Incorporation of Purine Nucleoside 5′-[γ-S]Triphosphates as Affinity Probes for Initiation of RNA Synthesis in Vitro†

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ABSTRACT: Synthetic DNA templates were transcribed by *Escherichia coli* RNA polymerase using nucleoside 5′-[γ-S]triphosphates as one of the nucleotide substrates. Substitution of the thiol analogues for the normal nucleotides had no effect on the rate of RNA synthesis. RNA synthesized with either adenosine 5′-[γ-S]triphosphate or guanosine 5′-[γ-S]triphosphate was isolated with high efficiency on mercury-agarose columns prepared by activation with low concentrations of cyanogen bromide. Sulfur was shown to be incorporated at the 5′ end of RNA chains by identification of the tetraphosphate HSpppA^{32}p liberated after alkaline hydrolysis of HS(A-^{32}pU)$_n$ (alternating copolymer synthesized by the action of *E. coli* RNA polymerase on d(A-T)$_n$·d(A-T)$_n$ with adenosine 5′-[γ-S]triphosphate and uridine 5′-[α-^{32}P]triphosphate as substrates). Transcripts elongated but not initiated with these thiol analogues did not bind to the affinity column. This technique provides an extremely sensitive assay for RNA synthesis initiation in vitro, since initiated transcripts containing radiolabel throught the entire transcript can be isolated.

The study of in vitro transcription requires a system which will faithfully initiate and elongate RNA chains. Investigations in this laboratory have indicated that mouse myeloma nuclei satisfy both these criteria at least for the abundant low-molecular-weight 4.5S and 5S RNA species (Marzluff et al., 1974). Verification of RNA chain initiation relied on the established method of detecting [γ-^{32}P]ribotriphosphates incorporated at the 5′ end of the transcripts (Maitra and Hurwitz, 1965). While this technique has been satisfactory for measuring RNA initiation at high frequency, the detection of very low levels of RNA initiation from single-copy sequences has been extremely difficult because of the lack of sensitivity of the assay.

Analogues of nucleoside triphosphates containing sulfur at the γ-phosphate position have been described by Goody and Eckstein (1971). We have used these analogues to develop an affinity chromatography system which allows the isolation of transcripts initiated in vitro. This method requires incorporation of thiol-nucleotide analogues ([γ-S]ATP or [γ-S]GTP)[1] into RNA such that only the 5′ ends of the transcripts are substituted with sulfur. Sulfur-terminated sequences may then be isolated by binding to a mercury-agarose column. Since chain elongation requires the elimination of the β and γ phosphates from the RNA ribonucleotide precursors during the polymerization process, it follows that only initiated transcripts will be bound to mercury-agarose. A distinction between chain initiation and chain elongation can therefore be readily made. Affinity binding increases the sensitivity of detection of RNA initiation, since such transcripts contain radiolabel throughout their chain length instead of only at the γ-phosphate. In addition, the ability to physically isolate only those RNA sequences initiated in vitro should be valuable in the study of the regulation of gene transcription.

The studies reported here have utilized the transcription of synthetic templates by *E. coli* RNA polymerase as a model system to test the possibility that RNA transcripts initiated with a 5′-sulfur terminus can be isolated by affinity chromatography on a mercury-agarose column. A brief description of these studies has recently been presented (Cold Spring Harbor Symposium, June, 1977).

Materials and Methods

Adenosine 5′-[γ-S]triphosphate was purchased from Boehringer Mannheim Biochemicals. Guanosine 5′-[γ-S]-triphosphate was a gift from Dr. W. Waessle of Boehringer. These compounds were purified from nucleoside diphosphate contaminants by binding to a mercury-agarose column (Affigel 501, Bio-Rad Laboratories) in 0.1 M ammonium formate, 0.01 M EDTA, and eluting with 0.1 M ammonium formate containing 0.005 M dithioerythritol. The sulfur nucleotides were lyophilized and stored in deionized water at −10 °C.

Radiolabeled nucleotides were obtained from both New England Nuclear Corp. and Amersham. DNA templates d(A-T)$_n$·d(A-T)$_n$ and d(G-C)$_n$·d(G-C)$_n$ were purchased from P-L Biochemicals Inc. *Escherichia coli* RNA polymerase (EC 2.7.7.6) prepared according to Burgess and Jendrisak (1975) was obtained from Miles Laboratories.

Preparation of Mercury-Agarose. Mercury-agarose was prepared essentially according to the methods used by Cuatrecasas (1970) and Ruiz-Carrillo and Allfrey (1973); however, much lower concentrations of cyanogen bromide were used during the gel activation. Bio-Gel A-15M (Bio-Rad Laboratories) was washed extensively and resuspended in an equal volume of 1.5 M potassium carbonate which had been adjusted to pH 10.8 with CO_2 gas. The slurry was stirred slowly in a well-ventilated hood and cyanogen bromide in dioxane at 500 mg/mL was added dropwise to give a final concentration of 2.5 mg/mL of packed gel. Potassium carbonate

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[1] Abbreviations used are: [γ-S]ATP, adenosine 5′-[γ-S]triphosphate; [γ-S]GTP, guanosine 5′-[γ-S]triphosphate; HS-RNA, RNA containing a sulfhydryl group at the γ-phosphate position; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl.

was used as a buffer during the activation, since the extent of reaction is dependent on the pH at which the activation is performed (Schnaar et al., 1977). The temperature of the reaction was kept at 20 °C by the addition of ice and the pH was maintained at 10.8 by titration with 10 M KOH. After 15 min, the slurry was quickly filtered through a coarse sintered-glass funnel and washed with a volume of ice-cold water (adjusted to pH 10 with KOH) ten times the packed gel volume. An equal volume of 2 M ethylenediamine (adjusted to pH 10 with HCl) was added to the washed gel and the slurry was stirred overnight at 4 °C. The resin was then washed by filtration with a 50-fold excess of ice-cold water and resuspended in an equal volume of 40% (w/v) *N,N*-dimethylformamide containing *p*-chloromercuribenzoate (2.5 g/100 mL). The pH was adjusted to 4.8 with concentrated HCl, 1-ethyl-3-dimethylaminopropylcarbodiimide (2.5 g/100 mL total volume) added, and the solution stirred overnight at room temperature. At the completion of the coupling, the gel was washed sequentially with an equal volume of 40% *N,N*-dimethylformamide, 20 volumes of 0.1 M sodium bicarbonate, pH 8.8, and 10 volumes of deionized water. The mercury–agarose gel was stored at 4 °C. The organomercurial content of this gel was 0.12 μmol of organomercurial/mL of packed gel as determined with 5,5′-dithiobis(2-nitrobenzoic acid) according to Sluyterman and Wijdenes (1970).

Transcription of RNA. Transcription reactions (0.1 mL) contained 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 M KCl, 1 μg of synthetic template, either $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ or $d(G\text{-}C)_n \cdot d(G\text{-}C)_n$, and 1 μg of *E. coli* RNA polymerase. The appropriate nucleotide triphosphates, either [α-^{32}P]UTP plus ATP or [γ-S]ATP, or alternatively [α-^{32}P]CTP plus GTP or [γ-S]GTP, were included at 0.1 mM in the reactions. Synthesis was at 37 °C for 60 min. Following synthesis, reactions were adjusted to 1 mL with 0.1 M sodium acetate buffer (pH 5), 0.1% sodium dodecyl sulfate, and extracted with 2 volumes of water-saturated phenol. RNA was ethanol precipitated from the aqueous phases with yeast RNA as carrier.

Preparation of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ Elongation Complexes. $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ elongation complexes were prepared essentially according to Rhodes and Chamberlin (1974). RNA synthesis reactions (0.1 mL) (see previous section) were incubated for 5 min at 37 °C and transcription was initiated by the addition of polymerase and terminated after 1 min by adding 0.025 mL of 0.1 M EDTA and immediately chilling on ice. The ternary complex was isolated at 4 °C by centrifuging through a 1-mL Bio-Gel P-6 column (prepared in a 1-mL disposable plastic syringe) directly into a 0.1-mL reaction solution containing the necessary salts and nucleotide precursors (see previous section). Rifampin (1 μg) was also included to prevent reinitiation. The elongation reaction was for 10 min at 37 °C and the synthesis was terminated by adding 0.8 mL of 0.01 M Tris-HCl (pH 7.9), 0.01 M EDTA, 0.1 M NaCl, 0.1% $NaDodSO_4$. Samples were phenol extracted and ethanol precipitated as above.

Affinity Chromatography of HS-RNA on Mercury–Agarose. HS-RNA was dissolved in 0.01 M Tris-HCl (pH 7.9), 0.1 M NaCl, 0.01 M EDTA, 0.1% $NaDodSO_4$ (TNE buffer), and desalted over Bio-Gel P-6 equilibrated in TNE buffer. The desalted HS-RNA was then applied directly to a 1.4 × 2.5 cm column of mercury–agarose also in TNE buffer. The column was washed with TNE buffer to remove unbound RNA and the bound fraction was displaced by eluting with TNE buffer containing 0.01 M dithioerythritol.

Mercury–agarose was regenerated after use by washing with 10 mM $HgCl_2$ and 20 mM EDTA in 50 mM sodium acetate at pH 4.8. Excess $HgCl_2$ was removed with 0.2 M NaCl and 1 mM EDTA in 0.1 M sodium phosphate, pH 6.0.

Identification of Incorporated Sulfur-Containing Nucleotides. Preparations of $HS(A\text{-}U)_n$ labeled with [α-^{32}P]UTP were alkaline digested in 0.3 M NaOH for 16 h at 37 °C. The digests were neutralized, adjusted to 0.1 M ammonium acetate, 0.01 M EDTA, and bound to a column of mercury–agarose equilibrated in the same buffer. The column was washed with 0.1 M ammonium acetate, and the bound HSpppA^{32}p eluted with 10 mM dithioerythritol, 0.1 M ammonium acetate and lyophilized.

For charge determination, nucleotide samples were dissolved in 0.5 mL of 50 mM Tris-HCl (pH 8), 7 M urea, 1 mM 2-mercaptoethanol, containing 1 mg of RNase A digested yeast RNA as charge markers. The sample was applied to a 0.8 × 20 cm column of DEAE-cellulose and eluted with a linear gradient of 0.05–0.3 M NaCl in 50 mM Tris-HCl (pH 8), 7 M urea, and 1 mM 2-mercaptoethanol. The elution of the yeast oligonucleotide peaks was measured by absorbance at 260 nm, while radioactivity was determined by direct Cerenkov radiation in the tritium channel of a Packard scintillation counter.

Thin-layer chromatography was performed as described by Goody and Eckstein (1971) on Brinkman polyethyleneimine thin layers. Samples were developed in 0.75 M KH_2PO_4 adjusted to pH 3.4 with concentrated HCl. Electrophoresis was run on Whatman 3MM paper in 5% glacial acetic acid, 0.5% pyridine, 5 mM EDTA at 60 V/cm for approximately 60 min. Radioactivity was determined by autoradiography using Kodak BB54 x-ray film.

Results

Incorporation of Nucleoside 5′-[γ-S]Triphosphates into RNA. The transcription of synthetic templates by RNA polymerase has been chosen as a model system to test the validity of the affinity chromatography procedure. Transcription of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ with [γ-S]ATP as a substrate has previously been reported (Goody et al., 1972). As shown in Figure 1, the rate of transcription of the alternating copolymers $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ and $d(G\text{-}C)_n \cdot d(G\text{-}C)_n$ was unchanged when the corresponding nucleoside 5′-[γ-S]triphosphates were used as substrates. Substitution of 2-mercaptoethanol for dithioerythritol had no effect on the reaction rate. No synthesis was observed if [γ-S]ATP was omitted from the reaction mixture. The synthesis of RNA with [γ-S]ATP had no effect on the final size of the transcripts as determined by sedimentation in denaturing dimethyl sulfoxide gradients.

Affinity Binding of [γ-S]ATP to Organomercurial Agarose. In order to establish that the γ-thiophosphate analogues reacted with mercury–agarose, a solution of [γ-S]ATP and [^{14}C]ATP was fractionated on Affigel 501. ATP was not bound to the mercury–agarose gel, while [γ-S]ATP was retained. A small amount of UV-absorbing material coeluting with [^{14}C]ATP was shown by thin-layer chromatography to be an ADP contaminant in the [γ-S]ATP preparation. Elution of the column with buffer containing dithioerythritol resulted in the displacement of the thiol nucleotide.

Affinity Binding of HS-RNA to Mercury–Agarose. Synthesis of RNA in the presence of a γ-sulfhydryl nucleoside triphosphate should result in the incorporation of sulfur exclusively at the 5′ end of the RNA molecules initiated with that nucleotide, since the β and γ phosphates must be eliminated during phosphodiester bond formation. Such sequences should then be easily isolated by covalently binding the HS-RNA transcripts to mercury–agarose.

Our attempts to bind HS-RNA to mercury– agarose were

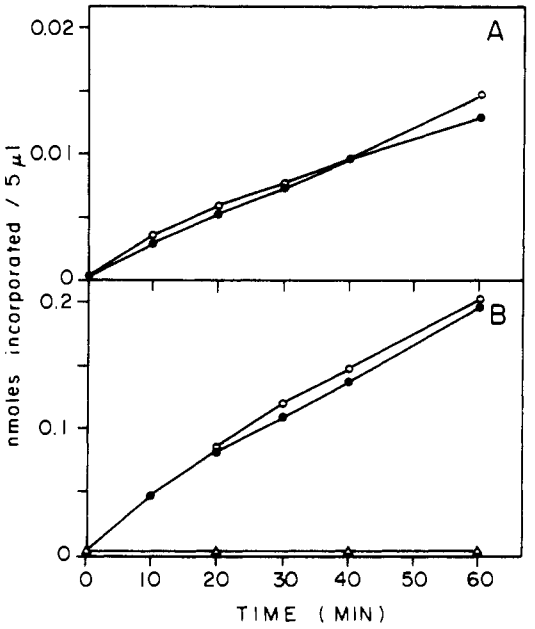

FIGURE 1: Incorporation of ribonucleoside triphosphates and their thiol analogues in the presence of the templates d(A-T)$_n$·d(A-T)$_n$ and d(G-C)$_n$·d(G-C)$_n$. Templates were transcribed with *E. coli* RNA polymerase as described under Methods, with [α-^{32}P]UTP (1 Ci/mmol) and [α-^{32}P]CTP (1 Ci/mmol) as radioactive labels. Samples were removed at indicated times and trichloroacetic acid insoluble radioactivity was determined. (a) d(G-C)$_n$·d(G-C)$_n$ template with GTP (●-●) and [γ-S]GTP (O-O). (B) d(A-T)$_n$·d(A-T)$_n$ template with ATP (●-●), [γ-S]ATP (O-O), and minus ATP control (Δ-Δ).

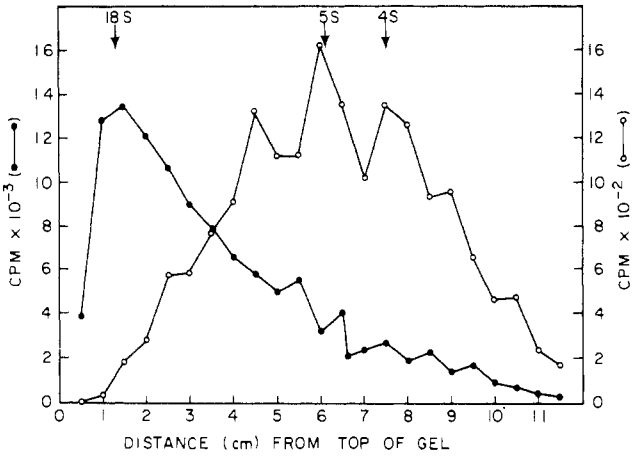

FIGURE 2: Polyacrylamide gel electrophoresis of total HS(A-U)$_n$ (●-●) and Affigel 501 bound RNA (O-O). RNA was synthesized for 60 min as described under Methods. Electrophoresis was performed on a 10-cm slab gel in 10% acrylamide–0.2% bisacrylamide containing 40 mM Tris-HCl (pH 7.4) and 2 mM EDTA. Samples were applied to the gel in 95% formamide and electrophoresis was at 4 V/cm for 1.5 passes of bromophenol blue marker dye. *Drosophila* marker RNAs were run in an adjacent slot on the slab gel. Gel slabs were dried on Whatman 3MM filter paper, cut into 0.5-cm slices, and counted by Cerenkov radiation in the tritium channel of a Packard scintillation counter.

initially performed using Affigel 501, a product of Bio-Rad Laboratories, prepared from a cross-linked derivative of Bio-Gel A-15M (4% agarose). HS(A-U)$_n$ was synthesized with *E. coli* RNA polymerase using d(A-T)$_n$·d(A-T)$_n$ as template and [γ-S]ATP and [α-^{32}P]UTP as substrates. The HS(A-U)$_n$ product of a 60-min synthesis sedimented in a broad peak at approximately 18 S in 99% Me_2SO sucrose density gradients and gave a number average molecular weight of approximately 550 nucleotides. This material gave only 15–25% binding to

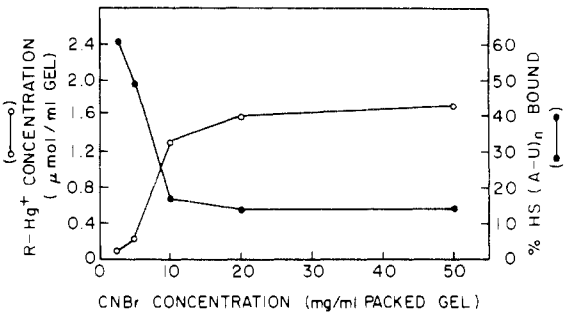

FIGURE 3: The binding of HS(A-U)$_n$ to a series of mercury gels prepared with varying amounts of cyanogen bromide. Bio-Gel A15M (10 mL packed volume) was converted to an organomercurial derivative as described under Methods. CNBr concentrations varying from 2.5 to 50 mg/mL were used in the activation step. HS(A-U)$_n$ isolated from a 60-min synthesis was chromatographed on 3-mL columns of the organomercurial derivatives, and the percentage bound was determined by radioactive counting (●-●). The organomercurial titer of each gel sample was determined with 5,5′-dithiobis(2-nitrobenzoic acid) according to Sluyterman and Wijdenes (1970) (O-O).

Affigel 501. The failure of the bulk of the RNA to bind to Affigel 501 was not due to a lack of sulfur-containing nucleotides, since the HS-RNA which fails to bind Affigel 501 contains 53% of the total sulfur-containing nucleotides. The fact that Affigel 501 bound 47% of the sulfur-containing nucleotides but only about 20% of the total HS-RNA suggested that the column had preferentially bound molecules of short chain length which contain a higher ratio of 5′ ends to chain length. Examination of the bound HS-RNA on polyacrylamide gels in 95% formamide (Figure 2) shows that indeed the RNA retained by Affigel 501 is considerably smaller than the total RNA.

These results suggested that the low-binding efficiency seen with larger molecular weight HS-RNA was due to the exclusion properties of the affinity gel. Since the concentration of organomercurial functions immobilized within the gel matrix is much higher than at the gel surface, the porosity of the beads must be an important factor in determining the binding efficiency. Lowe and Dean (1971) have shown, for example, that the binding of proteins to a Sephadex affinity matrix depends both on the size of the proteins and the porosity of the matrix.

In order to increase the binding efficiency of the affinity gel we attempted to obtain a matrix of higher porosity by using lower percentage agarose beads with a high-molecular-weight exclusion limit which would allow the diffusion of higher molecular weight species into the gel. Mercury–agarose was prepared according to Ruiz-Carrillo and Allfrey (1973) with 1% agarose beads (Bio-Gel A-150M) which should include all size classes of HS(A-U)$_n$ within the gel matrix. This affinity gel surprisingly gave very low binding of HS(A-U)$_n$ and, since no further binding was observed after incubating the gel and RNA for 1 h, it was unlikely that the decreased binding efficiency was due simply to a decrease in the organomercurial content of the gel.

These results suggested that, although the gel preparations began with high porosity agarose beads, extensive cross-linking during the cyanogen bromide activation step results in a reduction of the gel porosity (Axen et al., 1967; David et al., 1974; Lasch et al., 1975). We therefore prepared a series of organomercurial agarose gels activated with decreasing amounts of cyanogen bromide in an attempt to minimize this cross-linking. Figure 3 shows that at a critical cyanogen bromide concentration (less than 10 mg/mL) the binding of HS(A-U)$_n$ increased markedly, concomitant with a decrease

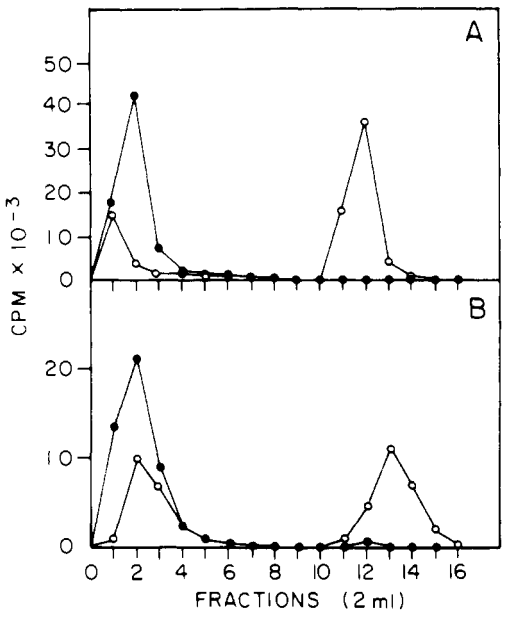

FIGURE 4: Chromatography of HS-RNA on mercury-agarose. RNA was synthesized for 60 min as described under Methods. At the completion of the synthesis (A) HS(A-U)$_n$ (○-○), (A-U)$_n$ (●-●) and (B) HS(G-C), (○-○) and (G-C)$_n$ (●-●) were phenol extracted and ethanol precipitated. The ethanol precipitate was redissolved in 0.2 mL of 0.01 M Tris-HCl (pH 7.9), 0.01 M EDTA, 0.1 M NaCl, 0.1% NaDodSO$_4$, and desalted on a Bio-Gel P-6 column using the same buffer. Fractions containing the eluted RNA were pooled and applied to a 3 × 1.4 cm mercury-agarose column (low CNBr activation, see Methods). The column was eluted with the Tris buffer for ten fractions to remove nonbound RNA and the bound RNA was then displaced with 0.01 M dithioerythritol. Fractions (2 ml.) were collected and counted for radioactivity.

in the organomercurial titer of the gel. Mercury-agarose gel prepared by activation with 2.5 mg/mL of cyanogen bromide binds 90% of the total sulfur-containing terminii and the bound HS-RNA shows the same size distribution as the total RNA. This gel derivative was chosen for most experiments as a consequence of its high binding efficiency and absence of size selection.

The binding of HS(A-U)$_n$ and HS(G-C)$_n$ to mercury-agarose is shown in Figure 4. These polymers were synthesized by transcription with *E. coli* RNA polymerase of d(A-T)$_n$·d(A-T)$_n$ and d(G-C)$_n$·d(G-C)$_n$ templates, respectively. It can be seen that, whereas the binding of RNA synthesized in the absence of the thiol nucleotides was extremely low, a large fraction of the HS-RNA was retained by the organomercurial agarose and subsequently displaced by the addition of a competing mercaptan such as dithioerythritol. Binding of HS(A-U)$_n$ was equally effective at either room temperature or 4 °C. To prevent competition for binding sites on the mercury-agarose by unincorporated thiol nucleotides, preparations of HS-RNA were routinely desalted by gel filtration prior to affinity chromatography. Since mercuric ions are known to form complexes with nucleic acids (Gruenwedel and Davidson, 1967), EDTA was included in the column buffers to minimize interactions between the immobilized organomercurial and the polynucleotides. Recovery of the RNA under these conditions was greater than 95%.

Recycling the retained RNA on mercury-agarose resulted in very high levels of binding. After a third passage through the affinity column, 87% of HS(A-U)$_n$ was bound. Quantitative retention of the recyled HS-RNA was not expected, since there is a small amount of chain scission during the elution of the RNA through the organomercurial agarose. Table I shows that when [^{3}H]RNA nascent chains initiated with

TABLE I: Binding of Nascent RNA to Mercury-Agarose.[a]

Initiation	Elongation	% Bound ^{3}H	% Bound ^{32}P
[γ-S]ATP + [^{3}H]-UTP	ATP + [α-^{32}P]-UTP	78	65
ATP + [^{3}H]-UTP	[γ-S]ATP + [α-^{32}P]-UTP	0.04	0.24

[a] d(A-T)$_n$·d(A-T)$_n$ elongation complexes were formed with ATP (or [γ-S]ATP) and [^{3}H]UTP as substrates and subsequently elongated with [γ-S]ATP (or ATP) and [α-^{32}P]UTP in the presence of rifampin (see Methods). The RNA products were chromatographed on mercury-agarose as described under Methods, and the percentage of RNA bound was determined.

[γ-S]ATP and subsequently elongated with [α-^{32}P]nucleotide precursors are chromatographed on mercury-agarose, there is a preferential binding of [^{3}H]RNA. Since the [^{32}P]nucleotides are at the 3′ end of the transcripts, we conclude that some chain scission occurs during the affinity chromatography step.

Identification of Sulfur at the 5′ Terminus. If γ-sulfhydryl nucleoside triphosphates are to serve as affinity probes for RNA initiation, the sulfur incorporated into the polynucleotide must be located exclusively at the 5′ γ-phosphate position. Alkaline digestion of such RNA should result in the release of a [γ-S]nucleotide tetraphosphate as the only sulfur-containing product.

To examine the site of incorporation of sulfur, HS(A-U)$_n$ synthesized using [α-^{32}P]UTP as the radioactive label was alkaline digested and neutralized, and the digestion products were fractionated over a mercury-agarose column. Approximately 0.35% of the radioactivity was retained on the column, whereas elution of an alkaline digested sample (A-U)$_n$ resulted in negligible binding (0.009%). The column-bound product was analyzed by chromatography on DEAE-cellulose-urea, electrophoresis on 3MM paper, and chromatography on PEI-cellulose thin layers.

Chromatography on DEAE-cellulose in 7 M urea separates nucleotide products on the basis of net charge (Tener, 1967). As shown in Figure 5, the major product migrates with a net charge of −6, consistent with its being a tetraphosphate. Some material with lower charge is also seen, perhaps through partial degradation of the γ-thiophosphate; however, no −2 charged material corresponding to nucleoside monophosphate is detected, indicating that no sulfur could have been incorporated internally in the phosphodiester bonds.

Figure 6 illustrates the electrophoresis of the sulfur-containing digestion product on Whatman 3MM paper in pyridine–acetate (pH 3.5). The main product migrates as a tetraphosphate with a secondary slower migrating band. Both species migrate more rapidly than marker ATP and no nucleoside mono- or diphosphate product is detected.

Finally, the sulfhydryl nucleotide was examined by thin-layer chromatography on PEI plates (Figure 7) (Goody and Eckstein, 1971). Sulfur-containing nucleotide analogues migrate slower than their normal counterparts as illustrated by the ATP and [γ-S]ATP markers. When treated with mercury acetate, the [γ-S]ATP then migrates faster with an R_f near that of ATP. Mercury acetate has no effect on the migration of ATP or ADP. The sulfur-containing alkaline digestion product migrates slower than [γ-S]ATP. When treated with mercury acetate, its migration is increased; however, it still chromatographs well behind mercury-treated [γ-S]ATP. These results are fully consistent with the sulfur-containing

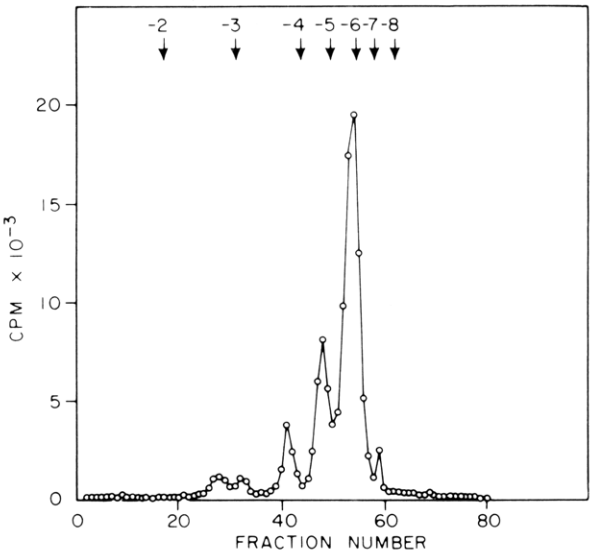

FIGURE 5: Chromatography of incorporated sulfur nucleotides on DEAE-cellulose in 7 M urea. HS[A-U]$_n$ was synthesized with [α-^{32}P]-UTP and [γ-S]ATP. The HS-RNA was alkaline digested and the sulfur-containing nucleotides were isolated on mercury–agarose as described under Methods. The isolated radioactivity was applied to a column of DEAE-cellulose in 50 mM Tris-HCl (pH 8), 7 M urea, and 1 mM 2-mercaptoethanol. The nucleotides were eluted with a linear gradient of 0.05–0.3 M NaCl. The arrows indicate the position of optical density oligonucleotide markers derived from the RNase A digestion of yeast RNA. Radioactivity was measured by Cerenkov radiation in the tritium channel of the scintillation counter.

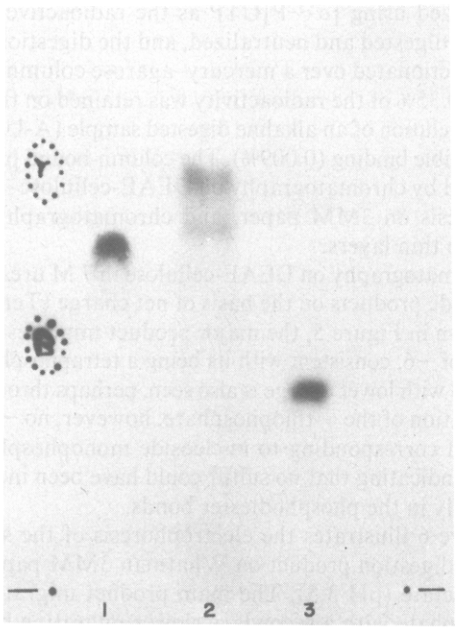

FIGURE 6: Paper electrophoresis of incorporated sulfur-containing nucleotides. Electrophoresis was done on Whatman 3MM paper in pyridine–acetate (pH 3.5) as described under Methods. The radioactivity was detected by autoradiography. Lane 1, [^{14}C]ATP marker; lane 2, radioactivity retained on mercury–agarose from the alkaline digestion of HS[A-U]$_n$ synthesized with [γ-S]ATP and [α-^{32}P]UTP (purification is described under Methods); lane 3, [2′, 3′-^{32}P]AMP marker. B and Y represent the positions of the marker dyes xylene cyanol and orange G, respectively.

product being a nucleoside tetraphosphate.

From the above analysis it is clear that the sulfur from the nucleotide analogues is incorporated predominantly at the 5′ terminus. Of the 125 000 cpm of radiolabeled product applied to the DEAE-cellulose column of Figure 5, only 59 cpm could

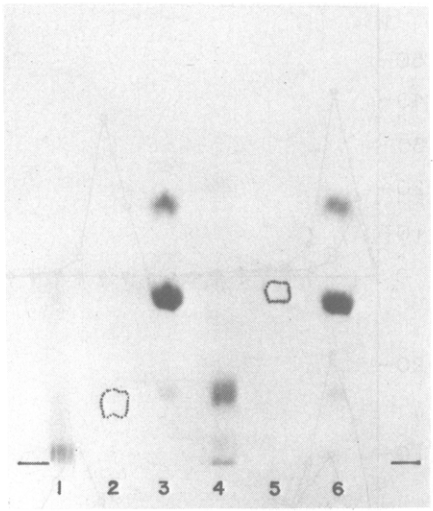

FIGURE 7: Thin-layer chromatography of incorporated sulfur-containing nucleotides. Thin-layer chromatography was done on PEI plates as described under Methods. Samples were applied either in deionized water (lanes 1–3), or in a solution containing 10 mM mercury acetate, 10 mM EDTA (lanes 4–6). Lanes 1 and 4, radioactivity retained on mercury–agarose from the alkaline digestion of HS[A-U]$_n$. Purification is described under Methods. Lanes 2 and 5, authentic [γ-S]ATP. The positions shown were detected by UV absorbance and outlined with radioactive ink. Lanes 3 and 6, [^{14}C]ATP marker, also containing [^{14}C]ADP as a contaminant.

be detected in the −2 charge material of fractions 15–19 and these gave no evidence of a peak. Therefore, no more than 0.05% of the sulfur-containing nucleotides could have had sulfur in the α position. If the total incorporated nucleotides are considered, no more than one nucleotide in 500 000 could have had an internal sulfur atom. Although the sensitivity of detection in the autoradiograms of Figures 6 and 7 is not as high as the DEAE-cellulose column, sulfur-containing nucleoside monophosphates would have been detected at the level of 0.5% and none was observed.

As additional proof that sulfur is not incorporated into phosphodiester bonds during chain elongation, elongation complexes were prepared by polymerization with [γ-S]ATP. Rhodes and Chamberlin (1974) have shown that ternary complexes consisting of *E. coli* polymerase, d(A-T)$_n$·d(A-T)$_n$ template and nascent r(A-U)$_n$ can be isolated free of ribonucleotide precursors by gel filtration and further incubation of the isolated complexes with substrates and rifampin (to prevent reinitiation) results in the elongation of nascent RNA chains.

Elongation complexes were formed with [γ-S]ATP and [^{3}H]UTP as substrates and subsequently elongated with ATP and [α-^{32}P]UTP. Table I shows that after isolating the RNA, ^{3}H and ^{32}P are both bound to the organomercurial agarose affinity column. In contrast to this experiment, very low levels of binding were observed when complexes formed with ATP and [^{3}H]UTP were elongated with [γ-S]ATP and [α-^{32}P]-UTP. This result therefore confirms that sulfur is not incorporated internally during chain elongation.

Discussion

The enzymatic synthesis of RNA with nucleoside 5′-[γ-S]triphosphates as substrates results in the incorporation of sulfur exclusively at the 5′ terminus. Since sulfur is not incorporated into phosphodiester bonds during chain elongation, transcripts initiated during the synthesis may be rapidly selected for by binding covalently to a mercury–agarose column.

The extent to which HS-RNA is bound to the affinity column depends on the porosity of the agarose matrix. Thus, gels extensively cross-linked during preparation by high concentrations of cyanogen bromide bind RNA only 100–200 nucleotides in length. Although an upper limit has not been defined, mercury–agarose prepared by activation with low concentrations of cyanogen bromide binds RNA molecules containing as few as 1 sulfur atom per 2000 nucleotides. Even though the organomercurial titer of this gel is reduced considerably by the use of low cyanogen bromide concentrations, the RNA binding capacity is still extremely high because the binding mode involves a single-point attachment of the RNA.

A large fraction of RNA transcribed with [γ-S]ribonucleotides is bound to mercury–agarose prepared by activation with low concentrations of cyanogen bromide. There are several reasons for the nonbinding of RNA to the affinity column. RNA not bound might arise from either a small amount of chain scission during the chromatographic procedure or as a result of initiation by contaminating non-sulfur-containing nucleotides such as ADP (Chamberlin, 1974). In addition, it is apparent from sulfur analyses of the bound and nonbound RNA that even though binding of RNA sulfur terminii is very efficient a small fraction of the HS-RNA is not retained, possibly as a result of limitations in the chromatography procedure.

The purification of sequences initiated with a 5′-sulfhydryl terminus can be utilized in several ways. For example, by choosing the appropriate thiol nucleotide, RNA sequences can be purified according to the identity of the initiating nucleotide. In this regard, Smith et al. (in preparation) have recently shown that bacteriophage lambda 4S RNA transcribed from the minor leftward promoter in vitro in the presence of [γ-S]GTP can be purified from ATP-initiated transcripts by binding to mercury–agarose. The ability to purify in vitro initiated transcripts means that sequences initiated in vitro may be detected directly by hybridization, since contaminating endogenous sequences are not bound to the affinity column. In contrast to initiation assays which involve the incorporation of only one ^{32}P atom at the 5′ terminus of initiated chains (Maitra and Hurwitz, 1965), the affinity chromatography assay is several orders of magnitude more sensitive because transcripts can be labeled throughout the entire length of the molecule. The measurement of transcripts initiated at low frequencies should therefore be expedited.

The thiol nucleotide [γ-S]ATP has been shown to be resistant to phosphatase cleavage (Goody and Eckstein, 1971). Since this property should facilitate the isolation of RNA transcripts with the 5′-end sequences intact, the application of the methodology described in this paper should be useful in the study of RNA precursor–product relationships in vitro. Such studies are currently under investigation.

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