Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1873–1877, March 1991
Biochemistry

Affinity-purified CCAAT-box-binding protein (YEBP) functionally regulates expression of a human class II major histocompatibility complex gene and the herpes simplex virus thymidine kinase gene

(DNA-binding proteins/gene regulation/*in vitro* transcription/*HLA-DRA* gene)

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Communicated by Ray D. Owen, December 12, 1990 (received for review October 19, 1990)

ABSTRACT **Efficient major histocompatibility complex class II gene expression requires conserved protein-binding promoter elements, including X and Y elements. We affinity purified an *HLA-DRA* Y-element (CCAAT)-binding protein (YEBP) and used it to reconstitute Y-depleted *HLA-DRA in vitro* transcription. This directly demonstrates a positive functional role for YEBP in *HLA-DRA* transcription. The ability of YEBP to regulate divergent CCAAT elements was also assessed; YEBP was found to partially activate the thymidine kinase promoter. This functional analysis of YEBP shows that this protein plays an important role in the regulation of multiple genes.**

An understanding of major histocompatibility complex (MHC) class II gene regulation is important for understanding and modulating immune regulation (1, 2). Promoter elements important in the inducible and basal expression of class II MHC genes, including the V, W, X, Y, and octamer elements, have been defined by deletion and mutation analyses (3–13). These elements bind multiple proteins as determined by gel mobility shift analysis and by direct cloning from expression libraries (14–16). Most pertinent to this study is the class II box which is found in all MHC class II genes and consists of the X [15-base-pair (bp)] and Y (8-bp) elements separated by a spacer of 18–21 bp (see Fig. 1). The purpose of this study is to analyze the function of a Y-element-binding protein (YEBP) in transcription of the MHC class II *HLA-DRA* gene (called *DRA* hereafter).

The Y element contains a CCAAT box in reverse orientation to the direction of transcription. Proteins bind to the CCAAT sequence, and mutations within this element disrupt *in vitro* protein binding and *in vivo* function (6, 14, 17, 18). CCAAT-element-binding proteins all bind to a core "CCAAT" sequence, with flanking bases determining the binding heterogeneity of these proteins (17, 19, 20). Multiple CCAAT-binding proteins have been described, including CTF/NF1 (19), C/EBP (21), and CP1 and CP2 (20) among others. Binding specificities of the CCAAT-binding proteins CP1, CP2, and NF-1 (20) and NF-Y, NF-1, and C/EBP have been compared (17). In particular, the murine MHC class II Y-binding protein NF-Y was distinct from NF-1 and C/EBP by differential competition in gel mobility shift analysis (17). These comparisons demonstrate CCAAT-binding protein heterogeneity.

Although Y-binding proteins have been characterized by binding assays and described by limited biochemical analysis (18, 22, 23), direct functional analysis of these Y-binding proteins has not been described. Furthermore, it is not known if the proteins that bind to Y can also regulate through other CCAAT elements. Recent development of a human B-cell *in vitro* transcription system (8, 24) makes possible the direct functional analysis of MHC class II regulatory proteins.

In this paper we describe the partial purification of one human MHC class II Y-element (CCAAT)-binding protein (YEBP) and we directly assess the ability of YEBP to regulate *DRA* gene transcription. We also demonstrate that YEBP can regulate transcription from another CCAAT-containing promoter, the thymidine kinase (TK) gene of herpes simplex virus (HSV). The implications of these results will be discussed.

MATERIALS AND METHODS

Plasmids and Oligonucleotides. The 5′Δ-56(X+Y) plasmid, which contains 109 bp of the wild-type *DRA* promoter upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene, the 5′Δ-56, the 5′Δ-56mut1X, 5′Δ-56mutY, and 5′Δrev(X+Y) plasmids were described (18). The pDHF-210 plasmid contains 210 bp of the hamster dihydrofolate reductase (DHFR) promoter upstream of the CAT reporter gene (25). The plasmid TK71CAT, which contains 200 bp of the HSV TK promoter, and pH-2K^{b}Δ15, which contains base pairs −65 to +3 of the mouse MHC class I *H2K^b* gene, were kindly provided by A. Baldwin (University of North Carolina).

Double-stranded competitors were prepared as described (18). For competition, the sequences of the upper (5′ to 3′) strand of the double-stranded oligonucleotides are Y, TAAAAAGACTAACCGGTTTCT and Sp1, GATCGGGGCGGGGC. X+Y (50 bp), X+spacer (36 bp), and Y+spacer (33 bp) were as described previously (18).

Nuclear Extracts. Namalwa B-lymphoblastoid cells (from R. Roeder, Rockefeller University) and BALB/c 3T3 cells were used to prepare nuclear extracts, using a modification (25) of the Dignam procedure (26) with the following addition. Protease inhibitors at final concentrations of 2 mM 3,4-dichloroisocoumarin, 2 mM 1,10-phenanthroline, and 0.4 mM E-64 (I-mix) (Pharmacia LKB) were added to buffers C and D. The extract was frozen in aliquots at −70°C.

***In Vitro* Transcription Assays.** Transcription reactions (25 μl final volume) were performed essentially as described (25). Template DNA was linearized with *Nco* I for all plasmids, except pDHF-210 plasmid, which was digested with *Sca* I, to produce discrete-sized transcripts. An internal standard radiolabeled 494-nucleotide Sp6 transcript (supplied by R. Kole, University of North Carolina) was added to each

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Abbreviations: MHC, major histocompatibility complex; YEBP, Y-element-binding protein; TK, thymidine kinase; HSV, herpes simplex virus; CAT, chloramphenicol acetyltransferase; DHFR, dihydrofolate reductase; BSA, bovine serum albumin.

sample to detect differences in recovery. Size markers were an end-labeled 0.16–1.77-kilobase (kb) RNA ladder (BRL). Transcription reactions were reconstituted with affinity-purified YEBP (200 ng), AC100 buffer (see below), 3 μg of bovine serum albumin (BSA) in AC100, a protein-containing flow-through affinity column fraction, or 3 μg of unfractionated Namalwa nuclear extract in AC100. Transcript production was quantitated by densitometry of autoradiographs using an LKB Ultrascan densitometer.

Affinity Purification of YEBP. Affinity columns were prepared (27), using 50-bp X+Y oligonucleotides (18). Namalwa nuclear extract was first fractionated by heparin-agarose (Sigma) chromatography. YEBP activity, monitored by gel mobility shift reactions with the X+Y probe, was eluted with a linear gradient from HA100 [25 mM Tris·HCl, pH 8/20% (vol/vol) glycerol/0.5 mM dithiothreitol/0.2 mM EDTA/100 mM KCl/I-mix] to HA500 (HA100 except 500 mM KCl) and the fractions were dialyzed against HA100. YEBP activity was quantitated by scintillation counting of shifted bands and free probe in the gel shift assay. YEBP-containing fractions were chromatographed over an X+Y-affinity column twice, and YEBP activity was eluted with a linear gradient of AC100 (HA100/0.1% Nonidet P-40) to AC2000 (2 M KCl). BSA (0.3 mg/ml) was added to the fractions before dialysis against AC100. Aliquots were stored at −70°C.

RESULTS

Accurate Transcription from a *DRA* Template *in Vitro* Requires Both X and Y Elements. Nuclear extracts from the Namalwa human B-cell line supplied factors necessary for transcription from the MHC class II *DRA* promoter *in vitro*. The template for transcription was the plasmid 5′Δ-56(X+Y), which contains 109 bp of the wild-type *DRA* promoter (the basal promoter) fused to the CAT reporter gene (Fig. 1) (18). The plasmid was digested with *Nco* I, which cleaves in the CAT reporter gene, resulting in a 595-base run-off transcript (Fig. 2, lane 2). In contrast, no detectable transcript was obtained from the 5′Δ-56 plasmid, which lacks the X and Y elements (Fig. 2, lane 1).

Elements defined by transient transfection analysis do not necessarily function in transcription systems *in vitro* (28, 29). To determine whether these elements regulate transcription *in vitro*, plasmids containing either mutated X element or mutated Y element were analyzed (Fig. 1). Mutation of the X element resulted in a 95% decrease in transcription as determined by densitometry scanning of autoradiographs (Fig. 2, lane 3). Mutation of the Y element resulted in a 90% decrease in transcription (Fig. 2, lane 4). The orientation of X and Y elements must also be conserved *in vitro*, since a plasmid with the X, spacer, and Y elements in reverse orientation, 5′Δ-56rev(X+Y), yielded greatly reduced transcription product (Fig. 2, lane 5). These results parallel previous *in vivo* findings (18).

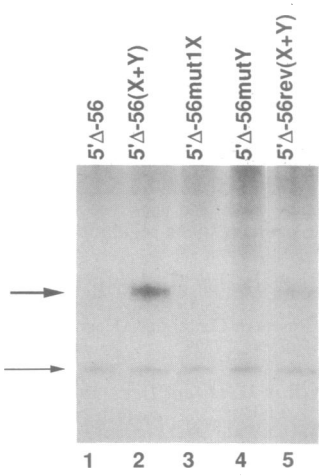

FIG. 2. Mutation of X or Y elements decreases *DRA* transcription. *In vitro* transcription samples using wild-type or mutant template DNA linearized with *Nco* I were electrophoresed on a denaturing 4% polyacrylamide gel. Correctly initiated *DRA* transcript is 595 nucleotides (upper arrow). Transcript level is quantitated relative to a radiolabeled internal standard 494-nucleotide transcript (lower arrow). Template plasmids used are indicated.

Oligonucleotides Representing *DRA* Promoter Elements Compete for *DRA* Transcription Factors *in Vitro*. Efficient DRA transcription depends on intact X and Y elements, which bind to proteins as shown by gel shift analysis (18). This suggests that disrupting protein binding to these elements might disrupt transcription. To functionally deplete transcription extracts of X- and Y-binding proteins, an excess of double-stranded oligonucleotides corresponding to the X and Y elements was added to an *in vitro* transcription extract. An alternative approach employing double-stranded oligonucleotide-coupled Sepharose to physically deplete the specific DNA-binding proteins was tested, but it did not work well because this manipulation significantly (more than 90%) reduced general transcription (N.J.Z.-L., unpublished observations).

In vitro DRA transcription from the 5′Δ-56(X+Y) *DRA* template (Fig. 3, lanes 1 and 11) was specifically reduced by the addition of excess competitor corresponding to the elements X (lanes 2–4), Y (lanes 5–7), or X+Y (lanes 8–10) in a dose-dependent fashion. Higher amounts of competitor resulted in transcription from incorrect initiation sites (lane 4). This observation is consistent with our previous S1 nuclease mapping of transient transfectants, which showed

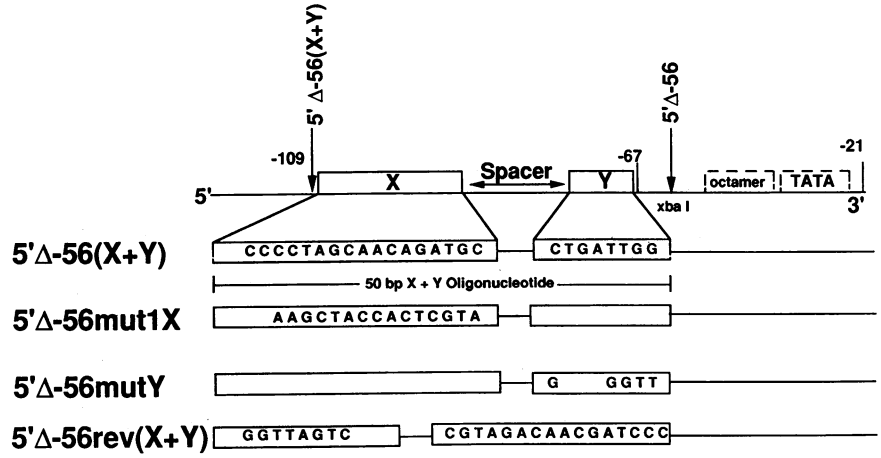

FIG. 1. Schematic representation of the *DRA* promoter and mutant plasmids. The promoter elements X, Y, octamer, and TATA are contained in 109 bp of the *DRA* promoter. The 5′Δ-56 plasmid contains 56 bp of the *DRA* promoter upstream of the CAT gene. 5′Δ-56(X+Y) has the 50-bp X+Y oligonucleotide inserted upstream of the octamer element into the 5′Δ-56 plasmid. 5′Δ-56mut1X and 5′Δ-56mutY have the indicated base substitutions in the X and Y elements, respectively. 5′Δ-56rev(X+Y) has the 50-bp X+Y oligonucleotide inserted in reverse orientation into the 5′Δ-56 plasmid.

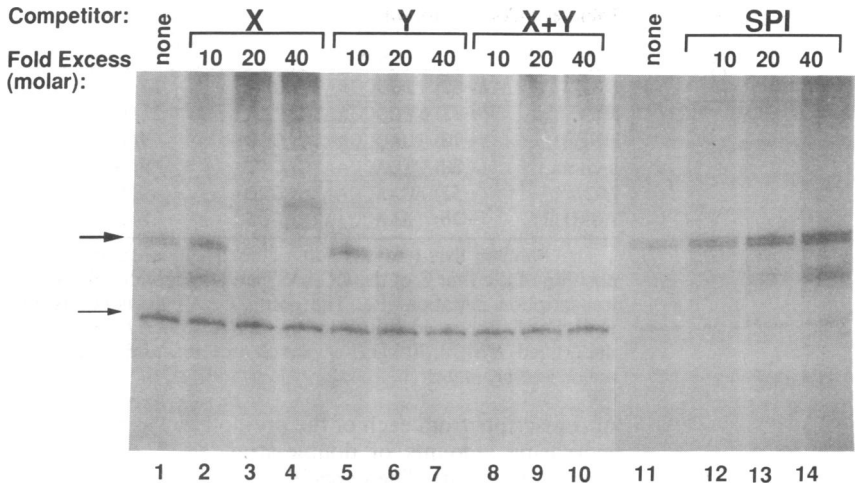

FIG. 3. Specific oligonucleotide competition of *DRA* transcription. Increasing quantities (10- to 40-fold molar excess) of double-stranded oligonucleotides corresponding to the *DRA* X element (lanes 2–4), Y element (lanes 5–7), X+Y elements (lanes 8–10), or to the Sp1 element (lanes 12–14) were added to *in vitro* transcription assays with the wild-type *DRA* template, 5′Δ-56(X+Y). The level of *DRA* transcript (upper arrow) was compared between reactions with and without added competitors, relative to an internal standard radiolabeled 494-nucleotide transcript (lower arrow). Lanes 1–10 and lanes 11–14 were from different gels.

that mutations in X or Y elements resulted in aberrant *DRA* transcripts (18).

As a specificity control, we used a competitor corresponding to the Sp1 element, which is not present in the *DRA* promoter. It does not decrease *DRA* transcription (lanes 12–14) and actually reproducibly increases the level of DRA transcript. In addition, Y oligonucleotide does not compete with transcription from an unrelated plasmid containing the hamster DHFR promoter (25) at the same concentrations (see Fig. 5*B*, lane 3).

Reconstitution of *DRA* Transcription with Affinity-Purified YEBP. We next tested purified YEBP for its ability to reconstitute the Y-depleted transcription system. YEBP was enriched from Namalwa nuclear extracts by chromatography on heparin-agarose, followed by two passages over an X+Y-Sepharose column (Table 1). The resulting highly enriched YEBP fraction was assayed for specificity by the gel mobility shift assay (Fig. 4). The purified fraction had seven protein bands by silver stain of an SDS/polyacrylamide gel (data not shown). Efforts to elute and renature individual protein bands alone and in combination resulted in loss of Y-element-binding activity. Therefore, for further studies, the partially purified YEBP affinity column fraction was used. YEBP protein was purified 3400-fold with an increase in the specific activity from 68 to 45,200 fmol of [^{32}P]DNA bound specifically per mg of protein and an overall yield of 19% (Table 1). This preparation was used for the reconstitution experiments described below.

The amount of Y oligonucleotide competitor added to functionally deplete the extract was titrated to achieve 50–80% reduction in transcription. Utilizing suboptimal levels of competitors was important to ensure that a positive or negative effect of added YEBP in transcription would be observed. Subsequently, affinity-purified YEBP was added to the functionally depleted extract. Purified YEBP protein could reconstitute *in vitro* transcription (Fig. 5*A*, lane 5). The level of reconstitution averaged 49% (range 28% to 69% from four experiments) as determined by densitometry. The experimental samples were quantitated relative to the level of internal 494-nucleotide standard. In contrast, buffer alone (lane 2) or a flow-through column fraction (lane 3) was unable to reconstitute transcription. In fact, the added flow-through column fraction inhibited *DRA* transcription. Addition of 3 μg of crude nuclear extract (lane 4) minimally reconstituted DRA transcription (average of 5% in three experiments), whereas addition of buffer containing 3 μg of BSA had no effect on the level of *DRA* transcript (compare lanes 7 and 8).

To determine if the effect of YEBP on *DRA* transcription was specific, similar experiments were done with a plasmid containing the DHFR promoter. The DHFR promoter does not contain any elements resembling the *DRA* Y element, and Y competitor does not block DHFR transcription (Fig. 5*B*, lane 3). Addition of purified YEBP to the DHFR template in the presence of excess Y competitor also had no effect on DHFR transcription (lane 4). As a second level of control, specific synthesis of DHFR transcripts (lanes 1 and 5) was blocked with excess Sp1 competitor (lanes 2 and 6) (25). However, addition of YEBP could not reconstitute the transcription of this gene (Fig. 5*B*, lane 8). Therefore, the enriched YEBP reconstitutes *DRA* transcription in a specific fashion.

Regulation of Other CCAAT-Element-Containing Promoters by YEBP. The Y element in the promoter of all MHC class

Table 1. Affinity purification of YEBP

Fraction	Protein, mg	Vol, ml	Specific activity*	Purif., fold	Yield, %
Nuclear extract	813	132	68	1	100
Heparin-agarose	113	96	515	7.2	96
First X+Y affinity	2.3	53	15,900	359	64
Second X+Y affinity	0.24	10	45,200	3400	19

*[^{32}P]DNA bound specifically, fmol/mg of protein.

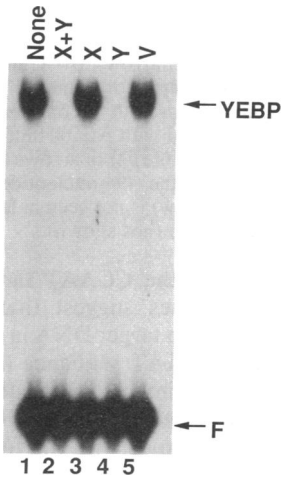

FIG. 4. Gel mobility shift analysis of highly enriched YEBP. Samples of the highly enriched YEBP-containing fraction and a ^{32}P-labeled *DRA* X+Y oligonucleotide were electrophoresed on a nondenaturing 6% polyacrylamide gel. Shifted YEBP-oligonucleotide complex (YEBP) and free oligonucleotide probe (F) are indicated. Competitors (200-fold molar excess) were added to the gel shift reaction mixture to assess the specificity of YEBP. Competitors used were none (lane 1), X+Y (lane 2), X+spacer (lane 3), Y+spacer (lane 4), and a nonspecific competitor corresponding to base pairs −153 to −179 of the wild-type *DRA* promoter (lane 5).

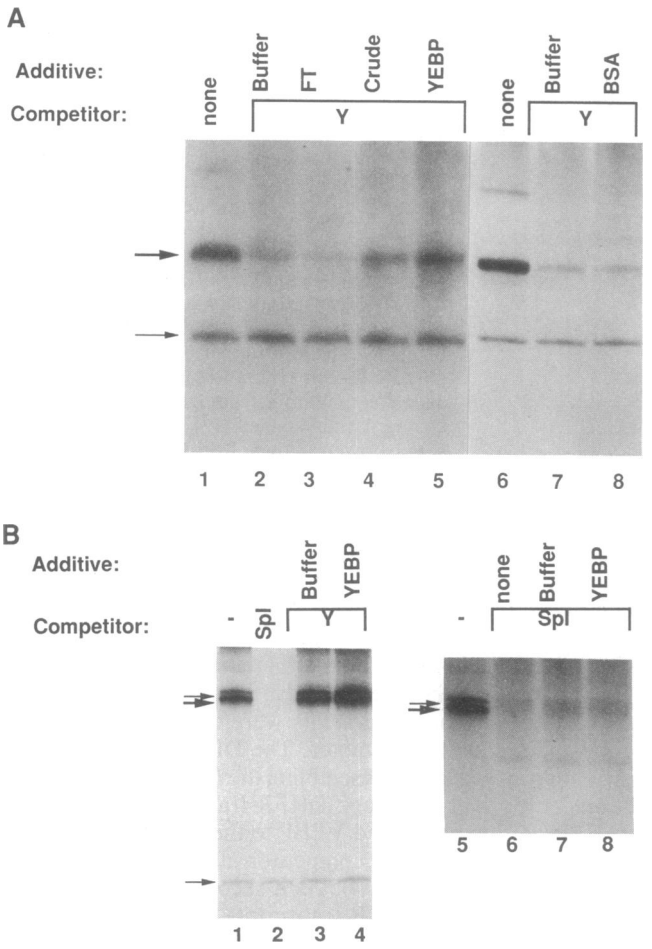

FIG. 5. Specific reconstitution of *DRA* transcription with purified YEBP. (*A*) Transcription of *DRA* template (upper arrow) without competitor (lanes 1 and 6) or with Y competitor (lanes 2–5, 7, and 8). Transcription was reconstituted with AC100 buffer (Buffer) (lanes 2 and 7), with an affinity column fraction lacking YEBP (flow-through, FT) (lane 3), with 3 μg of crude nuclear extract (Crude) (lane 4), with 3 μg of BSA (lane 8), or with 200 ng of highly enriched YEBP (lane 5). Lanes 1–5 and lanes 6–8 are from different experiments. *DRA* transcript levels were quantitated relative to an internal standard 494-nucleotide transcript (lower arrow). (*B*) Transcription of control DHFR template without competitor (lanes 1 and 5), with Spl competitor (lanes 2 and 6–8), or with Y competitor (lanes 3 and 4). Transcription was reconstituted with AC100 buffer (lanes 3 and 7) or with YEBP (lanes 4 and 8). Double upper arrows designate major and minor start sites. Note that the 494-nucleotide internal standard shown in lanes 1–4 (lower arrow) is not seen in lanes 5–8, but it was observed on a longer exposure (not shown).

II genes is a member of the CCAAT family of promoter elements. Gel-shift analyses suggest that the binding of CCAAT-binding proteins to target DNA is influenced by the sequence flanking the CCAAT sequence (17, 19). The purpose of the following experiments was to determine if YEBP could reconstitute transcription from other CCAAT element-containing templates. Plasmids with the following CCAAT-containing promoters were used: human ε-globin, 70-kDa human heat shock protein, HSV TK, adenovirus major late promoter, and MHC class I *H-2K* genes (Table 2). All of these promoters contain a CCAAT element needed for transcription, but with variable flanking sequence. Homology to the *DRA* CCAAT sequence is shown (Table 2).

We addressed the capacities of the *DRA* Y oligonucleotide to compete with transcription and of enriched YEBP to reconstitute transcription from these different promoters. Except for adenovirus major late promoter, *in vitro* synthesis

Table 2. Comparison of CCAAT elements

Gene	Sequence	*DRA* identity	Ref.
DRA	(−67) TTGGCCAATCAGAA	14/14	5
HSV TK	(−82) TTCGCCAATGACAA	11/14	20
HSP70	(−70) TGAGCCAATCACCG	9/14	20
ε-Globin	(−80) TTGACCAATGACTT	9/14	30
H-2K^b	(−51) AGAACCAATCAGTG	8/14	20
AdMLP	(−76) TAAACCAATCACCT	8/14	20

The number listed to the left of each sequence indicates the position of the first C of the CCAAT pentanucleotide relative to the transcription initiation site. The core CCAAT sequence is double underlined. Bases identical to the *DRA* CCAAT sequence are underlined. HSP70, 70-kDa heat shock protein; AdMLP, adenovirus major late promoter.

of transcripts from each of the promoters was inhibited with increasing amounts of double-stranded Y oligonucleotide (data not shown). The capacity of purified YEBP to reconstitute transcription from the above promoters was examined by using the same strategy described for *DRA* (Fig. 5). Of all the constructs tested, enriched YEBP could reconstitute transcription only from the HSV TK promoter (Fig. 6, lane 6). The reconstitution was low but reproducible, averaging 9% (range 8% to 10%). Reactions reconstituted with buffer (lane 3), with protein-containing flow-through column fraction (lane 4), or with unfractionated nuclear extract (lane 5) did not reconstitute HSV TK expression. In contrast, YEBP was unable to reconstitute *DRA* transcription from the MHC class I promoter (Fig. 6, lanes 7–12) or from the other promoters mentioned above (data not shown).

DISCUSSION

Several X- and Y-element-binding proteins have been identified by gel mobility shift analysis and by cloning, but the role of these specific proteins in MHC class II transcription has not yet been identified. The type of *in vitro* transcription analysis described in this paper enables the direct functional analysis of purified or cloned proteins alone or in combination. Only after such functional analysis will we begin to understand the requisite protein–DNA and protein–protein interactions necessary for accurate and efficient MHC class II gene transcription.

The experiments described here were designed to ascertain the role of YEBP, a *DRA* Y (CCAAT)-element-binding protein, in MHC class II gene transcription. YEBP is likely similar to murine NF-Y (6) and to the Y proteins described by other laboratories (11, 23). Results of *in vitro* transcription analysis (Fig. 2) confirmed previous *in vivo* results that the X and Y elements are required for efficient *DRA* transcription (18). In addition, functional depletion of YEBP activity decreased the level of specific *DRA* transcript (Fig. 3). Most importantly, we directly demonstrated a positive functional role for YEBP in *DRA* transcription. We know of no earlier direct functional analysis of any purified or cloned MHC class II regulatory protein.

YEBP is of broad importance because it binds to a CCAAT element. CCAAT-element-binding proteins are a family of proteins; however, their functional specificities in gene expression have not been determined. Here we examined whether YEBP could function in the transcription of other CCAAT-containing promoters. These promoters varied in the extent of similarity in the regions immediately adjacent to the CCAAT sequence (Table 2). In addition to the *DRA* promoter, YEBP could partially reconstitute transcription from the HSV TK promoter. Analysis of the murine MHC class II Eα binding protein NF-Y had demonstrated that its binding activity was partially blocked with a HSV TK CCAAT competitor (17). This might be analogous to the

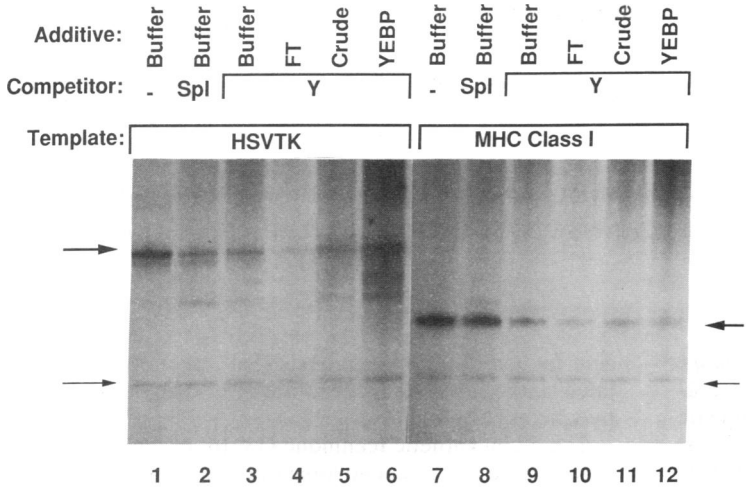

FIG. 6. Capacity of YEBP to reconstitute transcription from the TK promoter but not from another CCAAT element-containing promoter. Correct transcripts from the CCAAT element-containing promoters HSV TK and MHC class I are designated by the upper arrows. Transcript synthesis was in the absence of competitor (lanes 1 and 7) or in the presence of Sp1 competitor (lanes 2 and 8) or Y competitor (lanes 3–6 and 9–12). The transcription reactions were reconstituted with AC100 buffer (Buffer), with an affinity column fraction lacking YEBP (FT), with 3 μg of crude nuclear extract (Crude), or with 200 ng of highly enriched YEBP. Transcript levels were quantitated relative to an internal standard 494-nucleotide transcript (lower arrows).

results we observed, since YEBP partially reconstituted Y-depleted transcription from the TK promoter (Fig. 6) but could more fully reconstitute *DRA* transcription (Fig. 5*A*). Interestingly, both MHC class II and TK genes are cell cycle regulated (31–33). YEBP also has a role in the cell cycle regulation of gene expression (N.J.Z.-L., unpublished data).

There are several possible reasons why *DRA* Y competitor could block transcription from several CCAAT-containing promoters but YEBP could not reconstitute transcription from most of these promoters. One explanation is that proteins that normally bind to other CCAAT elements cross-react with the *DRA* Y (CCAAT) competitors and that this binding is not physiologically relevant. Another possibility is that the different CCAAT-binding proteins contain a common DNA-binding domain but different activation domains. This second possibility seems more likely, since YEBP could bind to the CCAAT elements from *DRA*, MHC class I *H-2K^b*, HSV TK, and adenovirus major late promoter by the gel mobility shift assay (data not shown). This suggests that although YEBP could bind to each of these CCAAT elements, transcription could be activated from only a subset of them. Although we have not directly addressed whether YEBP added to our depleted system acts indirectly by decreasing the effective level of competitors, we feel that this interpretation is unlikely. If YEBP were acting in this manner, one would have expected YEBP to increase the transcription from each of the CCAAT-containing promoters tested, since YEBP is capable of binding to the various promoters. This was not observed.

In this report we described the partial purification of YEBP, a human MHC class II Y-element (CCAAT)-binding protein. Second, and most importantly, we directly demonstrated that YEBP positively regulates *DRA* transcription as well as transcription from another CCAAT-element-containing promoter. Thus, YEBP could have a broad role in the regulation of multiple genes.

The authors gratefully acknowledge Drs. A. Baldwin, S. Bachenheimer, P. Sherman, T. Tlsty, and J. Vos for reviewing the manuscript; and Drs. A. Baldwin and R. Kaufman for CCAAT-element-containing plasmids. This work was supported by National Institutes of Health Fellowships 5T32CA09156-14 and IF32-CA08857 (N.J.Z.-L.), National Institutes of Health Grant RO1-CA48185, American Cancer Society Grant IM-591, a Jefferson Pilot Award (J.P.-Y.T.), and American Cancer Society Grant CD-317A,B (J.C.A.).

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