Purification and Characterization of a DNA Single Strand Specific Endonuclease from Human Cells†

E. C. Wang, J. J. Furth, and J. A. Rose*

ABSTRACT: An endonuclease with DNA single-strand specificity has been purified from KB cells. The enzyme has a pH optimum at 9.2, requires Mg^{2+} for activity, and is inhibited by mono- or divalent cations. Its sedimentation coefficient of 4.6 S is based on sucrose gradient sedimentation, and it has a molecular weight of 54 000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme specifically catalyzes the endonucleolytic cleavage of denatured DNA, yielding acid-soluble oligonucleotides which contain 5′-phosphoryl termini. The rate of hydrolysis of poly(dT) is approximately eightfold greater than that observed with denatured DNA, although the K_m for both substrates is 1.74×10^{-5} M. The relative rates of hydrolysis of homopolymers by the endonuclease are: poly(dG) > poly(dT) > poly(dA) > poly(dC). Purified enzyme preparations also hydrolyze poly(U), releasing acid-soluble products. This activity cosediments in sucrose gradients with the DNA endonuclease activity, suggesting that both activities are contained in the same enzyme molecule.

A recent report describes the purification and properties of a novel endonuclease obtained from calf thymus, DNase V (Wang and Furth, 1977). This enzyme, which possesses a uniquely high isoelectric point (p*I* = 10.3), introduces a limited number of single-strand nicks into native DNA, whereas treatment of denatured DNA yields acid-soluble oligonucleotides. In addition, there is some degree of base specificity, since breaks are preferentially introduced at pyrimidine residues, and homopolymers are hydrolyzed at different rates. In a preliminary study an endonuclease with similar physical properties also was found in KB cells (Wang and Furth, 1977), a continuous human cell line originally derived from a pharyngal carcinoma (Eagle, 1959). Our interest in this latter enzyme relates particularly to the mechanism of DNA replication by small, DNA-containing animal viruses, the adenovirus-associated viruses (AAV). These viruses, which can be readily propagated in KB cells, are defective parvoviruses whose replication depends upon coinfection with a helper adenovirus (Rose, 1974). Previous studies revealed that the single-stranded AAV genome arises via processing of longer, double-stranded concatemeric intermediates (Straus et al., 1976a). Processing of concatemers to unit length molecules implies the action of at least one endonuclease, an activity that could reside in an AAV, adenovirus, or cell-specified protein. The AAV genome, however, has a restricted coding capacity (Rose, 1974), and concatemeric processing also has been found to occur during synthesis of the DNA of parvoviruses not needing a helper adenovirus (Gunther and May, 1976; Rhode, 1977). This suggests that the required endonuclease is of host origin. In the present report we describe the purification of the KB endonuclease, define some of its physical and enzymic properties, and discuss its possible role in AAV DNA replication.

Materials and Methods

Cells and Reagents. KB cells were propagated in suspension culture in Eagle's medium supplemented with 5% heated horse serum. ^{32}P-labeled or ^{3}H-labeled adenovirus 5 DNA and ^{3}H-labeled KB DNA were prepared by methods used previously (Straus et al., 1976b). ^{3}H-labeled fd viral DNA and [^{3}H]poly(dT) were obtained from Miles Laboratories. Homopolymers and oligo(dT) were purchased from P-L Biochemicals. Ampholines were obtained from LKB, acrylamide and *N*,*N*′-methylenebisacrylamide were obtained from Bio-Rad Laboratories, *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) was purchased from Worthington Biochemicals, and polynucleotide kinase was obtained from P-L Biochemicals.

Assay for Endonuclease Activity. The reaction mixture (0.1 mL) contained: 10 mM sodium glycine buffer (pH 9.2), 0.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1.1 nmol of [^{3}H]poly(dT) (specific activity, 8.1×10^6 cpm/μmol) and nuclease. After incubation for 30 min at 37 °C, release of acid-soluble nucleotides was determined by acid precipitability. One unit of enzyme is defined as the amount of enzyme which results in degradation of 1 nmol of [^{3}H]poly(dT) into acid-soluble form in 30 min.

Isoelectric Focusing. The procedures were carried out as previously reported (Wang and Furth, 1977) but with a slight modification. A 110-mL column was employed using a 5–50% sorbitol gradient. The dense gradient solution (40 mL) was composed of 50% sorbitol, 1.6 mL of ampholines (pH 9 to 11), and enzyme fraction, previously dialyzed against 10 mM sodium glycine buffer (pH 9.2) which contained 20% glycerol, 2 mM 2-mercaptoethanol, and 0.1 mM EDTA. The light gradient solution (40 mL) was composed of 5% sorbitol with 0.3 mL of ampholines (pH 9 to 11). The sorbitol gradient was layered on a cathode solution (60% sorbitol, 0.20 N NaOH, pH 12.3), and the remainder of the column was filled with an anode solution (0.01 N acetic acid, pH 3.4).

Sodium Dodecyl Sulfate–Gel Electrophoresis. Electrophoresis was carried out according to Shapiro et al. (1967). Enzyme and protein standards (bovine serum albumin, IGG albumin, α-chymotrypsinogen, cytochrome *c*), each in 50-μL volumes containing 0.01 M sodium phosphate (pH 7.6), 0.1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol, were heated in a boiling water bath for 2 min, adjusted to 10% sucrose, and then applied to a 10% acrylamide gel (5 × 90 mm). Electrophoresis was carried out at 3 mA/gel for 18 h at room temperature. The gels were stained with 0.25% Coomassie blue

† From the Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014 (E.C.W. and J.A.R.), and the Department of Pathology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19174 (J.J.F.). *Received September 9, 1977.*

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TABLE I: Purification of KB Endonuclease.

Fraction	Units[a]	Protein (mg/mL)	Spec act. (units/mg)	Yield (%)	Volume (mL)
Crude extract	3976	16	4.1	100	60
DEAE, PC cellulose	1232	5.6	14.7	31	15
Isoelectric focusing	640	0.5	320	16	4

[a] One unit of enzyme activity is defined as that amount of enzyme which hydrolyzes 1 nmol of [^{3}H]poly(dT) into acid-soluble form in 30 min at 37 °C.

in methanol:acetic acid:water (5:1:5) and destained with 7% acetic acid.

Denaturing Polyacrylamide Electrophoresis. The limit product of digestion of DNA by KB endonuclease was analyzed by polyacrylamide gel electrophoresis containing 7 M urea by the procedure of Maniatis et al. (1975). KB endonuclease digested DNA and marker deoxynucleotides $(dTp)_5$, $(dTp)_{11}$, and $(dTp)_{17}$ and tracking dyes (bromophenol blue and xylene cyanole FF) in 0.2 mL were layered onto a 12% acrylamide TBE gel containing 7 M urea. After electrophoresis, positions of the marker oligonucleotides were determined by scanning the gels at 260 nm, the gels sliced (1-mm thickness), slices digested with H_2O_2, and radioactivity was assayed.

End-Group Analysis. The method of Weiss et al. (1968) was utilized. Calf thymus DNA (30 nmol as nucleotide) was incubated with KB nuclease at 37 °C for 30 min in a 0.1-mL reaction mixture containing 10 mM sodium glycine buffer (pH 9.2), 0.5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. The mixture was then adjusted to 40 mM Tris-HCl (pH 8.0) and incubated for 30 min at 65 °C with 20 μg of alkaline phosphatase (previously heated at 75 °C for 10 min to inactivate contaminating nuclease). Additional alkaline phosphatase (20 μg) was added and incubation continued for 30 min at 37 °C. The reaction mixture was then heated at 100 °C for 5 min, cooled quickly by immersion in ice-water, and adjusted to 1.1 mM sodium phosphate (pH 7.6) containing 10 mM $MgCl_2$ and 15 mM 2-mercaptoethanol. Two nanomoles of [γ-^{32}P]ATP (1.6×10^6 cpm/nmol) and 4 units of polynucleotide kinase were added (final volume 0.225 mL), and the reaction mixture was incubated at 37 °C for 30 min. Additional polynucleotide kinase (4 units) was added and incubation continued for another 30 min. The reaction was terminated by adjusting to 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate. Bovine serum albumin (20 μg) was added and the precipitate collected by centrifugation and dissolved in 0.1 mL of 0.2 M NH_4OH at 0 °C. DNA was reprecipitated with 1 mL of 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate and the precipitate collected by centrifugation and resuspended in 0.2 M NH_4OH. This "wash" procedure was repeated three times and the DNA then dissolved in 0.04 mL of 0.2 M NH_4OH and the radioactivity determined.

Other Analytical Methods. Sucrose gradient sedimentation of protein was performed in a 5 to 20% gradient according to the procedure of Martin and Ames (1961). Protein concentration was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as standard. The protein concentration of the isoelectric focusing fractions of KB endonuclease was determined by the ratio of the absorbance at 280 nm and 260 nm (Warburg and Christian, 1941). Alkaline sucrose gradient sedimentation of DNA was performed in a 10 to 30% sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl, and 1 mM EDTA.

Results

Isolation of Enzyme

Crude Extract. KB cells, wet weight 23 g, were suspended in 46 mL of 20 mM Tris-HCl buffer (pH 8.5) containing 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA and subjected to four 20-s bursts of sonication with a Cell Disruptor Sonifer (Model W 140D) while immersed in an ice-water bath. The sonicate was then centrifuged at 40 000 rpm for 90 min in a Beckman 60 Ti rotor at 5 °C and the supernatant taken as the crude extract. All subsequent procedures also were carried out at 0–5 °C.

DEAE-Cellulose–Phosphocellulose Chromatography. The crude extract (60 mL) was applied to a DEAE-cellulose column (40×2.5 cm^2) in series with a phosphocellulose column (20×2 cm^2). After washing with 400 mL of 20 mM Tris-HCl buffer (pH 8.5), which contained 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA, the two columns were disconnected, and enzyme which had adsorbed to phosphocellulose was eluted with 100 mL of 20 mM Tris-HCl buffer (pH 8.5) containing 0.2 M NaCl, 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA. The enzyme activity was precipitated by 0–60% ammonium sulfate, resuspended in 20 mM Tris-HCl buffer and dialyzed against 10 mM sodium glycine buffer (pH 9.2) containing 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA (DEAE-cellulose–phosphocellulose fraction).

Isoelectric Focusing. The DEAE-cellulose–phosphocellulose fraction (15 mL) was mixed with dense gradient solution as described in Materials and Methods, and electrophoresis was carried out at 200 V for 15 min, 400 V for 15 min, 600 V for 15 min, and 800 V for 68 h. After focusing, water was pumped into the column from the top and 0.8-mL fractions were collected from the bottom of the column. Absorbance at 280 nm, pH, and enzyme activity were then determined for each fraction. Fractions with enzyme activity (isoelectric point at pI = 10.3 ± 0.2) were pooled and dialyzed against 10 mM sodium glycine buffer (pH 9.2) which contained 0.1 M NaCl, 20% glycerol, 2 mM 2-mercaptoethanol, and 0.1 mM EDTA. The enzyme pool was then concentrated for 16 h by dialysis against 10 mM sodium glycine buffer (pH 9.2), containing 50% glycerol, 2 mM 2-mercaptoethanol, and 0.1 mM EDTA (isoelectric focusing fraction).

The enzyme was stable for at least 7 months at −20 or −90 °C. The results of a typical preparation are summarized in Table I. Since the assay is based on degradation of poly(dT) into an acid-soluble form, any deoxyribonuclease(s) present in the crude preparation would falsely enhance the specific activity of the enzyme.

Purity of Enzyme. Although purified enzyme preparations revealed only one protein band on electrophoresis in 0.1% sodium dodecyl sulfate–polyacrylamide gels, these preparations also contained ribonuclease activity as determined by the

TABLE II: Requirement for Enzyme Activity.[a]

Condition	Activity (%)
Complete	100
$-Mg^{2+}$	<1
−2-Mercaptoethanol	98
−2-Mercaptoethanol + *N*-ethylmaleimide (5 mM)	33
$-Mg^{2+} + Mn^{2+}$	<1
$-Mg^{2+} + Ca^{2+}$	<1
$-Mg^{2+} + Cu^{2+}$	<1
$-Mg^{2+} + Hg^{2+}$	<1
$-Mg^{2+} + Zn^{2+}$	<1

[a] Standard assay conditions with 0.2 unit of enzyme were used, except as indicated. The divalent cations were tested from 0.5 mM to 10 mM.

TABLE III: Inhibition of KB Endonuclease Activity by Divalent Cations.[a]

Conditions	Poly(dT) ion concn 0.5 mM	Poly(dT) ion concn 1.0 mM	Denatured DNA ion concn 0.5 mM	Denatured DNA ion concn 0.1 mM
Control	100		100	
Mn^{2+}	8.4	6.5	12.7	6.5
Ca^{2+}	74.7	50.9	52.0	35.1
Hg^{2+}	48.2	33.2	89.2	31.5
Cu^{2+}	56.0	18.0	71.4	46.0
Zn^{2+}	65.2	2.1	74.4	33.7
Fe^{2+}	<1		43.8	47.7

[a] Standard assay conditions were used except that 0.2 unit of enzyme was added with [^{3}H]poly(dT) as substrate and 0.5 unit of enzyme was added with denatured KB [^{3}H]DNA as substrate. Percent of enzyme activity in the presence of 0.5 mM or 1.0 mM concentrations of the indicated cations is tabulated.

formation of 0.16 nmol of acid-soluble material after incubation of 0.2 unit of enzyme with 1.1 nmol of [^{3}H]poly(U) (specific activity, 2.4×10^7 cpm/μmol) for 30 min at 37 °C under standard assay conditions. Thus, the rate of degradation of poly(U) was approximately 80% of that observed with poly(dT) as substrate. In addition, both enzyme activities [i.e., degradation of poly(dT) or poly(U)] cosedimented in a 5–20% sucrose gradient, and a 40% loss of both activities occurred after heating the enzyme fraction at 50 °C for 5 min. These results suggest that a single enzyme molecule may contain DNase and RNase activities, although we still cannot exclude the possibility of contamination by an exogenous RNase activity. It is not likely that the enzyme attacks DNA exonucleolytically since (a) it degraded single-strand, circular fd DNA (data not shown), (b) the product of a limit digest of denatured adenovirus DNA was larger than a tetranucleotide (Figure 4), and (c) with a small amount of enzyme and a short incubation period, acid-soluble material was not released from poly(dT) or denatured DNA.

Physical Properties and Requirements

Physical Properties. The sedimentation coefficient of the KB endonuclease was 4.6 S as estimated by sucrose gradient sedimentation with and without an internal bovine serum albumin marker. As already noted, electrophoresis of purified enzyme preparations in sodium dodecyl sulfate–polyacrylamide gels yielded only a single protein band. When the distance of migration of the protein band in the gel is plotted relative to the distance of migration of internal marker proteins, the purified enzyme had an estimated molecular weight of 54 000.

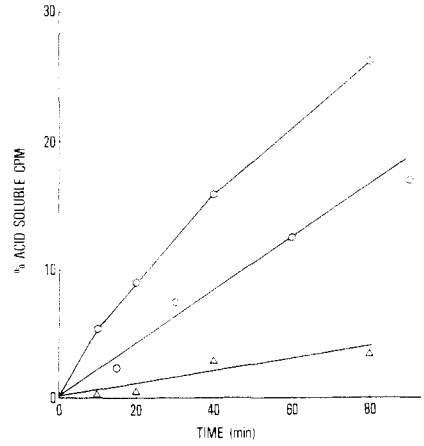

FIGURE 1: Rate of degradation of poly(dT) and denatured KB DNA by KB endonuclease. Standard assay conditions were used except that the reaction mixtures (0.6 mL) containing 1 unit of enzyme were incubated for different periods of time. Samples (0.1 mL) were removed at the indicated times. Poly(dT) (○—○) as substrate; denatured DNA (△—△) as substrate; denatured DNA (○—○) as substrate with three times more enzyme added.

Requirement for the Enzyme Activity. The endonuclease has a pH optimum at 9.2 with either poly(dT) or denatured DNA as substrate. Activities with poly(dT) as substrate at pH 4.5, 5.5, 6.5, 7.5, 8.0, 8.5, 9.5, 10.0, and 10.5 with citric-phosphate (pH 4.5–7.5), Tris-HCl (pH 7.0–9.0), and sodium glycine (pH 8.5–10.5) buffers were 12.3, 20.8, 38.3, 70.2, 79.9, 89.0, 94.2, 73.4, and 43.3%, respectively, of that observed at the optimum pH.

As shown in Table II, less than 1% activity was observed in the absence of Mg^{2+} ion. Maximal activity was observed at a Mg^{2+} concentration of 0.5 mM and was independent of substrate concentration. Activities at 0.2, 1, 2, 5, 10, and 20 mM Mg^{2+} were 88.3, 92, 58.9, 36.4, 25.4, and 9.4%, respectively, of that observed at 0.5 mM. No activity was detected when Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} from 0.5 mM to 10 mM were used instead of Mg^{2+}. The addition of 5 mM *N*-ethylmaleimide with 2-mercaptoethanol omitted resulted in a 67% reduction of hydrolysis, suggesting that a sulfhydryl group is required for enzyme activity. In addition, NaCl was found to inhibit the ability of enzyme to degrade the poly(dT). At 10, 20, 30, 40, and 50 mM NaCl about 82, 59, 44, 32, and 25%, respectively, of control activity remained. Unlike pancreatic DNase I, the NaCl inhibition could not be reversed by further addition of Mg^{2+}. The divalent metal ions, Mn^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, and Fe^{2+}, are not only excluded as cofactors for enzyme activity, but also can be shown to inhibit the formation of acid-soluble nucleotides resulting from enzyme degradation of either poly(dT) or denatured KB DNA (Table III). Mn^{2+} was more inhibitory than the other divalent ions, and this inhibition was similar with each substrate. Though exhibiting less inhibition, Cu^{2+}, Zn^{2+}, and Fe^{2+} were relatively more inhibitory with poly(dT) as substrate than with DNA as substrate. When the kinetics of inhibition by these ions with poly(dT) as substrate was analyzed, Mn^{2+}, Hg^{2+}, and Ca^{2+} were found to be competitive inhibitors of enzyme activity, whereas Zn^{2+}, Fe^{2+}, and Cu^{2+} acted noncompetitively (E. C. Wang and J. A. Rose, unpublished results).

Mechanism of Reaction

Kinetic Studies of Enzyme Activity. The kinetics of enzyme activity on poly(dT) and denatured DNA revealed that the K_m

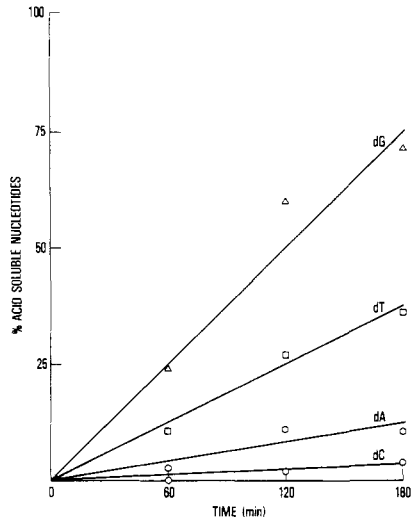

FIGURE 2: Rate of hydrolysis of homopolymers by KB endonuclease. Standard assay conditions were used, except that 0.5-mL reaction mixtures were incubated with 3 units of enzyme activity. After incubation at 37 °C at the indicated times, samples (0.1 mL) were removed and the release of acid-soluble nucleotides was determined spectrophotometrically. Poly(dG) (Δ—Δ); poly(dT) (□—□); poly(dA) (○—○); and poly(dC) (○—○).

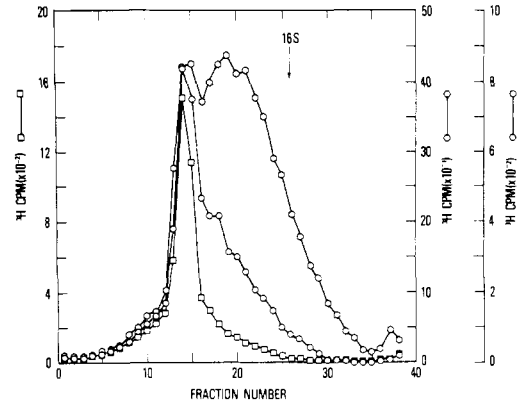

FIGURE 3: Alkaline sucrose density gradient centrifugation of KB endonuclease-treated native adenovirus DNA. Standard assay conditions, except different amounts of KB endonuclease and NaCl were added as indicated and incubated with 1.3 nmol of adenovirus [^{3}H]DNA (specific activity, 6×10^6 cpm/μmol). After 30 min at 37 °C, 30 μL of 0.2 M EDTA was added and the reaction mixtures were centrifuged in 11.5 mL of a 10-30% alkaline sucrose gradient at 30 000 rpm (Beckman SW41 rotor) for 16 h at 5 °C. Fractions (0.3 mL) were collected and radioactivities assayed. (□—□) Control experiment; (○—○) 0.16 unit of enzyme added; (○—○) 0.8 unit of enzyme and 50 mM NaCl were added. The arrow indicates the position of marker AAV [^{14}C]DNA (16S) sedimented in a parallel gradient.

for both substrates is 1.74×10^{-5} M, and the V_{max} is 0.53 nmol/30 min for poly(dT) and 0.26 nmol/30 min for denatured DNA. In the later case, however, there was a fourfold greater enzyme input into the reaction mixture. We, therefore, conclude that, although the enzyme has the same affinity for both poly(dT) and denatured DNA, poly(dT) is degraded about eightfold faster than denatured DNA. When the rates of hydrolysis of poly(dT) and denatured DNA were determined by measuring the release of acid-soluble material from ^{3}H-labeled substrate (Figure 1), enzyme activity with poly(dT) was seven- to eightfold that observed with denatured DNA. This finding is in agreement with the result of the kinetic study. A comparison of the rates of hydrolysis of four homopolymers is shown in Figure 2. The relative rates of homopolymer hydrolysis are: poly(dG) > poly(dT) > poly(dA) > poly(dC). According to these measurements, the rate of degradation of denatured DNA lies between those of poly(dA) and poly(dC). These results suggest that the enzyme exhibits some specificity in the degradation of different nucleotide bonds.

Single-Strand Specificity. Although the enzyme degrades native DNA as indicated by alkaline sucrose gradient analysis, it is possible that cleavage might be due to exposure of single-stranded regions as a result of partial denaturation during incubation. To stabilize the native DNA structure (i.e., to enhance double strandedness), the reaction mixture was adjusted to 50 mM NaCl and fivefold more enzyme added. Correcting for NaCl inhibition by addition of more enzyme and based on the release of acid-soluble oligonucleotides with either poly(dT) or denatured DNA as substrate, there was about 1.7 times more enzyme activity than in the control experiment in which NaCl was absent. As shown in Figure 3, the added NaCl and increased enzyme resulted in far less degradation than that observed under control conditions. This result suggests that the enzyme is a single-strand, polynucleotide-specific nuclease.

Products of Hydrolysis of Denatured DNA. A maximum of 70 to 75% of single-stranded circular fd viral DNA or denatured adenovirus DNA (17 nmol) was rendered acid-soluble by the enzyme (3.2 units/0.1 mL) when incubations were continued for longer periods of time (up to 5 h). Since 90% of

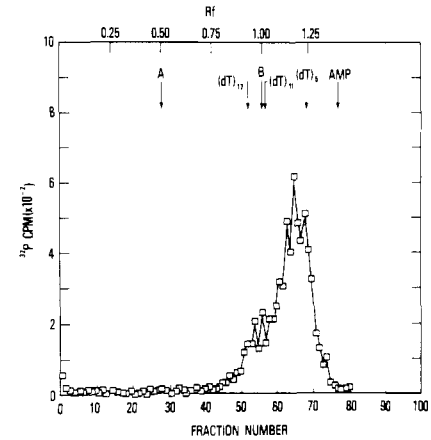

FIGURE 4: Denaturing polyacrylamide gel electrophoresis of the limit product produced by KB endonuclease digestion of denatured adenovirus [^{32}P]DNA. Standard assay mixtures (0.4 mL), containing 1.5 μg of denatured adenovirus [^{32}P]DNA (specific activity, 50 000 cpm/μg) and 6.7 units of enzyme, were incubated at 37 °C for 6 h. Portions (0.1 mL) were removed and marker deoxynucleotides [AMP, $(dTp)_5$, $(dTp)_{11}$, and $(dTp)_{17}$], tracking dye (bromophenol blue and xylene cyanole FF) and sucrose were added. The mixtures were then applied to denaturing polyacrylamide gels (12%) as described in Materials and Methods. Arrows indicate the marker deoxynucleotides and tracking dyes [(A) bromophenol blue; (B) xylene cyanole FF)].

the preparation of fd viral DNA did not have any free ends, the enzyme must catalyze an endonucleolytic cleavage of DNA.

The size of the product of hydrolysis of denatured adenovirus [^{32}P]DNA was analyzed by electrophoresis in a denaturing gel (Figure 4). No detectable mononucleotide was formed, very little, if any, dinucleotide was formed and most of the radioactively labeled oligonucleotides were longer than tetranucleotides. Similar results were obtained when incubations were continued for 18 h.

End-Group Analysis. To determine what end group exists at the 5′ termini following hydrolysis, denatured DNA, after degradation by the enzyme, was incubated with or without alkaline phosphatase. The 5′ ends were then incubated with

TABLE IV: End-Group Analysis.[a]

KB endonuclease	Alkaline phosphate	Polynucleotide kinase	^{32}P incorp (pmol)
+	+	+	21.6
−	+	+	15.3
+	−	+	5.9

[a] Denatured calf thymus DNA as substrate.

[γ-^{32}P]ATP and polynucleotide kinase. With alkaline phosphatase treatment, a significant increase in the amount of ^{32}P incorporated into DNA was observed (Table IV). These results indicate that the enzyme produces 5′-phosphoryl termini.

Discussion

Several deoxyribonucleases, both endonucleases and exonucleases, have been purified from mammalian cells. DNase I is an endonuclease that generates 5′-phosphoryl termini, has a neutral pH optimum, produces "nicks" on one strand in preference to scission of both strands (Zamenhof et al., 1954; Young and Sinsheimer, 1965) and, when acting on native DNA, exhibits autoretardation (Laskowski, 1967). It has a molecular weight of 31 000 (Lindberg, 1967). DNase II also is an endonuclease but has an acid pH optimum (pH 4.6). It shows a preference for native DNA over denatured DNA, generates 3′-phosphoryl termini and has a molecular weight of 38 000 (Laskowski, 1967; Bernardi, 1968). DNase III is an exonuclease with a pH optimum of 8.5. It hydrolyzes denatured DNA, poly(dT), and poly(dA) at approximately the same rate, whereas native DNA is hydrolyzed at about one-fourth the rate of denatured DNA. This enzyme has a molecular weight of 52 000 (Lindahl et al., 1969b). DNase IV, another exonuclease, specifically degrades double-stranded DNA from the 5′-terminal end and also shows optimal activity at pH 8.5. Its molecular weight is 42 000 (Lindahl et al., 1969a). DNase V was isolated from calf thymus and has a uniquely high isoelectric point. It introduces a limited number of single-strand nicks into native DNA but extensively hydrolyzes denatured DNA, yielding acid-soluble oligonucleotides. The molecular weight of this enzyme is 53 000 (Wang and Furth, 1977). Finally, an endonuclease isolated from adenovirus-infected KB cells or uninfected KB cells has recently been reported (Reif et al., 1977). This enzyme has its maximal activity at pH 4.0, and, in crude extracts, can be activated or enhanced by treatment of the extracts with proteolytic enzymes such as Pronase or trypsin. The KB cell endonuclease we describe in the present study differs from the pH 4.0 KB endonuclease on the basis of pH optima (other characteristics of the pH 4.0 KB endonuclease have not yet been reported). Although the KB endonuclease we have characterized shares certain similarities with calf thymus DNase V, it also possesses some distinctively different physical, chemical, and enzymatic properties. The enzymes are similar with respect to chromatographic properties, uniquely high isoelectric point, requirement of Mg^{2+} for activity, and a molecular weight of 53 000 to 54 000. Both enzymes degrade poly(dT) much faster than denatured DNA, and the products of digestion of denatured DNA are mostly larger than tetra- or pentanucleotides. Furthermore, both enzymes generate 5′-phosphoryl ends. On the other hand, the pH optimum of the KB enzyme is 9.2 as compared with 6.6 for calf thymus DNase V, and their respective Mg^{2+} optima are 0.5 mM and 5 mM. Mn^{2+} is not an effective cofactor for the KB endonuclease, whereas it is effective for calf thymus DNase V. The sedimentation coefficient of the KB endonuclease is 4.6 S but is 3.9 S for calf thymus DNase V, and it appears that the KB enzyme is composed of a single polypeptide, whereas the calf thymus enzyme is composed of four subunits. Finally, the relative rates of solubilization of homopolymers by each enzyme are different. The rates of hydrolytic activity with KB enzyme are: poly(dG) > poly(dT) > poly(dA) > poly(dC), whereas the rates of activity for the calf thymus enzyme are: poly(dT) > poly(dA) > poly(dC) > poly(dG).

More relevant to the presently described KB endonuclease are the recently reported single-stranded DNA specific endonucleases isolated from mouse cells (Otto and Knippers, 1976) and an established line of human cells (EUE cells) (Pedrini et al., 1976). The endonuclease isolated from mouse cells distinctly differs from KB endonuclease in several respects: (1) the mouse cell enzyme has a higher molecular weight (70 000) as determined by both sucrose gradient centrifugation and sodium dodecyl sulfate gel electrophoresis; (2) its pH optimum is near 7 as compared with 9.2 for the KB enzyme; (3) one-third of activity remains when Ca^{2+} is substituted for Mg^{2+} as a cofactor for mouse endonuclease, whereas no detectable activity is obtained with Ca^{2+} as a cofactor for KB endonuclease; and (4) there is no detectable RNase activity associated with the mouse endonuclease, but the KB enzyme degrades poly(U) as well as ribosomal RNA isolated from HeLa cells (Wang and Rose, unpublished results). On the other hand, the endonuclease characterized by Pedrini et al. (1976) is clearly more similar to the KB endonuclease (pH optimum, Mg^{2+} requirement, monovalent salt inhibition, affinity constant, K_m, substrate specificity and 5′-end group generated). These enzymes, however, appear to differ with respect to certain other properties: (1) the molecular weight of the EUE enzyme is smaller (45 000 as measured on Sephadex G-100), and sucrose gradient sedimentation yielded sedimentation coefficients of 3.6 and 4.6 S for the EUE and KB endonuclease, respectively; (2) no significant difference in EUE endonuclease activity could be detected at Mg^{2+} concentrations ranging from 0.5 and 2 mM, but KB endonuclease activity is nearly halved at 2 mM Mg^{2+}; furthermore, while 30% of EUE enzyme activity is obtained with Mn^{2+}, this ion was not an effective cofactor for the KB enzyme; (3) omission of the sulfhydryl reagent resulted in a 55% decrease in EUE enzyme activity, but a similar omission produced no significant change in activity with the KB enzyme; additionally, a sulfhydryl blocking agent completely inhibited EUE endonuclease activity, but only a partial inhibition (67%) was achieved with KB endonuclease; (4) although the natural DNA substrates employed were obtained from different sources, the EUE enzyme completely hydrolyzed denatured DNA into acid-soluble form, whereas the KB enzyme yielded a maximal hydrolysis of 70–75%; (5) the rate of poly(dT) degradation vs. denatured DNA was about twofold faster with EUE endonuclease than with KB endonuclease; and (6) the limit digestion product with the EUE enzyme contained 5% mononucleotides and 65% di- and tetranucleotides whereas the KB enzyme generated no detectable mononucleotides, and over 90% of the product was larger than tetranucleotides. Finally, we have observed, in KB cells, at least three other DNases with high pH optima and a requirement for Mg^{2+}. They also degrade denatured DNA much faster than native DNA (Wang and Rose, unpublished results), and it might be that the EUE endonuclease could correspond to one of these enzymes.

The biological function of the KB endonuclease is not known. Recent experiments, however, have shown that the KB endonuclease activity is not enhanced with DNA synthesis in synchronized KB cells (E. C. Wang and J. A. Rose, unpub-

lished results). This observation suggests that the enzyme may not be involved in cellular DNA synthesis, but there is still a possibility that it might play a role in AAV DNA replication. For example, the enzyme could act in conjunction with AAV structural or nonstructural polypeptides to promote endonucleolytic cleavages at specific sites. Although we have obtained no evidence to indicate that AAV-coded polypeptides possess endonucleolytic activity (R. M. L. Buller and J. A. Rose, unpublished results), current studies have demonstrated that AAV DNA processing is at least 80% reduced in the absence of demonstrable AAV protein synthesis (E. Sebring and J. A. Rose, unpublished results). Additionally, it should be noted that when purified unit length AAV DNA hairpins (Straus et al., 1976a) were incubated with the KB endonuclease in 50 mM NaCl, approximately half the hairpins were converted to molecules sedimenting as unit length monomers in an alkaline sucrose gradient (E. C. Wang and J. A. Rose, unpublished results). Most likely, the endonuclease cleaved the single-stranded hairpin loop, a result which would not be surprising in view of the observed single strand specificity of the enzyme (Figure 3). At present, we are continuing to characterize this as well as additional endonucleases we have now obtained from uninfected, adenovirus-infected, and adenovirus plus AAV-coinfected KB cells.

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