Simian Virus 40 Transcriptional Complexes Incorporate Mercurated Nucleotides into RNA In Vitro

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Simian virus 40 Sarkosyl transcriptional complexes incorporated mercurated nucleotide precursors into preinitiated RNA chains. Synthesis with mercurated precursors was three- to fivefold slower than with nonmercurated nucleotides (12 to 20 pmol per 10^6 cells per h at 25°C), and 50 to 70% of the RNA product bound specifically to sulfhydryl-agarose. The amount of mercurated CMP incorporated into RNA was measured by specific binding of [35 S]cysteine to the mercury residues. More than 90% of the mercurated RNA hybridized to simian virus 40 DNA.

Sarkosyl extracts of simian virus 40 (SV40)-infected cells contain most of the viral DNA, including transcriptional complexes (TCs) composed of host RNA polymerase II and nascent RNA bound to SV40 DNA templates (4-6, 14, 15, 20, 24, 27, 31, 32, 39). Because the viral TCs elongate RNA in vitro under conditions in which neither initiation nor post-transcriptional processing occurs (14, 20, 31), the RNA made reflects the in vivo distribution of nascent chains present at the time of extraction (1, 4, 5, 14, 20, 30, 31, 39). Presently, available systems are limited because they neither separate nascent viral RNA from completed chains nor remove the bulk of nontemplate DNA molecules from active TCs. To overcome this limitation, we have adapted Sarkosyl extracts for use with mercurated nucleotide precursors so that both nascent SV40 RNA and SV40 TCs should become amenable to purification by affinity chromatography on sulfhydryl resins.

We have modified the procedure reported by Gariglio and Mousset (20) to increase the efficiency of transcription by SV40 transcriptional complexes, as described in the legend of Fig. 1. In our system, a reaction mixture containing the TCs from 10^6 cells incorporates from 12 to 20 pmol of total nucleotide into RNA per h at 25°C, whereas others have reported only 0.16 to 5 pmol (5, 6, 14, 31, 39).

To measure the efficiency of RNA elongation with mercurated precursors, we compared synthesis in a reaction mixture containing no mercurated precursors with that of a reaction in which mercurated CTP (HgCTP) was substituted for CTP. In both cases RNA synthesis proceeded linearly for at least 2 h, but the rate

with CTP was three to five times higher than with HgCTP (Fig. 1). Substitution of HgUTP for UTP caused a similar reduction in rate. Similarly, use of mercurated precursors for transcription in isolated nuclei has consistently resulted in lower levels of RNA synthesis (34, 35, 38, 40, 42). In parallel control reactions which contained Sarkosyl extracts from uninfected cells, we detected no synthesis (data not shown).

The reduced synthesis with mercurated precursors was not due to an insufficient nucleotide concentration. When we varied the concentration of CTP, HgCTP, or UTP over the range of 0.015 to 2 mM, RNA synthesis was maximal for concentrations above 0.03 mM in each case. Similarly, Beebe and Butterworth (2) have shown that rat liver polymerase II has a K_m of 10 μ M for both HgUTP and UTP when tested with purified DNA templates. Thus, for the experiment shown in Fig. 1, the concentrations of both CTP and HgCTP were far in excess of the levels required for a maximal rate of synthesis.

We also measured the kinetics of RNA synthesis when CTP and HgCTP were present in the same reaction mixture (Fig. 2). For example, with CTP and HgCTP each at a concentration of 0.3 mM (reaction c), the rate of synthesis was intermediate between the rates for CTP alone at 0.6 mM (reaction a) and HgCTP alone at 0.6 mM (reaction e). Since the 0.3 mM concentration of CTP was 10 times that needed for the maximal rate of synthesis in the absence of HgCTP, we conclude that synthesis is slowed by the presence of HgCTP. These data are consistent with the incorporation of HgCTP at a rate approximately one-fourth that for CTP.

The reduced rate of RNA synthesis with mercurated precursors might result from a change

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FIG. 1. RNA synthesis by SV40 transcriptional complexes either with nonmercurated precursors or with HgCTP substituted for CTP. We infected confluent monolayers of BSC-1 cells with SV40 (strain 776) at a multiplicity of 10 to 15 PFU/cell (12). At 55 to 65 h post infection, we rinsed each monolayer three times with ice-cold buffered sucrose (0.22 M sucrose–50 mM Tris-hydrochloride, pH 7.9–10 mM NaF–1 mM MgCl_2 –0.5 mM CaCl_2 –2 mM 2-mercaptoethanol), added 5 ml of buffered sucrose to each dish, and scraped the cells into a centrifuge tube. After pelleting the cells, we isolated nuclei as described (36), using 0.05% (vol/vol) Nonidet P-40 in buffered sucrose, 0.17 ml per 10^6 cells. We suspended the nuclei in 10 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonate) (pH 7.9)–6 mM NaF–2 mM 2-mercaptoethanol (0.125 ml per 10^6 cells), lysed them by adding an equal volume of 0.8 M NaCl–0.5% (vol/vol) Sarkosyl NL30 (Geigy), and pelleted the cellular chromatin for 30 min at $48,000 \times g$. SV40 transcriptional complexes could be efficiently concentrated up to 10-fold by dialysis against a saturated solution of polyethylene glycol 6000 followed by dialysis against 0.15 M $(\text{NH}_4)_2\text{SO}_4$ –30 mM Tris-hydrochloride, pH 7.9–5 mM KCl–2 mM 2-mercaptoethanol. We prepared HgCTP and HgUTP by a modification of the procedure of Dale et al. (10) as detailed by Feist and Danna (manuscript in preparation). As evidenced by binding to sulfhydryl-agarose, at least 98% of the nucleotide in each of our preparations was mercurated. RNA synthesis reaction mixtures, incubated at 25°C, contained 24 mM Tris-hydrochloride, pH 7.9–0.12 M $(\text{NH}_4)_2\text{SO}_4$ –4 mM KCl–5 mM CaCl_2 –6 mM MnCl_2 –14 mM 2-mercaptoethanol–1.8 mM ATP–0.6 mM GTP–0.6 mM CTP (or HgCTP)–0.03 mM $[5,6\text{-}^3\text{H}]\text{UTP}$ (10 Ci/mmol). The nuclear extract isolated from infected cells comprised 80% (vol/vol) of each reaction mixture. Each 400- μl reaction mixture contained the Sarkosyl extract from a total of 1.5×10^7 cells, and at each time point, a 10- μl sample was spotted onto a DE-81 filter disk, which was washed as described (7). Approximately 11,000 cpm represents 1 pmol of $[^3\text{H}]\text{UMP}$. (●) Nonmercurated precursors; (Δ) HgCTP substituted for CTP.

in the rate of initiation, elongation, or termination of transcription. A reduced rate of initiation is an unlikely explanation because RNA polymerase II does not initiate synthesis in most nuclei systems nor in Sarkosyl extracts (14, 20, 31). After determining the uridine-to-UMP ratio of RNA synthesized in nucleoplasmic extracts of rat cells, Beebe and Carty (3) concluded that a threefold reduction in elongation rate accounted for the observed decrease in RNA synthesis with mercurated precursors. It is likely that a reduced elongation rate also occurs with the SV40 tran-

scriptional complexes. However, an increased frequency of termination cannot be ruled out at this time.

We used two approaches to demonstrate that the RNA synthesized with HgCTP contains mercury residues: affinity chromatography on sulfhydryl-agarose and specific binding of $[^{35}\text{S}]$ -cysteine to the RNA.

RNA synthesized by TCs with HgCTP bound efficiently to a column of sulfhydryl-agarose and was eluted with 0.1 M 2-mercaptoethanol. In contrast, very little RNA bound that was syn-

FIG. 2. Kinetics of incorporation of [^3H]UMP into RNA in the presence of CTP and HgCTP in various ratios. Transcriptional complexes and reaction mixtures were prepared as described in the legend of Fig. 1. Each 90- μl mixture contained the Sarkosyl extract from 1.7×10^8 cells and [^3H]UTP at 40 μM (20 Ci/mmol). Samples of 8 μl were removed for analysis at each time point. The concentrations of CTP and HgCTP in the reaction mixtures were as follows: (a) 0.6 mM CTP, no HgCTP (∇); (b) 0.4 mM CTP, 0.2 mM HgCTP ($\square$); (c) 0.3 mM CTP, 0.3 mM HgCTP (Δ); (d) 0.2 mM CTP, 0.4 mM HgCTP ($\circ$); (e) no CTP, 0.6 mM HgCTP ($\blacklozenge$).

thesized with CTP as precursor. Figure 3 shows the results of a double-label experiment in which RNA synthesized with HgCTP was labeled with [^3H]UTP, and nonmercurated RNA, used as a control, was labeled with [α - ^{32}P]UTP. The two RNA preparations were mixed in a mass ratio of approximately 1:1 and chromatographed on sulfhydryl-agarose. Only 2.7% of the ^{32}P -labeled, nonmercurated RNA bound to the column, whereas 56% of the [^3H]RNA made with HgCTP bound. With other preparations of RNA synthesized with HgCTP, we found that from 50 to 70% bound to sulfhydryl-agarose and that the RNA that did not bind initially never bound, even when applied to a fresh column. Binding of RNA synthesized with nonmercurated precursors was generally less than 1% when it was chromatographed alone. Other investigators have also reported efficiencies of binding between 50 and 70% (35, 38, 40), although some have reported 90 to 100% (2, 28).

We developed a quantitative assay for mer-

curation of RNA, based on the suggestion by Dale and Ward (11) that cysteine can be used as a counterligand for mercury groups in nucleotides. Our method involves incubation of mercurated [^3H]RNA with excess [^{35}S]cysteine, separation of free cysteine from that bound to the RNA by gel filtration, and determination of the molar ratio of bound [^{35}S]cysteine to [^3H]UMP residues in the RNA. Because of the high affinity between mercury and sulfhydryl groups, one cysteine molecule should be bound to each mercurated nucleotide in the RNA, thus allowing one to calculate the molar ratio of HgCMP to [^3H]UMP in RNA synthesized *in vitro*.

We used [^{35}S]cysteine binding to examine the RNA synthesized by TCs with [^3H]UTP and HgCTP as precursors. Results of a typical experiment are shown in Fig. 4. Approximately the same number of counts per minute of [^3H]RNA made with HgCTP (Fig. 4A) or with CTP (Fig. 4B) was incubated with [^{35}S]cysteine and chromatographed on Sephadex G-50. A significant amount of [^{35}S]cysteine eluted with the RNA made with HgCTP, whereas no detectable [^{35}S]cysteine eluted with the nonmercurated RNA. However, when we used six times as much nonmercurated RNA, a small amount of [^{35}S]cysteine eluted with the RNA (Fig. 4C). In this last incubation, the total mass of RNA, which included unlabeled cellular RNA from the Sarkosyl extract, *Escherichia coli* rRNA used as carrier during the RNA purification, and the nonmercurated [^3H]RNA, was about twice that used for the experiment in Fig. 4A. Therefore, although the background binding of [^{35}S]cysteine to nonmercurated RNA was dependent on total RNA concentration, it was negligible when compared with the binding to mercurated RNA.

We also found that when sodium dodecyl sulfate (SDS) was included in the column buffer, a small but constant background of [^{35}S]cysteine (100 to 175 cpm for the experiment shown in Fig. 4) was found throughout the column fractions in the control experiments with nonmercurated RNA. This background, which was subtracted before the data were plotted, was dependent on the presence of RNA and was greatly reduced when SDS was omitted from the column buffer.

From the data shown in Fig. 4A, we calculated the molar ratio of [^{35}S]cysteine to [^3H]UMP for the mercurated RNA to be 0.67. Assuming that the RNA has the base composition of the late strand of SV40 DNA (9, 16–18, 26, 29, 37, 41), the theoretical molar ratio should be 0.69 if all CMP were replaced with HgCMP. Therefore, about 97% of the CMP residues were replaced with HgCMP in the mercurated RNA used in

FIG. 3. Sulfhydryl-agarose chromatography of mercurated [^3H]RNA and nonmercurated [^{32}P]RNA synthesized by SV40 transcriptional complexes. Nonmercurated RNA was synthesized with CTP and [$\alpha\text{-}^{32}\text{P}$]UTP (9.1 Ci/mmol); mercurated RNA was synthesized with HgCTP and [^3H]UTP (10 Ci/mmol). We purified the RNA by chromatography on Sephadex G-50 in 0.1 M sodium acetate (pH 6.0)–1% (wt/vol) SDS. We pooled fractions that contained RNA, added an equal volume of SDS-pronase (1 mg of pronase [Sigma] per ml in 1% SDS–0.2 M NaCl–40 mM Tris-hydrochloride, pH 7.5–10 mM EDTA, self-digested at 37°C for 2 h), incubated the mixture for 3 h at 37°C, extracted it twice with phenol-chloroform-isoamyl alcohol, 24:24:1 [vol/vol] [saturated with 0.1 M Tris-hydrochloride, pH 7.9], and ethanol precipitated the nucleic acids three times. We treated the final pellet with 20 μg of DNase I (Worthington; RNase-free DNase I, purified further by affinity chromatography [33]) per ml for 30 min at 37°C, reextracted the sample with phenol-chloroform-isoamyl alcohol, and ethanol precipitated the RNA three times. We mixed about 26,000 cpm of [^3H]RNA and 20,000 cpm of [^{32}P]RNA and loaded the mixture onto a 1-ml column of sulfhydryl-agarose (P. L. Biochemicals) in 0.1 M sodium acetate (pH 6.0)–0.1% SDS. After allowing the RNA to adsorb for 2 h at 37°C, we washed the column with loading buffer and eluted the bound RNA with 0.1 M 2-mercaptoethanol–0.1 M sodium acetate (pH 6.0)–0.2% SDS. The arrow indicates the beginning of the elution with 2-mercaptoethanol. (●) ^{32}P -labeled, nonmercurated RNA; (Δ) ^3H -labeled, mercurated RNA.

this experiment. For three different preparations of mercurated RNA, the estimated level of HgCMP substitution ranged from 88 to 117%. In contrast, the amount of [^{35}S]cysteine bound to the nonmercurated RNA used for Fig. 4C was equivalent to a substitution of 1.8%. For RNA made with 0.3 mM each of CTP and HgCTP (reaction c, Fig. 2), the percentage of HgCMP substitution was approximately half that for the RNA made with 0.6 mM HgCTP. These results indicate that HgCTP is used as an RNA precursor even when CTP is present at a concentration well above the K_m , suggesting that the polymerase has no preference for CTP over HgCTP in SV40 TCs. This result is strikingly different from that of Mory and Geffer (34), who suggested that RNA polymerase II in isolated mouse nuclei

has an eightfold preference for UTP over HgUTP.

As mentioned above, a substantial fraction (30 to 48%) of RNA synthesized with HgCTP did not bind to sulfhydryl-agarose. Using [^{35}S]cysteine binding, we compared the amount of HgCMP in two RNA samples taken from a single experiment: RNA that did not bind to the column and RNA that bound and was eluted with 2-mercaptoethanol. Surprisingly, both samples had HgCMP substitutions of 97%. In addition, since the RNA eluted with 2-mercaptoethanol maintained a high level of HgCMP substitution, chromatography on sulfhydryl-agarose does not appear to cause significant demercuration.

Because we detected no RNA synthesis in

FIG. 4. $[^{35}\text{S}]$ cysteine binding to RNA. We prepared $[^{35}\text{S}]$ cysteine by incubating 10 nmol of $[^{35}\text{S}]$ cysteine (38.7 Ci/mmol) with 200 nmol of glutathione in 0.05 M sodium acetate (pH 6.0) for 1 h at 37°C . We loaded the mixture onto a 0.15-ml column of diethyl-(2-hydroxypropyl)aminoethyl-Sephadex (QAE-Sephadex), prewashed with 0.1 M NaCl-0.01 M Tris-hydrochloride (pH 7.5) and equilibrated with 0.01 M NaCl, and collected the $[^{35}\text{S}]$ cysteine, which eluted in 0.01 M NaCl. Excess glutathione remained bound to the resin. Samples of mercurated (A) and nonmercurated (B and C) RNA (2 to 16 ng/ml), synthesized in parallel reaction mixtures from the same Sarkosyl extract with $[^3\text{H}]$ UTP (11.7 Ci/mmol), were incubated with $[^{35}\text{S}]$ cysteine (14 μM , 10.8 Ci/mmol) in 40 mM NaCl-56 mM sodium acetate (pH 6.0)-0.1% SDS. Input RNA was as follows: (A) 500 cpm of mercurated RNA; (B) 670 cpm of nonmercurated RNA; (C) 3,200 cpm of nonmercurated RNA. The following $[^{35}\text{S}]$ cysteine background values were subtracted from all fractions: (A) 174 cpm; (B) 128 cpm; and (C) 107 cpm. Based on our efficiencies of counting for ^3H (19%) and for ^{35}S (69%), approximately 4,900 cpm represents 1 pmol of $[^3\text{H}]$ UMP, and 16,100 cpm represents 1 pmol of $[^{35}\text{S}]$ cysteine. (●) ^3H ; (Δ) ^{35}S .

vitro when we used Sarkosyl extracts from uninfected cells, the RNA made by infected-cell extracts should be SV40 specific. We confirmed

this by demonstrating that both mercurated and nonmercurated RNA hybridized to SV40 DNA. About 90% of the RNA proved to be SV40 specific regardless of whether HgCTP or CTP was used as precursor (Table 1). We also tested a sample of RNA that had been bound to and eluted from a sulfhydryl-agarose column. In this case (preparation 2, Table 1), 92% of the RNA hybridized to SV40 DNA, whereas less than 0.2% hybridized to rat DNA.

TABLE 1. Hybridization of RNA made by SV40 TCs^a

Sarkosyl prepn	Input RNA ^b	Hybridized RNA (%) ^c	
		SV40 DNA filters	Rat DNA filters
1	Nonmercurated	92	0.6
	Mercurated	92	0.2
2	Nonmercurated	93	0.2
	Mercurated	90	0.2
	Mercurated (eluted from sulfhydryl-agarose) ^d	92	0.3

^a We used the procedure of Gillespie and Spiegelman (21) to immobilize DNA on nitrocellulose filters. Each 0.5-cm filter contained either 2 μg of SV40 DNA prepared by the Hirt procedure (25), purified (12), and digested with *EcoRI* (13), or, as a control, 1 μg of sheared, unique-sequence rat DNA (8). We boiled the RNA samples in 0.15 ml of 82 mM NaCl-60 mM Na_2HPO_4 -60 mM NaH_2PO_4 -0.1% SDS-10 mM EDTA for 1 min, added a DNA filter, and gently agitated the sample on a rotary shaker at 68°C . After 18 to 24 h, we removed the filter, replaced it with a fresh filter of the same type, and continued the hybridization for an additional 18 to 24 h. We washed the filters for 30 min at 37°C in 5 ml of $6 \times \text{SSC}$ (0.9 M NaCl-0.09 M sodium citrate, pH 7.0) and then 10 times for 1 min in $6 \times \text{SSC}$ at 25°C . To determine the amount of bound radioactivity, we used Filter-Solv (Beckman).

^b The input counts per minute varied from 13,000 to 18,000 for these hybridization reactions.

^c The percentage of RNA hybridized was calculated as (total counts per minute bound to the filters divided by the total counts per minute recovered for the hybridization) $\times 100$. The total hybridized counts equal the sum of counts on the two filters; the second filter in each set contained about 5% of the counts bound to the first filter. The total recovered radioactivity was calculated as the sum of the hybridized counts per minute and the counts per minute remaining in solution at the end of the hybridization. The total recovered radioactivity was always greater than 65% of the input, but varied somewhat for different hybridizations.

^d Mercurated RNA from Sarkosyl preparation 2 was bound to and eluted from sulfhydryl-agarose as described in the legend of Fig. 3. The RNA eluted with 2-mercaptoethanol was then ethanol precipitated and used for hybridization.

For the experiments shown in Table 1, we did not treat the filters with RNase after hybridization. When the filters were treated with RNase, 60 to 70% of the RNA bound to the SV40 DNA filters, and less than 0.2% bound to the control, rat DNA filters.

Together with the data on sulfhydryl-agarose chromatography and [³⁵S]cysteine binding, these hybridization results indicate that most of the RNA made with HgCTP as a precursor is both SV40 specific and mercurated.

Recently, others have reported artifacts associated with the use of mercurated nucleotides for in vitro transcription of eucaryotic chromatin by exogenous *E. coli* RNA polymerase. These include the copying of RNA as a template (43, 44) and aggregation of the mercurated RNA products (28). Our system should be free from these artifacts since they have not been observed under conditions in which RNA is elongated but not initiated by endogenous polymerases (34, 35).

In contrast to other in vitro SV40 transcriptional systems, ours should allow the isolation of nascent chains, as Weinmann and Aiello (42) have done for adenovirus RNA elongated in nuclei. Since RNA prelabeled in vivo and elongated in vitro with mercurated nucleotides can be purified via its mercurated tail, the structure of the prelabeled nascent chains can be examined. Therefore, our system should facilitate the study of possible in vivo processing events that precede transcriptional termination. Our system might also be used to separate intact transcriptional complexes from nontemplate DNA so that the nature of the active polymerase and the structure of the viral template DNA can be studied directly. If SV40 nucleoprotein transcriptional complexes prepared by Triton X-100 extraction (19, 22, 23) also incorporate mercurated nucleotides efficiently, proteins other than the polymerase found in transcriptional complexes might be isolated and characterized. We are currently investigating these applications of our system.

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