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Electron microscopic visualization of protein–DNA interactions at the estrogen responsive element and in the first intron of the *Xenopus laevis* vitellogenin gene

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Stable protein–DNA complexes can be assembled *in vitro* at the 5′ end of *Xenopus laevis* vitellogenin genes using extracts of nuclei from estrogen-induced frog liver and visualized by electron microscopy. Complexes at the three following sites can be identified on the gene B2: the transcription initiation site, the estrogen responsive element (ERE) and in the first intron. The complex at the transcription initiation site is stabilized by dinucleotides and thus represents a ternary transcription complex. The formation of the complexes at the two other sites is enhanced by estrogen and is reduced by tamoxifen, an antagonist of estrogen, while this latter effect is reversed by adding an excess of hormone. No sequence homology is apparent between the site containing the ERE and the binding site in intron I and functional tests in MCF-7 cells suggest that these two sites are not equivalent. Finally, we made use of previously characterized deletion mutants of the 5′ flanking region of the gene B1, a close relative of the gene B2, to demonstrate that the 13-bp palindromic core element of the ERE is involved in the formation of the complexes observed upstream of the transcription initiation site.

Key words: electron microscopy/DNA bending/liver nuclear extract/MCF-7 cells/protein–DNA complexes/CAT assay

Introduction

The regulation of gene expression in differentiated cells is achieved in a variety of ways. The induced or repressed state of certain genes can be very stable while the transcriptional control of others can be modulated in response to specific signals, such as hormones. In all cases it seems likely that protein–protein and protein–DNA complexes are intimately involved. Cell-free extracts, especially those capable of initiating transcription *in vitro*, have proven useful to identify a number of general and specific transcription factors (for reviews see Dynan and Tjian, 1985; McKnight and Tjian, 1986). Recently tissue-specific transcription has been achieved *in vitro*, thus demonstrating the possibility of analyzing the mechanisms of the control of differential gene activity by *cis*- and *trans*-acting factors (Tsuda and Suzuki, 1981; Bazett-Jones *et al.*, 1985; Schöler and Gruss, 1985; Gorski *et al.*, 1986; Suzuki *et al.*, 1986).

We have chosen the 5′ end region of *Xenopus laevis* vitellogenin genes to study interactions of DNA with *trans*-acting factors. The vitellogenin genes are expressed in the liver in response to estrogen stimulation (for reviews on this system see Wallace, 1985; Wahli and Ryffel, 1985). Comparative analysis of the 5′ end region of the *Xenopus* and chicken genes has revealed regions of homology at similar positions (Walker *et al.*, 1983, 1984). The core of one of the blocks of homology is a 13-bp palindromic element GGTCANNNTGACC that is also present upstream of the transcription initiation site of the apo-VLDLII gene, another liver estrogen-responsive gene. This element has recently been identified as an essential part of the vitellogenin estrogen responsive element (ERE) by transient expression of vit-tk-CAT chimeric genes in the estrogen-sensitive human breast cancer cell line MCF-7 (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986). Here we report on the visualization of complexes resulting from the interaction between *trans*-acting factors present in a soluble protein extract prepared according to Gorski *et al.* (1986) from nuclei of estrogen-stimulated *X. laevis* liver and several DNA sequence elements, including the ERE, of the 5′ end of the vitellogenin gene.

Results

Visualization of protein–DNA complexes in the electron microscope

Our previous studies showed that the promoter of the *X. laevis* vitellogenin genes can be faithfully transcribed in a HeLa whole cell extract (Wahli *et al.*, 1982; Germond *et al.*, 1983; Walker *et al.*, 1983). More recently we have been able to visualize, using electron microscopy, ternary transcription complexes assembled *in vitro* on this promoter, starting with the same extract (ten Heggeler and Wahli, 1985). Stable complex formation on the template DNA, a 2.7-kb fragment of the 5′ end of the gene B2, was dependent on transcription factors present in the extract, as well as RNA polymerase II and at least two nucleoside triphosphates. Figure 1A shows, for reference purposes, such a complex at the transcription initiation site of the gene B2.

This assay was used as a means to search for tissue-specific complexes that might be involved in the estrogen-regulated expression of the vitellogenin genes in the liver. Since we assumed that the HeLa cell extract does not contain the hepatocyte-specific factors involved in this regulation, soluble extracts were prepared from purified liver nuclei that were isolated from estrogen-stimulated frogs as described by Gorski *et al.* (1986) for rat liver. The same gene B2 template as above was incubated in this extract under *in vitro* transcription conditions, along with estrogen (5×10^{-9} M) and the dinucleotide ApU to stabilize the ternary transcription complex at the gene B2 transcription initiation site (ten Heggeler and Wahli, 1985). The protein–DNA complexes were purified by chromatography, during which they were lightly fixed, and then prepared for electron microscopic analysis.

An example of the types of complexes observed is shown in Figure 1B, and Figure 2A gives their distribution on the 2.7-kb gene B2 fragment. The complex found at position 770 represents a ternary transcription complex and the site at which the RNA polymerase II is stably bound (PBS) gives the orientation of the fragment (see also ten Heggeler and Wahli, 1985). The presence of this complex is strictly dependent on the addition of the dinucleotide (Figure 2A,B), which is consistent with the results obtained previously with the HeLa whole cell extract. In addition,

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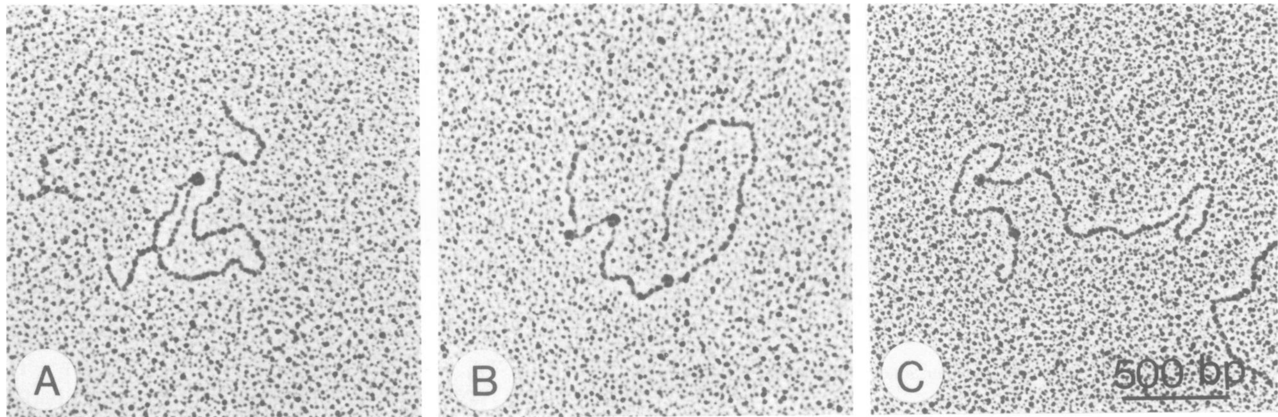


Fig. 1. Visualization of protein–DNA complexes in the promoter region of the *Xenopus laevis* vitellogenin gene B2. The DNA template is a 2.7-kb *Eco*RI fragment spanning from position −769 to +1963 (see Figure 2). (**A**) Ternary transcription complex assembled *in vitro*, using a HeLa whole cell extract at the transcription initiation site of the gene (ten Heggeler and Wahli, 1985). (**B**) Complexes seen after incubation of the template in the presence of estrogen in a soluble protein extract from estrogen-induced liver nuclei (see Figure 2A). (**C**) Same as above but without the dinucleotide ApU (see Figure 2B).

two other complexes are observed, one at 319 ± 23 bp upstream of the transcription initiation site and the other 375 ± 19 bp downstream of it, in the first intron of the gene (see also Figure 3). The presence of these two complexes was not affected by the omission of the dinucleotide, unlike the ternary transcription complex mapping between them (Figures 1C and 2B). At the position of the complexes, we observe a kink in the DNA in 40–50% of the molecules (stippled histograms, Figure 2A,B), which may have interesting mechanistic implications (Griffith *et al.*, 1986; Liu-Johnson *et al.*, 1986; Ptashne, 1986; Shuey and Parker, 1986). In our case this remains to be substantiated by further experiments.

The upstream binding site (UBS) maps at a position characterized by its homology to the ERE identified in the closely related gene B1 and in the gene A2 flanking sequences (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986). In fact, this region contains two copies of the 13-bp palindromic element (Walker *et al.*, 1984) found to be the core of the ERE (Figure 3A,B). In contrast, the downstream binding site (DBS) shows no obvious homology to the ERE, although it does contain sequences showing dyad symmetry (Figure 3A,C). The possible functional significance of these two sites will be addressed later.

Hormone-dependent formation of the complexes at the upstream and downstream binding sites

If the formation of the protein–DNA complex visualized upstream of the transcription initiation site involves the ERE, then the appearance of this complex should be dependent upon the presence of estrogen in the binding reaction. To test this hypothesis, the template was incubated as above, but in the absence of estrogen. In addition, tamoxifen, an antagonist of the hormone, was included in the mixture to counteract any residual estrogen present in the liver extract. This led to a decrease in the number of both upstream and downstream complexes to background levels, while the number of ternary transcription complexes was not significantly affected (Figure 2C). Thus the loss of these two complexes was not due to a general inhibition of binding by the antagonist. Further evidence that the effect of tamoxifen is specific was obtained in reactions containing both the antagonist and an excess of estrogen, which should compete out the effect of tamoxifen. Under these conditions the two complexes reappeared at similar levels to those seen in the presence of estrogen alone (Figure 2D). Thus it appears that the formation of the upstream complex, at a site containing the ERE, is indeed hormone-dependent. This is also true for the formation of the downstream complex, at a site showing no apparent sequence homology to the ERE. These two complexes have been obtained with four separate extracts prepared from both hormonally treated females and males. Furthermore, two apparently similar complexes form at the same sites on the gene B2 in nuclear extracts from estrogen-induced MCF-7 cells, which were used to identify the ERE (not shown). In all instances, the upstream complex appears at a slightly higher frequency than the downstream one. In contrast, first experiments with extracts from livers of uninduced males resulted in very little complex formation, which is consistent with the regulation of these genes *in vivo*. The latter result, however, remains to be reproduced.

Assay for estrogen-responsiveness in MCF-7 cells of the upstream and downstream binding sites

The vitellogenin gene ERE was identified upstream of the genes A2 and B1 by introducing chimeric vit-tk-CAT constructs into MCF-7 cells, which contain a functional estrogen receptor, and assaying their transient expression in the presence and absence of hormone (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986). To test whether one or both binding sites can function independently as an ERE, we have constructed similar chimeric genes with DNA fragments of the gene B2 containing either the UBS or DBS (Figure 4A). These have been inserted in both orientations upstream of the Herpes tk promoter, which in turn is linked to the CAT reporter gene. In these constructs either binding site can form a complex when incubated in the extracts from liver nuclei (data not shown). The four constructs were then transfected independently into MCF-7 cells, as was the parental plasmid lacking an insert upstream of the tk promoter (pBL-CAT8+). The transfected cells were either left unstimulated or treated with estrogen. After 20 h, cell extracts were prepared and their CAT activity was measured. As shown in Figure 4b, estrogen has no effect on CAT expression from the control parental plasmid. In contrast, if the DNA fragment spanning the UBS is present, a substantial rise is observed. This effect is independent of the orientation of the fragment. However, the DNA sequences encompassing the DBS do not lead to a similar increase of CAT activity upon estrogen stimulation. Thus only the fragment containing the UBS can confer hormone responsiveness to the tk promoter in MCF-7 cells. This is not surprising since

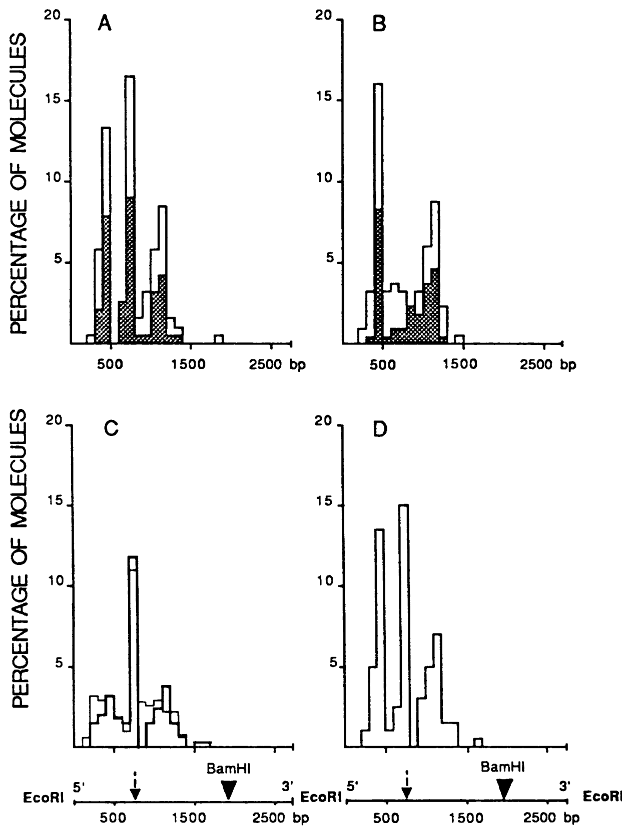


Fig. 2. Distribution of the protein–DNA complexes on the 2.7-kb *Eco*RI fragment from the gene B2. The template DNA was incubated in the soluble protein extract from estrogen-induced liver nuclei in the presence of 5×10^{-9} M estrogen. **(A)** Standard incubation mixture. **(B)** The dinucleotide ApU was omitted. No stable ternary transcription complexes at the transcription initiation site can be obtained under these conditions (ten Heggeler and Wahli, 1985; see Figure 1C). **(C)** Incubation in the presence of 10^{-6} M (thick line) or 10^{-5} M (thin line) tamoxifen, an estrogen antagonist. **(D)** Incubation in the presence of 10^{-5} M tamoxifen and 10^{-7} M 17β-estradiol. Four hundred molecules were screened for the presence or absence of complexes and the percentage of binding was 53% in A, 43% in B, 29.5% and 32% in C for 1 μM and 10 μM tamoxifen, respectively, and 50% in D. For each histogram a randomly selected subset, consisting of 100 molecules with complexes, was photographed in order to map their positions. The molecules were oriented using the following criteria. The first was the position of the ternary transcription complex. About 10% of the molecules analyzed in each case showed either the upstream or the downstream complex present with the ternary transcription complex. In addition, we used the fact that the regulatory sequences, as determined by functional assays, are located asymmetrically on the 2.7-kb fragment. To confirm further the positions of the complexes in this way, they were also mapped on a 1.9-kb *Eco*RI/*Bam*HI fragment (data not shown), which is 800 bases shorter on the 3′ end, i.e. in the coding region (ten Heggeler and Wahli, 1985). The percentage of molecules in the ordinates takes into account the percentage of total binding calculated from 400 molecules. For example, in Figure 2A 100 molecules were mapped from a sample of 400 showing 53% binding. The percentages were then calculated on the basis that this represents 100/0.53 or 188 molecules in all. The same calculation was used for each histogram. The map position of the kink observed in approximately half of the complexes is given by the stippled area in panels A and B. The schemes below the histograms show a map of the template DNA, where the arrow indicates the transcription initiation site of the gene B2 as determined by Germond *et al.* (1983) and Walker *et al.* (1984).

it contains two copies of the 13-bp element that is a key part of the ERE (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986). In contrast, the fragment from intron I that contains the DBS does not function as an ERE in the same context. From this preliminary analysis it appears that the two binding sites, which show no apparent sequence homology (Figure 3B), are not functionally equivalent.

The 13-bp element is necessary for upstream complex formation

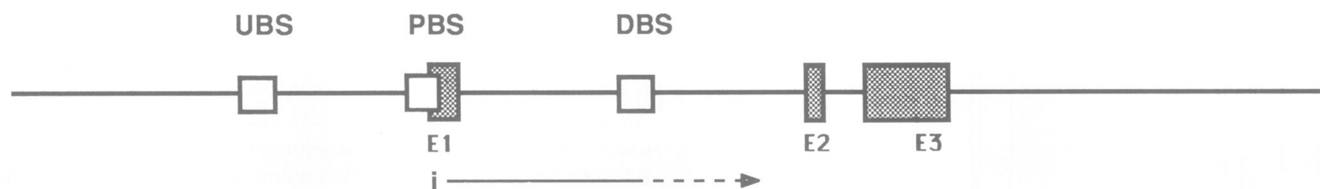
As shown above (Figure 3), the UBS of the gene B2 contains two copies of the ERE 13-bp core element, thereby suggesting that it is involved in the formation of the complexes in the 5′ flanking region. This was tested using different vit-tk-CAT constructs containing deletion mutations of the 5′ end region of the gene B1 (Seiler-Tuyns *et al.*, 1986), which is closely related to the gene B2. Furthermore, the 5′ flanking region of the gene B1 contains a third copy of the 13-bp element located at position −555. The two others are found at −334 and −314, similar to the other vitellogenin genes (Walker *et al.*, 1984). Several of the deletions were incubated with the liver extract, together with estrogen and the dinucleotide ApC, which corresponds to the initiation site of the tk gene. The complexes were visualized as above. The chimeric plasmid with the vit DNA fragment containing all three copies of the element (vit[−596/−42]-tk-CAT) shows three complexes (Figure 5A). One is found at the tk promoter transcription initiation site, representing a ternary transcription complex, while the two others are localized at the site of the −314/−334 double-element and of the far upstream −555 element. Binding is observed at this upstream site in the absence of the promoter-proximal copies of the element (vit[−564/−349]-tk-CAT mutant, not shown). Complex formation could also be demonstrated at both the −314 and −334 copies of the element when tested separately. Figure 5B,C shows that the −314 element is directly involved in binding. Indeed, in addition to the ternary transcription complex, a second complex maps at this upstream site in the vit[−313/−42]-tk-CAT construct (Figure 5B), while this second complex is no longer found when this element is deleted (vit[−301/−42]-tk-CAT, Figure 5C). Binding at the −334 element was tested using a subclone in pEMBL8+ containing only this 13-bp element. After linearization of this subclone and incubation in the liver extract a complex was observed over the vit[−334/ −320] insert (Figure 5D).

In conclusion, these results indicate that all three copies of the 13-bp element present in the 5′ flanking region of the gene B1 are capable of forming complexes similar to those observed with the gene B2.

Discussion

Extracts of nuclei from liver of estrogen-induced *X. laevis* were prepared to identify protein–DNA interactions in the 5′ end region of the vitellogenin gene. The extraction procedure used was developed by Gorski *et al.* (1986), who showed a tissue-specific utilization of the mouse albumin promoter in extracts from rat liver nuclei. Incubation of the 5′ end of the *Xenopus* vitellogenin gene B2, spanning positions −769 to +1963, in the *Xenopus* soluble nuclear extracts revealed stable complexes at three locations. One corresponds to the transcription initiation site of the gene, and is only found under conditions allowing the formation of stable ternary transcription complexes (Ackerman *et al.*, 1983). This is the only complex we can find when the same template is incubated in a HeLa whole cell extract (ten Heggeler and Wahli, 1985). It consists of the gene B2 transcription initiation site, transcription factors in the extract, RNA polymerase II and the dinucleotide ApU. Stable complexes at two additional locations are observed after incubation of the template, in the presence of estrogen, in extracts prepared from estrogen-

A) Structural Organisation



B) Nucleotide sequence at the upstream binding site (UBS)

-350	-340	-330	-320	-310	-300
AGAGCTGATG	CCTAAAGTCA	CTTTGACCCA	ACCCAAGTTA	TCATGACCTC	TTAGTTAGCT
TCTCGACTAC	GGATTTCAGT	GAAACTGGGT	TGGGTTCAAT	AGTACTGGAG	AATCAATCGA

C) Nucleotide sequence at the downstream binding site (DBS)

340	350	360	370	380	390	400	410	420
TTGGATGCAC	GTTATTGATT	ATAGTCTGTT	TTGTAATGTA	ATGCCAACAT	AATACTTAGC	ATTATACAGA	GAGTTTGCTG	GCATATATAT
TACCTACGTG	CAATAACTAA	TATCAGACAA	ACAATTACAT	TACGGTTGTA	TTATGAATCG	TAATATGTCT	CTCAAACGAC	CGTATATATA

Fig. 3. Structural organization of the 5′ end region of the gene B2 and nucleotide sequence of the upstream and downstream binding site regions. **(A)** Scheme indicating the position shown by open boxes of the upstream binding site (UBS), the polymerase binding site (PBS) and the downstream binding site (DBS). The stippled boxes represent exons 1−3 (E1−E3). The transcription initiation site (i) and the direction of transcription (arrow) are indicated. **(B)** and **(C)** Nucleotide sequence of the regions defined by electron microscopy as the UBS (B) and DBS (C). Palindromes are indicated by arrows. The binding sites as defined by electron microscopic analysis are underlined. The nucleotides are numbered according to their position relative to the transcription initiation site (position +1).

induced liver in which the vitellogenin genes are actively transcribed (reviewed by Wahli and Ryffel, 1985).

One of the three complexes observed maps within the first intron of the gene B2. The fact that estrogen promotes binding at this site whereas tamoxifen blocks this interaction suggests that the high-affinity estrogen receptor of the hepatocyte nuclei (Westley and Knowland, 1978; Hayward *et al.*, 1980) or another estrogen binding protein mediates the formation of this stable complex. The region around the binding site contains sequences showing dyad symmetry, but our analysis is not sensitive enough to reveal if the complex maps at one of those sequence elements. However, preliminary footprinting experiments with similar extracts show binding at one of the dyad symmetrical elements (R.Hipskind and W.Wahli, unpublished). Since a number of eukaryotic regulatory proteins have been shown to bind to DNA sites containing a palindromic element (Borgmeyer *et al.*, 1984; Wu, 1984a,b; Dynan and Tjian, 1985; Treisman, 1986; Baldwin and Sharp, 1987), experiments are underway to address the regulatory role of this binding site. Nevertheless, the results presented here demonstrate that this element alone cannot function as an ERE. Recently, Jost *et al.* (1987) have identified a protein in extracts from induced chicken liver nuclei that can bind to intron III of the chicken vitellogenin gene. Although no binding can be found in uninduced extracts, the binding reaction itself does not require estrogen. It will be interesting to see if the complex identified by Jost *et al.* (1987) and our intron complex are related structurally and functionally.

The complex that can be visualized upstream of the transcription initiation site is also increased in the presence of estrogen and is reduced by the inclusion of tamoxifen in the reaction mixture. The results obtained further suggest that binding requires the 13-bp GGTCANNNTGACC estrogen-responsive core element. When this element is present in several widely separated copies, complexes map to each site containing at least one of these copies. When one or the other is deleted, the corresponding complex disappears, and finally binding can be visualized to copies cloned singly, either in the presence or the absence of a promoter. Furthermore, we observe that the binding site from the gene B2, which contains two copies of the 13-bp element, can function as ERE independent of its orientation. Similar results have been found for the corresponding ERE from the gene B1 (E.Martinez and W.Wahli, unpublished).

Several lines of evidence further suggest, albeit indirectly, that complex formation involves the high-affinity estrogen receptor First, as seen above, the binding is enhanced by low estrogen concentrations and it is blocked by tamoxifen. Similarly to the results obtained with liver nuclear extracts, binding at the same sites can be visualized in the nuclear extracts of MCF-7 cells

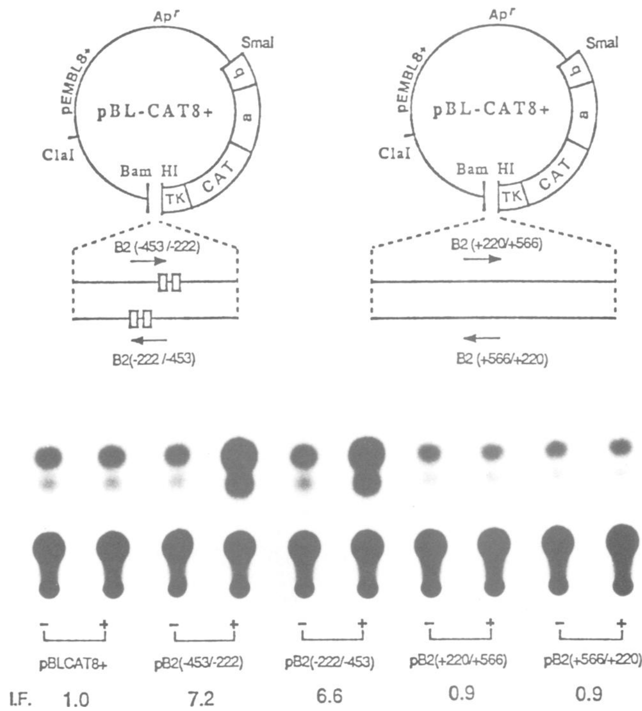


Fig. 4. Structure and expression of vitellogenin B2-CAT vectors. **(A)** Structural features of the vectors. Thin lines represent vector sequences. Open boxes stand for: (CAT) chloramphenicol acetyl transferase gene; (a) SV-40 small t splicing sequences. (b) SV-40 polyadenylation signals. (TK) Herpes simplex virus thymidine kinase gene promoter, positions −105 to +51. The B2 DNA fragments, B2 (−453/−222) containing the UBS (the boxes indicate the two 13-bp palindromic elements) and B2 (+220/+566) containing the DBS have been inserted in both orientations as indicated, immediately upstream of the tk-promoter. **(B)** CAT activities in extracts of MCF-7 cells transfected with the four constructs shown in (A). The transfected cells were left unstimulated (−) or stimulated with 2×10^{-7} M 17β-estradiol in the culture medium (+). Each reaction contained 40 μg protein. IF is the induction factor calculated from the mean CAT activity from four independent experiments. One of these experiments is shown.

that contain the human estrogen receptor (B.ten Heggler-Bordier, F.-X.Claret and W.Wahli, unpublished). Furthermore, extracts prepared from *Xenopus* oocyte nuclei that have been injected with an expression clone encoding the human ER (Green *et al.*, 1986) also stimulate binding at the UBS and DBS (I.Theulaz, B.ten Heggler-Bordier, R.Hipskind and W.Wahli, unpublished). However, it remains to be directly demonstrated that the receptor is part of the complexes.

Binding appears to be similar to all three copies of the 13-bp element from the gene B1 when tested in a liver extract. However, the regions containing these elements do not seem to be equivalent with regard to their ability to confer estrogen responsiveness on a heterologous promoter (Seiler-Tuyns *et al.*, 1986). This suggests that the sequences around these elements play a role in their function *in vivo*.

Our results lead to several possible interpretations that are currently being tested experimentally: (i) the hormone receptor binds at two sites that are clearly not equivalent functionally; (ii) two different proteins, e.g. the receptor and another unidentified estradiol-binding protein, are involved in the formation of the upstream and downstream complexes; (iii) the receptor itself is not a component of the complexes but is involved in their formation, though this last possibility seems the least likely.

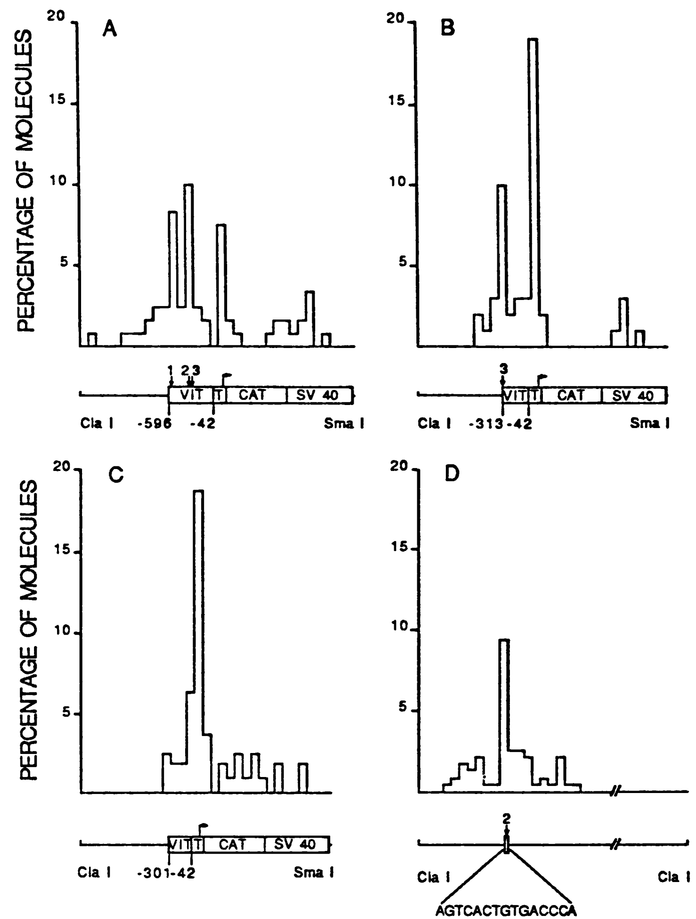


Fig. 5. Distribution of the protein–DNA complexes in the 5′ flanking region of the vitellogenin gene B1. The construction and characteristics of the Vit-tk-CAT chimeric genes shown schematically in **panels A, B** and **C** have already been described (Seiler-Tuyns *et al.*, 1986). T indicates the tk-promoter. The sequence of the element number 3 (−314) is AGTTATCATGACC; the construction of the −313 deletion **(panel B)** introduced a G in place of the deleted A at position −314 thus increasing the homology with the 13-bp consensus element. The arrows over the Vit boxes in the schemes give the position of the three copies of the 13-bp palindromic element GGTCANNNTGACC (Walker *et al.*, 1984). The scheme in **panel D** gives the position and nucleotide sequence of the copy number 2 of the element subcloned in pEMBL8+, which lies asymmetrically in the subclone linearized with *Cla*I. Incubation of the four constructs in the soluble protein nuclear extract was done under standard conditions in the presence of 5×10^{-9} M 17β-estradiol. In each experiment, 200 molecules were screened for the absence or presence of protein–DNA complexes, whose positions were determined in 50–65 molecules.

Materials and methods

Plasmid construction and DNA preparation

The 2.7-kb *Eco*RI fragment spanning the 5′ end region of the gene B2 was isolated from a subclone in pBR322 (Germond *et al.*, 1983). The construction of the different vit-tk-CAT constructs, containing portions of the 5′ end flanking region of the gene B1, have been described (Seiler-Tuyns *et al.*, 1986). The construct containing the only B1 palindromic element number 2 (positions −334 bp to −320 bp) cloned into the *Bam*HI site of pEMBL8+ (Figure 5D) resulted from the elimination of the tk-CAT-SV40 sequences by *Eco*RI digestion of the clone pB1(-334/-297)TK-CAT8+ and a subsequent *Bal*31 deletion to remove the remaining 26 bp of the tk promoter and the B1 sequences next to it, i.e. from positions −297 to −320. The vector was then recircularized with a *Bgl*II linker at the end point of the deletion, cloned and identified by sequencing. For electron microscopic visualization of protein–DNA complexes, this clone was linearized at the unique *Cla*I site in the vector so that the B1 palindromic insert sequence (−334 bp/−320 bp) was placed asymmetrically in the linear vector.

The plasmid DNAs used in transfection experiments were prepared by the Triton lysis or alkaline lysis methods (Grosveld *et al.*, 1981; Holmes and Quigley, 1981) followed by two CsCl/ethidium bromide gradients and finally a NaCl or gel filtration through Biogel A50. The DNA restriction fragments used for the formation of the protein–DNA complexes were isolated from agarose gels and purified as described by Maniatis *et al.* (1982).

MCF-7 cells, transfection with chimeric genes and hormonal induction

Most of the procedures used have been described (Seiler-Tuyns *et al.*, 1986) and were used with slight modifications as follows. MCF-7 cells (Soule *et al.*, 1973) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 4 μg/ml insulin, 200 IU/ml penicillin and 0.2 μg/ml streptomycin. For at least 24 h before transfection, the cells were grow in DMEM containing 10% new born calf serum (NBCS), 10^{-6} M tamoxifen (an estrogen antagonist), insulin and antibiotics as above. These cells were transfected with ~ 20 μg chimeric plasmid DNA per million cells (Wigler *et al.*, 1979; van der Eb and Graham, 1980). After the glycerol shock, the transfected cells were transferred into DMEM (as above) but containing 10% FCS (instead of NBCS) that had been stripped of endogenous steroids (Prud'homme *et al.*, 1985). Incubation was without hormone (uninduced cells) or in the presence of 2×10^{-7} M 17β-estradiol (induced cells).

Cell-free extracts and CAT assays

Cells were harvested 15–20 h after transfection. The washed cell pellet was lysed by three cycles of freezing and thawing in 50 mM Tris–HCl (pH 7.8). Cell debris were removed by a short centrifugation and the protein concentration in the supernatants was determined using the Bio-Rad Protein Assay. Aliquots containing identical amounts of protein were used for the determination of the CAT activity (Gorman *et al.*, 1982).

Preparation of soluble protein extracts from liver nuclei

Adult *Xenopus* females were injected 1–2 days prior to extract preparation in their dorsal lymph sac with 1 mg 17β-estradiol (Sigma) in 1,2-propanediol. Excised livers were perfused on ice with 75% phosphate-buffered saline containing 0.5 mg/ml heparin. Liver cell nuclei and soluble protein extracts thereof were prepared according to Gorski *et al.* (1986). These extracts were stored in aliquots in liquid nitrogen. Protein concentrations were determined using the Bio-Rad Protein Assay and ranged between 5 and 8 mg/ml.

Formation of protein–DNA complexes

A reaction mixture (3.5 μl) containing 70 mM KCl, 18 mM Hepes (pH 7.9), 70 nM EDTA, 0.7 mM DTT, 12% glycerol, 24% nuclear liver extract and 150 ng poly(dI-dC) was preincubated for 15 min at 0°C. It was then brought to a volume of 6 μl giving final concentrations of 7 mM $MgCl_2$, 10 mM creatine phosphate, 11 mM Hepes (pH 7.9), 7.5% glycerol, 40 nM EDTA, 45 mM KCl, 0.4 mM DTT, 15% liver extract, 0.5 mM ApU (vitellogenin-promoter) or ApC (tk-promoter), 25 ng DNA and 5×10^{-9} M 17β-estradiol unless indicated otherwise. The incubation was continued for 15 min at 30°C and then the reaction mixture was diluted 1:1 in 30 mM triethanolamine–HCl (pH 7.9), 10 mM $MgCl_2$ and 1 M KCl. After another 5-min incubation at 30°C, the samples were prepared for electron microscopy.

Specimen preparation for electron microscopy

The protein–DNA complexes (12 μl reaction mixture) were separated from unbound protein by gel filtration on pipette tip columns containing Sepharose 2B equilibrated with 30 mM triethanolamine–HCl (pH 7.9), 10 mM $MgCl_2$, 500 mM KCl and 0.1% glutaraldehyde. The fractions containing the complexes were prepared for electron microscopic analysis according to the protein-free spreading method of Dubochet *et al.* (1971). Five-microliter samples were adsorbed on previously positively charged carbon films. After 1 min the grids were washed by floating on two drops of bidistilled water, stained with 1% uranyl formate for 1 min (Brack and Pirotta, 1975) and blotted dry on filter paper. The samples were then rotary shadowed with platinum-palladium at an angle of 6°.

Electron microscopy and data analysis

The observations were made with a Philips 300 electron microscope and micrographs were taken at a magnification of 20 000. Measurements of the molecules were done on 10-fold enlarged negatives with a Summagraphic Digitizer connected to an ABC computer.

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