Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7878–7882, October 1990
Biochemistry

Estrogen receptor level determines sex-specific *in vitro* transcription from the *Xenopus* vitellogenin promoter

(hepatocytes/vaccinia virus/liver-specific gene expression)

BLAISE CORTHÉSY*, FRANÇOIS-XAVIER CLARET, AND WALTER WAHLI

Institut de Biologie Animale, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland

Communicated by Igor B. Dawid, July 16, 1990

ABSTRACT **Female-specific expression of the *Xenopus laevis* vitellogenin gene was reconstituted *in vitro* by addition of recombinant vaccinia-virus-produced estrogen receptor to nuclear extracts from male livers, in which this gene is silent. Transcription enhancement was at least 30 times and was selectively restricted to vitellogenin templates containing the estrogen-responsive unit. Thus, in male hepatocytes, estrogen receptor is the limiting regulatory factor that in the female liver controls efficient and accurate sex-specific expression of the vitellogenin gene. Furthermore, the *Xenopus* liver factor B, which is essential in addition to the estrogen receptor for the activation of this gene, was successfully replaced in the *Xenopus* extract by purified human nuclear factor I, identifying factor B of *Xenopus* as a functional homolog of this well-characterized human transcription factor.**

In oviparous vertebrates, vitellogenin genes, which encode the precursor of yolk proteins, are transcribed under estrogen control in the liver of adult females. In contrast, males normally never produce vitellogenin, although hormone treatment of these animals can lead to a *de novo* activation of the vitellogenin genes in preexisting differentiated and nonproliferating hepatocytes (1, 2). Earlier studies also demonstrated that in adult male liver, estrogen treatment increases the estrogen receptor (ER) level from 100–200 molecules per uninduced hepatocyte to about 1500 molecules per fully stimulated cell. Progressive activation of vitellogenin gene transcription follows the increase of ER with a 4- to 6-hr lag (3, 4). However, it is not known whether the absence of vitellogenin gene expression in male livers results solely from the low liver estrogen concentration (0.18 nM) and the accompanying very low level of receptor (see above) or whether other essential components of the vitellogenin gene stimulation machinery must first be produced *de novo* in hepatocytes as a primary effect of hormone preceding activation of the gene.

To address this point, which is crucial for the understanding of the sex-specific expression of the vitellogenin gene, we used tissue-specific nuclear extracts. Such extracts prepared from estrogen-induced *Xenopus laevis* female liver are capable of directing steroid hormone-dependent *in vitro* transcription (5). Analysis of the *Xenopus* vitellogenin gene B1 promoter region revealed that in addition to the estrogen-responsive element, at least two sequence elements play crucial roles in the regulation of this promoter in female liver (6). One of them, a negative regulatory element (NRE), is responsible for the lack of promoter activity in the absence of the hormone and interacts with a liver-specific transcription factor. The second is similar to the nuclear factor I (NF-I) consensus recognition sequence and is required in association with the estrogen-responsive element (ERE) to mediate hormonal induction. It is recognized by the *Xenopus* liver factor B.

Using a similar *in vitro* transcription system but derived from male liver, we demonstrate that in male hepatocytes ER is the limiting regulatory factor which in the female liver regulates the expression of the vitellogenin gene. Furthermore, the *Xenopus* liver factor B, which is essential in addition to the ER for the activation of this gene, was identified as the functional homolog of NF-I.

MATERIALS AND METHODS

Templates for *in Vitro* Transcription. All deletion mutants used are diagramed in Fig. 1 *Upper* and all contain the vitellogenin B1 promoter up to position +8. The 5′ deletion mutants have been described previously (6). The 3′ deletion mutant fragments of the gene B1 promoter region were cloned in front of the minimal (−41/+8) B1 promoter containing the natural transcription initiation site and the TATA box of the vitellogenin B1 gene (7).

Preparation of Nuclear Extracts from *Xenopus* Livers and Regulatory Factor Depletion Procedure. Nuclear extracts for transcription were prepared from *X. laevis* livers by the method of Gorski *et al.* (8) with the modifications described (9). The nuclear proteins were stored in liquid nitrogen in the nuclear extract dialysis buffer containing 25 mM Hepes (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, and 10% (vol/vol) glycerol. Protein concentration in the extracts was determined by the Bradford procedure using bovine serum albumin as the standard.

Depletion of the liver factor B from the *X. laevis* nuclear extracts was carried out with an affinity matrix containing the binding site for the NF-I activity as described (10). The depleted extracts were not stored but immediately used for *in vitro* transcription. To get the affinity matrix, CNBr-activated Sepharose CL-2B (Pharmacia) was prepared according to the manufacturer's directions. Coupling with the double-stranded NF-I Ad2 oligonucleotide (binding site underlined) 5′-TATACCTTATTTTGGATTGAAGCCAATATGATAATGAG-3′ in multiple copies (10- to 40-mer) was done by the method of Kadonaga and Tjian (11).

Production of *Xenopus* ER (xER) with Vaccinia Virus. HeLa S3 cells grown in suspension were infected either with nonrecombinant vaccinia viruses (WR) or with a recombinant vaccinia virus expressing the xER (the construction of this recombinant virus will be described elsewhere). After 12–14 hr of incubation in a culture medium containing 10 nM 17β-estradiol, whole cell extracts were prepared as described

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Abbreviations: ER, estrogen receptor; xER, *Xenopus* estrogen receptor; ERE, estrogen-responsive element; ERU, estrogen-responsive unit; NF-I, nuclear factor I; CAT, chloramphenicol acetyltransferase; NRE, negative regulatory element; Vit, vitellogenin.

*Present address: Howard Hughes Medical Institute Research Laboratories, Stanford University School of Medicine, Stanford, CA 94305-5425.

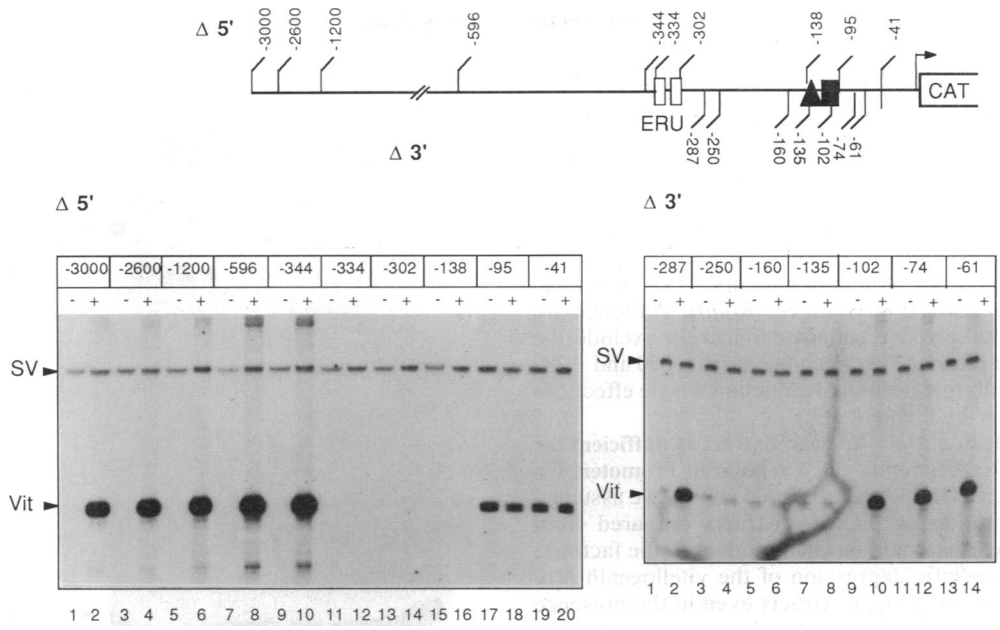

FIG. 1. Liver-specific and estrogen-controlled *in vitro* transcription from the *X. laevis* vitellogenin B1 promoter. (*Upper*) Diagram of the vitellogenin–chloramphenicol acetyltransferase (Vit-CAT) constructs used. The B1 promoter region cloned upstream of the CAT coding sequence is depicted as a black line. The open boxes in this region represent the two imperfect palindromic elements forming the estrogen-responsive unit (ERU). The filled rectangle and filled triangle correspond to the previously mapped binding site for the hepatic NF-I-like factor B and for the repressor C, respectively (6). The 5′ end positions of the different 5′ deletion mutants used (Δ5′) are indicated above the line. The 3′ deletion mutants (Δ3′) were obtained by removing DNA fragments between position −41 and the positions indicated on the diagram (−61, −74, −102, . . ., −287). (*Lower*) One microgram of the −596 Vit-CAT construct or the stoichiometric equivalent of the other templates, 5′ (*Lower Left*, Δ5′) and 3′ (*Lower Right*, Δ3′) deletion mutants, was transcribed *in vitro* with induced male liver nuclear extract (35 μg) in the presence (+) or the absence (−) of 2.5 nM 17β-estradiol. To allow direct comparison of the level of transcription the pSV2CAT template (250 ng) was added as internal control in each reaction. All deletion mutants contain the homologous vitellogenin B1 promoter up to position +8. The 3′ constructs −287 and −250 extend up to nucleotide −564, while the five others end at position −596. All 3′ deletions are fused to the vitellogenin promoter at position −41. The arrows indicate the position of RNA correctly initiated from the vitellogenin promoter (Vit) and from the simian virus 40 promoter (SV).

(12), except that buffers contained benzamidine, leupeptin, pepstatin A, chymostatin, and antipain at a final concentration of 1 μg/ml.

Partial purification of the xER protein was performed by loading 10 mg of the xER-containing whole cell extract in buffer B [20 mM Tris·HCl, pH 7.5/20% (vol/vol) glycerol/2 mM dithiothreitol/5 μM $ZnCl_2$/0.1 M KCl] onto a 1-ml heparin-Sepharose CL-6B column (Pharmacia) equilibrated with the same buffer. The bound proteins were eluted stepwise with buffer B but containing increasing concentration of KCl (0.1–1 M). The fractions (200 μl) containing the xER activity were identified by the mobility shift assay using a 3-μl aliquot in a 20-μl reaction volume. Pools of fractions corresponding to the different elution steps were dialyzed separately and progressively against buffer B (0.1 M KCl) and assayed (3-μl aliquot of pooled fraction) for xER binding activity as above.

***In Vitro* Transcription and Transcript Analysis.** *In vitro* transcription was performed as described (6). The amount of specific Vit-CAT mutant constructs was as indicated in the figure legends and that of the control template pSV2CAT (250 ng) was maintained constant in all reaction mixtures. The products of transcription were analyzed by primer extension and resolved by 6% polyacrylamide/7.5 M urea gel electrophoresis.

Gel Retardation Assay. Mobility shift assays were performed as described (12) in the presence of 50 nM hormone with a 3′-labeled double-stranded oligonucleotide containing the vitellogenin gene A2 ERE (minimal element is underlined) 5′-GATCCGGGGTCACAGTGACCTA-3′ (13) as probe.

RESULTS AND DISCUSSION

Hormone-Dependent *in Vitro* Transcription in Extracts from Estrogen-Induced Male Liver. We first tested a nuclear extract prepared from hormone-stimulated male livers (5) for its ability to transcribe templates consisting of 5′ and 3′ deletion constructs of the vitellogenin gene B1 promoter region fused to the CAT reporter gene in the pEMBL8+ plasmid (Fig. 1 *Upper*). As an estrogen-independent internal control we included a chimeric gene in which the simian virus 40 early promoter is linked to the CAT gene. The expression of the test and control genes was assayed by extension of a primer complementary to a 5′-proximal region of the CAT sequence.

The 5′-deletion series reveals that templates containing the estrogen-responsive unit (ERU, positions −334 to −302 from the *vitB1* gene cap site) formed by two imperfect EREs (13, 14) gave efficient induced transcription only in the presence of hormone, with the exception of construct −334, which remained silent under any condition (Fig. 1 *Lower Left*). Interestingly, this latter construct is inducible by estrogen after transfer into hormone-responsive cell lines (15), revealing subtle differences between different transcription systems. Additional experiments with an inducible 5′ mutant extending up to position −339 revealed that a few extra nucleotides upstream of the ERU are needed for proper activation in the *in vitro* system (B.C. and W.W., unpublished results). The level of induced activity was only slightly changed with increasing length of 5′ end vitellogenin sequences, suggesting that no additional activating element other than the ERU, which could be identified in this system, is located between positions −300 to −3000.

The mutants lacking the ERU are not inducible; however, the −95 and −41 constructs are expressed constitutively at a rate about 1/10th of that of ERU-containing templates in the presence of hormone. In contrast, the −138 deletion is silent under all conditions, consistent with the occurrence of a NRE between positions −138 and −119 in the B1 promoter (ele-

ment C, filled triangle, in Fig. 1 *Upper*; ref. 6). These results are very similar to those obtained previously with female extracts and suggest that induced male liver nuclear extracts are functionally equivalent to female extracts (6).

Analysis of the 3′ deletion mutants further demonstrated that an additional element, called B (filled rectangle in Fig. 1 *Upper*), is required in association with the upstream ERU to mediate hormonal induction (Fig. 1 *Lower Right*). Consistently, the ERU is unable to confer inducibility in the absence of element B unless the former is placed immediately upstream of the TATA box (compare mutants −135, −160, and −250 to mutant −287, Fig. 1 *Lower Right*). Although we consider it as unlikely, we cannot definitively exclude the possibility of the presence between positions −250 and −287 of an element inhibiting hormonal induction whose effect can be overcome only by element B.

Addition of ER to a Male Nuclear Extract Is Sufficient for Female-Specific Expression of the Vitellogenin Promoter. On the basis of the crucial finding presented above, we assumed that complementation of nuclear extracts prepared from noninduced male liver could be used to identify the factor(s) involved in sex-specific expression of the vitellogenin promoter, which is silent in such extracts even in the presence of hormone. Because unstimulated and estrogen-treated hepatocytes contain the same set of basal transcription factors (6), we tested the hypothesis that the receptor is the limiting factor in the noninduced male extract. To this end, xER (16) was produced in HeLa cells via a recombinant vaccinia virus expressing the full-length receptor. The specific binding of the xER to the ERE was tested by gel retardation. Using the xER-containing extract, we observed the formation of a complex with a consensus ERE probe whose intensity increased with the amount of protein assayed (Fig. 2*A*, lanes 8–13). The presence of xER in this complex was confirmed by coincubation of the complex with the monoclonal antibody H222 raised against the hormone-binding domain of the human ER (17) (Fig. 2*A*, lane 14). In addition, the binding was shown to be sequence specific, since the xER–probe association was prevented by excesses of the homologous oligonucleotide (Fig. 2*B*, lanes 2–4) but not by a 50-fold molar excess of an unrelated oligonucleotide (Fig. 2*B*, lane 5). These results suggest that the xER produced from the recombinant virus binds specifically to its cognate binding site in the vitellogenin promoter. In contrast, when the control HeLa extract lacking xER (WR) was used no specific retarded band was observed (Fig. 2*A*, lanes 1–5). These two crude HeLa extracts containing or lacking receptor were used to complement uninduced male liver extracts (Fig. 3). The −596 ERU-containing template was strongly activated after complementation of the liver nuclear extract with the whole-cell extract containing the receptor (Fig. 3*A*, lane 4) but not after complementation with the control extract from cells infected with the virus lacking receptor sequences (Fig. 3*A*, lane 3). As expected, no transcription of the −301 template lacking the ERU was observed in either of these two complemented extracts, indicating that the activation seen with the −596 template is dependent on the receptor and the ERU.

To enrich the preparation in receptor, an extract from HeLa cells infected with the recombinant virus was fractionated on heparin-Sepharose (Fig. 4). The different fractions were tested for ERE binding and for transcriptional complementation of the male liver extract. Only the column fraction containing the enriched receptor as identified by bandshift complemented the male extract, resulting in selective activation of the −596 template but not of the −301 template (Fig. 3*B*).

Since the xER preparation contained no *Xenopus* liver-specific regulatory factors except the ER, our complementation experiments indicate that the uninduced male liver

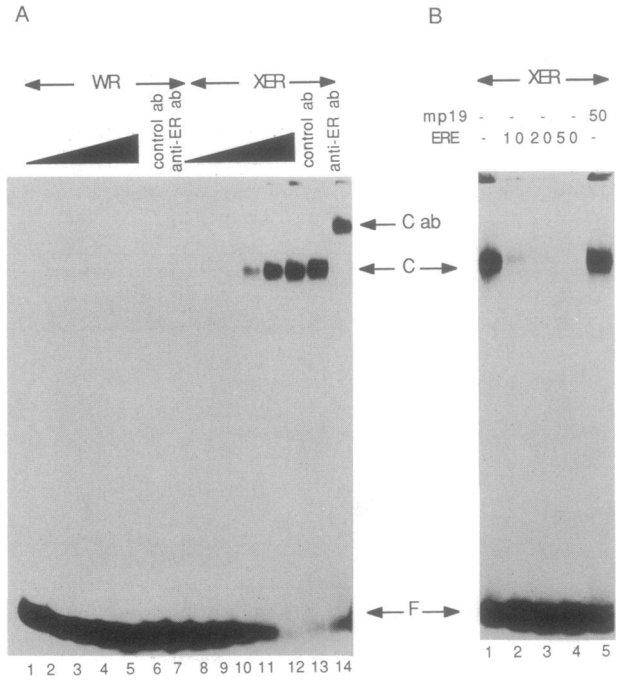

FIG. 2. Vaccinia recombinant xER expressed in HeLa cells binds specifically to its cognate response element *in vitro*. (*A*) No retarded bands were observed with incubation of increasing amounts of the WR extract proteins (0, 1, 5, 10, and 20 μg of protein in lanes 1, 2, 3, 4, and 5, respectively), whereas increasing amounts of the xER extract (0, 1, 5, 10, and 20 μg of protein in lanes 8, 9, 10, 11, and 12, respectively) induce the formation of a specific complex. The presence of the xER in the complex was demonstrated by adding the rat monoclonal antibody H222 to human ER (12) to the assay (lane 14). No further retarded band was detected with the rat normal IgG control antibody (lane 13) or in lanes corresponding to WR proteins (lanes 6 and 7). Bands: C, the specific DNA–protein complex; C ab, antibody shifted C; F, free probe. (*B*) Gel retardation competition analysis was performed with 10 μg of xER extract in the presence of increasing quantities (0, 10, 20, and 50-fold molar excess) of the unlabeled gene A2 ERE as specific competitor (see above and lanes 1–4), or a 50-fold molar excess of nonspecific competitor (mp19 polylinker, lane 5).

extract, in which the vitellogenin promoter is silenced via the NRE contains all the specific factors involved in the derepression and induction of the vitellogenin promoter except a sufficient amount of ER. Furthermore, complementation of the nontreated male liver extract with the crude or the enriched receptor preparations resulted in a promoter activity which was comparable to that observed with a female liver extract. Altogether, these observations indicate that the relevant difference between the sexes resides in the control of ER gene expression.

Functional Substitution of the *Xenopus* Liver Factor B by Human NF-I. As mentioned above, element B of the vitellogenin promoter (Fig. 1) binds a factor required to mediate estrogen responsiveness when the ERU is at a distance from the TATA box. This element, highly similar to a NF-I-binding site, is indeed able to bind purified human NF-I. Reciprocally, factor B recognizes the NF-I-binding site (6). To further understand the involvement of specific regulatory factors in the vitellogenin promoter activation mechanism we asked whether the *Xenopus* liver factor B could be replaced with the well-characterized human NF-I (18). First, a female liver extract and an induced male liver extract were depleted of factor B by incubation with an affinity matrix containing the NF-I-binding site. These extracts were then complemented with human NF-I purified as described (18). Remarkably, this factor is able to functionally complement the

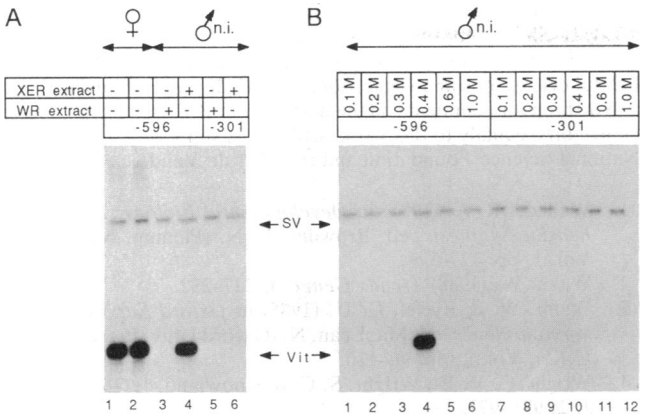

FIG. 3. The recombinant xER enhances transcription from the ERU-containing vitellogenin promoter in *X. laevis* noninduced male liver nuclear extracts. (*A*) Transcription with two Vit-CAT constructs either containing (−596) or lacking (−301) the ERU (see Fig. 1 *Upper*), in the presence of the pSV2CAT template as internal control. To standardize the system, we first incubated the −596 construct (1 μg) and pSV2CAT (250 ng) with female liver crude nuclear extracts (35 μg, lane 1). Replacement in the reaction of the nuclear extract dialysis buffer by the buffer used for the preparation of HeLa whole cell extracts does not change the level of transcription (lane 2). Complementation transcription reactions were performed with *X. laevis* noninduced (n.i.) male liver nuclear extracts (17 μg), the ERU-containing template (1 μg; lanes 3 and 4) or the ERU-lacking plasmid (1 μg; lanes 5 and 6), the pSV2CAT template (250 ng), the whole cell extract (10 μg) derived from HeLa cells infected with nonrecombinant vaccinia viruses (WR extract, lanes 3 and 5) or the whole cell extract (10 μg) obtained from cells infected with the recombinant virus expressing the xER (xER extract, lanes 4 and 6). Reactions were carried out in a final volume of 20 μl. Nuclear extract (35 μg for female or 17 μg from male liver) and whole cell extracts (10 μg) with or without receptor were mixed, then the templates were added, and the mixture was incubated for 15 min on ice. Transcription was initiated by addition of ATP, CTP, GTP, and UTP and proceeded for 45 min at 30°C. Continuation of the protocol has been as described elsewhere (6). (*B*) Complementation experiment using the noninduced male liver nuclear extract as before but complemented with 3 μl of the pooled heparin-Sepharose fractions used in the bandshift assay of Fig. 4 *Lower*. Eluting KCl concentration is given above each lane. The order of addition of components was the same as for experiments with crude WR and xER extracts. Comparison of the strength of the signals obtained with the crude and fractionated receptor preparations (lanes 4 in *A* and *B*, respectively) indicates that, taking into account the amount of protein used for the complementation, the ER is enriched 5 times. This is in agreement with its enrichment in ERE-binding activity (see Fig. 4) and indicates that there is no loss of transactivation activity, compared to ERE-binding activity, during chromatography. Thus, the heparin column represents a valuable first step towards the biochemical characterization and purification of the xER.

depleted female and induced male extracts in mediating the hormonal stimulation with the same efficiency as the endogenous *Xenopus* factor B (Fig. 5). Addition of the human factor to extracts that have not been depleted does not increase their hormone responsiveness, indicating that the endogenous factor B is not limiting in the transcription assay. To test if a complementation requiring more than one exogenously added factor is possible, we analyzed the effect of adding back to a factor-B-depleted, hormone-untreated male liver extract NF-I, xER, or both factors together. Consistent with the results above, neither protein added alone was sufficient for inducing transcriptional activation. In contrast, when a combination of the two factors was provided, the vitellogenin promoter was strongly activated. Together, these experiments show that the *Xenopus* liver factor B is homologous to human NF-I in both its DNA-binding properties (6) and its capacity to functionally cooperate with the ER in the sex- and tissue-specific promoter activation process.

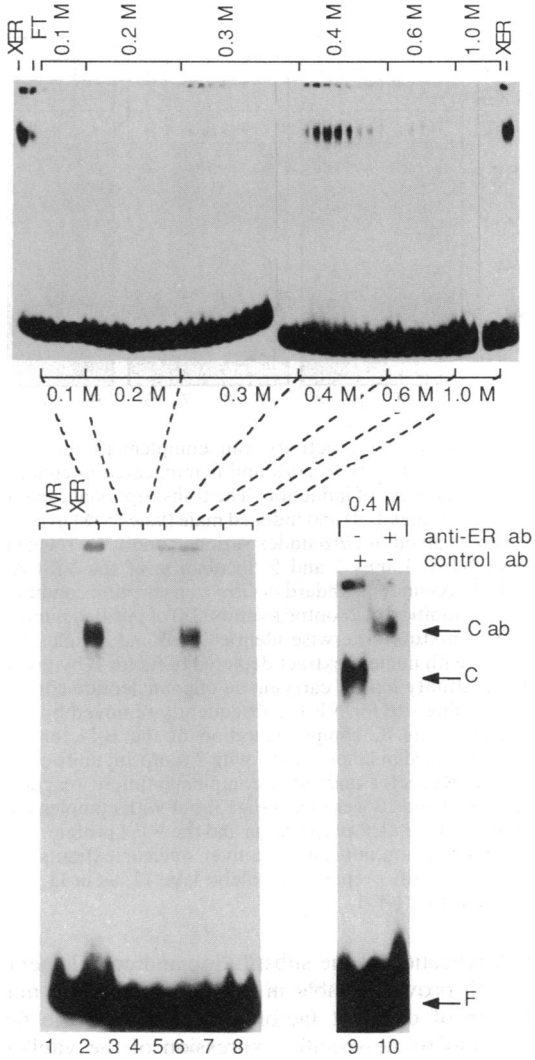

FIG. 4. Partial purification of xER by heparin-Sepharose chromatography. (*Upper*) Fractionation of the xER-containing extract was performed as described in *Materials and Methods*. (*Lower*) Pools of elution fractions were dialyzed against buffer B and 3-μl samples containing 1 μg of protein (0.1 and 0.2 M KCl steps, lanes 3 and 4), 2 μg of protein (0.3 M step, lane 5), 1.8 μg of protein (0.4 M step, lane 6), 0.7 μg of protein (0.6 M step, lane 7), and 1.2 μg of protein (1.0 M step, lane 8) were assayed for xER-binding activity. The protein–ERE complex generated by the 0.4 M KCl fraction (lane 6) is due to specific binding of xER to the probe, since the band can be further shifted by the anti-hER monoclonal antibody (lane 10) and not by the control IgG (lane 9). Comparison of the signals in lane 2 (10 μg of crude extract) and lane 6 (1.8 μg of 0.4 M KCl fraction) indicates that the ER, as measured by its ERE-binding activity, is enriched about 5 times by chromatography.

In summary, the use of homologous liver extracts for the analysis of the ER-dependent *in vitro* transcription of the unmodified natural vitellogenin promoter demonstrates that ER is the only limiting factor responsible for the biologically highly significant lack of vitellogenin gene expression in male liver. This implies that female-specific expression of the vitellogenin gene is based on a simple mechanism in which an increase of liver estradiol concentration elevates the ER sufficiently to turn on the vitellogenin gene. In agreement with this conclusion, it has recently been reported that induction in *Xenopus* liver of ER mRNA by estrogen results in the establishment of a stable autoregulatory loop which might play an important role in *Xenopus* sex determination (19). Finally, substitution of NF-I for the *Xenopus* liver factor B suggests that the protein domain interacting with the ER is well con-

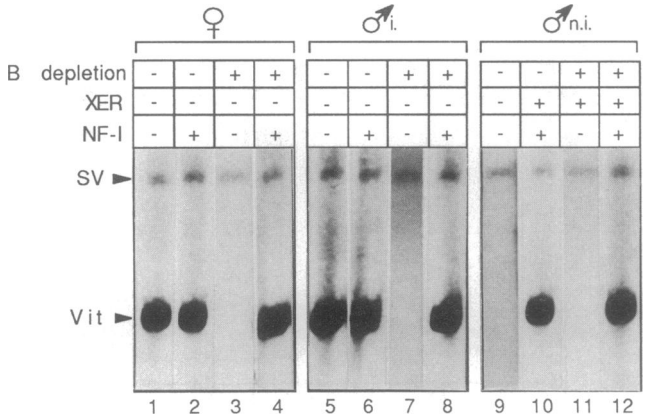

FIG. 5. Human NF-I activity can complement the *X. laevis* transcriptional machinery *in vitro* and is implicated in concert with the xER in the process of induction. The transcriptional capability of induced female (lanes 1–4) and induced male (lanes 5–8) liver nuclear extracts was assayed *in vitro* under various conditions revealing the function of NF-I. Lanes 1 and 5, incubation of the Vit-CAT and pSV2CAT DNAs under standard *in vitro* transcription conditions (9); lanes 2 and 6, addition of 2 footprint units (18) of purified human NF-I to a reaction mixture otherwise identical to 1 and 5; lanes 3 and 7, transcription with nuclear extract depleted of factor B by preincubation with an affinity matrix carrying an oligonucleotide corresponding to the binding site for NF-I, subsequently removed by centrifugation; lanes 4 and 8, complementation of the B-factor-depleted nuclear extract used in lanes 3 and 7 with 2 footprint units of purified human NF-I. Receptor and NF-I complementation: reaction mixtures for lanes 9 and 10 were as in Fig. 3, but with complementation (lane 10) by both the xER preparation and the NF-I protein. Lane 11, B factor-depleted noninduced male liver nuclear extracts complemented with the xER preparation solely; lane 12, as in 11, but with addition of purified NF-I.

served. Application of the substitution and complementation strategy will prove valuable in further defining the minimal combination of different factors necessary for the faithful hormone- and tissue-specific expression of the vitellogenin gene *in vitro*.

We thank N. Mermod and R. Tjian for supplying us with purified NF-I; D. Shapiro for the gift of the xER cDNA; C. Bertholet for the recombinant vaccinia virus expressing this receptor; N. Mermod for helpful discussions and suggestions; and G. Krey, E. Martinez, and J. Northrop for comments on the manuscript. B.C. and F.-X.C. have contributed equally to this work, which was supported by the Swiss National Science Foundation and the Etat de Vaud.

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