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Immunology

The octamer motif is a B-lymphocyte-specific regulatory element of the *HLA-DRα* gene promoter

(class II major histocompatibility complex genes/cell-free transcription system)

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ABSTRACT **The human class II gene, *HLA-DRα*, contains an octanucleotide sequence ATTTGCAT located ≈40 base pairs upstream of the transcription initiation site. We have investigated the transcriptional function of the *DRα* octamer in human B-lymphoblastoid cells and non-B cells. Deletion and substitution mutagenesis of the octamer sequence greatly reduced the activity of the *DRα* promoter in both *in vivo* and *in vitro* cell-free transcription systems of B-cell origin. Conversely, these mutations did not affect promoter activity in several non-B-cell lines that express the *DRα* gene. Removal of octamer-binding proteins by *in vivo* titration with an octamer-containing competitor plasmid reduced *DRα* promoter activity in B-lymphoblastoid cells. These results suggest that a protein–octamer interaction, most likely involving the B-cell-specific octamer binding protein (OTF-2), is required for *DRα* promoter function in B-lymphoblastoid cells but not in non-B cells.**

The promoter regions of class II major histocompatibility complex genes contain several DNA sequence motifs, which probably function as transcriptional regulatory elements. These include the class II box consensus sequences (1) in addition to the canonical CCAAT and TATA boxes. The human class II gene, *HLA-DRα*, also contains an octanucleotide sequence (ATTTGCAT) located ≈40 base pairs (bp) upstream of the transcription initiation site. This octamer motif is an essential transcriptional regulatory element in a wide variety of eukaryotic promoters, including immunoglobulin light- and heavy-chain gene promoters (2, 3). The *HLA-DRα* gene may be unique among class II genes, in that the octamer motif is not found in the promoters of any other human or murine class II genes sequenced to date. The murine *Eα* gene does contain a similar sequence (CTTTGGAT) at a location identical to the *HLA-DRα* octamer sequence.

DNA regulatory elements in eukaryotic promoters act as binding sites for proteins, and the interaction of these proteins with their target sequences and with each other determines promoter activity. At least two proteins that specifically recognize the octamer motif have been identified in nuclear extracts of mammalian cells. OTF-1 (NF-AI, OBP100, NF III) is a ubiquitous protein (4–7) that has been shown to regulate the cell-cycle expression of a human histone *H2B* gene (4). OTF-2 (NF-A2, OTF-2A) is a lymphoid-specific octamer-binding protein that may account for the B-cell-specific activity of immunoglobulin promoters (8–11). We have previously demonstrated that two nuclear proteins similar to OTF-1 and OTF-2 bind specifically to the octamer motif in the *DRα* gene (12).

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Since *in vitro* binding of a protein to DNA does not necessarily signify an *in vivo* transcriptional function for that protein, we have taken several experimental approaches to demonstrate that the octamer does function as a transcriptional regulatory element in the *HLA-DRα* promoter. These include deletion and substitution mutational analysis, *in vivo* competition assays, and *in vitro* transcriptional analysis. Using these approaches, we have determined that the octamer is crucial for *DRα* gene expression in B cells but is dispensable for its expression in non-B cells.

MATERIALS AND METHODS

Cell Lines. Raji is a human Epstein–Barr virus-positive Burkitt lymphoma cell line. Namalwa is a B-lymphoblastoid cell line. Sk-Mel13 (ref. 13; provided by A. Houghton, Sloan–Kettering) and Du-Mel17 (ref. 14; provided by M. Carrington and F. Ward, Duke University) are *DR*-expressing melonoma lines. U373MG and U105MG are *DR*-expressing glioblastoma lines (15).

DNA Constructs. Deletion mutants 5′Δ-109, 5′Δ-56, and 5′Δ-43 containing 5′ flanking sequences of the *HLA-DRα* gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene are described in detail in ref. 12 (see Fig. 1). In 5′Δ-109, the conserved class II boxes (X+Y), the octamer, and all downstream regulatory elements through the cap site are present. In 5′Δ-56, the class II boxes are deleted but the octamer and all downstream regulatory elements including the cap site are present; in 5′Δ-43, the octamer is further deleted. 5′Δ-56X+Y is a reconstructed plasmid that contains the class II boxes and the octamer. It was obtained by linearizing 5′Δ-56 with *Xba* I; this was followed by treatment with Klenow enzyme to form blunt ends and insertion of a single copy of a 50-bp oligonucleotide containing the X and Y consensus sequences. 5′Δ-43X+Y is a reconstructed plasmid that contains the class II boxes but lacks the octamer element. It was prepared in an identical fashion to 5′Δ-56X+Y, except that the starting plasmid was 5′Δ-43.

Constructs containing substitution mutations in the octamer were obtained by inserting a single copy of a 74-bp oligonucleotide containing the class II boxes and either the *Eα* octamer-like sequence (CTTTGGAT, underlined nucleotides differ from the *DRα* octamer) or a second mutated octamer sequence (CGGGTCAT) into linearized 5′Δ-43. The latter mutations were chosen to conserve the trinucleotide CAT at the 3′ end of the octamer, which in one report has been designated as a putative CCAAT box (16). These

Abbreviations: CAT, chloramphenicol acetyltransferase; MHC, major histocompatibility complex.

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constructs were called, respectively, pEαoct and pMUToct. Finally, a parallel construct (designated pWToct) containing the wild-type octamer sequence was prepared by inserting a 74-bp oligonucleotide containing the class II boxes and the wild-type octamer (ATTTGCAT) into 5′Δ-43. As shown in Fig. 1, pWToct is equivalent to 5′Δ-56X+Y, except for some sequence differences resulting from the cloning operations in the region between the Y box and the octamer (addition of an *Xba* I linker and deletion of the sequence AGTA in 5′Δ-56X+Y), and in the region between the octamer and the TATA box (addition of an *Xba* I linker and duplication of the sequence TTAATGG in pWToct). Constructs were characterized by dideoxynucleotide DNA sequence determination directly from the double-stranded plasmid DNA (17), using a 20-base oligonucleotide complementary to nucleotides 15–34 of the coding sequence of the CAT gene (18) as a primer for reverse transcriptase. pD164-2, the parental plasmid for all of the *DRα*–CAT recombinants, does not contain any *DRα* sequences (19).

pH2bl-12 contains the human histone *H2B* sequences from −54 to +117 inserted into the *Sma* I site of puc13. pH2b-OMA is an octamer double-point mutant containing the *H2B* sequences from −59 to +128, in which the sequence ATTTGCAT (−49 to −42) was mutated to AGTTGAAT (20). pGEM-4CPE was obtained by subcloning a *Pst* I/*Eco*RI fragment containing *H2B* sequences from +230 to −151 (20) between the *Eco*RI and *Pst* I sites of pGEM-1.

For *in vivo* competition studies, a 24-bp oligonucleotide containing the octamer motif flanked by sequences unrelated to the *DRα* gene was inserted into the *Sma* I site of pUC18.

Transfection. All cells were transiently transfected by using a Bio-Rad Gene Pulser electroporation apparatus. Cells (10^6) were collected by centrifugation and resuspended in 0.3 ml of complete RPMI medium. The cell suspension was then placed into the electroporation chamber; this was followed by addition of DNA, mixing, and application of the voltage pulse (200 V, 960 μF). The cells were immediately transferred to 10 ml of complete RPMI medium. Approximately 48 hr after transfection, cells were counted, harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in 0.25 M Tris·HCl (pH 7.6) (50 μl per 10^6 cells). Cells were lysed by several freeze–thaw cycles, cellular debris was removed by centrifugation, and the resulting extracts were assayed for CAT activity as described (21).

Preparation of Nuclear Extracts for *in Vitro* Cell-Free Transcription. Namalwa cells were grown in spinner flasks in RPMI 1640 medium supplemented with nonessential amino acids, glutamine, and 10% fetal bovine serum. HeLa cells were grown in spinner flasks in Joklik's minimal essential medium supplemented with 5% calf serum. Cells were harvested at 2 × 10^6 cells per ml and nuclear extracts were prepared as described (22), except that Tris was substituted for Hepes.

***In Vitro* Cell-Free Transcription.** Transcription reaction mixtures (25 μl) contained 20 mM Tris·HCl (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.75 mM ribonucleoside triphosphates, 0.5 μg of supercoiled template DNA, and 10 μl (≈80 μg of protein) of Namalwa or HeLa extracts. Transcription was carried out for 1 hr at 30°C; this was followed by digestion with RNase-free DNase I to destroy the template DNA. *In vitro* transcribed RNAs from DRα-CAT templates were mapped by primer-extension analysis as follows. The primer, a 20-base oligonucleotide complementary to nucleotides 15–34 of the coding sequence of the CAT gene (18), was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. RNA plus 4 pmol of primer were ethanol precipitated, resuspended in 3 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA, and incubated at 70°C for 5 min. Two microliters of 250 mM Tris·HCl, pH 8.0/250 mM KCl/200 mM dithiothreitol/40 mM $MgCl_2$ was added, and the samples were incubated at 42°C for 1 hr. This was followed by incubation for 30 min at 42°C in the presence of 1 mM deoxynucleoside triphosphates and 20 units of avian myeloblastosis virus reverse transcriptase (total reaction vol, 10 μl). Products were purified by ethanol precipitation and analyzed on an 8% denaturing polyacrylamide gel. *In vitro* synthesized RNAs from H2B templates were mapped by RNase protection analysis (23), using an SP6 probe derived from pGEM-4CPE cut with *Eco*RI.

RESULTS

Deletion and Substitution Mutagenesis of the Octamer Sequence Reduces *HLA-DRα* Promoter Activity in B-Lymphoblastoid Cells but Not in *DR*-Expressing Glioblastomas and Melanomas. The minimal *HLA-DRα* promoter contains the

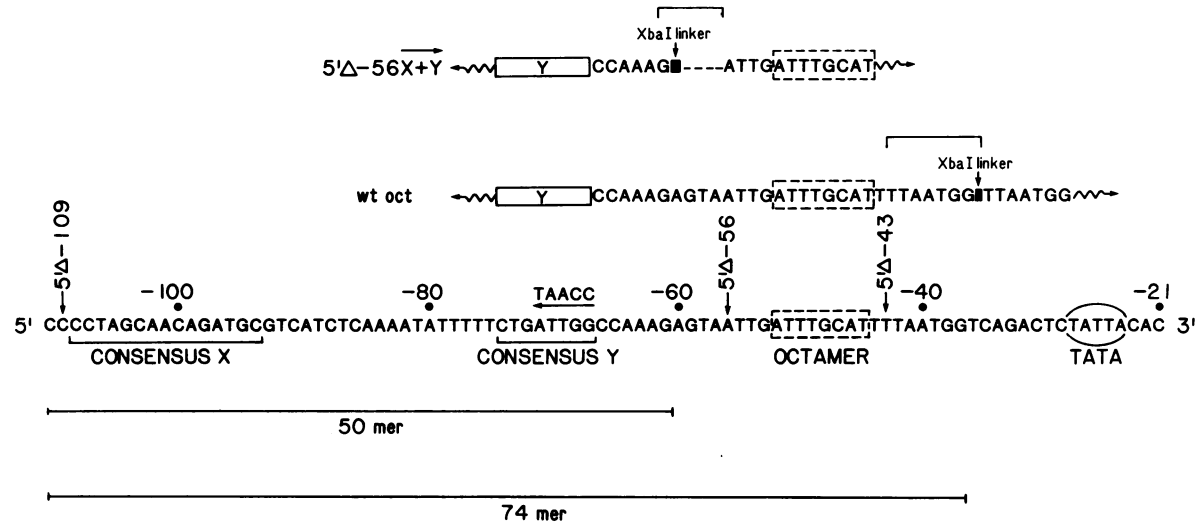

FIG. 1. The promoter region of the *HLA-DRα* gene. Nucleotides are numbered relative to the cap site. Potential cis-acting regulatory elements include the class II boxes (X and Y), the octamer sequence, and a TATA box. A CCAAT box is located on the opposite strand of the Y consensus sequence. 5′ deletion mutants contained *DRα* sequence extending from positions indicated by the arrows above the sequence to +30. Differences (indicated by brackets) between 5′Δ-56X+Y and pWToct are also shown above the parental sequence. The 50-bp oligonucleotide (contains the class II boxes) and the 74-bp oligonucleotide (contains the class II boxes and the octamer) used in promoter reconstructions are shown below the sequence.

class II boxes, the octamer, and the TATA box (Fig. 1). When fused to the CAT gene, these DNA sequences can function as a tissue-specific promoter (12, 24). We investigated the contribution of the octamer to *DRα* promoter activity by altering selected bases of this sequence or by completely removing it. Octamer deletion and substitution mutants were prepared by a promoter reconstruction strategy, which involved inserting oligonucleotides containing the desired changes into an *Xba* I restriction site created at the 5′ end of a DRα–CAT recombinant 5′Δ-43 (a deletion mutant in which DRα 5′ flanking sequences through the octamer were removed). These constructs were introduced by electroporation into a B-lymphoblastoid cell line, Raji, that constitutively expresses high levels of the HLA-DR antigen. The results of the transient transfection analyses are given in Tables 1 and 2. Both deletion (Table 1) and substitution mutagenesis (Table 2) of the octamer sequence reduced *DRα* promoter activity in Raji cells by 80–90%. Interestingly, the octamer-like sequence from the *Eα* gene promoter could not substitute for the *DRα* octamer in our system.

In contrast to the observations with Raji cells, neither deletion nor substitution mutations in the octamer sequence decreased CAT gene expression in *DR*-expressing non-B-cell lines. This was consistently demonstrated in melanoma and glioblastoma lines (Tables 1 and 2).

Titration of Octamer-Binding Proteins Reduces *HLA-DRα* Promoter Activity in Raji Cells. We used an *in vivo* competition assay to further define the role of the octamer sequence in the *DRα* promoter. In this approach, sequence-specific regulatory proteins that are present in limiting amounts in the cell are titrated by introducing a large excess of DNA containing the binding sequence of interest (25). If protein binding to the octamer sequence activates the *HLA-DRα* gene promoter, titration of octamer-binding proteins should reduce promoter activity. The results of an *in vivo* competition assay in which increasing amounts of a plasmid containing the octamer sequence were cotransfected with a DRα–CAT recombinant are shown in Fig. 2. CAT expression from the *DRα* promoter was considerably reduced by increasing quantities of the competitor plasmid. The octamer-containing oligonucleotide that was used in the competitor plasmid also competed for protein binding to the DRα octamer in an electrophoretic mobility shift assay (data not shown). This result lends support to the conclusion that the octamer is an important functional element of the *HLA-DRα* promoter in B cells. In addition, the function of this element is dependent on its interaction with a trans-acting molecule.

Table 1. Analysis of *HLA-DRα* octamer deletion mutants in Raji cells, melanomas, and a glioblastoma line

Cell type	No. of exp.	Plasmids	Relative CAT activity
Raji (B cells)	3	5′Δ-56X+Y	1.00 (14.8%)
		5′Δ-43X+Y	0.23 ± 0.10
		5′Δ-56	0.20 ± 0.04
		5′Δ-43	0.16 ± 0.09
Sk-Mel13 (melanoma)	2	5′Δ-56X+Y	1.00 (10.7%)
		5′Δ-43X+Y	1.21 ± 0.20
		pD164-2	0.06 ± 0.01
Du-Mel17 (melanoma)	3	5′Δ-56X+Y	1.00 (2.6%)
		5′Δ-43X+Y	1.50 ± 0.20
		pD164-2	0.15 ± 0.02
U105MG (glioblastoma)	2	5′Δ-56X+Y	1.00 (63%)
		5′Δ-43X+Y	0.84 ± 0.01
		pD164-2	0.31 ± 0.07

5′Δ-56X+Y is a reconstructed plasmid that contains the class II boxes (X+Y), the octamer element, and *DRα* sequence 3′ to the octamer element. In 5′Δ-43X+Y, the octamer element is deleted. pD164-2 is identical to the above two plasmids except it lacks all *DRα* 5′ sequences. Raji cells were transfected with octamer deletion and substitution mutants. Extracts of transfected cells were analyzed for CAT activity by TLC and autoradiography. Conversion of chloramphenicol to its acetylated forms was quantified by cutting out and counting sections of the TLC plates. Data for deletion mutants and pD164-2 were normalized to CAT activity measured for 5′Δ-56X+Y; data for substitution mutants were normalized to pWToct. The average percent acetylation is given in parentheses.

Table 2. Analysis of *HLA-DRα* octamer substitution mutants in Raji cells, melanomas, and glioblastomas

Cell type	No. of exp.	Transfected plasmids	Relative CAT activity
Raji (B cells)	2	pWToct	1.00 (16.9%)
		pEαoct	0.12 ± 0.03
		pMUToct	0.10 ± 0.01
		5′Δ-43	0.10 ± 0.03
Sk-Mel13 (melanoma)	4	pWToct	1.00 (4.4%)
		pMUToct	1.55 ± 0.25
		pD164-2	0.13 ± 0.03
Du-Mel17 (melanoma)	3	pWToct	1.00 (3.2%)
		pMUToct	1.18 ± 0.40
		pD164-2	0.18 ± 0.04
U373MG (glioblastoma)	2	pWToct	1.00 (3.15%)
		pMUToct	0.89 ± 0.04
		pD164-2	0.23 ± 0.07
U105MG (glioblastoma)	4	pWToct	1.00 (90%)
		pMUToct	1.60 ± 0.36
		pD164-2	0.16 ± 0.07

pWToct is a reconstructed plasmid that contains the class II boxes (X+Y), the octamer element and *DRα* sequences 3′ to the octamer element. pEαoct and pMUToct were prepared in an identical fashion to pWToct except an *Eα* octamer or a mutated octamer, respectively, were used in place of the wild-type octamer. See Table 1 legend and *Materials and Methods* for information on the other plasmids; see Table 1 legend for CAT activity data.

The Octamer Sequence in the *DRα* Promoter Is Required for *in Vitro* Transcription in a B-Cell Extract. The *in vivo* transcription results described above were complemented and extended by analyzing the function of the *DRα* octamer in an *in vitro* cell-free transcription system. Nuclear extracts that accurately and efficiently transcribed from RNA polymerase II promoters were prepared from a *DRα*-expressing cell line

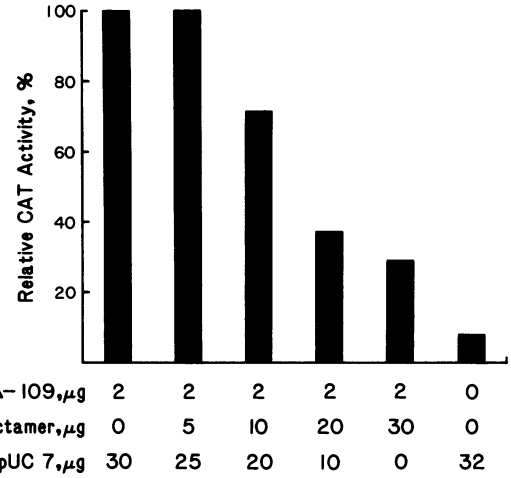

FIG. 2. *In vivo* competition analysis. Increasing amounts of a competitor plasmid containing the octamer sequence were cotransfected into Raji cells with 2 μg of 5′Δ-109 (*DRα*–CAT recombinant). Plasmid pUC 7 was added as indicated to yield a total of 32 μg of DNA per transfection. Forty-eight hours posttransfection, cell extracts were analyzed for CAT activity. Enzyme activity was calculated as the percentage of chloramphenicol converted to the acetylated forms and was normalized to the activity measured for 5′Δ-109 (100% relative CAT activity). Values from quadruplicate transfections were averaged.

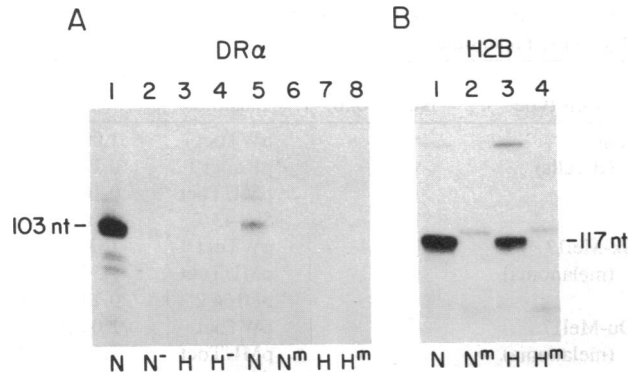

FIG. 3. *In vitro* transcriptional activity of the *HLA-DRα* promoter and the *H2B* promoter. (*A*) Autoradiograph of an 8% denaturing polyacrylamide gel of CAT RNA transcribed from the *DRα* promoter and mapped by primer-extension analysis. Transcripts correctly initiated at the *DRα* cap site are indicated by an arrow. The templates for transcription were 5′Δ-56X+Y (wild-type octamer, lanes 1 and 3), 5′Δ-43X+Y (octamer deletion mutant, lanes 2 and 4), pWToct (lanes 5 and 7), and pMUToct (octamer with a 5-base mutation) (lanes 6 and 8). DNA constructs are described in detail in *Materials and Methods*. Lanes 1, 2, 5, and 6, Namalwa nuclear extract (N); lanes 3, 4, 7, and 8, HeLa nuclear extract (H). (*B*) Autoradiograph of *H2B* transcripts analyzed by RNase protection mapping. The templates were pH2bl-12 (wild-type octamer, lanes 1 and 3) and pH2b-OMA (double point mutant octamer, lanes 2 and 4). Protected RNA transcribed from the former construct was 117 nucleotides (nt) as indicated. The latter yielded a weak 128-nucleotide transcript. Lanes 1 and 2, Namalwa extract; lanes 3 and 4, HeLa extract.

(Namalwa, a human Burkitt lymphoma) and a *DR*-negative cell line (HeLa). The Namalwa cell extracts produced one predominant transcript, correctly initiated at the *DRα* cap site, for both 5′Δ-56X+Y (Fig. 3*A*, lane 1) and for pWToct (lane 5). Both of these constructs contain the class II boxes, the octamer element, and all downstream regulatory sequences inclusive of the cap site. Deletion of the octamer sequence (lane 2) and changing the first 5 bases of the octamer sequence (lane 6) greatly reduced transcription from the *DRα* promoter by the Namalwa nuclear extract.

HeLa nuclear extracts exhibited very low transcriptional activity with constructs containing the wild-type *DRα* promoter, in accord with the B-cell-specific expression of the *DRα* gene (Fig. 3*A*, lanes 3 and 7). The transcription of a control template by the HeLa extract was examined to ensure that this HeLa extract exhibited transcriptional activity. A construct containing a wild-type histone *H2B* promoter, which is not tissue specific, was transcribed equally well by the Namalwa (Fig. 3*B*, lane 1) and HeLa (lane 3) nuclear extracts. The octamer element is required for *H2B* promoter function. Accordingly, mutations in the octamer abrogated the production of correctly initiated transcripts from the *H2B* promoter (lanes 2 and 4).

DISCUSSION

In a previous study, we demonstrated by *in vitro* DNA–protein binding assays that at least two nuclear proteins bind specifically to the octamer motif ATTTGCAT in the *DRα* gene (12). One of these proteins is B-cell specific, and the other is not cell specific. There is some evidence that a B-cell-specific octamer-binding protein is important in immunoglobulin gene regulation (10), while a ubiquitous octamer-binding protein is involved in the cell-cycle-dependent expression of the histone *H2B* gene (4). The class II major histocompatibility complex (MHC) genes are similar to immunoglobulin genes in that they are expressed at high levels in B lymphocytes. In contrast to immunoglobulin genes, class II MHC genes are also expressed by macrophages, some T cells, and in γ-interferon-treated cells of a number of other lineages. In this report, we have assessed the role of the octamer sequence in the expression of the *HLA-DRα* gene in a B-lymphoblastoid cell line and several non-B-cell lines.

The function of the octamer sequence in the *DRα* promoter was evaluated in both *in vivo* transfection and *in vitro* cell-free transcription systems. Similar results were obtained with these two approaches. Deletion and substitution mutagenesis of the octamer sequence resulted in a large reduction in promoter activity in B cells. Although these data do not exclude the possibility that additional DNA regulatory elements, upstream or downstream of the octamer sequence, are important for the B-cell-specific expression of the *HLA-DRα* gene, it does establish that the octamer motif is a positive transcriptional regulatory element in B cells. Importantly, mutations in the octamer sequence did not affect indicator (CAT) gene expression in *DRα*-expressing cells of glioblastoma and melanoma lineages. This observation supports the hypothesis that diverse, cell-specific regulatory pathways are involved in the expression of class II MHC genes. It is noteworthy that other studies with somatic hybrids have reached a similar conclusion (26).

In addition to establishing the function of the octamer sequence in the *DRα* gene, the functional role of protein binding to this sequence was assessed. It is generally accepted that cis-acting DNA regulatory elements control gene transcription via their interaction with trans-acting factors. Our data show that removal of octamer-binding proteins by *in vivo* titration with an octamer-containing competitor plasmid reduces *DRα* promoter activity in Raji cells, thus providing evidence that protein–octamer interactions are important for *DRα* gene expression in B cells. The individual contributions of the B-cell-specific (OTF-2) and the ubiquitous (OTF-1) octamer-binding proteins in *DRα* gene regulation were not assessed.

Based on the observations in this study, we propose that B-cell-specific *DRα* gene expression is regulated in part by the interaction of OTF-2 with the octamer element. This in combination with protein–DNA interactions involving other known regulatory elements of the *DRα* gene such as the class II boxes (24) and the W(Z) element (ref. 27; J. Cogswell, P.V.B., and J.P.-Y.T., unpublished observation) result in *DRα* gene expression in B cells. *DRα* gene expression in non-B cells is controlled primarily by interactions of nuclear proteins with these latter regulatory elements. In accord with this hypothesis, data from our laboratory indicate that the requirements for the W, X, and Y regions in *DRα* gene regulation are similar for all cell types examined (J. Cogswell, P.V.B., and J.P.-Y.T., unpublished observation). The octamer element of the *DRα* gene appears to be dispensable in non-B cells. This would imply that OTF-1, which is produced by these cells, does not function in *DRα* gene control. The simplest explanations for this are (*i*) intracellularly, OTF-1 does not bind to the *DRα* octamer element due to its inability to interact properly with other regulatory proteins that bind to DNA elements proximal to the octamer; or (*ii*) OTF-1 binds to the octamer element, but this binding does not regulate gene transcription.

It is enigmatic that the octamer sequence plays an important role in the B-cell expression of the *DRα* gene yet the upstream regions of other class II MHC genes do not contain this sequence. The murine homologue of the *DRα* gene, *Eα*, contains an octamer-like sequence (CTTTGGAT) at a location identical to the *HLA-DRα* sequence. However, the *Eα* sequence was not transcriptionally active when it was substituted for the wild-type octamer in the *DRα* promoter.

In conclusion, this report provides evidence that the octamer sequence in the *HLA-DRα* gene is a positive transcrip-

tional regulatory element in B cells. Conversely, it appears to be dispensable for *DRα* transcription in non-B cells. This provides the molecular basis for the diverse cell-specific regulatory pathways that are involved in class II MHC gene control. The function of the octamer in B lymphocytes is mediated by interactions with DNA binding molecules, one of which is likely the B-cell-specific octamer-binding protein. The availability of isolated octamer-binding proteins, in combination with a homologous cell-free transcription system, should greatly facilitate our understanding of class II MHC gene regulation.

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