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

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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 19-fold) turnover number for this degradation than the S species. In the present article, 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 CH3HgOH, and by electron microscopic characterization of duplex partially degraded molecules. The apparent Michaelis constant and turnover number have been determined. At equimolar enzyme concentrations in the limit of high substrate concentrations, the F nuclease will degrade duplex RNA at a rate 0.021 ± 0.010 (S.D.) times that for a duplex DNA of comparable guanine + cytosine content. The S species, by contrast, shows very little activity against the duplex RNA substrate relative to that of the F enzyme.

The nuclease activities present in the culture fluid of the marine bacterium Alteromonas espejiana sp. BAL 31 (American Type Culture Collection 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 these purified species include hydrolysis of single-stranded DNA (1), cleavage of negatively supercoiled DNA to the linear duplex form (1), and cleavage of duplex DNA in response to the presence of apurinic sites (2). 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 (3) and this reaction is catalyzed at lesser efficiency by the S species. Pronounced stability upon extended storage and resistance to inactivation in the presence of high concentrations of salt or denaturing agents have been demonstrated for highly purified preparations of the S species (4), 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 (3). Both species are active near pH 8 (1, 4).

The “killer” system of Saccharomyces cerevisiae is correlated with the presence of stable cytoplasmic virus-like particles containing several species of duplex linear RNA (reviewed in Ref. 5). A strain of this yeast which is an overproducer of the largest of the three size classes of RNA (L dsRNA) has been used as a source of linear duplex RNA substrate for the BAL 31 nucleases. In the case of the fractions corresponding to the F nuclease, a novel terminally directed hydrolysis of the RNA, which effects the removal of nucleotides from or near both the 3’ and the 5’ termini so as to result in a gradual shortening of these molecules, is demonstrated and kinetically characterized in this article. The S species apparently also catalyzes this reaction but does so with far less efficiency than the F nuclease.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

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 S. cerevisiae, the profile was nearly parallel with both the profiles of A\textsuperscript{LdsRNA} 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\textsuperscript{LdsRNA} is not considered significant since the two most active

1 M. W. Kilpatrick, C. F. Wei, H. B. Gray, Jr., and R. D. Wells, unpublished work.
2 The abbreviation used is: L dsRNA, the highest-molecular-weight linear double-stranded RNA species from the virus-like particles of S. cerevisiae.
3 Portions of this paper (including "Materials and Methods," part of "Results," and Figs. 3 and 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 3950 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-0196, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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Exonucleolytic Degradation of Duplex RNA

The appearance of the dsRNA sample of Fig. 2 after very brief exposure to the nuclease (Fig. 2B) indicated, when compared to the material not exposed to the nuclease (Fig. 2A), that significant cleavage to yield a broad distribution of shorter duplexes occurred after very brief exposure to BAL 31 nuclease. It appears (see Miniprint Supplement) that the dsRNA sample used in the electron microscopic analysis represented in Fig. 2 contained a low level of strand interruptions per molecule, which gave rise to internal scissions to produce shorter duplexes after very brief incubation with F nuclease. Such internally cleaved molecules were present at much lower levels in other dsRNA samples that were shown to contain a much lower level of strand interruptions. If the strand interruptions arising upon storage of dsRNA samples consist of breakages of single internucleotide bonds, it appears that the nuclease can cleave in response to pre-existing scissions in duplex RNA. The analogous activity has been demonstrated for highly purified preparations of S nuclease with duplex DNA containing pre-existing strand scissions (16, 21). Also, both species can cleave at the site of a strand scission or gap (missing nucleotides) in duplex DNA introduced by the nuclease itself (1).

The RNA sample used in the kinetic studies did not display evidence of strand breaks when subjected to electrophoresis under both denaturing and non-denaturing conditions nor when partially degraded samples were examined by band sedimentation under non-denaturing conditions. Hence, the values for the molar concentrations of duplex termini (substrate concentration) used in calculation of the kinetic parameters, which are based on the assumption that additional termini are not generated during the course of the digest by internal cleavages, should be valid. It is noted that S. cerevisiae L dsRNA can consist of up to three species of identical size (22), suggesting possible heterogeneity of the substrate used in this work. However, all of these RNAs have very nearly the same base composition (22), so that this substrate is essentially homogeneous with respect to degradation of substantial portions of the genome by the nuclease.

The kinetic parameters for the exonucleolytic hydrolysis of L dsRNA are presented in Table 1 along with those determined for F nuclease-catalyzed hydrolysis of linear duplex DNA (1) for comparison. The unit of enzyme activity refers to that using single-stranded DNA as substrate. The turnover number \( k_p \) has the usual definition \( k_p = V_{max}/[E] \), where \( V_{max} \) is the maximum reaction velocity corresponding to total molar enzyme concentration [E]. In order to calculate the number of nucleotide residues released per RNA terminus/unit of time, Equation 1 of Ref. 1 is used where \( V_{max} \) is calculated by multiplying the appropriate value in the first column of Table 1 by the number of units/liter. As noted for the hydrolysis of duplex DNA (1), the reaction velocity did decrease with time of reaction, presumably due to product inhibition by the released nucleotides. Hence, the reaction velocity calculated in Equation 1 of Ref. 1 will overestimate the amount of degradation of duplex RNAs if the degradation proceeds to a significant extent (e.g. 15% degradation gave rise to a noticeable decrease in reaction rate under our conditions).

It should be noted that the comparison in Table 1 is made between RNA and DNA substrates of comparable base composition (46 and 42% G + C for L dsRNA and PM2 phage

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Values corresponding to double-stranded DNA are modified from those of Ref. 1 as noted under "Materials and Methods."
TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Maximum velocity/unit/liter</th>
<th>Michaelis constant</th>
<th>$10^{-3} \times$ turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear duplex RNA</td>
<td>0.034 ± 0.004</td>
<td>9.7 ± 1.4</td>
<td>0.091 ± 0.013</td>
</tr>
<tr>
<td>Linear duplex DNA</td>
<td>1.6 ± 0.7</td>
<td>58 ± 41</td>
<td>4.3 ± 1.9</td>
</tr>
</tbody>
</table>

DNA, respectively (22–24)) because the kinetics of exonucleolytic degradation of duplex DNA are strongly dependent on this factor (1). A significant decrease in reaction velocities with increasing G + C content is also fully expected for linear duplex RNA. Estimation of the rate of removal of nucleotides for a known number of units/ml of F nuclease from the kinetic parameters presented here should be done 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 5–10% 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 (25) did not block BAL 31 nuclease-mediated degradation.

The ratio of the turnover numbers for F nuclease-mediated degradation of duplex RNA and DNA is 0.021 ± 0.010, 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 $k_{r, RNA}K_{m, DNA}/k_{r, DNA}K_{m, RNA}$ if the concentrations of both types of duplex termini are equal and has the value 0.13 ± 0.11. Even with the large uncertainty in these values, it is evident that the enzyme is much more efficient as a deoxyriboexonuclease than as a riboexonuclease against duplex substrates.

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 6 base pairs would be removed per terminus/min. This may be compared to approximately 90 base pairs released per terminus/min for the F nuclease under the same reaction conditions. It is thus 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. It should be noted that the commercially supplied samples of BAL 31 nuclease may be mixtures of the two species; several such samples analyzed in this laboratory 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 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. Samples were prepared for microscopy using the formamide modification of the aqueous Kleinschmidt procedure described under "Materials and Methods."
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 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.

Aliquots of the pooled S and F nuclease samples of this study also readily degraded mixtures of 16 and 23 S Escherichia coli ribosomal RNA and M13 phage RNA as monitored by hyperchromicity. This was also found to be the case for other samples of these nuclease species purified using the same procedure. Thus, fully duplex structure in the RNA substrate is not required for the BAL RNase activity. The activity analogous to that against single-stranded DNA (1) is therefore present in homogeneous preparations of S and F nuclease.

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. The recent development of a means for constructing recombinant RNA molecules and replicating these in an exponential fashion using Qβ replicase (26) provides a connection in which the controlled shortening of duplex RNA molecules could be useful. The separated shortened strands of various dsRNA species could be inserted into the recombinant RNA vector (26) and amplified for purposes such as use as labeled hybridization probes against DNA or RNA that may be homologous only to part of the original dsRNA sequence. This could also be useful in the manipulation of templates for studying RNA processing and/or translation in dsRNA genomes.

Acknowledgment—We thank Dr. Randy J. Legerski for aid with the computer analysis of the RNA histograms.

REFERENCES


Continued on next page.
Exonucleolytic Degradation of Duplex RNA

SUNITAL MATERIAL

TERMINALLY DIRECTED HYDROLYSIS OF DUPLEX KEMODERIVE ACID Catalyzed by a SPECIES OF THE BAL 31 NUCLEASE FROM ALTERNARIA ESPEJANA

Gerard M. Bencik, Chih-Fang Wei, Donald L. Robinson and Horace B. Gray, Jr.

MATERIALS AND METHODS

Materials - Metal-activated hydroxide was purchased as a 1 M solution from K & E Chemicals (Denver, MD). All other chemicals were reagent grade or better, unless otherwise indicated in the text.

Duplex RNA - Some of the individual steps in the procedure used are modifications of those described by Wickner and Leibovitz (7) and Vojnov et al. (13). All glassware was baked in a stream of dry air for 2 hours prior to use, and all solutions were deionized and passed through amberlite XAD needles. The RNA was prepared from straw mushroom (Agaricus bisporus) 8 days after inoculation according to the method described by Francieli et al. (11). The RNA was dissolved in 0.5 M NaCl, 1.5 M sodium acetate (pH 7.0) (0.5 M SSC), most of the sodium acetate was removed by filter sterilization and the RNA was precipitated with ethanol-amine-salt and acetic acid to a final concentration of 0.1 M, 0.1 M, and 2 M, respectively.

1) Gel Electrophoresis and Band Sedimentation - Samples of reaction mixtures were separated on a 5% polyacrylamide gel slab after fractionation on a 5% polyacrylamide gel slab. The gels were prepared in a gel maker and were run at 5 V/cm for 5 hours. The gels were stained with ethidium bromide and photographed. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

2) Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

3) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

4) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

5) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

6) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

7) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

8) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

9) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

10) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

11) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

12) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

13) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

14) Urea-Polyacrylamide Gel Electrophoresis - The RNA was electrophoresed at 130 V for 2 hours and the gel was stained with ethidium bromide. The results were confirmed by urea-polyacrylamide slab gel electrophoresis. The samples were prepared as described (13) for use as an internal standard. The polycrystalline RNA was added to each sample prior to electrophoresis and the RNA concentration was determined by spectrophotometry after staining with ethidium bromide.

15) Urea-Polyacrylamide Gel Electrophoresi
Exonucleolytic Degradation of Duplex RNA

The population of shortened duplexes observed in Fig. 3 upon very brief exposure to nuclease was not found in the gel electrophoretic (Fig. 3) or spin sedimentation analysis of another RNA sample after extensive nuclease-mediated degradation. This suggested that the RNA sample used in the electron microscopy experiments of Fig. 2 contained a significant fraction of molecules with nuclease-sensitive lesions, possibly strand breaks arising during storage of the sample. To test this possibility, an aliquot of this RNA sample was depurinated in formamide-urea and the resulting single-stranded molecules subjected to electron microscopy (Materials and Methods). Length distributions of these populations yielded a distribution very similar to that of Fig. 2B (data not shown). Choosing comparable ranges of size in each case to represent the most prevalent distribution, 95% and 96% of the single-stranded end duplex molecules, respectively, were in this high molecular weight population. This indicated that it is very likely that the RNA sample used in fact contained a small number of strand breaks per gap, in which case one or more nucleotides would be lost at each gap per strand from calculation of the Poisson equations 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 at gaps arising spontaneously in that particular sample of RNA upon storage. That such strand interruptions are not necessarily characteristic of all RNA is evidenced from the lack of material migrating as shorter RNA in the gel electrophoresis and immunoblotting analysis of partially nuclease-degraded samples from other RNA preparations. If the increase in sedimentation to yield shorter duplexes were introduced in the presence of nuclease, it is clear, given the fraction of shorter molecules appearing after very brief incubation (Fig. 2B), that a predominant population of longer molecules such as in Fig. 2, C to E, would not exist after the much more extended digestions corresponding to these histograms.

This point was further addressed in a series of experiments with a different RNA preparation. The nondigested RNA, mounted for electron microscopy using the standard aqueous Karnovsky procedure (10), had 73% of its mass distributed in segments of 40,000 to 200,000 kilobase pairs (data not shown). When an aliquot of this sample was 25% degraded as monitored by hyperchromicity, electron microscopy revealed that 80% of the mass was in a single peak in the interval 3.50 to 4.75 kilobase pairs (294 molecules examined). The ratio of the average length of the molecules comprising this peak to that of those in the peak representing nondigested RNA was 0.80 ± 0.06, in agreement with the length ratios determined from hyperchromicity. In contrast, the average length of the predominant population of duplex RNA remaining as judged by scanning from the base for the RNA sample of Fig. 2C was 2.44 ± 0.19 kilobase pairs. By assuming that no additional ends are generated during the digestion, all the lengths determined in this manner are those from the termini of the smaller duplex fragments generated rapidly in this sample upon exposure to nuclease. Such a discrepancy was found between the fractional lengths, relative to that shown in Fig. 2B, of the predominant populations of Fig. 2C to E, compared to the estimates from hyperchromicity.