Determinants of Vitellogenin B1 promoter Architecture: HNF3 and Estrogen Responsive Transcription within Chromatin

Daniel Robyr¹,²,#, §, Anne Gegonne²,§, Alan P. Wolffe² and Walter Wahli¹*

¹ Institut de Biologie animale, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland

² Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Building 18T, Room 106, Bethesda, MD 20892-5431, USA.

* Corresponding author: Walter Wahli, Institut de Biologie animale, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland
tel. +41 21 692 41 10
fax +41 21 692 41 15
e-mail walter.wahli@iba.unil.ch

§ Both authors did collaborate equally on this work

# Present address: Department of Biological Chemistry, Molecular Biology Institute, University of California, Los Angeles, CA, 90095, USA

Copyright 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
SUMMARY

The liver-specific vitellogenin B1 promoter is efficiently activated by estrogen within a nucleosomal environment after microinjection into Xenopus laevis oocytes, consistent with the hypothesis that significant nucleosome remodeling over this promoter is not a prerequisite for the activation by the estrogen receptor (ERα). This observation lead us to investigate determinants other than ERα of chromatin structure and transcriptional activation of the vitellogenin B1 promoter in this system and in vitro. We find that the liver-enriched transcription factor HNF3 has an important organizational role for chromatin structure as demonstrated by DNase I hypersensitive site mapping. Both HNF3 and the estrogen receptor activate transcription synergistically and are able to interact with chromatin reconstituted in vitro with three positioned nucleosomes. We propose that HNF3 is the cellular determinant which establishes a promoter environment favorable to a rapid transcriptional activation by the estrogen receptor.

INTRODUCTION

Vitellogenin is the precursor of the yolk proteins that represent a major source of nutrients for the developing embryo in oviparous vertebrates (1). Its liver-specific expression is controlled by estrogen in adult females (2) through its receptor which interacts as a homodimer with the estrogen responsive element (ERE) in the promoter of the vitellogenin genes (3,4). Functional studies of the Xenopus vitellogenin B1 promoter in cultured human breast cancer cell lines have
revealed the presence of an estrogen responsive unit (ERU: region -334 to -302 relative to the start site of transcription) composed of two imperfect EREs (5). A third functional ERE was detected farther upstream in the region -555 to -543. The *Xenopus* vitellogenin B1 promoter (6) encompasses additional *cis*-acting elements for the liver-enriched factors HNF3 and C/EBP as well as for the ubiquitous protein CTF/NF1 (7). Tissue specificity may be achieved by a combination of these various transcription factors together with a specialized chromatin structure. Chromatin is intimately related to the expression of eukaryotic genes *in vitro* and *in vivo* (8). Nucleosome assembly over a promoter is implicated in establishing the repression of many inducible genes (9-11). Although nucleosomes were often reported to globally repress transcription, the precise position of a nucleosome on a promoter can also improve transcriptional activation (12-15). Such a nucleosome, positioned *in vitro* on the promoter of the vitellogenin B1 gene in a region devoid of transcription factor binding sites (between -300 and -140 relative to the start site of transcription), potentiates estrogen receptor dependent transcriptional activation.

We have suggested that the wrapping of DNA around the histone octamer allows a better interaction between the distal estrogen receptor and proximal *trans*-activators (such as HNF3 and CTF/NF1) and/or the basal transcription machinery (14). Interestingly, HNF3 and NF1 are both able to modulate the position of a nucleosome (16-18) and HNF3 can replace linker histone on the serum albumin gene enhancer (19). Since HNF3 and NF1 bind to the vitellogenin B1 promoter, they may have important roles in the assembly of this regulatory DNA into organized chromatin structure *in vivo*.

In this study we have analyzed the interplay between the estrogen receptor and HNF3 on the vitellogenin B1 promoter within chromatin following microinjection into oocytes. We show that
HNF3 induces DNase I hypersensitivity within the proximal regulatory elements concomitant to
the activation of transcription. In addition, when expressed before chromatin assembly, HNF3
and the estrogen receptor activate transcription synergistically suggesting either that HNF3
establishes a chromatin structure favorable for subsequent association of the estrogen receptor
binding or that they interact directly or through a common target. We have determined the
positions of three nucleosomes over the vitellogenin B1 promoter in vitro. These nucleosomes do
not interfere with ER and HNF3 recruitment and are not displaced.

EXPERIMENTAL PROCEDURES

Plasmid constructs

The pBS(-596/+8)VgB1 plasmid was obtained by subcloning the BamHI/RsaI fragment of
pB1(-596/+8)CAT8+ (5,20) into pBluescript KS- BamHI/EcoRV sites. The subcloned fragment
contains the vitellogenin B1 (-598/+8) promoter sequence and about 0.1 kb of the CAT reporter
gene. The mutated promoters were prepared as follows. The pB1noERE plasmid was constructed
by site directed mutagenesis of the pBS(-596/+8)VgB1 leading to the introduction of point
mutations in all 3 EREs. The oligonucleotides used for the mutagenesis are shown hereafter with
the underlined mutated residues : (-338/-297) 5’CTC CAG A
CA CTG TGA A
CC AAC CCA
AGA TAT CAT A
AC CTC TTA-3’; (-560/-539) 5’TGG AGG GA
C ACA GG
G AAC ATG
C3’. The mutations were verified by DNA sequencing. Additional EREs were also subcloned
upstream of the TATA box to generate the construct pB1(6ERE). Briefly, 2 ERU oligos designed
from the wild type promoter (position -337 to -300), which contains two imperfect EREs, were flanked by AvaI sites (top strand: 5′TCG GGT CCA GTC ACTG TG ACC CAA CCC AAG TTA CTC TGA CTT C3′; bottom strand: 5′CCC GAG AGA TCA TGA TAA CTT GGG TTG GGT CAC AGT GAC TGG A3′). The annealed oligonucleotides were multimerized and then introduced into the BglII site (position -41) of pBS(-596/+8)VgB1. This generated one construct with 1 ERU and one ERE. Since the wild type promoter contains already 3 EREs, the mutated promoter was named pB1(6ERE). The single stranded DNAs were prepared from phagemids induced with the VCS M13 helper phage (21).

The plasmids used for in vitro mRNA transcription were prepared as follows. The xERα coding sequence was amplified from pKCR2xERα (22) by PCR using oligonucleotides generating XbaI and PstI sites. The PCR fragment was then introduced into the same sites of the plasmid pSP64(polyA) (Promega) to generate the xERpSP64(polyA) construct. rHNF3αpMS(II) plasmid was obtained after introduction of the SacI/HindIII fragment (from pRB-HNF3α, gift from K.S. Zaret) into the same sites of pMS(II) which is a modified version of pSP64(polyA) containing additional unique restriction sites after the poly(A) segment (Melissa Stolow, unpublished).

In vitro mRNA synthesis and microinjection of Xenopus oocytes

Constructs for in vitro mRNA synthesis were linearized either with EcoRI (xERpSP64(polyA)) or with AvrII (rHNF3αpMS(II)). The mRNA in vitro synthesis was carried out with the SP6 mMessage mMACHINE Kit (Ambion) according to the recommended procedure. The isolation procedure of Xenopus laevis oocytes was performed as previously described (23). Stage VI oocytes were microinjected (13.8 nl)(Drummond nanoject) with the indicated amount of in vitro
transcribed mRNA. After an overnight incubation in MBSH buffer (23) at 18°C, the oocytes nuclei (GV) were microinjected with the pBS(-596/+8)VgB1 as ssDNA (1ng). The oocytes were processed as described below generally after 6 hours incubation at 18°C.

Transcription assay and DNA recovery

A group of 15 to 20 oocytes was collected per sample and homogenized in 10 µl per oocyte of 0.25M Tris-HCl at pH 8.0. The equivalent of 10 oocytes was used for RNA purification and transcription analysis and the remaining 5 oocytes equivalent was used for DNA recovery. Total RNA was prepared from the homogenate using the RNazol™ method (Tel-Test Inc.). The RNazol™ reagent (500 µl) together with chloroform (50 µl) was added to the homogenate (100 µl) which was then incubated on ice for 15 minutes before centrifugation. The clear supernatant was then transferred to a fresh tube and precipitated with 1 volume of isopropanol. The washed RNA pellet (70% EtOH) was resuspended in 100 µl of TE buffer and precipitated once again for at least one hour at 4°C with 30 µl of 10M LiCl. The RNA pellet was then washed with EtOH 70% and dissolved in 20 µl of DEPC-treated water. Half of the RNA sample (5 oocytes) was analyzed by primer extension.

The RNA (10 µl) was supplemented with 10 µl of a first strand synthesis buffer (2x, provided by Gibco-BRL), 5mM DTT and end-labeled CAT-(5GGT GGT ATA TCC AGT GAT TTT TTT CTC CAT3) and H4-(5GGC TTG GTG ATG CCC TGG ATG TTA TCC3') primers (0.2 pmole each). The H4 primer is used as an internal control and detect the endogenous histone H4 mRNA. Annealing occurred for 10 minutes at 65°C, 30 minutes at 55°C, 20 minutes at 37°C and 5 minutes at room temperature. The reverse transcription reaction was carried out at 42°C upon
addition of 1 µl 10 mM dNTPs and 100 Units of the Superscript”II RT (Gibco-BRL). The reaction was stopped after one hour by ethanol precipitation. The extension products were resolved on a 8% sequencing gel and visualized by autoradiography.

DNA was recovered from the remaining 5 oocytes equivalents of the homogenate (see above). DNA recovery after injection is a control monitoring the microinjection efficiency. This DNA was also used for the supercoiling assay (see below). One volume of 1% SDS and 20mM EDTA pH 8.0 was added to the sample which was then treated for 2 to 3 hours with Proteinase K (200 µg/ml) at 55°C. DNA was then extracted twice with phenol/chloroform and precipitated at room temperature with isopropanol. The washed DNA pellet (EtOH 70%) was then dissolved in 100 µl of TE buffer, treated with RNase A (100 µg/ml) for 1 hour at 37°C, extracted with phenol/chloroform and finally isopropanol precipitated. The recovered DNA was quantitatively analyzed by Southern Blot as described (21). Blots were then probed with random-primed labeled ssDNA from the vitellogenin B1 promoter.

DNase I hypersensitive site analysis

After injection, groups of 20 to 25 oocytes were collected and homogenized in 300 µl of digestion buffer (20 mM Tris-HCl pH 8.0, 50mM NaCl, 2mM MgCl2, 1mM EDTA, 1mM DTT and 5% glycerol). The homogenate was divided into three 100 µl fractions and digested for 1 minute (room temperature) with respectively 160 U, 120 U and 80 U of DNase I (Gibco-BRL). The reactions were stopped with 2 volumes of 1% SDS and 20mM EDTA and treated for 1 hour at 37°C with RNase A (100 µg/ml). The samples were then incubated with Proteinase K (200 µg/ml) for at least 3 hours. DNA was finally purified as described above (see Transcription assay and
DNA recovery). The DNA pellets were resuspended in 50µl of 1x BamHI buffer and digested with 10 U of BamHI for 2 hours (Gibco-BRL). The samples were then once again extracted with phenol/chloroform and isopropanol precipitated. The DNA fragments were resolved on a 1.5 % agarose gel, blotted to a Zeta-probe® GT membrane (BioRad) and probed with a random-primed labeled promoter fragment (-596/-445). As a control, free dsDNA (2ng per oocyte) was mixed with non injected oocytes and treated immediately with DNase I under the same conditions described above.

Micrococcal nuclease assay

The injected oocytes (20 per sample) were homogenized in 200 µl of MNase digestion buffer (10 mM Hepes pH 8.0, 50mM KCl, 2mM MgCl₂, 3mM CaCl₂, 1mM DTT, 0.1 % NP-40 and 8 % glycerol) and split into four tubes (50 µl aliquots). Increasing amounts of Micrococcal nuclease (Worthington) were added into the different aliquots (0.04, 0.2, 1 and 5 U) and digestion was allowed to proceed at room temperature for 20 minutes. The reaction was stopped with 1 volume of 1 % SDS and 20 mM EDTA pH 8.0. DNA was then purified according to the method described above (see Transcription assay and DNA recovery). The fragments following digestion were resolved on a 1.5 % agarose gel in 1x TBE and blotted into a Hybond-N+ membrane (Amersham) as indicated (21). The membrane was then probed with a random-primed labeled probe corresponding to the vitellogenin B1 promoter (region -596/+139).

Supercoiling assay for chromatin disruption

The supercoiling assay was performed according to a method described earlier (24). Generally,
the equivalent of DNA recovered from one injected oocyte was loaded onto a 1.1 % agarose gel in 1xTPE buffer (40mM Tris, 30mM NaH$_2$PO$_4$, 10mM EDTA). Chloroquine (90µg/ml) was included in the gel and in the running buffer. Under these conditions the different topoisomers will be positively supercoiled, reflecting the differences in negative supercoiling depending on the original variations in nucleosomes density on the minichromosomes. Electrophoresis was performed overnight in the dark at 3.5V/cm. DNA was subsequently blotted onto a Hybond-N+ membrane (Amersham) by capillary transfer as described (21). The membrane was then probed with random-primed labeled ssDNA from the vitellogenin B1 promoter.

Nucleosome reconstitution

Nucleosomes were reconstituted on radiolabelled DNA fragments by salt/urea dialysis as described earlier (25). After reconstitution, the oligonucleosome cores were loaded on 10 to 30% sucrose gradients and centrifuged for 24 hours at 38000 rpm at 4°C in a Beckman SW41 rotor. Fractions were collected and analyzed in 0.5X TBE nucleoprotein agarose (0.7%) gels. Fractions containing trinucleosomes and above were pooled together, concentrated through a Microcon-30 (Amicon), dialized overnight at 4°C against 10mM Tris-HCl (pH 7.5), 0.1mM EDTA, 1mM 2β-mercaptoethanol and then concentrated to 20µg/ml. Samples were stored at 4°C until use. Chicken erythrocyte oligonucleosomes were prepared after removal of linker histone (26) and used as control for isolation of the native trinucleosome complexes.

Micrococcal nuclease mapping

Reconstituted chromatin (100ng of DNA) was digested with 0.01, 0.02 and 0.04 U of MNase
(Worthington) for 5 minutes at 22°C in the presence of 0.5mM CaCl₂. Digestions were started by concomitant addition of MNase and CaCl₂ and stopped by addition of 5mM EDTA, 0.25% (w/v) SDS and 1mg/ml proteinase K. After phenol/chloroform extraction and precipitation, the recovered DNA was 5’ end-labeled with \([\gamma^{32}P]ATP\) and T4 polynucleotide kinase. End-labeled fragments were separated by electrophoresis in non denaturing 6% polyacrylamide gels. DNA fragments of nucleosome core and chromatosome products were recovered and digested with restriction enzymes to determine microccocal nuclease cleavage sites. The digestions were analysed on 8% denaturing gel.

Gel Mobility Retardation Assay

Recombinant HNF3α was purified by affinity chromatography from SF9 cells infected with an HNF3 recombinant baculovirus generated by the BaculoGold kit (Pharmingen). Purified hER was purchased at Panvera. Binding reactions were performed in a 15 µl final volume of 1X binding buffer (10mM Tris-HCl pH 8.0, 5mM MgCl₂, 50mM NaCl, 150µg/ml BSA, 100µg/ml polydIdC) with 100 fmoles of labeled DNA and 300 fmoles of HNF3α or hER. Protein-DNA complexes were separated on a 0.25X TBE nondenaturing agarose gel.

DNaseI Footprinting

Binding reactions were performed as described above, except that polydIdC was omitted in the
binding buffer. DNaseI digestions were carried out at a final MgCl₂ concentration of 2.5mM. Naked DNA was treated with 1U/ml of DNaseI for 20 seconds and reconstituted DNA with 10U/ml for 40 seconds. Reactions were stopped by addition of EDTA to 10mM and phenol/chloroform extraction. Footprints were analyzed by denaturing gel electrophoresis. For these experiments, E. Coli expressed and purified HNF3 was kindly provided by Dr K. Zaret.
RESULTS

Regulation of the vitellogenin B1 promoter by ERα and HNF3α

The estrogen receptor is only one of several transcription factors that regulate vitellogenin B1 gene expression. The basal promoter element (BPE, region -119/-32) encompasses multiple binding sites for liver-enriched factors like HNF3 and C/EBP and for the ubiquitous CTF/NF1 (Figure 1A) (7). The contribution of these proteins has been already analyzed in vitro or by transient transfection (20,27). However these assays provide limited insights on how these transcription factors might function within chromatin. We have therefore decided to take advantage of the Xenopus oocyte microinjection system to examine this issue. Microinjection of DNA into the nucleus of Xenopus leads to chromatin assembly (23) which has been shown to influence the transcriptional regulation of the Xenopus HSP70 and TRβ promoters (11,28). Oocytes also provide an efficient translational machinery which allows the study of the regulation of a given promoter by expressing specific transcription factors (29,30) by injecting mRNA encoding these components either before or after chromatin assembly.

The capacity of the intact ERα to activate transcription was tested after injection of a CAT reporter template fused to the vitellogenin B1 promoter (-596/+8), which contains three estrogen responsive elements (ERE) (Figure 1A). Since chromatin assembly occurs with more rapid kinetics on microinjected ssDNA during second strand synthesis in comparison to the injection of dsDNA (23), we made use of ssDNA template. The ssDNA was injected after the expression of
ERα, meaning that the receptor was present during chromatin assembly. In contrast to an earlier report (31) we find that the exogenous ERα activates transcription in injected oocytes (Figure 1B, compare lane 1 to lanes 3 and 4). In this particular experiment the addition of 17-β-estradiol (E2) did not stimulate transcription further (Figure 1B, lane 4). This is not surprising since mature oocytes already contain enough endogenous estradiol to ensure receptor activity (32,33). Alternatively, ligand-independent activation may be obtained through the receptor AF-1 activation domain (34) or through the interaction with other proteins such as cyclin D1 (35,36). In few other experiments, a modest additional two-fold stimulation by exogenous estrogen was observed reflecting probable variations among oocytes batches.

The liver-enriched transcription factor HNF3 has multiple binding sites on the proximal region of vitellogenin B1 promoter (Figure 1A) (7). One site lies just downstream of the nucleosome positioning sequence (14). HNF3 (37) significantly stimulates transcription when synthesized before chromatin assembly (Figure 1B, lanes 5 and 6). Co-injection of both HNF3 and ERα before chromatin assembly resulted in the strongest activity observed indicating that they might cooperate to facilitate transcription (Figure 1B, lanes 7 and 8). This demonstrates that the assembly of the promoter into chromatin does not prevent its capacity to regulate transcription.

Several lines of evidence from previous studies have suggested that NF1 somehow influences the activity of a chromatin template when the transcription factor is present during chromatin assembly. Depletion of an NF1-like activity reduces transcription by 5 fold from the vitellogenin B1 promoter during the assembly of chromatin in *Xenopus* oocyte extract (38). Moreover, the CTF/NF1 proline rich activation domain was shown to interact with the core histone H3 in yeast and mammalian cells in response to TGFβ (18). Interestingly, one NF1 DNA binding element is
located downstream of the positioned nucleosome in the vitellogenin B1 promoter (Figure 1A). NF1 cannot interact \textit{in vitro} with nucleosomal DNA over the MMTV promoter regardless of its binding site orientation and position (39). It was therefore important to test NF1 ability to affect transcription when expressed either before or after chromatin assembly. In our microinjection assay however, expression of NF1 had no effect on transcription in the oocyte nucleus (data not shown).

In summary, these results indicate that HNF3 and ER\(\alpha\) are both competent to activate transcription from a chromatin template when present during or after chromatin assembly (Figure 1B and data not shown). The crystal structure of the DNA binding domain of HNF3 has striking similarities with the structured globular domain of the linker histone H5 (40,41). Interestingly HNF3 can direct the assembly of a nucleosome on the serum albumin enhancer where it is able to replace the linker histone H1 \textit{in vivo} (16,17,19). Its presence during chromatin assembly process might therefore modify the nucleosomal organization on the vitellogenin B1 promoter. This suggests either that the vitellogenin B1 promoter chromatin organization is already preset for activation or that HNF3 may participate in nucleosomes remodeling, directly or indirectly. We next tested the role of HNF3 in the organization of chromatin structure directly.

HNF3 induces strong DNase I hypersensitive sites on the vitellogenin B1 promoter.

DNase I hypersensitive sites generally reflect the stable association of transcription factors with
nucleosomal DNA. This increased accessibility also correlates with the activation of transcription. We have investigated DNase I accessibility to the vitellogenin B1 promoter upon expression of ERα and HNF3 during chromatin assembly (Figure 2).

The vitellogenin B1 promoter is relatively resistant to DNase I digestion after injection as ssDNA into the *Xenopus* oocyte nuclei (Figure 2, compare lanes 1-3 to free dsDNA which was mixed with non injected oocytes shortly before DNase I treatment, lanes 13-15). The expression of ERα leads to no major transition in DNase I cleavage. There is a very weak cleavage by DNase I upstream of the -300 region potentially reflecting a low efficiency of ERα association with the ERU (Figure 2, lanes 4 to 6 and 10 to 12).

The strongest hypersensitive site is observed upon HNF3 expression and extends from the start site of transcription to the downstream boundary of the *in vitro* positioned nucleosome around -140 (Figure 2, lanes 7 to 9) (14). We speculate that the presence of HNF3 leads to chromatin modification and facilitates the interaction of ERα with the ERU.

Gene activation is often associated with an alteration or transition in chromatin structure. Such potential chromatin alterations on the vitellogenin B1 promoter were tested by monitoring DNA topology and by micrococcal nuclease digestion (MNase). Single stranded DNA was co-injected into the oocytes and chromatin assembly on the replicated duplex DNA was analyzed 6 hours later (Figure 3A). MNase treated chromatin samples were blotted to a nylon membrane and were probed with a DNA sequence encompassing the whole promoter region and downstream sequences (-596/+139). The MNase ladder observed in the absence of ERα is consistent with the assembly of spaced nucleosomes (Figure 3B, compare panels 1 and 3). The definition of the
nucleosomal repeat is reduced upon expression of ERα in the presence of estradiol (Figure 3B, panel 2). Such a loss of nucleosomal repeat is generally interpreted as representative of nucleosome disruption (42,43). However the nucleosomal repeat does not entirely disappear in this assay. We suggest that the vitellogenin B1 promoter segment containing EREs shows evidence of limited chromatin reorganization dependent on the activity of ligand-bound estrogen receptor as revealed using micrococcal nuclease.

The loss of nucleosomes or the alteration in the path of DNA around an histone octamer can be estimated from a supercoiling assay. DNA topoisomers can be resolved in agarose gels containing chloroquine (44). In our experimental conditions the loss of nucleosomes from the injected template in the oocytes will generate DNA molecules with distinct topologies compared to unaltered chromatin templates. We have injected a mutated vitellogenin B1 promoter containing an insertion of 3 additional estrogen response elements (ERE) upstream of the TATA box. Since the wild type promoter contains 3 EREs, this insertion will generate a construct with 6 EREs totally. Our data show that the expression of ERα before chromatin assembly leads to the loss of nucleosomes from the minichromosomes (Figure 3C, compare lanes 5 and 6 with lanes 7 and 8). The global change of topology is not dramatic and densitometric scans indicate the loss of only two to three nucleosomes. The addition of estradiol leads to a further small topological change towards loss of superhelicity. These data also indicate that changes in DNA topology is not proportional to levels of transcription since the alterations of topology are similar between both promoter constructs despite of the huge transcriptional activity (over 100 fold activation) observed with the promoter containing 6 EREs (Figure 3D, lanes 7 and 8). Surprisingly, the
introduction of point mutations in every single EREs of the wild type promoter did not prevent 
the receptor dependent change of DNA topology (Figure 3C, lanes 1 to 4). Thus, our MNase 
digestion and topology assays (Figure 3) suggest that a substantial alteration of the chromatin 
environment, dependent on the EREs, is not required for the activation of the vitellogenin B1 
promoter by ERα.

In vitro nucleosome mapping over the vitellogenin B1 promoter

The lack of dramatic chromatin remodeling dependent on the estrogen receptor is consistent with 
the receptor binding in a linker region as predicted by the in vitro mapping of a nucleosome 
downstream of the ERU and functioning most effectively within a nucleosomal environment (14). 
In order to gain more insight on the link existing between transcription and nucleosome 
positioning we decided to map nucleosomes in vitro over the entire promoter area. We also 
analysed the impact of linker histones on the position of nucleosomes.

We reconstituted nucleosome cores on a 724 bp template containing the vitellogenin B1 promoter 
(-596/+8) (Figure 4). Reconstituted chromatin was then fractionated on a sucrose gradient 
(Figure 4A). Fractions containing trinucleosomes were subsequently pooled and as a control, an 
 aliquot was refractionated on a second sucrose gradient with MNase treated erythrocyte 
chromatin (Figures 4B and 4C). Next we made use of these reconstituted templates to map the 
position of nucleosomes (Figure 5). DNA fragments from core particles were eluted from the 
polyacrylamide gel after MNase treatment and digested with a set of endonucleases in order to
map the translational positioning of nucleosomes relative to the vitellogenin B1 promoter sequences (Figure 5). Although there is no strong translational positioning of any nucleosomes, it is possible to distinguish several positions despite of the relative nucleosome mobility along the promoter (See Figure 5B for a summary). Two nucleosomes with prefered translational positionings are located over the TATA-box (-83/+63) and between the ERU and the upstream ERE (-522/-376) (thick arrows in Figure 5B). In a series of additional experiment, different fragments of the vitellogenin B1 promoter were reconstituted and analysed under the same conditions as above. Globally, the results obtained are similar than those observed with the full length promoter (data no shown). No strong positioning signal could however be mapped in the region -300/-140 where a mononucleosome was first described (14,45). Weak bands indicates that the edge of a nucleosome (-359/-213) covers the ERU (-334/-302) (Figure 5A, see Ssp I digestion). The lack of strong positions over this promoter area suggests that this third nucleosome is extremely mobile. Linker histones were shown to restrict nucleosome mobility on the *Xenopus* somatic 5S RNA gene correlating with transcriptional repression (25). We have also found that the inclusion of histone H1 on the reconstituted vitellogenin B1 promoter has no apparent influence on the multiplicity of nucleosome positions (data not shown).

We conclude that nucleosomes have multiple positions over the vitellogenin B1 promoter with two preferences for the regions (-522/-376) and (-83/+63) *in vitro*. Importantly the estrogen responsive elements are devoid of nucleosomes, except for one weak position over the ERU. This predicts that the estrogen receptor would be able to bind onto the promoter within chromatin. Similarly, one HNF3 recognition sites (-125/-119) is free of nucleosomes. However, two HNF3
DNA binding elements (-81/-75 and -58/-52) are embedded into the TATA-box nucleosome.

The estrogen receptor and HNF3 associate with the vitellogenin B1 promoter within chromatin

The nucleosome mapping described above is consistent with HNF3 and ER binding to internucleosomic regions. Therefore, these transcription factors should gain access to the promoter without difficulty. We have tested their DNA binding ability on the reconstituted vitellogenin B1 promoter (-418/+150) (Figure 6). We show that purified ER and HNF3 are indeed able to interact with their cognate DNA binding elements within reconstituted chromatin. This interaction is specific since it is competed away by specific oligonucleotides and not by random sequences (Figure 6, lanes 11 to 18). The specific complexes shifted by ER and HNF3 are different between free and reconstituted DNA indicating that nucleosomes are not displaced (Figure 6, compares lanes 2 to 9 with lanes 11 to 18). We conclude that both proteins are competent for binding onto chromatin.

It is however necessary to perform DNase I footprinting experiments in order to determine whether ER and/or HNF3 interact with nucleosomes or in linker regions (Figure 7). The reconstituted vitellogenin B1 promoter shows a clear 10 bp modulation in DNase I representative of the presence of a rotationally positioned nucleosomes (Figure 7, compare lanes 1 to 4 with lanes 5 to 8). The nucleosome boundaries are indicated by arrows on the left side of Figure 7 (as determined earlier in Figure 5). The addition of HNF3 induces partial protection as well as appearance of DNase I hypersensitive sites on both free and reconstituted DNA (Figure 7, lanes 2
to 4 and 6 to 8). These protections correspond to the location of the previously identified HNF3 binding sites (7). One of these DNA binding sites (-125/-119) is located in a region devoid of nucleosomes whereas the other two sites (-81/-75 and -58/-52) lies within a nucleosome, apparently without disturbing it. Interestingly, others have suggested that HNF3 is able to modulate the position of a nucleosome (16,17). Although we do not have such evidence for the vitellogenin B1 promoter, it is noteworthy that the -85/-75 HNF3 binding site maps precisely to the upstream edge of a nucleosome.
DISCUSSION

The vitellogenin B1 promoter was extensively analyzed in the past using either transient transfection or in vitro transcription assays (5,20) but little is known about its regulation in a chromatin context. Early experiments have analysed the effect of estrogen on chromatin opening in Xenopus and chicken liver nuclei (see discussion below). However, these previous studies gave no clear indications on the contribution of other hepatic nuclear factors. The Xenopus oocyte microinjection system allowed us to identify such a factor in a chromatin context. Here we show that the nuclear transcription factor HNF3 plays a major role in opening chromatin and helping hormonal regulation of the vitellogenin gene. We have also mapped the position of nucleosomes in vitro over the vitellogenin promoter in order to relate chromatin structure to transcription.

A function for HNF3 in opening the promoter of the vitellogenin B1 gene

HNF3 increases dramatically the accessibility of the promoter to DNase I from the start site of transcription to the downstream boundary of the nucleosome mapped in vitro (see Figure 2). This region contains several binding sites for HNF3. However, HNF3 has no recognition element on the vitellogenin B1 promoter upstream of this nucleosome and yet helps to promote DNase I accessibility in the ERU region. Although the ERU is about 180 bp apart from the closest HNF3 binding site, the folding of DNA around the histone octamer could bring them within a much
closer range (14). Alternatively, the increased DNase I accessibility over a long distance like observed on the vitellogenin B1 promoter, rather than on a discrete area, is consistent with the disruption of a higher order chromatin structure. Several lines of evidence suggest that HNF3 might influence the formation of higher order chromatin structures. First, the winged-helix domain of HNF3 is found in the linker histones H5 and H1 and they all share striking similarities (40,41,46). Second, HNF3 was shown to replace a linker histone on the edge of a positioned nucleosome on the serum albumin enhancer but was not able to compact internucleosomic DNA as does H1 or H5 (19). Although the Xenopus oocytes contain a linker histone variant (cleavage stage linker histone B4) (47), this protein binds to DNA with much reduced affinity compared to H1 (48) and it is likely that HNF3 would replace B4 in chromatin when a specific recognition site is present.

Since HNF3 is a liver-enriched transcription factor and that the vitellogenin B1 gene is expressed exclusively in hepatocytes, it is possible that HNF3 might preset the promoter for a rapid response to estrogen. A previous study indicated that a male liver extract contains all the specific factors involved in the derepression and induction on the vitellogenin B1 promoter, but sufficient ERα (49). More recently we have shown that the vitellogenin B1 proximal promoter is accessible to restriction enzyme in hepatocytes but not in erythrocytes where the gene is silent, indicating that chromatin structure is different in these cells (50). In the same study we could not detect any differences between estrogen induced females and uninduced males suggesting that a tissue-specific factor, not related to gender (possibly HNF3), is responsible for this defined chromatin structure. Furthermore, an in vivo footprinting study has revealed that the (-81/-70) HNF3 binding site is occupied in male and female hepatocytes but not in erythrocytes (7). As
importantly, it was shown that the amount of injected ER mRNA required to activate chromosomal vitellogenin genes in oocytes is reduced when receptor-free liver nuclear extracts are co-injected (33).

The vitellogenin B1 promoter is also regulated by the ubiquitous transcription factor CTF/NF1 which was reported to interact with the core histone H3 and to modulate the nucleosome position (18). However we observed neither an activation of transcription, nor an induction of DNase I hypersensitive site in the oocyte system (data not shown) even though NF1 was previously shown to activate transcription synergistically with ERα (27,51).

HNF3, nucleosome positioning and implications in the activation of the vitellogenin B1 gene.

HNF3 and ERα cooperatively activate transcription when present during chromatin assembly suggesting either that they somehow interact physically or that HNF3 helps the recruitment of ERα on a loosened chromatin environment. HNF3 was also implicated in directing the position of a nucleosome in vitro similar to that seen in vivo on the serum albumin enhancer (16,17).

In this study we show that HNF3 is indeed able to interact with chromatin in linker regions and on apparent nucleosomes without disturbing their positions. Therefore DNase I hypersensitivity (Figure 2) does not necessary reflect displacement of nucleosomes from DNA (11,52). HNF3, like TR/RXR generates a robust DNase I hypersensitive site without significant change in DNA topology (data no shown) suggesting that nucleosome numbers remain constant. Preferential DNase I cleavage might reflect direct recruitment by HNF3 by protein-protein contacts or local
disruption of chromatin folding. We propose that HNF3 has a dual function in the activation of
the vitellogenin B1 promoter. In a first step, HNF3 replaces the linker histones leading to a
decompaon of the chromatin fiber (19). In a subsequent step, HNF3 may prevent the sliding of
nucleosomes over important regulatory elements. The priming of the promoter by HNF3 during
liver differentiation (53) will elicit a rapid response to estrogen in females or hormone stimulated
males. It is likely that the nucleosome (-85/+60) positioned over the TATA box will be
significantly altered upon initiation of transcription but HNF3 is probably not involved directly in
this process since it does not displace this nucleosome (Figure 7). One can speculate that
additional cellular components would play an important role subsequent to the recruitment of ERα
to the promoter such as the SWI/SNF chromatin remodeling complex (54) or an histone
acyltransferase activity.

The estrogen receptor and alterations in chromatin structure

Our experiments indicate that ERα in isolation induces a weak DNase I hypersensitive site when
expressed during chromatin assembly (Figure 2). This is consistent with early in vivo
experiments showing that Xenopus vitellogenin genes from estrogen-treated male liver cells
display only a modest increase in DNase I sensitivity although transcription is efficient (55,56). A
more precise study of the chicken vitellogenin (VTG II) gene, which is already marked in liver by
internal and 3’ end hypersensitive sites, demonstrates the appearance of strong additional
hypersensitive sites in the 5’ end region of the gene upon estrogen treatment (57,58). It seems that
estrogen-related remodeling events are more important in chicken than in *Xenopus*, although we do not know whether this difference reflects more profound regulation specificities between both species. Importantly, the activation of the chromosomal *Xenopus* vitellogenin B2 gene in oocytes requires the expression of a huge excess of receptors in comparison to the level which is needed in a liver nucleus (33). However, co-injection of receptor-free liver nuclear extract significantly reduces the amount of receptor needed. This clearly suggests that the estrogen receptor does not easily activate transcription without other hepatic nuclear factors. This might also explain why estrogen induces strong DNase I hypersensitive sites in chicken liver but not in our oocytes experiments.

The estrogen receptor interacts with SWI2/SNF2 suggesting that the SWI/SNF complex participates in chromatin remodeling events (54). However, in the oocyte system, we could not detect a change of topology which depends directly on any of the described vitellogenin B1 promoter EREs (Figure 3) and the nucleosomal array is not dramatically altered by ERα. It is not clear whether the DNase I hypersensitive site stems only from the receptor binding to the promoter or from subsequent subtle chromatin alterations that are not detected in our crude assays. Activation of transcription by nuclear hormone receptors is believed to be mediated by a growing family of coactivators which includes SRC-1 (59), p300/CBP (60-62), ACTR (63) and P/CAF (64). All of the cofactors possess intrinsic histone acetyltransferase activity (63,65-67). Thus it is probable that part of ER dependent transcriptional activation of the vitellogenin B1 promoter will involve histone acetylation. The recent resolution of the nucleosome core particle has shown that one histone H4 tail makes intensive contacts with the face of an H2A/H2B dimer from an adjacent nucleosome (68). This is extremely relevent in the context of chromatin...
decompaction through acetylation.

Recently, two rotationally and translationally positioned nucleosomes were mapped in vivo on the human estrogen responsive pS2 promoter (69). Their positions were not affected by the estrogen receptor although the pS2 ERE is localized within the 5’ edge of one nucleosome. According to the in vitro assembly of tri-nucleosomes on the vitellogenin B1 promoter (Figure 5) the ERU lies in a linker region or at the edge of a nucleosome (-359/-213). Therefore, the receptor should gain access to the promoter without difficulty as we have seen with the gel shift experiments (Figure 6). Moreover, since the presence of this nucleosome in vitro potentiates activation of transcription by ERα (14), it seems unlikely that the receptor would favor a major chromatin perturbation in this region.

Unlike many promoters, the vitellogenin B1 gene does not require profound chromatin reorganisation at the nucleosomal level. We think however that local higher-order chromatin structure unfolding, probably by HNF3, is a key event in liver-specific activation of the vitellogenin B1 gene. Such a role for HNF3 in the regulation of a nuclear hormone regulated promoter is suggested for the first time.

ACKNOWLEDGMENTS

We thank K.S. Zaret for the kind gift of the HNF3 plasmid as well as for the bacterially expressed HNF3. We are grateful to A.K. Hihi and M. Vogelauer for helpful advice and critical reading of the manuscript. This work was supported by the Etat de Vaud, the Swiss National Science Foundation, the “Fondation du 450e Anniversaire” and the National Institutes of Health.
REFERENCES

1. Wahli, W., and Ryffel, G. U. (1985) *Oxf. Surv. Eukaryot. Genes* 2, 96-120

2. Tata, J. R., and Smith, D. F. (1979) *Recent Prog. Hormone Res.* 35(47), 95

3. Kumar, V., and Chambon, P. (1988) *Cell* 55(1), 145-156

4. Wang, H., Peters, G. A., Zeng, X., Tang, M., Ip, W., and Kahn, S. A. (1995) *J. Biol. Chem.* 270(40), 23322-23329

5. Seiler-Tuyns, A., Walker, P., Martinez, E., Mérillat, A.-M., Givel, F., and Wahli, W. (1986) *Nucleic Acids Res.* 14(22), 8755-8770

6. Wahli, W., Dawid, I. B., Wyler, T., Jaggi, R. B., Weber, R., and Ryffel, G. U. (1979) *Cell* 16(3), 535-549

7. Cardinaux, J.-R., Chapel, S., and Wahli, W. (1994) *J. Biol. Chem.* 269(52), 32947-32956

8. Wolfge, A. P. (1994) *Cell* 77(1), 13-16

9. Almer, A., Rudolph, H., Hinnen, A., and Hörz, W. (1986) *EMBO J.* 5(10), 2689-2696

10. Richard-Foy, H., and Hager, G. L. (1987) *EMBO J.* 6(8), 2321-2328

11. Wong, J., Shi, Y.-B., and Wolfge, A. P. (1995) *Genes Dev.* 9, 2696-2711

12. Lu, Q., Wallrath, L. L., Granok, H., and Elgin, S. C. (1993) *Mol. Cell. Biol.* 13(5), 2802-2814

13. Jackson, J. R., and Benyajati, C. (1993) *Nucleic Acids Res.* 21(4), 957-967

14. Schild, C., Claret, F. X., Wahli, W., and Wolfge, A. P. (1993) *EMBO J.* 12(2), 423-433

15. Stünkel, W., Kober, I., and Seifart, K. H. (1997) *Mol. Cell. Biol.* 17(8), 4397-4405

16. McPherson, C. E., Shim, E.-Y., Friedman, D. S., and Zaret, K. S. (1993) *Cell* 75, 387-
17. Shim, E. Y., Woodcock, C., and Zaret, K. S. (1998) *Genes Dev.* **12**, 5-10

18. Alevizopoulos, A., Dusserre, Y., Tsai-Pflugfelder, M., von der Weid, T., Wahli, W., and Mermod, N. (1995) *Genes Dev.* **9**(24), 3051-3066

19. Cirillo, L. A., McPherson, C. E., Bossard, P., Stevens, K., Cherian, S., Shim, E. Y., Clark, K. L., Burley, S. K., and Zaret, K. S. (1998) *EMBO J.* **17**(1), 244-254

20. Corthésy, B., Cardinaux, J.-R., Claret, F.-X., and Wahli, W. (1989) *Mol. Cell. Biol.* **9**(12), 5548-5562

21. Sambrook, J., Maniatis, T., and Fritsch, E. F. (1989) in *Molecular Cloning: A Laboratory Manual* (Press, C. S. H. L., ed), 2nd Ed. Ed., Cold Spring Harbour, NY

22. Weiler, I. J., Lew, D., and Shapiro, D. J. (1987) *Mol. Endocrinol.* **1**(5), 355-362

23. Almouzni, G., and Wolffe, A. P. (1993) *Genes Dev.* **7**(10), 2033-2047

24. Clark, D. J., and Wolffe, A. P. (1991) *EMBO J.* **10**(11), 3419-3428

25. Ura, K., Hayes, J. J., and Wolffe, A. P. (1995) *EMBO J.* **14**(15), 3752-3765

26. Lee, D. Y., Hayes, J. J., Pruss, D., and Wolffe, A. P. (1993) *Cell* **72**(1), 73-84

27. Chang, T., and Shapiro, D. (1990) *J. Biol. Chem.* **265**(14), 8176-8182

28. Landsberger, N., and Wolffe, A. P. (1995) *Mol. Cell. Biol.* **15**(11), 6013-6024

29. Wong, J., Shi, Y. B., and Wolffe, A. P. (1997) *EMBO J.* **16**(11), 3158-3171

30. Wong, J., Patterton, D., A., I., Gushin, D., Shi, Y.-B., and Wolffe, A. P. (1998) *EMBO J.* **17**(2), 520-534

31. Watson, C. S. (1991) *J. Steroid Biochem. Molec. Biol.* **38**, 423-431

32. Fortune, J. E. (1983) *Dev. Biol.* **99**, 502-509
33. McKenzie, E. A., Cridland, N. A., and Knowland, J. (1990) *Mol. Cell. Biol.* **10**(12), 6674-6682

34. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) *Cell* **59**(3), 477-487

35. Neuman, E., Ladha, M. H., Lin, N., Upton, T. M., Miller, S. J., DiRenzo, J., Pestell, R. G., Hinds, P. W., Dowdy, S. F., Brown, M., and Ewen, M. E. (1997) *Mol. Cell. Biol.* **17**(9), 5338-5347

36. Zwijsen, R. M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R. J. (1997) *Cell* **88**(3), 405-415

37. Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H., and Darnell Jr., J. E. (1990) *Genes Dev.* **4**, 1427-1436

38. Corthésy, B., Léonnard, P., and Wahli, W. (1990) *Mol. Cell. Biol.* **10**, 3926-3933

39. Blomquist, P., Li, Q., and Wrange, O. (1996) *J. Biol. Chem.* **271**(1), 153-159

40. Ramakrishnan, V., Finch, J. T., Graziano, V., Lee, P. L., and Sweet, R. M. (1993) *Nature* **362**, 219-223

41. Clark, K. L., Halay, E. D., Lai, E., and Burley, S. K. (1993) *Nature* **364**, 412-420

42. Tsukiyama, T., Becker, P. B., and Wu, C. (1994) *Nature* **367**, 525-532

43. Wall, G., Varga-Weisz, P. D., Sandaltzopoulos, R., and Becker, P. B. (1995) *EMBO J.* **14**(8), 1727-1736

44. Bates, A. D., and Maxwell, A. (1993) *DNA topology*. In Focus (Rickwood, D., and Male, D., Eds.), Oxford University Press Inc., New York

45. Tsai-Pfluggfelder, M., Gasser, S. M., and Wahli, W. (1998) *Mol. Endocrinol.* **12**, 1525-
46. Cerf, C., Lippens, G., Ramakrishnan, V., Muyldermans, S., Segers, A., Wyns, L., Wodak, S. J., and Hallenga, K. (1994) *Biochemistry* **33**(37), 11079-11086

47. Smith, R. C., Dworkin-Rastl, E., and Dworkin, M. B. (1988) *Genes Dev.* **2**(10), 1284-1295

48. Ura, K., Nightingale, K., and Wolffe, A. P. (1996) *EMBO J.* **15**(18), 4959-4969

49. Corthésy, B., Claret, F. X., and Wahli, W. (1990) *Proc. Natl. Acad. Sci. USA* **87**(20), 7878-7882

50. Marilley, D., Robyr, D., Schild-Poulter, C., and Wahli, W. (1998) *Mol. Cell. Endo.* **141**, 79-93

51. Martinez, E., Dusserre, Y., Wahli, W., and Mermod, N. (1991) *Mol. Cell. Biol.* **11**(6), 2937-2945

52. Wong, J., Li, Q., Levi, B. Z., Shi, Y. B., and Wolffe, A. P. (1997) *EMBO J.* **16**(23), 7130-7145

53. Zaret, K. (1998) *Curr. Opin. Genet. Dev.* **8**, 526-531

54. Ichinose, H., Garnier, J. M., Chambon, P., and Losson, R. (1997) *Gene* **188**(1), 95-100

55. Gerber-Huber, S., Felber, B. K., Weber, R., and Ryffel, G. U. (1981) *Nucleic Acids Res.* **9**(11), 2475-2494

56. Williams, J. L., and Tata, J. R. (1983) *Nucleic Acids Res.* **11**(4), 1151-1166

57. Burch, J. B., and Weintraub, H. (1983) *Cell* **33**(1), 65-76

58. Burch, J. B. E., and Evans, M. I. (1986) *Mol. Cell. Biol.* **6**(6), 1886-1893

59. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1995) *Science* **270**(5240),
1354-1357

60. Chakravarti, D., Lamorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. (1996) *Nature* **383**(6595), 99-103

61. Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996) *Proc. Natl. Acad. Sci. USA* **93**(21), 11540-11545

62. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell* **85**(3), 403-414

63. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90**(3), 569-580

64. Blanco, J. C. G., Minucci, S., Lu, J., Yang, X.-J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) *Genes Dev.* **12**, 1638-1651

65. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1997) *Nature* **389**(6647), 194-198

66. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87**(5), 953-959

67. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) *Nature* **382**(6589), 319-324

68. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) *Nature* **389**, 251-260

69. Sewack, G. F., and Hanssen, U. (1997) *J. Biol. Chem.* **272**(49), 31118-31129
FIGURE LEGENDS

Figure 1
HNF3 and ERα activate transcription synergistically in oocytes. (A) Schematic representation of the 5′ flanking region of the vitellogenin B1 promoter, subcloned upstream of a fragment of the CAT gene reporter (construct pBS(-596/+8)VgB1). The promoter is composed of one estrogen responsive unit (ERU) and one estrogen responsive element (ERE3). Previous work have shown the position of a nucleosome in vitro between the ERU and the NRE (negative regulatory element; -133/-119) (14,20). The basal promoter element (BPE; -119/-74) and the NRE encompass numerous cis-acting elements for the transcription factors CTF/NF1 (gray oval), HNF3 (dark rectangles) and C/EBP (black dot) (7). Additional putative CTF/NF1 recognition elements are located upstream of the nucleosome. (B) Groups of oocytes were injected with 3ng of ERα or HNF3 mRNA before chromatin assembly which occurs after injection of single stranded pBS(-596/+8)VgB1 (1ng) as indicated in the cartoon. Oocytes were incubated in the presence or absence of 50nM estradiol (E2) as indicated, shortly after the second injection. Transcription was assayed by primer extension 6 hours later. The positions of the vitellogenin B1 transcripts (VitB1) and of the endogenous H4 mRNA (internal control) are indicated.

Figure 2
HNF3 induces the formation of DNase I hypersensitive sites on the vitellogenin B1 promoter. Groups of oocytes were injected with 3ng of ERα or HNF3 mRNA before chromatin assembly as in Figure 1. Oocytes were incubated in the presence or absence of 50nM E2 as indicated, shortly
after the second injection. Samples were treated with increasing amounts of DNase I (80 U, 120 U or 160 U) as described in Materials and Methods. The free dsDNA control (pBS(-596/+8)VgB1) was mixed with uninjected oocytes and treated immediately with DNase I under the same conditions. The purified DNA was then subjected to BamHI restriction (-596) and after transfer, the filters were hybridized with a 151 bp probe (-596/-445). On the left side are indicated the various positions within the vitellogenin B1 promoter (see also Figure 1A). The arrowheads point to the DNase I hypersensitive sites.

Figure 3
ERα and chromatin disruption. (A) Groups of oocytes were injected with 3ng ERα mRNA as indicated. After an overnight incubation, ssDNA (1ng of pBS(-596/+8)VgB1) was injected and the ERα expressing oocytes were incubated with 50 nM E2. Then, 6 hours later, collected oocytes were assayed for MNase, DNA topology and transcription. (B) The global nucleosomal array from the whole plasmid is weakly disturbed by ERα when present during chromatin assembly. The injected oocytes were treated with increasing amounts of MNase (0.04U, 0.2U, 1U and 5U). The free DNA control (lane 3) corresponds to dsDNA (pBS(-596/+8)VgB1) mixed with uninjected oocytes (2ng per oocyte) shortly before MNase treatment. After DNA transfer, the filter was hybridized with a random-primed labeled promoter probe encompassing region -596/+139. Lane 3 is a shorter exposure of the same filter. (C) ERα does not alter the DNA topology through the EREs of the vitellogenin B1 promoter. Two promoter constructs were injected as indicated in (A) together with [α-32P]-dCTP (0.1 µCi/oocyte). These constructs contain either no ERE (point mutations introduced in all EREs of the wild type promoter pBS(-
596/+8) VgB1) or 6 EREs (3 additional EREs corresponding to the wild type ERU and one ERE subcloned upstream of the TATA box). DNA was purified from each group and the topoisomers distribution was analyzed in a chloroquine agarose gel as described in Materials and Methods. Lanes 9 to 12 are profiles of the corresponding lanes 5 to 7. (D) Transcriptional activation of the various promoter mutants. Groups of oocytes from the very same injection experiments used in (C) were analyzed by primer extension. The positions of the vitellogenin B1 transcripts (VitB1) and of the endogenous H4 mRNA (internal control) are indicated.

Figure 4
Preparation and characterization of nucleosome cores reconstituted on the vitellogenin B1 promoter. The fragment used in figures 4, 5, and 7 is composed of the vitellogenin B1 promoter (-596/+8) and of a portion of the chloramphenicol acetyl transferase (CAT) gene (-30/+90). (A) Radiolabelled DNA templates were reconstituted with increasing amounts of purified core histones (molar ratio of core histones to DNA: 0.8, 1 and 1.2). The products were fractionated on a 10-30% sucrose gradient and each fraction was analyzed by nucleoprotein gel electrophoresis. (B/C) Purified nucleosome cores co-sediment with native trinucleosome complexes. The fraction containing tri or more nucleosomes were combined and refractionated on sucrose gradient with unlabelled chicken erythrocyte oligonucleosomes (B). DNA from each fraction was run on a 1.5% agarose gel and stained with ethidium bromide. M, low DNA size marker. Fraction 1 corresponds to the bottom of the sedimentation gradient. Location after sedimentation of free DNA and mono-, di- or trinucleosomes are indicated. (C) Autoradiogram of the gel in (B) showing the position of the reconstituted nucleosome fragment in the gradient.
Figure 5
Micrococcal nuclease mapping of core and chromatosome positions along the vitellogenin B1 promoter fragment. (A) DNA from core particles (prepared from MNase digested trinucleosome complexes) was eluted from a preparative acrylamide gel (not shown) and digested with restriction enzymes in order to define the positions of the histones-DNA particles. Dots, white or black arrows represent the multiple positions occupied by the histone octamers on the promoter. These positions are pointed on the schematic diagram (B) by the same symbols marked with an asterisk. Digestions in lanes 1 to 8 refer to the particles located in the vicinity of the AccI and AvaII restriction sites whereas lanes 9 to 12 and lanes 13 to 15 correspond respectively to the SspI/DraI and BglII/AvaI promoter regions. The following enzymes were used: PvuII (P), AccI (A), AvaII (Av), AlwNI (Al), BspHI (Bs), SspI (Ss), DraI (D), StyI (St), BglII (B) and AvaI (A1). Thick arrows, depicted in the diagram (panel B), represent the major core or chromatosome positions. Black, dotted and dashed boxes correspond to the EREs and HNF3 or NF1 DNA binding sites respectively. The TATA-box is displayed as a white box.

Figure 6
ER and HNF3 interact with the reconstituted -418/+150 vitellogenin promoter fragment. Free (lanes 1 to 9) or reconstituted (lanes 10 to 18) DNA was incubated with either purified ER (lanes 2 to 5 and 11 to 14) or HNF3 (lanes 6 to 9 and 15 to 18). Binding was performed either in absence (ER, lanes 2 and 11; HNF3, lanes 6 and 15) or in presence of oligonucleotide competitors (ER, lanes 3 to 5 and 12 to 14; HNF3, lanes 7 to 9 and 16 to 18). erre (consensus
estrogen responsive element: AGC TTA GTT TAT TGG GTC ACA GTG ACC TTA CCA CAA GGA); ere* (mutated ere: AGC TTA GTT TAT TGG AAC ACA GTG TTC TTA CCA CAA GGA); h (HNF3 transthyretin binding site: GTT GAC TAA GTC AAT AAT CAG AAT CAG); h* (mutated HNF3 transthyretin binding site: GTT GAC AAA GTC AAT AAT CAG AAT CAG); r (random sequence: CAC AGT TGG CAC AGT GCC AAA AGC C). The different shifted complexes are indicated on the left of the figure. The open and solid arrows represent the binding of HNF3 and ER to free DNA respectively, whereas the tailed arrow corresponds to the interaction of either ER or HNF3 with a nucleosome.

Figure 7
DNase I footprinting of HNF3 on the reconstituted vitellogenin B1 promoter (-596/+150). Increasing amounts of purified HNF3 proteins (0.3, 0.6 and 1.2 pM) were added to 100ng of either naked (lanes 2 to 4) or reconstituted (lanes 6 to 8) DNA. Lanes 1 and 5 are control reactions for free and reconstituted DNA. Arrows represent the core particle positions along the vitellogenin B1 promoter and brackets correspond to the DNA binding sites protected by HNF3. M, Msp1-digested pBR322 size marker.
