Deoxyribonucleic Acid Single-Strand-Specific Endonucleases in Human Cells: Partial Purification of a Salt-Resistant Endonuclease with an Acidic Isoelectric Point†

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ABSTRACT: A second endonuclease with DNA single-strand specificity has been purified from KB cells, a continuous line of human epithelial cells. In contrast to other mammalian enzymes that cleave single-stranded DNA, this enzyme has an acidic isoelectric point (6.4 ± 0.2). Its pH optimum is 9.5, it requires Mg^{2+} or Mn^{2+} for activity, and it has a sedimentation coefficient of 3.2 S, based on sucrose gradient centrifugation. The enzyme specifically catalyzes the endonucleolytic cleavage of synthetic DNA homopolymers and denatured viral DNA but does not attack linear duplex viral DNA. The rate of hydrolysis of poly(dT) is approximately 8-fold greater than that observed with denatured DNA. The relative rates of hydrolysis of homopolymers by the endonuclease are poly(dA) > poly(dT) > poly(dC) > poly(dG). Unlike other DNA single-strand-specific endonulceases isolated from human cells, this endonuclease is relatively insensitive to inhibition by KCl.

Recent reports have described two endonucleases isolated from calf thymus and KB cells that specifically cleave single-stranded DNA (Wang et al., 1975, 1978; Wang & Furth, 1977). These enzymes were shown to possess some degree of specificity in their attack on different nucleotide bonds as well as a uniquely high isoelectric point (p*I* = 10.3). Although they can degrade duplex DNA under certain conditions, this degradation is apparently due to exposure of single-stranded regions as a result of partial denaturation (Wang et al., 1978). In the present study, we describe the partial purification and characterization of a second endonuclease obtained from KB cells (KB_2 endonuclease). Although this enzyme, like those previously found, specifically hydrolyzes single-stranded DNA and exhibits some specificity for different nucleotide bonds, there are several important differences with respect to the other enzymes. These include (i) an acidic isoelectric point (p*I* = 6.4), (ii) inability to cleave double-stranded DNA when KCl is omitted from the standard assay mixtures, and (iii) lack of inhibition by 40 mM KCl.

Materials and Methods

Cells and Reagents. KB cells were propagated as described before (Wang et al., 1978). ^{3}H-Labeled single-stranded fd virus DNA (3000 cpm/μg), [^{3}H]poly(dT) (20000 cpm/μg), and [^{3}H]poly(U) (20000 cpm/μg) were obtained from Miles Laboratories. [^{3}H]Adenovirus 5 DNA (32000 cpm/μg) was prepared by methods used previously (Straus et al., 1976). [^{3}H]SV40 component I DNA (3200 cpm/μg) was obtained from N. Chiu, and [^{14}C]φX174 RF DNA (3200 cpm/μg) was provided by L. Salzman. Homopolymers were purchased from P-L Biochemicals, and ampholines were obtained from LKB Instruments, Inc.

Assay for Endonuclease Activity. The reaction mixture (0.1 mL) contained 20 mM sodium glycine buffer (pH 9.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 30 mM KCl, 1.8 μg of [^{3}H]poly(dT), and nuclease. After incubation for 60 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 required to degrade 0.1 μg of [^{3}H]poly(dT) into acid-soluble form in 60 min.

Other Analytic Methods. Isoelectric focusing was carried out as previously reported (Wang & Furth, 1977; Wang et al., 1978), except that a 5–50% glycerol gradient and pH 5–7 ampholines were used. The cathode solution was 0.2 N NaOH, and the anode solution was 0.68% H_3PO_4. Sucrose gradient sedimentation of protein was performed in a 5–20% gradient (Martin & Ames, 1961), and alkaline sucrose gradient sedimentation of DNA was carried out in a 10–30% sucrose gradient that contained 0.3 M NaOH, 0.7 M NaCl, and 1 mM EDTA. Protein concentrations were estimated by the ratio of the absorbance at 280 and 260 nm (Warburg & Christian, 1941).

Results

Isolation of Enzyme

Crude Extract. KB cells grown in Eagle's medium (5×10^5 cells/mL) were collected by centrifugation at 600*g* for 10 min. The cell pellet (2-mL volume) was resuspended in 1 mL of medium plus 3 mL of 50 mM sodium glycine buffer (pH 9.2) containing 20% glycerol, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, and 1 M NaCl and subjected to four 20-s bursts of sonication with a cell Disruptor Sonifer (Model W140D) while immersed in an ice–water bath. The extract was then centrifuged at 40000 rpm for 2 h at 5 °C in a Beckman SW50.1 rotor, and the supernatant was taken as the crude extract. All subsequent procedures were carried out at 0–5 °C.

DEAE-cellulose Chromatography. The crude extract (5 mL) was dialyzed into a 25 mM sodium glycine buffer (pH 9.2) containing 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA for 16 h and then applied to a DEAE-cellulose (DE-52) column (20×1.5 cm^2). After the column was washed with 10 mL of dialysis buffer, the enzyme was eluted with a linear gradient of 10–500 mM NaCl, 100 mL each, in 50 mM sodium glycine buffer (pH 9.2) containing 20% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA. The material containing activity, which eluted at 90 ± 20 mM NaCl, was collected by ammonium sulfate precipitation (0–60%) and resuspended in a 50 mM sodium glycine buffer (pH 9.2) containing 20% glycerol, 50 mM NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA (DEAE-cellulose fraction).

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Table I: Purification of KB_2 Endonuclease

fraction	units	protein (mg/mL)	spec act. (units/mL)	yield (%)	volume (mL)
crude extract	3925	35.5	22.1	100.0	5.0
DEAE-cellulose	2582	17.6	56.4	65.8	2.6
isoelectric focusing	1922	2.1	339.9	49.0	2.7

Isoelectric Focusing. The DEAE-cellulose fraction (2.6 mL) was mixed with dense glycerol solution, and electrophoresis was carried out at 400 V for 15 min and 800 V for 47 h. After focusing, a single band of aggregated protein was visible. This band contained all the enzyme activity and had an isoelectric point of 6.4 ± 0.2. Fractions (0.5 mL of each) that contained enzyme activity were pooled and then dialyzed into 50 mM sodium glycine buffer (pH 9.2) containing 50% glycerol, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, and 50 mM NaCl (isoelectric focusing fraction). The results of a typical preparation are summarized in Table I. The actual purification achieved may be greater than indicated because the crude extract was obtained following an initial fractionation step (i.e., it represents a high-speed supernatant fraction).

Purity and Physical Properties of Enzyme. Enzyme preparations also contained ribonuclease activity as determined by the formation of 0.19 and 0.33 μg of acid-soluble material after incubation of 3.6 and 7.2 units of enzyme, respectively, with 2 μg of [^{3}H]poly(U) for 60 min at 37 °C. This latter activity is about one-third as active as that which degrades [^{3}H]poly(dT). However, both enzyme activities [i.e., degradation of poly(dT) or poly(U)] sedimented separately in 5–20% sucrose gradients. The sedimentation coefficient of the enzyme that degraded poly(dT) was 3.2 S whereas that of the poly(U)-degrading enzyme was 5.7 S. Poly(dT) enzyme activity was completely eliminated by incubation at 65 °C for 5 min.

Requirements for Activity. The pH of the reaction mixtures (adjusted with Tris-HCl) did not greatly affect the enzyme activity over a wide range. Activities at pH 8.0, 8.5, 9.0, 9.5, 10.0, and 10.5 were 89%, 94%, 97%, 100%, 95%, and 67%, respectively.

In the absence of Mg^{2+} ion, less than 3% activity was observed. Maximal activity occurred at a Mg^{2+} concentration of 5 mM. Activities at 1, 2, 10, and 20 mM Mg^{2+} were 90%, 96%, 95%, and 56% of that observed at 5 mM Mg^{2+}. Mn^{2+} at a concentration of 5 mM sustained an activity that was about 44% of that observed at the optimal Mg^{2+} concentration. Less than 3% of the activity was detected when 5 mM concentrations of other divalent ions (Hg^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+}) were used instead of Mg^{2+}. The omission of 2-mercaptoethanol resulted in a 65% reduction of hydrolysis, suggesting that a sulfhydryl group is required for enzyme activity. In addition, KCl had no significant effect on enzyme activity over a range of concentration from 5 to 40 mM; in each instance, enzyme activity was greater than 98% of that observed when KCl was absent in the reaction mixture.

Rates of Hydrolysis of Synthetic and Viral DNA. The rates of hydrolysis of poly(dT) and adenovirus DNA were determined by measuring the release of acid-soluble material from ^{3}H-labeled substrates (Figure 1). Enzyme activity with poly(dT) was 7- to 8-fold that observed with denatured viral DNA. The enzyme did not degrade native adenovirus DNA into acid-soluble form, even when KCl was omitted from the reaction mixture. In addition, as assayed by alkaline sucrose gradient sedimentation, the enzyme probably produced little or no nicking of the closed, circular double-stranded molecules (53 S) of SV40 component I DNA and φX174 RF DNA (Figure 2A–C). On the other hand, the enzyme clearly

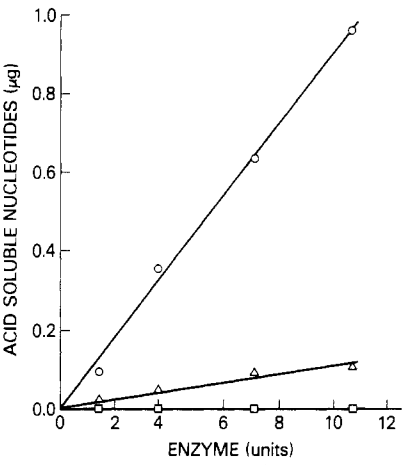

FIGURE 1: Rate of degradation of poly(dT) and denatured and duplex adenovirus DNA by KB_2 endonuclease. Standard assay conditions were used except that different amounts of enzyme and 2 μg of [^{3}H]adenovirus DNA were added as indicated. Poly(dT) as substrate (○); denatured DNA as substrate (△); native DNA as substrate (□).

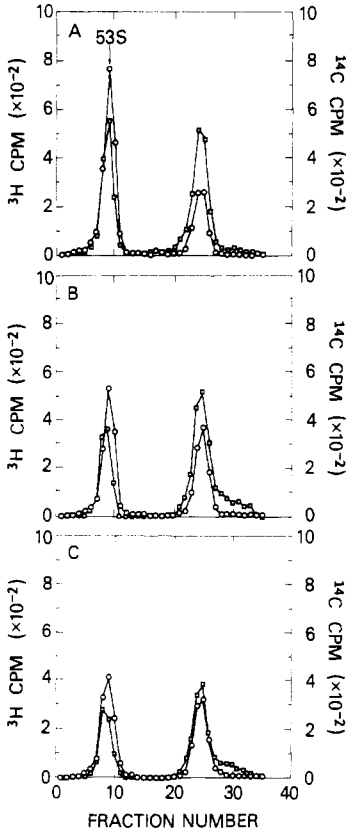

FIGURE 2: Endonuclease reactivity with SV40 and φX174 RF DNA. Standard assay conditions were used, except [^{3}H]SV40 DNA and [^{14}C]φX174 DNA were mixed together and different amounts of enzyme were added as indicated. The reaction was stopped with EDTA (0.06 M). Alkaline sucrose gradients were run at 50 000 rpm for 2 h at 25 °C in a Beckman SW50.1 rotor. (A) control; (B) 1 unit of enzyme; (C) 2 units of enzyme. φX174 DNA (□); SV40 DNA (○).

yielded an endonucleotic pattern of hydrolysis with closed, circular single-stranded fd virus DNA (Figure 3D–F) as well as the linear strands of alkali-denatured adenovirus DNA (not shown).

Analysis of the enzyme degradation product of poly(dT) in alkaline sucrose gradients revealed that the mean size of poly(dT) (initally 10.1 S) was decreased to 7.1 S and 5.7 S with 1 and 2 units of enzyme, respectively, during incubations

Table II: Comparison of Single-Strand-Specific Endonucleases

properties	enzymes			
	calf thymus[a]	EUE[b]	KB_1[c]	KB_2
physical and chemical properties				
isoelectric point	10.3		10.3	6.4
sedimentation coefficient	3.9	3.6	4.6	3.2
optimum pH	6.6	9.5	9.2	9.5
optimum Mg^{2+} (mM)	5.0	1.0	0.5	5.0
other cofactor	Mn^{2+}	Mn^{2+}	none	Mn^{2+}
salt inhibition	yes	yes	yes	no
sufhydryl group	yes	yes	yes	yes
catalytic properties				
hydrolysis rate: poly(dT)/denatured DNA	5	15	7–8	7–8
reactivity with native DNA	yes	yes	yes	no
relative rates of hydrolysis of homopolymers	dT > dA > dC > dG		dG > dT > dA > dC	dA > dT > dC > dG

[a] Wang & Furth (1977). [b] Pedrini et al. (1976). [c] Wang et al. (1978).

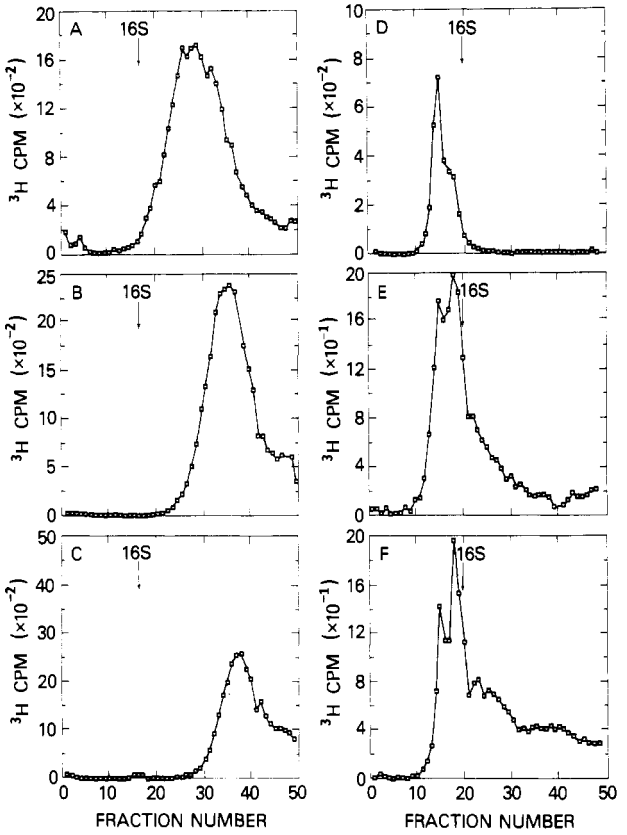

FIGURE 3: Endonuclease degradation of poly(dT) and fd virus DNA. For poly(dT), standard assay conditions were used, except different amounts of enzyme were added as indicated. Reactions were stopped with EDTA (0.06 M). Alkaline sucrose gradient sedimentation was performed as described under Materials and Methods, and gradients were run at 40 000 rpm for 18 h at 15 °C in a Beckman SW41 rotor. (A) Control. (B) 1 unit of enzyme. (C) 2 units of enzyme. For single-stranded circular fd viral DNA, standard assay conditions were used, except that [^{3}H]fd DNA and 1 unit of enzyme were added and the incubation time was varied as indicated. Reactions were stopped with EDTA (0.06 M). Alkaline sucrose gradients were run at 37 000 rpm for 19 h at 10 °C in the SW41 rotor. (D) Control. (E) 30 min. (F) 60 min. [^{14}C]AAV DNA was used as the marker.

at 37 °C for 60 min (Figure 3A–C). When additional enzyme was then added, more radioactivity shifted toward the top of the gradient. However, during the process of enzyme degradation, a distinct deoxyribonucleotide component did not accumulate at the top of these gradients. Taken together with viral DNA hydrolysis data (above), these results are consistent with the conclusion that the enzyme is an endonuclease and that enzyme preparations do not contain significant exonuclease activity.

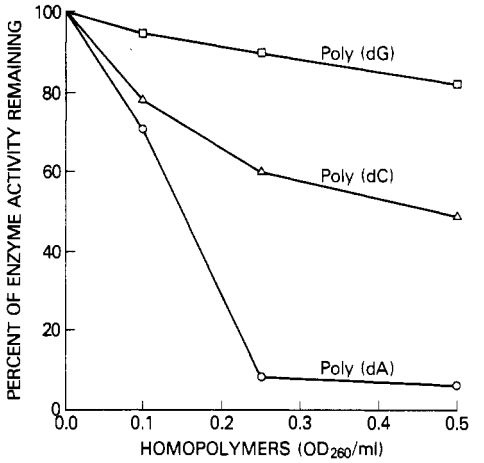

FIGURE 4: Inhibition of KB_2 endonuclease degradation of poly(dT) by other homopolymers. Standard assay conditions were used except that the homopolymers were added as indicated. Poly(dA) additions (○); poly(dC) additions (△); poly(dG) additions (□).

Not only does the enzyme degrade poly(dT), but it also degrades other homopolynucleotides into acid-soluble form. The slopes of the hydrolysis curves of four homopolymers are 120, 97, 39, and 31 ng/min for poly(dA), poly(dT), poly(dC) and poly(dG) (20 μg of each homopolymer and 35.6 units of enzyme in 5-mL volumes of reaction mixture). Moreover, in agreement with these results, differential inhibition of [^{3}H]-poly(dT) degradation by the other homopolymers can be readily demonstrated (Figure 4). The relative rates of reduction of degradation of poly(dT) by these homopolymers were poly(dA) > poly(dC) > poly(dG). These results indicate that the enzyme exhibits some specificity in the cleavage of different nucleotide bonds.

Discussion

Relatively few deoxyribonucleases with specificity for single-stranded DNA have been purified from mammalian cells (Wang et al., 1975, 1978; Pedrini et al., 1976; Wang & Furth, 1977). Although the presently described activity, KB_2 endonuclease, has not been completely purified, there is little doubt that it represents a single-strand-specific endonuclease that is distinct from those reported previously. A comparison of all four enzymes (Table II) reveals a common requirement for Mg^{2+} and a sulfhydryl reagent and all four more actively degrade poly(dT) than denatured duplex DNA. On the other hand, KB_2 endonuclease has an acidic isoelectric point (pI = 6.4) whereas the calf thymus (Wang et al., 1975; Wang & Furth, 1977) and KB_1 (Wang et al., 1978) enzymes have a uniquely high isoelectric point (pI = 10.3). Except for the calf thymus enzyme, which has a pH optimum at 6.6, all the

enzymes are most active at a relatively high pH (approximately 9.5). Although all four enzymes require Mg^{2+}, the optimum concentration for the KB_2 and calf thymus enzymes (5 mM) was greater than observed for either the KB_1 or EUE enzymes (0.5 and 1 mM). Furthermore, except for the KB_1 enzyme, all the other enzymes can utilize Mn^{2+} in place of Mg^{2+}. Additional distinctions among these enzymes are indicated by their different sedimentation coefficients and differences in relative rates of hydrolysis with poly(dT) as compared to denatured DNA. With regard to this latter feature, the two KB enzymes exhibited an intermediate ratio [about 7- to 8-fold greater degradation with poly(dT) as substrate] whereas the EUE enzyme had the highest ratio (15-fold) and the calf thymus enzyme the lowest degradation ratio (5-fold). Also, as shown in Table II, the relative rates of hydrolysis of homopolymers are clearly different among the calf thymus, KB_1, and KB_2 enzymes. A similar comparison was not included in the EUE enzyme study (Pedrini et al., 1976). Finally, it should be noted that there are two properties of the KB_2 enzyme that particularly distinguish it from the other three endonucleases: (i) degradation of linear double-stranded DNA does not occur with KB_2 endonuclease when KCl is omitted from the standard assay mixture whereas it does occur under similar conditions with each of the other enzymes and (ii) degradation of poly(dT) by the KB_2 enzyme is not inhibited by an increased salt concentration (up to 40 mM KCl), but similar increases in salt concentration markedly inhibit the degradation of poly(dT) by all the other enzymes.

Further questions relevant to KB_2 endonuclease specificity can be raised on the basis of several observations. First, alkaline sucrose gradient patterns suggest that the enzyme may have randomly nicked the single-stranded circular fd virus genome, yielding linear genome length molecules (Figure 3E,F; major slower sedimenting component). Alternatively, however, this cleavage could have been relatively site specific, a feature that might prove useful in the fractionation of certain single-stranded DNA molecules. An analysis of limit digestion products will be required to evaluate these possibilities. Secondly, unlike KB_1 endonuclease whose molecules appear to degrade both poly(dT) and poly(U) (Wang et al., 1978), KB_2 endonuclease can be readily purified away from any poly(U) degrading activity. Perhaps this difference relates to a difference in functions carried out by the two enzymes. Additional assessment to determine whether KB_2 endonuclease possesses any ribonucleolytic activity may thus be worthwhile.

In addition to the KB_1 and KB_2 endonucleases, we have observed that KB cells contain other endonucleases that hydrolyze poly(dT) more rapidly than denatured DNA. While little can be said at present concerning the specific cellular role of any of the endonucleases, there is a possibility that one or more of these enzymes may be involved in the replication of viral DNA (Wang et al., 1978).

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Inhibition of Double-Stranded Ribonucleic Acid Activated Protein Kinase and 2′,5′-Oligo(adenylic acid) Polymerase by Ethidium Bromide†

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ABSTRACT: The activation of two enzymes induced by interferon, a protein kinase and the 2′,5′-oligo(adenylic acid) polymerase [2′,5′-oligo(A) polymerase], is inhibited by ethidium bromide. The activation of these enzymes requires double-stranded RNA (dsRNA), and binding of ethidium to dsRNA inhibits the activation process. This was shown by determining the concentration of ethidium inhibitory for poly(A)·poly(U)- and poly(I)·poly(C)-activated reactions. Activation of both protein kinase and 2′,5′-oligo(A) polymerase is inhibited by much lower concentrations of ethidium with the former polymer as activator than with the latter polymer. Correspondingly, in the presence of magnesium, ethidium binds with much greater affinity to poly(A)·poly(U) than to poly(I)·poly(C). Synthesis of 2′,5′-oligo(A) with poly(A)·poly(U) as activator is arrested by adding low ethidium concentrations, but it is resumed upon addition of poly(I)·poly(C). Kinase activity, however, is not inhibited when ethidium is added after the activating dsRNA. This suggests that the kinase interacts with dsRNA in a manner different from the 2′,5′-oligo(A) polymerase interaction.

Two enzymatic activities are induced by interferon in mammalian and avian cells, an oligonucleotide polymerase and a protein kinase [see Baglioni (1979) for references]. these enzymes are present in an apparently inactive form in extracts of interferon-treated cells and are activated by double-stranded RNA (dsRNA). The polymerase converts ATP into a series of oligonucleotides characterized by 2′,5′-phosphodiester bonds (Kerr & Brown, 1978) and designated 2′,5′-oligoadenylic acid [2′,5′-oligo(A)]. These oligonucleotides in turn activate an endonuclease that degrades RNA (Baglioni et al., 1978; Clemens & Williams, 1978). The protein kinase phosphorylates two polypeptides of M_r 38 000 and about 70 000, re-

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