Characterization of the DNA-Binding Domain of the Avian Y-Box Protein, chkYB-2, and Mutational Analysis of Its Single-Strand Binding Motif in the Rous Sarcoma Virus Enhancer

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chkYB-2 is a sequence-specific, single-stranded DNA binding chicken Y-box protein that promotes Rous sarcoma virus long terminal repeat (RSV LTR)-driven transcription in avian fibroblasts. The DNA-binding domain of chkYB-2 has been mapped by characterizing the DNA binding properties of purified recombinant chkYB-2 mutant polypeptides. The data indicate that the invariant cold shock domain (CSD) is necessary but not sufficient for association with DNA and suggest that another conserved region, adjacent to the carboxyl boundary of the CSD, plays a role in high-affinity DNA binding. chkYB-2 binds to a tandem repeat of the 5'-GTACCACC-3' motif on the RSV LTR. Mutational analysis of this recognition sequence revealed the requirement of an essentially unaltered template for both high-affinity binding by chkYB-2 as well as maximal transcriptional activity of the RSV LTR in vivo. The single-stranded DNA binding activity of chkYB-2 is augmented by Mg²⁺. The possible significance of this finding for transactivation by a single-strand DNA binding protein is discussed.

The chicken Y-box protein, chkYB-2, is a sequence-specific single-stranded DNA binding protein that binds the octanucleotide motif 5'-GTACCACC-3' present on the noncoding strand of the Rous sarcoma virus (RSV) long terminal repeat (LTR) (7). chkYB-2 is expressed abundantly in avian fibroblasts and muscle tissue, the mesenchymal lineage host cells most permissive to infection and tumor formation by RSV. This property combined with its ability to function as a potent activator of RSV LTR-driven transcription in avian fibroblasts suggests an important role for this protein in the virus life cycle (40). We reported earlier on the cloning and characterization of chkYB-1b, another Y-box protein that closely resembles chkYB-2 in structure as well as in its ability to interact with specific motifs in the RSV enhancer (21). Recently, we also described the cloning of chkYB-1 homology protein, a potential regulator of Y-box transcription factors (31).

The Y-box proteins are a new class of DNA and RNA binding factors that have been shown to function as both transcriptional and translational regulators of gene expression (39, 47, 48). Genes encoding the eukaryotic Y-box proteins have been isolated from Xenopus, chicken, mouse, rat, and human cells. The modular nature of these proteins resembles that of proteins from the well-characterized families of transcription factors. What is unique, however, is the wide range of nucleic acid structures to which Y-box proteins have been reported to bind (2, 8, 9, 16, 18, 26, 34, 36, 42).

The nucleic acid binding properties of Y-box proteins are thought to reside primarily in the highly conserved cold shock domain (CSD) (14, 34, 42, 46). The CSD has been described to recognize diverse double-stranded motifs, especially sites with purine/pyrimidine asymmetry between strands, as well as different single-stranded DNA sequences, particularly pyrimidine-rich ones. The CSD also contains the RNA binding motif RNP-1 (28). While the Y-box proteins share a near identity over the CSD, they vary widely at their amino termini. The carboxyl termini, while divergent in primary amino acid sequence, still retain the charge-zipper motif wherein acidic and basic residues are organized as alternating islands (34).

Compelling evidence linking Y-box proteins to transcriptional regulation is accumulating, as the reports on roles played by single-stranded DNA binding proteins in activating transcription. Currently, information on the domain mapping of eukaryotic Y-box proteins is largely limited to work done on the Xenopus proteins FRGY1 and FRGY2 (4, 43), where it has been demonstrated that the functions of DNA binding, transcriptional activation, and multimerization can be localized to different domains. In this report, we describe the DNA binding properties of mutant polypeptides derived from chkYB-2. The results indicate that similar to classic CSD-containing proteins, chkYB-2 has an absolute requirement of the CSD for its DNA binding ability. However, mutant proteins lacking the carboxyl tail domain entirely or partially either were incapable of binding DNA or bound with markedly lower affinity, suggesting that while the CSD is necessary, it is not sufficient for high-affinity DNA binding.

5'-GTACCACC-3', the octanucleotide recognition motif for chkYB-2, is present as a nearly contiguous direct repeat on the noncoding strand of the RSV LTR. In this report, we also present the results of mutagenesis analysis of single-stranded oligonucleotides bearing this motif and correlate the nature of chkYB-2 interactions observed with these mutants in vitro with the transcriptional activity of RSV LTR reporter constructs carrying identical mutations.

MATERIALS AND METHODS

chkYB-2 mutants. Isolation of the chkYB-2 cDNA clone and its transfer into the bacterial expression vector pMAL-c2 have been described previously (7). The deletion mutants are named according to the amino acids they retain. The derivative denoted with a Δ lacks the segment delineated by the numbered amino acids.
acetylated chloramphenicol spots seen on the X-ray autoradiogram and counting it in the scintillation liquid. All of these experiments were repeated at least three times, and the averages of the results are presented.

**RESULTS**

chkYB-2 binds to several single-stranded motifs on the non-coding strand of the RSV LTR and acts as a potent activator of RSV LTR-driven transcription in avian fibroblasts. To localize the polypeptide region that is responsible for site-specific binding to single-stranded DNA, we performed deletion mutagenesis of YB-2 cDNA. Figure 1A is a line diagram of the mutants that were created by using standard recombinant DNA methods. Except for one nested deletion, YB-2 (Δ158-222), all constructs were either NH2-terminal or COOH-terminal deletions. The YB-2 (75-230) mutant has deletions at both termini.

Wild-type and mutant YB-2 polypeptides expressed in *E. coli* as MBP fusion proteins were column purified on amylose, and

<table>
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<tr>
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<tr>
<td>B ..............</td>
<td>GACATGATCCCTAGACGGGCGGAGGGGTA</td>
</tr>
<tr>
<td>C ..............</td>
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<tr>
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**DNA-binding assays.** Single-stranded oligonucleotides either containing the chkYB-2 wild-type binding site on the RSV LTR or carrying single- or double-point mutations or deletions in the core sequence were synthesized. These sequences are shown in Table 2. Electrophoretic mobility shift assays were carried out as described earlier (23). Binding reaction mixtures (total volume, 20 μl) included 0.1 ng of 5P-labeled synthetic oligonucleotide probe (−30,000 cpm), 2 μg of polyclonal rabbit IgG (diluted 1:100 in PBS), and 5% (vol/vol) glycerol. The reaction mixtures were incubated for 20 min at room temperature, and applied directly onto prerun nondenaturing 6% polyacrylamide gels, and electrophoresed in Tris-glycine buffer as described earlier.

Binding assays which examined the effects of other divalent cations like Zn2+, Mn2+, or Ca2+ or the polyvalent cation spermidine on DNA binding were carried out similarly except for the addition of the specified ion(s) as indicated in the figure legends. Binding reactions that compared the affinities of the chkYB-2 fusion protein to different oligonucleotides with mutations in the binding site were carried out after first equalizing the specific activities of all the labeled probes.

**Cell culture.** Chicken embryo fibroblasts were cultured in medium 199 supplemented with 10% tryptose phosphate broth, 10% calf serum, and 1% chicken serum in plastic dishes at 37°C and 5% CO2 in humidified air as reported earlier (23).

**Protein purification.** Protein preparations. The reporter vectors carrying the chloramphenicol acetyltransferase (CAT) gene under the control of the RSV LTR having point mutations in the chkYB-2 recognition site were constructed by using PCR-mediated mutagenesis as described earlier (40). The construction of E4 Del CAT, carrying a deletion in the E4 region, has also been described earlier (40). The incorporation of mutations in each of these constructs at the intended sites was confirmed by double-strand sequencing of the mutant constructs. Chicken embryo fibroblasts in the mid-log phase of growth were then transfected with 1 μg of each of these plasmids along with 1.0 μg of pSVGA as an internal control, using Lipofectamine as instructed by the manufacturer (Gibco BRL). CAT assays were performed with equal amounts of extracts from these cells, normalized to β-galactosidase activity (11, 15). Transcriptional activity of each of the LTR constructs was quantified by scraping the silica gel corresponding to the

<table>
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<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Binding</th>
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<td></td>
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<tr>
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<td>+++ +</td>
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<tr>
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**TABLE 2.** Mutational analysis of the chkYB-2 binding motif

Single-stranded oligonucleotides were synthesized. The first set includes all oligonucleotides from E4C1 through E4C2 M107. E4C1 is the wild-type sequence corresponding to the region from −103 to −123 on the noncoding strand of the RSV LTR. It contains the 12-mer, 5'-CTGGTACACTC-3' motif (boldface), a consensus sequence arrived at after aligning a panel of RSV LTR oligonucleotides that were described earlier to bind chkYB-2. Oligonucleotides E4C2 M101 to M111, E4C2, E4C3, E4C4, E4C2 AM2, and E4C2 AM3 represent the different mutations that were introduced. Mutant residues are shown with a double underline. The asterisk represents a deletion. + + + binding in this set refers to binding equivalent to that obtained with the wild-type E4C1. Binding of mutants is represented relative to this level. The second set includes X2/E4C1 and the others listed below it. These oligonucleotides differ from the first set in having a tandem repeat of the binding motif. X2/E4C1 is a synthetic construct representing a perfect duplication of the 12-mer, 5'-CTGGTACACTC-3' LTR −106−135 represents the −106 to −135 region on the noncoding strand of the RSV LTR. There is a 5'-GCTACACC-3' motif (underlined) immediately downstream of the octamer, 5'-GCTACACC-3', which could serve as an additional motif for chkYB-2 binding. The oligonucleotides E4M103-1C to -3C and E4M106-1C to -3C incorporate point mutations in either the 5' or the 3' octamer motif or both motifs simultaneously. + + + + binding in this set refers to binding obtained with the wild-type LTR −106−135 oligonucleotide. Binding of mutants is represented relative to this level.
aliquots electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (data not shown). In all cases, a protein of the expected size was obtained. Wild-type and mutant YB-2 proteins were examined for DNA binding activity by carrying out gel shift assays, using a radiolabeled single-stranded response element from the RSV LTR (LTR2106–2135 [Table 2]), shown in this report to be the maximum affinity binding site of chkYB-2.

In chkYB-2, the CSD extends from amino acids (aa) 86 to 155 (Fig. 1B). The first construct that we made was YB-2(D158-222). The internal deletion of 66 aa in the carboxyl-tail domain created a mutant with intact amino-terminal and CSDs. If the CSD is sufficient for DNA binding, this mutant polypeptide would be expected to bind DNA. As seen in Fig. 2 (lane 9), no binding to DNA was observed in an in vitro DNA binding assay. DNA binding was tested under different salt conditions, using up to 2 mg of the protein prepared from several independent clones, as well as after cleavage of the MBP fusion with factor Xa (data not shown). No binding was detected under any of these conditions. There are two possible explanations for the total abrogation of DNA binding upon the deletion of these 66 aa. This 66-aa stretch is immediate to the carboxyl side of the carboxyl boundary (aa 155) of the CSD. Given the proximity to the well-structured CSD, this deletion could have resulted in a major functional deformity in the protein leading to loss of DNA binding. An alternative explanation for the nonfunctionality of this mutant in terms of DNA binding is that the deleted aa 158 to 222, or at least some of them, are part of the minimum DNA binding domain, which in chkYB-2 could extend beyond the strict confines of the conserved residues of the CSD. To map the carboxyl boundary of the DNA binding activity of chkYB-2, we constructed the YB-2 (1-169) and YB-2 (158-298) mutants and assayed their ability to bind DNA. YB-2 (158-298) expresses only the carboxyl-tail domain of YB-2, the entire CSD and amino terminus having been deleted. As seen in Fig. 2 (lane 13), the YB-2 (158-298) mutant did not bind DNA. This result indicated that the carboxyl half of the protein cannot independently bind DNA. Although the residues in the 158–222 region are critical for DNA binding, they of themselves do not confer DNA binding activity in the absence of the CSD.

Mutant YB-2 (1-169) retains the CSD and, unlike YB-2 (Δ158-222), has 14 residues to the immediate carboxyl side of the CSD intact. As seen in Fig. 2 (lane 5), this polypeptide

FIG. 1. (A) Diagram of chkYB-2 mutant constructs. Wild-type (w/t) chkYB-2 (298 aa) is shown schematically. The filled-in region represents the highly conserved CSD. The various amino-terminal and carboxyl-terminal deletion mutants, and one internal deletion mutant (see Materials and Methods), are shown schematically below the wild type. The mutants are named according to the amino acids they retain. The derivative with a D lacks the segment delineated by the numbered amino acids. (B) Predicted 298-aa sequence of chkYB-2 protein. The boxed region extending from aa 86 to 155 is the invariant, nucleic acid binding CSD. Arginine clusters are present in the hydrophilic carboxyl-tail domain, while the amino-terminal region is rich in proline. (C) Protein structure analysis of chkYB-2 carried out with the Genetics Computer Group software package. Plots A and B represent the Kyte-Doolittle hydrophilicity predictions and surface probability (Emini method) predictions, respectively.

FIG. 2. Localization of the domains responsible for DNA binding. chkYB-2 deletion mutants were expressed as MBP fusion proteins in E. coli and partially purified on amylose columns. The ability of these mutant polypeptides to bind DNA was assessed by carrying out gel shift assays as described in Materials and Methods. A single-stranded DNA oligonucleotide (LTR–106–135 [Table 2]), corresponding to the –106 to –135 region of the noncoding strand of the RSV enhancer and bearing a chkYB-2 recognition motif, was end labeled and used as the probe. Proteins in the individual binding reactions are as follows: lane 1, probe alone; lane 2, 100 ng of MBP; lane 3, 100 ng of factor Xa-cleaved MBP–YB-2 protein. MBP fusion proteins were used in all remaining binding reactions. Lane 4, wild-type chkYB-2 (50 ng); lane 5, chkYB-2 (1-169) (200 ng); lane 6, chkYB-2 (1-167) (300 ng); lane 7, chkYB-2 (1-162) (300 ng); lane 8, chkYB-2 (1-155) (300 ng); lane 9, chkYB-2 (Δ158-222) (300 ng); lane 10, chkYB-2 (75-230) (300 ng); lane 11, chkYB-2 (75-298) (300 ng); lane 12, chkYB-2 (121-298) (300 ng); lane 13, chkYB-2 (158-298) (300 ng); lane 14, probe alone.
bound DNA, albeit with a 10-fold-lower affinity than the wild-type YB-2 protein (lanes 3 and 4, containing factor Xa-cleaved and MBP fusion proteins, respectively). The partial restoration of DNA binding activity upon preservation of residues 156 to 169 supports our belief that the domain subserving DNA binding in YB-2 extends beyond the CSD. Construction of mutants with their carboxyl termini at different positions between aa 169 and 298 is currently under way. DNA binding assays with these proteins would help in defining precisely the carboxyl boundary of the polypeptide displaying full restoration of DNA binding activity.

We were curious to know if all of the 14 residues in the short carboxyl tail of YB-2 (1-169) were necessary for DNA binding activity or whether small truncations in this region would be tolerated. Progressive C-terminal deletions of YB-2 (1-169) yielded the mutants YB-2 (1-167) and YB-2 (1-162). Gel shift assays were carried out with these mutant polypeptides to determine their ability to bind DNA. As seen in Fig. 2 (lanes 6 and 7), YB-2 (1-167) bound less avidly than YB-2 (1-169) and YB-2 (1-162) bound even less than YB-2 (1-167), showing that progressive deletions were deleterious to DNA binding. This effect was clearly seen when DNA binding was totally lost with the YB-2 (1-155) mutant (lane 8). The deletion in YB-2 (1-155) removes the entire carboxyl-tail domain of YB-2 up to the carboxyl boundary of the CSD. The total absence of DNA binding with the YB-2 (1-155) protein is similar to the behavior of the YB-2 (Δ158-222) mutant. Taken together, these results indicate that in chkYB-2, the CSD is necessary but not sufficient for DNA binding activity.

Interestingly, in gel shift assays, the DNA-protein complexes formed by the YB-2 (1-169), (1-167), and (1-162) mutants migrated more slowly than the complexes formed by the full-size protein. The YB-2 (1-162) protein in fact forms two complexes, one a faster-migrating complex similar to the wild-type YB-2 protein and the other a slower-migrating complex similar to those formed by the YB-2 (1-169) and (1-167) proteins. This was surprising, considering the fact that on SDS-polyacrylamide gels the migration of these polypeptides was proportional to their molecular weights. This pattern was reproduced even when DNA binding assays were carried out with factor Xa-cleaved protein, ruling out any artifacts introduced by the MBP moiety. The formation of multimeric complexes is a likely explanation for the above observation. Further experiments are, however, required to demonstrate unequivocally if these mutant proteins do indeed exist as multimers, either in solution or upon binding DNA.

We also constructed YB-2 mutants lacking portions of their N termini. The mutant YB-2 (121-298) disrupts the CSD and as expected showed negligible DNA binding ability (Fig. 2, lane 12). The mutant YB-2 (75-298), while carrying a large deletion at its amino terminus, still retains the entire CSD and could be expected to bind DNA. However, this mutant protein also showed minimal ability to bind DNA. A possible explanation for this could be the proximity of the deletion to the CSD. Given the behavior of this mutant, it was not surprising that the double-deletion mutant YB-2 (75-230) demonstrated no ability to bind DNA (Fig. 2, lane 10). We also tested the ability of the YB-2 (1-169) mutant to bind a series of unrelated single-stranded and double-stranded DNA oligonucleotides to which wild-type YB-2 had not bound. No binding was observed (data not shown). We also examined the ability of this mutant to bind the different RSV LTR mutant oligonucleotides shown in Table 2. The relative binding affinity of YB-2 (1-169) to these mutants (data not shown) always paralleled the results obtained with the full-size protein, indicating that although YB-2 (1-169) binds with less affinity than the wild-type YB-2, there is no relaxation in the sequence specificity. The components of the YB-2 protein molecule that are involved in sequence-specific recognition probably reside within the 1-169 region.

In summary, the DNA binding studies with the chkYB-2 mutants described above indicate that the CSD is important for DNA binding and that the carboxyl-terminal charge-zipper domain has no independent ability to bind DNA. The CSD mediates sequence-specific recognition as well as binding to single-stranded DNA. It is also evident that unlike the bacterial cold shock proteins wherein the CSD alone is adequate for DNA binding, the residues that make up the CSD in chkYB-2 are necessary but not sufficient for DNA binding. Apparently, the residues to the carboxyl side of CSD, even if not part of the binding domain, contribute to the generation of stable complexes with DNA, at least in vitro.

Some Y-box proteins are known to bind DNA more avidly in the presence of magnesium (18). Magnesium also appears to play a role in the nucleic acid interactions of several other RNA binding proteins (27). We carried out gel shift assays to examine the effects of different concentrations (0 to 20 mM) of several divalent cations (Mg²⁺, Ca²⁺, Mn²⁺, and Zn²⁺), as well as spermidine, a polyvalent cation. The results of these gel shift assays are shown in Fig. 3. Addition of magnesium chloride to final concentrations of 3 to 10 mM in the binding reaction increased DNA binding more than 10-fold, with maximum effect seen at 5 mM; 20 mM MgCl₂, however, had an inhibitory effect. A similar effect was noted with spermidine. While 3 or 5 mM CaCl₂ stimulated binding severalfold, concentrations of 10 mM and above were inhibitory. MnCl₂ at 3 and 5 mM promoted binding, although less than for the other ions. As found for CaCl₂, MnCl₂ concentrations of 10 mM or more were inhibitory. In contrast to the stimulatory effects of these cations, the addition of even 3 mM ZnCl₂ was inhibitory to the formation of DNA-protein complexes, with higher ionic strengths essentially eliminating binding. Figure 3 (lane 2) shows the binding of 6 ng of chkYB-2 protein to the radiolabeled LTR oligonucleotide −106/−135, in the absence of any divalent cation. Lanes 3 and 4 show the remarkable increase in DNA binding upon the addition of 5 mM MgCl₂ or spermidine, respectively. Lanes 6 and 7 show the stimulation of
binding in the presence of 3 mM CaCl$_2$ and 3 mM MnCl$_2$, respectively. ZnCl$_2$ at 3 mM inhibited binding (lane 5). This inhibition was, however, neutralized upon the addition of either 3 mM MgCl$_2$, (lane 9) or 3 mM each MgCl$_2$ and spermidine (lane 10) to the reaction.

The exact significance of the effect of a cationic environment on chkYB-2-DNA interactions is not known. We are not aware of any specific metal ion binding motifs on the chkYB-2 protein. We were curious to know if these results could be reproduced with any of the YB-2 mutants that we have made. We tested the DNA binding activity of the YB-2 (1-169) mutant protein either in the absence of cations or in the presence of MgCl$_2$, spermidine, or CaCl$_2$. As shown in Fig. 4, the addition of 5 mM MgCl$_2$ (lane 3), 5 mM spermidine (lane 4), or 3 mM CaCl$_2$ (lane 5) significantly promoted DNA binding compared to DNA binding carried out in the absence of any of these ions (lane 2). Lanes 2 to 5 contained 10 ng of the protein. The same effect was repeated when 50 ng of the protein was used in each binding reaction (lanes 7 to 10).

Effects of point mutations in the core binding site for chkYB-2

We have shown earlier that the E4 region in the RSV LTR is important for maximal enhancer activity (40). We also reported that the recognition motif for chkYB-2, the octamer 5'-GTACCACC-3', is located in this region. Also, transfection experiments using E4-deleted LTR constructs and chkYB-2 antisense oligonucleotides had demonstrated that the ability of chkYB-2 to act as an activator was mediated primarily through this octanucleotide motif. Our earlier work had shown that this protein bound with various affinities several different single-stranded oligonucleotides spanning the RSV LTR. We aligned the sequences of all these oligonucleotides to which chkYB-2 had bound and looked for a consensus sequence. This comparison revealed that the 12-mer 5'-TCGTACCCCTT-3' is the common motif. This is essentially the previously described octamer 5'-GTACCACC-3' extended by two nucleotides each in the 5' and 3' directions.

The 21-mer oligonucleotide E4C1, bearing this motif and corresponding to the region from −103 to −123 on the non-coding strand of the RSV LTR, was hence used as the wild-type binding motif, and systematic point mutations spanning the entire motif were introduced (Table 2). End-labeled oligonucleotides, adjusted for specific activity, were then used in gel shift assays. A summary of the binding results is presented in Table 2. The gel shift assay shown in Fig. 5 is representative of some of the oligonucleotides used. It is evident from these results that the binding of YB-2 to its recognition motif was abolished upon the introduction of any mutation in the core octamer, except for the mutant oligonucleotide E4C2 M101, where replacement of G with a C at position 3 appeared to be well tolerated. The other exception was the mutant E4C2 M106, where replacement of C with a T at position 9 did not affect binding. However, when the adjacent C was also replaced by a T, to yield the double mutant E4C2 M111, binding was abolished. Nucleotides at positions 11 and 12 did not appear to be critical, as shown by binding equivalent to wild-type binding by the mutant E4C2 M107. Binding to oligonucleotides with mutations at positions 1 and 2 (E4C2 M108 and E4C2 M109, respectively) was significantly less than binding to E4C1. These results indicate that the single-stranded DNA binding protein chkYB-2 binds its ligand in a sequence-specific manner and that maximum binding affinity requires the presence of at least the 5'-TCGTACCCACC-3' decamer motif.

In our earlier report (40), we had remarked on the ability of chkYB-2 to bind more than one site on the RSV LTR and had suggested that the appearance of multiple, slower-migrating complexes in gel shift assays carried out with the full LTR as the probe was probably due to occupancy of the other sites by additional molecules. To confirm this effect directly, we de-
signed the 30-mer oligonucleotide X2/E4C1 (Table 2), which is a direct repeat of the YB-2 binding motif. As shown in the gel shift assay (Fig. 5, lane 7), chkYB-2 bound avidly to this oligonucleotide, forming two DNA-protein complexes. The slower-migrating complex is a minor component and probably represents more than one molecule of YB-2 complexed to DNA.

An examination of the sequence of the noncoding strand of the RSV LTR, immediately to the 3′ side of the octamer motif 5′-GTACCACC-3′ (−112 to −119), revealed the presence of an almost identical 5′-CTACCACC-3′ (−123 to −130) motif. Also, the gel shift assays described above had shown that the replacement of the nucleotide G in the motif with a C, as in the mutant E4C2 M101, did not decrease the affinity of chkYB-2 binding (Table 2). Hence, the −112 to −130 region of the RSV LTR can be viewed as providing two potential sites for high-affinity binding by chkYB-2. To examine the affinity of chkYB-2 to DNA bearing such a double motif, we synthesized the oligonucleotide LTR (−GTACCACC-3′)(Table 2), which is a direct repeat of the YB-2 binding motif. As shown in the gel shift assay (Fig. 5, lane 7), chkYB-2 bound avidly to this oligonucleotide, forming two DNA-protein complexes. The slower-migrating complex is a minor component and probably represents more than one molecule of YB-2 complexed to DNA.

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The experiments described thus far helped in defining the requirements of the chkYB-2 binding motif. However, the exact relevance of results obtained from in vitro DNA binding assays to chkYB-2 interactions with the RSV LTR in vivo had to be determined. One approach was to correlate the mutations that abrogated protein binding in vitro with alterations in the in vivo transactivating potential of RSV LTR constructs carrying the same mutations in the YB-2 binding motif. Toward this end, we constructed a series of mutant RSV LTR reporter vectors. Mutations were made only within the −112 to −130 region described earlier as the site of two motifs for chkYB-2 binding. Furthermore, the point mutations introduced in these six constructs reflect exactly the changes made in the wild type.

Chicken embryo fibroblasts were chosen for the transfection experiments, as chkYB-2 is expressed abundantly in these cells and has been demonstrated to activate RSV LTR-driven transcription in these cells, primarily through its interaction with recognition motifs present within the −112 to −130 region. Cells in the mid-log phase of growth were transfected with 1 μg of each of these plasmids, along with 1 μg of the internal control plasmid, pSVGal. CAT assays were performed with protein extracts normalized to β-galactosidase activity. A representative autoradiogram (Fig. 6b) and the average results of three identical experiments (Fig. 6c) are presented. These results reveal that point mutations in either the 5’ or 3’ motif (M103-1CAT or M103-2CAT, respectively) reduced the transcriptional activity of the RSV LTR by about 20 to 25%. The decrease in transcriptional activity upon the introduction of point mutations in the two separate motifs is synergistic, as evidenced by the much greater reduction in transcriptional activity of the double mutant (M103-3CAT) compared with either single mutant alone. The activity of this double mutant (mutations are at positions −114 and −125) was, however, much higher than that observed with E4 Del CAT, an RSV LTR construct carrying a 14-nucleotide deletion (−114 to −127) that encompasses both chkYB-2 binding motifs. A possible interpretation of this finding is that although in vitro chkYB-2 bound negligibly to this double mutant (M103-3C [Fig. 6a]), in vivo, low-affinity interactions with this mutant YB-2 motif probably occur and contribute to transactivation of the LTR, albeit less efficiently. Even these low-affinity interactions apparently cannot take place when both motifs are completely deleted, as in E4 Del CAT. Alternatively, this result could reflect the fact that protein-DNA interactions, other than those mediated by chkYB-2, contribute to transactivation from this deleted region. Figure 6c also shows that compared to the M103-CAT constructs, the M106-CAT constructs did not show significant reductions in transcriptional activity, with even the double mutant M106-CAT displaying transcriptional activity comparable to wild-type RSV LTR.

In summary, the results presented above show a correlation between the transcriptional activities of RSV LTR constructs carrying point mutations in the chkYB-2 binding motifs and the relative affinities of the corresponding mutant oligonucleotides as assayed by DNA-protein complex formation in vivo. Additionally, our results also show that (i) both of the chkYB-2 recognition motifs contribute to RSV LTR-driven transcription and (ii) the interaction of chkYB-2 with these adjacent motifs is probably just additive and not cooperative in nature, since the reduction in transcription observed from the double mutant (M103-3CAT) was no more than the cumulative reduction in transcriptional activities of the single mutants. These results are also in agreement with our interpretation of the chkYB-2 binding assays with LTR −106/−135, which suggested that the −112 to −130 region is best viewed as providing a single high-affinity binding site for chkYB-2, with each octamer motif behaving as a half-site.

DISCUSSION

chkYB-2 was originally isolated by screening a chicken embryo fibroblast cDNA expression library by using a probe corresponding to the U3 enhancer region of the RSV LTR (7). We have since demonstrated that purified, recombinant chkYB-2 specifically recognizes the 5’-GTACCACC-3’ single-stranded motif on the noncoding strand of the RSV enhancer and acts as an activator of RSV LTR-driven transcription in avian fibroblasts (40). An understanding of the mechanism by which a factor like chkYB-2, which binds only single-stranded DNA templates yet acts as a transcriptional activator, would be facilitated by studies delineating the functional domains in the protein involved in DNA binding, multimerization, and transactivation. As a first step toward this, we report here the DNA binding properties of bacterially expressed and partially purified, recombinant chkYB-2 mutant polypeptides to a single-stranded response element (−106 to −135) of the RSV enhancer.

The specific DNA binding activities of several transcription factors have been localized to relatively small domains consisting of 60 to 100 aa. chkYB-2, an avian Y-box protein, is a 298-aa polypeptide. Like other Y-box transcription factors, chkYB-2 is characterized by the presence of the invariant CSD located in an intermediate position and flanked by variable amino and carboxyl-terminal domains. Wistow (46) initially proposed that the CSD is a structural motif involved in protein-nucleic acid interactions. The CSD is 43% identical to CS7.4, the 70-aa major cold shock protein of E. coli (14). Unlike the Y-box proteins, the E. coli cold shock proteins do not have the additional tail domain. CS7.4, nevertheless, binds DNA in a sequence-specific manner, suggesting that this domain is adequate for DNA binding. Studies on CspB (38), a cold shock protein of Bacillus subtilis, revealed the three-dimensional structure of its nucleic acid binding domain, and suggested that the structural organization of the CSD was best suited for interaction with single-stranded nucleic acids.

Mutagenesis analysis of Xenopus Y-box proteins had shown the CSD to be the primary domain responsible for both DNA and RNA binding (43). However, there are few data on the domain analysis of other eukaryotic Y-box proteins. Notwithstanding the strong conservation of the CSD, a remarkable feature of the Y-box proteins characterized thus far has been their ability to bind diverse double- and single-stranded DNA sequences, as well as RNA. It is hence likely that although the highly conserved amino acids in the CSD confer a structural framework for DNA binding, the determinants of binding specificity reside in the variable amino and carboxyl regions of the protein. The highly hydrophilic, carboxyl-tail domain (Fig. 1B) is a more likely candidate because, despite the divergence in primary amino acid sequence (e.g., only 50% identity between chkYB-2 and chkYB-1b), the organization of the residues into alternating clusters of acidic and basic residues to create a charge-zipper motif is a feature that is conserved among all Y-box proteins.

Similarities between classic Y-box proteins and chkYB-2 predicted that DNA binding would be mediated by CSD. However, a mutant chkYB-2 protein, lacking a 60-aa region in the carboxyl-tail domain, was found incapable of binding DNA. It therefore became interesting to determine the potential contribution of the carboxyl domain for interactions with DNA. The data presented in this study provide evidence that even in...
chkYB-2, the CSD is indeed indispensable but not sufficient for DNA binding. While low-affinity, site-specific binding is obtainable with carboxyl-domain truncation mutants that have an intact CSD and as few as 10 to 14 adjacent residues of the carboxyl tail, high-affinity binding requires that apart from the CSD, larger segments of the carboxyl domain remain intact in the protein. Tafuri and Wolfe (43) had reported that progressive deletion of the carboxyl terminus of FRGY2, a *Xenopus* Y-box protein, resulted in a reduction in the number of complexes formed with DNA. Binding to DNA, however, remained specific, even where the entire carboxyl-tail domain was removed. Removal of the CSD, however, led to a loss of specific DNA binding. They obtained similar results with the closely related FRGY1 protein and concluded that the CSD was essential for specific DNA binding, whereas the hydrophilic carboxyl-tail domain facilitated the formation of multiple protein-DNA complexes. It was also demonstrated that in both FRGY1 and FRGY2, the CSD was adequate for stimulation of transcription, both in vitro and in vivo (35, 42, 43).

In chkYB-2, the CSD extends from aa 86 to 155. However, we detected no DNA binding with the mutant chkYB-2 (1-155), which has an intact CSD but no carboxyl tail. Low-affinity DNA binding ability returned incrementally upon the progressive lengthening of the carboxyl tail, as evidenced by the complexes formed by the chkYB-2 mutants (1-162), (1-167), and (1-169). Significantly, we found no evidence of a relaxation in site specificity when the chkYB-2 (1-169) mutant was tested for its ability to bind several unrelated oligonucleotides, both single-stranded and double-stranded. While the lack of binding of the YB-2 (1-155) mutant could be explained by the proximity of the truncation to the carboxyl boundary of the CSD, the fact that even the chkYB-2 (1-169) mutant bound with only a 10-fold-lower affinity than the wild type suggests that the residues in the carboxyl domain beyond the CSD either are part of an extended DNA binding domain or contribute indirectly to the ability of chkYB-2 protein to complex with DNA. This possibility is further strengthened by our finding that the strong amino acid sequence conservation between eukaryotic Y-box proteins was not confined to the 70-aa CSD alone. A comparison of the sequence of the carboxyl-tail domain of chkYB-2 with those of other vertebrate Y-box proteins revealed an additional 32 aa conserved region contiguous with the carboxyl boundary of the classic CSD. Except for a single amino acid change, the aa 156–187 region showed 100% identity to human dbpA and greater than 70% identity to the *Xenopus*, chicken, mouse, and human YB-1 proteins.

The basic islands in the carboxyl-tail domain contain arginine clusters, which are frequently found in several RNA binding proteins and are thought to increase the potential for nucleic acid binding (5). We have recently demonstrated that chkYB-2 binds single-stranded RNA in a sequence-specific manner and that YB-2 mutants [including YB-2 (1-169)] that lack the carboxyl tail fail to bind RNA (unpublished results). These results are in agreement with the recent report by Bouvet et al. (4) which shows that RNA binding by FRGY2 is facilitated by both the amino- and carboxyl-terminal regions flanking the CSD, indicating contributions from regions beyond the CSD for optimal nucleic acid interactions.

The chkYB-2 (158-298) mutant did not bind DNA, showing that the carboxyl-tail domain had no independent ability to bind DNA. This is similar to the results obtained by Murray (30), who found that recombinant FRGY2 proteins expressing only the carboxyl-tail domain were incapable of binding DNA. In contrast to FRGY2, wherein the presence of the carboxyl-tail domain facilitated the formation of multiple protein-DNA complexes (43), we found that chkYB-2 mutants lacking the carboxyl-tail domain appeared to multimerize upon DNA binding.

The ability of Y-box factors to bind both single- and double-stranded DNA distinguishes them from other transcription factors. Impressive evidence implicating Y-box proteins in transcriptional regulation has accumulated in recent years. Reports of Y-box factors transactivating from viral promoters include the role of EF1A (12, 17) and chkYB-2 (40) in RSV LTR transcription and that of YB-1 in stimulating transcription from the human T-cell lymphotropic virus type I, human immunodeficiency virus, and JCV virus promoters (22, 24). Y-box factors are also involved in the regulation of cellular genes. For example, YB-1 represses transcription of major histocompatibility complex class II genes (44) and acts as an activator of the *MDR1* gene (I), and FRGY2 promotes transcription from the *Xenopus* hsp70 promoter (43).

Our earlier experiments have shown that chkYB-2 binds the single-stranded motif 5’-GTACCCACC-3’ on the noncoding strand of the RSV enhancer and promotes RSV LTR-driven transcription. In this study, we present the results of systematic mutational analysis of this motif. Gel shift assays using a series of oligonucleotides carrying point mutations spanning the entire 5’-GTACCCACC-3’ motif revealed that almost every nucleotide in the octamer was absolutely essential for high-affinity binding. A closer examination of the RSV LTR sequence showed that this octanucleotide motif (5’-[G/C]TACCCACC-3’) was in fact present as a tandem repeat in the −112 to −130 region. DNA binding assays carried out with a single-stranded oligonucleotide spanning this region showed a severalfold augmentation of DNA binding compared to oligonucleotides bearing a single motif. Also, the nature of complexes formed on gel shift assays suggested that the repeats behave more like two half-sites rather than two independent binding motifs. The importance of this region for chkYB-2 binding was further confirmed by introducing point mutations in the two half-sites in RSV LTR reporter constructs. Our results clearly demonstrate that mutations that decreased binding affinity in vitro also led to a decrease in the transcriptional activity of the corresponding mutant RSV LTR constructs.

chkYB-2 binds single- but not double-stranded DNA. The *B. subtilis* Y-box protein CspB has been shown to be capable of binding single-stranded but not double-stranded DNA (38). Also, NSEP-1 binds pyrimidine-rich single-stranded DNA (26), and YB-1 binds single-strand motifs with greater affinity than double-strand ones (29, 33). Apart from the Y-box proteins, the characterization of several other eukaryotic single-stranded DNA binding proteins has been reported (37, 41, 45). Other members of this growing family include the single-strand binding protein that complexes with the noncoding strand of the TSH receptor gene promoter and stimulates transcription (32), a pyrimidine single-strand-specific protein (ssPyrBF) that interacts with the androgen receptor gene promoter (6), and FUSE-binding protein, a single-stranded DNA binding protein whose role in transcription regulation of the c-myc gene has been well documented (10).

Experiments carried out to determine the effect of magnesium on DNA binding revealed the remarkable increase in affinity upon the addition of 3 to 5 mM Mg\(^{2+}\). Enhancement of DNA binding upon the addition of MgCl\(_2\) has been reported for two closely related Y-box proteins, human dbpA and dbpB (18). The effect of magnesium on FRGY2 binding to RNA has also been described (27). While the authors found Mg\(^{2+}\) to interfere with binding by the CSD over a range of 1 to 5 mM, it appeared to favor binding by the tail domains. Unr is a recently described DNA and RNA binding protein that is char-
acterized by the presence of a fivefold repeat of the CSD but with no tail domains (20). Interestingly, the interaction of Unr with either DNA or RNA was very sensitive to even low concentrations of magnesium. Addition of Mg$^{2+}$ markedly decreased the affinity of binding, which was in contrast to the results we observed with chkYB-2. In fact, even chkYB-2 (1-169), a mutant with a large truncation in the tail domain, demonstrated increased DNA binding in the presence of Mg$^{2+}$.

Magnesium is the most abundant intracellular divalent cation and is known to be required for the activity of several DNA repair enzymes. A recent report (13) that describes the effect of Mg$^{2+}$ on protein binding and structural transitions in a retroviral promoter, however, suggests that Mg$^{2+}$ may also be physiologically relevant for the optimum activity of single-stranded DNA binding transactivators. The authors reported that in the presence of Mg$^{2+}$, the binding of a nuclear factor to a polypurine/polypyrimidine DNA sequence element (NRE1) in the mouse mammary tumor virusLTR led to the appearance of single-stranded regions upstream. Also, factor binding to single-stranded DNA was facilitated by the presence of Mg$^{2+}$. As the authors pointed out, in order for single-strand binding proteins to successfully bind and regulate transcription, there needs to be a mechanism that will expose single-stranded regions in the promoters of genes. Binding of a protein factor(s) that induces Mg$^{2+}$-dependent structural transitions that allows a second, Mg$^{2+}$-dependent single-stranded DNA binding factor(s) to make contact with the exposed single strand is a possible solution to this problem. chkYB-2 binds selectively to the pyrimidine-rich strand of the −112 to −130 region of the RSV LTR. The only other region (−142 to −161) in the LTR exhibiting a greater degree of purine/pyrimidine strand asymmetry is located about 10 bp upstream. Regions of strong purine/pyrimidine strand asymmetry that can assume an H-DNA conformation have been identified in the promoter elements of the c-myc and γ-globin genes (19, 25). The pyrimidine-rich single strand is apparently accessible for interactions with proteins in these H-DNA regions. YB-1 has been shown to induce or stabilize single-stranded regions in a major histocompatibility complex class II gene promoter (29), and FUSE-binding protein has been reported to induce targeted melting of c-myc promoter prior to binding its single-strand recognition element on the noncoding strand (3). The regions of strand asymmetry in the RSV LTR may be too short to form H-DNA. However, localized melting of double-stranded DNA could expose the CT-rich strand for chkYB-2 binding.

In summary, we have characterized the functional domains in chkYB-2 responsible for its DNA binding activity, examined the effects, both in vitro and in vivo, of mutations in its binding motif, and discussed the possible relevance of Mg$^{2+}$-dependent binding for transcriptional activation by a sequence-specific, single-stranded DNA binding protein.

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