Addition of Poly(adenylic acid) to RNA Using Polynucleotide Phosphorylase: An Improved Method for Electron Microscopic Visualization of RNA–DNA Hybrids†

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ABSTRACT: We have observed that the enzyme polynucleotide phosphorylase from *M. luteus* or from *E. coli* will polymerize adenosine (A) from adenosine diphosphate onto 3′ ends of RNA molecules. For gene mapping, the poly(A)-tailed RNA is hybridized to its complementary sequence on a longer DNA strand. The position of the poly(A) tail, and thus the position of the 3′ end of the RNA on the DNA strand, can then be observed by electron microscopy. Our preferred mapping technique involves the synthesis of a poly(A)-specific label by polymerization of a poly(dBrU) tail onto one or both ends of a linear duplex DNA of defined length (a restriction fragment) and hybridization of this label to the poly(A) tail. In test experiments with a plasmid containing a *Drosophila* DNA sequence coding for 5S rRNA genes, overall labeling efficiencies of 70–80% were achieved.

Electron microscopic mapping of the position of RNA–DNA hybrid regions is a valuable method for determining the positions of genes on defined DNAs. One method in use in this laboratory involves the attachment of an electron opaque label such as ferritin (Angerer et al., 1976; Hershey et al., 1977; Yen et al., 1977; Broker et al., 1978) or polymer spheres (Manning et al., 1975; Sodja and Davidson, 1978) to the 3′ end of the RNA to assist in visualization of the RNA–DNA hybrid. This general approach, while effective, has some disadvantages. Several complex synthetic steps are necessary for the preparation of the modified RNA and the derivatized electron opaque label. More importantly, the overall efficiency of labeling is usually less than 50%.

An efficient simple method of mapping poly(A) segments on RNA molecules has been developed (Wensink et al., 1974; Hsu et al., 1973; Bender and Davidson, 1976). The technique used in this laboratory involves polymerization of poly(dT) tails onto internal 3′ termini of nicked circular duplex DNAs, such as SV40 DNA, with the enzyme terminal deoxynucleotidyltransferase. The poly(dT) pairs with the poly(A) on the RNA; the circular duplex DNA is a readily recognized label in the electron microscope. It was later shown that poly(dBrU) is a still better affinity reagent for reaction with poly(A) tails, because the stability of A–BrU base pairs is greater than that of A–T base pairs (W. W. Bender, personal communication). The A–T and A–BrU association reactions are rapid and require only a slight excess of poly(dT) or poly(dBrU) to poly(A). Used in this way, a labeling efficiency of greater than 75% is achieved for poly(A) ends on natural poly(A)-containing RNAs (Bender and Davidson, 1976).

In order to extend this simple and efficient gene-mapping approach, we have developed techniques for adding poly(A) tails to the 3′-OH end of RNAs—for example, tRNA, 5S RNA or poly(A)$^-$ mRNA—that do not naturally have a poly(A) tail. We find that the enzyme polynucleotide phosphorylase will add A residues (from adenosine diphosphate) onto the 3′ ends of all RNA molecules tested. Furthermore, our tests indicate a high efficiency of gene mapping with these RNA molecules.

We also report on the preparation of linear restriction fragments with one or two poly(dBrU) tails at the ends. These labels are particularly useful for the kinds of mapping experiments described here.

Materials and Methods

Chemicals. rADP, r[^{3}H]ADP, and oligo(dT)-cellulose (T3) were purchased from Boehringer-Mannheim, Schwarz-Mann, and Collaborative Research, respectively.

Enzymes. Polynucleotide phosphorylase (PNPase[1]) preparations were the generous gifts of Drs. T. Colburn and J. Dodgson (*E. coli* B) and C. Klee (*M. luteus*). The *E. coli* PNPase (from J.D.) had been purified through the Sephadex G-200 step (Dodgson and Wells, 1977); the polymerization activity was 600 μmol of rADP incorporated per 15 min per A_{280} protein. The *M. luteus* PNPase-I (primer independent) was purified as described (Klee and Singer, 1968) and had an activity of approximately 300 μmol of rADP incorporated (at 37 °C) per 15 min per A_{280} protein; the PNPase-T (primer dependent) was obtained by mild trypsin treatment of PNPase-I and then blocking sulfhydryl groups with *N*-ethylmaleimide (Klee, 1968); PNPase-T activity was approximately 200 μmol of rADP incorporated per 15 min per A_{280} enzyme. Terminal deoxynucleotidyltransferase ("minimal nuclease") was purchased from P-L Biochemicals. Restriction endonucleases *Eco*R1, *Hin*dIII, and *Bam*H1 were purchased from New England Biolabs. Bacterial alkaline phosphatase (BAPF) was purchased from Worthington Biochemicals.

Gel Electrophoresis. Neutral agarose (Helling et al., 1974), 8% acrylamide, 98% formamide (Maniatis et al., 1975), and CH_3HgOH–agarose slab gels (Bailey and Davidson, 1976) were run as described in the figure legends and text.

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[1] Abbreviations used are: nt, nucleotides (chain length); PNPase, polynucleotide phosphorylase from either *E. coli* or *M. luteus* (EC 2.7.7.8) (specified in text); divalent and monovalent labels, poly(BrU) polymerized onto one or both ends of a plasmid DNA restriction fragment (see text); kb, kilobase (1000 nucleotides); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; Pipes, 1,4-piperazinediethanesulfonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

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DNA and RNA. D. melanogaster RNAs ([^{3}H]uridine labeled and unlabeled) were isolated as described by Hershey et al. (1977). Col E1, pCIT19, and pDm103 plasmid DNAs were the generous gifts of P. Yen, S. Conrad, and M. Cohen. pMB9 DNA was prepared by a modification of the procedure of Hirt (1967; M. So, personal communication).

For experiments on poly(A) labeling of rRNA, *Drosophila melanogaster* (18 + 28)S rRNA was cleaved to an average length of about 250 nt by incubation at 70 °C in 0.1 M sodium borate, pH 9.2, for 25 min (Bock, 1967). The hydrolyzed RNA was fractionated by gel filtration over a Sepharose 2B column. The fraction used had an average size of 250 nt and a range of approximately 100 to 600 nt, as estimated by gel electrophoresis in the presence of CH_3HgOH. This RNA was treated with bacterial alkaline phosphatase to remove 3′-phosphates before poly(A) addition.

Enzymatic Addition of Poly(A) to RNA. These reactions were carried out in a medium of 10 mM sodium citrate, 1.6 mM $MgCl_2$, 50 mM Tris (pH 8.0), 200 μM rADP, and RNA and PNPase concentrations as given under Results. Unless otherwise specified, incubations were for 6 min at 37 °C. The reaction was stopped by the addition of an equal volume of neutral EDTA-saturated phenol. The aqueous phase was withdrawn and the phenol–aqueous interphase was reextracted once with 0.1 M sodium borate, pH 9.2. This high pH extraction of the interphase has been shown to release protein-bound poly(A) (Brawerman et al., 1972). The combined aqueous phases were adjusted to 0.2 M in NaCl and passed directly over an oligo(dT)-cellulose column which had been equilibrated with 100 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1% $NaDodSO_4$, pH 7.6. The column was washed with the above buffer until no more counts appeared; the poly(A)-containing RNA was then eluted with 5 mM Tris, 0.1 mM EDTA, pH 7.6; this solution was desiccated to dryness and stored at −20 °C.

Poly(dBrU) Addition to Restriction Fragments. PM2 DNA was cleaved by the restriction endonuclease *Hin*dIII and electrophoresed on a 1.5% preparative agarose slab gel. The 0.93 kilobase pair (kbp) band (Parker et al., 1977) was cut out and frozen, and the frozen gel was squeezed to recover buffer-containing DNA (Thuring et al., 1975). The DNA fragment was purified by absorption to and elution from DEAE-cellulose (Hirsh and Schleif, 1976). For enzymatic addition of dBrU tails by terminal transferase, the DNA solution was desiccated to dryness, redissolved at a concentration of 40 μg/100 μL in 50 mM Tes, pH 7.0, 40 mM KCl, 3 mM dBrUTP, 0.1 mM DTT, and 1 mM $CoCl_2$. The solution was protected with an atmosphere of argon (to prevent air oxidation of the Co^{2+}-DTT complex) and deoxynucleotidyl terminal transferase added (~100 units/100 μL). Incubation was for 30 min to 1 h at 37 °C.

pMB9 DNA was cleaved by the enzyme *Bam*H1 and dBrU tails were added by the same enzymatic reaction.

Hybridizations. RNA–DNA hybridizations were carried out as reported by Casey and Davidson (1977).

Heteroduplex hybridization of poly(A)·5S RNA to pCIT19 was performed as follows: randomly X-ray nicked pCIT19 (120 ng) and Col E1 (110 ng) DNAs were alkali denatured, neutralized, and then made up to 80% formamide, 0.4 M NaCl, 0.1 M Pipes, pH 6.9. This mixture was added to 150 ng of poly(A)·5S RNA, and the reaction (final volume 250 μL) was allowed to proceed for 15 min at 25 °C. This incubation time corresponds to a C_0t of 2.5×10^{-3} M^{-1} s for the Col E1 DNA sequences of Col E1 plus pCIT19 and a R_0t of 1.65×10^{-3} M^{-1} s for the 5S RNA driven reaction. We estimate from observed rate constants in this solvent (Casey and Davidson, 1977; P. Chandler, personal communication) that the respective $C_0t_{1/2}$ and $R_0t_{1/2}$ are 2.5×10^{-3} and 5.6×10^{-4} M^{-1} s. The hybridization was terminated by addition of the whole reaction mixture to a 0.7 × 14 cm Sepharose 2B column equilibrated with 5 mM Tris, 0.1 mM EDTA, pH 7.6; the effluent contents were monitored by UV absorption and radioactivity. The excluded volume (heteroduplexes) was pooled, desiccated to 2.5% of the original volume, diluted into spreading solution, and spread for electron microscopy. The included volume [poly(A)·5S RNA] was pooled and repassaged over an oligo(dT)-cellulose column (see below) to determine what, if any, breakdown of RNA had occurred during the hybridization.

Hybridization of poly(A)·(18 + 28)S rRNA to double-stranded pDm103 was performed as follows (Thomas et al., 1976; White and Hogness, 1977): 1 μg of pDm103 was partially digested with *Eco*R1, and the solution was deproteinized with phenol. Partial digestion was confirmed by native agarose gel electrophoresis (Hershey et al., 1977). To this DNA, 0.7 μg of poly(A)·(18 + 28)S rRNA was added, and the solution was dialyzed into 80% formamide, 0.4 M NaCl, 0.01 M Pipes, pH 6.9. The hybridization mixture was then incubated at 48 °C for 18 h. This condition allows RNA–DNA strand displacement association (R-loop formation) to occur without complete DNA–DNA denaturation. The reaction was terminated by addition of the mixture to a 1.5 × 10 cm Bio-Gel A150-M column. The column was eluted with 100 mM NaCl, 10 mM Pipes, 0.1 mM EDTA (pH 6.9) at 4 °C. The excluded peak was pooled, desiccated to 40% of the original volume, and directly spread for electron microscopy.

Electron Microscopy. DNA samples were prepared for electron microscopy by a modification of the standard formamide spreading conditions (Davis et al., 1971). The hyperphase was adjusted to 0.1 M monovalent cation concentration plus 50% formamide and DNA, as usual, but the hypophase was either aqueous 0.01 M cation or 0.01 M cation plus 5% formamide. This condition resulted in consistently improved single-strand/double-strand contrast (P. Wellauer, personal communication).

Results

Poly(A) Addition to RNA. Experiments were performed to determine optimal conditions for poly(A) addition to RNA. In these experiments, t[^{3}H]RNA or 5S RNA was used directly. (18 + 28)S rRNA was subjected to limited alkaline hydrolysis and treatment with bacterial alkaline phosphatase to increase the number of 3′-OH groups.

The effects of enzyme to RNA ratio and of time of poly(A) incorporation are illustrated in the experiment of Figure 1, using 5S [^{3}H]RNA and the primer-independent polynucleotide phosphorylase (PNPase-I) from *M. luteus*. The optimum PNPase/RNA ratio is about 2:1, and the percent RNA labeled is 14%. At a 1:1 ratio, about 10% of the RNA is labeled.

In our hands, the primer-dependent *M. luteus* enzyme (PNPase-T; prepared by mild proteolysis and reduction of native PNPase; Klee and Singer, 1968) was relatively inactive for poly(A) addition onto *Drosophila* 5S RNA. The greatest yield we obtained was 2.5% conversion to poly(A)·5S RNA despite the fact that PNPase-T was at least tenfold more active than PNPase-I with $(Ap)_3A$ as a primer (Klee, 1969; Engel, J. D., unpublished observations).

Yields comparable to those shown in Figure 1 were achieved using two different sources of the *E. coli* enzyme. The efficiency of the polymerization reaction proceeded to 10.2 and 12.8% in two separate reactions using, as substrate, hydrolyzed (18 + 28)S rRNA (average size, 250 nt) and 10.1 and 4.5%

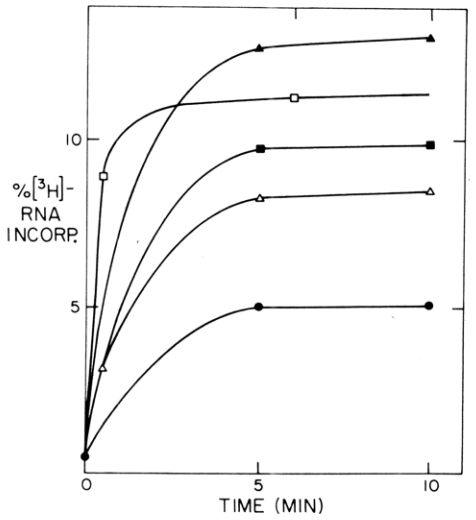

FIGURE 1: Percent of oligo(dT)-cellulose-bound poly(A)·5S RNA as a function of PNPase concentration. In this example, 26 pmol of 5S [^{3}H]-RNA was incubated in a 20-μL reaction mixture containing 10.2 (●), 19.8 (△), 26 (■), 52 (▲), or 320 pmol (□) of PNPase-I from *M. luteus*.

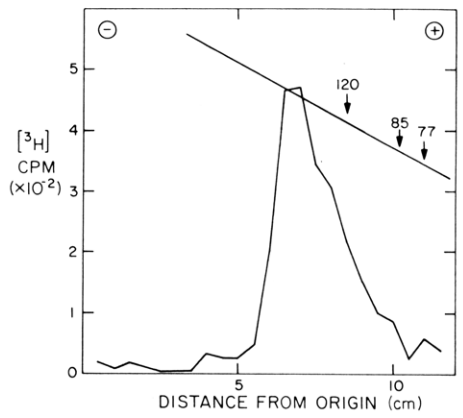

FIGURE 2: Denaturing 8% polyacrylamide, 98% formamide gel electrophoresis of poly(A)·5S [^{3}H]RNA (Maniatis et al., 1975). Migration from cathode (left) to anode (right). Visual RNA markers run in parallel were *E. coli* tRNAs (77 and 85 nucleotides) and *D. melanogaster* 5S RNA (120 nucleotides). Gels were stained in 0.1 μg/mL EtBr, photographed, and sliced into 0.5-cm pieces. The slices were solubilized in 10% NCS tissue solubilizer plus 90% Aquasol overnight at room temperature and then counted in a Beckman liquid scintillation spectrophotometer. Counting efficiency was approximately 15%.

in two separate reactions using *Drosophila* tRNA as substrate. The efficiencies in eight separate preparative reactions using *Drosophila* 5S RNA as substrate varied from 10.1 to 20.7%. Concentrations of RNA substantially lower than those used in Figure 1 resulted in a greatly decreased yield (data not shown). The RNA which is not bound to the oligo(dT) cellulose column can be (re)treated with alkaline phosphatase and reused in the polymerization reaction with a comparable final yield of product.

Length of Poly(A) Tail on 5S RNA. Poly(A)·5S RNA was electrophoresed on 98% formamide, 8% acrylamide slab gels to determine the number of adenosine residues added to 5S [^{3}H]RNA. The poly(A) addition reaction was conducted with the *M. luteus* PNPase-I enzyme at an enzyme/RNA ratio of 3, with an ADP concentration of 20 μM, and a 6-min incubation time. As shown in Figure 2, the increase in size of the RNA after poly(A) addition indicates that the average number of AMP residues incorporated per 5S RNA is about 60. It may be observed in Figure 2 that a small percentage of the counts migrate ahead of the 5S RNA marker. Since the counts are indicative of the amount 5S [^{3}H]RNA present and since only RNA with a minimum of 15 adenosine residues would be displayed on the gel (since poly(A)$^-$ material had been selected out on the oligo(dT)-cellulose column step), this result shows that there is some ribonuclease contaminating the PNPase used in the experiment.

The poly(A) addition reaction for the experiment of Figure 2 was conducted in the presence of 20 μM ADP. In all later experiments, we used 200 μM ADP. The average length of the poly(A) tails in these later experiments was estimated, by electron microscopy, to be approximately 400 nucleotides (see below).

Stability of Poly(A)·RNA. After hybridization of the poly(A)·RNA with DNA for the electron microscope mapping experiments, the excess unhybridized RNA was separated from the RNA–DNA hybrids by gel filtration as described under Materials and Methods. The unhybridized RNA in the included volume was dialyzed against 100 mM NaCl, 1 mM EDTA, pH 7.6, and then repassaged over the oligo(dT)-cellulose column to determine the amount of 5S [^{3}H]RNA which still bound by virtue of the poly(A) tail. In all cases, in excess of 80% (usually >90%) of the labeled material would rehybridize to the oligo(dT)-cellulose column in high salt and could

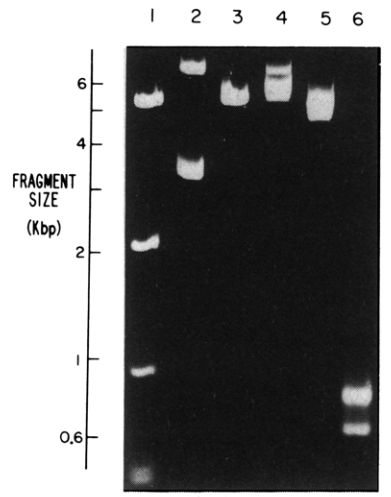

FIGURE 3: Synthesis of the poly(BrU)-monovalent fragment. One percent neutral agarose gel (Seakem ME) in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.6. The gel was run for 6 h at 80 V and then stained in 0.1 μg/mL ethidium bromide in H_2O. Lane 1, PM2 DNA cleaved with *Hin*dIII (size standard, Parker et al., 1977); lane 2, pMB9 DNA [form I (closed circular) and II (nicked circular); fast and slow migrating, respectively]; lane 3, pMB9 DNA cleaved with *Bam*H1 [form III (linear)]; lane 4, pMB9 DNA (linear) as in lane 3 reacted with terminal transferase and BrUTP; lane 5, DNA as in lane 4 after reaction with *Eco*R1; lane 6, isolated monovalent fragment plus unreacted fragment after sucrose gradient sedimentation.

be eluted with 5 mM Tris, 0.1 mM EDTA, pH 7.6. Recovery of the poly(A)·RNA from the Sepharose 2B or Bio-Gel A150-M column varied from 70% to over 90%. Thus, the enzymatically linked poly(A) tails are stable to the high formamide hybridization conditions.

Divalent Label. Conditions used for the addition of dBrU tails to both ends of the 0.93-kbp *Hin*dIII fragment of PM2 DNA are given under Materials and Methods. The amount of incorporation, as assayed by incorporation of dBr[^{3}H]UTP into material excluded by Sephadex G-50, was approximately 220 dBrU residues per fragment end per hour (W. Bender, personal communication).

Monovalent Label (Figure 3). pMB9 DNA (lane 2) was cleaved at the unique *Bam*H1 site (lane 3). Poly(dBrU) tails were added to the ends as above (lane 4). The DNA was then

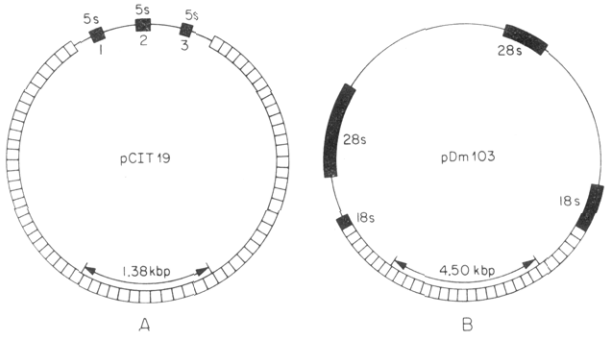

FIGURE 4: Schematic representations of plasmids pCIT19 (A, Hershey et al., 1977) and pDm103 (B, Glover and Hogness, 1977). Cross-hatched area depicts the vector (procaryotic) portion of the DNA, and solid lines depict the *Drosophila* portion of the recombinant plasmids. The RNA coding portions of the inserted *Drosophila* DNAs are depicted as black boxes.

restricted with *Eco*R1, producing two fragments of 0.61 and 4.6 kbp (lane 5). The fragments were separated by 12–30% isokinetic sucrose gradient sedimentation in an SW41 rotor (26 h, 15 °C, 35 000 rpm). The fractions containing the 610-bp monovalent fragment were pooled, ethanol precipitated, resuspended in 10 mM Tris, 1 mM EDTA, pH 7.6, and stored at −20 °C. The gel-electrophoretic analysis of this sample is shown in lane 6. There is a faint band due to part of the sample which has not acquired a dBrU tail. There is a more intense slower band due to the dBrU-tailed 610-bp fragment. From the displacement, assuming that single strands migrate two times as fast as duplex DNA of the same length in agarose, we estimate that the length of the dBrU tail is approximately 260 nt.

Electron Microscopy

pCIT19 Heteroduplexes. The plasmid pCIT19 contains a *Drosophila* insert of length 1.4 kbp with three equally spaced 5S genes, attached to Col E1 DNA (Hershey et al., 1977). The structure of the pCIT19 plasmid is depicted in Figure 4A.

Single strands of pCIT19 and of Col E1 DNA were incubated with poly(A)·5S RNA under the conditions given under Materials and Methods. These conditions are chosen to give a readily observable number of pCIT19/Col E1 heteroduplexes without too many tangled structures caused by overrenaturation and an estimate of 87% saturation of the 5S genes in the heteroduplexes with poly(A)·5S RNA.

Two methods of observing the RNA–DNA hybrids were used. In one, the poly(A) tails extending from the RNA–DNA hybrids were observed directly. In the second method, the PM2 DNA restriction fragment with dBrU tails at both ends was included in the spreading hyperphase, and the poly(A) tails were identified as points of attachment of this duplex fragment to the single strand of pCIT19 of the heteroduplex. Figure 5A shows a micrograph of a typical molecule observed by the first method. There are two 5S genes labeled with poly(A) tails at positions expected for the 5S genes labeled 1 and 3 in Figure 4A. These two poly(A) tails have a measured length of 400 nucleotides each. The average length of the 23 poly(A) tails measured was 380 ± 80 nucleotides. Figures 5B and 5C are micrographs of heteroduplexes labeled with the divalent dBrU-tailed restriction fragment. In Figure 5B, the two ends of one restriction fragment are attached to genes 1 and 3 (Figure 4A). In Figure 5C, the ends of one fragment are attached to sites 1 and 2; the ends of the other fragment are attached to sites 2 and 3. Many structures were seen with more than one restriction fragment attached to one gene site. Therefore, some of the poly(A) tails were sufficiently long so

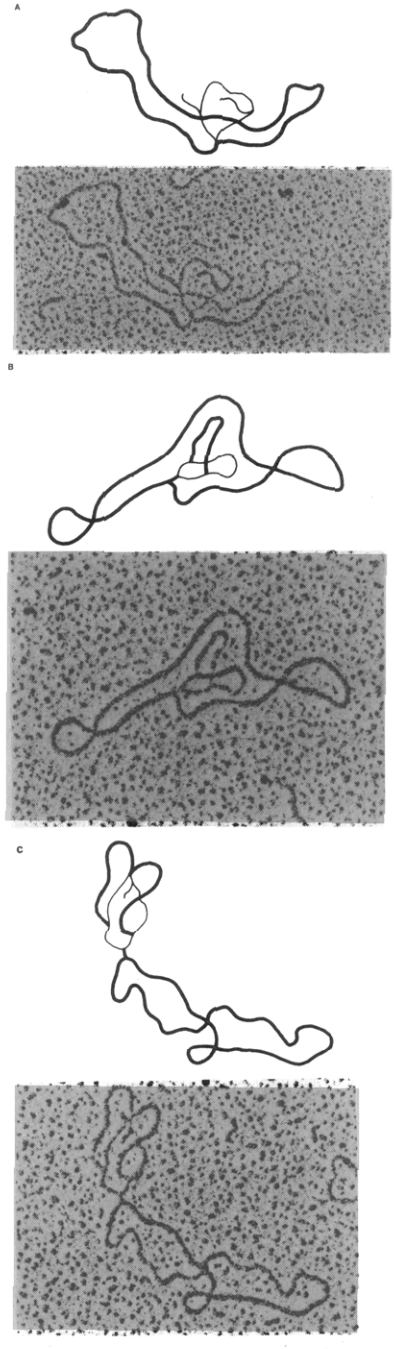

FIGURE 5: (A) Electron micrograph of two poly(A) tails protruding from the single-stranded *Drosophila* DNA insert into Col E1 coding for 5S RNA. (B) Electron micrograph of a divalent BrU restriction fragment labeling the analogous sites seen on the recombinant plasmid as in A. (C) Electron micrograph of two poly(BrU)-tailed restriction fragments labeling all three 5S genes.

that several dBrU tails could pair with them. Some highly tangled uninterpretable structures were seen in these experiments; these are presumably due to multiple attachments of the divalent labels to the three genes hybridized to poly(A)·5S RNA.

A histogram of the peak positions of the genes labeled by both methods is presented in Figure 6. These data on the positions of the genes are in complete agreement with those of Hershey et al. (1977). Efficiencies of mapping were estimated as follows. In the experiments with poly(A)·RNA, but without added dBrU label, all heteroduplex molecules with any recognizable tails were scored in random scanning of a grid. Thirty-four tails were observed on 21 molecules, leading to an

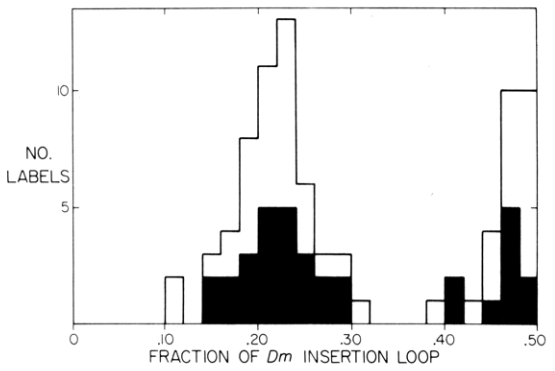

FIGURE 6: Histogram of the positions of the three 5S RNA genes on pCIT19. Since the second gene is located almost exactly in the middle of the Dm DNA insert, there exists a central ambiguity in the orientation of the genes (5′ to 3′). The positions are therefore plotted to extending halfway around the Dm DNA insert; the existence of two genes proximal to the A–T "neck" of the plasmid is confirmed by the 2:1 ratio ($n = 58/28$) of labeling of the proximal to distal genes, as well as by Figure 5C. The filled area indicates positions mapped using the divalent BrU label; the open area indicates positions mapped using poly(A) tails alone.

approximate labeling efficiency of $34/(3 \times 21)$ or 54% (Figure 6, open area). In the experiments with dBrU labeling, random fields were scanned and all interpretable heteroduplexes photographed. Of 66 such heteroduplexes, 30 molecules were labeled: 6 had one site labeled, 13 had two sites labeled, 10 had three sites labeled, and 1 appeared to have four sites labeled. Assuming that one of the latter sites is false, we have 65 genes labeled on 30 heteroduplexes (Figure 6, filled area). The observed labeling efficiency is therefore approximately 65/90 or 72%. Tangled molecules were disregarded in the observations; as stated above, many of these were probably molecules with several dBrU tails attached to one poly(A) tail. It is probable that the lengths of some of the poly(A) tails were too short to be positively identified in the micrographs; this explains the lower efficiency of labeling in the first method (i.e., without a restriction-fragment label).

R-Loop Mapping. The plasmid pDm103 contains one copy each of *Drosophila* 18S and 28S genes, as depicted in Figure 4B. R loops can be formed by hybridization of intact 18S and in vivo singly cleaved 28S Dm rRNA to the plasmid under controlled high formamide conditions (White and Hogness, 1977). We wished to test whether randomly cleaved rRNA with an average length of 250 nt (and thus approximately 8 and 16 fragments per 18S and 28S gene, respectively), each poly(A) tailed, would form R loops with reasonable efficiency to pDm103. Poly(A)-tailed short rRNA was hybridized to pDm103 under R-looping conditions, which gave greater than 85% efficiency using intact rRNA. The poly(A) tails were labeled with the monovalent poly(dBrU) duplex DNA fragment prepared as described under Materials and Methods.

Typical micrographs of the resulting molecules are shown in Figure 7A,B. One rRNA fragment of length 450 bp (Figure 7A) and of length 420 bp (Figure 7B) has formed an R loop, and each is labeled by the monovalent poly(dBrU) label. Most of the pDm103 molecules had from one to three small RNA pieces forming R loops, but we never observed molecules with the entire 18S or 28S region involved in R loops with multiple RNA fragments and multiple poly(A) tails.

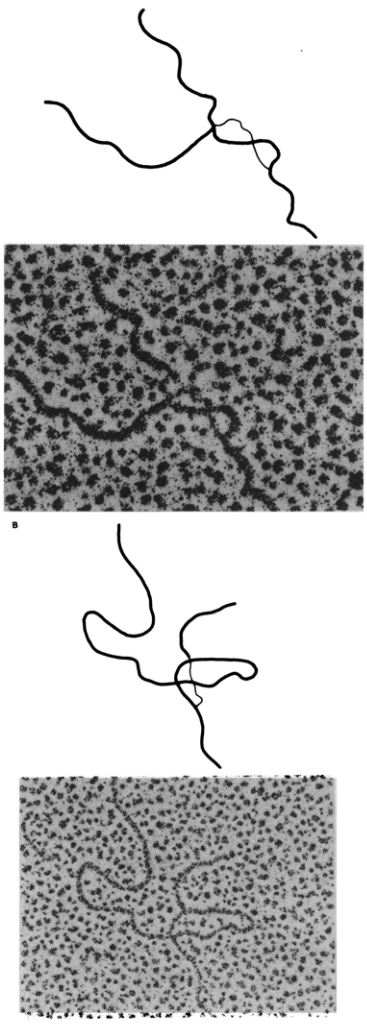

FIGURE 7: RNA displacement loops with monovalent poly(BrU) restriction fragments hybridized to the poly(A) tails on the 3′ end of the RNA.

Discussion

Our major points are the following. (1) Polynucleotide phosphorylase from either *E. coli* or *M. luteus* will polymerize poly(A) tails on to the 3′-OH ends of RNA molecules. We do not know whether under our reaction conditions the enzyme adds poly(A) tails processively or distributively (Moses and Singer, 1970). Between 10 and 20% efficiency of reaction was achieved with tRNA, 5S RNA, and cleaved (18 + 28)S rRNA [i.e., 10–20% of the initial RNA input was converted to poly(A)·RNA]. The reaction requires stoichiometric or greater amounts of enzyme.

(2) RNA–DNA hybrid regions along a single strand of DNA can be positively identified in the electron microscope by the poly(A) tailing method. The mapping efficiencies achieved are higher than those achieved by other labeling techniques, and the preparative operations are relatively simple.

(3) Linear duplex DNA with dBrU or dT tails makes an efficient, simple poly(A) label, but tangled structures sometimes occur on closely spaced genes with a divalent label. Our preliminary tests with the monovalent label (which was developed in the later stages of this work) suggest that it will be an excellent label with fewer tangled structures.

(4) The efficiency of formation of R loops by many short poly(A)-tailed fragments of RNA is much lower than that achieved with one intact RNA molecule (White and Hogness, 1977; Thomas et al., 1976; Engel, J.D., unpublished).

Several additional salient features of the poly(A)-tailing technique should be pointed out.

First, our inclination would be to recommend the monovalent label for poly(A) mapping studies. Qualitatively, we found

far fewer tangles when mapping poly(A) tails with the monovalent label, and further, in the great majority of cases (i.e., >75%) where an R loop was visualized in the pDm103 studies, the R loop was labeled with a monovalent label.

An interesting aspect of the R-loop experiments was the inability of small pieces of rRNA to form displacement loops efficiently, whereas in control experiments with full-length rRNA greater than 90% displacement was observed. One possible explanation might be that the poly(A) tail might somehow physically impede the formation of such structures. Another explanation is that short pieces of RNA are more effective in escaping the kinetic "trap" which is created after R loops are removed from the thermodynamically stable state (Casey and Davidson, 1977). A final possibility is that the R loops are anchored by some very stable RNA-DNA displacements in certain parts of the hybrid regions and these hold the whole R loop in place. Thus, in the absence of complete rRNA molecules hybridizing, only the most stable loops will remain paired, the rest being strand displaced when the molecules are exposed to lower formamide spreading conditions.

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