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Isolation and sequence determination of the 3'-terminal regions of isotopically labelled RNA molecules

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ABSTRACT

The method which was developed for the selective isolation of 3'-terminal polynucleotides from large RNA molecules on columns of cellulose derivatives containing covalently bound dihydroxyboryl groups has been modified and adapted for use on radioactively labelled RNAs. The 3'-terminal polynucleotide fragments which result from specific ribonuclease digestion of isotopically detectable quantities of RNA can be selectively obtained in both high yield and purity by the modified procedure and can be subsequently analyzed by standard electrophoretic and chromatographic techniques. In addition, when the extent of enzymatic fragmentation of the RNA is controlled, the procedure permits the selective isolation of discrete "sets" of fragments of variable chain length, all of which derive from the 3'-terminus of the RNA molecule. These overlapping polynucleotides can be used directly to obtain extensive sequence information regarding the primary structure in the 3'-region of the RNA.

INTRODUCTION

A method for the selective isolation of 3'-terminal polynucleotides from high molecular weight RNAs has been previously described[1]. The isolation procedure involves the selective binding of the 3'- terminal nucleotide fragments which result from specific enzymatic cleavage of an RNA to columns of cellulose derivatives containing covalently bound dihydroxyboryl groups[2, 3]. As originally described the method was applicable only to those RNA species which could be obtained in relatively large quantity (e.g. bacteriophage RNAs, bacterial rRNA and tRNA, etc.), so that optical density amounts of their 3'-terminal polynucleotides could be isolated. Since a variety of RNA species can be obtained only in isotopically detectable quantities, it was desirable to adapt the existing procedures to the structural examination of these molecules. An important consideration in this adaptation was to insure that the labelled end fragments selectively isolated could be subsequently

analyzed by standard electrophoretic and chromatographic techniques. Several procedural modifications were required to achieve the desired results and these will be described. In addition, it will be demonstrated that this approach has general application to the direct determination of extensive nucleotide sequences adjoining the 3'-region of any RNA molecule.

MATERIALS AND METHODS

2-(N-Morpholino)ethanesulfonic acid and E. coli tRNA were purchased from Calbiochem, San Diego, Calif. The tRNA was repurified by three extractions with phenol followed by precipitation with cold ethanol. Morpholine was obtained from the Aldrich Chemical Co., Milwaukee, Wis., and redistilled before use. The N-[N'-(m-dihydroxyborylphenyl)succinamyl]-aminoethylcellulose (DBAE cellulose) was prepared as previously reported[2]. Homochromatography solvents a and b were prepared according to the procedure described by Brownlee and Sanger[4].

The 6S RNA species was prepared by transcribing λ phage DNA (in vitro) with purified E. coli RNA polymerase (as described by Lebowitz, et al[5]). The [α-^{32}P] labelled nucleoside triphosphates used in the syntheses were purchased commercially from New England Nuclear Corp., Boston, Mass. The 6S RNA transcript was purified by electrophoresis on polyacrylamide gels (5%) which contained 8M urea.

The discrete T7 RNA species which had been uniformly labelled in vivo with ^{32}P and purified on polyacrylamide gel (2.5%, 0.5% agarose) was kindly supplied by R. Kramer.

Complete enzymatic digestion of RNA with T_1 ribonuclease (E.C. 3.1.4.8) was carried out by incubating the RNA for 45 to 60 minutes at 37°C with T_1 RNase in 0.01M Tris-HCl, 0.001M EDTA, pH 7.5 at an enzyme to substrate ratio of 25 units of T_1 RNase per 100 μg of RNA.

Partial enzymatic fragmentation of RNA with T_1 ribonuclease was performed by incubating the RNA for 10 to 20 minutes at 4°C with T_1 RNase in 0.01M Tris-HCl, 0.02M $MgCl_2$, pH 7.5 at enzyme to substrate ratios of 0.5 to 1.0 unit of T_1 RNase per 100 μg of RNA.

RESULTS AND DISCUSSION

A. Isolation of 3'-Terminal Polynucleotides from Extensively Digested RNAs.

The procedures for the selective isolation of 3'-terminal nucleotide fragments from isotopically labelled RNAs have all been performed on columns of the N-[N'-(m-dihydroxyborylphenyl)succinamyl]aminoethyl-cellulose derivative (DBAE cellulose). This material consistently exhibits high efficiency in retaining the terminal fragments and also demonstrates extremely low levels of background radioactive contamination when proper elution conditions are used. Initial use of the DBAE cellulose columns with small quantities of isotopically labelled RNAs can result in substantial losses of nucleotide material. These losses, which are apparently due to the irreversible binding of nonspecific nucleic acid material to the cellulose derivative, can be significantly reduced by application of the chromatographic procedure to several milligrams of unlabelled ribonucleotide fragments prior to use with a labelled sample (~5.0 mg of RNA for each 15 ml bed volume of DBAE cellulose). This step effectively "saturates" the problematic binding sites and does not have to be subsequently repeated. Sample losses and background contamination can be further reduced by the addition of small quantities (50-150 μg) of unlabelled carrier RNA to each radioactively labelled sample. Prior to each use, the columns are thoroughly prewashed first with Solvent B (0.05M sodium 2-(N-morpholino)ethane sulfonate; 1.0M NaCl, 20% dimethylsulfoxide, pH 5.5) and then with Solvent A (0.05M morpholinium chloride, 0.1M $MgCl_2$, 1.0M NaCl, 20% dimethylsulfoxide, pH 8.7). The DBAE cellulose derivative is relatively stable and can be stored in Solvent B. Columns of this material have been used repeatedly at room temperature for over a year with little apparent loss of retention capacity.

1. Elution procedure

A typical elution profile demonstrating the selective isolation of a 3'-terminal nucleotide fragment from an isotopically labelled RNA is shown in Figure 1. In this case, a discrete 6S RNA species of known nucleotide sequence[5] is used to demonstrate the procedure. The RNA ($4x10^5$cpm) is dissolved

in 0.01M Tris-HCl, pH 7.5 (10μl) containing non-radioactive carrier tRNA (50μg) and digested to completion with ribonuclease T_1. The mixture is then made 0.1M $MgCl_2$, 1.0M NaCl, 20% in dimethylsulfoxide (DMSO) and the pH adjusted to 8.7 by the addition of morpholine (final sample volume 1.0 ml). The digest is applied to a column (30x0.6 cm) of DBAE cellulose which has been pretreated as described above. Eltuion is then carried out with Solvent A at a flow rate of 4 ml/hr until most of the radioactive material (Peak A, Fig. 1) has been eluted from the column (i.e. column effluent counts less than 400 CPM/ml). A sharp change to Solvent B is then made at the top of the column and elution is continued (flow rate 6 ml/hr) until the pH of the column effluent drops to pH 5.5. Two peaks of radioactive material (Peaks B and C, Fig. 1) usually appear in the column effluent during this second chromatographic elution step. Peak B, which may vary considerably in quantity, elutes from the column one void volume after the change to Solvent B and appears to consist of nonspecific oligonucleotide material derived from the nuclease digested RNA. Peak C, however, elutes approximately 1 2/3 void volumes after the solvent change and consists specifically of the purified 3'-terminal oligonucleotide product. The absence of Mg^{++} in chromatography Solvent B during the second elution step is apparently responsible for the chromatographic separation of Peaks B and C. If the elution is carried out with Solvent B containing 0.1M $MgCl_2$, then Peak B co-chromatographs with Peak C, thereby substantially reducing the purity of the isolated terminal fragment. Monitoring the Mg^{++} concentration and the pH of the column effluent during the elution procedure (Fig. 1) indicates that Peak B elutes precisely at the drop in effluent Mg^{++} concentration whereas Peak C elution coincides with the abrupt change in effluent pH.

2. Characterization of the terminal fragment

The 3'-terminal oligonucleotide product (Peak C from Fig. 1, volume$\sim$ 1.0 ml) is desalted by dialysis for 18 hours against 3 changes of 6 liters each of distilled water. Several other desalting techniques have been examined (e.g. Sephadex G-25 chromatography, Bio-Gel P-2 chromatography, Amicon

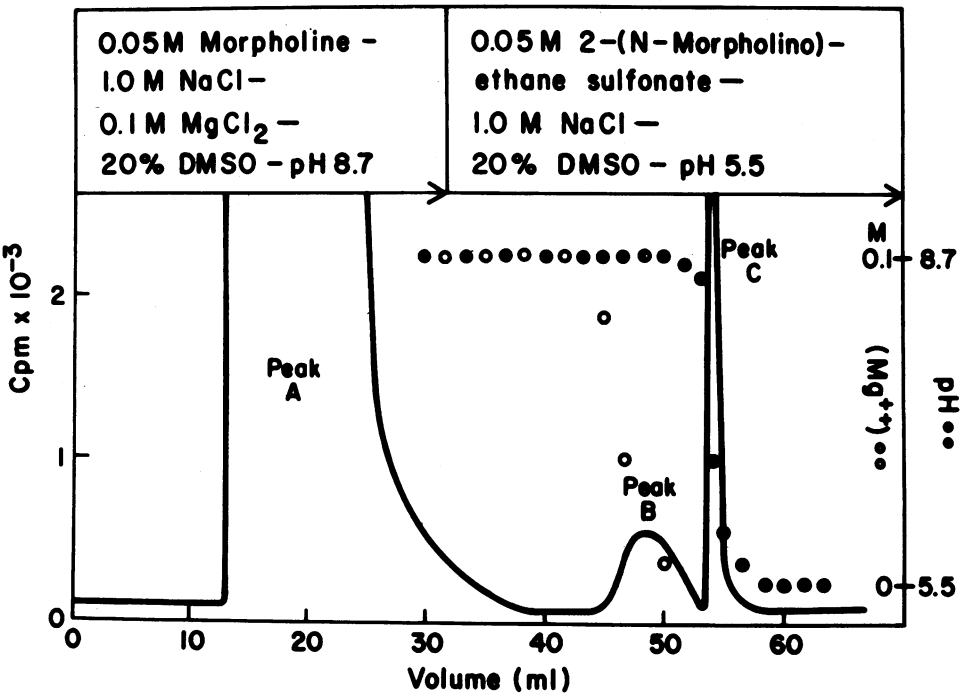


Figure 1

Elution pattern obtained from the chromatography of a ribonuclease T_1 digest of the isotopically labelled 6S RNA on a column (30 x 0.6 cm) of DBAE cellulose. The elution was effected with the two solvents as shown at flow rates of 4 ml/hr and 6 ml/hr, respectively. The Mg^{++} concentration (ooo) and the pH (●●●) of the effluent were monitored.

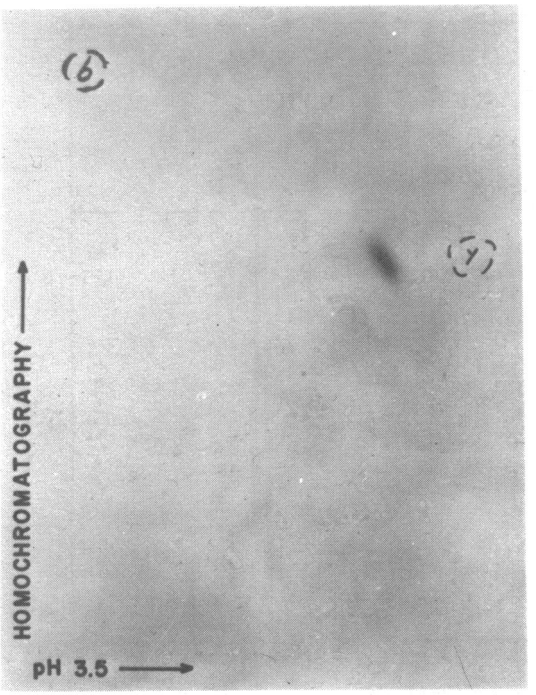


Figure 2A

Autoradiograph of a two-dimensional fractionation of the terminal oligonucleotide (Peak C, Fig. 1) selectively isolated from the 6S RNA labelled with (α-^{32}P) UTP. First dimension: electrophoresis on Cellogel in 8·0 M urea at pH 3·5[6,7]. Second dimension: ascending thin layer chromatography on plates of DEAE cellulose (9:1 cellulose to DEAE cellulose, 40 x 20 cm) using homochromatography solvent b[4]. Marker dyes xylene cyanol (b) and methyl orange (y) are indicated.

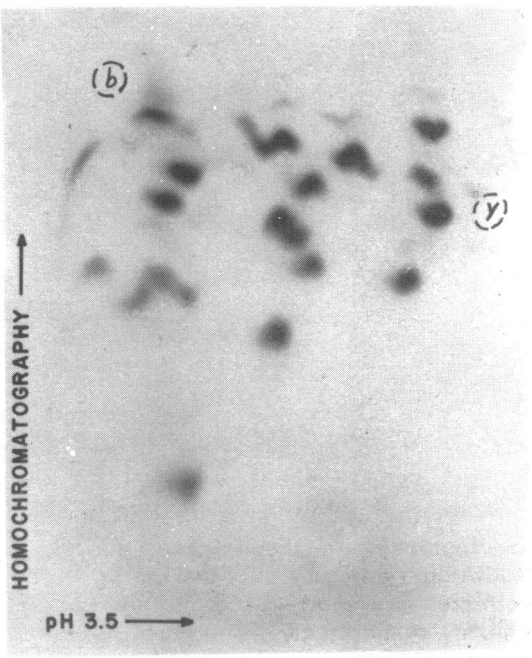


Figure 2B

Autoradiograph of a similar two-dimensional fractionation of the oligonucleotide products resulting from a complete T_1 RNase digestion of the 6S RNA labelled with (α-^{32}P) UTP.

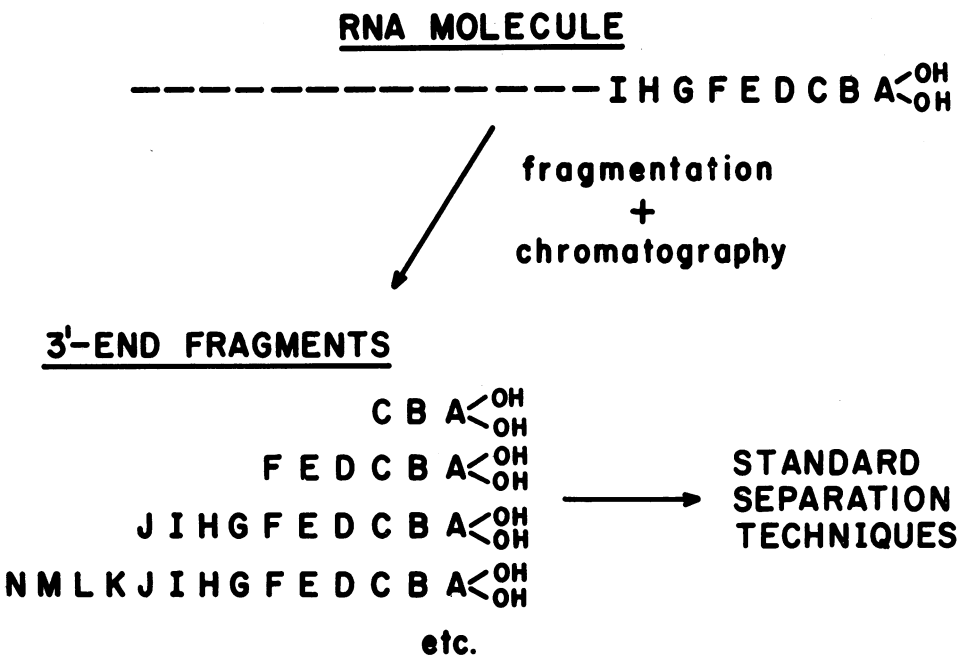


Figure 3

Diagramatic outline describing the general procedure for the selective isolation of discrete "sets" of overlapping polynucleotide fragments all of which derive from the original 3'-terminus of the RNA. A designates nucleotide sequence information at the 3'-terminus of the RNA. B, C, D, etc. designate additional sequence information extending into the RNA molecule.

ultrafiltration) however the dialysis procedure most consistently resulted in good yield of a product which could be readily characterized by standard electrophoretic and chromatographic techniques. The dialysate (volume ~ 1.2 ml) is concentrated to 10µl by rotary evaporation in vacuo at 35°C and then directly analyzed by a two-dimensional fractionation procedure. The first dimension consists of electrophoresis on cellulose acetate (Cellogel) in 8M urea at pH 3.5[6, 7] followed by a second dimension homochromatographic separation[4] on TLC plates of DEAE cellulose (9:1 cellulose/DEAE cellulose, 40 x 20cm). In the case of the 6S RNA species this procedure results in the resolution of a single, highly purified oligonucleotide product (Fig. 2A). Subsequent sequence analysis of this oligonucleotide confirmed that it was an octanucleotide with a primary structure identical to that previously determined for the 3'-terminus of the 6S RNA[5]. Comparison of the two-dimensional separation of the isolated end fragment with an identical "fingerprint" analysis of all the oligonucleotide products resulting from the T_1 RNase digestion of the 6S RNA (Fig. 2B) indicates the selectivity of the chromatographic isolation procedure. This was further demonstrated by two-dimensional fractionation of the oligonucleotide material which is not retained on the DBAE cellulose (Peak A, Fig. 1). This "fingerprint" is identical to that shown in Figure 2B except for the distinct absence of the 3'-terminal oligonucleotide.

The 3'-terminal isolation procedure has now been applied to a number of isotopically labelled discrete RNA species from a variety of in vivo and in vitro sources[8, 9, 10]. These RNAs have ranged in size from 80 to 3500 nucleotide residues with the 3'-end fragments representing as little as 0.1% of the total nucleotide material. In each case the 3'-terminal oligonucleotide fragment(s) was selectively isolated in both high purity and yield (70-90% of theoretical) by the described procedures. In addition, the high sensitivity of the procedure has allowed the detection of minor terminal sequences which may occur at levels as low as 5% of molar quantity. This has proved especially useful in examining the termini of certain RNA species which apparently contain varying degrees of heterogeneity at their 3'-ends[8, 9].

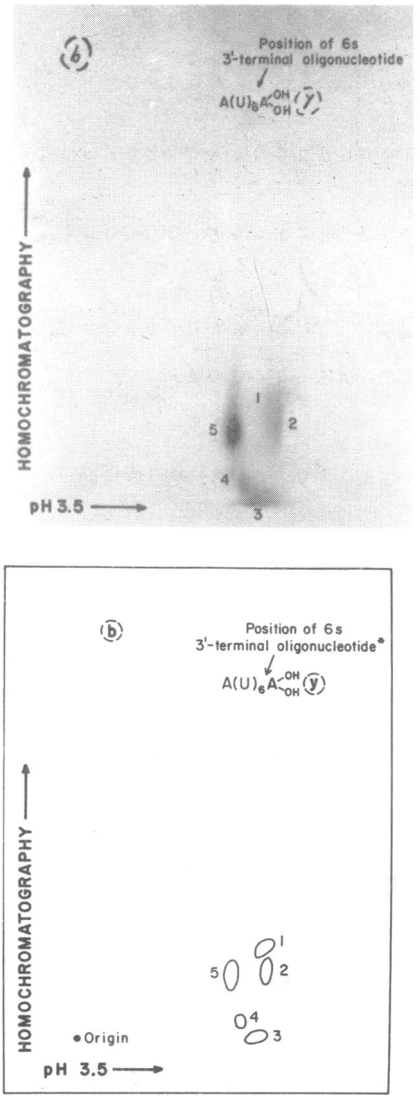


Figure 4

Two-dimensional fractionation of the polynucleotide fragments selectively isolated on DBAE cellulose which result from a limited T_1 RNase digestion of the 6S RNA labelled with (α-^{32}P) ATP. Fractionation conditions were similar to those described in Figure 2 except that the DEAE thin layer chromatography was effected with homochromatography solvent a[4]. A, autoradiograph. B, schematic sketch of autoradiograph.

*the 3'-terminal oligonucleotide which results from complete enzymatic digestion of the 6S RNA with T_1 RNase (as shown in Fig. 2A).

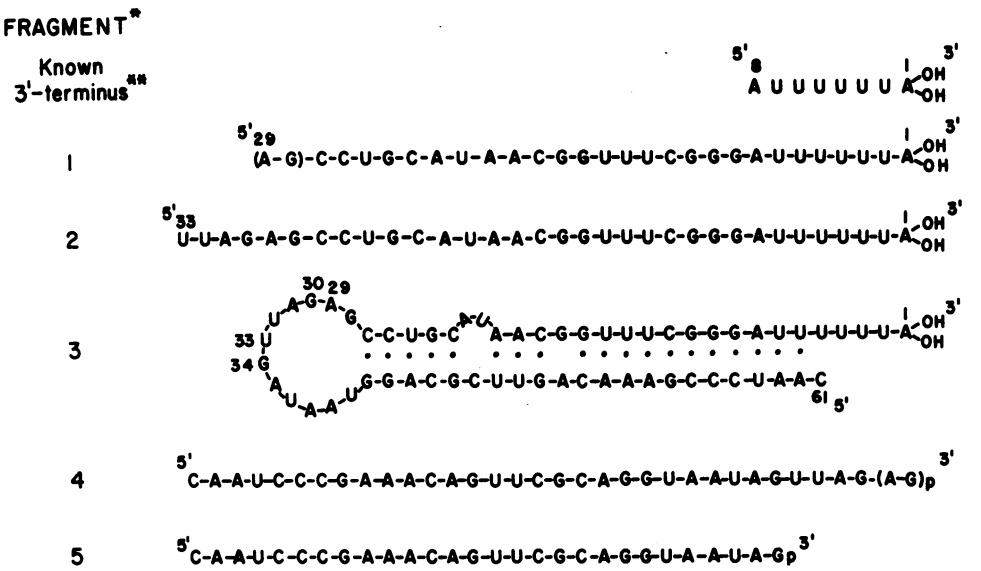


Figure 5

Nucleotide sequences derived for each of the polynucleotide fragments selectively obtained from the 6S RNA (as shown in Fig. 4). Each fragment was redigested to completion with T_1 RNase and the component products further analyzed by digestion with pancreatic RNase. All enzymatic fragmentation products were characterized by standard electrophoretic procedures[6, 7, 11]. The sequences could be deduced by comparison of the results of these compositional analyses to the known nucleotide sequence of the 6S RNA[5]. The sequence of fragment 3 is drawn in a possible pattern of base-pairing for the 3'-terminal region of the 6S RNA (as originally proposed by Lebowitz, et al)[5]. Solid dots represent Watson-Crick type base-pairing. Nucleotide residues are numbered consecutively starting at the 3'-terminus of the RNA.

* Fragment number corresponds to numbered designation given in Figure 4.
** 3'-terminal oligonucleotide which results from complete enzymatic digestion of the 6S RNA with T_1 ribonuclease.

Selective hybridization techniques are now being used to isolate a variety of unique RNA species in sufficient quantity and purity for structural examination. These procedures usually result in the introduction of numerous single strand scissions within an RNA molecule. It is possible that the new termini generated by these scissions would contain terminal 2', 3'-diol groups and therefore appear as contaminants when the original 3'-terminus of the RNA was isolated. Preliminary results suggest that 3'-terminal nucleotide fragments from RNAs which have been subjected to rather severe conditions of hybridization (20 hours at 75°C in 2 x SSC) can be selectively isolated in both high purity and yield. Apparently most of the strand breaks which occur in the RNA result in the formation of polynucleotide fragments which contain phosphate moieties at their 3'-termini and thus do not interfere with the isolation procedure.

B. Extension to Partially Fragmented RNAs and Application to Direct Sequence Studies.

The approach for obtaining specific 3'-terminal oligonucleotides from isotopically detectable quantities of RNA can also be applied to the selective isolation of larger polynucleotide fragments from the 3'-terminal regions of these RNAs. The procedure (diagramatically outlined in Fig. 3) can be used to obtain discrete "sets" of overlapping polynucleotide fragments all of which derive from the original 3'-end of the RNA. The RNA is first enzymatically fragmented under conditions which specifically limit the extent of nuclease digestion (e.g. low enzyme concentration, low temperature, short time of incubation). Subsequent to digestion the enzyme is removed or inactivated and the partially fragmented nucleotide products chromatographed on columns of DBAE cellulose. Those polynucleotide fragments which derive from the original 3'-terminus of the RNA are selectively retained and subsequent to elution are easily fractionated by standard techniques. The terminal fragments isolated by this procedure will all contain a common 3'-terminus, which by virtue of the isolation procedure will be the original 3'-end of the RNA. Variations in chain length among these polynucleotides must occur only at their 5'-ends. Thus, successively longer fragments can be used

directly to sequentially order the additional primary structural information contained in the overlapping segments. Relative placement of this sequence information within the fragment, in turn, directly establishes the position of these sequences within the original RNA molecule relative to its 3'-terminus.

Application of this procedure to certain RNA species, especially those known to contain considerable secondary structure in their 3'-terminal regions (e.g. tRNAs, 5S RNAs, etc.) may also result in the selective isolation of specific polynucleotide fragments which neither derive from the 3'-terminus of the RNA nor contain a nucleoside residue at their 3'-ends (see Figures 4 and 5). These additional fragments are retained on the DBAE cellulose columns not by their direct interaction with the derivatized cellulose matrix, but rather by their apparent ability to form stable base-paired complexes with the 3'-terminal polynucleotides. Fragments bound in this manner must contain nucleotide sequences which specifically complement sequence information present in the 3'-terminal polynucleotides. These complementary regions must be of sufficient length to result in base-paired complexes which remain stable throughout the limited enzymatic fragmentation and subsequent chromatographic isolation procedures. The entire duplex structure is then selectively obtained by virtue of the single cis-diol function on the 3'-nucleoside residue of the terminal polynucleotide fragment. The components of the complex are subsequently resolved by standard two-dimensional fractionation procedures and are readily identified as 3'-terminally derived or non-terminally derived. Polynucleotides containing the original 3'-terminus of the molecule will characteristically possess a 3'-terminal nucleoside residue (...$N\begin{matrix}OH\\OH\end{matrix}$), whereas internally derived polynucleotides will contain an easily distinguishable phosphoryl moiety at their 3'-ends (...Np).

1. Application to an RNA of known nucleotide sequence

The 6S RNA species described previously was used to demonstrate the general isolation procedure. The RNA (6×10^5 CPM) was dissolved in 0.01M Tris-HCl, 0.02M $MgCl_2$, pH 7.5 (20µl) which contained non-radioactive carrier tRNA (50µg) and was enzymatically fragmented under limiting conditions

with T_1 ribonuclease (see experimental section). The digestion mixture was then extracted twice with water saturated phenol containing 0.1% SDS (0.1 ml aliquots). Excess phenol was removed from the sample by extraction with ether and the remaining ether evaporated off under a stream of nitrogen. An equal volume of 0.1M Morpholinium-HCl, 0.2M $MgCl_2$, 2.0M NaCl, 40% DMSO, pH 9.0 was added and the total volume brought to 1.0 ml with Solvent A. The digest was then applied to a column of DBAE cellulose (30 x 0.5 cm) and processed as described previously.

The elution profile obtained was similar to that shown in Figure 1 except that a greater proportion of nucleotide material was selectively retained on the column (approximately 25%), indicative of the isolation of larger terminal polynucleotide fragments. Subsequent to elution, the material obtained was fractionated and characterized by a two-dimensional procedure similar to that described earlier, but capable of resolving polynucleotides of somewhat longer chain length[4]. In the case of the 6S RNA species a number of discrete polynucleotide fragments were obtained (Fig. 4). These fragments were eluted from the DEAE cellulose plate, redigested to completion with T_1 ribonuclease, and the products analyzed by electrophoresis in one dimension on DEAE cellulose paper at pH 1.7[6, 11]. Each of the complete T_1 ribonuclease digestion products was further examined by digestion with pancreatic ribonuclease and subsequently characterized by standard electrophoretic fractionation on DEAE paper at pH 3.5. By comparison of the results of these compositional analyses with the known primary structure of the 6S RNA, each of the isolated polynucleotide fragments designated in Figure 4 could be readily identified and its complete nucleotide sequence established (Fig. 5).

In this particular experiment all of the fragments selectively isolated from the 6S RNA were relatively large polynucleotides containing several internally located guanosine residues that had not been cleaved during the initial limited enzymatic fragmentation. The 3'-terminal oligonucleotide known to result from a complete T_1 ribonuclease digestion of this RNA[5] was

not observed, however its characteristic position and sequence are shown in Figures 4 and 5. Polynucleotide fragments designated 1, 2, and 3 contain this terminal oligonucleotide and were consequently shown to derive specifically from the 3'-end of the 6S RNA. These three fragments contain identical 3'-termini and vary in chain length only at their 5'-ends, extending approximately 29, 33, and 61 nucleotide residues respectively into the RNA molecule. Examination of these successively longer polynucleotides indicates that the sequence information contained in the additional nucleotide segments of increasingly larger fragments must be positioned at the 5'-end of the fragment. This information can be applied to the direct sequential ordering of primary structure both within a particular fragment and, in turn, within the original RNA molecule relative to its 3'-terminus. This rationale has been used to determine primary structure in the 3'-terminal regions of a number of RNA species and will be demonstrated in greater detail in the following section.

In addition to the 3'-terminal fragments, two other discrete polynucleotides (designated 4 and 5, Figures 4 and 5) were obtained which neither derive from the 3'-terminus of the 6S RNA nor contain nucleoside residues at their 3'-ends. These fragments were shown to be positioned internally in the RNA molecule approximately 30 to 60 nucleotide residues distal to the 3'-end and were found to contain guanosine 3'-phosphate residues (Gp) at their 3'-termini. As predicted, examination of the primary structure of these polynucleotides indicated that their nucleotide sequences would permit extensive base-pairing with the polynucleotide fragments derived from the 3'-terminus (as originally proposed by Lebowitz et al[5], and indicated for fragment 3 in Figure 5). These structural interactions apparently result in the formation of stable, non-covalently bound, double standard complexes between the internally derived fragments (fragments 4 and 5) and the terminal polynucleotides (fragments 1 and 2).* These duplex structures are selectively retained

*Structures of this type are readily generated by specific enzymatic cleavage of fragment 3(Fig. 5) at positions 28, 30 and/or 34.

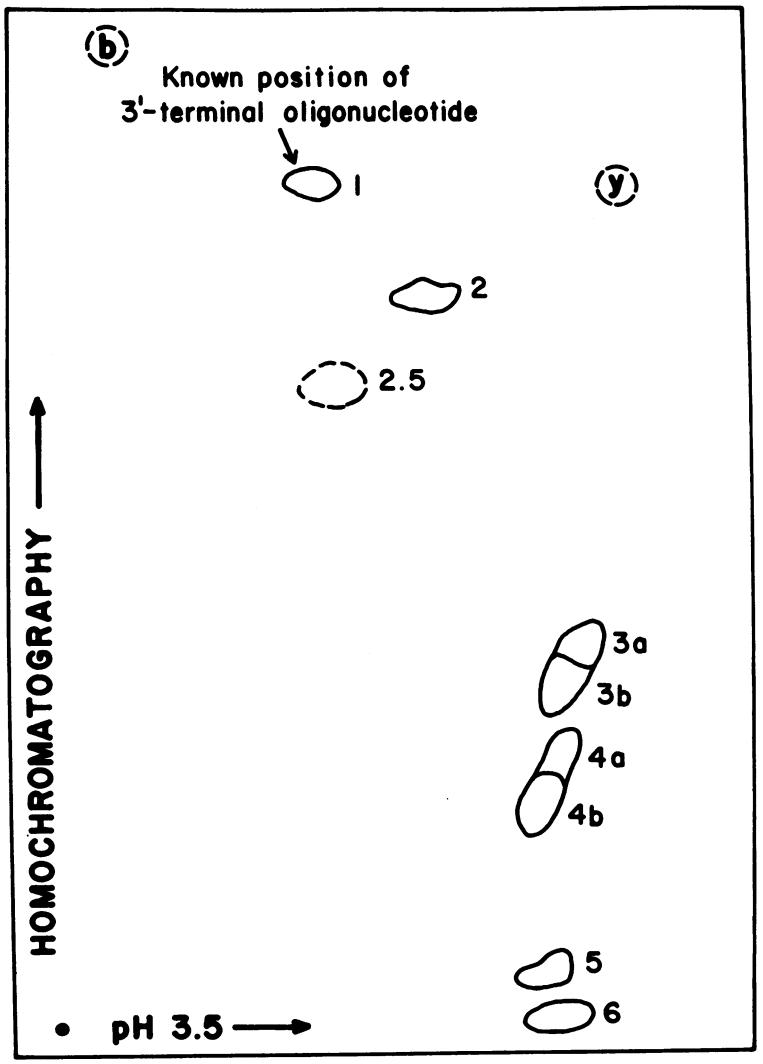
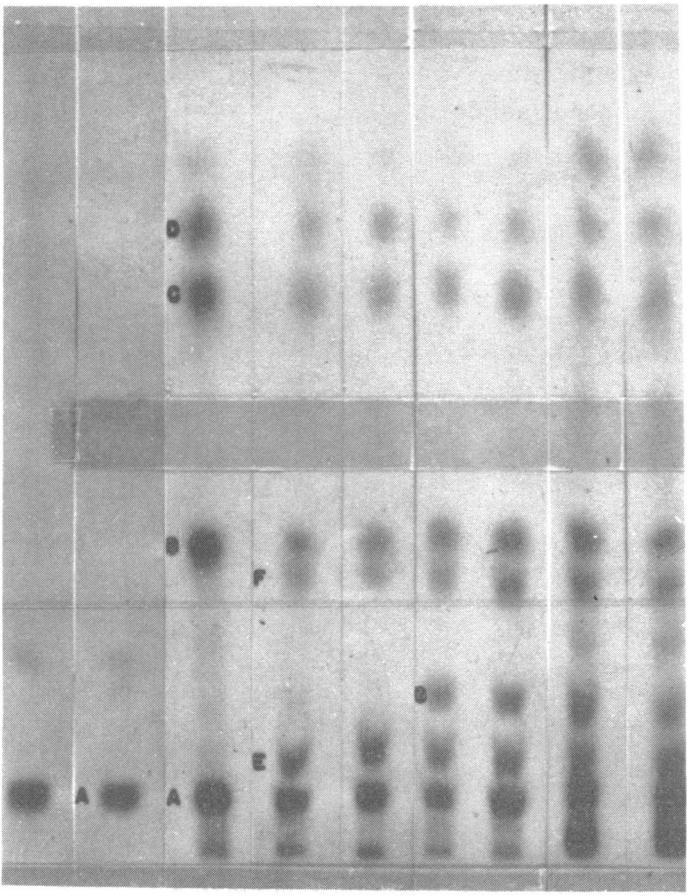


Figure 6

A composite sketch of two autoradiographs obtained from separate experiments which shows a two-dimensional fractionation of the polynucleotide fragments isolated on DBAE cellulose from limited T_1 RNase digestions of the G 0.3 T7 RNA uniformly labelled with ^{32}P. Fractionation conditions were identical to those described in Figure 4. Spots 3 and 4 tended to streak in the second dimension and were divided into a and b components (as indicated) for subsequent analyses. Fragment designated 2.5 was never present in sufficient quantity for secondary analysis. (•) origin of electrophoresis



Fragment*	Derived sequential order of oligos
1	$A<^{OH}_{OH}$
2	$(B,C,D)A<^{OH}_{OH}$
3	$(E,F)(B,C,D)A<^{OH}_{OH}$
4	$G(E,F)(B,C,D)A<^{OH}_{OH}$
Etc.	

Figure 7

Autoradiograph of a one-dimensional separation of the component oligonucleotides which resulted from the complete T_1 RNase digestion of each of the polynucleotide fragments indicated in Figure 6. The digestion products are fractionated on DEAE paper by electrophoresis at pH 1.7[6]. The alphabetical designation assigned to the component oligonucleotide products and the derivation of their sequential order are explained in the text.

*Fragment number corresponds to numerical order designated in Figure 6.

on the DBAE cellulose and the component products resolved only after two-dimensional fractionation.

The ability of certain polynucleotide fragments to specifically form stable complexes with the 3'-terminal fragments apparently does not interfere with the effective isolation or subsequent analyses of the 3'-terminal polynucleotides. In fact, the selectivity of isolation and the relative ease of separation and identification of these additional fragments can be readily applied to the structural examination of specific regions of an RNA molecule which potentially interact with the 3'-terminal region. Thus, extensive primary structural information is obtained as well as possible information which pertains to specific secondary structural characteristics of the molecule and overall molecular configuration.

2. Application to an RNA of unknown nucleotide sequence

The procedures described for the 6S RNA were applied to the examination of the 3'-terminal region of a discrete high molecular weight RNA species of unknown primary structure. The RNA species used was approximately 750 nucleotide residues in chain length and was known to correspond to the bacteriophage T7 G 0.3 early messenger RNA transcript[9, 12, 13].

The 3'-terminal oligonucleotide fragment which resulted from a complete T_1 ribonuclease digestion of this RNA was initially isolated and extensively characterized[9]. This unique oligonucleotide was subsequently used to identify larger polynucleotide fragments which derived specifically from the 3'-terminal region of the T7 transcript. The RNA was subjected to limited enzymatic fragmentation with T_1 ribonuclease (see experimental section) and chromatographed on DBAE cellulose. Several polynucleotide fragments were selectively isolated and initially characterized by standard two-dimensional fractionation[4] (Fig. 6). Each fragment obtained (1, 2, 3, etc., numbered in order of increasing chain length) was redigested to completion with ribonuclease T_1 and analyzed by electrophoresis in one dimension on DEAE cellulose paper at pH 1.7[6, 11]. The results of this analysis are shown in Figure 7.

Fragment 1 underwent no further cleavage upon reincubation with T_1 ribonuclease and subsequent analysis of this product has identified it as the limit T_1 digestion product derived from the 3'-terminus of the RNA. Redigestion of

fragment 2 resulted in the identification of four oligonucleotide fragmentation products designated oligos A, B, C, and D(see Fig. 7). Oligo A corresponded to fragment 1, the original 3'-terminus of the RNA, whereas oligos B, C, and D contained guanosine 3'-phosphate residues at their 3'-termini and therefore represented additional enzymatic cleavage products of fragment 2. The sequence information contained in these additional oligonucleotides must be positioned to the 5'-side of the terminal oligonucleotide (as indicated at the bottom of Fig. 7). A similar analysis of fragment 3 resulted in the identification of six oligonucleotide components. Four of these, oligos A(the 3'-terminus), B, C, and D were identical to those found in fragment 2, thereby indicating that this fragment was derived from the original 3'-terminus of the RNA. Since all chain length variations among terminal fragments must occur at their 5'-ends, the two new additional oligonucleotide components of fragment 3, oligos E and F, must be positioned at the 5'-end of this fragment immediately adjacent to the 5'-terminus of fragment 2. A similar examination of the oligonucleotide products derived from polynucleotide fragment 4 resulted in the identification of a single, new oligonucleotide component (G), which could be unambiguously positioned at the 5'-end of this fragment, immediately adjacent to oligos E and F.

Continued application of this rationale would permit the identification and relative sequential positioning of many of the component oligonucleotide products contained within the terminal polynucleotide fragments. By simply altering the conditions of the initial limited enzymatic fragmentation of the RNA and by varying the specific enzymes used for both the partial digests and the subsequent analyses of the polynucleotide products, it would be readily possible to obtain enough sequence information to directly establish the sequential order of extensive regions of primary structure near the 3'-terminus of this RNA.

This methodology is now being effectively applied to the primary structural investigation of the 3'-terminal regions of a variety of isotopically labelled RNAs. Partially fragmented polynucleotide products up to approximately 60

nucleotide residues in chain length have been selectively isolated and characterized using these procedures. The ability to specifically retain intact $tRNA^{14}$ and 5S rRNA (unpublished result) on columns of the DBAE cellulose and subsequently recover them in good yield indicates that 3'-terminal polynucleotide fragments 100-150 nucleotide residues in chain length are potentially obtainable. This will permit the examination of rather extensive stretches of RNA structure in the 3'-terminal region of an RNA molecule.

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