A unique nucleoprotein structure associated with the *Drosophila melanogaster* 18–28 S rDNA nontranscribed spacer

Louis F. Levinger and G. Scott Nass

School of Life and Health Sciences, University of Delaware, Newark, DE 19716, USA

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We have detected unique nucleoprotein particles specific for the 18–28 S rDNA nontranscribed spacer of *Drosophila melanogaster*. The particles migrate between di- and trinucleosomes on nucleoprotein gels, and are between mono- and dinucleosomal in DNA length. These migration properties suggest that the nontranscribed spacer particles could have a protein component larger than a histone core. The variant nucleoprotein structures map primarily within the nontranscribed spacer 235 base pair internal subrepeat, which is AT-rich and possesses a 50 base pair sequence homologous to the RNA polymerase I binding site.

Nucleoprotein particle *18–28 S rDNA* (D. melanogaster)

1. INTRODUCTION

Like other eukaryotic genes, 18–28 S ribosomal RNA genes are regulated by the binding of RNA polymerase and other factors. Efficient and faithful initiation of rDNA transcription presumably depends on the binding of ancillary factors to the promoter sequence (discussed in [1]). *Drosophila* and *Xenopus* contain internal subrepeats within the 18–28 S rDNA nontranscribed spacers (The term nontranscribed spacer, although historically useful, has been questioned by authors who found that the 18–28 S rDNA spacer may be transcribed in *D. melanogaster* [2] and in *Xenopus* [3].) [4,5] which display an approx. 50 bp homology to the respective RNA polymerase I initiation sequences. Internal sequences homologous to the RNA polymerase I binding site, repeated in tandem upstream from the initiation sequence in *Drosophila*, serve as additional sites for rDNA transcription initiation in vitro [6]. The analogous RNA polymerase I binding site homologues in *Xenopus* probably do not normally serve an initiation function in vivo [7]. Internal subrepeats could, however, function as loading sites for RNA polymerase I [5], or for a protein which directs RNA polymerase I binding [7], increasing by several-fold its local concentration upstream of the transcription start site.

We have performed two-dimensional hybridization mapping [8–10] to explore the nucleoprotein structure of the 18–28 S ribosomal DNA repeat in *D. melanogaster*. Soluble chromatin released by micrococcal nuclease digestion is separated on first-dimension nucleoprotein and second-dimension DNA gels. Hybridization using cloned DNA probes is used to search for selective arrangement of DNA-binding proteins on specific DNA sequences. Nucleoprotein particles are resolved on the basis of mass, charge and shape, with the protein composition making a major contribution to particle migration in the first dimension. After removal of proteins with SDS and pronase, the second dimension separates free DNA on the basis of DNA length. The pattern of spots in two-dimensional gels show off-diagonal spreading which results from the addition of proteins associated with certain nucleosome subpopula-

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tions. This technique has enabled us to detect a unique nucleoprotein structure specifically associated with the repeated promoter-like element in the nontranscribed spacer (NTS) region of the 18–28 S tandem repeat.

2. EXPERIMENTAL

2.1. *Cell culture*

D. melanogaster Kc cells were maintained in continuous culture in Eschalier's D22 medium supplemented with Pen-Strep and 10% fetal calf serum, at a density of 1–20 × 10^6/ml in spinner flasks at 25°C. Around 2 × 10^9 cells were harvested in exponential growth phase at a density up to 1 × 10^7/ml.

2.2. *Nuclei and soluble chromatin preparation*

Cells were pelleted at 2500 × *g*, washed once with Tris-buffered saline at 4°C, and lysed by pipetting up and down 10 times in 10 ml buffer containing 10 mM Na-Hepes, pH 7.5, 0.1% NP40, 10 mM NaCl, 3 mM $CaCl_2$, 1 mM freshly added, freshly dissolved PMSF, 1 mM DTT, 0.3 M sucrose, and made up to a final volume of 50 ml using the same buffer. Crude nuclei were pelleted at 3500 × *g* for 10 min at 4°C and resuspended in 15 ml of 10 mM Na-Hepes, pH 7.5, 0.05% NP40, 10 mM NaCl, 3 mM $CaCl_2$, 0.1 mM PMSF, 0.1 mM DTT and 0.3 M sucrose by pipetting up and down. This nuclear suspension was layered over a cushion containing 10 mM Na-Hepes, pH 7.5, 10 mM NaCl, 0.5 mM $CaCl_2$, 0.1 mM PMSF, 0.1 mM DTT and 1.8 M sucrose, and centrifuged at 20000 × *g* for 45 min at 4°C in a swinging-bucket rotor. The nuclear pellet was resuspended in 25 ml digest buffer (10 mM Na-Hepes, pH 7.5, 10 mM NaCl, 0.5 mM $CaCl_2$, 0.1 mM PMSF, 0.1 mM DTT lacking sucrose), sedimented for 10 min at 3500 × *g*, and resuspended in 1.5 ml digest buffer to a DNA concentration in nuclei of approx. 1.5 mg/ml.

The nuclei were prewarmed to 37°C and digested with micrococcal nuclease at 5 μg/ml for 3 or 10 min. Digestions were stopped with EDTA and EGTA added to a final concentration of 1 and 0.5 mM, respectively. Digests were sedimented for 5 min at 4°C (12500 × *g*) in a microfuge, digest supernatants removed, and the pellets resuspended in an equal volume of TE (10 mM Tris-Cl, pH 8, 1 mM EDTA). The yield of DNA in soluble chromatin in the digest supernatant was around 1 mg/ml by diphenylamine assay, with acid-soluble DNA in the range 2–10%. The digest supernatant was, in some cases, additionally clarified by 30 min ultracentrifugation at 100000 × *g* in an SW 50.1 rotor at 4°C.

2.3. *Gel electrophoresis*

For one-dimensional DNA gel electrophoresis, digest supernatants and pellets were deproteinized successively with phenol, phenol/chloroform/isoamyl alcohol (50:48:2) and with chloroform/isoamyl alcohol (24:1), ethanol-precipitated, washed with 70% ethanol, and redissolved in TE. An equal volume of nucleic acid sample was loaded on each channel. Since nucleic acid samples were not treated with RNase, RNA appears on these gels (see high-M_r bands in fig.3A, especially channel 4). RNA present on the gel can interfere with DNA-DNA hybridization of a transcribed probe (not shown), but only in the first use of the DPT paper, since RNA is hydrolyzed off the paper when hybridized [^{32}P]DNA is removed between uses with 0.4 M NaOH (see below).

The first-dimension 5% nucleoprotein gels were run as described [9]. A sample of up to 1 ml (1 mg chromatin DNA) was loaded on a channel 3 cm wide × 3 mm thick. First-dimension strips were subdivided vertically into four equal sections and run on parallel second-dimension DNA gels, as in [9]. In recent second-dimension runs, proteins were removed from DNA by pronase digestion [11]. Denaturation of DNA in the gels and electrophoretic transfer to DPT paper [12] were as described in [8].

2.4. *Hybridization*

The 18–28 S rDNA tandem repeat is illustrated schematically in fig.1A. LE 1 is a 28 S rDNA coding region which starts at the left-end *Eco*RI site of DM103 ([13]; obtained from P. Wensink) and runs approx. 5 kb to the *Hin*dIII site close to the boundary of the type I genetic element. LE 1 was subcloned into pBR322 using standard procedures [14] and is identical to the subclone DM103A1 [15]. PC225 is a type I genetic element subcloned from DM103, which runs from the *Hin*dIII site to the right-hand *Bam*HI site ([16]; obtained from D. Glover). The type I genetic ele-

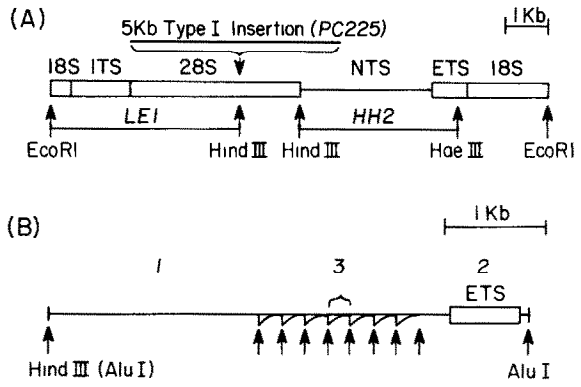

Fig.1. (A) A map of the 18–28 S rDNA tandem repeat of *D. melanogaster* (redrawn from [25]), showing restriction sites used to generate the subclones for hybridization. (B) A detail map of the subclone HH2 [4], illustrating the *Alu*I sites and the 235 bp NTS subrepeats.

ment interrupts around half of the ribosomal repeats on the X chromosome and apparently inactivates transcription [17]. About 2/3 of the type I element DNA is found at sites other than an interruption of the 28 S rDNA coding sequence, largely in heterochromatic blocks flanking the nucleolus organizer [15].

HH2 is a nontranscribed spacer subclone from the *Hin*dIII site to the right-hand *Hae*III site in the external transcribed spacer, in the vector pAT 153 ([4]; obtained from G. Dover). Plasmids were propagated in *E. coli* HB101 and purified using standard procedures [14]. Nick translation and hybridization were performed as in [8].

Hybridized [^{32}P]DNA was removed from the papers with three 15-min washes of 0.4 N NaOH, 1 mM EDTA at room temperature. The papers were then rinsed exhaustively with TE to remove NaOH, blotted dry and reset to prehybridize. Several rehybridizations were performed without a noticeable deterioration of the hybridization signal.

3. RESULTS

3.1. *A nucleoprotein particle specific for the 18–28 S rDNA nontranscribed spacer*

The probes illustrated in fig.1A were used for two-dimensional hybridization mapping of nucleosomes [8] as illustrated in fig.2. DNA from the two-dimensional map displayed in fig.2A was used for hybridization with three regions of the 18–28 S rDNA gene cluster (fig.2B–D). These regions (see fig.1) are a coding region (LE 1, fig.2B), the type I genetic element (PC 225, fig.2C) and the nontranscribed spacer (HH2, fig.2D). The three ribosomal probes hybridize to the two-dimensional map of total nucleosomal DNA (fig.2A) in similar ways.

The major mononucleosomal particles detected in the ethidium bromide staining pattern, which is a faithful representation of relative abundance of subtypes in the total digest supernatant, are:

(i) The core mononucleosome containing 146 bp of DNA and an octamer of core histones (MN1);
(ii) A larger particle which contains 20–60 bp extra (linker) DNA associated with a molecule of histone H1 (MN2); and
(iii) A particle with chromosomal protein D1 in addition to the core particle (MN_{D1} [9]).

Identification of variant nucleosome subtypes is based on correlation of second-dimension DNA and protein patterns [9,19,20] and strengthened in the case of D1-mononucleosomes by hybridization mapping with heterochromatic, AT-rich DNA sequences [21] and by nucleosome solubility in 0.1 M NaCl [10].

Two new spots appear in the pattern obtained with a nontranscribed spacer probe (fig.2D, arrow). These spots have never been detected with other probes, even with autoradiographic overexposure, and thus appear to be specific for the nontranscribed spacer, and are therefore referred to as nontranscribed spacer particles (NTSPs). In the first (nucleoprotein) dimension of migration, the leading edge of the faster migrating particle (NTSP1) is close to the leading edge of dinucleosomes, and the slower migrating of the two particles (NTSP2) appears to comigrate with the leading edge of trinucleosomes.

3.2. *Distribution of rDNA in soluble and insoluble fractions*

Nucleic acids from the digests (fig.2) were extracted and electrophoresed on 5% gels (fig.3). The digest supernatants and digest pellets (soluble and insoluble chromatin) from two different times of digestion were analyzed. The three ribosomal probes, coding, type I insertion, and NTS

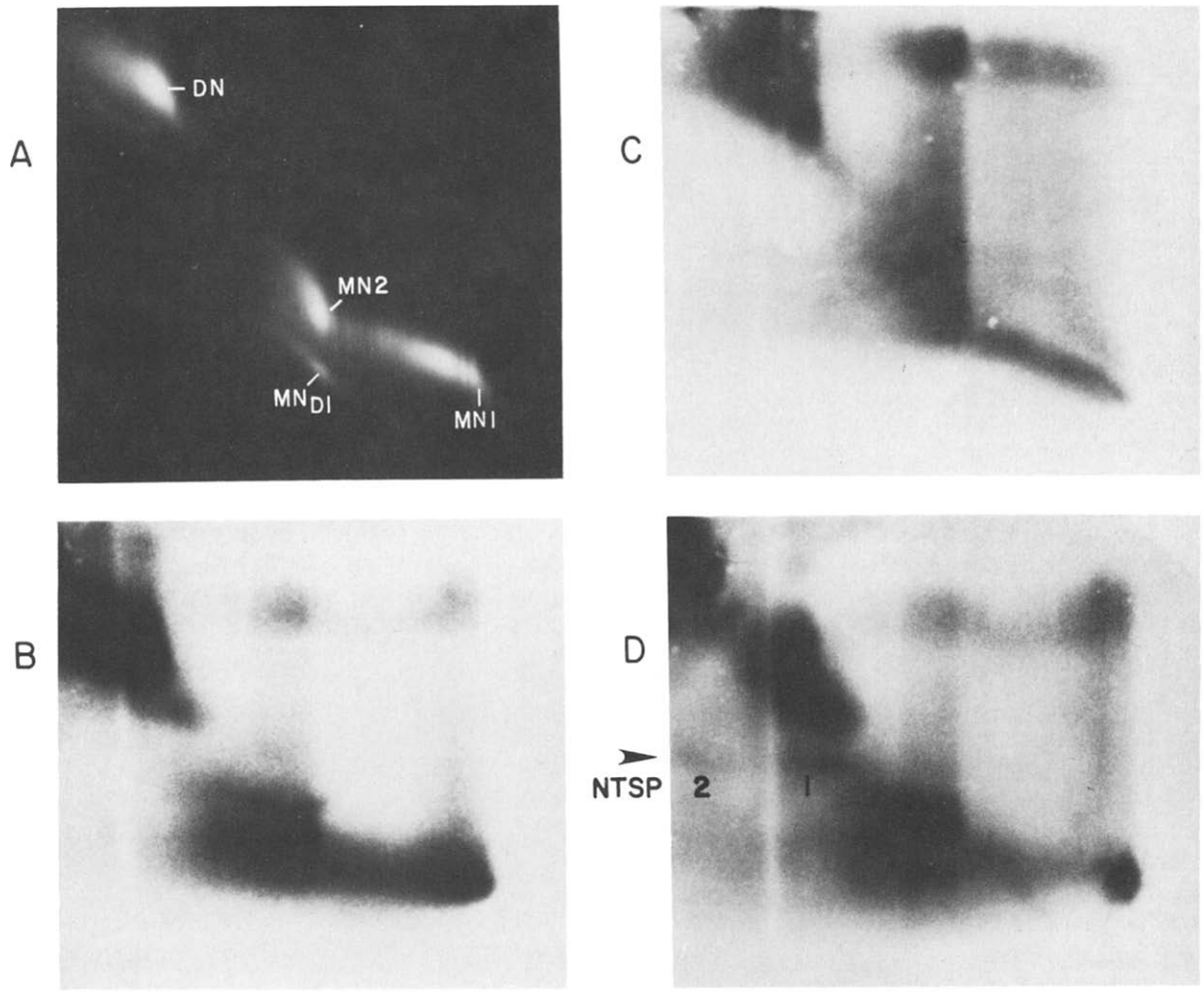

Fig.2. 18–28 S rDNA hybridization mapping. (A) Soluble chromatin was electrophoresed on a first-dimension 5% polyacrylamide nucleoprotein gel (left to right). DNA was electrophoresed free of protein from the first dimension strip on a second-dimension DNA gel (top to bottom), and displayed by ethidium bromide staining. MN1, MN2, MN_{DI} and DN identify core mononucleosome, H1 mononucleosome, D1 mononucleosome, and dinucleosome DNA as described in the text. (B) Hybridization was performed with LE1 probe for 28 S rDNA. (C) Hybridization with PC225, the type I genetic element probe. (D) The paper in B was rehybridized with HH2, the NTS-ETS probe. Arrow on the left of D identifies the position in second-dimension DNA gel migration of nontranscribed spacer particles 1 and 2 (NTSP1 and 2).

(fig.3B–D), are about equally represented in the digest supernatant and pellet. In both fractions, most of the DNA falls into the canonical nucleosomal DNA repeating pattern.

An arrow to the left of fig.3D identifies the most prominent member of a set of bands between mononucleosome and dinucleosome DNA homologous to the NTS probe. The apparent molecular size of the band marked in fig.3D is around 275 bp. These bands correspond in migration with the DNA released from NTSPs identified in fig.2, and with the 235 bp NTS subrepeat, which migrates anomalously slow on polyacrylamide gels.

Additional bands are noticeable in the original one-dimensional NTS autoradiograms, down to

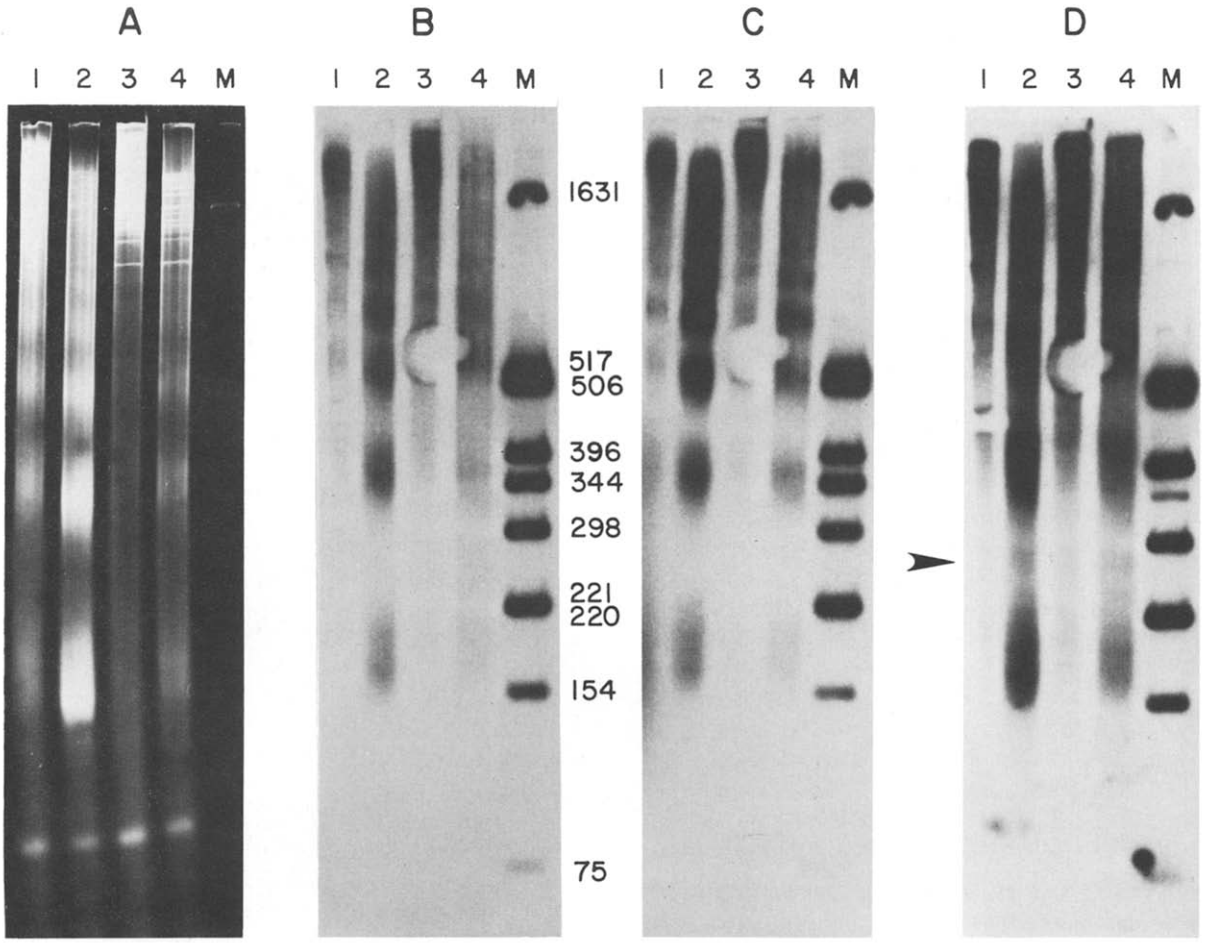

Fig.3. One-dimensional hybridization analysis of the 18–28 S rDNA region. (A) Nucleic acids were purified from micrococcal nuclease digests of nuclei, separated on a 5% polyacrylamide gel, and displayed by ethidium bromide staining. (1,2) Digest supernatant, 3′ and 10′ digestion. (3,4) Digest pellets, 3′ and 10′. (M) pBR-*Hin*fI marker. Nucleic acids were denatured and transferred to DPT paper for hybridization using (B) LE1, (C) PC225 and (D) HH2 probes. 1–4 and M are the same as in A. Numbers to the right of B identify the pBR-*Hin*fI marker DNA fragment lengths in bp. Arrow to the left of D identifies an internucleosomal DNA sequence homologous to the NTS, which displays similar DNA length to NTSPs (fig.2).

the region of mononucleosome DNA and up into the dinucleosome DNA region and beyond. These bands all seem to be specific for the NTS, since they have not been detected with the other two probes (or with the heterochromatic satellite probes [10]) over a wide range of autoradiographic exposures.

3.3. *NTSPs appear to be derived predominantly from the 235 bp subrepeats*

*Alu*I was used to map the NTSP (see restriction map in fig.1B; not shown). The results of two-dimensional (DNP ⟶ DNA) and one-dimensional (DNA) NTS subfragment hybridization mapping suggest that $3 \approx 1 \gg 2$ is the order of relative NTSP hybridization intensity. Subregion 1, from the left end of the NTS, contains 340 bp tandem repeats which consist of 235 bp and 95 bp subrepeats, and therefore would be expected to cross-hybridize with 235 bp subrepeat DNA [2]. The proportion of nontranscribed spacer DNA which exists as the NTSP, measured by densitometry, is approx. 2% of the intensity in the mono- to dinucleosome DNA region. The bulk

DNA in the genome as detected by ethidium bromide staining (figs 2,3A) shows a normal nucleosomal pattern in which the NTS subrepeat is undetectable.

3.4. *NTSPs disappear from the nontranscribed spacer when nucleosomes are prepared with a sulfhydryl inactivating reagent*

The use of DTNB (a sulfhydryl modifying reagent) in nuclei and soluble chromatin preparations was introduced in the hope of stabilizing the ubiquitinated core mononucleosome [9]. The effects of DTNB on chromatin are still not understood, but seem to be due, in part, to the loss of histone H1 from MN2; the proportion of MN2/MN1 in DTNB preparations is lower than that obtained in the absence of DTNB (fig.2A. cf. [10]). *Drosophila* histone H1, which contains a sulfhydryl residue [22], could be rendered unable to bind DNA when modified by DTNB. When DTNB is included in the nuclei preparation, the NTSPs are absent from the NTS hybridization pattern (not shown). Perhaps like *D. melanogaster* histone H1, NTSP proteins are labile to sulfhydryl modification.

4. DISCUSSION

4.1. *The NTS subrepeat has a novel nucleoprotein structure*

We have discovered a soluble nucleoprotein particle which migrates like a dinucleosome on the nucleoprotein dimension, although it is between mono- and dinucleosomal in DNA length (fig.2). These particles map within the 18–28 S rDNA nontranscribed spacer, and are thus known as NTSPs (figs 2,3). Finer structure mapping suggests that the NTSPs are localized within the 235 bp subrepeats of the NTS (not shown). Furthermore, NTSP DNA migration appears equal to NTS subrepeat DNA migration.

These results confirm and extend results from Schedl's laboratory [23]. They obtained a band between mono- and dinucleosome DNA specific for the NTS (fig.2 in [23]) similar to the one we observe in one-dimensional DNA hybridization patterns (our fig.3). Furthermore, DNase I and micrococcal nuclease hypersensitive sites map around 30 bp downstream from the repeated *Alu*I sites [23], close to the distal border of the RNA polymerase I homology region [4,6], while micrococcal nuclease hypersensitive sites in naked DNA occur approx. 120 bp away from the exposed sites in chromatin [23].

NTS subrepeat DNA of unit length (235 bp) appears in two regions of the nucleoprotein gel (see section 3; fig.2D). NTSP2 contains DNA the same length (and probably sequence) as NTSP1, although it is lower in migration rate than NTSP1 on the nucleoprotein gel. NTSP2 could be NTSP1 with which additional protein molecules are associated. Alternatively, NTSP2 could arise from an internal DNA break in an NTS trinucleosome (or structure of equivalent size) held together by noncovalent protein-protein interactions during nucleoprotein gel electrophoresis [24].

Although the NTSP is a small proportion of the NTS hybridization intensity, we believe it could be significant for the following reasons:

(i) Occupancy of the NTS by a nonhistone protein factor may be a transient phenomenon of regulatory importance;
(ii) The NTSP might be a labile structure which decays during nuclei and soluble chromatin preparation.
(iii) NTS subrepeat DNA hypersensitive sites map out of phase with the chromatin hypersensitive sites in the NTS [23], suggesting that singularities of nucleoprotein structure which generate NTSPs would not be solely due to nuclease susceptibility of free DNA.

We are presently trying to identify proteins which specifically bind the NTS 235 bp subrepeat. NTS subrepeat-specific DNA-binding activity could be responsible for formation of chromatin hypersensitive sites, NTSPs, and for regulatory functions associated with the RNA polymerase I upstream and initiation regions.

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REFERENCES

[1] Brown, D.D. (1984) Cell 37, 359–365.
[2] Tautz, D. and Dover, G.A. (1986) EMBO J. 5, 1267–1273.
[3] Labhart, P. and Reeder, R.H. (1986) Cell 45, 431–443.
[4] Coen, E.S. and Dover, G.A. (1982) Nucleic Acids Res. 10, 7017–7026.
[5] Moss, T. (1983) Nature 302, 223–228.
[6] Kohorn, B.D. and Rae, R.M.M. (1982) Nucleic Acids Res. 10, 6879–6886.
[7] Reeder, R.H. (1984) Cell 38, 349–351.
[8] Levinger, L., Barsoum, J. and Varshavsky, A. (1981) J. Mol. Biol. 146, 287–304.
[9] Levinger, L. and Varshavsky, A. (1982) Cell 28, 375–385.
[10] Levinger, L. (1985) J. Biol. Chem. 260, 11799–11804.
[11] Mirzabekov, A.D., Shick, V.V., Belyavsky, A.V. and Bavykin, S.G. (1978) Proc. Natl. Acad. Sci. USA 75, 4184–4188.
[12] Seed, B. (1982) Nucleic Acids Res. 10, 1799–1810.
[13] Glover, D. and Hogness, D. (1977) Cell 10, 167–176.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[15] Peacock, W.J., Appels, R., Endow, S.E. and Glover, D. (1980) Genet. Res. 37, 209–214.
[16] De Cicco, P.V. and Glover, D.M. (1983) Cell 32, 1217–1225.
[17] Long, E.O. and Dawid, I.B. (1979) in: Eucaryotic Gene Regulation (Axel, R. and Maniatis, T. eds) Academic Press, New York.
[18] Rigby, P.W.J., Rhodes, D., Dieckmann, M. and Berg, P. (1977) J. Mol. Biol. 113, 237–251.
[19] Bakayev, V.V., Bakayeva, T.G. and Varshavsky, A.J. (1977) Cell 11, 619–629.
[20] Todd, R.D. and Garrard, W.T. (1977) J. Biol. Chem. 252, 4729–4738.
[21] Levinger, L. and Varshavsky, A. (1982) Proc. Natl. Acad. Sci. USA 79, 7152–7156.
[22] Rodriguez Alfageme, C., Zweidler, A., Mahowald, A. and Cohen, L.H. (1974) J. Biol. Chem. 249, 3729–3736.
[23] Udvardy, A., Louis, C., Han, S. and Schedl, P. (1984) J. Mol. Biol. 175, 113–130.
[24] Levinger, L. and Varshavsky, A. (1980) Proc. Natl. Acad. Sci. USA 77, 3244–3248.
[25] Long, E.O. and Dawid, I.B. (1979) Nucleic Acids Res. 7, 205–215.