The EMBO Journal vol.8 no.8 pp.2343 – 2351, 1989

Nucleosomes inhibit both transcriptional initiation and elongation by RNA polymerase III *in vitro*

Randall H.Morse

Laboratory of Cellular and Developmental Biology, Bldg.6, Room Bl-26, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Communicated by A.Travers

To examine the effect of nucleosomes on *in vitro* transcription, purified chicken erythrocyte core histones and plasmid DNA bearing the *Xenopus* 5S RNA gene were assembled into nucleosomes and used as templates for transcription in a *Xenopus* oocyte nuclear extract. Plasmids having a nucleosome incorporating a specific region of the gene were selected by treating the reconstituted molecules with restriction endonucleases. In this way, it was shown that a nucleosome on or close to the internal control region of the 5S RNA gene inhibits transcription. Furthermore, experiments with 5S maxigenes showed that RNA polymerase III, in contrast to SP6 RNA polymerase, will not transcribe through a nucleosome *in vitro*.

Key words: RNA polymerase III/transcription/chromatin/nucleosome/5S rDNA

Introduction

The template for transcription in eukaryotes is not naked DNA, but chromatin. This has led a number of investigators to address the question of whether chromatin, and more specifically nucleosome core particles, present an impediment of any sort to transcription *in vitro*. Early studies using bacterial polymerases (Williamson and Felsenfeld, 1978; Wasylyk *et al.*, 1979) as well as eukaryotic polymerases I and II (Meneguzzi *et al.*, 1979; Wasylyk and Chambon, 1979) suggested that nucleosomes inhibit transcription, in the sense that shorter transcripts were observed from reconstituted than from naked templates, and rates of elongation were decreased. At the same time, transcripts of length corresponding to several nucleosomes were observed, leading to the inference that nucleosomes did not completely impede transcriptional elongation. However, these studies were performed with heterogeneously assembled templates (i.e. not every template had the same number of or, presumably, identically positioned nucleosomes) and, as has recently been pointed out (Lorch *et al.*, 1987), the conclusions were based on statistical correlations and were therefore indirect.

More recent studies have attempted to rectify these shortcomings by using templates with unique initiation sites (Lassar *et al.*, 1985; Knezetic and Luse, 1986; Matsui, 1987; Workman and Roeder, 1987) and investigating the effect on transcription of a single positioned nucleosome (Lorch *et al.*, 1987; Losa and Brown, 1987). These investigations have succeeded in distinguishing between effects of nucleosomes on transcriptional initiation and elongation. A prokaryotic (SP6) polymerase has been shown to be incapable of recognizing a promoter incorporated into a nucleosome, but capable of transcribing through a single positioned nucleosome on a short linear template (Lorch *et al.*, 1987; Losa and Brown, 1987). Similar results have been obtained with eukaryotic RNA polymerase II (Lorch *et al.*, 1987; Knezetic and Luse, 1986); however, it could not be determined whether the block to initiation by nucleosomes was due to direct interference with RNA polymerase II or to inhibition of binding of ancillary transcription factors (e.g. Matsui, 1987; Workman and Roeder, 1987).

Ideally, one would like to study the effect on transcription of an array of nucleosomes on a gene which resides on a topologically closed template, and to explore the effect of nucleosome position on initiation as well as on the interaction of *trans*-acting factors with DNA. In this respect, the experiments cited above suffer from one of two deficiencies: they either do not allow investigation of a homogeneous template or they do not allow the investigation of an array of nucleosomes on a topologically closed template. The importance of studying an array of nucleosomes is indicated by the work cited earlier which suggested that T7 RNA polymerase could elongate through one or two nucleosomes, but not more, before coming to a halt (Williamson and Felsenfeld, 1978). Thus, although SP6 RNA polymerase and eukaryotic RNA polymerase II will elongate through a single nucleosome, the question remains whether these enzymes will behave similarly towards an array of nucleosomes. The use of a closed circular template is desirable because it more closely reflects the topological state of chromatin found *in vivo* (Benyajati and Worcel, 1976). Moreover, the transient creation of supercoiled domains by a transcribing RNA polymerase (Liu and Wang, 1987) could be more easily explored using closed circular rather than linear templates.

For these reasons, I have devised a protocol for studying the effect that nucleosomes have on *in vitro* transcription when they incorporate specific regions of a closed circular, transcribed template. In this paper, I report on the effect that nucleosomes on or close to the internal control region of the 5S RNA gene of the frog *Xenopus borealis* have on *in vitro* transcription. I also report on the effect of nucleosomes downstream from this region in maxigenes constructed from the same parent 5S RNA gene.

Results

Experimental strategy

Reconstitution of nucleosomes onto a closed circular template at low histone:DNA ratios is likely to give a heterogeneous population in which the DNA sequences incorporated into nucleosomes vary from one molecule to the next (Poljak and Gralla, 1987; Stein, 1987). Reconstitution at higher histone: DNA ratios, on the other hand, while being likely to yield a more homogeneous population of assembled plasmids, is

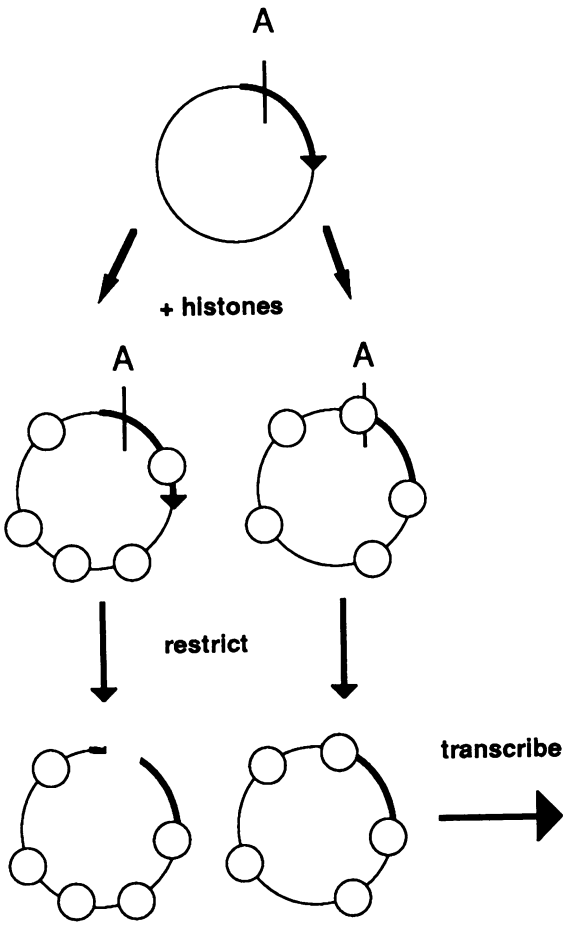

Fig. 1. Experimental protocol. Nucleosomes are reconstituted from plasmid DNA and purified core histones, yielding a mixed population of assembled molecules. Some molecules, for example, will incorporate restriction site A into a nucleosome, and others will not, as shown by two reconstituted plasmids. Treatment with the restriction enzyme A results in this population being divided into linearized molecules and molecules incorporating the recognition sequence for A in a nucleosome. If the restriction site used lies within a transcribed sequence (the heavy line), the effect of a nucleosome on transcription can be assessed.

also likely to yield a template which is completely refractory to transcriptional initiation (Weisbrod *et al.*, 1982; Knezetic and Luse, 1986; Shimamura *et al.*, 1988). I have attempted to circumvent this difficulty by selecting, from a heterogeneous population assembled at relatively low histone:DNA ratios, a population of molecules having a nucleosome at a particular region in or close to a transcribed region.

The protocol used is diagrammed in Figure 1. When a plasmid including the transcribed sequence of interest is assembled into nucleosomes, individual plasmid molecules will have different sequences incorporated into nucleosomes. Restriction of the reconstituted plasmids at a unique restriction site will cut those molecules which lack a nucleosome at the restriction site, while some or all of the molecules which incorporate the restriction site in a nucleosome will remain intact (Lorch *et al.*, 1987). If the restriction site lies within an essential promoter, or within the transcribed region, the effect of a nucleosome at that site on transcription can be readily assessed, since only the uncut molecules, which have the specific site within a nucleosome, will retain a length of DNA capable of producing full-length transcripts.

Nature of the reconstituted template

In the experiments described below, plasmid DNA was assembled into nucleosomes by mixing with purified core histones from chicken erythrocytes at 1.5 M NaCl and dialyzing against solutions of decreasing salt concentration. This method of reconstitution yields nucleosome core particles which are identical to those isolated from living cells as assayed by biochemical criteria, e.g. sedimentation coefficient, protection of DNA against digestion by micrococcal nuclease, protection of histone proteins against trypsin digestion and change in linking number of the DNA. Such reconstituted plasmids, lacking linker histone H1 or H5 and assembled at relatively low histone:DNA ratios so that on average only one nucleosome is present per 300–400 bp of DNA, show no apparent tendency towards precipitation or aggregation. Nucleosome assembly appears to be random rather than cooperative, as judged both by electron microscopy (Germond *et al.*, 1975; Simpson *et al.*, 1985) and the low intensity of bands corresponding to higher-order oligonucleosomes in micrococcal nuclease digests (Morse and Cantor, 1986). Nucleosome formation appears equally likely to occur, with some exceptions, over all regions of a given DNA molecule (Zimmerman and Levin, 1975; Poljak and Gralla, 1987). Whether nucleosomes are precisely positioned at individual DNA sequences has been studied for very few cases, and would appear to be dependent on the particular sequence (Simpson *et al.*, 1985; Stein, 1987).

As noted above, when a restriction endonuclease recognition sequence is in a nucleosome, digestion by the enzyme is likely to be inhibited. However, it seemed possible that treatment of reconstituted plasmids with a restriction endonuclease might be biased towards leaving a subpopulation of uncut molecules which are not representative of the original assembled material. For example, if two close-packed nucleosomes were much more resistant than a nucleosome monomer with no nearby neighbors, restriction endonuclease treatment would select for dinucleosomes. Since a bias of this sort would affect the interpretation of experiments involving the protocol of Figure 1, I decided to test this possibility. This was done by labeling the unique *Nco*I site of closed circular pXbs1185 (see Materials and methods) with ^{32}P, assembling the labeled plasmid into nucleosomes, and digesting with micrococcal nuclease after incubation with or without *Nco*I. The purified DNA was then electrophoresed and the gel analyzed by autoradiography. If, for example, DNA in a nucleosome monomer were less resistant than DNA in higher-order nucleosomes to *Nco*I digestion, the gel band corresponding to mononucleosome DNA would be depleted relative to higher-order oligonucleosome bands in the sample treated with *Nco*I. Instead, the digestion patterns were the same for the *Nco*I-treated as for the unrestricted reconstituted plasmid, with both showing a strongly protected DNA fragment corresponding to a nucleosome monomer and varying amounts of higher-order oligomers, depending on the individual reconstitution (Figure 2). This indicates that the same population of nucleosomes incorporates the *Nco*I site before and after digestion with *Nco*I, and therefore suggests that the structural feature leading to inhibition of restriction endonuclease cutting occurs at the level of the nucleosome monomer.

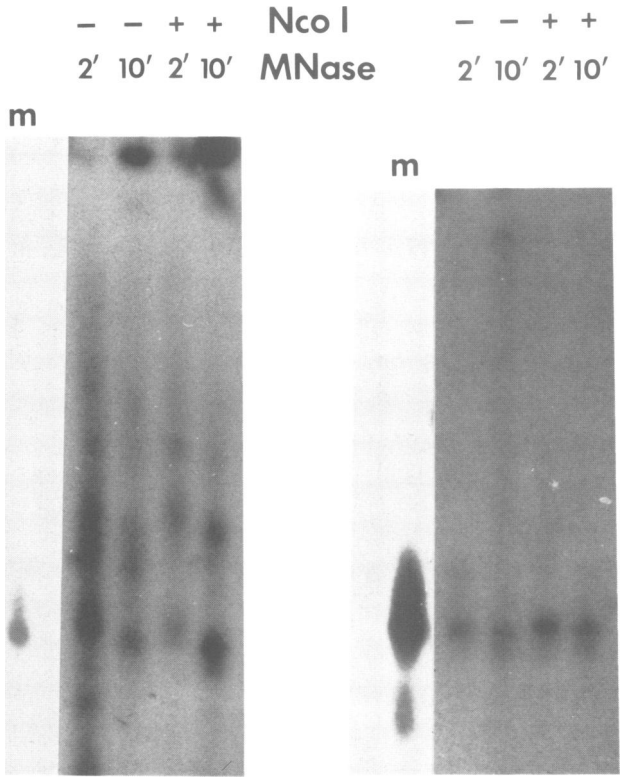

Fig. 2. Digestion of reconstituted plasmid chromatin by a restriction endonuclease does not alter the pattern of micrococcal nuclease digestion products. The plasmid pXbs1185 (see Materials and methods) was cut at the unique *Nco*I site, labeled by treatment with phosphatase and then polynucleotide kinase in the presence of [γ-^{32}P]ATP and religated. Closed circular plasmids were purified from an agarose gel and reconstituted into nucleosomes with purified core histones. The resulting plasmid chromatin was incubated in high-salt restriction buffer (Maniatis *et al.*, 1982) with or without *Nco*I at 37°C for 1 h. Following addition of $CaCl_2$ to 2 mM, the samples were immediately digested with micrococcal nuclease (0.5–1.0 U/μg DNA) for the indicated times, the DNA purified and electrophoresed on a 6% polyacrylamide gel, and the gel analyzed by autoradiography. The lanes labeled 'm' contain ^{32}P-labeled DNA from purified chicken erythrocyte mononucleosomes, and are derived from shorter exposures of the same gels. The samples analyzed in the left-hand panel originated from a more highly reconstituted sample (more nucleosomes per length of DNA) than those in the right-hand panel.

Effect of nucleosomes on transcription by SP6 RNA polymerase

To demonstrate the utility of the protocol shown in Figure 1, the plasmid pGEM4/XK, depicted in the upper part of Figure 3, was used to investigate the effect of nucleosomes on elongation by the RNA polymerase from bacteriophage SP6. Naked pGEM4/XK was linearized at the *Hpa*I site and mixed with reconstituted pGEM4/XK which had been treated with *Bgl*II. When this mixture was transcribed by SP6 RNA polymerase, two prominent transcripts of ~510 and 610 nucleotides (nt) were observed, corresponding to polymerase runoff at the two restriction sites (Figure 3, lane 1). Two longer transcripts of ~750 and >1500 nt, not observed when *Hpa*I-cut pGEM4/XK was transcribed alone (data not shown) are also present in lane 1, suggesting that SP6 RNA polymerase was able to elongate through nucleosomes protecting the *Bgl*II site in the reconstituted plasmid. The transcript of ~750 nt, labeled F in Figure 3, evidently arises from an SP6 termination site which is coincidentally present

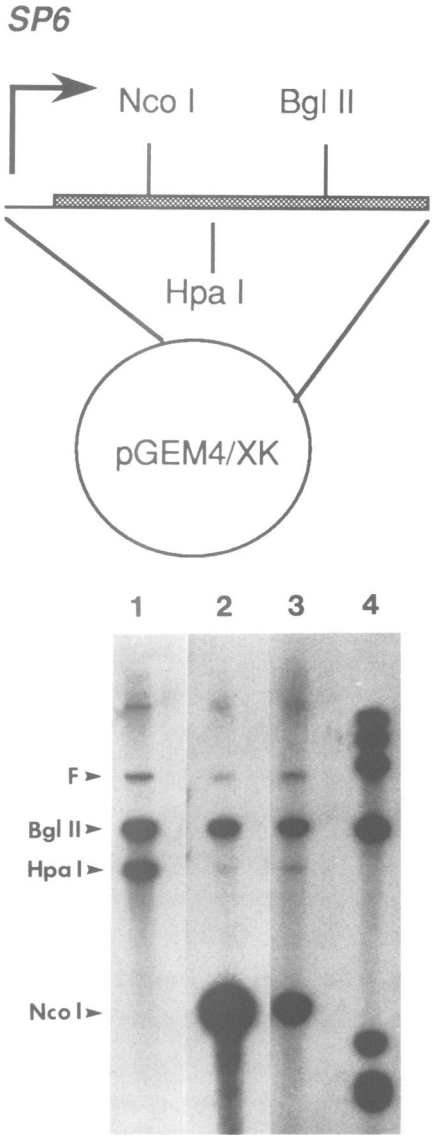

Fig. 3. SP6 RNA polymerase transcribes through a nucleosome. The plasmid pGEM4/XK (see Materials and methods), assembled into nucleosomes and treated with *Bgl*II, was mixed with an equal amount of naked pGEM4/XK which had been linearized with *Hpa*I. The mixture was then transcribed with SP6 RNA polymerase for 30 min at 31°C after incubating without (**lane 1**) or with (**lanes 2** and **3**) *Nco*I. The runoff transcripts are indicated according to the enzyme cut which gives rise to each, and F indicates a primary transcript due to the uncut plasmid. **Lanes 1** and **2**, 100 ng DNA (total) plus 19 U SP6 RNA polymerase; **lane 3**, 2 ng DNA plus 76 U SP6 RNA polymerase. **Lanes 1–3** were roughly normalized for the *Bgl*II runoff signal by varying the exposure time of the autoradiogram. **Lane 4** is a marker lane containing a *Hae*III digest of ΦX174 DNA.

in the XK endo B sequence of pGEM4/XK; the longer transcript must be due to SP6 transcribing through this site. To show more conclusively that SP6 RNA polymerase is capable of elongating through a nucleosome, as has been reported (Lorch *et al.*, 1987; Losa and Brown, 1987), this same mixture was treated with *Nco*I and subsequently transcribed. The prediction was that the *Hpa*I runoff transcript, arising from the naked plasmid, would be eliminated in favor of the shorter *Nco*I runoff transcript; at the same time, some of the reconstituted plasmid should be resistant to *Nco*I digestion, by virtue of having a nucleosome

at the *Nco*I site, and if SP6 RNA polymerase were capable of elongating through a nucleosome, some of the *Bgl*II runoff transcript should still be observed.

The data in Figure 3 show that this prediction is confirmed. Following treatment of the mixture with *Nco*I, a new runoff transcript of 335 nt is seen in lanes 2 and 3, and the *Hpa*I runoff transcript due to the naked plasmid in lane 1 is virtually completely eliminated, as expected. The original *Bgl*II runoff transcript due to the assembled plasmid, in contrast, is still readily apparent in both lanes 2 and 3. The difference between these two lanes is the ratio of SP6 polymerase to template used. In agreement with Losa and Brown, it can be seen that transcription of the nucleosomal template relative to transcription of naked DNA is considerably more efficient at a ratio of polymerase to DNA template of ~35 units per nanogram (lane 3) than at 0.4 units per nanogram (lane 2). Moreover, some full-length transcript is still visible, which means that SP6 RNA polymerase is able to transcribe through two nucleosomes, one at the *Bgl*II site and one at the *Nco*I site, on a closed circular template.

RNA polymerase III

SP6 RNA polymerase is a prokaryotic enzyme and therefore normally functions in an environment entirely devoid of nucleosomes. To explore the effect of nucleosomes on transcription by a eukaryotic polymerase, the 5S RNA gene from the frog *X.borealis* provides a useful template. This gene can be transcribed efficiently *in vitro* (Birkenmeier *et al.*, 1978; Wolffe *et al.*, 1986), its requirements for transcription factors are well understood, and the sequences at the 5′ end of the gene along with the upstream sequences are known to strongly position a nucleosome (Rhodes, 1985; Shimamura *et al.*, 1988). This latter feature, although not taken advantage of in the present study, seems likely to be useful in more detailed studies of the effect of nucleosome position on transcription of this gene.

The 5S RNA gene is diagrammed in Figure 4. Transcription of this gene by RNA polymerase III requires binding of the transcription factor TFIIIA to the internal control region extending from positions +50 to +97 relative to the transcription start site. Binding of TFIIIA is the first step in the formation of an active transcription complex, which also includes factors TFIIIB and TFIIIC (reviewed by Wolffe and Brown, 1988).

Effect of nucleosomes on or close to the internal control region

I first examined the effect that nucleosomes which protected either the *Eco*RV site at +33 or the *Sca*I site at +76 had on transcription of the 5S RNA gene. The closed circular plasmid pXbs201 was assembled into nucleosomes by salt dialysis, using purified core histones from chicken erythrocytes. By varying the ratio of histones to DNA in the reconstitution, an average of 11−21 nucleosomes were assembled per 4.3 kb plasmid, as determined by measuring the average decrease in linking number relative to relaxed, naked plasmid DNA (Germond *et al.*, 1975; Keller *et al.*, 1977; Simpson *et al.*, 1985). When the reconstituted material was treated with restriction enzymes which recognize a single site on the plasmid, the amount of linearization was inversely related to the average number of nucleosomes present, as expected (Figure 4, middle panel, and Table I).

Transcription of a plasmid assembled with an average

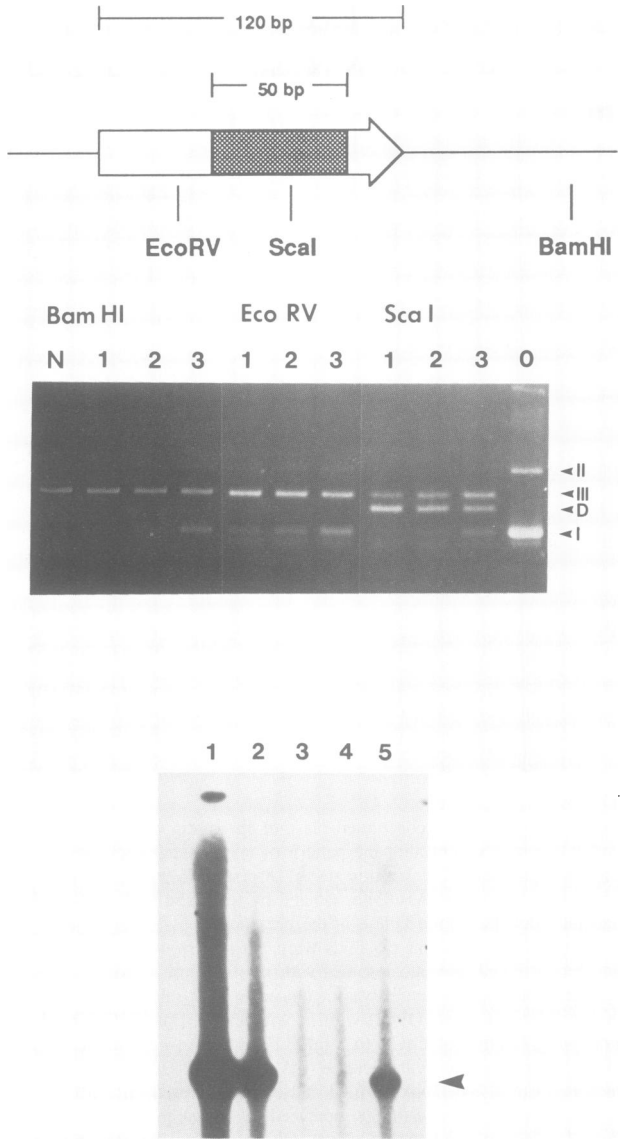

Fig. 4. Nucleosomes at the *Sca*I or *Eco*RV site of the 5S RNA gene block transcription. **Top.** The open arrow represents the 120 bp 5S RNA gene transcribed by RNA polymerase III. The shaded box represents the internal control region, and the solid lines indicate the sequences 5′ and 3′ to the gene and plasmid sequences beyond in pXbs201. The *Eco*RV and *Bam*HI sites are each present only once in this plasmid; *Sca*I has one other site in the plasmid, not shown. **Middle.** Agarose gel electrophoresis of pXbs201 assembled into an average of 11 (reconstitution 1), 14 (reconstitution 2) or 21 (reconstitution 3) nucleosomes and restricted with *Bam*HI, *Eco*RV or *Sca*I. The lane labeled N is naked DNA, and the lane marked 0 is uncut plasmid. Indicated are: I, supercoiled plasmid; II, relaxed or nicked circles; III, linear plasmid; D, the larger fragment resulting from pXbs201 which has been cut at both *Sca*I sites. The faint band just below supercoiled pXbs201 is an unidentified contaminant. **Bottom.** Reconstitution 2 from the middle panel was transcribed in oocyte nuclear extract in the presence of [α-^{32}P]CTP without further treatment (**lane 2**), or after treatment with *Eco*RV (**lane 3**), *Sca*I (**lane 4**) or *Bam*HI (**lane 5**). The purified RNA was electrophoresed, and the gel autoradiographed. **Lane 1** represents transcription of an approximately equal mass (100 ng) of naked pXbs201. The arrow indicates the 120 nt 5S RNA transcript.

of 14 nucleosomes (reconstitution 2 from Figure 4) gave rise to abundant transcript in an oocyte nuclear extract, as seen in Figure 4, bottom panel, lane 2. However, when transcription was preceded by treatment of the reconstituted

Table I. Quantitation of resistance to endonuclease cutting conferred by nucleosomes reconstituted onto pXbs201

No. of nucleosomes	Percent uncut by[a]:		
	*Bam*HI	*Eco*RV	*Sca*I[b]
11	13 ± 0.5	16 ± 2	18 ± 3
14	23 ± 3	25 ± 4	27 ± 0.5
21	39 ± 1	36 ± 15	43 ± 1

[a]Reconstituted plasmids were treated with restriction endonuclease and electrophoresed on 1% agarose gels. The amounts of supercoiled (uncut) and linearized plasmid DNA were measured by densitometric scanning of photographic negatives taken of the gels after staining with ethidium bromide. Three independent experiments were performed for each value in the table.
[b]There are two *Sca*I sites present in pXbs201. The average resistance at each site was calculated as (percent supercoiled + 1/2 × percent linear).

template with *Eco*RV or *Sca*I, transcription was eliminated (Figure 4, bottom panel). A control experiment in which the reconstituted plasmid was restricted with *Bam*HI shows that the presence of linearized plasmid does not abolish transcription, although there is some inhibitory effect (Figure 4, bottom panel, lane 5), probably due to sequestration of RNA polymerase at linear DNA termini (Berg *et al.*, 1965). Since the 5S RNA gene was left intact in ~25% of the reconstituted plasmids after treatment with *Eco*RV or *Sca*I (Figure 4, middle panel, and Table I), ~25 ng of input plasmid in lanes 3 and 4 of Figure 4, bottom panel, had full-length, transcribable sequences. This amount of naked or reconstituted plasmid gives rise to abundant transcription in the oocyte nuclear extract (data not shown, but see Wolffe *et al.*, 1986). The complete absence of specific 5S transcript therefore implies that nucleosomes which block *Eco*RV or *Sca*I digestion within the 5S RNA gene also inhibit transcription.

Effect of nucleosomes on elongation

Nucleosomes incorporating the *Eco*RV or *Sca*I recognition sequences within the 5S RNA gene most likely inhibit transcription by interfering with formation of the active transcription complex or with transcriptional initiation. To test whether nucleosomes block elongation by RNA polymerase III, 5S maxigenes were constructed as described in Materials and methods. One of these genes, Xbs1185, gives rise to a transcript of 1185 nt when transcribed in the oocyte nuclear extract. The transcribed region of this gene is therefore long enough to accommodate nucleosomes well downstream from the internal control region.

When the plasmid pXbs1185, which bears this maxigene, was assembled into nucleosomes and transcribed in the oocyte nuclear extract, a transcript nearly 1200 nt long was observed (Figure 5B, lane 1). When the reconstituted plasmid was treated with *Bgl*II, *Hpa*I or *Nco*I a proportion of the reconstituted plasmids was resistant to digestion, as expected (Figure 5A). Incubation of the assembled and restriction enzyme-treated plasmid in the oocyte nuclear extract led to the accumulation of runoff transcripts of approximately the predicted lengths (519, 617 or 797 nt after treatment with *Bgl*II, *Hpa*I or *Nco*I, respectively; Figure 5B, lanes 2–4). Control experiments showed that no additional linearization of the plasmid occurred during the transcription reaction (data not shown). If RNA polymerase III were

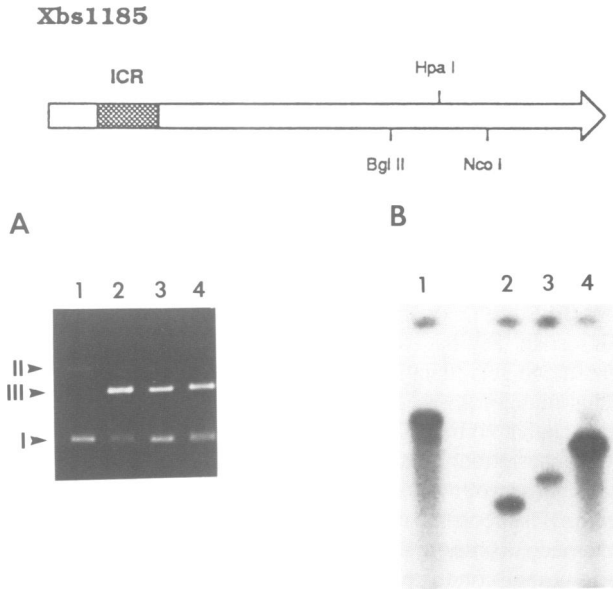

Fig. 5. Nucleosomes block elongation by RNA polymerase III. **Top:** the transcribed unit of pXbs1185. The *Bgl*II, *Hpa*I and *Nco*I sites are respectively 519, 617 and 797 bp downstream from the transcriptional start site. ICR, internal control region. **Panel A:** agarose gel electrophoresis of pXbs1185 assembled into an average of 10–12 nucleosomes and treated with *Bgl*II (**lane 2**), *Hpa*I (**lane 3**) or *Nco*I (**lane 4**). **Lane 1** contains supercoiled pXbs1185. The Roman numerals indicate: I, supercoiled plasmid; II, relaxed or nicked circular plasmid; III, linearized plasmid. **Panel B:** 20 ng of reconstituted pXbs1185 (from panel A) was transcribed in oocyte nuclear extract without restriction endonuclease treatment (**lane 1**) or after treatment with *Bgl*II (**lane 2**), *Hpa*I (**lane 3**) or *Nco*I (**lane 4**). Transcription reactions included 90 ng of pBR322. The sizes of the runoff transcripts in **lanes 2–4** were verified by comparison with single-stranded DNA markers, not shown.

able to transcribe through nucleosomes, transcription in the oocyte nuclear extract of material treated with restriction endonucleases should have yielded full-length transcripts from the uncut plasmids in addition to the runoff transcripts observed, similar to what was seen for reconstituted, restricted pGEM4/XK transcribed with SP6 RNA polymerase (Figure 3). However, no such full-length transcripts were observed (Figure 5B, lanes 2–4). Similarly, when pXbs520 (which gives rise to a 520 nt transcript; see Materials and methods) was assembled into nucleosomes and treated with *Eco*RI, which cuts only 50 bp 5′ from the native 5S termination signal, the full-length transcript was again virtually eliminated (data not shown).

These results strongly suggest that nucleosomes inhibit elongation by RNA polymerase III. This raises a question as to the origin of the full-length transcript of Figure 5B, lane 1. That is, if nucleosomes on or close to the 5S DNA promoter, as well as nucleosomes further downstream, inhibit transcription, how can a nucleosomal template give rise to any full-length transcript at all? It would seem that for some fraction of the reconstituted plasmids, the entire 1185 bp gene sequence must be free of nucleosomes. Is this reasonable? Random placement of nucleosomes onto a plasmid should lead to their being distributed according to Poisson statistics. Such a distribution, at the level of reconstitution employed in Figure 5, leads to 2–5% of the gene sequence being unoccupied. This amount of free template would be expected to produce a detectable amount

of transcript in the oocyte nuclear extract. It is also possible that a small proportion of the reconstituted plasmids have very few nucleosomes, or even none at all. In either case, of course, treatment with a restriction endonuclease that cuts within the gene would cause such molecules to give rise to only runoff transcripts, or no transcripts at all, depending on the restriction site.

Another aspect of the experiment of Figure 5 that merits consideration is the apparent absence of prematurely terminated transcripts from the reconstituted template. Such transcripts are not visible on low percentage polyacrylamide gels, such as that of Figure 5, nor on higher (10%) percentage gels (data not shown). One possible explanation is that nucleosomes adopt enough different positions along the gene that no individual stop or pause site stands out above the transcriptional background. Another possibility is that nucleosome assembly is cooperative, so that those molecules having a nucleosome at the downstream restriction sites also have nucleosomes in the vicinity of the TFIIIA binding site, thereby inhibiting transcriptional initiation. A prediction of the hypothesis that RNA polymerase III is able to initiate on a proportion of these reconstituted templates, and then terminates at many different sites within the transcribed region depending on where it encounters a nucleosome, is that there ought to be a molar excess of 5′ over 3′ sequences in the RNA produced from transcription of the reconstituted maxigene. This prediction was tested in two separate experiments.

In the first experiment, pXbs1185 was restricted to give fragments (among others) corresponding to the 5′ and 3′ ends of the 5S maxigene. This DNA was electrophoresed and blotted to a nylon filter. The filter was then successively hybridized, under conditions of DNA excess, with two radiolabeled RNA probes, both synthesized in *Xenopus* oocyte nuclear extract. The first probe was transcribed from naked pXbs1185; the second was transcribed from the same template assembled into an average of eight nucleosomes per 3.6 kb plasmid. Hybridization with the probe synthesized from naked RNA resulted in a stronger signal from the 3′ than from the 5′ fragment (Figure 6), reflecting target size and, presumably, the relative efficiency of the DNA fragments binding to the filter. When the same filter was washed free of probe and rehybridized with the probe from the nucleosomal template, a much stronger signal was seen from the 5′ relative to the 3′ fragment than when the probe was derived from naked DNA (Figure 6). Similar results were obtained when the order of hybridization was reversed. These results suggest that a molar excess of 5′ over 3′ ends were synthesized from the nucleosomal template, in accordance with the idea that RNA polymerase III, being unable to transcribe through a nucleosome, should produce prematurely terminated transcripts from a nucleosomal template.

In the second experiment, RNA was again made from both naked pXbs1185 and the same template assembled into an average of eight nucleosomes. Half of each reaction was allowed to proceed without addition of radioactive nucleotide, and half was labeled as usual. Figure 7A shows the transcription products of the labeled reactions; clearly, much more full-length product was synthesized from the naked than from the nucleosomal template. To measure the relative amounts of shorter transcripts in the two reactions, an aliquot of unlabeled RNA from each reaction

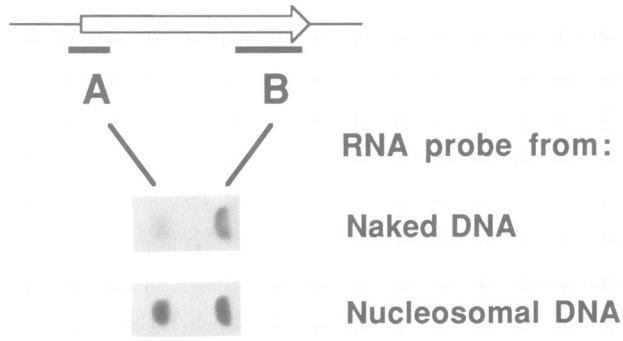

Fig. 6. Prematurely terminated transcripts from nucleosome-assembled pXbs1185 detected by hybridization analysis. The plasmid pXbs1185 was digested with *Eco*RI, *Hin*dIII, *Nco*I and *Nsi*I to give a 5′ fragment extending from −50 to +150 and a 3′ fragment from +797 to +1135 relative to the transcription start site, with other fragments being <120 bp or >600 bp in length. These fragments were electrophoresed on 1.8% agarose, and the gel stained with ethidium bromide to identify the 5′ and 3′ fragments. This part of the gel was blotted to a nylon membrane and successively hybridized with ^{32}P-labeled RNA probes derived from transcription of naked pXbs1185 and the same template assembled into nucleosomes, as indicated.

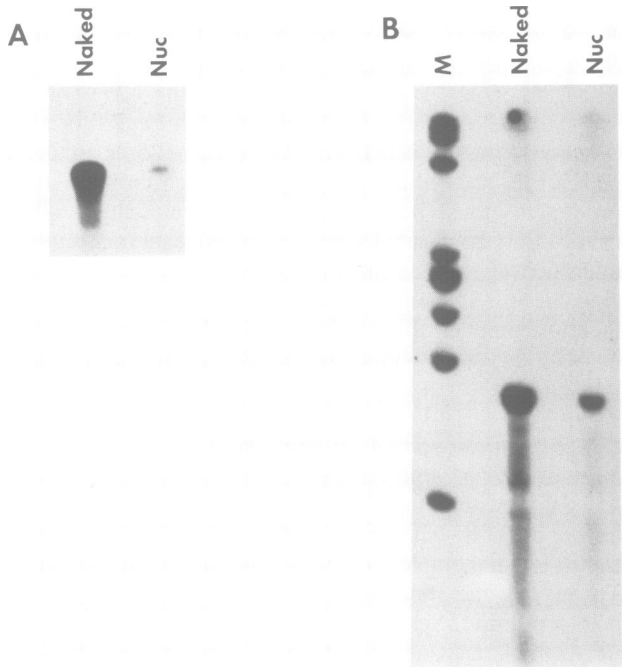

Fig. 7. Prematurely terminated transcripts from nucleosome-assembled pXbs1185 detected by primer extension. RNA was transcribed from 150 ng of naked pXbs1185 or the same mass of plasmid assembled into nucleosomes, with 7.5 μl of the oocyte nuclear extract. After the initial 30 min incubation (see Materials and methods), the reactions were divided in half and 15 μCi of labeled nucleotide added to half, while the remainder was allowed to continue transcription in the absence of label. **Panel A:** analysis of the labeled, full-length transcripts by autoradiography following electrophoresis on a 6% polyacrylamide−7 M urea gel. **Panel B.** equal fractions of RNA from the unlabeled reactions were hybridized with a labeled 16 nt primer complementary to nucleotides 161−176 of the pXbs1185 transcript, and extended with AMV reverse transcriptase. The extended products were electrophoresed on an 8% polyacrylamide−7 M urea gel and autoradiographed. **Lane M** contains labeled fragments from a *Hae*III digest of ΦX174 DNA.

was analyzed by primer extension. The primer used was complementary to a region 161−176 nt from the transcriptional start site, so that any transcripts longer than this would

be measured. The results, seen in Figure 7B, show an increased ratio of RNA products derived from nucleosomal versus naked DNA, when compared with the ratio of full-length products seen in Figure 7A. Again, this indicates the presence of prematurely terminated transcripts deriving from the nucleosomal template.

Discussion

The results presented in this paper show that nucleosome core particles which include sequences within or close to the internal control region of a 5S RNA gene from *Xenopus* completely inhibit transcription by RNA polymerase III. Moreover, RNA polymerase III, in contrast to SP6 RNA polymerase, will not progress through nucleosomes downstream of the promoter region. This interpretation of the results rests on the assumption that the protection against restriction of reconstituted templates must be due directly to DNA–histone interactions, and not on some gross morphological property such as aggregation or overall compaction of the reconstituted templates. At the relatively low levels of reconstitution employed in these studies, and without histone H1, one would not expect such effects to be important; but additionally, several observations suggest that direct histone–DNA interactions at the sites of interest are responsible for the protection against digestion by restriction endonucleases. (i) The protection against restriction for a variety of enzymes is proportional to the average number of nucleosomes reconstituted onto each plasmid molecule (Figure 4A and Table I). (ii) If a reconstituted plasmid is treated with two restriction enzymes, each of which cuts the plasmid at a single site, the distribution of uncut, singly cut and doubly cut molecules is precisely as one would expect if nucleosomes were deposited randomly and all the plasmid molecules (but not all the restriction sites) were accessible by the enzyme (unpublished data). (iii) When reconstituted and restricted plasmid molecules are centrifuged prior to being transcribed, in order to remove any aggregated or precipitated material, uncut plasmids are still observed and still fail to be transcribed (the experiment of Figure 5A and B was done in just this way). Since it is known that histone–DNA interactions can prevent restriction endonuclease digestion (see for instance Lorch *et al.*, 1987; Stein, 1987), the assumption that reconstituted plasmids uncut by a given restriction endonuclease have the recognition sequence for that enzyme sequestered within a nucleosome seems justified.

The finding that nucleosomes incorporating the *Sca*I or *Eco*RV sites of the 5S RNA gene inhibit its transcription by RNA polymerase III is consistent with earlier results indicating that nucleosome cores are capable of inhibiting 5S RNA transcription (Gottesfeld and Bloomer, 1982; Weisbrod *et al.*, 1982; Lassar *et al.*, 1985). In contrast, nucleosome cores do not repress transcription of 5S genes isolated in the form of chromatin from tissue culture cells (Schlissel and Brown, 1984). The work reported here, as well as a recent study by Shimamura *et al.* (1988) suggests that these contrasting observations are probably due to differences in the precise position of the nucleosome cores in the various studies. Establishing the effect on 5S RNA transcription of varying precise nucleosome position with respect to gene sequences should help to clarify the reasons for these differing observations, and may also help to delineate the mechanism for the selective repression of *Xenopus* oocyte 5S genes *in vivo* (Wolffe and Brown, 1988; Wolffe, 1989).

The inhibition of transcription due to nucleosomes at the *Sca*I or *Eco*RV sites could be due to interference with TFIIIA binding, with subsequent binding by TFIIIC or TFIIIB, or with transcriptional initiation. Rhodes (1985) has reported that a nucleosome and TFIIIA can simultaneously bind to the internal control region. On the other hand, Shimamura *et al.* (1988) reconstituted 5S DNA into nucleosomes in an oocyte S150 extract, obtained the same positioned nucleosome, and found the resulting templates to be refractory to transcription. I do not yet know whether the nucleosomes that protect the *Sca*I or *Eco*RV sites and also block transcription occupy the same position as these other workers have found (see also Simpson and Stafford, 1983). Reconstituting nucleosomes in the presence of the transcription complex and footprinting the resulting assembly should allow determination of the mechanism by which inhibition of transcription takes place.

RNA polymerase III, in being inhibited by nucleosomes assembled on or close to the promoter, is similar to SP6 RNA polymerase (Lorch *et al.*, 1987) and RNA polymerase II (Knezetic and Luse, 1986; Lorch *et al.*, 1987; Matsui, 1987; Workman and Roeder, 1987). In contrast, the finding that nucleosomes block elongation by RNA polymerase III is opposite to results obtained with both SP6 and RNA polymerase II (Lorch *et al.*, 1987; Losa and Brown, 1987). This difference is unlikely to be due to a difference in the nature of the templates used (short linear versus closed circular) in the light of the results of Figure 3. Losa and Brown report a reduced efficiency of transcription of nucleosomal templates relative to naked templates at low levels of SP6 RNA polymerase. A similar effect is apparent in the experiment with SP6 RNA polymerase reported here (Figure 3, lanes 2 and 3). This leads one to wonder whether levels of RNA polymerase III higher than those present in the oocyte nuclear extracts used here would allow transcription through nucleosomes. The oocyte nuclear extracts used in this work yielded 5–10 transcripts/gene/h under non-rate-enhanced conditions (Wolffe *et al.*, 1986), which is 2- to 4-fold lower than generally reported for such extracts, and ~100-fold lower than transcription rates for the 5S RNA gene thought to occur *in vivo* (Korn and Gurdon, 1981). Moreover, the presence of linear template reduced rates by a factor of 5–10. Thus, it is conceivable that at higher activities of RNA polymerase III, transcription might proceed through a nucleosome.

The inability of RNA polymerase III to transcribe through a nucleosome may, however, reflect a genuine difference in its properties from SP6 RNA polymerase or RNA polymerase II. Perhaps RNA polymerase III lacks whatever feature allows SP6 RNA polymerase and RNA polymerase II to disrupt protein–DNA contacts in the nucleosome, or perhaps it is more sensitive to the structural changes accompanying DNA incorporation into nucleosomes (Morse and Simpson, 1988), and reads such changes as signals for termination. Obviously, more work will be required to determine the mechanism by which nucleosomes cause transcriptional termination by RNA polymerase III.

The genes transcribed by RNA polymerase III *in vivo* are all short genes, mostly <200 bp in length, and most have internal promoters (Geiduschek and Tocchini-Valentini,

1988). These genes may therefore be free of histones *in vivo*, and so may present no impediment to RNA polymerase III. The apparent exceptions to this categorization of class III genes, 7SK and U6 RNA, have promoters in some ways characteristic of class II genes (Murphy *et al.*, 1987; Das *et al.*, 1988). Perhaps these genes are transcribed by a modified form of RNA polymerase III which is able both to recognize a TATA-like sequence and to traverse a nucleosome. It is also possible that, although the internal portions of these genes can be dispensed with *in vitro*, there may be non-histone proteins bound *in vivo* which exclude nucleosome formation.

Electron microscopy and cross-linking experiments suggest that class I and II genes differ *in vivo*, with the former being free of nucleosomes when actively transcribed (reviewed in Amero *et al.*, 1988), whereas the latter appear to retain a regular nucleosome structure even during transcription (DeBernardin *et al.*, 1986). Class III genes may differ from either of these: an individual class III gene may be either free of nucleosomes and active, or else constitutively repressed by incorporation into chromatin (Schlissel and Brown, 1984; Wolffe, 1989). Placement of a 5S maxigene into cells should address the question as to whether any mechanism exists *in vivo* for transcribing a class III gene which is partially incorporated into nucleosomes.

Finally, it should be pointed out that although the protocol shown in Figure 1 was used here to study transcription by RNA polymerase III, it should be useful in studying the effect of nucleosomes at various positions along genes transcribed by RNA polymerases I and II as well.

Materials and methods

Plasmids

Plasmid pGEM4/XK was made by ligating the 2 kb *Eco*RI fragment of XK endo B cDNA (LaFlamme *et al.*, 1988) into the *Eco*RI site of pGEM4 (Promega), in the anti-sense orientation with respect to the SP6 promoter. This plasmid was a gift of Dr Susan LaFlamme.

The 5S maxigenes used in this study were derived from the plasmid pXbs115/77', a gift of Dr Donald Brown. This plasmid was constructed by filling in the *Hin*dIII sites of the genic *Hin*dIII fragment from pXbs115/77 (Bogenhagen and Brown, 1981), adding *Eco*RI linkers and ligating the resulting fragment into the *Eco*RI site of pARA (Hartley and Gregori, 1981). Maxigene constructions began from this plasmid by ligating a 142 bp *Bam*HI fragment from pUC19 into the intragenic *Bam*HI site of partially digested pXbs115/77'. The 142 bp *Bam*HI fragment was made by purifying the 142 bp *Ban*I fragment from pUC19, ligating and re-cutting with *Bam*HI. The orientation in the resulting plasmid, called pXbs310, was such that the *Sma*I site from pUC19 was 5 bp from the 3' end of the inserted fragment.

Construction of the plasmid pXbs1185 then began with pXbs310 and the plasmid Bluescript/XK, which consists of the *Pst*I–*Eco*RI fragment of XK endo B cDNA cloned into Bluescript (Stratagene). The XK endo B fragment was excised with *Pvu*II and *Eco*RV, and the resulting 875 bp fragment ligated into the unique *Sma*I site of pXbs310; the construction having the XK endo B fragment in the sense orientation relative to the 5S gene has no natural internal termination sites for RNA polymerase III. Finally, the pBR322 sequences from the *Ssp*I to *Nru*I sites (4170–972 on the pBR322 map) and from the *Bal*I to *Nde*I sites (1444–2297) were removed, and the resulting plasmid was named pXbs1185. Plasmid pXbs520 was made by deleting the *Sal*I–*Nco*I fragment from pXbs1185, filling in the ends with Klenow and religating.

Nucleosome reconstitution

Chicken erythrocyte core histones were isolated from fresh red blood cells (Poolesville Animal Farm) (Stein and Bina, 1984) and found to be free of histones H1 and H5 by SDS–PAGE. Nucleosomes were reconstituted by mixing core histones with supercoiled plasmid DNA at wt:wt ratios of 0.5–0.9 in 1.5 M NaCl, 10 mM Tris, pH 8.0, 0.05 mM EDTA at 0.1 μg DNA/μl, and dialyzing at 20°C against 0.8 M NaCl, 10 mM Tris, pH 8.0, 0.05 mM EDTA for 2 h, 0.17 M NaCl, 10 mM Tris, pH 8.0, 0.05 mM EDTA for 2 h and 10 mM Tris, pH 8.0, 0.05 mM EDTA for 2 h. The average number of nucleosomes per plasmid was determined by relaxing with nicking–closing extract (Bina-Stein *et al.*, 1976) and measuring the decrease in linking number relative to relaxed, naked plasmid (Simpson *et al.*, 1985). Samples were stored at 4°C and used for up to 2 weeks.

Transcription

For all experiments involving restriction endonuclease treatment of reconstituted plasmids, parallel samples were divided following dilution into restriction buffer (Maniatis *et al.*, 1982) at 10–50 ng DNA/μl and incubated at 37°C for 1 h with or without restriction enzyme. Aliquots of 1–2 μl were then used in transcription reactions and SDS added to 0.2% to the remainder prior to analysis by agarose gel electrophoresis.

Transcription reactions with SP6 RNA polymerase (Promega) were performed in 20 μl reactions containing 40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 0.5 mM each of ATP, CTP, GTP and UTP, 10 μCi [α-^{32}P]CTP (3000 Ci/mmol, Amersham) and 10 U RNasin at 31°C for 30 min. *Xenopus* oocyte nuclear extract was prepared and used in transcription reactions essentially as described (Birkenmeier *et al.*, 1978) except that 0.3 mM each of ATP, GTP and UTP, either 75 or 8 μM CTP, and 10 U of RNasin were included. After 30 min incubation at 20°C, 5–10 μCi [α-^{32}P]CTP (3000 Ci/mmol, Amersham) in J buffer (Birkenmeier *et al.*, 1978) was added and the reaction mixture incubated at 20°C for an additional 30 min. All transcription reactions were terminated by addition of 180 μl of 1% SDS, 10 mM Tris, pH 8.0, extracted with phenol and $CHCl_3$ and ethanol precipitated. RNA was dissolved in 15 μl 90% formamide, denatured at 90°C for 3 min and electrophoresed on 5 or 10% polyacrylamide–7 M urea gels in 90 mM Tris–borate, 90 mM boric acid, 2 mM EDTA, pH 7.9, at 200–250 V for 3–4 h and the gels analyzed by autoradiography.

Hybridization and primer extension analysis

For the experiment of Figure 6, DNA was blotted to nylon membrane (Zetaprobe) by capillary action (Maniatis *et al.*, 1982), hybridized overnight at 65°C with [α-^{32}P]labeled RNA probes and washed according to Church and Gilbert (1984) prior to autoradiography. The labeled probe was removed from the filter by washing in 0.5 M NaOH, 1 mM EDTA for 30 min, in 1% SDS, 2 mM Tris–Cl, pH 8.0, for 1–2 h and in 10 mM NaP_i, pH 7.2, for >10 min, all at 20°C. The filter was then rehybridized directly.

For the primer extension analysis of Figure 7, unlabeled RNA was hybridized with a 20–100 times molar excess of a labeled 16 nt primer (no. 1201 reverse sequencing primer, New England Biolabs) complementary to nucleotides 161–176 of the pXbs1185 transcript by incubation at 37°C for 30 min following denaturation at 92°C for 5 min. The primer was then extended with AMV reverse transcriptase (BioRad) at 37°C for 30 min according to the manufacturer's specifications. The products were purified and analyzed identically to RNA from transcription reactions.

Acknowledgements

I would like to thank Dr Igor Dawid for donating frogs and materials; Drs Susan LaFlamme and Alan Wolffe for materials, invaluable help and stimulating discussions; and Dr Donald Brown and Dr Gary Felsenfeld for critical readings of the manuscript. I am especially grateful to Dr Robert Simpson, in whose laboratory this work was done, for generous encouragement, advice and support. I am also indebted to the members of the Laboratory of Cellular and Developmental Biology for their critical input, and to the National Research Council for support in the form of a National Research Council–NIH fellowship.

References

Amero,S.A., Montoya,V.L., Murdoch,W.L., Ogle,R.C., Keating,J.L. and Grainger,R.M. (1988) *J. Biol.Chem.*, **263**, 10734–10744.

Benyajati,C. and Worcel,A. (1976) *Cell*, **9**, 393–407.

Berg,P., Kornberg,R.D., Fancher,H. and Dieckmann,M. (1965) *Biochem. Biophys. Res. Commun.*, **18**, 932–942.

Bina-Stein,M., Vogel,T., Singer,D.S. and Singer,M.F. (1976) *J. Biol. Chem.*, **251**, 7363–7366.

Birkenmeier,E.H., Brown,D.D. and Jordan,E. (1978) *Cell*, **15**, 1077–1086.

Bogenhagen,D.F. and Brown,D.D. (1981) *Cell*, **24**, 261–270.

Church,G. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.

Das,G., Henning,D., Wright,D. and Reddy,R. (1988) *EMBO J.*, **7**, 503–512.

DeBernardin,W., Koller,T. and Sogo,J.M. (1986) *J. Mol. Biol.*, **191**, 469–482.
Geiduschek,E.P. and Tocchini-Valentini,G.P. (1988) *Annu. Rev. Biochem.*, **57**, 873–914.
Germond,J.E., Hirt,B., Oudet,P., Gross-Bellard,M. and Chambon,P. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 1843–1847.
Gottesfeld,J. and Bloomer,L.S. (1982) *Cell*, **28**, 781–791.
Hartley,J.L. and Gregori,T.J. (1981) *Gene*, **13**, 347–353.
Keller,W., Muller,U., Eicken,I., Wendel,I. and Zentgraf,H. (1977) *Cold Spring Harbor Symp. Quant. Biol.*, **42**, 227–243.
Knezetic,J.A. and Luse,D.S. (1986) *Cell*, **45**, 95–104.
Korn,L.J. and Gurdon,J.B. (1981) *Nature*, **289**, 461–465.
LaFlamme,S.E., Jamrich,M., Richter,K., Sargent,T.D. and Dawid,I.B. (1988) *Genes Dev.*, **2**, 853–862.
Lassar,A.B., Hamer,D.H. and Roeder,R.G. (1985) *Mol. Cell. Biol.*, **5**, 40–45.
Liu,L.F. and Wang,J.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7024–7027.
Lorch,Y., LaPointe,J.W. and Kornberg,R.D. (1987) *Cell*, **49**, 203–210.
Losa,R. and Brown,D.D. (1987) *Cell*, **50**, 801–808.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Matsui,T. (1987) *Mol. Cell. Biol.*, **7**, 1401–1408.
Meneguzzi,G., Chenciner,N. and Milanesi,G. (1979) *Nucleic Acids Res.*, **6**, 2947–2960.
Morse,R.H. and Cantor,C.R. (1986) *Nucleic Acids Res.*, **14**, 3293–3310.
Morse,R.H. and Simpson,R.T. (1988) *Cell*, **54**, 285–287.
Murphy,S., DiLiegro,C. and Melli,M. (1987) *Cell*, **51**, 81–87.
Poljak,L.G. and Gralla,J.D. (1987) *Biochemistry*, **26**, 295–303.
Rhodes,D. (1985) *EMBO J.*, **4**, 3473–3482.
Schlissel,M.S. and Brown,D.D. (1984) *Cell*, **37**, 903–913.
Shimamura,A., Tremethick,D. and Worcel,A. (1988) *Mol. Cell. Biol.*, **8**, 4257–4269.
Simpson,R.T. and Stafford,D.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 51–55.
Simpson,R.T., Thoma,F. and Brubaker,J.M. (1985) *Cell*, **42**, 799–808.
Stein,A. (1987) *J. Biol. Chem.*, **262**, 3872–3879.
Stein,A. and Bina,M. (1984) *J. Mol. Biol.*, **178**, 341–363.
Wasylyk,B. and Chambon,P. (1979) *Eur. J. Biochem.*, **98**, 317–327.
Wasylyk,B., Thevenin,G., Oudet,P. and Chambon,P. (1979) *J. Mol. Biol.*, **128**, 411–440.
Weisbrod,S., Wickens,M.P., Whytock,S. and Gurdon,J.B. (1982) *Dev. Biol.*, **94**, 216–229.
Williamson,P. and Felsenfeld,G. (1978) *Biochemistry*, **17**, 5695–5705.
Wolffe,A.P. (1989) *EMBO J.*, **8**, 527–537.
Wolffe,A.P. and Brown,D.D. (1988) *Science*, **241**, 1626–1632.
Wolffe,A.P., Jordan,E. and Brown,D.D. (1986) *Cell*, **44**, 381–389.
Workman,J.L. and Roeder,R.G. (1987) *Cell*, **51**, 613–622.
Zimmerman,S.B. and Levin,C.J. (1975) *Biochem. Biophys. Res. Commun.*, **62**, 357–361.

Received on April 10, 1989