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Transcription of *Xenopus* Chromatin by Homologous Ribonucleic Acid Polymerase: Aberrant Synthesis of Ribosomal and 5S Ribonucleic Acid†

Tasuku Honjo‡ and Ronald H. Reeder*

ABSTRACT: Both homologous DNA-dependent polymerases I and II can transcribe the genes for ribosomal RNA and for 5S RNA when given frog (*Xenopus laevis*) chromatin as a template. Both polymerases, however, transcribe these genes aberrantly. They transcribe the wrong strand and the spacer sequences which are not read *in vivo*. We conclude that still other factors are necessary for accurate *in vitro* transcription to occur.

Complete understanding of the control mechanisms for gene transcription in eukaryotes will require reproduction of those controls *in vitro*. With this goal in mind we have isolated the two major DNA-dependent RNA polymerases from the frog, *Xenopus laevis*, and have studied their ability to transcribe the genes for ribosomal RNA and for 5S RNA using homologous chromatin as a template.

Materials and Methods

Isolation of RNA Polymerase. RNA polymerases I and II were purified from *X. laevis* tissue culture cells and separated from each other as described by Roeder *et al.* (1970) with a slight modification. Polymerases I and II were eluted stepwise from DEAE-Sephadex by 0.19 and 0.28 M ammonium sulfate, respectively. The two polymerase fractions were purified to be free of each other by their α-amanitin sensitivity. Polymerase I thus purified was totally insensitive to α-amanitin, while polymerase II was inhibited completely by the toxin, suggesting very little contamination by polymerase I or III.

Preparation of Chromatin. Chromatin was prepared from *X. laevis* liver as described previously by Reeder (1973) and used within 6 hr after its preparation.

Preparation of DNA and RNA. Purified ribosomal DNA and unlabeled 5S RNA were gifts of Dr. D. D. Brown (Dawid *et al.*, 1970). Purified 5S DNA, strand-separated 5S DNA, and ^{3}H-labeled 5S RNA complimentary to either H or L strand of 5S DNA were gifts of Dr. K. Sugimoto (Brown and Sugimoto, 1973). The strands of rDNA[1] were separated by centrifugation in CsCl gradients as previously described (Dawid *et al.*, 1970). DNA from each fraction of such a strand separation gradient was caught on a separate nitrocellulose filter. A stack of filters from a single gradient was hybridized with various RNA preparations to assess the strand selectivity of transcription under different conditions. Labeled and unlabeled 18S and 28S rRNAs were prepared according to Brown and Weber (1968).

† From the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210. *Received October 5, 1973.*

‡ Present address: Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. 20014.

[1] Abbreviations used are: rDNA, DNA containing the genes for 18S and 28S ribosomal RNA plus spacer sequences; 5S DNA, DNA containing the genes for 5S RNA plus spacer sequences; rRNA, 18S and 28S ribosomal RNA.

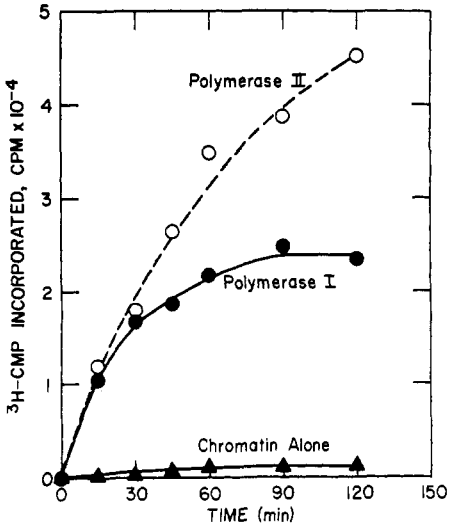

FIGURE 1: Time course of the reaction. RNA polymerase activity was assayed as described in methods except that 4.7 μg of chromatin DNA, 2.5 units of polymerase I, and 2.7 units of polymerase II were employed.

Assay of RNA Polymerase Activity. RNA polymerase activity was assayed in a reaction mixture (40 μl) containing 2.8 μg of DNA, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 50 mM Tris-Cl (pH 8), 50 mM $(NH_4)_2SO_4$, 0.63 mM each of ATP, UTP, and GTP, 0.05 mM [^{3}H]CTP (specific activity 880 cpm/pmol), and enzyme. The mixture was incubated at 30° and acid-insoluble radioactivity was determined according to the procedure of Bollum (1968). When chromatin was employed as template the reaction was terminated by the addition of 4 μg of DNase 1 and 10 μg of yeast tRNA. One unit of the enzyme was defined as that amount of enzyme required to incorporate 1 pmol of CMP in 1 min under the above conditions.

Synthesis and Purification of in Vitro RNA. The reaction mixture for *in vitro* transcription of chromatin was as described above except that its total volume was scaled up to 800 μl and the specified amount of chromatin and [α-^{32}P]CTP were employed instead of DNA and [^{3}H]CTP. Incubation was performed at 30° for 60 min. Purification of RNA was as described previously (Reeder and Roeder, 1972).

RNA–DNA Hybridization. DNA was immobilized on filters and hybridized with radioactive RNA in 50% formamide, 0.6 M NaCl, 0.2 M Tris-Cl (pH 8), and 4 mM EDTA at 40° for 18 hr. The radioactive RNA was denatured by heating at 100° in distilled water for 5 min immediately before starting the reaction. Backgrounds were estimated by including a blank (no DNA) filter in each reaction. Blank values ranged from 25 to 40 cpm and have been subtracted in the experiments reported here. Other details have been described (Brown and Weber, 1968).

Results

General Properties of Chromatin Transcription by Amphibian RNA Polymerases. Both polymerases I and II can transcribe chromatin actively as shown in Figure 1. The initial rate of reaction of polymerase I was almost as fast as that of polymerase II. Polymerase II continued to synthesize RNA at least 120 min whereas polymerase I reached a plateau after about 80 min. The endogenous polymerase associated with chromatin reached a plateau by 20 min (not shown) in agreement with earlier reports (Reeder and Roeder, 1972).

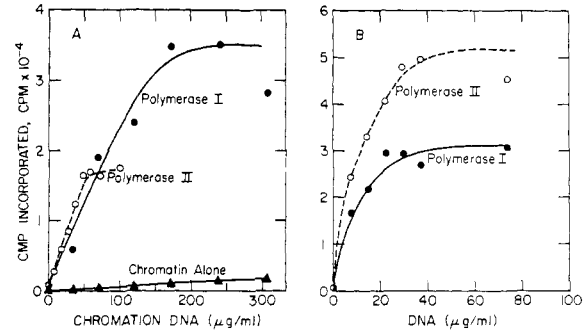

FIGURE 2: Effect of varying concentrations of template. RNA polymerase activity was assayed as described in Methods and incubation time was 30 min. In A where chromatin was employed as template, 3.0 and 2.7 units of polymerases I and II were used, respectively. In B where DNA was employed as template, 2.3 and 3.8 units of polymerases I and II were employed, respectively.

With polymerase I the reaction was saturated by 170 μg/ml of chromatin (Figure 2) while only 20 μg/ml of deproteinized DNA was required for the saturation. The plateau level of velocity per unit enzyme with chromatin as template was approximately 90% of that with DNA as template. Polymerase II was saturated by 50 μg/ml of chromatin or 25 μg/ml of deproteinized DNA under the same conditions. The plateau level of velocity per unit of enzyme with chromatin as template was about 50% of that with DNA as template. The lower amount of chromatin required for the saturation of polymerase II as compared to polymerase I may suggest that more binding sites on chromatin are available for polymerase II than for polymerase I.

The RNA transcribed from chromatin was relatively low molecular weight with sedimentation coefficients ranging from 4 S to 6 S. This suggests the presence of some RNase in our reactions.

Fidelity of 5S Gene Transcription. The RNAs synthesized by polymerases I and II were hybridized to purified, strand-separated 5S DNA in the presence and absence of an excess amount of cold 5S RNA. The results are summarized in Table I. Chromatin alone synthesized only a negligible amount of 5S RNA while polymerase I transcribed the 5S RNA genes in chromatin in significant quantity. However, 5S gene transcript made by polymerase I hybridized to both L and H strands. Since 5S RNA synthesized *in vivo* is complementary to the L strand, it is clear that the exogenous polymerase I initiated transcription of the wrong (H) strand *in vitro*. It has been shown by Brown and Sugimoto (1973) that when purified 5S DNA is hybridized to total pulse-labeled nuclear RNA all of the hybridization occurs to the L strand and is completely competed by unlabeled 5S RNA. From this we conclude that 5S DNA isolated by this procedure is clean enough that bulk DNA transcripts will not hybridize to it and therefore the noncompetable hybridization shown in Table I is due to transcription of spacer regions of the 5S DNA.

Essentially similar observations were made with respect to polymerase II. Polymerase II seems not to be responsible for 5S RNA synthesis *in vivo* (Price and Penman, 1972), but it transcribed 5S DNA in chromatin *in vitro*. The control experiment with native 5S RNA shows that the 5S DNA used in this experiment was completely strand separated.

The above results indicate that polymerases I and II transcribe 5S genes in chromatin but their transcripts contain RNA sequences which are not made in living cells.

Fidelity of Ribosomal Gene Transcription. ^{32}P-labeled RNA transcribed by polymerase I from frog liver chromatin was

TABLE I: 5S RNA Synthesis by Chromatin and RNA Polymerases I and II.[a]

Exptl System	Total RNA Synthesized (cpm)	RNA Hybridized to 5S RNA (cpm)			
		L Strand		H Strand	
		−5S RNA	+5S RNA	−5S RNA	+5S RNA
A chromatin alone	136,000	8	0	0	7
chromatin + polymerase I	1,504,000	233	161	462	507
B chromatin alone	107,600	16	3	5	2
chromatin + polymerase II	1,668,000	475	441	464	384
C *in vivo* 5S RNA	152,000	2031	1	14	0

[a] RNA was synthesized as described in methods except that 40 μCi of [^{32}P]CTP (specific activity 20 Ci/mmol), chromatin (94 μg of DNA for expt A and 62 μg of DNA for expt B) and enzymes as indicated (54 units of polymerase I and 40 units of polymerase II) were employed. In expt C ^{3}H-labeled 5S RNA isolated from *X. laevis* tissue culture cells were used. Labeled RNA was divided into two portions and hybridized to 0.1 μg each of strand-separated 5S DNA either in the presence or in the absence of 30 μg of cold 5S RNA. The efficiency of hybridization was monitored by the addition of [^{3}H]RNA transcribed from either H or L strand of 5S DNA by *E. coli* RNA polymerase. The efficiency ranged from 14 to 19% for L strand and from 15 to 18% for H strand.

purified and hybridized to strand-separated rDNA as shown in Figure 3. ^{3}H-labeled rRNA was added to the hybridization mixture to show the position of the H strand. It is clear that

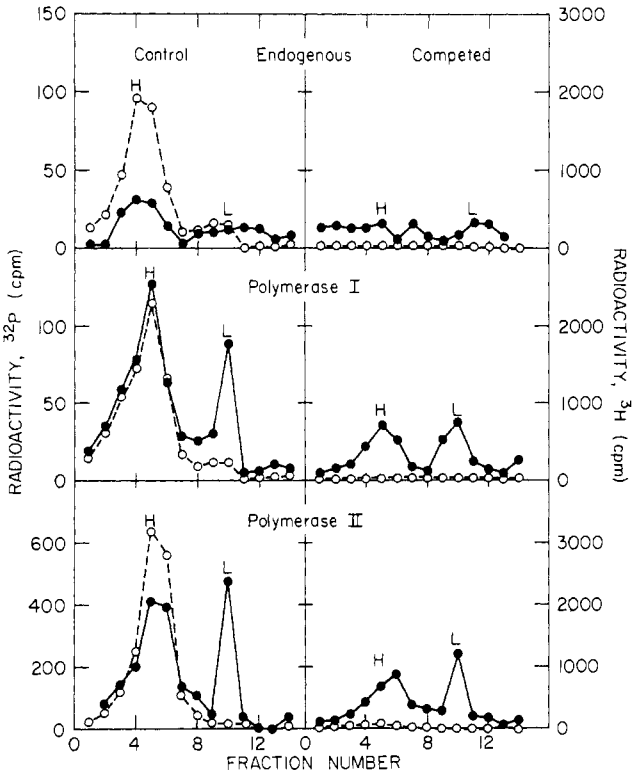

FIGURE 3: Hybridization of *in vitro* RNA to strand-separated rDNA. In three separate reaction mixtures (800 μl) equal amounts of chromatin (68.8 μg) were transcribed by either endogenous polymerase, polymerase I (43 units), or polymerase II (40 units) with 39 μCi of [^{32}P]CTP (specific activity 23.4 Ci/mmol). After 60-min incubation total [^{32}P]RNA was purified from each reaction mixture, divided into two portions, and hybridized to strand-separated rDNA (1.0 μg/filter set) either in the presence or in the absence of 100 μg of unlabeled rRNA. Total [^{32}P]RNA synthesized by endogenous polymerase and polymerases I and II were 113,000, 1,156,800, and 2,226,100 cpm, respectively. As an internal standard 73,000 cpm (8 ng) of *in vivo* synthesized [^{3}H]rRNA was added to each hybridization reaction: (●) *in vitro* [^{32}P]RNA; (○) *in vivo* [^{3}H]rRNA. H and L represent the position of H and L strands, respectively.

both H and L strands were transcribed by polymerase I. The fact that an excess amount of cold rRNA could reduce only partially the RNA hybridized to H and L strands indicates that both gene and spacer sequences on both the H and L strands were transcribed. RNA transcribed by the endogenous polymerase alone contained much less rRNA and all of it derived from theg ene sequence of H strand in agreement with previous results in isolated nuclei (Reeder and Roeder, 1972).

Discussion

Fidelity of Transcription by Eukaryote Polymerase. Several investigators (Butterworth *et al.*, 1971; Keshgegian and Furth, 1972; Maryanka and Gould, 1973) have suggested that eukaryote polymerase transcribes chromatin more accurately than does bacterial polymerase. Their evidence is summarized as follows. (1) Eukaryote polymerase transcribes chromatin more efficiently than bacterial polymerase (Butterworth *et al.*, 1971). (2) Kinetic analysis indicates that eukaryote and bacterial polymerases bind to different sites (Butterworth *et al.*, 1971) and that the former binds to more specific sites than the latter (Keshgegian and Furth, 1972). (3) RNA transcribed by eukaryote polymerase has a larger molecular weight (18 S–45 S) which is close to that of nuclear heterogenous RNA while the product of bacterial polymerase has a smaller molecular weight (below 19 S) (Maryanka and Gould, 1973). All of these experiments only demonstrate that eukaryote and bacterial polymerases are different. They offer no evidence that the eukaryote polymerase was more accurate in selecting sequences to transcribe.

The experiments reported here show that both polymerases I and II transcribe the genes for 5 S and rRNA in an aberrant fashion even when the chromatin proteins are present. Recent experiments (D. D. Brown, unpublished) indicate that most of the 5S genes in *Xenopus* code for oocyte 5S RNA which is not transcribed in somatic cells (Brownlee *et al.*, 1972; Wegnez *et al.*, 1972). Thus it is likely that the 5S gene transcription which we observe *in vitro* is also occurring on genes which are turned off in liver cells. We suspect that these polymerases also read other genes, aberrantly and, in fact, are probably no more accurate in transcribing chromatin than is *Escherichia coli* RNA polymerase (Reeder, 1973). Factor(s) other than the chromatin proteins are apparently

necessary before eukaryote transcription can fruitfully be studied *in vitro*.

Differences between Polymerases I and II. Butterworth *et al.* (1971) have reported that polymerase I from rat liver is unable to transcribe rat liver chromatin under a variety of conditions. We have never observed this phenomenon in our system. There is no doubt that in our system the RNAs transcribed from chromatin are synthesized *de novo* since they contain parts of the sequences which are not transcribed *in vivo*. At all concentrations of ionic strength and divalent cation that we have tested, both polymerases have roughly the same activity on chromatin. The salt conditions used in the present experiments are a compromise designed to produce reasonable enzyme activity while minimizing protein displacement from the chromatin. Except for the difference in our source materials we have no explanation for the discrepancy between ours and Butterworth *et al.*'s results.

A number of experiments (Reeder and Roeder, 1972; Price and Penman, 1972; Zylber and Penman, 1971) suggest that polymerase II is not responsible for transcription of 5S RNA and rRNA *in vivo*. Our experiments were unable to reproduce this difference *in vitro*. Polymerase II transcribed 5 S and rRNA genes as actively as did polymerase I.

Restriction of Template Activity in Chromatin. Although the chromatin proteins were unable to enhance the fidelity of transcription in our experiments, the data suggest that the number of sites for RNA synthesis are restricted in chromatin as compared to deproteinized DNA (Figure 2). This observation has been made for bacterial polymerase (Cedar and Felsenfeld, 1973) and also for eukaryote polymerase (Keshgegian and Furth, 1972). However, in the case of rDNA and 5S DNA this restricted synthesis does not represent proper initiation and termination.

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