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Limited accessibility of chromatin satellite DNA to RNA polymerase from *Escherichia coli*

(*in vitro* transcription/satellite sequences)

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ABSTRACT **An attempt was made to elucidate some of the factors influencing the fidelity with which isolated chromatin from mouse L-cells is transcribed by RNA polymerase from *Escherichia coli* by analyzing the *in vitro* transcript for the presence of satellite sequences. These sequences are absent from cellular RNA and therefore reflect aberrant transcription. The results indicate that satellite sequences are underrepresented in chromatin transcripts relative to those of DNA. This selectivity is insensitive to many variables in procedures for the isolation and transcription of chromatin. However, lowering the ratio of enzyme to template further reduced the proportion of satellite sequences in the transcript. We conclude that a primary factor influencing the extent of aberrant transcription is the level of enzyme used. Under limiting enzyme conditions, an efficient selection against satellite sequences is observed. However, under conditions of enzyme excess, the enzyme initiates chains at weaker secondary promoters localized in regions of the chromatin containing satellite DNA.**

An understanding of the mechanisms of selective gene expression in eukaryotes depends on our ability to isolate chromatin in a state resembling the *in vivo* state and to manipulate the components by dissociation and reconstitution. With this in mind, numerous studies have been conducted with prokaryotic and eukaryotic polymerases in attempts to characterize the fidelity of *in vitro* transcription. These include filter hybridization studies showing the tissue-specific restriction of transcription (1–5), as well as experiments showing that the presence or absence of a defined sequence in the *in vitro* transcript correlated with the source of the chromatin (6–10). However, in other studies designed to assay for sequences known to be repressed *in vivo*, it has been found that a considerable amount of aberrant transcription occurred *in vitro* with either prokaryotic or eukaryotic enzymes. These studies include those on ribosomal and 5S gene transcription (11, 12) in which both strands as well as spacer were transcribed *in vitro*, globin gene transcription (13) in which both strands were transcribed, and satellite DNA transcription (11) in which significant levels of satellite sequences were detectable in the *in vitro* transcript.

Therefore, despite numerous studies aimed at characterizing *in vitro* transcription, there remains some doubt as to the fidelity of the process. The possibility exists that variations in the conditions of chromatin preparation or transcription would influence the fidelity with which *Escherichia coli* RNA polymerase (RNA nucleotidyltransferase, nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) transcribes. Experiments were conducted in which chromatin from mouse L-cells was transcribed with RNA polymerase from *E. coli*. The fidelity of *in vitro* transcription was determined by monitoring the hybridization of the product with a labeled satellite DNA probe. Because satellite sequences are not transcribed *in vivo*, although they represent 10% of the total genome, these reactions provide a sensitive assay for aberrant transcription. With this approach, an attempt was made to elucidate some of the factors important to the fidelity of *in vitro* transcription.

MATERIALS AND METHODS

Cells. Mouse L-cells (line A9) were grown in suspension at 37° in Joklik's modified essential medium containing 5% calf serum and penicillin and streptomycin (100 units/ml each). For experiments, cells from mid to late logarithmic-phase growth were used.

Enzymes. DNA-dependent RNA polymerase was prepared from *E. coli* K-12 by the procedure of Burgess (14), except that the final glycerol gradient step was omitted.

S1 nuclease was purchased from P-L Biochemicals. DNase I from bovine pancreas (RNase free, Sigma) was resuspended at 1 mg/ml in 10 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ and stored at −20° in 0.5-ml aliquots. Pancreatic RNase (Sigma) was resuspended in H_2O at 2 mg/ml, boiled for 10 min to destroy residual DNAse activity, and stored at −20°.

Chromatin Preparation. Chromatin was prepared either by the method of Bonner *et al.* (15), except that chromatin was not pelleted through sucrose, or by the method described by Biessmann *et al.* (16). One liter of cells usually yielded 1–2 mg of chromatin. Chromatin was used within 24 hr for transcription experiments.

Reconstitution of Chromatin. Chromatin was reconstituted by the method of Kleiman and Huang (17). In a typical preparation, 2 mg of chromatin in about 2 ml of 10 mM Tris·HCl, pH 8, was dissociated by dialysis overnight against 100 volumes of 5 M urea/2 M NaCl/10 mM Tris·HCl, pH 8. Reconstitution was accomplished by stepwise reduction of the NaCl concentration to 0.1 M followed by dialysis into 10 mM Tris·HCl, pH 8.

Preparation of DNA for Transcription. Chromatin in 10 mM Tris·HCl, pH 8, was made 0.5% in sodium dodecyl sulfate and 0.1 M in NaCl. The chromatin was then extracted once with phenol/chloroform, 1:1 (vol/vol), equilibrated in 10 mM Tris·HCl, pH 8/0.1 M NaCl and several times with chloroform/4% isoamyl alcohol, and the DNA was purified by standard methods.

Preparation of 5-Mercuriuridine Triphosphate (Hg-UTP). Hg-UTP was prepared as described (16, 18).

Transcription. This and all subsequent procedures involving RNA were performed with sterile buffers and with glassware that had been heated at least 2 hr at 180° to destroy RNase. Mouse chromatin and DNA were transcribed *in vitro* by using the procedure of Axel *et al.* (7) or the procedure of Astrin (8) modified to use Hg-UTP as described by Biessmann *et al.* (16).

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Abbreviations: Hg-UTP, 5-mercuriuridine triphosphate; $C_{r0}t$, product of total RNA concentration (mol of nucleotide per liter) and time (sec).

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Transcription was terminated by the addition of 0.5% sodium dodecyl sulfate and 10 mM EDTA. RNA was extracted as described below. RNA synthesized under these conditions was approximately 6–9 S (7, 16).

Purification of *In Vitro* Synthesized RNA. RNA was extracted as described by Palmiter (19). In the case of transcripts prepared in the absence of Hg-UTP, the precipitated nucleic acids were resuspended in 10 mM Tris·HCl, pH 7.4/0.1 M NaCl/5 mM $MgCl_2$ and freed of template by treatment with DNase I (40 μg/ml) for 1 hr at 37°. The mixture was re-extracted as before, precipitated with ethanol, and subjected to a second cycle of DNase I treatment. Such treatment was sufficient to degrade greater than 99% of the DNA to acid-soluble material. The mixture was again extracted and ethanol precipitated. The precipitated nucleic acids were then freed of residual triphosphates and monophosphates by filtration over a column of Sephadex G-50 equilibrated in sterile H_2O.

In the case of transcripts prepared in the presence of Hg-UTP, the precipitated nucleic acids were purified by gel filtration on Sephadex G-50 followed by affinity chromatography on sulfhydryl-Sepharose 6B as described by Biessmann *et al.* (16).

L-cell RNA was prepared as described elsewhere (16).

Preparation of Radioactive Probes for Hybridizations. ^{3}H-Labeled mouse satellite DNA, separated into heavy and light strands, was prepared as described and donated by Gerhart Ryffel.

Hybridization. Hybridization kinetics with satellite heavy-strand DNA were carried out in 0.3 M NaCl/10 mM Tris·HCl, pH 7.4, with about 500 cpm of DNA (2×10^5 cpm/μg). Calf thymus or bacterial DNA (20 μg/ml) was added as carrier. Chromatin transcripts were present in at least 1000-fold excess over satellite heavy strand, and DNA transcripts were present in at least 100-fold excess over satellite DNA. Reactions volumes were either 0.5 ml in sealed tubes or 50 μl in sealed capillaries. At the end of the incubation, the samples were cooled on ice, diluted into 4 ml of 0.3 M NaCl/3 mM $ZnCl_2$/30 mM NaOAc, pH 4.5, and challenged with S1 nuclease (1000 units/ml) as described elsewhere (16).

Hybridization to equilibrium was carried out with 1000–2000 cpm of heavy strand and a 10-, 25-, or 100-fold excess of RNA or DNA.

Alkali treatment of transcripts was accomplished by treating them with NaOH, pH 13, for 10 min in a boiling water bath.

RESULTS

Analysis of chromatin transcription under low-salt conditions

Chromatin was prepared for these experiments by the method of Bonner *et al.* (15). In this procedure, possible perturbations of the system were minimized by avoiding the use of divalent cations in the preparation of nuclei and by avoiding exposure of chromatin to ionic strengths higher than that of 10 mM Tris. For solubilization, chromatin was sheared at 3000 pounds per square inch (2.1×10^7 Pa) in a French pressure cell, a treatment that produced fragments of chromatin containing DNA of double-stranded molecular weight up to about 3×10^6 (5000 base pairs).

Chromatin or DNA was transcribed under low-ionic strength conditions as described by Axel *et al.* (7). Saturating levels of enzyme were used (1 mg of enzyme to 1 mg of template). Under these conditions, transcription continued for about 45 min, producing about 30–40 μg of RNA per mg of chromatin template. For each chromatin transcription reaction, a parallel reaction was run in the absence of added RNA polymerase. Because no endogenous RNA polymerase activity was detectable under these conditions, this allowed determination of the amount of endogenous RNA present in the chromatin preparation. With most preparations, 1 mg of chromatin transcribed *in vitro* yielded 60 μg of RNA after isolation, of which 30 μg was present endogenously. Both chromatin and DNA yielded RNA with a broad size distribution, between 5 and 10 S, as we had observed previously (16).

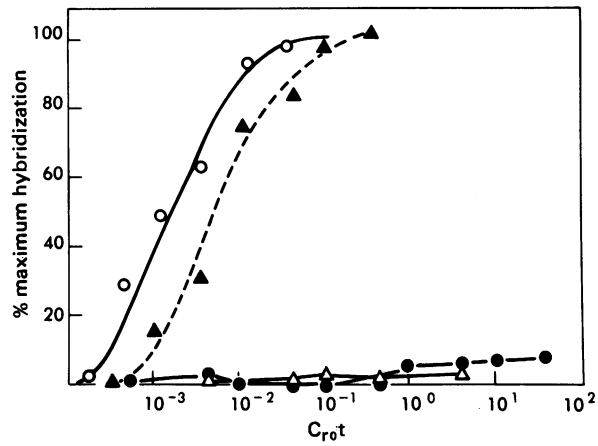

FIG. 1. Kinetics of hybridization of [^{3}H]thymidine-labeled satellite heavy strand (ordinate) with total L-cell DNA (○), DNA transcript (▲), endogenous RNA (●), and total L-cell RNA (△). The data are plotted as percent maximum hybridization, which was 65% of the input counts. $C_{ro}t$, product of total RNA concentration (mol of nucleotide per liter) and time (sec).

Fig. 1 shows the hybridization kinetics of the labeled satellite DNA probe with a 100-fold excess of L-cell DNA or L-cell DNA transcript or a 1000-fold excess of endogeneous chromatin RNA or total L-cell RNA. The heavy-strand probe was driven some 2-fold faster by total DNA than by the transcript of DNA. Allowing for the fact that the rate of RNA·DNA hybrid formation was probably slower than for DNA·DNA reactions (20), this result is consistent with essentially random representation of satellite sequences in the DNA transcript. This suggests that the DNA is transcribed randomly by *E. coli* RNA polymerase, in agreement with the results of Maio and Kurmit (21). The satellite probe did not hybridize to any significant extent with endogenous RNA or total L-cell RNA. From the saturation values obtained, we calculated that satellite sequences comprise less than 1 part in 10^5 of total L-cell RNA. This is consistent with previous studies that failed to detect significant levels of satellite sequences in cellular RNA (22).

Fig. 2 shows the hybridization kinetics of the labeled satellite probe to a 100-fold excess of chromatin transcript. The DNA transcript curve from Fig. 1 is included for comparison. The chromatin transcript hybridized about one-fourth the rate of the DNA transcript, indicating that satellite sequences are preferentially repressed in chromatin. Taking the fraction of satellite sequences in mouse DNA to be 10% (22), it is clear that satellite sequences in the transcript represent only 2.5% of the total.

Chromatin that had been reconstituted was also transcribed. As shown in Fig. 2, the reconstituted chromatin transcript hybridized with kinetics similar to those of the transcript from native chromatin. This indicates that the dissociation and reassociation process does not affect the degree to which satellite sequences are expressed.

In order to control for the possible presence of residual DNA in the transcript preparations, a portion of the transcript was treated with alkali. Fig. 2 shows that this treatment abolished all hybridization, proving that removal of DNA was successful.

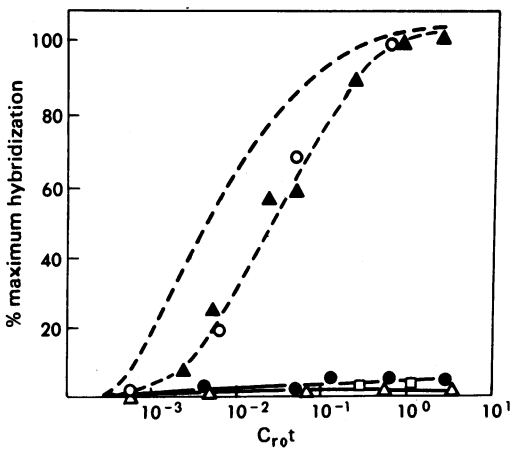

FIG. 2. Kinetics of hybridization of [^{3}H]thymidine-labeled satellite heavy strand (ordinate) with chromatin transcript (○), reconstituted chromatin transcript (▲), alkali-treated DNA transcript (●), alkali-treated chromatin transcript (□), and alkali-treated reconstituted chromatin transcript (△). A reference curve from Fig. 1 for DNA transcript hybridization kinetics is also included (---).

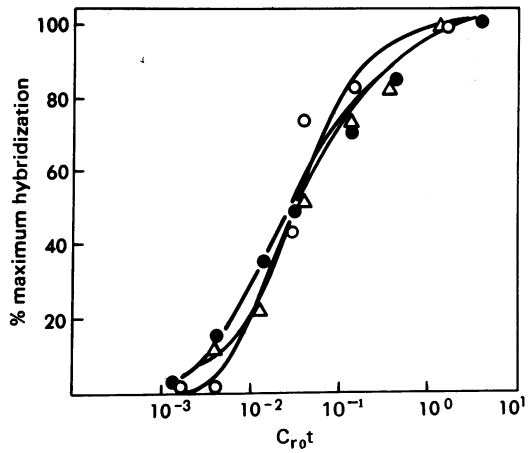

FIG. 3. Kinetics of hybridization of satellite heavy strand with transcript from unsheared chromatin (△), with transcript synthesized in the absence of Mn^{2+} (○), and with transcript synthesized at 25° (●).

Analysis of chromatin under physiologic salt conditions

Chromatin was prepared for these experiments as described (16). This procedure differed from the one used above in that sucrose and divalent cations were present during the preparation of nuclei. For most preparations, nuclei were lysed by shearing in a French pressure cell at 1000 psi (6.9×10^6 Pa), a procedure that produced chromatin fragments containing double-stranded DNA of molecular weight up to about 14×10^6 (25,000 base pairs) (16). When unsheared chromatin was desired, nuclei were lysed by homogenization in hypotonic buffer (10 mM Tris-HCl, pH 8).

Chromatin was transcribed under conditions more closely resembling the physiologic state (8)—i.e., salt concentrations were set at 150 mM. In addition, Hg-UTP, prepared as previously described (16), replaced UTP in the reaction mixture. The use of this precursor enabled the transcript to be purified free of DNA and endogenous RNA (18). Saturating levels of *E. coli* RNA polymerase were used. Under these conditions, transcription continued for 4 hr and produced about 400 μg of RNA from 1 mg of chromatin template.

Fig. 3 depicts the results of investigation of several possible sources of artifact in the *in vitro* transcription system. These include: the shearing process, which has been reported to abolish the characteristic nuclease digestion pattern of chromatin (23); the temperature of transcription, which could effect the activity of regulator proteins or of endogenous nucleases and proteases; and the use of Mn^{2+} in the transcription reaction mixture. This ion has been reported to promote incorrect initiation by yeast polymerase I on ribosomal DNA (24). As shown in Fig. 3, transcripts from unsheared chromatin, from chromatin transcribed at 25° rather than at 37°, or chromatin transcribed in the absence of Mn^{2+} all displayed hybridization kinetics similar to those of the transcript synthesized under normal conditions from sheared chromatin.

In order to investigate the possibility that the levels of aberrant transcription of satellite sequences were due to the high levels of enzyme used during transcription, mouse chromatin was transcribed with lower enzyme-to-template ratios (1:1, 0.5:1, and 0.1:1, wt/wt) and then the transcripts were hybridized to satellite DNA. Hybridization of total mouse DNA to satellite DNA was used as a control. Fig. 4 shows an experiment in which different amounts of transcript were hybridized to equilibrium with a constant amount of satellite DNA. The initial slopes of the curves give an indication of the proportion of satellite sequences in the RNA or DNA (25). Satellite sequences represented a lower proportion of the total transcripts than they did in total DNA, and as the level of enzyme to template was decreased so was the proportion of satellite sequences in the *in vitro* transcript.

DISCUSSION

The mechanism by which chromosomal components combine with DNA to effect transcriptional regulation is basic to our understanding of gene expression in eukaryotes. In the studies described here, we have examined some of the variables in the preparation and transcription of chromatin in an attempt to define the conditions that allow maximum fidelity of *in vitro* transcription. Quantitation of satellite DNA sequences offers several advantages for the characterization of the fidelity of chromatin transcription. In the first place, it is an unambiguous assay for aberrant transcription because heterochromatic regions containing satellite DNA are not transcribed *in vivo* (22). By the same token, the absence of such sequences in cellular RNA ensures that satellite sequences scored in the transcript do represent initiation of chains in satellite DNA regions of

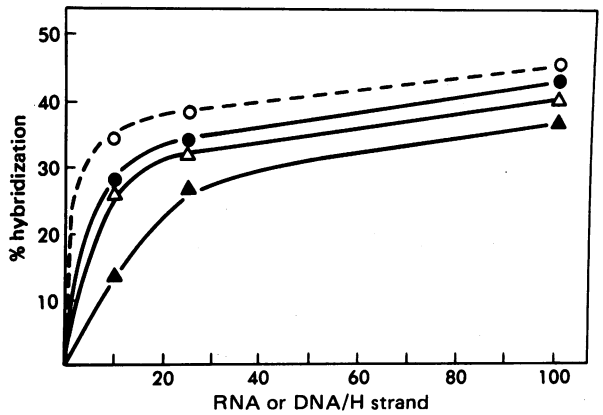

FIG. 4. Equilibrium hybridization of [^{3}H]thymidine-labeled satellite heavy strand H with mouse L-cell DNA or with transcripts synthesized at various ratios of enzyme to template (mg/mg). Hybridization to DNA (○), to transcript synthesized at enzyme-to-template ratio of 1:1 (●), to transcript synthesized at enzyme-to-template ratio of 0.5:1 (△), and to transcript synthesized at enzyme-to-template ratio of 0.1:1 (▲).

chromatin rather than contamination by endogenous RNA or extension of incomplete nascent chains. Nevertheless, although production of transcripts of satellite DNA is certainly a measure of aberrant transcription, we cannot conclude that it typifies all possible types of lack of fidelity.

Consistent with earlier studies (22), satellite sequences were found to be present in insignificant levels in cellular RNA (less than 0.001%), although they comprise 10% of the DNA. When purified DNA was transcribed with RNA polymerase from *E. coli*, satellite sequences accounted for about 10% of the transcript, suggesting random transcription. Chromatin isolated by either of two procedures produced a transcript with a reduced representation of satellite sequences compared to the transcript from DNA. In both cases, satellite sequences comprised only about 2% of the transcript. It was also of interest to know whether chromatin could be dissociated and reconstituted and still retain the properties of native chromatin. Several lines of evidence suggest that reconstitution of chromatin is possible (4–6, 26) but none of these experiments used probes for aberrant transcription. In the case of satellite chromatin, one might imagine that higher-order coiling resulting from long-range interactions would be involved in the formation of this structure and might be difficult to reconstitute. Nevertheless, the results of Fig. 2 demonstrate that this is not the case, because satellite sequences are equally underrepresented in the transcript of native and reconstituted chromatin. In fact, we wish to stress that, although satellite sequences were detectable in all chromatin transcripts examined, these heterochromatic regions were always transcribed with no more than one-fourth the efficiency relative to other regions.

Although satellite sequences are specifically repressed in chromatin, there is some artifact common to both methods of chromatin preparation and transcription that gives rise to a low level of expression. However, both methods call for mechanical shearing, a procedure that may alter the periodic structure of chromatin so as to abolish the characteristic nuclease digestion pattern (23). Transcription from unsheared chromatin was less efficient than that from sheared chromatin (about 150 μg of RNA per mg of template). Nevertheless, the proportion of satellite sequences in the transcript was the same as that from sheared chromatin. We conclude that the use of shearing forces of 1000–3000 psi ($0.69–2.1 \times 10^7$ Pa), while causing DNA breaks that could obscure the typical nuclease digestion patterns, nevertheless leaves intact some critical aspects of the structure that limit accessibility of satellite DNA sequences to RNA polymerase. Satellite sequence expression was also unaffected by lowering the temperature to 25° or by eliminating Mn^{2+} from the transcription reaction. It is unlikely, therefore, that temperature-dependent regulatory elements or Mn^{2+}-sensitive initiation signals play any substantial role in the selectivity of *in vitro* transcription.

When the ratio of enzyme to template was decreased, however, there was a marked decrease in the proportion of satellite sequences in the transcript. Thus, a substantial fraction of the satellite transcription observed here, as well as other types of aberrant transcription reported elsewhere, could be due to the use of high levels of enzyme. Such a situation would enhance the chance of the enzyme initiating in regions of the chromatin of secondary preference,—that is, regions that do not serve as initiating regions *in vivo*.

In conclusion, a critical variable influencing the fidelity of *in vitro* transcription is the ratio of enzyme to template. Other variables such as temperature or the ionic conditions of transcription have little effect. It appears, therefore, that the essential regulatory features of chromatin are a stable manifestation of its *in vivo* structure, a structure that is retained *in vitro* and that is amenable to analysis with heterologous enzyme. Under limiting enzyme conditions, the heterologous enzyme serves well to probe this structure, not necessarily because it recognizes correct initiation signals but rather because the regions of chromatin most accessible to it are regions correlated with transcriptional capacity *in vivo*. In this way, the interpretation would be in accordance with that of experiments in which two other DNA-binding proteins, DNase I (27) and II (28), were shown to attack preferentially those regions of chromatin that are transcribed *in vivo*. This analogy is strengthened by our unpublished observation that satellite DNA sequences in chromatin are resistant to attack by DNase I relative to regions transcribed *in vivo*.

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