Cloning and Expression of a Novel Dominant-Negative-acting Estrogen Response Element-binding Protein in the Heterogeneous Nuclear Ribonucleoprotein Family*

(Received for publication, August 18, 1998)

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Most genera of New World primates exhibit a compensated form of resistance to steroid hormones produced by the adrenal gland, gonads, and kidney. Estrogen resistance in New World primate cells is associated with the relative overexpression of a nonreceptor-related estrogen response element-binding protein (ERE-BP) that competes with estrogen receptor for ERE binding. Using the concatamerized ERE half-site (AGGTCAcag) in DNA affinity chromatography, we purified to homogeneity a 40–42-kDa ERE-BP. The affinity-purified ERE-BP bound specifically to either single- or double-stranded DNA bearing the consensus ERE half-site motif AGGTCA. Four distinct internal tryptic peptides from this protein were generated and shown to exhibit sequence similarity to proteins in the heterogeneous nuclear ribonucleoprotein family. These tryptic peptide fragments were used to generate a series of degenerate oligonucleotides that were successfully employed in isolating a full-length ERE-BP cDNA by polymerase chain reaction. Although a member of a family of proteins generally recognized for their ability to bind single strand RNA, the estrogen resistance-associated protein ERE-BP can effectively bind double strand DNA and competitively squelch estrogen receptor-directed transactivation.

Compared with Old World primates like man, New World primates, which reside in South and Central America, exhibit a compensated form of gonadal steroid hormone resistance (1). In female monkeys, this resistant state is characterized biochemically by increased circulating levels of 17β-estradiol (2) and progesterone (3), and anatomically by gonadal hypertrophy (4). In their native environment New World primates have obviously adapted well to this state of gonadal steroid resistance, as they exhibit normal reproductive capabilities (5). However, the reason why New World primate species require such high circulating levels of gonadal steroids has been unclear and debated (6–8). Chrousos et al. (2, 3) were the first to suggest a defect in expression of the estrogen and progesterone receptor proteins, indicating that there was either a change in affinity of the receptor for ligand, a decrease in the total number of receptors for hormone on a per cell basis, or some combination of the two events. More recently Brandon et al. (1) found that, when removed from the New World primate cell and transplanted into an Old World primate cell, the ability of the New World primate estrogen receptor (ER) to bind hormone and transactivate a reporter gene was not compromised. These data indicate that there exists in New World primate cells another protein(s) that interferes with the transactivating potential of the endogenous ER. We recently discovered such a protein that was greatly overexpressed in New World primate cells compared with Old World primate cells (9). This protein competes with ER for binding to the estrogen response element (ERE) and has been coined the “estrogen response element-binding protein” or ERE-BP. Here we report the successful purification and characterization of the 40–42-kDa ERE-BP as a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. We further demonstrate that endogenously expressed ERE-BP in estrogen-resistant primate cells as well as transient transfection of the ERE-BP cDNA in estrogen-responsive primate cells squelches estrogen-induced ER-ERE-directed transactivation.

EXPERIMENTAL PROCEDURES

Cells—The B95-8, owl monkey kidney (OMK), and Vero cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as described previously (10). The B95-8 cell line was established from the common marmoset (Callithrix jacchus), a New World primate that exhibits gonadal steroid hormone resistance. The OMK cell line, also from a New World primate is estrogen-responsive. The Vero cell line originated from the kidney of an African green monkey, which is an Old World primate and estrogen-responsive. Transient Transfection—A eukaryotic expression vector containing cDNA sequence encoding the full-length, wild-type human ERα and a reporter plasmid PERMLuc containing a single ERE cloned upstream from the adenovirus major-late promoter sequence were generously provided by Dr. Elizabeth A. Allegratto (Ligand Pharmaceuticals, Inc., San Diego, CA) (11). 5 × 10^5 B95-8 or OMK cells were seeded into wells of 6-well plates in phenol red-free medium containing 10% charcoal-stripped fetal calf serum and allowed to proliferate for 24 h. Transfections were performed in triplicate with the following DNA suspension: 6 μg of ERE-BP cDNA in the 3.1 expression vector (Invitrogen, Carlsbad, CA) and/or 0.5 μg of ER expression vector, 5 μg of ERE luciferase reporter plasmid, and 5 μg β-galactosidase plasmid with pGEM™-3Z vector as carrier DNA (Promega, Madison, WI) to a final concentration of 20 μg DNA/ml Lipofectamine solution (Stratagene, La Jolla, CA). An equal volume of 20% fetal calf serum-supplemented,

* This work was supported by National Institutes of Health Grants DK08903 (to H. C.) and AR57399 (to J. S. A.) and is dedicated to the memory Bayard D. Catherwood. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AF093414.

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1 D. Brandon, personal communication.

2 The abbreviations used are: ER, estrogen receptor-α; ERE, estrogen response element; ERE-BP, ER-binding protein; RACE, rapid amplification of cDNA ends; hnRNP, heterogeneous nuclear ribonucleoprotein; EMSA, electromobility shift assay; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; OMK, owl monkey kidney; ss, single strand; RBD, RNA binding domain.
antibiotic-free medium was added to each well 5 h after transfection followed by the addition of 10 mM 17β-estradiol. After an additional 48 h at 37 °C, the cells were lysed, and luciferase and β-galactosidase activities were measured.

Preparation of Cellular Extracts—Confluent cultures of cells were harvested, pelleted, and washed twice in ice-cold phosphate-buffered saline (PBS; 20 mM NaHPO₄ and 150 mM NaCl, pH 7.2) and cell extracts prepared as described previously by us (9). Postnuclear extracts were microcentrifuged and desalted through a Microcon-30 filter (30 kDa molecular mass cut-off; Amicon, Beverly, MA). The protein concentration of extracts was determined by the method of Bradford (12).

DNA Affinity Chromatography—A DNA affinity column was described as developed by Kadonaga and Tjian (13). Concatamers of two complimentary, gel-purified oligodeoxynucleotides (30-mers containing 26 nucleotides of complementary sequence and having four base pair, cohesive ends; 5'-GATCCCCAGGTCACAGGTCACAT-3' and 5'-GATCATGTATCTTCTGACCAATC3'-3') were coupled to cyanogen bromide (CNBr)-activated-Sepharose. The DNA-coupled resin was used to purify ERE-BP as described previously by us (9).

Electromobility Shift Assay (EMSA)—Oligonucleotide sequence for the consensus ERE probe was 5'-CTAGAGTATCTGAGCTCAGGATCCTGTGACCTGACTTTCTAG-3' and 5'-CTAGAAGATCTGCAGTACAGGATCCTGTGCACT-3' for the ERE half-site probe. The double strand oligonucleotides used as specific competitor (EMSA) were prepared by annealing complimentary sequence. The above-mentioned single-strand consensus oligonucleotide was used to prepare both single- and double strand labeled probes. The ERE 32-mer, either annealed with complementary strand or not, was radiolabeled with [32P]ATP (NEN Life Science Products) by T4 Kinase (Life Technologies, Inc.) to a specific activity of 10⁶ cpm/µg of DNA. Extract (~10 µg of protein) was incubated on ice with 2 µg of poly(dI-dC) (Boehringer-Mannheim). 20 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl₂ in 10% glycerol for 15 min. Radiolabeled probe (50 fmol), with or without a 50-fold molar excess of radioinert, competing ERE probe, was added and the incubation continued at room temperature for 15 min. Aliquots of the reaction were subjected to electrophoresis in a 6% polyacrylamide, 0.5% TBE gel in 0.5 × TBE running buffer at 100 V. The gels were dried and exposed to Kodak X-OMAT AR film.

**Polymerase Sequencing—**Desalted and microconcentrated fractions eluted from DNA affinity chromatography were loaded on 10% SDS-polyacrylamide gel electrophoresis and visualized with copper staining (Bio-Rad). The dominant 40–42-kDa band was excised from the gel, destained, and washed twice with 50% acetonitrile, 50% distilled water. The protein band was subjected to "in gel" digestion with trypsin and extracted (14). The resultant peptide fragments were separated by narrow bore high performance liquid chromatography using a Vydac C18 2-×150-mm reverse phase column on a Hewlett-Packard 1090 chromatograph equipped with a 1040 diode array detector. Eluted peptides were screened for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry on a Finnigan Laser mass spectrometer. Selected fractions were then subjected to automated Edman degradation and amino acid sequencing on an Applied Biosystems 477A or Hewlett Packard G1005 protein sequencer (Harvard Microchemistry Facility, Cambridge, MA) as described previously (15).

**Southwestern and Western Blot Detection of ERE-BP—**Extracts from estrogen-resistant B95-8 and estrogen-responsive OMK cells were suspended in 2× Laemmligh buffer and heated at 97 °C for 5 min. Portions and samples were loaded onto 10% SDS-polyacrylamide gels (5 µg of protein per lane), electrophoresed for 1.5 h at 100 V, and electroblotted onto nitrocellulose membrane overnight at 4 °C. The nitrocellulose membrane was blocked in 5% nonfat dry milk, 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol at room temperature for 1 h, and then hybridized in baking buffer (20 mM Hepes (pH 7.0), 100 mM KCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 15 µg/ml salmon sperm DNA) containing 10⁶ cpm/ml of the radiolabeled ERE half-site double strand probe at 23 °C for 2 h. The membrane was washed in the binding buffer at room temperature for 20 min and autoradiographed with an intensifying screen at ~80 °C. For Western blot analysis nitrocellulose membranes prepared as just described were incubated with mouse monoclonal anti-ER (Santa Cruz Biotech, Santa Cruz, CA) or monoclonal anti-ER antibody (16), both diluted 1:1000, horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma), and chemiluminescence detection reagent (ECL, Amersham Pharmacia Biotech).

**RT-PCR—**Approximately 200 ng of total RNA, isolated with total RNA isolation reagents (Life Technologies, Inc.), was used as template. Successful amplification of 351-base pair cDNA sequence was achieved with 30 cycles of reverse transcription-PCR using two of the primers, 5'-GTTATGAGGCGCTTGCTGGAGCAC-3' and 5'-TCCATAGGCTTCCCCATGGACAAACG-3'. This PCR product was ligated into the PCR 2.1 (Invitrogen) and the cDNA sequence verified.

**Amplification of a Full-length cDNA for ERE-BP—**B95-8 poly(A)+ RNA (2.5 µg) was used as template to generate the 5'- and 3'-ends of the ERE-BP cDNA with the Marathon cDNA amplification kit (CLONTECH Laboratories Inc., Palo Alto, CA). Second strand cDNA synthesis and adapter ligation were performed as instructed in the enclosed manual. The adapter-ligated cDNA was then used as a template for annealing amplifier- and ERE-BP-specific primers for the RACE reaction: 5'-AGATCCCGACTTGTGCTGCTG-3' and 5'-GCTGCGCTTTGTCTCCAG-ATACACTT-3' for the 5'- and 3'-RACE reactions, respectively. Full-length cDNA was generated by end-to-end amplification using specific 5' and 3' primers. The amplified products were then separately subcloned into the PCR 3.1 expression vector and sequenced.

**RESULTS**

**Squelching of Estrogen Receptor-Estrogen Response Element Transactivation in New World Primate Cells—**Most genera of New World primates are estrogen resistant in vivo (2) and crude New World primate cell extracts bearing ERE-BP have been shown to competitively inhibit binding of the ER to the ERE (9). However, resistance to estrogen action has not been previously shown in cells overexpressing the endogenous ERE-BP. Expression of a reporter gene bearing an ERE promoter region was reduced by 63% when transiently transfected into estrogen-resistant New World primate cells compared with similarly transfected, estrogen-responsive, wild-type cells (Fig. 1, panel A); the endogenous ER content of both cell lines was comparable (Fig. 1 insert). This difference in basal reporter activity between hormone-resistant and hormone-responsive cells was markedly amplified when cells were co-transfected with the ERE-reporter and wild-type human ER constructs (Fig. 1, panel B). Moreover, basal reporter activity did not increase after incubation of transfected hormone-resistant B95-8 cells with the stimulatory ligand 17β-estradiol as estrogen-responsive OMK cells.

**Affinity Purification and Functional Characterization of ERE-BP—**The result of the above described experiment suggested that endogenous ERE-BP in the hormone-resistant New World primate cell line was competing with the estrogen-activated ER for binding to the transfected ERE and inhibiting transactivation. However, it did not exclude the possibility that there was more than a single protein overexpressed in New World primate cells that participates in the squelching of estrogen action. To rule out this possibility, we created a double strand DNA affinity support containing concatamers of the estrogen affinity ligand ERE-BP half-site motif AGGTCAcag shown by us to greatly enrich New World primate cell extracts for ERE-binding proteins (9). Moreover, to avoid contamination of extracts with ERE-binding ER, we employed "low-salt," postnuclear extracts of B95-8 cells devoid of endogenous ER. An aliquot of each preparation of crude extract was examined for its ability to bind to the ERE in EMSA, and the specificity of ERE-BP-ERE binding was confirmed by the complete displacement of labeled ERE from the ERE-BP in the presence of a 50-fold excess of radiolabeled probe.

Preparations of B95-8 cell extracts with documented specific ERE binding activity were pooled and subjected to microconcentration (molecular mass cut-off of 30 kDa). The retentate was subjected to DNA affinity chromatography and eluted through a stepwise linear gradient of KCl. As previously described (2), we found one symmetrical peak of ERE binding activity, unaffected by anti-ER antibody or by pre-exposure to 100 nM 17β-estradiol, eluted from the central portion of the gradient with most specific activity measured in the 0.5 M KCl fraction. Response element-binding protein(s) present in DNA affinity-purified extracts of B95-8 cells was further characterized for its ability to compete with equimolar concentrations of human ER.
for binding to the ERE (Fig. 2, panel A). EMSA of affinity-purified ERE-BP and wild-type human ER binding to human ERE probe, through increasing concentrations of radioinert competitive ERE, yielded very similar displacement plots. An aliquot of the affinity-purified ERE-BP was committed to polyacrylamide gel electrophoresis, and the presence of a 40–42-kDa species was revealed with silver staining (Fig. 2, panel B).

Using the labeled “half-sited” double strand ERE oligonucleotide for detection, Southwestern blot analysis of proteins in crude (Fig. 2, panel C) as well as in affinity-purified New World primate extracts showed that a 40–42-kDa peptide(s) was responsible for ERE binding. ERE-BP, which cross-reacts with anti-hnRNP-C antibody (16), was detected by Western blot analysis and confirmed the presence of a plentiful 40–42-kDa protein in the postnuclear and nuclear extracts of the estrogen-resistant New World primate cell line B95-8 (Fig. 2, panel D). By contrast, ERE-BP was absent by polyacrylamide gel electrophoresis, Southwestern, and Western blot analysis of postnuclear extracts of any of the hormone-responsive primate cell lines.

Amino Acid Sequencing of ERE-BP Tryptic Peptides—Gels containing the ERE-BP were collected and the 40–42-kDa bands excised from the gels. The gel slices were then subjected to in-gel proteolytic digestion and peptides were generated for amino acid sequencing. Four tryptic peptides from the parent protein were reproducibly recovered from the digest (Table I). All retained a high degree of sequence homology (range 57–100%) with at least four members of the large hnRNP family, hnRNP-A, -C, -D, and UP2 (17, 18). Three of the highly conserved RNA binding motifs characteristic of hnRNPs (17) were identified in three of our peptides. The RNP-bearing tryptic peptides also possessed sequence identity with a single strand DNA-binding protein in the hnRNP-A/B family, called single strand D-box binding factor (19).

DNA Binding Properties of Affinity-purified ERE-BP—ERE-BP retarded a double strand DNA probe containing the AGGTCA half-site motif (Fig. 3, left, panel A). Addition of either excess, unlabeled single or double strand oligonucleotide competed away ERE-BP binding to double strand probe. If ERE-BP is similar in structure to single strand D-box binding factor, then it is reasonable to assume that it can also bind to acrylamide gel electrophoresis, and the presence of a 40–42-kDa species was revealed with silver staining (Fig. 2, panel B). Using the labeled “half-sited” double strand ERE oligonucleotide for detection, Southwestern blot analysis of proteins in crude (Fig. 2, panel C) as well as in affinity-purified New World primate extracts showed that a 40–42-kDa peptide(s) was responsible for ERE binding. ERE-BP, which cross-reacts with anti-hnRNP-C antibody (16), was detected by Western blot analysis and confirmed the presence of a plentiful 40–42-kDa protein in the postnuclear and nuclear extracts of the estrogen-resistant New World primate cell line B95-8 (Fig. 2, panel D). By contrast, ERE-BP was absent by polyacrylamide gel electrophoresis, Southwestern, and Western blot analysis of postnuclear extracts of any of the hormone-responsive primate cell lines.

**Table I**

| Peptide sequence |
|------------------|
| MFIGGLSWDTTKK |
| IFVGLSPDTPEEK |
| GFCFITFKEEEPVKK |
| IREYFGGFGEVESIELPMDNKTNK |

**Fig. 2.** Characterization of the DNA affinity-purified ERE-BP. Panel A shows competitive displacement of the wild-type human ER and ERE-BP from the ERE probe by increasing molar concentrations of excess unlabeled ERE. Each point is the mean of duplicate determinations of band density. Panel B is a silver-stained gel showing the ERE-BP that eluted from an ERE half-site DNA affinity support in 0.5 M KCl. Panel C demonstrates detection of a 40–42-kDa protein(s) by a labeled oligonucleotide containing the ERE half-site motif AGGTCA only in estrogen-resistant B95-8 cells but not in estrogen-responsive OMK cells. Panel D demonstrates preferential expression of a 40–42-kDa anti-hnRNP-C-reactive protein in the estrogen-resistant New World primate (platyrrhine) B95-8 cell line compared with the estrogen-responsive New World primate cell line OMKp and estrogen-responsive Old World primate (catarrhine) cell line Vero. Although detectable in the nuclear extract of OMK cells, cross-reactive protein was absent from lanes bearing postnuclear cell extracts of wild-type cells and control bovine serum albumin (BSA).
specific cis-acting sequences in single strand DNA; this was the case (Fig. 3, right, panel A). ERE-BP bound to a single strand probe containing the consensus ERE and was displaced with the addition of excess unlabeled of either single strand or double strand oligonucleotides containing one or two copies of the ERE half-site motif AGGTCA. In fact, unlabeled single strand probe bearing only a single ERE half-site appeared to be most effective in competing away ERE-ERE-BP binding; no competitive displacement of ERE-BP binding was observed with single strand oligonucleotides of similar size lacking the AGGTCA motif (Fig. 3, panel B).

ERE-BP cDNA Cloning—The fact that the 40–42-kDa ERE-BP was a member of the hnRNP family was confirmed by cloning a cDNA that codes for the entire open reading frame of the protein. An initial 351-base pair cDNA was cloned by RT-PCR using degenerate oligonucleotides based on the amino acid sequence of two of our tryptic peptides and total B95-8 cell RNA as template (see Fig. 4). This cDNA was then employed to develop 5’- and 3’-nested primers for use in cloning by 5’- and 3’-RACE a full-length cDNA for ERE-BP (Fig. 4). Compared with other members of the hnRNP family, including those known to bind to double strand DNA and to influence transcription (19–22), the deduced amino acid sequence for ERE-BP retained a high degree of sequence conservation in the two RNA binding domains, RBD-1 and RBD-2 (Fig. 5). However, outside of the highly conserved RNA binding domains, ERE-BP sequence diverged considerably from that of other members of the hnRNP family, particularly in the C-terminal extent of the protein.

Expression of Transiently Transfected ERE-BP—If the actions of the ERE-BP in vitro to compete with the ER for ERE binding are operative in vivo, then squelching of ER-ERE-directed transactivation should take place in estrogen-responsive, wild-type cells that have been induced to transiently overexpress the protein. This finding was confirmed by the results
of the experiment shown in Fig. 6. Hormone-responsive OMK cells and hormone-resistant B95-8 New World primate cells were equivalently co-transfected with ERE-BP cDNA, human estrogen receptor-α cDNA, and an estrogen receptor-responsive promoter driving a luciferase reporter, into estrogen-responsive OMK cells (wt) and hormone-resistant B95-8 cells (rst) not treated or treated with 10 nM 17β-estradiol (E2). Data are the mean of duplicate determinations of luciferase activity.

FIG. 6. Ability of transfected ERE-BP to squelch ER-directed ERE transactivation in wild-type cells. Co-transfection of plasmids bearing the human ERE-BP cDNA, human estrogen receptor-α cDNA, and an estrogen receptor-responsive promoter driving a luciferase reporter, into estrogen-responsive OMK cells (wt) and hormone-resistant B95-8 cells (rst) not treated or treated with 10 nM 17β-estradiol (E2).

We recently reported that steroid hormone- and vitamin D-resistant New World primate cells overexpress a protein that has the capacity to compete with the human ER for binding to the consensus ERE (9). We showed that this protein, which we call the ERE-BP, was distinct from endogenous ER and, unlike the ER, was not dependent upon the presence of the standard tandem half-site motif for DNA binding. Rather, it appeared that the ERE-BP was bound most avidly by the single ERE half-site motif, AGGTCA. Taking advantage of this fact we constructed an affinity support bearing concatamers of this motif for purification of ERE-BP. Here we show that through the use of this affinity support we were able to purify in a single chromatographic step a 40–42-kDa protein(s) that binds to the ERE. Generation and amino acid sequencing of four different tryptic peptides from the native protein showed ERE-BP to be a member of the large family of single strand nucleic acid-binding proteins whose most prominent members are in the subfamily of RNA-binding proteins termed hnRNPs.

There are now at least twenty different hnRNPs, designated hnRNP-A through -U, that are known to reside principally in the nucleus of vertebrate cells (17). In general, hnRNPs are considered RNA-binding proteins designed specifically for the metabolism and transport of pre-mRNA (23, 24). They are among the most abundant nuclear proteins, usually present in quantities several orders of magnitude greater than that of most DNA-binding proteins including transcription factors. Recently, proteins in the hnRNP family have also been shown to exist outside the nuclear compartment of the cell, being capable of shuttling back and forth between the nucleus and the cytoplasm (23). hnRNPs have also been shown not to be specific for binding only RNA (24). Tomonaga and Levens (25, 26) have provided evidence that hnRNPs are capable of binding single strand DNA; this suggests that the cis-acting elements do not require uracil as a component of the nucleic acid binding motif.

In this report we show that ERE-BP is an example of an hnRNP-related protein that can interact specifically with either single or double strand DNA (Fig. 3) containing the AGGTCA motif.

The deduced amino acid sequence of ERE-BP indicates the presence of two sets of the two consensus nucleic acid binding motifs, RNP-1 and RNP-2 (Fig. 5), which are characteristic of the hnRNP family of proteins (17). Although the central RNP-containing domain of ERE-BP bears a high degree of sequence similarity to other hnRNPs, it is not yet known whether these same RNA binding sites are also responsible for the binding of ERE-BP to DNA. Regardless of the primary structural similarities among the hnRNPs and ERE-BP, there are at least three ways in which ERE-BP differs from the classical profile of an hnRNP. First, ERE-BP is not confined to the nuclear compartment of the cell. We isolated the protein in low-salt, post-nuclear extracts of New World primate cells as well as from both the cytoplasmic and nuclear compartments of estrogen-resistant cells. Second, it is not ubiquitously present in all vertebrate cells. As we have previously shown (9) and confirm...
here (Fig. 2), ERE-BP is expressed at very low to nondetectable levels in cells from hormone-responsive primates, including man. And third, ERE-BP appears to be versatile in its ability to bind nucleic acid (Fig. 3); ERE-BP can bind to single- or double-stranded DNA and, although yet unproved, the presence of the conserved RNA binding motifs in ERE-BP suggests that it may also interact with RNA.

The survival advantage conferred on primates of the New World by overexpression of the ERE-BP and the development of relative estrogen resistance remains an intriguing question. Part of the answer may lie in the fact that there is overexpression of another family of proteins in New World primate cells that also has the potential to alter cellular responsiveness to 17b-estradiol (27). These proteins are structurally related to the proteins in the hsp-70 family of chaperone proteins and to the recently discovered intracellular vitamin D-binding protein (27, 28). The latter is a high capacity, relatively low affinity (compared with the vitamin D receptor), largely cytoplasmic protein. The vitamin D results in morbid rachitic bone disease and death of New World primates (including Homo sapiens) homologs of these New World primate proteins.

Acknowledgments—We thank B. Sharifi and L. Pei for helpful discussions and comments on the manuscript, and G. Dreyfuss for anti-hnRNP-C1/C2 antibody.

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