Heparin Binds to Intact Mononucleosomes and Induces a Novel Unfolded Structure†

Timothy W. Brotherton,* M. V. Jagannadham, and Gordon D. Ginder
Department of Internal Medicine, Division of Hematology-Oncology, and Department of Biochemistry, University of Iowa and Veterans Administration Medical Center, Iowa City, Iowa 52242
Received September 6, 1988; Revised Manuscript Received December 7, 1988

ABSTRACT: It has been previously shown that heparin can bind to chromatin and enhance transcriptional activity. To characterize this phenomenon further, we have studied the interaction of heparin with isolated core mononucleosomes from avian reticulocytes. The results of these studies suggest that heparin bound reversibly to intact core mononucleosomes to induce a new structure, identified by decreased electrophoretic mobility and altered circular dichroism spectra. This altered nucleosome conformation exhibits 3–5-fold increased sensitivity to digestion by the nuclease, DNase I, and allows more efficient passage of RNA polymerase. At higher concentrations of heparin, core histones were completely removed from DNA. The finding of a reversible nucleosome–heparin complex in which core DNA is readily accessible to both RNA polymerase and the nuclease DNase I is discussed in the context of transcriptionally active chromatin.

Transcriptionally active genes of higher eukaryotes reside in chromosomal domains that are more sensitive to digestion by DNase I than surrounding inactive chromatin (Cartwright et al., 1982). However, both active and inactive genes are predominantly found in tight association with histone protein in structures called nucleosomes (Cartwright et al., 1982). Although there is strong evidence that nucleosomes are absent from DNase I hypersensitive regions (McGhee et al., 1981) and that this absence is important for transcription initiation (Knezetic & Luse, 1986; Lorch et al., 1987; Losa & Brown, 1987), it is not clear that nucleosomes remain in place (or intact) during passage of the RNA polymerase complex. For example, Lorch et al. (1987) reported that either SP6 RNA polymerase or mammalian RNA polymerase II was capable of reading through a nucleosomal template in vitro, but in the course of read-through transcription, histones were displaced from the DNA. By contrast, Losa and Brown (1987) found no displacement of histone octamer from DNA during transcription by SP6 RNA polymerase, and Solomon et al. (1988) demonstrated that histone H4 is retained on a highly transcribed gene in vivo. Other groups have studied the interaction of mononucleosomes with *Escherichia coli* (Bustin, 1978; Shaw et al., 1978; Hodo et al., 1980) and mammalian (Baer & Rhodes, 1983; Sakuma et al., 1984) RNA polymerases. It has been concluded from these studies that the polymerase can proceed completely through the nucleosome core but at a very slow rate compared to naked DNA. In some of these studies, it has been shown that mononucleosomes bound to polymerase lack one histone H2A–H2B dimer (Baer & Rhodes, 1983; Gonzalez et al., 1987). However, this finding has not been universally obtained (Bustin, 1978; Hodo et al., 1980; Sakuma et al., 1984).

Various polyanions have been reported to modify the interaction between DNA and core histone (Ansevin et al., 1975). Heparin, in particular, has been shown to decrease the melting point of DNA in chromatin (Ansevin et al., 1975) and to increase the activity of RNA and DNA polymerases (Kraemer & Coffey, 1970; dePomeraci et al., 1974; Warnick & Lazarus, 1975; Graner et al., 1975). In light of these observations, we sought to determine if heparin might alter the structure of nucleosomes and whether this structural change is related to the potentiation of RNA polymerase activity previously described. Our results suggest that heparin can bind reversibly to core nucleosomes. Upon binding, the nucleosome assumes a new structure as identified by gel electrophoresis and circular dichroism in which DNA is more accessible to enzymes, such as DNase I and RNA polymerase I, than in native mononucleosomes. These findings are discussed in light of potential nucleosome structures reported to be present during transcription.

Experimental Procedures

Isolation of Nuclei. Chickens were injected intramuscularly with 1-acetyl-2-phenylhydrazine (20 mg kg^{-1} day^{-1}) for 5 days. Peripheral blood was collected into 5% final volume acid/citrate/dextrose anticoagulant (Aster & Jandle, 1964) and washed twice in 1 × SSC (0.15 M NaCl/0.015 M sodium citrate) with removal of the buffy coat. Reticulocyte counts were between 50 and 95%. Cells were lysed in 0.35% Triton X-100 in RSB [10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 7.0, 10 mM NaCl, and 3 mM $MgCl_2$] and washed in RSB. All buffers contained a final concentration of 0.2 mM PMSF (phenylmethanesulfonyl fluoride) and 2 mM sodium butyrate, pH 7.0. Unless indicated otherwise, all procedures were carried out at 4 °C.

Preparation of Mononucleosomes. Mononucleosomes were prepared as described previously (Brotherton & Ginder, 1986). Nucleosome preparations, at 0.4–0.5 mg/mL (as determined by A_{260} in 1 N NaOH), contained over 90% mononucleosomes and were dialyzed into 0.2 × TBE (18 mM Tris, 18 mM boric acid, and 0.5 mM Na_2-EDTA) for subsequent use.

Treatment of Mononucleosomes with Heparin. Sodium heparin (porcine intestinal mucosa, grade II, Sigma) or sodium

† This was supported by the Veterans Administration and by Grant IN-122I from the American Cancer Society, administered through the University of Iowa Cancer Center, to T.W.B., who is a Research Associate of the VA. G.D.G. is an Established Investigator of the American Heart Association.

* Address correspondence to this author at the Department of Internal Medicine, University of Iowa.

[1] Abbreviations: RSB, reticulocyte standard buffer; PMSF, phenylmethanesulfonyl fluoride; PIPES, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism.

0006-2960/89/0428-3518$01.50/0

[^{3}H]methylheparin (0.43 mCi/mg, NEN Research Products) was diluted to yield a stock solution of 5 mg/mL with 2 × TBE. Heparin was added to mononucleosomes as indicated for each experiment; samples were incubated for 15 min at 25 °C prior to electrophoresis.

Gel Electrophoresis. First-dimension electrophoresis of nucleohistone particles was carried out in 5% polyacrylamide gels [acrylamide:bis(acrylamide) ratio of 25:1] in 0.5–2 × TBE. Second-dimension electrophoresis in 18% acrylamide gels was carried out in SDS as described by Laemmli (1970) and in Triton–urea–acetic acid gels using protamine displacement (Richards & Shaw, 1982) as previously described (Brotherton et al., 1981). Gels were processed for fluorography as previously described (Brotherton & Ginder, 1986). Second-dimension electrophoresis of single-strand DNA was carried out in 7 M urea–polyacrylamide gels [10% acrylamide; acrylamide:bis(acrylamide) ratio of 20:1] as described elsewhere (Maniatis et al., 1982). Gel lanes from the first-dimension nondenaturing gel were incubated in 90% formamide/1 × TBE for 1 h at 45 °C and then boiled in fresh 7 M urea, 1 × TBE, 0.02% bromophenol blue, and 0.02% xylene cyanole for 20 min prior to being loaded over the 8 M urea gel. After electrophoresis, bands were stained with 0.05% (w/v) Stains-All (Eastman-Kodak) in 50% (v/v) formamide and destained in water.

Spectroscopic Analysis. Circular dichroism measurements were recorded at 25 °C in an AVIV 60DS dichroism spectrophotometer in a 1.0-cm path-length cell. Concentrations of DNA nucleotide residue were determined by the absorption at 258 nm using $\epsilon_{258} = 6800$ cm^{-1} (mol of nucleotide)$^{-1}$ (Sasi et al., 1982). The concentrations of the nucleosome samples tested were 2.09×10^{-4} M/nucleotide residue in 1 × TBE. The reported spectra represent averages of 10 repeat recordings from 1 preparation for each heparin–nucleosome incubation mixture. Heparin does not demonstrate circular dichroism in the wavelength range examined (250–310 nm).

Electroblot and Hybridization Analysis. Nucleohistone gels were prepared for transfer as we have described (Brotherton & Ginder, 1986) and transferred in 5 mM NaCl/10 mM NaOH to nylon membranes (Zeta-Probe, Bio-Rad). Cloned DNA probes for the chicken adult β-globin (FKK36) and ovalbumin (pOV_1)genes are described elsewhere (Brotherton & Ginder, 1986). Nick translation and all hybridization procedures are as previously described (Brotherton & Ginder, 1986).

DNase I Digestion of Nucleosomes in Acrylamide Gels. After electrophoresis, nondenaturing 5% acrylamide gels (see above) were incubated in 250 mL of 100 mM NaCl, 50 mM Tris-HCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1 mM dithiothreitol, pH 7.8, for 30 min at 37 °C, stained with ethidium bromide (1 μg/mL), and photographed. Gels were then placed in 100 mL of fresh buffer without ethidium bromide at 37 °C, and DNase I (Sigma) was added in the amounts and for the incubation times indicated in each experiment. The reaction was stopped by changing the buffer to 10 mM Na_2-EDTA, pH 7.0. Gels were then restained (1 h) and photographed. In selected experiments, serial photographs were taken during the course of DNase I digestion. In other experiments, gel lanes were excised, and DNA fragment length was determined by second-dimension 7 M urea gels (see above). Bands in photographs of the gels and autoradiograms were quantitated by scanning densitometry (LKB laser densitometer).

Transcription Assays. Transcription assays were performed by the method of Gonzalez et al. (1987), using *E. coli* RNA polymerase purchased from Boehringer (1 unit/μL) or NEB (5 units/μL). Mononucleosomal-length free DNA was prepared as previously described (Brotherton & Ginder, 1986). Transcription was stopped by boiling incubation mixtures for 2 min in 2 volumes of 90% formamide/1 × TBE, followed by electrophoresis on 7 M urea–12% polyacrylamide gels as described by Bustin (1978).

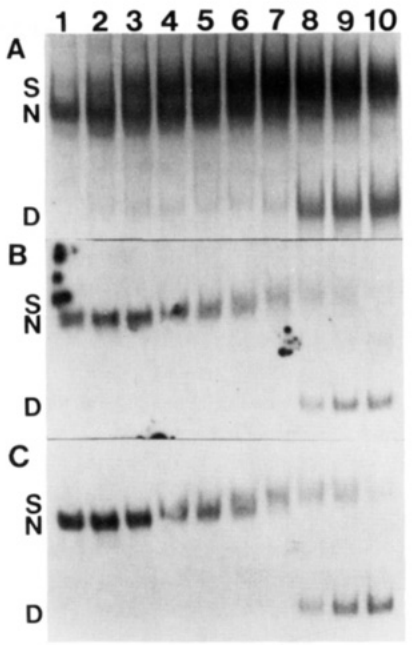

FIGURE 1: Acrylamide gel electrophoresis and hybridization analysis of mononucleosomes incubated with heparin in high-salt buffer. Avian erythrocyte mononucleosomes (24 μg/lane in 80 μL), stripped of noncore histone proteins and trimmed to about 146 bp of DNA, were incubated with increasing amounts of sodium heparin in 2 × TBE. The mixtures were then subjected to electrophoresis in 2 × TBE (see Experimental Procedures). Panel A is the photograph of the ethidium bromide stained gel. DNA from this gel was electroblotted to nylon and hybridized with ^{32}P-labeled probes. Autoradiograms are shown for β-globin (panel B) and ovalbumin (panel C). Lane 1 is mononucleosomes without heparin. Heparin content by lane is as follows: lane 2, 1 μg; lane 3, 1.5 μg; lane 4, 2 μg; lane 5, 2.5 μg; lane 6, 3 μg; lane 7, 4 μg; lane 8, 8 μg; lane 9, 10 μg; lane 10, 12 μg. N, native mononucleosomes, S, slow-moving nucleohistone–heparin complex; D, free core DNA.

Results

Mononucleosomes Bind Sodium Heparin and Have Altered Electrophoretic Mobility. Mononucleosomes, stripped of noncore histone proteins by washing in 0.45 M salt, were incubated with heparin in high-salt buffer (2 × TBE) and subjected to nondenaturing polyacrylamide gel electrophoresis. As shown in Figure 1A, treatment with heparin resulted in the appearance of two new electrophoretic bands, a slow-moving band that stained with both ethidium bromide and Coomassie blue and a fast-moving band with the mobility of free nucleosomal-length DNA that stained only with ethidium bromide. The slow-moving band was found at a heparin to DNA weight ratio, R, of 0.05 (heparin concentrations above 10–25 μg/mL) and was the major band present by $R \geq 0.15$ (heparin concentration 50–75 μg/mL). As the concentration of heparin was increased, an increasing proportion of total nucleosomal DNA was found migrating as free DNA. At heparin to DNA weight ratios above $R = 0.4$ ([heparin] ≥200 μg/mL), all nucleosomal DNA migrated as free DNA. Similar results were obtained in 1 × TBE and 0.5 × TBE (data not shown). Hybridization analysis of DNA in the bands present after heparin treatment reveals that transcriptionally active genes, such as β-globin, were found in the free DNA and slow-moving bands (Figure 1B) at similar concentrations

Table I: Histone Content of the Slow-Moving Nucleohistone–Heparin Complex[a]

lane	band	rel DNA content (%)	fractional content of histones				rel protein content (%)
			H3	H2B	H2A	H4	
1	N	100	0.27	0.27	0.19	0.27	100
2	N	57	0.21	0.25	0.17	0.37	58
	S	43	0.20	0.26	0.16	0.38	42
3	N	36	0.17	0.27	0.17	0.40	32
	S	64	0.24	0.26	0.16	0.35	68
4	N	12	0.24	0.28	0.12	0.35	28
	S	82	0.20	0.27	0.18	0.35	72

[a] The bands in the ethidium bromide and Coomassie blue stained gels shown in Figure 3 were determined by scanning densitometry. N, native mononucleosomes. S, slow-moving nucleohistone–heparin complex.

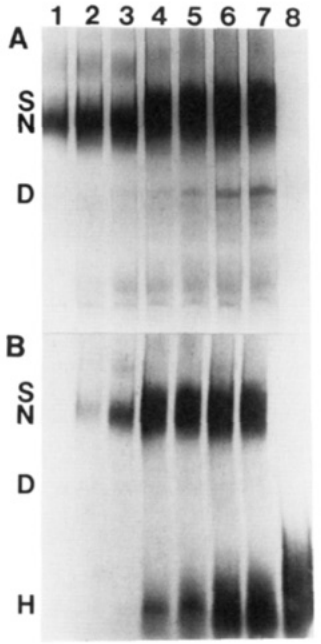

FIGURE 2: Heparin binds to nucleohistone particles, but not free core DNA. Core mononucleosomes (11 μg/lane in 20 μL) were incubated with [^{3}H]heparin in the amounts indicated for each lane and submitted to gel electrophoresis in 2 × TBE. The gel was stained with ethidium bromide (panel A) and then fixed and stained with Coomassie blue and subjected to fluorography (panel B). Lane 1 is nucleosomes without heparin. Lane 8 is 2.5 μg of heparin without nucleosomes. Heparin content by lane is as follows: lane 2, 0.5 μg; lane 3, 1 μg; lane 4, 2.5 μg; lane 5, 2.5 μg; lane 6, 2.75 μg; lane 7, 4.4 μg; H, free heparin. Other bands are labeled as in legend to Figure 1.

of heparin as inactive genes, such as ovalbumin (Figure 1C).

Heparin binding to mononucleosomes was determined by fluorography of [^{3}H]heparin–nucleohistone complexes isolated by acrylamide gel electrophoresis. As shown in Figure 2, heparin was present in the native nucleosome and the slow-moving nucleohistone bands but did not bind to free DNA. There was a threshold level of heparin binding before the slow-moving complex was detected. With increasing heparin, the slow-moving band appeared, but heparin-associated nucleosomes with the mobility of native nucleosomes persisted. Once the slow-moving band appeared, additional added heparin was found as free heparin at the bottom of the gel.

Histone Content of Heparin-Treated Nucleosomes. To characterize the protein composition of the new gel bands generated by the addition of heparin, nondenaturing nucleohistone gel lanes were excised, incubated in SDS gel layering buffer, and applied to second-dimension SDS–acrylamide gels. As shown in Figure 3, the slow-moving band contains all four core histones. When treated with heparin in high-salt buffers, the ratio of histone to DNA was unchanged as determined by comparison of the densitometric scans of Coomassie blue and ethidium bromide stained gels (Table I). In particular, no

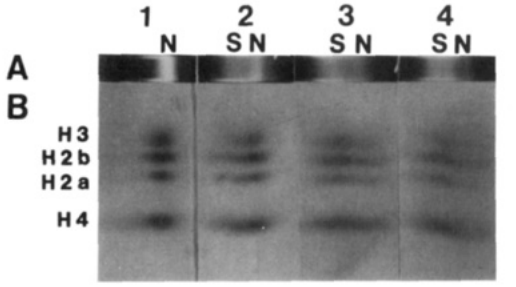

FIGURE 3: Slow-moving nucleohistone–heparin complex is not depleted of histone proteins. Mononucleosomes (22.5 μg in 50 μL) were incubated with no heparin (lane 1) or with 1.25 μg (lane 2), 2.5 μg (lane 3), or 3.75 μg (lane 4) of heparin in 2 × TBE and subjected to electrophoresis in 2 × TBE. After being stained with ethidium bromide (panel A), lanes were excised, incubated in SDS gel layering buffer, and placed over a second-dimension on SDS–18% acrylamide gels (panel B). The position of native nucleosomes (N) and slow-moving particle (S) in the first- and second-dimension gels is indicated, and core histone bands in panel B are identified.

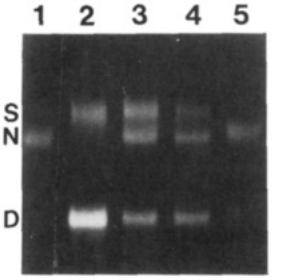

FIGURE 4: Protamine reversal of heparin binding to the slow-moving particle. Core mononucleosomes (7 μg/lane in 20 μL) were incubated with 2 μg of heparin in 2 × TBE. After 15 min, protamine was added and the mixture immediately subjected to electrophoresis in 2 × TBE. Lane 1 is native mononucleosomes and lane 2 mononucleosomes with heparin. Lanes 3–5 are heparin-treated mononucleosomes with 2 μg (lane 3), 2.5 μg (lane 4), and 3 μg (lane 5) of protamine. Bands are indicated as in the legend to Figure 1. Note that free DNA is bound by protamine and does not enter the gel.

loss of H2A or H2B was detected. This is relevant as several studies have suggested that a loss of H2A–H2B occurs with reaction of monunucleosomes with heparin in very low salt (Doenecke, 1981), and with RNA polymerase (Baer & Rhodes, 1983). Triton–urea–acetic acid gel electrophoresis demonstrated that histones in nucleosomes of the slow-moving band were modified by acetate to a similar degree as histones from control mononucleosomes (data not shown). To confirm that no significant loss of histones from the slow-moving nucleohistone–heparin particle had occurred, nucleosomes were treated with heparin and then incubated with increasing amounts of protamine. As can be seen in Figure 4, there is recovery of a band with identical mobility of native nucleosomes with sufficient protamine. In the gel system used, mononucleosomes depleted of one H2A–H2B dimer migrate as a distinct band ahead of native mononucleosomes

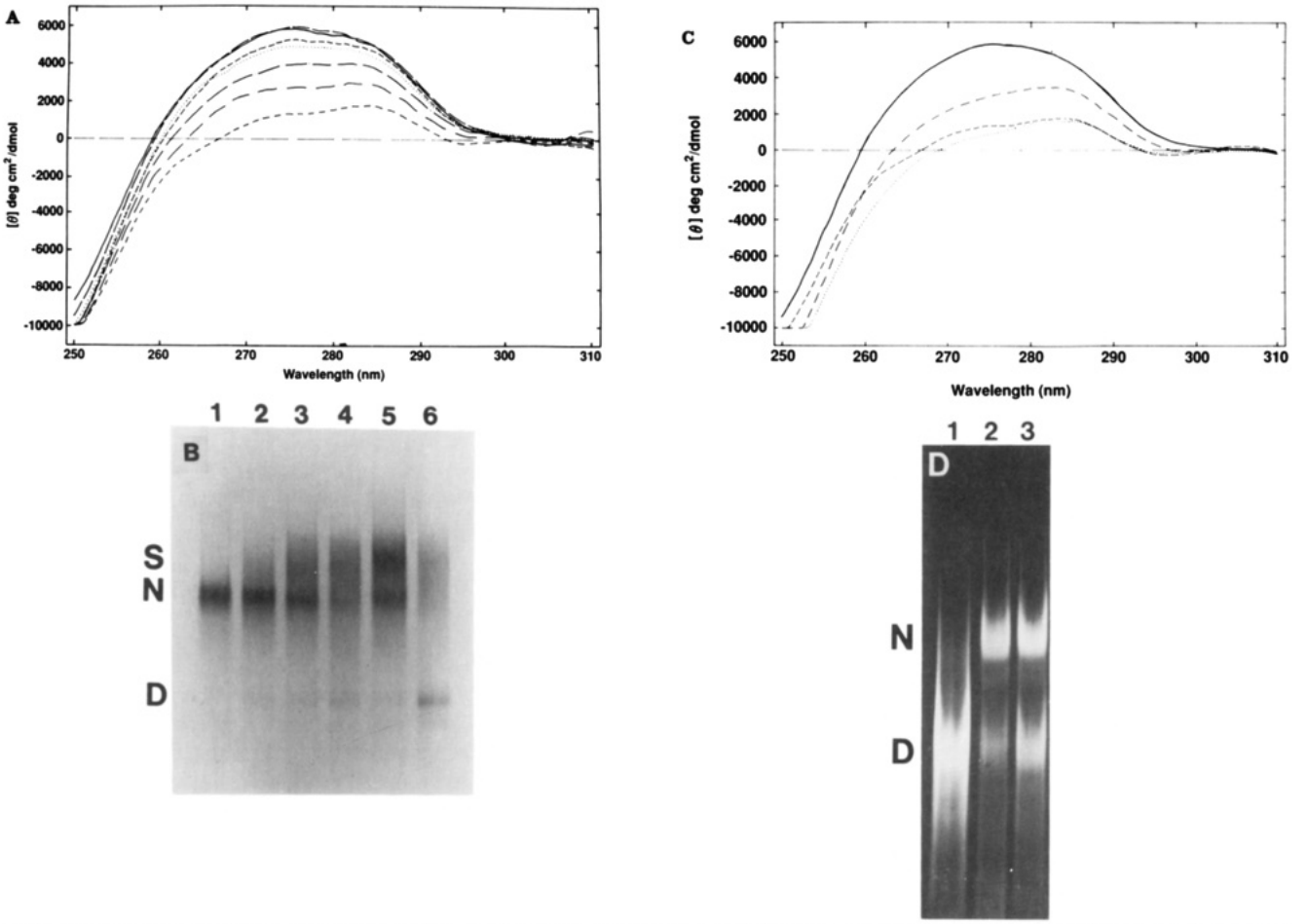

FIGURE 5: Circular dichroism spectra of stripped mononucleosomes and mononucleosome–heparin complexes. (A) Mononucleosomes in 1 × TBE at A_{258} = 1.6, with 1-cm path-length cells at 25 °C. Mononucleosomes alone (- - -) or with heparin at 12.5 μg/mL (−··−), 25 μg/mL (−·−), 37.5 μg/mL (···), 50 μg/mL (- - -), 100 μg/mL (—), and 200 μg/mL (– –). (B) After use for CD spectral analysis shown in panel A, samples (50 μL) were subjected to gel electrophoresis and stained with ethidium bromide. Lane 1, mononucleosomes alone. Lanes 2–6, mononucleosomes with heparin: 12.5 μg/mL (lane 2), 25 μg/mL (lane 3), 50 μg/mL (lane 4), 100 μg/mL (lane 5), and 200 μg/mL (lane 6). Bands are labeled as in Figure 1. (C) Mononucleosomes and free mononucleosomal-length DNA in 1 × TBE were mixed to give a final concentration at A_{258} = 1.6, with 1-cm path-length cells at 25 °C. Mononucleosomes alone (···); mononucleosomes with DNA (5:1 ratio, - - -); mononucleosomes with DNA (3:1 ratio, −·−); DNA alone (—). (D) After use for CD spectral analysis shown in panel C, samples (50 μL) were subjected to gel electrophoresis. Lane 1, mononucleosome-length DNA alone; lane 2, mononucleosomes with DNA (5:1 ratio); lane 3, mononucleosomes with DNA (3:1 ratio). Bands are labeled as in Figure 1.

(McMurry & van Holde, 1986). As protamine can displace histone from DNA (Richards & Shaw, 1982), it is unlikely that H2A–H2B dimers, or other core histones potentially displaced by heparin, would recombine quantitatively prior to electrophoresis. We interpret the data in Figure 4 to be further evidence that in the buffer conditions used, no loss of histone, including histone H2A–H2B dimers, occurred in the slow-moving heparin–mononucleosome complex.

Circular Dichroism Studies of Heparin–Nucleosome Complex. The circular dichroism (CD) spectrum of chromatin above 250 nm is entirely due to the DNA component of chromatin and thus can be used as a sensitive probe of DNA conformational changes in whole chromatin as well as in mononucleosomes (Sasi et al., 1982). The CD spectra of mononucleosomes with or without heparin were obtained in 1 × TBE buffer. Molar ellipticity peaks at $[\theta]_{272.5}$ and $[\theta]_{282.5}$ are shown in Table II and spectra in Figure 5A. Peak heights with no added heparin are low relative to published results (Sasi et al., 1982). This may be due to use of 1 × TBE buffer (total molarity, 0.18 M), as it has been shown that increasing salt decreases the ellipticity peak heights for mononucleosomes in the 260–300-nm region (Sasi et al., 1982; Oohara & Wada, 1987). Despite this overall decrease, the shoulder in the spectra at 275 nm characteristic of mononucleosomes and reported

Table II: Circular Dichroism Results of Mononucleosomes with Heparin[a]

[heparin] (μg/mL)	$[\theta]_{272.5}$	$[\theta]_{282.5}$
0	1119	1718
5	1376	1975
12.5	2553	2926
25	3606	3959
37.5	4604	4651
50	4839	4931
100	5587	5582
200	5554	5554

[a] Mononucleosomes were diluted to 2.09 × 10^{-4} M in 1 × TBE buffer with heparin at concentrations indicated, and spectra were taken at 25 °C. [θ], molar ellipticity values, expressed in degrees centimeter squared per decimole of nucleotide.

to be due to DNA wrapping around core histones (Sasi et al., 1982) was present. Thus, for control mononucleosomes, $[\theta]_{272.5}$ was lower [1119 deg cm² (dmol of nucleotide)$^{-1}$] than $[\theta]_{282.5}$ [1718 deg cm² (dmol of nucleotide)$^{-1}$]. Added heparin had two major effects on the CD spectra of mononucleosomes. First, the shoulder at 275 nm was changed so that the ellipticity at 272.5 nm became equal to that at 282.5 nm (Table II). Second, the total ellipticity was increased with increasing heparin up to 100 μg/mL. Additional heparin (200 μg/mL)

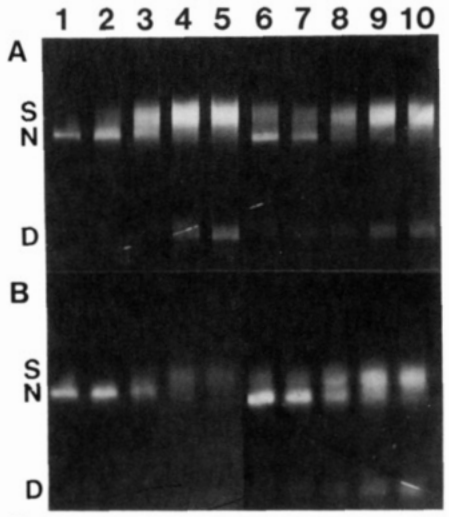

FIGURE 6: Slow-moving complex is susceptible to digestion with DNase I. Core mononucleosomes (9 μg/lane in 20 μL) were reacted with heparin and subjected to electrophoresis in a 5% acrylamide gel in 2 × TBE. After electrophoresis, the gel was incubated for 30 min at 37 °C in DNB with ethidium bromide and photographed (panel A). The gel was divided and placed in fresh DNB alone or in DNB with 1.5 μg/mL DNase I. After incubation for 90 min at 37 °C, the gels were placed in 10 mM Na-EDTA and restrained with ethidium bromide (panel B). Lanes 1–5, DNase I treated; lanes 6–10, control. Lane 1, no heparin. Lanes 2 and 7, 0.5 μg of heparin; lanes 3 and 8, 1 μg of heparin; lanes 4 and 9, 1.5 μg of heparin; lanes 5 and 10, 2 μg of heparin. Lane 6 had no added heparin but was contaminated by spillage from lane 5. Bands are indicated as in the legend to Figure 1.

did not alter the spectra from that at 100 μg/mL (Table II and Figure 5A). Samples used for CD analysis were then examined by gel electrophoresis (Figure 5B). As shown, the proportion of slow-migrating heparin–nucleosome complex increased with heparin concentrations up to 100 μg/mL. The proportion of mononucleosomal DNA migrating as free DNA did not substantially increase. At 200 μg/mL, about 50% of total mononucleosomal DNA migrated as free DNA, with the remainder as a smear in the mononucleosome and slow-moving heparin–mononucleosome complex regions of the gel. To control for the small amount of free DNA in the samples (7–9% relative to native mononucleosomes in lanes 1 and 5, and 14% in lane 4), the CD spectra of mixtures of mononucleosomes and mononucleosome-length genomic DNA were recorded. Figure 5C shows the CD spectra, and Figure 5D shows the gel electrophoresis pattern for these control samples. Free DNA to mononucleosome ratios were 20–35% as assessed by densitometric scanning of the stained gel. As shown, the CD spectra of mononucleosome samples with up to 35% free DNA had moderately higher molar ellipticity values compared to those of native mononucleosomes but did not lose the shoulder at 275 nm. As heparin does not alter the quantitation of either free DNA or native mononucleosomes, it is unlikely that the presence of free DNA can account for the CD spectra of heparin–mononucleosome complexes shown in Figure 5A. Therefore, these results show that the slow-moving heparin–mononucleosome complex has CD spectra similar to that of free DNA and indicates that, in this complex, the interaction between DNA and core histones has been substantially decreased.

Heparin Alters DNase I Sensitivity in Intact Nucleosomes. The results of gel electrophoresis and circular dichroism studies suggest that the reduction in mobility of the slow-moving band is due to heparin-induced relaxation of the interaction between core histone and DNA. If this prediction were correct, the DNA of intact nucleosomes bound by heparin should be more

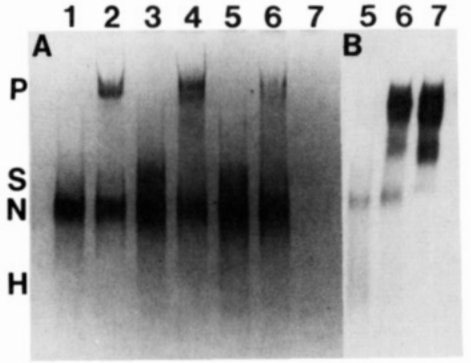

FIGURE 7: RNA polymerase binds heparin–mononucleosome particle. Mononucleosomes (1.7 μg/lane) (lanes 1–6) were incubated with heparin (0.25 μg) (lanes 3–7) and RNA polymerase (25 units) (lanes 2, 4, 6, and 7) in 25 μL of 10 mM Tris-HCl, pH 8, 0.1 mM Na_2-EDTA, 0.2 mg/mL BSA, 2 mM DTT, 5% glycerol, and 0.2 mM PMSF for 10 min at 30 °C and then subjected to electrophoresis in 1 × TBE. The heparin in lanes 5–7 was tritium labeled. P, RNA polymerase; S, heparin–nucleosome complex; N, native nucleosome; lane 4, free heparin. The ethidium bromide stained gel is shown in panel A and the fluorogram of lanes 5–7 in panel B. The band intermediate between bands P and S comigrates with albumin.

susceptible to digestion by nucleases, such as DNase I. To test this hypothesis, nucleosomes were reacted with heparin and separated by acrylamide gel electrophoresis. The gel, which contained separated nucleohistone bands, was incubated with DNase I (see legend of Figure 6). As shown in Figure 6, DNase I treatment resulted in a more rapid loss of DNA from the slow-moving band compared to native nucleosomal DNA. After incubation with DNase I for 90 min, 80–100% of the slow-moving band was digested, whereas only 30–35% of the native nucleosome was digested. Bulk DNase I sensitivity was also demonstrable at the level of hybridization analysis for active β-globin and an inactive repeat sequence gene probes (data not shown). Electrophoresis in 7 M urea denaturing gels of DNA isolated from heparin-bound nucleosomes digested with DNase I gave identical band patterns with those generated by equivalent digestion of native nucleosomes (data not shown). Thus, heparin does not expose new sites on the nucleosome core DNA to DNase I.

Heparin Alters Mononucleosome Interaction with RNA Polymerase. It has been shown that both *E. coli* and eukaryotic RNA polymerases can bind to mononucleosomes and transcribe mRNA that is full-length (i.e., 145 nt) (Bustin, 1978). However, binding by polymerase to mononucleosomes is 6–20-fold lower then binding to mononucleosome-length DNA (Bustin, 1978; Shaw et al., 1978), and the efficiency of full-length transcription is greatly reduced even with prolonged periods of incubation (Bustin, 1978; Shaw et al., 1978; Sakuma et al., 1984). We sought to determine if heparin could alter the interaction of mononucleosomes with RNA polymerase. As shown in Figure 7, the slow-moving heparin mononucleosome particle was preferentially bound by *E. coli* RNA polymerase. However, heparin alone can bind to polymerase (lane 7, Figure 7) and can compete with free DNA for binding (data not shown). To test if heparin altered the length of RNA transcripts or total transcription directed by mononucleosomes, mononucleosomes and mononucleosome-length DNA were incubated with *E. coli* RNA polymerase in the presence or absence of heparin. The results of these experiments are shown in Figure 8. Using free DNA as a template, it can be seen that full-length transcripts were present after 10-min incubation, and increased after 30 min. No transcription from DNA occurred in the presence of heparin, which is consistent with the observation that free heparin binds RNA polymerase

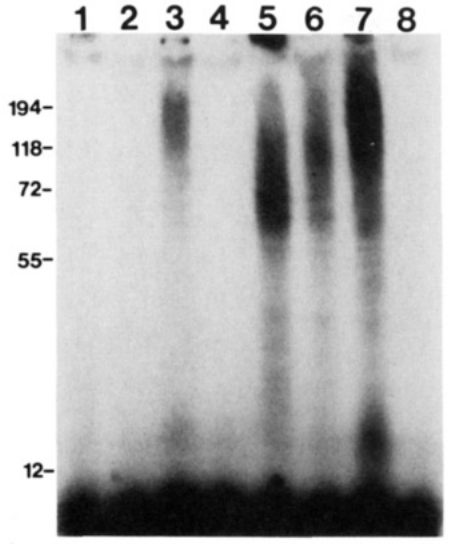

FIGURE 8: RNA transcripts from heparin–mononucleosome complexes, mononucleosomes, and mononucleosome-length DNA. Mononucleosomes (5 μg) or mononucleosome-length DNA (5 μg) was incubated at 37 °C in the buffer described in Figure 7 (100 μL), with RNA polymerase (5 units), 0.2 mM each of ATP, CTP, and UTP, 0.09 mM GTP, and 5 μCi/mL [^{32}P]GTP (450 Ci/mmol), with or without heparin (0.25 μg). After 5 and 30 min, 50-μL aliquots were removed, mixed with 100 μL of 90% formamide/1 × TBE, and boiled 2 min. Samples were then subjected to electrophoresis in 7 M urea/1 × TBE on a 12% acrylamide gel. Size was determined by labeled *Hae*III–φX174 markers and marker dye mobility. Lanes 1–4 are of 10-min incubation; lanes 5–8, 30-min incubation. Lanes 1 and 5, nucleosome control; lanes 2 and 6, nucleosome with heparin; lanes 3 and 7, DNA control; lanes 4 and 8, DNA plus heparin.

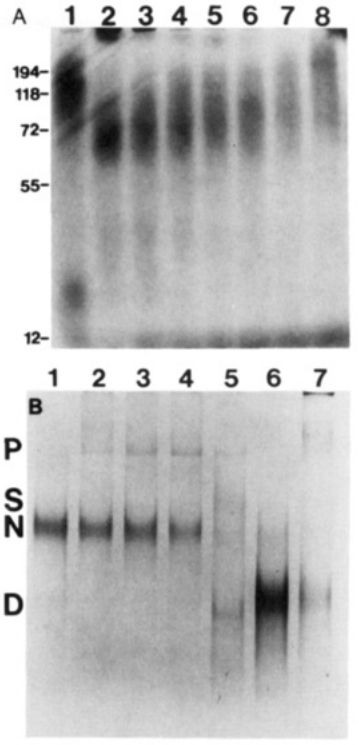

FIGURE 9: RNA transcript length from mononucleosome template increases as heparin concentration increases. (A) Mononucleosomes (5 μg) or mononucleosomal-length DNA (5 μg) was incubated for 30 min with RNA polymerase as described in Figure 8, in the presence or absence of heparin. Lane 1, DNA alone; lanes 2–8, mononucleosomes with 0, (lane 2), 0.125 μg (lane 3), 0.25 μg (lane 4), 0.375 μg (lane 5), 0.5 μg (lane 6), 0.75 μg (lane 7), and 1.0 μg (lane 8) of heparin. (B) Mononucleosomes or DNA (both at 2.5 μg in 50 μg of RNA polymerase buffer) and heparin were incubated with RNA polymerase (2 units) and unlabeled ribonucleotide precursors in parallel with samples in panel A. After 30 min at 37 °C, samples were made 1 × TBE and run on a 5% acrylamide gel. Lane 1, mononucleosomes alone; lane 2, mononucleosomes with polymerase; lane 3, mononucleosomes with 0.25 μg of heparin and polymerase; lane 4, mononucleosomes with 0.5 μg of heparin and polymerase; lane 5, mononucleosomes with 1 μg of heparin and polymerase; lane 6, DNA alone; lane 7, DNA with polymerase. Bands are labeled as in Figure 1.

(see above). With mononucleosomes as template, the level of transcription was markedly reduced at 10 min, compared to that with DNA as template; and even at 30 min, most transcripts were only about 70 nt in length. With mononucleosomes in the presence of heparin, total transcription was somewhat reduced compared to that found with mononucleosomes alone, but the relative proportion of longer transcripts increased to lengths approaching that found with free DNA as template (about 140 nt). With both free DNA and heparin–mononucleosomes, a significant proportion of transcripts was found to be longer than 145 nt, presumably due to fold-back transcription. Titration of mononucleosomes with heparin revealed that the increase in transcription length occurred at heparin concentrations that do not increase free DNA content (Figure 9). The reduction in total transcription with increasing heparin indicates that the apparent effect on mononucleosome-directed transcription was not due to inhibition of RNase activity by heparin. The possibility remains that small amounts of free DNA direct RNA polymerase activity, due to the release of histones from mononucleosomes by heparin. This is unlikely for the following reasons. First, if contaminating free DNA were present, then the rate of RNA synthesis should have increased as the heparin concentration was increased. However, this did not occur, as shown by the relative proportions of short and full-length transcripts in lanes 3 through 7 in Figure 9. Free DNA was not detected, even after prolonged incubation of heparin–mononucleosome in the presence of polymerase and ribonucleotide precursors (Figure 9B). Second, the transcripts directed by heparin–mononucleosomes were intermediate in length compared to those directed by mononucleosomes of free DNA alone (Figures 8 and 9A). Transcripts directed by free DNA contaminating heparin–mononucleosome samples should still be of the same length as transcripts directed by control DNA templates, even if the rate of transcription were slowed by polymerase occupation by heparin and mononucleosomes (see Figure 8, lane 3 and 7). Third, examination of polymerase complexes with mononucleosomes, heparin–mononucleosomes, and free DNA shows that the major ethidium bromide staining bands differ between these samples (Figure 9B). As heparin does not bind free DNA (see Figure 2), and polymerase–heparin complexes do not stain with ethidium bromide (see Figure 6), these results suggest that free DNA cannot be present in sufficient quantity to account for the quantity of longer transcripts using heparin–mononucleosome templates. For example, when 0.75 μg of heparin was added to 5 μg of mononucleosomes (lane 7, Figure 9A), total RNA transcripts were about 45% of that directed by 5 μg of free DNA (lane 1, Figure 9A). As 60% of free DNA was bound to polymerase (lane 7, Figure 9B), it is reasonable to conclude that an equally large portion of mononucleosomal DNA should have been converted to free DNA to support the high level of longer transcripts detected. However, no band with gel mobility similar to that of the major polymerase–DNA complex was found.

Discussion

The principle finding of this paper is that native nucleosomes react with sodium heparin to form several products, as de-

termined by gel electrophoresis. One product, with slowed gel migration, has a full complement of core histones but altered DNA–histone interactions as determined by circular dichroism studies and increased susceptibility to digestion by DNase I. This heparin–mononucleosome particle supported full-length RNA transcription more efficiently than did mononucleosomes alone. These characteristics suggest that this heparin–mononucleosome particle may share certain features in common with actively transcribed chromatin in vivo.

The features that distinguish actively transcribed chromatin domains from inactive are poorly characterized. Active chromatin is more readily digested by DNase I in intact nuclei (Weisbrod & Weintraub, 1979; Gazit et al., 1980). In vitro, active chromatin appears to have an unfolded structure as evidenced by sucrose gradient centrifugation (Kimura et al., 1983), histone H3–sulfhydryl exposure (Prior et al., 1983; Allegra et al., 1987), and RNA polymerase binding studies (Baer & Rhodes, 1983; Sakuma et al., 1984). The physical basis for this unfolding is not known. Studies designed to examine histone H3–sulfhydryl exposure have revealed a strong correlation between mononucleosomes containing hyperacetylated histones and the unfolded state (Prior et al., 1983, Allegra et al., 1987). Likewise, histone acetylation has proven to be a marker for active chromatin in terminally differentiated avain erythrocytes (Brotherton & Ginder, 1986; Nelson et al., 1986; Ridsdale & Davie, 1987; Hebbes et al., 1988). However, we (Brotherton & Ginder, 1986) and others (Perry & Chalkley, 1982) have shown that histone acetylation is not a property unique to active chromatin in more metabolically active cells. Other investigators have reported that active nucleosomes are depleted of one histone H2A–H2B dimer (Baer & Rhodes, 1983; Hutcheun et al., 1980). The results of the studies presented in this paper indicate that the bulk of mononucleosomes can acquire an unfolded state upon interaction with heparin. This state is not unlike that of active chromatin, as indicated by increased DNase I accessibility, the support of full-length RNA transcription, and decreased sedimentation rate in sucrose gradients (Ansevin et al., 1975; Donecke, 1981). It is also known that in low salt, histones H2A and H2B are partially removed by heparin (Donecke, 1981) and that in reconstituted SV40 minichromosomes transcription termination occurs at a nucleosome that is particularly resistant to heparin denaturation (Poljak & Gralla, 1987). Therefore, it is reasonable to suggest that the heparin–mononucleosome particle may serve as a useful model for the study of histone–DNA interaction in transcriptionally active chromatin. The results presented here also lend further support to the hypothesis that RNA polymerase can transcribe through inactive nucleosome cores in vivo, without displacement or removal of core histones (Losa & Brown, 1987). Furthermore, our findings suggest that polymerase passage may require ancilliary molecules, such as heparin, whose function is to relax the interaction between histone and DNA. Candidate molecules include the following: RNA, which has been shown to have effects on the DNA melting profile of chromatin similar to that of heparin (Ansevin et al., 1975); RNA polymerase complex (Baer & Rhodes, 1983); and the high mobility group (HMG) proteins, which can selectively bind to active chromatin in vitro (Sandeen et al., 1980; Brotherton & Ginder, 1986; Druckmann et al., 1986; Drobic & Wittig, 1986) and mediate DNase I sensitivity in intact nuclei (Weisbrod & Weintraub, 1979; Gazit et al., 1980).

ACKNOWLEDGMENTS

We acknowledge the technical assistance of C. Noftsger and the excellent secretarial skills of V. Weidner and J. Schoof.

Registry No. Heparin, 9005-49-6; RNA polymerase, 9014-24-8.

REFERENCES

Allegra, P., Sterner, R., Clayton, D. F., & Allfrey, V. G. (1987) *196*, 379–388.

Ansevin, A. T., MacDonald, K. K., Smith, C. E., & Hnilca, L. S. (1975) *J. Biol. Chem. 250*, 281–289.

Aster, R. H., & Jandle, J. H. (1964) *J. Clin. Invest. 43*, 843–855.

Baer, B. W., & Rhodes, D. (1983) *Nature 301*, 482–488.

Brotherton, T. W., & Ginder, G. D. (1986) *Biochemistry 25*, 3447–3454.

Brotherton, T. W., Covault, J., Shires, A., & Chalkley, R. (1981) *Nucleic Acids Res. 9*, 5061–5073.

Bustin, M. (1978) *Nucleic Acids Res. 5*, 925–932.

Cartwright, J. L., Abmayr, S. M., Fleishmann, G., Lowenhoupt, K., Elgin, S. C. R., Keene, M. A., & Howard, G. C. (1982) *CRC Crit. Rev. Biochem. 13*, 1–86.

de Pomerai, D. I., Chesterton, J., & Butterworth, P. H. W. (1974) FEBS Lett. *42*, 149–153.

Defer, N., Kitzis, A., Kruh, J., Brahms, S., & Brahms, J. (1977) *Nucleic Acids Res. 4*, 2293–2306.

Doenecke, D. (1981) *Biochem. Int. 3*, 73–80.

Drobic, T., & Wittig, B. (1986) *Nucleic Acids Res. 14*, 3363–3376.

Druckmann, S., Mendelson, E., Landsman, D., & Bustin, M. (1986) *Exp. Cell Res. 166*, 486–496.

Gazit, B., Panet, A., & Cedar, H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1787–1790.

Gonzalez, P. J., Martinez, C., & Palacian, E. (1987) *J. Biol. Chem. 262*, 11280–11283.

Groner, Y., Monroy, G., Jacquet, M., & Hurwitz, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 194–199.

Hebbes, T. R., Thorne, A. W., & Crane-Robinson, C. (1988) *EMBO J. 7*, 1395–1402.

Hodo, H. G., Sahasrabuddhe, C. G., Plishker, M. F., & Saunders, G. F. (1980) *Nucleic Acids Res. 8*, 3851–3864.

Hutcheon, T., Dixon, G. H., & Levy-Wilson, B. (1980) *J. Biol. Chem. 255*, 681–685.

Kimura, T., Mills, F. C., Allan, J., & Gould, H. (1983) *Nature 306*, 709–712.

Knezetic, J. A., & Luse, D. S. (1986) *Cell 45*, 95–104.

Kraemer, R. J., & Coffey, D. S. (1970) *Biochim. Biophys. Acta 224*, 553–567.

Laemmli, U. K. (1970) *Nature 227*, 681–683.

Lorch, Y., LaPointe, J. W., & Kornberg, R. D. (1987) *Cell 49*, 203–210.

Losa, R., & Brown, D. D. (1987) *Cell 50*, 801–808.

Maniatis, T., Fritsch, T. F., & Sambrook, J. (1982) *Molecular Cloning. A laboratory manual*, pp 184–185, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McGhee, J. D., Wood, W. I., Dolan, M., Engel, J. D., & Felsenfeld, G. (1981) *Cell 27*, 45–55.

McMurry, C. T., & van Holde, K. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8472–8476.

Nelson, D. A., Ferris, R. C., Zhang, D., & Ferenz, V. R. (1986) *Nucleic Acids Res. 14*, 1667–1682.

Oohara, I., & Wada, A. (1987) *J. Mol. Biol. 196*, 399–411.

Perry, M., & Chalkley, R. (1983) *J. Biol. Chem. 256*, 3313–3318.

Poljak, L. G., & Gralla, J. D. (1987) *Biochemistry 26*, 295–303.

Prior, C. P., Cantor, C. R., Johnson, E. M., Littau, V. C., & Allfrey, V. G. (1983) *Cell 34*, 1033–1042.

Richards, R. G., & Shaw, B. R. (1982) *Anal. Biochem. 121*, 69–82.

Ridsdale, J. A., & Davies, J. R. (1987) *Nucleic Acids Res. 15*, 1081-1096.
Sakuma, K., Matsumura, Y., & Senshu, T. (1984) *Nucleic Acids Res. 12*, 1415-1426.
Sandeen, G., Wood, W. I., & Felsenfeld, G. (1980) *Nucleic Acids Res. 8*, 3757-3778.
Sasi, R., Huvos, P. E., & Fasman, G. D. (1982) *J. Biol. Chem. 257*, 11448-11454.
Shaw, P. A., Sahasrabuddhe, C. G., Hodo, H. G., & Saunders, G. F. (1978) *Nucleic Acids Res. 5*, 1999-3012.
Solomon, M. J., Larsen, P. L., & Varshavsky, A. (1988) *Cell 53*, 937-947.
Warnick, C. T., & Lazarus, H. M. (1975) *Nucleic Acids Res. 2*, 735-744.
Weisbrod, S., & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 631-635.

Chloroplast Ribosomal Protein L12 Is Encoded in the Nucleus: Construction and Identification of Its cDNA Clones and Nucleotide Sequence Including the Transit Peptide[†,‡]

Klaus Giese and Alap R. Subramanian*
Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, West Germany
Received September 16, 1988; Revised Manuscript Received December 21, 1988

ABSTRACT: An architectural feature found in all classes of ribosomes is a thin, 10-nm-long protuberance in the large subunit, generated by multiple copies of r-protein L12. The primary structure of spinach chloroplast r-protein L12 is known [Bartsch, M., Kimura, M., & Subramanian, A. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6871-6875], but the location of its gene, whether in the organelle or in the nucleus, has not been determined. Therefore, we synthesized four oligodeoxynucleotides based on the amino acid sequence data and used them to probe a spinach cDNA library we constructed in λgt11 vector. cDNA inserts from four of the hybridizing recombinant clones were characterized and sequenced. The data showed that they are reverse transcripts of varying length, all derived from a single poly(A+) RNA species. The longest cDNA molecule is 900 base pairs (bp) long and includes a 5′ noncoding sequence followed by two neighboring AUG codons both in the consensus, eukaryotic initiator context, a 56-codon-long transit peptide sequence (starting from the first AUG codon), the amino acid sequence of mature L12 protein, and a 238 bp long 3′ downstream noncoding sequence including a polyadenylation signal and the start of the poly(A) tail. The transit peptide sequence has an unusual amino acid composition similar to that of other known chloroplast transit peptides. Northern blot analysis of the poly(A+) RNA isolated from spinach seedlings and probed with the cDNA insert revealed the occurrence of a strong, broad, 950-nucleotide-long band of the corresponding poly(A+)-containing mRNA species. These results thus show that chloroplast L12 is nuclear-encoded. The possible significance of the two AUG codons in the presequence, and of the codon usage pattern of L12 (different from that of chloroplast-encoded r-proteins), is discussed.

Genes encoding the ribosomal proteins (r-proteins)[1] and rRNAs of the chloroplast ribosome are located in two cellular compartments. The rRNAs and several r-proteins are encoded in the organelle DNA, but the majority of the r-protein genes are believed to be located in the nuclear DNA (Bogorad, 1975). Because of this bicameral gene distribution, biosynthesis of chloroplast ribosomes must include regulatory features which would be unnecessary for the biosynthesis of bacterial or cytoplasmic ribosomes, whose structural components are encoded in single genomes. The gene dosage ratio of nuclear to chloroplast genes is also dependent on the cell type and development. It is greater than $1:10^4$ in mature leaf cells but lower in other plant cells which contain developmentally arrested proplastids or etioplasts in place of chloroplasts (Hoober, 1984).

The subcloning and characterization of organelle-located chloroplast r-protein genes have been reported by us [e.g., see Subramanian et al. (1983) and Giese et al. (1987)] and by several other groups [tabulated in Prombona et al., 1989)]. The determination of the complete nucleotide sequence of two chloroplast genomes (Shinozaki et al., 1986; Ohyama et al., 1986) has, however, shifted the interest from organelle-located genes to those which are located in the nucleus. These nuclear genes are particularly interesting from three points of view. (1) Their mRNA is likely to be polyadenylated and monocistronic. The transcriptional regulation of such species would differ considerably from that of the prokaryotic-type, polycistronic mRNA produced in the chloroplast from the organelle DNA. (2) The chloroplast proteins encoded by nuclear genes and synthesized on the cytoplasmic 80S ribosomes always carry a transit sequence necessary to enter the organelle [reviewed in Schmidt and Mishkind (1986)]. The isolation of nuclear-located chloroplast r-protein genes will provide more transit sequences (and clones) useful to understand this process.

[†] This paper is part of the doctoral dissertation (Biochemistry) of K.G. to be submitted to the Free University of Berlin.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02849.
* Address correspondence to this author.

[1] Abbreviations: r-protein, ribosomal protein; SSC, 0.15 M NaCl/0.015 M sodium citrate (pH 7.0); Denhardt's, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% poly(vinylpyrrolidone); HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate.

0006-2960/89/0428-3525$01.50/0