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Modulation of Transcription from Chromatin Assembled in Vitro†

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ABSTRACT: A small plasmid DNA was assembled into chromatin in vitro by incubation in an extract prepared from eggs of *Xenopus laevis*. The plasmid DNA contained the regulatory region of the *Escherichia coli lac* operon, the transcription of which is under positive regulation by catabolite activator protein (CAP) and negative regulation by *lac* repressor. After incubation in the egg extract the plasmid DNA acquired ~60% of the predicted maximum number of nucleosomes. Chromatin was treated with protein and DNA cross-linking agents prior to transcription in order to demonstrate that regions of the DNA organized into nucleosomes served as templates for transcription. Cross-linking abolished transcription of chromatin but had no effect on transcription of DNA, suggesting that transcription of untreated chromatin was not solely attributable to nucleosome-free regions. In support of this conclusion, the average size of the RNA transcribed from chromatin was ~1000 bases, which was ~5 times longer than the average distance between nucleosomes. Transcription of in vitro assembled plasmid chromatin by *E. coli* RNA polymerase was stimulated by catabolite activator protein. The CAP-mediated stimulation of transcription was detectable as an increase in total transcription that was specific to chromatin made from a plasmid containing the *lac* regulatory DNA sequences. The specific increase in the amount of RNA whose synthesis was initiated within the *lac* region was demonstrated by hybridization of transcription products to complementary DNA fragments bound to nitrocellulose filters. Preliminary investigation of the action of *lac* repressor suggested that it also modulated transcription from the chromatin template.

The DNA in both transcribed and untranscribed regions of eucaryotic chromosomes is associated with histones to form nucleosomes [Lacy & Axel, 1975; for a review, see Chambon (1978)]. However, it is clear from the appearance in the electron microscope and from sensitivity to nuclease digestion that transcriptionally active chromatin differs in organization from inactive chromatin (Foe, 1978; McKnight et al., 1978; Weintraub & Groudine, 1976; Garel & Axel, 1976; Bonner et al., 1974; Gottesfeld & Partington, 1977). In order to examine how transcription occurs through nucleosomes and what structural alterations underly changes in template activity, efforts have been directed toward the development of in vitro transcription systems (Williamson & Felsenfeld, 1978; Wasylyk et al., 1979b; Ng et al., 1979).

In this paper we describe an in vitro transcription system in which the template was chromatin that had been assembled in vitro by incubating DNA in a crude extract prepared from the eggs of the toad *Xenopus laevis*. Laskey et al. (1977) showed that this extract contains the histones and all other factors required for the assembly of DNA into nucleosomes. It seems likely that chromatin is assembled in the egg extract under conditions similar to those for synthesis of chromatin in vivo.

The DNA used for the assembly into chromatin was a bacterial plasmid containing a segment of DNA with the regulatory region of the *Escherichia coli lac* operon (Miller

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& Reznikoff, 1978). Expression of the *lac* operon is under the control of *lac* repressor and catabolite activator protein. Therefore, this system allowed the analysis of the influence of regulatory proteins on transcription of chromatin. This system preserves the homology of the specific protein–DNA interactions for both regulatory proteins and RNA polymerase. The question was posed whether such sequence-specific regulatory mechanisms function in vitro within a chromatin-like template.

Materials and Methods

Bacterial Strains and Plasmids. The bacterial strains are derivatives of *E. coli* K12. RR1 is a *rec*A$^+$ derivative of HB101, F$^-$ *pro leu thi lac*Y *Str*^{r} $r_K^-m_K^-$ EndoI$^-$ *rec*A$^-$ (Bolivar et al., 1977a). LH1 is a low thymine requiring derivative of HB101 which was obtained by selection with trimethoprim (Miller, 1972). Cultures of HB101 grown under nonselective conditions were plated and restreaked onto selective plates containing 10 μg/mL trimethoprim (Sigma) and 50 μg/mL thymine. The resultant high thymine requirers were grown in liquid culture with 10 μg/mL trimethoprim and 50 μg/mL thymine followed by plating and restreaking onto plates with 4 μg/mL thymine. Supplemented M9 without casamino acids was used throughout the selection.

The bacterial host for the plasmids pMB9 (Bolivar et al., 1977a) and pBGP100 (Polisky et al., 1976) was RR1. The bacterial hosts for the plasmids pBR322 (Bolivar et al., 1977b) and pBH20Δ(*Eco*RI–*Sal*I) were the *rec*A$^-$ strains HB101 and LH1 in order to eliminate the possibility of formation of multimeric plasmid DNAs.

The plasmid pBH20 (Itakura et al., 1977) is a derivative of pBR322. pBH20Δ(*Eco*RI–*Sal*I) was constructed by removing the small *Eco*RI–*Sal*I fragment from pBH20. The nucleotides complementary to the single-stranded ends of the large fragment produced by *Eco*RI and *Sal*I digestion were filled in by using T4 DNA polymerase, and the resulting blunt ends were ligated with T4 DNA ligase (H. Heyneker, unpublished experiments).

Purification of Plasmid DNA. Cells were grown in supplemented M9 which contained, in addition to the M9 salts described by Miller (1972), 0.4% glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 μg/mL vitamin B_1, 100 μg/mL proline, 100 μg/mL leucine, 0.4% casamino acids, and 20 μg/mL ampicillin or tetracycline, the latter being used only for pMB9. When cells were grown for radioactive labeling of the DNA, the casamino acid solution was adsorbed with activated charcoal (BDH). Strain LH1 was used for all radioactive labeling in order to obtain plasmid DNA with a specific activity greater than 250 000 cpm/μg. At OD_{650} = 1 chloramphenicol was added to 250 μg/mL (Clewell, 1972). For unlabeled DNA, the cells were harvested 15–20 h later by centrifugation at 8000 rpm for 10 min in a Sorvall SS34 rotor. For labeled DNA, 20 μCi/mL [^{3}H]thymine or [^{3}H]thymidine was added 1 to 2 h after the chloramphenicol addition and the cells were harvested 6 h later. The cell pellets were washed with 10 mM Tris, pH 7.9, and 1 mM EDTA (TE).[1] (All Tris solutions were adjusted to the given pH at room temperature as 1 M Tris-HCl. NaEDTA solutions were adjusted to pH 7.5 in 0.1 M stocks. When EDTA was used together with Tris, only the pH of the Tris is given.)

The cleared lysate was prepared from a frozen cell pellet as described by Clewell & Helinski (1969) except that 0.1% Triton X-100 replaced Brij 58 and deoxycholate (Kupersztoch & Helinski, 1973). Centrifugation was performed for 60 min at 18 000 rpm in a Sorvall SS34 rotor. The cleared lysate was digested with 10 μg/mL RNase A (Sigma) for 30 min at 37 °C, extracted once with an equal volume of phenol–chloroform (1:1) and once with chloroform–isoamyl alcohol (24:1), adjusted to 0.3 M NaCl, and precipitated with 2 volumes of ethanol. The DNA was sedimented in CsCl–propidium iodide gradients (Smith et al., 1971) to remove residual bacterial chromosomal DNA. The DNA was dissolved in 10 mL of TE, pH 7.9. Then 11 g of CsCl and 0.75 mL of propidium iodide (2 mg/mL) were added and centrifugation was performed for at least 24 h in a Beckman 50Ti at 43 000 rpm. The gradient was illuminated with light of wavelength 375 nm, the lower DNA band was collected, and the propidium iodide was removed by extraction 10 times with CsCl-saturated 2-propanol (Sebring et al., 1971), while maintaining the volume with TE, pH 7.9. Contaminating RNA was removed by chromatography on a Bio-Gel A50 (Bio-Rad) column in TE, pH 7.9, plus 0.1 M NaCl. The plasmid DNA was ethanol precipitated and dialyzed extensively against 10 mM Tris, pH 7.9, and 0.1 mM EDTA.

Purification of Proteins. (*a*) *Catabolite Activator Protein.* Catabolite activator protein was purified from *E. coli* KLF41/JC1553 with modification of the procedure described by Anderson et al. (1971). The cells were grown in a high-density fermentor in 10 g of tryptone, 22 g of yeast extract, and 4 mL of glycerol per L. The cell pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 M KCl, 0.1 mM DTT, 0.1 mM NaEDTA, and 5% glycerol and were broken by sonification twice for 30 s each. After digestion for 30 min at 0 °C with 3 μg/mL DNase, the supernatant was recovered following centrifugation in the Beckman 35 rotor at 30 000 rpm for 2.5 h. Ammonium sulfate precipitation was performed first by the addition at 0 °C of 28 g of solid $(NH_4)_2SO_4$ per 100 mL, while maintaining pH 7.5 with NH_4OH. The precipitate was resuspended in and dialyzed against buffer B of Anderson et al. (1971) to which was added 0.05 M KCl. Phosphocellulose chromatography was performed as described except that the starting elution buffer contained 0.05 M KCl; cAMP binding activity eluted at ~0.7 M KCl. Precipitation with 60% $(NH_4)_2SO_4$ and gel filtration on Sephadex G-100 were as described except that the buffer was 60 mM Tris-HCl, pH 8, 0.05 M KCl, 0.5 mM DTT, and 5% glycerol. Fractionation on DEAE-cellulose was the last step and was carried out by passing the pooled material from the Sephadex column through a 0.7-mL column of DE52 (Whatman) equilibrated with the same buffer as was used for the Sephadex column. The flow through was concentrated by dialysis against 20 mM potassium phosphate, pH 7, 50 mM KCl, 0.1 mM NaEDTA, 0.5 mM DTT, and 50% glycerol and was stored at −20 °C. Purity was estimated at 70% by gel electrophoretic analysis.

(*b*) *Lac Repressor.* *Lac* repressor, purified as described by Rosenberg et al. (1977), was generously provided by A. Riggs.

(*c*) *Histones.* *X. laevis* histones were purified from blood as described by Destree et al. (1973).

Preparation of Extract from Eggs of X. laevis. The procedure developed by Laskey et al. (1977) for the preparation of the egg extract for chromatin assembly was followed with

[1] Abbreviations used: Ap^r, ampicillin resistance; βME, β-mercaptoethanol; cAMP, adenosine cyclic 3′,5′-monophosphate; CAP, catabolite activator protein; DNase, deoxyribonuclease; DTT, dithiothreitol; kb, kilobase pairs; RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 0.015 M sodium citrate; Cl_3AcOH, trichloroacetic acid; Tc^r, tetracycline resistance; Tc, designates sequence homology with Tc^r regions but plasmid does not confer resistance to tetracycline; TE, 10 mM Tris and 1 mM EDTA; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

minor modifications which are described below. The toads were injected in the dorsal lymph sac with 500 units of human chorionic gonadotropin (Sigma) 8–12 h prior to collection of the eggs; for most preparations the animals had also been injected previously with pregnant mare serum. The toads were kept in modified Ringer's solution, and the eggs that accumulated in the tank were collected. In some cases eggs were also squeezed from the animals. The eggs were dejellied with 1.2% cysteine, pH 7.8, and were washed with modified Ringer's solution (0.1 M NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.5 mM Tris-HCl, pH 7.8, and 0.01% $NaHCO_3$). Homogenization was done in a glass Dounce with a pestle with a clearance of 0.025 in. The final extract, which was stored in liquid nitrogen, was stable to freezing and thawing at least 3 or 4 times.

Chromatin Assembly. The conditions used for chromatin assembly were those described by Laskey et al. (1977). The specific activity of the DNA was adjusted to 250000 cpm/μg by mixing tritiated and unlabeled DNA. The DNA was lyophilized to dryness before the addition of egg extract; the contribution of buffer to the final reaction was less than 10 mM Tris, pH 7.9, and 0.1 mM EDTA. The usual reaction conditions were a 3-h incubation at 19 to 20 °C with 20 μL of egg extract per μg of DNA, except as noted. The dilution buffer used in some experiments, 10 mM Hepes, pH 8, 0.12 M KCl, and 2 mM $MgCl_2$, is a modification of that described by Laskey et al. (1978). *E. coli* ligase (500 units/mL) was obtained from New England Biolabs. T4 ligase (500 units/mL) was purified as described by Panet et al. (1973). The ligase reaction conditions are described in the figure legend.

Sucrose Gradient Sedimentation. The conditions for sucrose gradient sedimentation were those described by Laskey et al. (1977); 5-mL 5–20% linear sucrose gradients contained 60 mM KCl, 20 mM Tris, pH 7.5, and 1 mM EDTA. The samples to be loaded on the gradients, generally 50–150 μL, were adjusted to 10 mM EDTA, pH 7.5, 0.25% Triton X-100, and 10 mM Tris, pH 7.9. Centrifugation was performed at 4 °C in a Beckman SW50.1 at 44000 rpm for 1.75 h, except as noted. Gradients were fractionated by collection from the top with a Densi-flow gradient collector (Buchler). The fractions were collected into polypropylene tubes to avoid loss of chromatin in glass tubes. Tritiated DNA was located by spotting aliquots from the fractions directly onto glass fiber filters and counting in 0.4% Omnifluor in toluene.

Preparation of Chromatin for Transcription. The chromatin-containing fractions from the sucrose gradient were pooled in 2-mL ($^5/_{16} \times 1^{15}/_{16}$ in.) cellulose nitrate tubes and centrifuged at 4 °C for 12–24 h in a Beckman 50Ti at 40000 rpm. The pellet was resuspended in a small volume, generally 10–20 μL, of 10 mM Tris, pH 7.9, and 0.1 mM EDTA. Approximately 35–65% recovery of chromatin was achieved through the gradient and pelleting steps, as measured by radioactivity in DNA.

Conditions for Fixing Chromatin. Fixation with formaldehyde and glutaraldehyde was performed by following the procedure of Christiansen & Griffith (1977). The sample was adjusted to 0.01 M NaCl and 0.02 M sodium phosphate, pH 7.5. One-tenth volume of 10% formaldehyde, freshly diluted in H_2O, was added for 15 min at 0 °C. Then 0.1 volume of 6% glutaraldehyde, also freshly diluted in H_2O and filtered through a 0.45-μm nitrocellulose filter, was added and the incubation at 0 °C was continued for 15 min.

Transcription of DNA and Chromatin. DNA and chromatin templates were transcribed at 37 °C with *E. coli* RNA polymerase using the reaction conditions described by Burgess (1969): 40 mM Tris-HCl, pH 7.9, 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 0.1 mM NaEDTA, pH 7.5. Each of the four nucleoside triphosphates was present at 50 μM, except as noted. The radioactively labeled nucleoside triphosphate, either [^{3}H]CTP or [^{32}P]UTP, was present at 1–5 mCi/mL. *E. coli* RNA polymerase (specific activity ~800 units/mg) was obtained from Miles Research Laboratories. The concentration of chromatin is given in terms of the DNA content, as determined from the specific activity of the tritiated DNA used to prepare the chromatin. When the regulatory proteins were included, the order of addition was repressor, CAP, RNA polymerase, and nucleoside triphosphates, with brief incubation at 20 or 37 °C between each step. Since glycerol was introduced into the reactions with CAP, the glycerol in all samples was adjusted to the same concentration, to a maximum of 5%. RNA synthesis was measured by incorporation of radioactivity into material that was precipitated in 10% Cl_3AcOH.

Purification of RNA for Hybridization and Gel Electrophoresis. In preparation for filter hybridization, the DNA transcription reactions were terminated by sevenfold dilution into 20 mM Tris, pH 7.5, and 10 mM $MgCl_2$ and digestion with 10 μg/mL pancreatic DNase (Worthington, DPFF) for 30 min at 37 °C. DNase digestion was repeated following ethanol precipitation in the presence of 0.2 M sodium acetate, pH 5, and carrier tRNA. Extraction with chloroform–isoamyl alcohol (24:1) and a second ethanol precipitation were performed. To terminate chromatin transcription, the reactions were diluted to a final concentration of 10 mM EDTA, pH 7.5, 1% sodium lauroylsarcosinate, 0.2 M sodium acetate, pH 5, and 0.5 mg/mL carrier tRNA. The samples were extracted with an equal volume of chloroform–isoamyl alcohol (24:1) and precipitated with 2 volumes of ethanol. The precipitates were resuspended in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 10 μg/mL DNase. After digestion for 30 min at 37 °C, the samples were adjusted to 0.2 M sodium acetate, pH 5, and ethanol precipitated. DNase digestion and chloroform–isoamyl alcohol extraction were repeated, followed by three ethanol precipitations to decrease the amount of unincorporated [^{32}P]UTP.

For gel electrophoresis of RNA, the transcripts from both DNA and chromatin were processed identically. The reactions were terminated as described above for preparation of chromatin transcripts for hybridization. Extraction with chloroform–isoamyl alcohol was performed, followed by ethanol precipitation and digestion once with DNase. Then three ethanol precipitations were carried out to reduce the amount of residual [^{32}P]UTP.

Restriction Endonuclease Digestion. Digestions were carried out at 37 °C with 1 unit of enzyme per μg of DNA in the following buffers: *Hpa*I (New England Biolabs) in 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 60 mM KCl, and 5 mM βME; *Eco*RI [prepared according to Greene et al. (1978)] in 100 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 100 mM NaCl.

Gel Electrophoresis. Slab gels containing 1% agarose (Seakem) were used for the separation of restriction enzyme fragments of DNA. The gel and electrode buffer was 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.7 (Loening, 1969). Electrophoresis was conducted at 65 V for 7 h at 4 °C with recirculation of the buffer.

Composite gels of 2% acrylamide–0.5% agarose were prepared by following the procedure described by Peacock & Dingman (1968). *N,N,N′,N′*-Tetramethylethylenediamine (Temed, Eastman) was added last to the other prewarmed, degassed components; the final composition was 0.5% agarose,

1.9% acrylamide, 0.1% *N,N'*-methylenebis(acrylamide), 0.05% ammonium persulfate, and 0.0078% Temed.

Supercoiled DNA molecules were resolved on acrylamide–agarose gels as described by Germond et al. (1975) with Tris–phosphate buffer as described above. The gels were run at 50 V, 4 °C, with recirculation of the buffer. For the resolution of RNA on acrylamide–agarose gels the buffer was 10 mM sodium phosphate, pH 7. Denaturation of RNA and DNA with glyoxal was accomplished as described by McMaster & Carmichael (1977). Electrophoresis was carried out at 100 V for 2.5 h at room temperature with recirculation of the buffer. The portion of the gel with the DNA marker fragments was soaked in 0.1 M NaOH for 1 h and neutralized in 1 M Tris, pH 7.5, for at least 30 min before staining with ethidium bromide.

Photography, Fluorography, and Autoradiography. DNA was visualized by staining the gel in 0.5 μg/mL ethidium bromide in H_2O for ~30 min. Following brief destaining in H_2O, gels were photographed over an ultraviolet light of wavelength 254 nm, using a yellow filter (Kodak No. 9 Wratten gelatin filter) and Polaroid film (Type 55). For the detection of [^{3}H]DNA, the acrylamide–agarose gels were impregnated with Omnifluor (NEN) by following the procedure of Bonner & Laskey (1974). The gels were dried onto heavy paper (Hoefer) under vacuum and with heat. Kodak X-Omat film, which had been hypersensitized by incubation at 65 °C for at least 4 h, was exposed at −70 °C. Kodak No-Screen film, exposed at room temperature, was used for the detection of [^{32}P]RNA.

Densitometric scans of autoradiograms were obtained with a Gilford spectrophotometer at a wavelength of 600 nm. These measurements were performed with autoradiograms in which the intensity of the bands was within the linear range of the sensitivity of the film. A Du Pont 310 curve resolver was used for resolving slightly overlapping peaks and computing relative areas.

Nucleic Acid Hybridization. Transfer of DNA from agarose gels to nitrocellulose filters (Millipore, 0.45 μm) was performed as described by Southern (1975) except that 6 × SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate) was used for the transfer. Hybridization of [^{32}P]RNA to the filter-bound DNA was carried out at 37 °C in the following hybridization mixture: 50% formamide, 3 × SSC, 10 mM Hepes, pH 8, 0.1% $NaDodSO_4$, 1 mM EDTA, 100 μg/mL carrier tRNA, and 0.02% each of Ficoll, poly(vinylpyrrolidone), and bovine serum albumin (Calbiochem, cryst. A grade) (Denhardt, 1966). Following hybridization for 12–24 h the filters were placed in 400 mL of 2 × SSC at room temperature and then heated to 65 °C for 30–60 min. They were then transferred to 400 mL of 2 × SSC with 5 μg/mL RNase A (Sigma) and incubated 30–60 min at 37 °C. The final wash was in either 1 × SSC at room temperature or 2 × SSC and 0.5% $NaDodSO_4$ at 65 °C.

Results

In studies of chromatin transcription it is important to determine whether the observed transcription actually occurs from the chromatin template or from small amounts of free DNA in the chromatin preparation. Therefore, we will describe first the assembly of chromatin and the experiments which suggest that regions of the chromatin organized into nucleosomes do serve as templates for transcription. Secondly, the experiments which demonstrate regulation of *lac*-specific transcription from chromatin will be presented.

Characterization of the Template. (a) Chromatin Assembly. For the conversion of plasmid DNA to chromatin, the method of Laskey et al. (1977) was used; the plasmid DNA designated pBH20Δ(*Eco*RI–*Sal*I) was incubated with egg extract at 20 °C for 3 h at a ratio of 20 μL of extract per μg of DNA. This reaction resulted in the conversion of the DNA to a more rapidly sedimenting complex on a sucrose gradient (Figure 1). Whereas the nucleohistone complex sedimented approximately three-fourths of the distance down the gradient, free DNA remained near the top of the gradient. When DNA was mixed with egg extract in the presence of 10 mM Na-EDTA, pH 7.5, and 0.25% Triton X-100, conditions under which nucleosomes fail to assemble onto DNA (Laskey et al., 1977), the DNA was shifted to an intermediate position on the gradient. Therefore, in assessing the acquisition of chromatin structure, we compared the position of sedimentation to this intermediate species rather than to free DNA. In the studies described in this paper the DNA was uniformly converted to the rapidly sedimenting nucleohistone complex. No second peak or shoulder was observed at the position of the apparent nonnucleosomal DNA–protein complex.

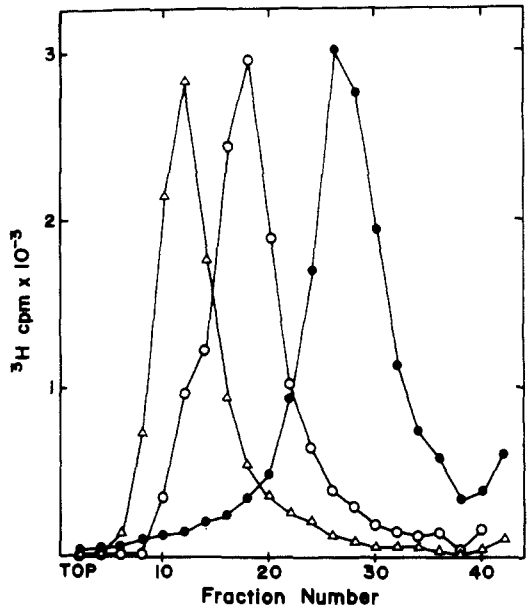

FIGURE 1: Sucrose gradient sedimentation of pBH20Δ(*Eco*RI–*Sal*I) DNA after incubation in *Xenopus* egg extract. (●) 0.25 μg of ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was incubated in 5 μL of egg extract for 3 h at 19 °C as described under Materials and Methods. Then 100 μL of stop mix (10 mM NaEDTA, pH 7.5, 0.25% Triton X-100, and 10 mM Tris-HCl, pH 7.9) was added. (○) 0.25 μg of ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was first mixed with 100 μL of stop mix. Then 5 μL of egg extract was added immediately before loading the sample on the gradient. (△) 0.25 μg of ^{3}H-labeled pBH20Δ(*Ec*RI–*Sal*I) DNA was mixed with 100 μL of stop mix. The samples were sedimented through 5–20% sucrose gradients for 1.75 h, and fractions were collected directly onto glass fiber filters as described under Materials and Methods.

The insertion of superhelical turns into the plasmid DNA as a result of incubation in the egg extract was another criterion by which the acquisition of chromatin structure was assessed. Germond et al. (1975) described a gel electrophoresis system which resolves small supercoiled DNA molecules based on the number of superhelical turns. These investigators showed that a correlation exists between the number of nucleosomes and the number of superhelical turns such that each nucleosome causes the DNA to acquire the equivalent of ~1.25 superhelical turns.

When pBH20Δ(*Eco*RI–*Sal*I) DNA was incubated with egg extract and then displayed on a gel, a regular array of supercoiled molecules was seen. The DNA in lane A of Figure 2 had been incubated with egg extract under the standard

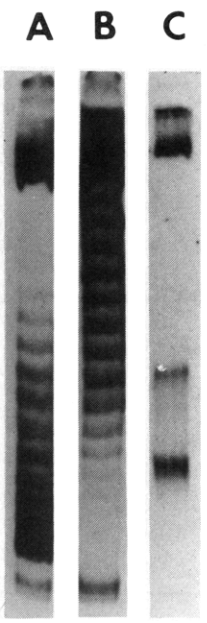

FIGURE 2: Acquistition of superhelical turns in DNA during incubation in egg extract. (A) 0.25 μg of ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was incubated for 3 h with 5 μL of egg extract. (B) 0.25 μg of ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was incubated for 3 h with 2.5 μL of egg extract and 0.25 μg of *Xenopus* histones in 2.5 μL of dilution buffer (see Materials and Methods). (C) 0.05 μg of ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA, without incubation in egg extract. The reactions were stopped by the addition of 15 μL of 10 mM Tris, pH 7.9, 1 mM EDTA, and 1% sodium lauroylsarcosinate and adjusted directly (without organic extraction) to the conditions for loading on the gel. Electrophoresis was performed for 68 h as described under Materials and Methods.

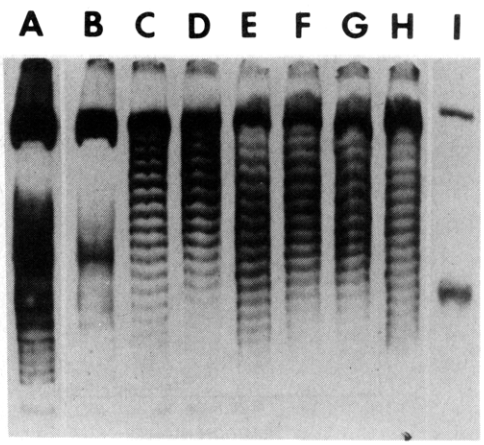

FIGURE 3: Effect of *E. coli* and T4 DNA ligase on assembly of chromatin. 0.25 μg of ^{3}H-labeled pBR322 DNA was incubated for 3 h with 5 μL of *Xenopus* egg extract and 2.5 μL of dilution buffer (see Materials and Methods) in the presence of 0.5 unit of DNA ligase under the following conditions. (A) Same as lane B except 3 times longer exposure of film to show supercoiled molecules; lanes B–I are all from the same film exposure. (B) No ligase; (C) T4 ligase present throughout 3-h incubation; (D) *E. coli* ligase throughout 3-h incubation; (E) T4 ligase added for last hour of incubation; (F) *E. coli* ligase added for last hour of incubation; (G) T4 ligase added for last hour of incubation, at which time reaction was adjusted to 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.25 mM ATP, 5 mM DTT; (H) *E. coli* ligase added during last hour of incubation and reaction adjusted to 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, and 26 μM NAD; (I) 0.06 μg of [^{3}H]DNA, no incubation with egg extract. The samples were prepared for the gel as described in the legend of Figure 2, and electrophoresis was performed for 56 h.

reaction conditions. The DNA in lane B was assembled into nucleosomes under conditions different from the standard ones and is included in the figure in order to show that 17 bands of superhelical DNA were resolved between nonsupercoiled DNA and the most rapidly migrating species. No conditions were found in which all the DNA acquired the maximum superhelix density without extensive nicking. Under our standard conditions the DNA acquired an average superhelix density of ~14 turns, corresponding to ~11 nucleosomes. Since pBH20Δ(*Eco*RI–*Sal*I) has ~3900 base pairs, these 11 nucleosomes must be spaced at intervals such that the average internucleosome distance is 210 base pairs, assuming 140 base pairs per nucleosome core.

In addition to the supercoiled DNA, some material migrated as nonsupercoiled DNA near the top of the gel. Laskey et al. (1977) reported that the DNA at that position represents both nicked circles and covalently closed relaxed circles. Distinguishing between the two species is important because nicked DNA could have been organized into nucleosomes before being deproteinized for electrophoresis whereas covalently closed relaxed circles must have been present as free DNA before deproteinization.

We added purified *E. coli* or T4 DNA ligase to the assembly reaction as a means of reducing the amount of nicked DNA. Figure 3 shows the result of adding the ligases under a variety of conditions. Under all conditions of ligase addition the intensity of the band at the position of nicked or covalently closed relaxed DNA was significantly reduced; the most effective reduction occurred with T4 ligase when the buffer conditions were adjusted to those optimal for the enzyme (lane F). However, in all samples with ligase the average superhelix density was lower than that achieved in the absence of ligase.

Although ligase was not included in our standard assembly reactions because of the decrease in the average superhelix density, these results indicate that most of the DNA at the position of nicked circles and covalently closed relaxed circles was nicked. These data are consistent with the idea that most of the DNA at that position was organized into nucleosomes at the time that the nick was repaired. In conjunction with the observation that, upon incubation with egg extract, all the DNA was converted to a more rapidly sedimenting complex on a sucrose gradient and the reported noncooperative nature of the assembly process (Laskey et al., 1977), these data argue against the presence of free DNA molecules within these preparations of chromatin.

(*b*) *Preparation of Chromatin for Transcription.* A two-step sedimentation protocol was employed for purifying and concentrating the chromatin in preparation for transcription. The first step was sedimentation in a 5–20% sucrose gradient such that the peak of chromatin was about three-fourths of the distance down the gradient and the bulk of the proteins remained near the top of the gradient. In Figure 4 the position of chromatin is shown with respect to the sedimentation pattern of the proteins in the egg extract; the distribution that resulted from loading either 50 or 160 μL of egg extract is shown. There was considerable trailing of proteins into the fractions containing chromatin when 160 μL of egg extract was loaded on the gradient. Therefore, no more than 100 μL was loaded on each gradient for all subsequent experiments. A rough estimate of the amount of protein that cosedimented with the chromatin was obtained by summing the protein content of the chromatin-containing fractions. There was ~10–20 μg of protein per μg of DNA, representing 5–10% of the total protein.

The fractions from the sucrose gradient that contained chromatin were pooled, the total volume being ~2 mL from a 5-mL gradient, and a second sedimentation was performed as described under Materials and Methods. The pellet was resuspended in a small volume of 10 mM Tris-HCl, pH 7.9, and 0.1 mM EDTA. This material was readily solubilized and

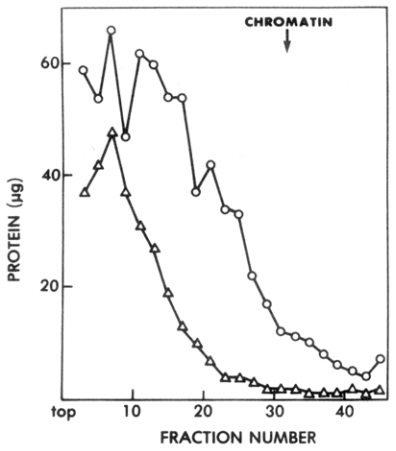

FIGURE 4: Sucrose gradient sedimentation of proteins in *Xenopus* egg extract. (○) 160 μL of *Xenopus* egg extract was incubated with 8 μg of ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA. After 3 h the reaction was adjusted to 10 mM NaEDTA, pH 7.5, 0.25% Triton X-100, and 10 mM Tris-HCl, pH 7.9, and sedimented for 2 h through a 5–20% sucrose gradient as described under Materials and Methods. (△) 50 μL of egg extract, without incubation with DNA, was sedimented as described above. The arrow marks the position of the peak of the chromatin-containing fractions. 50-μL aliquots from the fractions were assayed for protein content by using the Amido Schwartz procedure (Schaffner & Weissmann, 1973). Values greater than ~30 μg are estimates, since they were off the scale of the standard curve.

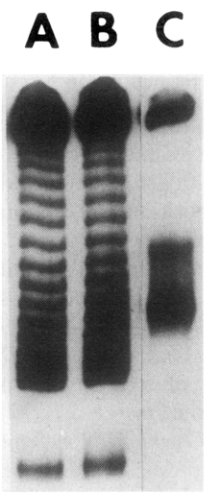

FIGURE 5: Retention of superhelix density through steps in preparation for transcription. ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was incubated for 3 h with egg extract at a ratio of 20 μL of extract per μg of DNA. The complex was sedimented through a 5–20% sucrose gradient. From the pooled chromatin-containing fractions an aliquot was removed and prepared for electrophoresis as described below. The remainder of the chromatin was sedimented and the pellet resuspended as described under Materials and Methods. Both samples were extracted with chloroform–isoamyl alcohol in the presence of 1% sodium lauroylsarcosinate and 5 μg of carrier tRNA and ethanol precipitated. Electrophoresis was performed for 57 h as described under Materials and Methods. (A) Sample from pooled sucrose gradient fractions; (B) sample from resuspended chromatin pellet; (C) ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA, no incubation in egg extract.

was not removed from solution by brief centrifugation, observations consistent with the absence of histone H1 (Griffith & Christiansen, 1978). The recovery of chromatin through these steps varied from 35 to 65%. The superhelix density of the chromatin remained unchanged from the gradient through the pelleting step, although there was some increase in the amount of nicked material (Figure 5). The chromatin prepared in this way also retained its shifted migration with respect to DNA when rerun on a sucrose gradient. (See the section on treatment of chromatin with fixing agents.)

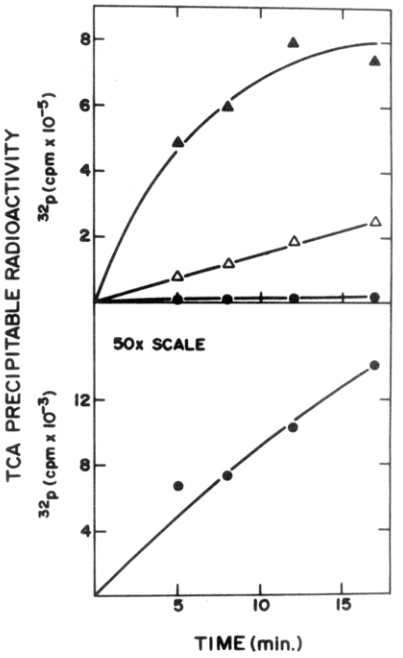

FIGURE 6: Transcription of pBH20Δ(*Eco*RI–*Sal*I) DNA, nonnucleosomal complexes, and chromatin. Transcription was carried out as described under Materials and Methods with 10 μg/mL template, 100 μg/mL RNA polymerase, and 2 mCi/mL [^{32}P]UTP. Transcription was measured by the incorporation of radioactivity into Cl_3AcOH-precipitable material per 2 μL. (▲) DNA; (△) nonnucleosomal complexes (DNA mixed with egg extract in the presence of Triton X-100 and EDTA; see text); (●) chromatin.

(*c*) *Transcription of Chromatin.* Figure 6 shows the relative levels of transcription from DNA and chromatin. In this experiment the initial rate of transcription from DNA was 100-fold greater than that from chromatin; this value varied between about 30- and 100-fold. However, when DNA that had been incubated with egg extract in the presence of Triton X-100 and EDTA as described earlier was transcribed under the same conditions, a fivefold reduction in transcription resulted. Therefore, we estimate that the reduction in the rate of transcription attributable to the deposition of nucleosomes was about 10–20-fold. These findings are consistent with the degree of reduction in transcription that has been reported previously for histone–DNA complexes (Cedar & Felsenfeld, 1973; Bustin, 1978; Williamson & Felsenfeld, 1978; Wasylyk et al., 1979b). It is unlikely that this transcription was due to free DNA because chromatin from the leading and trailing edges of the sucrose gradient peak had similar initial rates of transcription.

(*d*) *Analysis of Transcripts by Gel Electrophoresis.* The size of the RNA transcribed from DNA and chromatin in the experiment shown in Figure 6 was determined by gel electrophoresis of RNA that had been denatured with glyoxal (McMaster & Carmichael, 1977). Figure 7 displays the RNA transcribed from chromatin and DNA as a function of time. For both templates the size of the transcripts increased with time up to 6.5 min (see lanes A–C for each template). The size distribution of RNA molecules was centered at ~800–1000 bases by 6.5 min and remained relatively constant up to 15 min, as shown in lanes D and E.

It appears that transcription from chromatin proceeded with a slower rate of elongation since after 3 min the size of the transcripts from chromatin was less than those from DNA (compare lanes B). However, the data displayed in lane F show that when DNA transcripts were mixed with chromatin

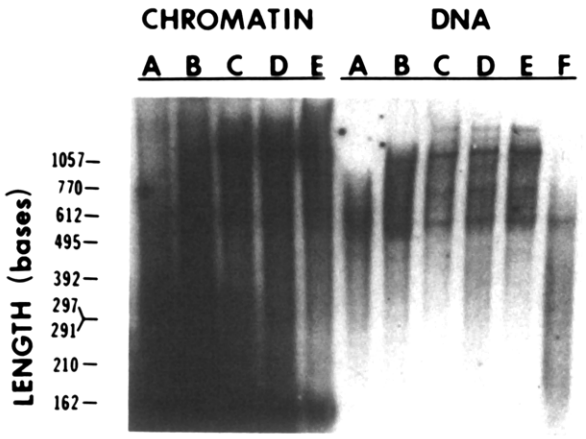

FIGURE 7: Gel electrophoresis of RNA transcribed from pBH20Δ(*Eco*RI–*Sal*I) DNA and chromatin. RNA was transcribed as described under Materials and Methods with 10 μg/mL template, 100 μg/mL RNA polymerase, and 2 mCi/mL [^{32}P]UTP. The samples were prepared for the gel, treated with glyoxal, and subjected to electrophoresis as described under Materials and Methods. φX174 DNA that had been digested with *Hin*cII and treated with glyoxal was used for the size markers. A broad band at the position of 345, 341, and 335 bases has not been designated in the figure. Aliquots were removed from each reaction mix at (A) 1.5, (B) 3.0, (C) 6.5, (D) 9.5, and (E) 15 min. The material in lane F of the DNA transcripts was prepared by transferring at 3.5 min a 2-μL aliquot from the DNA transcription reaction into a 22.5-μL reaction mix containing all the components of a chromatin transcription reaction except the [^{32}P]UTP. Incubation was continued for 15 min.

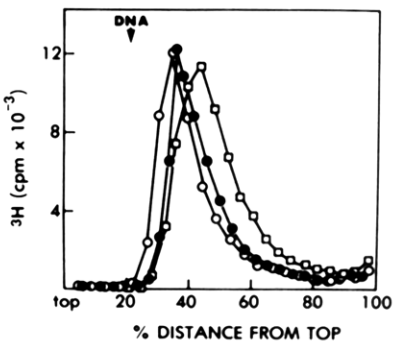

FIGURE 8: Sucrose gradient sedimentation of chromatin treated with fixing agents. ^{3}H-Labeled pBH20Δ(*Eco*RI–*Sal*I) chromatin was prepared, purified through a sucrose gradient, and pelleted as described under Materials and Methods. The pellet was resuspended in 15 μL of 0.2 mM NaEDTA, pH 7.5. One-third of this material was prepared directly for the second sucrose gradient sedimentation. The remaining 10 μL was treated with formaldehyde and glutaraldehyde, in a total volume of 120 μL, as described under Materials and Methods. The solution was adjusted to 10 mM NaEDTA, pH 7.5, 0.25% Triton X-100, and 10 mM Tris-HCl, pH 7.9; then to half of it was added $NaDodSO_4$ to a concentration of 1%. Each of these samples was then sedimented through a 5–20% sucrose gradient for 90 min as described under Materials and Methods. (□) Untreated chromatin (resuspended pellet after first sucrose gradient); (○) chromatin cross-linked with formaldehyde and glutaraldehyde; (●) cross-linked chromatin plus $NaDodSO_4$. The arrow marks the position of free DNA in a separate gradient.

after 3.5 min of transcription and incubated for an additional 15 min, degradation of the RNA occurred. Therefore, the apparent elongation rate of chromatin transcripts is a minimum estimate because of simultaneous degradation. Differences in the elongation rate cannot account for the 100-fold difference in template activity between DNA and chromatin. We conclude that this difference was primarily due to a decrease in initiation of transcription from chromatin.

Several discrete RNA species were apparent in the transcripts from both templates (shown in Figure 7), with the most prominent bands appearing at approximately 1100, 600, and 150 nucleotides. The 150-nucleotide transcript was very commonly observed in DNA transcripts, although in this particular experiment it appeared as a faint band. Although no attempt was made to assign these transcripts to particular portions of the plasmid DNA, their appearance does provide evidence that discrete initiation and termination events occurred during transcription of chromatin.

The data on the average size of chromatin transcripts suggest that transcription occurred along DNA that was organized into nucleosomes. There were on the average 11 nucleosomes/DNA molecule of 3900 base pairs (see Figure 2), giving an average of 210 base pairs of internucleosomal DNA and 140 base pairs of core DNA per nucleosome. If only stretches of internucleosomal DNA were transcribed, we would expect the RNA transcripts to average less than 210 bases. Alternatively, if nucleosomes were not evenly spaced along the DNA, it is possible that large RNA molecules were transcribed from long nucleosome-free stretches. It is conceivable that nucleosomes could apply constraints to the local melting required for RNA synthesis. However, since the denaturation of less than 10 base pairs is required for the initiation of RNA synthesis by *E. coli* RNA polymerase (Chamberlin, 1974), it seems unlikely that a distance of greater than 1000 bases (the average size of the chromatin transcripts) would be the minimum separation required between nucleosomes before RNA synthesis could begin. Although these data do not eliminate the possibility that nucleosomes slide along the DNA, previous studies have found that sliding is undetectable or occurs only very slowly under physiological salt conditions (Beard, 1978; Mathis et al., 1978; Weischet, 1979).

(*e*) *Treatment of Chromatin with Fixing Agents.* The effect on transcription of prior exposure of the template to fixing agents was examined to approach the question of whether the observed transcription might have occurred from free DNA rather than from the chromatin template. The rationale behind this approach is that if cross-linking blocks transcription from chromatin but does not affect transcription from DNA, then the observed RNA synthesis from chromatin is probably not due to naked DNA in the chromatin preparation.

The conditions employed for fixation of chromatin were those of Christiansen & Griffith (1977). These investigators showed that fixation of the SV40 nucleohistone complex with formaldehyde and glutaraldehyde allowed the retention of the native sedimentation behavior and appearance under the electron microscope. In Figure 8 the sedimentation of fixed chromatin through a sucrose gradient is shown compared to that of untreated chromatin. The fixation caused a slight shift in the sedimentation position, but the pattern of sedimentation was very similar to that of untreated chromatin; most significantly, the fixing did not cause the chromatin to aggregate. The fixed chromatin was completely resistant to dissociation by $NaDodSO_4$, whereas when unfixed chromatin was treated with $NaDodSO_4$, it sedimented at the same position as free DNA, which is designated in Figure 8 by an arrow.

The effect of fixation on transcription is demonstrated in Figure 9. Transcription of chromatin was completely blocked by the fixing treatment (Figure 9A). On the other hand, fixation had little or no effect on transcription of free DNA or DNA that had been exposed to the fixing conditions in the presence of chromatin, as shown in parts B and C of Figure 9.

These data suggest that the proteins in chromatin became fixed where they were bound to the DNA. If there were naked regions of DNA within the chromatin preparation, proteins probably did not become fixed there during the cross-linking.

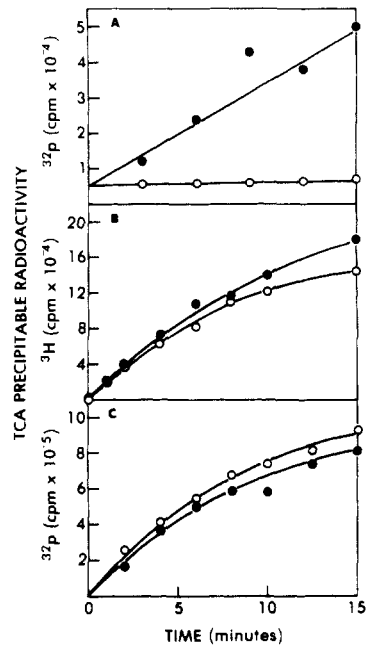

FIGURE 9: Transcription of chromatin and DNA treated with fixing agents. (●) Unfixed template; (○) fixed template. (A) Transcription of chromatin. The preparation of chromatin from ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA, purification through the two-step sedimentation protocol, and treatment with fixatives were as described under Materials and Methods. Both fixed and unfixed chromatin samples were then pelleted through a cushion of 20% sucrose in the same buffer as the sucrose gradients, under the same conditions as for the first pelleting step. The pellets were resuspended in 0.2 mM NaEDTA, pH 7.5. Transcription was carried out with 10 μg/mL template, 100 μg/mL RNA polymerase, and 2 mCi/mL [^{32}P]UTP. (B) Transcription of DNA. ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was exposed to fixing agents and pelleted through sucrose as described above. Unfixed DNA was prepared in parallel. The pellets were resuspended in 10 mM Tris, pH 7.9, and 0.1 mM EDTA. Transcription was carried out with 2 μg/mL DNA, 20 μg/mL RNA polymerase, and 1 mCi/mL [^{3}H]CTP. (C) Transcription of DNA fixed in the presence of chromatin. Chromatin was prepared from unlabeled DNA in parallel with ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA under standard conditions. After the chromatin was pelleted, ^{3}H-labeled pBH20Δ(*Eco*RI–*Sal*I) DNA was mixed with unlabeled chromatin, in an amount estimated to be approximately equal to the amount of DNA in chromatin. Fixation and pelleting through sucrose were performed as described above. An identical sample that was not exposed to fixing agents was prepared in parallel. The pellets were resuspended in 10 mM Tris, pH 7.9, and 0.1 mM EDTA. Transcription was performed as described under Materials and Methods with 3 μg/mL [^{3}H]DNA template, 100 μg/mL RNA polymerase, and 5 mCi/mL [^{32}P]UTP.

The possibility of sliding of proteins onto regions that had been naked has not been eliminated, but such an event would leave other regions relatively deficient in protein.

It is clear that the transcription that occurred from chromatin was not due to molecules of DNA that were entirely free of nucleosomes. On the other hand, if all the transcription of chromatin were due to synthesis between nucleosomes, it seems likely that at least some of this synthesis in large nucleosome-free regions could have still occurred after fixation. In this regard, Williamson & Felsenfeld (1978) have shown that transcription can occur from a fixed chromatin template with large nucleosome-free regions that are on the average 800–900 base pairs long. Unless fixation of a single nucleosome to the DNA is sufficient to block all transcription, our results support the view that transcription of untreated chromatin proceeded through nucleosomes.

Regulation of Transcription from Chromatin. (*a*) *Description of the Template.* The plasmid pBH20Δ(*Eco*RI–*Sal*I) contains the *E. coli lac* promoter and operator, the CAP

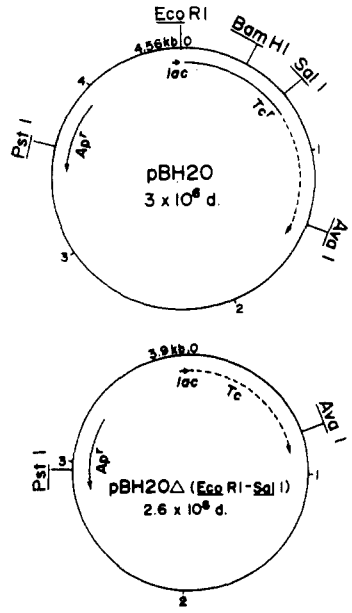

FIGURE 10: Diagrams of the plasmids pBH20 and pBH20Δ(*Eco*RI–*Sal*I). The molecular weights of the plasmids are given in the center of the circles, and around the outside the number of kilobases are shown. The locations of the genes specifying ampicillin (Ap^r) and tetracycline (Tc^r) resistance, as well as the directions in which they are transcribed, are marked by arrows. Note that the deletion of the *Eco*RI–*Sal*I fragment renders pBH20Δ(*EcoRI–SalI*) sensitive to tetracycline, but the region of homology with the Tc^r region of pBH20 is indicated by a dashed line. The position of the *lac* sequences and the direction in which the 60 bases of *lac*-specific RNA are synthesized are designated by the arrow labeled *lac*.

binding site, and the first 60 nucleotides of the β-galactosidase gene. The position of the *lac* sequences, the directions of transcription of *lac* and plasmid genes, and the locations of restriction enzyme sites of this plasmid and pBH20, from which pBH20Δ(*Eco*RI–*Sal*I) was derived, are shown in Figure 10. Modification of pBH20 to pBH20Δ(*Eco*RI–*Sal*I) removed the promoter for the gene(s) for tetracycline resistance (Tc^r) and therefore offered the possibility of reduced transcription downstream from the *lac* promoter. This allows readthrough transcription from the *lac* promoter to be assayed by hybridization to DNA fragments containing Tc^r sequences with a lower background of hybridization due to RNA that was initiated in that region.

(*b*) *CAP-Mediated Stimulation of Transcription.* Transcription of pBH20Δ(*Eco*RI–*Sal*I) DNA in the presence of CAP was stimulated by the addition of the effector ligand cAMP; this stimulation was detectable as an increase in total RNA synthesis (Figure 11A). No CAP- and cAMP-dependent stimulation of transcription occurred with pBR322 DNA, which is the parent plasmid containing no *lac* sequences (Figure 11B). Therefore, we interpret the cAMP-dependent stimulation of transcription in the presence of CAP as an increase in synthesis initiating at the *lac* promoter.

Total transcription of chromatin prepared from pBH20Δ(*Eco*RI–*Sal*I) was also stimulated by CAP and cAMP as shown in Figure 11C. The effect was again specific for the *lac*-containing template, since no CAP- and cAMP-dependent stimulation occurred with pBR322 chromatin (Figure 11D). In the absence of CAP, cAMP had no stimulatory effect on transcription of pBR322 or pBH20Δ(*Eco*RI–*Sal*I) DNA or chromatin prepared from either DNA (data not shown).

CAP- and cAMP-mediated stimulation of total transcription was used to study the dependence of the effect on the concentration of CAP. The concentration of CAP was varied from 0 to 20 μg/mL. At each CAP concentration, two samples were

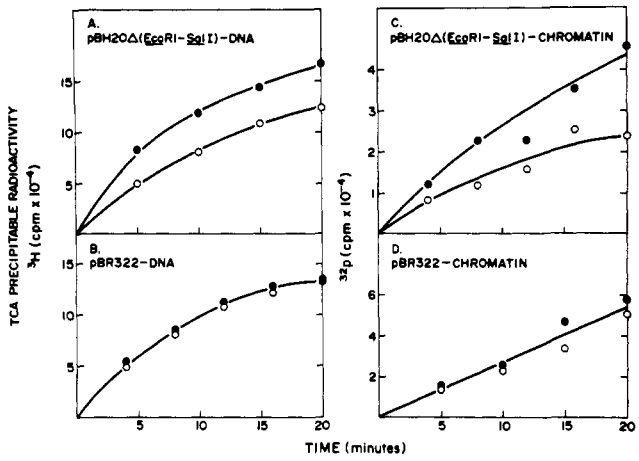

FIGURE 11: Effect of CAP and cAMP on transcription from DNA and chromatin prepared from pBH20Δ(*Eco*RI–*Sal*I) and pBR322. (A) pBH20Δ(*Eco*RI–*Sal*I) DNA. (B) pBR322 DNA. DNA transcription was carried out as described under Materials and Methods with 5 μg/mL template, 10 μg/mL RNA polymerase, and 1 mCi/mL [^{3}H]CTP. Aliquots of 3 μL were assayed for Cl_3AcOH-precipitable radioactivity. (C) pBH20Δ(*Eco*RI–*Sal*I) chromatin. (D) pBR322 chromatin. Chromatin transcription reactions contained 10 μg/mL template, 100 μg/mL RNA polymerase, and 2 mCi/mL [^{32}P]UTP. Aliquots of 3 μL were assayed. cAMP was used at 10^{-3} M and CAP was present at 10 μg/mL in the DNA transcriptions and 20 μg/mL in the chromatin reactions. (○) +CAP, no cAMP; (●) + CAP, + cAMP.

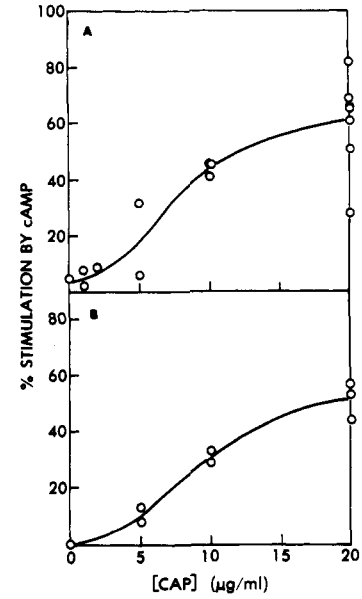

FIGURE 12: cAMP-mediated stimulation of transcription of DNA and chromatin as a function of CAP concentration. (A) pBH20Δ-(*Eco*RI–*Sal*I) DNA was transcribed with 5 μg/mL DNA, 10 μg/mL RNA polymerase, and 1 mCi/mL [^{3}H]CTP. (B) Chromatin prepared from pBH20Δ(*Eco*RI–*Sal*I) DNA was transcribed as described in the text with 5–10 μg/mL template, 100 μg/mL RNA polymerase, and 2 or 5 mCi/mL [^{32}P]UTP. Transcription was measured by incorporation of radioactivity into Cl_3AcOH-precipitable material. Percent stimulation by cAMP was determined as described in the text.

prepared which were identical except that one contained no cAMP and the other contained 10^{-3} M cAMP. Several time points were taken from each reaction which generated rate curves analogous to those shown in Figure 11. In Figure 12A the percent stimulation by cAMP after 10 min of transcription is plotted as a function of CAP concentration, with pBH20Δ(*Eco*RI–*Sal*I) DNA as the template. In this system half-maximal stimulation occurred at a CAP concentration of ~7 μg/mL. An exact comparison with other in vitro transcription systems cannot be made since the concentration of active CAP molecules is not known; however, this value is in good agreement with the expected value based on the range of half-maximal CAP concentrations determined by de Crombrugghe et al. (1971). These investigators found that the CAP concentration for half-maximal stimulation of *lac* synthesis varied with template concentration. When the correction is made for the size of their template compared to pBH20Δ(*Eco*RI–*Sal*I), a value between ~2 and ~10 μg/mL can be estimated for the expected half-maximal CAP concentration in this system.

When this assay was carried out with chromatin prepared from pBH20Δ(*Eco*RI–*Sal*I) DNA, the dependence of cAMP-mediated stimulation of transcription on CAP concentration was very similar to that observed for naked DNA as shown in Figure 12B. The determination of CAP-dependent stimulation of transcription of chromatin was carried out with a different concentration of RNA polymerase than that used in the DNA transcription assay, and it is possible that this may affect the level of stimulation. However, it should have no effect on the concentration of CAP required for half-maximal stimulation of transcription and this value was very similar for chromatin and DNA. This observation suggests that the affinity of CAP for its DNA binding site was not dramatically altered by the presence of nucleosomes.

(*c*) *Characterization of Transcripts by Hybridization.* Characterization of the RNA synthesized in response to CAP and cAMP was accomplished by hybridization to DNA bound to filters by the Southern procedure. Since pBH20Δ(*Eco*RI–*Sal*I) contains only 60 nucleotides of the β-galactosidase gene, most of the RNA initiated at the *lac* promoter will be complementary to the Tc region of the plasmid, due to readthrough transcription. A DNA fragment containing sequences complementary to the Tc region of pBH20Δ(*Eco*RI–*Sal*I) was obtained from the related plasmid pMB9 in order to assay transcription from that region. Digestion of pMB9 with *Eco*RI and *Hpa*I yields two fragments, the larger of which contains sequences homologous to pBH20Δ(*Eco*RI–*Sal*I) from position 0 to ~1.1 kb on the map in Figure 10; this is slightly beyond the Tc region. All other regions of homology are contained on the other *Eco*RI–*Hpa*I fragment of pMB9.

RNA that was transcribed from pBH20Δ(*Eco*RI–*Sal*I) DNA in the absence or presence of 10 μg/mL CAP and 10^{-3} M cAMP was hybridized to filters containing the two *Eco*RI–*Hpa*I fragments of pMB9 (Figure 13A). The effect of CAP is clearly seen as an increase in the amount of RNA hybridized to pMB9 fragment A, which contains sequences homologous to the Tc region of pBH20Δ(*Eco*RI–*Sal*I). Densitometric scans of the autoradiograms were normalized to the level of hybridization to pMB9 fragment B in Figure 13A in order to quantitate the degree of stimulation of transcription by CAP. The area under peak A relative to peak B increased 4.7-fold, from 0.11 to 0.52, in response to CAP.

The transcripts from chromatin prepared from pBH20Δ-(*Eco*RI–*Sal*I) were analyzed in the same way for CAP-mediated stimulation of transcription. Figure 13B shows that transcription of the Tc region increased in the presence of CAP; hybridization to pMB9 fragment A relative to fragment B increased twofold from 0.15 to 0.30. That the degree of CAP-mediated stimulation of transcription is less for chromatin than for DNA is probably in part due to the slower apparent rate of elongation observed for chromatin (Figure 7). This assertion implies that the degree of stimulation of *lac*-specific initiation of transcription was more similar for the two templates than was revealed by this assay.

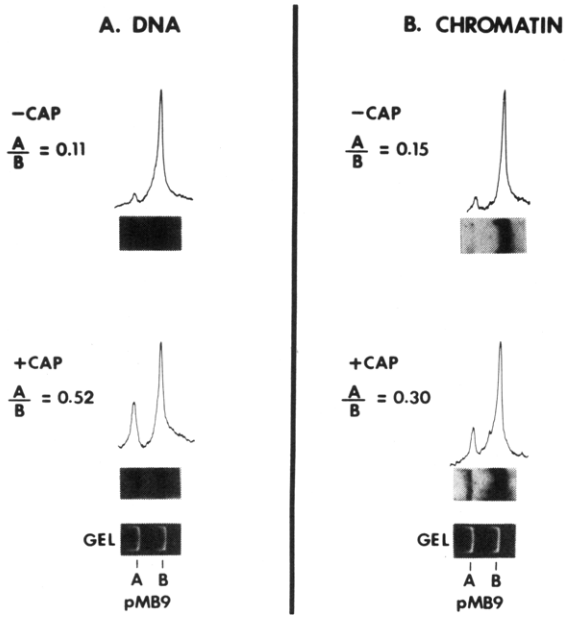

FIGURE 13: Hybridization of RNA, transcribed from pBH20Δ-(*Eco*RI–*Sal*I) DNA and chromatin in the absence or presence of CAP, to restriction enzyme fragments of pMB9. (A) pBH20Δ(*Eco*RI–*Sal*I) DNA transcripts. Transcription was carried out as described under Materials and Methods with 100 μg/mL DNA, 10 μg/mL RNA polymerase, 30 μM nucleoside triphosphates, 1.3 mCi/mL [^{32}P]UTP, and with or without 10 μg/mL CAP and 10^{-3} M cAMP. Transcription proceeded for 4 min. (B) pBH20Δ(*Eco*RI–*Sal*I) chromatin transcripts. Transcription was carried out with 12 μg/mL template, 100 μg/mL RNA polymerase, 5 mCi/mL [^{32}P]UTP, and with or without 10 μg/mL CAP and 10^{-3} M cAMP. Transcription proceeded for 5 min. The preparation of samples, separation of pMB9 fragments by electrophoresis, hybridization, and densitometric scans were performed as described under Materials and Methods. For each filter strip, 100 ng of pMB9 DNA was transferred from the gel. The hybridization mixes with DNA transcripts contained 1.3×10^6 Cl_3AcOH-precipitable cpm in 300 μL; those with chromatin transcripts contained 500 000 precipitable cpm in 400 μL.

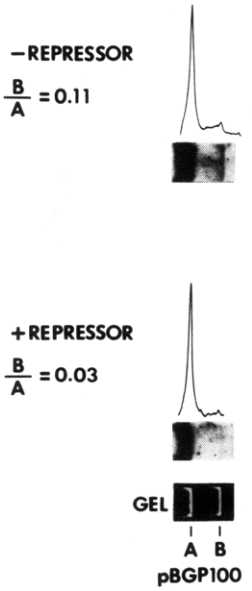

FIGURE 14: Hybridization of RNA, transcribed from pBH20Δ-(*Eco*RI–*Sal*I) chromatin in the absence or presence of *lac* repressor, to restriction enzyme fragments of pBGP100. Transcription was carried out with 12 μg/mL template, 100 μg/mL RNA polymerase, 5 mCi/mL [^{32}P]UTP, 10^{-3} M cAMP, 10 μg/mL CAP, and with or without *lac* repressor at 100 μg/mL. Transcription proceeded for 5 min. The preparation of samples, separation of pBGP100 fragments by electrophoresis, hybridization, and densitometric scans were performed as described under Materials and Methods. For each filter strip, 400 ng of pBGP100 DNA was transferred from the gel. The hybridization mixes contained 500 000 Cl_3AcOH-precipitable cpm in 400 μL.

(*d*) *Action of Lac Repressor.* A preliminary investigation of the action of *lac* repressor revealed that it also regulated *lac*-specific transcription from chromatin. The assay was carried out by hybridization of transcripts to *lac*-specific DNA. The source of the *lac* DNA was an *Eco*RI fragment of λp*lac*5 phage DNA which has been inserted into the *Eco*RI site of the plasmid RSF2124 to generate a plasmid designated pBGP100. Digestion of pBGP100 with *Eco*RI generates two fragments, the smaller of which contains only *lac* sequences in common with pBH20Δ(*Eco*RI–*Sal*I). The larger *Eco*RI fragment of pBGP100 contains sequences homologous to the Ap^r region but not the Tc region of pBH20Δ(*Eco*RI–*Sal*I).

RNA was transcribed from pBH20Δ(*Eco*RI–*Sal*I) chromatin in the absence or presence of *lac* repressor, with CAP and cAMP present in both cases. Figure 14 shows the hybridization of this RNA to the two *Eco*RI fragments of pBGP100. Hybridization to fragment B which contains the *lac* sequences was reduced to a low level when repressor was present during transcription. Comparison of relative areas under peaks A and B can only give an imprecise estimate of the degree of repression since hybridization to fragment B is small compared to fragment A; nevertheless, this analysis suggests that *lac* repressor decreased transcription of *lac* sequences nearly fourfold.

Discussion

Efforts have been directed toward developing in vitro systems which preserve the fidelity of transcription of the in vivo systems in order to elucidate the mechanisms of specific gene expression in eucaryotes. It has been possible in only a few systems, however, to demonstrate specific, accurate initiation of transcription in minimally disrupted isolated nuclei (Sklar & Roeder, 1977; Jaehning & Roeder, 1977; Smith et al., 1978; Tekamp et al., 1979) and from chromatin (Parker et al., 1978; Tekamp et al., 1979). Reconstituted systems have been developed in which specific initiation was detected when transcription of purified DNA was carried out in extracts from *Xenopus* oocytes (Birkenmeier et al., 1978; Schmidt et al., 1978; Ng et al., 1979) or extracts from cultured human cells (Wu, 1978). It seems likely that the DNA became organized into nucleosomes in the extracts from *Xenopus* oocytes since the conditions were very similar to those described by Laskey et al. (1977) and used for the work described in this paper. However, the structure of the template was not investigated in those studies. In some cases the kinetics of total RNA synthesis were not consistent with a progressive decrease in the available template by organization into nucleosomes which would be predicted from the observations described in this paper. Williamson & Felsenfeld (1978) described a system in which T7 DNA was shown to be organized into chromatin, but, contrary to the results described here, they observed a loss of fidelity of transcription by *E. coli* RNA polymerase of the reconstituted T7 template.

No direct evidence exists for sequence-specific protein–DNA interactions in transcriptional regulation in eucaryotes. However, genetic studies have revealed that both positive and negative regulation of transcription does occur in eucaryotes and some of the findings can be interpreted in terms of precedents offered by protein–DNA interactions in procaryotes (Thuriaux et al., 1972; Arst & MacDonald, 1975; Chovnick et al., 1977; Thompson et al., 1977; Scazzocchio & Arst, 1978; Perlman & Hopper, 1979). In particular, examples of cis-acting regulatory loci are often interpreted in terms of sites

for the binding of sequence-specific DNA binding proteins. Despite such circumstantial evidence, no such regulatory protein has yet been identified or isolated for testing in an in vitro transcription system.

In an attempt to circumvent this problem, we have developed a model system in which the control of transcription of a chromatin template by bona fide *E. coli lac* operon regulatory proteins could be studied. This approach offers the advantage that regulation of the *lac* operon is well understood and the relevant RNA polymerase and regulatory proteins are available. With this template it becomes feasible to ask whether a regulatory DNA binding protein can function when the DNA is organized into nucleosomes and whether transcription can proceed through nucleosomes. In fact, the environment created by chromatin reconstitution may not be so foreign to the *E. coli* proteins since the bacterial chromosome does contain a histone-like protein which can affect condensation and supercoiling of DNA in vitro (Rouviere-Yaniv et al., 1979). In order to maximize the possibility of fidelity of transcription, we chose conditions of transcription optimal for the *E. coli* proteins rather than others used previously for transcription of chromatin.

We have assumed that the *lac* control region was organized into nucleosomes as a result of random assembly of nucleosomes with respect to DNA sequence (Polisky & McCarthy, 1975; Cremisi et al., 1976), although specific cases of nonrandom distribution of nucleosomes on in vivo assembled SV40 have been described (Ponder & Crawford, 1977; Scott & Wigmore, 1978; Varshavsky et al., 1979). Also one case of preferential in vitro reconstitution of nucleosomes on AT-rich regions has been observed (Wasylyk et al., 1979a), and evidence for preferential points of assembly of nucleosomes on *lac* DNA has been presented (Chao et al., 1979). The question of whether transcriptional regulation occurred when the regulatory proteins were bound to DNA within the nucleosome or to DNA between nucleosomes can now be investigated. This may best be approached by studying the binding and action of regulatory proteins on small fragments of DNA containing *lac* sequences so that the density of nucleosomes in the region of interest may be more accurately determined. The only other investigations of the interaction of sequence-specific DNA binding proteins with DNA in nucleosomes have been with restriction enzymes, and it has been concluded that cleavage of the DNA occurred only in the regions between nucleosomes (Polisky & McCarthy, 1975; Cremisi et al., 1976; Ponder & Crawford, 1977). These results may be relevant to the interaction of other sequence-specific DNA binding proteins with chromatin and suggest that they may also bind between nucleosomes.

This same experimental strategy may be appropriate for the study of effects of alteration of nucleosome structure on the binding and activity of regulatory proteins. Analysis of the binding of regulatory proteins and RNA polymerase to chromatin that has been assembled with acetylated histones (Vidali et al., 1978; Mathis et al., 1978) and nonhistone chromosomal proteins such as those of the high mobility group (Goodwin & Johns, 1973; Levy W. et al., 1977) may provide insight into structural changes in chromatin which are related to changes in transcriptional activity.

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