Nucleosome Cores Assembled in Vitro Occupy Two Preferred Frames Flanking the Histone H1 Gene from *Psammechinus miliaris*†

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ABSTRACT: The histone h22 gene quintet of *Psammechinus miliaris* contains two simple sequence motifs. One sequence, $[d(C\text{-}T)]_{23}$, is located in the H2A–H1 spacer area, and another, $[d(G\text{-}A)]_{16}$, is located in the H1–H4 spacer area. Fragments containing these sequences were inserted into pGV403 plasmids to allow strand-specific end labeling. Nucleosome cores assembled "in vitro" in the presence of poly(glutamic acid) on these labeled linear fragments occupy in each of the fragments a preferred frame which includes the repetitive sequence motif. The frames are characterized by an approximately 10 base pair DNase I digestion periodicity. On the remainder of the fragments, nucleosome cores are assembled, either in a random manner or in a number of complex frames that cannot be resolved.

Under "in vivo" assembly conditions, nucleosome cores can take up precisely defined positions (Samal & Worcell, 1981; Zhang & Hörz, 1984; Benezra et al., 1986). The forces involved in the positioning reaction are obscured inter alia by the number of proteins that may be involved in such a process and the complexity of the in vivo assembly reaction at the replication fork (Herman et al., 1981) or following transcription (Baer & Rhodes, 1983). This is also true for "in vitro" assembly promoted by crude nuclear extracts [for a review see Zachau and Igo-Kemenes (1981)]. The use of defined in vitro assembly systems, on the other hand, reduces the number of unknowns and provides the possibility of investigating the specificity of the interactions between the histones and DNA. In such defined in vitro systems nucleosomal cores have been assembled in specific positions on unique DNA by using poly(glutamic acid) or high ionic strength protocols (Simpson & Stafford, 1983; Simpson et al., 1985).

Since, of the reaction partners in the in vitro assembly, in a given experiment all the histone octamers are identical, the position-determining force must be sought in DNA. To investigate this in more detail, we have chosen chicken histone octamers and two DNA fragments, both possessing two conformational domains. One domain should be fairly rigid by the criteria established by Hogan et al. (1983), and the second domain, consisting of the remainder of the fragment, should be of predominantly random flexibility. The rigid areas in both fragments comprise simple homocopolymer sequence motifs which occur commonly in a number of eukaryotic genomes (Tautz & Renz, 1984). The selected fragments were derived from the spacer areas flanking the histone H1 coding area of the h22 histone gene quintet of the sea urchin *Psammechinus miliaris* (Hentschel, 1982). The fragment upstream from the H1 coding area contains the homocopolymer motif $[d(C\text{-}T)]_{23}$ and the downstream stretch $[d(G\text{-}A)]_{16}$ (Hentschel, 1982). Considering the conformational constraints present in a double-stranded pyrimidine–purine homocopolymer, it is obvious that without strand separation such an area is rigid (Hogan et al., 1983), making DNA–octamer interaction difficult. These two motifs can become S1 sensitive under certain conditions, due to the formation of single-stranded loops by a unique slippage mechanism (Hentschel, 1982) that ultimately may lead to transposon-like genetic rearrangement events (Portmann & Busslinger, 1979). The in vitro conditions for strand separation are, however, such that this should not occur during core assembly by using a poly(glutamic acid) protocol. Discontinuity of double strandedness resulting in loops has been reported to exclude nucleosome core positioning on such regions during in vitro assembly (Nickol & Martin, 1983). On the other hand, the flanking regions to these motifs appear to be conformationally less committed and should be considerably more flexible (Dickerson & Drew, 1981). Using these fragments with potentially two simultaneously expressed conformations as substrates for in vitro assembly should reveal effects of DNA conformation on positioning. In order to achieve precise measurements of the positioning of cores on these unique DNA fragments, a mapping procedure with the resolution power of single base pairs would be desirable.

Unique DNA fragments propagated in pGV403 plasmids can be labeled strand specifically (Volkaert et al., 1984). These plasmids had been developed to facilitate chemical DNA sequencing, but we have found them to be particularly suitable for studying DNA–protein interactions at high resolution. We have used this technique in conjunction with a poly(glutamic acid)-assisted assembly reaction (Stein et al., 1979; Retief et al., 1984) to faithfully assemble nucleosome cores on such strand specifically end labeled fragments. The analysis of the protection pattern of the core-associated DNA, by DNase I digestions followed by denaturing DNA gel electrophoresis, can then be used to accurately map the positions of the assembled cores on unique DNA sequences.

Experimental Procedures

The pGV/JR/h1– and pGV/JR/h1+ plasmids were constructed according to Figure 1. All restriction digests, plasmid preparations, Southern hybridizations, and cloning steps were carried out according to Maniatis et al. (1982). pGV403 plasmids, *Sma*I digested and phosphatase treated, were obtained from Amersham. Fragments inserted in the *Sma*I site

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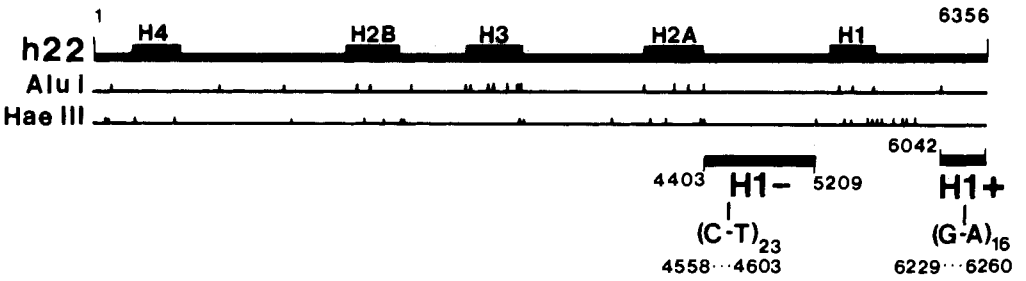

FIGURE 1: Construction of pGV/JR/h1− and pGV/JR/h1+ plasmids. The pGV/JR/h1− plasmid was constructed by digesting the h22 histone gene battery with *Hae*III, collecting the motif containing fragments from an agarose gel, and inserting the fragment containing the motif upstream from the H1 coding area into the *Sma*I site of pGV403. The fragment containing the motif downstream from the H1 coding area was also collected, digested further by *Alu*I, and purified on an agarose gel. This fragment is flanked by a *Hin*dIII and an *Alu*I site. The *Hin*dIII site was blunt-ended by a fill-in reaction with the Klenow fragment of DNA polymerase, and the pGV/JR/h1+ plasmid was constructed by inserting this fragment into the *Sma*I site of the pGV403. The *Sma*I site is 10 base pairs removed from the *Tth*111I labeling site. This, plus the additional base added by the fill-in labeling reaction, places the motifs an extra 11 bases from the labeled end of the fragment. The numbers indicate the positions of the cutting sites and the motifs according to the unpublished complete h22 sequence (see Acknowledgments).

of these plasmids can be excised by digestion with *Tth*111I, resulting in the formation of a single base pair overhang at each end. These overhangs are formed by G at one end and A at the other strand. This allows the excised insert to be labeled strand specifically by a Klenow fill-in reaction utilizing [α-^{32}P]dCTP or [α-^{32}P]dTTP, respectively (Volkaert et al., 1984). The vector will not be labeled during this process and thus does not have to be removed after the labeling reaction. Size standards for denaturing DNA gels are a *Hpa*II digest of pBR322, labeled with a Klenow fill-in reaction with [α-^{32}P]dGTP and [α-^{32}P]dCTP. This leads to an increase by 2 base pairs (bp) in the size of the fragments.

Histone octamers were prepared and stored at −20 °C in 50% (w/v) glycerol, 1 M NaCl, and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0 (Greyling et al., 1983). Nucleosome core assembly reactions were carried out by mixing 10 μg of poly(glutamic acid), from a stock solution of 10 mg/mL in 10 mM phosphate buffer, pH 6.9, to 5.4 μg of histone octamers (Retief et al., 1984). The NaCl concentration of the mixture was then adjusted to 125 mM NaCl from a 0.5 M NaCl stock solution. Eight micrograms of DNA was then added from a stock solution in 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris-HCl, pH 7.6. The assembly mixture (10 μL) was then gently agitated and incubated for 3 h at 37 °C. This octamer to DNA ratio was chosen in order to reduce the formation of extensive tracts of tight polycores which may obscure specific histone–DNA interactions. For DNase I digestions the assembly mixture was cooled to 20 °C for 30 min, followed by incubation at 16 °C for 15 min to limit the mobility of the assembled nucleosome cores (Beard, 1978). The magnesium concentration was then adjusted to 10 mM free magnesium ions, and the DNase I was added. Incubation was continued for varying periods and terminated with excess EDTA. Micrococcal nuclease digestions were carried out in 1.4 mM excess calcium ions, the reaction was similarly terminated with excess EDTA, and the DNA was twice extracted with phenol and chloroform prior to precipitation with ethanol. The DNA used for assembly reactions was purified via phenol extraction and cesium chloride gradient centrifugation. DNA was dissolved in 1 mM EDTA, 1 μg of proteinase K/mg of DNA and 10 mM Tris-HCl, pH 7.6, and digested for 1 h at 37 °C. DNA was then further purified by additional phenol extractions and a cesium chloride centrifugation step.

Denaturing DNA gels were prepared and electrophoresed on gels of 20 cm × 40 cm and varying in thickness from 0.45 mm at the top to 0.90 mm at the bottom (Maxam & Gilbert, 1980). The gels were scanned with a custom-built densitometer. To linearize the densitometer traces, the standards lane was scanned and the log of DNA length vs. distance traveled by each fragment plotted. A suitable polynomial, normally eighth order, was fitted and used to recalculate the positions of every point on the trace to a linear scale on the x axis (unpublished results).

RESULTS

Two plasmids were constructed (Figure 1). pGV/JR/h1− contains the h22 homocopolymer motif upstream from the H1 coding area, and pGV/JR/h1+ contains the motif downstream from the H1 coding area of ph22 (Schaffner et al., 1978). The inserts comprise 806 base pairs for the h1− fragment and 314 base pairs for the h1+ fragment, respectively. We had previously shown that under the assembly conditions used the 10 base pair protection periodicity against DNase I digestion of the assembled cores is indistinguishable from that of natural cores (Retief et al., 1984). Additional evidence for the conformational identity of natural and assembled cores is provided by the elution of histone octamers from the in vitro assembled cores bound to hydroxyapatite (Figure 2). In this experiment the labeled free DNA is bound to the column (Figure 2A). Free histones, on the other hand, remain essentially unbound, and the bulk can be recovered at low ionic strength (Figure 2B). The remainder elutes at higher ionic strength, possibly due to nonspecific interactions with the matrix. When histones are incubated with DNA in the absence of poly(glutamic acid), under conditions identical with those used for assembly reactions, the random complexes formed behave in much the same way as free histones (Figure 2C). However, after incubation of the histones and DNA in the presence of poly(glutamic acid), 81% of the labeled histones are recovered at NaCl concentrations appropriate for the elution of histone octamers from natural cores (Faulhaber & Bernardi, 1967; Simon & Felsenfeld, 1979; Greyling et al., 1983). To further characterize the assembly products, the pBRh22 plasmid was digested with *Hin*dIII, assembled with histone octamers to form nucleosome cores, and digested with micrococcal nuclease. The fragments were electrophoresed on a 2% agarose gel, Southern transferred, and probed with nick-labeled, purified h22. The assembled h22 quintet produced a characteristic range of well-defined bands typical for cores (McGhee & Felsenfeld, 1980), tight dimers and trimers (Steinmetz et al., 1978). Free h22 DNA treated in the same way was almost completely digested and did not reveal any prominent size classes.

DNase I digestion of the free, uniquely end labeled fragments, reveals a complex pattern of bands (Figures 3 and 4),

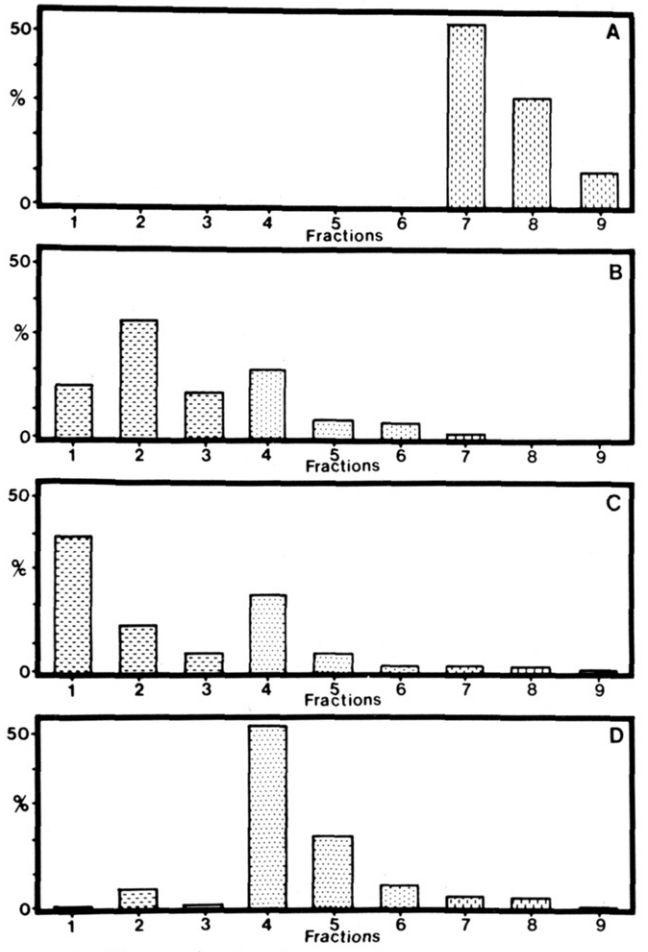

FIGURE 2: Chromatography of assembled cores. A miniature column containing 0.2 mL of hydroxyapatite was charged in successive experiments (A–D) with the labeled components of the assembly reactions and eluted with 200 μL/fraction of the following solutions: fractions 1–3, 0.5 M NaCl and 10 mM phosphate, pH 6.9; fractions 4–6, 2 M NaCl and 10 mM phosphate buffer, pH 6.9; fractions 7–9, 0.5 M phosphate buffer, pH 6.9. (A) [α-^{32}P]dCTP-labeled h1– fragment (total recovery = 94%). (B) ^{125}I-Labeled histones (total recovery = 88%). (C) ^{125}I-Labeled histones incubated with the h1– fragment in the absence of poly(glutamic acid) under conditions otherwise identical with those used in the assembly reaction (total recovery = 91%). (D) ^{125}I-Labeled histones assembled on the h1– fragment in the presence of poly(glutamic acid) (total recovery = 95%).

due to the sequence specificity of the nuclease (Drew, 1984). After the assembly of cores on the end-labeled fragments, a number of differences between the digestion patterns of free DNA and assembled DNA are apparent. For both the h1– and the h1+ fragments, core assembly results in a reduced rate of digestion compared to that for free DNA, requiring a 10-fold increase of DNase I to yield digestion patterns similar to those of free DNA. In addition, on the assembled DNA an approximately 10-bp ladder is apparent in the sequence motifs in both these fragments. The results for the h1+ fragment are given as an example. This demonstrates the presence of a periodic protection pattern which is expected from one-face protected DNA partially digested by DNase I. To elucidate any protection periodicity extending beyond the motifs, the autoradiograms were scanned and the traces linearized with respect to fragment size (Figure 4). From the linearized trace it is clear that the assembly has converted the equal accessibility to DNase I of all internucleotide linkages in the sequence motif to a new prominent feature of periodic protection, resulting in an approximately 10-bp register. This extends into the DNA sequences flanking the motif. The modulation of the typical digestion pattern of the sequence

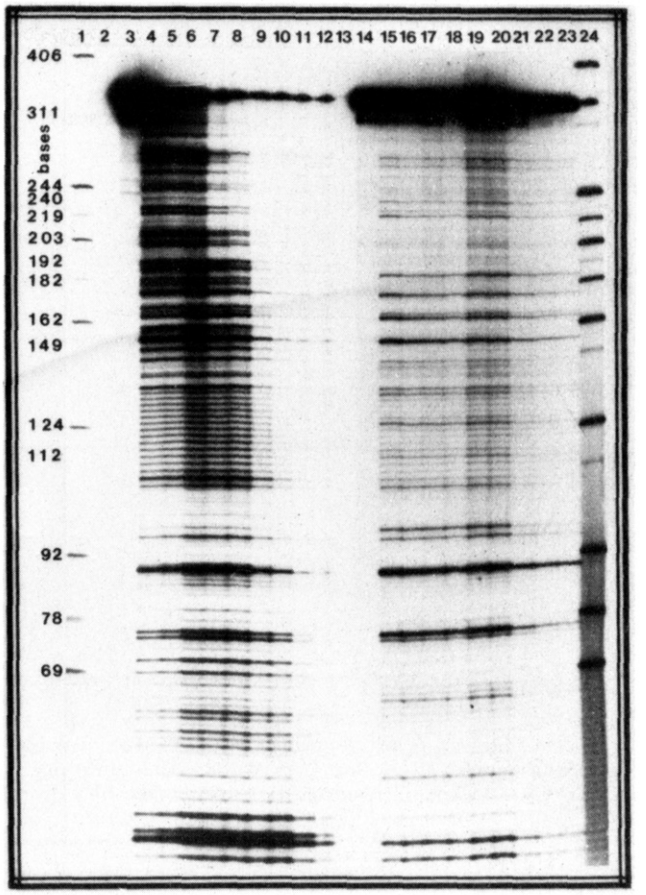

FIGURE 3: Autoradiogram of the h1+ fragment digested with DNase I. The h1+ fragment was labeled with [α-^{32}P]dCTP and digested with DNase I: lanes 3–12, free DNA digested at 1.25 units of DNase I/μg of DNA; lanes 14–23, DNA assembled at a 0.6 to 1 histone to DNA ratio digested at 12.5 units of DNase I/μg of DNA. The digestions were carried out at 16 °C for 0, 15, 30, 60, 90, 120, 240, 480, 720, and 1200 s. Lanes 1, 2, and 24, pBR322 digested with *Hpa*II and labeled with [α-^{32}P]dGTP by a fill-in reaction. The DNA was analyzed on a 6% acrylamide denaturing DNA gel.

motifs serve as an orientation point to determine the extent of the protection repeat both up- and downstream from the motif. For the h1+ fragment this pattern is particularly distinct between 40 and 190 base pairs (Figure 4). For the h1– fragment a similar but less well delimited periodicity extends from approximately 80–220 base pairs (results not shown). In the preferential assembly frame in the h1– fragment, two turns of the DNA helix can be easily discerned in the $[d(C\text{-}T)]_{23}$ motif, with an apparent pitch of closer to 10.5 than to 10 bp (Figure 5). Since there are only two turns of the DNA helix on the motif measurable at single base resolution, general conclusions as to the size of the protection periods over the entire length of the core DNA can not be drawn. The postulated regular lateral disposition of the DNase I cutting sites (Klug & Lutter, 1981) may not apply as a consequence of the irregular distortion of the DNA helix on the surface of the core (Richmond et al., 1984). The presence of such protection periodicities indicates a high abundance of cores assembled in register with the motifs in these two frames. The digestion protection pattern in the remainder of the fragments, though exhibiting some periodicity, does not allow the determination of the size or the position of preferential frames with the desirable precision. This indicates predominantly random distribution of cores in those areas. This randomness is also responsible for the formation of tight dimers and trimers (see above), which do not occur in native chromatin (Steinmetz et al., 1978). In an attempt to reduce this

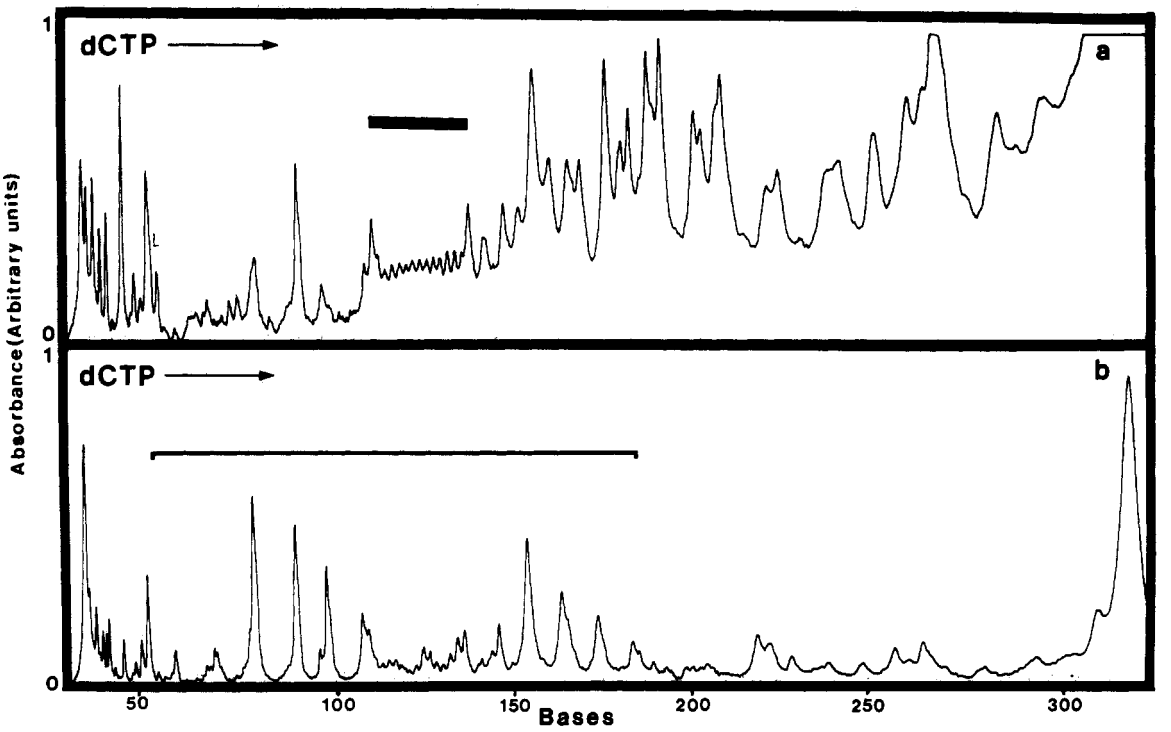

FIGURE 4: Linearized densitometer trace of the h1+ fragment. The 30-s digestion is from Figure 3. (a) Free DNA and (b) assembled DNA were scanned. The base numbering refers to the number of bases from the [α-^{32}P]dCTP label (see also legend to Figure 1). The bracket indicates the preferred assembly frame, and the horizontal bar indicates the extent of the sequence motif.

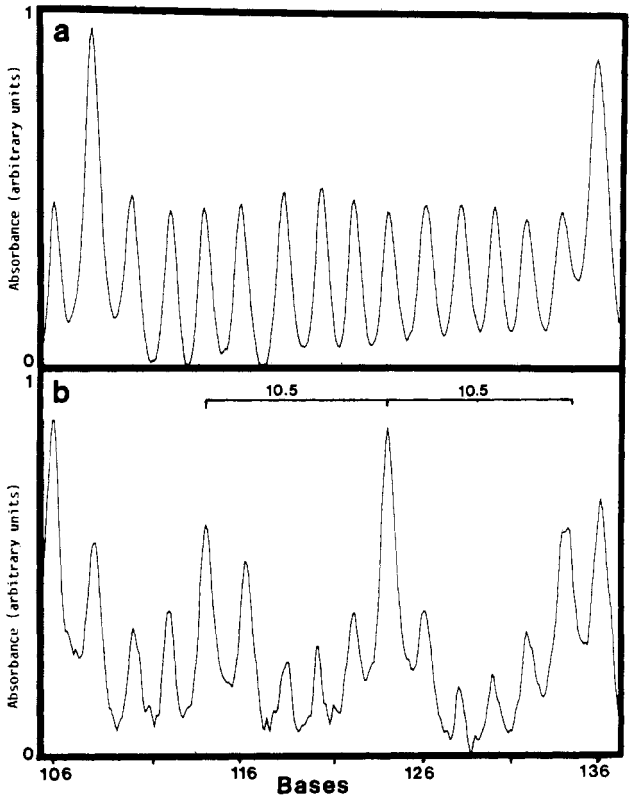

FIGURE 5: DNase I digestion pattern of the motif of the h1- fragment. Linearized densitometer trace of the h1- fragment labeled with [α-^{32}P]dTTP: (a) free DNA; (b) assembled DNA. The bracket indicates two adjacent DNA helix repeats. The base numbering indicates the number of bases from the label.

nonspecific interaction of DNA with excess octamers, all assembly reactions were also carried out at histone to DNA ratios of 0.6 to 1 and 0.3 to 1. However, no increase in preferential assembly occurs at reduced histone to DNA ratios (results not shown).

Discussion

A precisely positioned core should on DNase I digestion result in a well-defined 145 base pair protected area exhibiting a 10 base pair periodicity, which reflects the turns of the DNA helix on the surface of the core. Randomly assembled cores, on the other hand, only yield an overall protection of DNA, but the digestion pattern produced should be essentially identical with that of free DNA. The assembly to cores of the fragments did not comply exclusively with either of the models. The DNase I digestion patterns of the fragments investigated after assembly of cores are best interpreted as the result of two simultaneous assembly processes competing for positions on DNA. The one process is nonspecific. This becomes evident from the roughly equal protection of DNA over the entire fragment, which has resulted in the reduced digestion rate requiring a 10-fold increase of DNase I to achieve a comparable degree of digestion. This is obviously the result of the random occupation of DNA by cores. However, against this background of what appears to be a random association, a second more specific process becomes apparent. Two preferential assembly frames are recognizable, one more pronounced in the h1+ fragment at positions 40–180 bp (Figures 3 and 4) and one in the h1- fragment at positions 80–220 bp.

Since the structure of the octamers used in the assembly reactions is invariant, any selectivity with respect to the interaction between octamers and DNA must rest in DNA.

The sequence motifs, comprising poly(purine)–poly(pyrimidine) stretches, are relatively inflexible (Hogan et al., 1983) and should not associate easily with histones to form cores (Simpson & Kunzler, 1979; Prunell, 1982; Kunkel & Martinson, 1981; Haeutle & Martinson, 1983). However, these sequences are flanked by conformationally less committed, more flexible areas. The junction between these two may act as a nucleation point for the assembly of cores. Once an octamer has collided with the junction area and has interacted with DNA, possibly ionically, it sits in a privileged locus vis

à vis other octamers, in that it denies them, due to its size, access to its flexible immediate neighborhood, whereas the other flank is protected by the rigidity of the poly(purine)–poly(pyrimidine) rod. The rigidity of the rod may then be overcome through a caterpillar-like movement along the rod, based on the interaction between the strong positive charges on the octamer surface and the phosphate backbone. A similar interaction will force the DNA on the other side of the junction to bend in the same direction and thus follow the core. Such a process may be the reason why the sequence motifs have become, contrary to expectation, part of a preferential assembly frame. More distant from the junction area, the assembly becomes random, namely, on all faces of DNA. Here DNA bends in all directions equally well and octamers are competing for DNA without occupying preferential positions.

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