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Superhelical stress and nucleosome-mediated repression of 5S RNA gene transcription *in vitro*

David J.Clark and Alan P.Wolffe[1,2]

Laboratory of Molecular Biology, NIDDK and [1]Laboratory of Molecular Embryology, NICHD, National Institutes of Health, Room 131, Building 6, Bethesda, MD 20892, USA

[2]Corresponding author

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Nucleosomes were assembled on a plasmid carrying a *Xenopus* somatic 5S RNA gene prepared at different superhelix densities. The gene was preferentially assembled into a positioned nucleosome which was stable to superhelical stress. No evidence for a conformational change in the nucleosome was found, even under extreme negative superhelical stress. Transcription in an extract from *Xenopus* oocyte nuclei was repressed to a degree which depended on the number of nucleosomes assembled. Topoisomerase activity in the extract was effectively inhibited by camptothecin, which had no effect on transcription. Transcription of reconstitutes remained repressed relative to naked plasmids, and was independent of superhelix density. Transcripts from reconstitutes were derived solely from nucleosome-free genes. Thus, a histone octamer positioned on the gene was sufficient to block its transcription. Tryptic removal of the core histone tail domains had no effect on transcription at any superhelix density. Transcription of reconstitutes containing H3/H4 tetramers was also repressed, but not eliminated (unlike reconstitutes containing octamers), and repression was independent of superhelix density. We suggest that removal of histones H2A/H2B from the nucleosome facilitates activation of transcription in the extract. We conclude that superhelical stress alone does not activate transcription of a 5S RNA gene assembled into a nucleosome *in vitro*.

Key words: DNA supercoiling/histone octamer/histone tetramer/nucleosome-mediated repression/5S RNA gene transcription

Introduction

In prokaryotes there is good evidence that changes in DNA topology, involving the opposing activities of DNA gyrase and topoisomerase I, alter the efficiency of transcription from many promoters (Gellert, 1981). In contrast, the role of DNA supercoiling in eukaryotes is highly controversial. Although the bulk of DNA in eukaryotic nuclei is relaxed (for a review, see Esposito and Sinden, 1988), there may be regions in chromosomes containing supercoiled DNA, perhaps corresponding to certain chromatin loops (Luchnik *et al.*, 1988). Another possibility is that superhelical stress is transient, present only as genes are actively transcribed or as DNA is replicated, as suggested by Liu and Wang (1987). For example, processive enzyme complexes such as DNA and RNA polymerases may transiently unwind the DNA duplex, or cause the DNA to become positively supercoiled in front of, and negatively supercoiled behind, the complex. Thus, superhelical stress could have important consequences for the regulation of transcription of both naked DNA and DNA in chromatin.

The 5S RNA genes have served as a useful model system for examining the relationship between gene expression and chromatin structure. Assembly of the 5S RNA gene into chromatin results in repression of transcription (Schlissel and Brown, 1984). More recently it has been suggested that a nucleosome on the 5S RNA gene inhibits its transcription *in vitro* (Shimamura *et al.*, 1988; Morse, 1989; Felts *et al.*, 1990; Tremethick *et al.*, 1990). If nucleosomes are not closely packed, as is usually the case *in vivo*, complete repression requires the binding of histone H1 (Schlissel and Brown, 1984; Shimamura *et al.*, 1989; Wolffe, 1989a).

The structure of transcriptionally repressed chromatin is well-defined, but this is not true for either the mechanism of activation or the chromatin structure of actively transcribing genes. In one model, a gene assembled into chromatin is in a stable repressed state and may only be activated during replication following a disruption of chromatin structure, when a competition occurs between transcription factors to form an active complex and the core histones to form a nucleosome (see Almouzni *et al.*, 1991, and references therein). An earlier model invoked a more direct mechanism of activation in which transcription is facilitated by alterations in chromatin structure following the introduction of superhelical stress (Glikin *et al.*, 1984; Ryoji and Worcel, 1984, 1985). In this report, we have tested the effect of superhelical stress on transcription of the *Xenopus* somatic 5S RNA gene assembled into a nucleosome *in vitro*, and examined the consequences of removing either the core histone tail domains or histones H2A/H2B.

Results

Experimental approach

To examine the effects of DNA supercoiling on transcription we used a plasmid carrying a somatic 5S RNA gene from *Xenopus borealis* (pXP10, 3247 bp; Wolffe *et al.*, 1986) prepared at different superhelix densities with defined mean excess linking numbers ($\Delta\bar{L}$) and narrow distributions of topoisomers. We prepared relaxed (excess linking, $\Delta\bar{L} = 0$; superhelix density, $\sigma = 0$), moderately negatively supercoiled ($\Delta\bar{L} = -12$, $\sigma = -0.04$), highly ($\Delta\bar{L} = -35$; $\sigma = -0.11$) and very highly ($\Delta\bar{L} = -50$; $\sigma = -0.16$) negatively supercoiled pXP10. Thus, the superhelix density ranged from 0 to -0.16. However, in reconstitutes the effective superhelix density differs from these values because the formation of a nucleosome must satisfy a linking number change of -1 (Simpson *et al.*, 1985); a redistribution of supercoils within the plasmid is required, such that one *positive* supercoil must be accommodated within the plasmid

for every nucleosome formed (see Clark and Felsenfeld, 1991). Thus, the formation of 12 nucleosomes (see below) would effectively relax a negatively supercoiled plasmid with $\Delta\bar{L} = -12$; initially relaxed plasmid would become positively supercoiled by 12 turns ($\sigma = +0.04$); and a plasmid with $\Delta\bar{L} = -50$ would be reduced to $\Delta\bar{L} = -38$ ($\sigma = -0.12$). To put these numbers into perspective, under the most favourable conditions *in vitro*, prokaryotic DNA gyrase is able to supercoil DNA to a maximum superhelix density of −0.12 (Bates and Maxwell, 1989). Thus, we have selected a reasonable range of superhelix density to test effects on transcription.

Nucleosomes were reconstituted by salt/urea dialysis (Camerini-Otero *et al.*, 1976) because this is the most reliable and efficient method available, using purified core histones from adult chicken erythrocytes, because they can be obtained very pure, protease-free, and have few post-translational modifications and so are relatively homogeneous. Although the *Xenopus* egg extract has the advantage that nucleosomes are deposited with physiological spacing (Almouzni and Méchali, 1988; Shimamura *et al.*, 1988, 1989; Almouzni *et al.*, 1991), we did not use it to assemble nucleosomes because any effects on transcription would not be unambiguously attributable to nucleosomes (rather than other factors in the extract) (Blanco *et al.*, 1989; Tremethick *et al.*, 1990). An extract of *Xenopus* oocyte nuclei was used because very high rates of 5S RNA gene transcription (by RNA polymerase III) can be obtained, approaching those observed *in vivo* (Wolffe *et al.*, 1986). The *X.borealis* somatic 5S RNA gene positions a nucleosome very precisely (Rhodes, 1985; Losa and Brown, 1987; Hayes *et al.*, 1990), making it possible to footprint a histone octamer on the gene. Transcription of this gene in this extract has been extremely well-characterized (Birkenmeier *et al.*, 1978; Bogenhagen *et al.*, 1982; Wolffe *et al.*, 1986).

Characterization of reconstitutes containing DNA at different superhelix densities

We reconstituted nucleosomes on relaxed ($\Delta\bar{L} = 0$), moderately negatively supercoiled ($\Delta\bar{L} = -12$), highly negatively supercoiled ($\Delta\bar{L} = -35$) and very highly negatively supercoiled plasmid ($\Delta\bar{L} = -50$) at an input ratio of core histones to DNA equal to 12 histone octamers: plasmid. With this number of nucleosomes:plasmid, partial repression of transcription was expected (see Figure 3), and it should be possible to observe activation or inhibition of transcription due to superhelical stress.

Analysis of the reconstitutes in a nucleoprotein gel (Figure 1A) showed that the DNA is complexed with histone, and that no free plasmid is detectable. The number of nucleosomes reconstituted per plasmid was measured by relaxation of the DNA in the reconstitutes (with nicking–closing extract) and resolution and identification of the resulting topoisomers in gels containing chloroquine. The reconstitutes contained an average of 11 nucleosomes, close to the 12 expected from the input ratio (not shown). We confirmed that nucleosomes had been assembled by digestion with micrococcal nuclease (Figure 1B): protected DNA fragments of about 145 bp were observed (and some larger protected fragments indicating some closely packed nucleosomes: dimers and trimers). Core histones in the reconstitutes were present in the correct stoichiometries and were not degraded (see Figure 6).

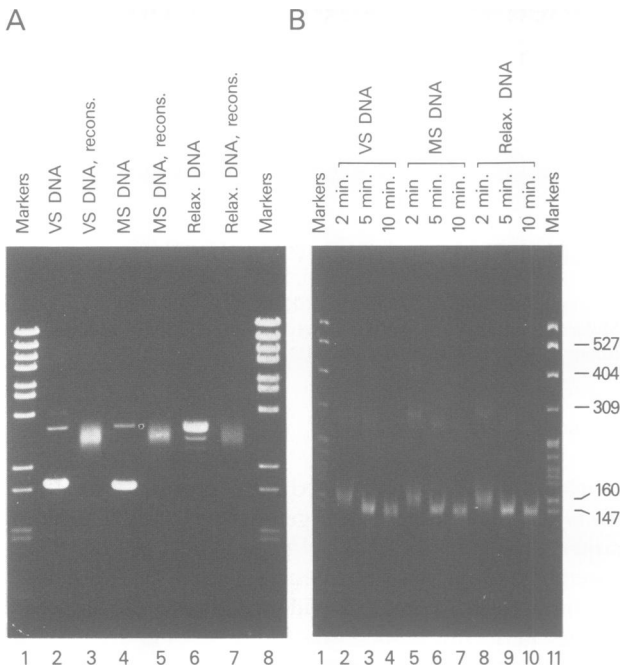

Fig. 1. Characterization of reconstitutes containing 12 nucleosomes and plasmid at different superhelix densities. Nucleosomes were assembled on relaxed ($\Delta\bar{L} = 0$), moderately negatively supercoiled (MS) ($\Delta\bar{L} = -12$), and very negatively supercoiled (VS) ($\Delta\bar{L} = -50$) plasmid (pXP10). **A.** Analysis of reconstitutes in a nucleoprotein gel. (Marker: λ-phage DNA digested with *Bst*EII. **B.** Time courses of digestion of reconstitutes with micrococcal nuclease. (Marker: pBR322 DNA digested with *Msp*I.)

We determined whether a nucleosome is positioned on the 5S RNA gene and whether it is stable to superhelical stress. We used reconstitutes prepared with pXP10 at bacterial superhelix density (a wide range, with $\Delta\bar{L} = -15$, $\sigma = -0.05$), and then introduced negative superhelical stress using DNA gyrase. Reconstitutes with an average of 6 and 10 nucleosomes contained plasmid with $\Delta\bar{L} = -32$ ($\sigma = -0.10$) and $\Delta\bar{L} = -29$ ($\sigma = -0.09$), respectively (not shown), after supercoiling with DNA gyrase. These highly supercoiled reconstitutes were lightly digested with DNase I and the nicks were mapped by primer extension to yield a footprint of the nucleosome on the 5S RNA gene (Figure 2B). The fact that a footprint was observed demonstrates that a nucleosome is indeed rotationally positioned on the 5S RNA gene, although multiple translational positions with the same rotational setting cannot be ruled out because the borders of the nucleosome are not defined. The DNase I cleavage pattern was unaltered even after the reconstitutes had been highly supercoiled by DNA gyrase, suggesting that neither the apparent twist of the DNA in this nucleosome nor its position were irreversibly altered at high levels of superhelical stress. The structure of the nucleosome on *supercoiled* DNA is reflected by the *first* nick; subsequent nicks may reflect the structure after relaxation. The fractions of plasmid molecules which had one, two or three nicks were calculated to be 35%, 24% and 11%, respectively, using the Poisson distribution and the fact that ~25% of the molecules were still supercoiled after DNase I treatment (Figure 2C). We calculate that ~40% of the signal (i.e. the fraction of all nicks which are

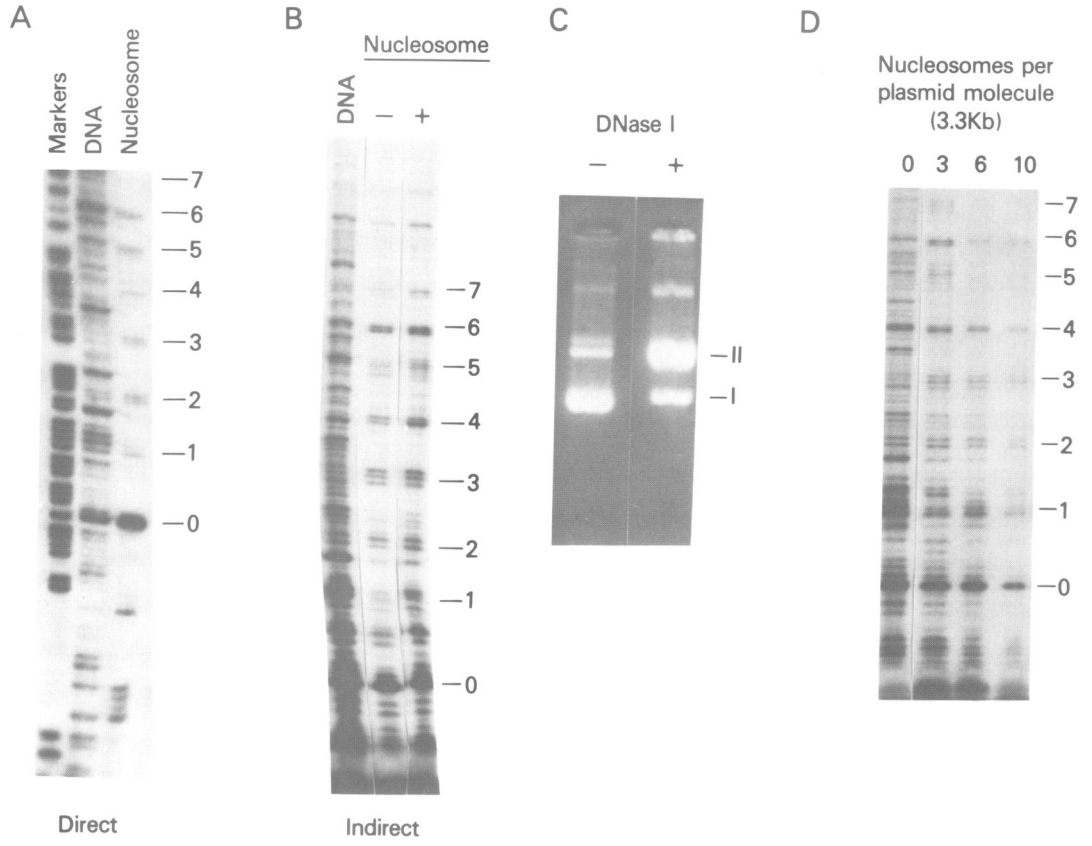

Fig. 2. DNase I footprinting of a nucleosome on the 5S RNA gene at high negative superhelical stress. **A**. Control experiment showing the DNase I footprint of a nucleosome positioned on the 5S RNA gene. A nucleosome was assembled on a short end-labeled linear DNA fragment carrying the 5S RNA gene, and lightly nicked with DNase I ('nucleosome'). The free fragment was also digested ('DNA'). The numbers at the side indicate the 10−11 bp separation of DNase I cleavage sites, moving away from the dyad axis of the nucleosome (labeled 0) (Rhodes, 1985). The markers are a G+A chemical sequencing reaction (Maxam and Gilbert, 1977). **B**. Footprints of the nucleosome on the 5S RNA gene in reconstitutes before (−) and after (+) supercoiling with DNA gyrase obtained by primer-extension. A reconstitute containing 10 nucleosomes/plasmid was highly supercoiled with DNA gyrase and then lightly digested with DNase I. **C**. Extent of digestion of the reconstitute supercoiled with DNA gyrase. Plasmid extracted from the reconstitute used in the analysis of **B** before (−) and after (+) digestion with DNAase I was analyzed in a 1% (w/v) agarose gel. Nicked circular pXP10 (form II) and supercoiled pXP10 (form I) are indicated. **D**. Occupancy of the 5S RNA gene by the histone octamer as a function of the number of nucleosomes/plasmid. Primer-extension footprint of the nucleosome on the 5S RNA gene in reconstitutes containing plasmid at bacterial superhelix density and 3, 6 or 10 nucleosomes as indicated.

first nicks) actually reflects the structure of the nucleosome on supercoiled DNA.

Other assays of nucleosome conformation within highly negatively supercoiled reconstitutes, including circular dichroism spectra, chemical cross-linking and chemical modification as described (Clark and Felsenfeld, 1991), were unable to detect any conformational differences between the reconstitutes (not shown). In conclusion, apparently normal nucleosomes are formed over a wide range of negative superhelix density, and a dramatic structural change in the nucleosome in response to negative superhelical stress seems very unlikely, although subtle conformational changes cannot be ruled out.

We investigated the fraction of 5S RNA genes occupied by histone octamer as a function of the number of nucleosomes assembled, using footprinting analysis with DNAase I (Figure 2D). Even with only three nucleosomes reconstituted per plasmid (one for every 1080 bp), a nucleosome footprint is discernable, suggesting that a nucleosome forms preferentially on the gene. The footprint is stronger in reconsitutes containing 6 and 10 nucleosomes, indicating increased occupancy. Thus, a very high proportion of the 5S RNA genes in reconstitutes containing 12 nucleosomes would be expected to be incorporated into a nucleosome (considerably higher than 50%, the proportion expected if 12 histone octamers were deposited randomly on the plasmid).

Nucleosome-mediated repression of 5S RNA gene transcription

We reconstituted increasing numbers of nucleosomes on pXP10 at bacterial superhelix density (up to 20; this corresponds to one nucleosome every 165 bp). These reconstitutes were transcribed in an extract of *Xenopus* oocyte nuclei in the presence of a naked plasmid carrying a maxi-5S RNA gene (pXbs 115/105; Bogenhagen and Brown, 1981). 5S RNA gene transcription decreased and maxi-5S RNA gene transcription increased as the number of nucleosomes increased (Figure 3). The 5S RNA gene was completely inactive in reconstitutes containing 20 nucleosomes. This was not due to the presence of a general inhibitor of transcription, because the naked maxi-5S RNA gene was transcribed at a high rate. Maxi-5S RNA transcription increased presumably because the fraction of 5S RNA genes able to compete with the naked maxi-5S RNA genes for rate-limiting amounts of transcription factors was

reduced. Thus, 5S RNA gene transcription is repressed on nucleosome assembly.

Camptothecin protects DNA from relaxation in the extract

Extracts of *Xenopus* oocyte nuclei contain topoisomerase activity which causes rapid relaxation of naked DNA, and of reconstitutes at a slower rate (Figure 4), making it difficult to determine the effects, if any, of changes in superhelix density on transcription. However, we found that camptothecin, a specific inhibitor of topoisomerase I (Hsiang *et al.*, 1985), inhibited relaxation of DNA in the extract (Figure 4). A large increase in the amount of nicked circle was not observed in the presence of camptothecin; this was unexpected because this drug is thought to inhibit topoisomerase I by blocking the strand re-joining reaction (Hsiang *et al.*, 1985). Perhaps the addition of Na-EDTA to stop transcription before SDS affects this process. This result also implies that the topoisomerase activity in the extract is topoisomerase I (in agreement with Almouzni and Méchali, 1988), because camptothecin does not inhibit topoisomerase II (Hsiang *et al.*, 1985). In the transcription experiments described below we included camptothecin (at 0.5 mM) in the extract to protect the plasmid from relaxation.

Reconstitutes containing DNA at different superhelix densities are transcribed at very similar rates

Reconstitutes containing 12 histone octamers and plasmid at different superhelix densities were transcribed in the presence and absence of 0.5 mM camptothecin (Figure 5A, Table I). Transcription of naked plasmid and reconstitutes was unaffected by this drug. Naked DNA was transcribed at essentially the same rate over a wide range of negative superhelix densities. Transcription of the 5S RNA gene in reconstitutes was heavily repressed (85–90% relative to naked DNA). The degree of repression was not affected by superhelical stress. The same result was obtained when the experiment was repeated in the presence of a naked maxi-5S RNA gene as a control for the absence of a general inhibitor of transcription (not shown, but see Figure 5B,C), and when reconstitutes formed on plasmid at bacterial superhelix density were supercoiled with DNA gyrase and then transcribed in the extract (not shown). We conclude that transcription of the 5S RNA gene is unaffected over a wide range of superhelix density.

However, in the experiment described in Figure 5A, the proportion of templates which were actively transcribing is likely to be very small; even for naked plasmids, the rate observed is only ~5% of the rate of 5S RNA gene transcrip-

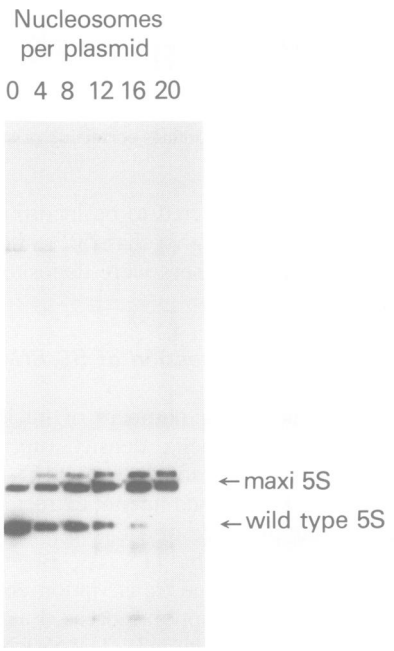

Fig. 3. The degree of repression of 5S RNA gene transcription depends on the number of nucleosomes reconstituted on the plasmid. Reconstitutes containing plasmid pXP10 (3247 bp) at bacterial superhelix density (ΔL = −15; a wide range of topoisomers) and 4, 8, 12, 16 or 20 nucleosomes as indicated, were transcribed in an extract from *Xenopus* oocyte nuclei in the presence of a naked plasmid carrying a maxi-5S RNA gene (pXbs115/105).

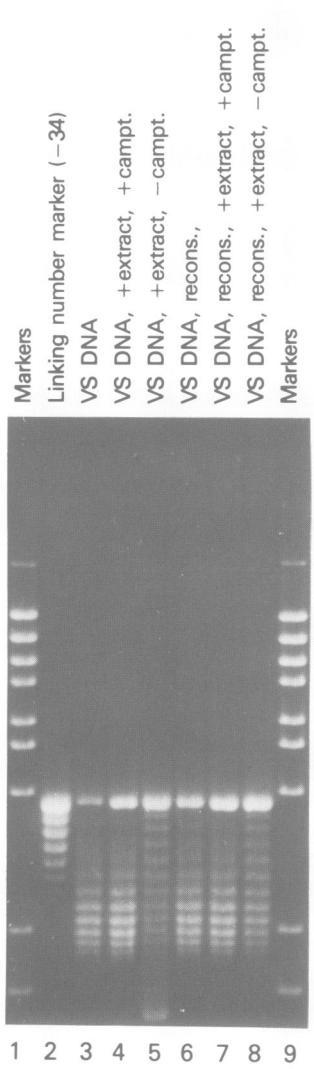

Fig. 4. DNA remains supercoiled after transcription in the extract in the presence of camptothecin. Analysis of very highly supercoiled (ΔL = −50) naked plasmid (VS DNA) and reconstitutes (VS DNA, reconst.) with or without incubation in the extract (± extract) in the presence or absence of 0.5 mM camptothecin (± campt.). Topoisomers were resolved in a gel containing 400 μg chloroquine/ml, and all are migrating as negative supercoils, except the linking number marker (ΔL = −34), and some relaxed DNA in the '+extract, −campt.' lane. Markers are λ-phage DNA digested with *Bst*EII.

tion *in vivo*, which is about 200−300 transcripts/gene/h (Korn and Gurdon, 1981; Wolffe *et al.*, 1986). Transcription rates for the reconstitutes were only ~1−2 transcripts/gene/h, or just 0.5% of the rate *in vivo*. This raises the possibility that the small subfraction of genes which are actively transcribing could have a structure different from that of the bulk. We repeated the experiment under conditions in which the fraction of active genes is much higher ('rate enhancement' conditions; Wolffe *et al.*, 1986) (Figure 5B). The concentration of the plasmid containing the 5S RNA gene was reduced 10-fold and naked vector DNA (pSP64; Melton *et al.*, 1984) was added to maintain the DNA concentration. We included a naked plasmid which carries a satellite I gene (pE190; Lam and Carroll, 1983), as a

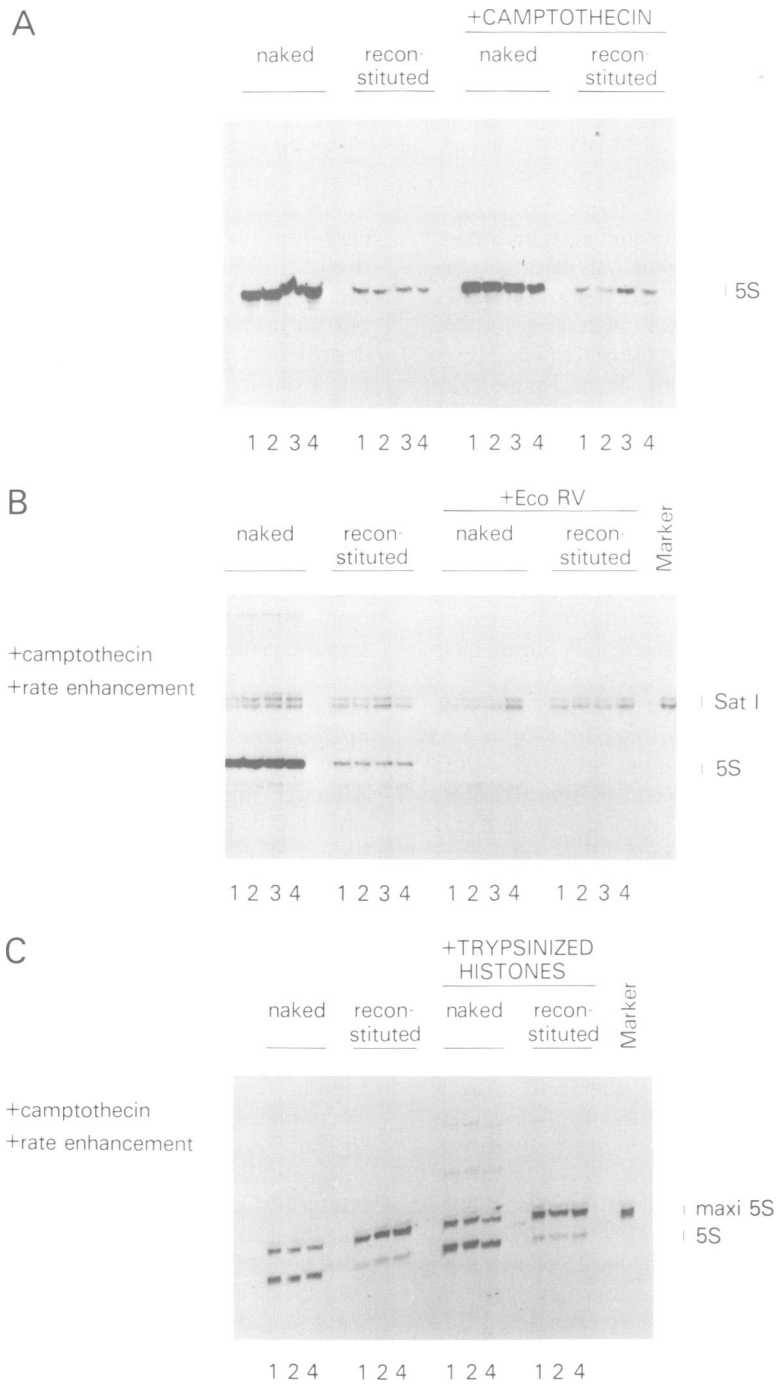

Fig. 5. Effect of DNA superhelix density on transcription of the 5S RNA gene in reconstitutes containing 12 octamers, and in naked plasmids. Reconstitutes contained 12 octamers and (1) relaxed ($\Delta\bar{L}$ = 0) plasmid, (2) moderately negatively supercoiled ($\Delta\bar{L}$ = −12) plasmid, (3) highly negatively supercoiled ($\Delta\bar{L}$ = −35) plasmid, or (4) very highly negatively supercoiled ($\Delta\bar{L}$ = −50) plasmid. Transcription of the naked plasmids and reconstitutes: (**A**) At 15 μg pXP10/ml in the presence or absence of camptothecin (at 0.5 mM). (**B**) With or without prior digestion with *Eco*RV, in the presence of camptothecin and naked pE190 (which carries the *Sat*I RNA gene), under conditions of rate enhancement. Marker: *Sat*I RNA. (**C**) With or without prior digestion with trypsin to remove the core histone tail domains, in the presence of camptothecin, trypsin inhibitor and pXbs115/105 (which carries the maxi-5S RNA gene), under rate enhancement conditions. (See Figure 6A for analysis of the histones after tryptic digestion.) Marker: maxi-5S RNA. Transcription rates are given in Table I.

Table I. Transcription of the 5S RNA gene as a function of DNA supercoiling

	Figure 5A				Figure 5B				Figure 5C				Figure 6					
			+ camp.				+*Eco*RV				+ Trypsin					+ *Eco*RV		
ΔL	DNA	Oct.	DNA	Oct.	DNA	Oct.	DNA	Oct.	DNA	Oct.	DNA	Oct.	DNA	Oct.	Tet.	DNA	Oct.	Tet.
0	15	1.5	14	1.3	122	14	0	0	97	13	107	13	124	11	11	0	0	10
−12	15	1.4	14	1.3	125	13	0	0	98	15	106	15	–	–	–	–	–	–
−35	13	1.5	16	1.6	130	14	0	0	–	–	–	–	97	12	13	0	0	8
−50	16	1.2	13	1.4	129	13	0	0	92	16	111	14	111	13	16	0	0	7

Rates are given in transcripts/gene/h for each of the experiments described in Figures 5 and 6. Rates were determined by cutting bands out of gels and measuring radioactivity in a scintillation counter. DNA, naked plasmid; Oct., reconstitutes containing 12 histone octamers; Tet., reconstitutes containing 12 H3/H4 tetramers; camp., camptothecin; (−), not determined. The average number of superhelical turns in the plasmid is ΔL, the mean excess linking number. In experiments involving *Eco*RV, rates were not corrected for the genes destroyed by this enzyme.

control for transcription. Under these conditions transcription rates per gene increased greatly, presumably because non-specific inhibitors of transcription were removed by the carrier DNA, and transcription factors were concentrated on 5S RNA genes, forming active transcription complexes more efficiently (Wolffe *et al.*, 1986). Transcription rates for naked plasmids and reconstitutes increased to 120−130 and to about 14 transcripts/gene/h, respectively (Table I). If it is assumed that active genes cannot be transcribed faster than the rate *in vivo*, then at least 40−60% of naked genes and 4−6% of genes in reconstitutes may be active. It seems unlikely that just a few genes in an altered conformation were being transcribed. The rate of transcription of naked DNA and reconstitutes was independent of superhelix density (Figure 5B; left panels), confirming the results presented in Figure 5A.

Transcription rates for reconstitutes were only 10−15% of those for the naked plasmids under standard (Figure 5A) and rate enhancement conditions (Figure 5B), and ~90% of genes may have been assembled into nucleosomes (Figure 2D). Assembly of the gene into a nucleosome may be sufficient to prevent its transcription. If so, transcripts from reconstitutes must derive solely from genes which happen to be nucleosome-free. This possibility was tested using the restriction enzyme *Eco*RV to destroy nucleosome-free genes in the reconstitutes before transcription (Morse, 1989).

pXP10 contains a unique *Eco*RV site, within the 5S RNA gene, which is protected from digestion if assembled into a nucleosome (the dyad axis is at −3; the *Eco*RV site is at +33). We digested reconstitutes containing 12 octamers with *Eco*RV under conditions in which free plasmid was completely linearized (not shown). Only 30−40% of the plasmid was stable to digestion, instead of the expected 50% (random deposition of octamers) or even 90% (preferential deposition). Morse (1989) also found that the protection from digestion conferred by nucleosomes was less than expected. Presumably the *Eco*RV site is accessible even when in a nucleosome, but digestion is much slower. Alternatively, if multiple translational positions with respect to the 5S RNA gene are possible, then the *Eco*RV site may be protected in only some nucleosomes; however, the unique position observed on DNA fragments containing this gene (Hayes *et al.*, 1990) makes this possibility seem unlikely.

Reconstitutes and naked plasmids were digested with *Eco*RV and immediately transcribed in the extract, in the presence of naked pE190 and camptothecin, under rate enhancement conditions (Figure 5B; right panels). Transcription of 5S RNA gene was completely abolished; only satellite I RNA was observed. This result is, of course, the expected one for naked plasmid because all the 5S RNA genes were destroyed by *Eco*RV, but the complete abolition of transcription from the reconstitutes (even though 30−40% of the genes are intact) suggests that all the 5S RNA transcripts were derived from nucleosome-free genes (although if multiple translational positions do exist, we cannot rule out the possibility that genes in nucleosomes sensitive to *Eco*RV are transcribable). We conclude that the assembly of a 5S RNA gene into a nucleosome is sufficient to block its transcription completely. Furthermore, this nucleosome-mediated repression was unaffected over a wide range of superhelix density.

Removal of the core histone 'tail' domains from the nucleosome does not affect 5S RNA gene transcription

Although mildly positive or highly negative superhelical stress had no effect on the ability of the nucleosome to repress transcription, it is possible that removal or modification of the core histone tail domains (these contain most of the post-translational modification sites) is required before supercoiling is effective. We examined the possible role of the core histone tail domains in repression of transcription by removing them with trypsin. Tryptic digestion of reconstitutes containing 12 histone octamers and DNA at different superhelix densities proceeded at the same rate yielding the same limit products, as judged by SDS−gel electrophoresis (Figure 6A), consistent with an octamer structure resistant to superhelical stress.

Reconstitutes which had been digested with trypsin were transcribed in the presence of trypsin inhibitor, camptothecin and the plasmid carrying the maxi-5S RNA gene, under conditions of rate enhancement. Removal of the core histone tails had no effect on 5S RNA gene transcription (Figure 5C, compare the second and fourth panels; Table I). Moreover, transcription remained independent of superhelix density. Thus, removal of the tail domains did not facilitate transcription of the 5S RNA gene assembled into a nucleosome.

The H3/H4 tetramer represses transcription, but does not eliminate it completely

The H3/H4 tetramer binds to DNA forming the 'kernel' of the nucleosome (Camerini-Otero *et al.*, 1976; Camerini-Otero and Felsenfeld, 1977); the nucleosome is completed

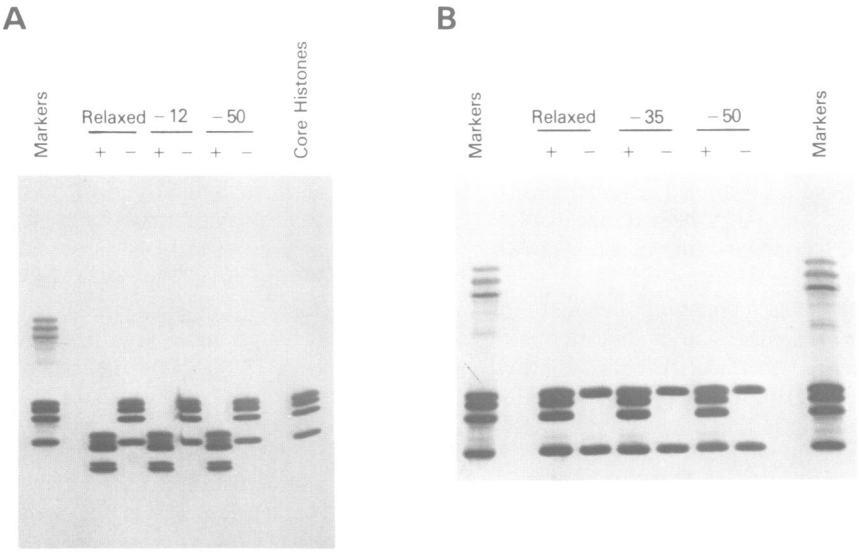

Fig. 6. Analysis of core histones from reconstitutes containing octamers or tetramers and DNA at different superhelix densities. The excess linking number ($\Delta\bar{L}$) of the plasmid in each reconstitute was 0, −12, −35 or −50 as indicated. **A**. Analysis of core histones from reconstitutes before (−) and after (+) limited digestion with trypsin to remove the core histone tail domains. **B**. Analysis of core histones from reconstitutes containing 12 octamers (+H2A/H2B) or 12 H3/H4 tetramers (−H2A/H2B). Markers are histones extracted from native adult chicken erythrocyte chromatin.

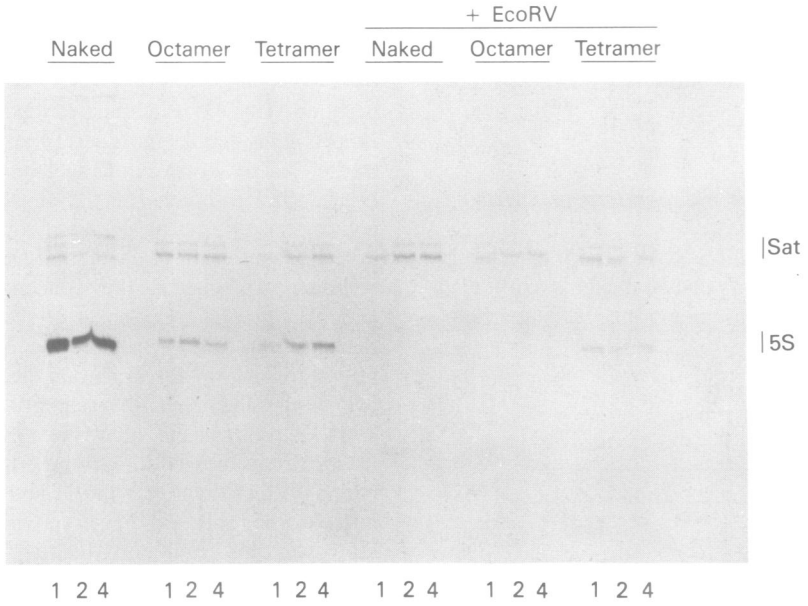

Fig. 7. Effect of DNA superhelix density on transcription of the 5S RNA gene in reconstitutes containing 12 tetramers, and in naked plasmids. Reconstitutes contained 12 tetramers or octamers, and (1) relaxed ($\Delta\bar{L}$ = 0) plasmid, (2) moderately negatively supercoiled ($\Delta\bar{L}$ = −12) plasmid, or (4) very highly negatively supercoiled ($\Delta\bar{L}$ = −50) plasmid. Transcription was in the presence of camptothecin and naked pE190 (which carries the *Sat*I RNA gene), under rate enhancement conditions. The same reconstitutes were transcribed after digestion with *Eco*RV. (See Figure 6B for analysis of histones in an SDS gel.) Transcription rates are given in Table I.

when two H2A/H2B dimers bind, one each side of the tetramer (Richmond *et al.*, 1984). We determined whether the tetramer is as efficient in repressing transcription as the octamer, and whether transcription depends on superhelix density. Reconstitutes containing 12 H3/H4 tetramers were prepared by omission of H2A/H2B from the reconstitution (Figure 6B).

Reconstitutes containing 12 tetramers or octamers were transcribed in the extract in the presence of camptothecin and naked pE190, under conditions of rate-enhancement (Figure 7). Reconstitutes containing tetramers were transcribed at similar rates to those containing octamers (Table I). The tetramers protects only about half the DNA the octamer protects from digestion by micrococcal nuclease, about 68 bp (Camerini-Otero *et al.*, 1976). Thus, if octamers and tetramers were deposited randomly, a higher transcription rate would be expected for reconstitutes containing tetramers. However, the tetramer recognizes the nucleosome positioning signals inherent in the 5S RNA gene (Hayes *et al.*, 1991), and so it might be expected to form preferentially on the 5S RNA gene, like the octamer. If so, similar efficiencies of repression might be expected. Thus, formation of a tetramer on the 5S RNA gene is apparently sufficient to repress its transcription (but see below).

We repeated the *Eco*RV protection experiment with the reconstitutes containing tetramers. We found that only 20–30% of the DNA was protected from digestion, and that this was much less stable than protected DNA in reconstitutes containing octamers, and was eventually linearized completely. This relatively weak protection can be accounted for if most of the genes have a positioned tetramer (68 bp would cover the region −37 to +31), because the *Eco*RV site (at +33) is right at its boundary (the octamer covers the region −76 to +70).

We transcribed reconstitutes containing octamers or tetramers in the extract, immediately after digestion with *Eco*RV for the time required to linearize naked plasmid completely (Figure 7; Table I). Surprisingly, transcription of the reconstitutes containing tetramers was only slightly reduced after digestion with *Eco*RV; transcription of reconstitutes containing octamers was completely abolished, as above (Figure 5B). This result indicates that most of the transcripts observed derive from reconstitutes which do have a tetramer on the 5S RNA gene, and possibly all are transcribed at a very slow rate. (If this is true, there must be very few tetramer-free genes.) This may be because a tetramer positioned on the gene with its dyad axis at −3 would not block the internal control region (ICR), the binding site for TFIIIA (Figure 8). We conclude that transcription of reconstitutes containing tetramers is independent of superhelix density, and that a tetramer on the 5S RNA gene represses but does not eliminate transcription completely.

Discussion

5S RNA gene transcription is independent of DNA superhelix density

We have shown that the rate of 5S RNA gene transcription in an extract made from *Xenopus* oocyte nuclei is unaffected by DNA supercoiling over a wide range of superhelix density, from mildly positive to very negative values. This is the case for genes assembled into a nucleosome (with or without the core histone tail domains) or bound to an H3/H4 tetramer, and for naked genes.

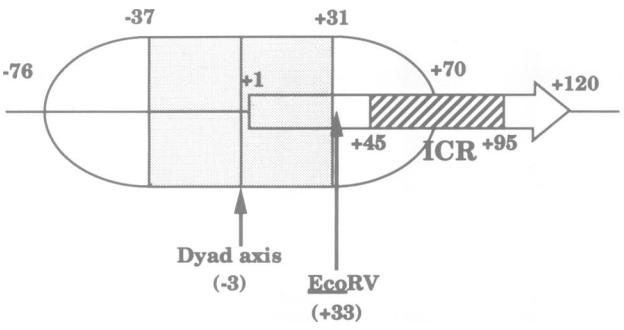

Fig. 8. Repression of transcription of the 5S RNA gene by a positioned octamer or tetramer *in vitro*. Both a positioned histone octamer (−76 to +70; 146 bp) or H3/H4 tetramer (grey box; −37 to +31; 68 bp, Camerini-Otero *et al.*, 1976) with the dyad axis at −3 (Hayes *et al.*, 1990), block the transcription initiation site (+1) of the 5S RNA gene (+1 to +120), but only the octamer blocks the internal control region (ICR) (striped box; +45 to +95). The octamer need not be precisely positioned to account for our observations. TFIIIA binds to the ICR and directs binding of TFIIIB and C to form a stable transcription complex, which has a large footprint extending from −30 to +159 (Wolffe and Morse, 1990). Whether the tetramer must be displaced before transcription can occur is not known. Note that the hydroxyl radical footprint suggests that the octamer (Hayes *et al.*, 1990) and the tetramer (Hayes *et al.*, 1991) influence DNA structure outside the central 146 bp or 68 bp, respectively, which are strongly protected from micrococcal nuclease digestion. The *Eco*RV site is at +33.

We found no evidence for a conformational change in the nucleosome in response to high levels of negative superhelical stress. Similarly, no conformational change was found in nucleosomes under a high degree of positive superhelical stress (Clark and Felsenfeld, 1991). However, a subtle conformational change in the nucleosome may not be detected with the assays used, and there is some evidence for such a change under high negative superhelical stress (Garner *et al.*, 1987). If this is occurring, clearly it has no effect on transcription of the 5S RNA gene.

Pioneering work led to a model in which putative gyrase activity supercoils minichromosomes, disrupting chromatin structure and activating transcription (Ryoji and Worcel, 1984, 1985; Glikin *et al.*, 1984). Evidence for this model included the fractionation of two different populations of minichromosomes, one with the properties of bulk ('static') chromatin, the other apparently giving rise to a half-nucleosome repeat ('dynamic' chromatin). The formation of dynamic chromatin was dependent on Mg^{2+} and ATP, and was blocked by novobiocin, an inhibitor of type II topoisomerases. DNA isolated from dynamic minichromosomes was relaxed, rather than highly supercoiled as predicted, because, it was argued, supercoils introduced by DNA gyrase were relaxed by topoisomerase during isolation. This model stimulated much research and a number of problems have emerged. Firstly, linear templates are transcribed in oocyte extracts, albeit at reduced rates (Wolffe *et al.*, 1987; Worcel, 1987). Secondly, a eukaryotic gyrase activity has not yet been identified. Thirdly, novobiocin is now known to inhibit chromatin assembly as well as other cellular processes at the concentrations generally used (Edenberg, 1980; Cotten *et al.*, 1986; Gottesfeld, 1986; Sealy *et al.*, 1986; Broyles and Moss, 1987). There is, however, general agreement on the importance of ATP and Mg^{2+} in the chromatin assembly process (Glikin *et al.*, 1984; Almouzni and Méchali, 1988).

Our results clearly do not support this model; supercoiling had no effect on transcription and we did not see a half-nucleosome repeat on digestion with micrococcal nuclease. However, we used core histones from transcriptionally repressed chicken erythrocytes rather than the *Xenopus* histones used in many of the studies above, and our reconstitutes differ from chromatin assembled *in vivo* in that nucleosomes are not regularly spaced and do not contain histone H1. More importantly, post-translational modifications of the core histones may be critical in the activation process. This seems less likely because proteolytic removal of the core histone tail domains (which contain most of the post-translational modification sites) did not affect transcription, and the absence of H2A/H2B had little effect on the overall transcription rate. We conclude that neither negative supercoiling nor mild positive supercoiling alone are sufficient to activate transcription of a 5S RNA gene in a nucleosome or bound to a tetramer. Whether supercoiling affects transcription by other RNA polymerases remains to be tested.

Nucleosome-mediated repression of transcription

We have demonstrated that a nucleosome is positioned on many if not most of the 5S RNA genes in our reconstitutes

(even those containing only three nucleosomes). This strongly suggests that the 5S RNA gene is preferentially assembled into a positioned nucleosome, as expected (Shrader and Crothers, 1989).

We have shown that assembly of the gene into a nucleosome is sufficient to block its transcription completely. The transcripts observed from reconstitutes probably derive solely from the small number of nucleosome-free genes. This nucleosome-mediated repression of transcription is not relieved by removal of the core histone tails. These results confirm and extend those of Shimamura *et al.* (1988, 1989) and Morse (1989). A nucleosome positioned on the 5S RNA gene may well repress transcription by physical blockage of the transcription initiation site and the ICR (Figure 8). TFIIIA may bind to the ICR even when it is part of a positioned nucleosome (Rhodes, 1985), but if so, it does not activate transcription (Felts *et al.*, 1990; Tremethick *et al.*, 1990). However, a nucleosome need not be precisely positioned on the gene to repress transcription (Shimamura *et al.*, 1989; Almouzni *et al.*, 1990b, 1991). In fact, it is not known whether a nucleosome is positioned on the gene *in vivo*. Some transcription was observed from minichromosomes containing physiologically spaced nucleosomes assembled in a *Xenopus* egg extract, but this was repressed by histone H1, which presumably bound to genes which happened to be in linker DNA (Shimamura *et al.*, 1989). Thus, the role of nucleosome-positioning on the 5S RNA gene *in vivo* is unclear.

The H3/H4 tetramer represses transcription almost as effectively as the octamer. Tremethick *et al.* (1990) found that tetramers assembled using N1-protein did not inhibit transcription at moderate levels, but repression was observed with larger numbers of tetramers. We did not determine whether a tetramer was positioned on the gene in our reconstitutes, but it does recognize the nucleosome positioning signals in the 5S RNA gene (Hayes *et al.*, 1991). Thus, it may bind preferentially to the gene like the octamer, blocking the transcription initiation site (Figure 8), accounting for their similar repressive properties. But, unlike the octamer, a positioned tetramer would leave the ICR free to bind TFIIIA. This is a necessary first step in the assembly of a transcription complex (Setzer and Brown, 1985; Bieker *et al.*, 1985), and may lead to events in the extract which render some of the genes in tetramers transcribable. These events may mimic those during replication *in vivo*, when the tetramer is deposited first, followed at a later stage by the dimers (Senshu *et al.*, 1978; Worcel *et al.*, 1978; Fotedar and Roberts, 1989; Almouzni *et al.*, 1990a, 1991), and may determine whether a gene is assembled into a transcription complex or a nucleosome.

Materials and methods

Preparation of supercoiled plasmid pXP10 with a narrow linking number distribution

Plasmid preparations with a defined $\Delta\bar{L}$ containing relatively few topoisomers were prepared (Keller, 1975) from highly purified pXP10 at 'bacterial' superhelix density (a wide range of topoisomers centred on $\Delta\bar{L} = -15$, ranging from -8 to -22): 60 μg pXP10 at 100 μg/ml in 0.2 M NaCl, 20 mM Tris–HCl, pH 8.0, 1 mM Na-EDTA, 1 mM Na-EGTA, 5% (v/v) glycerol, for relaxed pXP10 ($\Delta\bar{L} = 0$), or with ethidium bromide added to 4.3 μg/ml ($\Delta\bar{L} = -12$), or 12.1 μg/ml ($\Delta\bar{L} = -35$) or 15.0 μg/ml ($\Delta\bar{L} = -50$), and treated with 18 μl nicking-closing extract for 1 h in the dark at 37°C. Nicking-closing extract (a gift from Mary O'Dea; 0.3 μl was sufficient to relax 1 μg pBR322 in 30 min at 37°C) was made from chicken erythrocytes as described (Camerini-Otero and Felsenfeld, 1977). Ethidium bromide was extracted with 1-butanol. Protein was extracted with phenol and chloroform, and the DNA resuspended in 10 mM Na-HEPES, pH 8.0, 1 mM Na-EDTA after precipitation with ethanol. Linking numbers of pXP10 were determined in gels containing chloroquine as described (Clark and Felsenfeld, 1991).

Assembly of nucleosomes on plasmid DNA

Core histones were purified from chicken erythrocyte nuclei using hydroxylapatite chromatography, and nucleosomes were reconstituted as described (Camerini-Otero *et al.*, 1976; Simon and Felsenfeld, 1979; Clark and Felsenfeld, 1991). Different input ratios of core histones to DNA were used to obtain different numbers of nucleosomes/plasmid. Tetramer reconstitutes were prepared by omitting H2A/H2B from the reconstitution. Reconstitutes were finally dialysed into 70 mM NH_4Cl, 10 mM Na-HEPES (pH 8.0), 1 mM Na-EDTA, 2 mM DTT (J-Buffer), and stored at 4°C. Nucleoprotein gels, relaxation assays and micrococcal nuclease digestions were as described (Clark and Felsenfeld, 1991). Reconstitutes (2 μg DNA at 40 μg/ml J-Buffer) were treated with trypsin from bovine pancreas (TPCK-treated; Sigma) (1.6 μg/ml) for 10 min at 25°C, and put on ice. Aliquots containing 1.6 μg DNA were immediately precipitated with TCA and analyzed in an SDS–18% polyacrylamide gel, stained with silver as described (Clark and Felsenfeld, 1991). The remainder of the reaction was immediately made 0.1 mg/ml in hen egg white trypsin inhibitor (Boehringer) and transcribed in the extract. Reconstitutes containing octamers or tetramers (1 μg DNA at 50 μg/ml J-Buffer with $MgCl_2$ added to 7 mM) were digested with *Eco*RV (4 units; Boehringer) for 20 min at 25°C (conditions were determined from time courses of digestion), and put on ice. Aliquots were analyzed in an agarose gel or transcribed immediately in the extract.

DNase I footprinting of reconstitutes supercoiled by DNA gyrase

Reconstitutes containing 6 or 10 nucleosomes were supercoiled by DNA gyrase as described (Garner *et al.*, 1987) but with some modifications which increased the degree of supercoiling (D.J.Clark, Mary O'Dea, Gary Felsenfeld and Martin Gellert, unpublished), incubated at 25°C for 4 h, and briefly put on ice before digestion with DNase I (Worthington). Digestion was either light (600 units) or heavier (1500 units) for 90 s at 22°C.

The nucleosome on the 5S RNA gene was footprinted using the indirect method described by Gralla (1985), with minor modifications. The oligonucleotide primer was identical in sequence to the region -39 to -19 in the somatic 5S RNA gene of *X.laevis* (5′-GAAGGCAGCACAAGAGGAGG-3′); consequently it has a single base mismatch with the same region in the *X.borealis* gene in pXP10. Hybridization was allowed to occur as the mixture of primer (5′ end-labeled with T4 polynucleotide kinase) and plasmid (extracted from reconstitutes digested with DNase I) cooled slowly from 65°C to room temperature over 2 h. DNA was passed through a Sephadex G50 spin column pre-equilibrated in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT. Deoxyribonucleotides were added to 0.5 mM each and the primer extended by M-MLV reverse transcriptase (200 units; BRL) at 37°C for 20 min. As a control, the footprint of the nucleosome positioned on the 5S RNA gene was obtained by the direct method. An end-labeled 583 bp *Hha*I–*Eco*RI fragment was obtained by digestion of pXP10 at the unique *Eco*RI site in the polylinker, labelling at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase, digesting with *Hha*I, and gel purification. A nucleosome was assembled on this fragment in the presence of linear pUC9 as carrier by salt/urea dialysis, and lightly digested with DNAase I (90 s, 0.15 μg enzyme from Worthington). The reaction was stopped by adding EDTA to 10 mM. DNAase I footprints were analysed in 6% polyacrylamide gels containing 7 M urea.

Transcription in extracts from oocyte nuclei

Extracts from oocyte nuclei were prepared from ovaries of *X.laevis* as described (Birkenmeier *et al.*, 1978; Wolffe, 1989b). Transcription reactions were carried out in a final volume of 20 μl containing 15 μg DNA/ml, 10 μCi of [α-^{32}P]GTP, 0.1 mM GTP, 0.5 mM each of ATP, UTP and CTP in J buffer (70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM Na-EDTA, 2 mM DTT, 8% (v/v) glycerol, 10 mM Na-HEPES, pH 7.4), and initiated on addition of 15 μl extract from oocyte nuclei, left at room temperature for the times indicated, and stopped by adding EDTA to 10 mM and cooling on ice. Camptothecin was obtained as a solid from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, and was dissolved in methylsulphoxide at 5 mg/ml (14.4 mM) before use. Control plasmids containing the satellite I or maxi-5S RNA genes were added to 10 μg DNA/ml. In 'rate enhancement' experiments, pXP10 and pSP64 were mixed before addition of extract, to give final DNA concentrations of 1.5 and 13.5 μg/ml, respectively, and control plasmids pE190 or pXbs115/105 were added just before the reaction was terminated. Radiolabeled RNA was extracted with phenol, precipitated with ethanol, and resuspended in 99%

(v/v) formamide, 1 mM Na-EDTA, 0.03% (w/v) each of xylene cyanol and bromophenol blue. RNA was electrophoresed in polyacrylamide gels containing 7 M urea at 2000 V for 2–4 h.

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