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11 December 1989; accepted 27 April 1990

Induction of Neonatal Tolerance to Mls^a Antigens by CD8$^+$ T Cells

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Antigen-specific tolerance of T cells to minor lymphocyte stimulatory (Mls) antigens can be induced in mice by neonatal injection of foreign lymphohematopoietic cells. Although immune responses to Mls^a antigens are controlled by B cells, CD8$^+$ T cells were the most effective cell type for induction of Mls^a tolerance. Tolerance was evident in both thymus and lymph nodes and could be induced by as few as 2×10^4 CD8$^+$ T cells; these cells were 50 to 100 times as potent as CD4$^+$ cells or B cells in causing functional tolerance and deletion of $V_\beta 6^+$ T cells. Thus, intrathymic contact with antigens expressed on CD8$^+$ T cells may play an important role in controlling the normal development of tolerance.

THE SELECTIVE CAPACITY OF T LYMPHOCYTES to respond to foreign antigens while maintaining tolerance to self is one of the hallmarks of the immune system. Although self tolerance has generated much investigation, the mechanisms involved are not well understood, and it is unclear which cell types are responsible for presenting antigen in tolerogenic form. The prevailing view is that tolerance is induced intrathymically and reflects T cell contact with specialized antigen-presenting cells (APCs) such as macrophages and dendritic cells (*1*). This question is most easily addressed with the model of Billingham *et al.* for neonatal tolerance induction (*2*). We investigated the cell types controlling the induction of neonatal tolerance to Mls antigens in mice. The high precursor frequency of T cells reactive to Mls antigens and the availability of antibodies specific for Mls-reactive T cells makes the Mls system useful for probing tolerance induction.

Mls antigens are poorly characterized cell surface molecules that are immunogenic for unprimed T cells (*3, 4*). These antigens show limited polymorphism, and only two stimulatory forms, Mls^a and Mls^c, are known to exist. T cell reactivity to Mls antigens (anti-Mls response) correlates with expression of particular T cell receptor (TCR) molecules (*5, 6*). Thus, anti-Mls^a responses are controlled mainly by T cells expressing $V_\beta 6$ and $V_\beta 8.1$ TCRs, whereas anti-Mls^c responses involve $V_\beta 3^+$ T cells. Conversely, *Mls^a* and *Mls^c* mice show selective deletion of $V_\beta 6^+$ plus $V_\beta 8.1^+$ cells and $V_\beta 3^+$ cells, respectively.

Because Mls molecules cannot be detected serologically (*3, 4*), information on the tissue distribution of Mls molecules has depended on defining the cell types capable of stimulating Mls-reactive T cells and T hybridomas. Mls antigens are presented effectively by B cells but not by T cells or typical APCs such as macrophages and dendritic cells (*7–9*). In the case of T cells, these data have to be viewed with caution, however, because recognition of Mls antigens requires

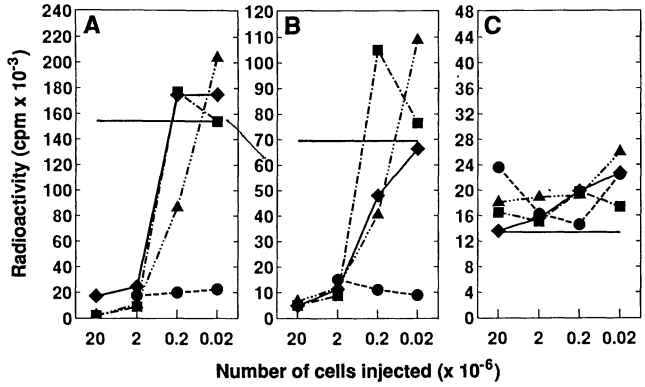

Fig. 1. Relative efficiency of different cell types in inducing functional tolerance to (**A**) Mls^a and (**B**) Mls^c antigens. (**C**) Response to H-2^p. B10.BR (*H-2^k*, *Mls^b*) neonates were intravenously injected with various numbers of (B10.BR × CBA/J)-F_1 (*H-2^k*, *Mls^b* × *H-2^k*, *Mls$^{a/c}$*) cells within 24 hr of birth. T cell–depleted spleen cells (■) (T$^-$ spleen), CD4$^+$ T cells (◆), and CD8$^+$ T cells (●) were purified as described in Table 1. B cells (▲) were purified from T$^-$ spleen by subsequent passage over two sequential G10 columns followed by 2 hr of adherence to plastic tissue culture dishes to remove adherent cells. MLR were carried out 6 weeks later as described for Table 1 with CD4$^+$-enriched T cells (LN cells treated with anti-Ia, J11d, anti-CD8, anti–Lyt 1.1, anti–Lyt 2.1, and complement) as responders (1×10^5 cells per well). Spleen stimulators (5×10^5 cells per well) were mitomycin C–treated before culture. The response of normal (uninjected) B10.BR mice is indicated by a horizontal line on each panel.

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co-recognition of major histocompatibility complex (MHC) class II (Ia) molecules (*3, 4*). Thus the failure to detect Mls antigens on T cells might simply reflect lack of Ia on T cells.

Large doses of unfractionated Mls^a spleen cells (2×10^7 to 1×10^8) injected into Mls^b neonates induce tolerance to Mls^a antigens accompanied by a reduction in the precursor frequency of Mls^a-responsive T cells and deletion of T cells expressing $V_\beta 6$ (*10*). Mls^a tolerance was also induced in Mls^b mice by injecting at birth cells from an Mls^a T cell line (*11*). These data suggest that Mls^a antigens can be expressed on T cells.

To investigate this possibility, we injected BALB/c (*H-2*^{d}, Mls^c) neonates with highly purified BALB/c or DBA/2 (*H-2*^{d}, $Mls^{a/c}$) T cells or T cell–depleted spleen in a dose of 2×10^7 cells per mouse. $CD4^+$ T cells were isolated from lymph nodes (LN) of the recipients 5 to 7 weeks later; contaminating donor-derived T cells were removed with the aid of antibodies that discriminated between host and donor T cells. Mls^a-specific tolerance was assessed functionally by mixed-lymphocyte reactions (MLR) in vitro as well as by monitoring the proportion of $V_\beta 6^+$ T cells (Table 1, experiment 1). BALB/c cells injected into syngeneic BALB/c mice had little effect on the percent of $V_\beta 6^+$ T cells or on the anti-Mls^a response to NZB (*H-2*^{d}, $Mls^{a/c}$) stimulators. In contrast, Mls^a DBA/2 cells, either T cell–depleted spleen or purified T cells, induced >90% tolerance in terms of both $V_\beta 6$ expression and anti-Mls^a MLR. This finding thus confirmed the above report that T cells can induce Mls^a tolerance. We subsequently tested which T cell subsets, $CD4^+$ or $CD8^+$ cells, were tolerogenic for Mls^a-reactive T cells.

The purity of the cell types used was important. Highly purified $CD4^+$ and $CD8^+$ cells were prepared from LN cells by nylon wool filtration, positive panning on plates coated with antibodies to CD4 or CD8, and two rounds of negative selection with a mixture of monoclonal antibodies (MAb) plus complement; contamination with non–T cells and T cells of the opposite subset was undetectable (<0.1%). Neonatal injection of Mls^b B10.BR mice with purified $CD4^+$ or $CD8^+$ cells from Mls^a (B10.BR × AKR/J)F_1 mice induced tolerance to Mls^a (AKR/J) antigens in MLR and marked deletion of $V_\beta 6^+$ cells (Table 1, experiment 2). Tolerance was Mls^a-specific since there was little or no reduction in the response to Mls^c (C3H/HeJ) or to an H-2 difference (B10.P). Tolerance was partly nonspecific when $CD8^+$ cells were transferred in high doses (2×10^7), but not with a lower dose (2×10^6).

Because quite low doses of $CD8^+$ cells induced Mls^a tolerance in the above experiment, we compared the tolerogenic properties of graded doses of $CD8^+$ cells, $CD4^+$ cells, and B cells. B10.BR (*H-2*^{k}, Mls^b) mice were injected neonatally with lymphoid subsets prepared from [B10.BR × CBA/J (*H-2*^{k}, $Mls^{a/c}$)]F_1 mice, thereby enabling us to examine tolerance to both Mls^a and Mls^c. With injection of purified $CD4^+$ cells, purified B cells, or T cell–depleted spleen cells, profound tolerance to Mls^a and Mls^c in MLR (Fig. 1) and deletion of $V_\beta 6^+$ cells (Fig. 2) were observed with cell doses ranging from 2×10^7 to 2×10^6; only minimal tolerance was observed with lower doses. In contrast, injection of $CD8^+$ cells induced functional tolerance with deletion of $V_\beta 6^+$ T cells at a dose of 2×10^4 cells. $CD8^+$ cells thus appeared to be 50 to 100 times as potent at inducing Mls^a tolerance as the other three populations.

To determine where tolerance was induced, we examined tolerance in $CD4^+CD8^-$ mature thymocytes and LN cells from B10.BR mice injected at birth with lymphoid cells from Mls^a (B10.BR × CBA/J)F_1 or (B10.BR × AKR/J)F_1 mice Injection of purified $CD4^+$, $CD8^+$, or T cell–depleted spleen was compared and tolerance was tested at 2 to 7 weeks after injection. Each cell population injected caused functional Mls^a tolerance and deletion of $V_\beta 6^+$ cells in thymus as well as LN (Table 2) (*12*). Tolerance of thymocytes was more pronounced with injection of $CD8^+$ cells than $CD4^+$ cells; doses of 2×10^5 $CD8^+$ cells caused strong tolerance. Exposing $CD8^+$ cells to light irradiation (1000 rad) before injection abrogated tolerance

Table 1. Analysis of cell types that induce tolerance to Mls antigens. Neonatal BALB/c (*H-2*^{d}, Mls^c) and B10.BR (*H-2*^{k}, Mls^b) mice were injected within 24 hours of birth with 2×10^7 cells from DBA/2 (*H-2*^{d}, $Mls^{a/c}$) (experiment 1) and (B10.BR × AKR/J)F_1 (*H-2*^{k}, Mls^b × *H-2*^{k}, Mls^a) (experiment 2) mice, respectively. The injected cells were highly purified: T cell–depleted spleen cells (T^- spleen) were prepared by two cycles of treatment with MAb to T cell antigens (3.168, anti-CD8; RL.172, anti-CD4; and J1j or T24, anti-Thy-1) and complement (*6, 16*); T cells were purified from LN by passage through nylon wool columns and then depleted of residual B cells and other cells with MAb (BP107, anti–I-A^d; 14-4-4, anti–I-E; or 10-2-16, anti–I-A^k; and J11d, anti–B cell) and complement (*17*). $CD4^+$ and $CD8^+$ cells were further purified by adherence to plates coated with either antibodies to CD8 or antibodies to CD4 and elution after removal of nonadherent cells, followed by another treatment with MAbs to Ia, CD4, or CD8 and complement. The injected mice were killed at 7 weeks after transfer to assess tolerance. Primary MLR were performed as described (*6*). LN responders were enriched for $CD4^+$ T cells by treatment with MAb (anti-Ia, J11d, and anti-CD8) and complement; to remove contaminating donor-derived T cells, the LN suspensions used as responders were also treated with anti–Lyt 1.1 plus anti–Lyt 2.1 (experiment 1) or anti–Thy 1.1 (experiment 2) MAb and complement. This treatment removed all chimeric donor cells detectable by FACS analysis. For FACS analysis, 0.3×10^6 to 1×10^6 cells were incubated with unlabeled primary MAb [anti-CD8 (3.168), anti-CD4 (RL172), or anti-$V_\beta 6$ (RR47) (*18*)]. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)–conjugated mouse antibody to rat immunoglobulin (Pel Freez). After blocking with rat serum the cells were stained with phycoerythrin (PE)–conjugated GK1.5 (anti-CD4, Becton Dickinson).

Neonatally injected mice		Percent $V_\beta 6^+$ ($CD4^+$)	MLR* (cpm × 10^{-3}) with stimulators			
Recipient	Cells injected					
		Experiment 1	BALB/c (*H-2*^{d}, Mls^c)	NZB (*H-2*^{d}, $Mls^{a/c}$)	B10.P (*H-2*^{P}, Mls^b)	
BALB/c	None	12.2	4.1	188.1	127.0	
	BALB/c T^- spleen	12.0	4.1	168.9	70.0	
	BALB/c T cells	10.0	4.8	173.7	85.7	
	DBA/2 T^- spleen	0.6	4.7	5.3	63.3	
	DBA/2 T cells	1.3	5.1	11.5	97.4	
		Experiment 2	B10.BR (*H-2*^{k}, Mls^b)	AKR/J (*H-2*^{k}, Mls^a)	C3H/HeJ (*H-2*^{k}, Mls^c)	B10.P (*H-2*^{P}, Mls^b)
B10.BR	None	9.3	0.7	93.7	48.9	44.4
	(B10.BR × AKR/J)F_1 $CD4^+$ T cells	1.9	1.5	21.2	113.3	43.8
	(B10.BR × AKR/J)F_1 $CD8^+$ T cells†	1.9	1.2	15.0	72.9	36.1
	(B10.BR × AKR/J)F_1 $CD8^+$ T cells	1.4	0.8	4.4	38.6	17.9
	(B10.BR × AKR/J)F_1 T^- spleen	1.0	1.7	7.3	107.6	34.8

*MLR were set up with 1×10^5 responders and 5×10^5 mitomycin C–treated spleen stimulators; cultures were pulsed with [^{3}H]thymidine (1 μCi per cell) 18 hours before harvest on day 4; the data show means of triplicate cultures. †In this case, 2×10^6 cells were injected; for all others, 2×10^7 cells were injected.

induction and did not cause $V_\beta6^+$ cell deletion (Table 2).

Our data provide direct evidence that Mls^a (and Mls^c) antigens are expressed on T cells, especially on $CD8^+$ cells. The finding that $CD8^+$ cells were 50 to 100 times as effective as B cells or T cell–depleted spleen cells at inducing tolerance probably rules out the objection that the tolerogenicity of $CD8^+$ cells reflected contamination with other cells. The reverse possibility must now be considered, that the tolerance induced by injection of B cells or $CD4^+$ cells might have reflected minor (<1%) contamination with $CD8^+$ cells. Despite the procedures used for cell purification, this possibility is difficult to exclude.

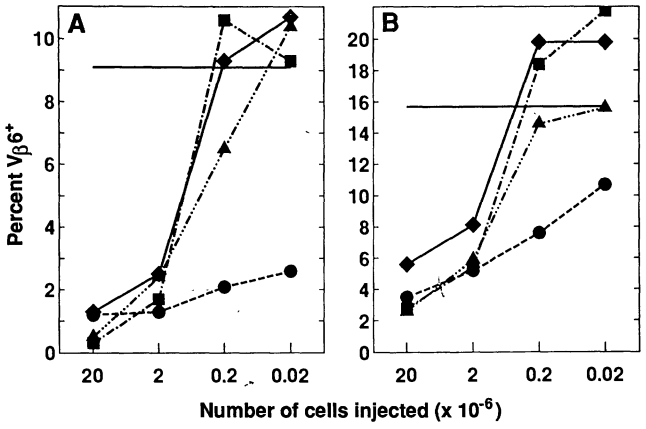

Fig. 2. $V_\beta6$ expression by LN $CD4^+$ and $CD8^+$ T cells taken from the neonatally tolerized mice described in Fig. 1. FACS analysis was as described in Table 1. The percent of $V_\beta6^+$ cells in normal uninjected B10.BR mice is indicated as a horizontal line across the panels. The percent of $KJ16^+$ ($V_\beta8.1$ + 8.2) T cells in the tolerized mice showed a moderate (15 to 20%) decrease, consistent with depletion of Mls^a-reactive $V_\beta8.1^+$ cells.

The mechanism by which $CD8^+$ cells induce Mls^a tolerance is still unclear. In the expectation that tolerance would depend on chimerism (*2, 13*), each T cell–injected mouse was tested for persistence of the injected cells [by fluorescence-activated cell sorter (FACS) analysis with anti–Thy 1.1, anti–Lyt 1.1, or anti–Lyt 2.1 MAbs]. Nearly all of the mice injected with purified $CD8^+$ cells or $CD4^+$ cells showed a variable but significant degree of chimerism (5 to 30%) in LN. The findings that tolerance and $V_\beta6^+$ deletion were prolonged (≥7 weeks) and were evident within the thymus itself imply that the injected $CD8^+$ cells rapidly entered the thymus and remained there as a constant source of tolerogen for newly generated host T cells. Detailed evidence on the extent of chimerism in thymus is not yet available; low but detectable thymic chimerism (0.4% of cortisone-resistant thymocytes) was observed in one preliminary experiment (tested at 7 weeks after injection of 2×10^6 $CD8^+$ cells). The close correlation between functional tolerance (unresponsiveness in MLR) and $V_\beta6$ deletion in both thymus and LN suggests that tolerance was largely a reflection of clonal deletion with little or no contribution from other forms of unresponsiveness, for example, anergy or suppression. The inefficiency of irradiated cells implies that tolerance required contact with intact viable cells.

Given that anti-Mls^a responses are Ia-restricted, one has to explain how tolerance to Mls^a antigens can be induced by $CD8^+$ cells, that is, by Ia^- cells. There are several possibilities. First, Mls^a antigens may be released from $CD8^+$ cells and absorbed as tolerogenic peptides by Ia^+ APCs, the superior potency of $CD8^+$ cells reflecting a much higher expression of Mls^a on $CD8^+$ cells than on other cells. This idea hinges on the assumption that Mls^a antigens are capable of moving from one cell to another. Although this is a popular concept (*14*), in our view there is no direct evidence that Mls^a antigens can be recognized in processed form. In fact, studies on double bone marrow (Mls^b + Mls^a) chimeras suggest that Mls^a antigens are not cross-presented (*9*). Second, despite evidence to the contrary, $CD8^+$ cells may express (or absorb) significant amounts of Ia molecules (*15*): the complex of Mls^a-Ia on $CD8^+$ cells is directly tolerogenic for host T cells, perhaps through a veto effect (*16*). According to this possibility, immunogenic expression of Mls^a antigens should be detectable on T cells, especially T blast cells. In our experience, however, all attempts to detect Mls^a expression on T cells with T hybridoma cells as responders have proved negative (*17*). Third, tolerance may reflect joint contact with Mls^a antigens on donor $CD8^+$ cells and Ia molecules on host APCs. This model is compatible with the hypothesis that Mls^a antigens are not recognized as processed peptides, but as integral cell-membrane molecules (*4, 9, 18*). The notion that Mls^a and Ia molecules can be recognized on different cells (T cells and APCs) accommodates the data in this paper and provides an alternative explanation for experiments purporting to show that Mls^a antigens are recognized in processed form (*14*). Experi-

Table 2. Analysis of tolerance in thymus of neonatally tolerant mice. B10.BR mice were injected intravenously at birth with purified cells (T^- spleen, $CD8^+$ T, or $CD4^+$ T) from (B10.BR × CBA/J)F_1 ($Mls^{a/c}$) mice (experiment 1) or (B10.BR × AKR/J)F_1 (Mls^a) mice (experiments 2 and 3). Cells were purified as described in Table 1. Tolerance was tested at 7 weeks (experiment 1), 2 weeks (experiment 2) or 4 weeks (experiment 3) after injection. Thymocytes were enriched for $CD4^+CD8^-$ mature host cells by treatment with anti-CD8, anti–Thy 1.1 (or anti–Lyt 1.1 or Lyt 2.1) and complement. NT, not tested.

B10.BR injected neonatally with	No. of cells injected (× 10^{-6})	Analysis of $CD4^+$ cells from	Percent of $V_\beta6^+$ $CD4^+$ cells	MLR* (cpm × 10^3) with stimulators		
				B10.BR (syngeneic)	AKR/J (Mls^a)	B10.P (allo ($H\text{-}2^P$)
		Experiment 1				
None	None	Thymus	NT	0.2	184.6	33.1
$Mls^{a/c}$, $CD4^+$ T	2	Thymus	NT	0.1	35.4	21.7
	0.2			0.2	186.4	28.6
$Mls^{a/c}$, $CD8^+$ T	2	Thymus	NT	0.1	6.5	23.1
	0.2			0.1	1.4	26.8
$Mls^{a/c}$, T^- spleen	2	Thymus	NT	0.1	3.5	37.7
		Experiment 2				
None	None	LN	9.8	0.8	95.9	48.9
Mls^a, T^- spleen	2	LN	1.8	1.6	1.2	15.3
Mls^a, $CD8^+$ T	2	LN	2.2	3.2	3.6	21.1
None	None	Thymus	7.0	0.4	53.2	38.8
Mls^a, T^- spleen	2	Thymus	3.3	0.7	19.8	78.6
Mls^a $CD8^+$ T	2	Thymus	2.3	0.1	8.1	89.5
		Experiment 3				
None	None	LN	9.8	1.7	111.1	56.9
Mls^a, $CD8^+$	2	LN	1.6	1.7	4.7	49.0
Mls^a, $CD8^+$ irrad.†	2	LN	9.7	2.1	107.7	48.5
None	None	Thymus	7.5	0.5	223.4	50.8
Mls^a, $CD8^+$	2	Thymus	2.5	0.3	0.6	43.7
Mls^a, $CD8^+$ irrad.†	2	Thymus	7.7	0.3	268.0	70.8

*Response of 1×10^5 responder cells cultured with 5×10^5 mitomycin C–treated spleen stimulator cells. [^{3}H]thymidine uptake was measured on day 5. †1000 rad.

ments designed to test this three-cell model for tolerance induction are under way. At present, none of the above models can be ruled out.

Three other aspects of the data warrant comment. First, the minute quantities of cells ($CD8^+$ cells) required to induce Mls^a tolerance contrast with the relatively massive numbers of cells (5×10^7 spleen cells) needed to induce MHC tolerance in the classic system of Billingham *et al.* (*2*). The simplest explanation for this discrepancy is that tolerance to Mls^a and MHC antigens is controlled by different cells: whereas Mls^a tolerance is controlled by a common cell type ($CD8^+$ cells), full induction of MHC tolerance probably requires contact with dendritic cells, a relatively rare cell type (*1*). Whatever the explanation, the ~1000-fold difference in cell numbers required for Mls^a versus MHC tolerance indicates that the rules governing MHC tolerance induction do not necessarily apply to other antigens. Second, the finding that $CD8^+$ cells are highly effective at inducing Mls^a tolerance but are nonstimulatory for mature T cells indicates that the cell types controlling tolerogenicity and immunogenicity can be distinctly different. Third, the potency of $CD8^+$ cells in mediating Mls^a tolerance supports the view (*19*) that these cells may play a critical role in inducing tolerance to various self components.

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21. We thank O. Kanagawa for providing the RR47 MAb, B. Marchand for preparation of the manuscript, and D. McQuitty, M. Rehse, and W. Wiltse for flow cytometry. Supported by grants CA 25803, CA 38355, and CA 41993 from the National Cancer Institute and AI 21487 from the National Institute of Allergy and Infectious Diseases. This is publication no. 6251-IMM from the Research Institute of Scripps Clinic.

2 February 1990; accepted 27 April 1990

Cloning of a Transcriptionally Active Human TATA Binding Factor

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Transcription factor IID (TFIID) binds to the TATA box promoter element and regulates the expression of most eukaryotic genes transcribed by RNA polymerase II. Complementary DNA (cDNA) encoding a human TFIID protein has been cloned. The human TFIID polypeptide has 339 amino acids and a molecular size of 37,745 daltons. The carboxyl-terminal 181 amino acids of the human TFIID protein shares 80% identity with the TFIID protein from *Saccharomyces cerevisiae*. The amino terminus contains an unusual repeat of 38 consecutive glutamine residues and an X-Thr-Pro repeat. Expression of DNA in reticulocyte lysates or in *Escherichia coli* yielded a protein that was competent for both DNA binding and transcription activation.

INITIATION OF TRANSCRIPTION IN EUkaryotes by RNA polymerase II is a complex process that requires the orchestrated function of several factors (*1*). The binding of a protein, TFIID, to the TATA box promoter element is the first step in the assembly of the transcription complex at the promoter (*2*). Because it has been difficult to purify, the mammalian TFIID protein has been poorly characterized. However, the gene from *S. cerevisiae* that encodes the yeast TFIID protein (YIID) has been cloned (*3–6*), and the YIID protein can substitute for mammalian TFIID in transcription assays conducted in vitro in HeLa nuclear extracts (*7*).

YIID consists of 240 amino acids with a molecular size of ~27 kilodaltons (kD). The carboxyl terminus has a partially repeated sequence between amino acid residues 67 to 131 and 157 to 222 (*6, 8*), which may form a helix-turn-helix structure (*5*). Nearly perfect direct repeats occur between amino acid residues 109 to 127 (PKTTALIFASGKMVVTGAK) and 200 to 218 (PKIVLLIFVSGKIVLTGAK) (*3, 9*).

Although YIID can substitute for mammalian TFIID in an in vitro transcription assay, it is unclear why YIID responds to upstream activator proteins for some promoters (*10*), but not others (*11*). To understand fully the function of TFIID in regulating transcription in mammalian cells, we cloned and expressed the gene that encodes a mammalian TFIID protein.

Our approach for isolating the human TFIID gene was to identify regions in the protein that are highly conserved in evolution by determining the sequence of the TFIID gene from several species. We then designed oligonucleotide primers based on the conserved regions and used these primers to amplify a fragment of the human TFIID gene by the polymerase chain reaction (PCR). YIID DNA was used to probe digested human, mouse, *Drosophila*, and *Lemna* genomic DNA (*12*), and no specific hybridizing sequences were observed. We next looked for cross-hybridizing sequences in more closely related species of budding yeast. All 13 species that we examined had

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