Transcription in vitro of immunoglobulin kappa light chain genes in isolated mouse myeloma nuclei and chromatin

(RNA nucleotidyltransferase/mercurated nucleotides/affinity chromatography)

M. MITCHELL SMITH AND RU CHIH C. HUANG
Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Communicated by James Bonner, December 18, 1975

ABSTRACT Messenger RNA sequences for immunoglobulin kappa light chain were synthesized in vitro in isolated mouse myeloma nuclei using endogenous RNA polymerase (RNA nucleotidyltransferase; nucleoside triphosphate:RNA nucleotidyltransferase; EC 2.7.7.6) and from isolated myeloma chromatin using exogenous Escherichia coli RNA polymerase. The in vitro RNA was transcribed using 5-mercuriuridine triphosphate and separated from in vivo RNA by chromatography on an agarose sulfhydril affinity column. Template restriction is retained in vitro since synthesis of kappa chain messenger RNA, as determined by hybridization with complementary DNA, was much more efficient in nuclei and chromatin isolated from myeloma 66.2 tissue culture cells, a kappa-chain-producing cell line, than from MOPC 315 tissue culture cells, a lambda-chain-producing cell line. Transcription of kappa chain messenger RNA was 25 times more efficient in myeloma 66.2 nuclei than in myeloma 66.2 chromatin.

In three recent publications (1–3) we have reported that isolated nuclei and gently prepared chromatin from myeloma 66.2 tissue culture cells, a kappa chain producer (4), actively synthesize RNA in vitro for an extended period of time. Among many different species of RNA synthesized, we have demonstrated synthesis, correct chain initiation and termination of 5S RNA and precursor tRNA, and correct DNA strand selection for the 5S genes.

In this communication, we describe studies related to immunoglobulin kappa chain mRNA (mRNAK) sequence synthesis by nuclei and chromatin isolated from myeloma tissue culture cells. The in vitro synthesis was carried out using 5-mercuriuridine triphosphate as one of the substrates (5). By means of sulfhydril-agarose affinity chromatography, de novo synthesized product was separated from cellular RNA.

MATERIALS AND METHODS

Isolation of mRNAK. Messenger RNA for mouse kappa chain immunoglobulin was purified from MOPC 41 tumor by a modification of methods reported previously (6). About 2000 A260 units of sodium dodecyl sulfate-extracted microsomes were centrifuged on a 5–20% exponential sucrose gradient containing 0.5% sodium dodecyl sulfate, 0.1 M NaCl, 2 mM EDTA, and 10 mM Tris-HCl (pH 7.4) in a Beckman Ti-14 zonal centrifuge rotor at 40,000 rpm for 16 hr (7). The fractions that synthesized kappa chain in a rabbit reticulocyte cell-free system (8) were pooled and adsorbed to oligo(dT)-cellulose as described (6). The oligo(dT)-cellulose bound RNA was centrifuged on 0–20% linear sucrose gradients in 99% dimethyl sulfoxide, 10 mM Tris-HCl (pH 7.1), 1 mM EDTA in a Beckman SW 27 rotor at 25°, for 88 hr at 25,000 rpm (9). After precipitation of the RNA, the gradient fractions were tested for kappa chain synthesis in a rabbit reticulocyte cell-free system, and the fractions with the highest specific activity were pooled. The mRNA, isolated migrates as a single electrophoretic band of about 4 × 10^6 daltons on 5% acrylamide, 9% formamide gels (10).

Preparation of cDNAK. Complementary DNA (cDNAK) was synthesized from mRNAK in a 0.1-ml reaction mixture containing 5 μmol of Tris-HCl (pH 8.5), 0.6 μmol of magnesium acetate, 5 μmol of NaCl, 20 nmol each of dATP, dCTP, dGTP, 5 nmol of [α-32P]dGTP at about 113 Ci/mmol, 1 μmol of dithiothreitol, 5 μg of actinomycin D, 0.2 μg of oligo(dT)12-18, 5 μg of mRNAK, and about 100 units of avian myeloblastosis virus reverse transcriptase (EC 2.7.7.7). The cDNAK was centrifuged on 5–20% sucrose gradients containing 0.1 M NaOH, 0.9 M NaCl, and 2 mM EDTA in a Beckman SW 41 rotor at 40,000 rpm for 14 hr. Fractions larger than 7 S were pooled. When resedimented on analytical alkaline sucrose gradients, this cDNAK showed a sedimentation of 8.2 S, corresponding to approximately 3 × 10^6 daltons. This larger molecular weight cDNAK was hybridized with excess purified mRNAK to a Cm = 2 × 10^-4, diluted with 10 volumes of 0.14 M sodium phosphate (pH 6.8), and applied to a column of Bio-Rad HTP hydroxyapatite at 60°. (Cm is the product of RNA concentrations in mol of nucleotides/liter and time in sec.) Hybridized cDNAK was eluted with 0.3 M phosphate buffer, dialyzed, and precipitated with ethanol, with yeast RNA as carrier. The mRNAK was alkali-hydrolyzed and the cDNAK again precipitated with ethanol and yeast carrier RNA. This purified [32P]cDNAK was used as probe for examining the presence of mRNAK sequences in RNA populations in vivo and in vitro.

Mercury-Substituted RNA (Hg-RNA) Synthesis and Isolation. 5-Mercuricuridine triphosphate (Hg-UTP) was prepared by Dr. E. Dobrzynski according to Dale et al. (5).

Nuclei were prepared from myeloma 66.2 and MOPC 315 tissue culture cells as described (1) except that 2 mM 2-mercaptoethanol replaced dithiothreitol in the 0.3 M and 2 M sucrose solutions. The conditions for RNA synthesis were as reported previously (1) except that a final concentration of 12 mM 2-mercaptoethanol replaced dithiothreitol and Hg-UTP replaced uridine triphosphate. Synthesis was for 60 min at 25°.

Chromatin was prepared according to Shaw and Huang (11) from 66.2 and MOPC 315 tissue culture cell nuclei. The chromatin synthesis reaction was as described (12) and contained 0.2 M KCl with Hg-UTP replacing UTP. Isolation of the RNAs after synthesis has been described (1).
RESULTS

Synthesis of Hg-RNA In Vitro. Fig. 1 illustrates the effect of Hg-UTP on the kinetics of nuclear transcription. The rate of synthesis is reduced by 30–35% in the presence of the mercury-substituted nucleoside triphosphate; nevertheless, synthesis parallels the unsubstituted reaction for at least 60 min. Hg-UTP had little effect on transcription from isolated chromatin using E. coli RNA polymerase. As would be predicted from the results of Dale et al. (5), synthesis is inhibited by approximately 90% in both systems if dithiothreitol is used instead of 2-mercaptoethanol.

Since the RNA newly transcribed in vitro is mercury substituted, it can be separated from endogenous in vivo RNA by binding it to a sulphydryl affinity column (Bio-Rad Affi-Gel 401). As shown in Fig. 2, all of the endogenous nuclear RNA, as detected by absorbance at 260 nm, and all of the radioactivity incorporated in the absence of Hg-UTP, fails to bind to the column. In contrast, 60–70% of the radioactivity incorporated by nuclei in reactions containing Hg-UTP is bound by the column and can be eluted by buffer containing 0.1 M 2-mercaptoethanol. Ninety percent of the radioactivity incorporated during transcription of chromatin with E. coli RNA polymerase using Hg-UTP is bound by the column. Recovery of RNA from the columns is greater than 98%, and the column may be regenerated if 2-mercaptoethanol is removed by washing the column with 0.1 M sodium acetate buffer.

In some studies, RNA transcripts were isolated after synthesis using both [3H]GTP and [γ-32P]GTP in the presence of Hg-UTP. When the product was examined by binding it to Affi-Gel 401, the bound Hg-RNA contained incorporated 32P in the molar ratio of approximately 700 3H per 32P label. The incorporated 32P radiolabel was sensitive to alkaline phosphatase, while the incorporated 3H radiolabel was not. Although the exact extent of chain initiation cannot be calculated from these data alone, it is clear that some Hg-RNA molecules can be initiated with pppGp.

Specificity of the cDNA. The [32P]cDNA, used as probe in the RNA synthesis studies was taken from the heavy side of alkaline sucrose gradients to give a molecular weight of approximately 3 X 10^6, ranging from about 700 to 1100 nucleotides long. It was further purified by hybridization back to its template mRNA, and elution as hybrid from hydroxyapaptite (10).

The specificity of the cDNA, is shown in Fig. 3. Hybridization with purified mRNA, from MOPC 41 gives homogeneous reaction over approximately a 2 logarithm range of C value and has a C=1/2 = 1.7 X 10^-3. RNA isolated from mouse liver microsomes does not hybridize with cDNA, even at very high C value. Hybridization is also not seen using E. coli or yeast RNA (10).

MOPC 315 tissue culture cells produce lambda light chains, and its mRNA is not expected to hybridize with cDNA specific for mRNA (15). As shown in Fig. 3, RNA from MOPC 315 tissue culture cell microsomes shows little hybridization.

Microsomal RNA from MOPC 41 solid tumor and 66.2 tissue culture cells, both of which produce kappa chains, yield good hybrids with cDNA, giving C=1/2 = 1.6 and C=1/2 = 3.7, respectively. Hybridization with 66.2 microsomal RNA, however, only protects about 65% of the cDNA against S1 nucleas digestion (EC 3.1.4.21). That this due to sequence heterogeneity in the variable region of MOPC 41 and 66.2 mRNA, and not to impurity of the cDNA, is shown in Table 1. Whereas the cDNA, in 66.2 RNA hybrids is only 65% resistant to S1 nuclease, it is 85% retained as hybrid on hydroxyapatite. Furthermore, when reverse transcribed cDNA, of small molecular weight is hybridized, it is more completely protected from S1 nucleas digestion by the 66.2 microsomal RNA, implying that the mismatching is towards the 5'-end or variable region of the mRNA, (16, 17); the cDNA, molecules must begin synthesis from the oligo(dT) primer at the 3'-end of the messenger. Melting profiles of the hybrids formed from either MOPC 41 or 66.2 RNA had identical Tm, except for a small fraction of low melting hybrid with 66.2 RNA (10).

We conclude that the cDNA, shows excellent specificity.
for RNA populations containing kappa chain mRNA.

Synthesis of mRNA In Vitro. Isolated nuclei from kappa-chain-producing or lambda-chain-producing myeloma tissue culture cells retain template restriction with respect to mRNA synthesis. Fig. 4A illustrates the hybridization of Hg-RNA preparations transcribed in vitro. Hg-RNA from 66.2 nuclei efficiently hybridizes cDNA, with a $C_{t1/2}$ = 5.2, and the shape of the reaction curve is very similar to that obtained with in vitro 66.2 microsomal RNA (Fig. 3). Hg-RNA from MOPC 315 nuclei, however, has a much lower concentration of mRNA sequences. The half-reaction can only be estimated as $C_{t1/2}$ > 200. This implies that the mRNA concentration in MOPC 315 nuclei in vitro Hg-RNA can be no more than 2% of that in 66.2 nuclear transcripts.

To ascertain that, in fact, the hybridization seen using 66.2 in vitro nuclear RNA is not due to endogenous RNA, a mRNA, seeding control was done. Synthesis was done with a small number of MOPC 315 nuclei using Hg-UTP and $[3H]GTP$ (8.5 Ci/mmol). These nuclei were then added to 2 × 10$^7$ 66.2 nuclei that had been isolated identically but never incubated for RNA synthesis. The nuclei were extract- ed together and the RNA was fractionated over Affi-Gel 401. The bound RNA, traced by the MOPC 315 Hg- $[3H]RNA$, was then hybridized and plotted on the $C_{t}$ values equivalent to those that would have been obtained if Hg-RNA had actually been synthesized from that number of 66.2 nuclei. As can be seen in Fig. 4A, this preparation shows negligible hybridization to the cDNA$\text{a}$.

Synthesis of mRNA, sequences by E. coli RNA polymerase from isolated chromatin was also examined. The chromatin used in these experiments was extensively washed, swelled in distilled water, and moderately sheared to solubi- lize it, and retains no endogenous polymerase activity. RNA was transcribed using Hg-UTP, and the in vitro Hg-RNA was purified over Affi-Gel 401 as for the nuclear synthesis.

Fig. 4B illustrates that a certain level of template restriction for mRNA synthesis is preserved in isolated chromatin from myeloma cells. The Hg-RNA transcribed from 66.2 chromatin hybridizes cDNA, with $C_{t1/2}$ = 1.2 × 10$^3$. Hybridization with Hg-RNA transcribed from MOPC 315 chromatin has approximately a $C_{t1/2}$ = 1.1 × 10$^3$. Furthermore, although mRNA sequences are transcribed from 66.2 chromatin, their concentration is about 25 times lower than in Hg-RNA transcribed from isolated 66.2 nuclei using their endogenous RNA polymerase activity.

A comparison of the kinetic data and the concentrations of mRNA, sequences in the various RNA populations is given in Table 2. In all of the hybridization studies, the amount of mRNA, sequences was in at least 10-fold excess over cDNA$\text{a}$.

**Table 1. Hybridization of MOPC 41 and 66.2 RNA with cDNA$\text{a}$ of different nucleotide lengths**

<table>
<thead>
<tr>
<th>Source of microsomal RNA</th>
<th>S1</th>
<th>Hydroxyapatite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large $M_f$ cDNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOPC 41</td>
<td>94.9</td>
<td>91.9</td>
</tr>
<tr>
<td>66.2</td>
<td>66.4</td>
<td>83.2</td>
</tr>
<tr>
<td>Small $M_f$ cDNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOPC 41</td>
<td>97.5</td>
<td>93.4</td>
</tr>
<tr>
<td>66.2</td>
<td>89.4</td>
<td>82.5</td>
</tr>
</tbody>
</table>

The $[32P]cDNA$, fractions were isolated from alkaline sucrose gradients as described in Materials and Methods. The large molecular weight ($M_f$) cDNA represents molecules 700 to 1100 nucleotides long. The small molecular weight ($M_s$) cDNA represents molecules 150 to 500 nucleotides long. Reactions were hybridized to a $C_{t1/2}$ = 10$^3$. Hydroxyapatite hybrids represent those molecules eluting above 0.14 M sodium phosphate at 60°.

**DISCUSSION**

Since our initial observation (8, 18), workers in many laborato- ries have isolated mRNA activities for a variety of light chain immunoglobulins (19, 16, 20–24). The estimation of the purity of this mRNA is difficult to determine precisely (17). The kinetic rate of $C_{t1/2}$ = 1.7 × 10$^{-3}$ for the purified cDNA, is extremely close to that which would be predicted (25) from hemoglobin mRNA hybrid standards (6). Never- theless, the kinetics of RNA-DNA hybridization is subject to a number of parameters (25) and may not be a reliable indicator of purity here.
In fact, as initially synthesized and isolated, the cDNA is only hybridized to 60% by its template mRNA_b at values below C_r = 5 × 10^{-1}. When this hybrid is purified by hydroxyapatite chromatography, the resulting cDNA is sharply hybridized to 98% with mRNA_b. It is probable, then, that the mRNA as isolated is approximately 60% pure and is contaminated with sequences of similar molecular weight containing poly(A) but having, as a group, a very high complexity. However, the cDNA isolated by hybridization with template mRNA_b to low C_r and purification over hydroxyapatite is a highly purified sequence, specific for κ-chain mRNA.

Recently, several reports have been published on the synthesis of simian virus 40 RNA in vitro (26, 27) and of globin mRNA from chromatin assayed by hybridization with globin cDNA probes (28-31). A major problem in studies of this kind is to insure that the hybridization seen is due to in vitro synthesized RNA and not endogenous cellular RNA sequences isolated along with the nuclei or chromatin.

Dale, Livingston, and Ward (5) first demonstrated that 5-mercuriuridine triphosphate could be utilized by several enzymes, including E. coli RNA polymerase. Since mercury-substituted RNA, like other organomercurials, exhibits a high affinity for mercaptans, it was clear that the use of a sulphydryl-containing agarose affinity column (32) might allow separation of newly synthesized Hg-RNA from in vitro sequences. As shown in the present study and by Dale et al. (33, 34), elimination of endogenous nuclear RNA contamination is possible.

Interpretation of the experiments reported here is limited since we do not know what fraction of the isolated Hg-RNA represents sequences initiated in vitro and what fraction is

Table 2. Kinetics of hybridization and mRNA_b content of various RNA populations

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>C_r1/2</th>
<th>Fractional mRNA_b content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo RNA:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOPC 41 mRNA_b</td>
<td>0.0017</td>
<td>1.0</td>
</tr>
<tr>
<td>MOPC 41 microsomes</td>
<td>1.6</td>
<td>110 × 10^{-5}</td>
</tr>
<tr>
<td>66.2 microsomes</td>
<td>3.7</td>
<td>46 × 10^{-5}</td>
</tr>
<tr>
<td>66.2 nuclei</td>
<td>2.6</td>
<td>65 × 10^{-5}</td>
</tr>
<tr>
<td>MOPC 315 microsomes</td>
<td>4000</td>
<td>0.04 × 10^{-5}</td>
</tr>
<tr>
<td><strong>In vitro Hg-RNA:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66.2 nuclei</td>
<td>5.2</td>
<td>32 × 10^{-5}</td>
</tr>
<tr>
<td>66.2 chromatin</td>
<td>120</td>
<td>1.4 × 10^{-5}</td>
</tr>
<tr>
<td>MOPC 315 nuclei</td>
<td>200</td>
<td>0.85 × 10^{-5}</td>
</tr>
<tr>
<td>MOPC 315 chromatin</td>
<td>1100</td>
<td>0.15 × 10^{-5}</td>
</tr>
</tbody>
</table>

In vitro nuclear Hg-RNA was synthesized using retained endogenous nuclear RNA polymerase activity. In vitro chromatin Hg-RNA was synthesized using added exogenous E. coli RNA polymerase.
completion of in vitro initiated nascent chains. The kinetics of synthesis is consistent with considerable re-initiation in vitro, and it is clear from the incorporation of \([\gamma-32P]GTP\) that some of the Hg-RNA sequences can be initiated with pppGp in vitro. However, there is no direct evidence regarding initiation of the specific mRNA, sequence hybridized.

Template restriction for mRNA synthesis is retained in the in vitro systems. Both nuclei, using endogenous RNA polymerase, and chromatin, using E. coli RNA polymerase, synthesize 10 to 40 times more mRNA, sequences when they are isolated from the kappa-chain-producing cell line than when isolated from the lambda chain producer.

Isolated nuclei are capable of highly preferential synthesis of mRNA, in vitro. Assuming two gene copies per haploid genome (6, 35–37, 16), the fraction of the DNA that is kappa chain gene is about 1.04 \times 10^{-6}. The fraction of the in vitro RNA synthesized by 66.2 nuclei that is mRNA, is 3.2 \times 10^{-4} (Table 2), which thus represents approximately a 300-fold enrichment of mRNA sequences over the haploid genome. Highly preferential synthesis of specific sequences in vitro has also been reported by Jacquet et al. (38) for avian myeloblastosis virus RNA, and by Rymo et al. (39) for Rous sarcoma virus RNA.

The concentration of mRNA, in RNA synthesized by 66.2 chromatin is about 25 times less than in 66.2 nuclear transcripts. This decrease in mRNA synthesis relative to the total transcript in chromatin may be due to several factors. It could be the result of differences in chain completion, as discussed above. Alternatively, isolated chromatin may transcribe 25 times more of the genome than nuclei. Finally, the lower relative mRNA synthesis by chromatin may represent differences in the operation of the myeloma polymerases and E. coli polymerase due to either intrinsic differences in affinities for promoter regions or ability to recognize regulatory mechanisms, or it may be due to the loss of labile control functions during isolation of the chromatin.

The excellent technical assistance of Mrs. Sue Auyung, Mrs. Shilling Huang, and Ms. Roslyn Miller is gratefully acknowledged. We also wish to thank Drs. Yong Shin and E. Dobrzensky for consultation on the mercuration reactions. This work was supported by NIH Grants 2-R01-CA19553 and 5-R01-AO00472.

7. Lingrel, J. B. (1972) in Methods in Molecular Biology, eds.