Chicken YB-2, a Y-Box Protein, Is a Potent Activator of Rous Sarcoma Virus Long Terminal Repeat-Driven Transcription in Avian Fibroblasts

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We have previously reported on the cloning and characterization of chk-YB-2, a novel member of the Y-box family of proteins, that binds to the sequence 5'-GTACCACC-3' present on the noncoding strand of the Rous sarcoma virus (RSV) long terminal repeat (LTR) in a single-strand-specific manner. Here, we demonstrate that deletion or mutation of this motif not only eliminates chk-YB-2 binding in vitro but also down-regulates RSV LTR-driven transcription in avian cells. Selective abrogation of chk-YB-2 expression by using antisense oligonucleotides decreased RSV LTR-driven transcription in a promoter-specific manner. This inhibition was not observed when a reporter construct with a deletion in the chk-YB-2 binding site was used. Depletion of cellular chk-YB-2 by transfecting the cells with excess of its recognition sequence oligonucleotides also resulted in reduced transcription from the RSV LTR. Taken together, these results suggest that chk-YB-2 acts as an activator of LTR-promoted transcription in avian cells and that this activation is mediated primarily through the sequence 5'-GTACCACC-3'.

The U3 enhancer region of the long terminal repeat (LTR) of Rous sarcoma virus (RSV) contains the determinants for the tissue-tropic expression of viral genes (9, 24, 30). Multiple *cis*-acting elements have been identified within this region (5, 6, 14, 17, 19, 22, 25, 26, 44). Protein factors that bind to these sequences have been identified and partially characterized (2, 11, 16, 19, 31, 32, 34, 35). Cellular genes encoding three factors which bind to the RSV LTR have been cloned and characterized in detail (3, 15, 18). However, the involvement of these cloned gene products in the regulation of LTR-promoted transcription has not been clearly established. Also, a clear picture as to how a multitude of these factors regulate transcription from the LTR under variable physiological conditions is yet to emerge.

Members of the Y-box family of proteins share a well-conserved nucleic acid binding domain (reviewed in references 36 and 42). Diverse biological roles have been proposed for these proteins in different systems. They are thought to play a role in the cold shock response in *Escherichia coli*, positive or negative modulation of transcription (4, 7, 10, 27, 38), modification of chromatin (20), translational masking of mRNA in vertebrate gametes (29, 36, 37, 39), and cell proliferation (21). The involvement of Y-box proteins in the regulation of transcription of class II major histocompatibility complex (MHC) genes has been studied in detail (reviewed in reference 1).

We have recently reported on the cloning and characterization of avian protein chk-YB-2, a novel member of the Y-box family of proteins which binds to the RSV LTR in a sequence-specific manner (3). Further, we had shown that this protein recognizes the single-strand sequence 5'-GTACCACC-3' in the noncoding strand of the RSV LTR. Earlier, we showed that chk-YB-2 acts as a repressor of LTR-driven transcription in mouse NIH 3T3 cells (3). In this report, we show that blocking the expression or depletion of chk-YB-2 in permissive avian cells leads to a specific reduction in the transcriptional activity

of the RSV LTR. We also show that chk-YB-2 exerts its influence on the RSV LTR through the sequence 5'-GTACCACC-3', identified earlier as the principal DNA binding motif for chk-YB-2 in vitro. Taken together, these results suggest that chk-YB-2 acts as an activator of RSV LTR-driven transcription in avian cells.

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblast cells (CKY cells) were cultured in medium 199 (M199) 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 (19).

Labeling of oligonucleotides and EMSAs. The sequences of oligonucleotides used in electrophoretic mobility shift assays (EMSAs) are given in Fig. 1. Single-stranded oligonucleotides were end labeled as described earlier (19). EMSAs were carried out by adding about 40,000 cpm (~0.2 ng) of radiolabeled oligonucleotides and 50 to 200 ng of partially purified Mal-chk-YB-2 fusion protein cleaved with factor Xa that was prepared as described previously (3). The reaction mixtures were incubated for 20 min in buffer A (5% glycerol, 10 mM Tris [pH 7.5], 1 mM EDTA [pH 7.5], 1 mM dithiothreitol, 100 mM KCl, 2 µg of dI-dC as nonspecific DNA) at room temperature and then run on a 6% polyacrylamide gel in Tris-glycine buffer as described earlier (19). Following electrophoresis, gels were dried and exposed to X-ray films at -70°C with intensifying screens.

The LTR fragment probes used in EMSAs were prepared as follows. The CAT-1C oligonucleotides were end labeled by using T4 polynucleotide kinase and used in PCRs with E5 as the upstream primer and either an intact LTR or an E4Del LTR (LTR with a 14-nucleotide deletion in E4 region) as the template (see the legend to Fig. 4 for the sequences of primers used). This way, only the complementary strand of the LTR was labeled. The PCR products were eluted from a 6% polyacrylamide gel and purified by phenol-chloroform extraction and ethanol precipitation. The eluted products were dissolved in a minimal volume of 0.1 M NaOH to keep the probe in a single-stranded form. These probes were diluted 1:200 during EMSAs, which were performed as described above except that the gel was run for 4 h.

Synthesis of sense and antisense oligonucleotides to chk-YB-2. Phosphorothioate-modified 17-mer oligonucleotides were designed based on the sequence corresponding to chk-YB-2 coding region and synthesized by Oligo Therapeutics Inc. (Wilsonville, Oreg.). Antisense oligonucleotides were synthesized as the reverse complementary sequences for the coding sequences. The sequences of these oligonucleotides are given in Fig. 7. Concentrations of the oligonucleotides were determined by spectrophotometry.

Construction of reporter plasmids. Site-specific deletion or mutagenesis in the E4 or E3 region of the RSV LTR was achieved through PCR using linearized pRSV-LTR-CAT plasmid (14) as the template and specific oligonucleotides as

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FIG. 1. Schematic representation of the U3 region of the RSV Prague-C strain LTR. The sequence of U3 region (-181 to -98), which includes the E3 and E4 oligonucleotides, is given. Sequences of oligonucleotides used in this study are given below. Oligonucleotides with the suffix "C" represent the negative strand of the LTR.

primers, the sequences of which are given in Fig. 1. The PCR-generated fragments were purified, restricted with specific restriction enzymes, and used to replace the corresponding fragments from the reporter vector containing the chloramphenicol acetyltransferase (CAT) gene under the regulation of wild-type RSV LTR, using standard molecular biological methods (33). Two independent isolates of each construct were tested in our experiments.

Plasmid and oligonucleotide transfection into CKY cells and CAT assays. CKY cells in mid-log phase of growth were transfected with the plasmids alone or, in the case of antisense and chk-YB-2 depletion experiments, with the plasmids and the oligonucleotides in a sequential manner, using lipofectAMINE as suggested by the manufacturer (GIBCO-BRL). Briefly, 100 μ l of serum-free, antibiotic-free M199 containing 10 μ g of lipofectAMINE was mixed with 100 μ l of serum-free, antibiotic-free M199 containing 1 μ g of each of the reporter plasmids (pRSV-LTR-CAT or its E4 deletion or mutation derivatives) and the internal control plasmid pSV-gal and incubated at room temperature for 45 min. This mix was then diluted up to 2 ml with M199 without serum and antibiotics and layered onto CKY cells in mid-log phase of growth. In antisense experiments, the cells were subjected to a second transfection with 1.2 μ g of each of the oligonucleotides for 4 h soon after the first transfection with reporter plasmids as described above. In experiments aimed at depleting the cellular chk-YB-2 also, the cells were subjected to sequential transfections with oligonucleotides first and then with the plasmids pRSV-LTR-CAT and pSV-gal. After 6 h of transfection, the transfection mix was removed and the cells were allowed to recover in complete M199 (M199 with 10% calf serum, 1% chicken serum, antibiotics, and 10% tryptose phosphate broth) for 48 h, after which they were harvested and lysed by three cycles of freezing and thawing. The cell debris was removed by centrifugation, and the protein concentration in the clear supernatant was estimated by the method of Bradford. β -Galactosidase assays were performed as described earlier with 10 μ g of the extract for 30 min at 37°C (8). CAT assays were performed with protein amounts normalized to β -galactosidase activity (13). The CAT assay reaction products were separated by thin-layer chromatography and visualized by X-ray autoradiography overnight. Relative CAT activity was quantitated by scraping the silica gel from the thin-layer chromatography plates corresponding to the acetylated and nonacetylated chloramphenicol as visualized by X-ray autoradiography and counting it in liquid scintillation counter.

RESULTS

Deletion or mutation of the chk-YB-2 binding site in the RSV LTR reduces RSV LTR-driven transcription. Earlier studies have shown that the nucleotide sequence between bp -139 and -109 in the RSV LTR is important for RSV LTR-driven transcription (5, 6). However, these studies did not identify the precise nucleotides within this region which regulate RSV LTR-driven transcription. We have shown that chk-YB-2 binds to the sequence 5'-GTACCACC-3' present in the noncoding strand of the RSV LTR within this region. To test whether the chk-YB-2 binding site is involved in regulating RSV LTR-promoted transcription, we constructed reporter vectors wherein the CAT reporter gene is transcribed from (i) an intact, wild-type LTR (pRSV-LTR-CAT), (ii) an LTR where the sequence 5'-GTACCACC-3' has been deleted (pD3E4Del-CAT), or (iii) an LTR with specific nucleotides

mutated within this region (pD3E4Mut-CAT; 5'-GTACCACC-3' is replaced by 5'-TTACGAAT-3'), using PCR-mediated mutagenesis. One microgram of each of these plasmids was transiently transfected into the chicken embryo fibroblast cells in mid-log phase of growth, using lipofectAMINE (GIBCO-BRL), and the activity of each of these promoters was measured through CAT assays as described earlier (12). The results shown in Fig. 2 indicate that the deletion or mutation in this motif decreased RSV LTR-driven transcription by 75 or 65%, respectively, compared to the transcription from the intact, wild-type RSV LTR. Thus, our results suggest that the chk-YB-2 binding site, 5'-GTACCACC-3', is essential for optimal activity of the RSV LTR.

Elimination of chk-YB-2 binding to E4-C region of the RSV LTR correlates with the reduction in efficiency of LTR-driven transcription. To determine whether the deletion or mutation in the sequence 5'-GTACCACC-3' which affected RSV LTR-

FIG. 2. Deletion or mutation of the chk-YB-2 binding site decreases RSV LTR-driven transcription. Chicken embryo fibroblasts were transfected with 1 μ g of either pRSV-LTR-CAT (wild type [WT]), pD3E4Del-CAT (Del), or pD3E4Mut-CAT (Mut) along with 1 μ g of pSV-gal. Transcriptional activity was measured by CAT assays done with protein amounts normalized with respect to β -galactosidase activity. The quantitative data shown are averages of six identical experiments carried out independently. The decrease in transcription from the LTR as a result of either deletions or mutations in the E4 region varied from 50 to 75% in individual experiments. The sequence of the relevant region in the RSV LTR is shown below, along with the deletions and the mutations in the chk-YB-2 binding site.

FIG. 3. Elimination of chk-YB-2 binding to E4C region of the RSV LTR by deletion or mutations in its binding site. Oligonucleotides representing chk-YB-2 binding site in the RSV LTR (E4 C) and either deletion (E4 Del C) or mutations (E4 Mut C) in this site were end labeled and used in EMSAs with bacterially expressed, partially purified chk-YB-2. Note that while the wild-type E4-C oligonucleotides are bound by chk-YB-2 with a high affinity (lanes 1 to 3), both deletion and mutations in the chk-YB-2 binding site eliminated this binding (lanes 4 to 9 compared to lanes 1 to 3). Similar results were obtained under conditions of very high affinity binding (last three lanes), where calf serum was included in the binding reaction at a final dilution of 1:15.

promoted transcription also affects the chk-YB-2 binding to this region, we performed EMSAs where bacterially expressed chk-YB-2 was allowed to bind to different oligonucleotides. The results shown in Fig. 3 indicate that while the wild-type oligonucleotides were bound with very high affinity (Fig. 3, lanes 1 to 3), both deletions (Fig. 3, lanes 4 to 6) and mutations (Fig. 3, lanes 7 to 9) in the sequence 5'-GTACCACC-3' eliminated the chk-YB-2 binding. We have noticed that inclusion of calf serum at a final dilution of 1:15 greatly enhances the affinity of interaction between chk-YB-2 and its binding site (Fig. 3, lane 11 compared to lane 2). We found that under these conditions also, chk-YB-2 failed to bind the deleted or mutated oligonucleotides (Fig. 3, last three lanes). These results suggest that the observed reduction in RSV LTR-driven transcription as a result of the deletions or mutations in the sequence 5'-GTACCACC-3' is most probably due to the failure of chk-YB-2 to bind this region.

chk-YB-2 binds to multiple sites on the LTR. We have shown previously that chk-YB-2 interacts with multiple sites on the RSV LTR, although it binds E4 region with highest affinity (3). Thus, it was interesting to compare the ability of chk-YB-2 to interact with an intact LTR with its ability to interact with an E4Del LTR. Toward this end, we generated probes by PCR using end-labeled CAT-1C and unlabeled E5 oligonucleotides as the primers and either an intact LTR or an E4Del LTR as the template DNA. As shown in Fig. 4, with increasing amounts of protein in the binding reaction, the DNA-protein complex is retarded in a progressive manner (lanes 1 to 7). Surprisingly, these results show that chk-YB-2 continues to bind the RSV LTR having a 14-nucleotide-long deletion in the E4 region (Fig. 4, lanes 8 to 14). The interaction between chk-YB-2 and E4Del LTR is sequence specific, because the reaction mix contained 2 μ g of the nonspecific competitor DNA. Thus, a lack of interaction between chk-YB-2 and E4-C region does not affect the ability of chk-YB-2 to bind other regions on the LTR.

FIG. 4. chk-YB-2 binds the RSV LTR at multiple sites. The complementary strand of the RSV LTR (bp -54 to -204) was labeled as described in Materials and Methods and used in EMSAs with increasing amounts of chk-YB-2 (lanes 1 to 7). Similar reactions were carried out with a fragment of the LTR having a 14-nucleotide deletion in the E4 region (E4 Del LTR). C1, C2, C3, and C4 refer to different complexes that are formed as a result of increasing amounts of chk-YB-2. Sequences of the primers used for preparation of the radiolabeled probe are 5'-TTGCAACATGCTTATGTAACGATGAGTTAG-3' (E5) and 5'-CAGTGGTTCGTCCAATCCATGTTAG-3' (CAT-1C).

The progressive retardation of the DNA-protein complex, when the U3 region of the LTR was used as the probe, could arise due to two different reasons. (i) The RSV LTR is bound at different sites by chk-YB-2, with different affinities. The highest-affinity site (E4-C) is bound first, when the amounts of chk-YB-2 are limiting. The other, low-affinity binding sites also are occupied when the amounts of chk-YB-2 are increased in the reaction, leading to a progressive retardation. (ii) Progressive retardation could also arise due to protein multimerization. However, protein multimerization does not appear to be the case here, since we do not observe a similar effect when the E4-C oligonucleotides are used in the binding reaction (Fig. 3, lanes 1 to 3). Moreover, the results of DNase footprinting reactions performed with the negative strand of the LTR as a probe indicate that chk-YB-2 interacts with multiple sites on the RSV LTR, with disparate affinities (36a).

chk-YB-2 binds to another pyrimidine-rich sequence in the RSV LTR. To determine the other sequences on the RSV LTR with which chk-YB-2 interacts, EMSAs were performed with overlapping oligonucleotides covering the entire U3 region of the RSV LTR. The results of several such EMSAs indicated that chk-YB-2 binds with low affinity to another region, 40 bp upstream of its high-affinity binding site identified earlier (Fig. 5, lane 4; data negative for other oligonucleotides are not shown). Here again, chk-YB-2 was found to recognize the pyrimidine-rich noncoding strand but not its purine-rich complementary coding strand (Fig. 5, lane 4 compared to lane 2).

To identify the specific sequence within this region which is recognized by chk-YB-2, we performed EMSAs with different oligonucleotides corresponding to this region but having specific deletions or point mutations (see Fig. 1 for the sequences of oligonucleotides). The results of these studies indicate that the pyrimidine-rich single-strand sequence -152 TTCTTTC CTT -161 on the noncoding strand of the RSV LTR is bound by chk-YB-2 with a low affinity (Fig. 5, lanes 5 to 10).

FIG. 5. chk-YB-2 interacts with another pyrimidine-rich region on the RSV LTR. Interaction of chk-YB-2 with the E3 region of the RSV LTR was studied by binding 50 ng of chk-YB-2 with different single-stranded oligonucleotides, sequences of which are given in Fig. 1. Note that chk-YB-2 interacts with the negative strand (lane 4) but not with the positive strand (lane 2) from this region. The precise sequence within this region with which chk-YB-2 interacts was identified by using different mutant oligonucleotides (lanes 5 to 10). Note that chk-YB-2 is capable of binding E3DelC (lane 6) but not to E3MutC (lane 8) or E3-1DC (lane 10). See Fig. 1 for sequences of oligonucleotides.

Transactivation of the RSV LTR is dependent on interaction of chk-YB-2 with the E4-C region. Having discovered that chk-YB-2 interacts with the pyrimidine-rich sequences on the noncoding strand of the LTR in the E3 region also, it was of interest to study if this interaction is important for RSV LTR-driven transcription. Toward this end, we constructed reporter vectors wherein the CAT gene is driven by (i) an LTR carrying specific mutations in the E3 region which abolished the ability of chk-YB-2 to bind that region (pD3-E3-Mut-CAT) and (ii) an LTR carrying these mutations in the E3 region along with deletion of the E4 region (pD3-E4Del-E3-Mut-CAT). Chicken embryo fibroblasts in mid-log phase of growth were transfected with these vectors, and relative transcriptional activity measured in CAT assays. The results presented in Fig. 6 show that mutations in the E3 region, which had eliminated the interaction of chk-YB-2 with this region, do not significantly reduce the LTR-driven transcription (compare lanes E3Mut LTR CAT and pRSV LTR CAT). Further, transcription from pD3E4Del-E3Mut-CAT (which had both E4 and E3 regions mutated) was not significantly lower than the transcription from pD3E4Del-CAT (Fig. 6; compare lanes E4Del-E3Mut LTR CAT and E4Del LTR CAT). Thus, interaction of chk-YB-2 with the pyrimidine-rich sequences in the E3 region is not of any direct relevance to the transcriptional activity of RSV LTR under the physiological conditions used in this study.

RSV LTR contains two CA₂G boxes, one between nucleotides -104 and -95 and the other between nucleotides -167 and -158 (2, 44; for a review on LTR binding factors, see reference 30). Point mutations that we introduced in pD3E3Mut-CAT disrupt the CA₂G box between -167 and -158. Interestingly, however, these mutations do not lead to a significant reduction in the transcriptional activity of the RSV LTR (Fig. 6). It is possible that this observation reflects the redundancy in having two CA₂G boxes where the presence of one CA₂G box is sufficient for a high level activity of the LTR.

FIG. 6. Interaction of chk-YB-2 with E4-C region alone is relevant to RSV LTR-driven transcription. CKY cells in mid-log phase of growth were transfected with 1 μ g each of pRSV-LTR-CAT (lane 1), pD3E4Del-CAT (lane 2), pD3E3Mut-CAT (lane 3), or pD3E4Del-E3Mut-CAT (lane 4) along with 1 μ g of pSV-gal. CAT assays were performed with protein amounts normalized with respect to β -galactosidase activity. The quantitative data shown on the right side are averages of results obtained in three independent experiments. The decrease in transcription due to a deletion in the E4 region of the LTR varied from 65 to 75% in individual experiments.

chk-YB-2 activates LTR-driven transcription in avian cells.

Infection and production of RSV are known to occur predominantly in avian fibroblast cells. chk-YB-2 is overexpressed in cultured avian fibroblasts and muscle tissue (3). This pattern of expression of chk-YB-2 parallels the tissue tropism of RSV infection. Hence, it was interesting to study the effect of blocking the expression of chk-YB-2 on LTR-promoted transcription in avian fibroblast cells. Toward this goal, we designed specific sense and antisense oligonucleotides to the chk-YB-2 mRNA. While designing these oligonucleotides, care was taken to select only those sequences that were sufficiently divergent from other related gene sequences, so that nonspecific interferences are minimized. The sequences and positions of these oligonucleotides are presented in Fig. 7.

Chicken embryo fibroblast cells in mid-log phase of growth were transfected sequentially with 1.2 μ g of either sense or antisense oligonucleotides and 1.0 μ g of plasmid pRSV-LTR-CAT, using lipofectAMINE (GIBCO-BRL). The CAT activity was measured following recovery of the transfected cells as described previously (13). The results presented in Fig. 7 (bottom left) reveal a 30 to 70% decrease in CAT activity in the cells treated with various antisense oligonucleotides compared to the cells transfected with sense oligonucleotides, which served as a good control for any nonspecific toxicity that might alter gene expression.

To determine whether the alterations in gene expression resulting from transfection with these oligonucleotides extend to transcription from a promoter other than the RSV LTR, we performed similar experiments where the reporter plasmid pRSV-LTR-CAT was substituted by pColCAT. In pColCAT, the CAT gene is driven by a rat α -1 collagen promoter, which has been shown in this laboratory to be transcribed efficiently in chicken fibroblasts. Results of these studies showed that treatment with chk-YB-2-specific oligonucleotides did not alter the rat α -1 collagen promoter activity (Fig. 7, bottom right), suggesting that down-regulation of chk-YB-2 gene expression in chicken embryo fibroblasts specifically decreases RSV LTR-promoted transcription.

FIG. 7. Blocking the expression of chk-YB-2 leads to a specific reduction in the RSV LTR transcriptional activity. CKY cells in mid-log phase of growth were sequentially transfected with 1.2 μ g of either sense oligonucleotides or one of three different antisense oligonucleotides, followed by the reporter vector, as described in Materials and Methods. An X-ray autoradiogram showing the effects of sense and antisense oligonucleotides on transcription from pRSV-LTR-CAT is shown at the top. The sequences of oligonucleotides used are given below. The quantitative data are averages of three independent experiments conducted in a similar manner but with different reporter vectors. Note that the antisense oligonucleotides to chk-YB-2 mRNA had a specific repressive effect on RSV LTR-driven transcription (bottom left). Repression varied between 30 and 60% in individual experiments. This repressive effect was not seen when the reporter vector used was pD3E4Del-CAT (CAT gene under the control of the RSV LTR carrying a deletion in the E4 region) (bottom middle) pCol-CAT (CAT gene under the control of the rat α -1 collagen promoter) (bottom right).

chk-YB-2 activates RSV LTR-driven transcription through its LTR binding site. Having thus determined a possible role for chk-YB-2 in LTR-driven transcription, we sought to establish the relevance of the observed *in vitro* interaction of chk-YB-2 with its LTR binding site to its transactivating potential *in vivo*. To this end, we tested the hypothesis that if chk-YB-2 activates transcription principally through the 5'-GTACCAC C-3' motif, transactivation from an LTR with this motif deleted (E4Del LTR) would remain unaffected even upon decreasing the cellular levels of chk-YB-2. Similar transient transfection experiments were done with chk-YB-2-specific antisense oligonucleotides and plasmid pD3E4Del-CAT. As shown in Fig. 7 (bottom middle), unlike the inhibitory effect on the wild-type RSV LTR, the application of chk-YB-2-specific antisense oligonucleotides did not affect the transcription from an RSV LTR where the chk-YB-2 binding site has been deleted. Thus, the observed strong binding of chk-YB-2 to the 5'-GTACCACC-3' motif on RSV LTR in EMSAs appears to be of significance *in vivo* also. These results confirm that chk-YB-2 contributes significantly to the RSV LTR transcriptional activity in avian cells through the 5'-GTACCACC-3' motif.

Depletion of cellular chk-YB-2 affects RSV LTR-driven transcription. We have used the approach of depleting cellular chk-YB-2 activity by using nuclease-resistant phosphorothioate oligonucleotides having binding sites for chk-YB-2 to study the function of chk-YB-2. We transfected the cells first with single-

stranded oligonucleotides containing the chk-YB-2 recognition sequence (E4-C PT) or its complementary strand (E4-PT, as the control). These transfections were followed by a second transfection with the reporter plasmid pRSV-LTR-CAT and the internal control pSV-gal. Results of the CAT assays done with protein amounts normalized with respect to β -galactosidase activity indicate that concomitant with an increase in the amount of oligonucleotide E4-C PT transfected into the cells, there is a progressive decrease in LTR-driven transcription (Fig. 8, lanes 4 to 6). In contrast, increasing amounts of the E4-PT oligonucleotides did not have any inhibitory effect on LTR-driven transcription (Fig. 8, lanes 1 to 3). Thus, depleting the cellular chk-YB-2 activity by providing the cells with excess of its recognition sequence containing oligonucleotides leads to a nonavailability of the transactivating potential of chk-YB-2 for LTR-driven transcription. That this effect is recognition sequence dependent is proved by the absence of inhibitory effect when similar amounts of E4-PT oligonucleotides were used in the control experiments (Fig. 8, lanes 1 to 3).

DISCUSSION

Various functions have been ascribed to the members of Y-box family of proteins, including transcriptional activation and repression, translational masking of vertebrate mRNA, etc. (36, 42). Here, we have provided evidence that chk-YB-2,

FIG. 8. Depleting the availability of cellular chk-YB-2 reduces RSV LTR-driven transcription. CKY cells in mid-log phase of growth were transfected with 0, 1, 3.3, and 10 µg of E4 or E4-C oligonucleotides. Control-S oligonucleotide was used as filler, to maintain the amounts of oligonucleotides used in each treatment the same. Following the first transfection, cells were subjected to a second transfection with 1 µg each of the reporter plasmid pRSV-LTR-CAT and the internal control for the efficiency of transfection, pSV-gal. CAT assays were done with protein amounts normalized for galactosidase activity. The quantitative data shown are averages of four similar experiments performed independently. The decrease in transcription as a result of depletion of chk-YB-2 varied between 50 and 75% in individual experiments. The sequence of the control-S oligonucleotide is 5'-TGGATCCGACATGTCAGA-3'.

a unique member of the Y-box family of proteins, acts as an activator of RSV LTR-driven transcription in avian cells. We have shown that chk-YB-2 mediates this transactivation through the sequence 5'-GTACCACC-3' present on the non-coding strand of RSV LTR, using three different approaches: (i) deletion or mutation of the sequence to which chk-YB-2 binds in the RSV LTR led to a reduction in the transcriptional activity of the RSV LTR (Fig. 1), (ii) depletion of cellular chk-YB-2 activity by using a specific recognition sequence containing oligonucleotides resulted in a reduced transcription from the LTR (Fig. 8), and (iii) transfection of avian cells with antisense oligonucleotides specific to chk-YB-2 mRNA decreased RSV LTR-driven transcription (Fig. 7).

Specificity of the antisense effect in our experiments was demonstrated by the following data: (i) chk-YB-2-specific antisense oligonucleotides targeting three different regions of the chk-YB-2 mRNA had inhibitory effect, while the sense oligonucleotides had no effect; (ii) The antisense oligonucleotides which reduced RSV LTR-driven transcription had no adverse effect on the control collagen promoter-driven transcription; and (iii) the inhibitory effect of the antisense oligonucleotides on RSV LTR-driven transcription was abolished by a deletion in the RSV LTR in the chk-YB-2 binding site. While these results do not rule out a contribution by other protein factors, they clearly demonstrate that endogenous chk-YB-2 promotes RSV LTR transactivation and that this effect is mediated principally via the sequence 5'-GTACCACC-3' on the RSV LTR. Whether this effect is direct or is aided by other factors remains to be tested.

We have exploited the approach of depleting a DNA binding protein in vivo by transfecting the cells with excess of its rec-

ognition sequence containing oligonucleotides, to study its function. A similar approach has earlier been used to study the function of C/EBPs (41). In our experiments, we have used phosphorothioate oligonucleotides, which are relatively stable due to their resistance to degradation by cellular nucleases. Transfection of the recognition sequence containing oligonucleotide E4-C PT led to a specific, concentration-dependent reduction in the transcription from RSV LTR (Fig. 8, lanes 4 to 6). This effect was not seen when nonspecific oligonucleotides (Fig. 8, lane 7) and the complementary sequence E4-PT to which chk-YB-2 does not bind were transfected at similar concentrations (Fig. 8, lanes 1 to 3). Thus, the observed reduction in LTR-driven transcription is a reflection of the nonavailability of the transactivating potential of chk-YB-2. Our results provide evidence that this technique of depleting the cellular factors by providing excess of their ligands in vivo can be applied more widely to study the functions of putative transcription factors.

Earlier, we reported on the cloning and characterization of RSV-EF-I (hereafter referred to as chk-YB-1b), another member of the Y-box family of proteins (18). chk-YB-1b has significant homology with chk-YB-2 and has some of the DNA binding properties of chk-YB-2 (3, 18). Considering that both chk-YB-1b and chk-YB-2 bind to the same sequences on the RSV LTR, it is possible that these factors contribute equally to the RSV LTR promoted transcription. However, under the conditions used in this study, the contribution of chk-YB-2 to LTR-promoted transcription is proved beyond doubt by using the antisense approach. The antisense oligonucleotides used in this study were designed specifically against chk-YB-2; they are not complementary to the chk-YB-1b mRNA and hence do not block its synthesis. Thus, the reduction in LTR-promoted transcription observed in our antisense experiments is a measure of the contribution of chk-YB-2, but not of chk-YB-1b, to LTR-promoted transcription. However, it remains possible that chk-YB-1b also contributes to the LTR-driven transcription, but under conditions different from those tested in this study. Another interesting possibility which remains to be explored is that chk-YB-1b is a transcriptional repressor whereas chk-YB-2 is a transcriptional activator. In such a situation, the competition between chk-YB-1b and chk-YB-2 for the common binding site determines whether the RSV LTR is transcribed.

It will be interesting to study the mechanism by which chk-YB-2 regulates transcription. Involvement of Y-box proteins in regulation of MHC class II genes is well known (1). NF-Y, which is required for class II MHC transcription, is thought to mediate its action by interacting with and stabilizing the binding of other proteins to the promoter (43). There is evidence that another Y-box protein, YB-1, regulates transcription of human polyomavirus JC through its interaction with the p65 subunit of NFκB (28). It is pertinent that YB-1 is known to promote the formation or stabilization of single-stranded regions in the DRA gene promoter (23). Y-box proteins are known to act as both transcriptional activators and repressors. The levels of YB-1 are known to be inversely related to the levels of MHC class II gene expression (7). Overexpression of YB-1 is known to repress the gamma interferon-mediated activation of MHC class II genes (40). We have shown that chk-YB-2 acts as a repressor of transcription in a cell system that is nonpermissive to RSV infection (3). In contrast, the results presented here indicate that chk-YB-2 acts as a positive regulator of RSV LTR-driven transcription in a cell system permissive to infection by RSV. Thus, the same protein acts as either a transcriptional activator or repressor in a context-dependent manner. It is possible that this context-dependent

behavior of chk-YB-2 is based on the presence or absence of some other factor(s) with which chk-YB-2 interacts either before or after binding to DNA.

The DNA binding characteristics of chk-YB-2 are interesting. Like some other Y-box proteins, it binds avidly to single-stranded DNA but not to double-stranded DNA. Binding to single-stranded DNA is sequence specific, with a marked preference for pyrimidine-rich sequences. Curiously, the same protein appears to bind with widely different affinities to two different sites on the LTR. Both the sites are pyrimidine rich and reside on the noncoding strand of the LTR. We have no clue as to what advantage is conferred to the RSV LTR by the presence of two adjacent sequences which are recognized by the same protein but with different affinities.

We have demonstrated that chk-YB-2 is capable of interacting with multiple sites on the LTR (Fig. 4 and 5). However, a significant reduction in LTR-promoted transcription was observed only in constructs having deletions or mutations in the E4 region (Fig. 6). Thus, the interaction of chk-YB-2 with E4 region alone appears to contribute significantly to LTR-promoted transcription. Binding of chk-YB-2 to other places on the LTR has no significant effect on the transcription from the LTR. Thus, the data presented in this report suggest that steric interactions of chk-YB-2 with other members of the transcription machinery play a major role in regulating RSV LTR-driven transcription in a manner reminiscent of the protein-protein interactions known to occur on the class II MHC gene promoter where these different sequence elements work in concert to direct high-level expression (reviewed in reference 1).

The RSV LTR is interesting for its unusually high density of protein recognition sequences in the U3 region. More than six protein factors are known to bind the LTR (30). What makes it much more interesting is the fact that many of these sites are repeated within a short stretch of DNA. For instance, there are two C/EBP binding sites (32), two inverted CCAAT boxes (Y boxes), two serum-responsive elements (2, 44), and two 5'-G CAACATG-3' motifs to which EF-IV binds (17), and now we have evidence that chk-YB-2 also binds to multiple positions on the RSV LTR—all within the U3 region of the LTR, which stretches for 180 bp of DNA. The presence of this unusually high density of protein binding sites on the U3 region of the LTR indicates that LTR-driven transcription is very well regulated and that it is highly fine-tuned to suit the varying physiological conditions that the virus may encounter.

While strengthening and explaining the earlier observations, our results provide an important insight into the regulation of RSV LTR-driven transcription in avian cells. In addition, our work raises several interesting questions. Does the region which chk-YB-2 binds in the LTR exist *in vivo* in a single-stranded state? If so, how far does this single-stranded region extend? Is this single-stranded nature an intrinsic property of the RSV LTR, or are there factors which generate or aid in the formation and/or stabilization of the single-stranded region once it is formed? Further work is necessary before we begin to understand these interesting aspects in the life cycle of RSV.

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ADDENDUM

We found that the name RSV-EF-II, which we had used to describe the protein reported in our recent manuscript (3), was earlier used by others to describe sequences on the RSV LTR. Potential confusion in this matter has been avoided by referring to our protein as chicken YB-2 (chk-YB-2) henceforth. The name of RSV-EF-I, which we had described earlier (18), also has been changed to chicken YB-1b (chk-YB-1b).

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