Limitations of the Poly(glutamic acid) Reconstitution Method in the Reassembly of Mono- and Dinucleosomes†

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Received September 23, 1985; Revised Manuscript Received March 3, 1986

ABSTRACT: Reconstitution of mononucleosomes and dinucleosomes at physiological ionic strength by means of poly(glutamic acid) is not efficient at physiological histone octamer:DNA ratios, unlike that with the salt dialysis method. The shorter the DNA is, the less transfer of octamers from poly(glutamic acid) to DNA occurs. By increasing the octamer:DNA ratio it is possible to involve all the DNA in the assembly, but for DNA longer than core particle length, nucleoprotein particles containing extra histones are concomitantly generated. Except for core particle and chromatosome lengths of DNA reassembled at 0.6:1 or 1:1 octamer:DNA ratio (and thus with low yield), reconstituted nucleoprotein particles proved to be different from native nucleosomes by their insolubility upon isolation. In the aggregates, DNA ends seemed to be sufficiently loose to allow exonuclease III digestion up to a certain limit. This resulted in patterns that for some cloned DNA fragments could give the impression, without knowledge of the above, of resulting from a unique octamer position. In view of the small range of length of DNA and the low yield of faithful reconstitution, the assembly method using poly(glutamic acid) is only of limited use in mono- or dinucleosome reconstitution experiments, at least in our hands.

A question that has been haunting chromatin research for some years now is whether nucleosomes are positioned on specific DNA sequences. Since the nucleosome conformation is thought to impair the accessibility of the DNA to RNA polymerase action (among others), sequenced-directed nucleosome positioning is believed to have some regulatory implications.

This sequence specificity, however, cannot be very stringent, considering the variety of DNA sequences in the genome that nucleosomes must be accommodated to. Although it is now clear that nucleosomes are positioned in a nonrandom way on the DNA, the clues determining that positioning are still obscure. For instance, on mouse and rat satellite sequences, nucleosomes seem to occupy a limited number of different, well-defined locations (Zhang & Hörz, 1984; Böck et al., 1984). Several experiments searching for a relationship between histone octamers and random DNA sequences have been carried out on reconstituted nucleosomes (Tatchell & Van Holde, 1979).

Using cloned DNA for reconstitution offers the opportunity to obtain homogeneous samples of identical particles. Preparations of native nucleosomes always contain various DNA sequences and some DNA length variation, which can make results from experiments of the "footprinting" type difficult to interpret.

Reconstitution experiments using DNA containing 5S rRNA genes (Louis et al., 1981; Gottesfeld & Bloomer, 1981; Simpson & Stafford, 1983), SV40 DNA (Wasylyk et al., 1979), and procaryotic DNA (Ramsay et al., 1984; Chao et al., 1979) indicated a sequence dependence for nucleosome positioning, though not a simple one. Due to the lack of "averaging" that random DNA confers to results, several sequences allowing nucleosome formation will have to be compared in order to discover the features that determine the position of the histone octamer on the DNA. Recent findings (Drew & Travers, 1985) suggest the importance of sequence-dependent bendability of the DNA.

Essentially three methods are used to reconstitute mononucleosomes: dialysis through a salt gradient, reconstitution using nucleoplasmin or poly(L-glutamic acid), and direct-mixing procedures.

Working with reconstituted nucleosomes imposes several controls to check if composition and structure of these particles do not differ from those of native nucleosomes, and one can never be sure whether the in vitro position will reflect the in vivo one. Indeed, some end positioning on DNA fragments has been reported at least for the salt dialysis method (Tatchell & Van Holde, 1979; Linxweiler & Hörz, 1984). Apart from reconstitution artifacts, positioning in vivo could well be dependent on additional factors (histone H1, non-histone proteins).

In the reconstitution method using poly(L-glutamic acid), this acidic polypeptide is thought to act as a chaperon that combines with the histones and allows them to associate to the DNA in "physiological" salt conditions. It was during control experiments on particles obtained by this method that we became aware of its low efficiency when reconstituting at physiological histone:DNA ratios (in the mono- and dinucleosomal range). Our attempts to improve yield resulted in the observations presented in this paper, including indications that this method can generate aberrant nucleoprotein particles.

Materials and Methods

Isolation of Histone Octamers. Histone octamers were prepared largely according to the method described by Rhodes (1979). Chicken erythrocyte nuclei were briefly digested with micrococcal nuclease and lysed. The long chromatin was depleted from H1 and H5 on a Sepharose 2B column in a 650 mM NaCl, 0.2 mM EDTA,[1] 50 mM phosphate (pH 6.0)

† This work was financially supported by a fund from the Belgian National Bank and by a fund from the Belgian IIKW. S.P. is a Fellow of the NFWO (Nationaal Fonds voor Wetenschappelijk Onderzoek).

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; OD, optical density.

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buffer. The histone octamers were isolated from the depleted chromatin by passing it over a hydroxyapatite column in 2 M NaCl, 0.2 mM EDTA, and 50 mM phosphate (pH 7.0). SDS protein gel electrophoresis and sedimentation value ($s_{20,w} \sim 4.0$) proved that the octamers were intact. Protein concentration was determined according to Thomas and Butler (1977) ($A^{1\%}_{280} = 4.2$).

Preparation of Core Particles and Chromatosomes. Long chromatin, obtained by brief micrococcal nuclease digestion of chicken erythrocyte nuclei, was depleted from H1 and H5 on a Sepharose 2B column, as above. The depleted chromatin was once again digested with micrococcal nuclease to core particles with 145-bp length of DNA. These core particles were further isolated by preparative rate zonal centrifugation [see Muyldermans et al. (1985)], in 5 mM NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl (pH 7.4).

Chromatosomes were prepared by digesting long H1, H5-containing chromatin to a mononucleosome mixture of core particles and chromatosomes with 166–176-bp length of DNA. The chromatosomes having H1 or H5 were precipitated by incubation for 2 min at 0 °C in 130 mM NaCl and pelleted. Pellets were resolubilized in 5 mM NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl (pH 7.4). Chromatosomes were further isolated by rate zonal centrifugation.

DNA Extraction. Core particles (145-bp DNA) or chromatosomes (166–176-bp DNA) were treated with proteinase K. The DNA was subsequently extracted twice with phenol in 1 M NaCl.

Preparation of Radiolabeled DNA of Different Lengths. Mono- and dinucleosome samples were proteinase K treated and run on a preparative Tris–borate–EDTA–5% polyacrylamide gel. Following ethidium bromide staining, thin strips containing the desired DNA lengths were cut out of the gel and soaked in the dark in 0.2 mM EDTA–10 mM Tris-HCl (pH 7.4) buffer at 4 °C for several days. Then the ethidium bromide was removed by butanol washes and the DNA ethanol precipitated. The DNA 5′-OH ends of these micrococcal nuclease generated fragments were labeled with [γ-^{32}P]ATP (7000 mCi/mmol, New England Nuclear) by use of T4 polynucleotide kinase (Boehringer), according to Maniatis et al. (1982).

Reconstruction Procedure. Poly(L-glutamate) (Miles, average M_r 30 000) was solubilized in 100 mM Tris (pH 8.0) and mixed with octamers in a 4:1 (w/w) ratio. This mixture and the DNA were dialyzed overnight to 100 mM NaCl, 1 mM EDTA, 1.4 mM mercaptoethanol, and 10 mM Tris-HCl (pH 8.0) (Simpson & Stafford, 1982). The DNA was added to the histones and poly(L-glutamate) in the proper ratio, and the samples were incubated for 60 min at 37 °C and then cooled. Reconstructions were carried out at OD_{260} 5, unless indicated otherwise. Radiolabeled DNA was present in trace amounts, when used.

Reconstituted samples were analyzed on 12-mL (10–30%) linear sucrose gradients in a Beckman ultracentrifuge using an SW 41 rotor. Following centrifugation, gradients were fractionated from the bottom and the 260-nm absorption profiles recorded. Radioactivity profiles were obtained by scintillation counting of aliquots from 0.6-mL gradient fractions.

Salt Dialysis Reconstitution. DNA and histone octamers were dialyzed to 2 M NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl (pH 7.4) and then mixed in the proper ratio. This reconstitution mixture was dialyzed against a series of buffers with decreasing NaCl concentration (1.5 M–1 M–0.75 M, in the presence of 5 M ureum, and 0.75 M–0.5 M–0.05 M, without ureum), each time for 1 h. Reconstituted samples were analyzed on sucrose gradients.

Sedimentation Analysis. Sedimentation values were obtained from centrifugation at 20 °C, 45K rpm in a Beckman L8 ultracentrifuge equipped with a Prep UV scanner. Corrections to obtain $s_{20,w}$ were made when necessary.

Histone Quantification in Reconstituted Particles. All samples were in 5 mM NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 8). The protein contents within each sample were estimated by two methods.

(*A*) *Isoabsorbance Method* (*Groves et al., 1967*): *Spectrophotometric Determination of Microgram Quantities of Protein without Nucleic Acid Interference.* In the 220–240-nm range, where DNA passes through an absorption minimum, two wavelengths were selected at which absorbance of DNA was equal. By subtraction from each other the absorbances for nucleosomes at these wavelengths, the contribution of nucleic acid was cancelled. These absorbance differences were then evaluated in terms of histone concentration, with a standard plot for pure histone.

(*B*) *Coomassie Brilliant Blue G Assay* (*Sedmak & Grossberg, 1977*): *Assay for Microgram Quantities of Protein Depending on Conversion of Dye from Brown to Blue.* Coomassie G250 was prepared as a 0.6% solution in 3% perchloric acid. Equal volumes of G250 solution and nucleoprotein solution were mixed, and absorbance at 600 nm was determined immediately. Histone content was estimated from a chromatosome standard plot.

Labeling of Poly(glutamic acid). The N-terminal of poly(glutamic acid) was amidinated by Wood's reagent (methyl *p*-hydroxybenzimidate hydrochloride, Pierce) according to Wood et al. (1975) and then iodinated (^{125}I) by the chloramine T method (Hunter, 1973). Incorporation of label was verified on a Sephadex G-75 column.

Nucleoprotein Gel Electrophoresis. Electrophoresis of nucleoprotein particles was performed on 5% polyacrylamide gels with a 28:1 acrylamide:bis(acrylamide) ratio. Gels were run overnight at 4 °C. The running buffer [0.2 mM EDTA, 1 mM Tris (pH 7.4)] was stirred and recirculated continuously. Samples were applied in the sucrose gradient buffer. The gels were stained with ethidium bromide and dried and autoradiographed.

Denaturing Polyacrylamide Gels. The 7 M ureum–5% polyacrylamide gels and ultrathin 98% formamide–7% polyacrylamide gels, for electrophoresing single-strand DNA, were according to Maniatis and Efstratiadis (1980). Gels were soaked in a 10% acetic acid–10% methanol solution, dried, and autoradiographed.

Cloning of Nucleosomal DNA. DNA extracted from mononucleosomes was made blunt by exonuclease III and S1 digestion and then ligated into the *Sma*I site of M13mp9 and cloned (Messing, 1977). The inserted fragment was excised by *Eco*RI and *Bam*HI and isolated on a preparative Tris–borate–EDTA (1.5%) agarose gel.

RESULTS

Poly(glutamic acid) Reconstitution with Core Particle Length of DNA at Different Histone to DNA Ratios. Core histones in the presence of poly(glutamic acid) were incubated with core particle DNA at different ratios. The samples were then sedimented through (10–30%) linear sucrose gradients in 5 mM NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl (pH 7.4). The profiles of the gradients were recorded at 260 nm.

The gradient profiles exhibited a peak at 11 S and one at 5 S. In going from an octamer to DNA ratio of 0.6:1 (w/w) up to one of 3:1, the material from the 5S peak gradually

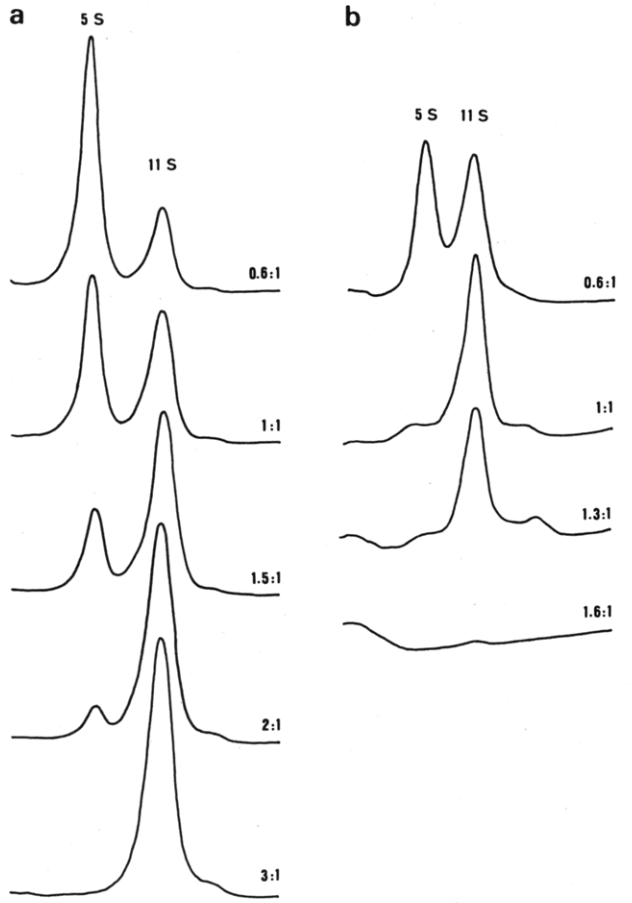

FIGURE 1: (a) Poly(glutamic acid) reconstitution of histone octamers with core particle DNA at different octamer:DNA ratios. Shown are the 260-nm absorption profiles from (10–30%) linear sucrose gradients. Sedimentation (38K rpm, 18.5 h, 5 °C) is from left to right. Histone:DNA ratios were (from top to bottom) 0.6:1, 1:1, 1.5:1, 2:1, and 3:1 (w/w). (b) Salt dialysis reconstitution with chromatosome DNA at histone:DNA ratios (from top to bottom) of 0.6:1, 1:1, 1.3:1, and 1.6:1 (same profiles for core particle DNA, not shown).

disappeared into the 11S peak (Figure 1a). The fraction migrating at 5 S proved to be free, not reconstituted, DNA: it comigrated with the starting DNA in sucrose gradients and DNA gels (not shown) and on nucleoprotein gel (Figure 2). No degradation nor any difference whatsoever could be detected.

The necessity of using completely deproteinized DNA, obtained by proteinase K digestion and subsequent phenol extraction in high salt (1 M), should be emphasized. Short peptides, resulting from proteinase K digestion, seem to stick strongly to DNA, creating particles that sediment at 3 S (instead of 5 S). This DNA does not reconstitute at all. Reassembly was expected to occur at a ratio of 1:1, about the ratio in native core particles. For the salt gradient dialysis method, this is indeed the case (Figure 1b). At 1:1 ratio however, about half of the DNA still remained not reconstituted. Shortage of histones does not seem the main reason for this phenomenon. Even in the 5S free DNA fraction, the presence of small amounts of histones was revealed by protein gels and histone quantification, although these were not associated to the DNA, as judged from nucleoprotein gel (Figure 2).

It was only at ratios of more than 2:1, even 3:1 (w/w) octamer to DNA, that all DNA was reconstituted into 11 S particles, which comigrate with native core particles. The histone complement of the 11 S fraction was comparable to that of native core particles and octamers (Figure 3).

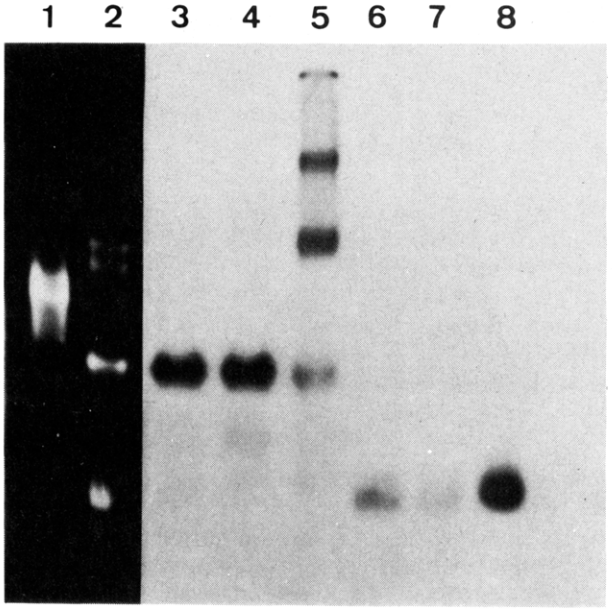

FIGURE 2: Reconstitution of core particles. Sucrose fractions on low ionic strength nucleoprotein gel (5% polyacrylamide): (lane 1) native chromatosomes; (lane 2) free DNA, native core particles, and compact dinucleosomes; (lane 3) core particles reconstituted at 0.6:1 ratio; (lane 4) core particles reconstituted at 1:1 ratio; (lane 5) core particles reconstituted at 2:1 ratio; (lanes 6 and 7) material sedimenting at 5 S; (lane 8) start 5′-labeled core particle DNA. The left side of the gel shows the UV image after ethidium bromide staining; the right part of the same gel is shown as an autoradiogram.

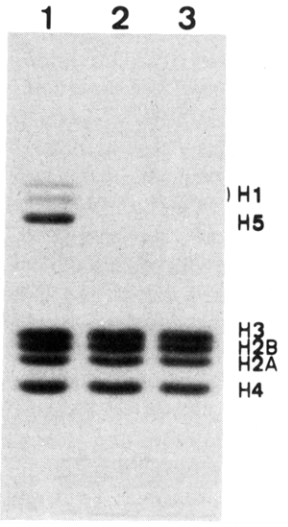

FIGURE 3: The 17.5% SDS–polyacrylamide gel electrophoresis of histones: (lane 1) histone complement of native chromatin; (lane 2) from the isolated histone octamer; (lane 3) histone complement from reconstituted monosomes.

Throughout the ratio range, no precipitation was found on gradient centrifugation, though this might have been expected for particles with a 3:1 octamer to DNA ratio. Poly(glutamic acid) has been reported for keeping chromatin with additional histones soluble that otherwise would precipitate (Stein et al., 1985). Still, it is difficult to imagine how core particles could take up more than the usual complement of histones without changing the S value. A more logical explanation would be that the poly(glutamic acid), which has been combined to the histone octamers prior to reconstitution, does not release all its histones to the DNA when the latter is added. This explanation, however, proved not to be completely satisfactory.

Effect of Total Concentration on Poly(glutamic acid) Reconstitution. We examined if total concentration during

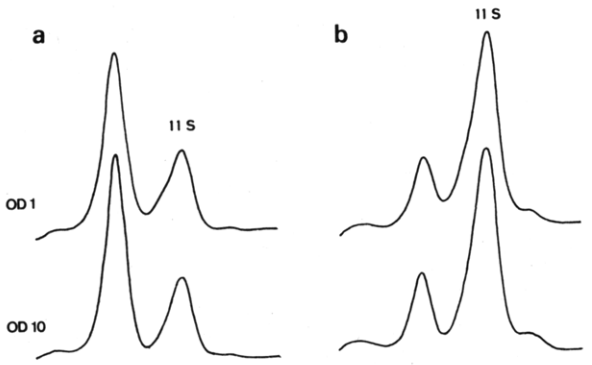

FIGURE 4: Effect of total concentration on reconstitution yield—sucrose profiles (as in Figure 1): (a) poly(glutamic acid) reconstitution at 0.6:1 ratio, OD_{260} 1 (upper profile) and OD 10 (lower profile); (b) reconstitution at 1.5:1 ratio, OD 1 (upper profile) and OD 10 (lower profile).

the assembly had an effect on reconstitution yield. Reconstitutions were carried out at concentrations ranging from OD_{260} 0.5 to 10, for different octamer:DNA ratios, with the same preparations of DNA and octamers. In the range tested, no significant effect on reconstitution yield could be detected (Figure 4).

Sedimentation Analysis. The S values had to be determined on complete assembly mixtures. Core particle reconstitution samples [still including poly(glutamic acid)] at 1:1 and 2:1 ratios were compared with native core particles. The "free DNA" portion of the 1:1 sample exhibited the same S value as naked core particle DNA (5.0 ± 0.1 S compared to 5.0 ± 0.03 S). For the reconstituted particles, at both ratios no significant difference in S value was observed compared to native core particles (11.3 ± 0.1 S for 1:1 and 11.0 ± 0.1 S for 2:1 compared to 11.0 ± 0.1 S for native core particles).

Poly(glutamic acid) Reconstitution of Nucleoprotein Particles with Different Lengths of DNA. DNA fragments of 100 bp, core particle length (145 bp), chromatosome length (166–177 bp), long monosome length (180–200 bp), compact disome length (about 280 bp), and disome length (about 400 bp) were obtained from mono- and dinucleosome samples through a preparative polyacrylamide gel and labeled (Figure 5).

Reconstitutions were carried out with cold core particle DNA, to which the labeled DNA of different lengths was added in trace amounts. Not only does this ensure identical ratio conditions during reconstitution, but on sedimentation the cold profiles offer an internal reference to which radioactivity profiles can be compared. The octamer:DNA ratios tested were 0.6:1, 1:1, 2:1, and 3:1 (w/w). Radioactivity profiles of the sucrose gradients are shown in Figure 6.

As might be expected, the 100-bp sample is not a good candidate for reconstruction; the ratio of nonreconstituted to reconstituted DNA was always higher than in the profiles from cold core particle DNA. The nucleoprotein particles that were generated exhibited a smaller S value (approximately 8 S).

For the other lengths of DNA, reconstitution efficiency seemed to improve with increasing length, judging from the diminishing free DNA fraction (Figure 6a,b). The increase in sedimentation rate of the free DNA peak for longer DNA (Figure 6a,b) is expected in view of the calculated S values [according to Eigner & Doty (1965)], and for dinucleosomal length of DNA, the position in sucrose gradients was verified (not shown).

At a 0.6:1 ratio (Figure 6a), the reconstituted particles of all lengths of DNA migrated at the same position (11 S), even the two DNA samples of dinucleosomal length. In the latter,

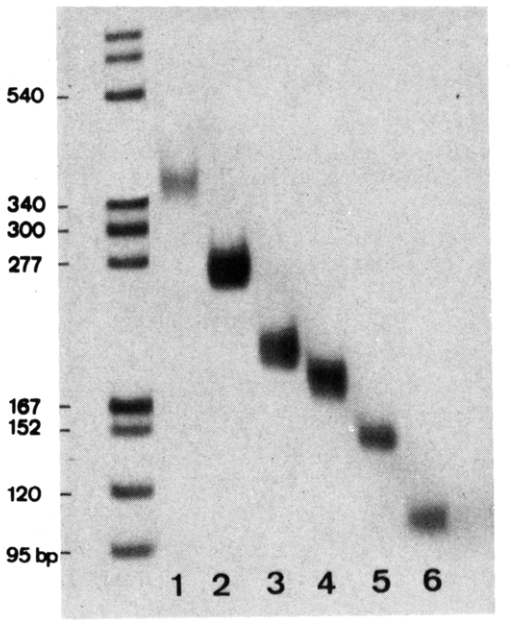

FIGURE 5: The 5% polyacrylamide gel of 5′-labeled DNA: (lane 1) dinucleosomal DNA; (lane 2) compact dinucleosomal DNA; (lane 3) long mononucleosomal DNA; (lane 4) chromatosome DNA; (lane 5) core particle DNA; (lane 6) 100-bp DNA. Marker: PM2 *Hae*III.

however, the profiles showed a shoulder of faster migrating particles. Due to fractionation, the free DNA peak sometimes interfered with the 11S peak in the case of dinucleosomal DNA.

At a 1:1 ratio (Figure 6b), more DNA seemed to be reconstituted, and again the material sedimented at 11 S regardless of the DNA length. The faster migrating shoulder for dinucleosomal length of DNA was more prominent.

At a 2:1 ratio (Figure 6c), the free DNA seemed to disappear, even for the 100-bp sample. Surprisingly, for the reconstituted particles a gradual peak shift appeared as a function of DNA length. These peak shifts were apparent from 100 bp, over 145 bp (where the peak coincided with the 11S peak of the cold reconstituted core particle), to dinucleosomal DNA length.

At a 3:1 ratio (Figure 6d), these peak shifts were even more important; though the 145-bp particle peak still sedimented at 11 S. The disome length particle now sedimented at an S value (about 17.5 S) exceeding the S value of native disome. For comparison, the same experiment was performed by the salt dialysis method. When reconstitution was complete (at 1:1 ratio), all mononucleosomal length of DNA associated into nucleoprotein particles migrating at the mononucleosome position (except for the 100-bp particle). dinucleosomal length of DNA reassembled into particles migrating at the dinucleosome position (Figure 6e).

When cold chromatosome DNA was reconstituted by the poly(glutamic acid) method at increasing octamer:DNA ratios, the profiles showed that the 11S peak was gradually converted into a faster migrating peak of approximately 13.5 S at a 3:1 ratio (Figure 7).

With the salt dialysis method, it was not possible to generate considerable amounts of faster migrating material, as precipitation occurs when the histone:DNA ratio is driven beyond the point of complete reconstitution (Figure 1b).

Histone Quantification. In order to quantitate the histone content in the two differently sedimenting particles generated by the poly(glutamic acid) method, we isolated nucleoprotein populations from 1:1 and 3:1 octamer:DNA reconstitutions on chromatosome DNA. All sucrose fractions and start DNA

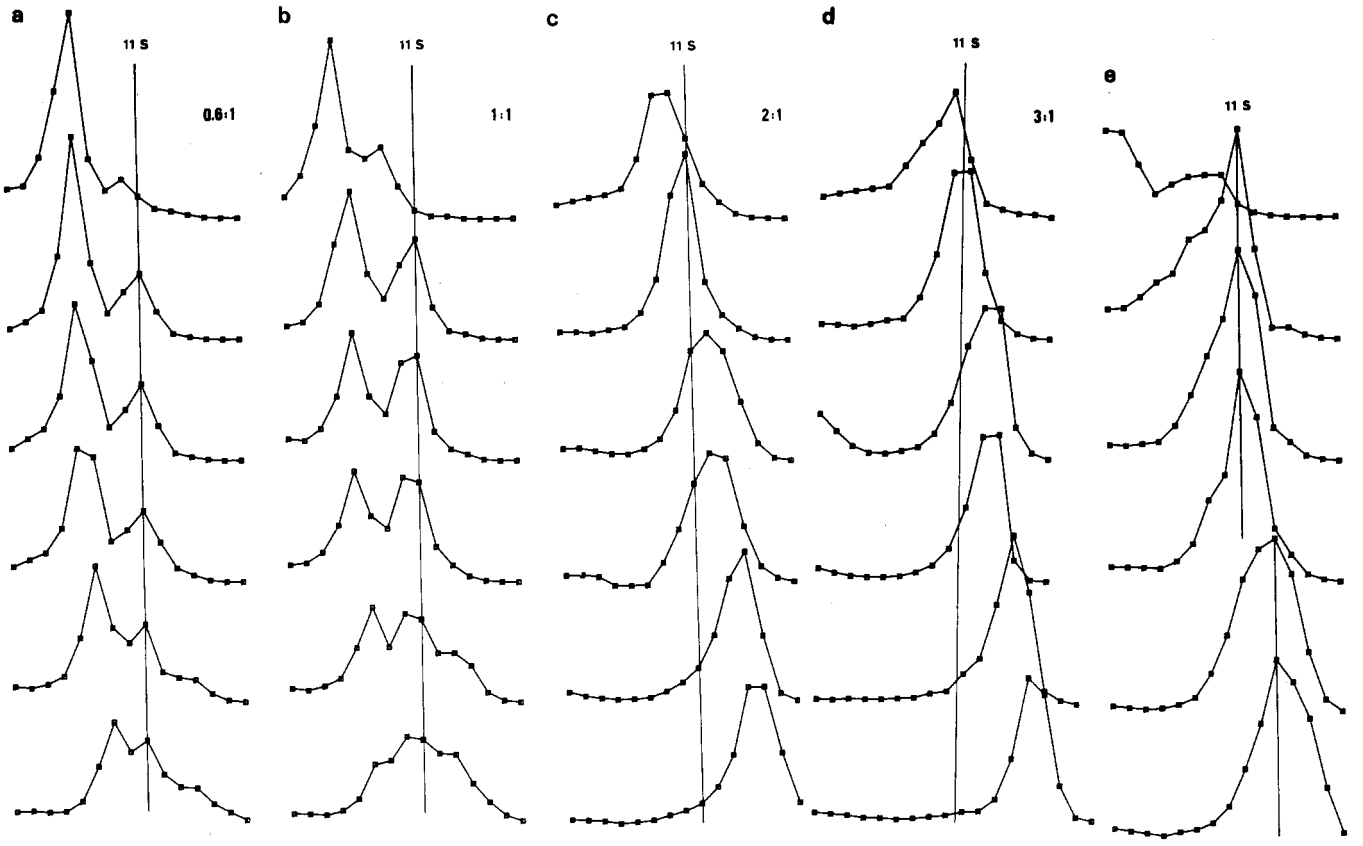

FIGURE 6: Reconstitution with different lengths of DNA and at different octamer:DNA ratios. Radioactivity profiles from fractionated sucrose gradients (10–30%). Sedimentation (38K rpm, 18.5 h, at 5 °C) is from left to right. Reconstitutions were carried out at histone:DNA ratios of 1:1 (a), 1.5:1 (b), 2:1 (c), and 3:1 (d) (left to right). The core particle position is indicated by a bar. (e) Salt dialysis reconstitution at 1:1 ratio. Bars indicate the core particle and dinucleosome position. The labeled fragments reconstituted in each series were (from top to bottom) 100 bp, core particle DNA, chromatosome DNA, long mononucleosomal DNA, compact dinucleosomal DNA, and dinucleosomal DNA. A total of 4×10^5 cpm of radiolabeled fragment was used in each reconstitution.

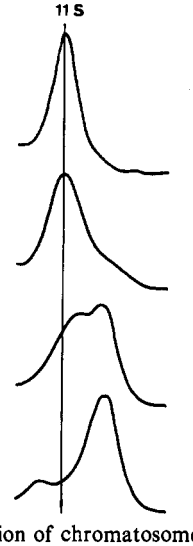

FIGURE 7: Reconstitution of chromatosome DNA at different octamer:DNA ratios: 260-nm absorption profiles from a (10–30%) sucrose gradient. Sedimentation (38K rpm, 18.5 h, at 5 °C) is from left to right. Octamer:DNA ratios were (from top to bottom) 1:1, 1.5:1, 2:1, and 3:1.

were dialyzed and concentrated to the same OD_{260}. The two quantification methods used—the isoabsorbance method (Groves et al., 1967) and the Coomassie brilliant blue G assay (Sedmak & Grossberg, 1977)—essentially yielded the same results.

(*A*) *Isoabsorbance Method.* For the start DNA, the chosen isoabsorbance wavelengths were 224.0 and 240.7 nm. For the nucleoprotein samples, the absorption difference between these two wavelengths was determined. Native chromatosome samples were included for comparison and pure histone samples for the standard plot. The 3:1 particles contained 2.0 times more histone than 1:1 particles, for the same DNA concentration; the 1:1 particles did not significantly differ from native chromatosomes (1.1 times more histone).

(*B*) *Coomassie G250 Assay.* Each sample was quantitated for histones at a few concentrations, together with native chromatosome samples, in the range were 600-nm absorption is proportional to histone concentration. Again, this method indicated that 3:1 reconstituted particles had 2 times more histone for the same DNA concentration and that 1:1 particles had the same octamer:DNA ratio as in native chromatosomes.

Fate of Poly(glutamic acid) during Reconstitution. ^{125}I-Labeled poly(glutamic acid) was added in trace amounts to cold poly(glutamic acid), and reconstitutions were carried out at ratios of 1:1, 2:1, and 3:1, with core particle DNA. The samples were then separated and fractionated on sucrose gradients. ^{125}I was only found in the gradient top fractions. We thus confirm the results of Retief et al. (1984) obtained from reconstitutions on long DNA, who showed that the poly(glutamic acid) aiding the assembly is only associated with the octamer in a transient fashion.

Nucleoprotein Gels. Nucleoprotein particles generated by poly(glutamic acid) reconstitution were isolated on sucrose gradients and analyzed directly on very low ionic strength polyacrylamide slab gels. The particles formed at different octamer:DNA ratios and for different lengths of DNA were compared on these gels.

The assembly products at 0.6:1 and 1:1 ratios for 145 bp comigrated with native core particles (Figure 2). Fractions

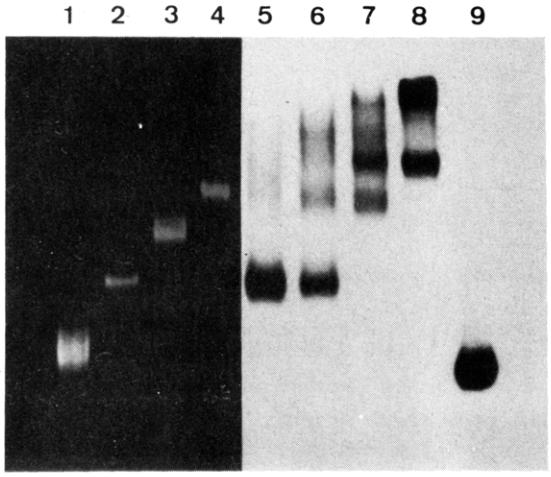

FIGURE 8: Chromatosome reconstitution—sucrose fractions on low ionic strength nucleoprotein gel (5% polyacrylamide): (lane 1) free chromatosome DNA; (lane 2) native core particles; (lane 3) native chromatosomes; (lane 4) compact dinucleosomes; (lane 5) chromatosomes reconstituted at 1:1 ratio; (lane 6) chromatosomes reconstituted at 1.5:1 ratio; (lane 7) chromatosomes reconstituted at 2:1 ratio; (lane 8) chromatosomes reconstituted at 3:1 ratio; (lane 9) start chromatosome DNA.

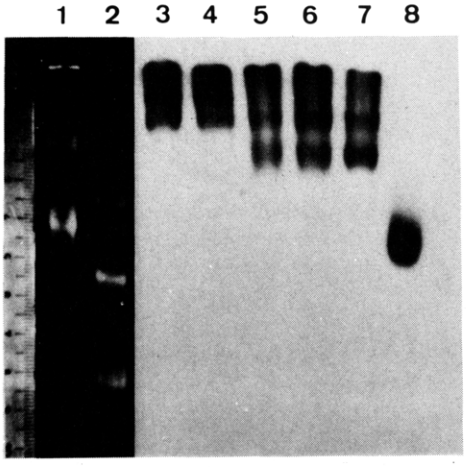

FIGURE 9: Dinucleosome reconstitution. Low ionic strength nucleoprotein gel (5% polyacrylamide). (Lane 1) Native chromatosomes and disomes; (lane 2) free DNA, core particles, and compact disomes; (lane 3) particles reconstituted at 0.6:1 ratio (peak); (lane 4) particles reconstituted at 1:1 ratio (peak); (lane 5) particles reconstituted at 0.6:1 ratio (faster migrating shoulder); (lane 6) particles reconstituted at 1:1 ratio (shoulder); (lane 7) reconstitution at 2:1 ratio (peak); (lane 8) start disome DNA.

sedimenting at 5 S in sucrose gradients comigrated with free DNA. At a ratio of 2:1, where all the DNA takes part in the reconstitution, a different pattern appeared in nucleoprotein gel. Only part of the sample comigrated with native core particles. Most of it was found at the compact dinucleosome position (dinucleosome with no linker) and at the trinucleosome position. Some material could not even enter the gel. The presence of particles carrying two or three octamers is hard to reconciliate with an S value, equal to that of core particles. The complex of 145 bp and two octamers has been reported to sediment at 13 S (Stein, 1979). So, we concluded that the 2:1 reconstituted particles were subject to aggregation after isolation on sucrose gradient. This would imply that although reconstitution seems most complete at a 2:1 ratio, the particles carry features alien to normal core particles that allow aggregation.

Nucleoprotein gels of particles reconstituted on longer DNA confirmed the observations. For a chromatosomal length of DNA, the 1:1 assembly product comigrated with core particles, as is expected for chromatosomes not carrying a histone H1 (Figure 8). At 1.5:1, 2:1, and 3:1 ratios, aggregation seemed to have occurred again.

For dinucleosomal length of DNA, the reconstitution products were susceptible to aggregation from the low ratios on (Figure 9). A considerable part of the samples did not enter the gel, and the remainder migrated as disomes and larger. Not all nucleoprotein particles showed the same predisposition to aggregation. The 11S fractions aggregated in larger structures than did the faster migrating fractions.

As mentioned earlier, poly(glutamic acid) has been reported for keeping chromatin with additional histones soluble that otherwise would precipitate. On the other hand, the poly(glutamic acid) is left on top of the gradient on centrifugation.

These observations could explain why the formed nucleoprotein particles that seem perfectly soluble during separation on sucrose gradient aggregate after fractionation. Upon storage at 4 °C, the material migrating above "trisome" in nucleoprotein gel even came out of solution.

Exonuclease III Digestion on Poly(glutamic acid)-Reconstituted Particles. Exonuclease III exhibits a 3′–5′ single-strand exonuclease action that is impaired at the points of interaction with, in casu, the histones. Analysis of the

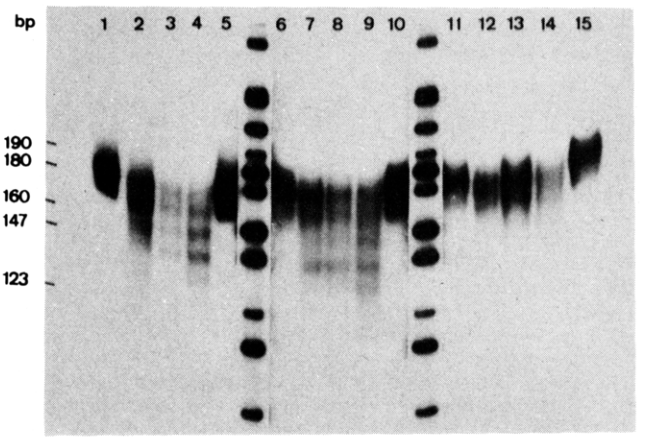

FIGURE 10: ExoIII digestion of radiolabeled chromatosomes—7% single-strand formamide gel. Digestions were carried out in 0.1 mM EDTA, 0.6 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) with 100 units/mL exonuclease III (Boehringer) at 37 °C. The digestion was stopped by adding EDTA to a final concentration of 5 mM and by cooling on ice. (Lanes 1–5) Labeled native chromatosomes; (lanes 6–10) chromatosomes reconstituted at 0.6:1 ratio; (lanes 11–15) chromatosomes reconstituted at 2:1 ratio. Marker: PBR *Msp*I. (Lanes 1, 6, and 11) Start sample; (lanes 2, 7, and 12) 2-min digestion; (lanes 3, 8, and 13) 4-min digestion; (lanes 4, 9, and 14) 8-min digestion; (lanes 5, 10, and 15) blank 8-min incubation at 37 °C.

digestion fragments on a denaturing polyacrylamide gel indicates the position of the octamer on the DNA.

Reconstituted particles, containing chromatosomal DNA labeled at its 5′ ends, were recovered from sucrose gradients. The digestion pattern from these particles was compared to that from native H1,H5-depleted chromatosomes, also 5′ labeled.

Digestion of native chromatosomes leads to discrete sets of single-stranded fragments that migrate as a 10-bp ladder on denaturing gel (Figure 10). For the 0.6:1 reconstituted particle, the general appearance of the digestion pattern was the same, but the bands looked somewhat diffuse and the 145-bp band was more prominent. Since the 145-bp band reflects the positioning of the octamer on one end of the DNA fragment, this indicates that some end positioning is induced by the reconstitution method.

The 2:1 reconstituted particle showed a different pattern.

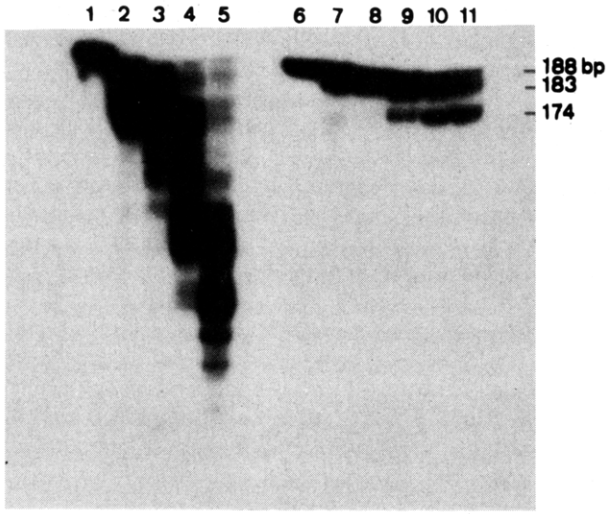

FIGURE 11: Exonuclease III digestion of a nucleoprotein particle reconstituted from a cloned 186-bp fragment (obtained from nucleosomal DNA) and excess octamers. The DNA was labeled on one 5′ end. A 7% single-strand formamide gel was used. (Lanes 1–5) Free DNA digestion: start and 1-, 2-, 5-, and 9-min digestion. (Lanes 6–11) Nucleoprotein digestion: start and 1-, 2-, 5-, 9-, and 14-min digestion. Digestions were as in Figure 10.

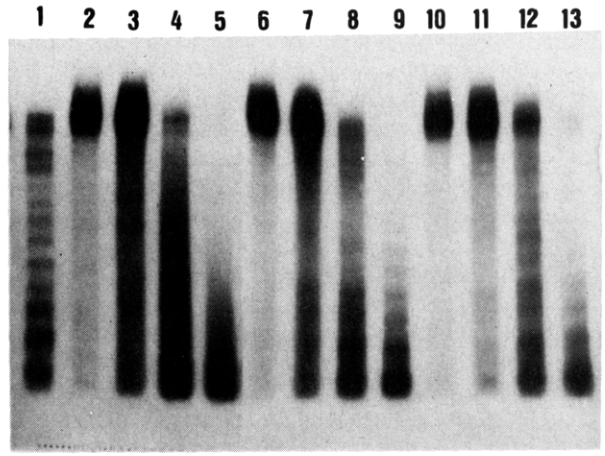

FIGURE 12: DNase I digestion of reconstituted chromatosomes—single-strand 5% polyacrylamide–ureum gel. Digestions were carried out in 0.1 mM EDTA, 0.6 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8) at 37 °C and stopped by EDTA (5 mM) on ice. (Lane 1) typical digestion pattern from native chromatosomes; (lanes 2–5) start DNA; (lanes 6–9) particles reconstituted at 1:1 ratio; (lanes 10–13) particles reconstituted at 3:1 ratio; (lanes 2, 6, and 10) start; (lanes 3, 7, and 11) 1-min digestion (6 units/mL DNase I); (lanes 4, 8, and 12) 1-min digestion (60 units/mL); (lanes 5, 9, and 13) 10-min digestion (60 units/mL).

The DNA seemed more protected against digestion and bands were only vaguely imposed on a smear.

Both reconstituted samples, but in particular the 2:1 particle, were found turbid, after they had been dialyzed to digestion buffer and concentrated. This was not the case for native chromatosomes.

As turbidity usually indicates the occurrence of aggregates, this could explain the protection of the DNA against exo III digestion, and so the presence of some extra octamer (Figure 7c) could have an amplified effect. That this protection produces digestion stops that could be mistaken for resulting from an octamer position is shown in Figure 11, a digestion pattern from a particle containing a cloned (nonidentified) chromatosome DNA fragment, labeled at one 5′ end and saturated with octamers.

Instead of finding full protection, digestion is halted after three strong bands, which are also found in the exo III pattern on free DNA. Under these, a faint 10-bp ladder was visible. With the label on the other 5′ end, a similar pattern was generated. Comparable results were found on another cloned fragment (not shown).

DNase I Digestion on Poly(glutamic acid)-Reconstituted Particles. DNase I, a single-strand endonuclease that is inhibited at sites where DNA is bound to protein, can be used to take a "footprint" of the DNA pathway on the histone octamer (Lutter, 1978).

Chromatosomes reconstituted at 1:1 and 3:1 ratios were isolated on sucrose gradients and dialyzed. After digestion and deproteinization, the DNA was labeled at the 5′-OH termini from the original micrococcal nuclease cuts (DNase I cuts require dephosphatizing prior to labeling). The DNase I digestion kinetics from these particles and start DNA were compared on a denaturing gel (Figure 12).

The occurrence of the 10-bp ladder (reflecting the helix repeat of the DNA bound on the octamer) proves that reconstitution is in any case not random but that at least the orientation of the DNA along its axis is uniform. Although 3:1 particles always seemed somewhat more protected against nuclease action, the general appearance of the digestion patterns was the same. This indicates that the additional histones do not cover much of the outer DNA surface nor that much DNA is buried inside in the aggregates.

Discussion

Direct mixing in vitro of DNA and histones at physiological ionic strength results in very inefficient reconstitution if not precipitation, although assembly in vivo occurs in similar ionic conditions. Therefore, the requirement for an "assembly factor" has been put forward. In *Xenopus laevis*, an acidic "assembly protein" (nucleoplasmin) that binds histones and transfers them to the DNA was purified from egg extract (Laskey et al., 1978; Earnshaw et al., 1980). Acidic polypeptides have been shown to display similar properties in vitro (Stein et al., 1979).

Poly(glutamic acid) makes it possible to avoid the high-salt buffers that are used in the salt dialysis method. Even direct mixing and methods involving redistribution of octamers from native chromatin to (labeled) DNA fragments require 0.6–0.8 M NaCl buffers. Poly(glutamic acid) also permits very fast reconstitution. After 30 min, the association maximum is attained (Retief et al., 1984). Yet, in spite of its advantages, our studies show that this method may not be well suited for reconstituting mono- and dinucleosomal length of DNA.

First of all, at a 1:1 (w/w) ratio of octamer to DNA (which is the ratio in native core particles), it does not seem possible to reconstitute more than half of the DNA into 11S nucleoprotein particles. Total concentration during assembly had no effect on reconstitution yield. We found the poly(glutamic acid):octamer ratio not to be very critical in the excess range (personal observation). For core particle assembly, similar inefficient reassociation was observed by Oohara et al. (1983), using longer (average M_r) poly(glutamic acid) preparations at lower poly(glutamic acid):octamer (w/w) ratios.

Octamer:DNA (w/w) ratios of at least 2:1 are needed before all free DNA is assembled. On the other hand, with the salt dialysis method we obtained almost complete reassociation into 11S particles at 1:1 ratio, using the same preparations of octamers and DNA.

For core particle DNA, the S value of the nucleoprotein particles did not shift as a function of histone:DNA ratio. This and the presence of histones in fractions near the top of the

gradients argue in favor of the conclusion that the transfer of histones from poly(glutamic acid) to DNA is not complete. Judging from the series of reconstitutions with different lengths of DNA, this transfer becomes more efficient as the DNA is longer. This is in accordance with the remark of Retief et al. (1984) that core particle yield from reconstitution on 145 bp is lower than that from long DNA. In native particles, 145 bp is clearly sufficient to stabilize and accommodate a histone octamer. At pH 8, poly(glutamic acid) adopts an almost completely ionized random coil conformation (Imahori & Tanaka, 1959). If poly(glutamic acid) reconstitution involves competition of DNA with this polyanion for octamers, then it seems plausible that increasing DNA length will favor nucleoprotein formation. Not only does reconstitution efficiency increase with DNA length, but also the ability to accommodate additional histones does. Assembly at histone:DNA ratios that allow participation of all free DNA and with DNA longer than core particle length results in particles that sediment faster in sucrose gradients. The peak shifts increase as the DNA is longer and also with histone:DNA ratio. For chromatosome DNA, maximum packing seems to be reached at a 3:1 ratio, as reconstitution at much higher ratios does not produce any further shifts. Reconstitution using cold chromatosome DNA shows that the peak shifts should be interpreted as resulting from gradual forming of populations of faster sedimenting particles. For chromatosome DNA, the S value of this heavier complex and histone quantification argue in favor of a two-octamer particle. Protein gels always show the normal histone contents, which indicates that additional core histones are acquired in stoichiometrical amounts. This is in agreement with the notion that poly(glutamic acid) binds and transfers core histones as octamers, unless, as cannot be excluded, individual histones have an equal probability of getting involved as extra histones. With the salt dialysis method, it was not possible to generate particles with extra histones without provoking an overall precipitation.

Analysis of isolated sucrose fractions on low ionic strength nucleoprotein gels showed that for DNA lengths in the mononucleosomal range, the 2:1 assembled particles were highly susceptible to aggregation. Only particles reconstituted at low octamer:DNA ratios had a normal mobility. Also due to precipitation, sedimentation analysis had to be carried out on complete reconstitution mixtures. Assembled particles containing dinucleosomal DNA were always susceptible to aggregation. It seems as if poly(glutamic acid), which remains at the top of the sucrose gradient, is needed to keep the formed nucleoprotein particles soluble. Exonuclease III digestion of aggregated samples resulted in aberrant digestion patterns with digestion stops, which in some cases could easily be mistaken as a result of a particular octamer position. The presence of incompatible single-strand stops and the mobility of the particles argued in favor of large nucleoprotein complexes with less tightly bound DNA ends.

The fact that isolated reconstituted particles are not soluble at low ionic strength, even when they do not carry extra histones, makes the faithfulness of their reconstructed conformation questionable. It is not very likely that they aggregate due to excess free histones as these are not expected to be present in an 11S fraction. Rearrangement within particles is also unlikely at low ionic strength.

In the case of core particles reconstituted at 2:1 ratio, it could be that in the reconstitution mixture 145 bp can actually be packed with an extra octamer (Oohara et al., 1983) but that the octamer with the fewest DNA interactions is torn off during centrifugation. This would leave an aberrant 11S particle with a protruding DNA end. One could imagine for instance the DNA being partially "screwed out" of the solenoidal pathway on the octamer. Such a position has indeed been reported for a 145-bp salt-reconstituted particle (Ramsay et al., 1984). Particles like this would have sticky ends available for aggregation. DNA excess seems to cause aggregation: for disome DNA, aggregation is more severe in slower sedimenting fractions than in faster, presumably octamer-saturated, particles. Our DNase I results could indicate that the accommodation of extra octamers and aggregation may occur by protein core to core stacking.

Thus, only short monosome DNA can be faithfully reconstituted with octamers by poly(glutamic acid), but unfortunately even then only at ratios resulting in a low efficiency of reconstitution. This also implies that when random DNA is being worked with, some DNA sequences might be favored in the reconstituted fraction.

ACKNOWLEDGMENTS

We thank Dr. I. Lasters, G. Meersseman, and Prof. R. Hamers for helpful discussions and suggestions and W. Verheulpen for preparing the histone octamers.

Registry No. Poly(glutamic acid), 25513-46-6; poly(glutamic acid), SRU, 24991-23-9.

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High-Resolution Analysis of *Lac* Transcription Complexes inside Cells†

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Received March 21, 1986

ABSTRACT: A new primer extension analysis is used to determine the methylation pattern over the *lac* UV5 promoter when dimethyl sulfate is added to growing *Escherichia coli*. The high-resolution analysis reveals altered methylation of 15 bases when the transcription machinery occupies the promoter inside the cell and shows a striking dichotomy in the distribution of methylated bases. Four protected guanosines lie on the side of the helix shown previously to be closely bound by RNA polymerase in vitro [Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell (Cambridge, Mass.) 20*, 269–281]. By contrast, the 11 hyperreactive bases lie on the side of the DNA directly opposite from that bound by protein. Those not in the melted region form two distinct "back-side" patches near −35 and −16. We suggest that such hyperreactive patches can be caused by proteins bending the DNA toward themselves to allow a full range of contacts, thus distorting the helix grooves on the "back" side and facilitating attack by the methylating reagent. This leads to a proposal for the formation of transcription complexes in which RNA polymerase interacts with deformed and torsionally stressed DNA.

The characterization of transcription complexes at promoters is a major goal of molecular biology. Much valuable information has come from the study of transcription systems reconstructed in vitro from isolated components [for references to the *lac* system, see Meiklejohn & Gralla (1985)]. One potential problem in this approach is that one can never be certain that all of the important components have been included or that the in vitro conditions reflect the milieu of the cell. In recognition of this potential problem, several recent reports describe methods aimed at studying the state of regulatory DNA in vivo (Becker & Wang, 1984; Nick & Gilbert, 1985). These methods involve isolating DNA from cells containing nicks or modifications introduced in vivo and mapping these relative to nearby restriction cleavage sites.

Recently we described a new method of DNA footprinting in vitro that does not require the close proximity of restriction enzyme cleavage sites or blotting techniques (Gralla, 1985). In addition to yielding especially high-resolution data and being particularly rapid, this technique allows the simultaneous probing of many regions of a DNA sample. DNA samples are probed by primer extension, with the synthetic primer sequences chosen to flank regions of interest. When applied to the interaction of RNA polymerase with supercoiled (i.e., torsionally stressed) *lac* DNA in vitro, this technique confirmed the importance of DNA supercoiling in certain reconstructed systems [see Borowiec & Gralla (1985a,b) for a discussion]. The attacking reagent used in the initial study, DNase I, does not reveal the intimate contacts between protein and DNA as do the chemical probes of DNA developed by Gilbert and colleagues [see Siebenlist et al. (1980)]. We have now adapted the primer extension method to allow the use of these and other probes in vivo or in vitro on supercoiled DNA with single nucleotide resolution.

Materials and Methods

Materials. Escherichia coli proteins RNA polymerase, *lac* repressor, and CRP were all purified in this laboratory [see Meiklejohn & Gralla (1985) for references]. CRP (catabolite activator protein) was a gift of A. Meiklejohn. *Lac* plasmids carried a 207 base pair (bp) fragment containing a *lac* control region inserted into the *Eco*RI site of pAS21 (a modified pBR322 plasmid; Stefano et al., 1980). Commercial sources were used for Klenow fragment of DNA polymerase (BRL) and pBR322 sequencing primers (*Hin*dIII, top strand primer; *Eco*RI, bottom strand primer; Pharmacia P-L Biochemicals), which can be extended through the *lac* insert.

In Vitro Methylation. Lac plasmid DNA (0.5–2.5 μg) was diluted into 100 μL of transcription buffer containing 30 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM dithiothreitol,

† This research was supported by U.S. Public Health Service Grants CA19941 and GM35754 and by U.S. Public Health Service National Research Service Award GMO7104 (to J.A.B.).

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0006-2960/86/0425-5051$01.50/0