is involved in the nonspecific and operator binding processes (Bourgeois and Pfahl, 1976), the interaction with operator is not simply the superposition of specific interactions between nucleic acid bases and repressor and the nonspecific interactions of the RD complex. Instead a comparison of the RO and RD results indicates that possibly one less protonation event is required in the RO interaction (Riggs et al., 1970b), and the RO complex contains only ~70% of the ionic interactions of the RD complex.

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Composition and Template Activity of Chromatin Fractionated by Isoelectric Focusing†

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ABSTRACT: HeLa cell interphase chromatin has been sheared and fractionated by isoelectric focusing. Chromatin fractions are obtained with a wide range of isoelectric points. No free DNA is observed. While protein/DNA ratios are similar in the various fractions, they appear to contain different nonhistone chromosomal proteins. A minor chromatin fraction with isoelectric point ≃7.0 does not contain histone H1. This fraction is considerably more active as template with different RNA polymerases than the other fractions. Kinetic studies, in which RNA polymerase activity is assayed at various concentrations of chromatin, indicate that the greater activity of *Escherichia coli* RNA polymerase 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 rate of transcription by calf-thymus RNA polymerases II and III is due to a decrease in chromatin concentration required for half-maximal rate of transcription rather than an increased rate of transcription at saturating concentrations of template. These results suggest that chromatin with isoelectric point ≃7 offers a greater frequency of binding sites for mammalian RNA polymerases, as would be expected for a "transcriptionally active" fraction.

Recent observations on the fine structure of chromatin (Axel et al., 1974; Kornberg and Thomas, 1974; Olins and Olins, 1974) and the reconstitution of active transcribing chromatin (Barrett et al., 1974; Chan et al., 1973; Gilmour and Paul, 1969) have made it increasingly desirable to separate transcriptionally active chromatin from total chromatin. Fractionation has been attempted by a variety of techniques including mechanical breakage (Arnold and Young, 1974; Chesterton et al., 1974; Clark and Felsenfeld, 1971) or nuclease digestion (Gottesfeld et al., 1974) followed by fractionation techniques based on differences in size (Janowski et al., 1972), density (Frenster et al., 1963; McCarthy et al., 1974; Henner et al., 1975), and charge (Simpson, 1974).

Isoelectric focusing (IEF)[1] has been used extensively in the

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[1] Abbreviations used are: IEF, isoelectric focusing; p*I*, isoelectric point; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

fractionation of proteins (Vesterberg, 1971), nucleic acid (Drysdale and Richetti, 1972), and metaphase chromosomes (Landel et al., 1972). Since the IEF procedure routinely used has technical disadvantages, such as the long period of time required to focus macromolecules and the possibility of degradation during the procedure, we have used a sucrose gradient in a microcolumn (Kint, 1975) to shorten the focusing time, making it possible to study materials available in small amounts, without the limitations due to gel porosity and problems of osmoelectrolysis.

Here we report results of studies on the fractionation of postnucleolar chromatin of HeLa cells. Fractions of chromatin with different isoelectric points (p*I*) have been observed. Characterization of these fractions in terms of chemical composition as well as template activity for different RNA polymerases (RNA nucleotidyltransferase, EC 2.7.7.6) has been carried out. We have found no free stretches of DNA in chromatin thus fractionated.

Materials and Methods

Cell Culture. HeLa cells were maintained in spinner cultures in Eagle's minimal essential medium supplemented with penicillin–streptomycin and 5% calf serum. Cultures were diluted to 1.5×10^5 cells/mL every other day.

Chromatin Preparation. Exponentially growing HeLa cells were used. Procedures were carried out at 0–5 °C with all the buffer solutions containing 0.05 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) as protease inhibitor.

Cells, harvested by centrifugation, were resuspended in 10 mM Tris-Cl (pH 7.4) containing 10 mM NaCl and 1.5 mM $MgCl_2$–10 mM Tris (pH 7.4) and broken by 10–15 strokes of a tight fitting Dounce glass homogenizer. After adding an equal volume of 1% Triton X-100 containing 80 mM NaCl and 20 mM EDTA (pH 7.4), the nuclei were collected by centrifugation for 1 h through 2.2 M sucrose containing 3 mM $MgCl_2$ and 10 mM Tris-Cl (pH 7.5). The nuclear pellets were washed with 0.15 M NaCl containing 10 mM Tris-Cl (pH 7.0) and resuspended in water. After swelling for 15 min, the nuclei were broken by Dounce homogenization. The nucleoli were separated from bulk chromatin by sedimentation of the nuclear lysate through 0.8 M sucrose at low speed (Busch and Smetana, 1970). The nucleoli-depleted nuclear lysate was layered onto a 1.7 M sucrose cushion and the chromatin collected by centrifugation (Marushige and Bonner, 1966).

Shearing of Chromatin. Chromatin was sheared in three different ways: (a) sonication in the cold with a Branson sonifier for 20 s at 50 W; (b) shearing with a Virtis "45" homogenizer at 80 V in two bursts of 45 s; (c) mild digestion with DNase II as described by Gottesfeld et al. (1974). After 2 min of enzyme treatment (at 10 enzyme units/A_{260} unit of chromatin at pH 6.6), the reaction was terminated by addition of $MgCl_2$. The supernatant, after centrifugation, was saved and the pellet resuspended in 25 mM sodium acetate (pH 6.6) and subjected to another 5 min of DNase II digestion. The supernatant and pellet were then combined.

Determination of DNA Molecular Weight. Sheared chromatin was deproteinized with sodium dodecyl sulfate and phenol and the size of DNA was determined by sucrose gradient sedimentation (Burgi and Hershey, 1963). DNA sheared to a size of 2.3×10^5 daltons (350 nucleotides) as determined by Model E sedimentation analysis (Studier, 1965) was used as a marker.

Formaldehyde Fixation of Chromatin. The procedure of Doenecke and McCarthy (1975) was followed. The chromatin was fixed with 1% formaldehyde in 50 μM triethanolamine (pH 7.8) for 2 h at 4 °C. The chromatin was then dialyzed at 4 °C for 24 h against 50 μM triethanolamine.

Isoelectric Focusing of Chromatin. The procedure of Kint (1975) was used. Sheared chromatin was mixed with the proper volume of stock (LKB) ampholytes with pH ranges of 3.5–10, 2.5–4, 4–6, and 9–11 (volume ratio 1:3:3:3) and sucrose (80%) solutions. Chromatin in 5 mL of 2% ampholyte in a 10–35% sucrose gradient was held in the tube by a 20% acrylamide gel plug. Cathode and anode electrode buffers were 0.02 M sulfuric acid and 2% ethanolamine, respectively. To avoid any possible extreme pH effect from electrode buffers, 0.3 mL of 5 and 40% sucrose in 2% ampholytes was placed on the bottom and top of the gradient, respectively. The apparatus was maintained at 4 °C by circulating thermal regulated coolant. Constant voltage (150 V) was applied for 12 h. Gradient fractions were collected from the top of the gradient after the electrode buffer had been removed.

Chemical Analysis. 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 calf-thymus histone (Sigma) as a standard (Bonner et al., 1968).

RNA Polymerase Assay. Reaction mixtures contained (in 0.5 mL) 0.05 M Tris-Cl (pH 7.5), 1 mM $MnCl_2$, 12 mM $MgCl_2$, 0.2 M KCl, 0.5 mM dithiothreitol, 320 μM each of ATP, GTP, and CTP, 30 μM [^{3}H]UTP (148×10^3 counts min^{-1} nmol^{-1}), calf-thymus DNA or HeLa cell chromatin, and enzyme. After 15 min at 37 °C, the reaction was terminated by the addition of 3 mL of cold 10% trichloroacetic acid in 40 mM sodium pyrophosphate. Incorporation of labeled nucleoside triphosphate into acid-insoluble material was determined by filtering each assay through Whatman GF/C disk filter paper. After washing 4 times with 10 mL of 10% trichloroacetic acid in 40 mM sodium pyrophosphate, the filter was incubated wtih 1 mL of Protosol (NEN) for 10 h, and then 10 drops of acetic acid and 15 mL of Triton–toluene (Austin et al., 1973) scintillation fluid were added. RNA polymerase preparations were obtained as described by Henner et al. (1975).

Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out according to a modification of Laemmli's procedure (1970). Electrophoresis in polyacrylamide gels in the presence of urea at pH 2.7 was performed as described by Panyim and Chalkley (1969).

Results

Size of DNA Fragments in Sheared Chromatin. The size of DNA in sheared chromatin was determined as described in Materials and Methods. The sizes were in a bell-shaped distribution ranging from 800 to 2000 nucleotides (data not shown).

Isoelectric Focusing Profile of [^{3}H]Thymidine-Labeled Interphase Chromatin. As shown in Figure 1, interphase chromatin of HeLa cells consists of a heterogeneous population of chromatin with various isoelectric points ranging from 2.8 to 8.5. Approximately 28% of the chromatin possesses isoelectric points between 2.8 and 4, 52% possesses isoelectric points between 4 and 5.5, and 18% is found between 5.5 and 8.5. Free DNA, which under these conditions would precipitate at the bottom of the gradient, is not observed.

Further evidence that fractionation had been observed was obtained by three additional experiments: (a) rerun of fractionated chromatin; when fractionated chromatin was run separately on a second gradient, it refocused at the same po-

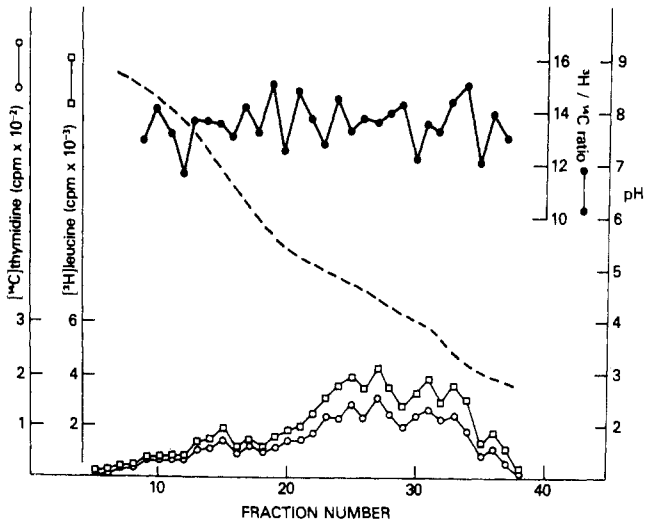

FIGURE 1: Isoelectric focusing of chromatin labeled with [^{14}C]thymidine and [^{3}H]leucine. Cell cultures labeled with either 1 μCi/mL [^{3}H]leucine or 0.05 μCi/mL [^{14}C]thymidine were mixed at a ^{3}H/^{14}C ratio of 15, sheared, and focused as described in Materials and Methods. To each fraction 50 μg of bovine albumin and 5 mL of 10% trichloroacetic acid were added and filtered through GF/C filters. The filters were dried and radioactivity content was determined in a Packard liquid scintillation spectrometer. There was essentially no spillover of ^{3}H counts in the ^{14}C channel. Results were corrected for spillover (20%) of ^{14}C counts in the ^{3}H channel.

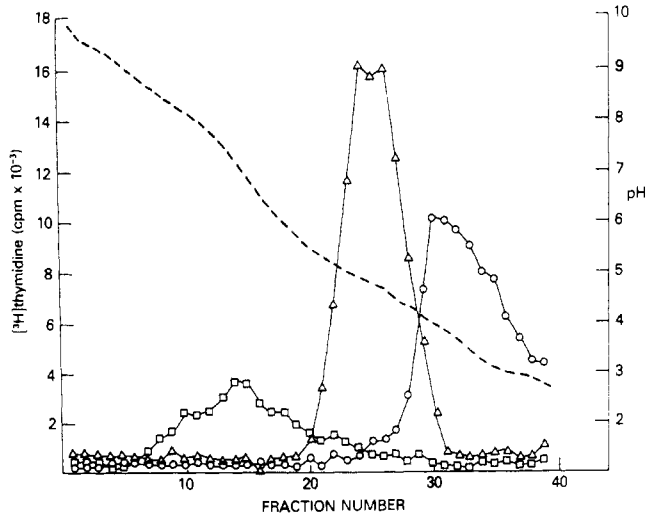

FIGURE 2: Isoelectric focusing of fractionated chromatin. Fractions (obtained in an experiment similar to that shown in Figure 1) with pH ranges 2.8–4, 4–5.5, and 5.5–8 were combined, concentrated with solid RNase-free sucrose, and dialyzed for 3 h against H_2O. The combined chromatin fractions were focused as described in Materials and Methods and analyzed as described in the legend to Figure 1: (○) combined fractions, pH 2.8–4.0; (△) combined fractions, pH 4.0–5.5; (□) combined fractions, pH 5.5–8.5.

sition as in the first run (Figure 2); (b) focusing of chromatin which had been formaldehyde fixed and then sheared; the profile of fixed chromatin was similar to that observed with unfixed chromatin; moreover, on a second rerun, each fraction possessed the same isoelectric point as in the first run (data not shown); (c) IEF of chromatin sheared by different methods. With chromatin sheared by mild digestion by DNase II or by VirTis "45" homogenization, the same IEF profile was obtained as was obtained with chromatin sheared by sonication (data not shown); these experiments suggest that the results obtained reflect a true fractionation of chromatin.

Simultaneous Focusing of [^{3}H]Leucine- and [^{14}C]Thymidine-Labeled Chromatin. As shown in Figure 1, the profile of [^{3}H]leucine-labeled chromatin is essentially superimposable with chromatin labeled with [^{14}C]thymidine. A similar ^{3}H/^{14}C ratio throughout the gradient suggests that the fractions of chromatin with different isoelectric points have very similar protein/DNA ratios.

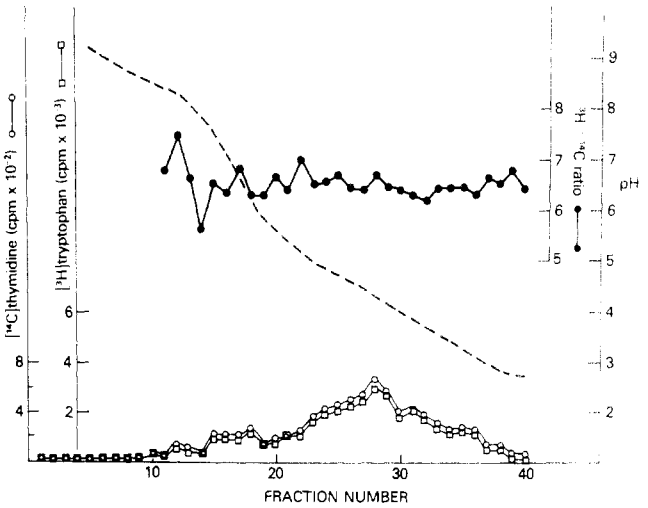

FIGURE 3: Isoelectric focusing of chromatin labeled with [^{14}C]thymidine and [^{3}H]tryptophan. Cell cultures were exponentially labeled with either 1 μCi/mL [^{3}H]tryptophan or 0.05 μCi/mL [^{14}C]thymidine, mixed at a ^{3}H/^{14}C count ratio of 10, sheared, and focused as described in Materials and Methods. Fractions were collected and counted as described in the legend to Figure 1.

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

Chromatin fraction, p*I* =	DNA	Protein	Histone
2.8–4.0	1.0	1.97	1.02
4.0–5.5	1.0	2.01	1.04
5.5–8.5	1.0	1.92	0.89

[a] DNA and protein content were determined as described in Materials and Methods.

Simultaneous Focusing of [^{3}H]Tryptophan- and [^{14}C]-Thymidine-Labeled Chromatin. Tryptophan is found in nonhistone proteins but not in histone proteins (Daly et al., 1950; Crampton et al., 1955). The profile of [^{3}H]tryptophan is similar to chromatin labeled with [^{14}C]thymidine (Figure 3) suggesting that fractions with different isoelectric points have similar nonhistone chromosomal protein to DNA ratios.

Chemical Composition of Fractionated Chromatin. As shown in Table I, the chromatin fraction with p*I* $\simeq$ 7 has a somewhat lower histone/DNA ratio. Gel electrophoresis, reported in the following section, indicates that this difference is significant.

Gel Electrophoresis of Proteins of Fractionated Chromatin. The results of acid-urea gel electrophoresis of histone of chromatin with different p*I* values are presented in Figure 4A. The distribution of the four histones H2a, H2b, H3, and H4 is similar among the different chromatin fractions. However, chromatin focused between 5.5 and 8.5, unlike the other two fractions, does not contain histone H1.

The results of electrophoresis of proteins of each chromatin fraction in 10% polyacrylamide gels containing sodium dodecyl sulfate are shown in Figure 4B. Here, again, H1 is missing in chromatin with p*I* = 5.5–8.5.

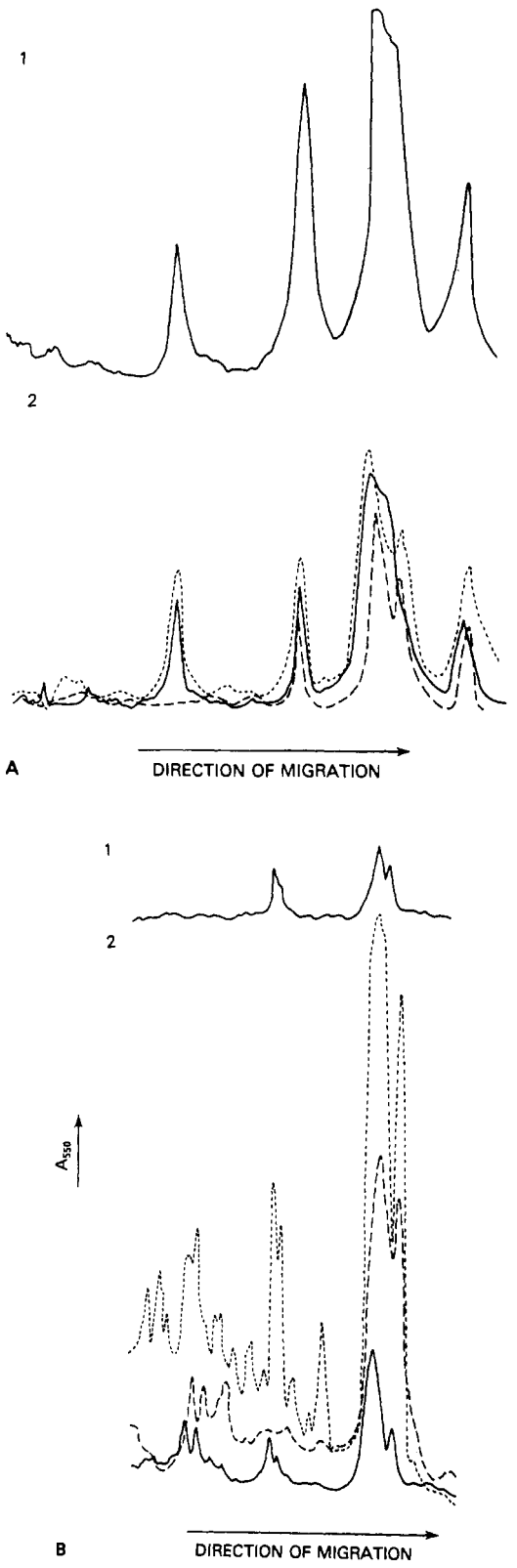

FIGURE 4: (A) Acid-urea gel electrophoresis of histones of fractionated chromatin; 5 OD_{260} (optical density) units of chromatin was fractionated by IEF. Histones of each fraction were obtained by acid extraction: (1) calf-thymus histones (Sigma); (2) (—) chromatin with pI = 2.8–4.0; (···) chromatin with pI = 4.0–5.5; (- - -) chromatin with pI = 5.5–8.5. (B) Sodium dodecyl sulfate gel electrophoresis of proteins from fractionated chromatin. Chromatin was fractionated by IEF, dialyzed against 0.5% acetic acid, and lyophilized. Proteins were extracted by Tris–sodium dodecyl sulfate–β-mercaptoethanol buffer and applied to 10% sodium dodecyl sulfate–acrylamide gel with 2.5% stacking gel as described by Laemmli (1970); (1) calf-thymus histones (Sigma); (2) (—) chromatin with pI = 2.8–4.0; (···) chromatin with pI = 4.0–5.5; (- - -) chromatin with pI = 5.5–8.5.

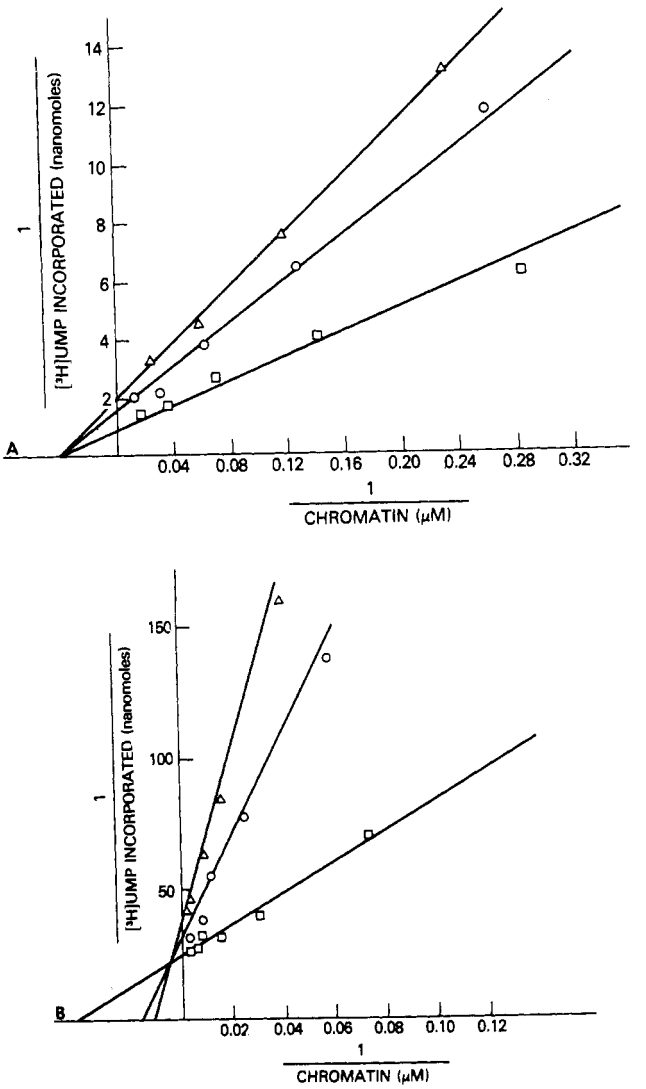

FIGURE 5: (A) RNA synthesis by *E. coli* RNA polymerase as a function of chromatin concentration; comparison of various fractions. Sheared chromatin was fractionated, concentrated, and dialyzed as described in the legend to Figure 1. Assays of each fraction with varying concentration of template and 1.5 unit of enzyme were performed as described in Materials and Methods: (○) pI = 2.8–4.0; (△) pI = 4.0–5.5; (□) pI = 5.5–8.5. (B) RNA synthesis by calf-thymus RNA polymerase II as a function of chromatin concentration; comparison of various fractions. Sheared chromatin was isoelectric focused. Chromatins with pH ranges 2.8–4, 4–5.5, and 5.5–8.5 were combined, concentrated with solid RNase-free sucrose, and dialyzed against 5 mM sodium phosphate buffer (pH 7.5). Template activity of each chromatin fraction was assayed with 0.05 unit of enzyme as described in Materials and Methods: (○) pI = 2.8–4.0; (△) pI = 4.0–5.5; (□) pI = 5.5–8.5.

Chromatins focusing at different pI values differ in their nonhistone protein population (Figure 4B). Chromatin with a pI of 4–5.5 contains the greatest amount of high molecular weight nonhistone proteins, while chromatin focusing between 2.8 and 4 contains less high molecular weight nonhistone proteins. Chromatin focusing between 5.5 and 8.5 contains very little high molecular weight nonhistone proteins and relatively more proteins of lower molecular weight.

Transcription of Chromation Fractions. The fractionated chromatin was examined as a template for *Escherichia* RNA polymerase and calf-thymus RNA polymerases II and III at low (rate limiting) chromatin concentrations (Figure 5, Table II). 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

TABLE II: Kinetic Parameters of Transcription of Chromatin Fractions.[a]

Template	K_m (μM)	V_{max} (nmol)
E. coli RNA polymerase		
Chromatin with p*I* = 2.5–4.0	25	0.666
Chromatin with p*I* = 4.0–5.5	25	0.555
Chromatin with p*I* = 5.5–8.5	25	1.176
Calf-thymus RNA polymerase II		
Chromatin with p*I* = 2.5–4.0	66.6	0.033
Chromatin with p*I* = 4.0–5.5	100.0	0.029
Chromatin with p*I* = 5.5–8.5	24.4	0.042
Calf-thymus DNA	41.6	0.063
Unfractionated chromatin	90.9	0.029
Calf-thymus RNA polymerase III		
Chromatin with p*I* = 2.5–4.0	33.3	0.059
Chromatin with p*I* = 4.0–5.5	66.6	0.059
Chromatin with p*I* = 5.5–8.5	7.1	0.059
Calf-thymus DNA	17.5	0.123
Unfractionated chromatin	39.4	0.053

[a] RNA polymerase assays were performed as described in Materials and Methods and the legend to Figure 5.

polymerase (Keshgegian and Furth, 1972), kinetic parameters (apparent K_m and V_{max}) have been determined for different 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 the chromatin fraction with different p*I* values (Figure 5A). The values for V_{max} and K_m calculated from this plot are shown in Table II. As previously reported (Marushige and Bonner, 1966; Keshgegian and Furth, 1972), the apparent K_m for chromatin is the same as that of native DNA with *E. coli* RNA polymerase. The transcription of chromatin fractions with different p*I* values 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 chromatin with a p*I* of 5.5–8.5. The increase in template activity of this 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 template concentration with calf-thymus polymerases (II and III) and IEF fractionated chromatin, a pattern distinctly different from the result with *E. coli* RNA polymerase is observed. For RNA polymerase II (Figure 5B) chromatin with a p*I* of 5.5–8.5 shows increased template activity in comparison to both more acidic as well as unfractionated chromatin. Calculated K_m values (Table II) indicate that this increase in template activity is due not to an increase in V_{max} but rather to a large decrease in the apparent K_m.

Similar results are obtained for RNA polymerase III (Table II). While the V_{max} for chromatin with different p*I* values is the same, 0.059 nmol, the K_m for p*I* = 5.5 to 8.5 chromatin is 7.1 μM compared to 33.3 and 66.6 μM for the more acidic fractions.

For both eukaryotic polymerases, the basic chromatin fraction has a lower K_m than DNA. This could be due to the ability of RNA polymerase to initiate at some DNA sites only when chromatin proteins are present or, alternatively, to binding of the polymerase at ends or nicks in the DNA of sheared chromatin. However, this does not account for the differences between the various chromatin fractions, for on alkaline sucrose gradients, DNA isolated from the various fractions showed identical profiles.

Discussion

It is generally believed that the proteins of chromatin play a major role in limiting the template activity of the DNA to which they are bound, and that this limitation is related to gene regulation. The recent discovery of nucleosome structure has thrown new light on the understanding of chromatin structure.

We have used isoelectric focusing to fractionate nucleoli depleted chromatin because of its simplicity and power of resolution. The IEF profile of the interphase chromatin of HeLa cells is very different from that of metaphase chromosomes of HeLa cells which focus at pH 3.5–4.3 (Landel et al., 1972). There appears to be a heterogeneous population of chromatin fragments with various p*I* values. Strikingly, we cannot find any stretches of free DNA 1000 base pairs or larger in length. This is in agreement with the recent work of Varshavsky et al. (1976) who studied fixed chromatin by CsCl gradient centrifugation. Their previous work (Varshavsky et al., 1974), suggesting the existence of long stretches of free DNA in chromatin, may be due to chromosomal protein rearrangement at elevated ionic strength in vitro.

It is interesting that three major fractions of chromatin with p*I* of 2.8–4, 4–5.5, and 5.5–8.5, respectively, are obtained by IEF. From the rerun and gel profile of chromosomal proteins we consider it very unlikely that chromatin with p*I* = 4–5.5 is a mixture of the other two major fractions. It can be theorized that there is really no absolute demarcation between chromatin of p*I* = 2.5–4, 4–5.5, and 5.5–8.5. More likely there is a gradual transition from one to the other. (We have used shallower gradients and indeed obtain further broadening of the profile.)

The possibility of rearrangement of chromosomal proteins during mechanical fragmentation of chromatin in our experimental procedure seems unlikely. We have obtained similar IEF results with nuclease digested chromatin. In addition, when chromatin is fixed with formaldehyde and then subjected to brief mechanical shearing, no differences can be found in the resultant IEF profiles. The use of the protease inhibitor $PhCH_2SO_2F$ also reduced the possible effect of proteases released during chromatin preparation. This would argue against the notion that differences in the gel profiles of chromatin fractions are artifactual.

Basic chromatin, with a p*I* of 5.5–8.5, does not contain H1 histone. It has been suggested that H1 histone is responsible for forming the superhelical structure of the chromosome and the folded structure may decrease the accessibility of the genome to RNA polymerases. While the nonhistone protein/DNA ratio of chromatin with different p*I* values appears to be similar, gel electrophoresis patterns suggest that there are different populations of nonhistone proteins in different chromatin fractions. Acidic fractions, with p*I* of 2.8–4.0 and 4–5.5, contain more high molecular weight proteins than basic fractions. These high molecular weight proteins may be the structural proteins of chromatin which bind to repressed chromatin fragments (Comings and Harris, 1975).

The protein composition analysis strongly suggests that basic chromatin with p*I* of 5.5–8.5 may be involved in active transcription. When different fractions of chromatin were assayed by both bacterial and mammalian RNA polymerase, basic chromatin possesses much higher template activity. This would be expected if the basic chromatin contains the transcriptionally active fraction, since RNA polymerase II is thought to transcribe mRNA and RNA polymerase III is thought to transcribe tRNA and 5S RNA (Weinman and Roeder, 1974).

From our enzyme kinetic data, there appear to be important

differences between bacterial and mammalian RNA polymerases in their interactions with chromatin during transcription, as previously suggested by the kinetic studies of Keshgegian and Furth (1972) and Henner et al. (1975). To reiterate, *E. coli* polymerase does not interact with more sites on active chromatin than inactive chromatin since the K_m values for different fractions of chromatin 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 the transcription of the basic chromatin. In contrast, while calf-thymus RNA polymerases II and III demonstrate essentially the same V_{max} for different chromatin fractions, they both show a much lower K_m for the basic chromatin than for the acidic fractions. This suggests that the basic chromatin offers a much greater frequency of binding sites for the mammalian RNA polymerases, as would be expected for an "active" chromatin fraction. We conclude that genes are maintained as active and inactive in chromatin by controls of a type not present in the bacterial cell and eukaryotic RNA polymerase responds to these controls.

There is a constant proportion of histone H2a, H2b, H3, H4, and DNA in both the active and nonactive regions. There is no dramatic change in the nonhistone population but rather a gradual transition from the active to inactive portions and the nonhistone proteins do not simply expose more free DNA to the RNA polymerase.

Since shearing can alter chromatin (Noll et al., 1975; Nicolini et al., 1976), it would be of interest to perform kinetic studies with chromatin fractions obtained by limited nuclease digestion. Shearing was utilized in the present experiments as nuclease digestion must be carefully controlled to prevent extensive degradation of DNA as well as inevitable introduction of single-strand breaks. It should also be noted that the nucleosome structure of chromatin is preserved in chromatin dispersed by ultrasonication (Oudet et al., 1975), and brief shearing need not cause a substantial change in histone binding to DNA or a change in the structure of both histones and DNA (Maciewicz and Li, 1977).

The basic fraction of chromatin obtained in the present experiments is only 28% of total chromatin. In the experiments of Henner et al. (1975), transcriptionally active chromatin obtained by sucrose gradient centrifugation constituted 5–10% of total chromatin. We currently are determining the relationship of the basic fraction to active chromatin isolated by sucrose gradient centrifugation and whether cells which are transcriptionally more active have an increased proportion of this fraction.

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Properties of Rabbit Muscle Phosphofructokinase Modified with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole†

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ABSTRACT: A single sulfhydryl group per polypeptide chain of rabbit muscle phosphofructokinase has been specifically labeled with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) at pH 7. The rate of reaction is similar in the presence of fructose 6-phosphate, fructose 1,6-bisphosphate, Mg^{2+}, and low ATP concentrations (100 μM) but is markedly decreased by high concentrations of MgATP (5 mM). At higher molar ratios of reagent to protomer (5:1 vs. 2.5:1) more than a single sulfhydryl group is labeled. The labeled enzyme has a specific activity 15-30% that of the native enzyme. Removal of NBD from the enzyme by dithiothreitol only partially restores the activity (up to 60%) although the circular dichroism and aggregation state of the protein are unchanged by the chemical modification. The modified enzyme has a considerably larger Michaelis constant for MgATP (six- to tenfold) than the native enzyme at both pH 7.0 and 8.0. The inhibition constant for adenylyl imidodiphosphate also increases. The modified enzyme is strongly inhibited by MgATP at pH 7.0 but at concentrations approximately twice as large as with the native enzyme. The Michaelis constant for fructose 6-phosphate is approximately the same for both the native and modified enzyme at pH 8.0. However, at pH 7.0 the cooperativity associated with fructose 6-phosphate binding is considerably reduced with the Hill coefficient changing from 4.0 to 1.4. The location of the modified sulfhydryl group with respect to the MgATP catalytic site and inhibitory site is uncertain since modification has a major effect on both sites.

Rabbit muscle phosphofructokinase is a key enzyme in the regulation of glycolysis. The enzyme activity is altered by a number of metabolites (Passoneau and Lowry, 1962, 1963) and the steady kinetic properties of the enzyme are complex at pH 7 and below (Hofer and Pette, 1968), suggesting the enzyme has allosteric properties. The enzyme is made up of apparently identical subunits of molecular weight 80 000 (Leonard and Walker, 1972; Pavelich and Hammes, 1973; Coffee et al., 1973), and its regulation apparently involves both aggregation phenomena and conformational changes within a tetramer or higher aggregate (Lad et al., 1973; Hill and Hammes, 1975; Frieden et al., 1976).

In the work reported here, a single sulfhydryl group (per subunit of mol wt 80 000) has been modified with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, NBD-Cl,[1] and the effects of this modification on the kinetic properties of the enzyme have been determined. The results obtained indicate that the primary effects of modifying the sulfhydryl group are to decrease the affinity of the enzyme for MgATP (also for AMP-PNP and MgAMP-PNP) at the catalytic site, to reduce the cooperativity in the fructose 6-phosphate-enzyme interaction at pH 7.0, and to reduce the catalytic activity to 15-30% of that of the native enzyme.

Experimental Section

Chemicals. The ATP, ADP, fructose 6-phosphate, fructose 1,6-bisphosphate, dithiothreitol, aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, and bovine serum albumin were purchased from Sigma Chemicals. The AMP-PMP was obtained from P-L Biochemicals. The NBD-Cl was obtained from Pierce Chemical Co. All other chemicals were the best available commercial products, and all solutions were prepared with deionized distilled water.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling et al. (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate (pH 8.0), 1.0 mM EDTA and dialyzed against the same buffer to give a stock solution of 10-14 mg/mL. The enzyme concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.02 mL mg^{-1} cm^{-1} (Parmeggiani et al., 1966). The specific activity of the enzyme at 23 °C (33 mM Tris-Cl, 2 mM fructose 6-phosphate, 2 mM ATP, 5 mM $MgCl_2$, pH 8.0) was 100-120 units/mg. (A unit of enzyme activity is defined as the formation of 1 μmol of product/min.) After a period of about 4 weeks, the specific activity of the enzyme stock solution declines significantly. Only enzyme of specific activity greater than 90 units/mg was employed to obtain the results presented here.

Reaction of Phosphofructokinase with NBD-Cl. The reaction of the enzyme with NBD-Cl was carried out in 25 mM diglycine, 25 mM potassium phosphate (pH 7.2), 0.4 mM fructose 6-phosphate, 0.1 mM ATP, 1 mM EDTA at 23 °C or in the same buffer at pH 7.0 with 5 mM fructose 6-phos-

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[1] Abbreviations used are: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AMP-PNP, adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid.