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Distribution of RNA Polymerase Binding Sites in Fractionated Chromatin

(transcription/ECTHAM-cellulose /sucrose gradient sedimentation)

ROBERT T. SIMPSON

Developmental Biochemistry Section, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT Calf-thymus chromatin was fractionated by ion-exchange chromatography on ECTHAM-cellulose and sucrose gradient sedimentation. [ECTHAM-cellulose is a cationic adsorbent prepared by coupling tris(hydroxymethyl)aminomethane to cellulose with epichlorohydrin.] The capacity of these fractionated chromatins to support RNA synthesis by DNA-dependent RNA polymerase of *Escherichia coli* was examined, using procedures that permit measurement of binding site frequency. Unfractionated calf thymus chromatin has 5–10% as many binding sites as protein-free DNA. By combination of the two fractionation methods, chromatin samples were obtained containing as few as 2% and as many as 47% of the number of binding sites found on protein-free DNA.

Chromatin fractionated by ion-exchange chromatography on ECTHAM-cellulose has been extensively characterized in terms of its composition and physicochemical properties. The material that elutes early from ECTHAM-cellulose has a full complement of histones, is condensed or supercoiled in structure, and virtually lacks any sequences which melt (denature) below 70° in 0.25 mM ethylenediaminetetraacetate, pH 7.0. In contrast, the material eluted late is depleted in histone content, especially f1, has a different content of nonhistone proteins, is more extended and DNA-like in structure, and is enriched in low-melting sequences (1–5). On the basis primarily of their thermal denaturation properties, early- and late-eluted chromatins have been considered to be repressed and transcribable, respectively, although direct evidence of functional diversity between the disparate physical types of chromatin has not been offered previously.

In this communication, I report the capacity of isolated chromatin fractions to support RNA synthesis by *Escherichia coli* DNA-dependent RNA polymerase under conditions where the rate of RNA synthesis is a measure of the frequency of binding sites for the enzyme on the template. Additionally, functional studies are described for early and late ECTHAM-cellulose chromatin samples further fractionated by the sucrose gradient sedimentation system of Murphy *et al.* (6).

EXPERIMENTAL SECTION

Calf-thymus chromatin was prepared from frozen thymus (Pel-Freez Biologicals) as previously described for rabbit-liver chromatin (1). After sonication for 2 min at 0–2° and 75 W with a Branson model W185 Sonifier equipped with a microtip, the chromatin was chromatographed on ECTHAM-cellulose at a preparative level (2). Where I discuss the characteristics of early and late ECTHAM-cellulose fractions, these correspond to pooled material taken from the first 10% and the last 10% of the eluate, respectively.

Samples for subsequent fractionation by sucrose gradient sedimentation were concentrated 4-fold with a Minicon B15 membrane concentration apparatus (Amicon Corp.) and dialyzed to 0.01 M Tris·HCl, pH 8.0. Linear 5–50% (w/v) gradients of ultrapure sucrose (Schwarz-Mann) containing 0.01 M Tris·HCl, pH 8.0, were prepared with an LKB Instruments Ultragrad. Generally, 50-ml gradients were prepared, overlaid with 5 ml of sample, and centrifuged at 20,000 rpm for 20 hr at 2° in an SW 25.2 rotor in a Beckman L5-65 preparative ultracentrifuge. Gradients were fractionated by pumping from the bottom of the tube. Prior to transcription by RNA polymerase, all samples were dialyzed against a large excess of 1 mM Tris·HCl, pH 7.5, for at least 6 hr at 4°.

Transcription was measured using *E. coli* RNA polymerase (Miles Laboratories) under the conditions utilized for chromatin transcription by Cedar and Felsenfeld (7). After a 15-min low-salt initiation period at 37° with template and enzyme in 10 mM Tris·HCl, pH 7.9, 1 mM $MnCl_2$, 0.8 mM each ATP and GTP, and 0.02 mM [^{3}H]UTP (500 cpm/pmol), the reaction mixture was made 0.4 M in $(NH_4)_2SO_4$ and 5 mM in $MgCl_2$. A zero-time sample was withdrawn and then the propagation reaction begun by adding CTP to 0.063 mM. Generally, assays were made in 1.34 ml with 2.5 μg of DNA or chromatin DNA and 8 units of RNA polymerase. These conditions are such that all available binding sites on the DNA are saturated (i.e., the rate is identical when the enzyme concentration is doubled). During the initiation phase, polymerase binds to DNA or chromatin, and initiates transcription, but propagation is blocked by the absence of CTP. When CTP is added, propagation proceeds but the presence of high concentrations of ammonium sulfate precludes further initiation or reinitiation (7). Hence, the rate of incorporation of labeled UMP reflects the number of binding sites present on either chromatin or DNA (7). Duplicate 100-μl samples were withdrawn at various times to ice-cold tubes containing serum albumin carrier, precipitated with 10% trichloroacetic acid–0.01 M sodium pyrophosphate, plated on Whatman GF/A filters, washed four times with 5% trichloroacetic acid–0.01 M sodium pyrophosphate, dried at 75° for 2 hr, incubated overnight at 37° with a mixture of 0.2 ml of NCS (Amersham/Searle) and water (9:1) and 10 ml of 0.42% rpi scintillator (Research Products International) in toluene, and finally counted in a Beckman LS-250 liquid scintillation counter. Incorporation in the absence of added template was

Abbreviation: ECTHAM-cellulose is a cationic adsorbent prepared by coupling tris(hydroxymethyl)aminomethane to cellulose with epichlorohydrin.

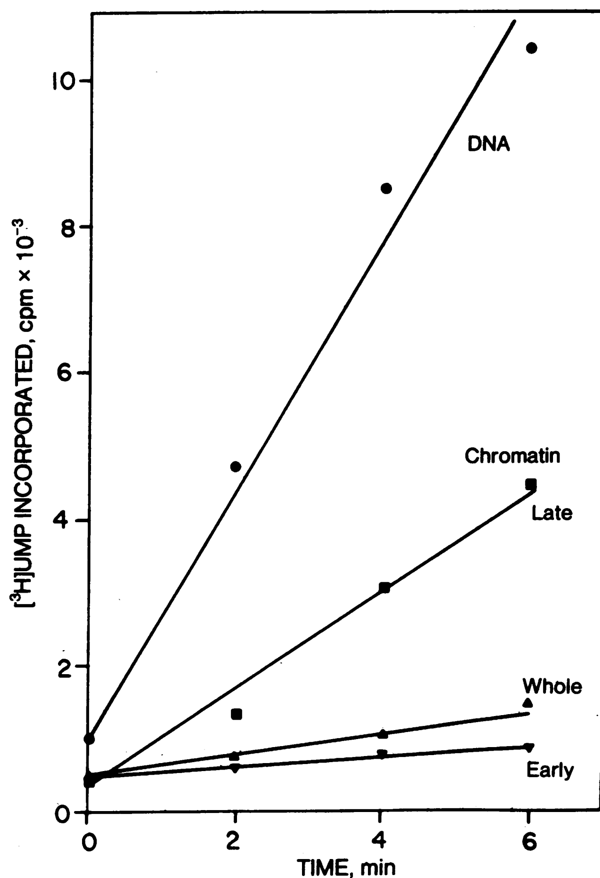

FIG. 1. Transcription of chromatin fractions and DNA by *E. coli* RNA polymerase. Rates of RNA synthesis under the conditions detailed in the *Experimental Section* are shown for DNA (●), whole chromatin (▲), and early (▼), and late (■) ECTHAM-cellulose fractions.

less than 1–2 pmol per assay and has been subtracted from the data. The rate of incorporation with DNA as template, 185 pmol/min per μg of DNA, agrees well with that presented by others, e.g., 200 pmol/min per μg of DNA (7). By a variety of techniques, Cedar and Felsenfeld (7) have determined that free DNA possesses one polymerase binding site per 1000 nucleotide pairs and I have used this figure in calculating binding site frequency for the various chromatin samples.

RESULTS

Incorporation of labeled UMP into RNA is generally linear for 6–8 min under these assay conditions for RNA polymerase. With DNA as template, as noted above, the rate of RNA synthesis (Fig. 1) is near that previously reported by Cedar and Felsenfeld (7). Whole chromatin is transcribed by RNA polymerase at a rate 10- to 20-fold less than that for protein-free DNA (Fig. 1). Fractionation of chromatin into early and late ECTHAM-cellulose fractions reveals that this depressed rate of RNA synthesis is in part due to the presence of a mixture of nucleoproteins in sonicated chromatin. Thus, early chromatin is transcribed even less well than unfractionated chromatin, the rate being 2–4% of that for DNA. In contrast, transcription of late chromatin fractions is markedly enhanced compared to whole chromatin (Fig. 1), with rates 25–41% of that for DNA being observed for several different preparations. Across the elution profile of an ECTHAM-cellulose column,

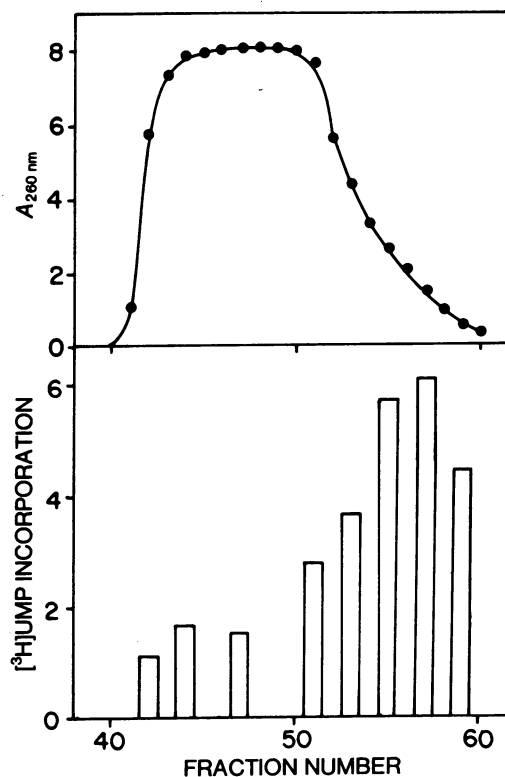

FIG. 2. Distribution of RNA polymerase initiation sites on chromatin fractions from chromatography. The rates of RNA synthesis under the conditions described in the *Experimental Section* were determined for a series of fractions from ECTHAM-cellulose chromatography. The elution pattern from the column is shown in the *upper* portion of the figure, while the histogram in the *lower* section represents synthetic rates expressed as pmol of UMP incorporated per min/μg of DNA. The whole chromatin employed for fractionation had a synthetic rate of 1.8 pmol/min per μg of DNA.

the rates of transcription of the various chromatin fractions vary from low rates initially to high rates at the end of the elution (Fig. 2). This gradual transition is that expected from arguments about the nature of the heterogeneity of sonicated chromatin which have been discussed previously (5).

Further subfractionation of chromatin was performed using sucrose gradient sedimentation of early and late ECTHAM-cellulose fractions. As reported previously (5), early chromatin sediments more rapidly and late chromatin sediments more slowly than whole chromatin, both in preparative sucrose gradients and in analytical self-generating D_2O density gradients. Here, sedimentation in steep sucrose gradients, previously employed by Chalkley and Jensen (8) and Murphy and collaborators (6) for chromatin fractionation, has been utilized. These workers have reported that the more slowly sedimenting fractions represent chromatin enriched in transcribable regions.

The sedimentation patterns for these experiments are shown in Fig. 3 and the template capacities of the various fractions are reported in Table 1. Whole sonicated chromatin sediments as a main band with a faster shoulder. Early ECTHAM-cellulose chromatin is enriched in its content of the fast shoulder, and the slow band is displaced to the centrifugal side of the corresponding peak for whole chromatin. In contrast, late ECTHAM-cellulose chromatin is depleted in its content of the

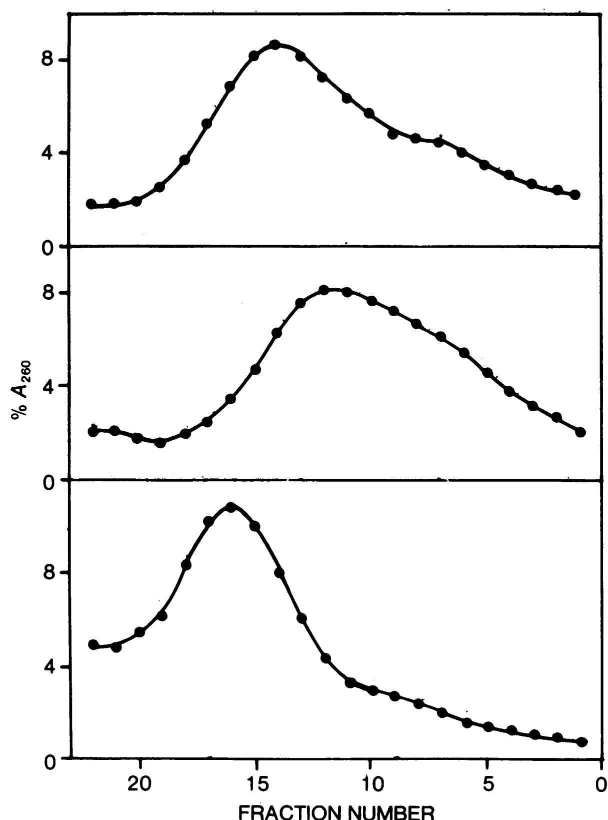

FIG. 3. Sucrose gradient sedimentation of whole chromatin (*top*) and early (*middle*) and late (*bottom*) ECTHAM-cellulose chromatin fractions. Absorbancies are plotted as percent A_{260} recovered. Sedimentation was from *left* to *right*. Fractions analyzed for template activity were: whole, 17 = slow, 7 = fast; early, 13 = slow, 7 = fast; and late, 17 = slow, 9 = fast.

fast shoulder and the main band sediments more slowly than that in bulk chromatin. Samples from the fast shoulder and the centripetal side of the main peak of whole, early, and late chromatin were assayed as templates.

The results are tabulated in Table 1, both as actual incorporation rates (pmol of UMP per min/μg of DNA and % of incorporation relative to DNA) and as frequency of occurrence of polymerase binding sites, based on Cedar and Felsenfeld's assignment of whole calf-thymus DNA as having one site per 1,000 nucleotide pairs (7). Binding site frequencies for slowly and rapidly sedimenting fractions of whole chromatin differ by 3-fold, being 1/8,000 and 1/23,000, respectively. Early ECTHAM-cellulose chromatin, having a site frequency of 1/23,000 base pairs, is further fractionated by the sedimentation method. The slow peak has a slight increase in frequency to 1/18,000, while the fast shoulder is further diminished to a frequency of 1/61,000 nucleotide pairs. A similar fractionation in template capacity is seen for late ECTHAM-cellulose chromatin. Thus, the fast sedimenting late chromatin has a site frequency of 1/23,000 while that for the slow late is 1/2,100. This latter, most active fraction, has a binding site frequency increased by about 15% over late chromatin alone and nearly half the site frequency observed for protein-free DNA.

DISCUSSION

A number of methods purporting to separate repressed from transcribed chromatin have been reported, supported by various criteria of functional differences between the isolated fractions (6, 8–11). Where the functional evidence has involved transcription by *E. coli* RNA polymerase, the measurements have all been made under conditions wherein template insolubility, reinitiation, protein exchange, and measurement of both binding site frequency and propagation rate all contribute to, and complicate the interpretation of, the final results. However, conditions for separate measurement of the initiation and propagation phases of polymerase activity were defined by Hyman and Davidson in 1970 (12), and this methodology was applied to chromatin transcription by Cedar and Felsenfeld (7), further defining conditions where solubility and protein exchange were not problems. It then became possible to demonstrate that the primary *in vitro* manifestation of repression in whole chromatin is a reduction of 10- to 15-fold in the frequency of binding sites, with a less dramatic 2-fold decrease in propagation rate relative to DNA (7). Thus, the probable basis for the *in vivo* restriction of transcription of DNA in chromatin is reduction of the number of sites at which RNA polymerase can initiate RNA synthesis. Using this unambiguous method of assessment of the functional capacity of chromatin, I have examined the transcription of chromatin fractionated first on ECTHAM-cellulose and subsequently by sucrose gradient sedimentation.

TABLE 1. *Transcription of DNA and chromatin fractions by E. coli RNA polymerase*

Sample	Transcription rate: pmol/min per μg DNA	Transcription rate: % of DNA Rate	(Binding site frequency)$^{-1}$, nucleotide pairs
DNA	18.5	100	1,000
Whole chromatin	1.1	6	16,000
Slow sedimenting	2.4	13	8,000
Fast sedimenting	0.8	4	23,000
Early ECTHAM-cellulose fraction	0.8	4	23,000
Slow sedimenting	1.0	5	18,000
Fast sedimenting	0.3	2	61,000
Late ECTHAM-cellulose fraction	7.5	41	2,400
Slow sedimenting	8.6	47	2,100
Fast sedimenting	0.8	4	23,000

In agreement with Cedar and Felsenfeld (7), the frequency of binding sites in chromatin is about 5–10% of that for DNA. Chromatographic fractionation of chromatin leads to a marked disparity in site frequency for early- and late-eluted fractions, to 2–4% of DNA for early- and 25–41% of DNA for late-eluted material. The average of 10-fold enrichment in late- versus early-eluted chromatin suggests that the previously described properties (1–5) of these separated nucleoproteins ought to reflect accurately the structure and composition of transcribable and repressed regions of the genome.

Addition of sucrose gradient sedimentation to the primary ion-exchange fractionation allows a small but significant further separation of template-active and template-inactive portions of chromatin. Chromatin that elutes late from ECTHAM-cellulose and sediments slowly has an increased frequency of binding sites compared to that fractionated by either procedure alone. Similarly, chromatin that elutes early

from ECTHAM-cellulose and sediments rapidly has a decreased frequency of polymerase binding sites compared to material separated by either method alone. Overall, it is possible to obtain fractions of a whole chromatin that differ by nearly 25-fold in frequency of binding sites for *E. coli* RNA polymerase.

Chromatin fractions that elute late from ECTHAM-cellulose and sediment slowly on sucrose gradients have a frequency of polymerase binding sites nearly half that of protein-free DNA, yet still contain about 0.8 g of histone per g of DNA. This is nearly 80% of the histone content of whole chromatin which is, in the main, repressed. We have previously shown that late ECTHAM-cellulose chromatin is characterized by a less condensed conformation and looser histone-DNA interactions than either whole or early chromatin (2, 3, 5). The current studies correlate condensed conformation with repression of *in vitro* transcription and correlate extended conformation with *in vitro* transcribability. Whether *in vitro* transcribable chromatin is the same as chromatin that is transcribed *in vivo* could be answered by hybridization studies.

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