In Vitro Transcription of Moloney Leukemia Virus Genes in Infected Cell Nuclei and Chromatin: Elongation of Chromatin Associated Ribonucleic Acid by *Escherichia coli* Ribonucleic Acid Polymerase†

Thomas Y. Shih,* Howard A. Young, Wade P. Parks, and Edward M. Scolnick

ABSTRACT: The in vitro transcription of viral specific DNA sequences in nuclei and chromatin isolated from mouse cells chronically infected with Moloney murine leukemia virus (Mo-MuLV) has been studied. The in vitro RNA synthesized by *Escherichia coli* RNA polymerase has been isolated by sulfhydryl affinity column following reaction in the presence of 5-mercuriuridine triphosphate. By comparison of the C_rt curves of the in vitro RNA with that of 70S viral RNA, the content of viral sequences is found to be 1.3% in nuclei product and 0.24% in chromatin product which is lower than the 2.5% found in chromatin associated RNA. This latter value, however, is very close to the in vivo viral RNA content in pulse-labeled [^{3}H]RNA of the infected cells. Unexpectedly, it is observed that over 20% of the chromatin associated RNA prelabeled in vivo with [5-^{3}H]uridine is elongated and tagged with Hg atoms during RNA synthesis catalyzed by the exogenous *E. coli* RNA polymerase in the presence of Hg-UTP. The elongation reaction is dependent on the presence of all four nucleotide triphosphates and appears to be due to *E. coli* RNA polymerase per se. It is suggested that most of the viral specific sequences observed in the in vitro RNA products are very likely initiated and derived from the chromatin associated species. The implication of the present findings for in vitro RNA synthesis in nuclei and chromatin as related to regulation of gene expression is discussed.

In eukaryotic cells, nuclear DNA is associated with histone and nonhistone proteins and some RNA in complexes known as chromatin. It is generally thought that these proteins have important functions not only for maintaining the structure of chromatin but also for gene regulation. As a step toward analysis of the components and their mechanisms in transcriptional regulation, cell-free systems of RNA synthesis involving isolated chromatin as well as nuclei have been extensively studied. Many of these studies suggest that chromatin and nuclei appear to retain their in vivo template specificities in transcription of specific genes (Axel et al., 1973; Gilmour and Paul, 1973; Astrin, 1973; Shih et al., 1973; Steggles et al., 1974; Rymo et al., 1974; Jacquet et al., 1974; Wilson et al., 1975; Gilmour et al., 1975; Marzluff and Huang, 1975; Stein et al., 1975; Tsai et al., 1976). Recent reports on the use of mercurated nucleotide triphosphate as the substrate for RNA synthesis in isolated nuclei or chromatin and purification of the Hg-tagged RNA by sulfhydryl affinity column have been met with interest (Smith and Huang, 1976; Crouse et al., 1976; Beebee and Butterworth, 1976; Biessmann et al., 1976). It seems to offer a novel method of resolving the newly synthesized in vitro RNA from any preexisting nuclear and chromatin RNA, and to permit the use of specific radioactive cDNA probes to analyze the in vitro products. Using this method, we have studied the in vitro viral RNA synthesis in Moloney murine leukemia virus (Mo-MuLV)[1] infected NIH 3T3 cells. The unexpected finding that, even in reactions catalyzed by the exogenous *E. coli* DNA-dependent RNA polymerase, a very large fraction of chromatin associated RNA is elongated by the enzyme during RNA synthesis poses questions concerning the fidelity and specificity of in vitro RNA synthesis.

Materials and Methods

Cells and Viruses. The NIH 3T3 mouse cell line has been previously described (Scolnick et al., 1975). A clonal derivative of NIH 3T3 cells was infected with a cloned strain of the Moloney murine leukemia virus and this chronically infected culture was employed in the present studies (Parks et al., 1976; Scolnick et al., 1975). A bat cell (CCL88) was from the American Type Culture Collection (Rockville, Md.), and the normal rat kidney cell, NRK, has been described (Scolnick et al., 1974).

Chemicals. 5-Mercuriuridine triphosphate (Hg-UTP) was prepared from UTP by mercurization with mercuric acetate according to the procedure of Dale et al. (1973, 1975). The mercurated UTP purified by DEAE-cellulose was characterized by a 5-nm red shift of the 261-nm absorption maximum. When the Hg-RNA was freshly prepared, 95 to 99% of Hg-RNA transcribed from calf thymus DNA with *E. coli* RNA polymerase was retained by the sulfhydryl column. The sulfhydryl affinity column, Affi-Gel 401, was purchased from Bio-Rad, Richmond, Calif. *E. coli* DNA-dependent RNA polymerase was isolated both from *E. coli* B to the DEAE-cellulose step according to the procedure of Chamberlin and Berg (1962), and from *E. coli* K12 by the method of Burgess and Jendrisak (1975) (purchased from Miles Lab., Elkhart, Ind.), with specific activities of 500 and 800 units/mg, respectively. The purified enzyme was free of endogenous template activity and was shown to contain δ factor by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The avian myeloblastosis virus reverse transcriptase was purified from

† From the Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Maryland 20014. *Received November 30, 1976.* This work was supported by the Virus Cancer Program of the National Cancer Institute.

[1] Abbreviations used: Mo-MuLV, Moloney murine leukemia virus; Hg-UTP, 5-mercuriuridine triphosphate; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; PERT, phenol emulsion reassociation technique; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)-aminomethane.

disrupted virion by Sephadex G-200 (Kacian et al., 1971). The sources of the following chemicals were: nucleotide triphosphates (P-L Biochemicals); α-amanitin (Boehringer, West Germany); [^{3}H]UTP or [^{32}P]GTP (New England Nuclear or Schwarz/Mann); pancreatic DNase I (Sigma); yeast RNA (Sigma); calf thymus terminal transferase (P-L Biochemicals); RNase A (Sigma); chick DNA (Calbiochem); mercuric acetate (Fisher); ATP, GTP, UTP, and CTP (P-L Biochemicals).

Isolation of Nuclei and Chromatin. All cells were monitored for *Mycoplasma* using both aerobic and anaerobic culture techniques by Flow Laboratories, Rockville, Md., and were found to be negative. The cells were grown to confluence in roller bottles at 37 °C in Dulbecco-Vogt modified Eagle's minimal essential medium supplemented with 10% calf serum. After decanting the medium, the cells were scraped with rubber policemen into cold (4 °C) phosphate-buffered saline (PBS) and collected by low-speed centrifugation (1500*g*, 3 min). The cell pellets were then washed three more times with PBS.

To isolate the nuclei, the cells were suspended in 20 volumes of a buffer containing 0.01 M Tris-HCl (pH 7.4), 0.01 M NaCl, 1.5 mM magnesium acetate, 0.3 M sucrose, 1 mM dithiothreitol, and 0.01% Triton X-100 (v/v). They were disrupted with 15 strokes in a tight-fitting glass Dounce homogenizer, and nuclei were pelleted at 2000*g* for 15 min. They were resuspended and pelleted three more times in the homogenization buffer (20 volumes). Finally, the nuclei were resuspended in 5–10 volumes of storage medium containing 0.01 M Tris-HCl (pH 7.4), 0.01 M NaCl, 1.5 mM magnesium acetate, 5 mM dithiothreitol, and 20% glycerol and were stored in small aliquots in liquid nitrogen. All the above procedures were carried out at 4 °C.

To isolate chromatin, the washed cells (5 g) were disrupted by Dounce homogenization with 15 strokes in 8 volumes of 0.02 M Tris-HCl (pH 7.5), 5 mM magnesium acetate, 1 mM dithiothreitol, 20% glycerol, and 0.5% (v/v) Triton X. The crude nuclei were pelleted twice at 5000*g* for 10 min in another 100-mL homogenization buffer and were washed three more times with 100 mL of 1 mM Tris (pH 8.0) and pelleted. The nuclei were then washed with 100 mL of 0.14 M NaCl and 0.01 M Tris (pH 8.0) in Dounce homogenizer and were stirred for 30 min. They were resuspended and swelled twice in 100 mL of 1 mM Tris (pH 8.0). After Dounce homogenization at a concentration of 0.3 mg DNA/mL, it was layered on an equal volume of 40% glycerol in 0.01 M Tris (pH 8.0) and was sedimented at 190 000*g* for 2 h. The chromatin pellet was resuspended twice in 0.01 M Tris (pH 8.0) at DNA concentration of 1 mg/mL and was used within the next 2 days. All procedures were at 4 °C (Howk et al., 1974).

Synthesis and Isolation of RNA Tagged with Hg-UTP in Nuclei and Chromatin. Nuclei (2.6×10^7/mL, 0.26 mg of DNA/mL) or chromatin (0.1 mg of DNA/mL) were incubated at 25 °C for 60 min in a 10-mL reaction mixture containing 30 mM Tris-HCl (pH 7.9), 1 mM manganese acetate, 5 mM magnesium acetate, 12 mM β-mercaptoethanol, 50 mM ammonium sulfate, 0.4 mM each of ATP, CTP, GTP, and Hg-UTP, and 8% glycerol from the nuclei storage medium. *E. coli* DNA-dependent RNA polymerase (0.2 to 0.4 mg/mL, 500 units/mg) was added to the reaction mixture in the presence of α-amanitin (10 μg/mL). At the end of incubation, sodium dodecyl sulfate was added to 0.5% to lyse the nuclei and to disrupt chromatin in a Dounce homogenizer. A small amount (10^5 cpm) of Hg-tagged [^{32}P]RNA (for preparation see below) was added to the mixtures to monitor the efficiency of recovery of Hg-tagged RNA by the following procedure. The lysed reaction mixture was digested with Pronase (100 μg/mL) at 37 °C for 20 min. Sodium dodecyl sulfate was added to a final concentration of 1% and the reaction mixture was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (50:49:1) at room temperature. The organic phase and the interphase were further extracted with 1/5 volume of 0.05 M sodium acetate (pH 5) and 1% sodium dodecyl sulfate at 55 °C. The combined aqueous phase was dialyzed against water and was digested with RNase free DNase I (20 μg/mL) in 0.01 M Tris-HCl, pH 7.5, and 5 mM magnesium acetate for 1 h at 25 °C. It was again extracted with phenol–chloroform. After purification through Sephadex G-75, the RNA in the excluded volume was purified by the Affi-Gel 401 column according to the procedure of Smith and Huang (1976). After thorough washing the column with 30 volumes of 0.1 M sodium acetate (pH 6.0) and 0.1% sodium dodecyl sulfate, the Hg-tagged RNA was eluted with 0.1 M β-mercaptoethanol and was precipitated by 2.5 volumes of ethanol. In some experiments as indicated, RNA was precipitated with carrier yeast RNA. The precipitates were dissolved in 0.5 to 1.0 mL of distilled water. The background retention by Affi-Gel column of the [^{3}H]RNA prepared with ordinary UTP is less than 0.01%.

The Hg-tagged [^{32}P]RNA used as the internal standard was prepared in 0.5 mL of 40 mM Tris (pH 7.9), 4 mM Mg^{2+}, 1 mM Mn^{2+}, 12 mM β-mercaptoethanol, 150 mM NaCl, 25 μg of calf thymus DNA, 370 μg of *E. coli* RNA polymerase, 0.5 mM each of ATP, CTP, Hg-UTP, and 8 μM [α-^{32}P]GTP (13 Ci/mmol). After incubation at 30 °C for 60 min, it was deproteinized following DNase digestion and was collected in the excluded volume of Sephadex G-75. RNA, 99%, was retained by the Affi-Gel 401 column.

Analytical Hybridization of Unlabeled RNA with [^{3}H]-cDNA Probes. [^{3}H]cDNA was prepared by the endogenous reverse transcriptase reaction of Mo-MuLV (Scolnick et al., 1974). The cDNA was labeled with [^{3}H]dCTP to a specific activity of 2×10^7 cpm/μg. At a 1:1 molar ratio of [^{3}H]cDNA to ^{32}P viral 70S RNA, the probe protected 63% of the viral RNA and at 3:1 ratio, 85% of the viral RNA. RNA–DNA hybridization was performed by a modified phenol emulsion reassociation technique (PERT) of Kohne et al. (1977) to accelerate the rate of RNA–DNA reassociation. The hybridization mixture had a composition of 20 mM Tris-HCl, pH 7.2, 1.5 M NaCl, 0.05% sodium dodecyl sulfate, 0.05 mM EDTA, 100 μg/mL yeast RNA, 2500 cpm of [^{3}H]cDNA, and appropriate amounts of test RNA; 0.005 mL of 90% redistilled phenol was added to 0.05 mL of hybridization mixture in a capped test tube; the mixture was emulsified by vigorous shaking on a mixer and further incubated at 48 °C for 4 h with shaking every 30 min. At the end of incubation, hybrid was detected by S_1 nuclease treatment using procedures previously described (Leong et al., 1972).

Analytical Hybridization of [^{3}H]RNA by the Elongated Poly(dC)-cDNA Probes. Unlabeled cDNA was prepared from purified 70S RNA of Mo-MuLV by avian myeloblastosis virus reverse transcriptase primed by chick DNA fragments (Taylor et al., 1976). The purified cDNA was elongated with a stretch of poly(dC) by calf thymus terminal transferase. The [^{3}H]-RNA hybridized to poly(dC)-cDNA was scored on poly(I)-Sephadex column after RNase A treatment. The poly(dC)-cDNA represents more than 70% of the viral genome as determined by its hybridization to ^{32}P-labeled 70S RNA in DNA excess. The procedure followed was essentially that of Coffin et al. (1974) and Young et al. (1977). Mo-MuLV ^{32}P-labeled

TABLE I: In Vivo Viral RNA Content in Cells Pulse-Labeled with [^{3}H]Uridine.[a]

Cell Type	Poly(dC)-cDNA	% Internal Std 70S [^{32}P]RNA Hybridized	Input (cpm)	Hybridization of Cell [^{3}H]RNA: Hybridized[b] (cpm)	% Input Hybridized	% Viral RNA Content[c]
NIH 3T3	−	0	6.8×10^5	2	0.0003	
NIH 3T3	+	37	6.8×10^5	202	0.03	0
NRK	+	43	3.6×10^5	120	0.03	0
BAT (CCL88)	+	42	5.6×10^5	107	0.02	0
NIH 3T3/Mo-MuLV	+	18	4.2×10^5	2013	0.48	2.5

[a] Cells were labeled in vivo with [^{3}H]uridine (100 μCi/mL) for 1.5 h (30 000 cpm/μg of RNA). The viral-specific RNA was determined by hybridization with 0.8 μg of poly(dC)-cDNA prepared from Mo-MuLV 70S RNA, and the hybrid was recovered on a poly(I)-Sephadex column. ^{32}P-labeled 70S RNA (ca. 1500 cpm, 1×10^6 cpm/μg) was added to each assay mixture to monitor its hybridization efficiency. [b] Scintillation counter background of 20 cpm was subtracted. [c] Viral RNA content was calculated as follows: [% input hybridized − 0.03(NIH 3T3 background)]/% 70S [^{32}P]RNA hybridized × 100.

70S RNA (ca. 1000 cpm at 4×10^6 cpm/μg) was added as the internal standard to monitor the efficiency of hybridization.

Results

In Vivo Content of Viral Sequences in Pulse-Labeled RNA. We have studied a clonal derivative of NIH 3T3 mouse cells chronically infected with Mo-MuLV. Although the proviral DNA appears to be integrated into cell DNA at a frequency of only one to two copies per haploid cell genome (Benveniste and Todaro, 1974; Khoury and Hanafusa, 1976), the virus producer cells seem to commit themselves heavily to viral RNA synthesis as seen in Table I. To assess the in vivo content of viral RNA sequences in newly synthesized cell RNA, the cells were pulse-labeled with [5-^{3}H]uridine, and the viral specific sequences were determined by the procedure described by Coffin et al. (1974) and Young et al. (1977). Unlabeled Mo-MuLV cDNA was elongated with a stretch of poly(dC), it was then hybridized with the ^{3}H-labeled cell RNA, and the hybrids were scored by their retention on a poly(I)-Sephadex column. As shown in Table I, the viral RNA content is 2.5% of the pulse-labeled RNA after substracting for the nonspecific hybridization seen in virus-free cells. The viral RNA content remains fairly constant for labeling periods varying from 15, 30, 60, and 90 min, indicating that various RNA species are synthesized at constant rates (data not shown).

Conditions for RNA Synthesis in Isolated Nuclei and Chromatin. In vitro synthesis in isolated nuclei or chromatin was carried out in the presence of 5-mercuriuridine triphosphate (Hg-UTP). The rate of RNA synthesis is essentially the same either in the presence of Hg-UTP or ordinary UTP as measured by incorporation of [^{3}H]GTP in accord with previous observations by other investigators (Dale et al., 1973; Smith and Huang, 1976; Crouse et al., 1976; Beebee and Butterworth, 1976; Biessmann et al., 1976). Addition of saturating amounts of *E. coli* RNA polymerase to nuclei results in about 20-fold increase in RNA synthesis even in the presence of 10 to 20 μg/mL of α-amanitin (Figure 1). Therefore, the endogenous synthesis is essentially negligible. The incorporation is dependent on the presence of all four nucleotide triphosphates and has maximum activity in the presence of both Mg^{2+} and Mn^{2+}. If Mn^{2+} ion is omitted, the incorporation is reduced about 40%.

The Phenol Emulsion Reassociation Technique (PERT) for RNA·DNA Hybridization. Kohne et al. (1977) have devised a technique to accelerate the rate of DNA·DNA reassociation by emulsifying the hybridization mixture with phenol. Although RNA·DNA hybridization is not accelerated under

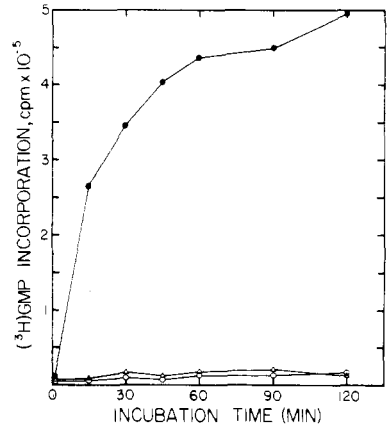

FIGURE 1: RNA synthesis in isolated nuclei with exogenous *E. coli* DNA-dependent RNA polymerase. Nuclei (1.3×10^7) were incubated at 25 °C in an 0.50-mL reaction mixture containing Hg-UTP as described in Materials and Methods for large scale RNA synthesis, except the reaction mixture was labeled with 0.75 mM [^{3}H]GTP (1.3 mCi/mmol). At indicated periods of incubation, 0.05 mL of reaction mixture was withdrawn and was precipitated with 10% Cl_3CCOOH. The radioactivity was counted on Millipore filters. (●—●) Reaction mixture with *E. coli* RNA polymerase (200 μg, 100 units) and α-amanitin (10 μg/mL); (Δ—Δ) endogenous synthesis by nuclei alone; (○—○) nuclei with α-amanitin (10 μg/mL).

their conditions, we have successfully adapted this technique for a moderate rate increase in RNA·DNA hybridization. The apparent rate of hybridization of Mo-MuLV 70S RNA to [^{3}H]cDNA prepared from the endogenous viral template is seen in Figure 2 to be accelerated by about 23 times in PERT compared with the conventional hybridization method. The 23 times apparent rate increase is greater than 2.5-fold the correction factor, attributable to the higher salt concentration of 1.6 M in PERT than 0.6 M in the conventional method (Britten et al., 1974). With more vigorous shaking, rates up to 100-fold faster could be achieved. The $C_rt_{1/2}$ value in Figure 2 for 70S RNA was accelerated from 1.1×10^{-2} in the conventional assay to 4.8×10^{-4} mol s L^{-1} in PERT. The reassociation kinetics in PERT were constant at different RNA concentrations. The effect of phenol on RNA-DNA hybridization has also been observed by Hill and Echols (1966).

To examine the fidelity of the hybrids formed by the PERT method vs. conventional hybridization conditions, thermal melting curves were performed on hybrids formed under both sets of conditions. Both the shape of the curve and the actual t_m were identical in both sets of hybrids (data not shown). The

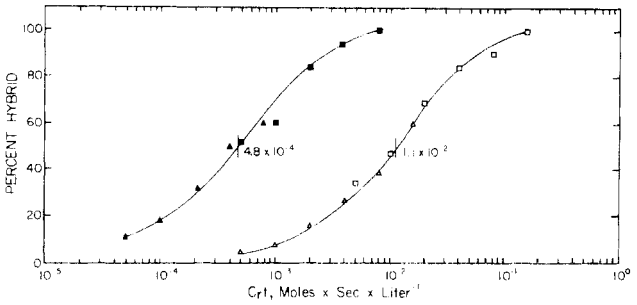

FIGURE 2: Acceleration of the rate of RNA-DNA hybridization by phenol emulsion reassociation technique (PERT). [^{3}H]cDNA (about 2500 cpm) was hybridized with Mo-MuLV 70S RNA by the PERT described in Materials and Methods or by the conventional hybridization method under the following conditions: 20 mM Tris-HCl (pH 7.2), 0.6 M NaCl, 0.05 mM EDTA, 0.05% sodium dodecyl sulfate, 3 μg of yeast RNA and calf thymus DNA in a 0.05-mL reaction mixture. PERT hybridization was carried out at RNA levels of 1 ng (▲) and 10 ng (■) for a period of 15 min to 4 h at 48 °C. The conventional hybridization was at 65 °C for 15 min to 8 h at 10 ng (△) and 100 ng (□) of 70S RNA levels. Hybridized [^{3}H]cDNA was precipitated by Cl_3CCOOH after S_1 digestion (Leong et al., 1972). The actual maximum hybridizations were 83% in PERT and 75% in conventional. They were normalized to 100% in the figure.

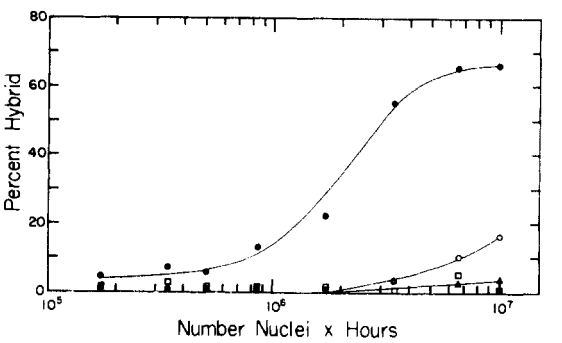

FIGURE 3: Viral sequences of in vitro RNA following incubation of isolated nuclei with *E. coli* RNA polymerase. RNA was synthesized with Hg-UTP as described in Materials and Methods. Nuclei, 2.6×10^8 (2.6 mg of DNA), of the Mo-MuLV infected NIH 3T3 cells were incubated in a 10-mL reaction mixture containing 4 mg of *E. coli* RNA polymerase (2000 units) and 10 μg/mL of α-amanitin at 25 °C for 1 h (●—●). All three other reaction mixtures were in 50-mL volume with proportional amounts of nuclei (13×10^8): (●—●) plus *E. coli* RNA polymerase; (△—△) the endogenous synthesis; (○—○) the control in the presence of α-amanitin (10 μg/mL); (□—□) the zero-time control without any incubation (α-amanitin, 10 μg/mL). The latter two extracts were precipitated with 200 μg of yeast RNA as carrier by 2 volumes of ethanol in the final step. The RNA synthesized from a given number of nuclei was hybridized with 2700 cpm of the whole Mo-MuLV [^{3}H]cDNA by PERT for 4 h. The data are presented on a semilog scale as the extent hybridization vs. the number of nuclei multiplied by hours of hybridization. Highly labeled [^{32}P]RNA (Hg), 10^4 cpm, was added to the reaction mixture prior to RNA extraction to monitor the efficiency of recovery of Hg-tagged RNA, which were all 33% of the initial inputs except the control with α-amanitin (50%). The nuclei number was corrected for the small influence of [^{32}P]RNA (Hg) recovery.

results indicate that the PERT yields faithful hybrids and thus permits smaller amounts of RNA samples to be assayed in a shorter period of time.

Hybridization Analysis of Viral Sequences of in Vitro RNA. A series of experiments were designed to demonstrate the synthesis of viral RNA sequences and their retention by Affi-Gel. (I) Nuclei of the infected cells were incubated for endogenous RNA synthesis at 25 °C for 60 min in the presence of Hg-UTP, and (II) in a parallel experiment, 10 μg/mL of α-amanitin was included to inhibit the endogenous RNA polymerase II activity. (III) In the third experiment, *E. coli* RNA polymerase was added to the nuclear mixture in the presence of α-amanitin. As already noted in Figure 1, RNA synthesis was greatly enhanced by the exogenous *E. coli* enzyme even at α-amanitin levels sufficient to completely inhibit the eukaryotic RNA polymerase II. These conditions permit us to study the RNA synthesis by the exogenous RNA polymerase from the nuclear template in its most native state in the isolated nuclei. (IV) As a further control for the nonspecific adsorption of preexisting RNA to the affinity column, a reaction identical with that in experiment II containing α-amanitin was also extracted for its RNA at the zero time of incubation.

To control for the gradual loss of Hg-tag during the RNA extraction, and thus the efficiency of RNA isolation, a small amount (approximately 10^5 cpm) of [^{32}P]RNA tagged with Hg was included in each reaction mixture immediately prior to RNA isolation. This internal marker also facilitated localization of RNA in Affi-Gel chromatography. The recovery of [^{32}P]RNA [Hg] in all the purified RNA samples was in the range of 33 to 50% of input, and it was very similar in a given set of experiments. Thus, the experimental protocol ensured that any differences in viral RNA content subsequently observed in hybridizations was not due to differential loss of Hg tag in in vitro RNA during isolation.

Thus, viral sequences synthesized from the comparable volumes of reaction mixtures containing the same number of nuclei were then compared by its hybridization to [^{3}H]cDNA probe as is seen in Figure 3. The results are first shown in Figure 3 as a function of the number of nuclei and time, and, in Figure 4, as RNA concentration and time. The RNA extracted from the zero time mixture (yeast RNA added as

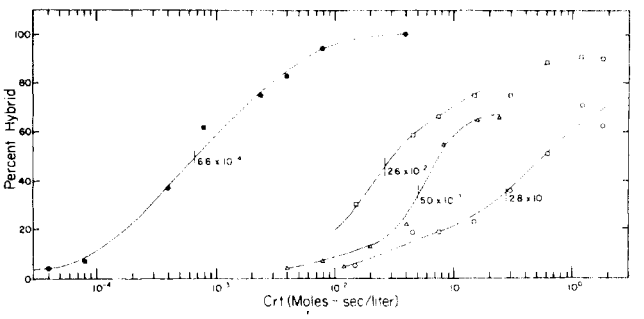

FIGURE 4: Hybridization of Mo-MuLV [^{3}H]cDNA to chromatin associated RNA and in vitro RNA from virus infected cells. In vitro RNA was synthesized from nuclei or chromatin in the presence of Hg-UTP and *E. coli* RNA polymerase and was purified by Affi-Gel as described in Materials and Methods. Chromatin-associated RNA was extracted from the same chromatin preparation by identical procedure except the Affi-Gel step was omitted. Hybridization was performed by the phenol emulsion reassociation technique by varying RNA concentration with constant amount of Mo-MuLV [^{3}H]cDNA (2700 cpm, 2×10^7 cpm/μg). Seventy-five percent input cDNA radioactivity was hybridized to 70S RNA in this experiment and the value was normalized to 100% in the figure. (●—●) To 70S viral RNA; (□—□) to chromatin-associated RNA; (△—△) to in vitro RNA from nuclei incubated with *E. coli* RNA polymerase; (○—○) to in vitro RNA from chromatin incubated with *E. coli* polymerase.

carrier) (experiment IV) contains virtually no detectable viral sequence, while RNA from the reaction mixture with *E. coli* polymerase (experiment III) reacted strongly with the [^{3}H]-cDNA probe to a saturating plateau under the same condition (Figure 3). RNA extracted from the endogenous reaction mixtures both in the absence (experiment I) and presence (experiment II) of α-amanitin both contain detectable viral sequences although at low levels. The half-saturation points for products of endogenous reactions both in the presence and

TABLE II: Binding of Nuclear and Chromatin RNA Prelabeled in Vivo with [^{3}H]Uridine to Affi-Gel Column after in Vitro Synthesis with *E. coli* RNA Polymerase in the Presence of Hg-UTP.[a]

Conditions of RNA Synthesis	Total Input RNA (cpm)	RNA Retained by Affi-Gel (cpm)	% Binding
A. Nuclei			
Complete mixture	820 000	21 000	2.6
No polymerase	450 000	420	0.1
No CTP, GTP, ATP	880 000	950	0.1
No Mn^{2+}	830 000	9 100	1.1
B. Chromatin			
Complete mixture	76 200	9 814	12.9
No polymerase	76 000	526	0.7
No CTP, GTP, ATP	73 500	3 651	5.0
No Mn^{2+}	75 200	5 346	7.1
C. Chromatin			
Complete mixture	1 080 000	110 000	10.2
No CTP, GTP, ATP	1 290 000	30 016	2.3

[a] Mouse cells were labeled in vivo for 18 h with [5-^{3}H]uridine (100 μCi/mL, 25 Ci/mmol) at 37 °C in Dulbecco-Vogt modified Eagle's minimal essential medium supplemented with 10% calf serum. Nuclei and chromatin were isolated and incubated for RNA synthesis, and the RNA was finally purified as described in Materials and Methods. The complete mixture (0.5 to 1.5 mL) containing 30 mM Tris-HCl (pH 7.9), 1 mM manganese acetate, 5 mM magnesium acetate, 12 mM β-mercaptoethanol, 50 mM ammonium sulfate, 0.4 mM each of ATP, CTP, GTP, and Hg-UTP and chromatin or nuclei at DNA concentration of 0.1 mg/mL. It was incubated at 25 °C for 60 min after addition of *E. coli* RNA polymerase (0.2 mg/mL, 800 units/mg) in the presence of α-amanitin (10 μg/mL). The other conditions are omission of indicated components from the complete mixture. Binding to Affi-Gel 401 was performed according to Smith and Huang (1976) as described in Materials and Methods. Experiments A, B, and C shown in the table are three sets of independent experiments.

absence of α-amanitin are indistinguishable at 3.7×10^7 nuclei-hours, while the zero-time control accounts for less than 10% of the background (data not shown). As compared with 2.2×10^6 nuclei-hours for the half-saturating point in the presence of *E. coli* polymerase, the endogenous products represent 6% of the observed hybridization of the in vitro transcript by *E. coli* enzyme. Thus it is clear that there is marked retention of viral sequences by Affi-Gel following incubation with the exogenous *E. coli* RNA polymerase while the level of endogenous synthesis is low. The results are in accord with [^{3}H]GTP incorporation in Figure 1. The comparison of the C_rt curve of in vitro RNA with that of 70S viral RNA is seen in Figure 4 under PERT condition. The $C_rt_{1/2}$ of 5.0×10^{-2} mol s L^{-1} of the in vitro RNA as compared with $C_rt_{1/2}$ of 6.6×10^{-4} of the viral 70S RNA in the same experiment suggests that the viral RNA content is 1.3% of the in vitro products.

Similar experiments were also performed for chromatin isolated from virus-infected cells. *E. coli* RNA polymerase dependent synthesis of viral sequences and their retention by Affi-Gel were similar to results in Figure 3. As is seen in Figure 4, the $C_rt_{1/2}$ value of the in vitro products is, however, slightly higher than that of in vitro nuclei products, suggesting lower viral RNA content (0.24%). As a control, chromatin associated RNA isolated from the same chromatin preparation by the same procedure except omitting the Affi-Gel purification step, reveals much higher viral RNA content (2.5%). The difference in chromatin associated RNA and the in vitro products is not due to the presence of Hg-tag interfering with RNA-DNA hybridization as observed by Beebee and Butterworth (1976). Further demercurization with 3 M β-mercaptoethanol (25 °C, 18 h) did not abolish this difference. Thus, the above observations seem to confirm the previous reports for resolving in vitro RNA from any preexisting nuclear and chromatin RNA containing no Hg-tag (Smith and Huang, 1976; Crouse et al., 1976; Beebee and Butterworth, 1976; Biessmann et al., 1976). As a matter of fact, the viral sequence content of the in vitro RNA is remarkably similar to the in vivo newly synthesized RNA as studied by pulse-labeling with [^{3}H]uridine seen in Table I.

Elongation by E. coli RNA Polymerase of RNA Prelabeled in Vivo. The close resemblance of in vitro products and the in vivo RNA, however, led us to suspect that *E. coli* RNA polymerase might by some unknown mechanism introduce the Hg-tag from Hg-UTP into some preexisting RNA chains allowing them to be retained by Affi-Gel. Results in Table II suggest that this may well be the case. Cellular RNA was labeled in vivo with 100 μCi/mL of [5-^{3}H]uridine in the culture medium at 37 °C overnight. Nuclei and chromatin were isolated and incubated for RNA synthesis in the presence of Hg-UTP as described in Materials and Methods. After incubation of the nuclei (RNA/DNA ratio = 0.40/1.0) for RNA synthesis in the presence of *E. coli* RNA polymerase, 2.6% of the RNA prelabeled in vivo with [^{3}H]uridine is retained by the Affi-Gel, while in the absence of exogenous RNA polymerase only 0.1% of the RNA is bound (experiment A). In the further purified chromatin, RNA is present in much lower amounts (RNA/DNA ratio = 0.06/1.0). However, after incubation with the enzyme, a much higher level of 10 to 13% of the chromatin associated RNA is retained (experiments B and C). Since chromatin contains much smaller amounts of RNA than nuclei, the higher percentage of retention suggests that RNA molecules being tagged by Hg are closely associated with chromatin, and most of the nuclear RNA is unaffected. To further ascertain whether the radioactivity retained by the Affi-Gel is indeed RNA in nature, the following tests were performed: (a) 100% of the trichloroacetic acid precipitable radioactivity of the bound and input RNA became acid soluble after alkaline hydrolysis in 0.5 N NaOH at 37 °C for 18 h; (b) 79% of the radioactivity was sensitive to pancreatic RNase A (heated at 90 °C for 10 min) following digestion at 30 μg RNase/mL in 0.1 M NaCl at 37 °C for 60 min; (c) 100% of the radioactivity was resistant to DNase digestion (30 μg/mL) in 0.1 M NaCl at 37 °C for 60 min. The very low levels of retention seen in Table II by incubation without added enzyme

rule out the simple exchange of Hg atom between Hg-UTP and RNA molecules. Furthermore, in the presence of RNA polymerase and Hg-UTP alone without CTP, GTP, and ATP, the level of retention is substantially reduced. The possibility of minute contaminating activities in RNA polymerase preparations such as polynucleotide phosphorylase which might incorporate Hg from trace amounts of Hg-UDP into RNA chains cannot totally account for the much higher incorporation in the presence of all four nucleotide triphosphates. The activity observed in incubation without Mn^{2+} ion also seems to exclude the template independent polymerization of ATP and UTP which requires Mn^{2+} and does not occur in the presence of Mg^{2+} alone (Smith et al., 1967). Similar retention of prelabeled [^{3}H]RNA in nuclei was also observed by incubation with *B. subtilus* RNA polymerase (kindly supplied by Dr. H. R. Whiteley, University of Washington, Seattle, Wash.).

Thus the results in Table II seem to suggest that *E. coli* RNA polymerase per se is capable of elongating more than 10% of chromatin associated RNA during RNA synthesis. Since only about 30 to 50% of the fully Hg-tagged RNA added to the incubation mixture was routinely recovered in the Affi-Gel fraction, the actual fraction of chromatin RNA elongated is over 20%. It is of interest to note that the 2.5% viral RNA content in chromatin-associated RNA (Figure 4) is extremely close to the value determined in pulse-labeled cell RNA (Table I), consistent with the observation on the tight association of the pulse-labeled nuclear RNA with chromatin (Kimmel et al., 1976). In conclusion, there is a strong possibility that most of the in vitro viral RNA sequences observed herein derived from the chromatin-associated species. The more native chromatin template in isolated nuclei yields the in vitro RNA with its C_rt curve very close to that of chromatin associated RNA. The higher shift of the C_rt curve of the in vitro products from isolated chromatin which possesses higher template activity than isolated nuclei may indicate higher extent of adventitious synthesis thus diluting out the viral sequences. If viral sequences are transcribed in vitro at all, it is transcribed less specifically than in vivo.

Discussion

Although DNA-histone complexes are less active than pure DNA in transcription by RNA polymerases, the roles of chromosomal nonhistone proteins and RNA in determination of cell and tissue specificities of template restriction have not been unequivocally established. Many attempts have been made to determine whether chromatin or nuclei retain their in vivo template specificity in transcription in vitro by either homologous or heterologous RNA polymerases. Basically, in vitro transcription studies of specific gene sequences have been approached by two ways. The first approach involves preparation of in vitro RNA with radiolabeled nucleotide triphosphates, and analysis of the labeled RNA products with cDNA gene probes by various hybridization methods (Jacquet et al., 1974; Rymo et al., 1974; Swetly and Watanabe, 1974). Although this approach ensures only the in vitro synthesized and labeled RNA products being analyzed, the relatively high background in these hybridization procedures prohibits detailed analysis of less abundant mRNA species. Another approach involves analysis of in vitro RNA products with highly radiolabeled probes prepared from mRNA by reverse transcriptase or from virions of DNA viruses (Axel et al., 1973; Gilmour and Paul, 1973; Astrin, 1973; Shih et al., 1973; Steggles et al., 1974; Wilson et al., 1975; Gilmour et al., 1975; Marzluff and Huang, 1975; Stein et al., 1975; Tsai et al., 1976). However, the contamination of in vitro products by preexisting RNA species in chromatin and nuclei in this approach cannot always be eliminated. The recent advance by carrying out RNA synthesis in the presence of Hg-UTP and subsequent purification of the in vitro RNA by sulfhydryl affinity chromatography apparently resolved this dilemma (Smith and Huang, 1976; Crouse et al., 1976; Beebee and Butterworth, 1976; Biessmann et al., 1976).

In this paper, we have presented evidence that, even in RNA synthesis catalyzed by the exogenous *E. coli* RNA polymerase, a very large fraction of chromatin-associated RNA is elongated and tagged by the Hg atoms during RNA synthesis in the presence of Hg-UTP. By studying the transcription of proviral DNA sequences of Moloney murine leukemia virus in nuclei and chromatin isolated from chronically infected cells, the in vitro RNA products have been found to be remarkably similar to the in vivo RNA. Furthermore, essentially the same results have been obtained by studying the transcription of mammary tumor virus genes in virus-producing mouse cells derived from mammary carcinoma (data not shown). It is most likely that these viral sequences in in vitro RNA products are initiated and derived from the preexisting chromatin-associated species.

DNA-dependent RNA synthesis primed and initiated by an oligoribonucleotide has been described in reaction involving a synthetic single-stranded polydeoxyribonucleotide template (Terao et al., 1972). The initiation of RNA synthesis by template associated RNA molecules in nuclei and chromatin by an exogenous RNA polymerase, however, has not been previously recognized largely due to unknown structure of the association of these RNA molecules with the template and partly due to lack of proper method of detection. The use of mercurated UTP in RNA synthesis with prelabeled nuclei or chromatin provides a convenient assay. These observations arouse caution and concern in reaching conclusions regarding fidelity of in vitro transcription. For meaningful studies pertaining to the specificity of in vitro transcription and its regulation by specific factors, it is imperative that observation should be made only on new RNA products initiated and transcribed de novo from the template. It can be argued that mere in vitro elongation of RNA chains being actively transcribed in chromatin in vivo only reflects the residual in vivo state and reveals very little on how that gene is regulated by in vitro factors. The question of initiation of RNA synthesis by chromatin-associated RNA is important not only for interpretation of experiments involving detection of specific sequences of in vitro RNA by radiolabeled cDNA probes, but also for experiments involving RNA labeled in vitro during RNA synthesis. If there is preferential initiation from in vivo RNA chain termini, the newly synthesized RNA merely reflects the in vivo state.

Acknowledgments

The authors thank Dr. Richard Howk, Anthony Anisowicz, and Maureen Weeks for preparation of the Mo-MuLV probes. We are indebted to Drs. David E. Kohne and John Taylor for access to their manuscripts before publication.

References

Astrin, S. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 2304-2308.

Axel, R., Cedar, H., and Felsenfeld, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 2029-2032.

Beebee, T. J. C., and Butterworth, P. H. W. (1976), *Eur. J.*

Biochem. 66, 543–550.
Benveniste, R. E., and Todaro, G. J. (1974), *Nature (London) 252,* 170–173.
Biessmann, H., Gjerset, R. A., Levy, B., and McCarthy, B. J. (1976), *Biochemistry 15,* 4356–4363.
Britten, R. J., Graham, D. E., and Neufeld, B. R. (1974), *Methods Enzymol. 29,* 363–418.
Burgess, R. R., and Jendrisak, J. J. (1975), *Biochemistry 14,* 4634–4638.
Chamberlin, M. J., and Berg, P. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 81–94.
Coffin, J. M., Parsons, J. T., Rymo, L., Haroz, R. K., and Weissmann, C. (1974), *J. Mol. Biol. 86,* 373–396.
Crouse, G. F., Fodor, E. J. B., and Doty, P. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1564–1567.
Dale, R. M. K., Livingston, D. C., and Ward, D. C. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2238–2242.
Dale, R. M. K., Martin, E., Livingston, D. C., and Ward, D. C. (1975), *Biochemistry 14,* 2447–2457.
Gilmour, R. S., and Paul, J. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3440–3442.
Gilmour, R. S., Windass, J. D., Affara, N., and Paul, J. (1975), *J. Cell. Physiol. 85,* 449–458.
Hill, C. W., and Echols, H. (1966), *J. Mol. Biol. 19,* 38–51.
Howk, R. S., Williams, D. R., Haberman, A. B., Parks, W. P., and Scolnick, E. M. (1974), *Cell 3,* 15–22.
Jacquet, M., Groner, Y., Monroy, G., and Hurwitz, J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3045–3049.
Kacian, D. L., Watson, K. F., Burny, A., and Spiegelman, S. (1971), *Biochim. Biophys. Acta 246,* 365–383.
Khoury, A. T., and Hanafusa, H. (1976), *J. Virol. 18,* 383–400.
Kimmel, C. B., Sessions, S. K., and MacLeod, M. C. (1976), *J. Mol. Biol. 102,* 177–191.
Kohne, D. E., Levison, S. A., and Byers, M. J. (1977), *Biochemistry* (in press).
Leong, J. A., Garapin, A. C., Jackson, N., Fanshier, L., Levinson, W., and Bishop, J. M. (1972), *J. Virol. 9,* 891–902.
Marzluff, W. F., and Huang, R. C. C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1082–1086.
Parks, W. P., Howk, R. S., Anisowicz, A., and Scolnick, E. M. (1976), *J. Virol. 18,* 491–503.
Rymo, L., Parsons, J. T., Coffin, J. M., and Weissmann, C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2782–2786.
Scolnick, E. M., Howk, R. S., Anisowicz, A., Peebles, P. T., Scher, C. D., and Parks, W. P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4650–4654.
Scolnick, E. M., Maryak, J. M., and Parks, W. P. (1974), *J. Virol. 14,* 1435–1444.
Shih, T. Y., Khoury, G., and Martin, M. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3506–3510.
Smith, D. A., Ratliff, R. L., Williams, D. L., and Martinez, A. M. (1967), *J. Biol. Chem. 242,* 590–595.
Smith, M. M., and Huang, R. C. C. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 775–779.
Steggles, A. W., Wilson, G. W., Kantor, J. A., Picciano, D. J., Falvey, A. K., and Anderson, W. F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1219–1223.
Stein, G. S., Park, W., Thrall, C. L., Mans, R. J., and Stein, J. L. (1975), *Nature (London) 257,* 764–767.
Swetly, P., and Watanabe, Y. (1974), *Biochemistry 13,* 4122–4126.
Taylor, J., Illmensee, R., and Summers, J. (1976), *Biochim. Biophys. Acta 442,* 324–330.
Terao, T., Dahlberg, J. E., and Khorana, H. G. (1972), *J. Biol. Chem. 247,* 6157–6166.
Tsai, S. Y., Harris, S. E., Tsai, M. J., and O'Malley, B. W. (1976), *J. Biol. Chem. 251,* 4713–4721.
Wilson, G. N., Steggles, A. W., Kantor, J. A., Nienhuis, A. W., and Anderson, W. F. (1975), *J. Biol. Chem. 250,* 8604–8613.
Young, H. A., Shih, T. Y., Scolnick, E. M., and Parks, W. P. (1977), *J. Virol. 21,* 139–146.