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Transcription of Fractionated Calf Thymus Chromatin by RNA Polymerase of Calf Thymus and *Escherichia coli*†

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ABSTRACT: Calf thymus chromatin has been sheared and fractionated on sucrose gradients. Approximately 5–10% of the chromatin is resolved from the bulk of the input chromatin as a slowly sedimenting fraction. The protein/DNA ratio of the slowly sedimenting fraction is not greatly different from the protein/DNA ratio of the more rapidly sedimenting chromatin fraction. Analysis of DNA of the chromatin fractions by CsCl equilibrium density gradient centrifugation indicates that DNA of the slowly sedimenting fraction is depleted in the satellite DNA banding at 1.716 g/cm^3. The template properties of the chromatin fractions have been examined with *Escherichia coli* RNA polymerase and with form II and form III RNA polymerases of calf thymus. At rate-limiting concentrations, the slowly sedimenting fraction is twofold more active than the rapidly sedimenting fraction as a template for *E. coli* RNA polymerase. Homologous form II and form III RNA polymerases are respectively 30-fold and 16-fold more active with the slowly sedimenting fraction than with the rapidly sedimenting fraction. The activity of form II RNA polymerase in transcribing the slowly sedimenting fraction exceeds its activity in transcribing an equal concentration of native DNA. Kinetic studies, in which RNA polymerase activity is assayed at various concentrations of chromatin, indicate that the greater activity of *E. coli* RNA polymerase with the slowly sedimenting fraction is due to an increased rate of transcription at saturating concentrations of template (V_{max}), and is not due to a lower concentration required for half-maximal rate of transcription (K_m). In contrast, the increased rates of transcription of the slowly sedimenting chromatin fraction by the homologous polymerases are due to a decrease in concentration required for half-maximal rate of transcription rather than an increased rate of transcription at saturating concentrations of template. The relative decrease of satellite DNA in the slowly sedimenting fraction of chromatin and the enhanced template activity of the slowly sedimenting fraction suggest that this fraction is equivalent to nuclear euchromatin while the more rapidly sedimenting chromatin is equivalent to nuclear heterochromatin.

Chromatin is a complex of DNA, histones, non-histone proteins, and possibly RNA, the interactions of which are believed to restrict transcription to only a selected set of genes in differentiated cells. On the microscopic level, genes selected for active transcription are visualized as euchromatin while genes which are inactive, either facultatively or constitutively, are visualized as highly condensed heterochromatin (see review by Frenster, 1974). These visualized structural differences between active and inactive chromatin have led to a number of experiments in which chromatin has been separated into portions active and inactive for transcription in vitro.

In vitro studies of transcription of chromatin have been performed on chromatin fractionated by differential centrifugation (Frenster et al., 1963; Chalkley and Jensen, 1968; Murphy et al., 1973; McCarthy et al., 1974), gel filtration (Janowski et al., 1972), differential solubility (Marushige and Bonner, 1971; Arnold and Young, 1974), and ion-exchange chromatography (Simpson, 1974). Except for the studies of Murphy et al. (1973) and Howk et al. (1975), *Escherichia coli* RNA polymerase (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) rather than homologous RNA polymerases was used.

Although isolated mammalian chromatin has been shown to yield tissue specific RNA when transcribed in vitro by *E. coli* RNA polymerase (Gilmour and Paul, 1973; Axel et al., 1973), it is reasonable to assume that at least some of the in vivo controls of transcription will not operate when chromatin is transcribed by bacterial RNA polymerase. In particular, controls affecting only one of the multiple forms of RNA polymerase found in mammalian cells (Roeder and Rutter, 1969) can be studied only by using homologous polymerases.

We have fractionated sheared calf thymus chromatin by sedimentation through sucrose gradients and have evaluated the fractions as templates for form II and form III RNA polymerases from calf thymus, as well as for *E. coli* RNA polymerase. Fractions which differ in template activity have been obtained and kinetic studies indicate that there are important qualitative as well as quantitative differences between *E. coli* and mammalian RNA polymerases in the

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manner in which they transcribe the various chromatin fractions.

A preliminary report of this work has appeared (Henner and Furth, 1975).

Experimental Section

Materials. Calf thymus DNA and ribonuclease A were purchased from Worthington Biochemical Corp. [5-^{3}H]UTP was purchased from New England Nuclear Corporation. Unlabeled nucleoside triphosphates were obtained from Sigma Chemical Co. and P-L Biochemicals.

Chromatin. Nuclei were prepared from frozen calf thymus by a modification of the method of Marzluff et al. (1973). Tissue was minced and thawed in 3 volumes of 0.3 *M* sucrose, containing 5 m*M* magnesium acetate, 3 m*M* $CaCl_2$, 10 m*M* Tris-Cl (pH 8.0), 0.1% Triton X-100, and 0.5 m*M* dithiothreitol. Tissue was homogenized with ten strokes of a Teflon pestle in a Potter homogenizer and filtered through 4 layers of cheesecloth. The filtrate was mixed with an equal volume of 2 *M* sucrose containing 5 m*M* magnesium acetate, 10 m*M* Tris-Cl (pH 8.0), and 0.5 m*M* dithiothreitol. The mixture was layered over 5 ml of 2 *M* sucrose buffer, and centrifuged for 60 min at 20,000 rpm in the SW 25.1 rotor. The nuclear pellet was resuspended and washed twice in 0.3 *M* sucrose containing 2 m*M* $MgCl_2$ and 10 m*M* Tris-Cl (pH 8.0) and stored (as a pellet) at −90°C.

Chromatin was prepared from isolated nuclei by the method of Paul and Gilmour (1968). Unless otherwise noted, chromatin at a concentration of 12 A_{260} units/ml was sheared in a VirTis "45" homogenizer at 60% of maximum rate for 6 min.

Sucrose Gradient Fractionation of Chromatin. Three milliliters of sheared chromatin solution was layered onto 27 ml of a 0.25–2.0 *M* sucrose gradient containing 10 m*M* Tris-Cl (pH 8.0) and 0.2 m*M* EDTA and centrifuged at 4°C for 15 hr at 20,000 rpm in a SW 25.1 rotor; 2 ml fractions were collected and pelleted material was resuspended in 0.2 m*M* NaEDTA. A_{260} was measured for each fraction and pooled fractions were dialyzed for 8 hr at 4°C against two changes of 2 l. of 0.2 m*M* NaEDTA (adjusted to pH 7 with NaOH). Chromatin fractions too dilute for kinetic studies of template activity were concentrated 5–10-fold by ultrafiltration of dialyzed fractions through an Amicon PM10 filter at 30 psi at 4°C. Recovery of chromatin was at least 95%.

Chemical Analyses. DNA was determined by the diphenylamine assay of Burton (1956). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Histones were measured as the 0.4 *N* H_2SO_4 extractable protein, using whole calf thymus histones as a standard.

Isolation of DNA. Sheared chromatin and pooled chromatin fractions were dialyzed against 0.2 m*M* NaEDTA, frozen, and lyophilized. The chromatin was suspended in 10 m*M* Tris-Cl (pH 9.0) containing 0.1 *M* NaCl and 0.1% sodium dodecyl sulfate, and extracted twice with phenol. Two volumes of 95% ethanol was added to the aqueous phase and DNA collected by centrifugation. The precipitate was dissolved in 15 m*M* NaCl containing 1.5 m*M* sodium citrate and treated with ribonuclease (20 μg/ml) at 37°C for 30 min. The DNA solution was then phenol extracted, precipitated, and redissolved as described above. *Micrococcus luteus* DNA was isolated as described by Marmur (1961).

Density Gradient Sedimentation of DNA and Chromatin. DNA from sheared chromatin or from chromatin fractions was analyzed by equilibrium sedimentation in cesium chloride density gradients for 48 hr at 31,400 rpm, 25°C, in the Spinco Model E analytical centrifuge. Buoyant densities were calculated based on a reference density of 1.731 g/cm^3 for *Micrococcus luteus* DNA (Schildkraut et al., 1962). Chromatin fractions, fixed by dialysis against 500 volumes of 0.5% formaldehyde in 2.5 m*M* sodium phosphate buffer (pH 7.0) for 24 hr, were banded in cesium sulfate density gradients, also in the Model E. Buoyant densities were determined using the gradient data of Ludlum and Warner (1965) after centrifugation for 24 hr, 44,770 rpm, 25°C. Cesium sulfate was found more convenient than cesium chloride for routine density gradient analysis of fixed chromatin since the entire range of buoyant densities, from protein (approximately 1.25 g/cm^3) to DNA (1.43 g/cm^3), can be encompassed in a single cell with only a small loss of resolution (R. Kelley, manuscript in preparation).

RNA Polymerase. Two forms of RNA polymerase were obtained from calf thymus by a procedure previously described for calf thymus and bovine lymphosarcoma (Furth et al., 1970; Keshgegian et al., 1975a). One of the enzymes is form II RNA polymerase as determined by its chromatography profile on DEAE-Sephadex, sensitivity to α-amanitin, and preference for transcription of denatured DNA. The other enzyme, termed $RNAP_L$ (Keshgegian et al., 1975a), is a form III type enzyme. Upon DEAE-Sephadex chromatography it elutes in a broad band from 0.17 to 0.28 *M* $(NH_4)_2SO_4$. All column fractions exhibit similar properties including a small preference for native DNA (up to twofold), and inhibition by α-amanitin at high (>80% at 100 μg/ml), but not low (<5% at 0.2 μg/ml), concentrations (Keshgegian et al., 1975b). Since some of the properties of the enzyme, such as low salt optimum (Keshgegian et al., 1975b), are dissimilar from other form III enzymes (Roeder and Rutter, 1969; Schwartz et al., 1974; Sklar et al., 1975), we shall refer to it as form III*.

E. coli RNA polymerase was prepared as described by Furth and Pizer (1966) followed by chromatography on DEAE-Sephadex. The *E. coli* RNA polymerase preparation contained σ factor.

RNA Polymerase Assay. Assay A contained (in 0.5 ml): 0.05 *M* Tris-maleate (pH 7.7), 2 m*M* $MnCl_2$, 2 m*M* 2-mercaptoethanol, 320 μ*M* each of ATP, GTP, and CTP, 40 μ*M* [^{3}H]UTP (15–60 × 10^3 counts min^{-1} $nmol^{-1}$), calf thymus DNA or chromatin (120 μ*M* deoxynucleotides), and enzyme. The $(NH_4)_2SO_4$ concentration in the reaction was 0.05 *M* for form III* and *E. coli* RNA polymerases and 0.08 *M* for form II RNA polymerase. Assay B differed from assay A in that $(NH_4)_2SO_4$ was omitted, the concentration of $MnCl_2$ was reduced to 1 m*M*, and 12 m*M* $MgCl_2$ and 0.2 *M* KCl were added.

After 10 min at 37°C, incorporation of labeled nucleotides into acid-insoluble material was determined as described previously (Austin et al., 1973). Units of enzyme are determined using assay A. One unit of form III* or *E. coli* RNA polymerase is defined as the amount of enzyme that converts 1 nmol of [^{3}H]UTP into acid-insoluble material in 10 min with native calf thymus DNA as template. One unit of form II converts 1 nmol of [^{3}H]UTP into acid-insoluble material with denatured calf thymus DNA as template. Endogenous RNA polymerase activity of chromatin was not detectable at the specific activity of [^{3}H]UTP used. Under assay A conditions, in 10 min, 10 nmol of calf thymus chromatin degrades less than 0.1 pmol

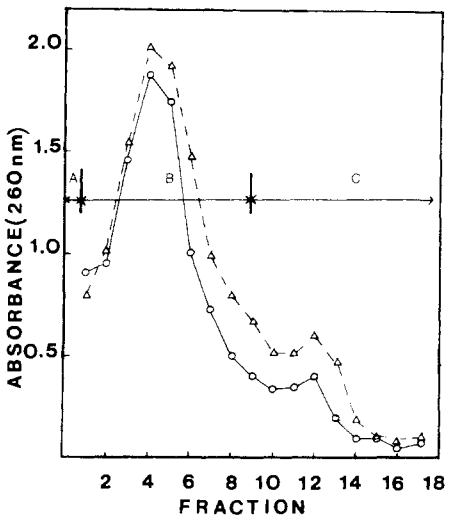

FIGURE 1: Sedimentation of sheared calf thymus chromatin; 38 A_{260} units of chromatin was sedimented on sucrose gradients as described in the Experimental Section. Sedimentation is from right to left. Unsheared chromatin is completely pelleted under these conditions. (O—O) 3 min shear, (Δ- - -Δ) 6 min shear.

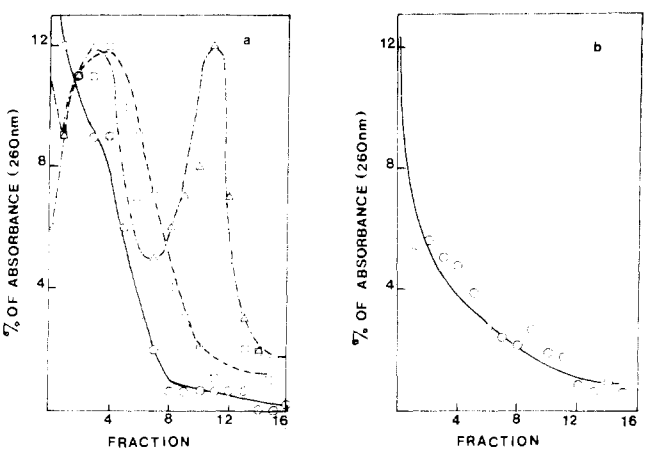

FIGURE 3: Resedimentation of chromatin fractions. Sucrose gradient chromatin fractions, pooled as in Figure 2, were dialyzed and recentrifuged on identical gradients. A_{260} per fraction is reported as a percent of A_{260} of input chromatin. Upon resedimentation 48, 15, and 6% of fraction A, fraction B, and fraction C, respectively, were recovered as pelleted material. (a) (O) Fraction A, pelleted chromatin; (□) fraction B, fast sedimenting peak; (Δ) fraction C, slowly sedimenting peak; (b) resedimentation of fraction A chromatin after reshearing for 6 min.

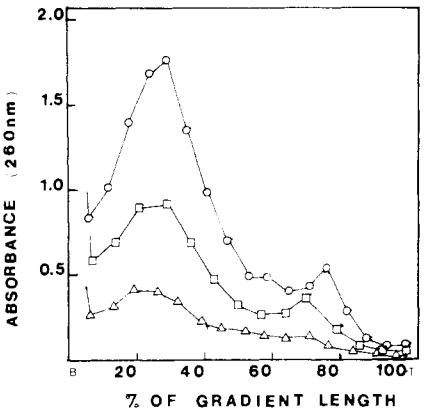

FIGURE 2: Effect of chromatin concentration on chromatin fractionation. Calf thymus chromatin was fractionated on 0.25–2.0 *M* sucrose gradients in parallel tubes. (Δ) 9 A_{260} units input; (□) 18 A_{260} units input; (O) 36 A_{260} units input.

of ^{3}H-labeled RNA, synthesized in vitro by form III*, to acid-soluble material.

Results

Effect of Shearing Chromatin. Calf thymus chromatin was sheared as described in the Experimental Section and aliquots were removed at intervals. Template activity of each aliquot was measured with either 0.7 unit of *E. coli* RNA polymerase or 0.2 unit of calf thymus form III* RNA polymerase, using assay B with chromatin at 120 μM (as deoxynucleotides). With unsheared chromatin, incorporation was 98 pmol for *E. coli* RNA polymerase and 67 pmol for form III* RNA polymerase. After 0.5, 1, 3, and 6 min of shear, chromatin was 1.4-, 2.3-, 2.2-, and 2.7-fold as effective a template for *E. coli* RNA polymerase and 1.8-, 1.6-, 1.9-, and 2.3-fold as effective a template for form III* RNA polymerase; 6 min of shear, a time at which the effect of shear on transcription appears to level off, was chosen as the standard time for further studies.

Sucrose Gradient Fractionation of Chromatin. Sheared calf thymus chromatin sediments at a wide range of rates (Figure 1). Under these conditions approximately 40% of the chromatin is pelleted during centrifugation. The nonpelleted chromatin sediments as two distinct peaks. The bulk of the chromatin is found in a peak sedimenting ⅔ the length of the gradient. A second fraction of chromatin is found ⅓ the length of the gradient. This second slowly sedimenting fraction is consistently present and contains approximately 5% of the chromatin. Chromatin that has not been sheared prior to centrifugation is completely sedimented under these conditions. Chromatin sheared for 3 min demonstrates peaks at ⅓ and ⅔ the length of the gradient; an increase of shear time to 6 min does not shift the position of the peaks and causes a small increase in the amount of chromatin in the position of the slowly sedimenting peak (Figure 1). The pattern of sedimentation is not dependent on the amount of chromatin placed upon the gradient over the range of 9–36 A_{260} units (Figure 2).

To test whether chromatin fractions with different sedimentation rates retain their sedimentation properties upon resedimentation, regions of a gradient were pooled as shown in Figure 1, dialyzed against 0.2 m*M* NaEDTA (adjusted to pH 7.0 with NaOH), and recentrifuged on identical gradients. A large portion of the pelleted material (fraction A) again pellets and only small amounts of chromatin sediment less than 50% the length of the gradient (Figure 3a). Fraction B, which contains the fast sedimenting fraction, resediments to the same position as the original fraction, approximately ⅔ the length of the gradient. Fraction C, which contains the slowly sedimenting fraction and the trailing edge of the fast sedimenting fraction, splits into two distinct components of approximately equal amounts; one component sedimenting at ⅓ and the other sedimenting at ⅔ the length of the gradient. Thus, fraction C is approximately 50% pure "slowly sedimenting fraction". To test whether fraction A chromatin could be converted into a more slowly sedimenting form by further shear, it was resuspended, dialyzed, and resheared for 6 min. The resheared fraction A chromatin was centrifuged again. Comparison of Figure 3a and b shows that after reshearing, fraction A chromatin still mostly sediments to the bottom of the tube. Also, no peaks of chromatin at ⅓ and ⅔ of the length of gradient are generated by shearing a second time.

Table I: Protein and Histone Composition of Chromatin Fractions.[a]

Chromatin Fractions	Buoyant Density (g/cm^3)	DNA	Protein (±SD)	Histone (±SD)
Fraction A (pelleted chromatin)	1.3028	1.00	1.52 ± 0.12	1.04 ± 0.05
Fraction B (fast sedimenting peak)	1.3069	1.00	1.38 ± 0.21	1.07 ± 0.05
Fraction C (slowly sedimenting peak)	1.3126	1.00	1.36 ± 0.20	0.83 ± 0.12

[a] Amounts of DNA, protein, and histones were determined as described in the Experimental Section. Values are expressed as the average ratio of total protein and histone protein to DNA. Densities of formaldehyde fixed chromatin fractions were determined by Cs_2SO_4 density gradient centrifugation as described in the Experimental Section.

Protein Composition of Chromatin Fractions. Chromatin fractions, pooled as shown in Figure 1, were dialyzed against 0.2 m*M* NaEDTA, lyophilized, and analyzed for total protein, DNA, and histone protein (Table I). The (average) protein/DNA ratios for the chromatin fractions used in kinetics studies ranges from 1.36 for fraction C, the slowly sedimenting peak, to 1.52 for fraction A, the pelleted chromatin. While substantial variation in the chemically determined protein/DNA ratios is observed, the relative order of the fractions was the same in all determinations.

The extraction of chromatin fractions with 0.4 *N* H_2SO_4 indicates that the slowly sedimenting chromatin fractions contain approximately 80% the amount of histones as rapidly sedimenting chromatin. This is consistent with the results of Simpson and Reeck (1973) for calf thymus chromatin fractionated by ECTHAM-cellulose chromatography. In their experiments, the 20% decrease in histone content of active chromatin fractions was found to be due to a depletion of histone f1.

The buoyant densities of formaldehyde fixed chromatin provide a second method for comparing the protein to DNA ratios of the chromatin fractions (Brutlag et al., 1969). Consistent with the chemical determinations, the three chromatin fractions have buoyant densities in the inverse order of their rates of sedimentation (Table I). The weighted buoyant density average for the three fractions, 1.3056, is, within experimental error, identical with the buoyant density of the sheared, unfractionated chromatin, 1.3059. No material was detected at the density of free DNA. The apparent discrepancy between the similar chemically determined protein contents of fraction B and fraction C and their significantly different buoyant densities may be explained by the fact that histones, which are relatively depleted in fraction C, have a very low buoyant density (1.19–1.21 g/cm^3) compared to non-histone proteins (1.23–1.28 g/cm^3) in cesium sulfate (R. Kelley, manuscript in preparation). Hence fraction C bands at a greater density than fraction B even though their total protein contents are almost the same.

Satellite DNA of Chromatin Fractions. DNA was isolated from pooled chromatin fractions (Figure 1) and sedimented to equilibrium in CsCl (Figure 4). Calf thymus DNA prepared from sheared, unfractionated chromatin has a satellite which bands at a density of 1.716 and a poorly defined band at 1.721. DNA from both fraction A and fraction B contains approximately the same amount of satellite DNA as total chromatin, while satellite DNA is depleted in DNA from fraction C, the slowly sedimenting fraction. Resolution of main band and satellite DNA was not sufficient to exclude the presence of some satellite DNA in fraction C. Since RNA complementary to satellite DNA is not detected among in vivo RNA sequences (Flamm et al., 1969), it was predicted that satellite DNA would not be found in DNA of "active" fractions of chromatin. Our results confirm this prediction; similar results have been reported by Yasmineh and Yunis (1971) and Duerksen and McCarthy (1971).

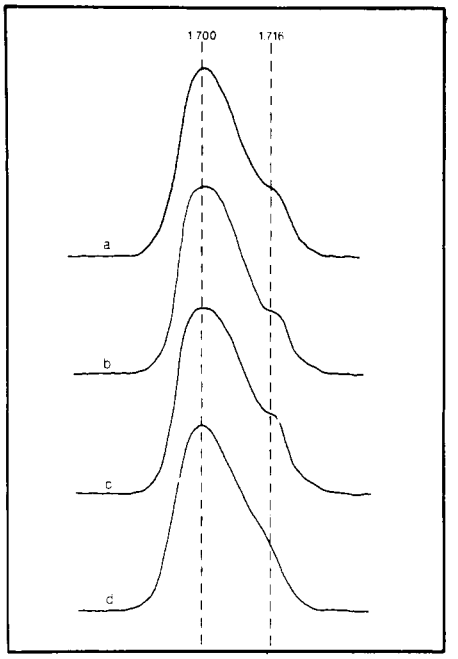

FIGURE 4: Equilibrium density centrifugation of DNA from sucrose gradient chromatin fractions. Chromatin fractions were pooled as shown in Figure 1. (a) DNA from unfractionated sheared calf thymus chromatin. (b) DNA from fraction A, pelleted chromatin. (c) DNA from fraction B, fast sedimenting peak of chromatin. (d) DNA from fraction C, slowly sedimenting peak of chromatin.

Transcription of Chromatin Fractions. The sucrose gradient fractions were next examined as templates for *E. coli* RNA polymerase and the two calf thymus RNA polymerases at low (rate limiting) chromatin concentrations (Figure 5). With *E. coli* RNA polymerase there is an increase in template effectiveness of the trailing edge of the fast sedimenting peak of chromatin and a larger and sharper increase coincident with the slowly sedimenting peak. However, the overall rise in template activity from rapidly to slowly sedimenting fractions is only 70%. Thus the fractionation of chromatin sequences active for *E. coli* RNA polymerase appears to be poor.

When the chromatin fractions are assayed with form III* RNA polymerase, there is also an increase in template activity on the trailing edge of the fast sedimenting peak and a larger increase in template activity coincident with the slowly sedimenting peak. The template activity increases 16-fold from pelleted material to the slowly sedimenting fraction.

Transcription of chromatin fractions by form II RNA polymerase shows a pattern of template activity similar to that of form III*. There is a 30-fold increase in template activity between pelleted chromatin and the slowly sedi-

Table II: Comparison of Native DNA and the Slowly Sedimenting Chromatin Fraction as Templates for *E. coli* and Calf Thymus RNA Polymerase.[a]

Template	[^{3}H]UMP Incorporated (pmol/nmol of DNA)	% Incorporation Rel to Native DNA
E. coli RNA polymerase		
Slowly sedimenting chromatin fraction	6.3	28
Native calf thymus DNA	22.1	100
Calf thymus form II RNA polymerase		
Slowly sedimenting chromatin fraction	1.1	258
Native calf thymus DNA	0.4	100
Calf thymus form III* RNA polymerase		
Slowly sedimenting chromatin fraction	6.8	52
Native calf thymus DNA	13.2	100

[a] Slowly sedimenting chromatin was fraction 11 of the sucrose gradient shown in Figure 5. Chromatin or DNA was present at a concentration (15 μM) which is rate limiting. Assay conditions and units of enzyme are as described in the legend to Figure 5.

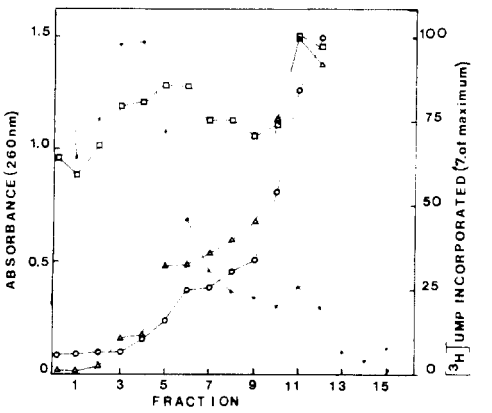

FIGURE 5: Template activity of sucrose gradient fractions of chromatin. Sheared calf thymus chromatin was fractionated on a 0.25–2.0 M sucrose gradient. Chromatin from fractions, at a concentration of 15 μM, was assayed for template activity using assay A, and (□) 0.5 unit of *E. coli* RNA polymerase; (△) 0.2 unit of form II RNA polymerase; or (○) 0.12 unit of form III*. (- - - -) A_{260}/ml. Maximal incorporation (100%) represents 94, 8, and 63 pmol of [^{3}H]UTP incorporated for *E. coli* RNA polymerase, form II, and form III*, respectively.

Table III: Kinetic Parameters of Transcription of Chromatin Fractions.

Template	K_m (μM)	V_{max} (nmol)
E. coli RNA polymerase		
Chromatin fraction A	31	0.22
Chromatin fraction B	31	0.31
Chromatin fraction C	31	0.53
Calf thymus form II RNA polymerase		
Chromatin fraction A	>200	>0.005
Chromatin fraction B	91	0.020
Chromatin fraction C	36	0.017
Calf thymus form III* RNA polymerase		
Chromatin fraction A	109	0.028
Chromatin fraction B	14	0.028
Chromatin fraction C	6	0.029

[a] RNA polymerase assays were performed as described in the Experimental Section and the legend to Figure 6, with 0.5 unit of *E. coli* RNA polymerase, 0.2 unit of form II RNA polymerase, or 0.06 unit of form III* RNA polymerase.

menting fraction. The 30-fold increase is a conservative estimate of the actual difference in template activities for form II RNA polymerase, since incorporation with pelleted chromatin could not be reliably measured at this low chromatin concentration. When pelleted chromatin is resuspended and resheared, the template activity does not increase for any of the three RNA polymerases used in these studies (data not shown).

Comparison of the template activity of the slowly sedimenting chromatin fraction with the template activity of native calf thymus DNA, at the same concentration, indicates that *E. coli* RNA polymerase utilizes the slowly sedimenting fraction 28% as effectively as native calf thymus DNA (Table II). With calf thymus form II and III* polymerases, respectively, the slowly sedimenting chromatin is 52 and 258% as effective a template as native DNA.

In view of the preference of form II RNA polymerase for denatured DNA, we attempted to exclude the presence of significant amounts of denatured DNA in the slowly sedimenting fraction by measuring the hyperchromicity on denaturation. Chromatin from the pellet, fraction 4, and fraction 11 of the gradient shown in Figure 5 all show the same degree of hyperchromicity, 32%, when heat denatured in 0.2 mM NaEDTA. The ability of form II to transcribe the slowly sedimenting chromatin is therefore not due to significant amounts of single-stranded regions of DNA but rather to a complex of native DNA and chromatin proteins which facilitate transcription by form II RNA polymerase.

Kinetic Data. Since kinetic parameters have indicated a different mechanism of restriction of template activity of chromatin for *E. coli* RNA polymerase as compared with mammalian RNA polymerase (Keshgegian and Furth, 1972), kinetic parameters (apparent K_m and V_{max}) have been determined for active and inactive chromatin fractions. Data for *E. coli* RNA polymerase activity at various chromatin concentrations were plotted by the double reciprocal method for calculation of the V_{max} and apparent K_m (Lineweaver and Burk, 1934) for three regions of a chromatin sucrose gradient (Figure 6a). The values for V_{max} and K_m calculated from this plot are shown in Table III. As previously reported (Marushige and Bonner, 1966; Keshgegian and Furth, 1972), the apparent K_m for chromatin is identical with that of native DNA with *E. coli* RNA polymerase. The transcription of chromatin from all three regions of the gradient gives an apparent K_m identical with that of native DNA and unfractionated chromatin. However, the V_{max} of *E. coli* RNA polymerase is greater when assayed with the more slowly sedimenting fractions. The increase in template activity of the slowly sedimenting fraction of chromatin is, therefore, due to an increase in the rate at which the enzyme can transcribe chromatin in the presence of saturating concentrations of template.

When RNA synthesis is measured as a function of chromatin concentration with calf thymus form III* and the slowly and rapidly sedimenting fractions, a pattern distinctly different from the result with *E. coli* RNA polymerase is observed (Figure 6b). The calculated K_m and V_{max} values show that the increase in template activity observed with the slowly sedimenting chromatin fractions is not due to an increase in V_{max} but rather to a large decrease in the apparent K_m (Table III). Chromatin fractions at various concentrations were also assayed with form II RNA polymerase and K_m and V_{max} calculated for fraction B and fraction C. K_m and V_{max} for fraction A could not be accurately as-

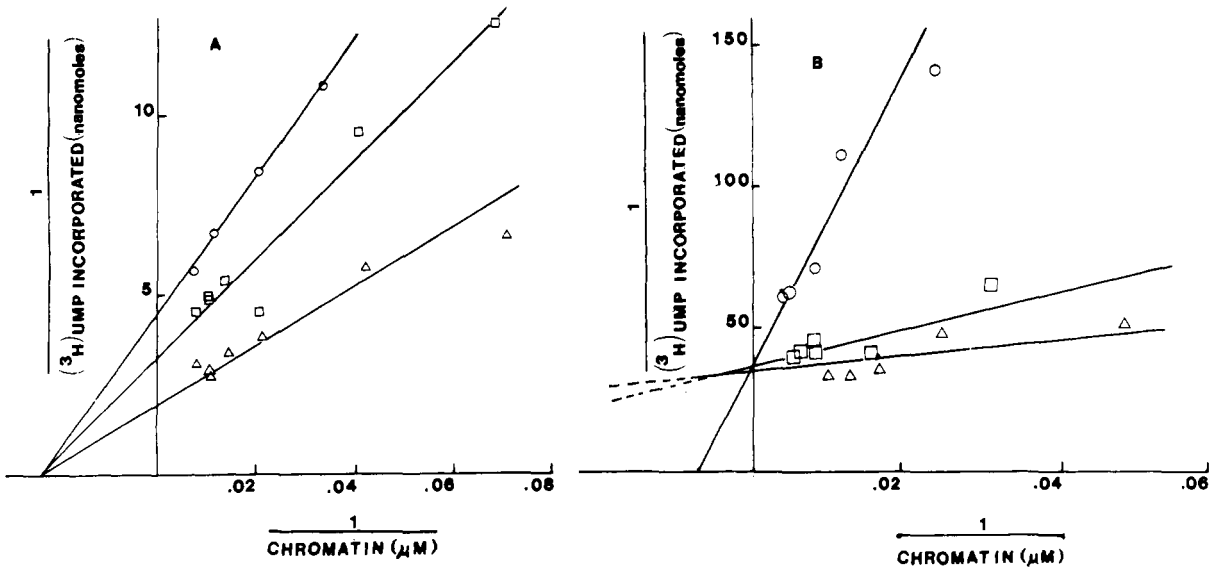

FIGURE 6: RNA synthesis by *E. coli* RNA polymerase or calf thymus form III* RNA polymerase as a function of chromatin concentration. Calf thymus chromatin was fractionated and pooled as described in the legend to Figure 1. The indicated concentration of chromatin was assayed with (a) 0.5 unit of *E. coli* RNA polymerase, or (b) 0.06 unit of calf thymus form III* RNA polymerase using assay B. (Incorporation of [^{3}H]UTP vs. chromatin concentration is displayed as the double reciprocal plot of the data.) The lines drawn through the data points have been calculated according to the method of least squares. (○) Fraction A; (□) fraction B; (△) fraction C. K_m and V_{max} values obtained are shown in Table III.

sessed because the K_m appeared to be higher than 200 μM, the highest chromatin concentration tested. The kinetic parameters for form II (Table III) indicate that the increase in template activity in the slowly sedimenting fraction of chromatin is due to a decrease in K_m with no change in V_{max}.

Discussion

These results indicate that sucrose gradient centrifugation can be used to achieve a separation of calf thymus chromatin into components active and inactive for RNA synthesis in vitro.

The amount of chromatin in the slowly sedimenting, active fraction was consistently between 5 and 10% of total chromatin in a series of more than 15 separate determinations. We consider this slowly sedimenting fraction of chromatin to be analogous to the euchromatin observed microscopically in intact nuclei, while the bulk of the chromatin, including both the rapidly sedimenting fraction and the pelleted chromatin, to be analogous to the more highly condensed heterochromatin. This proportion of DNA that segregates with our slowly sedimenting, "active" chromatin is comparable to the fraction of mammalian DNA transcribed in vivo in other tissues as estimated by DNA–RNA hybridization (Grouse et al., 1972). The majority of the rapidly sedimenting peak appears to be inactive for transcription; the small amount of transcription that is supported resides in the trailing border (Figure 5) and probably represents incomplete resolution of the two peaks or possibly, tags of euchromatin not removed from the heterochromatin by shearing.

Although Noll and his coworkers (Noll et al., 1975) have suggested that nuclease digestion is preferable to shearing in preparing chromatin, we have chosen not to use the nuclease method for two reasons: (1) the small percentage of transcriptionally active chromatin may be more susceptible to nuclease degradation because of a structurally more "open" conformation, and (2) the presence of nuclease in chromatin preparations would hinder the interpretation of experiments involving the incubation of chromatin with RNA polymerase in the presence of divalent cation. Furthermore, we do not believe that the slowly sedimenting, active fraction is an artefact of the shearing process because: (1) only a small proportion of total chromatin is found in the slowly sedimenting fraction and the proportion is not increased by further shearing, and (2) the DNA sequences which are released into the slowly sedimenting fraction are not random segments of total DNA since satellite DNA content is significantly reduced. Whether or not the relative decrease in the amount of satellite DNA in the slowly sedimenting fraction is representative of nontranscribed sequences remains to be determined.

Studies with *E. coli* RNA polymerase indicate that chromatin which sediments more slowly is generally more active as a template by 2–10-fold (Chalkley and Jensen, 1968; McCarthy et al., 1974). Murphy et al. used both *E. coli* and homologous form I and form II RNA polymerases and found that the slowly sedimenting chromatin was approximately 7-fold, 60-fold, and 20-fold more active as a template for *E. coli*, form I, and form II polymerases, respectively. The apparent purification of transcriptionally active calf thymus chromatin by sucrose gradient centrifugation is much greater when assayed with form II or form III* calf thymus RNA polymerases than when assayed with *E. coli* RNA polymerase. This would be expected if the slowly sedimenting fraction is composed of euchromatin, since form II is thought to transcribe mRNA and form III is thought to transcribe tRNA and 5S RNA (Weinman and Roeder, 1974). The inability of *E. coli* RNA polymerase to show the same preference for chromatin that is so active for homologous polymerase suggests that it is not able to respond to factors which control the calf thymus RNA polymerases. The tissue specific transcription of the globin gene from chromatin by *E. coli* RNA polymerase (Axel et al., 1973; Gilmour and Paul, 1973) may be due to very gross changes in the accessibility of a gene to binding and transcription by *E. coli* polymerase, while a greater degree of specificity might be expected, and has been reported

(Steggles et al., 1974), with homologous polymerase.

In general, we obtain a good fit of our data to the Michaelis–Menten equation and have found it useful to express them in terms of V_{max} and K_m, even though the assumptions of the Michaelis–Menten model may not be strictly valid for a reaction as complex as that catalyzed by RNA polymerase. From this type of analysis, we conclude that *E. coli* RNA polymerase does not interact with more sites on active chromatin than inactive chromatin since the K_m's for the two templates are similar. (It is not possible, however, to distinguish between an increased rate of elongation or a greater fraction of bound, transcribing polymerase as the cause of the greater V_{max} for transcription of the slowly sedimenting, active chromatin.) In contrast, while the homologous forms II and III* polymerases demonstrate essentially the same V_{max} for both slowly and rapidly sedimenting chromatin fractions, they both show a much lower K_m value for the slowly sedimenting chromatin than for the rapidly sedimenting fraction. This suggests that the slowly sedimenting chromatin offers a much greater frequency of binding sites for the homologous RNA polymerase, as would be expected for an "active" chromatin fraction. We conclude that genes are maintained as active or inactive in chromatin (and presumably in vivo) by controls of a type not present in the bacterial cell and which therefore do not affect *E. coli* RNA polymerase in the same manner as they affect homologous form II or form III* polymerases.

Consistent with the conclusions from our data, other investigations have demonstrated that: (1) *E. coli* RNA polymerase transcripts contain a lower proportion of RNA sequences complementary to repeated DNA sequences than transcripts produced by either form II (Dupras and Bonner, 1974) or form III* (Attikan, 1974) RNA polymerases, and (2) the initial nucleotides of RNA synthesized by *E. coli* and form III* RNA polymerase differ significantly (Keshgegian et al., 1973).

Taken together these investigations suggest that there are important differences between bacterial and mammalian RNA polymerases in their interactions with chromatin during transcription. The separation of active and inactive chromatin has allowed us to more clearly demonstrate such differences. We believe that the ability to isolate the small proportion of chromatin which is transcriptionally active will be useful in further in vitro studies of transcription, and that a fully homologous system of chromatin and RNA polymerases will be required to obtain in vitro transcription which is identical with that occurring in vivo.

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Osmotic Pressure Induced Pores in Phospholipid Vesicles†

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ABSTRACT: We report a comparative study of the leakage of hydrophilic molecules from vesicles of egg lecithin (EL) and of dipalmitoyllecithin (DPL). The effect of osmotic pressure differences on leakage is consistent with a model for a statistical pore nucleation process. The major difference in osmotic pressure induced leakage from DPL and EL is that the number of pore creation sites is much greater in DPL. We suggest that the difference in number of these sites also accounts for other differences in the properties of DPL and EL, namely for differences in vesicle fusion and apparent rate of "flip-flop".

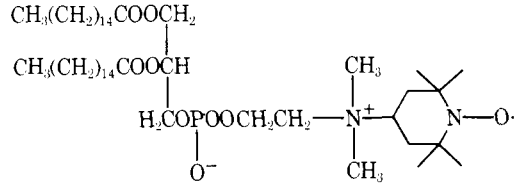
When dipalmitoyllecithin (DPL)[1] vesicles are preserved at a temperature above the "chain-melting point" (41°C) they undergo fusion. Evidence for this process based on the increase of internal volume, the transfer of phospholipids from the outer to the inner layer, and the mixing of the internal contents of the "fusing vesicles" has been presented by Taupin and McConnell (1972). Since then, different techniques have been used to confirm the existence of a fusion process in the case of vesicles made of saturated phospholipids (Papahadjopoulos et al., 1974; Prestegard and Fellmeth, 1974). On the other hand, egg lecithin (EL) vesicles do not fuse (Kornberg et al., 1972). Better knowledge of the bilayer properties is clearly required if we are to explain the different behavior of these two types of vesicles.

In this article, we present comparative measurements on DPL and EL vesicles which suggest the presence of a great number of defects in the DPL bilayer, these defects or pores being responsible for the observed leakage of hydrophilic solutes and an apparent increase in flip-flop rate. We propose a simple model for the pore nucleation process which leads to specific laws for leakage as a function of stress induced by an osmotic pressure difference across the vesicle wall. Experiments confirm this model.

Experimental Procedure

Dipalmitoyllecithin was purchased from Fluka. Egg lecithin was kindly provided by C. M. Gary Bobo (Laboratoire de Physiologie Cellulaire, Collège de France). The DPL (50 m*M* aqueous dispersion) was sonicated in buffered salt solutions at 50–55°C using titanium probes and then centrifuged to remove titanium debris. The conditions for egg lecithin were the same except that during sonication the tube was cooled in an ice bath. The buffer was 0.1 *M* phosphate (pH 6.9). The ionic strength of the buffer was varied by addition of sodium chloride for the tension-induced leakage measurements.

Several labels were used in the present work. Spin label I (dipalmitoylphosphatidyltempocholine) was synthesized by one of us (M.D.) following the general scheme of Kornberg and McConnell (1971) except for the production of phosphatidic acid which was prepared from (*S*)-(−)-1,2-dipalmitin (Fluka) following the process of Uhlenbroek and Verkade (1953).



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The rotatory power of dipalmitoyl-L-α-glycerophosphoric acid thus obtained, $[\alpha]^{22}D$ +3.7° (*c* 7.9, dry $CHCl_3$), can be compared with the value of Baer (1951), $[\alpha]^{26}D$ +4.0° (*c* 9.6, $CHCl_3$). The condensation was performed by shaking the mixture of phosphatidic acid, tempocholine, and 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine–chloroform for 4 hr in a closed flask containing glass beads. The spin label was purified by preparative silica thin-layer chromatography, in chloroform–methanol–NH_4OH (*d* = 0.90)–water (70:30:4:1, v/v). Anal. Calcd for $C_{48}H_{96}N_2O_{10}P\cdot 2H_2O$: C, 62.1; H, 10.85; N, 3.0; P, 3.3. Found: C, 62.0; H, 10.1; N, 3.0; P, 3.2.

The aqueous internal compartments of the vesicles were labeled using the water-soluble bilayer impermeable tempocholine (label II), prepared following the method of Kornberg and McConnell (1971). The label was dissolved

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[1] Abbreviations used are: DPL, dipalmitoyllecithin; EL, egg lecithin.