Bhargava, P., and Shanmugam, G. (1971), *Prog. Nucleic Acid Res. Mol. Biol. 11,* 103–192.
Bogdanovsky, D., Hermann, W., and Schapira, G. (1973), *Biochem. Biophys. Res. Commun. 54,* 25–32.
Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83–88.
Buckingham, M. E., Caput, M. E., Whalen, P. G., and Gros, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1466–1470.
Chen, G. S., and Siddiqui, M. A. Q. (1975), *J. Mol. Biol. 96,* 153–171.
Colby, C., Stoller, B. D., and Simon, M. I. (1971), *Nature (London), New Biol. 229,* 172–174.
Darnbrough, C., Hunt, T., and Jackson, R. J. (1972), *Biochem. Biophys. Res. Commun. 48,* 1556–1564.
Davidson, E. H., and Britten, R. J. (1971), *J. Theor. Biol. 23,* 123–130.
Davidson, E. H., and Britten, R. J. (1973), *Q. Rev. Biol. 48,* 565–613.
Deshpande, A. K., Jakowlew, S. B., Arnold, H. H., Crawford, P. A., and Siddiqui, M. A. Q. (1977), *J. Biol. Chem. 252,* 6521–6527.
Deshpande, A. K., and Siddiqui, M. A. Q. (1977), *Dev. Biol. 58,* 230–274.
Dickson, E., and Robertson, H. D. (1976), *Cancer Res. 36,* 3387–3393.
Diener, T. O., Smith, D. R., and O'Brien, M. J. (1972), *Virology 48,* 844–846.
Hardesty, B., Miller, R., and Schweet, R. (1963), *Proc. Natl. Acad. Sci. U.S.A. 50,* 924–931.
Heywood, S. M., and Kennedy, D. S. (1976), *Biochemistry 15,* 3314–3319.
Hunt, T., and Jackson, R. J. (1974), Modern Trends in Human Leukemia, Neth, R., Gallo, R. C., Spiegleman, S., and Stohlman, F., Ed., Munich, Lehmanns Verlag, pp 300–307.
Hunter, A. R., Hunt, R. T., Jackson, R. J., and Robertson, H. D. (1972), Synthese, Strucktur and Funktion des Hämoglobins, Marlin and Nowicki, Ed., Munich, Lehamanns Verlag, p 133.
Laemmli, U. K. (1970), *Nature (London) 227,* 680–685.
Lee-Huang, S., Sierra, J. M., Naranjo, R., Filipowicz, W., and Ochoa, S. (1977), *Arch. Biochem. Biophys. 180,* 276–287.
Pelham, H. R. B., and Jackson, R. J. (1976), *Eur. J. Biochem. 67,* 247–256.
Pestka, S. (1977), Molecular Mechanism of Protein Biosynthesis, Weissbach, H., and Pestka, S., Ed., New York, N.Y., Academic Press, pp 467–554.
Pilch, Y. H., Fritze, D., Waldman, S. R., and Kern, D. H. (1975), *Curr. Top. Microbiol. Immunol. 72,* 157–190.
Rao, N. J., Blackstone, M., and Busch, H. (1977), *Biochemistry 16,* 2756–2762.
Schimke, R. T., Patacios, R., Sullivan, D., Kiely, M. L., Gonzales, C., and Taylor, J. M. (1974), *Methods Enzymol. 30,* 631–648.
Siddiqui, M. A. Q., Deshpande, A. K., Arnold, H.-H., Jakowlew, S. B., and Crawford, P. A. (1977), *Brookhaven Symp. Biol. 29,* 62–81.
Sleger, H., Mattrie, R., and Kondo, M. (1977), *FEBS Lett. 80,* 390–394.

Selectivity of RNA Chain Initiation in Vitro. 1. Analysis of RNA Initiations by Two-Dimensional Thin-Layer Chromatography of 5′-Triphosphate-Labeled Oligonucleotides†

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ABSTRACT: A method for the rapid and quantitative analysis of 5′-terminal oligonucleotides of RNAs made in vitro is described. The method involves synthesis of RNA in the presence of [γ-^{32}P]ATP or GTP, isolation of the RNA, and digestion with T1 or pancreatic ribonucleases to release labeled 5′-triphosphate terminated oligonucleotides. The oligonucleotides are then subjected to chromatography on a polyethylenimine cellulose thin-layer system using 2 M LiCl, 0.01 M EDTA (pH 6.5) in the first dimension and 1.5 M LiCl, 1.8 M formic acid, 0.005 M EDTA (pH 2.0) in the second. RNAs made with *E. coli* RNA polymerase and λcb2, T7, T4, and adenovirus 2 DNA yield characteristic fingerprint patterns. The utility of this method in studying selectivity of in vitro RNA chain initiation is discussed.

Synthesis of specific functional RNAs is dependent on the correct initiation of transcription by RNA polymerase at a specific site or sites on the DNA template. The region of DNA which signals the start of a new RNA is called the promoter (Reznikoff, 1972). Each promoter gives rise to an RNA transcript beginning with a characteristic 5′-triphosphate terminal sequence. Purified *E. coli* RNA polymerase is capable of transcribing a DNA template selectively in vitro. It has been demonstrated by hybridization, electron microscopy, and direct sequence techniques that these transcripts are selectively initiated and are homologous to in vivo RNAs from the same DNA. The factors governing this selectivity of initiation of transcription are not completely understood (for review, see Chamberlin, 1974). In order to study the selectivity of initiation, it is necessary to be able to measure initiation of the RNA at different promoters. The number and quantity of 5′-terminal

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ends present as the result of transcription of a DNA template under a specific set of conditions may be indicative of selectivity of transcription or its loss due to the conditions of synthesis, the template, or the RNA polymerase used. Several available techniques used to examine 5′-terminal oligonucleotides are difficult to use quantitatively and to apply to multiple samples (Sugiura et al., 1969; Konrad, 1973; Smith et al., 1974; Takeya & Fugisawa, 1974).

A rapid and quantitative method has been developed for examining the 5′ ends of RNAs made in vitro. Since most RNA chains are initiated by ATP or GTP, [γ-^{32}P]ATP or GTP has been used to label specifically the 5′ ends of RNAs (Maitra et al., 1967). This paper describes the synthesis of RNA with [γ-^{32}P]ATP or GTP, the isolation of this RNA, cleavage of the RNA to yield 5′ oligonucleotides, and the two-dimensional separation of these oligonucleotides on polyethyleniminecellulose thin-layer chromatography. This provides a method for the recovery, fractionation, and analysis of 5′-terminal oligonucleotides which is essential for investigating the variables and control elements which affect initiation. The utility of this method will be described and its limitations discussed.

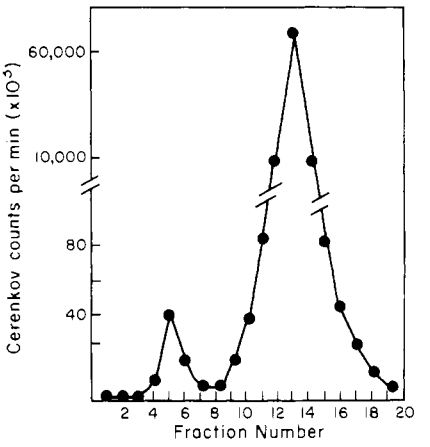

FIGURE 1: Profile of a typical Sephadex G-50 column chromatography of λcb2 RNA synthesized in vitro in the presence of [γ-^{32}P]ATP. The RNA was synthesized as described in Experimental Procedures, treated with DNase, and phenol extracted, and 200 μL of the aqueous phase was applied to a 10-mL column equilibrated in 10 mM Tris (pH 7.9) and 0.1 M NaCl. Fractions (0.6 mL) were collected and Cerenkov radiation was counted to localize the peaks.

Experimental Procedures

Synthesis of [γ-^{32}P]ATP and [γ-^{32}P]GTP. [γ-^{32}P]ATP and [γ-^{32}P]GTP were synthesized by the method of Schendel & Wells (1974) with the following modifications. The reaction was run and chromatography carried out on a DEAE-Sephadex A-25 column as described, through the 0.23 M ammonium bicarbonate step. The column was then washed with 30 mL of sterile double-distilled water to free the column of ammonium bicarbonate. The labeled triphosphate was eluted from the column with 1 M ammonium acetate (pH 7.0) into a siliconized scintillation vial. Ammonium acetate was substituted for ammonium bicarbonate because ammonium acetate is more volatile and requires only two to three washes with water to completely desalt the sample. The product was lyophilized and washed with sterile water until no salt residue remained. The product was stored in 50% ethanol at −20 °C at 1 mCi/mL. The yield was 60–80%. The nucleotide remained stable for at least 2 weeks as judged by thin-layer chromatography.

Phage Strains and DNA Isolation. λcb2 was the gift of W. Szybalski. Phage T7 was grown according to the method of Studier (1969). Phage T4 was grown according to Terzaghi et al. (1966). DNA was isolated from phage by extraction with phenol (Thomas & Abelson, 1961), dialyzed extensively against several changes of 0.01 M Tris-HCl (pH 7.9), 0.01 M NaCl, and stored at 5 °C. Adenovirus 2 DNA was prepared essentially by the method of Pettersson & Sambrook (1973) and was the gift of W. Dynan. DNA concentration was determined by measuring the OD_{260} and assuming that a 50 μg of DNA/mL solution would have an OD_{260} of 1.0.

Preparation of RNA Polymerase. E. coli RNA polymerase was purified as described (Burgess & Jendrisak, 1975) from PR 7, an RNase I lacking, polynucleotide phosphorylase deficient strain of *E. coli* K 12 (Reiner, 1969). No impurity bands were visible when 10 μg of enzyme was analyzed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$.[1] The enzyme was 60–80% saturated with sigma subunit. Addition of extra sigma to the polymerase did not affect the results of the experiments. The enzyme preparations used in these studies lacked RNase and phosphatase activity when assayed as follows. To assay RNase, a batch of ^{3}H- or ^{14}C-labeled RNA was made using conditions described below for in vitro synthesis of RNA. Transcription was terminated after 5 min by adjusting the reaction mixture to 20 mM EDTA. Portions of the labeled RNA were Cl_3CCOOH-precipitated and counted at various times after EDTA addition. We judged that there was no significant RNase contamination if there was no loss of Cl_3CCOOH-precipitable radioactivity in 60 min. Phosphatase activity was assayed by incubating 5 μg of enzyme with [γ-^{32}P]ATP in transcription buffer for 15 min at 37 °C. The mixture was treated with Pronase and then subjected to the first dimension of chromatography on polyethyleniminecellulose thin layer as described below. No inorganic phosphate appeared on the chromatogram.

In Vitro Synthesis of RNA. A standard reaction mixture for RNA synthesis (0.25 mL) contained the following: 0.03 M Tris-HCl (pH 7.9) at 25 °C, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM potassium phosphate, 10 mM $MgCl_2$, 0.12 mM γ-^{32}P-labeled ATP or GTP, 0.24 mM of the other three nucleoside triphosphates, 0.15 M KCl, 30 μg of DNA, and 30 μg of RNA polymerase. The final specific activity of the γ-^{32}P-labeled nucleotide was 15 000–30 000 dpm/pmol. The amount of [γ-^{32}P]NTP to be used in the reaction was measured into a siliconized test tube and lyophilized to remove the ethanol and water. The rest of the components, except the $MgCl_2$, were added and mixed well. The reaction mixture was preincubated in the absence of Mg^{2+} for 10 min at 37 °C. $MgCl_2$ was added to a final concentration of 10 mM and the synthesis carried out for 10–15 min at 37 °C. Rifampicin was then added to a concentration of 20 μg/mL and the mixture incubated for 5 min more to allow initiated RNA chains to be completed. This rifampicin treatment does not appear to alter the 5′-terminal oligonucleotide fingerprints. Incorporation of label into RNA ranged from 0.01% to 0.05% depending on conditions used. No detectable incorporation occurred in a control in which no $MgCl_2$ was added.

All solutions were sterilized by autoclaving or Millipore filtering to inactivate or remove contaminating ribonucleases. Test tubes were siliconized by rinsing them with 2% (v/v) dimethyldichlorosilane (Matheson, Coleman and Bell) in carbon tetrachloride and heating to at least 120 °C for 5 h.

Isolation of RNA. Fifteen microliters of RNase-free DNase (Worthington) at 1 mg/mL in sterile double-distilled water

[1] Abbreviations used: PEI, polyethylenimine; NMP, nucleoside monophosphate; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; $NaDodSO_4$, sodium dodecyl sulfate.

was added to the reaction. Digestion was carried out for 15 min at 25 °C. An equal volume of phenol was added and the mixture was incubated for 15–30 min at 37 °C with intermittent vortexing and then centrifuged at low speed for 5 min. To separate the RNA product from unincorporated substrate, 200 μL of the aqueous phase was applied to a 10-mL Sephadex G-50 (superfine) column equilibrated in 10 mM Tris-HCl (pH 7.9) and 0.1 M NaCl. The columns were run by gravity at room temperature in disposable 10-mL syringes at a flow rate of approximately 30 mL per h. Several columns were run at once and 0.6–0.7-mL fractions were collected by hand. Figure 1 shows a typical profile of radioactivity measured by Cerenkov counting. The fractions comprising the void volume which contained the labeled RNA were pooled into siliconized 30-mL Corex tubes. Yeast tRNA (from Miles, which had been phenol extracted twice, ethanol precipitated, and redissolved in sterile water to 10 mg/mL) was added to a final concentration of 15 μg/mL. Ethanol (2.5 volumes) was added and the samples precipitated at −70 °C for 60 min or at −20 °C overnight. The precipitates were pelleted by centrifugation for 20 min at 10 000 rpm in a Sorvall SS-34 rotor. The pellets were then redissolved in 50 μL of sterile double-distilled water.

Digestion of RNA. A 10-μL aliquot of the redissolved RNA pellet was dried on parafilm under reduced pressure. The dried sample was dissolved in either: (a) 2 μL of T1 RNase (Sigma, at 0.5 mg/mL, dialyzed against 0.01 M Tris-HCl (pH 7.9), 0.02 M EDTA) and 3 μL of 0.01 M Tris-HCl (pH 7.9), 0.02 M EDTA; or (b) 2 μL of bovine pancreatic RNase A (Worthington, at 0.8 mg/mL in 0.01 M Tris-HCl (pH 7.9), and 3 μL of 0.01 M Tris-HCl (pH 7.9). Digestions were carried out in 5-μL Microcaps (Drummond, Biolabs, Inc.) in a humidified 37 °C incubator for 30 min. Longer times of digestion did not alter the fingerprint pattern, suggesting that complete digestion was occurring by 30 min. Since the T1 RNase can inactivate on storage, we suggest that it be tested occasionally for its ability to digest RNA completely.

Two-Dimensional Thin-Layer Chromatography. Polyethyleniminecellulose thin-layer sheets (Brinkmann, PEI MN 300, 20 × 20 cm) were washed by sequential immersion in 10% (w/v) NaCl, double-distilled water, and 50% (v/v) methanol and were allowed to dry at room temperature for 12 h. The solvents used in the two-dimensional chromatography system were based upon two described by Randerath & Randerath (1964). The first dimension uses a solvent of 2.0 M LiCl (Mallinckrodt) and 0.01 M EDTA (added from a stock solution, 0.2 M EDTA, pH 7.9), with a final pH of 6.5. The solvent for the second dimension is made by adding 500 mL of 3 M LiCl, 0.01 M EDTA to 500 mL of 3.6 M formic acid, and adjusting to pH 2.0 with solid LiOH (Fisher). The digested sample (5 μL) was spotted on a washed PEI sheet 1-in. from each edge on the lower left hand corner. Best resolution was obtained by spotting 1 μL at a time with intermittent drying. The sheet was soaked for 2 min in anhydrous methanol to remove residual salts and then dried at room temperature for 15 min or for 5 min at 32 °C. The chromatogram was developed at room temperature in a Kodak thin-layer chromatography chamber until the front had reached the edge of the sheet. The sheet was then desalted by soaking twice for 5 min in 500 mL of anhydrous methanol, dried, and developed in the second dimension. The sheet was dried thoroughly and subjected to autoradiography as described below.

Migration of nucleotides in the two-dimensional system is dependent both on base composition and charge. In the first dimension charge effects are such that the migration order is NMP > NDP > NTP and base effects cause the migration order to be UTP > CTP > ATP > GTP. The second dimension migration is similar to the first dimension regarding charge, but base effects result in migration in the order ATP = CTP > UTP > GTP.

Protein, salt, divalent cations, and DNA interfere with the resolution. Too much protein (greater than 3 μg) will prevent migration of the sample away from the origin. EDTA is included in the buffers to chelate divalent cations and thus to minimize streaking caused by strong interaction between the PEI-cellulose and the triphosphate moiety of the oligonucleotide. DNA in the sample also results in streaking and a high concentration will prevent migration of the sample. Considerable variability in the batches of thin-layer sheets had been observed. In most cases groups of experiments have been carried out using sheets with the same lot number. Thin-layer sheets have been stored at 4 °C for up to 4 months with no change in properties.

The patterns of the 5′-oligonucleotide fingerprints obtained with a particular DNA template are highly reproducible and characteristic and the migration of each spot relative to the other spots in the fingerprint is constant from chromatogram to chromatogram.

Autoradiography. Autoradiography of the chromatograms and the gels was carried out using Kodak No-Screen x-ray film, NS 2T. Gels and chromatograms were placed face down on the outside of a Kodak "Ready-Pak" and were exposed for 2–5 days. The dried chromatograms were marked with radioactive ink prior to exposure for identification and proper orientation of the chromatogram.

Quantitation. The amount of radioactivity in each oligonucleotide spot was determined as follows: the location of each spot was determined from the autoradiogram of the chromatogram. An outline of each spot was made on the thin-layer sheets. Each spot was moistened with water and then scraped into a scintillation vial with a flat ended spatula. The samples were counted in 10 mL of Scintosol (Isolab, Inc.) with an efficiency of about 80%.

Polyacrylamide Gel Electrophoresis. Slab gels containing 20% acrylamide were prepared as described by Burd & Wells (1974) except that the bisacrylamide concentration was reduced to 0.5% to make the gels less brittle. Following electrophoresis, the gels were wrapped in Saran Wrap and the origin and the bromophenol blue marker dye position marked with radioactive ink. Autoradiography was carried out as described above. Standard curves of oligonucleotide chain length vs. gel migration distance were determined using oligonucleotides of dC of known length (gift of J. Dodgson).

Results

Fingerprint Patterns of 5′ Ends of RNAs Made from Various Templates. Comparison of the fingerprints of RNAs synthesized from T7, λcb2, T4, and adenovirus 2 DNA is shown in Figure 2. A–D show the 5′ ends of RNAs labeled with [γ-^{32}P]ATP and digested with T1 RNase, while E–H show the fingerprints of RNA labeled with [γ-^{32}P]GTP and digested with pancreatic RNase. This figure shows that the patterns of the 5′ ends of RNAs made from various templates are clearly different, and that the spots vary in intensity. The assumption is made that the amount of label in a spot is proportional to the number of times an RNA with that particular 5′-terminal oligonucleotide sequence is initiated. The limitations of this assumption are discussed in the following papers (Miller & Burgess, 1978a,b).

T7 has three major promoters, two beginning RNA synthesis with ATP and one with GTP (Dunn & Studier, 1973). Figure 2A shows two major ATP-labeled 5′ ends as judged by the darkness of the spots. One major GTP oligonucleotide and

2 minor ones are observed (Figure 2E). The minor spots observed in Figures 2A and 2E may correspond to the minor bands observed on polyacrylamide gels of T7 RNA made in vitro (Dunn & Studier, 1973), may correspond to minor promoters B, C, D, E, and F (Minkley & Pribnow, 1973; Stahl & Chamberlin, 1977), may be due to aging of the polymerase (Grohmann et al., 1975), or in some cases may be the result of abortive initiation (Johnston & McClure, 1976).

In λ phage, λcb2, three major ATP and one major GTP-initiated RNAs have been identified and sequenced (Blattner & Dahlberg, 1972; Dahlberg & Blattner, 1973; Lebowitz et al., 1971). The fingerprint of λ ATP starts shows 5–6 major spots (Figure 2B). One major spot and several minor spots are observed with GTP labeled RNA (Figure 2F). Recently, several other λ transcripts have been observed in vitro under certain conditions (Meyer et al., 1975; J. Salstrom & E. Rosenvold, personal communication). The extra spots on the chromatograms may represent the 5′ ends of these RNAs.

The fingerprint of [γ-^{32}P]ATP-labeled T4 RNA (Figure 2C) shows a pattern of 10–11 spots, of which 3 to 4 are more intense than the rest. Only one spot is seen when the RNA is initiated with GTP (Figure 2G).

Adenovirus 2 DNA transcribed with *E. coli* RNA polymerase yields 4 unique oligonucleotides with ATP starts and 2 with GTP (Figures 2D and 2H). Surzycki et al. (1976) have found between 5 and 6 promoters on adeno 2 DNA for *E. coli* RNA polymerase, 3 initiating RNA synthesis with ATP and 2 to 3 initiating with GTP.

It was demonstrated that these spots represent 5′-triphosphate ends of RNAs by treating the T1 RNase digested RNA with 0.3 μg of alkaline phosphatase (Worthington) for 30 min at 37 °C. The sample was run on PEI-cellulose as described above and autoradiography performed. No oligonucleotides were seen and all of the label migrated in the position of inorganic phosphate. This demonstrated that the oligonucleotides were actual 5′-terminal ends of RNAs and not the result of incorporation from possible contaminating [α-^{32}P]NTPs.

Quantitation. Our goal in developing this method was to be able to examine initiation of RNA synthesis both qualitatively and quantitatively. In order for the procedure to be quantitative, it was necessary to determine whether losses of RNA were occurring at the various steps involved.

To examine possible losses of small oligonucleotides on the G-50 column, the void peak (fractions 4–7, Figure 1) and the front part of the substrate peak (fractions 8–10) were pooled separately and ethanol precipitated. The precipitated RNAs were loaded on 20% polyacrylamide gels and subjected to electrophoresis and autoradiography as described above. Figure 3 shows the results of this experiment. Column A shows the distribution of RNAs made from λcb2 DNA contained in the void peak. The oligonucleotides ranged in size from greater than 80 (not entering the gel) to 10 nucleotides in length. Column B shows RNA from the front of the substrate peak and indicates that oligonucleotides containing 10 to 15 residues are slightly retarded on the G-50 column. This indicates that it is necessary to pool more than the void volume if precise quantitation of every initiated chain is desired. The fingerprints shown in this paper in Figure 2 were from the void peak and therefore represent primarily RNAs greater than about 15 nucleotides in length.

Recoveries of the samples in the steps of the procedure after the G-50 column were determined by counting portions of the samples after the G-50 column and after the ethanol precipitation, and by counting material recovered as discrete spots on the chromatograms. Recovery of material from the void peak after ethanol precipitation was 80–90%. Loss at this stage

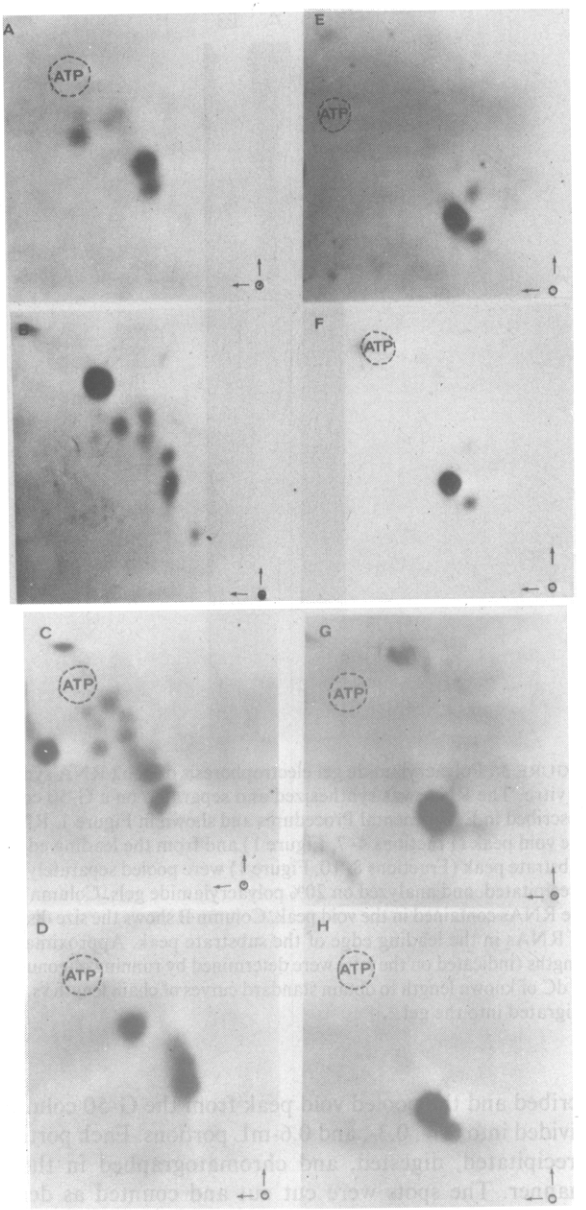

FIGURE 2: Two-dimensional fractionation of the 5′-triphosphate terminal ends of RNAs made from various DNA templates. RNAs were synthesized, isolated, and digested as described in Experimental Procedures. The digested samples were chromatographed on polyethyleniminecellulose thin-layer sheets. First dimension was 2.0 M LiCl, 0.01 M EDTA (pH 6.5). The second dimension was 1.5 M LiCl, 1.8 M formic acid, 0.005 M EDTA (pH 2.0). The darkest spot in B and the dashed circles represent added [γ-^{32}P]ATP marker. A–D are autoradiograms of T1 RNase digests of [γ-^{32}P]-ATP-labeled RNAs. E–G are pancreatic RNase A digests of [γ-^{32}P]GTP-labeled RNAs. (A and E) T7 RNA; (B and F) λcb2 RNA; (C and G) T4 RNA; (D and H) adenovirus 2 RNA. A small amount of $^{32}PO_4$ occasionally occurs due to breakdown of the γ-labeled oligonucleotide and migrates slightly faster than the ATP marker in both directions. The spots on the left side of C and top of G are artifacts and are not normally seen.

is almost entirely of nucleoside triphosphates contaminating the void peak. Recovery of material from the chromatograms was 65–75% of the void peak for oligonucleotides labeled with ATP and 80–90% for oligonucleotides labeled with GTP. Recovery of the ATP-initiated oligonucleotides on the chromatogram is lower because the ATP oligonucleotides appear to streak more.

To demonstrate that the amount of radioactivity in a spot is proportional to the amount of sample applied to the chromatogram, the following experiment was done. λcb2 RNA synthesized under standard conditions was isolated as de-

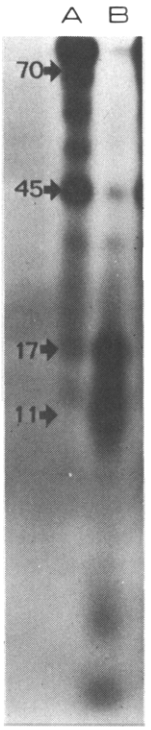

FIGURE 3: Polyacrylamide gel electrophoresis of λcb2 RNA synthesized in vitro. The RNA was synthesized and separated on a G-50 column as described in Experimental Procedures and shown in Figure 1. RNA from the void peak (Fractions 4–7, Figure 1) and from the leading edge of the substrate peak (Fractions 8–10, Figure 1) were pooled separately, ethanol precipitated, and analyzed on 20% polyacrylamide gels. Column A shows the RNAs contained in the void peak. Column B shows the size distribution of RNAs in the leading edge of the substrate peak. Approximate chain lengths (indicated on the left) were determined by running oligonucleotides of dC of known length to obtain standard curves of chain length vs. distance migrated into the gel.

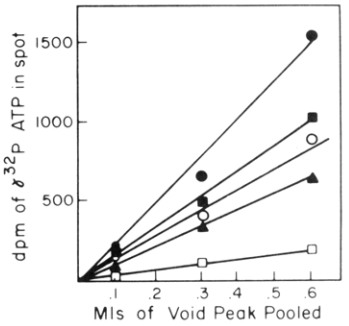

FIGURE 4: Effect of increasing amounts of sample on relative amounts of 5′-terminal oligonucleotides detected. RNA was synthesized from λcb2 DNA using [γ-^{32}P]ATP and isolated as described in Experimental Procedures. The pooled void peak from the G-50 column was divided into various sized samples. These samples were ethanol precipitated, digested with T1 RNase, and chromatographed as described. The spots were located by autoradiography, scraped into scintillation vials, and counted as described. Each symbol represents a different spot on the chromatogram.

scribed and the pooled void peak from the G-50 column was divided into 0.1-, 0.3-, and 0.6-mL portions. Each portion was precipitated, digested, and chromatographed in the usual manner. The spots were cut out and counted as described above. Figure 4 shows the results of quantitating several labeled oligonucleotides from λcb2 and demonstrates the linearity of the system with respect to increasing amounts of 5′-terminal oligonucleotides applied to the chromatogram.

Discussion

A method has been described which allows the rapid and quantitative analysis of 5′-terminal oligonucleotides of RNA made in vitro. This method involves labeling the γ phosphate of the 5′-triphosphate end of RNA transcripts during synthesis, isolating and digesting the RNA, and fractionating the 5′-oligonucleotides on a two-dimensional thin-layer chromatogram system. In one long day it is possible to do the complete analysis of 8–12 separate transcription reactions up to the beginning of the autoradiography.

It has been shown that the RNA synthesized in vitro off of a variety of DNA templates yields characteristic fingerprint patterns which are qualitatively different. RNAs initiating with either ATP or GTP have been analyzed and the number of major spots appears to correlate approximately with the number of promoters thought to be active in vitro with each template. It has also been demonstrated that the recovery of labeled oligonucleotides is high and that one can quantitate the amount of radioactivity in each spot and in that way measure the amount of various 5′-triphosphate ends present in the RNA population.

In the following paper (Miller & Burgess, 1978a), it is demonstrated that these spots can be correlated with particular 5′-terminal oligonucleotide sequences and with particular known promoters of bacteriophages λ and T7.

While providing a direct and rapid method of examining the 5′ ends of in vitro synthesized RNAs, this system has some obvious limitations. One is that it is unable to distinguish two RNAs which give rise to 5′-terminal oligonucleotides which are identical or which have identical mobilities in this system. This limits studies to templates with a small number of promoters of different starting sequences or to systems in which the transcripts can be partially fractionated prior to 5′-end analysis. Fortunately most of the bacteriophage templates are amenable to this type of study. This limitation is more severe for GTP-initiated transcripts digested by pancreatic RNase than by ATP-initiated transcripts digested by T1 RNase, because pancreatic RNase generates shorter oligonucleotides possessing less possible sequence diversity. In addition, abortive initiation and ambiguous starting might occur at some promoters leading to an underestimation of the initiations at those promoters. This point is discussed in more detail in the following papers (Miller & Burgess, 1978a,b).

Recently another paper has appeared describing a procedure for utilizing γ-^{32}P-labeled triphosphate terminal oligonucleotides to study in vitro transcription but which employs a chromatographic fractionation with less resolving power than that described here (Lebowitz et al., 1977).

Of the possible methods for quantitating the amount of in vitro transcription of a gene, the direct measurement of the amount of 5′ end corresponding to its transcript allows the least ambiguous measure of the amount of specific initiation at its promoter. Hybridization methods cannot distinguish a change in initiation rate at a promoter from a change in the read-through into the region from a region upstream from that promoter. They are also subject to variable hybridization efficiencies and the inability to detect very short transcripts quantitatively. Measuring in vitro transcription by fractionation on the basis of size requires complete freedom from RNase activity, efficient specific termination, and good resolution of the transcripts.

The method described here will allow much more detailed analyses of the effects of a number of variables on initiation of RNA synthesis, in vitro. Such a study is reported in an accompanying paper (Miller & Burgess, 1978b).

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References

Blattner, F., & Dahlberg, J. (1972) *Nature (London), New Biol. 237,* 227-232.
Burd, J., & Wells, R. (1974) *J. Biol. Chem. 249,* 7094-7101.
Burgess, R., & Jendrisak, J. (1975) *Biochemistry 14,* 4634-4638.
Chamberlin, M. (1974) *Annu. Rev. Biochem. 43,* 721-775.
Dahlberg, J., & Blattner, F. (1973) *Virus Research,* pp 533-543, Academic Press, New York, N.Y.
Dunn, J., & Studier, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 1559-1563.
Grohmann, K., Smith, L. H., & Sinsheimer, R. L. (1975) *Biochemistry 14,* 1951-1955.
Johnston, D., & McClure, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 413-428, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Konrad, M. (1973) *Anal. Biochem. 54,* 213-222.
Lebowitz, P., Weissman, S., & Radding, S. (1971) *J. Biol. Chem. 246,* 5120-5139.
Lebowitz, P., Stern, R., Ghosh, P. K., & Weissman, S. (1977) *J. Virol. 22,* 430-445.
Maitra, U., Nakata, Y., & Hurwitz, J. (1967) *J. Biol. Chem. 242,* 4908-4918.
Meyer, B. J., Kleid, D., & Ptashne, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4785-4789.
Miller, J. S., & Burgess, R. R. (1978a) *Biochemistry 17* (2nd of series of three papers in this issue).
Miller, J. S., & Burgess, R. R. (1978b) *Biochemistry 17* (3rd of series of three papers in this issue).
Minkley, E. G., & Pribnow, D. (1973) *J. Mol. Biol. 77,* 255-277.
Pettersson, U., & Sambrook, J. (1973) *J. Mol. Biol. 73,* 125-130.
Randerath, K., & Randerath, E. (1964) *J. Chromatogr. 16* 111-125.
Reiner, A. M. (1969) *J. Bacteriol. 97,* 1437-1443.
Reznikoff, W. (1972) *Annu. Rev. Genet. 6,* 133-156.
Schendel, P, & Wells, R. (1974) *J. Biol. Chem. 248,* 8319-8321.
Smith, L. H., Grohmann, K., & Sinsheimer, R. L. (1974) *Nucleic Acid Res. 1,* 1521-1529.
Stahl, S. J., & Chamberlin, M. J. (1977) *J. Mol. Biol. 112,* 577-601.
Studier, F. W. (1969) *Virology 39,* 562-574.
Sugiura, M., Okamoto, T., & Takanami, M. (1969) *J. Mol. Biol. 43,* 299-315.
Surzycki, S. J., Surzycki, J. A., DeLorbe, W., & Gussin, G. N. (1976) *Mol. Gen. Genet. 143,* 177-184.
Takeya, T., & Fugisawa, H. (1974) *Biochim. Biophys. Acta 324,* 110-119.
Terzaghi, E., Okada, Y., Streisinger, G., Emrich, J., Inouye, M., & Tsugita, A. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56,* 500-507.
Thomas, C., & Abelson, J. (1961) *Proc. Nucleic Acid Res. 1,* 553-561.

Selectivity of RNA Chain Initiation in Vitro. 2. Correlation of 5′-Triphosphate-Labeled Oligonucleotides on Polyethyleniminecellulose Thin-Layer Chromatography with RNA Transcripts of Bacteriophage λcb2 and T7†

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ABSTRACT: Methods are described for the correlation of 5′-terminal oligonucleotides separated by two-dimensional polyethyleniminecellulose thin-layer chromatography with specific RNA transcripts made in vitro from DNA of phages T7 and λcb2. The 5′-terminal oligonucleotides transcribed from DNA containing mutations and alterations which affect the RNA transcripts from specific promoters were compared with fingerprints of RNA from wild type DNA. Specific RNAs were purified on polyacrylamide gels and digested and their 5′-terminal oligonucleotides subjected to chromatography. Transcription of DNA fragments containing specific promoters was carried out and the 5′-oligonucleotide fingerprints of the RNA products compared with fingerprints of the RNA from the whole DNA. Using these methods and information known about T7 and λcb2 RNA 5′-terminal sequences, it was possible to identify many of the oligonucleotides separated on the polyethyleniminecellulose chromatography system.

The *E. coli* RNA polymerase can selectively initiate and transcribe in vitro specific RNA transcripts on DNA templates from many bacterial and viral sources. To determine the parameters influencing selectivity, one would like to measure initiations of specific RNAs rapidly and quantitatively.

A thin-layer chromatography method has been described which allows the rapid and quantitative analysis of 5′-triphosphate oligonucleotides labeled in the γ position and generated by RNase digestion of transcripts synthesized in vitro (Miller & Burgess, 1978a). This procedure results in a fin-

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