Nucleic Acid-Binding Properties of the *Xenopus* Oocyte Y Box Protein $mRNP_{3+4}$[†]

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ABSTRACT: Y box proteins contain the conserved cold shock domain (CSD) and several basic/aromatic (B/A) islands that are rich in arginine and aromatic residues. The binding of purified *Xenopus* oocyte 6S Y box protein, $mRNP_{3+4}$, to Y box RNA, single-stranded (ss) DNA, and double-stranded (ds) DNA was studied by gel mobility shift and nitrocellulose filter binding assays. $mRNP_{3+4}$ specifically bound Y box ssDNA or RNA, while binding of dsDNA was not detected. Y box ssDNA and RNA did not efficiently cross-compete for $mRNP_{3+4}$ binding, and no evidence for ternary complex formation was detected. However, Y box ssDNA binding was competed by high concentrations of Y box RNA or nonspecific RNA competitors, indicating that the ssDNA-binding site has a lower affinity for RNA. $mRNP_{3+4}$ demonstrated similar affinity for either Y box RNA or ssDNA. However, at elevated ionic strength RNA binding was markedly greater than ssDNA binding, indicating that RNA binding involves nonionic interactions that are not utilized for ssDNA binding. Recombinant polypeptides containing B/A islands bound Y box RNA exclusively, but inclusion of the CSD led to preferential ssDNA binding. The results demonstrate that the B/A islands are exclusively RNA-binding, while the CSD exhibits preferential binding of ssDNA. The inability of Y box RNA and ssDNA to efficiently cross-compete for $mRNP_{3+4}$ binding suggests that isoforms exhibit preferential ssDNA or RNA binding.

Y box proteins are a highly conserved family of nucleic acid-binding proteins that have been identified from human (Didier et al., 1988; Sakura et al., 1988; Kolluri et al., 1992), mouse (Gai et al., 1992), rat (Ozer et al., 1990), *Xenopus* (Tafuri & Wolffe, 1990), and chicken cDNAs (Grant & Deeley, 1993). The Y box proteins are reported to be transcriptional activators that bind various germ line-specific and tissue-specific gene promoter elements containing the inverted CCAAT sequence known as the Y box (Didier et al., 1988; Tafuri & Wolffe, 1990), as well as other CCAAT-containing promoter elements (Ozer et al., 1990; Cohen & Reynolds, 1991; Spitkovsky et al., 1992), and a nuclease-sensitive element of the c-*myc* gene (Kolluri et al., 1992). Alternative functions in DNA repair and replication have also been suggested (Hasegawa et al., 1991; Grant & Deeley, 1993).

Two germ line members of the Y box multigene family are also known as the most abundant RNA-binding proteins associated with *Xenopus* untranslated maternal mRNAs (Darnbourgh & Ford, 1981; Richter & Smith, 1983; Cummings & Sommerville, 1988). These two polypeptides are highly phosphorylated (Crawford & Richter, 1987; Murray et al., 1991), exhibit anomalous and variable electrophoretic mobility (Deschamps et al., 1992), and have been named $mRNP_3$ and $mRNP_4$, p54 and p56, and pp56 and pp60 by the aforementioned groups. In the *Xenopus* oocyte, $mRNP_3$ and $mRNP_4$ are predominantly cytoplasmic (Murray et al., 1992), existing as a soluble free pool of protein particles as well as in assembled mRNPs (Murray et al., 1991). A 6S protein particle composed of $mRNP_3$ and $mRNP_4$ dimers, termed $mRNP_{3+4}$, has been identified from *Xenopus* ovaries and shown to bind mRNA *in vitro* (Murray et al., 1991). Cloning and direct protein sequencing demonstrated that $mRNP_3$ and $mRNP_4$ are Y box proteins of 36 and 37 kDa that share 87% amino acid identity and may represent alleles in *Xenopus* (Murray et al., 1992). $mRNP_{3+4}$ is identical to FRGY2 (Deschamps et al., 1992).

Members of the Y box proteins contain a conserved domain of 85 amino acids (having 90% identity between *Xenopus* and man) that shares at least 40% identity with the cold shock proteins of *Escherichia coli* and *Bacillus subtilis* (Wistow, 1990; Willimsky et al., 1992). This cold shock domain (CSD)[1] is found in the amino-terminal portion of the vertebrate Y box proteins, and in most cases it is followed by four interspersed stretches rich in arginine and aromatic amino acids that have been termed basic/aromatic (B/A) islands. The *E. coli* cold shock protein CspA activates transcription of cold shock-responsive genes both *in vitro* (La Teana et al., 1991) and *in vivo* (Jones et al., 1992). No role for the cold shock protein in RNA regulation has been defined, although one is suggested, on the basis of the accumulation of 70S ribosomes that accompanies cold shock-induced translational repression (Broeze et al., 1978).

The structure of the bacterial cold shock proteins is a five-stranded β-barrel (Schindelin et al., 1993, 1994). The three N-terminal antiparallel β-strands form a surface rich in aromatic residues that represents the nucleic acid-binding domain. The second β-strand contains amino acids conserved from the RNP-1 motif (Landsman, 1992), in a conformation identical to that of RNP-1 in snRNP protein U1A, while the third β-strand contains amino acid interactions that are similar to those of RNP-2 (Nagai et al., 1990; Schindelin et al., 1993; Schnuchel et al., 1993). In addition, these studies showed binding of the bacterial cold shock proteins to Y box ssDNA (Schindelin et al., 1993, 1994).

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[1] Abbreviations: CSD, cold shock domain; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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The nucleic acid-binding domain of the cold shock proteins is greatly conserved in the vertebrate Y box proteins, suggesting that the same domain mediates DNA binding. Deletion analysis of *Xenopus* (Tafuri & Wolffe, 1992) and human (Kolluri et al., 1992) Y box recombinant proteins demonstrated that the CSD is required for DNA binding and that additional C-terminal sequences are required for transcriptional activation (Tafuri & Wolffe, 1992).

This study describes the binding of purified *Xenopus* $mRNP_{3+4}$ to either Y box ssDNA or RNA. These nucleic acid substrates were inefficient in cross-competition studies, suggesting either that independent binding sites exist or that different isoforms determine nucleic acid-binding specificity. No binding of $mRNP_{3+4}$ to Y box dsDNA could be detected, suggesting that transcriptional activation requires Y box elements in single-stranded form. The nucleic acid-binding properties of recombinant polypeptides containing B/A islands and the universal cold shock domain were also analyzed. The results (i) define the B/A islands as exclusive RNA-binding elements of the Y box proteins, (ii) demonstrate that the cold shock nucleic acid-binding domain has a higher affinity for Y box ssDNA than RNA, and (iii) suggest that alternate conformations and/or posttranslational modifications of $mRNP_{3+4}$ determine its nucleic acid-binding selectivity.

MATERIALS AND METHODS

Isolation of $mRNP_{3+4}$. $mRNP_{3+4}$ was purified in the course of purifying a 15S ATPase from *Xenopus* ovary S100 supernatants and was generously provided by Dr. J. M. Peters (Peters et al., 1990). The S100 extract was fractionated by ion-exchange chromatography; the fractions containing the 15S ATPase also contained 6S $mRNP_{3+4}$. These different particles were separated on density gradients to yield highly enriched 6S $mRNP_{3+4}$ (Peters et al., 1990). This 6S $mRNP_{3+4}$ is slightly contaminated by two smaller polypeptides as shown in Figure 1A. These minor contaminating polypeptides had no appreciable nucleic acid-binding activity because fractions lacking these demonstrated the same $mRNP_{3+4}$ binding activity as the pooled fractions. These preparations of 6S $mRNP_{3+4}$ contain a mixture of highly phosphorylated $mRNP_{3+4}$ isoforms similar to those of ovary S100 extracts (Murray et al., 1991), but do not contain kinase activity.

Protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA), and the polypeptide content was analyzed as previously described (Murray et al., 1992). $mRNP_{3+4}$ molar values were calculated assuming a heterodimer of 36- and 37-kDa polypeptides.

Y Box Nucleic Acid Probes. The oligonucleotide GAATCCTACTGATTGGCCAAGGTGCTGGTGGTAC, termed the Y box (34 nt; the Y box is underlined), and its overlapping complement TCGACTTAGGATGACTAACCGGTTCCACGACCAC were annealed, phosphorylated, and cloned into *Kpn*I/*Sac*I sites of pBluescript (Stratagene, La Jolla, CA). The sequence of the pYbox clone selected for further study showed that transcription from the T7 promoter would yield an RNA with the sequence GGCGAAUUGGAGCUGAAUCCUACUGAUUGGCCAAGGUGUGGUGGUAC (47 nt) from plasmid linearized with *Kpn*I. Radiolabeled Y box RNA was synthesized by incorporation of [α-^{32}P]CTP. Single-stranded DNA probes were radiolabeled at the 5′ ends using T4 polynucleotide kinase and [γ-^{32}P]ATP. The annealed ds Y box oligonucleotide was radiolabeled by filling in the overhanging ends using Klenow enzyme and [α-^{32}P]dCTP. All probes were purified by polyacrylamide gel electrophoresis (Latham et al., 1990).

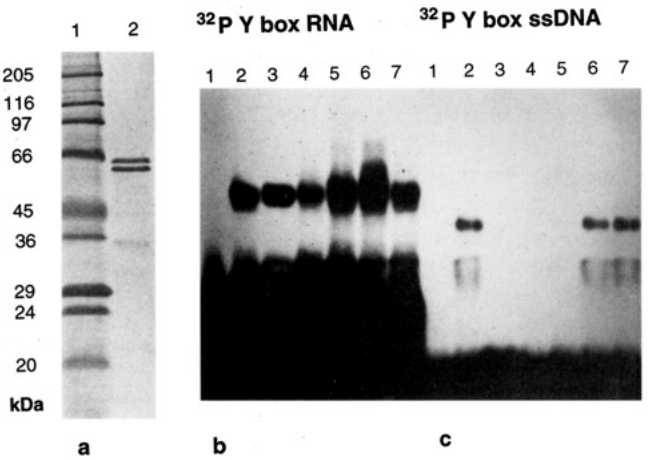

FIGURE 1: $mRNP_{3+4}$ binding to Y box RNA or single-stranded DNA in the presence of nonspecific competitors. (a) The protein composition of the 6S $mRNP_{3+4}$ preparation used for binding studies is shown in lane 2. Material was separated by 18% SDS−PAGE and stained with Coomassie blue. Molecular mass markers were separated in lane 1. (b, c) Purified 6S $mRNP_{3+4}$ (0.2 μM) binding to either Y box RNA probe (panel b, lane 2; 0.8 nM) or Y box ssDNA probe (panel c, lane 2; 1.5 nM) was tested by gel mobility shift assay (lane 2). Lane 1 contains substrate incubated in the absence of $mRNP_{3+4}$. $mRNP_{3+4}$ binding in the presence of nonspecific competitors is shown in lanes 3−7, using heparin at 5 mg/mL (lane 3), calf liver tRNA at 0.5 mg/mL (lane 4), poly(A) at 1 mg/mL (lane 5), and either ds poly(dI:dC) or ss poly(dI), poly(dC) at 0.5 mg/mL (lanes 6 and 7, respectively). The products were separated on a 5% gel.

To prepare Y box oligomers, the overhanging ends of the annealed Y box oligonucleotides were filled using Klenow enzyme, the 5′ ends were phosphorylated, and the oligonucleotides were ligated. Ligation products were separated on NuSieve agarose (FMC Corp., Rockland, ME), and the oligomers larger than 4*n* were isolated. *Eco*RI adapters were ligated to the oligomers, and they were cloned into pBluescript. Chain-termination sequencing determined that pY-box$_6$ contains a 6*n* oligomer of the Y box sequence (204 nt) oriented for T7 polymerase transcription of Y box$_6$ RNA. For Y box$_6$ DNA probe, the *Bam*HI/*Hin*dIII insert containing Y box$_6$ was isolated and radiolabeled by the Klenow end-filling reaction to minimize the contribution of single-stranded components.

Gel Mobility Shift Assays. The gel shift assay was carried out in buffer A (20 mM Hepes, pH 7.9, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP) and 24 μg/mL BSA, to maintain conditions similar to those in previous studies (Murray et al., 1991). For mobility shift assays, 5 × 10^4 cpm of radiolabeled probe (Y box RNA = 2.5 × 10^{12} cpm/μmol; Y box ssDNA = 1.25 × 10^{12} cpm/μmol) was mixed on ice with any competing nucleic acid or analog, the reaction was started by addition of 5 pmol of 6S $mRNP_{3+4}$ in a final volume of 25 μL, and the mixture was incubated at 22 °C for 15 min. The mixture was returned to ice, loading dye was added, and the samples were electrophoresed on prerun polyacrylamide gels containing 30 mM Tris-HCl, pH 8.3, and 30 mM sodium borate. Gels were transferred to DE 81 paper (Whatman), dried, and exposed to Kodak XAR film using intensifying screens.

Nitrocellulose Filter Binding Assays. The nucleic acid probe was incubated with the indicated amount of protein or peptide in 50 μL of buffer A and 24 μg/mL BSA at 22 °C for 30 min. The nucleic acid probe (5×10^3 cpm) was used with the following specific activities: Y box RNA = 8×10^{12} cpm/μmol; Y box ssDNA = 1.25×10^{12} cpm/μmol. Samples were returned to ice, and 150 μL of cold buffer A was added. Each sample was filtered through 0.4-μm nitrocellulose filters (HAWP, Millipore, Middlebourgh, MA) prewetted with buffer A, and 200 μL of buffer A was then used to wash the reaction vial and filter. Cherenkov counting of the filters was performed in a Packard 1600 TR counter. Background binding in the absence of protein was determined (typically 3−7% of input) and subtracted from the filter-retained counts for each sample. Each data point was determined in triplicate. The specifically retained counts were then expressed as a percent of the input probe and used to generate binding isotherms. The apparent association constant (K_a') was calculated by estimating the concentration of protein when the binding was 50% of the observed maximum (Draper et al., 1988; Gregory et al., 1988). This method closely approximates K_a' because the total protein concentration was always more than 1000-fold larger than the nucleic acid−protein complex concentration.

For the analysis of binding under conditions of increasing ionic strength, the samples were incubated under standard conditions that were supplemented with KCl. The background binding was determined at 0.1 M KCl. At high KCl concentrations, the filter-retained DNA was reduced to values below the 0.1 M KCl background binding.

Recombinant Polypeptides. Fragments from $mRNP_3$ cDNA p54 (Murray et al., 1992) were subcloned into either plasmid pQE (Quiagen, Chatsworth, CA) or plasmid pRSET (InVitrogen, San Diego, CA) expression vectors, and recombinant polypeptide was induced and isolated by nickel chelate affinity chromatography according to the manufacturer's protocol. Recombinant plasmids encoding polypeptides CBA1 and BA4 were engineered using partial cDNAs of p54, while BA2 utilized a *Hae*III fragment of p54. CBA1 corresponds to nucleotides 65−668 encoding amino acids 19−229, BA2 includes nucleotides 419−229 encoding amino acids 134−229, and BA4 includes nucleotides 728−1218 encoding amino acids 240−324 of p54. Recombinant polypeptides were denatured in 2 M guanidinium HCl and 57 mM Tris·HCl, pH 8.0, for 1 h on ice and then dialyzed into buffer D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 20% glycerol). Purity of the recombinant protein was estimated to be >95% for BA2, >90% for BA4, and >80% for CBA1 from Coomassie-stained gels.

RESULTS

Purified Native $mRNP_{3+4}$ Binds Y Box RNA or Single-Stranded DNA. The objective of this study was to determine the RNA-binding sites of $mRNP_{3+4}$ and to analyze the nucleic acid-binding properties of native $mRNP_{3+4}$. Figure 1a shows the composition of a representative preparation of 6S $mRNP_{3+4}$. 6S $mRNP_{3+4}$ contains a mixture of highly phosphorylated isoforms similar to that previously reported (Murray et al., 1991). $mRNP_{3+4}$ RNA and DNA binding was tested using the Y box sequence, CTGATTGGCCAAG. The Y box sequence was present in a 34-nt oligodeoxynucleotide or a 47-nt RNA prepared by *in vitro* transcription. Gel mobility shift assays were used to characterize 6S $mRNP_{3+4}$ binding to Y box RNA or Y box ssDNA.

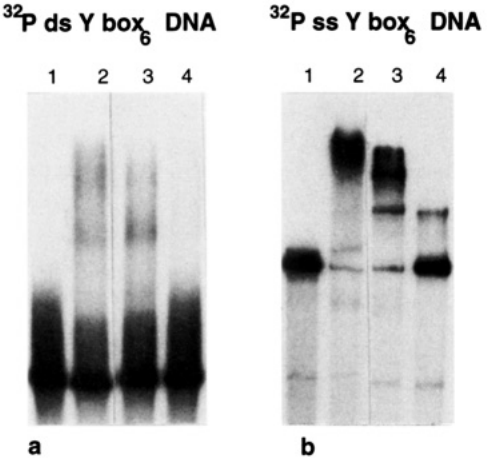

FIGURE 2: $mRNP_{3+4}$ binding to double-stranded and single-stranded Y box$_6$ DNA. $mRNP_{3+4}$ binding to 6*n* oligomerized Y box dsDNA (a) or ssDNA (b) was tested in the absence (lane 2) or presence of competitors. The ds Y box$_6$ DNA or the heat-denatured ss Y box$_6$ probe incubated in the absence of $mRNP_{3+4}$ is shown in lane 1. Binding was tested in the presence of ds and ss Y box$_6$ plasmid DNA at 0.5 μM (lanes 3 and 4, respectively). Products were separated on an 8% gel.

6S $mRNP_{3+4}$ binding to either Y box RNA or ssDNA was readily detected in the absence of nonspecific competitor (Figure 1b,c, lane 2). $mRNP_{3+4}$ binding of Y box RNA was unaffected by either nonspecific RNA or DNA competitors (Figure 1b, lanes 3−7). In contrast, $mRNP_{3+4}$ ssDNA binding was competed by the nonspecific RNA competitors heparin, tRNA, and poly(A) (Figure 1c, lanes 3−5), but was unaffected by either poly(dI:dC) or poly (dI) and poly(dC) (lanes 6 and 7). DNA binding was sequence-specific, as none was detected to the ssDNA Y box complement (not shown).

For tests of double-stranded (ds) DNA binding, the probes were radiolabeled by the end-filling reaction in order to minimize the contribution of single-stranded components. Repeated attempts to detect 6S $mRNP_{3+4}$ binding of Y box dsDNA failed. Because the Y box proteins protect large segments of promoter regions that often contain multiple binding sites (Wolffe et al., 1992), a larger, multicopy Y box dsDNA substrate was prepared. A tandem direct repeat of six copies of the Y box sequence (Y box$_6$) was prepared and tested for $mRNP_{3+4}$ binding in the presence of specific nucleic acid competitors (Figure 2).

6S $mRNP_{3+4}$ binding to Y box$_6$ dsDNA (Figure 2a) was compared to binding to the heat-denatured, ssDNA Y box$_6$ form (Figure 2b). 6S $mRNP_{3+4}$ bound Y box$_6$ ssDNA avidly (Figure 2b, lane 2), forming two complexes that probably represent one and two dimers of $mRNP_{3+4}$. Extended autoradiographic and photographic exposure was needed to reveal apparent $mRNP_{3+4}$ Y box$_6$ dsDNA binding (Figure 2a, lane 2). Competition experiments clarify that this is artifactual because a vast excess of cold ds Y box$_6$ plasmid DNA failed to compete the binding (Figure 2a, lane 3). It has been reported that the Y box proteins have a higher affinity for ssDNA than dsDNA (Tafuri & Wolffe, 1992). However, the inability of excess cold dsDNA to compete dsDNA binding indicates that $mRNP_{3+4}$ efficiently bound partial single-stranded regions of the Y box$_6$ dsDNA probe.

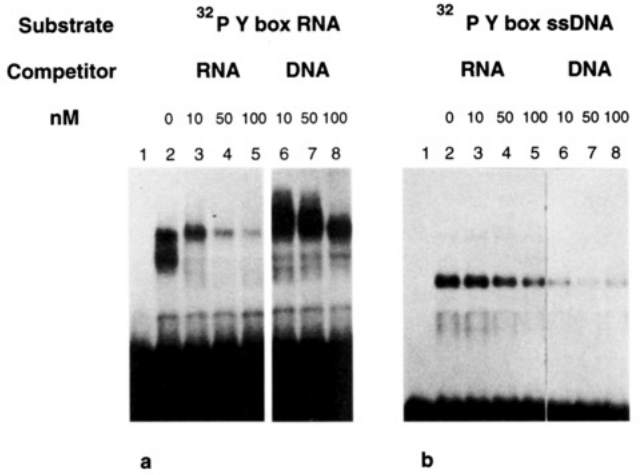

FIGURE 3: Specific competition of $mRNP_{3+4}$ binding to Y box RNA or ssDNA. Binding of purified 6S $mRNP_{3+4}$ (0.2 μM) to Y box RNA (panel a; 0.8 nM) or ssDNA (panel b; 1.5 nM) probes was tested in the presence of 10, 50, and 100 nM oligonucleotide competitor Y box RNA (lanes 3−5) or Y box ssDNA (lanes 6−8). Lane 1 contains the Y box RNA or ssDNA probes incubated in the absence of protein. Lane 2 contains Y box RNA or ssDNA probes incubated with $mRNP_{3+4}$. The same number of counts of Y box RNA and ssDNA probes were used. However, the specific activity of the RNA probe was twice that of the DNA probe; therefore, the molar excess of competitor over RNA probe was 13-, 66-, or 130-fold, and the excess over DNA probe was 6.6-, 33-, and 66-fold. Products were separated on a 5% gel.

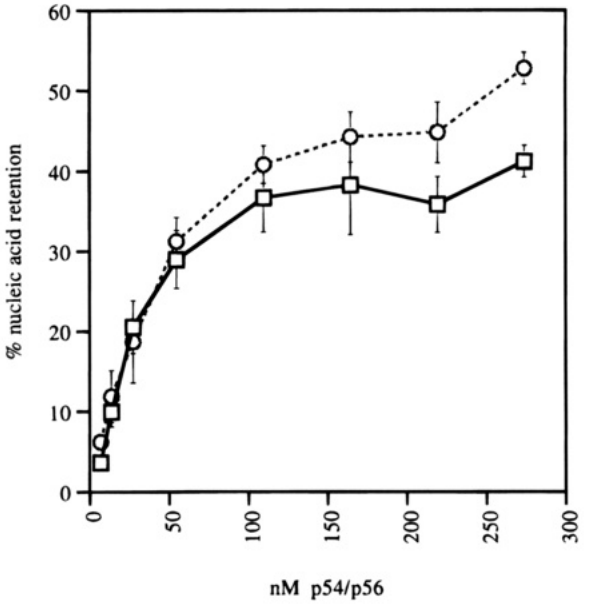

FIGURE 4: Nucleic acid-binding curves of $mRNP_{3+4}$ for Y box RNA or ssDNA. Solution binding assay mixtures were filtered through nitrocellulose, and the specifically retained nucleic acid−protein complex was expressed as a percentage of the input radioactivity. Typical binding curves are shown for Y box RNA (○) and Y box ssDNA (□). Each sample was determined in triplicate, and the average value was plotted with error bars denoting the standard deviation.

It should be noted that these observations of dsDNA binding are only applicable to this germ line Y box protein. This is in contrast to a somatic Y box protein, NSEP-1, which binds both dsDNA and ssDNA (Kolluri et al., 1992).

Purified $mRNP_{3+4}$ Independently Binds Y Box RNA and Single-Stranded DNA. The nucleic acid-binding specificity of $mRNP_{3+4}$ for Y box ssDNA and RNA was tested by competition gel shift assays using excess Y box RNA or ssDNA (Figure 3). Addition of 10 nM cold self-competitor resulted in significant competition, but this same concentration was ineffective in cross-competition. Thus, $mRNP_{3+4}$ Y box RNA binding was only efficiently competed by RNA (Figure 3a), while ssDNA binding was only efficiently competed by Y box ssDNA (Figure 3b). This inefficient cross-competition for $mRNP_{3+4}$ binding was also observed in the presence of excess nonspecific competitors (i.e., poly(dI), poly(dC); not shown). Higher levels of competitor RNA demonstrated significantly greater competition for ssDNA binding than the reverse case (Figure 3b, lanes 4 and 5 at 33- and 66-fold molar excess versus Figure 3a, lane 7, at 66-fold). Together with the observations of RNA analogs competing ssDNA binding (Figure 1b), these data indicate that $mRNP_{3+4}$ ssDNA-binding domains also demonstrate a lower affinity for RNA.

The selectivity of native $mRNP_{3+4}$ for the ribo or deoxyribo form of the Y box sequence may reflect multiple, independent nucleic acid-binding sites in $mRNP_{3+4}$. If Y box RNA and ssDNA binding occur through entirely independent binding sites, then the protein should be capable of dual binding. The competitive binding conditions that used a molar excess of competing nucleic acid would reveal any substantial $mRNP_{3+4}$ ternary complex formation. In particular, the [^{32}P]Y box ssDNA−$mRNP_{3+4}$ complex, which migrates as a single tight product, should be affected by additional binding of Y box RNA, which would produce a broader, more slowly migrating product. But 6S $mRNP_{3+4}$ did not demonstrate any significant dual nucleic acid binding under these conditions (Figure 3b, lanes 3−5). Therefore, the lack of efficient cross-competition may indicate that $mRNP_{3+4}$ assumes different conformations that are selective for either RNA or Y box ssDNA binding.

$mRNP_{3+4}$ binding of Y box ssDNA and RNA was further assessed under conditions of increasing $mRNP_{3+4}$ concentration and constant amounts of radioactive nucleic acid substrate. The formation of radioactive nucleic acid−protein complex was quantitated by retention on nitrocellulose filters, and the value of specifically retained nucleic acid−protein complex was expressed as a percentage of the total nucleic acid. These studies were used to generate binding isotherms (shown in Figure 4) which allow direct comparison of the affinity for either nucleic acid (Draper et al., 1988; Gregory et al., 1988). Binding was tested at protein concentrations ranging from 0.2 to 20 ng/μL (2.7 to 270 nM dimer $mRNP_{3+4}$). Control competition experiments verified that the conditions maintained $mRNP_{3+4}$ specificity for Y box RNA versus ssDNA binding, as no efficient cross-competition was observed. Saturation was typically observed when 30−60% of the total Y box ssDNA or RNA was bound. The percentage of nucleic acid bound at saturation varied with the nucleic acid probe preparation; however, the binding isotherms were similar.

The apparent affinity constant (K_a') of $mRNP_{3+4}$ was calculated from the binding isotherms by estimating the concentration of $mRNP_{3+4}$ at half-maximal binding. These $mRNP_{3+4}$ binding analyses were performed for Y box RNA ($n = 6$) and ssDNA ($n = 7$) using three different preparations of 6S $mRNP_{3+4}$. $mRNP_{3+4}$ had a similar affinity for Y box ssDNA and RNA, with an average K_a' of 3.6×10^8 M (SD $= 1.6 \times 10^8$) for Y box ssDNA and 3.0×10^8 M (SD $= 1.1 \times 10^8$) for Y box RNA.

Analysis of $mRNP_{3+4}$ nucleic acid binding at increasing ionic strength provided a clear distinction of binding proper-

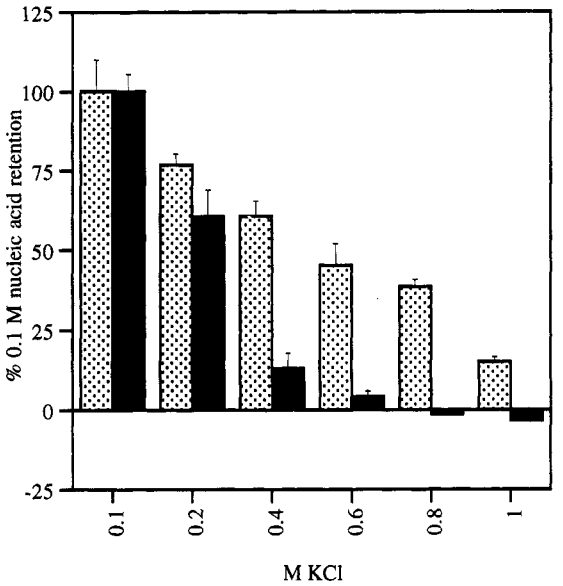

FIGURE 5: Nucleic acid binding of $mRNP_{3+4}$ at increasing ionic strength. Binding of $mRNP_{3+4}$ to either Y box RNA (stippled bars) or Y box ssDNA (solid bars) was determined from binding assays performed with increasing KCl concentrations. The binding was quantitated as described in Figure 4 and expressed as a percentage of the binding at 0.1 M KCl. At high levels of KCl, retention of DNA fell to levels below the background retention determined at 0.1 M KCl.

ties. The binding of $mRNP_{3+4}$ for Y box RNA or ssDNA was determined under binding conditions of increasing KCl concentration by nitrocellulose filter binding assays. The results are shown in Figure 5 as a percentage of binding at 0.1 M KCl. $mRNP_{3+4}$ RNA binding was relatively salt resistant as previously shown (Murray et al., 1991); however, the profile of DNA binding shows sensitivity to raised KCl concentrations. Over 60% of the RNA binding remained at 0.4 M KCl, while ssDNA binding dropped to less than 20%. At 0.8 and 1 M KCl, no ssDNA binding was detected, as the retained radioactivity fell below the 0.1 M KCl background retention level. These results indicate that RNA binding involves nonionic interactions that are not utilized for ssDNA binding.

Nucleic Acid-Binding Domains of $mRNP_{3+4}$. To identify the domains specifying RNA and Y box ssDNA binding, recombinant polypeptides corresponding to specific regions of $mRNP_3$ were employed. Recombinant polypeptides containing a single B/A island from either the center (BA2; aa 134−229) or the C-terminal end (BA4; aa 240−324) of $mRNP_3$ or the conserved domain and two B/A islands (CBA1; aa 19−229) were tested for specific binding of Y box ssDNA or RNA in the gel mobility shift binding assay (Figure 6). The same molar concentration of each recombinant polypeptide was used, thereby allowing an accurate assessment of the relative affinities for RNA. The results show that the polypeptides containing B/A island II or IV mediate RNA binding in a specific manner, as RNA binding is not competed by Y box ssDNA (Figure 6, lanes 7−18). Neither competition of RNA binding by ssDNA nor direct ssDNA binding was detected when higher concentrations of either BA2 or BA4 were used in other experiments (not shown). Therefore, the carboxy-terminal region of $mRNP_{3+4}$ contains nucleic acid-binding domains that are selective for RNA.

In order to test the effect of the presence of the CSD on the RNA binding of the B/A islands, a recombinant polypeptide containing the entire CSD and B/A islands I and II (CBA1; aa 19−229) was similarly tested. Previous studies have determined that the CSD and B/A I are the minimal sequence required for DNA binding (Kolluri et al., 1992; Tafuri & Wolffe, 1992). The results with CBA1 support and extend those findings, as inclusion of the conserved domain together with B/A I and II conferred Y box ssDNA binding that was unaffected by Y box RNA (Figure 6, lanes 4−6). Earlier studies with the human somatic Y box protein NSEP-1 described separate ssDNA- and dsDNA-binding properties, with the conserved RNP-1 motif of the CSD being essential for ssDNA binding (Kolluri et al., 1992). This notable difference in dsDNA binding may be attributable to sequence divergence between the germ line and somatic forms of the Y box proteins.

The analysis of recombinant CBA1 (aa 19−229) furthermore demonstrates that the cold shock domain influences B/A island RNA binding. CBA1 includes B/A II (aa 134−229) which demonstrated specific RNA binding as BA2 (Figure 6, lanes 7−9). Analysis of CBA1 revealed that CBA1 RNA binding was competed by Y box ssDNA as well as RNA (lanes 1−3). These results are unexpected and indicate that the CSD and the B/A islands, at least B/A I and II, do not behave in an autonomous fashion. The observation suggests that the cold shock domain preferentially binds ssDNA and that this occludes RNA binding by adjacent B/A islands.

DISCUSSION

A specific member of the Y box protein family, $mRNP_4$ (FRG Y2), has been described as both a transcriptional activator and a translational repressor. Oocyte $mRNP_{3+4}$ that is highly phosphorylated (Murray et al., 1991) was purified under conditions that maintained $mRNP_{3+4}$ phosphorylation and was used for *in vitro* nucleic acid-binding analysis. This native 6S $mRNP_{3+4}$ exhibited independent, stable, and mutually exclusive binding of Y box ssDNA and RNA. Studies using recombinant polypeptides were used to analyze nucleic acid-binding domains and provide some insight into the interactions that occur in the native $mRNP_{3+4}$. Because native 6S $mRNP_{3+4}$ is a complex molecule of undetermined structure, these analyses only provide a first step toward defining the multiple nucleic acid-binding domains of this protein.

Dedicated RNA- and ssDNA-Binding Populations. The binding studies performed here with purified native 6S $mRNP_{3+4}$ determined that the Y box protein binds either Y box RNA or Y box ssDNA in a specific manner. Binding was unaffected by nonspecific nucleic acids, with the notable exception of nonspecific RNA analogs competing Y box ssDNA binding. Specific competition analysis demonstrated that $mRNP_{3+4}$ can independently bind Y box RNA and ssDNA, in that binding of either nucleic acid was not efficiently competed by the other nucleic acid. In addition, no evidence for ternary complex formation was detected in the specific cross-competition mobility shift assays, suggesting that the two modes of nucleic acid binding are mutually exclusive. The stable non-cross-competitive nature of the nucleic acid binding, together with an apparent absence of dual nucleic acid binding supports the existence of $mRNP_{3+4}$

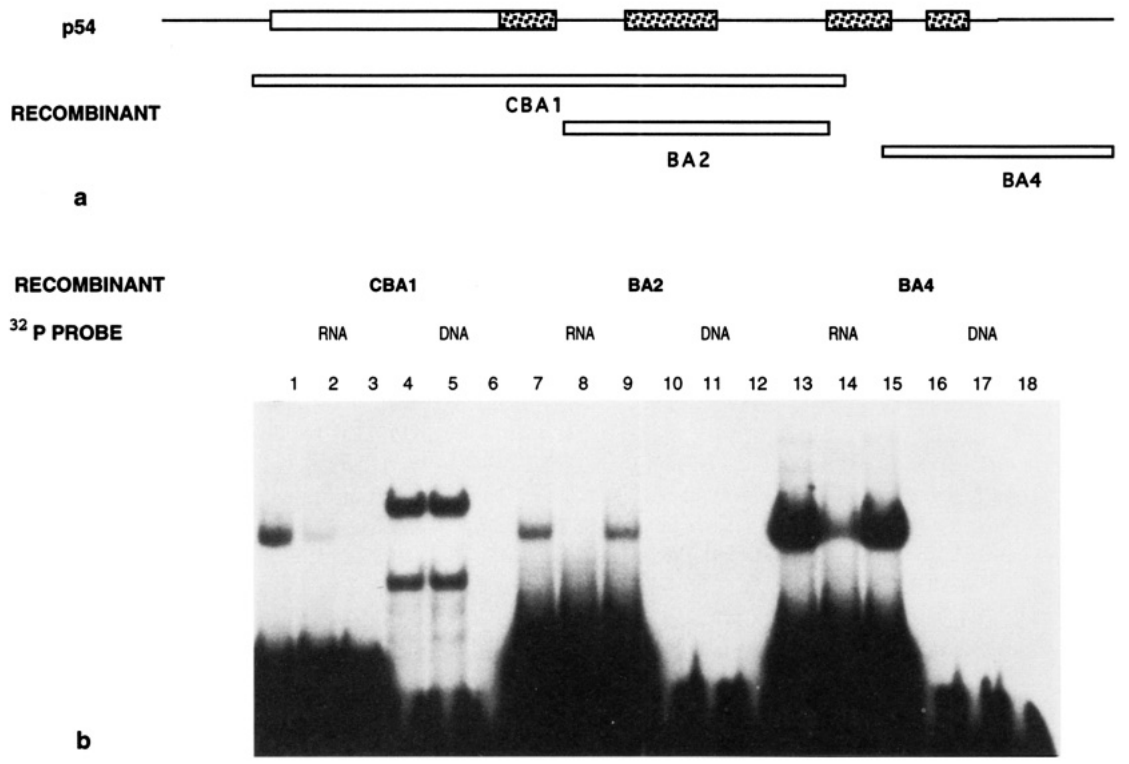

FIGURE 6: Recombinant $mRNP_3$ polypeptide binding of Y box RNA or ssDNA. (a) Scheme of $mRNP_3$ structure with conserved CSD (indicated by the open box) of $mRNP_3$ cDNA p54 and the four basic/aromatic islands (speckled boxes) that correspond to $mRNP_3$ B/A I−IV. Recombinant polypeptides of $mRNP_3$ cDNA p54 are aligned with the region of p54 present in recombinants CBA1, BA2, and BA4. (b) Nucleic acid binding of recombinant polypeptides CBA1, BA2, and BA4 (12.5 pmol; lanes 1−6, 7−12, and 13−18, respectively) was tested in the absence or presence of competitor Y box RNA or ssDNA. For each recombinant polypeptide, binding to Y box RNA (lanes 1−3, 7−9, and 13−15) or ssDNA (lanes 4−6, 10−12, and 16−18) was tested in the absence of competitor (the first of each probe series) or in the presence of 500 nM Y box RNA or DNA (the second and third of each probe series, respectively).

isoforms that preferentially bind either RNA or Y box ssDNA.

This behavior of 6S $mRNP_{3+4}$ in competition analysis of RNA and ssDNA binding is similar to that observed for the *in vitro* translation products of a somatic Y box protein, MSY-1 (Gai et al., 1992). In that study, the Y box sequence and two other DNA substrates that also contained an inverted CCAAT box were each efficiently bound by MSY-1. Each substrate showed efficient self-competition of MSY-1 binding but displayed inefficient cross-competition with the other two substrates. This suggested that multiple forms of MSY-1 were produced which had distinct DNA-binding specificity. It is presently unknown whether $mRNP_{3+4}$ exhibits specific binding of other DNA substrates or whether any relationship exists between non-Y box-specific DNA-binding forms and RNA-binding forms of $mRNP_{3+4}$.

Y Box Protein Basic/Aromatic Islands Mediate RNA Binding. Both somatic and germ line-specific members of the Y box protein family have basic/aromatic islands composed of amino acid double and triple repeats of arginine and tyrosine or phenylalanine, separated by flanking sequences rich in acidic residues. The amino acid character of the B/A islands is ideally suited for both ionic interactions with the nucleic acid phosphate backbone and stacking of aromatic amino acids with the bases. The two B/A-rich segments of $mRNP_3$ examined in this report are B/A I, PQQRPQRRRPPPFFYRRRFRRGPRPNNQQNQG, and B/A IV, PQRQRNRPYVQRRRAQQ. The efficient UV cross-linking of $mRNP_{3+4}$ to RNA *in vivo* (Swiderski & Richter, 1988) and *in vitro* (Murray et al., 1991; Marello et al., 1992) can be attributed to the B/A island's aromatic residues, which are the most photoreactive (Smith, 1969). The binding of $mRNP_{3+4}$ at high ionic strength also supports the contribution of the aromatic residues in the protein−RNA interaction.

Selective RNA binding was observed for the recombinant polypeptides containing either B/A island II or IV and the flanking acidic segments. These two B/A islands were analyzed because they are the most dissimilar with respect to their amino acid content, size, and location in $mRNP_{3+4}$. The inability of these B/A island recombinant polypeptides to bind ssDNA, either directly or in competition for RNA, demonstrates that the B/A islands are selective RNA-binding elements.

Sequence-independent mRNA binding may be essential for sequestering a wide variety of maternal mRNAs from translation and turnover. Sequence-independent mRNA binding of $mRNP_{3+4}$ has been observed *in vitro* (Richter & Smith, 1984; Murray et al., 1991; Marello et al., 1992), and this is consistent with the widespread occurrence of $mRNP_{3+4}$ in maternal mRNPs (Darnbourgh & Ford, 1981; Richter & Smith, 1983; Tafuri & Wolffe, 1993). Transfection of FRG Y2($mRNP_4$) into somatic cells has provided further evidence that it is an mRNA-sequence-independent translational repressor (Ranjan et al., 1993). *In vivo*, the specificity of translational repression and temporal regulation of mRNA mobilization (Stick & Hausen, 1985; McGrew et al., 1989; Paris & Phillippe, 1990; Simon et al., 1992) undoubtedly involves other mRNP proteins. One would anticipate that these proteins would be specific for the given mRNA (McGrew & Richter, 1990; Fox et al., 1992; Simon et al., 1992) and that they would either modify $mRNP_{3+4}$ or interact with $mRNP_{3+4}$ to achieve specific translational repression/

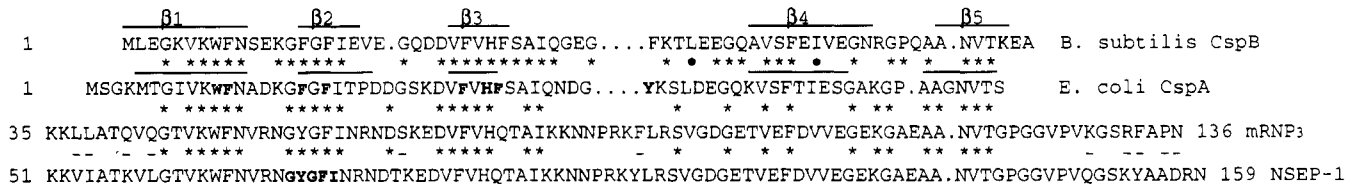

FIGURE 7: Alignment of *B. subtilis* and *E. coli* cold shock proteins with the conserved domain of the germ line Y box protein *Xenopus* $mRNP_3$ and a somatic Y box protein, human NSEP-1. The larger conserved domain of the Y box proteins (85 aa) is shown for $mRNP_3$ and NSEP-1. This region of NSEP-1 only contains a single amino acid substitution from the *Xenopus* somatic protein FRG Y1. The regions of the *B. subtilis* Csp B and *E. coli* CspA sequences that are found in β-strands are indicated by the bars. The bold residues of NSEP-1 indicate residues essential for ssDNA binding (Kolluri et al., 1992), and those in CspA denote aromatic amino acids perturbed by Y box ssDNA binding (Newkirk et al., 1994). Conserved residues are aligned with an asterisk; residues that diverge between $mRNP_3$ and NSEP-1 are aligned with a hyphen.

derepression.

Y Box Protein $mRNP_{3+4}$ Binds ssDNA. Binding of $mRNP_{3+4}$ to the Y box DNA element was examined because recombinant FRG Y2($mRNP_4$) supports *in vitro* transcriptional activation from Y box promoter-dependent gene constructs (Tafuri & Wolffe, 1990, 1992). This study demonstrates that native 6S $mRNP_{3+4}$ exhibits specific binding of the single-stranded form of the Y box. Specific competition experiments determined that the apparent binding of Y box dsDNA resulted from high-affinity binding of partial single-stranded components of the double-stranded probe. Consequently, it is necessary to consider this germ line Y box protein as a ssDNA-binding transcriptional activator along with a small but increasing number of ssDNA-binding proteins (Mukherjee & Chambon, 1990; Wilkison et al., 1990; Santoro et al., 1991; Quinn & McAllister, 1993). Therefore, a functional requirement for transcriptional activation by this Y box protein would be formation of single-stranded control regions, which are a feature of some transcriptionally active genes (Larsen & Weintraub, 1982; Nickol & Felsenfeld, 1983; Siebenlist et al., 1988; Kohwi & Kohwi-Shigematsu, 1991).

As reported for NSEP-1, other Y box proteins may exhibit dsDNA binding in addition to ssDNA binding. However, the minimal ability of $mRNP_{3+4}$ to bind ssDNA must be sufficient for transcriptional activation in this case. A requirement for single-stranded regions may explain the relatively low transcriptional activation observed with FRG Y2($mRNP_4$), both *in vitro* (Tafuri & Wolffe, 1990, 1992) and *in vivo* (Ranjan et al., 1993).

Previous analysis of FRGY2($mRNP_4$) demonstrated that regions necessary for Y box DNA binding are not sufficient for transcriptional activation (Tafuri & Wolffe, 1992). Transcriptional activation also requires C-terminal regions that are proposed to mediate protein−protein interactions via the short, acidic α-helices separating the B/A islands. In this situation, RNA binding by the B/A islands could interfere with the formation of extended protein interactions. Thus, suppression of RNA-binding activity may be necessary for formation of a functionally active transcriptional complex.

Cold Shock Nucleic Acid Binding Domain. The Y box proteins all contain a conserved domain that has at least 40% identity with the cold shock protein from *Escherichia coli* and *Bacillus subtilis* (Wistow, 1990; Willimsky et al., 1992). The sequence and structural determination of the bacterial cold shock proteins are compared with those of the larger conserved domains of *Xenopus* $mRNP_3$ and human NSEP-1 in Figure 7. Structural analysis of the bacterial cold shock proteins determined that the three N-terminal antiparallel β-strands represent a nucleic acid-binding domain (Schindelin et al., 1993, 1994).

In the Y box proteins, the CSD contains the central part of the RNA recognition motif RNP-1 sequence, GFGF ($mRNP_3$, aa 55−58) (Landsman, 1992), which is essential for ssDNA binding by the human somatic Y box protein NSEP-1 (Kolluri et al., 1992). Native $mRNP_{3+4}$ Y box ssDNA binding exhibited competition by excess Y box RNA (Figure 3b) or the nonspecific RNA competitors tRNA, poly(A), and heparin (Figure 1c). This direct competition of ssDNA by RNA supports an interaction between the native $mRNP_{3+4}$ CSD and RNA, albeit a weaker one than with ssDNA. Furthermore, the observed absence of RNA competition of ssDNA binding by the CSD-containing recombinant polypeptide CBA1 (Figure 6, lane 5) suggests that the CSD−RNA interaction of native $mRNP_{3+4}$ is promoted or stabilized by elements outside of the CSD. These may be the C-terminal B/A islands, the structure of the CSD in 6S $mRNP_{3+4}$, or protein modifications. While the results support previous conclusions that the CSD confers Y box DNA binding (Kolluri et al., 1992; Tafuri & Wolffe, 1992), they also raise the possibility that the CSD interacts with RNA following RNA binding by the B/A islands.

Conclusions. The *Xenopus* oocyte Y box protein, $mRNP_{3+4}$, binds either Y box ssDNA or RNA in a noncompetitive manner, indicating that different binding sites are primarily responsible for binding the different nucleic acids. The primary determinant for Y box ssDNA binding is the conserved cold shock domain, while individual B/A islands can act as primary RNA-binding sites. The stable non-cross-competitive binding of Y box ssDNA and RNA by native $mRNP_{3+4}$ suggests that separate dedicated subpopulations of $mRNP_{3+4}$ exist. One possibility is that alternate conformations, perhaps stabilized by covalent modification, result in either the CSD or the B/A islands conferring binding selectivity.

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