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Role of Adenovirus Structural Proteins in the Cessation of Host-Cell Biosynthetic Functions[1]

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Two of the adenovirus capsid proteins, the fiber and the hexon, complexed with either KB cell or type 5 adenovirus deoxyribonucleic acid (DNA). Maximal binding occurred at 0.01 M NaCl; increasing the ionic strength of the reaction mixture to 0.2 M NaCl resulted in a decrease in the association of either antigen to DNA. Variations of *p*H between 6.3 and 8.4 did not affect the binding of fiber antigen to DNA. Below *p*H 7.5, however, there was a small decrease in the ability of the hexon to bind nucleic acid. The association between the adenovirus structural proteins and DNA was reversible and was independent of whether the DNA was native or denatured. The fiber or hexon protein inhibited the DNA-dependent ribonucleic acid (RNA) polymerase and the DNA polymerase from KB cells. On a weight basis, the fiber protein inhibited enzymatic activity to a greater extent than the hexon. Increasing the template DNA concentration decreased this inhibition. The inhibition of the DNA-dependent RNA polymerase activity by either antigen could be reversed by increasing the ionic strength of the reaction mixture. After infection of KB cells with type 5 adenovirus, the levels of DNA and RNA polymerases remained unchanged for 15 to 20 hr. Thereafter, the specific activity of both enzymes decreased. By 30 hr post-infection, the polymerase activities were only about 30% of the enzyme activities in uninfected cells.

The fiber antigen, but not the hexon, from type 5 adenovirus inhibits multiplication of adenovirus, poliovirus, and vaccinia virus (25, 29). This phenomenon could not be attributed to inhibition of viral attachment, penetration, or uncoating. Highly purified fiber antigen blocks deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein biosynthesis beginning 20 hr after its addition to either uninfected or adenovirus-infected KB cells (25). The fiber antigen inhibits poliovirus replication even when RNA production in an antigen-treated cell is markedly reduced by actinomycin D (25). These data imply that the fiber antigen can act on some cellular biosynthetic process and, unlike interferon (21, 31), does not require the synthesis of one or more proteins for its effect.

The fiber antigen has been reported to be a basic protein which is found associated with DNA in crude cell extracts (2). Some basic proteins have been shown to possess unique biological properties and to inhibit the biosynthesis of nucleic acids both in vivo and in vitro. For example, Bukrinskaya et al. (8) reported that a class of histones inhibit RNA synthesis in chick embryo fibroblasts in cell culture and that these treated cells do not support either the propagation of fowl plague virus or the synthesis of viral RNA or hemagglutinin. Histones, as well as other basic proteins such as polylysine, can also inhibit the activities of DNA and RNA polymerases in vitro (1, 4, 20, 23). It was the objective of this investigation to study the mechanism by which fiber antigen inhibits intracellular DNA, RNA, and protein synthesis. Evidence will be presented to show that, although only fiber antigen can attach to intact KB cells (25), both antigens, fiber and hexon, can complex in vitro with KB cell DNA and type 5 adenovirus DNA. This noncovalent association of the viral proteins with DNA in vitro inhibits the activities of DNA-dependent RNA polymerase and DNA polymerase.

MATERIALS AND METHODS

Viruses. The prototype strain of type 5 adenovirus (24) was used throughout these studies.

Tissue culture. Spinner cultures of KB cells were

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employed. Cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 10% calf or human serum by methods previously described (10, 11).

Preparation of virus. Cells in suspension cultures were resuspended at a concentration of approximately 300,000 cells/ml of MEM supplemented with 5% calf serum. Cells were infected at an input multiplicity of about 100 plaque-forming units (PFU) per cell, and they were harvested after incubation at 36 C for 36 to 40 hr. The cells were concentrated 10-fold in maintenance medium (17), and the virus was released by six cycles of freezing and thawing. The clarified viral suspensions were stored at −28 C.

Preparation of antiserum. Rabbits were immunized with 10-fold concentrated extracts of KB cells infected with type 5 adenovirus. Three intramuscular injections were given with Freund's complete adjuvant (Difco) on days 0, 12, and 26 in amounts of 4, 2, and 2.5 ml into each hind leg. The rabbits were bled 15 days after the last injection.

Purification of adenovirus. Adenovirus was purified according to the procedure of Wilcox and Ginsberg (32).

Purification of adenovirus antigens. The purification procedure and the criteria for the purity of the adenovirus antigens were described previously (25).

Purification of DNA. KB cell DNA was extracted and purified by the procedure of Marmur (27). When this DNA was used as a primer for DNA and RNA polymerases, several additional steps were included. The DNA was extracted two times with water-saturated redistilled phenol. The phenol was removed with ether which had been treated with aluminum oxide, and the ether was removed by blowing nitrogen over the DNA solutions.

Adenovirus DNA was extracted from purified virus by a modification of the procedure of Borenfreund et al. with the following steps: 1% sodium dodecyl sulfate (SDS), for 30 min at 4 C to disrupt the virions; 0.25 M 2-mercaptoethanol, shaken for 30 min at 4 C; pronase, 1 mg/ml, shaken for 60 min at 37 C. DNA was then extracted with phenol as described above for KB cell DNA; after removal of the phenol, the DNA was precipitated with 2 volumes of ethyl alcohol.

Chemical determination of DNA and protein. DNA was measured by the Burton (9) modification of the diphenylamine reaction. Thymus DNA was used as a standard. The method of Lowry et al. (26) was employed to determine protein concentrations; crystalline bovine albumin was used as the standard.

Radioisotopes and techniques of measurement. ^{14}C-thymidine (0.11 mc/mg, New England Nuclear Corp., Boston, Mass.), ^{3}H-thymidine (0.28 mc/mg, New England Nuclear Corp.), and ^{32}P-orthophosphate (carrier-free, E. R. Squibb & Sons, New York, N.Y.) were employed to label DNA. These were measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) by methods previously described (25).

Measurement of the association of viral proteins and DNA. A typical reaction mixture contained 0.1 ml of purified protein (25) at the concentration designated in each experiment, 0.1 ml of isotopically labeled purified DNA, 0.1 ml of diluent, and 0.2 ml of undiluted antiserum. Unless specified, the diluent was adjusted to give a final concentration of 0.01 M NaCl. Usually, the first three components were mixed and incubated at 37 C for 10 min; an excess of type 5 adenovirus antibody was then added. The complete reaction mixture was incubated at 37 C for an additional 1.5 hr, and the precipitate which formed was sedimented by centrifugation at 1,000 × *g* for 30 min at 4 C. The supernatant fluid was assayed to determine the quantity of radioactive DNA that was unassociated with the antigen-antibody complex. Preimmune rabbit serum was used in place of specific antiserum in reaction mixtures prepared as described above to determine the 100% value of unassociated DNA.

Preparation of DNA and DNA-dependent RNA polymerases from KB cells. DNA (deoxynucleoside-triphosphate: DNA deoxynucleotidyl transferase, EC 2.7.7.7) and RNA (nucleosidetriphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) polymerases from KB cells were obtained by the method of Furth and Ho (14). About 10^{10} cells were suspended in 50 mM tris(hydroxymethyl)aminomethane (Tris)-chloride, *p*H 8.1, and 5 mM 2-mercaptoethanol, and the suspension was homogenized at 16,000 rev/min in a Servall Omni-mixer for 30 to 60 sec. The homogenate was centrifuged at 100,000 × *g* for 30 min, and protamine sulfate was added to a final concentration of 0.15% to the supernatant fluid. The protamine sulfate precipitate was extracted twice with 0.1 M sodium succinate buffer at *p*H 6.0 and then twice with 0.5 M sodium succinate, *p*H 6.0. All buffers contained 5 mM 2-mercaptoethanol. To both succinate solutions (0.1 and 0.5 M), a saturated ammonium sulfate solution, *p*H 7.0, was added to 40% saturation. The resulting precipitates were collected, and the supernatant fluid of the 0.1 M succinate solution was adjusted to 60% $(NH_4)_2SO_4$ saturation. All three $(NH_4)_2SO_4$ precipitates (0.1 M succinate, 0 to 40% and 40 to 60%, and the 0.5 M succinate, 0 to 40%) were resuspended in 0.2 ml of 10 mM Tris buffer, *p*H 8.1, containing 2 mM 2-mercaptoethanol and were dialyzed for 1 hr against several changes of this same buffer. DNA polymerase was present in the 0.1 M succinate fractions and RNA polymerase was present in the 0.5 M succinate solution. All enzyme preparations were assayed the day they were purified.

To determine the polymerase activities in KB cells infected with type 5 adenovirus, the enzymes were prepared by the method of Hopper, Ho, and Furth (22). For this procedure, 60 to 120 million cells from a growing culture were removed by centrifugation and washed once with Tris-saline, *p*H 7.9. The cell extract was prepared in a manner identical to that previously described, except that the protamine precipitation and succinate elution steps were not employed. The 100,000 × *g* supernatant fluid was adjusted to 60% saturation with ammonium sulfate at *p*H 7.0, and the resultant precipitate was resuspended and dialyzed as described.

The enzymes obtained from infected and uninfected KB cells had the requirements and specificities as described by Hooper et al. (22). Extract concentrations were proportional to enzyme activity up to 410 μg of extract protein for RNA polymerase and 675 μg of

extract protein for DNA polymerase. The RNA polymerase reaction was linear with time up to 20 min at 37 C, and the DNA polymerase reaction proceeded linearly for over 1 hr at 37 C.

RESULTS

Association of viral capsid proteins with DNA. The possibility that the fiber protein might inhibit synthesis of host macromolecules by reacting with nucleic acids which serve as templates suggested that the large intranuclear accumulation of viral structural proteins which are not assembled into virus (7) might combine with DNA during infection. To test the latter hypothesis, the following experiment was performed. ^{14}C-thymidine (10 μc/ml of culture) was added to a culture of exponentially growing KB cells for 24 hr at 36 C. The cells were then sedimented by centrifugation, resuspended in medium without isotope, and cultured for an additional 24 hr. At the end of this period, greater than 99% of the radioactivity was in cellular acid-precipitable material. Half of these cells were infected with type 5 adenovirus (200 PFU/cell), and the remainder of the culture served as an uninfected control. Two additional suspension cultures were treated in an identical manner except that the KB cell DNA was not labeled. At 36 hr after infection, all four cultures were harvested; the cells were washed and resuspended in 5 ml of phosphate-buffered saline (PBS). A sample from each labeled culture was taken to determine total trichloroacetic acid-precipitable counts. The concentrated cell suspensions were disrupted by sonic vibrations for 2 min at 0 to 4 C and centrifuged at 1,050 $\times$ *g* for 30 min. A sample of each supernatant fluid from the isotopically labeled cultures was counted to determine the amount of radioactivity made soluble by this procedure. A second sample was incubated with type 5 adenovirus antiserum for 1 hr at 37 C. The precipitate that formed was sedimented by centrifugation at 1,000 $\times$ *g* for 30 min, and a portion of the supernatant fluid was assayed for radioactivity.

The results of this experiment (Table 1) demonstrated that the presence of some viral product (or products) in the infected cell extracts permitted the major portion of the host-cell DNA to become soluble in 0.15 M NaCl and that antiserum to the viral proteins precipitated the host DNA. Both increased solubilization and precipitation of host-cell DNA occurred readily in vitro. When normal rabbit serum was used instead of immune antiserum, DNA was not precipitated. Additional evidence indicated that the labeled DNA precipitated by immune serum was not viral DNA: (i) host-cell DNA was not degraded to an acid-soluble form during infection, and (ii) host-cell labeled DNA could not be recovered in purified viral progeny (Bello, Lawrence, Levine, and Ginsberg, *unpublished observations*). However, viral DNA, which is extracted from infected cells as a nucleoprotein, is also soluble in 0.15 M NaCl (16).

TABLE 1. *Solubility of host-cell DNA in 0.15 M NaCl and the association of viral antigens with this DNA in KB cells infected with type 5 adenovirus*

Cell extract tested[a]	Percentage of counts solubilizing in 0.15 M NaCl[b]	Percentage of the counts precipitated with antiserum[c]
I*	71	75
C*	35	0
C + I*	63	79
I + C*	61	76

[a] Samples were obtained from cells prepared in the following manner: I*, host-cell DNA labeled with ^{14}C-thymidine and the cells infected for 36 hr; C*, host-cell DNA labeled with ^{14}C-thymidine and the uninfected cells cultured for 36 hr; I, infected for 36 hr; the DNA was not labeled; C, uninfected cells, cultured for 36 hr; DNA was unlabeled.

[b] Cells sonically treated for 2 min in 0.01 M phosphate-buffered 0.15 M NaCl, *p*H 7.2, and centrifuged at 1,050 $\times$ *g* for 30 min. Supernatant fluids were assayed.

[c] A sample of each supernatant fluid was incubated with an equal volume of undiluted type 5 adenovirus antiserum for 1 hr at 37 C; the precipitate was sedimented, and the supernatant fluid was assayed for radioactivity (unassociated DNA).

Characterization of the assay used to demonstrate binding of antigen with DNA. The data described (Table 1) imply that viral protein and host-cell DNA readily complex in vitro, possibly within the infected cell. Several attempts to prevent in vitro binding, so that the possible intracellular association could be studied, proved unsuccessful. Therefore, an investigation of the properties of the in vitro binding reaction was undertaken on the premise that it would reflect the association of antigen and DNA which might occur in vivo.

Table 2 summarizes the results of experiments carried out to establish the validity of the assay used to measure the binding of the viral proteins to DNA. The data demonstrate that incubation of antigen and antibody, before the addition of DNA, resulted in a greatly reduced precipitation of labeled DNA; that the maximal combination of DNA with viral antigens required that the protein be incubated with DNA before the addition of antiserum; and that incubation of purified DNA

TABLE 2. *Effect of the order of addition of the components of the reaction mixture on the binding of antigens to DNA*

Reaction mixture[a]	Percentage of DNA precipitated with	
	Fiber	Hexon
Antigen + DNA	0	0
Antiserum + DNA	0	0
(Antigen + DNA) + antiserum	80	48
(Antigen + antiserum) + DNA	20	13

[a] The reaction mixture consisted of 0.1 ml of purified antigen (50 μg), 0.1 ml of ^{14}C-labeled purified KB cell DNA (3.6 μg), 0.1 ml of diluent, and 0.2 ml of type 5 antiserum. When a component of the reaction mixture was omitted, the amount of diluent was adjusted so that the final volume was always 0.5 ml. The reaction mixtures were incubated at 37 C for 1.5 hr and then centrifuged at 1,000 × *g* for 30 min at 4 C. A sample of the supernatant fluid was assayed to determine the amount of free DNA.

either with protein alone or with antibody alone did not precipitate the purified labeled nucleic acid.

Antibodies present in a single antiserum precipitated both fiber and hexon antigens to the same degree (greater than 95%) in the presence or absence of DNA. Hence, it was possible to compare directly the ability of these two viral proteins to associate with DNA by use of the same immune serum.

To ascertain whether the DNA was precipitated because it was complexed with viral protein or was merely trapped in the precipitate, labeled DNA was added to a solution of hemocyanin before the addition of antihemocyanin antibody. The mere occurrence of a heavy antigen-antibody precipitation did not trap the DNA in the antigen-antibody complex.

Binding of fiber and hexon antigens to viral and KB cell DNA. The ability of each of the viral structural proteins to combine with viral and KB cell purified DNA was compared. Increasing concentrations of labeled DNA were added to 50-μg amounts of protein, and the percentage of DNA bound (DNA_b/DNA_t) was determined. Representative binding curves obtained are presented in Fig. 1.

There are two distinct portions of the curve. At lower levels of DNA, a twofold increase in the DNA concentration more than doubled the amount of DNA bound by either protein. The results indicate that, with the addition of increasing amounts of DNA to the reaction mixture, a greater proportion of the DNA is bound to protein. Several possible explanations for this cooperative effect will be considered in the Discussion. Above 7.2 μg/ml of DNA for the fiber antigen and 14.4 μg/ml for the hexon, the cooperative effects were lost and a simple linear relationship was observed. At DNA levels above 21.6 μg/ml, there was a decrease (not shown here) in the percentage of DNA bound by 50 μg of either protein.

At the lower DNA concentrations, the fiber protein (on a weight basis) associated with either viral or KB cell DNA about two times better than did the hexon. At a DNA concentration of 4.5 μg/ml, 50 μg of fiber protein bound 50% of the DNA. In comparison, a DNA concentration of 9.2 μg/ml was required for 50 μg of the hexon protein to associate with 50% of the DNA. When binding was compared on a molar basis, the association of each protein with DNA was similar. The DNA used, viral or host, did not influence the protein binding in the lower range of DNA concentrations. At higher DNA levels, however, 90% of the KB cell DNA and only 70% of the viral DNA was complexed by either antigen.

Effect of pH and ionic strength on the association of viral proteins and DNA. Maximal binding of fiber and hexon proteins to DNA occurred at NaCl concentrations lower than 0.01 M (Fig. 2). When NaCl concentrations were increased from

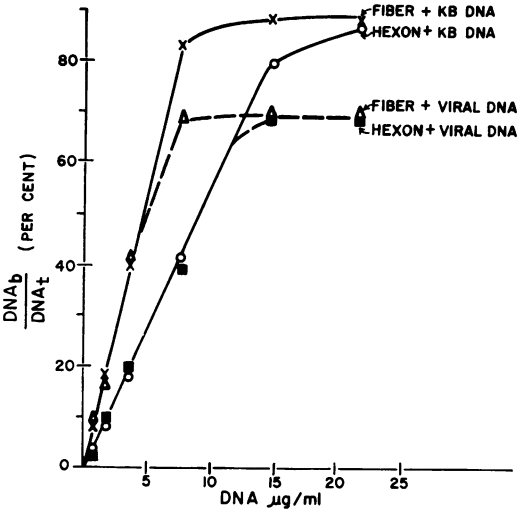

FIG. 1. *Binding of fiber and hexon antigens to viral and KB cell DNA. Amounts of 50 μg of either protein were incubated with increasing concentrations of labeled KB cell or viral DNA. Antiserum was added to the mixture, and the percentage of DNA bound (DNA_b/DNA_t) by the antigens was determined as described in the text.*

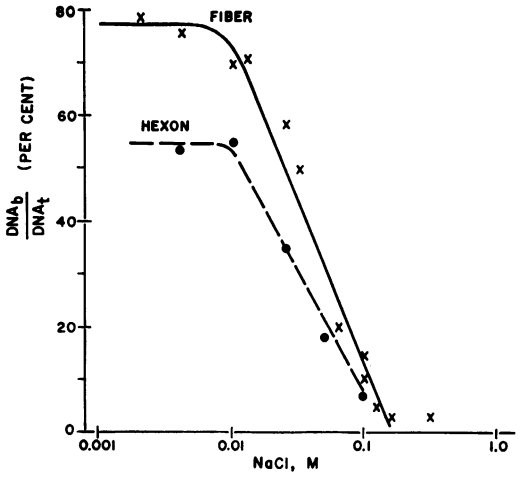

FIG. 2. *Effect of ionic strength on the binding of the viral antigens to the KB cell DNA. Quantities of 50 μg of either protein were incubated with 3.6 μg of labeled KB cell DNA, and various concentrations of NaCl were used as diluent. The percentage of DNA bound (DNA_b/DNA_t) was determined as described in the text.*

0.01 to 0.1 M, the association of either antigen with KB cell DNA was markedly reduced. Identical results were obtained with viral DNA. The ranges of ionic strength used in this experiment had no effect on the antigen-antibody precipitation reaction.

Experiments to determine the effect of *p*H on the binding of the two viral proteins to KB cell DNA were carried out at a constant ionic strength (0.005 M NaCl). The results (Fig. 3) indicated that over a *p*H range from 6.3 to 8.4 differences in the ability of the fiber antigen to associate with DNA could not be detected. However, the association between hexon and nucleic acid was decreased below *p*H 7.5. This decrease in binding of the hexon antigen at lower *p*H values was unexpected. The predicted effect of lowering the *p*H would be to increase the net positive charge of the protein and therefore to enhance the association. The reason for this unusual result remains obscure. The ranges of *p*H used in this experiment did not effect the antigen-antibody reaction, and identical results were obtained whether the antiserum was dialyzed before its use, precipitated with 40% ammonium sulfate and dialyzed, or remained untreated.

Reversibility of the antigen-DNA complex. The dissociation of antigen-DNA complexes was tested to ascertain whether or not the antigen-DNA complex was reversible and to obtain further evidence on the nature of the bonding between DNA and viral protein. Antigen (150 μg/ml) was incubated with labeled KB cell DNA (7.2 μg/ml) for 10 min at 37 C. A sample of this reaction mixture was diluted fivefold in 0.01 M NaCl or 0.4 M NaCl and incubated at 37 C for 10 min. Antiserum was then added, and the percentage of DNA bound by protein was determined. The data presented in Table 3 indicate that both fiber and hexon antigens were reversibly bound to DNA. During the 10-min period in which dissociation was permitted, the hexon-DNA complex was reversed more efficiently than the fiber at the lower salt concentration. At the higher electrolyte concentration, however, the dissociation between DNA and fiber or hexon protein was similar; approximately 85% of the complex dissociated in each case. These data did not result from inefficient precipitation of the antigen-DNA complex at low protein concentra-

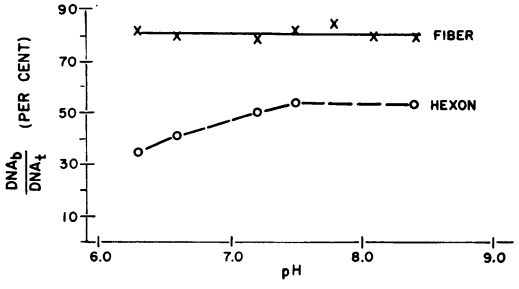

FIG. 3. *Effect of pH on the binding of the viral antigens to KB cell DNA. Quantities of 50 μg of either antigen were incubated with 3.6 μg of labeled KB cell DNA at different pH values. The pH of the reaction mixture was altered by varying the relative concentrations of mono- and disodium phosphate (5×10^{-3} M). The percentage of DNA bound (DNA_b/DNA_t) was determined as described in the text. Similar results were obtained with labeled DNA from type 5 adenovirus.*

TABLE 3. *Effect of dilution on the reversibility of the antigen-DNA complex*[a]

Antigen	Dilution	NaCl concn	Percentage of DNA bound
		M	
Fiber	None	0.01	81
	1:5	0.01	44
	1:5	0.4	14
Hexon	None	0.01	78
	1:5	0.01	20
	1:5	0.4	16

[a] Antigen (150 μg/ml) was incubated with ^{14}C-labeled KB cell DNA (7.6 μg/ml) for 10 min at 37 C. A sample of this reaction mixture was diluted into 0.01 or 0.4 M NaCl and incubated at 37 C for 10 min. The percentage of DNA bound by the viral protein was determined as described in Table 2.

tions, since greater than 95% of the antigen was precipitated with antiserum at the diluted protein concentration (30 μg/ml).

Effect of the secondary structure of the DNA on the formation of the protein-DNA complex. Denatured viral or KB cell DNA, heated at 100 C for 5 min and rapidly cooled, and the native DNA preparations were compared for the ability of each to bind antigen. The data (not presented) clearly indicated that there was not a significant difference between native and denatured DNA. The results were similar when either fiber or hexon antigen and viral or host cell DNA were employed.

Effect of fiber and hexon antigens on RNA polymerase activity. Since the fiber and hexon antigens combine with KB cell and viral DNA, it was postulated that these viral capsid proteins could block the template function of DNA and thus inhibit polymerase activity. The results of an experiment to test this hypothesis with RNA polymerase (Table 4) indicated that enzyme activity was reduced by both fiber and hexon proteins and that on a weight basis the fiber was a more effective inhibitor than the hexon. The fiber protein inhibited *Escherichia coli* and KB cell RNA polymerases equally.

The data presented (Table 4) imply that the antigens inhibited RNA polymerase activity because they complexed with DNA and prevented it from functioning as a template. If this interpretation is correct, the addition of more DNA to the reaction mixture should decrease the inhibition. The results of an experiment designed to test this prediction (Fig. 4) indicated that with increasing concentrations of primer DNA, at constant enzyme and antigen concentrations, there were comparable increases in uridine monophosphate (UMP) incorporation into an acid-precipitable form. The results of this experiment also confirm the findings that the enzyme preparation employed was completely DNA-dependent; that in the absence of antigen RNA polymerase activity increased linearly with increasing DNA concentrations up to about 26 μg/ml of DNA; and that the addition of fiber or hexon antigen had an inhibitory effect on the incorporation of UMP.

The inhibition of RNA polymerase activity by fiber or hexon proteins was identical whether the enzyme was obtained from infected (17 hr post-infection) or uninfected KB cells. In addition, the origin of the template DNA, whether viral or host cell, did not influence the results.

Effect of ionic strength on the ability of the viral structural antigens to inhibit RNA polymerase. Since the fiber and hexon proteins cannot complex

TABLE 4. *RNA polymerase activity[a] in the presence of fiber and hexon antigens*

Enzyme source	Antigen added[b]	UMP incorporated	Inhibition
		μμmoles	%
KB cells[c]	None	19	—
	Fiber	10	49
	Hexon	15	21
Escherichia coli[d]	None	292	—
	Fiber	127	56

[a] The complete reaction mixture contained: 60 μM $UT^{32}P$ (4×10^6 counts per min per μmole); 320 μM adenosine, cytidine, and guanosine triphosphates; and 12.8 μg of KB cell DNA in 0.5 ml. After incubation at 37 C for 20 min, the reaction was terminated by the addition of 1 ml of cold 10% trichloroacetic acid with 0.1 M sodium pyrophosphate. The precipitate was washed three times in cold 10% trichloroacetic acid and 0.1 M sodium pyrophosphate, dissolved in 1 N NH_4OH, and plated on metal planchets. Radioactivity was counted in a windowless gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

[b] Fiber or hexon protein (700 μg) added to the standard RNA polymerase reaction mixture before the addition of enzyme.

[c] KB cell extracts, 420 μg of protein.

[d] *E. coli* extracts, 42 μg of protein.

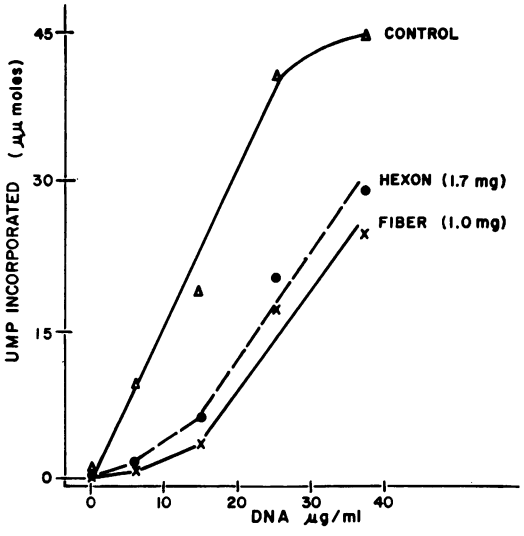

FIG. 4. *Activity of RNA polymerase from KB cells in the presence of the viral antigens. The incorporation of $UM^{32}P$ was measured in the presence of constant viral protein and enzyme (401 μg) as a function of increasing DNA concentrations; 60 μM $UT^{32}P$ (3.6×10^8 counts per min per μmole) was employed. For details of the reaction mixture, see Table 6.*

TABLE 5. *Effect of fiber and hexon antigens on the activity of RNA polymerase and the dependence of inhibition on ionic strength of the reaction mixture*

Treatment	UMP incorporation[a]			
	0.01 M NaCl		0.4 M NaCl	
	Amt	Inhibition	Amt	Inhibition
	μμmoles	%	*μμmoles*	%
None	19.2	—	14.6	—
Fiber antigen[b]	4.2	78	16.2	0
Hexon antigen[c]	6.0	69	15.0	0

[a] Details of the reaction mixture are given in Table 6. KB cell polymerase was employed.
[b] Protein, 1.0 mg.
[c] Protein, 1.7 mg.

DNA at high ionic strengths, the viral proteins should not inhibit RNA polymerase activity at ionic strengths above 0.2 M NaCl. The test of this deduction (Table 5) demonstrates that at low ionic strength (i.e., 0.01 M NaCl) the fiber and hexon antigens inhibited KB cell RNA polymerase activity by 78 and 69%, respectively. However, when the concentration of NaCl in the reaction mixture was increased to 0.4 M the antigens did not reduce enzymatic activity.

Effect of viral antigens on activity of DNA polymerase from infected and uninfected cells. It was predicted from the evidence described above that fiber and hexon proteins should also inhibit DNA polymerase. Indeed, the viral proteins did inhibit DNA polymerase activity, and the enzymes from infected and uninfected cells were inhibited to about the same extent (Table 6). In agreement with the results of previous experiments, the fiber antigen, on a weight basis, was a more effective inhibitor than was the hexon. Similar results were obtained when denatured type 5 adenovirus or KB cell DNA was used as template.

Levels of RNA and DNA polymerases after infection of KB cells with type 5 adenovirus. Results of the in vitro experiments indicated that, although the viral proteins inhibited RNA and DNA polymerase activities from infected or uninfected cell extracts to the same extent, there was a quantitative difference. Infected cell extracts (17 hr postinfection) always possessed lower specific activities (micromicromoles incorporated per milligram of protein) than the extracts from uninfected cells. To investigate the nature of this difference, the activities of the polymerases were followed after infection of KB cells with type 5 adenovirus. For these experiments, two 3-liter suspension cultures (220,000 cells/ml) of KB cells were used. One of these was infected at a multiplicity of 500 PFU/cell. Samples of 400 ml were taken at the start of the experiment and at intervals thereafter. Enzyme extracts were prepared by the procedure described in Materials and Methods, and RNA and DNA polymerase activities were assayed in the same cell extracts. At about 15 hr after infection, RNA polymerase activity began to decrease (Fig. 5) and reached about one-third the control value by 31 hr after infection. The activity of DNA polymerase 15 to 20 hr after infection (Fig. 6) began to decrease. By 31 hr postinfection, the specific activities of DNA polymerase in infected cell extracts were only about one-third of the controls. Green et al. (19) found a decrease in DNA polymerase of about 30% in cells 24 hr after infection with type 2 adenovirus.

DISCUSSION

A capsid protein of the adenovirus particle, the fiber, can halt biosynthesis of macromolecules in the host cell and through this mechanism reduce the propagation of adenoviruses as well as

TABLE 6. *Effect of viral antigens on the activity of DNA polymerase[a] from infected and uninfected cells*

Cell extract[b]	Antigen added	Incorporation of deoxyguanosine monophosphate	Inhibition
		μμmoles	%
Uninfected	None	72	—
	Fiber[c]	26	64
	Hexon[d]	35	52
Infected (17 hr)	None	36	—
	Fiber[c]	9	75
	Hexon[d]	13	64

[a] The complete reaction mixture contained: 14 μM dGTP-^{14}C (1,060 counts per min per μμmole); 160 μM of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate; 10 mM Tris-chloride buffer, *p*H 8.1; 4 mM $MgCl_2$; 2 mM 2-mercaptoethanol; 11.6 μg/ml of heat-denatured DNA from purified type 5 adenovirus (100 C for 5 min and rapidly cooled), and enzyme in 0.5 ml. After incubation for 30 min at 37 C, the reaction was terminated by the addition of 10% cold trichloroacetic acid and 0.1 M sodium phosphate. The washing and counting procedures were identical to those used for RNA polymerase (*see* Table 6).
[b] Amounts were 200 and 205 μg of protein, respectively, from infected and uninfected KB cell.
[c] Protein, 1 mg
[d] Protein, 1.5 mg.

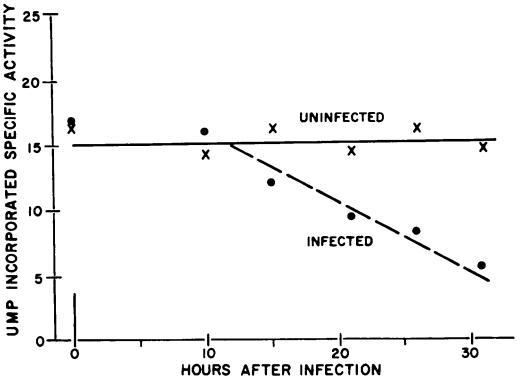

FIG. 5. *RNA polymerase activity in KB cells infected with type 5 adenovirus. One of two 3-liter spinner cultures was infected with 500 PFU/cell of type 5 adenovirus at 0 hr. Samples of 400 ml were taken from the infected and uninfected cultures at the indicated times, the cells were fractionated, and the RNA polymerase was assayed (see Table 6). The specific activity of the polymerase (μμmoles of UMP incorporated in 20 min at 37 C/mg of protein) is plotted as a function of the time after infection.*

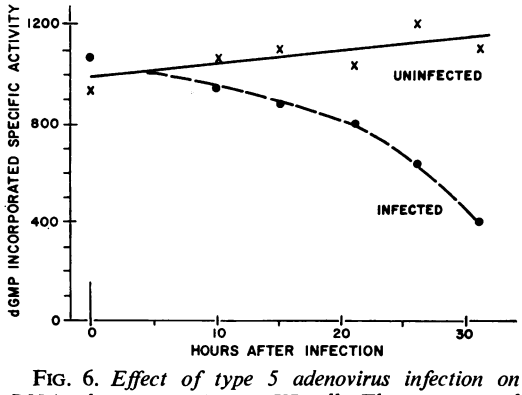

FIG. 6. *Effect of type 5 adenovirus infection on DNA polymerase activity in KB cells. The experimental design is the same as that described in Fig. 5. The specific activity of the polymerase is expressed as the μμmoles of dGMP incorporated in 30 min at 37 C/mg of protein. For details of the reaction mixture, see Table 6.*

unrelated viruses (25). These findings and the fact that the fiber antigen is a basic protein suggested that it might combine with nucleic acids, similar to histones and other basic proteins, and in this way exert its biological activity. The complexed DNA or RNA would presumably be unable to function as a template for replication or transcription.

Indeed, as demonstrated above, purified fiber and hexon proteins of type 5 adenovirus can combine with viral or KB cell DNA. This association, however, is quite complex. At lower DNA concentrations, the binding is cooperative, whereas at higher levels of DNA this effect is no longer observed. Several alternative explanations for this phenomenon can be formulated. (i) The binding of a second (or third, etc.) DNA molecule that binds to the protein may be facilitated by the presence of the first DNA molecule already bound to the protein. This facilitation of binding could be due to increasing binding constants or increasing rates of DNA-protein association. (ii) The antigen proteins tend to aggregate (Levine and Ginsberg, *unpublished observation*); if DNA dissociates these aggregates, then increasing the DNA concentration would increase the amount of protein available for binding to the DNA. (iii) The DNA added to the reaction may antagonize an inhibitor of the binding reaction. These hypotheses have in common the fact that more than one simple process is involved in the binding of DNA to viral proteins at low DNA concentrations. Whether this has any physical significance in vivo is not clear.

The DNA-protein complex is established by ionic bonds, and increasing the salt concentration above 0.01 M decreases the association of either viral protein with DNA to a lower limit of 3 to 5% binding at 0.2 M NaCl. Moreover, dilution as well as increasing the ionic strength of the solution reverses the adenovirus protein-DNA complex.

Addition of either viral capsid protein to DNA, KB cell or viral, inhibits the enzymatic activity of DNA and RNA polymerases. Increasing the ionic strength of the reaction mixture reverses the inhibitory effects of the protein. These data support the concept that association of the capsid protein with DNA blocks its function as a template for replication or transcription.

The internal protein of T2 bacteriophage and the phage DNA also form complexes, and the attractive forces involved possess about the same dependence upon ionic strength (28) as the association of adenovirus capsid proteins and DNA. The binding of T2 internal proteins or histones to DNA likewise reduces the capability of the DNA to serve as a template for polymerases (3, 23). However, the quantity of adenovirus proteins (i.e., the protein-DNA ratios on a weight basis) required to inhibit polymerase activities is higher than for histones or the T2 internal protein (3, 4, 20, 23).

The polymerases from infected and uninfected cells appear to be similar. The nature of the primer DNA, viral or KB cell, neither influences the characteristics of the enzymatic reactions studied nor alters the inhibitory capabilities of the viral proteins. The activities of the DNA and RNA

polymerases do not increase in cells infected with type 5 adenovirus, just as the rates of DNA and RNA syntheses do not differ between infected cells and uninfected cells growing exponentially (15, 18). These data probably reflect the fact that the optimal rate of biosynthesis of host-cell macromolecules is replaced by an equal rate of production of virus-specific nucleic acids in infected cells (15). The specific activities of both enzymes actually decrease 15 to 20 hr after infection. This decrease in enzymatic activity probably results from (i) cessation of host-cell protein synthesis 16 to 20 hr after infection (5), or (ii) inhibition of the enzymatic activities of the polymerases owing to the synthesis and accumulation of viral structural proteins (only relatively crude enzyme extracts were employed for these experiments).

Since the fiber proteins can block intracellular DNA, RNA, and protein synthesis (25), and both the fiber and hexon proteins can associate with DNA and inhibit DNA and RNA polymerase activities in vitro, it is tempting to speculate on the possible regulatory roles of these viral capsid proteins in adenovirus-infected cells. Biosynthesis of host DNA is blocked about 8 hr after infection with type 5 adenovirus, about the time that viral DNA synthesis commences and several hours before the viral capsid proteins are produced (15).

Relatively large amounts of hexon or fiber protein were required to inhibit DNA and RNA polymerases in vitro. Hence, it seems unlikely that the capsid proteins of the infecting virions could effect the interruption of host DNA synthesis directly unless there are preferred sites for binding or increased efficiency of binding to the native host DNA intracellularly. On the other hand, the biosynthesis of host RNAs begins to decline about 16 hr postinfection and after viral capsid proteins have begun to accumulate within nuclei of infected cells (15). At approximately the same time, synthesis of viral DNA and of virus-specific messenger RNA are also stopping. Therefore, inhibition of host RNA production does not require a highly specific reaction, and it is possible that the accumulation of large quantities of the viral fiber and hexon proteins could induce this suppression of DNA and RNA synthesis by complexing with both host and viral DNA intracellularly as it does in vitro. Thus, infection supplies to a cell viral subunits which, like histones, may potentially affect cell functions.

It should be pointed out that the presence of large quantities of viral antigens in the nuclei of infected cells does not *a priori* mean that the viral proteins associate with nucleic acids in vivo. There may be compartmentalization or other structural features of the cell nucleus which could prevent such interactions. However, numerous electron microscopic studies of adenovirus-infected cells fail to reveal compartmentalization or other unique geographic features in the infected nuclei.

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LITERATURE CITED

1. Allfrey, V. G., V. C. Littan, and A. E. Mirsky. 1963. On the role of histones in regulating ribonucleic acid synthesis in the cell nucleus. Proc. Natl. Acad. Sci. U.S. **49**:414–421.
2. Allison, A. C., H. G. Pereira, and C. P. Farthling. 1960. Investigation of adenovirus by agar gel diffusion techniques. Virology **10**:316–328.
3. Bachrach, U., and A. Friedmann. 1967. Purification and some possible functions of internal proteins. Biochem. Biophys. Res. Commun. **26**:596–601.
4. Barr, G. C., and J. A. V. Butler. 1963. Histones and gene function. Nature **199**:1170–1172.
5. Bello, L. J., and H. S. Ginsberg. 1967. Inhibition of host protein synthesis in type 5 adenovirus-infected cells. J. Virol. **1**:843–850.
6. Borenfreund, F., E. Fitt, and A. Bendich. 1961. Isolation and properties of deoxyribonucleic acid from mammalian sperm. Nature **191**: 1375–1377.
7. Boyer, G. S., F. W. Denny, and H. S. Ginsberg. 1959. Sequential cellular changes produced by types 5 and 7 adenovirus in HeLa cells and in human amniotic cells. J. Exptl. Med. **110**: 827–844.
8. Bukrinskaya, A. G., A. K. Gitelman, and I. M. Shapiro. 1965. Influence of heterologous histones on the synthesis of cellular and viral ribonucleic acid and product of haemagglutinin. Nature **208**:557–558.
9. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric determination of deoxyribonucleic acid. Biochem. J. **62**:315–322.
10. Eagle, H. 1955. Propagation in a fluid medium of a human epidermoid carcinoma strain KB. Proc. Soc. Exptl. Biol. Med. **89**:362–364.
11. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.

12. Flanagan, J. F., and H. S. Ginsberg. 1962. Synthesis of virus-specific polymers in adenovirus-infected cells: effect of 5-fluorodeoxyuridine. J. Exptl. Med. **116**:141–157.
13. Flanagan, J. F., and H. S. Ginsberg. 1964. Role of ribonucleic acid biosynthesis in multiplication of type 5 adenovirus. J. Bacteriol. **87**:977–987.
14. Furth, J. J., and P. L. Ho. 1965. The enzymatic synthesis of RNA in animal tissues. I. The DNA directed synthesis of RNA as catylized by an enzyme obtained from bovine lymphosarcoma tissue. J. Biol. Chem. **240**:2602–2606.
15. Ginsberg, H. S., L. J. Bello, and A. J. Levine. 1967. Control of biosynthesis of host macromolecules in cells infected with adenoviruses, p. 547–572. *In* J. S. Colter [ed.], Symposium on the molecular biology of viruses. Academic Press, Inc., New York.
16. Ginsberg, H. S., and M. K. Dixon. 1959. Deoxyribonucleic acid (DNA) and protein alterations in HeLa cells infected with type 4 adenovirus. J. Exptl. Med. **109**:407–422.
17. Ginsberg, H. S., E. Gold, and W. S. Jordan, Jr. 1955. Tryptose phosphate broth as a supplementary factor for the maintenance of HeLa cell tissue culture. Proc. Soc. Exptl. Biol. Med. **89**:66–71.
18. Green, M., and G. E. Daesch. 1961. Biochemical studies on adenovirus multiplication II. Kinetics of nucleic acid and protein synthesis in suspension cultures. Virology **13**:169–176.
19. Green, M., M. Pina, and V. Chogoya. 1964. Biochemical studies on adenovirus multiplication. V. Enzymes of deoxyribonucleic acid synthesis in cells infected with adenovirus and vaccinia virus. J. Biol. Chem. **239**:1188–1197.
20. Gurley, L. R., J. L. Irvin, and D. J. Halbrooks. 1964. Inhibition of DNA polymerase by histones. Biochem. Biophys. Res. Commun. **14**:527–532.
21. Heller, E. 1963. Enhancement of Chikungunya virus replication and inhibition of interferon production by actinomycin D. Virology **21**:652–656.
22. Hopper, D. K., P. L. Ho, and J. J. Furth. 1966. Detection of RNA and DNA polymerase in animal tissues by ammonium sulfate fractionation of cell-free extracts. Biochim. Biophys. Acta **118**:648–651.
23. Huang, R. C., and J. Bonner. 1962. Histone, a suppressor of chromosomal RNA synthesis. Proc. Natl. Acad. Sci. U.S. **48**:1216–1222.
24. Huebner, R. J., W. P. Rowe, T. G. Ward, R. H. Parrott, and J. A. Bell. 1954. Adenoidal-pharyngeal-conjuctural agents. A newly recognized group of common viruses of the respiratory system. New Eng. J. Med. **251**:1077–1086.
25. Levine, A. J., and H. S. Ginsberg. 1967. Biochemical studies on the mechanism by which the fiber antigen inhibits multiplication of type 5 adenovirus. J. Virol. **1**:747–757.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
27. Marmur, J. 1964. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
28. Minagawa, T. 1961. Some characteristics of the internal protein of phage T2. Virology **13**:515–527.
29. Pereira, H. G. 1960. A virus inhibitor produced in HeLa cells infected with adenovirus. Virology **11**:590–602.
30. Skallka, A., A. V. Fowler, and J. Hurwitz. 1966. Effect of histones on enzymatic synthesis of RNA. J. Biol. Chem. **241**:588–596.
31. Taylor, J. 1964. Inhibition of interferon action by actinomycin. Biochem. Biophys. Res. Commun. **14**:447–451.
32. Wilcox, W. C., and H. S. Ginsberg. 1963. The structure of type 5 adenovirus. I. Antigenic relationship of virus-structural proteins to virus specific soluble antigens from infected cells. J. Exptl. Med. **118**:295–306.