-hr control sample repaired the nicked DNA substrate with a $\bar{M}_w$ of 15.0×10^6 to a $\bar{M}_w$ of 21.8×10^6, indicating 59.1% repair (see Table I). The calculation for the residual activities is based on this repair value taken to represent 100% activity.

Between 12 and 32 hr after infection with adenovirus 2 or 12, the amount of newly synthesized HEK cell DNA decreased progressively, and at 30–40 hr, the newly synthe-

sized DNA was reduced by approximately 60–70% relative to that found in control cells (20). It is tempting to speculate that the apparent loss of ligase activity found in infected cells may be related to the adenovirus-induced inhibition of cell DNA synthesis. Growth of adenovirus was complete at 40–60 hr (26), at which times some ligase activity was still detected (Table I). If the joining reaction is essential for adenovirus DNA replication, then either the residual activity of the host ligase is sufficient, or a new adenovirus DNA-specific ligase is induced after infection.

Summary. Cell-free extracts prepared from human embryonic kidney (HEK) cultures after infection with adenovirus 2 or 12 had a decreased ability to repair single-strand breaks in the HEK cell DNA substrate. The apparent loss of polynucleotide ligase activity found in infected cells may be related to the adenovirus-induced inhibition of HEK cell DNA synthesis.

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