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Expression and Modulation of Voltage-Gated Calcium Channels After RNA Injection in *Xenopus* Oocytes

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Calcium ions flow into cells through several distinct classes of voltage-dependent calcium-selective channels. Such fluxes play important roles in electrical signaling at the cell membrane and in chemical signaling within cells. Further information about calcium channels was obtained by injecting RNA isolated from rat brain, heart, and skeletal muscle into *Xenopus* oocytes. Macroscopic currents through voltage-operated calcium channels were resolved when the endogenous calcium-dependent chloride current was blocked by replacing external calcium with barium and chloride with methanesulfonate. The resulting barium current was insensitive to tetrodotoxin but was completely blocked by cadmium or cobalt. With both heart and brain RNA at least two distinct types of calcium ion conductance were found, distinguishable by their time course and inactivation properties. In oocytes injected with heart RNA, the slowly inactivating component was selectively blocked by the calcium-channel antagonist nifedipine. Barium ion currents induced by heart RNA were modulated by isoproterenol, cyclic adenosine monophosphate, and acetylcholine.

ELECTRICALLY EXCITABLE Ca^{2+} CHANNELS are widespread in nerve, muscle, and several other eukaryotic cell types (*1*). These channels regulate the flow of Ca^{2+} into the cell, where it functions in the contraction of muscle, the release of neurotransmitters and hormones, and the activation of other channels. Several types of Ca^{2+} channels can be distinguished on the basis of the waveform of the currents, voltage- and Ca^{2+}-dependent inactivation, single-channel conductance, and sensitivity to blocking agents (*2*). Ca^{2+} channels can be modulated by neurotransmitters acting through intracellular second messengers. A multisubunit protein complex, presumed to be the Ca^{2+} channel, has been enriched from skeletal muscle by biochemical fractionations in which the binding of dihydropyridine Ca^{2+}-channel antagonists is used as an assay (*3*).

It would be desirable to characterize the individual Ca^{2+} channel types in a similar membrane environment that also permits the introduction of modulatory elements. *Xenopus* oocytes provide an excellent preparation for such studies because (i) they translate and process exogenous messenger RNA (mRNA) efficiently (*4*); (ii) they are amenable to modern electrophysiological measurements, including voltage- and patch-clamp methods; and (iii) several intracellular messenger systems have been identified and characterized (*5*).

To characterize Ca^{2+} currents in oocytes injected with mRNA isolated from electrically excitable tissues, we first identified the endogenous Ca^{2+} currents in uninjected oocytes. The oocytes were tested with a voltage-clamp circuit that used two intracellular microelectrodes. Stepping the voltage from a holding potential of −100 to 0 mV in a normal solution containing 1.8 m*M* Ca^{2+} resulted in both a transient outward current of amplitude 20 to 50 nA lasting for 0.5 to 1 second and a slowly activating K^+ current (Fig. 1). Previous studies have shown that the transient current is a chloride current, $I_{Cl(Ca)}$, evoked by the entry of Ca^{2+} through voltage-dependent Ca^{2+} channels; however, the underlying Ca^{2+} current has not been detected (*6*). To resolve currents through the putative Ca^{2+} channels, we found it necessary to replace the Ca^{2+} with Ba^{2+} (40 m*M*) and to replace the Cl^- with methanesulfonate (legend to Fig. 1). In this solution, both the leak and the slow outward currents were reduced, $I_{Cl(Ca)}$ disappeared, and a transient inward current (decay time constant, ~0.5 second) was observed (Fig. 1, C and D). This conductance was smaller than most conductances observed in *Xenopus* oocytes; the net peak amplitude (after subtracting the leak current) was usually less than 10 nA, and the currents of Fig. 1, C and D, were the largest recorded in more than 25 oocytes. This current also appeared in a Cl^--free solution with high concentrations of Ba^{2+} and *N*-methyl-D-glucamine substituted for Na^+; it was therefore not a Na^+ current. It was, however, completely inhibited by 0.5 to 1 m*M* Cd^{2+} or by 2 m*M* Co^{2+}. The current was activated at voltages more positive than −30 mV and had a bell-shaped current-voltage curve that peaked at about 10 mV (Fig. 1E). Taken together, these results suggest that the transient inward current was a Ba^{2+} current (I_{Ba}) through a Ca^{2+} channel. Like $I_{(Cl(Ca)}$ (*6*) I_{Ba} was almost fully inactivated by holding potentials more positive than −40 mV (Fig. 1F).

Xenopus oocytes were injected with either total RNA or polyadenylated [poly(A)$^+$] RNA isolated from rat liver, skeletal muscle, heart, or brain (legend to Fig. 2). After incubation for 48 to 72 hours, the oocytes were tested for $I_{Cl(Ca)}$ and I_{Ba} by voltage clamping. In oocytes injected with RNA from brain, skeletal muscle, and heart but not from liver, $I_{Cl(Ca)}$ was significantly increased, reaching amplitudes up to 2 μA. In addition, many oocytes injected with brain or heart RNA showed $I_{Cl(Ca)}$ with two peaks (Fig. 2A). In contrast to uninjected cells, injected cells displayed only partial (40 to 60 percent) inactivation of $I_{Cl(Ca)}$ when the membrane was depolarized from holding potentials more positive than −40 mV. Clearly, the injection of RNA isolated from excitable tissues altered several properties of $I_{Cl(Ca)}$.

In Cl^--free solution with high concentration of Ba^{2+}, large (up to 150 nA) voltage-sensitive inward currents were detected in oocytes injected with brain, skeletal muscle, or heart RNA. These currents were unchanged by addition of tetrodotoxin (1

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μM), a blocker of neuronal and muscular sodium channels, but were completely supressed by Cd^{2+} (0.2 to 2 mM) or by Co^{2+} (2 mM); this suggests that they were Ba^{2+} currents through Ca^{2+} channels. Oocytes were injected with RNA from rats of various ages. The largest I_{Ba} induced by heart or muscle RNA was obtained from tissues of 4- to 8-day-old rats (six different RNA preparations), and the largest I_{Ba} induced by brain RNA was obtained from tissues of 4- to 16-day-old rats (eight different RNA preparations). Little or no I_{Ba} was induced by heart or muscle RNA from prenatal, 1-, 14-, or 17-day-old rats (ten different preparations). Thus there is good agreement between the age at which each organ contains the most Ca^{2+}-channel RNA and the age of most rapid appearance of binding sites for the Ca^{2+}-channel antagonist nitrendipine (*7*). Injection of total, poly(A)$^-$, and poly(A)$^+$ RNA into the oocytes showed that poly(A)$^+$ RNA is responsible for encoding the Ca^{2+} channels.

With muscle and brain RNA, we observed transient, tetrodotoxin-sensitive Na^+ currents; with brain RNA, we observed functional γ-aminobutyric acid, kainate, serotonin, glycine, and glutamate receptors, and a transient outward K^+ current, basically as reported by others (*4*). To resolve I_{Ba} it was sometimes necessary to block the K^+ current by the intracellular injection of tetraethylammonium to a final cytoplasmic concentration of 2 to 4 mM.

Although uninjected oocytes had a single component that inactivated with a decay constant of about 0.5 second, oocytes injected with heart RNA displayed two additional distinct components. A "fast" transient component was studied in detail in batches of oocytes, where it was several times larger than the transient I_{Ba} in uninjected controls; this component inactivated with a time constant between 150 and 300 msec. The "slow" component inactivated little during a 2.5-second step from −100 to 0 mV (Fig. 2, B and C, and Fig. 3). The slow component was the dominant, and with some RNA preparations the only observable, component (Fig. 4). The current-voltage characteristics of the fast and slow components differed slightly, the fast component activating at voltages more negative than the slow component. The two components could also be distinguished pharmacologically with the Ca^{2+}-channel antagonist nifedipine (1 to 10 μM), which selectively inhibited the slow component (Fig. 3A). Therefore, subtraction of records obtained in nifedipine yielded currents representing only the slow component (Fig. 3B).

In addition to their sensitivity to nifedipine, the fast and slow components differed in their dependence on the holding potential. A holding potential of −40 mV resulted in little inactivation of the slow component, although the fast component was inactivated by 40 to 60 percent. Steps from a holding potential of −20 mV to +20 mV evoked only the slow current. When such traces were subtracted from the data obtained with steps from more hyperpolarized holding potentials to +20 mV, we obtained an inactivation curve for the fast component similar to that in Fig. 3D. The total I_{Ba} in oocytes injected with heart RNA was inhibited 50 to 90 percent by 10 to 20 μM Cd^{2+}; however, the available data were not precise

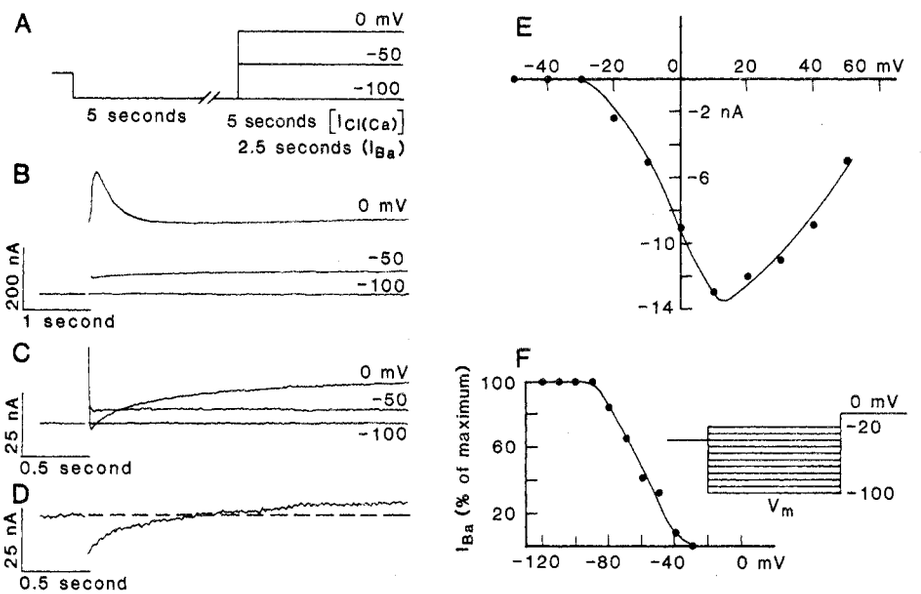

Fig. 1. Study of $I_{Cl(Ca)}$ and I_{Ba} in uninjected oocytes. Stage 5 or 6 oocytes (*11*) were defolliculated by treatment with collagenase (Type 1A, Sigma; 2 mg/ml) in Ca^{2+}-free solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes-NaOH (*p*H 7.5) for 2 to 3 hours at 22°C. The oocytes were washed several times with ND96 solution, and cells devoid of any external cellular layers were stored for up to 5 days in 70 percent Leibovitz L-15 culture medium (Irvine Scientific) supplemented with penicillin (100 unit/ml), streptomycin (100 μg/ml), and 0.5 mM theophylline [to prevent spontaneous maturation (*12*)]. (A) Voltage sequence for panels B through D. (B) Voltage-clamp currents recorded in normal physiological solution, ND96 [96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM Hepes-NaOH (*p*H 7.5)]. Holding potential, −60 mV. (C) Currents in the same cell in Cl^--free solution with high Ba^{2+} concentration [40 mM $Ba(OH)_2$, 50 mM NaOH or *N*-methyl-D-glucamine, 2 mM KOH, and 5 mM Hepes; titrated with methanesulfonic acid to *p*H 7.4). Holding potential, −40 mV. (D) The net active currents for the jump to 0 mV in (C). Currents for the jump to −50 mV have been scaled and subtracted. (E) Current-voltage relation for I_{Ba} in an uninjected cell. After the 5-second conditioning pulse to −100 mV, the voltage was stepped to various test values. Between −80 and −40 mV, only linear leak currents were observed; these were scaled appropriately and subtracted from the total currents recorded at all other voltages. (F) Steady-state inactivation of I_{Ba} in a control oocyte. The experimental protocol is shown to the right: membrane potential was stepped from the holding value (−40 mV) to various prepulse values for 5 seconds and then to 0 mV to elicit I_{Ba}. V_m, maximum potential.

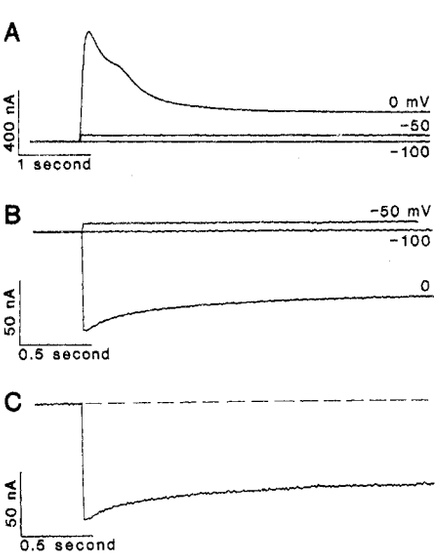

Fig. 2. $I_{Cl(Ca)}$ and I_{Ba} in oocytes injected with RNA isolated from rat heart. Poly(A)$^+$ RNA was isolated by passage over an oligo(dT) column. One to five hours after defolliculation (legend to Fig. 1), oocytes were injected with either 50 ng of poly(A)$^+$ RNA or 150 to 200 ng of total RNA. Recordings were made 2 to 4 days later. Traces in Figs. 2 and 3 are typical of results observed in at least ten oocytes under each condition. (A) The two $I_{Cl(Ca)}$ peaks in ND96 solution. Holding potential, −60 mV. (B) Inward current in the same cell in Cl-free solution with high Ba^{2+} concentration. Holding potential, −40 mV. (C) The net I_{Ba} of records in (B) after subtraction of leak current. Same protocol as in Fig. 1, A to D.

enough to determine whether the fast or slow component was selectively inhibited.

Oocytes injected with brain RNA also showed both transient and maintained components of I_{Ba}. The transient component displayed a decay time constant of 150 to 200 msec for voltage steps from −100 to 0 mV. As with heart I_{Ba}, brain I_{Ba} was activated at voltages more positive than −30 mV and peaked at about +10 mV.

Ca^{2+} currents in the heart are modulated by a number of neurotransmitters and drugs (*1*). In oocytes injected with heart RNA, the β-adrenergic agonist isoproterenol (10 μ*M*) increased the total I_{Ba} to 134 ± 7 percent of control in 1 to 2 minutes (mean ± SEM, $n = 4$; Fig. 4A, trace 2). The effect of isoproterenol desensitized within 5 to 10 minutes but could be repeated several times on the same cell with 10- to 15-minute intervals between applications. The effect of isoproterenol was inhibited by 10 μ*M* propranolol, suggesting that β-adrenergic receptors are involved in the response. The adenylate cyclase activator forskolin (40 to 50 μ*M*) increased the amplitude of I_{Ba} to 150 ± 5 percent of control in 15 to 30 minutes ($n = 3$; Fig. 4B). Intracellular injection of cyclic adenosine monophosphate (cyclic AMP; 2 to 4 pmol per oocyte) increased the amplitude of I_{Ba} to 127 ± 3 percent of control values ($n = 4$). Although a comprehensive study has not been carried out, it appears that cyclic AMP had a greater effect on the slow component of heart I_{Ba}. Furthermore, the total peak I_{Ba} was reduced to 64 ± 7 percent of control ($n = 7$) by application of 10 μ*M* acetylcholine (Fig. 4, trace 4). This decrease was observed regardless of whether the oocyte had been previously exposed to isoproterenol. I_{Ba} in uninjected control oocytes was too small for a systematic study of pharmacological effects, but there were no consistent effects of isoproterenol, forskolin, or acetylcholine.

Thus, the Ca^{2+} channels incorporated into the oocyte membrane after RNA injection respond to the same neurotransmitters and drugs that regulate cardiac Ca^{2+} channels. Several components of this modulatory pathway are already present *Xenopus* oocytes: for example, catecholamine responses, cyclic AMP–dependent protein kinase, and adenylate cyclase. However, these responses are not usually present after the collagenase treatment that defolliculates the oocytes (*5, 8*). In addition, *Xenopus* oocytes respond to muscarinic stimulation, primarily with a fluctuating Cl^- current (*9*). With oocytes injected with heart RNA and tested in normal physiological solutions, the response of Cl^- current to acetylcholine was enhanced compared to uninjected oocytes; this suggests a contribution to the response by heart RNA. We do not know whether the Ca^{2+} channels synthesized from the exogenous RNA are modulated by endogenous components (including receptors, guanosine 5′-triphosphate binding proteins, and kinases) or whether they require some components encoded by the injected RNA.

The results of this study demonstrate that

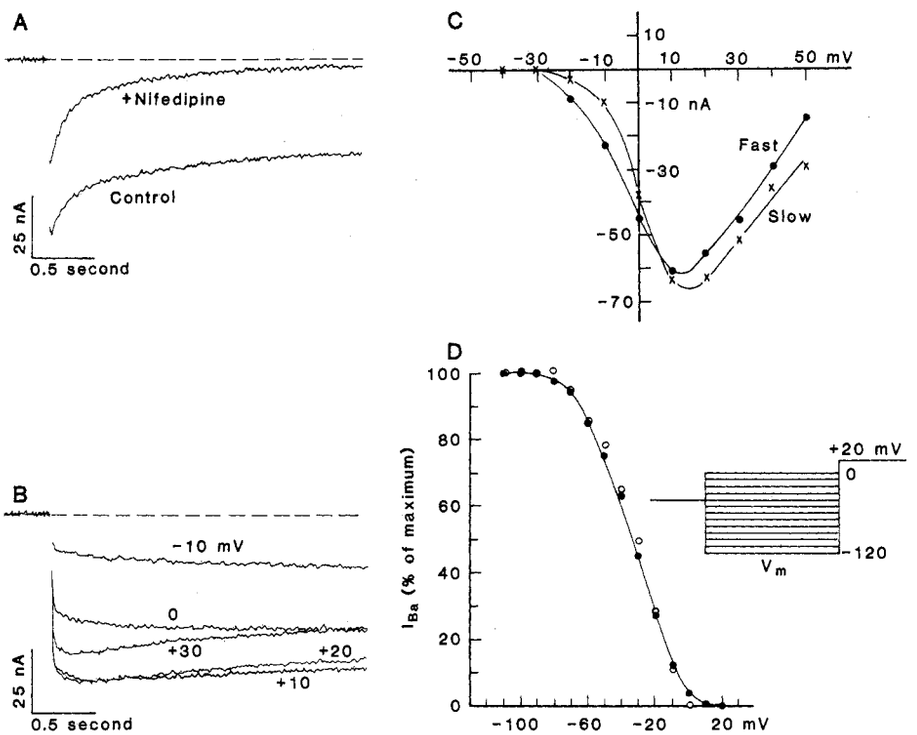

Fig. 3. Effect of nifedipine on fast and slow components of I_{Ba} in an oocyte injected with heart RNA. (A) I_{Ba} evoked by stepping the voltage from −100 to 0 mV, as in Fig. 1A. Nifedipine (10 μ*M*) reduced the current. I_{Ba} obtained with the same protocol in the presence of 1 m*M* $CdCl_2$ was subtracted from both traces. In the presence of Cd^{2+}, traces showed only the leak and an outward component of net amplitude of 4 nA at the end of the 2.5-second pulse. Nifedipine was freshly prepared as a stock solution of 10 m*M* in dimethyl sulfoxide (DMSO). (B) A family of slow I_{Ba}'s obtained by stepping the membrane potential to various levels after the 5-second conditioning pulse to −100 mV. Leakage currents and the fast components of I_{Ba} were eliminated by subtracting the records obtained in the presence of 10 μ*M* nifedipine. (C) Current-voltage relations for the two components of I_{Ba}. The curve for the slow component was obtained as explained in (B). The fast component was measured in the presence of 10 μ*M* nifedipine; leak and outward currents were eliminated by subtracting the records in the presence of 1 m*M* Cd^{2+}. Neither nifedipine nor Cd^{2+} had any obvious effects on the outward currents at voltages up to +50 mV. (D) Steady-state inactivation curve for the fast component. Nifedipine (10 μ*M*) was added to suppress the slow component of I_{Ba}. The experimental protocol (right) was similar to that used in Fig. 1F, but the test voltage was 20 mV. The net I_{Ba} values were calculated by subtraction of either the current for the step from 0 to +20 mV (●) or that recorded in the presence of 1 m*M* $CdCl_2$ (○).

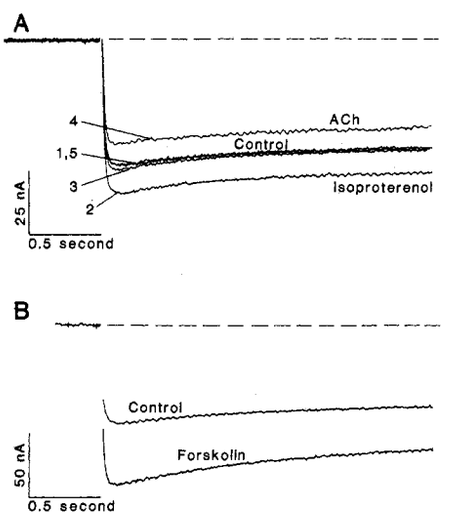

Fig. 4. Pharmacology of the slow component of I_{Ba} in oocytes injected with heart RNA. (A) The effects of isoproterenol (10 μ*M*) and acetylcholine (ACh, 10 μ*M*). Leakage currents have been subtracted from all traces (Fig. 1). Holding potential, −40 mV. (Trace 1) Control. (Trace 2) Two minutes after addition of isoproterenol. (Trace 3) Eight minutes after washing out isoproterenol; I_{Ba} had returned to its control level. (Trace 4) Fifteen minutes after addition of ACh. (Trace 5) Three minutes after ACh washout. (B) Effect of 40 μ*M* forskolin. I_{Ba} was evoked by a voltage step from −80 to +10 mV. Leakage currents were eliminated by subtracting records in the presence of 0.1 m*M* $CdCl_2$. Forskolin was stored as a stock solution (50 m*M*) in DMSO.

the injection of exogenous RNA obtained from electrically excitable tissues into *Xenopus* oocytes causes the appearance of voltage-operated Ca^{2+} channels in the oocyte membrane. These channels are distinct from the endogenous ones in respect to their time course and inactivation properties. Moreover, heart and brain RNA each encode at least two distinct types of Ca^{2+} channels. For the heart RNA, the slow current appears to show the appropriate sensitivity to and modulation by transmitters and intracellular messengers. Significantly, a rapidly inactivating, norepinephrine- and dihydropyridine-insensitive Ca^{2+} current has recently been reported in heart cells (*10*).

The injection of RNA from various tissues into *Xenopus* oocytes should aid in the characterization of different types of Ca^{2+} channels. Such data will complement experiments with isolated synaptosomes, peripheral neurons, cell lines, and reconstituted membranes. If specific RNA populations are used, it may also be possible to clarify the relations among components of Ca^{2+} channels and the interactions between channels and modulatory elements.

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Sequence and Expression of Human Estrogen Receptor Complementary DNA

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The mechanism by which the estrogen receptor and other steroid hormone receptors regulate gene expression in eukaryotic cells is not well understood. In this study, a complementary DNA clone containing the entire translated portion of the messenger RNA for the estrogen receptor from MCF-7 human breast cancer cells was sequenced and then expressed in Chinese hamster ovary (CHO-K1) cells to give a functional protein. An open reading frame of 1785 nucleotides in the complementary DNA corresponded to a polypeptide of 595 amino acids and a molecular weight of 66,200, which is in good agreement with published molecular weight values of 65,000 to 70,000 for the estrogen receptor. Homogenates of transformed Chinese hamster ovary cells contained a protein that bound [^{3}H]estradiol and sedimented as a 4*S* complex in salt-containing sucrose gradients and as an 8 to 9*S* complex in the absence of salt. Interaction of this receptor-[^{3}H]estradiol complex with a monoclonal antibody that is specific for primate ER confirms the identity of the expressed complementary DNA as human estrogen receptor. Amino acid sequence comparisons revealed significant regional homology among the human estrogen receptor, the human glucocorticoid receptor, and the putative v-*erb*A oncogene product. This suggests that steroid receptor genes and the avian erythroblastosis viral oncogene are derived from a common primordial gene. The homologous region, which is rich in cysteine, lysine, and arginine, may represent the DNA-binding domain of these proteins.

THE REGULATION OF GENE EXPRESsion in eukaryotic cells by estrogens and other steriod hormones involves the interaction of specific intracellular receptor proteins with the genome, resulting in the activation of selected sets of responsive genes (*1*). As a consequence, DNA synthesis in certain target cells is altered, and there are changes in the synthesis of specific RNA's and proteins involved in the regulation of cell proliferation, differentiation, and physiologic function in diverse tissues. In addition, steriod hormones and their receptors appear to be involved in the regulation of abnormal growth in various tumors and tumor cell lines (*2*). Recent data from several laboratories (*3*) suggest that steroids may exert their effects by binding directly to an intranuclear receptor molecule that is weakly associated with nuclear components in the absence of ligand. Binding of hormone to its receptor results in conversion of the receptor-steroid complex to a form that associates with high affinity to one or more nuclear components. The molecular nature of this association and of the subsequent modulation of specific gene transcription is not known, although a number of nuclear acceptor sites have been proposed. These include specific DNA sequences (*4*), the nuclear matrix (*5*), and acidic nonhistone protein-DNA complexes (*6*). Although distinct steroid- and DNA-binding domains have been postulated to exist in all steroid receptors, few data are available on the detailed structure, composition, and chemical properties of the subunit that binds both steriod and DNA, and virtually nothing is known about the possible involvement of other components that do not bind steroid.

Determination of the primary structure of the estrogen receptor (ER) and expression of this molecule in homologous and heterologous systems can provide valuable information about structure-function relationships at a molecular level. Although ER is distributed in a tissue-specific manner, many of these cell types also express receptors for several other steroid hormones (*7*). Thus, it is likely that the specificity of control of responsive elements by steroids is determined, at least in part, by the primary structure of the receptor protein. Like other steroid receptors, hormone-occupied ER appears to recognize discrete DNA sequences that are generally upstream of transcriptional start sites in responsive genes. In the prolactin gene, footprinting analysis revealed a specific binding site for ER about 2 kb upstream of the start site (*8*); similar analyses of genes responsive to progestins and glucocorticoids revealed binding sites

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