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Activation of κB-specific proteins by estradiol

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ABSTRACT **The κB enhancer serves as a recognition site for the nuclear transcription factor NF-κB and other κB-specific proteins which are activated in many cell types in response to a variety of extracellular signals. But a steroid-dependent activation of NF-κB or any other κB-specific protein has not previously been reported, to our knowledge. In this report we demonstrate that estrogen can activate κB-specific protein in its target tissue, uterus. We have done this by analyzing the interaction of nuclear extracts with κB enhancers, using DNA mobility shift assays. The activation by estradiol was time dependent, reaching a maximum at approximately 2 hr after steroid treatment, and was not inhibited by prior cycloheximide treatment. The protein–DNA complexes formed in response to estradiol did not contain NF-κB and, when compared with other κB enhancer motifs, had a higher affinity to the κB enhancer corresponding to the PRDII element present in duplicate motifs. These protein–DNA complexes also did not appear to contain estrogen receptor, since antibodies to estrogen receptor were without any effect on either their formation or their mobility. The protein–DNA complexes formed in response to estradiol, however, exhibited a high affinity for the estrogen-responsive element, suggesting the participation of an estrogen-receptor-like molecule in the DNA binding. In contrast, the protein–DNA complexes formed constitutively contained NF-κB, had equivalent affinities to various κB enhancers, and did not have a high affinity for the estrogen-responsive element. On the basis of these findings, we propose that estrogen-dependent activation of the as-yet-unidentified κB-specific protein involves the association of this protein with an estrogen-receptor-related molecule and binding of the resulting complex to PRDII. The high affinity and specificity of this binding to PRDII suggests that this may serve as a composite regulatory element in mediating estrogen-dependent gene expression. The potential significance of such a mechanism for steroid hormone action is discussed.**

The κB enhancer is a potent *cis*-acting regulatory sequence present in many inducible cellular and viral genes and serves as a recognition site for the promiscuous transcription factor NF-κB, which was originally identified in nuclei of mature B cells (1, 2). Recent studies have shown that NF-κB-binding activity corresponds to a family of inducible κB-specific proteins which, in turn, are structurally related to the family of *rel* proteins and the product of the dorsal gene, a ventral morphogen in *Drosophila* (3–6). In addition to the *rel* family of proteins (whose DNA-binding domain is as yet unclassified), κB enhancers are also recognized by the family of κB-specific proteins containing 'zinc finger" motifs—such as MBP-1 or PRDII-BP, αA-CRYBP-1 and AFIE-BP1, and RκB—and by the MyoD family of helix–loop–helix proteins (7–11).

In most cells, NF-κB and other κB-specific proteins are present in a latent inactive form in the cytoplasm, which, upon activation *in vivo* by an appropriate cellular signal, enter the nucleus in a DNA-binding form in the absence of any new protein synthesis (12, 13). Activation of this family of transcription factors is an efficient regulatory system for transmitting the signal for gene induction from the cytoplasm to the nucleus. A significant characteristic of κB-specific proteins is their ability to regulate the expression of multiple genes in eukaryotic cells in response to a variety of extracellular signals (1, 2). However, a steroid-dependent activation of NF-κB or any other κB-specific protein has not been reported as yet, although this possibility is suggested by a recent report that a steroid-dependent regulatory element of the chicken ovalbumin gene contains NF-κB-like sequences (14, 15). In this report, we present evidence that the female steroid hormone estradiol-17β can activate κB-specific proteins in its target tissue, the uterus, and we discuss the potential importance of κB-specific proteins in mediating steroid hormone action.

EXPERIMENTAL PROCEDURES

Tissues and Reagents. Uteri were obtained from ovariectomized BALB/c mice given a single subcutaneous injection of either saline or 1 μg of estradiol-17β.

Oligonucleotides were synthesized by the Microchemical Facility of the University of California at Berkeley. The sequences of the various oligonucleotides and their corresponding designations used throughout the paper are as follows: PRDII (wild type), 5′-GGGAAATTCCGGGAAATTCC-3′; PRDII-M_1 (mutant κB), 5′-AAGAAATTCCAAGAAATTCC-3′; IgκB, 5′-GGGACTTTCCGGGACTTTCC-3′; H-2K-κB, 5′-GGGATTCCCCGGGATTCCCC-3′; PRDII (0.5), 5′-GGGAAATTCCAAGAATTCC-3′; IgκB (0.5), 5′-TGGACAGAGGGGACTTTCCGAGAGGCTCGA-3′; H-2K-κB (0.5), 5′-GATCCTCTGGGGATTCCCCATGCA-3′; ERE, 5′-GATCCGTCAGGTCACAGTGACCTGATG-3′; mutant ERE, 5′-GATCCGTCAGGTCACAGTGGCCTGATG-3′. Underlines indicate the enhancer sequences when present as a single motif.

NF-κB affinity matrix was purchased from GIBCO/BRL. The matrix consisted of Sepharose 4B coupled to one strand of a 42-mer double-stranded oligonucleotide containing a triplicate repeat of the nucleotide sequence 5′-GGGGACTTTCCC-3′, corresponding to an IgκB enhancer (16). Purified preparations of NF-κB and the inhibitory protein IκB were provided by S. Ghosh (Yale University, New Haven, CT).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs). Nuclear extracts were prepared as described by Shapiro *et al.* (17). For EMSAs, aliquots of cell extracts were incubated at 25°C with ^{32}P-end-labeled oligonucleotide and poly(dI-dC) in a buffer composed of 20 mM Hepes at pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol, and 5% (vol/vol) glycerol. The resulting DNA–protein complexes were resolved by electro-

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Abbreviation: ERE, estrogen-responsive element.

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phoresis at room temperature on nondenaturing 5% polyacrylamide gels in a Tris–glycine buffer system. In experiments using IκB, cellular extracts were preincubated with a highly purified preparation of IκB in the presence of Nonidet P-40 for 20 min at room temperature prior to the addition of the DNA binding reaction mixture as described by Bauerle and Baltimore (18). In experiments using NF-κB affinity matrix, nuclear extracts were incubated with the matrix equilibrated with the DNA binding buffer for 30 min at 4°C. Subsequently, the unabsorbed proteins were recovered by centrifugation, incubated with specified labeled oligonucleotides, and processed for EMSA.

RESULTS

Effect of Estradiol on the Interaction of Uterine Cellular Extracts with κB Enhancer Sequence. To identify the estrogen-dependent activation of κB-specific proteins, we examined the interaction of uterine cellular extracts with κB enhancer by DNA mobility shift assays, using a synthetic oligonucleotide containing two repeats of the κB motif 5′-GGGAAATTCC-3′, corresponding to the virus-inducible element (PRDII) contained within the human β-interferon promoter (19). Such an assay revealed two discrete protein–DNA complexes (designated as N1 and N2) in nuclear extracts from estrogen-treated tissues, while in control extracts, only the faster-migrating complex, N2, was present (Fig. 1*A*; compare lane 2 with lanes 4, 6, and 8). The formation of N1 was dependent on the duration of treatment with estradiol, reaching a maximum at approximately 2 hr after steroid treatment. Densitometric scanning of autoradiographs from three separate experiments revealed that, at 2 hr after estradiol treatment, on an average, there was approximately a 9.5-fold increase in the radioactivity associated with N1 and a 1.5-fold increase with N2. The effect of steroid was not inhibited by prior cycloheximide treatment (Fig. 1*A*; compare lane 6 with lane 10). Cycloheximide, however, was effective in inhibiting total protein synthesis by >80% in both control- and estrogen-treated tissues, as reported almost 30 years ago (20). Competition experiments using mutant κB sequences confirmed the κB-specific nature of these interactions (Fig. 1*B*; compare lanes 2 and 4). In contrast to estradiol, there was no formation of steroid-specific protein–DNA complexes upon administration of the synthetic progestin promegestone (data not shown).

Relative Binding Affinities of N1 and N2 to Various κB Enhancer Motifs. Although the hallmark of all κB-specific proteins is their ability to bind with high affinity to the decameric consensus sequence GGGRHTYYCC (R = A or G; H = C, A, or T; and Y = T or C) (1), there are differences among these proteins with respect to their affinity for various κB enhancer elements (2). Therefore, for an initial characterization of the nature of κB-specific proteins responsible for the formation of N1 and N2, we examined the ability of various κB enhancer elements to compete with the binding of labeled PRDII. A typical fluorogram of the native gels from these experiments and the data representing a densitometric scanning of these gels are shown in Figs. 2 and 3, respectively. Homologous competition with excess unlabeled PRDII revealed that the proteins in N1 had approximately a 15-fold higher affinity to this motif as compared with those present in N2 (Fig. 2*A*, compare lane 13 with lane 9; Fig. 3*A*). In contrast, competition experiments with unlabeled κB motif GGGACTTTCC, corresponding to the sequence present on mouse Igκ enhancer (16), revealed a lower affinity (approximately 14-fold) to this motif by protein in N1 compared with N2 (Fig. 2*B*, compare lanes 5, 6, and 7 with lane 2; Fig. 3*B*). Competition with unlabeled κB motif GGGATTCCCC, corresponding to the motifs present on the major histocompatibility complex class I (*H-2K*^{b}) gene (21), revealed that the proteins in N1 did not have any significant affinity to this motif (Fig. 2*B*, compare lanes 11, 12, and 13 with lane 9; Fig. 3*C*), while the proteins in N2 had an affinity to the H-2K-κB enhancer motif equivalent to that observed with the mouse Igκ enhancer motif (compare Fig. 3 *B* with *C*). In these competition experiments we also compared the

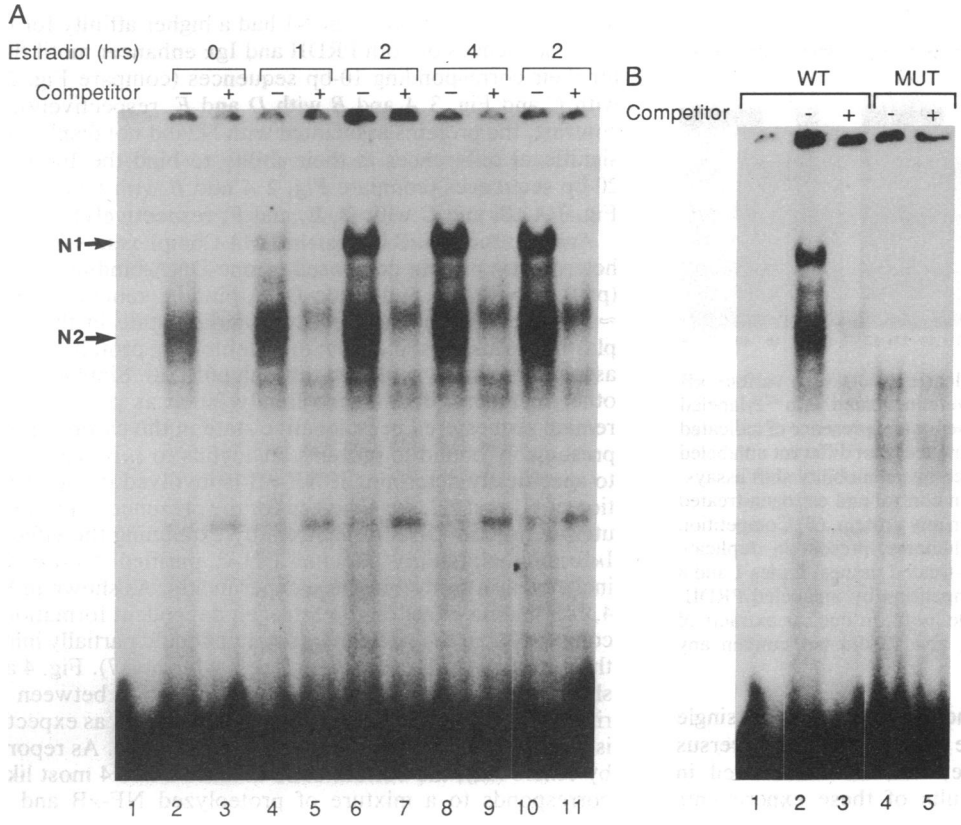

FIG. 1. Activation of uterine κB-specific proteins by estradiol. Uterine nuclear extracts from ovariectomized mice exposed to estradiol *in vivo* for the indicated times were incubated with either ^{32}P-labeled wild-type or mutant PRDII and analyzed by gel retardation assays. (*A*) Time course for estrogen-dependent formation of nuclear protein–DNA complexes using ^{32}P-labeled wild-type PRDII and a 25-fold excess unlabeled oligonucleotide for competition. Lane 1 did not contain any protein, and lanes 10 and 11 were loaded with extracts prepared from tissues treated with cycloheximide for 1 hr prior to exposure to estradiol. (*B*) Comparison of protein–DNA complexes formed in extracts from tissues treated with estradiol (for 2 hr) with ^{32}P-labeled wild-type and mutant PRDII in the absence or presence of a 25-fold excess unlabeled oligonucleotide. Lane 1, no protein.

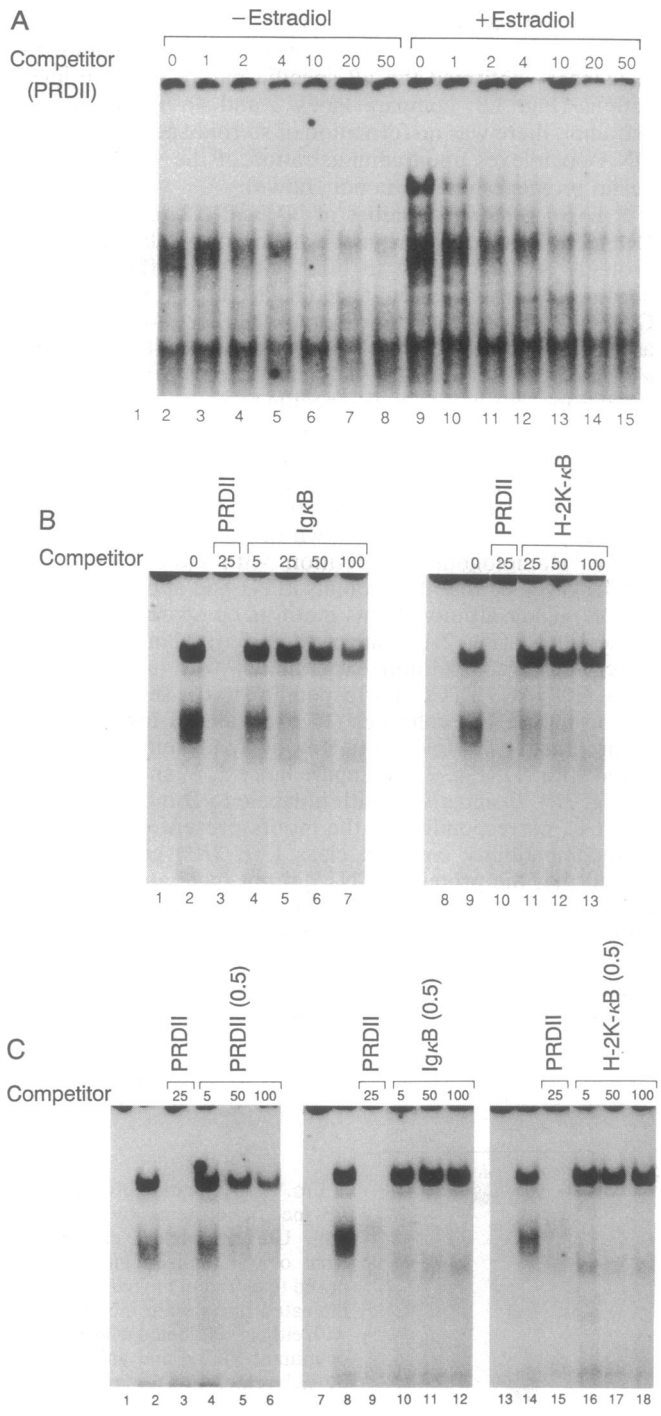

FIG. 2. Interaction of uterine nuclear extracts with various κB enhancer elements. Nuclear extracts were incubated with ^{32}P-labeled PRDII (duplicate motifs) both in the absence and presence of indicated concentrations (expressed as fold molar excess) of different unlabeled κB enhancers (competitor) and analyzed by gel mobility shift assays. (*A*) Competition by unlabeled PRDII in control and estrogen-treated nuclear extracts. Lane 1 did not contain any protein. (*B*) Competition by unlabeled IgκB and H-2K-κB enhancers, present in duplicate motifs, in nuclear extracts of estrogen-treated tissues. Lanes 1 and 8 did not contain any protein. (*C*) Competition by unlabeled PRDII, IgκB, and H-2K-κB present in a single motif, in nuclear extracts of estrogen-treated tissues. Lanes 1, 7, and 13 did not contain any protein.

ability of the proteins in N1 and N2 to bind to a single 10-base-pair (bp) sequence of the various κB motifs versus the two-repeat (20-bp) sequence, such as those used in preceding experiments. The results of these experiments

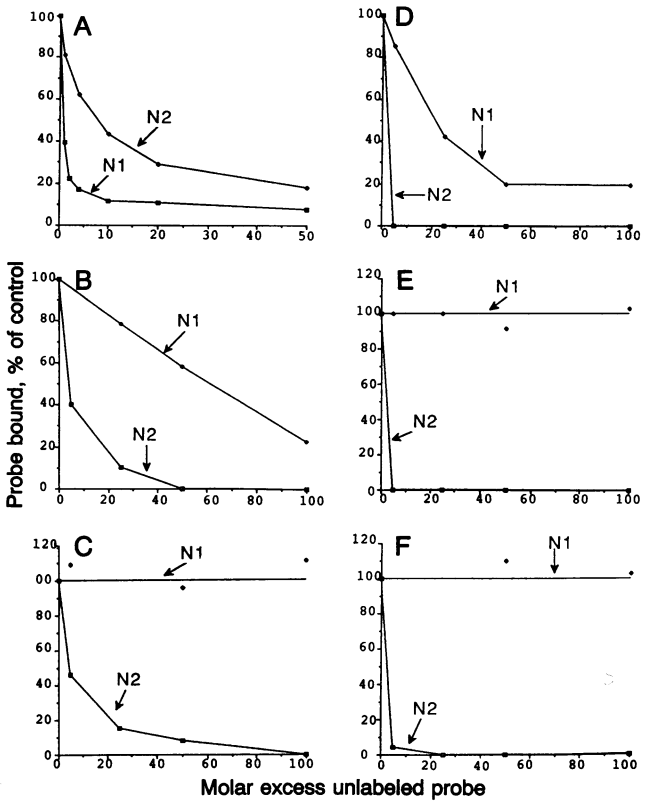

FIG. 3. Relative affinity of uterine protein-DNA complexes to various κB enhancer elements. The autoradiographs corresponding to the experiments shown in Fig. 2 were scanned with a computerized laser scanning densitometer to estimate the relative amounts of radioactivity associated with regions corresponding to N1 and N2. The data are presented as percentages of control samples without any competitor. Competitors were unlabeled PRDII in *A*, IgκB in *B*, H-2K-κB in *C*, PRDII (0.5) in *D*, IgκB (0.5) in *E*, and H-2K-κB (0.5) in *F*.

revealed that the proteins in N1 had a higher affinity for the 20-bp sequences of both PRDII and Igκ enhancer motif than for their corresponding 10-bp sequences (compare Fig. 2 *B* with *C* and Fig. 3 *A* and *B* with *D* and *E*, respectively). In contrast, the proteins associated with N2 did not display any significant differences in their ability to bind the 10-bp vs. 20-bp sequences (compare Fig. 2 *A* and *B* with *C*; compare Fig. 3 *A*, *B*, and *C* with *D*, *E*, and *F*, respectively).

Analyses for NF-κB in Protein-DNA Complexes. NF-κB is a heteromeric protein composed of one DNA-binding subunit (p50) of ≈50 kDa and a non-DNA-binding subunit (p65) of ≈65 kDa; in its nonactive DNA-binding state in the cytoplasm, it is also associated with an inhibitory protein, IκB, an association that is mediated through p65 (22). Similar to p50, other members of this gene family, such as p75, can also remain sequestered in an inactive state in the cytoplasm (6), presumably bound to proteins analogous to IκB. Therefore, to specifically determine if NF-κB is involved in the formation of N1 and N2, we analyzed for the presence of p65 in the uterine protein–DNA complexes by examining the effect of IκB on their ability to bind DNA; purified NF-κB was included as a control in these experiments. As shown in Fig. 4, IκB had no effect on the estrogen-dependent formation of complex N1 in the nuclear extracts but could partially inhibit the formation of N2 (compare lane 5 with lane 7). Fig. 4 also shows that the majority of complex formation between purified NF-κB and PRDII (the top band in lane 2), as expected, is inhibited by IκB (compare lane 2 with lane 4). As reported by others (22), the bottom band in lanes 2 and 4 most likely corresponds to a mixture of proteolyzed NF-κB and ho-

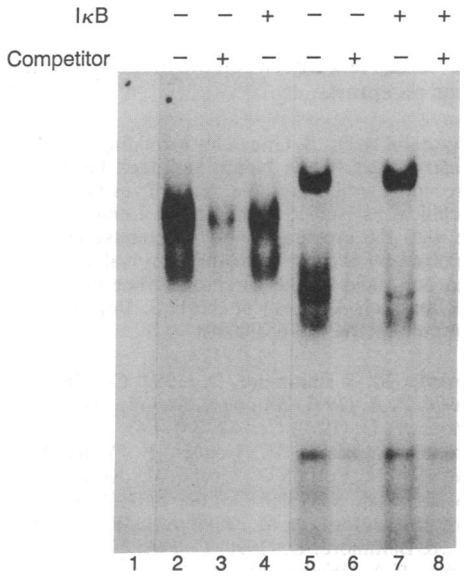

FIG. 4. Effect of IκB on the interaction of uterine nuclear extracts with PRDII. Nuclear extracts (lanes 5 to 8) from uteri exposed to estradiol for 2 hr were analyzed for their ability to bind PRDII either as is or after a pretreatment with IκB as discussed in text. Competitor is unlabeled PRDII. Lane 1 did not contain any protein; lanes 2–4 were loaded with purified NF-κB.

modimers of p50, which accounts for its faster mobility and the relative inability of IκB to inhibit its formation. Thus it appeared that the protein(s) responsible for the formation of N2 contained p65, thereby suggesting that NF-κB might be involved in the formation of these complexes. In contrast, the inability of IκB to inhibit the estrogen-dependent formation of N1 indicated that the proteins in these complexes did not contain p65.

Next, to determine if p50 or a related protein was involved in the formation of N1, uterine nuclear extracts were initially fractionated on a column of Sepharose 4B coupled to an oligonucleotide containing a triplicate repeat of IgκB enhancer and then tested for their ability to bind PRDII. As

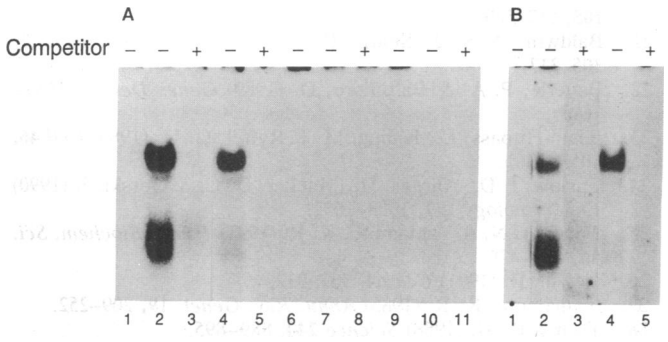

FIG. 5. Effect of fractionation on NF-κB affinity matrix on the formation of protein–DNA complexes by nuclear extracts. Nuclear extracts from uteri exposed to estradiol for 2 hr were analyzed, either as is or after fractionation on NF-κB affinity matrix, for their ability to bind various labeled κB enhancer motifs in the absence (−) or presence (+) of an excess unlabeled homologous competitor. (*A*) Lanes 1, 6, and 9 did not contain any protein; lanes 2 and 3 were loaded with unfractionated extracts; and lanes 4, 5, 7, 8, 10, and 11 were loaded with fractionated extracts. Samples in lanes 1–5 were incubated with labeled PRDII, in lanes 6–8 with labeled PRDII-M1 (mutant PRDII), and in lanes 9–11 with labeled PRDII (0.5). (*B*) Nuclear extracts were fractionated either on plain Sepharose 4B (lanes 2 and 3) or on NF-κB affinity matrix (lanes 4 and 5) prior to incubation with labeled PRDII in the presence or absence of a 25-fold excess of unlabeled PRDII.

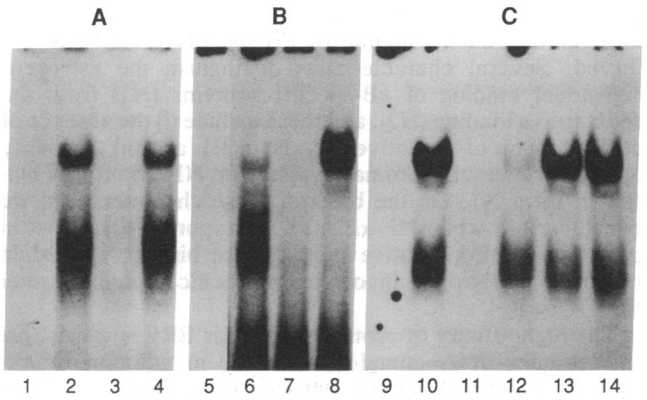

FIG. 6. Analyses for estrogen receptor in the uterine protein–PRDII complexes. Nuclear extracts from uteri exposed to estradiol for 2 hr were incubated with either ^{32}P-labeled PRDII (*A* and *C*) or the ERE (*B*) and analyzed on gel mobility shift assays. Lanes 1, 5, and 9 did not contain any protein. For competition, samples loaded in lanes 3 and 11 also contained 25-fold excess unlabeled PRDII; lanes 7 and 12, a 25-fold excess of wild-type ERE; lane 13, a 25-fold excess of mutant ERE; and lane 14, a 50-fold excess of mutant ERE. Samples loaded in lanes 4 and 8 had been incubated with anti-estrogen-receptor antibody (H222) prior to addition of ^{32}P-labeled DNA probes.

shown in Fig. 5*A*, in extracts chromatographed on NF-κB affinity matrix, there was no formation of protein–PRDII complexes corresponding to N2, while the formation of complexes corresponding to N1 remained unaffected (Fig. 5*A*, compare lane 4 with lane 2). Chromatography of nuclear extracts on plain Sepharose 4B had no significant effect on the formation of either N1 or N2 (Fig. 5*B*, lane 2). An examination of the oligonucleotide specificity for the binding observed in extracts chromatographed on NF-κB affinity matrix revealed it to be identical to that seen for N1 in nonchromatographed extracts (Fig. 2); i.e., it still bound PRDII better than PRDII (0.5) and had no affinity for PRDII-M1 (mutant κB enhancer).

Effect of Anti-Estrogen-Receptor Antibody and Estrogen-Responsive Element (ERE) on the Formation of Nuclear Protein–DNA Complexes. Finally, as a first step towards understanding the mechanisms underlying the estrogen-dependent activation of κB enhancer, we analyzed for estrogen receptor in N1 and N2 by incubating nuclear extracts with an anti-estrogen receptor antibody (H222) prior to analysis by gel mobility shift assays. As shown in Fig. 6*A*, H222 had no effect on either the formation or the mobility of N1 and N2. In parallel experiments, when a labeled synthetic oligonucleotide containing the ERE, corresponding to that found in vitellogenin A2 gene (23), was used as the probe (instead of PRDII), addition of H222 resulted in a discrete decrease in the mobility of protein–ERE complexes, indicating that the nuclear extracts did contain estrogen receptor capable of interacting with the antibody (Fig. 6*B*, compare lanes 6 and 8). Similar results were also obtained when, instead of H222, a polyclonal antiserum to estrogen receptor (24) was used (data not shown). If instead of an excess of unlabeled PRDII, an excess of unlabeled ERE was included in the reaction as a competitor, it effectively abolished the formation of N1 but not N2 (Fig. 6*C*, compare lane 10 with lane 12). In contrast to wild-type ERE, mutant ERE had no effect on the formation of either N1 or N2 (Fig. 6*C*, compare lane 10 with lanes 13 and 14).

DISCUSSION

In this report, we have demonstrated an estrogen-dependent interaction of uterine nuclear extracts with κB enhancer elements, establishing that a steroid hormone can activate

κB-specific proteins. In the uterus, both constitutive and an estrogen-dependent binding of κB-specific proteins is observed. Several characteristics distinguish the estrogen-dependent binding of κB-specific proteins (N1) from the constitutive binding (N2), and these include (i) the absence of p65 (a subunit of the active NF-κB) in N1, and (ii) the ability of nuclear extracts chromatographed on NF-κB affinity matrix to form N1. On the basis of these characteristics, we conclude that while NF-κB may be responsible, at least in part, for the constitutive binding, the binding elicited in response to estrogen involves κB-specific protein(s) other than NF-κB.

The high affinity of proteins in N1 for ERE suggests that this protein–DNA complex contains, in addition to κB-specific protein(s), either estrogen receptor or a protein closely related in structure to the receptor. However, both the H222 monoclonal antibody to estrogen receptor, known to interact with the steroid-binding domain of the receptor, and the polyclonal antiserum to estrogen receptor (raised against the hinge region of the receptor) were without any effect on either the formation or the mobility of N1. This strongly suggests that the protein(s) in N1 responsible for eliciting the competition with ERE may not be estrogen receptor itself but a protein closely related in structure. Regardless, it appears that it is the association of the κB-specific protein in question with the receptor or receptorlike molecule that must be responsible for dictating the high specificity and affinity of N1 for duplicate motifs of PRDII (shown in Figs. 2 and 3), since N2, which does not exhibit any significant competition with ERE, has similar affinity to various κB enhancer motifs. We believe that this is entirely possible if, in fact, the 20-bp repeat sequence of PRDII corresponds to a "composite regulatory element" defined as one to which regulatory factors from different gene families can bind and modulate transcription (25). Although at present we do not know the identity of the κB-specific protein activated by estrogen or the protein responsible for the observed competition with ERE, the biological significance of our observations is borne out as follows.

A hallmark of κB-specific proteins is their ability to activate a wide variety of genes, analogous to a second messenger system (26). Thus, an estrogen-dependent activation of this family of transcription factors through a composite regulatory element has the potential to mediate the widely documented pleiotropic responses associated with estrogen action in the female reproductive tissues. The likelihood that the 20-bp PRDII may indeed represent a composite regulatory element capable of regulating steroid-dependent gene expression is suggested not only by the observations reported herein but also because the NF-κB-like sequences present in the steroid-dependent regulatory element of the chicken ovalbumin gene in fact more closely resembles PRDII (14). In this context it is also worthwhile to mention that in the nuclear extracts of mammary glands, which are insensitive to estrogen action during lactation, there is no κB-specific binding to PRDII (unpublished observations).

It is becoming increasingly apparent that steroid receptors can regulate gene expression by binding both to simple steroid-responsive elements (27–30) and to composite regulatory elements through protein–protein interactions with other cellular transcription factors (31–33). From a recent report, it appears that a membrane receptor agonist such as dopamine can also activate members of superfamily of nuclear steroid hormone receptors such as COUP transcription factor (34). Therefore, considering that the activation of NF-κB and other κB-specific protein also occurs in response to membrane receptor agonists, κB-specific proteins may well represent a class of important intracellular signals triggering the "cross talk" between the membrane receptors and the nuclear receptors.

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