A DNA-binding Element for a Steroid Receptor-binding Factor Is Flanked by Dual Nuclear Matrix DNA Attachment Sites in the c-myc Gene Promoter*

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Andrea H. Lauber‡§, Thomas J. Barrett‡§, Malayannan Subramaniam¶, Mark Schuchard, and Thomas C. Spelsberg†**

From ‡Mayo Medical Ventures, Mayo Clinic, Rochester, Minnesota 55905, the †Department of Biochemistry and Molecular Biology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905, and the ¶Electrophoresis Department, Sigma Chemical Co., St. Louis, Missouri 63118

The receptor-binding factor (RBF) for the avian oviduct progesterone (Pg) receptor (PR) has previously been shown to be a unique 10-kDa nuclear matrix protein that generates high affinity PR-binding sites on avian DNA. This paper describes the use of Southwestern blot and DNA gel shift analyses with RBF protein to identify a minimal 54-base pair RBF-binding element in the matrix-associated region (MAR) of the Pg-regulated c-myc gene promoter. This element contains a 5'-CC-rich domain and a 3'-AT-rich domain, the latter of which has a homopurine/homopyrimidine structure. The gel shift assays required the generation of an RBF-maltose fusion protein (RBF-MBP), which specifically binds this element and is supershifted when the anti-RBF polyclonal antibody is added. Computer analysis of the full-length amino acid sequence for RBF predicts a DNA-binding motif involving a β-sheet structure at the N-terminal domain. Southern blot analyses using nuclear matrix DNA suggests that there are dual MAR sites in the c-myc promoter, which flank an intervening domain containing the RBF element. The co-transfection of this MAR sequence, containing the RBF element and cloned into a luciferase reporter vector, together with an RBF expression vector construct, into steroid treated human MCF-7 cells, results in a decrease of the c-myc promoter activity relative to control transfections containing only the parent vector of the RBF expression construct. These data suggest that a unique chromatin/nuclear matrix structure, composed of the RBF-DNA element complex which is flanked by nuclear matrix attachment sites, serves to bind the PR and repress the c-myc promoter.

The classic model of steroid hormone action suggests that ligand-bound receptors dimerize and bind to specific sites (acceptor sites) on the chromatin/DNA to alter gene expression. In many genes, the steroid receptors (SR)2 bind to specific cis-

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† The first two authors contributed equally to this work.

‡ To whom correspondence should be addressed: George M. Eisenberg Professor, Dept. of Biochemistry and Molecular Biology, 1801A Guggenheim Bldg., Mayo Clinic, Rochester, MN 55905, Tel.: 507-284-2480; Fax: 507-284-2053.

§ The abbreviations used are: SR, steroid receptor; bp, base pair(s); CAPS, 3-(cyclohexylamino)propanesulfonic acid; CAT, chloramphenicol acetyltransferase; E2, 17β-estradiol; EMSA, electrophoretic mobility shift assay; IC1, IC1 182,780, a pure antiestrogen (Zeneica Pharmaceuticals); MAR, nuclear matrix-associated region; MCF, maltose-binding protein; pAb, polyclonal antibody; PCR, polymerase chain reaction; Pg, progesterone; PMSF, phenylmethylsulfonyl fluoride; PR, progesterone receptor; RBF, receptor-binding factor; SAR, scaffold attachment region; Tricine, N-tris(hydroxymethyl)methylglycine.

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levels in these same tissues correlate with the RBF protein levels by Western blot analyses (28). A homologous RBF-like mRNA species was identified in human tissues.2 Earlier studies had indicated that specific DNA sequences were important when generating RBF/PR-binding sites. The PR-binding sites could only be generated when the purified RBF was bound to mammalian genomic DNA, but not when the RBF was complexed to prokaryotic, or lower eukaryotic genomic DNAs (29, 30). The PR-binding sites could also be generated with the genomic sequences of the c-myc gene but not those of the ovalbumin gene (31). Past studies in our laboratory demonstrated that the steady state levels of mRNA and the transcription rates of several nuclear proto-oncogenes, c-myc, c-fos, and c-jun, in the avian oviduct are rapidly regulated in vivo by Pg, glucocorticoids, and estrogen (24, 32–36). These nuclear proto-oncogenes have also been shown to be regulated by steroids in many other animal systems (37–43). In addition, Southern blot analyses revealed that the DNase-resistant nuclear matrix DNA had sequence homology with sequences in the 5'-ends of the steroid-regulated c-myc and the c-jun proto-oncogenes but not to genomic sequences of some other genes in the avian oviduct, suggesting that nuclear matrix is attached to 5'-flanking regions of those genes (23). Thus, a model is emerging, whereby the RBF-containing nuclear matrix is associated with c-myc and other proto-oncogene promoters and generates PR-binding sites (acceptor sites) through which steroids might regulate the transcription of these genes. It should be noted here that other nuclear matrix proteins have been reported to bind to promoter domains and alter gene expression (44, 45).

To challenge this model, it was necessary to determine whether RBF binds to a specific element in or near the same nuclear matrix attachment site in the c-myc 5'-flanking domain. This paper demonstrates that the RBF does bind to a specific DNA element within this nuclear matrix attachment site in the 5'-flanking domain of the avian c-myc gene. Further, the nuclear matrix is shown to attach to each side of this RBF-binding element. Primary sequence analyses of the RBF reveals structural motifs resembling other DNA-binding proteins. Preliminary studies show that the overexpression of RBF in transiently transfected human breast cancer cells inhibits reporter gene activity when the reporter gene construct contains the 1021-bp region of the c-myc promoter. The treatment of these cells with steroids ensures this inhibition. Thus, the RBF and steroid hormones may regulate c-myc gene promoter activity via this 1021-bp region of the promoter that contains the RBF element and nuclear matrix attachment sites.

EXPERIMENTAL PROCEDURES

Isolation and Purification of RBF by Preparative Gel Electrophoresis—Hen oviduct was excised and the chromatin isolated. Chromosomal proteins were size-fractionated by molecular sieve chromatography as described previously (46). Fractions containing proteins in the 4–20-kDa size range were pooled and separated by preparative SDS-polyacrylamide gel electrophoresis in a Tris-Tricine buffer as described previously (28). The gel was transferred to six sheets of polyvinylidene difluoride membrane using a CAPS buffer system (10 mM CAPS + 10% methanol, pH 11.0). The sixth sheet was immunostained for RBF using a RBF-specific monoclonal antibody (22, 46, 47). 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% trifluoroacetic acid (v/v) overnight; dialyzed against 5% (v/v) acetonitrile/H2O, 0.25 mM PMSE, and lyophilized. This protocol was tested and reported in detail (22, 28).

Reconstitution of Purified RBF—This method has been described in detail previously (22, 26, 48). Briefly, purified RBF was solubilized in 6 mM guanidine-HCl in reconstitution buffer (10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 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 (26, 30, 48). It was also found in these current studies to be important for achieving optimal DNA binding. Preparation specific binding. The fusion protein was loaded into 10-mm diameter dialysis tubing and dialyzed in a plastic cylinder filled with 200 ml of 6.0 mM guanidine-HCl against 1 liter of reconstitution buffer at a replacement rate of 1 ml/min to a guanidine concentration of 0 molarity, while rocking at 4°C. After 16 h, the protein solution was removed from the dialysis bags, homogenized, and used in the Southern blotting analysis.

Southern Blot Analyses—Synthetic DNA corresponding to regions of the 5'-c-myc gene were synthesized using PCR. However, the 1021-bp E fragment (see Fig. 1) was obtained by restriction digests of the c-myc plasmid with Smal. The nuclear matrix DNA was isolated, with modifications, as described in Schuchard et al. (23). Briefly, nuclei, isolated from hen oviduct, were resuspended in buffer A (10 mM Tris-HCl, 0.2 mM MgCl2, 0.1 mM PMSF, pH 7.5) and centrifuged. After a second resuspension and centrifugation, the pellet was resuspended in TM buffer (50 mM Tris-HCl, 5 mM MgCl2, 0.25 mM sucrose, pH 7.5). This solution was subjected to extensive DNase I and RNase digestion. SDS and proteinase K were then added (to 0.5%) and 50 μg/ml, respectively. The fractions were phenol-extracted and ethanol-precipitated. Matrix DNA was then gel-purified and fragments of 100–200 base pairs, representing the bulk of the residual/protected DNA, were excised. The DNA was dephosphorylated and the 5'-end labeled with γ-32PATP.

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 representing different regions of the 5'-upstream flanking domain of the c-myc gene. DNA was gel-purified and end-labeled with γ-32PATP by standard T4 kinase reactions. For the Southwestern blot analyses, the purified, reconstituted RBF (31 or 62 ng/slot) was diluted in reconstitution buffer and then slot blotted onto nitrocellulose under vacuum, using a slot blot apparatus. The RBF-MBP fusion protein was also slot blotted in some cases. The membrane was incubated with end-labeled DNA probes representative of nuclear matrix DNA or c-myc fragments of the upstream regions of the c-myc gene. Colipase C (50 ng; Sigma), a 10-kDa protein with a P.I. of 7.0, was also slot blotted and used as a negative control. In some cases, histones (histone type II-AS calf thymus; Sigma H7755) were blotted and used as a nonspecific DNA-binding protein. The nitrocellulose was cut into strips to be incubated with different probes. Blots were blocked for 1.5 h in standard Bowens buffer (50 mM Tris-Cl, pH 7.0, 0.1% bovine serum albumin, 0.01% sodium azide, 5% (v/v) Ficoll, 0.01% sodium pyrophosphatidylcholine) with 3–5 × 106 cpm end-labeled DNA probes, including 50 mM KCl and 1 mM CaCl2 (23). The blots were washed with Bowens binding buffer and exposed to autoradiographic film.

Southern Blot Analysis—Southern blot analysis using DNA/nuclear matrix was conducted according to the procedure described previously with some modifications (23). Equimolar amounts (10 pmol for 1021 and matrix; 20 pmol for 1, 1s, 1H, 1J, K, L, I1, and I10, corresponding to upstream regions of the avian c-myc gene, as described previously above), were diluted in SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) were heat- and alkali-denatured and slot blotted onto nitrocellulose. Blots were baked, prehybridized in Denhardt's medium, then hybridized with the end-labeled nuclear matrix DNA, and washed and exposed to x-ray film.

RBF-MBP Fusion Protein and Polyclonal Antibody—The coding region of the RBF gene was cloned, in frame, into the pMAL-c2 vector and expressed as MBP as specified by the brochure “Protein Fusion and Purification System” accompanying the kit provided by New England Biolabs.

RBF-MBP Fusion Protein and Polyclonal Antibody—The coding region of the RBF gene was cloned, in frame, into the pMAL-c2 vector and expressed as MBP as specified by the brochure “Protein Fusion and Purification System” accompanying the kit provided by New England Biolabs, Beverly, MA. After the sequence of the construct was verified, the vector was used to transform competent Escherichia coli whereby large scale amounts of the fusion protein were produced and purified by affin-gel chromatography as described in the kit. The fusion protein migrated in SDS-polyacrylamide gel electrophoresis 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 sera recovered. The pAb was then purified from the serum using the Affi-Gel Protein A MAPS II kit according to a brochure provided by Bio-Rad. Western blots showed that

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this polyclonal antibody (pAb 273) reacted against the RBF-MBP, as well as the purified reconstituted and factor Xa-cleaved RBF proteins.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility gel shift assays (EMSA) were conducted, with modification to the original protocol, reported by Fried and Crothers (49). Briefly, 200–500 ng of RBF-MBP was incubated with 10 ng of 32P-labeled DNA fragment (10 ng Tris-HCl, pH 7.8, 150 mM NaCl, 1.0 mM dithiothreitol, 2.0 mM MgCl2, 10% (v/v) glycerol) and incubated with 0.5 μg of poly(dI-dC) for 15 min at room temperature. The DNA probes were synthesized and labeled as above (500,000 cpm each) and added to the protein solution, which was incubated for 20 min at room temperature. The mixture was rocked with the RBF-MBP polyclonal antibody for 4 h at 4 °C. The DNA-antibody complexes were resolved by electrophoresis on 6% (w/v) native acrylamide gels (75:1 acrylamide: bisacrylamide) at 26 V/cm in 0.5 × TBE buffer (0.5 mM Tris borate, 0.001 M EDTA, pH 8.0) for 2.5 h at 4 °C. Gels were then dried and exposed to autoradiographic film.

Determining the Full Sequence of the RBF Protein—The N-terminal amino acid sequence analysis of the RBF protein was determined by the Mayo Protein Core Facility (Mayo Clinic, Rochester, MN). Briefly, purified RBF protein (500 pmol) or its tryptic peptides were applied to a Polybrene-treated glass fiber disk, and then microsequenced by the Edman degradation method on an Applied Biosystems (ABI) 476A Protein Sequencer using programmed parameters provided by ABI (Foster City, CA). The phenylthiobenzyldiamino acids from each cycle were identified on-line by reverse phase high performance liquid chromatography on an ABI 120A PTH Analyzer.

The cDNA for RBF was obtained using polymerase chain reaction procedures, as described previously (28). Briefly, poly (A) + mRNA from chicken oviduct was reverse transcribed using an oligo(dT) primer to obtain the first strand cDNA. The cDNA was used as a template in the PCR along with sense primer (oligonucleotides synthesized from the N-terminal sequence of RBF) and antisense primer (dT). One of the PCR primers also contained a T7 or SP6 phage promoter. The PCR-amplified DNA was transcribed and sequenced using RNA amplification with transcript sequencing (RAWTS) method described previously by Sarkar and Sommer (52). The cDNA sequence correlated with the amino acid sequence. 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 the sequence analysis software package, produced by the Genetics Computer Group (GCG) of the University of Wisconsin, Biotechnology Center, Madison, WI.

Transient Transfections into MCF-7 Cells—An RBF open reading frame was cloned into an expression vector (pSG5; Stratagene, La Jolla, CA), which was regulated by SV40 enhancer element. In all experiments, the pSG5 expression vector itself was transfected as a control to determine if the RBF protein itself would bind directly to the same E domain previously described under “Experimental Procedures” and outlined in Fig. 1B. As described in inset portion of Fig. 1, cloned into the pGL2 basic (Promega) luciferase vector. The RBF construct was cotransfected along with the 1021 pgl2 reporter construct. A tk-CAT reporter construct was included in all transfections to access and analyze the promoter activities in the transcription efficiency. The MCF-7 cells were cultured using Dulbecco’s minimal essential medium (DMEM) containing 1 × antibiotic/antimycotic solution (100 μg of penicillin, 0.1 μg of streptomycin, and 0.25 μg of amphotericin B/ml (Sigma)), 10% (v/v) fetal bovine serum (Sigma), and 10−8 M 17β-estradiol (E2) (Sigma). The E2 was included to maintain optimal cell proliferation and high PR levels.

For the transfections, the MCF-7 cells were trypsinized, plated into 100-mm dishes, and grown for ~48 h to approximately 50% confluence in the above described (DMEM) medium containing charcoal-stripped serum containing 10−8 M E2. The charcoal stripping of the serum was performed to remove endogenous steroids. The cells were then co-transfected with the 1021 pgl2 reporter gene construct and either the pSG5-RBF expression vector or the pSG5 vector alone, using the LipofectAMINE reagent according to the procedure provided by Life Technologies, Inc. The assays contained experimentally optimized quantities of DNA (represented per plate as: 10 μg of pSG5 and/or RBF expression vectors along with 2 μg of the 1021 pgl2 reporter gene construct and 2.4 μg of tk-CAT). The various DNAs and LipofectAMINE were added individually to serum-free, antibiotic-free media, mixed thoroughly, and plate coated with serum-free, antibiotic-free media. The optimal transfection incubation period was then determined to be 4 h at 37 °C. The transfection mixtures were then removed by aspiration and replaced with DMEM containing charcoal-stripped serum and 10−8 M E2. After 24 h, the transfected cells were then cultured in the same media containing 10−8 M of both Pg (Sigma) and E2. The 10−8 M estrogen was maintained in all media to maintain high levels of PR. The Pg was added to generate functional/activated PR.

Analyses of Reporter Gene Expressions—The transfected cells were then harvested into a solution containing 40 mg Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, and sedimented at 14,000 rpm for 30 s. The cell pellet was resuspended in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 1.0 mM dithiothreitol, 2.0 mM MgCl2, 10% (v/v) glycerol) and incubated with 0.5 μg of poly(dI-dC) for 15 min at room temperature. The DNA probes were synthesized and labeled as above (500,000 cpm each) and added to the protein solution, which was incubated for 20 min at room temperature. The mixture was rocked with the RBF-MBP polyclonal antibody for 4 h at 4 °C. The protein-DNA complexes were resolved by electrophoresis on 6% (w/v) native acrylamide gels (75:1 acrylamide: bisacrylamide) at 26 V/cm in 0.5 × TBE buffer (0.5 mM Tris borate, 0.001 M EDTA, pH 8.0) for 2.5 h at 4 °C. Gels were then dried and exposed to autoradiographic film.

Results

Previous data had shown that nuclear matrix DNA, which was protected from DNase and RNase digestion, hybridized to a specific 1021-bp region (the E domain) of the avian c-myc gene promoter (~676 to ~345 bp), supporting the presence of a sequence-specific binding factor (Ross). Since RBF has been identified as a nuclear matrix-associated protein, it was of interest to determine if the RBF protein itself would bind directly to the same E domain of the c-myc gene promoter. As described under “Experimental Procedures” and outlined in Fig. 1, Southwestern blotting techniques were used whereby purified, renatured RBF protein was slot blotted onto nitrocellulose and probed in the early experiments with radiolabeled restriction enzyme fragments representing various stretches of the c-myc gene. In subsequent studies, the blots were probed with PCR-generated DNAs representing smaller domains of the c-myc promoter. Optimal assay conditions for the Southwestern blot technique were first determined using the early generated (A–E) fragments of the c-myc gene.

The overall structure of the avian c-myc gene and the locations and sizes of the restriction enzyme-generated fragments are outlined in Fig. 1. As described in panel A, the PstI restriction fragments of the c-myc gene generated the A, B, and C fragments. The A fragment was subdivided by restriction enzymes into regions D, E, F, and G. The 1021-bp E fragment was in turn, subdivided into H through K regions. Finally, as shown in panel B, the smaller DNA fragments representing various regions of the I fragment were generated by PCR. Early studies using Southwestern blot analyses have revealed that RBF specifically bound to the A fragment and subsequently to the 1021-bp E fragment (~676 to ~345 bp) with no binding to the B, C, D, F, or G fragment of the gene (data not shown). More recent results, displayed in the inset portion of Fig. 1, indicate that RBF bound with the highest avidity to the 331-bp I fragment at ~583 to ~252 bp, with some binding to the 447-bp fragment K at ~102 to ~345 bp from the first exon. No binding was found with the H and J fragments. The specificity of the interactions between RBF and the DNA is supported by the failure of BS to bind DNA, while, conversely, the basic histone proteins bound indiscriminately to all the DNA fragments.

Subsequent studies focused on the I fragment since it displayed a greater level of RBF binding than the K fragment under the experimental conditions.

Southwestern blot analyses of RBF binding to the internal...
regions of the I fragment is shown in Fig. 2. Panel B of Fig. 1 gives the orientation and locales of the corresponding PCR fragments generated from the 331-bp I fragment of the c-myc gene promoter region. As shown in Fig. 2A, the RBF does not bind to the I1 or the I2 fragments. The RBF, however, does bind to the 152-bp I3 fragments and to the successively shorter I7 and I8 fragments. The RBF protein did not bind to the other regions within the I fragment (data not shown). The specificity of the RBF binding is further supported by the fact that RBF binds optimally to I8 fragment, while it does not bind to a 125-bp fragment of the human retinoblastoma gene promoter region which, by computer analysis, was shown to have 70% homology with the I8 fragment. Fig. 2B shows additional Southwestern blot analyses demonstrating that the 54-bp I10 fragment is the shortest fragment that binds the RBF. Deletions on either side of this 54-bp I10 fragment (I9 and I11) result in a complete loss of RBF binding. The 10-kDa colipase C served as a negative control in these experiments. The sequence of the 54-bp I10 (−404 to −340) fragment of the c-myc gene that binds RBF is shown in Fig. 3. This fragment contains a 5’-GC-rich region and a 3’-AT-rich region. A GC-rich sequence that is located on the 3’-end in the larger I8 fragment was shown to be nonessential for RBF binding, since its deletion to achieve the I10 fragment has no apparent effect on RBF binding. However, the 5’-GC-rich region of I8 is essential for RBF binding since its deletion to achieve the I9 fragment results in the loss of RBF binding.

To demonstrate the specificity of the RBF bound to the I10 element, electrophoretic mobility shift assays (EMSA) were performed. However, a larger protein complex containing RBF was needed to perform the EMSA. Therefore, a RBF-MBP fusion protein was constructed, expressed, and purified from E. coli. The purified RBF-MBP protein showed the same DNA sequence specificity in the Southwestern blots, as the native, reconstituted oviduct RBF. Fig. 4 shows that RBF-MBP fusion protein binds to the same c-myc regions as the native reconsti-
were performed using 32P-end-labeled purified nuclear matrix to assess the specificity of the RBF/I8 interaction. Which is a 10-kDa protein with a pH of 7.0 serves as a protein control to binding to the I9 and I11 probes, which do not bind the native DNA binding is further confirmed by the absence of RBF-MBP to the c-my e gene (23), the question arose as to whether the nuclear matrix, and the latter was shown to be attached to the nuclear matrix DNA. These results support that the RBF-binding element located in the I8/10 region, which flanks the RBF element located in the I8/10 region. To further test whether or not the RBF DNA element and the nuclear matrix DNA are distinct, Southern blot analyses for RBF binding were performed using 32P-labeled I9 and I8 fragments, which bind RBF; two fragments, I9 and H, which do not bind RBF, as well as the nuclear matrix DNA itself. The results in Fig. 7 again show that RBF does bind the avian c-myc gene K region which RBF shows weak binding (Fig. 2), also showed no homology to matrix DNA. The fact that the nuclear matrix is bound to the I9 and to the I6/J regions, but not the I9 and I11 fragments, suggests a dual attachment of the nuclear matrix, which flanks the RBF element located in the I9/I10 region. To answer this question, the same fragments of the c-myc gene 5'-flanking region were examined for homology with the nuclear matrix DNA. Southern blots of the c-myc DNA fragments were performed using 32P-end-labeled purified nuclear matrix DNA as a probe. As shown in Fig. 6, the fragments and, of course, the nuclear matrix DNA itself, displayed sequence homology to the nuclear matrix DNA fragment by Southern blotting, while the fragments which bind the RBF, I9 and I10, display little or no homology to the matrix DNA. The c-myc gene K region which RBF shows weak binding (Fig. 2), also showed no homology to matrix DNA. The fact that the nuclear matrix is bound to the I9 and to the I6/J regions, but not the I9/I10 fragments, suggests a dual attachment of the nuclear matrix, which flanks the RBF element located in the I9/I10 region. To further test whether or not the RBF DNA element and the nuclear matrix DNA are distinct, Southern blot analyses for RBF binding were performed using 32P-labeled I9 and I8 fragments, which bind RBF; two fragments, I9 and H, which do not bind RBF, as well as the nuclear matrix DNA itself. The results in Fig. 7 again show that RBF does bind the avian c-myc, I9 and I8 fragments, but not the I9 and H fragments, as shown earlier. Importantly, the RBF fails to bind the nuclear matrix DNA. These results support that the RBF-binding element, the specific I9 region in the avian c-myc gene promoter, is distinct from the nuclear matrix-associated DNA. In fact, the sequences of the nuclear matrix attachment sites in the I9 and the I6/J regions flank the I9 region containing the RBF element.

Since RBF appears to be a specific DNA-binding protein, the...
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Possible DNA-binding motifs of the RBF protein were then considered. Fig. 8 shows the primary sequence of RBF as determined by the nucleotide sequence of the c-myc DNA and by direct amino acid sequencing of the RBF and its peptide fragments. Peptide structural analyses predict an N-terminal domain with parallel and anti-parallel β-sheet structure. The C-terminal region contains uninterrupted amphipathic leucine/isoleucine-rich domain, reminiscent of a leucine zipper domain for homo- and hetero-dimer interactions. One possible structure derived from this preliminary structural analysis suggests a homodimer complex at the C-terminal region, with a dual β-sheet DNA-binding motif at the N-terminal region. As discussed later, the β-sheet is a reported DNA-binding motif.

DISCUSSION

Past studies have suggested an essential role for specific nuclear chromatin proteins in forming high affinity binding sites (acceptor sites) in the nuclei/chromatin of target tissues for a variety of steroid receptors (22, 23, 26, 54–66). In our laboratory, a specific “acceptor protein,” RBF, was isolated based on its ability to generate saturable, high affinity binding sites on avian and mammalian genomic DNAs and characterized as a component of the chromatin nuclear matrix acceptor sites for the avian oviduct PR (22, 23, 25, 26, 29, 30, 67, 68). The RBF failed to generate these sites when using insect and prokaryotic genomic DNA.

The data presented in this paper address several aspects of the nuclear matrix and RBF, their structure, the steroid receptor interactions, and transcriptional regulation of the c-myc gene. First, the nuclear matrix-associated “RBF” binds to a unique, specific, DNA element within a MAR-like region in the 5′-flanking domain of the c-myc proto-oncogene. Second, the nuclear matrix attachment sites are distinct from the specific DNA element for RBF, and actually flank this element. Third, the complete primary sequence of RBF is presented and shown to be unique with a structure similar to certain other DNA-binding proteins. Fourth, preliminary studies indicate that transient co-transfections of the RBF expression construct, together with c-myc promoter-reporter gene constructs, results in the inhibition of the reporter gene in the presence of steroids. The latter results mimic earlier in vivo studies whereby the endogenous avian c-myc gene expression is rapidly (within 30 min of injection) inhibited by Pg and glucocorticoids (32, 33). In these studies, the combined treatments of estrogen and progesterone were found to be essential for this down-regulation of c-myc promoter. We speculate that the presence of the steroid gene (Promega). As shown in Fig. 9, a 3–4-fold inhibition occurred only in the cells with the RBF expression construct. This inhibition required the presence of both E2 and Pg. A nonsignificant and variable inhibition of the c-myc promoter was observed in the absence of the steroids. In any case, these results suggest that RBF protein can interact and inhibit the c-myc gene promoter activity using the domain containing the RBF element in the presence of activated PR.

Fig. 10 presents a model of the nuclear matrix attachment site with the intervening RBF acceptor site based on the data presented in this paper. The RBF/DNA complex, at the nuclear “acceptor site,” –404 to –350 bp, is shown to be flanked by nuclear matrix attachment sites at ~582 to ~423 and at ~310 to ~252 bp. The nuclear matrix attachment extends into the J region (~253 to ~102 bp). The neighboring K fragment, which binds RBF but not the nuclear matrix, is also shown. Thus, while the RBF was reported to be associated with the isolated nuclear matrix (23), the protein appears to actually be bound to an intervening DNA region, as depicted in the figure, while the two nuclear matrix attachment sites flank this intervening element. Thus, the RBF acceptor site is made available by a dual nuclear matrix attachment. Interactions between steroid receptors and/or other factors with this RBF/DNA complex and may utilize the flanking nuclear matrix attachment sites to subsequently regulate the transcription of the c-myc gene expression.

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Fig. 8. The complete primary amino acid sequence of RBF with peptide structure analyses. The complete amino acid sequence for RBF and the structural analyses were performed as described under “Experimental Procedures.”
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Fig. 9. Effects of RBF expression on the activity of the c-myc gene promoter (E fragment) containing the RBF element. The MCF-7 cells were plated in media containing charcoal-stripped serum with 10^{-8} M E2, added and grown for 48 h to 50% confluence. Either the RBF-pSG5 expression vector or the vector (pSG5) alone were co-transfected into cells along with the pGL2 luciferase reporter plasmid containing the E fragment (containing the RBF element) of the c-myc promoter region as well as the tk-CAT control reporter gene. After 24 h, all the transfected cells were treated with both E2 and Pg at 10^{-8} M. The steroid treatment was carried out for 24 h, and the cells harvested and analyzed for luciferase and CAT activities. The former were normalized to the co-transfected tk-CAT signal. The asterisk (*) represents p < 0.05 with an n of 3. Pg, progesterone; pSG5, expression vector alone; RBF, the complete RBF expression vector.

Fig. 10. Proposed model of the nuclear matrix attachment and RBF complex for SR binding in the c-myc gene promoter. Evidence presented in this paper suggests that the nuclear matrix shares sequence homology with regions flanking the RBF-binding element in the 5'-flanking region of the c-myc gene. The interaction of nuclear matrix DNA and a dimer of RBF to specific regions in the c-myc promoter would form the secondary structures necessary for high affinity steroid receptor binding sites and subsequent regulation of the c-myc gene transcription by steroid hormones. The RBF dimer is included since the peptide structure indicates a possible leucine/isoleucine-like zipper structure in the C-terminal end of the protein.

generates functional PR required for the inhibition of the promoter activity wherein the estrogen induces PR levels in the MCF-7 cells and the Pg activates the PR. Further studies are required, however, to prove that the steroids and their receptors play a direct role in this inhibition. The RBF has been shown previously to bind to the genomic c-myc gene and generate specific binding sites for PR (31). Based on these data, as well as those described in this paper, a model was generated whereby the PR binds to the RBF-DNA element flanked by the nuclear matrix attachment sites in the c-myc promoter, which causes an inhibition of the c-myc gene transcription.

The computer-generated structure of RBF protein was based on its primary amino acid sequence. The analysis suggests a C-terminal end, which generates a homodimer via its amphipathic leucine-isoleucine-rich region. A similar leucine-isoleucine zipper-like structure has been shown for the human single-stranded DNA-binding protein (70). The primary sequence also predicts an N-terminal region with parallel and anti-parallel β-sheet stretches. These β-sheets are known DNA-binding motifs for some prokaryote and eukaryote transcription activators and repressors (71–76). Studies are under way to determine if RBF binds to its element via this β-sheet structure. In general, RBF was found to be unique with some homology to several other proteins such as bovine, rat, and mouse F1/F0ATPase subunit E (77–79) and a rapidly UV-induced nuclear protein mRNA in Chinese hamster ovary cells (80).

Evidence demonstrating the significance of the nuclear matrix in steroid hormone action, including the location of specific nuclear binding sites for steroid receptors, has been accumulating (7–10, 17, 18, 21, 67, 81–84). Previous studies have reported that certain nuclear matrix proteins are bound to MAR-like AT-rich DNA sequences, similar to the RBF element, as described in this paper (95, 96). The functions and properties of these and other nuclear matrix proteins are only now being elucidated. Estrogen has recently reported to significantly increase the levels of several nuclear matrix-intermediate filaments in steroid-responsive human breast cells but not nonresponsive cells (69). These effects may reflect a coordinated response of steroid action on the nuclear matrix. Our laboratory reported that the chromatin acceptor sites and the nuclear matrix binding sites for the avian PR were the same (12, 67). In fact, the isolated RBF protein and PR nuclear binding were localized solely to the oviduct nuclear matrix (22, 25). The nuclear matrix is an organized structure responsible for anchoring topological domains, called closed loop domains, in interphase nuclei and play a role in DNA replication, mRNA synthesis, DNA loop attachment sites, and topoisomerase activity (85) (for recent reviews see Refs. 14 and 86). The total genomic 10 nm chromatin filaments are subdivided into 50,000 loops, each containing an average of 60,000 base pairs of DNA. These loops are tightly anchored to the DNA at specific matrix attachment regions (MARs), also termed scaffold attachment regions (SARs) (87–89). These MARs/SARs are DNase-resistant regions of variable length and sequence with AT-rich stretches and some containing topoisomerase II cleavage sites (88, 90). Recently, evidence for two classes of MARs with matrix protein complexes bound to them has been reported. There is a “constitutive class,” which permanently anchors the loops and appears to be involved in DNA replication, and a “regulatory class,” which is bound to the flanking domains of genes to regulate transcription as cis-acting regulatory elements (15, 87, 91–94). Our results implicate the latter “regulatory MARS” as another class of nuclear acceptor (binding) sites for SRs which functions in the steroid regulation of the c-myc gene transcription. In support of this model, many species of SRs, as well as their high affinity nuclear binding sites, have been localized to the nuclear matrix in a variety of animal systems (5, 8–10, 17, 18, 20, 21, 23, 67, 83–85).

In this paper we also present data supporting the theory that the nuclear matrix-associated RBF (acceptor protein) for the avian oviduct PR binds between two matrix attachment domains in the 5′-promoter domain of the progesterone-regulated c-myc proto-oncogene. Since the nuclear matrix DNA represents those regions of DNA that are protected from DNase digestion of genomic DNA, the domains flanking the RBF element appear to be the domains actually protected from DNase action and thus bound by the nuclear matrix. The actual nuclear matrix attachment sites in these flanking regions have been localized to I_{10} (and the 3′-end of H) and to I_{2} (and the 5′-end of J). The RBF binds to an AT-rich domain located in an intervening sequence between these two nuclear matrix attachment sites. This novel dual nuclear matrix attachment structure is supported by the fact that not only does the RBF fail to bind to the nuclear matrix DNA, but the RBF element I_{10} fails...
to recognize any sequences in nuclear matrix DNA. The RBF-binding element has a 25-bp 5′-GC-rich domain and a 29-base AT-rich 3′-domain, the latter containing a 19-bp homopurine/homopyrimidine tract.

Thus, these flanking domain regions of the avian c-myc proto-oncogene do not have the typical MAR-like sequences, but rather the intervening RBF element in this structure has the previously reported MAR-like primary sequence of both GC- and AT-rich regions. The K domain of the c-myc promoter, which also binds the RBF in Southwestern blot assays (Fig. 1), contains two AT-rich regions with 10-bp and 15-bp homopurine/homopyrimidine (polyA/poly T) stretches. However, the nuclear matrix does not appear to bind to this region. Whether the PR binds to these sites of RBF binding is not currently known. The DNA structures generated by the MAR-binding proteins include DNA bending (97–98), minor groove narrowings, and the quaternary structure of the RBF-DNA complex are being studied (15).

The DNA duplex unwinding was found to be important for the generation of MAR sites, as well as the augmentation of RNA polymerase II and c-myc proto-oncogene expression. Further questions arise. 1) Are there other steroid receptors and other transcription factors, besides PR and progesterone, which also bind the RBF in Southwestern blot assays (Fig. 1), on the 54-bp DNA element, with its polyA/polyT tract, and the site of interaction of PR, are currently under study.

In summary, the data presented in this paper support the model presented in Fig. 10, whereby the nuclear matrix is attached on either side of the RBF element. The nuclear matrix could play a role either in regulating the availability of the RBF/DNA complex for steroid receptor binding, or in regulating the steroid-induced gene transcription after steroid receptor binding has occurred. This model should readily allow the nuclear matrix to alter the structure of the intervening domain with its AT-rich polypurine-polypyrimidine structure, and maintain the latter in a unique structure poised for the interaction with steroid receptors and other transcription factors. The exact secondary/tertiary structures of the DNA element and the quaternary structure of the RBF-DNA complex are currently unknown. In any event, this nuclear matrix structure and steroid receptor binding (acceptor site) are novel chromatins structures and possible participants in the steroid regulation of the c-myc proto-oncogene expression. Further questions arise.

1) Does this nuclear matrix attachment structure in the c-myc gene 5′-flanking domain occur in other gene promoters?
2) Is this structure utilized by the progesterone receptor as well as other steroid receptors in vivo and in other animal species?
3) Is this structure specific for regulatory MAR structures as opposed to constitutive MAR structures as per Bode et al. (15)?

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A DNA-binding Element for a Steroid Receptor-binding Factor

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Andrea H. Lauber, Thomas J. Barrett, Malayannan Subramaniam, Mark Schuchard and Thomas C. Spelsberg

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