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A nucleosome-dependent static loop potentiates estrogen-regulated transcription from the *Xenopus* vitellogenin B1 promoter *in vitro*

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We describe the transcriptional potentiation in estrogen responsive transcription extracts of the *Xenopus* vitellogenin B1 gene promoter through the formation of a positioned nucleosome. Nuclease digestion and hydroxyl radical cleavage indicate that strong, DNA sequence-directed positioning of a nucleosome occurs between −300 and −140 relative to the start site of transcription. Deletion of this DNA sequence abolishes the potentiation of transcription due to nucleosome assembly. The wrapping of DNA around the histone core of the nucleosome positioned between −300 and −140 creates a static loop in which distal estrogen receptor binding sites are brought close to proximal promoter elements. This might facilitate interactions between the *trans*-acting factors themselves and/or RNA polymerase. Such a nucleosome provides an example of how chromatin structure might have a positive effect on the transcription process.
Key words: enhancer/estrogen/NF1/nucleosome/receptor/transcription

Introduction

Specific chromatin structures play a role in the transcriptional regulation of several eukaryotic genes *in vivo* (Simpson, 1991; Felsenfeld, 1992). The association of the promoter elements of inducible genes with both transcription factors and histones changes dependent on whether transcription is activated or repressed (Fasher *et al.*, 1990; Reik *et al.*, 1991; Straka and Horz, 1991). Many experiments have attempted to reconstruct the interaction of transcription factors with chromatin templates *in vitro* (Perlmann and Wrange, 1988; Pina *et al.*, 1990; Archer *et al.*, 1991; Hayes and Wolffe, 1992a). Nucleosome formation has been found either to allow or to inhibit transcription factor binding dependent on both the type of transcription factor and the precise position of key DNA sequences in chromatin. These results suggest that the regulated transcription of a gene depends on transcription factors functioning correctly in a chromatin environment (Wolffe, 1991).

Chromatin structure has been found to have an apparently passive role in transcriptional regulation: normally nucleosomes compact DNA and prevent the basal transcriptional machinery gaining access to a template. However, when an inducible transcription factor binds to DNA between nucleosomes or within a nucleosome, a chain of events is initiated that eventually allows the basal transcriptional machinery access to DNA. In contrast to this general inhibitory role for nucleosomes, there are no defined examples in which their specific positioning actually facilitates transcription initiation. This is in constrast to the many examples in prokaryotic systems where proteins known to compact DNA facilitate processes such as transcription, replication and recombination (Schmid, 1990). However, Elgin (1988) has suggested a model for the facilitation of transcription by nucleosome formation. The constraint of the DNA sequence comprising the *Drosophila* hsp26 promoter into a nucleosome generates a static loop which brings widely separated transcription factor binding sites into juxtaposition. This clustering of factors may increase transcriptional efficiency through unknown mechanisms. Although such a nucleosome is positioned on the hsp26 promoter *in vivo*, any positive effect on transcription remains to be determined (Thomas and Elgin, 1988).

The vitellogenin genes of *Xenopus* and the chicken provide an attractive model system for investigating the molecular mechanisms of inducible gene expression by the hormone estrogen (Wahli, 1988). *In vitro* and *in vivo* experiments have suggested roles for several *trans*-acting factors, including the estrogen receptor (Theulaz *et al.*, 1988; Corthésy *et al.*, 1990a), a nuclear factor 1-like activity (Chang and Shapiro, 1990; Corthésy *et al.*, 1989, 1991), a leucine-zipper protein VBP (Lyer *et al.*, 1991), a USF-like activity (Seal *et al.*, 1991), a liver-specific repressor (Corthésy *et al.*, 1989) and the basal transcriptional machinery (Philipsen *et al.*, 1988). Alterations in chromatin structure over the promoter elements of the vitellogenin genes following the estrogen dependent induction of transcription have also been described (Burch and Weintraub, 1983; Burch and Evans, 1986; Burch and Fischer, 1990). Surprisingly, *in vitro* experiments have suggested that the concomitant assembly of chromatin during transcription complex formation potentiates transcription from the *Xenopus* vitellogenin B1 gene promoter (Corthésy *et al.*, 1990b). How nucleosome assembly facilitates the transcription process was not determined.

In this work we present evidence of a role for nucleosome formation in potentiating estrogen regulated gene expression from the vitellogenin B1 gene promoter *in vitro*. We suggest that the formation of a static loop dependent on a positioned nucleosome (Elgin, 1988) potentiates transcription activation by the estrogen receptor.

Results

Transcriptional potentiation of estrogen responsive transcription through chromatin assembly

We assembled linear plasmids containing either the vitellogenin B1 promoter or control herpes simplex virus thymidine kinase (HSV tk) or simian virus 40 (SV40) early promoters with purified core histones, such that different levels of nucleosome reconstitution might be predicted to

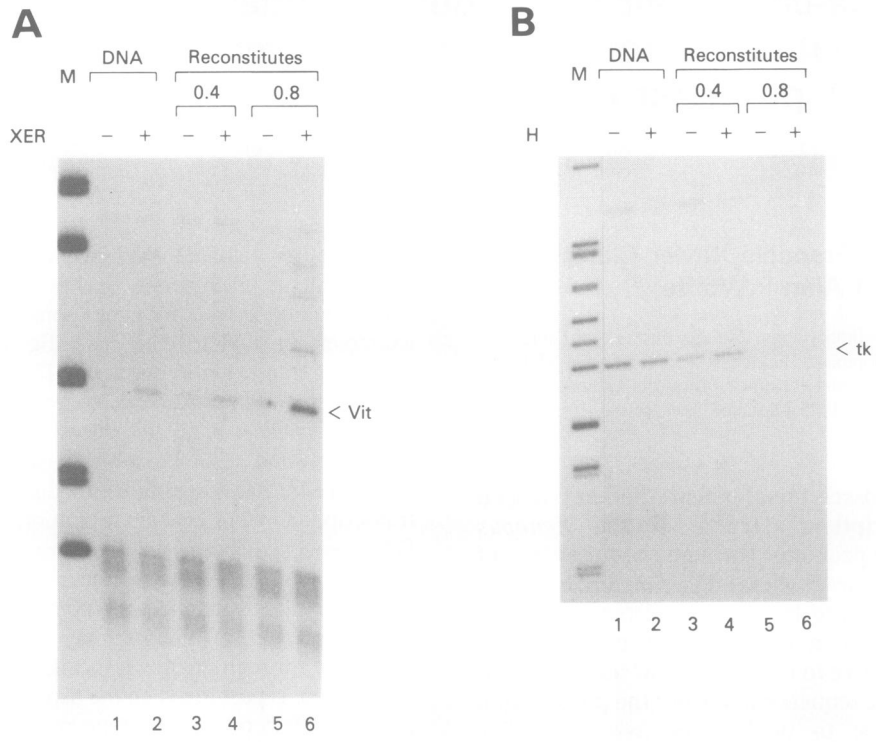

Fig. 1. Effect of nucleosome reconstitution on estrogen receptor dependent transcription in *X.laevis* liver nuclear extract. (**A**) Transcription from the vitellogenin B1 promoter is potentiated upon nucleosome reconstitution in the presence of estrogen receptor. 0.2 μg of linearized pB1 (−596/+8) CAT8+ alone (lanes 1 and 2) or reconstituted [low level (0.4), lanes 3 and 4; moderate level (0.8), lanes 5 and 6] were transcribed in the liver nuclear extract supplemented with 2.5 nM estradiol and 10 μg total protein of either wild-type HeLa cell extract (WR, lanes 1, 3 and 5) or *Xenopus* estrogen receptor containing HeLa cell extracts (XER, lanes 2, 4 and 6, see Materials and methods). Transcripts were analyzed by primer extension. (**B**) Transcription from the HSV tk promoter is repressed by nucleosomes. Linearized plasmid pBLCAT2 alone (lanes 1 and 2) or reconstituted [low level (0.4), lanes 3 and 4; intermediate level (0.8), lanes 5 and 6] were transcribed in the liver nuclear extract in the presence (+, lanes 2, 4 and 6) or absence (−, lanes 1, 3 and 5) of 2.5 nM estradiol (H). (Addition of WR or XER HeLa cell extracts does not affect the level of basal transcription, data not shown.) Transcripts were analyzed by primer extension.

occur on each template. Low (one nucleosome/400 bp, 0.4:1 histone:DNA by mass), moderate (one nucleosome/200 bp, 0.8:1 histone:DNA by mass) and high densities of nucleosomes [one nucleosome/100 bp (1.5:1 histone:DNA by mass; this would lead to close packed nucleosomes)] were assembled as indicated in Materials and methods. Sub-saturated arrays of nucleosomes were generated since we wished to avoid the non-physiological close-packing of nucleosomes, which might be expected to repress all DNA templated events (Clark and Wolffe, 1991). In practice, monitoring of nucleosome assembly by DNA supercoiling and micrococcal nuclease digestion revealed the assembly process to be 90% efficient (data not shown, Clark and Wolffe, 1991). This discrepancy is probably due to unavoidable loss of histones resulting from binding to dialysis tubing and microfuge tubes during reconstitution.

The reconstituted chromatin templates were then transcribed in the estrogen responsive *Xenopus* liver nuclear extract (Corthésy *et al.*, 1988). As previously reported, transcription of naked DNA containing the binding sites for estrogen receptor, the estrogen response elements (EREs), was stimulated by the addition of estrogen receptor and estrogen (Figure 1). A similar transcriptional stimulation was seen at low levels of nucleosome assembly, but the response was augmented at the moderate level of assembly. For the latter, quantification reveals a 7-fold potentiation over that seen in the absence of chromatin assembly. Estrogen receptor dependent transcriptional stimulation was 2-fold for naked DNA and for the low level (0.4) of reconstitution and 14-fold for the moderate level (0.8) of reconstitution. This and other experiments revealed a range of transcriptional potentiation attributed to moderate levels of nucleosome assembly between 2- and 8-fold. In contrast, reconstitution of control templates containing the HSV tk promoter reveal a dramatic reduction in transcriptional efficiency in response to nucleosome formation at these moderate nucleosome densities. There was a 4-fold reduction of transcription for the low level of reconstitution (0.4) and a >20-fold reduction of transcription for the moderate levels of reconstitution (0.8). At high levels of nucleosome reconstitution, which result in the close packing of nucleosomes in which no free DNA is present between nucleosomes, transcription was inhibited from both the vitellogenin B1 and HSV tk promoters (not shown).

Previous experiments had documented a similar transcriptional potentiation attributed to nucleosome assembly in mixtures of HeLa nuclear and *Xenopus* oocyte extracts (Corthésy *et al.*, 1990b). However, any potentiation of estrogen-stimulated transcription was not investigated. Thus, the nucleosome-mediated transcriptional stimulation in the *Xenopus* liver extract in the presence of estrogen probably represents a distinct phenomenon, although common chromatin structural features may exist in both expression systems (Figure 1, see below). To confirm our attribution of transcriptional stimulation to an estrogen receptor dependent effect, we repeated the experiment using plasmid DNAs containing the vitellogenin B1 promoter, but from which the EREs had been removed. No transcriptional

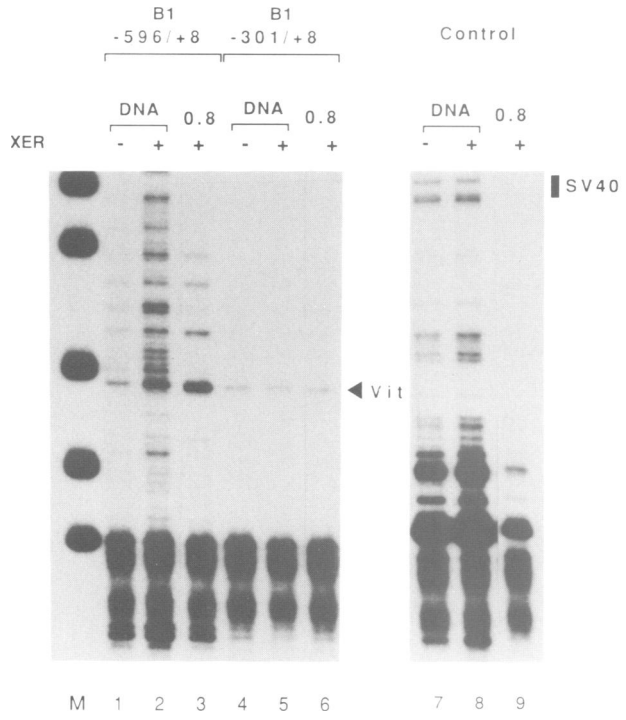

Fig. 2. Interaction of the estrogen receptor with the EREs is required for the stimulation of transcription in the presence of nucleosomes. A construct containing the EREs, pB1 (−596/+8) CAT8+ (lanes 1−3), a construct lacking EREs, pB1 (−301/+8) CAT8+ (lanes 4−6), and the SV40 early promoter, pSV2CAT (lanes 7−9) were transcribed in the liver nuclear extract after nucleosome assembly in the presence of 2.5 nM estradiol. The extract was supplemented with wild-type HeLa cell extract (WR, lanes 1, 4 and 7) or HeLa cell extract containing *Xenopus* estrogen receptor (XER, lanes 2, 3, 5, 6, 8 and 9). Transcription of free DNA (lanes 1, 2, 4, 5, 7 and 8) and DNA after moderate (0.8) levels of chromatin reconstitution (lanes 3, 6 and 9) is shown. Transcripts were analyzed by primer extension. Correctly initiated transcripts are indicated.

potentiation by nucleosome assembly is seen using the templates lacking the EREs (Figure 2, lanes 4−6). However, it is also important to note that no repression of transcription occurs on these ERE deficient templates, even though they are assembled into chromatin. The chromatin templates containing the EREs show a stimulation of transcription in the presence of estrogen that is greater than that seen on naked DNA (Figure 2, lanes 1−3). In contrast, transcription from the SV40 early promoter is not affected by estrogen and is repressed by nucleosome assembly (control). We conclude that the EREs, estrogen receptor complex and nucleosome assembly are required to obtain the maximum induction of transcription in this *in vitro* system.

Nucleosome positioning on the vitellogenin B1 promoter

We examined whether the specific positioning of nucleosomes was involved in the potentiation of estrogen stimulated transcription of the vitellogenin B1 promoter described above. Our preliminary experiments used linearized plasmid that was reconstituted into chromatin (see Materials and methods), then digested with micrococcal nuclease. Two new preferred micrococcal nuclease cleavage sites relative to the cleavage of naked DNA are found to exist at −300 bp and −140 bp relative to the start site of transcription on the vitellogenin B1 gene promoter after moderate levels of nucleosome assembly (data not shown). This is consistent with a nucleosome being positioned between these sites as the enzyme cuts preferentially in the linker regions between nucleosomes. Exo III nuclease digestion of reconstituted linearized DNA fragments reconstituted at moderate levels of nucleosome assembly was also consistent with nucleosomal boundaries at −140 and −300 (see Figure 5 later). We substantiated our analysis of nucleosome positioning between −140 and −300 using other DNA cleavage reagents: an enzyme, DNase I, and a chemical cleavage reagent, the hydroxyl radical. Both give ~10−11 bp periodic cleavage patterns when DNA is associated with the histone core of a nucleosome (Lutter, 1978; Hayes *et al.*, 1990, 1991a). As seen in Figure 3A and B (marked by the black bar), both strands of the vitellogenin B1 promoter show a clear 10−11 bp modulation in DNase I and hydroxyl radical cleavage between −140 and −300. This pattern of modulation becomes clearer at high nucleosome densities, but is apparent at moderate densities. We do not know exactly why the modulations of DNase I cleavage are less apparent in Figure 3B towards the −300 boundary of the nucleosome, but presume it is a consequence of the sequence specificity of DNase I since modulations in hydroxyl radical cleavage are clearly seen in this region. A short DNA region from −140 to approximately −120 appears free of histone DNA contacts at low and moderate nucleosome densities (Figure 3A and B, arrows).

Hydroxyl radical cleavage analysis is useful in determining sequence features of the double helix that might contribute to nucleosome positioning (Hayes *et al.*, 1990, 1991a). For example, reduction in minor groove width alters the efficiency of hydroxyl radical cleavage (Hayes *et al.*, 1991b). A periodic narrowing of minor groove width that is in phase with the helical periodicity of DNA will lead to intrinsic DNA curvature, a feature that has been shown to be associated with DNA sequences that position nucleosomes (FitzGerald and Simpson, 1985; Shrader and Crothers, 1989; Wolffe and Drew, 1989; Hayes *et al.*, 1990, 1991b). A periodic narrowing of minor groove width will lead to a periodic hydroxyl radical cleavage pattern. Such a pattern is apparent in the cleavage of the vitellogenin B1 promoter DNA between −140 and −300 (Figure 4). This feature is likely to account for the strong nucleosome positioning seen in this region (see below). As previously reported (Hayes *et al.*, 1990), nucleosome assembly enhances many of the pre-existing modulations in DNA structure since the intrinsic DNA curvature is further exaggerated as DNA is wrapped around the histone core in the nucleosome (Figure 4). Similar DNA structural features that might direct nucleosome positioning are found in the promoters of all four *Xenopus* vitellogenin genes and in the chicken vitellogenin II gene (Walker *et al.*, 1984, data not shown). The DNA sequence between −150 and −278 of the *Xenopus* vitellogenin A2 gene promoter has been shown to be curved, and has been proposed to be a nucleosome positioning element (Dobbeling *et al.*, 1988). In fact, sequence conservation in the region marked in Figure 4 by brackets has been previously noted (Walker *et al.*, 1984). This region shows strong variation in hydroxyl radical cleavage and contains runs of As and Ts that might be expected to lead to modulations in DNA minor groove width (Hayes *et al.*, 1991b).

We have tested the region likely to account for strong nucleosome positioning in the vitellogenin B1 promoter for

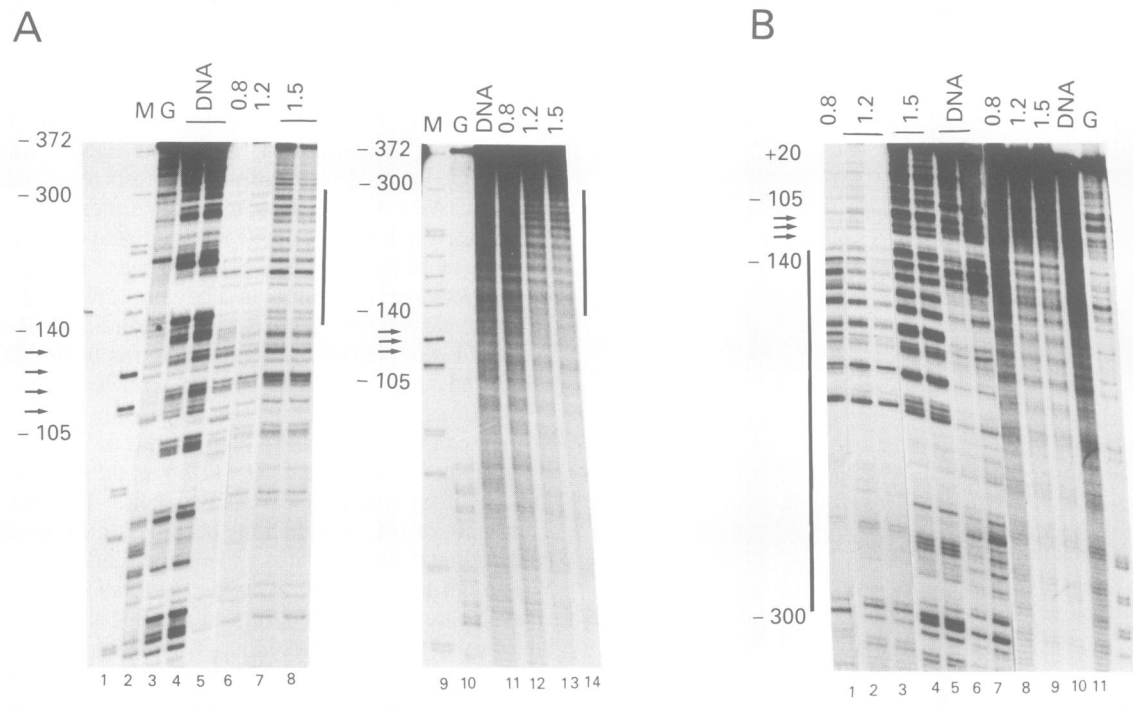

Fig. 3. DNase I and hydroxyl radical footprints of the reconstituted nucleosome. (**A**) DNase I and hydroxyl radical analysis of the B1 −372/+24 fragment labelled at the *Bam*HI site (lower strand). DNase I analysis is shown in lanes 1−8; hydroxyl radical analysis is shown in lanes 9−14. Cleavage patterns are shown for the free DNA (lanes 3, 4 and 11) and for reconstituted DNA at three different levels of reconstitution (0.8: lanes 5 and 12; 1.2: lanes 6 and 13; 1.5: lanes 7, 8 and 14). The arrows indicate the linker region where little change in the cleavage pattern is observed between the free DNA and the lowest levels of reconstitution. Lane M is an *Hpa*II digest of pBR322. Lane G is the Maxam−Gilbert sequencing reaction showing the guanines of the sequence. The products were analyzed on a 6% polyacrylamide gel containing 7 M urea. (**B**) DNase I and hydroxyl radical analysis of the B1 −372/+24 fragment labelled at the *Eco*RI site (upper strand). DNase I analysis is shown in lanes 1−7, and hydroxyl radical analysis is shown in lanes 8−11. The free DNA cleavage pattern is shown in lanes 6, 7 and 11. Cleavage patterns for three different levels of reconstitution are shown (0.8: lanes 1 and 8; 1.2: lanes 2, 3 and 9; 1.5: lanes 4, 5 and 10). The position of the putative linker region is indicated as in (A). G and M lanes are as in (A). Black bars indicate the position of the nucleosome.

its affinity for histone octamers by exchange reconstitution of nucleosomes (Jayasena and Behe, 1989; Shrader and Crothers, 1989). The change in free energy attributed to nucleosome formation (ΔΔG) has been calculated for the *Xenopus borealis* 5S rRNA gene (as a control) as well as for the vitellogenin B1 gene promoter compared with the random sequence DNA found in nucleosome core particles prepared from all the chromatin in a chicken erythrocyte nucleus (Table I). We have compared our results with data obtained by Shrader and Crothers (1989) for the ΔΔG values derived from the assembly of nucleosomes with other naturally occurring nucleosome positioning elements. The vitellogenin B1 promoter sequence shows a much higher affinity for the histone octamer than random core particle DNA. The ΔΔG value for the B1 promoter is intermediate to that measured for the various types of *Xenopus* 5S rRNA gene which are known to position nucleosomes *in vivo* and *in vitro* (Simpson and Stafford, 1983; Rhodes, 1985; Thoma and Simpson, 1985; Gottesfeld, 1987; Chipev and Wolffe, 1992). We conclude that the vitellogenin B1 promoter contains a strong nucleosome positioning element. Such a positioned nucleosome would potentially bring the EREs from −330 to −300 into juxtaposition with the proximal promoter elements at −120 to −100 relative to the start of transcription (see Figure 10).

Transcription factor interactions with the positioned nucleosome

We first examined whether we could detect the specific association of the *Xenopus* estrogen receptor with the EREs when a nucleosome was positioned between −140 and −300. Exo III nuclease digestion of linearized DNA fragments at moderate levels of nucleosome assembly is consistent with nucleosomal boundaries at these positions (Figure 5A and B). Exonuclease III is a useful reagent since a kinetic barrier to nuclease activity due to *trans*-acting factor binding at a specific sequence can be detected even when occupancy is not complete. Thus we detect an estrogen receptor dependent nuclease barrier over the EREs even on the nucleosomal template (Figure 5C, horizontal arrow). The major barrier due to nucleosome formation is also apparent in Figure 5C (at approximately −300). This experiment also indicates that the nucleosome is stable in the presence of the HeLa extract (see also Figure 9). Our next experiments used DNase I to examine whether binding of estrogen receptor might cause either protection from cleavage or the appearance of hypersensitive cleavage sites. An important control in these experiments are mobility shifts indicating that all of the DNA fragment is histone bound (Figure 6A; Pina *et al.*, 1990). These mobility shifts also indicate that non-specific protein−DNA interactions occur with the DNA fragments in HeLa extracts independent of the presence of estrogen receptor. These interactions are inhibited by prior association of the DNA with histones. However, receptor binding to the EREs, as detectable by the presence of a DNase I hypersensitive site as well as by a partial protection from cleavage is seen on naked DNA. At moderate levels of nucleosome reconstitution (Figure 6B), the hypersensitive site is clearly visible, but is progressively lost as the degree of chromatin assembly is increased. We conclude that at

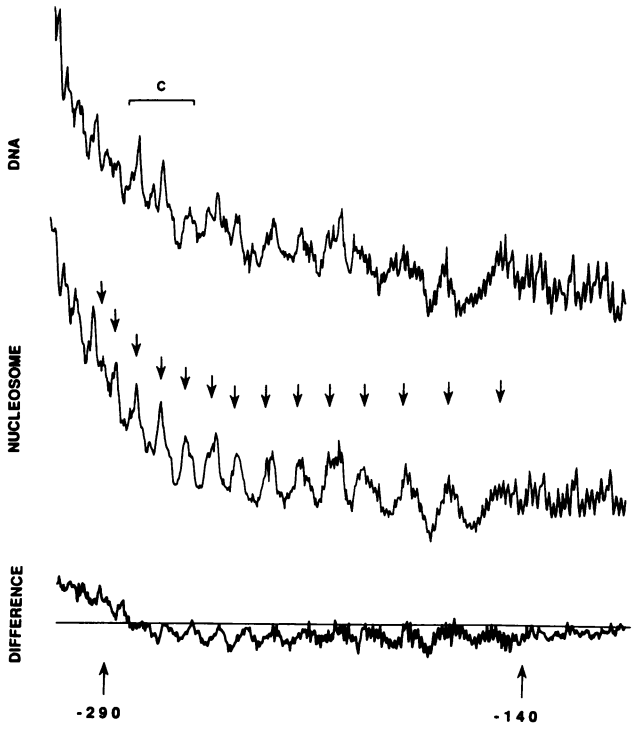

Fig. 4. Densitometric analysis of the hydroxyl radical analysis. Cleavage pattern for the lower strand is shown. The first two traces represent the cleavage pattern for free and reconstituted DNA (0.8, octamer) respectively. The lower trace represents the difference between the two previous scans. The small arrows mark the position of the maximal hydroxyl radical cleavage of the nucleosomal DNA. The borders of the nucleosome are indicated by the arrows on the lower scan. The bracket on the DNA scan (labelled C) indicates the highly conserved sequence between the four *Xenopus* and the chicken vitellogenin II genes (Walker *et al.*, 1984).

moderate levels of nucleosome assembly, such as those exhibiting transcription potentiation, the estrogen receptor can still bind specifically to the vitellogenin B1 promoter. Nucleosome assembly does not facilitate the binding of the estrogen receptor to its recognition site. Furthermore, the receptor does not appear to bind to the EREs when they are in direct contact with the histone core, a situation that exists at high levels of nucleosome assembly (see also Figure 9 later).

We next examined the consequences of estrogen receptor binding for the interaction of NF1 with the proximal promoter element. The binding of NF1 to the proximal promoter elements of the vitellogenin B1 promoter is seen at moderate, but not high levels of nucleosome assembly (Figure 7, compare lanes 5 and 8). However, the presence of HeLa extracts with or without the estrogen receptor inhibits NF1 binding (data not shown, Figure 7, compare lanes 5 and 6). This is presumably due to non-specific protein–DNA interactions competing for the correct binding site (see Figure 6A). We conclude that under our experimental conditions it is unlikely that the estrogen receptor is mediating transcriptional potentiation through a direct influence on the binding of NF1 alone to the vitellogenin B1 promoter. In the mouse mammary tumor virus LTR *in vivo*, glucocorticoid receptor binding is believed to facilitate the association of NF1 (Pina *et al.*, 1990; Archer *et al.*, 1991). However, *in vitro*, NF1 binding is prevented by a nucleosomal template (lanes 7–9) (Pina *et al.*, 1990; Archer *et al.*, 1991). In agreement with the MMTV results,

Table I. Comparative free energies in nucleosome formation

Gene	ΔΔG (cal/mol)
Xenopus borealis somatic 5S rRNA gene	−1750
Lytechinus variegatus 5S rRNA gene	−1600*
Xenopus laevis somatic 5S rRNA gene	−1500*
Xenopus laevis vitellogenin B1 promoter	−1310
Xenopus laevis trace oocyte 5S rRNA gene	−1250*
Mononucleosomal DNA	0

* Values from Shrader and Crothers (1989) adapted to the value of the mononucleosomal DNA.
The difference in the free energy of nucleosome formation was calculated for the *X.borealis* somatic 5S rRNA gene (583 bp fragment from the plasmid pXP-10) and for the *X.laevis* vitellogenin B1 promoter (413 bp fragment from the plasmid pB1, −371/+24) relative to mononucleosomal DNA. Each value represents the average of four different reconstitution experiments. The lower values indicate a higher tendency to reconstitute.

NF1 can bind to its recognition site in the vitellogenin B1 promoter when the binding site is outside the nucleosome but not when it is within the nucleosome.

Removal of the −138 to −297 DNA sequence from the vitellogenin B1 promoter eliminates the potentiation of estrogen responsive transcription through chromatin assembly

We have presented evidence consistent with the assembly of a nucleosome positioned between −140 and −300 on the vitellogenin B1 promoter (Figures 3, 4 and 5). The estrogen receptor and NF1 remain able to interact with this particular nucleosomal template (assembled at moderate levels of histone to DNA excess) (Figures 5, 6 and 7). Nucleosome assembly potentiates estrogen responsive transcription from the intact vitellogenin promoter (Figures 1 and 2). We next wished to substantiate that the transcriptional potentiation is due to the nucleosome, and that it is not due to some unknown factor added during the reconstitution process. This problem was approached by making deletions of either the entire nucleosomal region (−297 to −138) or of half of the nucleosomal region (−290 to −199). These templates were reconstituted into nucleosomes, and then the effect of reconstitution on estrogen-inducible transcription was examined and the nucleoprotein organization of the template was investigated.

When the intervening DNA between −297 and −138 is deleted from the vitellogenin B1 promoter, the estrogen response unit (ERU) is brought close to the proximal promoter elements. Estrogen-induced transcription initiated at the vitellogenin B1 promoter in the presence of receptor is very strong (Figure 8A, compare lanes 1 and 2). Quantification revealed a 5-fold induction of transcription in the presence of estrogen receptor. Thus stimulation was stronger than what was observed for the pB1 (−596/+8) CAT 8+ template, which was expected as the ERU is brought immediately adjacent to the basal transcription elements at −138. Transcription from a naked SV40 early promoter-containing DNA fragment, present as an internal control, is unaffected by the presence of estrogen receptor. Basal transcription from this deletion mutant is not significantly repressed by moderate levels of nucleosome reconstitution relative to transcription from a naked SV40 promoter internal control (lanes 3 and 4). Importantly, the

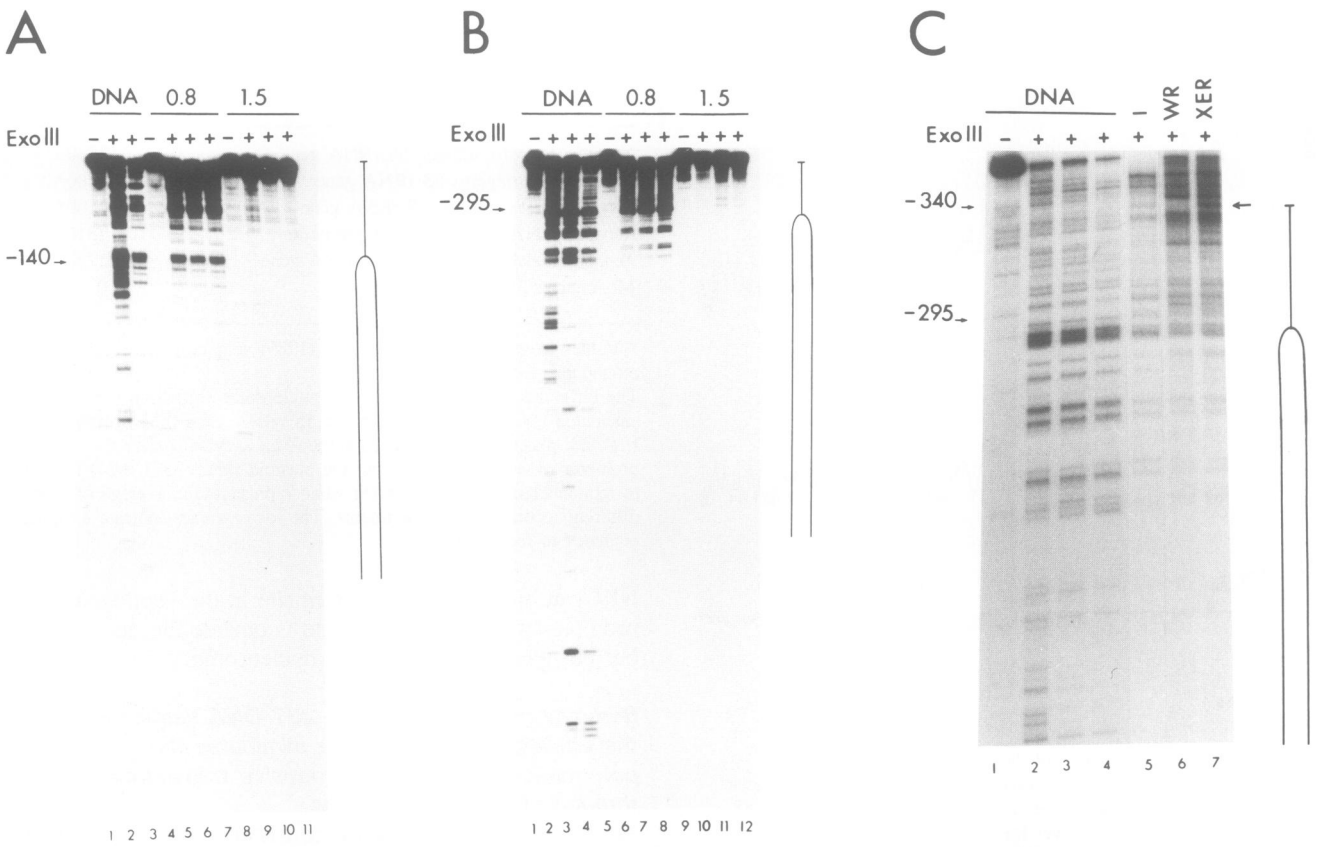

Fig. 5. Exonuclease III analysis of nucleosome reconstitutes alone and in the presence of the estrogen receptor. (**A**) The B1 −372/+24 fragment was labelled at the −372 *Eco*RI 1 site (upper strand). Free DNA was digested with increasing amounts of exonuclease III (lanes 2−3). Reconstitutions at a moderate histone:DNA ratio (0.8), (lanes 5−7), or at a high histone:DNA ratio (1.5), (lanes 9−11) were digested with increasing amounts of exonuclease III. (**B**) The B1 −372/+24 fragment was labelled at the +24 *Bam*HI site. The free DNA was digested with increasing amounts of exonuclease III (lanes 2−4). Reconstitutions at moderate histone:DNA ratio (0.8), (lanes 6−8), or at a high histone:DNA ratio (1.5) (lanes 10−12) were digested with increasing amounts of exonuclease III. (**C**) A higher resolution analysis of exonuclease III digestion using the same DNA fragment as in B as naked DNA or after reconstitution into nucleosomes with or without the estrogen receptor present. Free DNA was digested with increasing amounts of exonuclease III (lanes 2−4). Reconstitutes at a moderate histone:DNA ratio (0.8) (lanes 5, 6 and 7) were digested by exonuclease III in the absence (lane 5) or presence of 10 μg wild-type HeLa cell extract (lane 6) or of HeLa cell extract containing *Xenopus* estrogen receptor (lane 7). The schematics on the right indicate the position of the nucleosome in A, B and C and the horizontal arrow in C represents the estrogen receptor-dependent exonuclease III block.

presence of the estrogen receptor complex has no effect on transcription from this promoter after reconstitution into chromatin. Thus nucleosome assembly on a construct in which the ERU is contiguous with the proximal promoter elements prevents estrogen inducible transcription. Similar results were obtained using the construct in which half the nucleosome binding site (−290 to −199) was deleted (data not shown). The stimulation of transcription by estrogen receptor on naked DNA was between that of the wild-type B1 promoter and the mutant in which sequences from −297 to −138 were deleted. Basal transcription was not significantly repressed by moderate levels of nucleosome reconstitution relative to a naked SV40 control. However, estrogen receptor complex responsiveness was again abolished by moderate levels of nucleosome reconstitution. As a further control in this experiment we again examined the effect of nucleosome reconstitution on transcription using the promoter from which the ERU was deleted. Consistent with previous observations (see Figure 2), removal of the ERU prevented induction of transcription by estrogen and the estrogen receptor (Figure 8B, compare lanes 1 and 2), reconstitution of moderate levels of nucleosomes did not stimulate or inhibit transcription. We conclude that both the ERU, the estrogen receptor complex and the nucleosome positioning element are required for nucleosome assembly to potentiate estrogen inducible transcription. The reconstitution process itself does not potentiate transcription whether or not estrogen responsive conditions are established (Figure 8).

In order to interpret the transcription results shown in Figure 8A, we examined the nucleoprotein organization of the vitellogenin B1 promoter from which the nucleosome positioning sequence between −140 and −300 had been removed. At moderate levels of nucleosome reconstitution, DNA from −40 towards the 5′ end of the DNA fragment appears to be incorporated into a positioned nucleosome. Thus, the ERU previously located between −295 and −340 is wrapped on the surface of a histone octamer (Figure 9A). Unlike the glucocorticoid receptor (Pina *et al.*, 1990; Archer *et al.*, 1991), the estrogen receptor does not bind to DNA that is wrapped on the surface of the histone octamer (Figure 9B). The structural properties of the deletion construct reconstituted into nucleosomes are consistent with the transcription results. Basal transcription is not inhibited because the TATA box (−30) region is predominantly free of histone DNA contacts, yet estrogen responsive transcription is inhibited because the estrogen receptor cannot bind to the ERU in the nucleosome. The DNase I footprinting

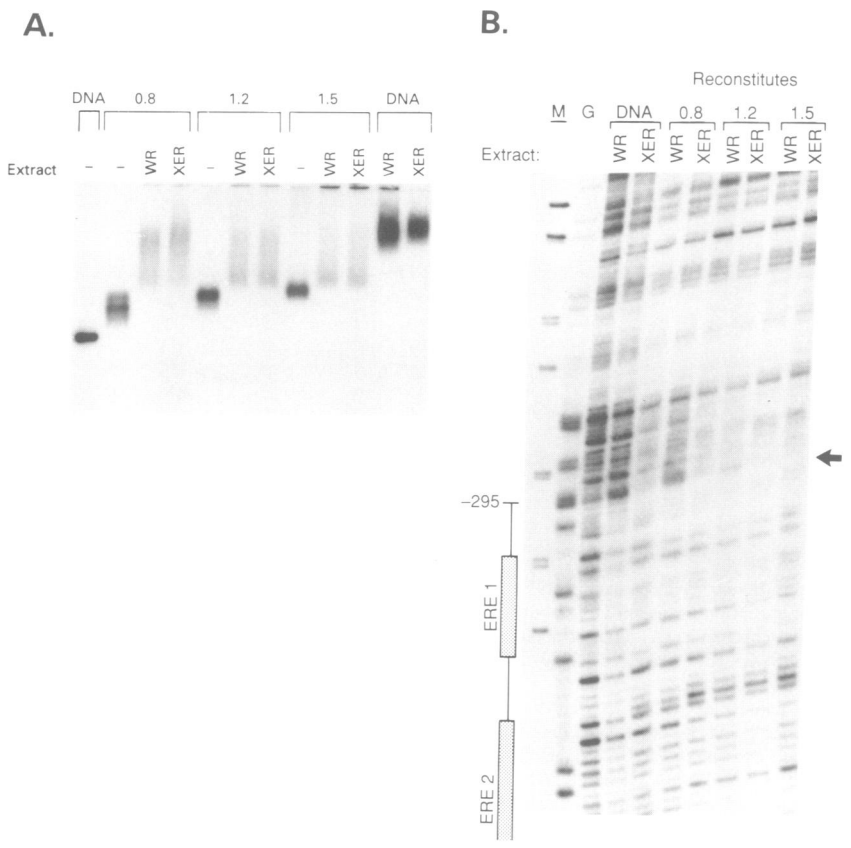

Fig. 6. The *Xenopus* estrogen receptor interacts with the reconstituted B1 promoter at moderate levels of nucleosome reconstitution. (**A**) Binding activity of the wild-type and recombinant HeLa cell extracts containing *Xenopus* estrogen receptor to the nucleosome reconstituted B1 −372/+24 fragment. Binding reactions were performed with free DNA or nucleosome reconstituted DNA (histone:DNA ratios of 0.8, 1.2 or 1.5) incubated with 10 μg of HeLa cell extract infected with the wild-type vaccinia virus (WR) or with the recombinant virus expressing the *Xenopus* estrogen receptor (XER). Samples were loaded directly on a 0.7% native agarose gel containing 0.5% TBE. (**B**) DNase I analysis of the estrogen receptor binding to the nucleosomal DNA. The −372 end-labelled DNA or reconstitutes [same histone:DNA ratios as in (A)] were incubated with either WR or XER extract and digested with DNase I as described in Materials and methods. DNase I digestion of reconstitutions and free DNA are shown. 'G' is the Maxam−Gilbert sequencing reaction. 'M' is an *Hpa*II digest of pBR322. The products were analyzed on a 6% polyacrylamide gel containing 7 M urea. The position of the EREs is shown. The arrow indicates the estrogen receptor-dependent hypersensitive site.

experiments shown in Figure 9 also demonstrate that the nucleosome positioned over the ERU is stable to the addition of HeLa extract with or without the estrogen receptor (see also Figure 5). Similar experiments using a DNA fragment from which half of the nucleosome positioning element (−290/−199) has been deleted also revealed nucleosome positioning over the ERU (data not shown).

Discussion

The major conclusion of this work is that a nucleosome-mediated static loop potentiates the transcriptional stimulation of the vitellogenin B1 promoter directed through the association of the estrogen receptor with the EREs. This occurs only at moderate levels of chromatin assembly reflecting physiological densities of nucleosomes (Wolffe and Schild, 1991). Furthermore, low and moderate densities of nucleosome assembly do not inhibit transcription from the vitellogenin B1 promoter (Figures 1 and 2). This may be contrasted with the transcriptional inhibition seen with our control templates, and numerous other examples (Knezetic and Luse, 1986; Workman and Roeder, 1987; see Felsenfeld, 1992). Like all genes studied so far, a non-physiological density of nucleosomes (~ one nucleosome/150 bp) inhibits transcription from the B1

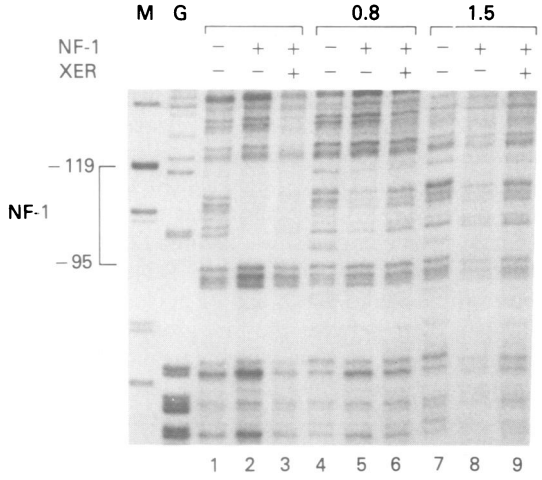

Fig. 7. NF-1 interacts with the reconstituted B1 promoter at moderate levels of nucleosome reconstitution. Free DNA (lanes 1−3) or reconstitutes (histone:DNA ratio 0.8: lanes 4−6 and 1.5: lanes 7−9) were incubated with purified NF-1 alone (lanes 2, 5 and 8) or with both purified NF-1 and 10 μg of XER extract (lanes 3, 6 and 9). Lanes 1, 4 and 7 are control reactions for free DNA and reconstitutes. Binding reactions and DNase I digestions were performed as described in Materials and methods. NF-1 protection is shown by brackets. Lane G is the Maxam−Gilbert sequencing reaction. Lane M is an *Hpa*II digest of pBR322.

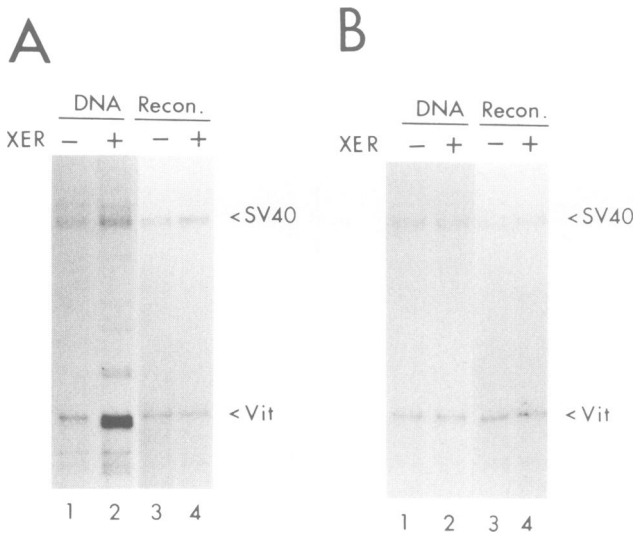

Fig. 8. Deletion of the nucleosome portioning element from −297 to −138 facilitates estrogen-induced transcription on naked DNA, but eliminates transcription potentiation in chromatin. (**A**) Transcription of a mixture of the pB1 ERU (−138/+8) CAT8+ plasmid (0.2 μg) and the SV40 early promoter (0.2 μg) in the liver nuclear extract supplemented with 2.5 nM estradiol and 10 μg total protein either of wild-type HeLa cell extract (−), (lanes 1 and 3), or of *Xenopus* estrogen receptor-containing HeLa cell extracts (XER, +), (lanes 2 and 4). The plasmid was transcribed as naked DNA, (lanes 1 and 2) or after reconstitution with a moderate level (0.8) of histones (lanes 3 and 4). The SV40 early promoter containing plasmid was always transcribed as naked DNA. (**B**) Like A except the pB1 (−301/+8) CAT8+ construct was used, the ERU is deleted from this construct.

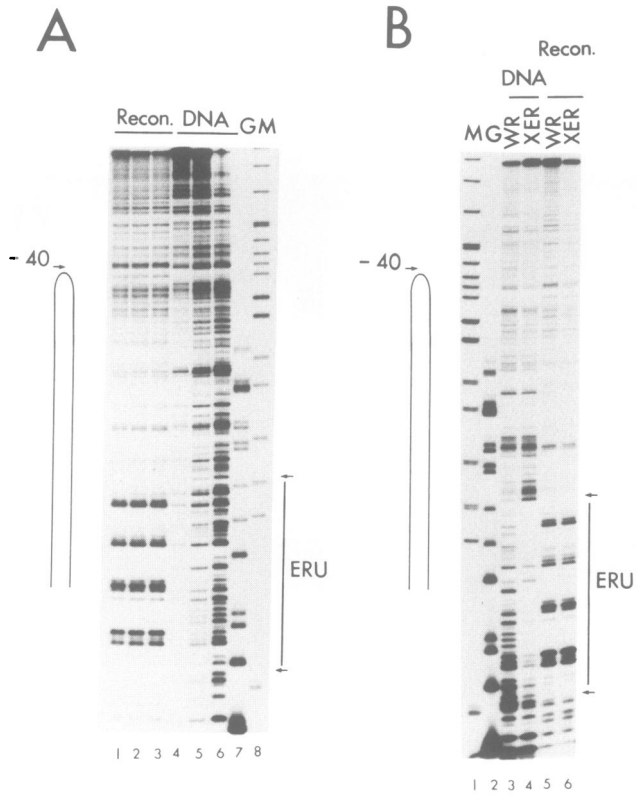

Fig. 9. Nucleoprotein organization of the vitellogenin B1 promoter from which the nucleosome portioning element (−297 to −138) is deleted. (**A**) DNase I footprinting of the pB1 ERU (−138/+8) fragment after reconstitution to a moderate level (0.8) with histones. DNase I cleavage of reconstituted (lanes 1−3) or of naked DNA (lanes 4−6) is shown together with markers (M) and a Maxam−Gilbert G-sequencing reaction (G). The approximate position of the nucleosome and its 5′ boundary (−40) are indicated together with the position of the ERU. (**B**) The estrogen receptor does not interact with the complex of the histone octamer with the vitellogenin B1 promoter from which the nucleosome positioning element is deleted. The DNase I digestion patterns of naked DNA in the presence of wild-type HeLa cell extract (WR, lane 3), or of HeLa cell extract containing *Xenopus* estrogen receptor (XER, lane 4) are shown. DNase I digestion patterns of the same DNA fragment after reconstitution with moderate levels of histone octamer in the presence of wild-type HeLa cell extract (WR, lane 5) or of HeLa cell extract containing *Xenopus* estrogen receptor (XER, lane 6) are shown together with markers (M) and a Maxam−Gilbert G-sequencing reaction (G). The approximate position of the nucleosome and its 5′ boundary (−40) are indicated together with the position of the ERU.

promoter (data not shown, see Clark and Wolffe, 1991).

We have described the structural features of the DNA sequences within the vitellogenin B1 promoter that position a nucleosome between the ERU from −295 to −340 relative to the start of transcription and the proximal promoter element from −140 to +1. Periodic modulations in minor groove width are consistent with the DNA between −300 and −140 containing strong nucleosome positioning elements (Figure 3). These structural features are maintained and exaggerated following nucleosome assembly (Figure 4, see also Hayes *et al.*, 1990). Micrococcal nuclease, DNase I, hydroxyl radical and exonuclease III cleavage data are consistent with a nucleosome being positioned here on both short (~400 bp) linear DNA fragments (Figures 3, 4 and 5) and long plasmid DNA molecules (data not shown). The vitellogenin B1 promoter contains a favored DNA sequence for association with a histone octamer (Table I).

It is interesting to note that a gap of nucleosome length (~180 bp) is present between the first group of EREs and the proximal promoter element which contains the binding sites for numerous *trans*-acting factors including NF-1 like activities (Corthésy *et al.*, 1990b; Seal *et al.*, 1991). A similar organization is found in all four *Xenopus* vitellogenin genes and in the chicken vitellogenin II gene (Walker *et al.*, 1984). This region has previously been proposed to represent a nucleosome positioning element (Dobbeling *et al.*, 1988). Moreover, the position of DNase I hypersensitive sites B1 and B2 in the chicken vitellogenin II promoter documented *in vivo* (at −300 and +1) are consistent with a nucleosome being positioned between them (Burch and Weintraub, 1983). Mutational studies of the chicken vitellogenin II promoter using transient assays in which the EREs are progressively brought closer to the proximal promoter element reveal an initial drop in transcription before transcription is potentiated (Seal *et al.*, 1991). Such studies would be consistent with the spacing between the EREs and the proximal promoter element playing an important role in transcription, perhaps due to a requirement for a nucleosomal length of DNA. Deletion of the DNA between the EREs and the proximal promoter element eliminates the potentiation of transcription following nucleosome assembly (Figure 8). Thus we suggest that a nucleosome-mediated static loop potentiates transcriptional stimulation by bringing the estrogen receptor bound to the EREs into juxtaposition with the proximal promoter element and factors bound to it (Figure 10, see also Hayes and Wolffe, 1992b). Such a model has been proposed by Elgin and colleagues for activation of the hsp26 gene in *Drosophila* (Elgin, 1988;

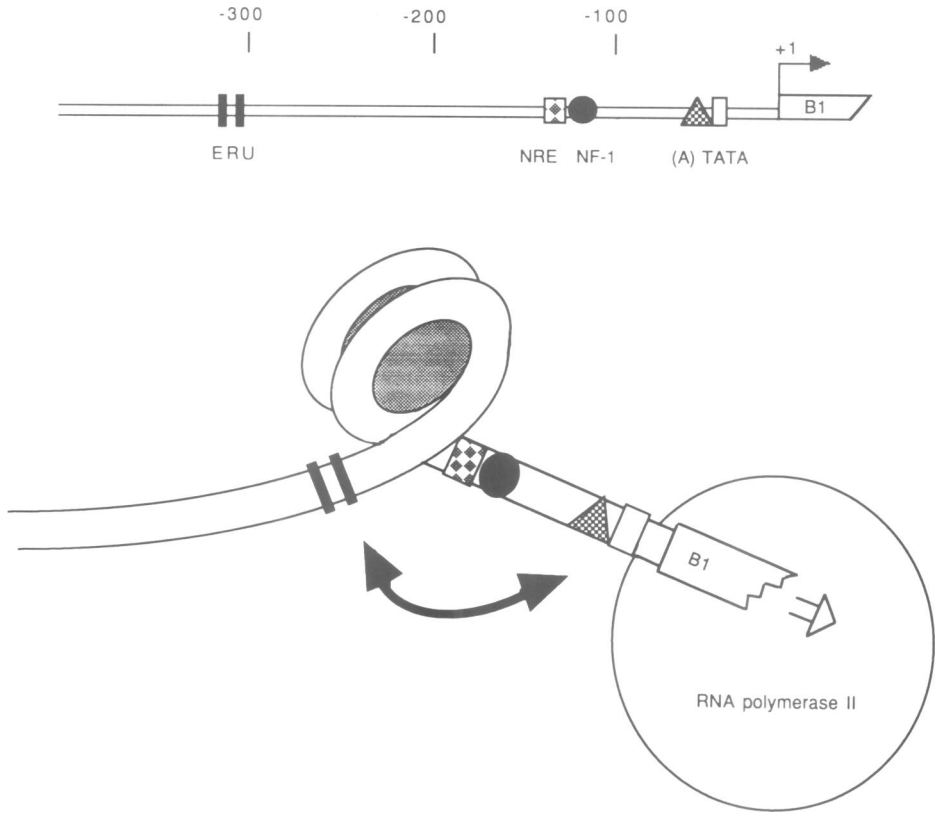

Fig. 10. Stimulation of transcription from the vitellogenin B1 promoter by a positioned nucleosome (see Hayes and Wolffe, 1992b). The positions of the promoter elements have been defined previously (Corthésy *et al.*, 1989). The positioning of a nucleosome in the region −300 to −140 from the initiation of transcription allows the estrogen receptor complex to interact with the estrogen responsive unit (−300/−330). The folding of the DNA around the core histones creates a static loop which is proposed to facilitate the interaction of the estrogen receptor complex with the transcription factors present at the proximal promoter elements (black arrow).

Thomas and Elgin, 1988), but a direct positive effect on transcription has yet to be demonstrated for this gene.

We have investigated how the potentiation of transcription observed through nucleosome positioning on the vitellogenin B1 promoter might occur. Estrogen receptor and NF1 will interact with their binding sites when they are located outside the nucleosome (Figures 5, 6 and 7). Neither protein will bind to DNA when histones completely cover the binding sites (Figures 6, 7 and 9). The estrogen receptor does not appear to facilitate NF1 binding through direct interaction, consistent with previous data obtained in HeLa cell extracts (Martinez *et al.*, 1991). However, this does not imply that these two proteins do not cooperate in transcriptional activation once bound. We therefore suggest that estrogen receptor facilitates transcription through interactions with some other *trans*-acting factor or with the basal transcriptional machinery. Our results are not consistent with models in which NF1 binding is facilitated by prior nucleosome assembly. It has also been proposed from experiments using different nucleosome assembly systems that NF1 binding might be facilitated during nucleosome formation (Corthésy *et al.*, 1990b); this remains to be tested. It is also possible that conditions that might facilitate NF1 binding in a small population of transcriptionally active templates are not reflected in the bulk population used to examine structure in our experiment.

It is well established that chromatin structure can direct the repression of genes (Wolffe, 1991; Felsenfeld, 1992), it is also known that nucleosome positioning can allow the activation of genes otherwise packaged with histones (Simpson, 1991; Wolffe, 1991). Here we have provided functional evidence for a third role for chromatin structure in gene regulation: the potentiation of transcription by a positioned nucleosome.

Materials and methods

Plasmid and probe preparation

The plasmids used for reconstitution and/or transcription were linearized by restriction digestion. Plasmid pB1(−596/+8) CAT8+ (6.2 kb) (Seiler-Tuyns *et al.*, 1986) was linearized with *Acc*I, pB1 (−302/+8)CAT8+ (5.9 kb) (Corthésy *et al.*, 1989) with *Hin*dIII, pBLCAT2 (4.5 kb) (Luckow and Schutz, 1987) with *Hin*dIII and pSV2CAT (5.0 kb) (Gorman *et al.*, 1982) with *Bam*HI. Plasmid pB1(−371/+24) (Corthésy *et al.*, 1989) was end-labelled either at +24 or at −371 with T4 polynucleotide kinase using [γ-^{32}P]ATP (3000 Ci/mMol, NEN) after linearization and dephosphorylation. The 413 bp fragment was then obtained by a second restriction digestion with either *Eco*RI or *Bam*HI, and isolated by electroelution after electrophoresis on agarose gel.

The plasmid pB1 ERU (−138/+8) CAT8+ was obtained by introducing a synthetic oligonucleotide corresponding to the B1 ERU (−298/−337) into the *Bam*HI site of the plasmid pB1 (−138/+8) CAT8+ (Corthesy *et al.*, 1989). For the transcription experiments this construct was linearized by *Hin*dIII in the polylinker upstream of the ERU. The deletion mutant pB1 (−596/−290/−199/+8) CAT8+ was obtained by site-directed mutagenesis using PCR as described by Imai *et al.* (1991). The plasmid pB1 (−596/+8) CAT8+ was used as template and two synthetic oligonucleotides (−313/−290 and −199/−175) were used as primers to create the −290/−199 deletion. The plasmid was linearized with *Acc*I for the transcription experiments.

The fragment pB1 ERU (−138/+8) used in Figure 9 is a 410 bp fragment isolated by *Hin*dIII and *Eco*RI digestion of the plasmid pB1 ERU (−138/+8)

CAT8+. The fragment was end-labelled at the *Hin*dIII site using T4 polynucleotide kinase as described above.

The *X.borealis* somatic 5S rRNA gene was isolated from the plasmid pXP-10 (Wolffe *et al.*, 1986). The 583 bp *Hha*I–*Eco*RI fragment was isolated after end-labelling at the *Eco*RI site.

All oligonucleotides used as probes were 5′ end-labelled using T4 polynucleotide kinase with [γ-^{32}P]ATP.

Nucleosome reconstitution and hydroxyl radical footprinting

Purified core histones were prepared from chicken erythrocyte nuclei using hydroxylapatite chromatography (Simon and Felsenfeld, 1979). Nucleosomes were reconstituted on radiolabelled DNA fragments or linearized plasmids by salt/urea dialysis (Camerini-Otero *et al.*, 1976). Different histone:DNA (mass to mass) ratios were used assuming that a 1:1 ratio corresponds to one nucleosome core particle per 160 bp of DNA. Final dialysis was into 10 mM Tris–HCl (pH 8.0), 1 mM EDTA for the labelled DNA fragments, whereas for the reconstituted linearized plasmid DNA, EDTA was omitted. The efficiency of reconstitution was monitored by electrophoresis on 0.7% agarose in 0.5×TBE.

OH· radical footprinting was performed as described by Hayes *et al.* (1991). Samples were analyzed on denaturing 8% polyacrylamide gels containing 7 M urea.

Autoradiographs were scanned with a Molecular Dynamics densitometer and the area of each band was determined using Imagequant software. The values were then smoothed by performing a three-bond running average throughout the entire data set (Hayes *et al.*, 1990). To determine the contribution of the nucleosome in the cleavage pattern, the naked DNA values were subtracted from the values of the nucleosome samples.

Competitive reconstitution

We used a procedure described by Jayasena and Behe (1989) and Shrader and Crothers (1989) with minor modifications. Chromatin stripped of linker histones was prepared according to standard procedures. Radiolabelled DNA (~10 ng) was mixed with 1 μg of stripped chromatin in 1 M NaCl, 10 mM Tris–HCl (pH 8.0), 0.1% NP-40, 0.1% BSA with various amounts of non-specific competitor DNA (calf thymus DNA digested with *Hae*III) in a final volume of 10 μl. After incubation at 37°C for 30 min, the salt concentration was gradually reduced to 100 mM by three additions of 30 μl of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (30 min apart, room temperature). Samples were resolved on 4.5% native polyacrylamide gels as described by Wolffe (1988).

Scannings of the bound and free DNA were performed with a Phosphorimager (Molecular Dynamics) and the area of each band was determined using Imagequant software. The difference in ΔG in nucleosome formation was calculated by reference to random sequence core DNA (~0 cal/mol, Hayes *et al.*, 1991b) from the equation $\Delta\Delta G = -RT \ln (K_b/K_a)$ where K_a is the ratio of bound to free random core DNA and K_b is the ratio of bound to free DNA of a particular DNA. The values from Shrader and Crothers (1989) shown in Table I are relative to the random core sequence DNA value.

Production of Xenopus estrogen receptor (XER) with vaccinia virus and binding conditions

HeLa cells were infected by either wild-type (WR) or recombinant vaccinia viruses expressing the *Xenopus* estrogen receptor (XER) and whole cell extracts were prepared as described by Kumar and Chambon (1988) and Corthésy *et al.* (1990a). Binding experiments were performed in 100 mM KCl, 10 mM Tris–HCl (pH 7.5), 10% glycerol, 1 mM DTT, 0.05% NP-40, 5 μM $ZnSO_4$, 50 nM estradiol. Binding reactions were performed for 30 min on ice and either loaded directly on gel or subjected to DNase I digestion.

DNase I analysis

Naked DNA and reconstituted DNA were digested in 1 mM $MgCl_2$ with 0.01 mg/ml DNase I. For the binding experiments with the HeLa cell extracts, digestions were performed in 2.5 mM $MgCl_2$ with 0.03 mg/ml DNase I. Reactions were stopped in 0.5% SDS, 10 mM Tris–HCl (pH 7.5), 15 mM EDTA, 500 μg/ml proteinase I was added and, after 30 min incubation at 37°C, the samples were phenol extracted and precipitated with ethanol. Analysis was performed on a denaturing polyacrylamide gel.

Mobility shift assay

Protein–DNA complexes were analyzed in non-denaturing agarose gels containing 0.5×TBE. Reconstituted samples were loaded directly on the gel after adding 10% glycerol. Electrophoresis was performed at 20–25 mA at 4°C or room temperature.

Micrococcal nuclease analysis of reconstituted DNA

Samples were digested in 3 mM $CaCl_2$ with 0.5–2 U MNase/μg DNA. Reactions were stopped in 0.5% SDS, 10 mM Tris–HCl (pH 7.5), 15 mM EDTA and precipitated directly with ethanol. Radioactive samples were analyzed on a native 2% agarose gel and directly autoradiographed after the gel had been dried. MNase analysis done with unlabelled plasmid pB1(–596/+8)CAT8+ was analyzed on 2% agarose gel and transferred onto nylon membrane by capillary blotting. Hybridization was done according to standard procedures (Sambrook *et al.*, 1989) with an oligonucleotide (3′-CTTAATCAATTTGATTTGAACCTGG-5′) complementary to the region –414/–438 of the B1 promoter.

Exonuclease III protection assay

DNA fragments or reconstituted samples (~20 ng DNA) were digested in 100 mM KCl, 10 mM Tris–HCl (pH 7.5), 10% glycerol, 1 mM DTT, 5 mM $MgCl_2$ for 15 min at room temperature with 0–500 U of exonuclease III (Promega). When the reaction was supplemented with HeLa cell extracts (WR or XER) 700 U of enzyme was added. Reactions were stopped in 0.5% SDS, 15 mM EDTA, and samples were then phenol extracted, ethanol precipitated and resolved on 6% polyacrylamide–7 M urea gel.

In vitro transcription in Xenopus liver nuclear extract and transcript analysis

Liver nuclear extracts were prepared from female *Xenopus* as described by Corthésy and Wahli (1990). *In vitro* transcriptions were performed essentially as described by Corthésy and Wahli (1990) with some modifications. Transcription reactions were supplemented with 10 μg of HeLa cell extract infected with either wild-type (WR) or recombinant (XER) vaccinia virus. The amount of template used in all transcriptions was 0.2 μg. Transcripts analysis was done by primer extension as described by Corthésy and Wahli (1990) with a CAT primer (3′-TACCTCTTTTTTT-AGTGACCTATATGGTGG-5′) homologous to the first 30 nucleotides of the CAT gene. Extension products were analyzed on 6% polyacrylamide–7 M urea sequencing gels.

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