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Evidence for Torsional Stress in Transcriptionally Activated Chromatin

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The existence of torsional stress in eukaryotic chromatin has been controversial. To determine whether it could be detected, we probed the structure of an alternating AT tract. These sequences adopt cruciform geometry when the DNA helix is torsionally strained by negative supercoiling. The single-strand-specific nuclease P1 was used to determine the structure of an alternating AT sequence upstream of the *Xenopus* β-globin gene when assembled into chromatin in microinjected *Xenopus* oocytes. The pattern of cleavage by P1 nuclease strongly suggests that the DNA in this chromatin template is under torsional stress. The cruciform was detected specifically in the most fully reconstituted templates at later stages of chromatin assembly, suggesting that negative supercoiling is associated with chromatin maturation. Furthermore, the number of torsionally strained templates increased dramatically at the time when transcription of assembled chromatin templates began. Transcription itself has been shown to induce supercoiling, but the requisite negative supercoiling for cruciform extrusion by $(AT)_n$ in oocytes was not generated in this way since the characteristic P1 cutting pattern was retained even when RNA polymerase elongation was blocked with α-amanitin. Thus, torsional stress is associated with transcriptional activation of chromatin templates in the absence of ongoing transcription.

A role for DNA supercoiling in gene activation is suggested by the observations that initiation of transcription is accompanied by local unwinding of DNA (71) and that such strand separation is facilitated by negative DNA supercoiling (reviewed in reference 39). Furthermore, both the formation of stable initiation complexes and the number of initiated transcripts have been shown to be greater on negatively supercoiled than on relaxed templates in vitro (64). However, although superhelicity has been shown to influence transcription of a number of prokaryotic genes in vivo (62), the situation in eukaryotes is much less clear.

Both prokaryotic (88) and eukaryotic (59) chromosomes appear to be organized as a series of independently supercoiled loops or domains. In prokaryotes, the supercoiling is only partially constrained by association with protein (11), so that partitioning between the writhing and twisting of the DNA helix can still take place. Two sources of supercoiling in bacteria are DNA gyrase, which generates negative supercoils (23), and the process of transcription, which introduces both negative and positive supercoils (90). The overall level of unconstrained supercoiling appears to be the result of the combined effects of transcription, DNA gyrase, and the relaxing enzyme DNA topoisomerase I (63, 65, 78). Fluctuations in superhelicity have been shown to affect the expression of several genes (62).

In contrast to the situation in prokaryotes, it is clear that the bulk of DNA in eukaryotic chromatin is not significantly torsionally strained (74). However, whether local regions of the chromatin contain torsionally unconstrained supercoiling is much less clear. The inability to identify a eukaryotic equivalent of the bacterial enzyme DNA gyrase has made it seem unlikely that torsional stress can be locally introduced into chromatin in a site-specific manner. However, there are a number of other potential sources of local supercoiling in eukaryotic chromatin, including the breakdown of higher-order structure (57), the loss of nucleosomes from nuclease-hypersensitive sites (32, 69), unfolding of the nucleosome itself (13, 61), histone acetylation (54), or the passage of transcribing RNA polymerase (10, 24, 55). To determine whether torsional stress could be detected in chromatin, we made use of the extreme sensitivity of alternating AT sequences to negative supercoiling.

Tracts of alternating AT exist as interspersed repeats in the *Xenopus laevis* genome, and a number have been mapped in the globin loci (25). A significant feature of these sequences is the ease with which they adopt cruciform geometry in negatively supercoiled plasmids (27, 44). The free energy of formation of these cruciforms is very low compared with that of other sequences described in the literature (40, 45, 48). The sensitivity of alternating AT tracts to DNA supercoiling suggested that their structure in eukaryotic chromatin might provide a readily detectable test for the presence of torsional stress. The formation of Z-DNA has been used as an analogous indicator of supercoiling in whole chromosomes (35, 87). The presence of an $(AT)_n$ tract in the promoter region of the *Xenopus* β-globin gene raised the possibility that the structure adopted by this sequence might enable the supercoil status of a specific gene to be determined.

Here we describe experiments using P1 nuclease to probe the structure of the $(AT)_n$ tract upstream of the *Xenopus* β-globin gene introduced into *Xenopus* oocyte nuclei by microinjection. Our results suggest the formation of a cruciform by this sequence in chromatin, thereby revealing torsional stress in the DNA. The supercoiling is associated with the later stages of chromatin assembly and, although not a consequence of transcriptional elongation, coincides with transcriptional activation.

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MATERIALS AND METHODS

Internal labelling of DNA. Constructs were linearized with *Eco*RI. The 5′ termini were ^{32}P labelled by using calf intestinal phosphatase and polynucleotide kinase (42). Unincorporated label was removed by Sephadex G-50 column centrifugation prior to recircularization. Part of each sample was restricted with *Nco*I to provide an end-labelled fragment from which A>C and G+A sequencing ladders were generated (43, 76). Ligations were performed at 1 μg of DNA ml^{-1} in 50 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–20 mM dithiothreitol (DTT)–1 mM rATP–50 μg of bovine serum albumin ml^{-1}–8 U of DNA ligase ml^{-1} at 4°C overnight. Ethidium bromide (1 μg ml^{-1}) was added when supercoiled molecules were to be generated. Samples were extracted with phenol, butanol, phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) and then isopropanol precipitated. ^{32}P label was removed from nonligated molecules by retreatment with calf intestinal phosphatase. The extent of religation and the final proportion of supercoiled, closed circular relaxed, and linear molecules still retaining label was assessed by agarose gel electrophoresis and autoradiography. Supercoiled, closed circular relaxed, and linear molecules for naked DNA digestion controls were isolated by preparative low-gelling-temperature agarose gel electrophoresis.

Oocyte injection and nucleic acid extraction. Isolation and injection of stage VI oocytes were done as described previously (85). Oocytes were removed from ovarian follicular material by treatment with collagenase, gently centrifuged to bring the nucleus to the surface, and injected with 20 nl of 500-μg ml^{-1} DNA (containing 1 mg of α-amanitin ml^{-1} where necessary). Oocytes were incubated at 19°C in modified Barth's saline for the periods described in the text. Total nucleic acid (for RNA and DNA analysis) was extracted from 20 to 30 pooled oocytes. Oocytes were homogenized in 300 mM NaCl–2% sodium dodecyl sulfate (SDS)–50 mM Tris-HCl (pH 7.5)–1 mM EDTA–1 mg of proteinase K ml^{-1}. The homogenate was extracted with chloroform-isoamyl alcohol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol and then ethanol precipitated. The pellet was washed with 70% ethanol–diethyl pyrocarbonate-treated H_2O, dried, and resuspended in 1 mM EDTA (diethyl pyrocarbonate treated) for storage at −70°C.

Nuclease digestions. P1 nuclease digestions were carried out in 50 mM Tris-HCl (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$–1 mM DTT (8) at 37°C for 20 min. Micrococcal nuclease (MNase) digestions were performed in 5 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 7.0)–85 mM KCl–1 mM $CaCl_2$–5% (wt/vol) sucrose (20) at 20°C for 0.5 to 5 min. Chromatin from injected oocytes was prepared for digestion by the standard method (20). Briefly, 25 injected oocytes were homogenized in 225 μl of the appropriate buffer, divided into 45-μl aliquots, and incubated with 5 μl of the appropriate nuclease (0.375 to 18.75 U of P1; 5 U of MNase). Naked DNA (20 μg ml^{-1}) control digestions were performed in the same buffers but with 1/10 the amount of nuclease. All reactions were terminated by the addition of an equal volume of 30 mM Tris-HCl (pH 7.9)–20 mM EDTA–1% SDS and were treated with proteinase K (50 μg ml^{-1}) overnight. Samples were extracted with phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol and then ethanol precipitated.

Polyacrylamide and agarose gel electrophoresis of DNA. For high-resolution determination of sites of nuclease cleavage, samples were cut with *Eco*RI at the site of internal labelling and analyzed on 5% denaturing polyacrylamide gels (alongside DNA sequencing ladders). For low-resolution analysis, samples were digested with *Bgl*II and electrophoresed on a 1% agarose gel (2 V cm^{-1}, 18 h) in Tris-phosphate buffer (30 mM Tris-HCl [pH 7.3], 36 mM NaH_2PO_4, 0.1 mM EDTA); 0.8% agarose gels containing chloroquine (0 to 7.5 μg ml^{-1}) were electrophoresed (4 V cm^{-1}, 24 h) in Tris-phosphate buffer for analysis of topoisomer distribution (34). Gels were transferred to nitrocellulose and hybridized with the *Nco*I-*Bgl*II β-globin gene fragment (*P in Fig. 4B), ^{32}P labelled by random-primed synthesis (18).

Analysis of RNA. Cap site-specific β-globin gene transcripts in oocyte samples were analyzed by primer extension (85). Two oocyte equivalents of total nucleic acid were mixed with 0.1 pmol of a 5′-end-labelled 20-residue oligonucleotide primer from the first exon of the β-globin gene (10 μl, final volume) in 400 mM NaCl–10 mM PIPES (pH 6.4). The mixture was heated briefly to 65°C and allowed to anneal at 52°C for 2.5 h. Reaction mixtures were transferred into 90 μl of extension buffer (55 mM Tris-HCl [pH 8.2], 11 mM DTT, 6.7 mM $MgCl_2$, 27.8 μg of actinomycin D ml^{-1}, 0.55 mM deoxynucleoside triphosphates), reverse transcriptase (10 U) was added, and extension proceeded at 42°C for 1 h. After ethanol precipitation, samples were analyzed on a 5% denaturing polyacrylamide gel. Total template-derived RNA was analyzed by RNA dot blot. Two oocyte equivalents of total nucleic acid were treated with RNase-free DNase (23 U, 37°C, 30 min) and ethanol precipitated. Dried pellets were resuspended in 30 μl of 0.1 mM EDTA, and 10 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 8 μl of 37% formaldehyde were added before heating to 60°C for 15 min. Samples were serially diluted (twofold) with 15× SSC, applied under vacuum to nitrocellulose, and washed with 6× SSC. Bound RNA was hybridized with linearized injection template, labelled by random-primed synthesis (18).

RESULTS

P1 nuclease cleavage of $(AT)_n$ is specific for supercoiled molecules. We and others have previously shown that alternating AT tracts become especially sensitive to single-strand-specific reagents when present in negatively supercoiled molecules (27, 44). The negative supercoils induce unwinding of the primary helix, and the self-complementarity of the melted strands leads to the formation of a cruciform (Fig. 1A). The cruciform loops are then preferentially cleaved by single-strand-specific reagents. Thus, by investigating the structure of alternating AT sequences in chromatin, we hoped to determine whether the requisite unwinding energy, or torsional stress, was present. With this in mind, it was important to use a structural probe active under reasonably physiological conditions in order to maximize chromatin stability. P1 nuclease is active at physiological pH and has been shown to recognize altered DNA conformations (19, 31), including cruciforms (8, 30).

Upstream of the *Xenopus* β-globin gene is an $(AT)_{24}$ sequence centered on nucleotide −408 with respect to the start of transcription (9). When naked DNA molecules containing this sequence were digested with P1 nuclease, cleavage in the alternating AT tract was observed only in supercoiled templates (Fig. 1B, lanes SC). Neither closed circular relaxed, nicked circular, nor linear DNA molecules were cleaved in this region, even at enzyme concentrations

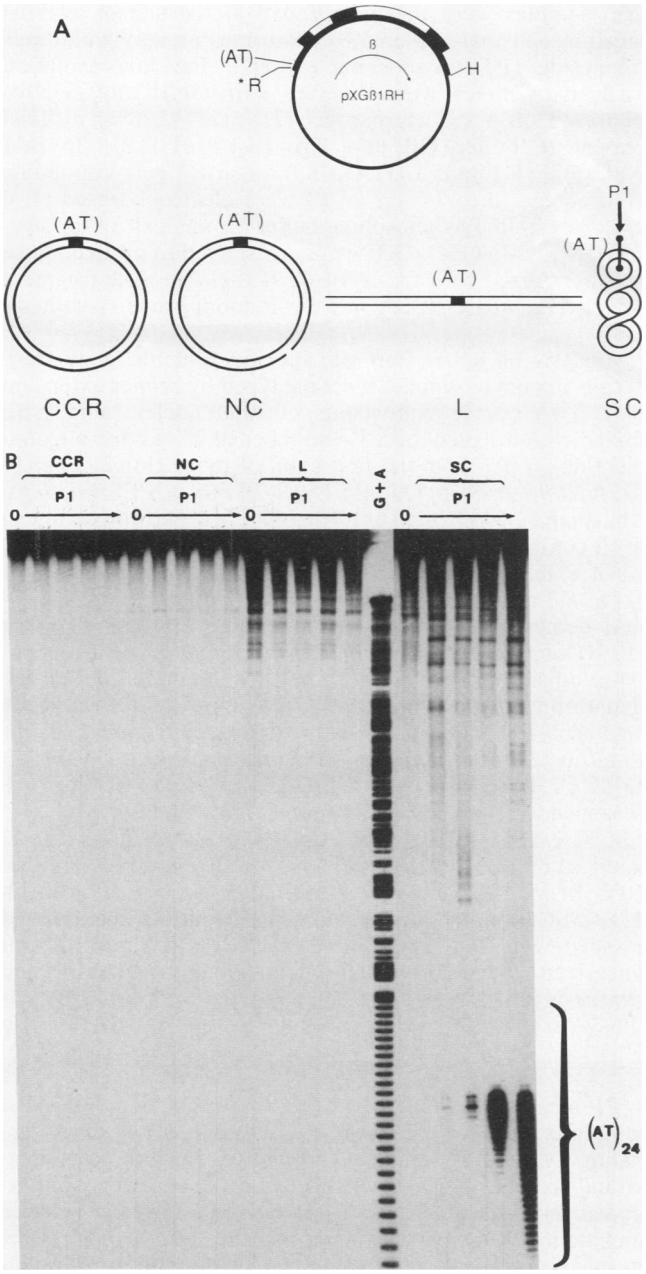


FIG. 1. Supercoil specificity of P1 nuclease cutting of $(AT)_{24}$ in naked DNA. (A) The internally labelled *Xenopus* β-globin gene construct used in this experiment (pXGβ1RH [17]). The thin line represents pAT153 bacterial vector DNA, and the thick line depicts *Xenopus* DNA (*Eco*RI [R]-*Hin*dIII [H]). Filled and open boxes in the β-globin gene depict exons and introns, respectively, and the arrow indicates the direction of β-globin gene transcription. The site of internal labelling (*R) and the alternating AT tract (AT) are marked. The alternating AT tract in this construct is $(AT)_{24}$. Previous reports have referred to the same sequence as $(AT)_{23}$ (9, 25). This reflects the tendency of these sequences to undergo small deletions, presumably as a consequence of slippage during replication. Beneath the construct diagram are depicted the four topological forms (listed below), with cruciform extrusion being supercoil (SC) dependent. (B) P1 nuclease digestion of internally labelled pXGβ1RH as closed circular relaxed (CCR), nicked circular (NC), linear (L), and supercoiled (SC) molecules. Samples were incubated without P1 nuclease (lanes 0) or with increasing amounts of the enzyme (indicated by horizontal arrows). The pXGβ1RH DNA was extracted and cleaved with *Eco*RI at the site of internal labelling. P1 cleavage sites were identified by polyacrylamide gel electrophoresis alongside DNA sequencing ladders (lane G+A). Since the chemical sequencing reactions cause base elimination and leave a 3′ phosphate, bands produced by cleavage at the same residues as P1 nuclease migrate as if they are 1.5 nucleotides smaller (77). The alternating AT tract is bracketed.

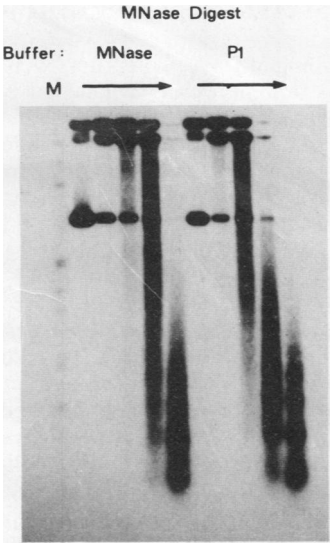


FIG. 2. Evidence that MNase digestion patterns of *Xenopus* β-globin gene chromatin reconstituted in oocyte nuclei are equivalent in P1 nuclease and MNase buffers. Oocytes injected with pXGβ1RH (Fig. 1A) were homogenized either in MNase buffer or in P1 buffer and incubated for the time used in P1 digestion but without P1 nuclease. Oocyte homogenates in P1 buffer were then supplemented with Ca^{2+}, and both homogenates were immediately digested with MNase for increasing periods of time (indicated by horizontal arrows). MNase digestion products were detected by hybridization to the *Nco*I-*Bgl*II β-globin fragment (*P in Fig. 4B). The molecular weight size marker (lane M) is a 3′-end-labelled pAT153 *Hpa*II digest yielding fragment sizes of 634, 494, 407, 244, 240, 219, 205, 192, 162 (doublet), and 149 bp and a number of smaller bands.

20 times higher than those required to initiate cleavage of supercoiled DNA (Fig. 1B, lanes CCR, NC, and L). In the supercoiled molecules it is clear, from the pattern generated at lower enzyme concentrations, that P1 nuclease preferentially cleaves in the middle of the alternating AT tract. In agreement with previously reported results with other single-strand-specific reagents (27, 44), this pattern of cleavage is indicative of cruciform geometry. The high degree of specificity for supercoiled molecules meant that P1 nuclease cutting of $(AT)_{24}$ was a very suitable assay for the existence of torsional stress in chromatin.

The *Xenopus* β-globin gene is under torsional stress in chromatin reconstituted in *Xenopus* oocyte nuclei. DNA injected into *Xenopus* oocyte nuclei is rapidly converted into canonical chromatin (20, 91). To determine the structure of $(AT)_{24}$ in chromatin, and thereby the torsional state of the DNA, the *Xenopus* β-globin gene was introduced into oocyte nuclei by microinjection. After 18 h of incubation to

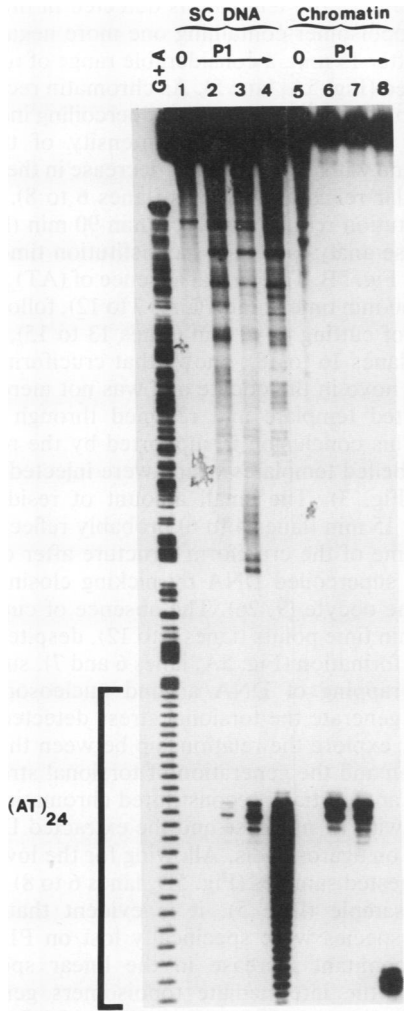


FIG. 3. Evidence that P1 nuclease digestion of $(AT)_{24}$ in internally labelled *Xenopus* β-globin gene chromatin reconstituted in oocyte nuclei resembles the pattern found in naked supercoiled (SC) DNA. Samples were incubated without P1 nuclease (lanes 0) or with increasing amounts of the enzyme (indicated by horizontal arrows). The pXGβ1RH DNA (Fig. 1A) was extracted and cleaved with *Eco*RI at the site of internal labelling. P1 cleavage sites were identified by polyacrylamide gel electrophoresis alongside a DNA sequencing ladder (lane G+A) and a naked supercoiled sample (lanes 1 to 4) as for Fig. 1. Migration differences between P1 and chemical cleavage products are explained in the legend to Fig. 1. The alternating AT tract is bracketed.

ensure that chromatin assembly was complete, oocytes were gently homogenized to extract the chromatin. This method of chromatin extraction has been shown previously to leave the chromatin intact (20). To confirm that this was the case for our experiments and to determine specifically whether the chromatin would survive homogenization in P1 buffer, injected oocytes were homogenized in either MNase buffer or P1 buffer supplemented with Ca^{2+} and treated with MNase (Fig. 2). The results show that in the buffer used for P1 digestion, MNase produced a canonical nucleosomal ladder with a repeat length of ~185 bp. The bulk of the DNA was converted to nucleosomal bands, showing that the majority of the templates remained fully reconstituted under these conditions.

To determine the torsional state of the *Xenopus* β-globin gene in chromatin-reconstituted in *Xenopus* oocytes, a β-globin plasmid (pXGβ1RH; Fig. 1A) internally labelled at the *Eco*RI site (see Materials and Methods) was injected into oocyte nuclei. The oocytes were incubated overnight, during which time the injected DNA was assembled into chromatin. After P1 nuclease digestion of the homogenized oocytes, DNA was extracted and cleaved at the site of internal labelling. Fractionation by polyacrylamide gel electrophoresis revealed cutting in the middle of the $(AT)_{24}$ tract (Fig. 3, lanes 5 to 8). This pattern of cleavage is characteristic of the supercoiled state (Fig. 3, lanes 1 to 4; Fig. 1) and suggests that the DNA is under torsional stress in this oocyte-reconstituted chromatin.

In this type of experiment, it is essential to show that the chromatin is stable and that the P1 nuclease cutting that we were seeing did not arise from supercoiled DNA released from minichromosomes during oocyte homogenization and subsequent incubation. In addition to the MNase digestion discussed above, the P1 cutting patterns both within the AT tract and elsewhere in the plasmid suggest that this is not the case.

First, it is evident from Fig. 3 that at low enzyme concentrations, P1 digestion in both the chromatin and the naked supercoiled DNA samples is confined to the center of the AT tract, reflecting cleavage of the cruciform loop. However, at higher enzyme concentrations, the P1 cleavage pattern of the chromatin samples is in marked contrast to that observed for naked supercoiled DNA. The absence of cleavage throughout the lower half of the AT tract (Fig. 3, lane 8) suggests that the cruciform stem was protected by some feature of the chromatin structure in this region.

Second, an even more marked difference in P1 cleavage patterns between chromatin and naked supercoiled DNA was detected in the ampicillin resistance gene of the plasmid vector (Fig. 4). Cleavage at $(AT)_n$ and within the plasmid vector was monitored simultaneously by fractionating the products of P1 digestion on an agarose gel and visualized by indirect end labelling after secondary cutting with *Bgl*II (Fig. 4). In agreement with the results from polyacrylamide fractionation of internally labelled templates (Fig. 3), the major site of P1 cleavage in the chromatin samples was in the $(AT)_{24}$ tract (Fig. 4A, lanes 1 to 3), and this region was cleaved only in supercoiled naked DNA (lanes 4 to 6), not in covalently closed relaxed (lanes 7 to 9) or linear (lanes 10 to 12) molecules. The additional site of P1 cleavage in supercoiled DNA (lanes 4 to 6) maps to the ampicillin resistance gene in the plasmid vector and has been reported previously for both S1 and P1 nucleases (8, 38, 56, 75). This sequence retained some sensitivity in relaxed DNA for reasons that are at present unclear (lanes 7 to 9). However, more importantly, whereas the ampicillin gene was the major site of P1 cleavage in naked supercoiled DNA (lanes 4 to 6), there was no preferential cleavage at this site in oocyte homogenates (lanes 1 to 3; see also Fig. 5B and 6C). Thus, the $(AT)_{24}$ sensitivity that we detect is not merely a consequence of supercoiled DNA released from chromatin under the conditions of P1 digestion. Rather, it appears genuinely to reflect torsional stress in the chromatin template.

Torsional stress is generated in the later stages of chromatin reconstitution. The experiments reported so far were performed on templates reconstituted in oocytes for extensive periods, thereby ensuring essentially complete chromatin assembly. To determine the relationship between the kinetics of chromatin assembly and the generation of torsional stress in the template, the sensitivity of $(AT)_{24}$ to P1 nucle-

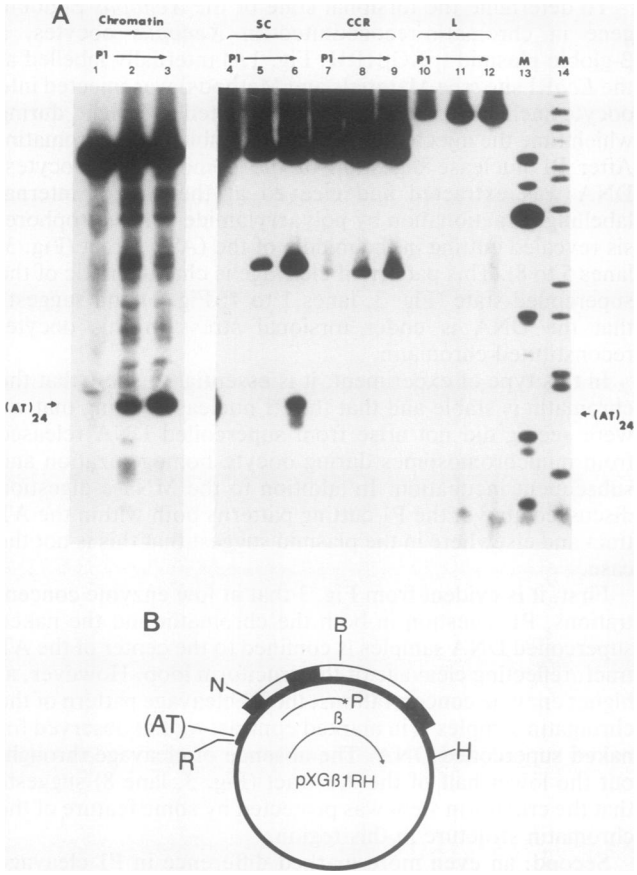


FIG. 4. (A) The additional site of P1 nuclease cleavage found in naked supercoiled DNA (SC; lanes 4 to 6) is not found in *Xenopus* β-globin gene chromatin reconstituted in oocyte nuclei (lanes 1 to 3). Closed circular relaxed (CCR; lanes 7 to 9) and linear (L; lanes 10 to 12) naked DNAs are included for comparison. Samples were incubated with increasing amounts of P1 nuclease (horizontal arrows). The pXGβ1RH DNA (Fig. 1 and panel B) was extracted and cleaved with *Bgl*II (B). The linear sample (lanes 10 to 12) was pXGβ1 (58), which differs from pXGβ1RH only by an extended 3′ flank, and was linearized with *Bgl*II prior to P1 digestion. Sites of P1 cleavage were identified by indirect end labelling (89) using the *Nco*I-*Bgl*II β-globin fragment as the probe (*P in panel B). The position of the alternating AT tract is indicated. The molecular weight size markers (M) are a 3′-end-labelled lambda *Eco*RI-*Hin*dIII digest (lane 13) showing fragment sizes of 5.1, 4.9, 4.3, 3.5, 2.0, 1.9, 1.6, 1.3, 0.98, 0.83, and 0.56 kb and a 3′-end-labelled lambda *Bst*EII digest (lane 14) showing fragment sizes of 7.2, 6.4, 5.6, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.3, and 0.70 kb. R, *Eco*RI; N, *Nco*I; H, *Hin*dIII.

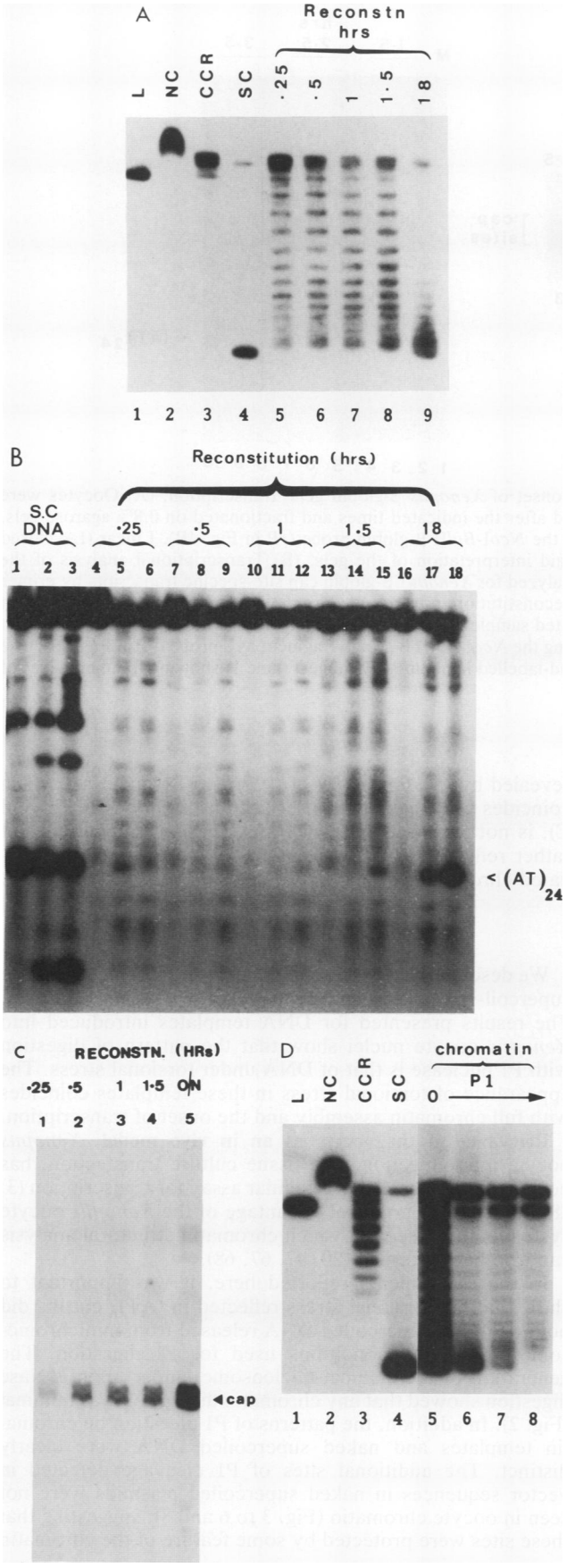
ase was monitored as a function of the time of reconstitution. The β-globin gene was injected into oocyte nuclei; oocytes were homogenized after 15, 30, 60, and 90 min, and the remainder were left for 18 h to ensure full reconstitution. Chromatin assembly was monitored by agarose gel electrophoresis of extracted templates in the presence or absence of chloroquine (34), and P1 sensitivity was monitored by indirect end labelling (Fig. 5A and B).

Chromatin assembly in *Xenopus* oocytes and eggs has been extensively studied (20, 67, 68). After less than 5 min in oocyte nuclei, injected templates are relaxed by the action of endogenous nicking-closing enzymes. Subsequent chromatin assembly introduces constrained supercoils, detected as distinct topoisomer bands on agarose gels. Each nucleosome reconstituted onto the template is detected in the extracted DNA as a topoisomer containing one more negative supercoil. Even after 15 min, a considerable range of topoisomers was generated (Fig. 5A, lane 5). As chromatin reconstitution proceeded to 90 min, the extent of supercoiling increased, as revealed by an increase in the intensity of the fastest-migrating band with a concomitant decrease in the number of closed circular relaxed molecules (lanes 6 to 8). However, full reconstitution required greater than 90 min (lane 9).

P1 nuclease analysis of this reconstitution time course is presented in Fig. 5B. The virtual absence of $(AT)_{24}$ cutting in the 30- and 60-min time points (lanes 7 to 12), followed by the appearance of cutting at 90 min (lanes 13 to 15), increasing up to 18 h (lanes 16 to 18), shows that cruciform extrusion occurred de novo in the oocyte and was not merely present in the injected template and retained through chromatin assembly. This conclusion is supported by the results with internally labelled templates which were injected as relaxed molecules (Fig. 3). The small amount of residual $(AT)_{24}$ cutting after 15 min (lanes 4 to 6) probably reflects the slow relaxation time of the cruciform structure after cleavage of the injected supercoiled DNA by nicking closing enzymes present in the oocyte (9, 26). The absence of cutting in the 30- and 60-min time points (lanes 7 to 12), despite significant nucleosome formation (Fig. 5A, lanes 6 and 7), suggests that the mere wrapping of DNA around nucleosomes is not sufficient to generate the torsional stress detected.

To further explore the relationship between the extent of reconstitution and the generation of torsional stress, a mixture of fully and partially reconstituted chromatin templates was treated with P1 nuclease and the extracted DNAs were fractionated on agarose gels. Allowing for the lower loading of the P1-digested samples (Fig. 5D, lanes 6 to 8) than of the undigested sample (lane 5), it is evident that the most supercoiled species were specifically lost on P1 digestion, with a concomitant increase in the linear species. The intensities of the intermediate topoisomers generated by partial chromatin assembly were unaltered by P1 digestion. The complete absence of cutting in the partially reconstituted templates excludes the possibility that the enzyme is itself inducing cruciform formation. We therefore conclude that the P1 sensitivity genuinely reflects torsional stress in the chromatin template and that it is detected only in templates in which reconstitution is essentially complete.

A dramatic increase in the number of torsionally strained templates coincides with the major onset of transcription. β-Globin transcripts were monitored during the reconstitution time course (Fig. 5C). A small amount of transcription occurred within 30 min (lanes 1 and 2), probably reflecting transcription of naked DNA templates immediately upon injection. However, no further transcription took place up to 90 min (lanes 2 to 4). After overnight incubation, an approximately 20-fold increase in the level of transcripts was apparent (lane 5), suggesting that the bulk of transcription takes place at times beyond 90 min. The production of these transcripts is sensitive to low levels (0.2 $\mu g\ ml^{-1}$) of α-amanitin (data not shown), indicating that these specifically initiated β-globin transcripts were generated by RNA polymerase II. A lag of approximately 3 h before the major onset of RNA polymerase II transcription in the oocyte has been reported previously, and this coincides with the time required for full chromatin reconstitution (47). Since our observations show that torsional stress in the DNA is also associated with full reconstitution (Fig. 5D), we decided to look more closely at the temporal relationship between the



generation of torsional stress in the template and the onset of transcription.

Time points of 1.5, 2.5, and 3.5 h were taken in a separate reconstitution experiment in injected oocytes (Fig. 6). In agreement with previous studies (47), the topoisomer gel shows that reconstitution was essentially complete by 3.5 h (Fig. 6A, lane 6). Primer extension analysis of β-globin RNA shows that a major increase in specifically initiated transcription is detected at 3.5 h (Fig. 6B), confirming the previously reported correlation of RNA polymerase II transcription with full chromatin assembly (47). P1 nuclease analysis of these chromatin templates (Fig. 6C) shows that although a proportion of the molecules was already sensitive in the AT_{24} tract after 1.5 h, in agreement with the data in Fig. 5B, a major increase in sensitivity was detected after 3.5 h. Thus, the major onset of transcription coincides with a major increase in torsional stress, and both coincide with full chromatin assembly.

The supercoiling of oocyte chromatin is not a consequence of transcription. The process of transcription has been shown to generate supercoiling in *Escherichia coli* (90) and in yeast cells (10, 24, 55). The coincidence of β-globin transcription and cruciform extrusion in the AT_{24} tract, detected in Fig. 6, suggested that there might be a causal relationship between transcription and supercoiling in our experiments. To determine whether the supercoils required for cruciform extrusion of $(AT)_{24}$ were generated by transcription of the adjacent β-globin gene, oocyte injections were performed in the

FIG. 5. Evidence that cruciform extrusion occurs de novo during chromatin assembly and only in fully reconstituted templates. (A) Agarose gel analysis of DNA extracted from *Xenopus* β-globin gene chromatin reconstituted for 0.25 to 18 h in *Xenopus* oocyte nuclei. The *Xenopus* β-globin construct used in this experiment was pXGβ1RHEO6-1 (86), which contains a simian virus 40 enhancer/origin segment located 3′ to the β-globin gene fragment. This construct behaves identically to pXGβ1RH in *Xenopus* oocytes (not shown). Oocytes were injected with pXGβRHEO6-1 DNA; after 0.25 to 18 h, DNA was extracted and fractionated on 0.8% agarose gels. Topoisomers of the injected template were identified by hybridization to the *Nco*I-*Bgl*II β-globin probe (*P in Fig. 4B). Linear (L), nicked circular (NC), closed circular relaxed (CCR), and supercoiled (SC) DNAs were included to aid interpretation of the gels. (B) P1 nuclease analysis of *Xenopus* β-globin gene chromatin reconstituted for 0.25 to 18 h in oocyte nuclei and comparison with naked supercoiled (SC) DNA. Chromatin samples from the oocytes in panel A were incubated with increasing amounts of P1 nuclease (increasing left to right for each bracketed sample). The template DNA was extracted and linearized with *Bgl*II. Sites of P1 cleavage were identified by indirect end labelling (89) using the *Nco*I-*Bgl*II β globin fragment as a probe (*P in Fig. 4B). The position of the alternating AT tract is indicated. (C) Primer extension analysis of *Xenopus* β-globin gene transcription during chromatin reconstitution in oocytes. Total nucleic acid from injected oocytes was analyzed for β-globin cap site-specific transcripts by primer extension (see Materials and Methods). O/N, overnight (18 h). (D) P1 nuclease analysis of fully and partially reconstituted *Xenopus* β-globin gene chromatin. Chromatin was assembled under conditions of DNA excess to ensure that some partially reconstituted templates were present. Samples were incubated without P1 nuclease (0; lane 5) or with increasing amounts of enzyme (lanes 6 to 8). DNA was extracted and fractionated on a 0.8% agarose gel run in the presence of chloroquine (2.5 μg ml^{-1}). Topoisomers of the injected template were identified by hybridization to the *Nco*I-*Bgl*II β-globin probe (*P in Fig. 4B). Linear (L), nicked circular (NC), closed circular relaxed (CCR), and supercoiled (SC) naked DNAs were included to aid interpretation of the gel.

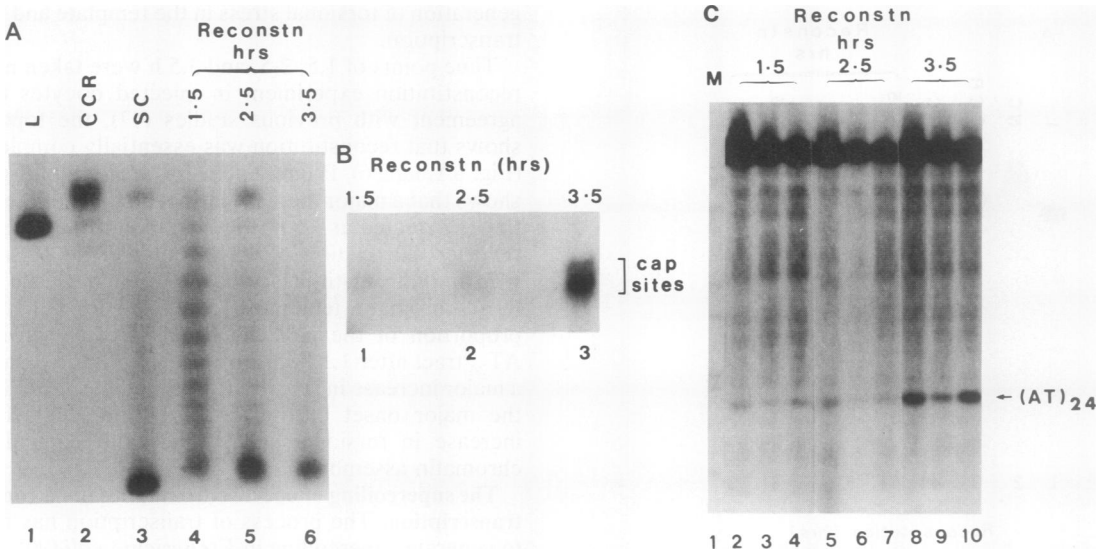


FIG. 6. Evidence that cruciform extrusion coincides with the major onset of *Xenopus* β-globin gene transcription. (A) Oocytes were injected with pXGβ1RHEO6-1 (see legend to Fig. 5). DNA was extracted after the indicated times and fractionated on 0.8% agarose gels. Topoisomers of the injected template were identified by hybridization to the *Nco*I-*Bgl*II β-globin probe (*P in Fig. 4B). Linear (L), closed circular relaxed (CCR), and supercoiled (SC) DNAs were included to aid interpretation of the gels. (B) Transcriptional analysis of the reconstitution in panel A. Total nucleic acid from injected oocytes was analyzed for *Xenopus* β-globin cap site-specific transcripts by primer extension (see Materials and Methods). (C) P1 nuclease analysis of the reconstitution in panel A. Chromatin samples were incubated with increasing amounts of P1 nuclease (increasing left to right for each bracketed sample). The template DNA was extracted and linearized with *Bgl*II. Sites of P1 cleavage were identified by indirect end labelling (89) using the *Nco*I-*Bgl*II β-globin fragment as a probe (*P in Fig. 4B). The position of the alternating AT tract is indicated. The marker (M) is a 3′-end-labelled lambda *Bst*EII digest (lane 1) showing fragment sizes of 7.2, 6.4, 5.6, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.3, and 0.70 kb.

presence of α-amanitin (28), an inhibitor of elongation by eukaryotic RNA polymerases (41, 66).

The *Xenopus* β-globin gene was injected into oocytes in the presence of α-amanitin at a concentration (20 μg ml^{-1}) previously shown to inhibit RNA polymerases II and III (28). To confirm that transcription was inhibited, RNA was assayed by primer extension to detect specifically initiated β-globin transcripts (Fig. 7A). RNAs initiated at the β-globin cap site (labeled mt), and those initiated upstream (85), were detected only in the absence of α-amanitin, confirming that transcription in the β-globin promoter region had been blocked by α-amanitin. This result is consistent with the data of Bendig and Williams (3), who showed that both cap site- and upstream-initiated transcription from *Xenopus* β-globin gene templates injected into *Xenopus* oocytes is catalyzed by RNA polymerase II. However, both RNA polymerases II and III have been shown to initiate transcription on eukaryotic repetitive sequences and even on bacterial vector DNAs injected into *Xenopus* oocytes (46). Furthermore, transcription by RNA polymerase I is not inhibited by α-amanitin (41, 66). Since any of these activities could generate supercoiling, it was important to monitor the transcriptional activity of all three polymerases outside the β-globin gene 5′ end. Therefore, dot blot analysis using the whole injected template as the probe was performed (Fig. 7B). The results confirm that α-amanitin dramatically reduced RNA production from all parts of the injected template.

To determine the structure of the $(AT)_{24}$ tract in chromatin from oocytes in which transcriptional elongation had been blocked by α-amanitin, oocyte homogenates were incubated with P1 nuclease (Fig. 8). The P1 cleavage pattern found in the absence of α-amanitin (lanes 1 to 5) was unaltered by the presence of the drug (lanes 6 to 12). Thus, the supercoiling revealed by the sensitivity of $(AT)_{24}$ to P1 nuclease, which coincides with the major onset of transcription (Fig. 6B and C), is not a consequence of transcriptional elongation, but rather reflects a structural feature of transcriptionally activated chromatin templates.

DISCUSSION

We describe experiments to determine the structure of the supercoil-sensitive sequence, $(AT)_{24}$, when in chromatin. The results presented for DNA templates introduced into *Xenopus* oocyte nuclei show that the pattern of digestion with P1 nuclease is that of DNA under torsional stress. The appearance of torsional stress in these templates coincides with full chromatin assembly and the onset of transcription.

Relevance of the oocyte as an in vivo model. *Xenopus* oocyte microinjection, like tissue culture transfection, has been used extensively as a cellular assay for transcription (3, 28, 47, 85). The principal advantage of the *Xenopus* oocyte system is the ease with which chromatin structural analysis can also be performed (20, 47, 67, 68).

In the experiments reported here, it was important to show that the torsional stress reflected in $(AT)_n$ cutting did not arise from supercoiled DNA released from minichromosomes under the conditions used for P1 digestion. The generation of a canonical nucleosome ladder upon MNase digestion showed that any chromatin disruption was minimal (Fig. 2). In addition, the patterns of P1 digestion on chromatin templates and naked supercoiled DNA were clearly distinct. The additional sites of P1 cleavage detected in vector sequences in naked supercoiled plasmids were not seen in oocyte chromatin (Fig. 3 to 6 and 8), suggesting that these sites were protected by some feature of the chromatin

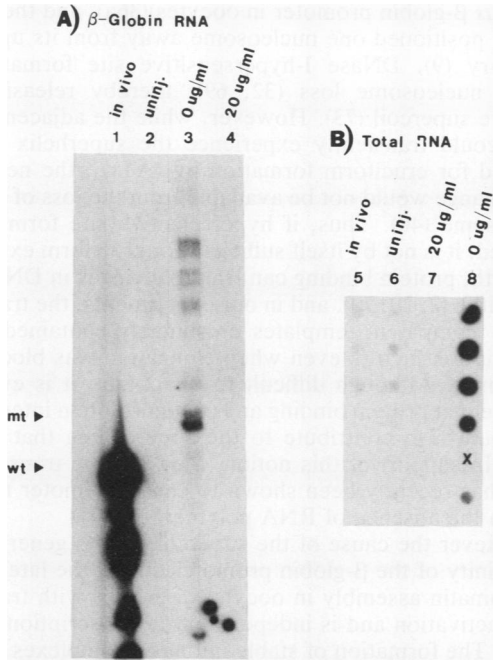


FIG. 7. Blockage of transcriptional elongation by coinjection of the template with α-amanitin (20 μg ml^{-1}). Total RNA was extracted from oocytes injected with pXGβ1RHEO6-1 (see legend to Fig. 5) or an equivalent volume of injection buffer (uninj.) and analyzed by primer extension (A) or dot blot hybridization (B). (A) Primer extension was performed on two oocyte equivalents of RNA, using a 5'-end-labelled oligonucleotide from the first exon of the β-globin gene. The β-globin gene in pXGβ1RHEO6-1 has been marked by insertional mutagenesis (4), giving rise to a different-size mRNA (mt) distinguishable from the wild type (wt) by primer extension. Total cytoplasmic RNA (100 ng) from *Xenopus* erythroblasts (in vivo; lane 1) was used as a control. Cap site-initiated transcripts are indicated by arrowheads; smaller products probably result from premature termination by reverse transcriptase, and larger species reflect the upstream initiations characteristic of RNA polymerase II genes injected into *Xenopus* oocytes (reference 85 and references therein). (B) Dot blot analysis was performed on a twofold dilution series of one oocyte equivalent of RNA (lanes 6 to 8) or of 100 ng of total cytoplasmic RNA from *Xenopus* erythroblasts (in vivo; lane 5). The filter was hybridized with the whole of the injected construct as probe. X, a failed sample loading.

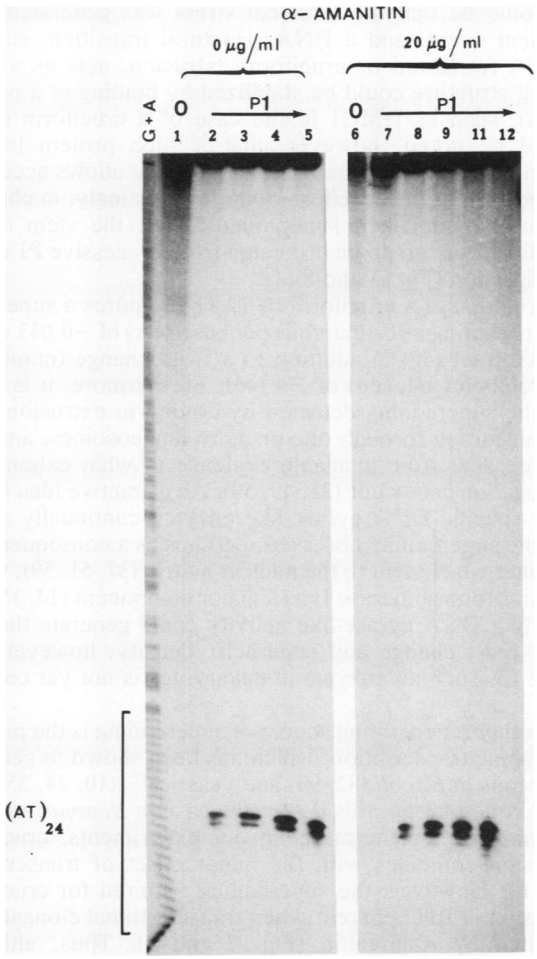


FIG. 8. Evidence that cruciform extrusion in *Xenopus* β-globin gene chromatin is unaltered by coinjection with α-amanitin (20 μg ml^{-1}). Chromatin samples from the oocytes analyzed in Fig. 7 were incubated without P1 nuclease (0; lanes 1 and 6) or with increasing amounts of the enzyme (indicated by horizontal arrows; lanes 2 to 5 and 7 to 12). The pXGβRHEO6-1 DNA (see legend to Fig. 5) was extracted and cleaved with *Eco*RI at the site of internal labelling. P1 cleavage sites were identified by polyacrylamide gel electrophoresis alongside a DNA sequencing ladder (lane G+A). Migration differences between P1 and chemical cleavage products are explained in the legend to Fig. 1. The alternating AT tract is bracketed.

structure. Thus, it seems likely that cleavage by P1 nuclease at the center of $(AT)_{24}$ is genuinely revealing torsional stress in β-globin gene chromatin assembled in oocytes.

Obviously it will be important to determine the torsional state of the endogenous β-globin gene in *Xenopus* erythroid cells, in which it is transcriptionally active, and to compare it with the situation in nonerythroid cells. However, thus far we have been unable to obtain P1 nuclease digestion of these nuclei for reasons that are at present unclear. S1 nuclease, which gives results qualitatively similar to those obtained with use of P1 nuclease with oocyte chromatin (not shown), will digest chromatin in intact nuclei, but this enzyme also recognizes other features of chromatin structure, such as nucleosome-free regions (36), making detection of the supercoil-dependent $(AT)_{24}$ cutting difficult (not shown).

It is likely that the formation of a cruciform would occur only if the $(AT)_{24}$ tract was positioned in linker DNA (12, 52, 53), which implies that the nucleosomes are phased relative to this sequence. There is now much evidence for the existence of phased nucleosomes on a variety of genes both in vivo (5, 80, 81) and in vitro (33, 72, 73), and preliminary results suggest that specific phasing of nucleosomes does occur in the *Xenopus* β-globin gene promoter region (36a). However, deletion of the $(AT)_{24}$ tract does not affect transcription of the *Xenopus* β-globin gene in oocytes, suggesting that cruciform extrusion is not itself the source of phasing in such episomal chromatin.

Potential sources of the supercoiling. The cruciform extrusion that we observe is taking place even in the presence of endogenous topoisomerases. Similarly unrelaxed torsional strain has also been inferred in yeast cells from the altered topoisomer distribution of recovered templates (55) and in mammalian cell nuclei with use of anti-Z-DNA antibodies (87). In order for the supercoils to survive in the presence of topoisomerases, the required supercoiling energy would need to be continually regenerated. An alternative possibil-

ity would be that the torsional stress was generated by a transient event, and a DNA structural transition, such as Z-DNA formation or cruciform extrusion, acts as a sink. Such a structure could be stabilized by binding of a protein (6, 16), such as HMG1 in the case of a cruciform (7). It should be noted, however, that if such protein binding occurs, it must take place in a manner that allows access by P1 nuclease to the cruciform loop. Interestingly, in chromatin but not in naked supercoiled DNA, the stem of the cruciform appears to be protected from processive P1 nuclease digestion (Fig. 3 and 8).

Formation of a cruciform by $(AT)_{24}$ requires a superhelix density (number of supercoils per base pair) of -0.033 within the AT tract (40) in addition to a twist change (number of supercoils introduced) of -4 (45). Furthermore, it is likely that the supercoiling detected by cruciform extrusion must be transmitted through one or more nucleosomes, and it is not yet clear from available evidence to what extent such propogation can occur (21, 49, 50). An attractive idea is that a site-specific DNA gyrase-like enzyme continually generates the supercoiling observed, perhaps as a consequence of template attachment to the nuclear matrix (37, 51, 59), which contains topoisomerase II as a major component (14, 15, 22). Clearly a DNA gyrase-like activity could generate the requisite twist change and superhelix density; however, evidence for such an enzyme in eukaryotes is not yet compelling.

Another regenerating source of supercoiling is the process of ongoing transcription, which has been shown to generate supercoils in *E. coli* (82, 90) and yeast cells (10, 24, 55), and we have found that this is also the case in *Xenopus* oocytes (not shown). Furthermore, in our experiments, cruciform extrusion coincides with the major onset of transcription (Fig. 6). However, the supercoiling required for cruciform extrusion is still apparent when transcriptional elongation is inhibited by α-amanitin (Fig. 7 and 8). Thus, although transcription results in additional supercoiling, it is not responsible for the torsional stress that induces cruciform extrusion in our experiments.

We have examined the relationship between torsional stress in the template and chromatin assembly, and it is clear that the supercoiling arises in the later stages of reconstitution. When a mixture of fully and partially assembled templates was treated with P1 nuclease, only those nearing their full complement of nucleosomes were sensitive to digestion (Fig. 5D). Thus, it is possible that the torsional stress we detect is generated by one or more of the processes involved in chromatin maturation (1) which precede transcriptional activation. A number of chromatin modifications could result in the transient release of supercoils.

First, histone acetylation is associated with transcriptionally active chromatin (60) and has recently been shown to alter the linking number change per nucleosome particle (54). This change in linking number could result in a superhelix density in adjacent linker DNA equal to that required to induce the structural transition in $(AT)_{24}$ (40). Furthermore, the requisite twist change of -4 (45) could be attained by hyperacetylation in 18 of the ~30 nucleosomes present on the constructs used here, although transmission of the supercoiling through the nucleosomes to the $(AT)_{24}$ tract would also be required.

Second, unfolding of the core histone octamer, whether or not as a consequence of acetylation (83), is associated with transcriptional activation (13, 61) and would be expected to release additional supercoiling.

Third, a DNase I-hypersensitive site is detected in the *Xenopus* β-globin promoter in oocytes (36a), and the $(AT)_{24}$ tract is positioned one nucleosome away from its upstream boundary (9). DNase I-hypersensitive site formation involves nucleosome loss (32, 69), thereby releasing one negative supercoil (73). However, while the adjacent linker DNA could transiently experience the superhelix density required for cruciform formation by $(AT)_{24}$, the necessary twist change would not be available from the loss of a single nucleosome (40). Thus, if hypersensitive site formation is involved, it is not by itself sufficient for cruciform extrusion.

Fourth, protein binding can lead to changes in DNA twist and writhe (2, 29, 70), and in our experiments, the transcriptionally competent templates presumably contained bound transcription factors even when elongation was blocked by α-amanitin. Although difficult to quantitate, it is evidently possible that protein binding and protein-protein interactions (79) could also contribute to the supercoiling that we observe. In support of this notion, a eukaryotic transcription factor has recently been shown to cause promoter melting, even in the absence of RNA polymerase (84).

Whatever the cause of the supercoiling, its generation in the vicinity of the β-globin promoter during the later stages of chromatin assembly in oocytes coincides with transcriptional activation and is independent of transcriptional elongation. The formation of stable initiation complexes and the number of initiated transcripts is greater on supercoiled templates in vitro (64). The data presented here are consistent with a role for supercoiling in facilitating strand separation (71) and stable initiation complex formation in eukaryotic chromatin in vivo.

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REFERENCES

1. **Almouzni, G., and M. Mechali.** 1988. Assembly of spaced chromatin: involvement of ATP and DNA topoisomerase activity. EMBO J. **7**:4355–4365.
2. **Baur, C.-P., and R. Knippers.** 1988. Protein-induced bending of the simian virus 40 origin of replication. J. Mol. Biol. **203**:1009–1019.
3. **Bendig, M. M., and J. G. Williams.** 1984. Fidelity of transcription of *Xenopus laevis* globin genes injected into *X. laevis* oocytes and unfertilized eggs. Mol. Cell. Biol. **4**:2109–2119.
4. **Bendig, M. M., and J. G. Williams.** 1984. Differential expression of the *Xenopus laevis* tadpole and adult β-globin genes when injected into fertilized *X. laevis* eggs. Mol. Cell. Biol. **4**:567–570.
5. **Benezra, R., C. R. Cantor, and R. Axel.** 1986. Nucleosomes are phased along the mouse β-major globin gene in erythroid and nonerythroid Cells. Cell **44**:697–704.
6. **Bianchi, M. E.** 1988. Interaction of a protein from rat liver nuclei with cruciform DNA. EMBO J. **7**:843–849.
7. **Bianchi, M. E., M. Beltrame, and G. Paonessa.** 1989. Specific recognition of cruciform DNA by nuclear protein HMG1. Science **243**:1056–1059.
8. **Blaho, J. A., J. E. Larson, M. J. McLean, and R. D. Wells.** 1988. Multiple DNA secondary structures in perfect inverted repeat inserts in plasmids. J. Biol. Chem. **263**:14446–14455.
9. **Brewer, A. C., T. Enver, D. R. Greaves, J. Allan, and R. K. Patient.** 1988. 5′ structural motifs and Xenopus β globin gene activation. J. Mol. Biol. **199**:575–585.
10. **Brill, S. J., and R. Sternglanz.** 1988. Transcription-dependent DNA supercoiling in yeast DNA topoisomerase mutants. Cell **54**:403–411.

11. **Broyles, S. S., and D. E. Pettijohn.** 1986. Interaction of the Escherichia coli HU protein with DNA: evidence for formation of nucleosome-like structures with altered DNA helical pitch. J. Mol. Biol. **187**:47–60.
12. **Cafferelli, E., L. Leoni, B. Sampaolese, and M. Savino.** 1986. Persistence of cruciform structure and preferential location of nucleosomes on some regions of pBR322 and ColE1 DNAs. Eur. J. Biochem. **156**:335–342.
13. **Chen, T. A., and V. G. Allfrey.** 1987. Rapid and reversible changes in nucleosome structure accompany the activation, repression and superinduction of murine fibroblast protooncogenes c-fos and c-myc. Proc. Natl. Acad. Sci. USA **84**:5252–5256.
14. **Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu.** 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell Biol. **100**:1706–1715.
15. **Earnshaw, W. C., and M. M. S. Heck.** 1985. Localization of topoisomerase II in mitotic chromosomes. J. Cell Biol. **100**: 1716–1725.
16. **Elborough, K. M., and S. C. West.** 1988. Specific binding of cruciform DNA structures by a protein from human extracts. Nucleic Acids Res. **16**:3603–3616.
17. **Enver, T., A. C. Brewer, and R. K. Patient.** 1988. Role for DNA replication in β-globin gene activation. Mol. Cell. Biol. **8**:1301–1308.
18. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **137**:266–267.
19. **Fowler, R. F., and D. M. Skinner.** 1986. Eukaryotic DNA diverges at a long and complex pyrimidine · purine tract that can adopt altered conformations. J. Biol. Chem. **261**:8994–9001.
20. **Gargiulo, G., and A. Worcel.** 1983. Analysis of the chromatin assembled in germinal vesicles of Xenopus oocytes. J. Mol. Biol. **170**:699–722.
21. **Garner, M. M., G. Felsenfeld, M. H. O'Dea, and M. Gellert.** 1987. Effects of DNA supercoiling on the topological properties of nucleosomes. Proc. Natl. Acad. Sci. USA **84**:2620–2623.
22. **Gasser, S. M., T. Laroche, J. Falquet, E. Boy de la Tour, and U. K. Laemmli.** 1986. Metaphase chromosome structure: involvement of topoisomerase II. J. Mol. Biol. **188**:613–629.
23. **Gellert, M., K. Mizuuchi, M. H. O'Dea, H. Ohmori, and J. Tomizawa.** 1978. DNA gyrase and DNA supercoiling. Cold Spring Harbor Symp. Quant. Biol. **43**:35–40.
24. **Giaever, G. N., and J. C. Wang.** 1988. Supercoiling of intracellular DNA can occur in eukaryotic cells. Cell **55**:849–856.
25. **Greaves, D. R., and R. K. Patient.** 1985. $(AT)_n$ is an interspersed repeat in the Xenopus genome. EMBO J. **4**:2617–2626.
26. **Greaves, D. R., and R. K. Patient.** 1986. RecBC, sbcB independent, $(AT)_n$-mediated deletion of sequences flanking a Xenopus laevis β globin gene on propagation in E. coli. Nucleic Acids Res. **14**:4147–4158.
27. **Greaves, D. R., R. K. Patient, and D. M. J. Lilley.** 1985. Facile cruciform formation by an $(A\text{-}T)_{34}$ sequence from a Xenopus globin gene. J. Mol. Biol. **185**:461–478.
28. **Gurdon, J. B., and D. I. Melton.** 1981. Gene transfer in amphibian eggs and oocytes. Annu. Rev. Genet. **15**:189–218.
29. **Gustafson, T. A., A. Taylor, and L. Kedes.** 1989. DNA bending is induced by a transcription factor that interacts with the human C-FOS and α-actin promoters. Proc. Natl. Acad. Sci. USA **86**:2162–2166.
30. **Haniford, D. B., and D. E. Pulleyblank.** 1985. Transition of a cloned $d(AT)_n$-$d(AT)_n$ tract to a cruciform in vivo. Nucleic Acids Res. **13**:4343–4363.
31. **Hanvey, J., J. Klysik, and R. D. Wells.** 1988. Influence of DNA sequence on the formation of non-B right handed helices in oligopurine · oligopyrimidine inserts in plasmids. J. Biol. Chem. **263**:7386–7396.
32. **Jakobovits, E. B., S. Bratosin, and Y. Aloni.** 1980. A nucleosome-free region in SV40 minichromosomes. Nature (London) **285**:263–265.
33. **Kefalas, P., F. C. Gray, and J. Allan.** 1988. Precise nucleosome positioning in the promoter of the Chicken β^A globin gene. Nucleic Acids Res. **16**:501–517.
34. **Keller, W.** 1975. Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. Proc. Natl. Acad. Sci. USA **72**:4876–4880.
35. **Lancillotti, F., M. C. Lopez, P. Arias, and C. Alonso.** 1987. Z-DNA in transcriptionally active chromosomes. Proc. Natl. Acad. Sci. USA **84**:1560–1564.
36. **Larsen, A., and H. Weintraub.** 1982. An altered DNA conformation detected by S1 nuclease occurs at specific regions in active chick globin chromatin. Cell **29**:609–622.

36a. **Leonard, M.** Unpublished data.

37. **Lewis, C. D., and U. K. Laemmli.** 1982. Higher order metaphase chromosome structure: evidence for metalloprotein interactions. Cell **29**:171–181.
38. **Lilley, D. M. J.** 1980. The inverted repeat as a recognizable structural feature in supercoiled DNA molecules. Proc. Natl. Acad. Sci. USA **77**:6468–6472.
39. **Lilley, D. M. J.** 1988. DNA opens up—supercoiling and heavy breathing. Trends Genet. **4**:111–114.
40. **Lilley, D. M. J., and J. A. McClellan.** 1987. Alternating adenine-thymine tracts in supercoiled and linear DNA molecules, p. 73–93. *In* M. H. Sarma and R. H. Sarma (ed.), Structure and expression, vol. 2. DNA and its drug complexes. Adenine Press, New York.
41. **Lindell, T., F. Weinberg, B. Morris, R. Roeder, and W. Rutter.** 1970. Specific inhibition of nuclear RNA polymerase II by α-amanitin. Science **170**:447–448.
42. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–560.
44. **McClellan, J. A., and D. M. J. Lilley.** 1987. A two-state conformational equilibrium for alternating $(A\text{-}T)_n$ sequences in negatively supercoiled DNA. J. Mol. Biol. **197**:707–721.
45. **McClellan, J. A., E. Palacek, and D. M. J. Lilley.** 1986. $(A\text{-}T)_n$ tracts embedded in random sequence DNA—formation of a structure which is chemically reactive and torsionally deformable. Nucleic Acids Res. **14**:9291–9309.
46. **Michaeli, T., and C. Prives.** 1987. pBR322 DNA inhibits simian virus 40 gene expression in Xenopus laevis oocytes. Nucleic Acids Res. **15**:1579–1594.
47. **Miller, T. J., and J. E. Mertz.** 1982. Template structural requirements for transcription in vivo by RNA polymerase II. Mol. Cell. Biol. **2**:1595–1607.
48. **Mizuuchi, K., M. Mizuuchi, and M. Gellert.** 1982. Cruciform structures in palindromic DNA are favoured by DNA supercoiling. J. Mol. Biol. **156**:229–243.
49. **Morse, R. H., and R. C. Cantor.** 1985. Nucleosome core particles suppress the thermal untwisting of core DNA and adjacent linker DNA. Proc. Natl. Acad. Sci. USA **82**:4653–4657.
50. **Morse, R. H., D. S. Pederson, A. Dean, and R. T. Simpson.** 1987. Yeast nucleosomes allow thermal untwisting of DNA. Nucleic Acids Res. **15**:10311–10331.
51. **Nelson, W. G., K. J. Pienta, E. R. Barrack, and D. S. Coffey.** 1986. The role of the nuclear matrix in the organization and function of DNA. Annu. Rev. Biophys. Biophys. Chem. **15**: 457–475.
52. **Nickol, J., and R. G. Martin.** 1983. DNA stem-loop structures bind poorly to histone octamers. Proc. Natl. Acad. Sci. USA **80**:4669–4673.
53. **Nobile, C., J. Nickol, and R. G. Martin.** 1986. Nucleosome phasing on a DNA fragment from the replication origin of simian virus 40 and rephasing upon cruciform formation of the DNA. Mol. Cell. Biol. **6**:2916–2922.
54. **Norton, V. G., K. W. Marvin, P. Yau, and E. M. Bradbury.** 1990. Nucleosome linking number change controlled by acetylation of histones H3 and H4. J. Biol. Chem. **265**:19848–19852.
55. **Osborne, B. I., and L. Guarente.** 1988. Transcription by RNA polymerase II induces changes of DNA topology in yeast. Genes Dev. **2**:766–772.
56. **Panayotatos, N., and R. D. Wells.** 1981. Cruciform structures in

supercoiled DNA. Nature (London) **289**:466–470.
57. **Patient, R. K., and J. Allan.** 1989. Active chromatin. Curr. Opinion Cell Biol. **1**:454–459.
58. **Patient, R. K., R. Harris, M. E. Walmsley, and J. G. Williams.** 1983. The complete nucleotide sequence of the major adult β globin gene of Xenopus laevis. J. Biol. Chem. **258**:8521–8523.
59. **Paulson, J. R., and U. K. Laemmli.** 1977. The structure of histone-depleted metaphase chromosomes. Cell **12**:817–828.
60. **Perry, M., and R. Chalkley.** 1981. The effect of histone hyperacetylation on the nuclease sensitivity and the solubility of chromatin. J. Biol. Chem. **256**:3313–3318.
61. **Prior, C. P., C. R. Cantor, E. M. Johnson, V. C. Littau, and V. G. Allfrey.** 1983. Reversible changes in nucleosome structure and histone H3 accessibility in transcriptionally active and inactive states of rDNA chromatin. Cell **34**:1033–1042.
62. **Pruss, G. J., and K. Drlica.** 1989. DNA supercoiling and prokaryotic transcription. Cell **56**:521–523.
63. **Pruss, G. J., S. H. Manes, and K. Drlica.** 1982. Escherichia coli DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell **31**:35–42.
64. **Richardson, J. P.** 1975. Initiation of transcription by Escherichia coli RNA polymerase from supercoiled and non-supercoiled bacteriophage PM2 DNA. J. Mol. Biol. **91**:477–487.
65. **Richardson, S. M. H., C. F. Higgins, and D. M. J. Lilley.** 1984. The genetic control of DNA supercoiling in Salmonella typhimurium. EMBO J. **3**:1745–1752.
66. **Roeder, R. G.** 1974. Multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in Xenopus laevis. J. Biol. Chem. **249**:241–248.
67. **Ryoji, M., and A. Worcel.** 1984. Chromatin assembly in Xenopus oocytes: in vivo studies. Cell **37**:21–32.
68. **Ryoji, M., and A. Worcel.** 1985. Structure of the two distinct types of minichromosomes that are assembled on DNA injected in Xenopus oocytes. Cell **40**:923–932.
69. **Saragosti, S., G. Moyne, and M. Yaniv.** 1980. Absence of nucleosomes in a fraction of SV40 chromatin between the origin of replication and the region coding for the late leader RNA. Cell **20**:65–73.
70. **Shuey, D. J., and C. S. Parker.** 1986. Bending of promoter DNA on binding of heat shock transcription factor. Nature (London) **323**:459–461.
71. **Siebenlist, J.** 1979. RNA polymerase unwinds an 11-base pair segment of a phage T7 promoter. Nature (London) **279**:651–652.
72. **Simpson, R. T., and D. W. Stafford.** 1983. Structural features of a phased nucleosome core particle. Proc. Natl. Acad. Sci. USA **80**:51–55.
73. **Simpson, R. T., F. Thoma, and J. W. Brubaker.** 1985. Chromatin reconstituted from tandemly repeated cloned DNA fragments and core histones: a model system for study of higher order structure. Cell **42**:799–808.
74. **Sinden, R. R., J. O. Carlson, and D. E. Pettijohn.** 1980. Torsional tension in the DNA double helix measured with trimethylpsoralen in living E. coli cells: analogous measurements in insect and human cells. Cell **21**:773–783.
75. **Singleton, C. K., J. Klysic, and R. D. Wells.** 1983. Conformational flexibility of junctions between contiguous B- and Z-DNAs in supercoiled plasmids. Proc. Natl. Acad. Sci. USA **80**:2447–2451.
76. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence of the E. coli gene coding for dihydrofolate reductase. Nucleic Acids Res. **8**:2255–2274.
77. **Sollner-Webb, B., and R. H. Reeder.** 1979. The nucleotide sequence of the initiation and termination sites for ribosomal RNA transcription in X. laevis. Cell **18**:485–499.
78. **Sternglanz, R., S. DiNardo, K. A. Voelkel, Y. Nashimura, Y. Hirota, K. Becherer, L. Zumstein, and J. C. Wang.** 1981. Mutations in the gene coding for the Escherichia coli DNA topoisomerase I affect transcription and transposition. Proc. Natl. Acad. Sci. USA **78**:2747–2751.
79. **Su, W., S. Jackson, R. Tjian, and H. Echols.** 1991. DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. Genes Dev. **5**:820–826.
80. **Thoma, F.** 1986. Protein-DNA interactions and nuclease-sensitive regions determine nucleosome positions on yeast plasmid chromatin. J. Mol. Biol. **190**:177–190.
81. **Thoma, F., and R. T. Simpson.** 1985. Local protein-DNA interactions may determine nucleosome positions on yeast plasmids. Nature (London) **315**:250–252.
82. **Tsao, Y.-P., H.-Y. Wu, and L. F. Liu.** 1989. Transcription-driven supercoiling of DNA: direct biochemical evidence from in vitro studies. Cell **56**:111–118.
83. **van Holde, K. E.** 1989. Chromatin. Springer-Verlag, New York.
84. **Vos, J. C., M. Sasker, and H. G. Stunnenburg.** 1991. Promoter melting by a stage-specific vaccinia virus transcription factor is independent of the presence of RNA polymerase. Cell **65**:105–113.
85. **Walmsley, M. E., and R. K. Patient.** 1987. Highly efficient β globin transcription in the absence of both a viral enhancer and erythroid factors. Development **101**:815–827.
86. **Wilson, A. C.** 1991. Ph.D. thesis. University of London, London, United Kingdom.
87. **Wittig, B., T. Dorbic, and A. Rich.** 1989. The level of Z-DNA in metabolically active, permeabilized mammalian cell nuclei is regulated by torsional strain. J. Cell Biol. **108**:755–764.
88. **Worcel, A., and E. Burgi.** 1972. On the structure of the folded chromosome of Escherichia coli. J. Mol. Biol. **71**:127–147.
89. **Wu, C., P. M. Bingham, K. J. Livak, R. Holmgren, and S. C. R. Elgin.** 1979. The chromatin structure of specific genes. I. Evidence for high order domains of defined DNA sequence. Cell **16**:797–806.
90. **Wu, H.-Y., S. Shyy, J. C. Wang, and L. F. Liu.** 1988. Transcription generates positively and negatively supercoiled domains in the template. Cell **53**:433–440.
91. **Wyllie, A. H., R. A. Laskey, J. Finch, and J. B. Gurdon.** 1978. Selective DNA conservation and chromatin assembly after injection of SV40 DNA into Xenopus oocytes. Dev. Biol. **64**:178–188.