Chicken Y-box proteins chk-YB-1b and chk-YB-2 repress translation by sequence-specific interaction with single-stranded RNA

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Y-Box proteins comprise a large family of multifunctional proteins with a wide spectrum of activities in both transcription and translational regulation of gene expression. Earlier, we have reported on the involvement of chk-YB-2 in transcriptional regulation of Rous sarcoma virus long terminal repeats and the involvement of chk-YB-1b in transcriptional regulation of alpha1(I) collagen genes. Here, we have investigated the potential role of chk-YB-2 and chk-YB-1b in RNA metabolism. We report that chk-YB-2 and chk-YB-1b are localized predominantly in the cytoplasm and that they both can bind single-stranded RNA in a sequence-specific and reversible manner. Well-conserved cold-

shock domain, N-terminal proline-rich domain and the alternating clusters of acidic and basic amino acids located in the C-terminal ends of these two proteins were all found to be necessary for their RNA-binding ability. Further, we demonstrate that these two proteins inhibit translation *in vitro* and that binding to RNA is required for this inhibition. The significance of these results is discussed.

Key words: cold-shock domain, RNA-binding proteins, Rous sarcoma virus.

INTRODUCTION

Several proteins are known to bind RNA in a non-specific and/or highly sequence-specific manner and influence properties such as stability and availability of RNA for translation in the cytoplasm [1–7]. These RNA-binding proteins belong to several different multigene families. Among them, Y-box proteins have emerged recently as an important family of multifunctional proteins. They are involved in regulation of transcription of a number of genes in several systems [8] and also in the regulation of stability and translatability of specific transcripts [1,6,9–16]. Y-Box proteins have been shown to be predominantly cytoplasmic and are usually complexed with different mRNA molecules as 50–60 S messenger ribonucleoprotein (mRNP) particles [16]. Repression of translation seems to be the most common effect of over-expression of Y-box proteins in the cytoplasm [1,17,18]. In one report, however, Y-box proteins have been shown to be necessary for translation [19]. Recently, a Y-box protein called *unr*, which contains five repeats of cold-shock domain (CSD), was shown to be required for internal initiation of translation of human rhinovirus RNA [20]. In *Xenopus* oocytes, spermatocytes and early embryos, two Y-box proteins (FRGY1 and FRGY2) have been demonstrated to bind specific mRNA sequences and mask their translation [21]. However, these RNAs are released during the development of the embryo in a regulated manner in a later stage when there is a need for these proteins but active transcription has not yet begun [22]. A mouse Y-box protein, MSY-1, is known to be associated with paternal RNA in spermatocytes [23].

Sequence comparison of RNA-binding proteins has revealed well-conserved domains that are required for RNA binding. The X-ray crystal structures of some of these domains have been

worked out [24]. One such domain is the RNP1 motif, a relatively short stretch of about eight amino acids buried in the middle of the well-conserved CSD found in Y-box proteins [9,25]. Other motifs which can confer RNA-binding ability are (i) the arginine-rich motif found in the HIV Rev and Tat proteins and (ii) the RGG box, closely spaced repeats of arginine and glycine interspersed with aromatic residues. Interestingly, most Y-box proteins also have these motifs. In addition to an RNP1 motif within the N-terminal CSD, they contain alternating clusters of acidic and basic amino acids at their C-terminal ends. The relative contributions of these different domains to RNA-binding ability of many different Y-box proteins is less understood. Only in the case of *Xenopus* Y-box protein FRGY-2 it is proposed that the C-terminal charge zippers confer non-specific RNA binding while the CSD confers sequence specificity [13,26].

Previously, we have shown that an avian Y-box protein chk-YB-2 is important for transcription from the highly potent promoter of Rous sarcoma virus (RSV) long-terminal repeats (LTR) in avian cells [27,28]. In addition, we characterized the DNA-binding domain of chk-YB-2 [29]. We also have presented evidence showing that another Y-box protein, chk-YB-1b, is involved in regulating the transcription of type-I collagen genes in several species [30,31]. Apart from the RNP1 motif found in the middle of the CSD, chk-YB-1b and chk-YB-2 also have well-conserved arginine-rich motifs and the RGG repeats in the C-terminal charge zipper region [27,30], suggesting that these two proteins may also bind to RNA. In this report, we show that (i) chk-YB-1b and chk-YB-2 are localized predominantly in the cytoplasm of chicken embryo fibroblasts (CEFs); (ii) chk-YB-1b and chk-YB-2 are capable of binding to single-stranded RNA oligonucleotides in a sequence-specific and reversible manner; (iii) both CSD and the C-terminal charge zippers are required for

Abbreviations used: RSV, Rous sarcoma virus; LTR, long terminal repeats; CSD, cold-shock domain; mRNP, messenger ribonucleoprotein; CEF, chicken embryo fibroblast; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; TNT, coupled transcription and translation; MBP, maltose-binding protein.

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this RNA-binding activity of chk-YB-2; and (iv) binding inhibits translation of the bound RNA.

MATERIALS AND METHODS

Cell culture, preparation of proteins and electrophoretic mobility shift assays (EMSAs)

CEF cells or RSV-infected CEF cells were grown as described previously [28]. Nuclear extracts and cytoplasmic extracts were prepared as described [32]. Each of these extracts (15 µg) was used in EMSAs. Fusion proteins maltose-binding protein (MBP)-chk-YB-1b or MBP-chk-YB-2, or their deletion mutants, were expressed in *Escherichia coli* and partially purified using amylose columns as described in [28]. These proteins were run on SDS/PAGE gels to confirm their size.

The sequences of oligonucleotides used in this study are presented in Table 1. All the oligonucleotides used were phosphodiester oligonucleotides with no modifications. Double-stranded oligonucleotides were prepared in the following way. Equimolar amounts of the constituent single-stranded oligonucleotide were mixed in 300 mM NaCl/10 mM Tris/1.0 mM EDTA (pH 8.0). This mix was heated to 95 °C and cooled down gradually to room temperature. The annealing mix was then run on a 6% acrylamide preparative gel in TBE buffer (45 mM Tris/borate/1 mM EDTA) and the double-stranded oligonucleotides were eluted from the gel. Oligodeoxyribonucleotides and oligoribonucleotides (Table 1) were obtained from commercial sources (Oligos, etc., Wilsonville, OR, U.S.A.) and radiolabelled on their 5' termini using polynucleotide kinase. EMSAs were performed as described previously [28]. Briefly, bacterially expressed proteins or nuclear extracts were incubated with approximately 50000 c.p.m. (≈ 0.5 ng) of DNA or RNA oligonucleotides in a buffer containing 20 mM Hepes (pH 7.9), 3 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, 2 µg of poly(dI-dC) and 10% glycerol for 20 min and then run on 6% polyacrylamide gels in Tris/glycine/EDTA buffer to separate the bound complex from the free oligonucleotides. Gels were dried and exposed to X-ray film for about 18 h. Competition assays were performed by incubating the proteins with the cold competitor oligonucleotides for 15 min before the addition of radiolabelled probe.

Polyclonal antibodies against chk-YB-1b and chk-YB-2 were raised in mice, using bacterially expressed chk-YB-1b or chk-YB-2, which were purified as before [28]. We have tested them and found that anti-chk-YB-1b and anti-chk-YB-2 antibodies do not cross-react with each other (S. K. Swamynathan and R. V. Guntaka, unpublished work). Super-shift assays were performed by incubating the proteins with respective antibodies for 15 min at room temperature before addition of the radiolabelled probe.

Table 1 Sequences of different oligonucleotides used

Oligonucleotide	Sequence
E4C-1 DNA	5'-GGCAGCATGCTACCACTTAC-3'
E4C-1 RNA	5'-GGCAGCAUGCUACCAUUAAC-3'
E4C-1-Mut RNA	5'-GGCAGCAUGCGACCGCUUAC-3'
E4-1 RNA	5'-GUAAGGUGGUACGAUCGUGGC-3'
E4-1 RNA-E4C-1 DNA heteroduplex	5'-GUAAGGUGGUACGAUCGUGGC-3'
	3'-CATTCCACCATGCTAGCACGG-5'
E4-1 DNA-E4C-1 RNA heteroduplex	5'-GTAAGGTGGTACGATCGTGCC-3'
	3'-CAUUCACCAUGCUAGCAGCG-5'
RSV Ψ RNA	5'-ACGACUGAGCAGUCCACCCAGGCGUAAU-3'

Construction of green fluorescent protein (GFP)-Y-box-protein fusion proteins and transfection

Vectors expressing fusion proteins of chk-YB-1b and chk-YB-2 with GFP under the influence of the RSV LTR promoter/enhancer were constructed following standard methods [33]. They were constructed such that the coding sequences of chk-YB-1 or chk-YB-2 were inserted in-frame, in the *Bsr*GI site at the 3'-end of the *EGFP* (enhanced GFP) gene (the plasmid pEGFP containing *EGFP* was from Clontech, Palo Alto, CA, U.S.A.). Equal amounts (1.0 µg) of these plasmid DNAs were transfected into CEFs in the log phase of growth using lipofectAMINE, following the protocol suggested by the manufacturers (Gibco-BRL, Grand Island, NY, U.S.A.), as described previously [28]. After transfection (24 h), cells were observed under a fluorescence microscope and photographed.

Coupled transcription and translation (TNT) reactions

Translation reactions *in vitro* coupled to T7 polymerase-mediated transcription reactions (TNT reactions) were performed using the rabbit reticulocyte lysate TNT kit following the protocol suggested by the manufacturer (Promega, Madison, WI, U.S.A.). The plasmid expressing p19 Gag protein of RSV under the influence of T7 promoter was constructed by directional cloning of the PCR-amplified fragment of RSV genome encompassing nucleotides 1–844 in pBSSK+. TNT reactions were performed using ³⁵S-methionine, following the manufacturer's protocol in a 25-µl volume. Fusion proteins MBP-chk-YB-1b or MBP-chk-YB-2 were included in the reactions at the concentrations indicated in the Figure legends. Following incubation for 2 h at 30 °C, the reactions were stopped by boiling in 1×SDS/PAGE sample buffer and the reaction products were separated on SDS/PAGE. The gels were dried and exposed to X-ray film for 20 h.

RESULTS

Both chk-YB-1b and chk-YB-2 bind RNA in a sequence-specific manner

Earlier, we studied in detail the interaction of chk-YB-1b and chk-YB-2 with single-stranded E4C-1 DNA having the sequence 5'-GTACCACC-3'. Here, we report the results of our experiments with chk-YB-1b and chk-YB-2 proteins for their ability to interact with single-stranded RNA oligoribonucleotides in a sequence-specific manner. We performed EMSAs with ³²P-labelled E4C-1 RNA and chk-YB-2 or chk-YB-1b, as described in the Materials and methods section. The sequence of E4C-1 RNA is shown in Table 1. This sequence is the same as that of E4C-1 DNA on the negative strand of RSV LTR (to which we have previously demonstrated a high-affinity binding of both chk-YB-2 and chk-YB-1b), except that ribonucleotides were used. We found that chk-YB-2 bound E4C-1 RNA with high affinity in a Mg²⁺-dependent manner (Figure 1, top panel). Inclusion of 3 mM Mg²⁺ stimulated RNA binding by chk-YB-2. There was no further stimulation or inhibition in binding upon increasing the Mg²⁺ concentration up to 10 mM in the binding reaction (Figure 1, top panel). We obtained similar results with chk-YB-1b also (results not shown).

chk-YB-2 and chk-YB-1b bind to RSV RNA within the leader region

RSV is a simple retrovirus which expresses Gag, Pol, Env and Src proteins. As the relevance of the above-demonstrated binding of chk-YB-1b and chk-YB-2 to E4C-1 RNA (which represents the

Figure 1 chk-YB-2 binds E4C-1 DNA, E4C-1 RNA and RSV Ψ RNA with equal affinity, in a Mg²⁺-dependent manner

(Top panel) Equal amounts (10 ng) of bacterially expressed chk-YB-2 were incubated with E4C-1 RNA in the absence or presence of increasing amounts of Mg²⁺ in the reaction, and EMSAs were performed as described. Note that bound complex formation is extremely dependent on the presence of Mg²⁺ in the reaction. Similar results were obtained with chk-YB-1b (results not shown). (Middle panel) Increasing amounts of bacterially expressed MBP-chk-YB-2 fusion protein were incubated with E4C-1 DNA, E4C-1 RNA or RSV Ψ RNA labelled to the same specific activity as described in the Materials and methods section. Note that the intensity of the bound complex is roughly the same in the three different kinds of probe, at any given concentration of chk-YB-2. (Bottom panel) Increasing amounts of unlabelled E4C-1 DNA, E4C-1 RNA or RSV Ψ RNA as shown were used to compete with labelled E4C-1 DNA for binding

Figure 2 Sequence specificity of interaction of chk-YB-2 with E4C-1 RNA

Similar amounts of chk-YB-2 were incubated with (reading left to right on the Figure) single-stranded E4C-1 RNA, single-stranded E4-1 RNA, double-stranded E4-1/E4C-1 RNA, double-stranded heteroduplex E4-1 DNA/E4C-1 RNA or double-stranded heteroduplex E4-1 RNA/E4C-1 DNA and the EMSAs were performed as described. Note that only single-stranded E4C-1 RNA was bound well by chk-YB-2. See Table 1 for the sequences of oligonucleotides used.

sequence on the non-coding strand of RSV LTR) is questionable, a search was made for similar sequence motifs in the RSV genomic RNA that would serve as a potential binding site for chk-YB-1b and chk-YB-2. We found that a site present within the RSV leader RNA (nucleotide positions 328–357, see Table 1) closely resembled the canonical single-stranded DNA sequence 5'-GTACCACC-3' present in the RSV LTR, with which both chk-YB-1b and chk-YB-2 interact [27–30]. We synthesized an oligoribonucleotide RSV Ψ RNA representing the sequence of RSV leader RNA, between nucleotides 328 and 357 and used in EMSAs with chk-YB-1b or chk-YB-2. We carried out EMSAs in which increasing amounts of chk-YB-2 were incubated with E4C-1 DNA, E4C-1 RNA or RSV Ψ RNA, which were labelled to the same specific activity (see Table 1 for sequences). As shown, chk-YB-2 bound these three different oligonucleotides with equal affinity (Figure 1, middle panel).

These results were confirmed further by competition experiments. Non-labelled E4C-1 DNA, E4C-1 RNA or RSV Ψ RNA oligonucleotides competed with equal efficiency with the labelled E4C-1 DNA oligonucleotides for binding to chk-YB-2 (Figure 1, bottom panel). Similar results were obtained with chk-YB-1b (results not shown).

In order to find out if this interaction of chk-YB-2 and/or chk-YB-1b with RNA is sequence-specific, we investigated if chk-YB-2 can interact with the purine-rich RNA sequence complementary to E4C-1 (5'-GGTGGTAC-3') or the double-stranded RNA sequence representing this region. As shown in Figure 2, chk-YB-2 binds only to the pyrimidine-rich single-stranded E4C-1 RNA and not the purine-rich complementary strand E4-1 RNA or the double-stranded RNA E4-1/E4C-1. Since the genome of RSV goes through a stage of RNA–DNA duplex in the host cell during its life cycle, it was important to test if chk-YB-2 is capable of binding different RNA–DNA heteroduplexes which, if found, will be of potential significance. However, we found that chk-YB-2 bound RNA–DNA heteroduplexes with very low

the same amount of chk-YB-2 (50 ng). Lane 1, free (unbound) E4C-1 DNA; lane 2, E4C-1 DNA bound with 50 ng of chk-YB-2. As can be seen, E4C-1 DNA, E4C-1 RNA and RSV Ψ RNA competed equally efficiently, indicating that the affinity of chk-YB-2 for the three competitors was the same.

Figure 3 CSD is essential but not sufficient for RNA-binding activity of chk-YB-2

(Top panel) Equimolar amounts of bacterially expressed MBP fusion proteins of various deletion mutants of chk-YB-2 were incubated with same amounts of radiolabelled E4C-1 RNA and EMSAs performed as described. Note that only MBP-chk-YB-2 showed an ability to bind the E4C-1 RNA under the conditions used. None of its deletion mutants could bind the E4C-1 RNA, indicating that essentially all of the full-length chk-YB-2 protein contributes to RNA binding. (Bottom panel) Presence of five repeats of eight amino acids in the C-terminus of chk-YB-2. Note that the position of arginine moieties is fairly well conserved within these repeats.

affinity (Figure 2, lanes marked E4/E4C-1 and E4-1/E4C; see Table 1 for sequence) in contrast to its high-affinity binding with the single-stranded RNA (E4C-1 RNA, Figure 2). These results suggest that chk-YB-2 binds with high affinity, only to single-stranded RNA or DNA in a sequence-specific manner but not to double-stranded DNA or double-stranded RNA or to RNA-DNA heteroduplexes.

CSD is necessary but not sufficient for conferring RNA-binding ability on chk-YB-2

We have shown previously that the CSD is necessary but not sufficient for conferring DNA-binding ability on chk-YB-2 [29]. In this report, we have used the deletion mutants of chk-YB-2 described earlier [29] to locate the RNA-binding domain in chk-YB-2. As can be seen, none of these mutant proteins was capable of binding to E4C-1 RNA, while the wild-type protein bound efficiently (Figure 3, top panel). It is interesting that the mutant containing the N-terminal 162 amino acids, which we have shown to be capable of interacting with DNA [29], fails to bind RNA [MBP-(1-162); Figure 3, top panel]. This suggests that the

domain required for binding RNA extends beyond the region required for interaction with DNA. Our results also show that the CSD is necessary but not sufficient for conferring RNA-binding ability on chk-YB-2 (Figure 3, top panel). An analysis of the amino acid sequence of chk-YB-2 revealed the presence of five arginine-rich repeats, each containing eight amino acids, in the C-terminal half of the protein (Figure 3, bottom panel). Our results suggest that, in addition to the CSD, the presence of all of these repeats in the charge zipper is necessary for chk-YB-2 to exhibit RNA-binding ability.

The host factor interacting with E4C-1 RNA and RSV Ψ RNA is chk-YB-2 and is cytoplasmic

All of the above experiments were performed with bacterially expressed, partially purified Y-box proteins. It was therefore important to demonstrate the corresponding activities in either the cytoplasm or the nucleus of the avian fibroblasts. Therefore we carried out EMSAs with cytoplasmic or nuclear extracts from CEF and radiolabelled RSV Ψ RNA. As expected, a complex was detected when cytoplasmic extract was used with radio-

labelled RSV Ψ RNA (Figure 4, top panel, lane 1), which could be competed out with 30-fold molar excess of unlabelled RSV Ψ RNA (Figure 4, top panel, lane 2). Higher amounts of non-specific RNA were required to compete out binding (Figure 4, top panel, lane 3). We did not observe the formation of any complex when similar amounts of nuclear extracts were used. This indicates that the complex seen in lane 1 (Figure 4, top panel) is the result of sequence-specific interaction of a cytoplasmic factor with RSV Ψ RNA.

We then performed super-shift assays, using anti-chk-YB-1b or anti-chk-YB-2 antibodies to identify the cytoplasmic factor binding to the E4C-1 RNA. Our results show that anti-chk-YB-2 antibodies either abolished the formation of the complex or caused a super-shift of the complex (Figure 4, bottom panel, lane 3). Anti-chk-YB-1b antibodies did not have a similar effect on the complex formation (Figure 4, bottom panel, lane 4). These results therefore suggest that the cytoplasmic factor binding to the E4C-1 RNA is predominantly chk-YB-2. We then confirmed the sequence specificity of this interaction by using a mutant E4C-1 RNA having changes, which when introduced in the E4C-1 DNA had abolished binding [29]. Our results show that the cytoplasmic factor fails to bind the mutant E4C-1 RNA (Figure 4, bottom panel, lanes 5–8). When RSV Ψ RNA was used in the binding reactions, we obtained essentially the same results as with E4C-1 RNA, again confirming that the cellular factor which interacts with the RSV Ψ region is predominantly chk-YB-2 (Figure 4, bottom panel, lanes 9–12).

GFP-chk-YB-1b and GFP-chk-YB-2 fusion proteins are localized to the cytoplasm

In order to confirm the cytoplasmic location of chk-YB-2 and chk-YB-1b, we constructed fusion proteins of chk-YB-2 or chk-YB-1b with GFP and expressed them under the influence of the RSV LTR in avian fibroblasts, by transient transfection. Fluorescence microscopic analysis indicated that GFP was present all over the cell in a diffuse manner (Figure 5A). In contrast, GFP-chk-YB-2 fusion protein was localized predominantly in the cytoplasm (Figure 5B) with very little in the nucleus. GFP-chk-YB-1b was also localized in the cytoplasm, but its appearance is quite different from chk-YB-2, as it is mainly found in discrete particles or in punctate form (Figure 5C). This punctate appearance of GFP-chk-YB-1b suggests that it probably is part of a large particulate complex, such as the 50–60 S mRNP storage particles that were documented earlier as containing homologues of YB1 proteins [16,23]. In order to find out whether infection by RSV causes any changes in the distribution of these proteins, we repeated the same experiments with CEF cells transformed by the Prague A strain of RSV (gift of Dr. Martin Stoltzfus, University of Iowa, Iowa City, IA, U.S.A.). We did not see any significant differences in the patterns of subcellular localization of these Y-box proteins in RSV-transformed CEFs (Figures 5D–5F). We have since confirmed the validity of using GFP fusion proteins to study subcellular localization of Y-box proteins by performing immunocytochemistry on CEFs and Prague A RSV-transformed CEFs using anti-chk-YB-1b and anti-chk-YB-2 antibodies (S. K. Swamynathan and R. V. Guntaka, unpublished work).

chk-YB-1b and chk-YB-2 repress translation

Our results showed that both chk-YB-1b and chk-YB-2 can interact with single-stranded RNA in a sequence-specific manner, so we were interested in understanding the functional significance of this interaction. Therefore, we performed translation reactions *in vitro* using the construct RSV 1-844, which produces the p19

Figure 4 Sequence-specific binding of RSV Ψ RNA by the cytoplasmic factor chk-YB-2

(Top panel) The cellular factor interacting with the RNA is cytoplasmic and the nature of interaction is sequence-specific. Lane 1, 15 μ g of CEF cytoplasmic extract bound with radiolabelled RSV Ψ RNA. Lane 2, as lane 1, but with 30-fold molar excess of unlabelled RSV Ψ RNA as specific competitors. Lane 3, as lane 1, but with 30-fold molar excess of a E4C-1 mutant RNA competitor. Lanes 4–6 are the same as lanes 1–3 respectively; however, in these reactions, CEF nuclear extracts were used. (Bottom panel) Super-shift experiments demonstrate that the cytoplasmic factor interacting with the RNA is chk-YB-2. Lanes 1, 5 and 9, free, unbound RNA as indicated at the top of the Figure. Lanes 2, 6 and 10, respective oligoribonucleotides bound with 15 μ g of CEF cytoplasmic extracts. Lanes 3, 7 and 11, similar to lanes 2, 6 and 10, but with anti-chk-YB-2 polyclonal antibodies (1:30 final dilution). Lanes 4, 8 and 12, similar to lanes 2, 6 and 10, but with anti-chk-YB-1b polyclonal antibodies (1:30 final dilution). Note that the bound complex formation is observed only with either E4C-1 RNA (lane 2) or RSV Ψ RNA (lane 10) but not with E4C-1-Mut RNA (lane 6). Also, only the anti-chk-YB-2 antibodies could super-shift or abolish the formation of the bound complex (lanes 3 and 11), whereas anti-chk-YB-1b antibodies failed to demonstrate a similar effect (lanes 4 and 12).

Figure 5 Subcellular localization of chk-YB-1b and chk-YB-2

Recombinant vectors expressing (i) GFP, (ii) GFP-chk-YB-2 fusion protein or (iii) GFP-chk-YB-1b fusion protein were constructed and transfected into CEF cells or RSV-transformed CEF cells. Localization of these proteins was visualized using a fluorescence microscope after 48 h of recovery from transfection. (A) GFP in CEF cells, (B) GFP-chk-YB-2 in CEF cells, (C) GFP-chk-YB-1b in CEF cells, (D) GFP in RSV-transformed CEF cells, (E) GFP-chk-YB-2 in RSV-transformed CEF cells and (F) GFP-chk-YB-1b in RSV-transformed CEF cells.

Figure 6 Both chk-YB-2 and chk-YB-1b are inhibitors of translation *in vitro*

(a) *In vitro* TNT reactions using T7 RNA polymerase and rabbit reticulocyte lysate were performed in either the absence or presence of increasing amounts of chk-YB-2 or chk-YB-1b, as described in the Materials and methods section. Note that both chk-YB-2 and chk-YB-1b inhibit translation *in vitro* of p19 Gag in a concentration-dependent manner. (b) RNA-binding ability is a prerequisite for the ability to repress translation. Only the full-length wild-type chk-YB-2 was found to be capable of repressing translation in TNT reactions (second lane from left). All the deletion mutants of chk-YB-2, which we have shown to be incapable of binding RNA (Figure 3), are also incapable of repressing translation (right-hand three lanes).

Gag protein of RSV when transcribed by T7 polymerase and translated *in vitro* using a rabbit reticulocyte extract system. This construct also contains the RSV Ψ sequence with which both chk-YB-1b and chk-YB-2 interact. TNT reactions *in vitro* in rabbit reticulocyte lysates showed a dose-dependent reduction in the amount of p19 Gag with either chk-YB-1b or chk-YB-2 (Figure 6).

We then asked whether binding to RNA is a prerequisite for repression of translation by Y-box proteins. We performed TNT reactions with different deletion mutants of chk-YB-2, which we have shown to be incapable of binding RNA. As shown in Figure 6(b), all the deletion mutants of chk-YB-2, which are unable to bind RNA, are also incapable of repressing translation of p19 Gag. Hence there is a perfect correlation between the ability of the Y-box proteins to bind RNA and their ability to repress translation. Therefore, this suggests that RNA binding is a prerequisite for Y-box-protein-mediated repression of translation.

Since transcription and translation reactions were coupled in the above experiments, we needed to ascertain that the observed reduction was due only to a reduced translation and not due to any reduction in the T7 polymerase-mediated transcription. Towards this, we performed transcription reactions *in vitro* using T7 RNA polymerase, in either the presence or absence of chk-YB-2 or chk-YB-1b. In these control reactions, no inhibitory effect of the Y-box proteins on T7 polymerase-mediated transcription was observed (results not shown). To confirm further the specificity of repression of translation by these Y-box proteins, we performed translations *in vitro* of total RNA from CEFs using rabbit reticulocyte lysates, either in the absence or presence of increasing amounts of chk-YB-2 or chk-YB-1b. We did not observe any general repression of translation in these reactions, as a result of inclusion of either chk-YB-1b or chk-YB-2 (results not shown). These results therefore suggest that chk-YB-1b and chk-YB-2 are not general repressors of translation.

DISCUSSION

In this report, we have shown that (i) bacterially expressed chk-YB-1b and chk-YB-2 are capable of interacting with single-stranded RNA in a sequence-specific and Mg^{2+} -dependent manner, (ii) CSD is necessary but not sufficient for imparting RNA-binding ability to chk-YB-2, (iii) both chk-YB-1b and chk-YB-2 are localized in the cytoplasm, (iv) in the cytoplasmic extracts from CEFs, chk-YB-2 is the predominant component of the E4C-1 RNA or RSV Ψ RNA-binding activity and (v) chk-YB-1b and chk-YB-2 inhibit translation *in vitro* in an RNA-binding-dependent manner. It is interesting to note that even though these two factors share a lot of homology and are both capable of binding E4C-1 and RSV Ψ RNA sequences in their bacterially expressed form, only chk-YB-2 demonstrated binding RNA in the cytoplasmic extracts from CEFs. Further, even though the two proteins are localized in the cytoplasm, chk-YB-1b is punctate in nature while chk-YB-2 is diffusely distributed all over the cytoplasm.

Results presented in this report show that both chk-YB-2 and chk-YB-1b are capable of interacting with single-stranded RNA in a sequence-specific manner. The sequence specificity of this interaction is proved by the fact that these proteins fail to bind to the complementary purine-rich single-stranded RNA or the double-stranded RNA having the same sequence. A comparison of the RSV Ψ sequence and the E4C-1 sequence shows that the sequence 5'-GTACCACC-3' is conserved among them. Pre-

viously, we have shown this sequence to be the primary recognition motif for these Y-box proteins on the RSV LTR [28,29]. Interestingly, we found no difference in the affinity of interaction of these proteins with either RNA or DNA, although there was a difference in the chk-YB-2 domain required for binding RNA as compared with DNA.

EMSA performed with the mutant proteins and E4C-1 RNA indicate that all the essential features of the protein chk-YB-2, like the proline-rich N-terminal domain, the CSD and the C-terminal charge zipper, are required for a high-affinity binding of E4C-1 RNA. This contrasts with the requirement of only the N-terminal 162 amino acids of chk-YB-2 for binding E4C-1 DNA [29]. An analysis of the structure of chk-YB-2 revealed the presence of five repeats of an eight-amino acid motif rich in arginine (Figure 3, bottom panel). Therefore, it appears as though these arginine-rich motifs and the RNP1 motif in the CSD co-operate to impart RNA-binding ability to chk-YB-2. Earlier it was suggested that the charge zippers in the C-terminal domain provide for non-specific interactions while the RNP1 and the amino acids surrounding that domain impart a sequence-specific interaction with the RNA [13]. However, in the case of chk-YB-2, both these domains are required for the ability to bind RNA in a sequence-specific manner. Further, we do not see any non-specific RNA binding by the full-length wild-type chk-YB-2, in contrast to FRGY2 [13].

It is believed that the translation-initiation sites in eukaryotic mRNAs are reached via a scanning mechanism by the ribosomes, which bind the mRNA at the 5' end [34]. In the genome of RSV, the sequence bound by Y-box proteins (nucleotides 328–357 in the leader) is located just upstream of the translation-initiation site at nucleotide 380. Therefore, it is possible that the Y-box proteins bound in this region pose a steric hindrance to the scanning of RNA for the initiation codon, by the bound ribosomes. However, we do not have direct evidence demonstrating this. We should, however, emphasize that inhibition of translation may not be mediated only through the RSV Ψ sequence described here. It is probable that additional sites such as the polypyrimidine motif at nucleotides 430–450 of RSV also may be bound by Y-box proteins. Therefore, different Y-box-protein-binding sites in this 1–844 RSV RNA have to be precisely mapped. These experiments are in progress.

Several proteins are known to be multifunctional, influencing both transcription and translation [10,25,35]. One such example is *Drosophila* sex lethal (SXL), which is known to regulate both splicing and translation [36]. TFIID is another protein which can bind both DNA and RNA, and can influence both transcription and translation of 5 S rRNA gene. Our results support the contention that Y-box proteins are multifunctional proteins and that the same regulatory protein can serve to control the expression of a protein at either the transcriptional or post-transcriptional stages, or both. This multifunctional capacity of Y-box proteins has been explained as an outcome of their variable distribution between nuclear and cytoplasmic compartments, as well as their ability to bind and stabilize or destabilize either single-stranded or double-stranded RNA and DNA [25].

The ability of chk-YB-1 and chk-YB-2 to bind RSV Ψ RNA raises important questions and interesting possibilities. In any retrovirus-infected cell, a fraction of the RNA transcripts is transported out of the nucleus in an unspliced form. The unspliced RNA in the cytoplasm is translated into Gag and Gag-Pol polyproteins. Further, a fraction of the unspliced RNA is encapsidated into virions [37,38]. In HIV, *rev* gene product is known to bind specific viral RNA sequence and help in stabilizing and export of the non-spliced RNA into the cytoplasm [39,40], inhibiting its splicing [41] and translation [42].

Tat is another HIV-encoded multifunctional protein that acts as an activator of transcription from the HIV LTR [43]. This protein (Tat) is also known to bind the HIV leader RNA, TAR [44]. Recently, it has been shown that the human YB1 interacts with TAR RNA as well as the viral protein Tat [45]. Simpler retroviruses such as RSV lack these accessory proteins and therefore need to rely on the cellular machinery for performing similar events in their life cycle. Results presented in this report have brought out a candidate protein in the host cell for such functions. Interestingly in this regard, a strong homology is known to exist between the HIV Tat protein RNA-binding domain and the YB1 homologous Y-box protein dbpB [46].

In summary, we have shown here that the two chicken Y-box proteins we have studied are both localized predominantly in the cytoplasm, are capable of binding single-stranded RNA in a sequence-specific manner and inhibiting translation, and that the domains required for RNA binding include the CSD and the C-terminal charge zipper region. Taken together with our earlier report on the involvement of chk-YB-2 in transcriptional activation of RSV LTR [28], these results suggest that the Y-box proteins are involved in more than one stage of the life cycle of RSV in the host cells. How they achieve a balance between the two seemingly opposite functions of transcriptional activation and translational repression is still not clear and it should be intellectually rewarding to find out.

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