Terminally Directed Hydrolysis of Duplex Ribonucleic Acid Catalyzed by a Species of the BAL 31 Nuclease from *Alteromonas espejiana*

A Thesis

Presented to

the Faculty of the Department of Biochemical

and Biophysical Sciences

University of Houston

In partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Gerard Henri Bencen

December, 1983

TO MY WIFE, MY FATHER, AND MOTHER

WITHOUT WHOSE LOVE AND SUPPORT

THIS WORK

WOULD HAVE NEVER BEEN COMPLETED

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. H. B. Gray, Jr., my advisor, for his continued support and tolerance over the period of my stay in his lab.

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ABSTRACT

The extracellular nuclease activities of Alteromonas espejiana sp. BAL 31 are mediated by at least two distinct protein species that differ in molecular weights and catalytic properties. The two species that have been purified to homogeneity and characterized, the "fast" (F) and "slow" (S) enzymes, both possess an exonuclease activity that shortens both strands of duplex DNA, with the F nuclease displaying a much greater (approximately 27-fold) turnover number for this degradation than the S species. In the present thesis, it is shown that the F species also mediates the terminally directed hydrolysis of a linear duplex RNA, gradually shortening molecules of this substrate through a mechanism that results in the removal of nucleotides from both the 3' and the 5' ends. This degradation proceeds with very infrequent introduction of scissions away from the termini as demonstrated by gel electrophoretic examination of the products of partial degradation, both in duplex form and after denaturation by reaction with CH_3HgOH, and by electron microscopic characterization of duplex partially degraded molecules. Tha apparent Michaelis constant and turnover number have been determined. At equimolar enzyme concentrations in the limit of high substrate concentration, the F nuclease will degrade duplex RNA at a rate 0.019 ± 0.004 times that for a duplex DNA of comparable guanine + cytosine content. The S species, by contrast, showed very little activity against the duplex RNA substrate in the photometric assay.

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INTRODUCTION

A. BAL 31 Nuclease

The nuclease activities present in the culture fluid of the marine bacterium Alteromonas espejiana sp. BAL 31 (American Type Culture Collection number 29659) are catalyzed by at least two protein species. The two species that have been purified to homogeneity and characterized are designated the "fast" (F) and "slow" (S) nucleases according to the relative rates at which they catalyze the terminally directed hydrolysis of duplex DNA (1). Other nuclease activities displayed by both these purified species include hydrolysis of single-stranded DNA (1), cleavage of duplex DNA in response to a pre-existing single strand break, cleavage of negatively supercoiled DNA to the linear duplex form (1) and cleavage of duplex DNA in response to the presence of apurinic sites (31).

Cleavage at the junctions between right-handed and left-handed helical segments in a closed circular duplex plasmid DNA has been reported for the F enzyme (2) and this reaction is catalyzed at lesser efficiency by the S species (Kilpatrick et al., unpublished). The nuclease has found numerous applications in nucleic acid biochemistry, including the controlled size reduction of linear duplex DNAs (1), restriction fragment mapping for small genome DNAs (24), and as a probe for lesions introduced into DNAs by various alkylating agents, carcinogens and mutagens (19). Pronounced stability upon extended storage and resistance to inactivation in the presence of high concen-

trations of salt or denaturing agents have been demonstrated for highly purified oreparations of the S species (3), while the F enzyme has been tested with respect to high concentrations of NaCl and shown to retain much of the activity observed in the standard reaction mixture (2).

The fact that the nuclease is highly single strand-specific allows the biochemist an alternative to using some of the other single strand specific nucleases currently available. The BAL 31 nuclease is optimally active near pH 8 (3) making it decidedly more advantageous to use this nuclease over the S_1 or mung bean nucleases which are active only at acidic pH where depurination of DNA may cause complications.

B. Duplex RNA

The existence of naturally occurring duplex RNA's has been reported for numerous viruses including, among others, the reoviruses (25), Aspergillus foetidus (26), and those of Saccharomyces cerevisiae (4).

The "killer" system of Saccharomyces cerevisiae is correlated with the presence of stable cytoplasmic virus-like particles (ScVLP's) containing several species of linear duplex RNA (reviewed in (4)). There are apparently three major size classes of duplex RNA expressed in Saccharomyces cerevisiae strains, designated L (large, M_r = 3.15 x 10^6), M (medium, M_r = 1.24 x 10^6), and S (small, M_r = 0.37 - 1.01 x 10^6) The M dsRNA has been shown to be susceptible to internal cleavage by S_1 nuclease (27) and shown to contain an internal region rich in A-T base pairs. Since it is likely that such regions would be recognized by the BAL 31 nuclease as substrate sites resulting in internal cleavage of the RNA, the L dsRNA species was chosen for kinetic and mechanistic studies as it is more resistant to S_1 cleavage than is the M species.

While the function of the S dsRNA is unknown, the M dsRNA has been shown to code for a heat labile, diffusible toxin and for the host's resistance to that toxin. In addition, it has been shown that M never occurs in the absence of L (29), but that L may occur in the absence of M ; this dependence of M on L has been attributed to the polypeptide encoded for by L which acts as the capsid protein for both L and

M. It was therefore possible to utilize a strain, JM 4 (5), which is an overproducer of L but contains no M, making JM 4 a non-killer and sensitive to the toxin.

The transmission of the ScV dsRNA is apparently horizontal and non-Mendelian in nature (4). However, numerous chromosomal elements have been identified which are responsible for the maintenance of the VLP's(30). It is beyond the scope of the present investigation to delve into this fascinating area of yeast genetics, but it is hoped nonetheless that the work presented here might in some way facilitate research into this phenomenon.

In the case of the F nuclease, a novel terminally directed hydrolysis of the RNA, which effects the removal of nucleotides from or near both the 5' and the 3' termini so as to result in a gradual shortening of these molecules, is demonstrated and kinetically characterized in this thesis. The S species apparently also catalyzes this reaction but does so with far less efficiency than the F nuclease.

MATERIALS AND METHODS

A. CHEMICALS

Methyl mercuric hydroxide was obtained as a 1 M solution from Alfa Chemicals (Danvers, MA). All other chemicals were reagent grade or specially manufactured for the uses in this work.

B. BAL 31 NUCLEASE

1. PURIFICATION

The marine bacterium Alteromonas espejiana was cultured in 15 to 45 l batches which were filtered free of cells and concentrated using the Millipore Pellicon ultrafiltration system as described (3). The concentrated culture supernatant was acetone-precipitated, the precipitate resuspended in 1ml/l original crude 1 x CAM buffer (5 mM $CaCl_2$, 5mM $MgCl_2$, 0.1 M NaCl, 20 mM Tris, 1 mM EDTA (pH 8.1)), subjected to ultracentrifugation (90,000 x g, 1 hr), and the supernatant passed through a 5 ml column containing 5'-AMP covalently bound to agarose through a hexane linker to C_8 (Sigma). The washed column was eluted with 20 mM 5'-AMP, 1 M NaCl in CAM buffer and the fractions dialyzed and assayed for nuclease activity. The peak fractions were pooled, concentrated over dry sucrose, and applied to a 2.5 cm x 75 cm column of Sephadex G 100 superfine resin and 2 ml fractions collected. Individual fractions were assayed for endonucleolytic and exonucleolytic activity and peak fractions were pooled (1). The homogenous

pooled samples of the F species of BAL 31 nuclease and the individual chromatographic fractions were those used in a previous study (1).

2. ENZYME ASSAY

(All assays were carried out in 0.6 M NaCl, 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$, 20 mM Tris-HCl, 1 mM EDTA, pH 8 (30°C).)

a. Standard Unit assay: The standard unit assay is a point by point assay using alkaline denatured calf thymus DNA as substrate in a reaction mixture which, after addition of 0.1 volumes of 1 x CAM buffer or enzyme in CAM, has the final concentrations of electrolytes indicated above, and DNA at a nominal concentration of about 600 µg/ml. Aliquots of 0.4 ml were withdrawn at appropriate intervals, and reactions were terminated by addition of 40 µl 0.5 M EDTA to chelate divalent metal cations (Mg^{2+}, Ca^{2+}), in the absence of which the enzyme is inactive. Eight-tenths ml of ice-cold 10% perchloric acid were added to each time-point and control (CAM buffer, no enzyme) to precipitate nondegraded DNA.

The acidified mixtures were filtered through 0.2 µm nitrocellulose filters (Amicon) and the absorbance of the acid soluble material was read at 260 nm on a Beckman Model 25 spectrophotometer. A TI-55 calculator was used to generate a linear least-squares fit to the data and the slope of the ΔA^{260} versus minutes of incubation curve thus generated converted into units per ml of enzyme activity by the factor: (slope/0.55)(16.7)(1.24/0.04)(dilution factor). This factor is dependent on the unit definition described by Vogt (11) according to which 1 unit will release 1 µg of nucleotides into solution in 1

min and the fact that 16.7 μg/ml of nucleotides in 6.7% (w/v) $HClO_4$ has an A^{260} of 0.55 (11). The total volume of each acidified aliquot is 1.24 ml, while the volume of enzyme sample added was 0.04 ml.

b. Exonuclease assay: For a known number of enzyme units, the rate of terminally directed hydrolysis of duplex DNA or RNA at a known substrate concentration was obtained by monitoring the increase in absorbance at 260 nm due to hyperchromicity.

Assays were performed in a Cary 118 C spectrophotometer with a cuvette thermostatted at 30°C in a volume of 0.2 ml.

Substrate concentrations were obtained directly prior to addition of 0.1 volumes of enzyme by measurement of the A^{260}, and converted into moles/l duplex termini for duplex DNA by the relation:

$$[S] = (0.90\ A^{260})(50\ \mu g/ml\text{-}A^{260})(1\ g/10^6\ \mu g)(10^3\ ml/l)(1/M_r)(2)$$

M_r is the molecular mass of the duplex DNA in g/mole and the factor 2 takes into account the presence of 2 moles of termini per mole of DNA duplex; 50 $\mu g/ml\text{-}A^{260}$ is taken to be the reciprocal weight extinction coefficient of duplex sodium DNA. For duplex RNA, the above relation holds with the modification that the reciprocal weight extinction coefficient becomes 52.4 $\mu g/ml\text{-}A^{260}$ (this assumes the average molar extinction coefficient for a ribo-nucleotide residue is the same as that for a deoxyribonucleotide and a ratio of the molecular mass of an RNA nucleotide residue to that of a DNA residue of 1.048. The relations assume that only the termini of the duplex are serving as substrate sites. The above reduce to:

$$[S] = 0.090\ A^{260}/M_r \text{ for DNA, and}$$

$$[S] = 0.0944\ A^{260}/M_r \text{ for RNA.}$$

Initial velocities of reactions for duplex DNA and RNA were obtained by measuring the initial slope in terms of ΔA^{260}/min and converting to moles nucleotides released per liter per minute using the relation:

$$v_o = (\Delta A^{260}/\text{min})(1/(0.60\ A^{260}))[S](M_r/M_o)(0.5)$$

where M_o is the average molecular mass of a DNA nucleotide (330 g/mole). This relation makes use of the measured figure of 60% hyperchromicity for the complete depolymerization of dsDNA. Since the calculation required to convert A^{260} into [S] includes M_r in the numerator, it is clear from the above calculation that the absolute velocity, is in fact independent of the substrate molecular weight. This relation is modified for dsRNA only by the insignificant difference that a hyperchromicity of 59.7% is used for the dsRNA of this study (the differnce in M_o values is cancelled by the difference in reciprocal weight extinction coefficients implicit in [S]). A single relation applicable to both DNA and RNA is

$$v_o = (2.27 \times 10^{-4})(\Delta A^{260}/\text{min})$$

3. Kinetics

The kinetics of the F and S nucleases were measured in the manner described above for the rate of exonucleolytic hydrolysis using constant, known enzyme concentrations and varying the concentration of PM2 form I DNA (1) or S cerevisiae L dsRNA. Lineweaver-Burk analysis

of kinetic data was done as described in the legend to Fig. 8. In the assays of the individual fractions of Fig. 1, 20 µl of each fraction were used in mixtures of 0.2 ml total volume at an RNA concentration near 15 µg/ml.

C. OTHER ENZYMES

S_1 nuclease was purchased from Seikaguku Kogyo Co., Japan and assayed according to Vogt (11). Digests of RNA samples that had been partially degraded by BAL 31 nuclease were precipitated with ethanol-ammonium acetate as described below, washed with 70% (v/v) ethanol in 20 mM Tris-HCl, 1 mM EDTA (pH 8.0) and the pellet resuspended in S_1 digestion buffer (30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM $ZnSO_4$, 5% (v/v) glycerol). Incubation at 37°C with 75 units/ml of nuclease at an RNA concentration of approximately 75 µg/ml proceeded for 5 min, at which time the samples were adjusted to 50 mM EDTA to stop the reaction and adjust the solution to near neutral pH. These samples were examined by gel electrophoresis as described below.

D. DUPLEX RNA

Some of the individual steps in the purification procedure used are modifications of those described by Wickner and Leibowitz (6) and Vodkin et al.(7).

All glassware was either autoclaved or treated with bentonite prepared according to Fraenkel-Conrat et al. (8). The Saccharomyces cerevisiae strain designated JM 4 by Vodkin (5), which is an overproducer of only the largest of the three major size classes of duplex

RNA associated with the "killer" phenotype in this organism, was the gift of Dr. G. A. Alianell.

For each preparation, 2 l of 1% yeast extract (Difco), 2% Bacto Peptone (Difco), 2% dextrose were inoculated with 10 ml of a fresh stationary culture and grown for 4 days at room temperature with constant mild agitation. The cells were pelleted and washed in 0.2 M Tris-HCl, 20 mM EDTA, 0.1 M 2-mercaptoethanol, 1.2 M sorbitol (pH 9.2), resuspended in this buffer, and incubated for 30 min at 37°C. The cells were again pelleted, washed twice in 10 mM EDTA, 1 M sorbitol, 0.1 M sodium citrate (pH 7.0), resuspended in 75 ml of this buffer and made 0.2 mg/ml in Zymolyase 5000 (samples of this enzyme were the gifts of Dr. G. A. Alianell and Kirin Brewery, Japan). After incubation at 37°C for 4 h, the mixture was centrifuged to pellet the spheroplasts, the supernatant was discarded and 8 ml of 20 mM EDTA, 4 ml of 5 M NaCl and 1 ml of 2% sodium dodecyl sulfate were added. Lysis was allowed to occur overnight at 4°C.

The debris was pelleted by centrifugation at 20,000 x g for 1 h at 4°C and the supernatant was extracted with distilled phenol saturated with 0.1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA (pH 8) (BE buffer) after which the aqueous phase was extracted with 24:1 chloroform/isoamyl alcohol. To the aqueous phase were added 2.5 volumes of 95% ethanol saturated with ammonium acetate and the nucleic acids were allowed to precipitate at -20°C for 12 h. The precipitate was pelleted by centrifugation at 10,000 x g for 15 min at 4°C and the pellets were dried in a stream of dry nitrogen. After resuspending each of the 4

pellets in 2.0 ml of 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0) (0.1 x SSC), most of the ribosomal RNA was precipitated (9) by the addition of an equal volume of 4 M LiCl in 2 x SSC and agitation at 4°C overnight followed by centrifugation at 7,000 x g for 10 min.

The supernatants were precipitated with ethanol as described above and the pellets, after drying in a stream of nitrogen, were resuspended in 10 mM Tris-HCl, 50 mM $MgCl_2$ (pH 7.4) which had been treated with 0.1% washed (8) bentonite and cleared by centrifugation. The solutions were pooled and adjusted to 100 µg/ml in pancreatic DNase I (RNase-free, Worthington) and incubated for 30 min at 37°C, followed by extraction with BE buffer-saturated phenol and precipitation of the aqueous phase with ethanol. The RNA was resuspended in 0.1 M NaCl, 50 mM Tris-HCl, 1 mM EDTA (pH 6.9) and 0.36 volumes of 95% ethanol were added. This mixture was applied to a 1.6 x 6 cm column of CF-11 cellulose (Whatman) equilibrated in the same solvent and stepwise elution of various nucleic acid species was carried out as described by Franklin (10). Elution was monitored at 260 nm using a flow cell in a Beckman model 25 spectrophotometer, with the elution at each step continued until zero absorbance was reached. Duplex RNA, which elutes last, was further purified by pooling the appropriate fractions, concentration by repeated cycles of dialysis against dry sucrose and BE buffer and centrifugation through a linear 5-20% (w/v) sucrose gradient containing 1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA for 19 h at 27,000 rpm at 4°C in a Beckman SW 27 rotor. The individual sucrose solutions successively overlayed to produce the gradient were treated

with bentonite overnight and cleared by centrifugation before use. The fractions corresponding to the duplex RNA, monitored by pumping the contents of the gradients through a flow cell, were ethanol-precipitated and resuspended in BE buffer. Purity was ascertained by electrophoresis in agarose gels as described below. Omission of the sucrose gradient step gave rise to samples contaminated with lower molecular weight material. These samples also displayed anomalous degradation kinetics in the early stages of nuclease digestion.

E. GEL ELECTROPHORESIS AND BAND SEDIMENTATION

To aliquots of reaction mixtures containing L dsRNA degraded to varying extents by the F nuclease, EDTA was added to 0.05 M to terminate the reaction. The extent of degradation was obtained from the hyperchromicity observed for reaction mixtures which were placed directly into a spectrophotometer cuvette. The RNA from the aliquots was precipitated with ethanol-ammonium acetate and washed as above and resuspended in borate buffer (50 mM H_3BO_3, 5 mM $Na_2B_4O_7$, 10 mM Na_2SO_4 (pH 7)) (13). Gel electrophoretic analysis was in 1.5% agarose slab gels (0.3 x 14 x 8 cm) for 16 h at 25 V at 7°C in borate buffer. The gels were cast either in the absence or presence of 10 mM $HgCH_3OH$ to achieve nondenaturing and denaturing conditions, respectively (13). Samples loaded onto denaturing gels were preincubated with 10 mM $HgCH_3OH$. Gels were stained for 30 min in 0.5 M ammonium acetate, 1 µg/ml ethidium bromide and photographed under 302 nm illumination as described (14). Samples were ethanol-precipitated as above and resus-

pended in BE buffer for analytical band sedimentation (15), which was carried out in neutral CsCl solution as described (16).

In an additional experiment, nontreated dsRNA was sedimented in the Beckman model E ultracentrifuge using 2 M $NaClO_4$ in the presence of 10 mM CH_3HgOH to determine if such a technique might allow resolution of complementary strands.

F. ELECTRON MICROSCOPY

Samples of undigested L dsRNA and dsRNA digested to various extents with the F nuclease were prepared for electron microscopy using a modified Kleinschmidt technique (17) after ethanol precipitation and washing. Fifty µl of a solution (pH 8.5) containing 0.5 µg/ml RNA, 40% (v/v) formamide, 2 M urea, 0.1 M Tris base, 1 mM EDTA and 100 µg/ml cytochrome c were spread onto a hypophase of deionized water containing 3 mM Tris-HCl, 0.3 mM EDTA (pH 7.7). Samples also included phage PM2 closed circular DNA prepared as described (14) to be used as an internal length standard. These spreading conditions are a modification of a previous procedure (18). Grids were rotary shadowed with Pt:Pd (80:20) and examined in a Philips 300 electron microscope. Length measurements were made from enlarged photographs taken on 35 mm film using a Numonics 1224 digitizer interfaced with a Hewlett-Packard computer and plotter. The average length of PM2 DNA (taken to be 10,250 base pairs) was determined and then converted to the equivalent number of base pairs of A-form duplex by multiplying by the ratio of linear translation per base pair of A form to that of B-

form duplex nucleic acid (0.255 and 0.346 nm, respectively). The double-stranded RNA is assumed to be in the A crystallographic form.

In other experiments, undigested L dsRNA was denatured and single-strand lengths determined using essentially the procedure described (18). Samples (100 µl) of RNA (0.5 µg/ml) in approximately 83% (v/v) formamide, 4 M urea were heated for 30 sec at 61°C. After cooling to room temperature, 0.5 µl of 1 M Tris base, 0.01 M EDTA (pH 8.5) and 1.5 µl of a 5 mg/ml solution of cytochrome c were added and 35 µl of the sample were spread onto deionized water. The translation per residue for single-stranded RNA was taken to be 0.26 nm per nucleotide residue (18) and actual lengths were determined on photographs as described above using a replica of a diffraction grating to determine magnification (18).

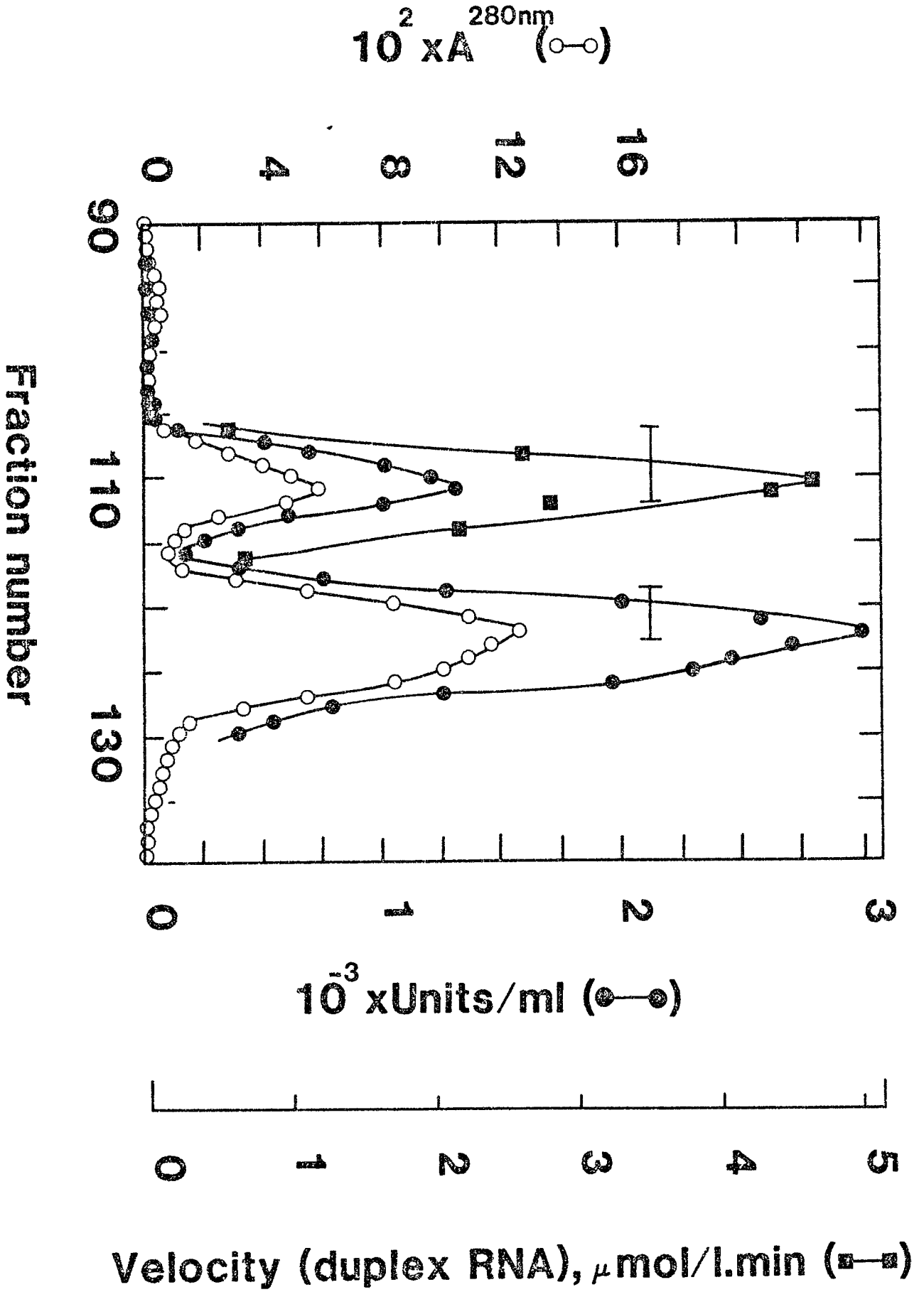
RESULTS

The F and S nucleases have been separated and obtained as homogeneous preparations from chromatography on a column of Sephadex G-100 Superfine resin (1). When the individual fractions corresponding to the peak of F nuclease were assayed for activity against the L dsRNA of Saccharomyces cerevisiae, the profile was nearly parallel with both the profiles of $A^{280\ nm}$ and nuclease activity against single-stranded DNA (Fig. 1). The fact that the fraction of apparent highest RNase activity is one fraction away from the peaks of DNase activity and $A^{280\ nm}$ is not considered significant since the two most active RNase fractions differed in reaction velocities by only approximately 6%. These profiles all coincide with that of the exonuclease activity against double-stranded DNA from the same column (1).

The individual fractions represented by a bar in Fig. 1, which were pooled to obtain the sample of F nuclease used in the earlier study (1) and in the present work, all displayed only a single band upon electrophoresis under denaturing conditions. This was also true of the pooled sample under both nondenaturing and denaturing conditions where considerably larger amounts of protein were used (1). It can accordingly be concluded that the protein species displaying the RNase activity corresponds to the BAL 31 F nuclease.

The individual fractions corresponding to the S nuclease (Fig. 1) were not assayed for activity against dsRNA because incubation of this RNA with the homogeneous S enzyme sample obtained from pooling the

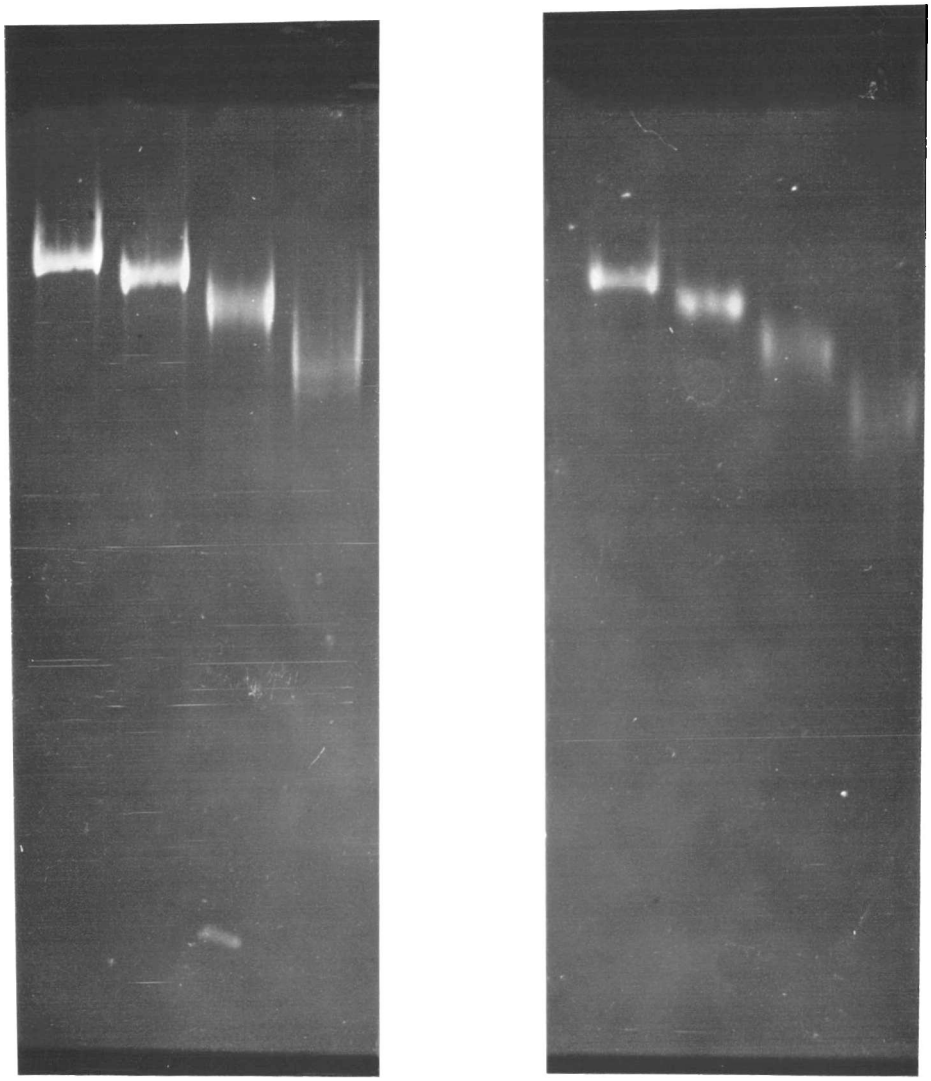
Fig. 1. Profiles of nuclease activities and absorbance of BAL 31 nuclease chromatographed on Sephadex G-100 Superfine gel filtration resin as described (1). ○ , $10^2 \times A^{280\ nm}_{1\ cm}$; ● , 10^{-3} x nuclease activity, units/ml; ■ , reaction velocity with L dsRNA substrate, mol nucleotide/l-min. Reaction conditions and assay for the activity against dsRNA were as described in Materials and Methods. Bars represent fractions pooled to obtain the homogeneous samples of F and S nucleases as described (1).


10^2 xA 280nm (○—○)
0
4
8
12
16
90
110
130
Fraction number
0
1
2
3
10^{-3} xUnits/ml (●—●)
0
1
2
3
4
5
Velocity (duplex RNA), μmol/l.min (■—■)

indicated fractions of Fig. 1 showed very little activity compared to that observed for F nuclease at considerably lower enzyme concentration under the same set of incubation conditions and RNA concentration. The rate of hyrolysis of duplex RNA for the S nuclease under the above conditions would be only 7% of that for the F nuclease at the same number of units/ml. The comparison involving the number of units/ml of activity against single-stranded DNA is valid in that the maximum velocities per unit per liter for the two nuclease species are very similiar for this substrate (1).

Evidence that the degradation of dsRNA proceeds predominantly through hydrolysis at or near the termini, as opposed to the introduction of scissions at random, was obtained by examination of the products of partial digestion by gel electrophoresis and band sedimentation. Aliquots from reaction mixtures in which up to 55% of the nucleotides would have been released as monitored by hyperchromicity were subjected to gel electrophoresis under nondenaturing and denaturing conditions (Fig. 2, A and B, respectively). Discrete bands are seen which migrate progressively faster with increasing extent of degradation under both conditions, which rules out a mode of hydrolysis in which a significant fraction of the cleavage takes place at random but is consistent with a gradual shortening of both strands of the duplex molecules. The migration rates of denatured and nondenatured RNA subjected to electrophoresis in the same experiment are discernibly different for a given sample in Fig. 2. That this difference corresponded to strand separation induced by $HgCH_3OH$ was con-

Fig. 2. Electrophoretic patterns of L dsRNA in agarose slab gels after incubation for various times with BAL 31 F nuclease. Electrophoresis was done under nondenaturing (A) and denaturing (B) conditions as described in Materials and Methods. The four lanes of each gel contain, from left to right, L dsRNA incubated with F nuclease (26 units/ml) for 0, 5, 13 and 20 min which incubations resulted in 100, 78, 61 and 45% remaining intact dsRNA, respectively, as calculated from the observed hyperchromicities.



A B

firmed in experiments in which the intact L dsRNA was subjected to electrophoresis in the presence of 0, 5, 10 and 15 mM $HgCH_3OH$, where it was observed that the migration rate of the RNA clearly increased in going from 0 to 5 mM denaturant and remained constant at the higher value at the higher $HgCH_3OH$ concentrations (Fig. 3). All subsequent denaturing gels were cast in the presence of 10 mM CH_3HgOH. The denaturing capacity of CH_3HgOH was independently confirmed in the band sedimentation experiments using 2 M $NaClO_4$ as solvent in the presence and absence of 10 mM CH_3HgOH, where a notable difference in the migration pattern was observed for the nontreated and treated samples. Whereas the RNA migrated as a single discrete band in the absence of CH_3HgOH, in the presence of 10 mM CH_3HgOH two bands were observed, possibly corresponding to the two separated complementary strands (Fig. 4). The scan in A was traced at a much later time (at least 16 16 min.) than that in B, making it unlikely that the trailing band in B, although it lines up with the band in A, represents non-denatured dsRNA. The presence of slowly sedimenting material near the meniscus is an indication of what is thought to be tRNA in the sample of dsRNA used in this experiment. This technique may provide a convenient method for separation of complimentary strands of RNA and requires further investigation. It may operate on the basis that CH_3HgOH preferentially binds to amino groups in U and G residues in RNA, thus separating complements by increasing one strands' molecular mass over that of the other on the basis of their differential G+U content. Band sedimentation at neutral pH of partially degraded samples

Fig. 3. Gel electrophoretic patterns of L dsRNA in the presence of varying concentrations of CH_3HgOH. One percent agarose tube gels were run in which 0, 5, 10, 15 mM CH_3HgOH (concentration increasing from left to right) was incorporated into the gel at the time of pouring. The gels were run for 15 hours at 30 volts, stained for 30 minutes in 0.5 M NH_4Cl, 1 µg/ml ethidium bromide, and photographed under ultraviolet light (302 nm). Migration was from top to bottom. Wherever possible these gels were confined to well ventilated fume hoods due to the highly toxic and volatile nature of this compound.

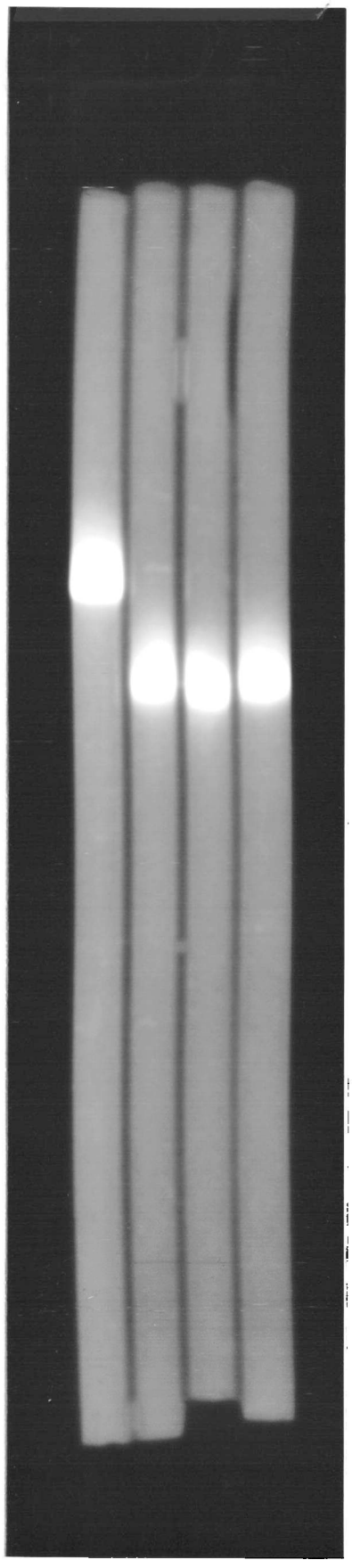
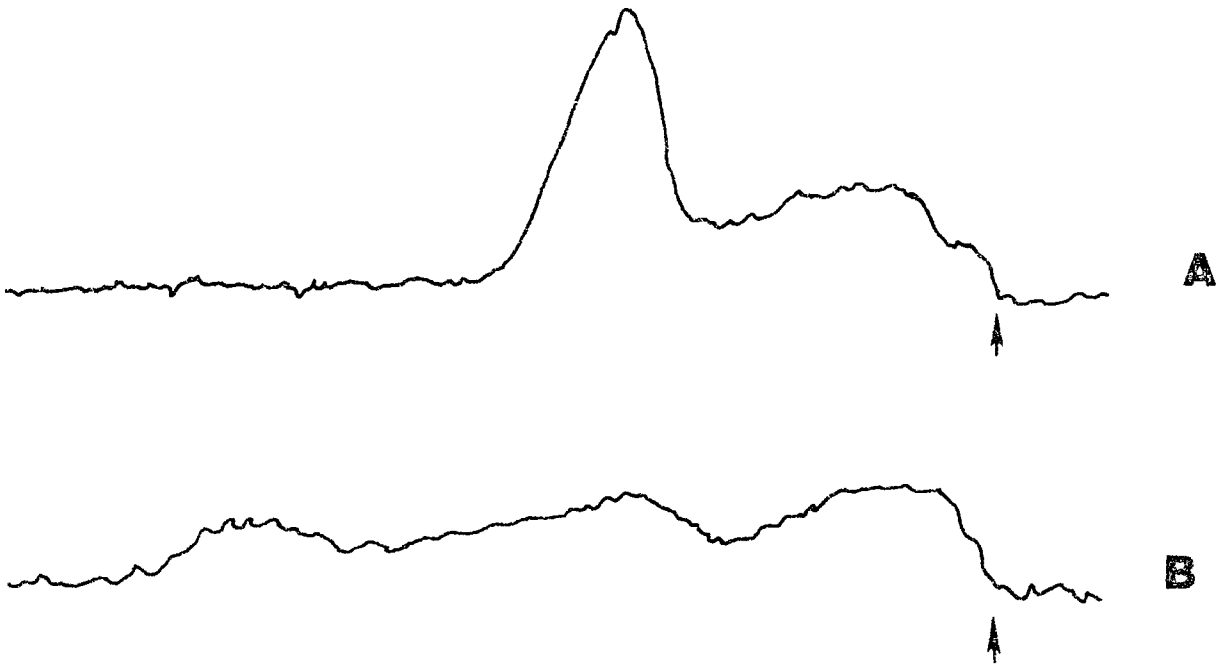


Fig. 4. Analytical Band sedimentation profiles, using photoelectric scanning system, of dsRNA using 2 M $NaClO_4$, 20 mM Tris (pH 7.5) as the banding medium. A, no denaturant, B CH_3HgOH present at 10 mM . Direction of sedimentation was from right to left. Meniscus positions of liquid columns are indicated by arrows. The slowly sedimenting species is taken to be low molecular weight contaminant in this dsRNA sample (possibly tRNA).


A
B

(maximum extent of 45% degradation) showed a single sedimenting band with no evidence of trailing material (Fig. 5), the sedimentation coefficient of which decreased with increasing extent of digestion. This is also consistent with an exonucleolytic mode of degradation of the duplex species.

A possible mode of terminally directed hydrolysis is that catalyzed by, e.g., E. coli exonuclease III, in which one strand only is degraded exonucleolytically, leaving a long single-stranded "tail." The presence of such very long single-stranded structures is not consistent, however, with the results of treatment of partially degraded dsRNA samples with S_1 nuclease as described in Materials and Methods. Such treatment altered the migration rate in the non-denaturing gels slightly, which is consistent with the removal of some single stranded material by S_1 nuclease (Fig 6). However, it is unlikely that the single stranded "tails" are any longer than a few hundred nucleotides in length, since tails of about 500 nucleotides are detectable by electron microscopy as "bushes"; such structures did not appear in the electron micrographs of partially degraded samples (below). The mode of exonucleolytic degradation in which both strands are shortened by attack at or near the termini is consistent with the known endonuclease activity against single-stranded DNA and the mode of degradation of duplex DNA, which is terminally directed and shortens both strands (1). It seems likely, therefore, that if single strand tails are produced by BAL 31 nuclease mediated degradation of only one of the strands, that these structures are be removed by the single-strand

Fig. 5. Analytical band sedimentation profiles of L dsRNA shortened by BAL 31 F nuclease. Profiles from top to bottom correspond to increasing periods of digestion (0, 5, 13 minutes respectively) with 26 units/ml of nuclease under conditions described in Materials and Methods . Sedimentation is from right to left.

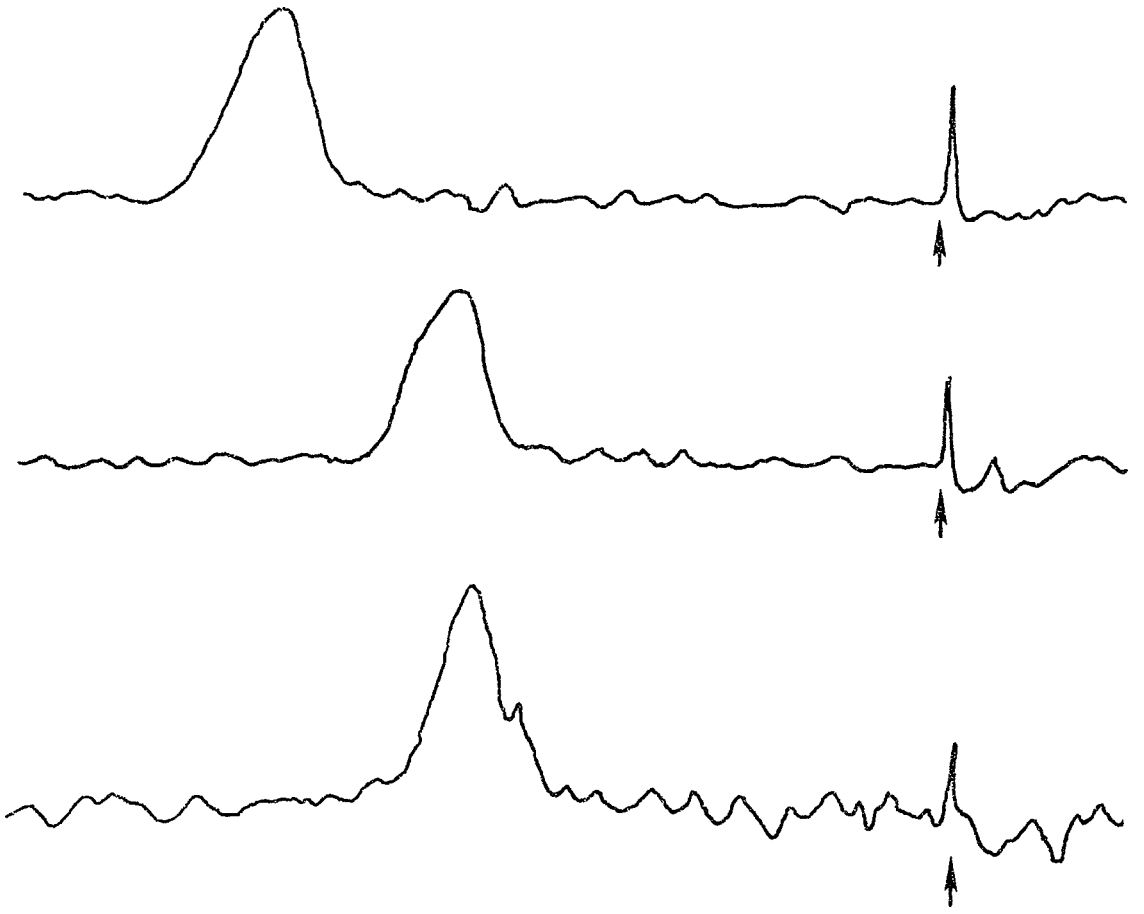
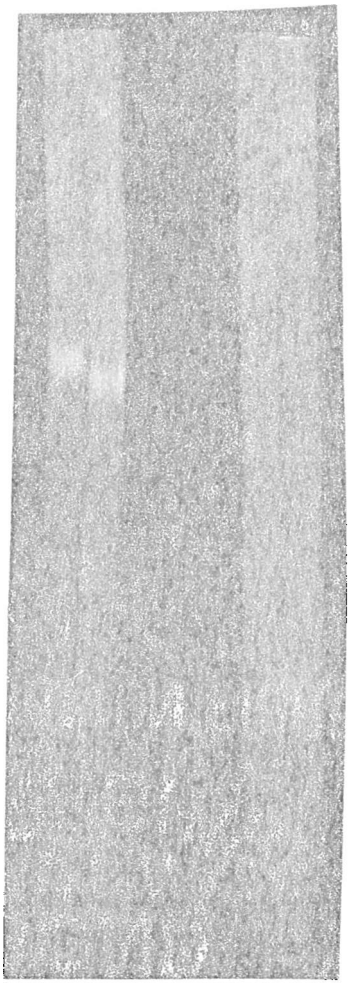


Fig. 6. L dsRNA degraded by the BAL 31 nuclease to (A) 80% and (B) 60% of the full molecular weight, followed by treatment with 1 µg S_1 nuclease/µg RNA. The S_1 treated RNA migrated more rapidly in the sample degraded to 80% but a substantial increase in the rate of migration of the sample degraded to 60% was not detectable.



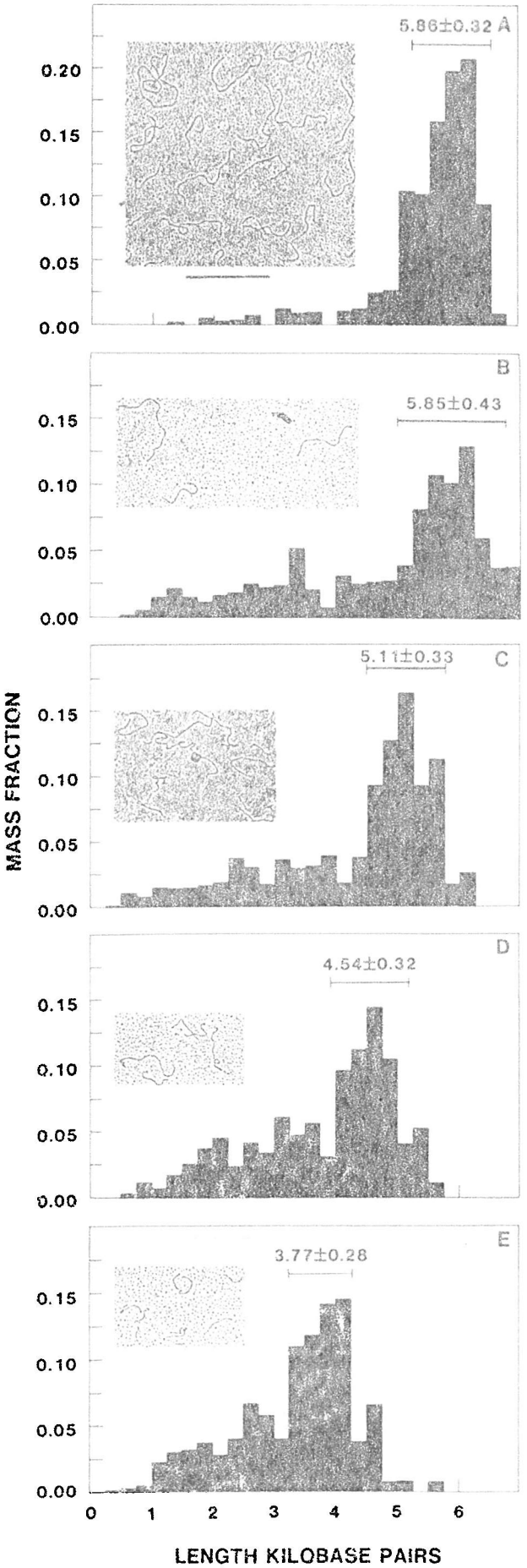
A B

specific endonuclease activity displayed by the same enzyme.

The course of BAL 31 F nuclease-catalyzed degradation of L dsRNA was monitored by electron microscopy (Fig. 7) using a different RNA preparation than that represented in Fig. 2. In the absence of any exposure to nuclease, the bulk of the RNA lies in a single length distribution with the average size indicated (Fig. 7A). After a very short "0 min" incubation (actually approximately 15 sec), most of the RNA still lies within a single distribution at the same average length but a very significant fraction now appears at shorter lengths (Fig. 7B). As degradation proceeds, the average length of the predominant population decreases with significant mass fractions of shorter molecules present in each case (Fig. 7, C to E).

The electron microscopic appearance and length analysis of the most predominant population in the dsRNA is as expected if the initially full length molecules are degraded exonucleolytically. However, the appearance of the molecular population after very brief exposure to the nuclease (Fig. 7B) indicated, when compared to the material not exposed to the nuclease (Fig. 7A), that significant cleavage to yield a broad distribution of shorter duplexes occurred after very brief exposure to BAL 31 nuclease. This shortened population was not observed in the gel electrophoretic (Fig. 2) or band sedimentation analysis (Fig. 5) of another RNA sample after extensive nuclease-mediated degradation. This suggested that the RNA sample used in the electron microscopy experiments contained a significant fraction of molecules with nuclease-sensitive lesions, possibly strand

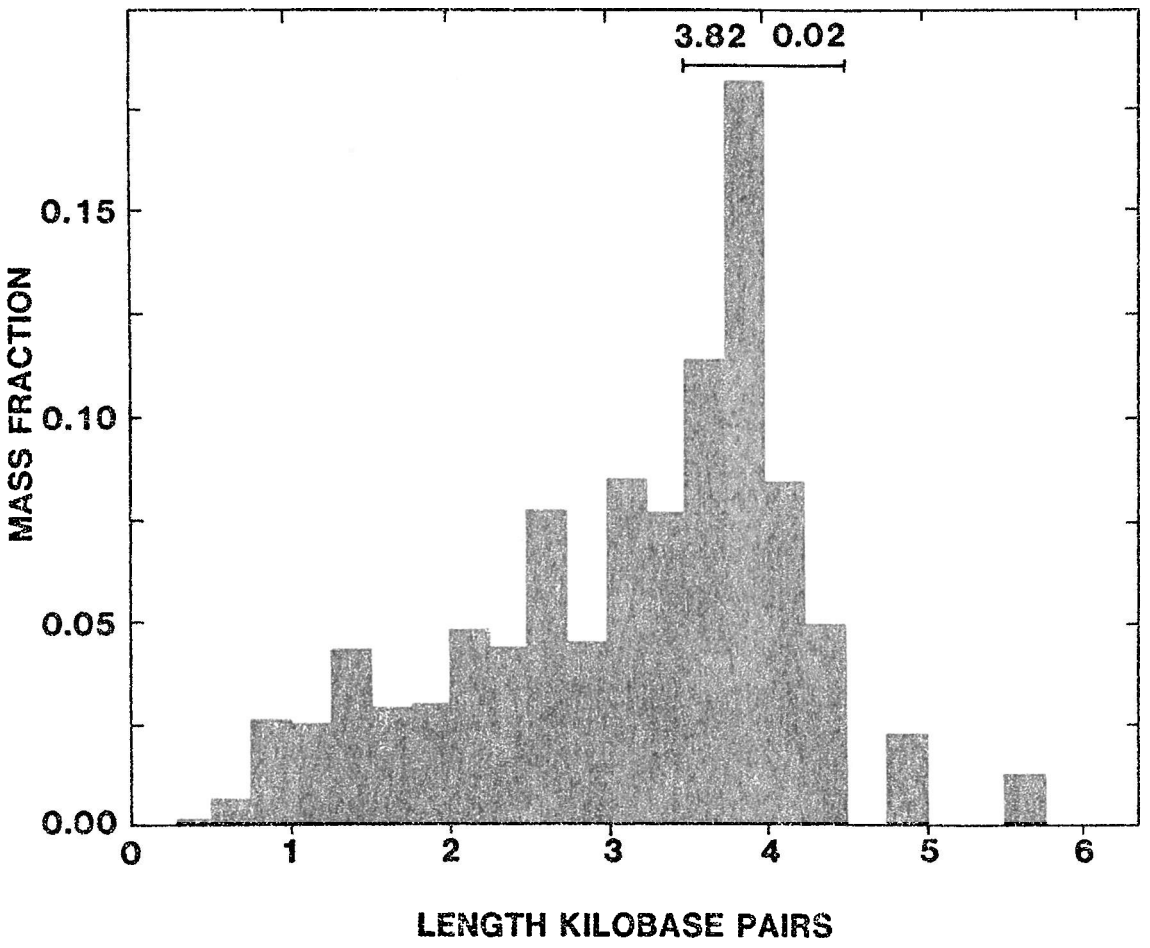
Fig. 7. Mass fraction distributions of the lengths of L dsRNA not treated with nuclease (A) and treated with nuclease (92 units/ml) for 0 (B), 2 (C), 4 (D) and 7 (E) min. The fractions of remaining intact duplex RNA as estimated from hyperchromicity are 100, 83, 72 and 51% for panels B to E, respectively. Average length ± one standard deviation over the length intervals indicated by the bars are shown in the histograms. Micrographs inset in each panel illustrate the appearance of the RNA molecules and are printed at the same magnification (indicated by the bar representing a length of 1 μm below the micrograph in A). The lengths of 140 (A), 127 (B), 196 (C), 156 (D) and 244 (E) molecules selected at random were determined. The mass fraction for each length interval is determined by the sum of lengths of molecules in that interval divided by the sum of all lengths of all molecules.


5.86±0.32 A
B
5.85±0.43
5.11±0.33
C
4.54±0.32
D
3.77±0.28
E
MASS FRACTION
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
LENGTH KILOBASE PAIRS

breaks arising during storage of the sample. Accordingly, an aliquot of this RNA sample that had not been exposed to nuclease was denatured in formamide-urea and the resulting single-stranded molecules subjected to electron microscopy (Materials and Methods). Histographic analysis of this population yielded a distribution very similiar to that of Fig. 7B (Fig.8)

Choosing comparable ranges of size in each case to represent the most predominant distribution, 52% and 58% of the single-stranded and duplex molecules, respectively, were in this high molecular weight population. This indicated that it is very likely that the RNA sample used did in fact contain a small number of strand breaks (or gaps, in which one or more nucleotides are missing) per strand. The number of such events is obtained as 0.65 breaks per strand from calculation in the Poisson equation using the fraction of single-stranded RNA that is apparently in the full length population. It is concluded that the appearance of the shorter RNA duplexes after very brief exposure to nuclease is the result of the presence of strand breaks or gaps arising spontaneously in that particular sample of RNA upon storage. That such strand interruptions are not necessarily characteristic of L dsRNA is evidenced from the lack of material migrating as shorter duplexes in gel electrophoretic and band sedimentation analysis of partially BAL 31 nuclease-degraded samples from other RNA preparations. If the cleavages to yield shorter duplexes were introduced in fully intact RNA by the nuclease, it is clear, given the fraction of

Fig. 8. Mass distribution of the length of L dsRNA not treated with nuclease and denatured in formamide-urea. Compare distribution with Fig. 7B.


3.82 0.02
0.15
0.10
0.05
0.00
MASS FRACTION
0
1
2
3
4
5
6
LENGTH KILOBASE PAIRS

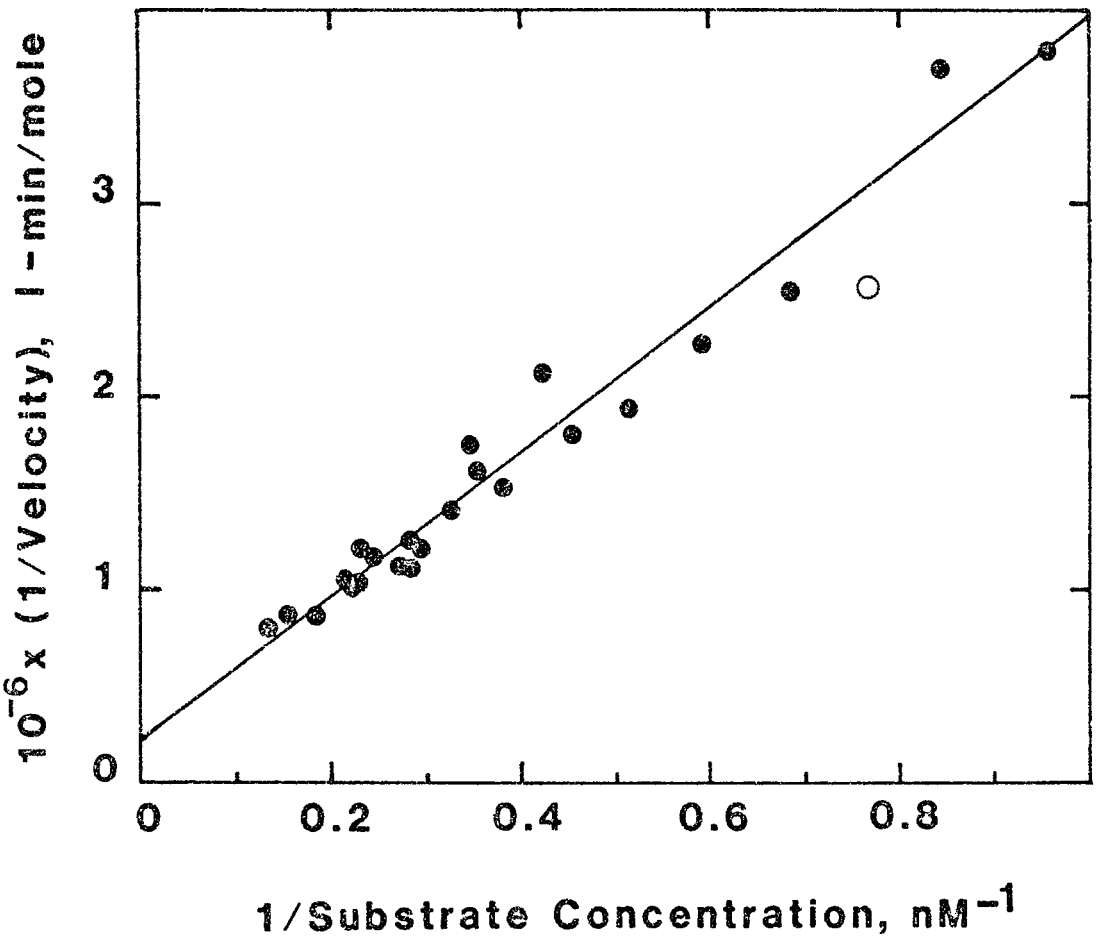
shorter molecules appearing after very brief incubation (Fig. 7B), that a predominant population of longer molecules such as in Fig. 7, C to E, could not exist after the much more extended digestions corresponding to those histograms.

Cleavage at the sites of pre-existing strand breaks in duplex DNA has been demonstrated to occur with high efficiency with highly purified preparations of S nuclease (14,19) and both species clearly can cleave at the site of a break or gap in DNA introduced by the action of the enzyme itself (1). As it seems likely that strand interruptions arising on storage of dsRNA represent breakages of single internucleotide bonds, it appears that BAL 31 F nuclease can cleave in response to pre-existing strand scissions in duplex RNA also.

Because of the degradation from the termini of the smaller duplex fragments generated very rapidly upon exposure to the nuclease, the average lengths of duplex RNA remaining at various times of digestion as calculated from the observed hyperchromicity, on the assumption that no additional ends are generated during the digestion, are expected to be underestimated. This was seen to be true when the fractional lengths, relative to that shown in Fig. 7B, of the predominant populations of Fig. 7, C to E, were compared to the estimates from hyperchromicity.

A Lineweaver-Burk plot representing the kinetics of exonucleolytic degradation of L dsRNA is presented in Fig. 9. It should be noted that reaction velocities are expressed in mol nucleotides/l-min while

Fig. 9. Lineweaver-Burk plot for the exonuclease activity of BAL 31 F nuclease (92 units/ml) against S. cerevisiae L dsRNA. Reciprocal of substrate concentration is expressed in terms of (molar concentration of duplex termini)$^{-1}$ and reciprocal of velocity is expressed in (moles nucleotide released per liter per minute)$^{-1}$. Reaction conditions and details of the photometric assay are given in Materials and Methods. Least squares analysis of the data to obtain values for K_m and V_{max} was done assuming a constant percentage error in substrate concentrations and weighting the reciprocals accordingly. The single open circle represents a data point that was identified as being more than two standard deviations away from the least squares straight line. This point was not used in the calculation of K_m and V_{max}.


10^{-6} x (1/Velocity), l-min/mole
0
1
2
3
0
0.2
0.4
0.6
0.8
1/Substrate Concentration, nM^{-1}

substrate concentrations are in mol RNA termini/l. The RNA sample used in the kinetic studies did not display evidence of the presence of strand breaks when subjected to electrophoresis under both denaturing and nondenaturing conditions nor when partially degraded samples were examined by band sedimentation under nondenaturing conditions (Figs. 3,5). Hence, the calculation of molar concentration of duplex termini, which is based on the assumption that additional termini are not generated during the course of the digest by internal cleavages, should be valid.

The kinetic parameters for the exonucleolytic hydrolysis of L dsRNA are presented in Table I along with those determined previously for F nuclease catalyzed hydrolysis of duplex DNA (1) for comparison. It is important to note that the comparison is made between RNA and DNA substrates of comparable base composition (46% and 42% G + C for L dsRNA and PM2 phage DNA, respectively) (20-22) because the kinetics of exonucleolytic digestion of DNA are strongly dependent upon this factor (1). The unit of enzyme activity refers to that using single-stranded DNA as substrate. The turnover number (k_p) has the usual definition $k_p = V_{max}/[E]_t$ where V_{max} is the maximum reaction velocity corresponding to total molar enzyme concentration $[E]_t$. In order to calculate the number of nucleotide residues released per RNA terminus per unit time, Eq. (1) of Reference 1 is used where V_{max} is calculated by multiplying the values in the first column of Table I by the number of units/l. As noted for the hydrolysis of duplex DNA (1), the

Table I

Kinetic properties of BAL 31 F nuclease with respect to linear duplex RNA and DNA

Substrate	Maximum velocity per unit per liter	Michaelis constant	10^{-3} x Turnover number
	nmol/unit-min	nM	min^{-1}
Linear duplex RNA	0.047±0.004	16±1.5	0.12±0.01
Linear duplex DNA[a]	2.3±0.3	96±15	6.2±1.0

[a]Data from Reference 1

reaction velocity did decrease with time of reaction, presumably due to product inhibition by the released nucleotides. Hence, the reaction velocity calculated in Eq. (1) of Reference 1 will overestimate the amount of degradation of duplex RNAs if the degradation proceeds to a significant extent. A significant decrease in reaction velocities with increasing G + C content is also fully expected although no data are available from which to estimate the magnitude of this effect.

The ratio of the turnover numbers for F nuclease-mediated degradation of duplex RNA and DNA is 0.019±0.014, which ratio corresponds to the limit of saturating substrate concentration. In the limit of low substrate concentration, where this concentration is negligible with respect to K_m, the corresponding ratio becomes $k_{p,RNA}K_{m,DNA}/k_{p,DNA}K_{m,RNA}$ if the concentrations of both types of duplex termini are equal and has the value of 0.12±0.03. It is evident that the enzyme is much more efficient as a deoxyriboexonuclease than as a riboexonuclease against duplex substrates.

DISCUSSION

The terminally directed hydrolysis of linear duplex RNA so as to progressively shorten both strands is not exhibited by any other nuclease acting upon duplex RNA substrates. This novel reaction catalyzed by the BAL 31 nucleases can be used in applications requiring the controlled exonucleolytic size reduction of linear duplex RNA. The rate of removal of nucleotides for a known number of units/ml of F nuclease can be estimated from the kinetic parameters presented here with the stipulations that the overall rate will decrease for RNAs of higher G + C content than that used in these studies and that the rate of nucleotide removal in a particular small region of a genome will depend upon local sequence features, such as the presence of consecutive G·C base pairs. By analogy with the results for the degradation of linear duplex DNA (1), it is estimated that a difference in G + C content of 5 to 10% would significantly alter the kinetics of degradation from those for S. cerevisiae L dsRNA under the same set of reaction conditions. It is noted that the 5'-triphosphate termini of the RNA used (23) did not block BAL 31 nuclease-mediated degradation.

Under the same set of reaction conditions, the observed reaction velocity for the F nuclease on dsRNA was approximately 14 times that of the S enzyme when normalized to the same number of enzyme units/ml. This is in the range of values for the ratio of the exonuclease reaction velocities against linear duplex DNA for the two nuclease species, which range from approximately 11 to 27 in going from the limit in which substrate concentration is negligible with respect to K_m to

the limit in which substrate concentration greatly exceeds K_m (1). Given that the riboexonuclease activity of the F species is at most 12% of the deoxyriboexonuclease activity at a given enzyme concentration (Results), it is apparent that the S nuclease will be the enzyme of choice for degradation of dsRNA only in applications in which a very limited extent of removal of RNA nucleotides is desired. From the very limited data obtained for the S nuclease, it is estimated that at a concentration of L dsRNA of 15 μg/ml (9.2×10^{-9} M termini) and an enzyme concentration of 100 units/ml approximately 9 base pairs would be removed per terminus per minute. This may be compared to approximately 130 base pairs released per terminus per minute for the F nuclease under the same reaction conditions. It should be noted that the commercially supplied samples of BAL 31 nuclease may be mixtures of the two species, and several such samples analyzed in this laboratory clearly corresponded much more closely to S than to F nuclease. Thus it may be necessary to purify the F species as described (1) for applications requiring extensive degradation of dsRNA.

In assaying the fractions of Fig. 1 corresponding to F nuclease for both activity against linear duplex DNA (1) and RNA, it was noted that the reaction velocities normalized by the number of units/ml were roughly constant across most of the fractions, including those pooled to provide the enzyme samples used here and in the earlier work (1), but that a significant increase in this value at fraction 114 occurred

in both assays. This increase seems to be larger than could be accounted for by errors in the assays of activity against either the single-stranded or duplex substrates. However, all the fractions in question have been examined by gel electrophoresis in denaturing polyacrylamide gels and no protein species other than that corresponding to the F nuclease was found. Thus, if another enzyme species is responsible for the above observations, it chromatographs on Sephadex G-100 at a different position than the peak fraction and yet is not revealed by electrophoresis as a species of different molecular weight than that designated as the F nuclease. The above observations are presented as they may be significant to investigators purifying the nucleases but are unexplained.

REFERENCES

1. Wei, C.-F., Alianell, G. A., Bencen, G. H., and Gray, H. B., Jr. (1983) J. Biol. Chem. (in press)
2. Kilpatrick, M. W., Wei, C.-F., Gray, H. B., Jr., and Wells, R. D. (1983) Nucleic Acids Res. 11, 3811-3822
3. Gray, H. B., Jr., Winston, T. P., Hodnett, J. L., Legerski, R. J., Nees, D. W., Wei, C.-F., and Robberson, D. L. (1981) in Gene Amplification and Analysis Vol. 2. Structural Analysis of Nucleic Acids (Chirikjian, J. G., and Papas, T. S., eds.) pp. 169-203, Elsevier North-Holland, New York
4. Bruenn, J. (1980) Annu. Rev. Microbiol. 34, 49-68
5. Vodkin, M. (1977) J. Bacteriol. 132, 346-348
6. Wickner, R. B., and Leibowitz, M. J. (1976) Genetics 82, 429-442
7. Vodkin, M., Katterman, F., and Fink, G. (1974) J. Bacteriol. 117, 681-686
8. Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961) Virology 14, 54-58
9. Avital, S., and Elson, D. (1969) Biochim. Biophys. Acta 179, 297-307
10. Franklin, R. M. (1966) Proc. Natl. Acad. Sci. U.S.A. 55, 1504-1511
11. Vogt, V. M. (1973) Eur. J. Biochem. 33, 192-200
12. Gray, H. B., Jr., Ostrander, D. A., Hodnett, J. L., Legerski, R., and Robberson, D. L. (1975) Nucleic Acids Res. 2, 1459-1492

13. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning A Laboratory Manual, pp. 204-206, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

14. Legerski, R. J., Gray, H. B., Jr., and Robberson, D. L. (1977) J. Biol. Chem. 252, 8740-8746

15. Vinograd, J., Bruner, R., Kent, R., and Weigle, J. (1963) Proc. Natl. Acad. Sci. U.S.A. 49, 902-910

16. Ostrander, D. A., and Gray, H. B., Jr. (1973) Bioploymers 12, 1387-1419

17. Davis, R. W., Simon, M., and Davidson, N. (1971) Methods Enzymol. 21, 413-438

18. Robberson, D. L., Aloni, Y., Attardi, G., and Davidson, N. (1971) J. Mol. Biol. 60, 473-484

19. Wei, C.-F., Legerski, R. J., Robberson, D. L., Alianell, G. A., and Gray, H. B., Jr. (1983) in DNA Repair: A Laboratory Manual of Research Procedures (Friedberg, E. C., and Hanawalt, P. C., eds.), Vol. 2, pp. 13-40, Marcel Dekker, New York

20. Bruenn, J., and Kane, W. (1978) J. Virol. 26, 762-772

21. Sommer, S. S., and Wickner, R. B. (1982) Cell 31, 429-441

22. Espejo, R. T., Canelo, E., and Sinsheimer, R. L. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 1164-1168

23. Bruenn, J., and Keitz, B. (1976) Nucleic Acids Res. 3, 2427-2436

24. Legerski, R.J., Hodnett, J. L., and Gray, H. B. Jr., (1978) Nucleic Acids Res. 5, 1445-1464

25. Shatikin, A. J., Sipe, J. D., Loh, P., (1968) J. Virol, 2, 986-991

26. Buck, K.W., Raffi, G., (1975) J. Gen. Virol, 27, 211-224

27. Meinhardt, F., Current Genetics (1982), 6, 171-172

28. Welsh, J. D., Leibowitz, M. J., (1982) PNAS, 79, 786-789

29. Bostian, K. A., Sturgeon, J. A., Tipper, D. J., (1980) J. Bacteriol., 463-470

30. Wickner, R. B., Leibowitz, M. J., (1976) J. Mol. Biol., 105, 427-434

31. Wei, C.F., Legerski, R. J., Alianell, G.A., Robberson, D.L., Gray, H.B. Jr., (manuscript submitted).