Interactions of the Nuclear Matrix-Associated Steroid Receptor Binding Factor with Its DNA Binding Element in the c-*myc* Gene Promoter†

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ABSTRACT: Steroid receptor binding factor (RBF) was originally isolated from avian oviduct nuclear matrix. When bound to avian genomic DNA, RBF generates saturable high-affinity binding sites for the avian progesterone receptor (PR). Recent studies have shown that RBF binds to a 54 bp element in the 5′-flanking region of the progesterone-regulated avian c-*myc* gene, and nuclear matrix-like attachment sites flank the RBF element [Lauber et al. (1997) *J. Biol. Chem. 272*, 24657−24665]. In this paper, electrophoretic mobility shift assays (EMSAs) and S1 nuclease treatment are used to demonstrate that the RBF−maltose binding protei (MBP) fusion protein binds to single-stranded DNA of its element. Only the N-terminal domain of RBF binds the RBF DNA element as demonstrated by southwestern blot analyses, and by competition EMSAs between RBF−MBP and the N-terminal domain. Mass spectrometric analysis of the C-terminal domain of RBF demonstrates its potential to form noncovalent protein−protein interactions via a potential leucine−isoleucine zipperlike structure, suggesting a homo- and/or possible heterodimer structure in solution. These data support that the nuclear matrix binding site (acceptor site) for PR in the c-*myc* gene promoter is composed of RBF dimers bound to a specific single-stranded DNA element. The dimers of RBF are generated by C-terminal leucine zipper and the DNA binding occurs at the N-terminal parallel β-sheet DNA binding motif. This complex is flanked by nuclear matrix attachment sites.

Previous work from this laboratory has demonstrated that whole nuclei chromatin and nuclear matrix from the avian oviducts contains saturable, high-affinity binding sites for activated progesterone receptors (PR)[1] (*1*−*5*). Pure genomic DNA did not display these high-affinity, saturable kinetics with PR or other steroid receptors (*6*−*13*). Other laboratories, studying other steroid receptor systems, have also reported specific receptor sites (acceptor sites) in the nuclear chromatin for a variety of steroid receptors (for reviews see refs *14*−*17*). From previous studies, using the avian PR system, a receptor binding factor (RBF) was isolated as a proteinaceous "activity" from avian oviduct chromatin. By reconstituting crude or subsequently purified RBF protein fractions to avian genomic DNA, saturable, high-affinity binding sites for the avian PR were obtained. The number of sites on the genome that could be generated by the excess RBF protein was also rate-limiting (*6*, *7*, *11*, *12*). These sites displayed in vivo-like patterns of binding using seasonally active and inactive PR (*2*, *7*, *10*, *12*). Moreover, this binding by RBF displayed a genomic DNA specificity in the reconstituted complex by not binding to insect or bacterial genomic DNA (*6*, *8*). These data support that the RBF appears to be an essential protein component of progesterone receptor nuclear acceptor sites, or, at least, a component of a subset of these sites in the chromatin structure and that specific DNA elements may be required for these sites. Immunohistochemical staining showed that RBF was located in the nucleus and colocalized in cells that also expressed progesterone receptor, such as avian oviduct and rat reproductive tissues (*18*).

This laboratory had previously hypothesized that steroid-induced gene regulation might involve early "regulatory" genes whose transcription would be rapidly modulated by steroid receptor binding and whose protein products would in turn regulate the transcription of genes involved in "later" cellular events (*4*, *14*, *16*, *19*). The rapidly responding c-*myc* gene was shown to fit this model. Fink et al. (*20*) showed that the transcription of the avian c-*myc* gene was rapidly down regulated by progesterone in vivo within 10−20 min, even though no steroid hormone response element is present in the avian 5′-flanking region of the c-*myc* gene. This rapid progesterone-mediated inhibition of the c-*myc* gene expression was also shown to be steroid dose-dependent and tissue-specific. Subsequently, Schuchard et al. (*11*, *12*) demonstrated by Southern blot analyses that the isolated nuclear

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[1] Abbreviations: RBF, receptor binding factor; PR, progesterone receptor; EMSA, electrophoretic mobility shift assays; MBP, maltose binding protein; BSA, bovine serum albumin; ABI, Applied Biosystems; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ESI-MS, electronspray ionization−mass spectrometry; PVDF, poly(vinylidene difluoride); CAPS, 3-(cyclohexylamino)propanesulfonic acid; PVP, polyvinylpyrrolidone.

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matrix DNA has sequence homology with the 5′-flanking regions of a few rapidly steroid-regulated genes, in particular c-*myc* and c-*jun*. There was no sequence homology with genomic sequences in several late steroid-regulated genes, such as ovalbumin or the α-actin gene.

Since the RBF protein is localized in the avian oviduct nuclear matrix, it was of interest to determine whether RBF also binds to the 5′-flanking region of the c-*myc* gene. Subsequent studies by Lauber et al. (*1*) describes that transient transfection of RBF in MCF-7 cells treated with estrogen and progesterone results in a decrease of c-*myc* promoter activity. Further, by southwestern blotting analyses, a 54 bp RBF binding element was identified that contains a predominantly GC-rich 5′-region and AT-rich 3′-region involving a long poly A−poly T track. This paper further characterizes the RBF and demonstrates that the RBF binds to either a single- or double-stranded DNA element, possibly as a dimer. The dimerization appears to occur via its C-terminal leucine−isoleucine zipperlike domain. Moreover, preliminary evidence suggests that RBF can bind to PRB with an affinity capable of inhibiting PRB binding to its PRE.

EXPERIMENTAL PROCEDURES

Southwestern Blot Analyses. The avian c-*myc* genomic clone was used as a template in standard PCR reactions. Primers were designed to yield DNA fragments (I_8, I_9, and I_{10}) representing different regions of the 5′-upstream flanking domain of the c-*myc* gene (*1*). The DNA was purified from the gel and end-labeled with [γ-^{32}P]ATP by standard T4 kinase reactions. For the southwestern blot analyses, the purified, reconstituted RBF was diluted in reconstitution buffer and then slot-blotted (500 ng/slot), under vacuum, to nitrocellulose with a slot-blot apparatus. The RBF-MBP fusion protein was also slot-blotted at 500 ng/slot. The nitrocellulose was cut into strips and the membrane was incubated with end-labeled DNA probes representative of c-*myc* fragments (I_8, I_9, and I_{10}) of the upstream regions of the c-*myc* gene. The blots were first blocked for 1.5 h in standard Bowens buffer [50 mM Tris-HCl, pH 7.0, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, and 0.1% (w/v) PVP] and then probed with $(3-5) \times 10^6$ cpm of end-labeled DNA probes, including 50 mM KCl and 1 mM $CaCl_2$ (*12*). The blots were washed with Bowens binding buffer and exposed to autoradiographic film. In some experiments, after the blots were probed and washed, the blots were further incubated with S1 nuclease (4, 10, and 20 units/mL) (Promega, Madison, WI) in digestion buffer for 1 h at 20 °C. The blots were rinsed and then exposed to X-ray film.

Southwestern Blot Analysis of Peptide Fragments A, B, and C. The Mayo Protein Core Facility (Rochester, MN) synthesized peptide fragments A, B, and C corresponding to the different structural domains of the RBF. The sequence of each fragment was verified by sequence analyses. The peptides were resuspended in distilled water and slotted at 150, 250 and 500 ng/slot. Then southwestern blot analyses were performed as previously described.

Isolation/Purification/Reconstitution of RBF: (A) Isolation of Avian RBF. The isolation of purified RBF has been reported in detail (*11*, *21*). Briefly, hen oviducts were excised, the chromatin was isolated, and the chromosomal proteins were size-fractionated by molecular sieve chromatography as described previously (*9*). Fractions containing proteins in the 4−20 kDa size range were pooled and separated by preparative SDS−PAGE in a Tris−tricine buffer as described previously (*21*). The gel was transferred to six sheets of PVDF membrane in a CAPS buffer system (10 mM CAPS + 10% methanol, pH 11.0). One sheet was immunostained for RBF with a RBF-specific monoclonal antibody (*9*, *11*, *22*). The five additional sheets were matched against the one with the visible immunoproduct. Bands encompassing RBF were excised and the protein was eluted from the membrane in 70% (v/v) 2-propanol/5% TFA (v/v) overnight, dialyzed against 5% (v/v) acetonitrile/H_2O containing 0.25 mM PMSF, and lyophilized.

(B) Reconstitution of Purified RBF. This method has been described in detail previously (*5*, *11*, *13*). Briefly, purified RBF was solubilized in 6 M guanidine-hydrochloride in reconstitution buffer [10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, and 0.2 mM PMSF] to a concentration of 250 ng/mL. This concentration of the RBF was found to be important for optimal protein renaturation and reconstitution of PR binding sites on the DNA (for reviews see refs. *5*, *13*, and *23*). It was also found in these current studies to be important for achieving the optimal DNA sequence-specific binding. The solution was loaded into 10 mm diameter dialysis tubing and dialyzed in a plastic cylinder filled with 200 mL of buffered 6.0 M guanidine-hydrochloride solution against 1 L of a solution representing reconstitution buffer containing no guanidine hydrochloride, at a replacement rate of 1 mL/min, while rocking at 4 °C. This process gradually decreased the guanidine hydrochloride concentration to 0.1 M by 16 h. After 16 h, the protein solution was removed from the dialysis bags, homogenized, and used in the southwestern blotting analysis.

RBF−MBP Fusion Protein and Polyclonal Antibodies. The coding region of the RBF gene was cloned, in frame, into the pMAL-c2 vector (New England Biolabs Inc.) and expressed as a maltose binding fusion protein (MBP) in *Escherichia coli* and purified as specified by the brochure New England Biolabs "Protein Fusion and Purification System". The fusion protein migrated in SDS−PAGE analysis as a 55 kDa protein. A new polyclonal antibody (pAb) was then prepared against the RBF-MBP fusion protein. Briefly, 1 mg of purified lyophilized protein was sent to Cocalico Biologicals, Inc. (Reamstown, PA), where two rabbits were inoculated and the sera were recovered and sent to us. One serum, pAb-273, was then purified in our laboratories from this serum by use of the Affi-Gel protein A MAPS II kit according to a brochure provided by Bio-Rad Laboratories, Hercules, CA. Western blots showed that pAb-273 reacted with the RBF-MBP, as well as with the purified, reconstituted, and factor Xa-cleaved RBF protein.

Electrophoretic Mobility Shift Assays. Electrophoretic mobility gel shift assays (EMSA) were conducted, with modification to the original procedure, reported by Fried and Crothers (*24*−*26*). Briefly, 200−500 ng of a fraction containing RBF-MBP was added to the protein−DNA binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1.0 mM dithiothreitol, 2.0 mM $MgCl_2$, and 10% (v/v) glycerol] and the solution was incubated with 0.5 μg of poly[(dI-dC)] for 15 min at room temperature. The DNA probes were labeled as described above and 500 000 cpm was carefully added to each of the protein solutions, which were then

incubated for 20 min at room temperature. In competition experiments, using RBF peptide fragments (A, B, and C), 150, 250, and 500 ng/reaction of each peptide were incubated for 30 min at room temperature in the binding buffer before the antibody (pAb-273) was added. The mixture was then incubated with the RBF-MBP polyclonal antibody for 2 h at room temperature. Control reaction mixtures had either no antibody and/or no competing peptide fragment added. The protein−DNA complexes were resolved by gel electrophoresis on 6% (w/v) native acrylamide gels (75:1 acrylamide:bis) at 26 V/cm in 0.5× TBE buffer (0.05 M Tris−borate and 0.001 M EDTA, pH 8.0) for 2.5 h at 4 °C. The gels were then dried and exposed to autoradiographic film.

Protein Structure Analyses. The Mayo Protein Core Facility (Mayo Clinic, Rochester, MN) determined the N-terminal amino acid sequence analysis of the RBF protein, its tryptic peptides, or its synthetic peptide sequence. Briefly, purified RBF protein (500 pmol), its tryptic peptides, or its synthetic peptides (used for quality controls) were applied to a Polybrene-treated glass filter disk. Then the peptides were microsequenced by the Edman degradation method on an Applied Biosystems (ABI) 476A protein sequencer using programmed cycles provided by ABI (Foster City, CA). The phenylthiohydantoin amino acids from each cycle were identified on-line by reverse-phase HPLC on an ABI 120A PTH Analyzer. Preliminary analysis of the primary and secondary structure of the RBF protein was performed by computer analysis using peptide structure and helical wheel algorithms contained in a sequence analysis software package. The Genetics Computer Group (GCG) of the University of Wisconsin, Biotechnology Center, Madison, WI, produced these algorithms.

Electrospray Ionization Mass Spectrometry. A Finnigan MAT (Bremen, Germany) Model 95Q of EBQ_1Q_2 configuration mass spectrometer was used throughout these studies. The *E* represents an electric field, the *B* a magnetic field, the Q_1 a rf-only octapole collision cell, and the Q_2 a quadrupole mass filter. This instrument was equipped with an "in-house" modified Finnigan MAT electrospray ionization source (*27*). Mass measurements were made in positive ion modes, by infusion of aqueous polypeptide solutions into the ESI source via 50 μm i.d. fused silica tubing, at a rate of 300 nL/min. A model 22 Harvard Apparatus syringe pump (South Natick, MA) was used throughout to deliver samples to the ESI. An ESI voltage of 2.4 kV reference to the instrument accelerating voltage (5 kV) was used throughout. The temperature of the ion source was maintained at 50 °C and the heated transfer capillary of the ESI source was used at 100 °C. An instrument resolution of ~1000 was used for all analyses, and the scan range was 600−2000 Da at a rate of 30 s per mass decade.

Statistical Analyses. A logarithmic transform was used to stabilize variance in the RBF peptide fragment A, B, and C competition EMSAs. Overall tests for effects were carried out with multivariate analysis of variance (MANOVA); in addition, treatments were compared with paired *t*-tests. No adjustments were made for multiple hypothesis tests.

RESULTS

RBF Binds to Double- and Single-Stranded DNA. Since the DNA binding element includes a long stretch of unstable poly-(A)poly-(T) region with a GC anchor, and since some DNA binding proteins as well as nuclear matrix proteins interact with single-stranded DNA, mobility shift assays were performed to examine whether RBF-MBP can bind to single-stranded DNA (ssDNA). As shown in Figure 1B (lanes 1 and 8), the RBF-MBP binds the double-stranded I_8 and I_{10} fragments and impedes their mobility in the gel (arrow 1). Pure MBP alone has previously been shown to not bind to the DNA in these gel shift assays (1). Further, the addition of antibody pAb-273 specific to RBF-MBP fusion protein results in a supershifted band (arrow 2, lanes 2 and 9), suggesting that the supershifted band is specific and comprises of RBF-MBP/DNA/Ab. All EMSAs were performed with the same amount of radiolabeled probe (500 000 cpm/lane), and the apparent differences of signal for the shift and supershift intensities should not be due to uneven loading but due to differences in RBF's affinity for the probes. Further control studies have demonstrated that there is no DNA binding by MBP alone, and the control antibody did not recognize the fusion protein nor form a supershifted complex (*1*). Panel B also shows that nonradiolabeled double-stranded I_8 and I_{10} DNA compete for radiolabeled probe binding, resulting in decreased detection of RBF-MBP/DNA/Ab complex signal (arrow 2, lanes 3−7 and 10−14),

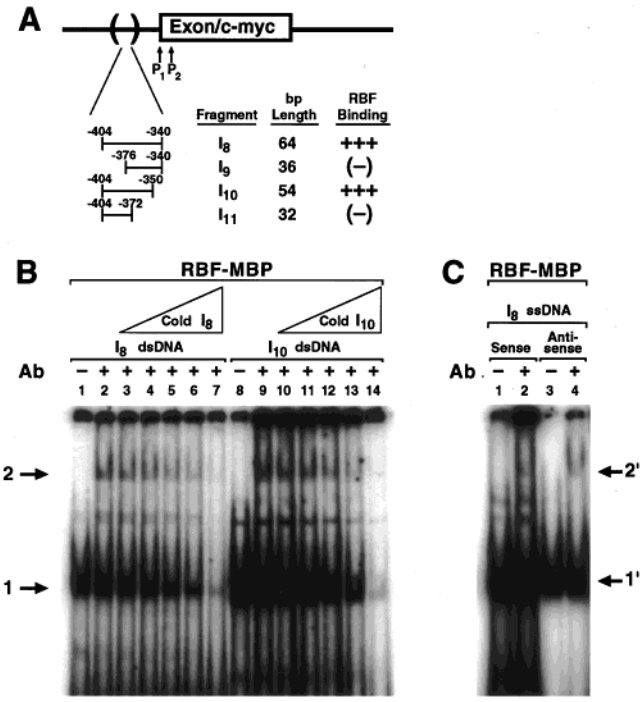

FIGURE 1: RBF binding to double and single stranded DNA. Panel A shows a diagram of the oligos used throughout the study and a summary of RBF-MBP DNA binding affinity. Panel B shows nonradiolabeled dsDNA I_8 and I_{10}, both fragments that contain the RBF element (*1*), compete with radiolabeled dsDNA I_8 and I_{10}, respectively, resulting in a decrease of detectable RBF-MBP complex (arrow 1, lanes 3−7 and 10−14) and supershifted complex (arrow 2, lanes 3−7 and 10−14) in a concentration dependent manner. Lanes 3 and 10, 4 and 11, 5 and 12, 6 and 13, and 7 and 11 were treated with 0.0125, 0.025, 0.05, 0.1, and 1.0 pmol, respectively, of nonlabeled oligos. Panel C demonstrates that RBF-MBP binds both the sense and antisense strand of I_8 DNA that contains the RBF binding element I_{10} (arrow 1′) and can be supershifted by pAb 273, a RBF-MBP-specific antibody (arrow 2′, lanes 2 and 4). Control lanes showing equivalent loading of the labeled DNA were performed in all experiments (data not shown). In all experiments 500 000 cpm was carefully added to each lane.

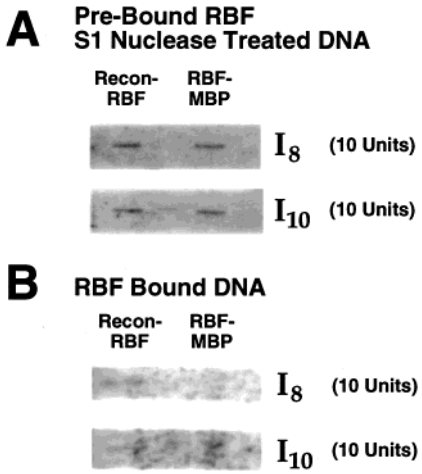

FIGURE 2: RBF induced S1 nuclease sensitivity. Panel A shows that radiolabeled I_8 and I_{10} are not sensitive to S1 nuclease treatment before RBF binding and are available to bind to reconstituted RBF (recon-RBF) and RBF-MBP. Panel B demonstrates that once I_8 and I_{10} DNAs were bound to recon-RBF and RBF-MBP, the DNA became S1 nuclease-sensitive.

supporting the specificity of these interactions. Half-maximum binding occurs at 0.025 pmol of nonradiolabeled dsDNA for both I_8 and I_{10} (determined by analyses using Storm 840 phosphoimager, Molecular Dynamics). Interestingly, RBF-MBP specifically binds very well to ssDNA (sense and antisense strand), as demonstrated in Figure 1C by the RBF-MBP/ssDNA I_8/Ab complex (arrow 2′, lanes 2 and 4). The binding is also observed with ssDNA I_{10} (data not shown). Thus RBF-MBP has affinity for the structures homologous to the RBF binding element in both dsDNA and ssDNA.

RBF Induced S1 Nuclease Sensitivity. Studies with S1 nuclease further support that RBF binds to ssDNA since RBF can generate ssDNA when bound to its dsDNA element (see Figure 2). Our laboratory previously demonstrated that purified reconstituted RBF (recon-RBF) and RBF-MBP fusion protein binds to a unique element, the I_{10} fragment, in the 5′-flanking region of the progesterone-regulated avian c-*myc* gene by southwestern blot analyses (*1*,*11*, *12*) and EMSA (ref 1 and Figure 1). The purified MBP alone did not bind (shift) the DNA band. This element has been located in two DNA fragments, I_8 and I_{10}, representing the same region of the c-*myc* 5′-flanking domain (*1*). In these papers we hypothesized that RBF interaction with its DNA element, along with the flanking dual matrix-attachment sites, could be forming a unique structure with RBF stabilizing a tertiary DNA structure. Consequently, a southwestern/S1 nuclease sensitivity assay (Figure 2) was devised and performed to test whether RBF can induce the formation of single-stranded DNA. The 54 bp I_{10} fragment of the c-*myc* gene is contained within the larger 64 bp I_8 fragment (*1*). The radiolabeled I_8 and I_{10} DNA fragments were treated with 4, 10, or 20 units/mL of the single-stranded nuclease, S1, in digestion buffer (1 h at 20 °C) prior to hybridization with the immobilized proteins (4 and 20 units/mL southwestern blots not shown). Panel A shows that the radiolabeled I_8 and I_{10} probes remain in a double stranded DNA (dsDNA) structure prior to hybridization (no digestion of the probes). The 4 and 20 units/mL nuclease enzyme treatments showed the same results. The radiolabeled I_8 and I_{10} were bound first by recon-RBF or RBF-MBP and then examined for single-strandedness by digestion with S1 nuclease. The blots were rinsed and 4, 10, or 20 units/mL of S1 nuclease was added to the various blots. Panel B shows that the I_8 and I_{10} fragments became nuclease-sensitive in the presence of both the recon-RBF and the RBF-MBP (10 units/mL blots shown). The blots in panels A and B were exposed to film for 24 h prior to development. Blots in panel B were purposely overexposed in order to detect any possible signal (data not shown). The 4 and 20 units/mL enzyme treatments showed the same results. The MBP was shown not to bind to I_8 or I_{10} (*1*), and mock nuclease treatments showed no decrease in signal (data not shown). A distinctly different DNA binding protein that would bind to this element and not show nuclease activity was not available as a control for these studies. These data suggests that RBF induces S1 nuclease sensitivity on the DNA, suggesting the possibility of single-stranded DNA formation.

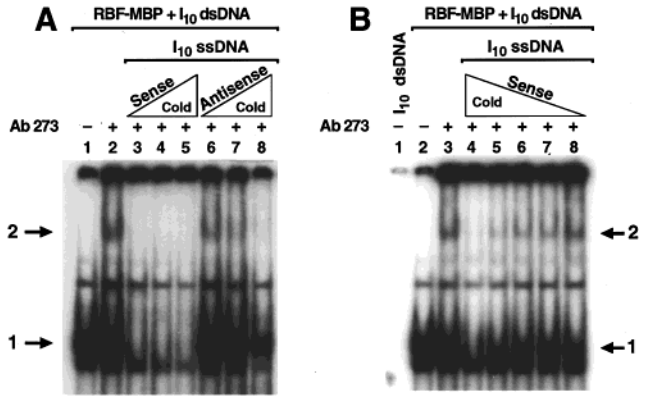

FIGURE 3: ssDNA competition EMSAs. In panel A, both sense and antisense nonradiolabeled ssDNA I_{10} competes with dsDNA I_{10} for RBF-MBP/I_{10} (arrow 1, lanes 3−5 and 6−8, respectively) and RBF-MBP/I_{10}/Ab (arrow 2, lanes 3−5 and 6−8, respectively) complex binding in a concentration-dependent manner. A pM (10^{-12} M) stock of nonradiolabeled ssDNA was diluted 1:750-fold, and 1, 2, and 3 μL were added to lanes 3 and 6, lanes 4 and 7, and lanes 5 and 8, respectively. The final reaction volume was 40 μL. In panel B, sense I_{10} ssDNA competes with dsDNA I_{10} for RBF-MBP/I_{10} (arrow 1, lanes 4−8) and RBF-MBP/I_{10}/Ab (arrow 2, lanes 4−8) complexes in a concentration-dependent manner. Serial dilutions of 1:2500, 1:5000, 1:10 000, 1:20 000, and 1:30 000 of a 1 pmol I_{10} ssDNA nonradiolabeled stock were performed and incubated at 1 μL per 40 μL reaction, respectively.

Strand-Specific Binding by RBF. To determine the avidity of RBF-MBP binding to ssDNA, oligonucleotides corresponding to the sense and antisense strand of the RBF binding element (I_{10}) were synthesized and used in competition gel shift analyses. The RBF-MBP forms a stable protein−ssDNA complex (Figure 3A, arrow 1) as well as a protein/ssDNA/Ab complex (arrow 2) with either strand of the I_{10} RBF binding element. Interestingly, the gel shift analysis suggests that RBF has a greater preference for the sense strand of the RBF binding element. The nonradiolabeled sense strand of the RBF binding element competes much better for DNA binding than does the antisense strand, as demonstrated by the ability of the former to completely abrogate all detectable signal from the RBF-MBP/dsDNA complex (arrow 1, lanes 3−5) and the RBF-MBP/dsDNA/Ab complex (arrow 2, lanes 3−5) at lower concentrations of competing ssDNA. There is a concentration-dependent titration of this competition by the sense DNA strand (Figure

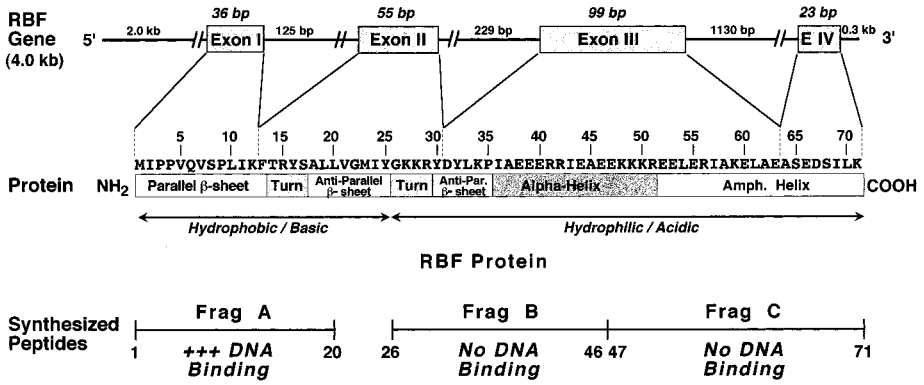

FIGURE 4: Gene and potential protein structure of RBF. Gene structure and complete amino acid sequence for RBF, along with a summary of the synthetic peptide fragment DNA binding activity. Structural analyses were performed as described under Experimental Procedures. [Taken in part from Lauber et al. (*1*)].

3B) (lanes 4−8). After comparing the half-maximal binding of RBF-MBP for the sense and antisense strand of DNA, it was calculated that the sense strand binds the RBF-MBP at a 10-fold lower concentration of DNA (Storm 840 phosphoimager, Molecular Dynamics). Thus, it appears RBF can bind both dsDNA and ssDNA, with the sense strand displaying a 10-fold higher affinity for RBF than the antisense strand. While the exact dissociation constant for the RBF-MBP/DNA complex has not been determined, these data do suggest that RBF-MBP, when bound to DNA in solution, has a preference for single-stranded DNA with a greater affinity for the sense strand of the RBF binding element.

DNA Binding by the N-Terminal Domain of RBF. The cloned and sequenced 4.0 kb RBF gene has four coding exons and three introns. The 5′-untranslated region (UTR) is nearly 2.0 kb in length, and the 3′-UTR is approximately 0.3 kb with a consensus polyadenylation signal (AATAAA) (Figure 4). Computer analysis of the primary amino acid sequence reveals RBF contains the following potential secondary structures (Figure 4): The N-terminus is hydrophobic/basic and contains a potential parallel β-sheet. The parallel β-sheet motif is common in various proteins that bind DNA (*28*−*32*). The C-terminus is hydrophilic/acidic and contains a potential leucine-isoleucine amphipathic helix. There is a potential proline-dependent kinase phosphorylation site (-VSPL-) in the N-terminus of RBF (*33*). The contribution of this phosphorylation site to RBF function has yet to be determined. There are no consensus glycosylation sites in the RBF amino acid sequence. To test which region of RBF binds DNA, peptide fragments of roughly 20 amino acids, representing one-third of the protein, were synthesized. These peptides were resuspended in distilled water and used in southwestern blot and gel shift analyses. The bottom of Figure 4 displays a summary of the peptide domains, where peptide fragment A = the N-terminal domain containing the β-sheet structure; peptide fragment B = the middle domain; and peptide fragment C = the C-terminal domain containing the amphipathic sequence (leucine zipper).

To determine the concentration of the peptide fragment required for potential DNA binding, each was slot-blotted onto nitrocellulose at concentrations of 150−500 ng of peptide fragment (Figure 5). These peptides were then incubated with the I_8 region of DNA, which was bound by both recon-RBF and RBF-MBP fusion protein (Figure 2 and bottom panel of Figure 5A). As seen in Figure 5A, peptide

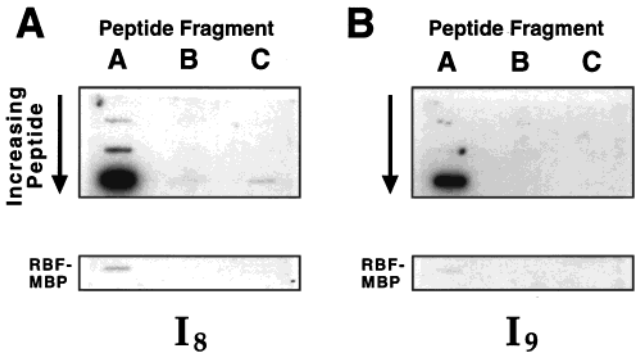

FIGURE 5: Southwestern blot analyses of RBF peptide fragments. Peptide fragments A, B, and C were each slotted at 150, 250, and 500 ng/slot. RBF-MBP was slotted at 500 ng/slot, as a positive control for I_8 binding and as a negative control for I_9 binding. Southwestern blot analysis shows that peptide fragment A binds to I_8 in a concentration-dependent manner (panel A). Some binding to I_9 was observed, but only at high concentrations of peptide fragment A (panel B). No specific binding by peptide fragments B or C was observed.

fragment A binds radiolabeled I_8 DNA in a concentration dependent manner. Peptide fragments B and C do not bind to the I_8 region of the c-*myc* gene. Panel B, Figure 5 shows that the DNA fragment I_9, a negative control that contains only part of the element and has been shown by southwestern blots and EMSAs not to interact with intact recon-RBF or RBF-MBP (*1*), is not bound by peptide fragments B or C. Interestingly, at very high concentrations, peptide fragment A also binds to the I_9 region of c-*myc*. This loss of specificity could be due the lack of modulating domains in the protein as a whole. These data support that the 20 amino acid residues of peptide fragment A are sufficient to define the DNA binding domain of RBF.

Peptide Fragment A Competes for DNA Binding. In support of the results obtained with southwestern blot analyses, the peptide fragments were used in gel shift competitions. The gel shift analyses were carried out as described under *Experimental Procedures* and elsewhere (*1*). In these studies increasing amounts of peptide fragment A, B, or C were added to the EMSA incubation mixtures. As shown in Figure 6A, peptide fragment A competes for RBF-MBP binding to the I_8 DNA, as shown by decreases in the RBF-MBP/DNA (arrow 1, lanes 3−5) and RBF-MBP/DNA/Ab (arrow 2, lanes 3−5) complexes that occur with increasing fragment A concentration. RBF peptide fragments B and C did not compete for RBF-MBP binding to the I_8 DNA

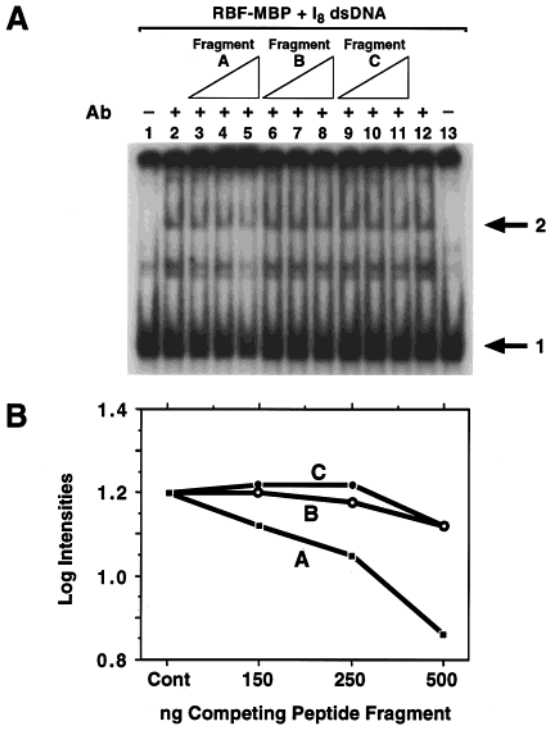

FIGURE 6: Peptide fragment A competes for DNA binding. In these studies, 0, 150, 250, and 500 ng/reaction of peptide fragment A, B, or C were added to the EMSA incubation mixtures. As seen in panel A and shown graphically for I_8 (panel B), peptide fragment A competes with RBF-MBP for DNA binding as shown by decreased signal of RBF-MBP/DNA (arrow 1) and RBF-MBP/DNA/Ab (arrow 2) complexes. Overall tests for effects were carried out with multivariate analysis of variance (MANOVA) using the RBF-MBP/DNA/Ab signal; in addition, treatments were compared with paired ***t***-tests as described under Experimental Procedures. Graph legend: (■) nanograms of competing peptide fragment A; (○) nanograms of competing peptide fragment B; (●) nanograms of competing peptide fragment C; (cont) the average of the two noncompeted supershift signals.

(lanes 6−11). Changes in the RBF-MBP/DNA/Ab complex signal were analyzed by statistical analyses. Statistical analyses were performed on five separate experiments, which utilized a single preparation of RBF-MBP. Other preparations of RBF-MBP show the same trend but were not subjected to statistical analysis. As suspected, there was no significant difference in the RBF binding to DNA fragments I_8 and I_{10} ($p > 0.19$, data not shown), using a paired *t*-test analysis and a multivariate analysis of variance (MANOVA). The overall MANOVA tests support a highly significant inhibition by peptide fragment A ($p = 0.002$), but no inhibition by either peptide fragment B ($p = 0.23$) or peptide fragment C ($p = 0.19$). As shown graphically in panel B, pairwise comparisons found significant suppression effects at each concentration of peptide fragment A: control vs 150 ng, p = 0.004; 150 vs 250 ng, p = 0.002; and 250 vs 500 ng, p = 0.0006. However, there is evidence of a slight effect at the highest concentration of competing peptides for peptide fragments B ($p = 0.06$) and peptide fragment C ($p = 0.03$).

Since we are measuring a loss of RBF/DNA/Ab complex signal, western blots were performed to determine if there was a peptide-specific immunoreaction of the polyclonal antibody (pAb-273) with the peptide fragments. The pAb-273 recognized all three peptide fragments with similar levels of staining (data not shown). Therefore, the loss of signal was *not* due to preferential antibody recognition of any one of the three peptides in the EMSA reaction mixtures. These data, together with those of the southwestern blot analysis, suggests that peptide fragment A may comprise part, if not all, of the DNA binding domain for RBF.

Peptide Fragment C Forms Homodimers. Electrospray ionization−mass spectrometry (ESI-MS) is a "soft" ionization technique that has significant applicability in the biological and biomedical sciences. This relatively novel technique allows accurate molecular weight measurement of biopolymers due to its mild conditions and rapid analyses. It also confirms the identity of the isolated protein and provides evidence of posttranslational modifications, dimerizations, and substrate adduction. In the present study, microspray ESI-MS was used to investigate the composition of aqueous solutions of RBF peptide fragments A, B, or C. By these experiments, we investigated the tendency for each of these peptides to form homodimers when dissolved in water. Following the report from Veenstra et al. (*34*), we initially evaluated the most appropriate microspray ESI-MS conditions for these studies (data not shown). From these investigations, we found that a heated transfer capillary of 100 °C was most appropriate, providing good instrument sensitivity and permitting the detection of homodimeric peptide responses. As shown in Figure 7, microspray ESI-MS spectra was obtained from analysis of aqueous solutions of the three RBF peptide fragments, under the developed conditions and at a concentration of 4 μM in water. Spectra characteristic of each of the monomeric peptides are clearly seen in these data (denoted by M in Figure 7). In all of these spectra, peaks indicative of the peptide homodimer (denoted by D in Figure 7) were observed. Some of the peaks observed in the Figure 7 (panels B and C) spectra are labeled M/D; they represent potential monomeric peptides and their homodimer. These result from the fact that charge states of each species gives rise to the same *m*/*z* value. For example, monomeric peptide fragment C is characterized by the ion $MH_2^{2+} = 1473.1$, and homodimeric peptide fragment C is characterized by an ion $MH_4^{4+} = 1473.1$. Clearly, at low instrument resolution both of these responses will overlap on the *m*/*z* scale (Figure 7C). Therefore, all subsequent studies dealt with those peaks that correspond only to the homodimers (labeled with only D in Figure 7).

In the current studies, the most abundant of the homodimeric signals was observed for peptide fragment C ($MH_5^{5+} = 1179.0$, Figure 7C). Indeed, while the homodimeric signal for peptide fragment C was detected under many experimental conditions (data not shown), similar responses were not observed for peptide fragments A or B. Peaks representing homodimers composed of peptide fragments A or B were minimal in the spectra acquired at a concentration of 4 μM in water (Figure 7, panels A and B) and could not be detected upon dilution of these peptides to 1 μM in water (data not shown). By contrast, the peptide fragment C homodimer response became a minor component of the microspray ESI-MS spectrum at this concentration (1 μM in water), but it was still visible above the background noise (data not shown). These results indicate that homodimer formation was specific to peptide fragment C. In summary, peptide fragment C formed a homodimer with high affinity, while peptide fragments A and B did not homodimerize when dissolved in water.

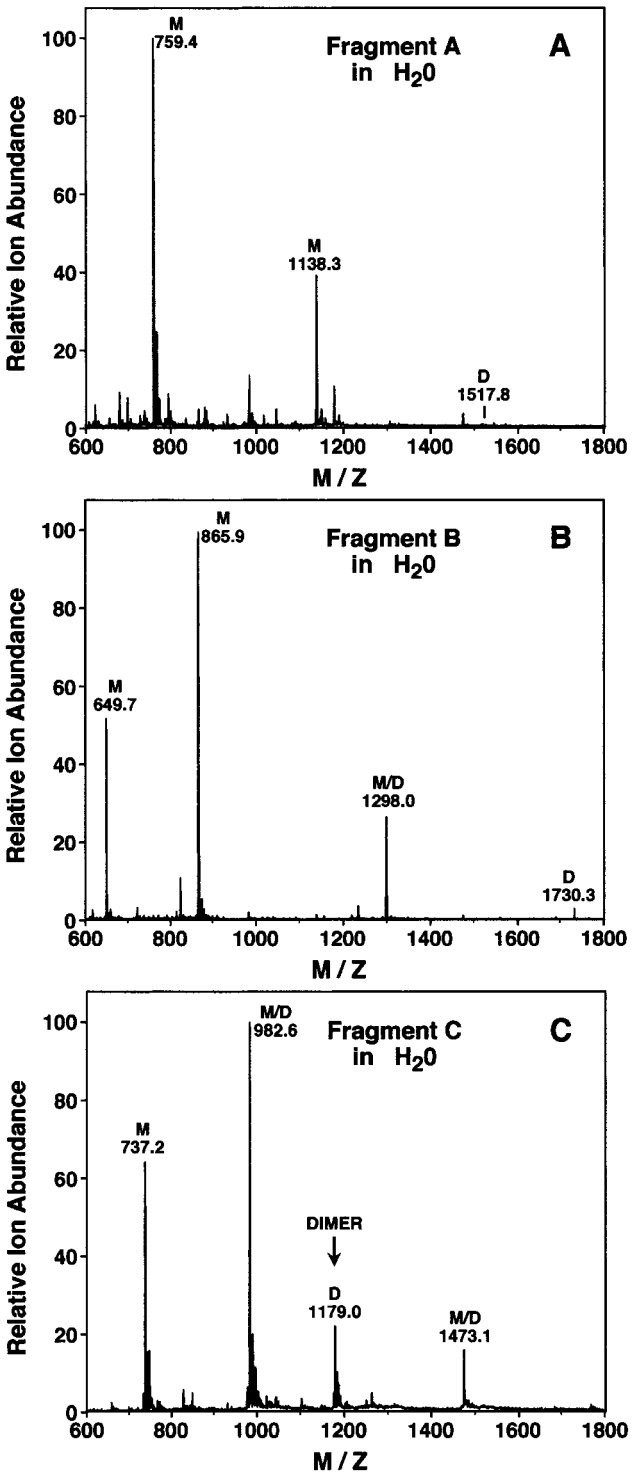

FIGURE 7: Peptide fragment C forms homodimers: Microspray ESI-MS spectra obtained by analysis of aqueous solutions of the RBF peptide fragments: panel A, peptide fragment A; panel B, peptide fragment B; panel C, peptide fragment C. All peptides were at a concentration of 4 μM in water. Sample flow rate was 300 nL/min, and the mass spectrometer was scanned over the range 600−2000 Da at a rate of 30 s/d. ESI voltage was 2.4 kV referenced to the instrument accelerating voltage (5 kV), and instrument resolution was ~1000. The source temperature was 50 °C and the heated transfer capillary was maintained at 100 °C. M above a peak represents an ion that is monomeric peptide, and D denotes a homodimer response. Those responses labeled M/D represent ions of overlapping *m*/*z* value (see text for discussion). These responses are representative of both monomeric peptide and its homodimer. Therefore, the reader is directed to those responses that represent only monomer (M) or only homodimer (D).

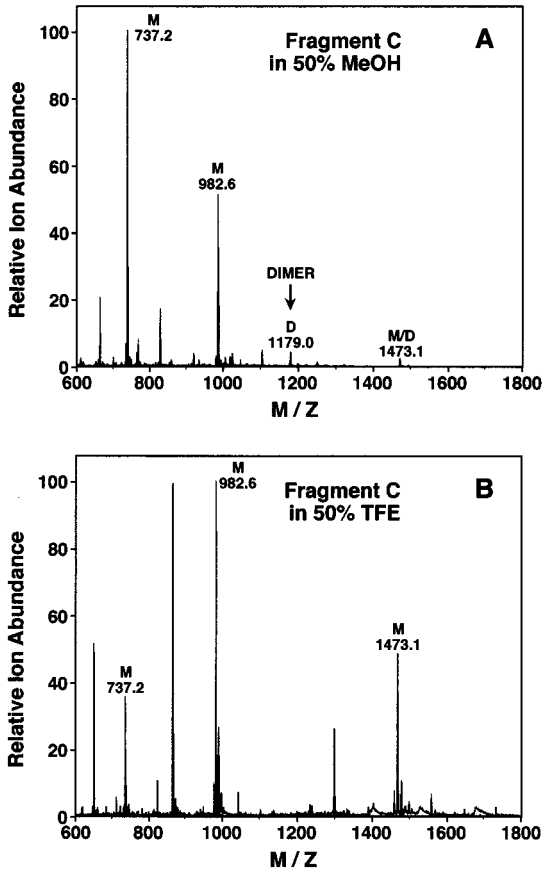

FIGURE 8: Microspray ESI-MS spectra of RBF peptide fragment. Peptide concentration was 4 μM for both experiments. In panel A, RBF peptide fragment C was dissolved in 50% aqueous methanol, and in panel B, RBF peptide fragment C was dissolved in 50% aqueous TFE. All other experimental conditions and the explanation of M and D are as Figure 7.

To substantiate these findings we prepared peptide fragments A and C at concentrations of 4 μM in a solution containing 50% methanol in water. As shown in panel A of Figure 8, responses are characteristic of the homodimer that are observed for peptide fragment C (compare with Figure 7C). Indeed, peptide fragment A homodimer was not detected (spectra not shown). Peptide fragment A homodimer was not detected even at a concentration of 10 μM in 50% aqueous methanol (results not shown). Next, peptide fragments A and C were prepared at concentrations of 4 μM in a solution containing 50% trifluoroethanol (TFE) in water (Figure 8B). Trifluoroethanol is known to encourage helical structure to some peptides (*35*) and might enhance the dimerization of these molecules via the amphipathic leucine−isoleucine zipper−like structure found in peptide fragment C. However, these effects are dependent upon the concentration of TFE. Above a concentration of 50%, TFE is known to disrupt the formation of a helical structure (*35*), which should also disrupt dimer formation. As expected, the microspray ESI-MS spectrum obtained for peptide fragment A in 50% TFE showed no response that was characteristic of a homodimer for this peptide (data not shown), so TFE and/or the ESI-MS was not inducing dimer formation. Therefore, as expected, the spectra characteristic of peptide fragment C homodimers were *not* observed when analyzed with 50% TFE (Figure 8, panel B). These results lead us to the conclusion that peptide fragment C forms a noncovalently

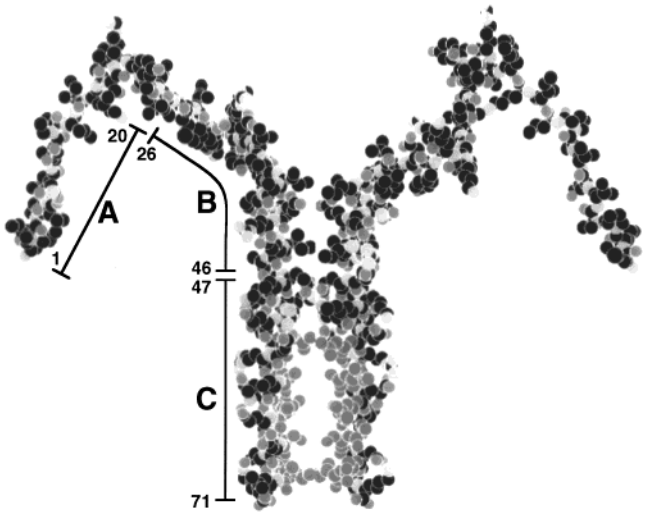

FIGURE 9: Potential three-dimensional dimer model of RBF. The primary amino acid sequence of RBF protein was analyzed for potential secondary structures using various computer programs (described under Experimental Procedures). After refinement and energy minimization of the proposed RBF structure a three-dimensional model of RBF was developed. Computer modeling suggest that RBF could exist as a homo- and/or heterodimer via a leucine−isoleucine zipperlike region in the C-terminus. RBF peptide fragments A, B, and C used in the previously described experiments are indicated.

bound homodimer in water, which is dependent on an α-helical structure. The other RBF peptides did not appear to be particularly susceptible to dimerization under the conditions examined, and as such provided reasonable negative controls for these experiments (spectra not shown). While the analyses of intact RBF by microspray ESI-MS conditions are needed, the apparent peptide fragment C dimer supports that RBF can potentially exist as a homodimer.

As a follow-up to these studies, the primary amino acid sequence of RBF protein was analyzed for potential secondary structures using various computer programs. After refinement and energy minimization of the proposed RBF structure, a three-dimensional model of RBF was developed (Figure 9). Modeling suggests that RBF could exist as homodimers (and possibly heterodimers) via the leucine−isoleucine zipperlike region in the C-terminus, as supported by the microspray ESI-MS data. This model also allows for the N-terminus of RBF protein to interact with DNA.

DISCUSSION

The nuclear matrix has been implicated in the actions of steroid hormones on gene expression (*37*−*40*). In vivo and in vitro analyses has demonstrated that steroid receptors are localized to the nuclear matrix in a wide variety of target tissues (*17*). Steroid hormones have been shown to regulate the mRNA and protein levels of a variety of nuclear protooncogenes and tumor suppressor genes, which are associated with the nuclear matrix (*41*). The regulation of c-*myc* expression can be attributed to various mechanisms including gene amplification (*42*, *43*), insertion of viral transcription regulating elements near the gene (*44*), chromosomal translocations (*45*), and removal of negative DNA enhancer elements normally associated in cis with the gene (*46*). Protein factors regulating control elements in the c-*myc* gene have been suggested since identification of DNase I hypersensitive sites in c-*myc* chromatin can be correlated to the transcription state of the gene (*47*−*51*). Furthermore, Chou et al. (*52*) have identified a region of the human c-*myc* gene containing the 3′-end sequence of exon 3 and a polyadenylation signal as a c-*myc* matrix attachment region (MAR). A nuclear protein, p25, was also shown to preferentially bind to the human c-*myc* MAR.

This laboratory has previously reported a unique nuclear matrix structure associated with the 5′-flanking region of the avian c-*myc* gene (*11*, *12*). These data, including the previously supported nuclear matrix DNA mapping experiments (*1*, *36*, *37*), lead to the potential model whereby the nuclear matrix DNA attaches to regions of DNA that flank the RBF element in the c-*myc* gene. This novel dual nuclear matrix attachment structure could play a role in regulating the availability of the RBF/DNA complex for rapid steroid receptor binding by forming twists, looping, or stabilizing regions of ssDNA. Alternatively, this structure could enable the RBF/DNA/PR complex to interact with other regulatory regions of the c-*myc* gene. This structure appears to be a nuclear matrix acceptor site (binding site) for PR, composed of the RBF bound to a specific RBF element, which is flanked on both sides by nuclear matrix DNA.

Unfortunately, DNA sequence database searches have not shown there to be a high degree of homology between the RBF binding element and the promoter regions of other steroid-regulated genes. However, Alvarez et al. (*53*) have shown that nuclear matrix protein 4 (NMP4) and soluble nuclear protein (NP) both bind to poly(dT) elements within the rat type 1 collagen α1 polypeptide chain (COL1A1) promoter. The COL1A1 promoter region that NP/NMP4 binds is similar to the RBF binding element in the chicken c-*myc* gene. Both contain a run of Ts interrupted by some G/Cs followed by three or more Ts. Like RBF-MBP, NP/NMP4 has a binding preference for the T-rich strand over the A-rich strand. This is similar to the yeast DNA-binding proteins PUB1, PAB1, and ssARS-T that may play role in replication initiation in yeast ARS elements (*54*, *55*). It should be emphasized that NP/NMP4 appear to be architectural factors that may or may not interact directly with the transcription machinery of the cell, whereas RBF appears to have dual roles: first, potentially stabilizing a single-stranded DNA MAR-like structure, and second, forming protein−protein interactions with steroid hormone receptors, i.e., serving as nuclear binding sites for the latter.

This paper expands on our earlier studies by showing that RBF-MBP binds to a single-stranded RBF DNA element, with a preference for the sense strand or T-rich strand of DNA. Work by various groups (*56*−*58*) has shown that matrix-associated DNA regions are DNase I hypersensitive and expose single-stranded DNA. Bode et al. (*59*) have shown that base-unpairing regions (BURs) are characteristic of S/MARs and may be important for function in vitro as well as in vivo. Many nuclear matrix-associated proteins have been shown to bind single-stranded DNA or BURs, in particular, T-cell specific S/MAR-binding protein (SATB1) (*59*, *60*), lamins (*61*), NuMA (*61*), and nucleolin (*61*). Furthermore, nuclear matrix proteins SATB1 and B-cell regulator of IgH transcription (Bright) recognize the minor groove of specific MAR sites (*62*, *63*). It remains to be determined whether RBF binds to the minor or major groove of dsDNA or only to ssDNA. Moreover, since there exists a

high degree of heterogeneity in S/MARs (*59*), it still has to be determined if RBF binding and the proposed DNA architecture are unique to the c-*myc* gene.

We have previously demonstrated that progesterone rapidly inhibits the c-*myc* gene expression in the avian oviduct system (*20*). However, other groups have reported rapid progestin stimulation of c-*myc* expression (*64*, *65*). However, the overexpression of transiently transfected RBF showed a decrease in reporter gene expression when driven by DNA sequences that contain the RBF element (*1*). The RBF/c-*myc* gene interaction and/or RBF/PR interaction might explain the paradoxical regulation of c-*myc* transcription by progesterone in different cellular systems, as supported by the different responses for the chick oviduct (*20*) versus human breast cancer cells (*64*).

Because we do not know the exact number of RBF molecules bound to the RBF DNA element or the affinity RBF has for itself or for other potential proteins, we can only speculate as to the significance of the homo- or heterodimer formation and function. Homodimerization would suggest that the RBF−RBF interaction is more important for interactions with the DNA. This will allow the RBF dimer to bind DNA with high affinity and stability, with the resulting DNA tertiary structure needed for PR binding. Homodimerization would also limit the concentrations of available RBF in the cell and result in increased specificity for sites on the genomic DNA with the proper nucleotide composition (sequence). This would ultimately result in a regulation by steroid receptors mediated by DNA structure where the nuclear receptor recognizes the overall structure created by RBF/DNA interactions.

Evidence is provided that the N-terminal region of RBF, containing a potential parallel β-sheet, is the region of the protein that binds the RBF DNA element. This protein structure has previously been shown to be a DNA binding motif in other single-stranded and double-stranded DNA binding proteins (*28*−*32*). In addition, data are provided by mass spectrometry that the leucine−isoleucine zipperlike structure in the C-terminus of RBF is capable of forming homodimers in solution. The RBF protein has an 86% similarity/76% identity to the mitochondrial ATP synthase ϵ chain. It is important to emphasize that Western blots demonstrated that RBF-specific antibodies do not cross-react to mitochondrial antigens (unpublished observation). The mitochondrial ATP synthase ϵ chain is a distinct polypeptide that is a nonenzymatic component of the multiprotein complex located in the mitochondrial membrane that synthesizes ATP (*66*). This identity supports that RBF protein would form protein−protein interactions, even though the two proteins have very distinct morphological functions.

Electrospray ionization-mass spectrometry (ESI-MS) is becoming a versatile tool. Recently, detection of noncovalently bound supramolecular complexes by ESI-MS has also been reported. Some of the different complexes observed with this technique include protein−protein (*67*, *68*), protein−ligand (*69*−*72*), protein−oligonucleotide (*67*, *73*), and protein−DNA (*74*, *75*). This application of ESI-MS has also recently been the subject of an extensive review (*76*). Recently, the emergence of microspray and nanospray ESI-MS interfaces and the use of a cooled interface have provided improved conditions for the analysis of noncovalently bound complexes (*34*, *76*). In these studies, a previously described microelectrospray ion source (*27*) was used to investigate the composition of aqueous solutions of RBF peptide fragments A, B, and C. Results indicate that only the C-terminus of RBF (peptide fragment C) had a tendency to form homodimers. Furthermore, we demonstrated by use of TFE that the detected peptide fragment C homodimer was a result of a noncovalent interaction. These results support our hypothesis that RBF can exist as a homodimer in cells using the leucine−isoleucine zipperlike structure in the C-terminal end. It is also highly conceivable that RBF can form heterodimers with other cellular proteins, like the steroid receptors, to help regulate gene transcription. Studies are underway to further test this hypothesis, as well as whether the RBF is bound to dsDNA or ssDNA in vivo.

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