A RNA-dependent RNA polymerase activity: implications for chromatin transcription experiments

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ABSTRACT

Mercurated nucleoside triphosphates have been used for transcription of chicken oviduct chromatin with E. coli RNA polymerase. The newly synthesized RNA was purified from preexisting RNA by SH-agarose chromatography and analyzed for the content of specific mRNA sequences. The apparent preferential production of ovalbumin mRNA sequences was not inhibited by actinomycin D, although total RNA synthesis was reduced by more than 90%. Furthermore, when globin mRNA alone, or added to oviduct chromatin, was incubated in the transcription assay, a significant fraction of this mRNA was retained on SH-agarose. The copurification of chromatin associated RNA with in vitro synthesized mercurated RNA was mainly due to a RNA-dependent synthesis of complementary sequences by the bacterial enzyme. Although denaturation of the transcripts prior to SH-agarose chromatography leads to a reduced contamination with endogenous ovalbumin specific RNA, we are unable to show that the messenger-specific RNA sequences purified with the newly mercurated RNA result from a DNA-dependent reaction.

INTRODUCTION

Much of the information concerning the mechanism by which eucaryotic genes are regulated has come from experiments in which isolated chromatin is transcribed in vitro by exogenous bacterial RNA polymerase. From experiments with a variety of systems it has been concluded that some tissue specific transcription is retained in isolated chromatin (1-9). The selectivity of transcription was analyzed by hybridization with radioactive DNA complementary to specific RNA sequences. However, hybridization to labeled DNA does not distinguish in vitro synthesized RNA from chromatin-associated RNA. Mercured ribonucleoside triphosphates as substrate for RNA polymerase have been introduced to allow the isolation of newly synthesized RNA by affinity chromatography on
sulphydryl agarose (10,11). This method was thought to offer the possibility to analyze in vitro transcripts free of endogenous RNA (12-16).

When mercurated nucleotides were used for transcription of chicken oviduct chromatin with E. coli RNA polymerase, we found that the apparent production of ovalbumin specific RNA sequences was not inhibited by actinomycin D at concentrations in which the total RNA synthesis was lowered to 10%. This lack of inhibition is caused mainly by a DNA-independent activity of the bacterial RNA polymerase leading to copurification of endogenous RNA with in vitro synthesized RNA. In this report we show that RNA, which is transcribed by E. coli RNA polymerase into complementary sequences, can be retained by the affinity column in a duplex with the in vitro synthesized Hg-RNA. Thus we feel that the presence of endogenous RNA in chromatin is a major difficulty for the quantification of in vitro synthesized specific RNA sequences even when mercurated nucleotides are used as an affinity label for the newly synthesized RNA. Experimental suggestions are given for overcoming some of the problems arising from the RNA-dependent activity of the bacterial RNA polymerase. During the preparation of this manuscript Zasloff and Felsenfeld (17) reported similar results from experiments with duck reticulocyte chromatin.

MATERIALS AND METHODS

Preparation of Chicken Oviduct Chromatin

Nuclei were prepared from laying hen oviduct tissue as described earlier (18) except that CaCl₂ was omitted and Mg²⁺ ions raised to 5 mM in the cell lysis buffer. Isolated nuclei from 10 grams of oviduct tissue were resuspended in 25 ml of 5 mM MgCl₂, 10 mM Tris-HCl, pH 8.0 and 0.3 M sucrose, repelleted at 1500 g and lysed in 50 ml of buffer A (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT) with 5-10 strokes in a teflon-glass homogenizer (1000 rpm). The chromatin was pelleted at 20,000 g and homogenized into 10 ml of buffer A plus 12.5% glycerol by treatment in a teflon-glass homogenizer (19,20). The yield of chromatin was approximately 20 A₂₆₀ units per gram of oviduct tissue. The chromatin had an A₂₆₀/A₂₈₀ ratio of 1.4.
Synthesis and Isolation of RNA

Chromatin containing 500 µg of DNA was incubated at 37°C in 10 ml of 25 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 1 mM MnCl₂, 150 mM NaCl, 10 mM 8-mercaptoethanol, 0.25 mM each of ATP and GTP, 0.08 mM Hg-CTP (prepared according to Dale et al., 11), 0.17 mM CTP and 0.25 mM [³²P]UTP (0.7 mCi/mMol), 12.5 mg creatin phosphate, 1.25 mg creatin phosphokinase (Boehringer), with 200 µg highly purified RNA polymerase holoenzyme from E.coli (1500 units/mg, purified by Dr. Sternbach, according to Sternbach et al., 21). After the indicated incubation time, 10 ml of 150 mM NaCl, 0.5% SDS, 50 mM Tris-HCl, pH 7.5 and 5 mM EDTA were added and the RNA extracted by the hot phenol method (22,23). The RNA was precipitated with 2 volumes of ethanol at -20°C, redissolved in 1 ml of 10 mM Tris-HCl, pH 7.5 and chromatographed on a Sephadex G-50 column (1 cm x 65 cm) equilibrated in 10 mM Tris-HCl, pH 7.5 and 0.1 M NaCl. The peak fractions of the excluded material (approximately 60% of the originally incorporated radioactivity) were combined and the RNA ethanol precipitated and redissolved in 0.5 ml H₂O.

RNA was also synthesized with E.coli RNA polymerase using globin mRNA as a template. Each 1 ml reaction volume contained 20 µg of RNA polymerase and 10 µg of chicken globin mRNA (purified from erythroblast polysomes by EDTA-detergent treatment and poly(U)-Sepharose chromatography as described previously, 24) in the same reaction mixture described for chromatin transcription. The reaction was stopped after 1 h at 37°C with 0.5% SDS and the RNA was extracted with 1 ml of chloroform, chromatographed on Sephadex G-50, ethanol precipitated with 40 µg of yeast RNA (Worthington) and redissolved in 0.5 ml of H₂O.

Analytical assays for the DNA-dependent and RNA-dependent RNA polymerase reaction were performed in 0.1 ml reaction volumes containing 5 µg of calf thymus DNA or 0.5 µg of chicken oviduct polysomal poly(A)-containing RNA (25), 2 µg of RNA polymerase, 50 µg of BSA, 0.25 mM [³H]UTP (80 mCi/mMol) and all other components as described for chromatin transcription. Incorporation of radioactivity into TCA precipitable material was determined on Millipore filters; where possible [³²P]RNA was measured by Cerenkov radiation.
SH-Agarose Chromatography of Hg-RNA

The isolated RNA was chromatographed on sulfhydryl aminoethyl agarose (prepared according to Cuatrecasas, 26), 0.17 mval SH/ml packed gel (27) after (a) partial or (b) total denaturation.

(a) Hg-RNA containing RNA samples were adjusted to 5 ml TN buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5) and 1 mM EDTA and 25% formamide, heated for 3 min to 65°C and diluted immediately to 10 ml with the same buffer. The RNA was applied to a 1 ml SH-agarose column at room temperature at a flow rate of 10 ml/h. The column was washed with 10 ml of application buffer, then successively with 30 ml of TN, 10 ml of 10 mM Tris-HCl, pH 7.5, 10 ml of TN containing 2 M NaCl plus 1 mM EDTA and 50 ml of TN at maximal flow rate. After the column was allowed to stand in the mercaptan solution for 15 min, the Hg-RNA was eluted with 5 ml of TN containing 0.2 M β-mercaptoethanol. Following the addition of NaCl to 0.3 M and 10 µg/ml yeast RNA, the eluted RNA was precipitated by 2.5 volumes of ethanol. RNA not bound to SH-agarose (the first 15 ml flow through) was precipitated in the same manner.

(b) The RNA samples were adjusted to 5 ml 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 50% formamide, heated for 3 min to 100°C, diluted immediately to 10 ml with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl and chromatographed as described above.

Of the applied [3H]UMP-labeled Hg-RNA synthesized with chromatin, between 30% and 40% bound to the SH-agarose column and was eluted with 0.2 M β-mercaptoethanol.

Determination of Specific RNA Content

The ovalbumin RNA and the globin RNA content in both RNA samples from the SH-agarose chromatography was determined by cDNA excess hybridization (25,28). Specific cDNA probes were prepared by reverse-transcription of purified mRNAs as described (25). Increasing amounts of RNA were hybridized to completion in 5 µg reactions containing excess amounts of specific cDNA (0.1 ng, 10^7 cpm/µg), as outlined previously (25).

RNase A Digestion

The content of double-stranded RNA was determined by diges-
tion with RNase A. Native or denatured RNA samples were adjusted to 1 ml containing 10 mM Mg-acetate, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5 (RNase A digestion buffer). 450 μl were incubated with 5 μg/ml RNase A and 450 μl were incubated without RNase for 1 h at 30°C and the degree of RNase A resistance determined by comparison of acid precipitable radioactivity in both samples.

RESULTS
1. The Apparent Synthesis of Ovalbumin mRNA is not inhibited by Actinomycin D

Hg-substituted nucleotides have been used in chromatin transcription experiments to separate in vitro synthesized RNA from pre-existing chromatin associated RNA sequences. To achieve a complete separation it is necessary to prove that no endogenous chromatin RNA binds to the sulfhydryl agarose column. When oviduct chromatin RNA was extracted before or after a one hour incubation at 37°C with RNA polymerase (Table 1, IIb and Ib) we found approximately 200 ng of endogenous ovalbumin specific RNA per 500 μg of chromatin DNA. Less than 2 pg of these ovalbumin sequences bound unspecifically to the sulfhydryl agarose column. To achieve this low level of unspecific binding, it was necessary to follow the washing procedure outlined in Materials and Methods. In all buffer systems used during synthesis and isolation of the RNA acetate ions were replaced by chloride ions. This is of importance for the chloride ion and not the acetate ion effectively prevents the covalent mercuration reaction, thus circumventing the potential problem of reutilization of any labilized Hg^{2+} ions (10). Under the conditions used, less than one in 10^5 molecules were retained on SH-agarose when purified unmercurated ovalbumin mRNA was applied to the column (29).

When oviduct chromatin was incubated with bacterial RNA polymerase (200 μg of enzyme per 500 μg of DNA) in the presence of 30% mercury substituted CTP, the SH-agarose bound RNA was found to contain an amount of ovalbumin specific RNA several 1000 times higher than that caused by nonspecific adsorption (Table 1, Ia and IIa).

The appearance of ovalbumin specific RNA in the bound RNA fraction is dependent on the function of E.coli RNA polymerase. If the enzyme is omitted or inhibited by rifampicin, a pronounced
reduction of column-bound ovalbumin RNA is found (Table 1, Ic and Id). When we titrated the SH-agarose bound RNA for globin RNA sequences, the product of a structural gene not actively expressed in the oviduct tissue, we found approximately 1000 times less globin specific RNA than ovalbumin specific RNA. However, this apparent specificity of in vitro synthesis of ovalbumin RNA is not due to a DNA-dependent enzymatic process. The presence of 50 μg/ml actinomycin D, an inhibitor of double-stranded DNA-dependent RNA synthesis, did not reduce the content of ovalbumin RNA in the RNA fraction bound to SH-agarose, although total RNA synthesis was lowered approximately ten-fold (Table 1, IIc). This also demonstrates that endogenous RNA is not retained significantly on SH-agarose through aggregation with Hg-RNA since the ovalbumin RNA in the retained RNA fraction is not related to the amount of Hg-RNA chromatographed (Table 1, IIa and IIc).

Purified ovalbumin mRNA or globin mRNA added to the chromatin before incubation with the polymerase caused an increase of ovalbumin specific RNA and globin specific RNA in the bound fraction (Table 1, Ie and Ifd).

These findings suggested that endogenous RNA contaminates the newly synthesized, mercury substituted RNA and pointed to the possibility that this contamination is due to a DNA-independent activity of the bacterial polymerase. We therefore studied whether a RNA-dependent polymerase activity could cause the effect.

2. The RNA-dependent RNA polymerase activity

It is known that bacterial DNA-dependent RNA polymerase can utilize a variety of synthetic polyribonucleotides as effective templates to synthesize their complementary polymers (30-33). Naturally occurring RNA also supports RNA synthesis by bacterial RNA polymerases, although to a lesser extent than DNA and synthetic ribopolymers (31,32,34-36). Because of the possible implication of this RNA polymerase side reaction for chromatin transcription experiments, we asked whether E.coli RNA polymerase could synthesize complementary RNA using poly(A)-containing mRNA as a template.

The rate of UMP incorporation into acid precipitable material in the presence of eucaryotic mRNA and of double-stranded DNA was measured (Fig.1). Whereas at 4 mM MgCl₂ the DNA-dependent incorporation
Table 1  Specific Messenger Sequence Content in the SH-Agarose Bound Hg-RNA Fraction
from in vitro Transcription of Oviduct Chromatin

<table>
<thead>
<tr>
<th>No.</th>
<th>Reaction Components</th>
<th>Total RNA Synthesized (µg)</th>
<th>Ovalbumin Specific RNA SH-nonbound (ng)</th>
<th>Ovalbumin Content SH-bound (ng)</th>
<th>Globin Specific RNA SH-nonbound (ng)</th>
<th>Globin Content SH-bound (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I a</td>
<td>RNAP + chromatin</td>
<td>24.0</td>
<td>220</td>
<td>6.0</td>
<td>0.04</td>
<td>&lt; 0.006</td>
</tr>
<tr>
<td>b</td>
<td>RNAP + chromatin — HgCTP</td>
<td>28.5</td>
<td>213</td>
<td>&lt; 0.002</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c</td>
<td>RNAP + chromatin + Rif.</td>
<td>0.57</td>
<td>229</td>
<td>1.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d</td>
<td>chromatin</td>
<td>0.28</td>
<td>205</td>
<td>0.065</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>e</td>
<td>RNAP + chromatin + mRNA^OA</td>
<td>27.1</td>
<td>1250</td>
<td>18.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II a</td>
<td>RNAP + chromatin</td>
<td>10.2</td>
<td>157</td>
<td>3.4</td>
<td>0.04</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td>b</td>
<td>chromatin + SDS</td>
<td>&lt; 0.05</td>
<td>171</td>
<td>&lt; 0.002</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c</td>
<td>RNAP + chromatin + Act. D</td>
<td>1.6</td>
<td>180</td>
<td>3.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d</td>
<td>RNAP + chromatin + mRNA^HB</td>
<td>12.7</td>
<td>180</td>
<td>2.8</td>
<td>154</td>
<td>2.9</td>
</tr>
</tbody>
</table>

RNA was transcribed from oviduct chromatin in the presence of 30% Hg-CTP (except for Ib, where only CTP was used) and E.coli RNA polymerase, where indicated. In Ic 6 µg/ml rifampicin, in IIc 50 µg/ml actinomycin D, in Ie 1.2 µg purified ovalbumin mRNA (mRNA^OA) and in IID approximately 0.2 µg purified chicken globin mRNA (mRNA^HB) was added to the incubation mixture, all other components were as indicated in Materials and Methods. Chromatin samples (500 µg DNA) were incubated at 37°C for 1 h in experiment I and for 30 min in experiment II, and the RNA isolated and chromatographed on SH-agarose columns as outlined in Materials and Methods. The total content of globin and ovalbumin specific sequences in the column flow-through and the bound RNA fraction was determined in cDNA excess hybridization reactions.
rate is independent of the presence of Mn\textsuperscript{2+} ions, the RNA-dependent reaction has a clear dependency on the concentration of MnCl\textsubscript{2}. The low level of RNA-dependent RNA synthesis in the absence of Mn\textsuperscript{2+} ions is significant, since omission of the polynucleotide leads to an even lower incorporation of \[^{3}\text{H}\]UMP. The RNA-dependent reaction is not due to an enzyme activity contaminating \textit{E. coli} RNA polymerase because it is totally inhibited by rifampicin. In addition, the RNA-dependent reaction is not inhibited by phosphate ions, an inhibitor of the polynucleotide phosphorylase polymerization reaction (37).

The experiment outlined in Table 2 was designed to decide whether the synthesized RNA is complementary and whether it is covalently bound to the mRNA. When globin mRNA and its \[^{3}\text{H}\]UMP or \[^{3}\text{H}\]CMP labeled product were deproteinized and digested with RNase A in the presence of 0.5 M NaCl, almost no radioactivity could be solubilized. After denaturation 95% of the \[^{3}\text{H}\]CMP labeled product and 67% of the \[^{3}\text{H}\]UMP labeled product could be digested by RNase A. These results show that the synthesized RNA is more than 90% complementary to its mRNA template and remains duplexed to the template RNA after synthesis. The \[^{3}\text{H}\]CMP labeled RNA did not become RNase A resistant by intracellular renaturation following heat denaturation and we conclude that at least 95% of the product is not covalently bound to its template.
The RNase A Resistance of RNA Synthesized with *E. coli* RNA Polymerase on Globin mRNA

<table>
<thead>
<tr>
<th>RNase A treatment of RNA</th>
<th>Degree of RNase A Resistance of Labeled RNA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[(^{3})H]CMP-RNA</td>
<td>[(^{3})H]UMP-RNA</td>
</tr>
<tr>
<td></td>
<td>Poly(U) added</td>
<td>Poly(U) added</td>
</tr>
<tr>
<td>non-denatured RNA</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>denatured RNA</td>
<td>92</td>
<td>98</td>
</tr>
</tbody>
</table>

RNA was synthesized with *E. coli* RNA polymerase in a 1 ml reaction containing 10 µg of purified chicken globin mRNA in the presence of \(^{3}\)H-labeled CTP or UMP (0.2 Ci/mMol) and subsequently deproteinized and recovered as described in Materials and Methods. The RNA samples containing approximately 0.6 nMol of incorporated \(^{3}\)HUMP or 0.2 nMol of incorporated \(^{3}\)H CMP, respectively, were split into four parts. To two of the aliquots 20 µg of commercial poly(U) (Miles) was added. One sample with and one without poly(U) was directly, without previous denaturation, adjusted to 1 ml 10 mM Mg-acetate, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5 (RNase A digestion buffer). The two other RNA samples were denatured by heating for 5 min to 100°C in 200 µl H2O and then immediately diluted and also adjusted to 1.0 ml RNase A digestion buffer. For the experiment in column 3 100 pg of \(^{3}\)H Poly(U) (6 Ci/mMol) were incubated alone or together with 2 µg of globin mRNA in 1 ml RNase A digestion buffer and treated as described above. The degree of RNase A resistance was determined for all samples as described in Materials and Methods.

cess unlabeled Poly(U) added before denaturation leads to the elimination of the partial RNase A resistance shown by the \(^{3}\)H-UMP labeled RNA. This RNase A resistance is most likely due to a rapid Poly(A):Poly(U) renaturation rather than to an intramolecular reassociation of the labeled sequence to its template RNA. Column 3 of Table 2 shows that 100 pg of \(^{3}\)H Poly(U) in one ml in the presence of 2 µg of globin mRNA are resistant to RNase A immediately after heat treatment. This suggests that Poly(U):Poly(A) renaturation is a rapidly occurring process under the conditions used here even at very low component concentrations.

3. **The Apparent Specificity of Chromatin Transcription is mainly due to an RNA-dependent RNA Polymerase Activity**

If synthesis of complementary RNA can occur in the presence of
Table 3  Retention of Globin mRNA on SH-Agarose after Incubation with RNAP in the Presence of Hg-CTP

<table>
<thead>
<tr>
<th>No.</th>
<th>Incubation Components</th>
<th>[3H]UMP Incorporated (pMol)</th>
<th>Globin RNA Bound to SH-Agarose (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>complete</td>
<td>46.6</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>+ Rifampicin (6 µg/ml)</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>+ Actinomycin D (50 µg/ml)</td>
<td>47.0</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>- MnCl₂</td>
<td>11.0</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>- RNA Polymerase</td>
<td>0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6</td>
<td>- Hg-CTP, + CTP</td>
<td>50.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

RNA was synthesized with E.coli polymerase in a 200 µl reaction containing 2 µg of purified chicken globin mRNA in the presence of [32P]-labeled UTP (22 mCi/mMol) and 0.25 mM Hg-CTP (except in No. 6: 0.25 mM CTP) and subsequently deproteinized, recovered and chromatographed on SH-agarose as described in Materials and Methods. The content of globin specific sequences in the column bound RNA fraction was determined in cDNA excess hybridization reactions.

Hg-CTP, chromatin associated RNA could be bound to SH-agarose via a duplex with newly synthesized mercurated RNA. The use of Hg-CTP instead of CTP in an E.coli RNA polymerase reaction with purified globin mRNA as template did not significantly alter the activity of the enzyme and the effect of rifampicin, actinomycin and Mn²⁺ ions were comparable (Table 3). Hg-RNA samples isolated from such reactions were chromatographed on SH-agarose and the globin mRNA content was determined in the column retained RNA fraction. Globin mRNA sequences are readily found (approximately 2% of input mRNA). This effect was inhibited by rifampicin but not by actinomycin D (Table 3). When the isolated transcripts were chromatographed after total heat denaturation, the amount of globin specific RNA was reduced approximately 30-fold (Table 4). These results demonstrate that the mercury substituted antisense RNA sequences are responsible for the retention of globin mRNA on the SH-agarose column.

The experiment outlined in Table 4 was performed in order to establish whether in chromatin transcription experiments the
Table 4  Effect of Denaturation on the Binding of Specific RNA to SH-Agarose

<table>
<thead>
<tr>
<th>Template</th>
<th>non-denatured</th>
<th>denatured</th>
<th>denatured with excess poly(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total RNA%</td>
<td>globin RNA%</td>
<td>ovalbumin RNA%</td>
</tr>
<tr>
<td>globin mRNA</td>
<td>78</td>
<td>82</td>
<td>-</td>
</tr>
<tr>
<td>oviduct chromatin</td>
<td>75</td>
<td>-</td>
<td>79</td>
</tr>
<tr>
<td>oviduct chromatin + 50 µg/ml actinomycin D</td>
<td>73</td>
<td>-</td>
<td>86</td>
</tr>
</tbody>
</table>

RNA was transcribed from globin mRNA and isolated as outlined in Table 3. Aliquots containing 50 pMol of UMP incorporated in total RNA and 236 ng of globin mRNA were rechromatographed on SH-agarose either non-denatured or after total denaturation as described in Materials and Methods. Oviduct chromatin was transcribed for 1 h (10 ml assay) in the presence of 30% Hg-CTP and 50 µg/ml actinomycin D where indicated. The RNA was isolated and chromatographed on SH-agarose without denaturation. SH-agarose bound RNA samples were then divided into 3 aliquots and rechromatographed on SH-agarose either non-denatured or after total denaturation. To one aliquot 10 µg of commercial poly(A) was added prior to the denaturation step. Each aliquot contained 1 µg of in vitro synthesized [32P]RNA and 2 ng of ovalbumin specific RNA. Aliquots derived from actinomycin D assays contained 0.1 µg of in vitro synthesized RNA and 2.2 ng of ovalbumin specific RNA. Values are expressed as ratio of RNA retained on SH-agarose over RNA applied to the column (in %).
same mechanism can cause contamination of in vitro transcribed Hg-RNA with endogenous RNA. Laying hen oviduct chromatin was incubated with RNA polymerase and mercurated CTP in the absence and presence of 50 µg/ml actinomycin D. Total RNA was extracted and the newly synthesized RNA purified by SH-agarose chromatography. Approximately equal amounts of ovalbumin specific RNA were found in the bound RNA fraction regardless of the presence or absence of actinomycin D during transcription. If this result is due to a copurification of endogenous ovalbumin RNA in a duplex with mercurated RNA, heat denaturation should separate the in vitro transcript from contaminating sequences. When we re-chromatographed the isolated RNA samples on a second SH-agarose column after heat denaturation in 50% formamide, we found an approximately 4-fold reduction in the amount of ovalbumin RNA. Poly(A)-containing mRNA sequences could be trapped by poly(U)-containing Hg-RNA sequences. When we prevented this by heat denaturation in the presence of excess poly(A), a further 2-fold reduction of column retained ovalbumin RNA could be achieved. The remaining ovalbumin specific RNA may represent the product of gene transcription and/or may still represent endogenous sequences retained on SH-agarose by a presently unknown mechanism. The data in Table 4 show, however, that RNA isolated from transcription reactions in the presence and absence of actinomycin D and heat-denatured in the same way did not contain different amounts of ovalbumin RNA. We thus have no evidence that the messenger-specific RNA sequences purified with the newly synthesized mercurated RNA result from a DNA-dependent reaction.

DISCUSSION

It has been widely demonstrated that transcription of chromatin with E. coli RNA polymerase gives rise to products which are specific to the tissue from which the chromatin originates (1-9). Far reaching conclusions about the regulation of gene activity in eucaryotes have been drawn from these results. Since it is unlikely, though not impossible, that exogenously added bacterial RNA polymerase faithfully transcribes eucaryotic chromatin, it is generally believed that the in vitro transcription of specific genes is a consequence of these DNA sequences being more access-
The RNA directed in vitro synthesized Hg-RNA sequences can retain endogenous chromatin RNA in form of complementary duplexes on the sulfhydryl agarose columns. About 80% of the bound ovalbumin RNA sequences are preexisting, chromatin-associated RNA since they can be eliminated by heat denaturation prior to the affinity chromatography (Table 4). Zasloff and Felsenfeld (17) have made similar findings and have previously attributed the contamination to the RNA-dependent activity of the bacterial RNA polymerase.

We show that an additional 10% of the SH-agarose bound ovalbumin RNA can be eliminated by heat denaturation in the presence
of excess poly(A). The retention of these sequences is most probably due to rapid reannealing of endogenous poly(A)-containing ovalbumin RNA to poly(U)-containing Hg-RNA. Therefore, the retention of chromatin associated RNA might be increased when Hg-UTP instead of Hg-CTP is used as a polymerase substrate (12-17,41). Even after denaturation in the presence of poly(A) no actinomycin D sensitive ovalbumin RNA synthesis could be detected. Thus using the action of actinomycin D as a criterion, we are unable to show that the messenger-specific RNA sequences purified with the newly mercurated RNA result from a DNA-dependent reaction.

It is possible that to a minor extent the added polymerase covalently attaches Hg-nucleotides to 3' OH ends of preexisting RNA molecules which are still duplexed to chromatin DNA. This activity would be similar to an activity described by Terao et al. (41) and could cause retention of chromatin associated RNA on SH-agarose. Shih et al. (42) have suggested that added polymerase tags preexisting RNA with mercurated nucleotides.

Our results indicate that different RNA-dependent polymerase activities interfere with the isolation of newly synthesized transcripts by the mercury method. The non-specific aggregation of endogenous RNA sequences to Hg-RNA described by Konkel and Ingram (43) does not contribute significantly to the contamination of mercurated RNA by non-mercurated RNA in our experiments. We did not observe retention of specific RNA proportional to the amount of Hg-RNA applied to the SH-agarose column in chromatin transcription experiments in the presence and absence of actinomycin D (Table 1 and 4). The omission of Mn$^{2+}$, the formation of RNA polymerase pre-initiation complexes with RNA elongation in the presence of rifampicin and the use of Hg-CTP instead of Hg-UTP were unsuccessful attempts to specifically eliminate the RNA-dependent side reactions of the E.coli enzyme. There are indications that in this respect the use of eucaryotic RNA polymerase offers no advantage compared with the bacterial enzyme (44). For the quantitation of in vitro synthesized specific mRNA sequences we therefore apply now a method which is independent of the separation of newly synthesized transcripts from preexisting RNA (45).

It is obvious that the RNA-dependent polymerase activity will complicate transcription analysis not only when mercurated
nucleotides are used. The finding that chromatin associated RNA can serve as a template for transcription by exogenous RNA polymerase sheds some doubt on the interpretation of experiments in which the number of initiation sites on chromatin and of experiments in which the degree of asymmetric transcription have been measured. It also suggests the use of double-stranded plasmid DNA containing eucaryotic gene DNA fragments for _in vitro_ transcription studies might be limited (46).

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* to whom reprint requests should be addressed.

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