THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 267, No. 11, Issue of April 15, pp. 7677-7682, 1992
Printed in U.S.A.

The B Cell-specific Nuclear Factor OTF-2 Positively Regulates Transcription of the Human Class II Transplantation Gene, DRA*

(Received for publication, November 22, 1991)

Nancy J. Zeleznik-Le‡, Yoshie Itoh-Lindstrom§, Jane Badley Clarke, Terry L. Moore, and Jenny P.-Y. Ting¶

From the Lineberger Comprehensive Cancer Center and the Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599-7295

The promoter of the major histocompatibility class II gene DRA contains an octamer element (ATTTGCAT) that is required for efficient DRA expression in B cells. Several DNA-binding proteins are known to bind this sequence. The best characterized are the B cell-specific OTF-2 and the ubiquitous OTF-1. This report directly demonstrates that OTF-2 but not OTF-1 regulates the DRA gene. *In vitro* transcription analysis using protein fractions enriched for the octamer-binding protein OTF-2 demonstrate a positive functional role for OTF-2 in DRA gene transcription. In contrast, OTF-1-enriched protein fractions did not affect DRA gene transcription although it functionally enhanced the transcription of another gene. Recombinant OTF-2 protein produced by *in vitro* transcription/translation could also enhance DRA gene transcription *in vitro*. *In vivo* transient transfection studies utilizing an OTF-2 expression vector resulted in similar findings: that OTF-2 protein enhanced DRA gene transcription, and that this effect requires an intact octamer element. Together these results constitute the first direct evidence of a positive role for the lymphoid-specific octamer-binding factor in DRA gene transcription.

Major histocompatibility (MHC)[1] class II proteins control the level of the immune response by functioning as ligands for the activation of antigen-specific T lymphocytes (1, 2). In addition, class II proteins transmit signals which activate B lymphocytes (3). MHC class II protein expression is limited to a few cell types, most notably on B cells, and is subject to transcriptional gene regulation. The transcriptional regulation of class II expression is mainly mediated by elements in their promoters, presumably via proteins that bind to these elements. Several cis-acting elements critical for MHC class II expression have been defined in B lymphocytes and other cell types including the W, X, and Y elements (4). Furthermore, the class II DRA promoter contains an octamer element, ATTTGCAT, that is required for its efficient expression in B cells (5, 6) (see Fig. 1*A*). This octamer element is identical to the octamer elements important for the expression of a variety of other genes including the immunoglobulin (Ig) light and heavy chains (7, 8), the human histone H2B (9), and the U1, U2, and U4 snRNA genes (10). The octamer element has therefore been determined to be a functional element for genes expressed in restricted cell types as well as for genes expressed ubiquitously.

Nuclear proteins have been defined that bind specifically to the octamer element. These include both the ubiquitous protein OTF-1 (also referred to as oct-1, NF-A1, OBP100, NFIII, and oct-B3) (11–17) and OTF-2, classically referred to as a lymphoid-restricted protein (also referred to as oct-2, NF-A2, oct-2A, and oct-B2) (13, 14, 16, 18, 19). Oct-3 through oct-7 have been identified in various mouse tissues at different stages of development (20, 21), and related proteins have been identified in adult brain (22). Since oct-3 through oct-7 are not present in the cell types used in this study, they will not be discussed further.

Both the OTF-1 and OTF-2 proteins bind to the octamer element with identical methylation interference and DNase I footprinting patterns (11, 19). Both proteins have been purified from cells (11, 19) and the corresponding genes have been isolated (17, 23–27). Because of its restricted tissue distribution, OTF-2 was hypothesized to be at least partially responsible for the restricted cell type expression of the Ig gene (16, 18, 19) and the MHC class II gene, DRA (5). In fact, it has been shown that purified OTF-2 can activate transcription of Ig heavy and light chain promoters *in vitro* (19, 28, 29). It was also demonstrated that OTF-1 could activate transcription of these same Ig promoters *in vitro*, but less efficiently (28, 29). The difference in OTF-2's *versus* OTF-1's ability to activate transcription from these two promoters has been suggested to be due to the requirement for an additional B-cell-specific component (29, 30) or due to quantitative differences in the amount of octamer-binding protein present (28). The context of the octamer element within the promoter may also be important (28). It has been hypothesized that the B cell-specific octamer-binding protein, OTF-2, is required for MHC class II DRA promoter function in B cells, but this has yet to be shown directly.

In this report, we demonstrate a functional role for the octamer-binding protein OTF-2 in MHC class II gene expression using both *in vitro* transcription and *in vivo* transient

* This work was supported in part by Grants CA 48185 and AI 29564 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Fellowship 5F32 CA08857 and is a Leukemia Society of America Fellow. Present address: Dept. of Medicine, University of Chicago, 5841 S. Maryland Ave., Box 391, Chicago, IL 60637.

§ Supported by a National Institutes of Health training grant and National Institutes of Health Fellowship 1F32 DK08575 postdoctoral award.

¶ To whom correspondence should be addressed: Lineberger Comprehensive Cancer Center, CB 7295, University of North Carolina, Chapel Hill, NC 27599-7295. Tel.: 919-966-5538; Fax: 919-966-3015.

[1] The abbreviations used are: MHC, major histocompatibility complex; CAT, chloramphenicol acetyltransferase; OBP, octamer-binding proteins; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; CMV, cytomegalovirus, early promoter; bp, base pair; TH2A, testis H2A histone; kb, kilobase.

transfection systems. This is similar to the positive functional role for OTF-2 observed in Ig gene transcription (19, 28, 29). In contrast to the Ig system, however, OTF-1 appears not to play a role in transcription from the MHC class II DRA promoter in a B cell system, whereas it can activate transcription of the testis H2A histone (TH2A) gene in our system. These findings underscore that even though there are several similarities between the Ig and the DRA genes, including their expression in B lymphocytes, there are significant differences in their transcriptional regulation. The differences observed may be due to the context of the octamer element in the promoters of these genes. The octamer element upstream of a TATA box seems sufficient to confer B cell-specific promoter activity of Ig genes (31, 32), whereas MHC class II genes require additional promoter elements for expression (33, 34).

MATERIALS AND METHODS

Plasmids, Probes, and Competitors—The construction of plasmids pWToct and pMUToct (Fig. 1*A*) have been described in detail (5). They contain wild type DRA X and Y elements and either a wild type or mutant octamer element placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene.

The human OTF-2 cDNA was isolated by polymerase chain reaction-based cloning from a human B lymphoblastoid cell line, Raji, using published DNA sequences (26). First strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase (Life Sciences Biologicals, St. Petersburg, FL) from 2 μg of oligo(dT) (Boehringer Mannheim Biochemicals, Indianapolis, IN; 10-mer) primed mRNA isolated by batch elution (35) from 10^8 log phase Raji cells. Five percent of the reaction mixture was subjected to 35 cycles of amplification by polymerase chain reaction in a DNA thermal cycler (Perkin-Elmer Cetus Corp., Emeryville, CA) using 50 pM each kinased primers of the sequence 5′-ACGATGGTTCACTCCAGCATGG-3′ and 5′-CTGCCATCAGGCTGGTAAGG-3′ (26). The temperature profile of amplification was 94 °C, 5 min, 55 °C, 1 min, 72 °C, 1 min for 1 cycle; 94 °C, 30 s, 55 °C, 30 s, 72 °C, 1 min for 30 cycles; and 94 °C, 30 s, 55 °C, 30 s, 72 °C, 7 min for 1 cycle. Products were separated on a 2% Nuseive low melt-agarose gel (Seakem, FMC Corp., Rockland, ME). Three microliters of remelted agarose containing the 1.4-kb DNA band predicted for OTF-2 was directly ligated to dephosphorylated *Sma*I-digested pGEM3Z (Promega, Madison, WI). Sequence of the resulting clone determined by dideoxy chain termination (36) was identical to the published sequence (26).

To obtain the OTF-2 expression vector, human OTF-2 cDNA cloned into pGEM3Z (described above) was digested with *Kpn*I and *Xba*I in the polylinker region to release the 1.4-kb OTF-2 cDNA, which was isolated electrophoretically and then electroeluted from the gel. The OTF-2 cDNA was then subcloned into the compatible cloning site of the plasmid pCMV5 (37). pCMV5 was a gift from D. W. Russell (University of Texas Southwestern Medical Center) and contains the human cytomegalovirus early promoter (CMV), a linker containing multiple cloning sites, and transcription termination and polyadenylation signals from human growth hormone (Fig. 1*B*). Ligation was performed at room temperature for 4 h in a 20-μl volume. The reaction was stopped by adding 1 μl of 0.5 M EDTA, pH 8.0. Southern and sequencing analyses revealed that the 1.4-kb OTF-2 was properly subcloned into pCMV5. This clone was named plasmid pCMV5-OTF-2.

The DNA probes and competitors used for gel mobility shift assays consisted of complementary oligonucleotides that were resuspended in annealing buffer (38), boiled 5 min, and allowed to cool slowly to room temperature to anneal. Probes were radiolabeled using T4 polynucleotide kinase (U. S. Biochemicals, Cleveland, OH) and [γ-^{32}P]ATP prior to annealing and purification on a NENsorb column (Du Pont-New England Nuclear, Boston, MA). The sequence of strand 1 of WToct is: 5′-AGAGTAATTGATTTGCATTTTAA-TGGTCAG-3′. The sequence of strand 1 of MUToct is: AGAGTAA-TTGCGGGTCATTTTAATGGTCAG-3′, where the mutated sequence is underlined.

Preparation of Nuclear Extracts and Octamer-binding Protein (OBP) Enrichment—Nuclear extracts were prepared using a modification (39) of the Dignam procedure (40). 2 Liters of log phase cells were pelleted, washed twice in cold phosphate-buffered saline, and resuspended in 5 packed cell volumes of Buffer A (10 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl). Cells were allowed to swell 5 min on ice, pelleted, and resuspended in 2 packed cell volumes of Buffer A. Cells were lysed using the B pestle of a Dounce homogenizer until 95% lysis had occurred as determined by trypan blue dye exclusion. Nuclei were separated by quick centrifugation at 16,000 rpm in a Beckman SS34 rotor for 30 s. The pellet was resuspended in 1 ml of Buffer C/10^9 cells (20 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 20% glycerol, 0.1 mM 1,10-phenanthroline, 0.1 mM 3,4-dichloroisocoumarin, 0.02 mM E-64). NaCl was then added to a final concentration of 0.4 M while vortexing the extract. The nuclei were agitated 20 min at 4 °C and the extract centrifuged at 150,000 × *g* for 90 min. The extract was dialyzed 2 × 90 min against 100 volumes of Buffer D (20 mM Hepes, pH 7.9, 17% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 12.5 mM $MgCl_2$, 2 μM 1,10-phenanthroline, 2 μM 3,4-dichloroisocoumarin, 0.4 μM E-64). Extracts were then centrifuged 20 min at 16,000 rpm in a Beckman SS34 rotor and the supernatant stored in small aliquots at −70 °C. Extracts were not freeze/thawed more than two times for use in *in vitro* transcription reactions. Protein concentrations were determined using the Bio-Rad protein assay (Richmond, CA).

Octamer-binding proteins were enriched by incubating 1 ml of nuclear extract with 1 ml of octamer-conjugated Sepharose affinity matrix. The affinity matrix was prepared according to the method of Kadonaga and Tjian (41) using concatamers of the double stranded oligonucleotides; 5′-GATCAGAGTAATTGATTTGCATTTTAA-TGGTC-3′ and 3′-TCTCATTAACTAAACGTAAAATTACCAGC-TAG-5′, to couple to the matrix. Extract was incubated with affinity matrix and rotated end-over-end for 1 h at 4 °C. Octamer-binding proteins were eluted with 200 μl of AC1000 buffer (Buffer D with 1 M KCl) and dialyzed against 100 volumes of Buffer D using a BRL microdialyzer (Gaithersburg, MD). Extracts were checked for complete depletion of octamer-binding proteins by gel mobility shift assay.

In Vitro Transcription/Translation of Recombinant OTF-2 Protein—*In vitro* transcription/translation of recombinant OTF-2 was performed as described by the manufacturer of the kit (Promega, Madison, WI). For transcription, 20 μl of 5 × transcription buffer, 10 μl of 100 mM DTT, 100 units of RNasin, 20 μl of 2.5 mM each of ATP, GTP, CTP, and UTP, 2 μl of plasmid linearized with either *Bam*HI or *Eco*RI (3 μg), 50 units of SP6 or T7 polymerase, and H_2O to a 100-μl final volume were incubated at 37 °C for 90 min. The DNA template was removed following transcription by adding RQ1 RNase-free DNase to a concentration of 1 unit/μg of DNA, incubating for 30 min at 37 °C, extracting sequentially with phenol:chloroform and chloroform, and precipitating the RNA with ethanol. RNA was resuspended in H_2O and stored at −70 °C. For *in vitro* translation of OTF-2 protein, 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI), 7 μl of H_2O, 1 μl of RNasin ribonuclease inhibitor (at 40 units/μl), 1 μl of 1 mM amino acid mixture (minus methionine), 2 μg of RNA substrate in H_2O that had been heated at 67 °C for 10 min and immediately cooled on ice to decrease secondary structure, and 4 μl of [^{35}S]methionine (1200 Ci/mmol) at 10 mCi/ml (Du Pont-New England Nuclear) or 4 μl of unlabeled methionine were mixed in a final volume of 50 μl and incubated at 30 °C for 60 min. Translation products were analyzed on 10% SDS-polyacrylamide gels and by gel mobility shift assay, and were stored at −70 °C. Translation products were chromatographed on Sephadex G-25 or G-10 prior to use to reconstitute *in vitro* transcription reactions.

Gel Mobility Shift and in Vitro Transcription—Gel mobility shift reactions were performed essentially as described (15). DNA binding was allowed to proceed for 30 min at room temperature in a 20-μl volume containing either 3 μg of nuclear extract or 2 μl of *in vitro* transcribed/translated protein, 20,000–50,000 cpm of radiolabeled probe, 1 μg of poly(dIdC)-poly(dIdC) (Pharmacia LKB, Piscataway, NJ) in a buffer containing 20 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 2.5 mM $MgCl_2$. Double stranded DNA competitor, when used, was added at 100-fold molar excess. Protein-DNA complexes were separated from free probe by electrophoresis on a 6% nondenaturing polyacrylamide gel in Tris glycine buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.3). After drying the gel, complexes were visualized by autoradiography.

Transcription reactions were performed essentially as described (39). The plasmids used as templates for *in vitro* transcription, pWToct and pMUToct, are schematically drawn in Fig. 1*A* and have been described in detail (5). They contain wild type DRA X and Y

elements and either a wild type or mutant octamer element upstream of the bacterial CAT gene. The TH2A-CAT plasmid contains 248 bp of the rat testis H2A histone promoter upstream of the CAT gene (42) and was kindly provided by Dr. Chi-Bom Chae (University of North Carolina, Chapel Hill, NC). The adenovirus major late promoter-CAT plasmid contains 170 bp of the adenovirus major late promoter upstream of the CAT gene (43), and was kindly provided by Dr. Al Baldwin (University of North Carolina, Chapel Hill, NC). Template DNA was linearized with *Nco*I, and precipitated prior to use in the reactions. The reaction consisted of template DNA (1.5 μg), 0.4 mM each ATP, UTP, and CTP, 1 mM creatine phosphate, 0.14 mM EDTA, 1 μl of [α-^{32}P]GTP, and 15 μl of extract in a final volume of 25 μl. Transcription was allowed to proceed for 60 min at 30 °C. The reaction was stopped by adding 275 μl of stop solution (8 M urea, 0.5% SDS, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA) plus 1000 cpm of an internal standard radiolabeled 494-nucleotide Sp6 transcript to detect differences in recovery as previously published (39). After extraction with phenol:chloroform:isoamyl alcohol (20:20:1), the phenol layer was back-extracted with 165 μl of urea extraction buffer (7 M urea, 350 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) plus 25 μg of tRNA carrier. The supernatants were combined and re-extracted with phenol:chloroform:isoamyl alcohol and precipitated with 95% ethanol. After washing in 70% ethanol, the RNA was heated at 90 °C for 2 min and electrophoresed on a 4% polyacrylamide gel containing 7 M urea. The gel was then autoradiographed.

Transient Transfection of Plasmid DNA and CAT Assay—Cells were transiently transfected using a Bio-Rad Gene Pulser electroporation apparatus which was set at 200 V and 960 microfarad. Five to six × 10^6 cells were resuspended in 0.3 ml of complete growth medium containing 7.5% heat-inactivated fetal calf serum: RPMI 1640 for HSB and Joklik's modified minimal essential medium for HeLa. The cell suspension was then placed into the electroporation chamber, followed by addition of DNA (10 μg of expression plasmid, 10 μg of reporter plasmid), mixing, and application of the voltage pulse. The cells were immediately transferred to a 25-cm^2 flask containing 10 ml of complete growth medium. Approximately 48 h after transfection, the cells were counted, harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in 50 μl of 0.25 M Tris-HCl, pH 7.6. Cells were then lysed by 3–4 freeze-thaw cycles, and cellular debris was removed by microcentrifugation for 5 min. After heat inactivating the extract at 65 °C for 10 min (44), the same cell number equivalents of the resulting extract were assayed for CAT activity. The CAT assay was performed as described (5), except that the final concentration of Tris-HCl was 0.25 M and the final volume of each reaction was 150 μl. The CAT activities were analyzed by TLC and autoradiography. Percent acetylation of chloramphenicol was quantified by cutting out and counting sections of the TLC plates in liquid scintillation fluid. The fold induction of DRA-CAT expression by OTF-2 protein was calculated as the percent acetylation produced by pWToct in the presence of pCMV5-OTF-2 divided by percent acetylation produced by pWToct in the presence of pCMV5. Additionally, the requirement for an intact octamer element for OTF-2 induction was determined as above except by using the pMUToct reporter plasmid.

RESULTS

OBPs from B Cells but Not from HeLa Cells Can Partially Reconstitute DRA Transcription in Vitro—To assess whether OBPs from B cells which contain both OTF-1 and OTF-2 or OBPs from HeLa cells which only contain OTF-1 could reconstitute OTF-depleted DRA gene transcription *in vitro*, we compared the effects of OBPs enriched from these two cell lines on DRA transcription in an *in vitro* run-off transcription assay. A plasmid containing the basal DRA promoter upstream of the bacterial chloramphenicol acetyltransferase gene was used as a template for transcription (Fig. 1*A*). A schematic depiction of the strategy used for the transcription experiments is shown in Fig. 2*A*. OBPs were enriched by eluting HeLa or B cell octamer-binding proteins from an octamer element DNA affinity column. In Fig. 2*B*, a gel mobility shift assay (15) was used to test for the presence and specificity of octamer-binding factors in the material eluted off the column. Shifted complexes corresponding to OTF-1

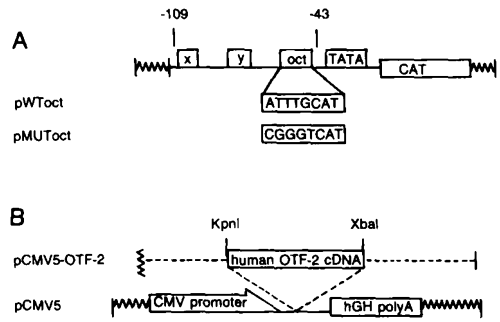

FIG. 1. *A*, schematic representation of wild type and mutant MHC class II DRA reporter plasmids. pWToct contains 109 bp of the DRA promoter placed upstream of the bacterial chloramphenicol acetyltransferase reporter gene. A 66-bp oligonucleotide coding for the DRA X, Y, and octamer elements was cloned into the 5′Δ-43CAT plasmid. pMUToct is identical to pWToct except mutant octamer sequence replaces wild type octamer sequence. *B*, schematic representation of OTF-2 expression plasmid, pCMV5-OTF-2. Human OTF-2 cDNA was cloned into the compatible cloning site of the plasmid pCMV5. pCMV5 contains the human cytomegalovirus early promoter, a multiple cloning site linker, and transcription termination and polyadenylation signals from human growth hormone.

and OTF-2 were identified based on published profiles from ours and other laboratories (6, 19, 28, 33). *Lanes 1* and *2* are whole nuclear extract from Namalwa B cells and HeLa cells, respectively. Both cells contain OTF-1 protein (*upper arrow*), but only the B cell line contains OTF-2 protein (*lower arrow*). Namalwa B cell OBPs eluted off the octamer element affinity matrix contain both OTF-1 and OTF-2 (*lane 3*) which are specifically competed with an excess of octamer competitor (*lane 4*), but not with the same amount of a mutant octamer competitor (*lane 5*). HeLa cell OBPs eluted off the octamer element affinity matrix contain OTF-1 (*lane 6*) and another complex, which can be specifically competed with an excess of octamer element competitor (*lane 7*), but not with mutant octamer competitor (*lane 8*). These OBP-enriched extracts were used as a source of OTF proteins to reconstitute DRA gene transcription in a B cell nuclear extract depleted of octamer-binding proteins. The extract was depleted of OBPs by passage over an octamer element affinity matrix, and the depletion of octamer-binding proteins confirmed by gel mobility shift assay (data not shown). DRA *in vitro* transcription using nondepleted B cell extracts as a source of transcription factors resulted in the correct 595-nucleotide run-off transcript (*left arrow*) (Fig. 2*C*, *lanes 1* and *4*), whereas octamer-depleted B cell extracts produced no DRA transcription (*lanes 2* and *5*). It is critical that any differences in OTF-1 and OTF-2 activity would not be due to different amounts of these proteins used for reconstitution, therefore equivalent binding activity was determined by titration of these OBP-enriched fractions in a gel mobility shift assay (data not shown). B cell and HeLa cell OBPs containing similar amounts of OTF-1 binding activity were added to the depleted transcription reaction (see Fig. 2*A*). B cell OBPs could partially reconstitute transcription (*lane 3*), whereas HeLa cell OBPs could not (*lane 6*). The partial reconstitution with B cell OBPs averaged 35% as quantitated relative to the level of internal 494-nucleotide standard (*arrowhead*) in three separate experiments as determined by densitometric scanning of autoradiographs. When the same type of experiment was done using a plasmid containing a mutated octamer element as a transcription template, no effect of B cell OBPs was seen (data not shown). As a control to demonstrate that the HeLa cell OBPs were functional, we determined that HeLa OBPs could reconstitute octamer-depleted transcription from the TH2A

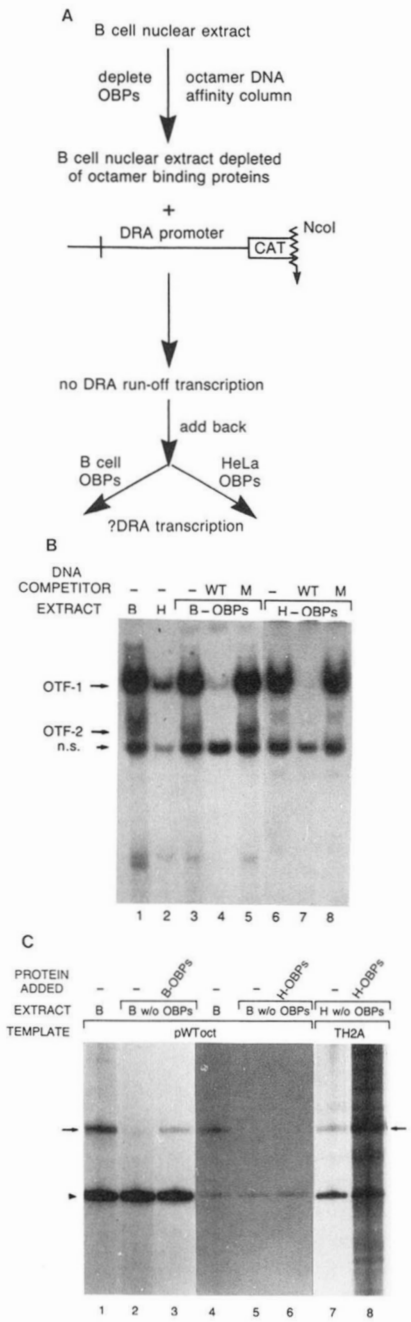

FIG. 2. *A*, flow diagram of the strategy used for DRA *in vitro* transcription reconstitution experiments. B cell nuclear extract was depleted of octamer-binding proteins by passage over an octamer DNA affinity column. This octamer-binding protein-depleted extract did not support DRA run-off transcription (see *C*). Octamer-binding proteins enriched from B cells (which contain OTF-2 and OTF-1) or from HeLa cells (which contain OTF-1) were assessed for their ability to reconstitute DRA gene transcription. *B*, gel mobility shift of octamer-binding proteins enriched from B cell and HeLa cell nuclear extracts. B cell (*lane 1*) or HeLa cell (*lane 2*) nuclear extracts, and octamer-binding proteins enriched from B cells (*lanes 3–5*) or octamer-binding proteins enriched from HeLa cells (*lanes 6–8*) were incubated with a radiolabeled octamer DNA probe in the presence of no competitor (*lanes 1–3* and *6*), wild type octamer competitor (*lanes 4* and *7*), or mutant octamer competitor (*lanes 5* and *8*). The resulting complexes were electrophoresed on a 6% nondenaturing Tris glycine polyacrylamide gel. *C*, functional analysis of B cell or HeLa cell OBPs in DRA *in vitro* transcription. DRA *in vitro* transcription using B cell nuclear extracts (*lanes 1* and *4*) or B cell nuclear extracts depleted of octamer-binding proteins (*lanes 2*, *3*, *5*, and *6*). B cell OBPs were added to *lane 3*, HeLa cell OBPs were added to *lane 6*, and no OBPs were added to *lanes 2* and *5*. The correct DRA run-off transcription product (*left arrow*) is indicated. As a control for HeLa cell OBP function, *in vitro* transcription from the TH2ACAT plasmid was analyzed using HeLa cell extracts depleted of octamer-binding proteins (*lanes 7* and *8*) without (*lane 7*) or with (*lane 8*) added HeLa cell OBPs. The correct TH2A run-off transcription product (*right arrow*) is indicated. The *arrowhead* indicates the 494-nucleotide internal standard included in all reactions.

gene (Fig. 2*C*, *lanes 7* and *8*). These experiments demonstrate that B cell-derived OBPs can partially reconstitute octamer-depleted DRA gene transcription, whereas HeLa cell-derived OBPs cannot. Additionally, this effect is dependent on an intact octamer element.

Cloned OTF-2 Protein Positively Affects DRA Gene Transcription in Vitro—Octamer-binding proteins enriched from B cells were able to partially reconstitute octamer-depleted DRA transcription, in contrast to octamer-binding proteins enriched from HeLa cells. Since B cells make OTF-2 protein and HeLa cells do not, this suggests that OTF-2 was responsible for the reconstitution of octamer-depleted DRA gene transcription that we had observed. We tested this directly using cloned OTF-2 protein. For these experiments, a plasmid coding for human OTF-2 was utilized as a source of recombinant OTF-2 protein. OTF-2 protein was transcribed and translated *in vitro* from this plasmid in the sense orientation, resulting in the correct OTF-2 band as assessed by gel mobility shift assay (Fig. 3*A*, *lane 2*). As a comparison, nuclear extracts from the B cell line Namalwa (*lane 1*) gave the expected OTF-1 (*upper arrow*) and OTF-2 (*lower arrow*) shifted bands. The recombinant OTF-2 shifted band was specific since it could be competed with an excess of wild type octamer element (*lane 3*) but was not competed with the same molar amount of mutant octamer element (*lane 4*). Protein transcribed and translated from the same plasmid, but in the antisense orientation did not result in a specific OTF-2 protein (*lane 5*). This antisense protein was used as a control for the cloned OTF-2 protein in the DRA transcription experiments. The extra bands in the sense and antisense recombinant OTF-2 lanes (*lanes 2* and *5*; *arrowheads*) are specific octamer-binding proteins produced by the rabbit reticulocyte lysate (compare *lanes 3* and *4*). HeLa cells do not contain OTF-2 protein (see Fig. 2*B*) and were used as a source of general transcription factors. We (5) have previously reported that HeLa extracts can only minimally support transcription from the DRA promoter. Cloned OTF-2 protein added to HeLa nuclear extracts allows for increased DRA gene transcription (Fig. 3*B*, *lane 2*, *left arrow*) over a control extract to which antisense OTF-2 has been added (*lane 1*). The *arrowhead* corresponds to the 494-nucleotide internal standard. When a plasmid containing a mutated octamer element is used as a template for transcription, OTF-2 protein in the sense or in the antisense direction has no effect (data not shown). As another control for specificity, we examined the effect of recombinant OTF-2 protein on transcription from the adenovirus major late promoter (*right arrow*), which does not contain an octamer element in its promoter. Recombinant OTF-2 protein in the sense or in the antisense direction had no effect on adenovirus major late promoter transcription (*lanes 3* and *4*). Thus, OTF-2 plays a positive role in DRA gene transcription. This effect is also dependent on an intact octamer element.

In Vivo Role of Recombinant OTF-2 Protein on DRA Gene Transcription—To further address the effect of OTF-2 protein expression on DRA gene transcription, an OTF-2 expression plasmid, pCMV5-OTF-2 (Fig. 1*B*), was co-transfected into the human T cell line HSB or into HeLa cells along with DRA-CAT reporter plasmids pWToct or pMUToct. HSB and HeLa cells lack endogenous OTF-2 protein and DRA expression is negligible (33). The reporter plasmids contain wild

type or mutant octamer elements in the context of 109 bp of DRA promoter sequence (see Fig. 1*A*). As a control for the OTF-2 expression plasmid, the same expression plasmid without OTF-2 sequence was used (pCMV5). The effect of OTF-2 protein on DRA promoter function was determined by comparing the levels of CAT enzyme produced by pWToct in the presence of the OTF-2 or the control CMV expression plasmids. A representative result of the transient transfection analysis using HSB cells (Fig. 4) is shown. DRA-CAT expression was induced 2.6-fold by CMV-OTF-2 in HSB cells (compare *lanes 2* and *1*). We have repeated the experiments using HSB cells 4 times and we have consistently observed a 2–3-fold induction of CAT expression by OTF-2. To determine if the effect of OTF-2 is dependent on the octamer element, a reporter plasmid containing a mutant octamer element, pMUToct, was used for similar experiments (Fig. 4, *lanes 3* and *4*). OTF-2 had little effect on CAT expression from the pMUToct plasmid, demonstrating that OTF-2 induction of DRA-CAT expression requires an intact octamer element. This effect was also observed when the similar experiment was performed using HeLa cells (data not shown).

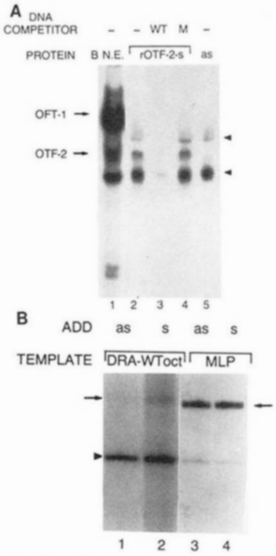

FIG. 3. *A*, gel mobility shift analysis of *in vitro* transcribed/translated human OTF-2. B cell nuclear extract (*lane 1*) or recombinant OTF-2 protein obtained from OTF-2 translated in the sense orientation (rOTF-2-s; *lanes 2–4*) or in the antisense orientation (*as*; *lane 5*) were incubated with a radiolabeled octamer DNA probe in the absence of competitor (*lanes 1, 2,* and *5*), wild type octamer competitor (*lane 3*), or mutant octamer competitor (*lane 4*). OTF-1 and OTF-2 shifted complexes are indicated. *Arrowheads* indicate octamer-binding proteins produced by the rabbit reticulocyte lysate. The protein-DNA complexes were electrophoresed on a 6% nondenaturing Tris glycine polyacrylamide gel. *B*, functional analysis of recombinant human OTF-2 protein in DRA *in vitro* transcription. *In vitro* transcription from the DRA promoter (*lanes 1* and *2*) or the MLP promoter (*lanes 3* and *4*) using HeLa nuclear extracts to supply general transcription factors plus either recombinant OTF-2 protein obtained from OTF-2 translated in the sense orientation (*s*; *lanes 2* and *4*) or in the antisense orientation (*as*; *lanes 1 and 3*). The correct DRA run-off transcription product (*left arrow*) and the correct MLP run-off product (*right arrow*) are indicated. The 494-nucleotide internal standard (*arrowhead*) is included in all reactions.

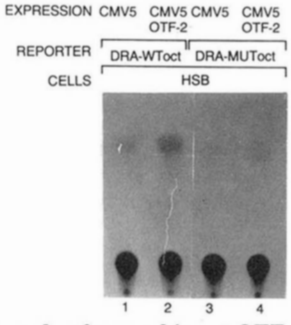

FIG. 4. ***In vivo* role of recombinant OTF-2 protein on DRA gene transcription.** Assay of CAT activity in extracts from HSB (*lanes 1–4*) cells transiently co-transfected with either DRA-WToct (*lanes 1* and *2*) or DRA-MUToct (*lanes 3* and *4*) reporter plasmids and pCMV5 (*lanes 1* and *3*) or pCMV5-OTF-2 (*lanes 2* and *4*) expression plasmids. Ethyl acetate-extracted material was separated by thin layer chromatography and subjected to autoradiography.

DISCUSSION

Transcriptional regulation is an important mechanism for the control of class II MHC gene expression. Several cis-acting elements in the promoters of MHC class II genes have been defined that mediate this transcriptional control. The octamer element, ATTTGCAT, is one of the cis elements defined that is necessary for DRA gene transcription in B cells, but not in non-B cells that express class II molecules (6). This conclusion was reached because mutagenesis of the octamer element in DRA greatly diminished gene expression in B cell lines but not in DR^+ non-B cell lines. We had previously demonstrated by gel mobility shift assays that two proteins bind to the DRA octamer element, one B cell-specific and the other not (33). These were thought to be the octamer-binding proteins OTF-2 and OTF-1, respectively. Although both OTF-2 and OTF-1 were capable of binding to the DRA promoter *in vitro*, it was not known whether these octamer-binding proteins functionally contributed to DRA gene transcription. In this report, we demonstrated the role of OTF-2 protein in DRA gene transcription.

OTF-2 protein function was assessed in both a cell-free *in vitro* transcription system and by *in vivo* transient transfection studies. Similar conclusions were obtained using both of these approaches. Octamer-binding proteins enriched from B cells which contain OTF-2 could partially restore octamer-depleted DRA transcription *in vitro*, whereas those from HeLa cells which do not contain OTF-2 protein had no effect (Fig. 2). Furthermore, recombinant human OTF-2 protein could potentiate DRA gene transcription, but to a lesser extent than enriched cellular OTF-2 (Fig. 3). This result is consistent with data from other laboratories that suggests a role for an additional B cell factor in Ig gene regulation (28–30). Perhaps this same or another B cell factor is also required for optimal MHC DRA gene transcription. Our *in vivo* studies demonstrated that OTF-2 protein enhanced DRA gene transcription 2–3-fold, and that this effect requires an intact octamer element (Fig. 4). Together these results demonstrate a role for the OTF-2 protein in DRA gene transcription.

Although the effect of OTF-2 did not seem very dramatic, contributing about 2–3-fold enhancement of DRA transcription, it was very reproducible and the extent of OTF-2 contribution was similar using two very different methods, *i.e. in vitro* cell-free transcription and *in vivo* transient transfection. This level of effect was not unexpected since protein binding to the octamer element was thought to act in concert with DNA-protein interactions at other known regulatory elements of the DRA gene, including the W, X, and Y elements (4). We have already previously demonstrated a positive functional role for one Y element-binding protein YEBP, in DRA gene transcription in B cells (39). It will be informative to study the effects of all of the MHC class II promoter-binding proteins individually as well as in combination with each other to discern their roles in transcription.

In our studies, the OTF-1 enriched preparation did not

affect DRA gene transcription (Fig. 2). This is not due to the inactivity of OTF-1 in these preparations because it enhanced transcription of the control TH2A promoter. At first glance, this seems to be in contrast to other studies where either OTF-1 or OTF-2 could positively regulate expression of the B cell-specific Ig gene (28–30, 45). Octamer-enriched extracts used for our reconstitution experiments contained similar levels of OTF-1 binding activity; therefore the differential ability of these two fractions to affect DRA gene transcription could not be due simply to differences in the amounts of octamer-binding proteins added. It is more likely that since the context of the octamer element in the DRA promoter is different from that in the Ig promoter, the proteins that bind to this element function differently in the two genes. Additionally, since octamer-enriched fractions from B cells were able to potentiate DRA gene transcription more than recombinant OTF-2 protein alone, another factor, potentially an additional B cell-specific factor similar to the one that acts in conjunction with OTF-2 in Ig transcription (29, 30), is also required for optimal MHC class II DRA gene transcription.

Although the octamer element and the OTF-2 protein play a role in MHC class II DRA gene transcription, other human MHC class II genes do not contain an obvious octamer element in their promoters. Interestingly, the DRA homologues in other primates, including old and new world monkeys, also contain octamer elements in similar locations in their promoter regions (46). Whether this suggests that the octamer-binding protein OTF-2 only affects transcription of the DRA gene (and potentially its primate homologues) but not other MHC class II genes, or whether the octamer-binding protein can bind to other DNA sequences in the promoters of these genes remains to be determined. The octamer-binding proteins have, however, been shown to bind to DNA sequences other than the canonical ATTTGCAT octamer element including the TAATGARAT (R = purine) sequence upstream of the herpes simplex virus immediate-early genes (47), the AAGAATAAATTAGA site in the Aγ-globin gene promoter (48), and the heptamer element CTCATGA in the Ig heavy chain promoter (49, 50). On the other hand, if OTF-2 is only able to regulate transcription from the DRA MHC class II promoter, this could explain a possible mechanism for the higher levels of DR protein relative to DP or DQ proteins that are normally found on class II expressing cells (51).

In conclusion, this is the first demonstration that the lymphoid-specific octamer-binding protein, OTF-2, can positively regulate expression of the MHC class II gene, DRA. Using both affinity-enriched protein fractions and *in vitro* translated OTF-2 protein in an *in vitro* transcription system, in addition to an *in vivo* approach, our results established a role for OTF-2 in DRA transcription. Experiments are ongoing to determine the role of other proteins which may interact with OTF-2 in DRA gene expression.

Acknowledgment—We thank Dr. Chi-Bom Chae for the TH2A plasmid.

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