A comparison of nucleoside (β -S)triphosphates and nucleoside (γ -S)triphosphates as suitable substrates for measuring transcription initiation in preparations of cell nuclei

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ABSTRACT

RNA chains initiated with nucleoside (β -S)triphosphates and (γ -S)triphosphates retain the thiol groups and can be separated from thiol-free RNA by chromatography on mercury-Sepharose. Thiol-containing mouse mammary tumor virus (MMTV) RNA synthesized by preparations of nuclei from virus-infected cells was quantitated by nucleic acid filter hybridization. With ATP β S and GTP β S, region-specific initiation of MMTV RNA chains was detected in the cell free system. However, with ATP γ S and GTP γ S, region-specific initiation was not clearly demonstrable. The nuclear preparations can also transfer thiol groups, presumably in the form of thiophosphate, from ATP γ S or GTP γ S onto preexisting RNA molecules; little or no thiol-transfer occurs with the two (β -S)-analogues. The thiophosphate transfer activity apparently interferes with the measurement of RNA chain initiation with ATP γ S and GTP γ S.

INTRODUCTION

Adenosine 5'-0-(3-thiotriphosphate) and guanosine 5'-0-(3-thiotriphosphate) (ATP γ S and GTP γ S) have been employed as a means of measuring initiation of RNA chains in cell free reaction systems where it is necessary to distinguish RNA molecules initiated in vitro from endogenous RNA that was elongated but not initiated in vitro (1-4). However, in attempting to use these substrates to measure cell free initiation of mouse mammary tumor virus (MMTV) RNA, we obtained evidence that a substantial fraction of the thiol-containing RNA products generated in our system were not initiated in vitro. In fact, generation of thiol-containing RNA in our cell free reaction was shown to be independent of continuing RNA synthesis, which was blocked with actinomycin D (5). Based on this evidence, we postulated that an oligonucleotide kinase enzyme in the nuclear preparations was transferring the γ -thiophosphate from ATP γ S and GTP γ S to preexisting RNA molecules. Because such a thiophosphate transfer activity would likely interfere with the measurement of cell free transcription initiation with

the (γ -S)-analogues, we have synthesized adenosine 5'-0-(2-thiotriphosphate) and guanosine 5'-0-(2-thiotriphosphate) (ATP β S and GTP β S) to test as substrates for measuring RNA chain initiation. In the (β -S)-analogues, the thiophosphate should not be accessible to transfer by a kinase type of mechanism.

We report here a more direct assay to test the thiophosphate transfer potential of the nucleoside (γ -S)triphosphates and (β -S)triphosphates. In addition we tested the suitability of each thiol analogue for measuring region-specific initiation of MMTV RNA chains in the cell free system.

EXPERIMENTAL PROCEDURES

Materials

ATP γ S, GTP γ S, ADP β S, GDP β S, pyruvate kinase, and *E. coli* RNA polymerase holoenzyme were purchased from Boehringer Mannheim. ATP β S, isomer a, and GTP β S, isomer a were synthesized from the thio-diphosphates by pyruvate kinase reactions and purified by DEAE-Sephadex chromatography (6-7). T7 bacteriophage DNA was kindly provided by R.L. Burke and B.N. Alberts, University of California, San Francisco. [5- 3 H]CTP (24 Ci/mmol) was purchased from ICN; [α - 32 P]UTP (400 Ci/mmol) was purchased from New England Nuclear. All other materials were described elsewhere (8).

Cloned MMTV DNA segments used for nucleic acid filter hybridization analyses

Clones I and II were kindly provided by J. Majors and H. Varmus (University of California, San Francisco); both fragments were inserted into the Pst I site of pBR322. Clone III was generously provided by V. Chandler and K. Yamamoto (University of California, San Francisco); this fragment was ligated to Bam HI linkers and then inserted into the Bam HI site of pBR322. Figure 1 illustrates the portion of the MMTV genome represented by each DNA clone. Clone I is a 1400-nucleotide Pst I fragment that contains one complete copy of the MMTV long terminal repeat and a short non-redundant sequence. The long terminal repeat is a 1327-nucleotide sequence found at each end of the MMTV provirus; it is composed of 1192 nucleotides representing the 3'-terminus of the virion RNA and 135 nucleotides representing the 5'-terminus (9-10). Clone II is a 1700-nucleotide Pst I fragment that represents sequences near the 3' end of the MMTV virion RNA. Clone III is a Sau3A-Pst I subfragment of Clone I; it contains approximately 110 nucleotides representing the 3'-terminus of the virion RNA and 270 nucleotides that represent the

5'-terminus. The nucleotide encoding the 5'-terminus is the presumptive site for transcription initiation.

Cell lines

M1.54, a clone of MMTV-infected rat HTC cells (11), and GR cells, a mouse mammary tumor cell line (12), were grown in monolayer culture on Dulbecco's modified Eagle's medium supplemented with 5% horse serum (Flow Laboratories); the cells were harvested before reaching confluency. WEHI7.2, a cloned mouse lymphoma cell line (13), and 66-2, a light chain producing clone of a mouse myeloma cell line derived from tumor line MPC-11 (14), were grown in suspension culture in Dulbecco's modified Eagle's medium with 10% horse serum; cells were harvested during log-phase growth.

Assay for initiation of MMTV RNA *in vitro*

The details of the assay are described elsewhere (8). A summary of the procedure follows: Nuclei were prepared from dexamethasone-stimulated M1.54 cells and incubated in cell free transcription reactions containing all four ribonucleoside triphosphates; [³H]CTP was the labeled precursor, and one of the four thiol analogues (ATPyS, ATP β S, GTPyS, or GTP β S) was substituted for the analogous natural substrate. After incubation for 1 hr at 25°C, RNA from the reaction was isolated by phenol extraction at pH 5, ethanol precipitated twice, and chromatographed on a column of mercury-Sepharose. Thiol-free RNA passed through the column directly; thiol-containing RNA bound to the column and was subsequently eluted with buffer containing dithiothreitol.

After ethanol precipitation, the abundance of MMTV RNA in the two column fractions was determined by nucleic acid filter hybridization, using cloned DNA segments representing three different regions of the MMTV genome (Fig. 1). Each assay contained one filter with MMTV DNA inserted into pBR322 and one control filter with non-recombinant pBR322 DNA. In addition to the [³H]RNA synthesized in the cell free transcription reactions, 1000-4000 cpm of purified MMTV [³²P]RNA isolated from virus particles was included in each reaction to monitor efficiency of hybridization. After the hybridization reaction, the filters were treated with ribonuclease and washed extensively to remove unbound RNA; bound RNA was eluted with base, neutralized, and counted in a liquid scintillation counter.

The hybridization efficiency sometimes varies from assay to assay. Therefore, it is necessary to measure this variable in each assay and

correct for it in subsequent calculations. Within a set of assays, the variation is never greater than two-fold. The relative percentage of MMTV RNA in the [^3H]RNA products was calculated as follows (8): The efficiency of hybridization is the fraction of the total ^{32}P cpm bound to the MMTV DNA filter minus that bound to the control pBR322 filter. The ^3H cpm bound to the MMTV DNA filter, minus the ^3H cpm bound to the control filter, was expressed as a percentage of total ^3H cpm in the hybridization reaction and then divided by the ^{32}P hybridization efficiency. Because the [^{32}P]RNA represents the entire MMTV genome, the final percentage for MMTV [^3H]RNA calculated by this method is automatically corrected for hybridization efficiency and normalized for the length of the MMTV DNA segment on the filter.

In control experiments, varying amounts of unlabeled competitor MMTV RNA were added to parallel filter hybridization assays containing [^3H]RNA synthesized by nuclei from dex-treated M1.54 cells. Although the efficiency of hybridization was reduced by as much as 50% in the presence of competitor RNA, the final corrected percentage of MMTV [^3H]RNA was the same (within 5%) for reactions with and without competitor (8).

Preparation of T7 bacteriophage [^{32}P]RNA

The transcription reaction contained, in a volume of 0.1 ml, 40 mM Tris-HCl, pH 8.0, 10 mM MgCl_2 , 2 mM dithiothreitol, 100 mM KCl, 0.1 mM CTP, 0.1 mM ATP, 0.1 mM GTP, 0.03 mM unlabeled UTP, 20 μCi [$\alpha\text{-}^{32}\text{P}$]UTP, 4.5 μg of T7 DNA, and 1.5 units of *E. coli* RNA polymerase holoenzyme. The reaction was incubated for 60 min at 37°C , then diluted with 2 ml of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM Na_2EDTA , 25 $\mu\text{g}/\text{ml}$ yeast RNA. RNA was purified from the reaction mixture by extraction at room temperature with 1 volume of water-saturated phenol (redistilled) mixed with 0.5 volume of chloroform; the RNA was precipitated from the aqueous phase by addition of NaCl to 0.25 M, and addition of two volumes of 95% ethanol. After standing at -20°C for several hours, the RNA was collected by centrifugation for 20 min at 10,000 g, dissolved in water and reprecipitated with salt and ethanol as before. The final RNA pellet was dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA .

Thiophosphate transfer assays

Nuclei from M1.54 cells were prepared and incubated in the cell free transcription reaction as described above, except that purified T7 [^{32}P]RNA (approximately 200,000 cpm) was added to each 0.5 ml reaction either prior to or following the 25°C incubation for cell free

transcription. RNA from the reaction was isolated by phenol extraction and two ethanol precipitations as described above. This procedure results in removal of all unincorporated [^3H]CTP. At this point, samples were counted to determine total [^3H]RNA synthesized and total [^{32}P]RNA recovered. The RNA was fractionated by mercury-Sepharose chromatography. The percentage of [^{32}P]RNA that bound to Hg-Sepharose served as a measure of thiophosphate transfer activity; the binding of [^3H]RNA to Hg-Sepharose served as a control to assure that the cell free transcription reaction and Hg-Sepharose fractionation worked properly for each assay.

RESULTS AND DISCUSSION

Thiophosphate transfer from ATP γ S and GTP γ S to exogenously added RNA

To test for thiol transfer, purified T7 bacteriophage [^{32}P]RNA was added to the complete cell free transcription reaction prior to the 25°C incubation, as described in Experimental Procedures. When only the four natural ribonucleoside triphosphate substrates were used for transcription instead of the thiol analogues, the Hg-Sepharose column bound less than 0.3% of the exogenously added T7 [^{32}P]RNA or of the [^3H]RNA synthesized by the nuclei in the cell free reaction (Table 1, line 1). This small amount of binding was presumably non-specific. When either ATP γ S plus GTP γ S or ATP β S plus GTP β S were substituted for the natural purine nucleoside triphosphates, 3 to 5% of the [^3H]RNA synthesized by the nuclei bound to Hg-Sepharose (Table 1, lines 2-5). With the (γ -S)-analogues, 2 to 3% of the T7 [^{32}P]RNA also bound to Hg-Sepharose (Table 1, line 2), indicating that some form of thiol transfer to the [^{32}P]RNA had occurred during the reaction. When either ATP γ S or GTP γ S was substituted alone into the transcription reaction, the percentages of binding of [^3H]RNA and [^{32}P]RNA were about half of those obtained when both (γ -S)-analogues were substituted together into the same reaction (data not shown). We detected similar levels of thiol transfer activity with nucleoside (γ -S)triphosphates in nuclear preparations from three other mammalian cell lines: GR, a mouse mammary tumor cell line; WEHI7, a mouse lymphoma cell line; and 66-2, a mouse myeloma cell line.

When the two (β -S)-analogues were substituted into the reaction, only background levels of [^{32}P]RNA bound to Hg-Sepharose (Table 1, line 3). In the same reaction, the [^3H]RNA binding was essentially the same

TABLE 1
Assay for Thiophosphate Transfer

	purine nucleotides used	Experiment	% bound to Hg-Sephadex				
			$[^3\text{H}]\text{RNA}$		$[^{32}\text{P}]\text{RNA}$		
			I	II	I	II	
1	ATP & GTP			.29		.25	$[^{32}\text{P}]\text{RNA}$ added <u>before</u> cell free incubation
2	ATPyS & GTPyS		3.8	5.4	2.4	3.1	
3	ATP β S & GTP β S		3.2	3.2	.39	.20	
4	ATPyS & GTPyS		3.2		.09		$[^{32}\text{P}]\text{RNA}$ added <u>after</u> cell free incubation
5	ATP β S & GTP β S			3.9		.05	

Thiophosphate transfer assays were performed as described in Experimental Procedures, using the indicated purine nucleotides. $[^3\text{H}]\text{RNA}$ (approximately 100,000 cpm) was synthesized by the nuclei in the cell free reaction. Purified T7 $[^{32}\text{P}]\text{RNA}$ (200,000 cpm total; $\sim 10^7$ cpm/ μg) was added to the cell free system either before (lines 1-3) or after (lines 4-5) the transcription reaction occurred.

as in the reaction with the (γ -S)-analogues; this showed that the cell free transcription reactions and Hg-Sephadex columns worked properly in both cases. In control experiments, the T7 $[^{32}\text{P}]\text{RNA}$ was added to the transcription reaction after completion of the 25°C incubation, just before phenol extraction (Table 1, lines 4 and 5). In these control experiments the $[^{32}\text{P}]\text{RNA}$ did not bind to Hg-Sephadex, but $[^3\text{H}]\text{RNA}$ binding demonstrated that proper transcription and Hg-Sephadex chromatography had occurred. Thus, the $[^{32}\text{P}]\text{RNA}$ did not bind to Hg-Sephadex unless it was incubated in the cell free reaction with ATPyS and/or GTPyS.

The results of this experiment are consistent with our hypothesis of thiophosphate transfer by a kinase type mechanism, in which only the γ -phosphate of the nucleoside triphosphate substrate is directly accessible for transfer. Oligonucleotide kinase activities have also been reported in other mammalian cell extracts (15-16). As an alternative explanation for the binding of $[^{32}\text{P}]\text{RNA}$ to Hg-Sephadex in our experiments, it seems extremely unlikely that 3 to 5% of the $[^{32}\text{P}]\text{RNA}$ could be degraded to mononucleotides, converted to $[\alpha\text{-}^{32}\text{P}]\text{triphosphates}$, and incorporated into new thiol-containing RNA chains in the cell free

reaction. Less than 1% of the total [^3H]CTP and less than 0.1% of the other nucleoside triphosphates in the cell free reactions are incorporated into RNA. Furthermore, the γ -thiophosphate does not appear to be exchanged onto other nucleoside triphosphate molecules in our cell free system at any measurable rate: when a complete nuclear transcription reaction containing ATP γ S and [^3H]GTP was incubated for 1 hr at 25°C and then analyzed by polyethyleneimine-cellulose thin layer chromatography with 1.6 M LiCl, most of the radioactivity still migrated with GTP, and none was observed in the GTP γ S spot (data not shown). In contrast, *X. laevis* nuclear preparations catalyze rapid exchange of thiophosphate from ATP γ S into other triphosphates (3).

Ribonuclease activity in the nuclear preparations

Recovery of [^{32}P]RNA from the cell free reactions was used as a crude measure of ribonuclease activity. When the [^{32}P]RNA was added to the cell free reaction after the 25°C incubation and just before the phenol extraction, about 60% of the original [^{32}P]RNA was recovered from the subsequent ethanol precipitation steps. However, when the [^{32}P]RNA was present in the cell free reaction during the 25°C incubation, only 20-30% was recovered after phenol extraction and ethanol precipitation. These results indicate a substantial amount of ribonuclease activity in the cell free system. This may account for the small size of the cell free transcription products, most of which are 5S (or about 120 nucleotides) or smaller, as measured by polyacrylamide gel electrophoresis (data not shown).

Testing for region-specific initiation of MMTV RNA with (γ -S)- and (β -S)-analogues of ATP and GTP

We have demonstrated that cell free initiation is not the only process by which RNA molecules can acquire thiol groups from ATP γ S and GTP γ S in our cell free system. In contrast, the (β -S)-analogues do not appear to support the thiol transfer that occurs with the (γ -S)-analogues. We have developed an assay with the thiol-analogues to quantitate region-specific initiation of MMTV RNA in the cell free system and to distinguish specific initiation from nonspecific initiation and thiol transfer.

The rate of MMTV RNA synthesis in MMTV-infected cells is stimulated 10 to 20-fold by glucocorticoid hormones (17). Similarly, nuclear preparations from glucocorticoid-treated cells synthesize MMTV RNA in vitro at a rate 10 to 20-fold higher than nuclei from control cells (18).

TABLE 2
Transcription Initiation Assays with Various Nucleoside Thio-Triphosphates

Thiol analogue used	A			B					
	MTV RNA, % of total [³ H]RNA assayed with			relative ratios of [³ H]RNA representing MTV DNA Clones					
	Clone I	Clone II	Clone III	Experiment 1		Experiment 2		Experiment 3	
[³ H]RNA containing thiols									
ATPyS	.31	.32	.48	1.0	1	1.5	.8	1	
ATPßS	.58	.11	1.6	5.3	1	15	2.4	1	
GTPyS	.25	.09	.82	2.8	1	9.1	1.1	1	.8
GTPßS	1.6	.30	5.9	5.5	1	20	6.3	1	2.9
Thiol-free [³ H]RNA									
ATPyS	.43	.53	.48	.8	1	.9	.6	1	
ATPßS	.57	.56	1.0	1.0	1	1.8	.9	1	
GTPyS	.64	.83	1.1	.8	1	1.3	.9	1	.8
GTPßS	.45	.51	.34	.9	1	.7	.7	1	.7

The assay for MTV RNA initiation in cell free nuclear preparations was performed as described in Experimental Procedures, using the thiol analogue shown in the left hand column. The top half of the table presents data for the RNA fractions that bound to Hg-Sepharose. Data in the bottom half of the table are for the corresponding thiol-free RNA fractions from the same reactions. Each RNA sample was analyzed by filter hybridization with 2 or 3 different cloned MTV DNA segments. Part A presents the relative amounts of MTV [³H]RNA representing each clone (for Experiment 1 only), calculated as described in Experimental Procedures. In Part B the same data is expressed as ratios, relative to the results for Clone II. The table includes data from a total of 10 assays performed in 3 separate experiments. For simplicity, only the ratios (Part B) are presented for Experiments 2 and 3.

Figure 1. MMTV DNA segments used for filter hybridization. The MMTV genome is depicted as it appears integrated into host cell DNA. The virion RNA is aligned colinearly with the corresponding DNA sequences. The vertical arrow indicates the 5'-end of the virion RNA, which is also the presumptive site for transcription initiation *in vivo*. LTR signifies the long terminal repeat sequence in the retroviral DNA. The LTR consists of 1192 nucleotides representing the 3'-terminus of the virion RNA and 135 nucleotides representing the 5'-terminus. Restriction endonucleases are represented by the following symbols: O, PstI; X, Sau3A (only those Sau3A sites within Clone I are shown). The bar (1 kb) represents the length of 1000 nucleotides.

Total RNA synthesis is unaffected by the hormone treatment both *in vivo* and *in vitro*. To test for region-specific initiation of MMTV RNA in nuclear preparations from glucocorticoid-stimulated M1.54 cells, four parallel cell free transcription reactions were performed; in each reaction one of the four thiol analogues (ATP γ S, ATP β S, GTP γ S, or GTP β S) was substituted for the analogous natural substrate. RNA was isolated from the reactions and fractionated on Hg-Sephadex. The thiol-containing and thiol-free RNA fractions from each reaction were analyzed for MMTV [3 H]RNA by filter hybridization with MMTV Clones I and II or Clones I, II, and III (Fig. 1), as described in Experimental Procedures. Table 2 shows the results of two experiments where each of the four thiol analogues was tested and a third experiment where only the two GTP analogues were tested. Part A under Experiment 1 lists the amount of MMTV [3 H]RNA as a percentage of the total [3 H]RNA in the hybridization reaction, corrected for hybridization efficiency and normalized for the length of the MMTV DNA segment on the filter. Part B expresses the same results as ratios, calculated relative to the result for clone II. For simplicity, only column B is shown for Experiments 2 and 3.

The thiol-free RNA fractions from all the transcription reactions in the three experiments represent the three different MMTV DNA clones approximately equally, no matter which thiol analogue was used (Table 2).

However, the thiol-containing RNA fractions from the ATP β S and the GTP β S reactions represent the three clones unequally, with III > I > II. This data suggests that region-specific initiation is occurring with ATP β S and GTP β S. If nonspecific initiation or thiol transfer were responsible for the binding of [3 H]RNA to Hg-Sepharose, the relative ratios of MMTV [3 H]RNA for the thiol-containing RNA should be the same as the ratios for the thiol-free RNA. Instead, we see from these ratios a clear difference between the thiol-containing and thiol-free fractions, for both the ATP β S and the GTP β S reactions. The ratios indicate that initiation is occurring primarily within region III of the MMTV genome, which is a subregion of Clone I; region III contains the site where transcription is believed to initiate in vivo (10, 19).

In contrast, the results from the ATP γ S and GTP γ S reactions do not give a clear indication of region-specific initiation. The thiol-containing RNA fractions from the GTP γ S reaction in Experiment 1 (Table 2) shows greater representation of Clones I and III than Clone II, but the ratios (I/II and III/II) are smaller than for the corresponding (β -S)-analogue. The thiol-containing RNA from the ATP γ S reaction in Experiment 1 shows no evidence of region-specific initiation: the [3 H]RNA is equally representative of all three genomic regions. In Experiments 2 and 3, there is no indication of region-specific initiation with either ATP γ S or GTP γ S.

To explain these results, we propose that random ribonuclease fragments of the cell free RNA products can serve as recipients for thiophosphate transfer from ATP γ S and GTP γ S. Thiophosphate transfer should generate a population of thiol-containing MMTV RNA molecules that equally represents all regions of the MMTV genome. In contrast, region-specific initiation generates thiol-containing MMTV RNA molecules that primarily represent the region where initiation occurred. The (γ -S)-analogues may support specific initiation as well as thiophosphate transfer; however, the (β -S)-analogues support initiation but not thiophosphate transfer, because the thiophosphate in the β -position is not accessible for a kinase-type of phosphorylation reaction. The thiophosphate transfer that occurs with the (γ -S)-analogues may partially or completely interfere with our ability to detect the region-specific initiation of MMTV RNA. Since more MMTV RNA initiation appears to occur with GTP than with ATP (Table 2, Part A), the initiation with ATP γ S is more easily obscured by the thiophosphate transfer. It is not clear why

the absolute percentage of MMTV [^3H]RNA obtained with the (γ -S)-analogues is lower than that obtained with the (β -S)-analogues (Table 2, Part A); perhaps γ -thiophosphates on the cell free RNA products are more labile than β -thiophosphates under our conditions for cell free transcription or RNA purification and fractionation.

Conclusion

We have demonstrated that nuclear preparations contain an activity that transfers thiol groups, presumably in the form of thiophosphate, from ATP β S and GTP β S to preexisting RNA molecules. ATP β S and GTP β S do not support the thiophosphate transfer reaction. A rigorous investigation of the mechanism for thiol transfer would require analysis of the thiol-labeled nucleotides resulting from the transfer.

At this time initiation of MMTV RNA in our cell free system is not efficient enough to obtain sufficient material for a 5'-terminal nucleotide analysis. However, we are currently beginning S1 nuclease mapping studies to compare the precise locations of the 5' ends of MMTV RNA initiated in vivo and in our cell free system. In addition, we are attempting to synthesize UTP β S and CTP β S to complete our analysis of nucleotide preference in MMTV RNA initiation. In a separate set of experiments with our cell free system, nuclei from cultured mouse cells demonstrated the expected nucleotide specificity (ATP β S in preference to GTP β S) for initiation of the 45S ribosomal RNA precursor (20).

In systems where nucleotide specificity of initiation has been demonstrated with nucleoside (γ -S)triphosphates (1-4), it appears unlikely that thiophosphate transfer is a significant problem. In our cell free system, neither nucleotide-specificity nor region-specificity of initiation is observed with nucleoside (γ -S)triphosphates, presumably because additional thiol-containing MMTV RNA molecules are generated by the non-specific thiol transfer reaction. The use of the (β -S)-analogues has allowed us to circumvent the thiol transfer problem and thus to observe nucleotide-specific, region-specific initiation of transcription.

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