Transcription of Chromatin by Human RNA Polymerase

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SUMMARY

DNA-dependent RNA polymerase isolated from lymphocytes of patients with chronic lymphocytic leukemia (CLL) was used to study the transcription of human DNA and chromatins. The α-amanitin sensitive CLL RNA polymerase responds to fewer binding sites on chromatin than on DNA, but both templates have about the same number of binding sites for Escherichia coli RNA polymerase. Both RNA polymerases synthesize RNA with approximately the same efficiency when human chromatin is used as template suggesting that the proportion of DNA available for transcription is the same for the polymerases. Molecular hybridization studies showed that human polymerase transcripts of purified DNA contain more repetitive sequences than do transcripts of DNA complexed in chromatin, whereas bacterial polymerase transcripts are composed of relatively equal amounts of repetitive sequences from both templates.

INTRODUCTION: Chromatin preparations from interphase cells have been extensively studied with respect to their abilities to serve as template for RNA synthesis using bacterial RNA polymerases. DNA-RNA hybridization competition experiments show that in vitro transcripts are similar to in vivo RNA (1-4). The value of these experiments is limited since there is no a priori reason why correct initiation sites for bacterial polymerase should be found on mammalian chromatin. Methods for isolating RNA polymerase from higher organisms have been recently developed and reasonable quantities of the enzyme can be prepared for use in studies of gene regulation (5-7).

Butterworth et al (8) and Chambon et al (9) obtained evidence suggesting that mammalian DNA-dependent RNA polymerase binds and initiates RNA synthesis at different sites on both DNA and chromatin, as compared to bacterial RNA

polymerase. This implies that studies of the template properties of chromatin must necessarily employ polymerase from eukaryotic cells. Preferably one should use chromatin and polymerase from the same cell type.

Kinetic studies of DNA reassociation and DNA-RNA hybridization have indicated that part of the genome of higher organisms consists of families of repeated or related DNA base sequences (10-12). Several models have been proposed in order to explain the function of these repeated sequences and their relationship to gene regulation (13-16). In general, these models proposed that repetitive DNA sequences are involved in gene regulation such as the binding of histones or other repressors (receptor gene of the Britten & Davison model (13), acceptor zone of Georgiev's model (14) and globular control site of Crick's model (15), or binding to non-histone chromosomal proteins (address site of Paul's model (16)). If any of these models is correct, it is reasonable to assume that the amount of total repetitive DNA sequences, which includes the highly repetitive satellite DNA classes, available to polymerase will be different in chromatin from that in deproteinized DNA. Thus, studies of the population of repetitive sequences in the transcript of DNA and chromatin generated by these two enzymes may provide some insight into understanding the mechanism of gene regulation.

In this communication, we report our findings of the differences between human RNA polymerase and Escherichia coli RNA polymerase with respect to the template activities and initiation sites of DNA and chromatin. In addition, studies of the population of repetitive sequences in the transcripts of DNA and chromatin synthesized by the two enzymes as well as their relationship to gene regulation will be discussed.

MATERIALS AND METHODS: Isolation of human DNA-dependent RNA polymerase II (B): DNA-dependent RNA polymerase II was isolated and purified from the lymphocytes of patients with chronic lymphocytic leukemia (CLL) by a slight modification of the method of Roeder and Rutter (5). Details of the method will be published elsewhere. Two forms of RNA polymerase were obtained from

DEAE-Sephadex A-25 columns. RNA polymerase II was further purified through phosphocellulose columns as described by Weaver et al (6). Polymerase II purified in this way is free of ribonuclease as judged by the kinetics of UMP incorporation. Escherichia coli RNA polymerase containing sigma factor was isolated according to the method of Burgess (17).

Assay for RNA polymerase activity: Standard incubation mixtures contained: 50mM Tris-HCl pH 7.9, 2mM $MnCl_2$, 2mM mercaptoethanol, 0.1M $(NH_4)_2SO_4$ (unless otherwise noted), 0.25mM ATP, GTP and CTP, 0.05mM ^{3}H-UTP (400μc/μmoles or 1.23×10^8cpm/μmoles), 4 to 40μg/ml of human placental DNA and 100 to 500 units of RNA polymerase. Reactions (0.25ml) were started by addition of enzyme to the reaction mixture and incubated at 37°C for 20 minutes. Acid precipitable material was collected on glass fiber membrane filters and counted in toluene base scintillation fluid. One unit of RNA polymerase is defined as the amount of enzyme required for incorporation of one pmole of UMP into TCA precipitable material in 20 minutes under standard assay conditions. The specific activity of the human RNA polymerase II was 5600 units per mg. protein.

Isolation of Chromatins: Chromatins were isolated from CLL lymphocytes, leukemic lymphosarcoma cells (LS), and lymphocytes from patients with acute lymphocytic leukemic (ALL) according to a modified method of Marushige and Bonner (18) as described before (4).

Isolation of human DNA: Human DNA was prepared from human placenta according to the method of Kirby (19). In Vitro RNA Synthesis: Incubation mixture is the same as described above with the exception that the ^{3}H-UTP specific activity is 8mc/μmole, and larger volumes of the reaction mixture were incubated at 37°C for one hour. At the end of the incubation period the reaction mixture was treated with DNase I (RNase free) (Worthington Biochemicals) at 10μg/ml at room temperature for 30 minutes. SDS was added to a final concentration of 0.5% and the mixture was extracted twice with an equal volume of 0.1xSSC (0.015M NaCl and 0.0015M Na_3Citrate, pH 7.0) saturated

phenol pH 7.0 at 60°C for 2.5 minutes. The RNA solution thus obtained was dialyzed for 4 hours at 4°C against 2 liters of 0.01xSSC containing 1μg/ml of polyvinyl sulfate. The RNA solution was again treated with DNase I, re-extracted with phenol and dialyzed versus 2 liters of 0.01xSSC and concentrated by flash evaporation.

Hybridization: DNA-RNA hybridization was carried out in 30% formamide and 2xSSC at 45°C for 25 hours as described by Sawada et al (4). The Tm of the hybrid formed under these annealing conditions is 70°C in SSC which corresponds to less than 10% mismatching of bases in the hybrid structures. The DNA content on the filter before and after hybridization was determined according to the procedure of Mizuno and Whiteley (20). Under our hybridization conditions only 5% or less of the DNA was lost either during incubation or in subsequent RNase and washing procedures. Details of the hybridization and thermal dissociation methods have been previously published (4).

RESULTS: Chromatin preparations from lymphocytes of CLL and LS patients showed very little endogeneous RNA polymerase activity in a range of 0.02 to 0.25 M $(NH_4)_2SO_4$. The template activities and Km's were computed from the double reciprocal plots of the rate of RNA synthesis as a function of template concentration by the method of weighted least squares.

Since the structure of chromatin and the rate of RNA synthesis are very sensitive to salt concentration, experiments for determination of template activity were carried out at three different $(NH_4)_2SO_4$ concentrations; 0.05M, 0.10M, and 0.15 M. Template activities of CLL and LS chromatin, Table I, were similar no matter what salt concentration and which kind of chromatin were used. These results contradict those reported by Keshgegian and Furth (21), who obtained 100% template activity in a homologous system of calf thymus, polymerase and chromatins. The reason for this difference is not known, but examination of the effect of salt concentration on the activity of the enzyme with DNA and chromatin template shows that at salt

Table 1

Template Activity of CLL and Lymphosarcoma Chromatin*

Enzyme	Chromatin	$(NH_4)_2SO_4$ (M) 0.05	0.10	0.15
CLL	CLL	7.30	6.86	5.30
	LS	9.14	-	6.42
E. coli	CLL	8.39	10.48	9.10
	LS	13.76	10.55	11.07

*Enzyme activity was assayed as described in Materials and Methods. Rate of RNA synthesis at infinite template concentration (Vmax) was calculated from the double reciprocal plots of rate of RNA synthesis as a function of template concentration using the weighted least squares method. Template activity of chromatin was calculated from the equation below:

$$\text{Template activity} = \frac{\text{Vmax of chromatin as template}}{\text{Vmax of DNA as template}} \times 100$$

CLL RNA polymerase used in these experiments was 337 units and E. coli RNA polymerase was 300 units

concentrations higher than 0.2M $(NH_4)_2SO_4$ it may be possible to get 100% template activity.

Table 2 shows the Km for DNA and CLL chromatin at 0.05M, 0.10M and 0.15M $(NH_4)_2SO_4$ concentration. The data for E. coli RNA polymerase support the earlier findings of Marushige and Bonner (18) that the template concentration necessary for half maximal reaction rate is the same for both DNA and chromatin. However, when CLL RNA polymerase was used as template the concentration of CLL chromatin necessary for half maximum reaction rate is 5 to 10 times higher than that of DNA except at high $(NH_4)_2SO_4$ concentration (i.e. 0.15M). As suggested by Bonner et al (22), Km is a function of the availability of sites at which RNA polymerase can bind to DNA. The high Km for chromatin in comparison with DNA when CLL polymerase was used in-

Table 2

Km* for E. coli and CLL RNA Polymerase

Exp.	Enz.	Template	$(NH_4)_2SO_4$ (M) 0.05**	0.10	0.15
A	CLL	DNA	2.96	3.36	-
		CLL Chromatin	29.0	35.5	-
	E. coli	DNA	-	2.56	4.96
		CLL Chromatin	-	1.12	1.76
B	CLL	DNA	2.80	5.76	39.1
		CLL Chromatin	22.0	51.8	29.4
	E. coli	DNA	-	1.12	3.28
		CLL Chromatin	-	1.44	3.20

*Km (template concentration at half maximum velocity) was calculated from double reciprocal plots of the rate of RNA synthesis as a function of template concentration as described in Table 1. Unit of Km is µgDNA/ml.

** In these experiments approximately the same units of each enzyme was used. With E. coli enzyme in 0.05M $(NH_4)_2SO_4$ template is in excess at the lowest amounts of template added and accurate Km values could not be computed.

dicates that CLL polymerase binds at fewer sites on chromatin than on DNA. Thus, it requires more chromatin to achieve the half maximal rate of RNA synthesis. This doesn't mean that the number of initiation sites are equal in the two cases. Presumably most of the polymerase bound by chromatin doesn't transcribe.

In vitro RNA synthesized from human placenta DNA, CLL and ALL chromatins by the human and bacterial polymerases were hybridized to increasing amounts of denatured immobilized DNA on nitrocellulose filters. These experiments enable us to compare the populations of repetitive sequences in the RNA transcripts. The results shown in figure 1, panel A, ^{3}H-RNA was synthesized from DNA while in panel B and C, ^{3}H-RNAs were transcribed from CLL and ALL chromatins. The results show that almost twice as much of the DNA transcripts of CLL RNA polymerase hybridized to DNA in comparison to DNA transcripts by E. coli RNA polymerase. With chromatin transcripts, the

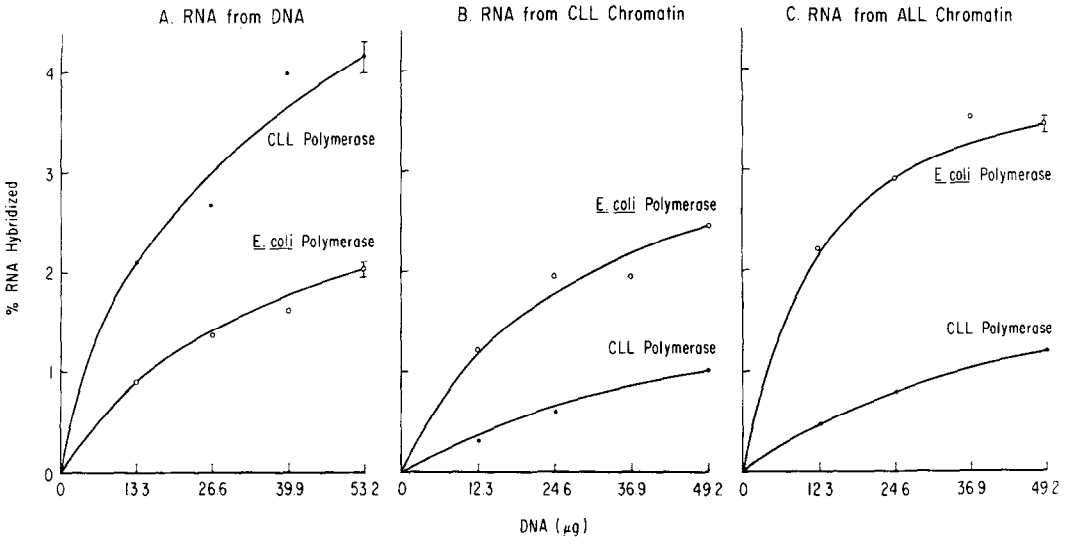

Figure 1: Hybridization of DNA and chromatin transcripts.

Hybridization of *in vitro* RNA to immobilized denatured DNA was carried out as described in Materials and Methods. 3 to 6μg of ^{3}H-RNA were used in these hybridization studies, reaction volumes were 0.3 ml.

Panel A: *In vitro* RNA synthesized from whole human DNA by either CLL or *E. coli* RNA polymerase.

Panel B: *In vitro* RNA synthesized from CLL chromatin.

Panel C: *In vitro* RNA synthesized from ALL chromatin. -●-●-, CLL RNA polymerase; -o-o-, *E. coli* RNA polymerase.

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reverse is true where *E. coli* RNA polymerase transcripts hybridized more to DNA than the CLL RNA polymerase transcripts. CLL polymerase transcribes more repetitive DNA sequences from DNA than those from either CLL or ALL chromatin, and *E. coli* polymerase transcribes relatively constant amounts of repetitive sequences from these templates. This shows that *E. coli* RNA polymerase is apparently more specific in its requirements for initiation than is the homologous polymerase. In the same hybridization conditions 23%

of the RNA transcripts from the isolated , highly repetitive (Cot 0→0.02) DNA sequences (12) by E. coli RNA polymerase can be hybridized to DNA.

The differences in extent of hybridization are not due to RNase contamination of the in vitro RNA preparations since at least 95% of the radioactivity remains acid precipitable following incubation under our hybridization conditions. When relatively short RNA molecules are used in hybridization studies, substantial differences in chain length can markedly affect the results obtained (23). The size of RNA synthesized was studied by electrophoresis in 2.4% polyacrylamide gels and showed that more than 95% of the ^{3}H-RNA was larger than 4S. Thus, the hybridization experiments should not be affected by the RNA size, since most of the RNAs were larger than 4-5S.

The possibility that some double stranded RNA was formed was also investigated by virtue of the known nuclease resistance of double stranded RNAs. In all RNA preparations used less than 3% of the radioactivity was resistant to pancreatic ribonuclease as measured by acid precipitable material. The same amounts were found both before and after hybridization. Since our H^3-RNA contained only one labeled nucleotide (H^3UTP), it was necessary to rule out the possibility that the differences in hybridization properties was due to homopolymer synthesis by the polymerases. This could be easily accomplished by adding a large excess of poly U in the hybridization competition studies. When up to $4x10^4$ fold (200μg) excess of poly U was added to the hybridization reactions no significant decrease in hybrid formation was observed with the transcripts generated by either enzyme on DNA or chromatin. There was also no significant amount of polypyrimidines in the population of non-hybridizable RNA sequences since 80% or more of the RNA was sensitive to ribonuclease T_1 digestion (50 units/ml for one hour at room temperature). Therefore, the difference in hybridization of the transcripts reflects properties of the two enzymes in transcribing DNA and chromatin.

DISCUSSION: Weaver et al have (6) have suggested that DNA-dependent RNA

polymerase of higher organism resembles E. coli RNA polymerase in structure; both enzymes are composed of two (or more in higher organism) large subunits and several small subunits. However, these two RNA polymerases behave somewhat differently in binding to and in transcribing DNA and chromatin This suggests that recognition of transcriptional sites may play an important role in gene regulation.

Our data show that CLL RNA polymerase transcripts of DNA have more repetitive sequences than those of chromatin. Does this reflect the true open DNA sequences which are available in both templates? This question can be answered if we know whether polymerase II transcribes randomly on the exposed portion of the genome. From the proposed structure of chromatin (13-16), it is expected that the sequences available for RNA polymerase in chromatin will be less than in DNA. This agrees with our results that DNA has ten times more binding sites for CLL RNA polymerase than chromatin, whereas DNA and chromatin have approximately the same number of binding sites for E. coli RNA polymerase. This implys that higher organism RNA polymerase may transcribe relatively more randomly than bacterial RNA polymerase. The recent finding of Butterworth et al (8), that rat liver RNA polymerase binds and transcribes more chromatin than Micrococcus luteus polymerase also supports this view. Thus, the transcripts of CLL RNA polymerase from chromatin may more closely reflect the available regions in chromatin. This fits very well with the hypothesis that the highly repetitive DNA sequences are complexed with histone or non-histone proteins and therefore are not available for transcription in vitro.

It has been reported that bacterial core polymerase, without sigma factor, transcribes template much more randomly (24), although with much lower efficiency, than enzyme plus sigma factor. The studies of transcripts of DNA and chromatin by the bacterial core polymerase will furnish new information for understanding this problem. These studies are now in progress.

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