age to 17q or genetic heterogeneity.

We anticipate that accurate phenotypic characterization of PBD in our kindreds will leadto linkage to candidate markers that can then be confirmed by analysis of breast cancer alone with an extended set of markers. Genetic mapping may in turn lead to molecular isolation of the locus or loci responsible for breast cancer susceptibility, which will permit further refinement of the underlying genetic model and analysis of interaction of genetic susceptibility with other risk factors. Four-quadrant fine-needle aspiration is a sensitive, rapid, minimally invasive test that could identify these high-risk lesions in the absence of a clinically identifiable mass and allow identification and close monitoring of genetically susceptible women. Women thus identified might also benefit from specific intervention therapy before the development of cancer, should this type of treatment become available.

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Induction by Antigen of Intrathymic Apoptosis of $CD4^+CD8^+$ TCR^{lo} Thymocytes in Vivo

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In order to examine the mechanisms by which clonal deletion of autoreactive T cells occurs, a peptide antigen was used to induce deletion of antigen-reactive thymocytes in vivo. Mice transgenic for a T cell receptor (TCR) that reacts to this peptide contain thymocytes that progress from the immature to the mature phenotype. Intraperitoneal administration of the peptide antigen to transgenic mice results in a rapid deletion of the immature $CD4^+CD8^+$ TCR^{lo} thymocytes. Apoptosis of cortical thymocytes can be seen within 20 hours of treatment. These results provide direct evidence for the in vivo role of apoptosis in the development of antigen-induced tolerance.

SELF TOLERANCE WITHIN THE T CELL repertoire is established in part by clonal deletion (clonal elimination) of self-reactive T cell clones (*1, 2*). Clonal deletion involves the presentation of self antigens and self–major histocompatibility complex (MHC) molecules to T cells that are developing within the thymus (*3–12*). Clonal deletion of I-E (MHC class II)–reactive T cells in I-E–expressing mice was first inferred by the absence of $V_\beta 17$-bearing T cells from the mature, but not the immature, thymocyte population (*3*). The deletion of $V_\beta 17$ from peripheral $CD8^+$- and $CD4^+$-bearing T cells suggested that deletion could occur at the $CD4^+CD8^+$ stage of thymocyte development (*4*). Tolerance induction to the Mls antigens involves deletion of reactive T cells from the mature thymocyte and peripheral T cell populations, but not from the immature pools (*5–9*).

The deletion of anti–H-Y and allospecific T cells was inferred from the absence of immature $CD4^+CD8^+$ thymocytes in mice expressing the H-Y or allospecific antigen (*10, 11*). The stage at which deletion is evident within thymocyte populations can depend on the antigen system examined. Pircher and co-workers (*12*) demonstrated this by examining deletion in a transgenic system having T cells of dual specificity—to lymphocytic choriomeningitis virus (LCMV) and to Mls^a. Tolerance to Mls^a deleted the mature but not the immature $CD4^+CD8^+$

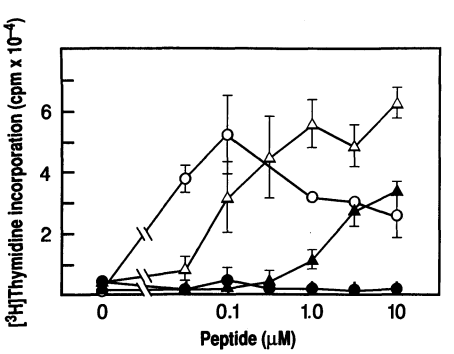

Fig. 1. Reactivity of peripheral transgenic T cells to OVA323-339 peptide analogs. Peptides used at the indicated concentration were OVA323-339 (○), OVA323-338 (△), OVA323-336 (▲), and OVA324-334 (●). Splenocytes from H-2^d haplotype mice expressing the DO11.10 αβ TCR were isolated as described (*15*) and cultured at 2.5×10^6 cells per milliliter with the indicated concentration of peptide. Triplicate 200-μl cultures were pulsed with 0.4 μCi of [^{3}H]thymidine on the second day of a 3-day culture. Data represent the mean and standard deviation of incorporated cpm of [^{3}H]thymidine. The experiment was repeated three times with similar results. Peptides were synthesized on an Applied Biosystems model 430 peptide synthesizer as described (*26*).

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thymocytes, whereas tolerance to LCMV led to drastic reduction in the $CD4^+CD8^+$ immature thymocyte population.

These systems provide direct evidence for clonal deletion as a mechanism for self-tolerance but do not suggest how or where the thymocytes are eliminated. Experiments with in vitro thymic organ culture show that apoptosis, an intrinsic program of cell death characterized by chromatin condensation and DNA degradation, results from the engagement of the TCR of immature thymocytes in culture (*13, 14*). We now present direct in vivo evidence that antigen-induced clonal deletion can proceed by means of apoptosis of thymocytes within the thymic cortex and, in this system, deletes immature $CD4^+CD8^+$ TCR^{lo} cells.

We developed a transgenic system (*15*) in which negative selection can be induced in mice in a synchronous fashion by administration of a deletional antigen that can circulate to the thymus for presentation to developing thymocytes. Thymocytes are then examined at fixed times after injection of antigen. Virtually all thymocytes and peripheral T cells in these mice express the transgenic TCR from a T cell hybridoma, DO11.10, that recognizes chicken ovalbumin (OVA) in the context of $I\text{-}A^d$ (*15*). The transgenic TCR expressed in this system is detected by the clonotypic monoclonal antibody (MAb) KJ1-26 and by the MAb to $V_\beta 8$, F23.1. Thymocytes expressing this TCR develop through an immature $CD4^+CD8^+$ TCR^{lo} stage comprising 70% of thymocytes and, in the $H\text{-}2^d$ haplotype, progress to mature $CD4^+CD8^-$ TCR^{hi} thymocytes and are exported to the periphery as OVA-reactive mature $CD4^+$ T cells (*15*). To identify potential tolerogenic peptide antigens capable of delivering a deletional signal to developing transgenic thymocytes, we examined responses of transgenic thymocytes and peripheral T cells to a series of structurally related peptides. OVA peptide fragment 323-339 (OVA323-339) (ISQAVHAAHAEINEAGR) (*16*) produced strong T cell responses from thymocytes and splenocytes of transgenic mice (Fig. 1). Successive COOH-terminal truncations to OVA323-339 reduce and eventually abolish reactivity by transgenic T cells (Fig. 1). OVA324-334 (SQAVHAAHAEI) binds $I\text{-}A^d$ (*17, 18*) but does not induce responses from transgenic T cells expressing the DO11.10 TCR (Fig. 1). OVA323-339, but not OVA324-334, can produce strong TCR engagement and could possibly provide a tolerogenic signal to developing thymocytes.

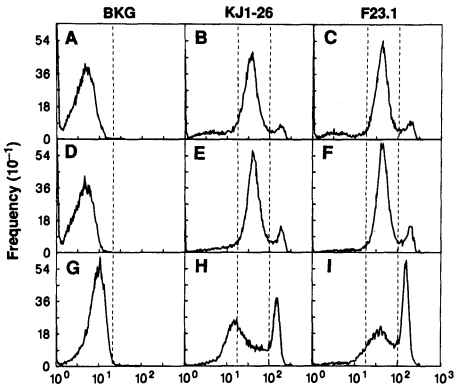

Fig. 3. Loss of clonotype but not of $V_\beta 8$ expression by immature thymocytes after peptide-antigen administration. Thymocytes from (**A** to **C**) PBS-, (**D** to **F**) OVA324-334–, or (**G** to **I**) OVA323-339–treated mice as described in Fig. 2 were isolated, and single-color flow cytometry was carried out to compare clonotype and $V_\beta 8$ expression by thymocytes. Cells were stained with b-KJ1-26 (B, E, H) or biotinylated-F23.1 (C, F, I) to label the αβ transgenic TCR clonotype or $V_\beta 8$-expressing thymocytes, respectively. SAPE was used as a secondary fluorochrome. Cell numbers (frequency) are represented on the vertical axis and fluorescence, on a logarithmic scale, is plotted on the horizontal axis. The dashed lines are set to indicate the upper and lower boundaries of the TCR^{lo} population for comparison between treatment groups. Background (BKG) staining (A, D, G) was determined in the absence of primary antibody addition. Data represent 10,000 events.

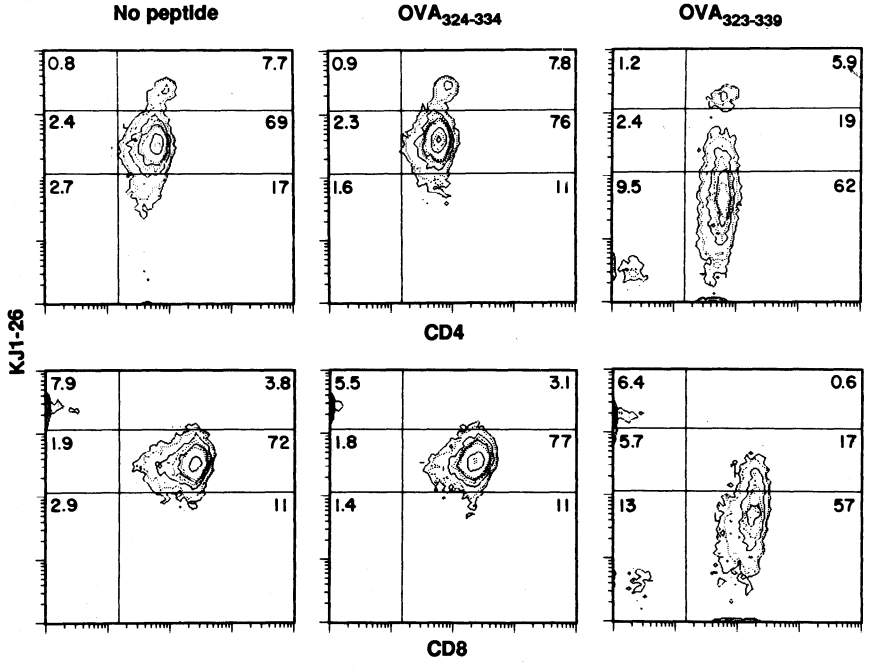

Fig. 2. Influence of peptide antigen on $CD4^+CD8^+$ TCR^{lo} thymocytes in vivo. OVA323-339, OVA324-334, or PBS (no peptide) was administered as 250 μl of a sterile 100 μM solution of peptide or PBS by intraperitoneal injection daily for 3 days. Flow cytometry of thymocytes and splenocytes was carried out as described (*15*). Two-color analysis used biotinylated-KJ1-26 (b-KJ1-26) and streptavidin-phycoerythrin (SAPE) conjugate (Southern Biotechnology, Birmingham, Alabama) to label the transgenic TCR and either FITC-conjugated GK1.5 (Becton Dickinson) or FITC-conjugated 53.6-7 (PharMingen) to label CD4 or CD8, respectively, as previously described (*15*). Stained cells were analyzed on a FACScan (Becton Dickinson). Gates were placed to define the boundaries for the $CD4^+CD8^+$ TCR^{lo} population as previously described (*15*). Data collection was gated on live thymocytes by forward- and side-angle scatter and data represent 25,000 events presented as probability isocontours. In the experiment shown, three mice were given OVA323-339, two mice OVA324-334, and two mice PBS, with similar results within groups.

To test this prediction, intraperitoneal administration of OVA323-339 was given to $H\text{-}2^d$ haplotype transgenic mice. Administration of OVA324-334 or of vehicle, phosphate-buffered saline (PBS), was used as a specificity control. Three days after beginning administration, the thymuses from OVA323-339–treated mice showed markedly reduced thymocyte numbers ($4.6 \times 10^7 \pm 1.9 \times 10^7$; mean ± SD, $n = 3$), whereas thymuses from OVA324-334– or PBS-treated mice had essentially normal numbers of viable thymocytes ($2.2 \times 10^8 \pm 0.5 \times 10^8$; $n = 2$, and $2.5 \times 10^8 \pm 0.2 \times 10^8$; $n = 2$, respectively). The loss was largely from the immature $CD4^+CD8^+$ TCR^{lo} thymocyte population (Fig. 2). In PBS- and OVA324-334–treated thymuses, $CD4^+CD8^+$ TCR^{lo} cells that express clonotype comprise 70 to 75% of all thymocytes, whereas in OVA323-339–treated mice, these are reduced to 15 to 20% ($n = 3$). $CD4^+CD8^-$ TCR^{hi} thymocytes in OVA323-339–treated mice are not greatly reduced as a percentage of thymocytes but are reduced in absolute numbers (5.9% of 4.6×10^7 in OVA323-339–treated mice, compared to 7.8% of 2.2×10^8 in untreated mice). This may either indicate a loss of

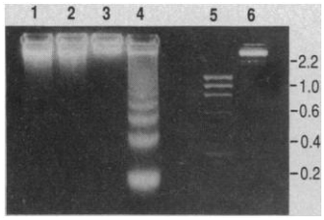

Fig. 4. DNA degradation induced in thymocytes by peptide-antigen administration. A single administration of 250 μl of sterile solution of 100 μM OVA323-339 or OVA324-334 in PBS by intraperitoneal injection was given to H-2^d haplotype transgenic and nontransgenic mice. After 20 hours, the total thymocytes and splenocytes were isolated. The thymus was divided for analysis by flow cytometry, electron microscopy, and immunohistochemistry, and for DNA preparation. Flow cytometry of thymocytes and splenocytes was carried out to confirm the transgene expression and haplotype. Total DNA was isolated from 1×10^6 thymocytes essentially as described (*13*). Samples were separated by electrophoresis on a 2.5% agarose gel containing ethidium bromide (0.1 μg/ml). Lane 1, nontransgenic control given OVA324-334; lane 2, nontransgenic control given OVA323-339; lane 3, transgenic mouse given OVA324-334; lane 4, transgenic mouse given OVA323-339. Size markers are lane 5, Hae III–digested Phi-X 174 DNA and lane 6, Hind III–digested λ phage DNA. (Weights are given in kilobases.) The experiment was repeated twice with similar results.

cells with a more mature phenotype or reflect exit of mature cells from the thymus. The immature thymocytes that remained after deletion by OVA323-339 were negative for clonotype expression (Fig. 3H), but they were positive for expression of the β chain transgene $V_\beta 8$ at levels normal for TCR^{lo} immature thymocytes (Fig. 3I). Thus, OVA323-339 treatment produced a loss of clonotype-positive TCR^{lo} thymocytes, but spared the normally small population of thymocytes that express only the β chain transgene, presumably with an endogenous α chain. The selective survival of thymocytes expressing only the β chain transgene argues that the OVA323-339–induced decrease in TCR^{lo} clonotype-expressing T cells is not a nonspecific toxic phenomenon affecting all immature thymocytes. Rather, the deletion is related to the precise antigen specificity of the complete αβ DO11.10 TCR. The β chain TCR by itself in single-construct transgenic mice does not result in OVA-peptide reactivity by T cells (*19*). Additional evidence of specificity is provided by the lack of cell loss or decrease in clonotype expression by thymocytes in response to OVA324-334 or PBS treatment (Fig. 3). Here, $CD4^+CD8^+$ TCR^{lo} thymocytes remain the majority population. These data show that peptide administration in this system leads to a rapid and specific loss of the reactive $CD4^+CD8^+$ TCR^{lo} thymocytes, similar to antigen-specific deletion in other system (*10, 11*).

To examine the processes occurring during this period of cell loss in vivo, we again administered OVA323-339 or OVA324-334 to H-2^d transgenic and nontransgenic control mice. Thymuses were examined 20 hours later for DNA degradation (Fig. 4) and histologic changes (Fig. 5). OVA323-339 induced degradation of transgenic thymocyte DNA into oligonucleosomal bands characteristic of apoptosis but did not induce this change in nontransgenic thymocytes. The control peptide, OVA324-334, produced no detectable DNA degradation in either transgenic or nontransgenic thymocytes. OVA323-339–induced apoptosis was clearly evident histologically (Fig. 5). Striking chromatin condensation and cell shrinkage were seen in the majority of cortical thymocytes from transgenic mice that received OVA323-339, but not in those that received OVA324-334. Apoptosis was seen among thymocytes throughout the thymus. The extent of involvement includes subcapsular and deep cortical areas. Identification of distinct cortical-medullary boundaries is difficult in the electron micrographs, but apoptotic cells may be present in some medullary areas. However, thymuses that have undergone 3-day treatments with OVA323-339 show virtually acellular cortex with relatively normal cellularity within the medulla. Apoptotic cells appeared to remain in situ, because no apoptotic cells were identified within the small vessels of the thymus to suggest that they might be exported.

Cytolytic T cells, glucocorticoids, and x-irradiation induce apoptotic cell death in lymphocytes (*20–23*). Anti-CD3 antibodies and the bacterial "superantigen" staphylococcal enterotoxin B induce apoptosis in an in vitro thymic organ culture system (*13, 14*). Further, the death of autoreactive thymocytes in vitro can be prevented by inhibition of RNA or protein synthesis (*24*). Administration of anti-CD3 antibody produced DNA degradation in thymocytes in vivo (*25*). This report demonstrates antigen-specific deletion by means of apoptosis in vivo. Our results directly demonstrate that the elimination of thymocytes caused by contact with antigen within the thymus proceeds in situ by apoptosis and largely involves the immature $CD4^+CD8^+$ cells. This system should prove useful in examining the roles of various thymic cells in the induction of tolerance.

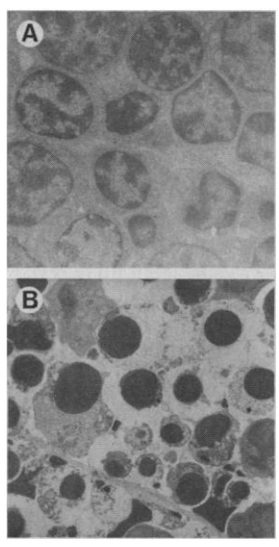

Fig. 5. Apoptosis of cortical thymocytes induced by peptide-antigen. (**A**) Thymic cortex (×33,000) of a transgenic mouse treated with OVA324-334. (**B**) Thymic cortex (×20,000) of a transgenic mouse treated with OVA323-339. Samples were obtained from thymuses as described in Fig. 4 and immediately were placed in an isotonic 3% glutaraldehyde solution. Electron micrographs of thin, plastic-embedded sections were taken on a JEOL 100CX model electron microscope. Lower power examination was carried out on thick plastic sections stained with toluidine-blue.

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Transport Protein Genes in the Murine MHC: Possible Implications for Antigen Processing

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T lymphocyte activation requires recognition by the T cell of peptide fragments of foreign antigen bound to a self major histocompatibility complex (MHC) molecule. Genetic evidence suggests that part of the class II region of the MHC influences the expression, in trans, of MHC class I antigens on the cell surface, by regulating the availability of peptides that bind to and stabilize the class I molecule. Two closely related genes in this region, *HAM1* and *HAM2*, were cloned and had sequence similarities to a superfamily of genes involved in the ATP-dependent transport of a variety of substrates across cell membranes. Thus, these MHC-linked transport protein genes may be involved in transporting antigen, or peptide fragments thereof, from the cytoplasm into a membrane-bounded compartment containing newly synthesized MHC molecules.

THE LIGAND RECOGNIZED BY THE antigen-specific T cell receptor consists of a peptide fragment of foreign antigen bound to a molecule encoded in the MHC. Peptides bound by MHC class I molecules are recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs), whereas peptides bound to MHC class II molecules are recognized by $CD4^+$ (primarily helper) T cells. Native antigens added exogenously to cells (which are taken up by endocytosis into membrane-bounded vesicles) preferentially yield peptide–class II complexes, whereas endogenously synthesized antigens, or antigens introduced directly into the cytoplasm of living cells, preferentially yield peptide–class I complexes (*1*). However, this distinction is not absolute (*2*). The intracellular pathways taken by antigens destined for processing and presentation to T cells, the exact molecular nature of the physiologic antigenic peptides bound by MHC molecules, and the mechanism of and subcellular compartment for the production of peptides remain unknown.

A mutated murine cell line designated RMA-S, originally selected for loss of class I surface expression (*3*), is defective in the assembly of peptide–class I complexes (*4*). Thus, virally infected RMA-S cells are resistant to lysis by specific CTLs. These cells produce normal class I and β_2-microglobulin mRNA and polypeptides, which, in the absence of bound peptide fragments, fail to associate in a stable manner and are rapidly lost from the cell surface at 37°C. Exogenously added antigenic peptides bind to and stabilize these "empty" class I molecules, thereby rescuing class I surface expression and rendering the cells sensitive to lysis by CTLs (*4*). Although the genetic defect in these cells has not been mapped, the defect in several mutant human cell lines with a similar phenotype (*5*) maps to the class II (*HLA-D*) region of the human MHC. The class II region of the rat MHC also contains a polymorphic, trans-acting gene (called *cim*, for class I modifier)(*6*). Mapping data in the rat system, in which all known MHC genes are colinear with the murine MHC, suggests that the murine equivalent of the *cim* gene maps between the *H-2K* and *I-*A_α genes, most likely between $A_{\beta 3}$ and $A_{\beta 2}$ (*7*). This location is consistent with the location of the deletions in the class I–defective human cell lines. The molecular and genetic evidence thus suggests that the class II region of the MHC contains a gene or genes involved in antigen processing and presentation to class I–restricted T cells. It is possible that the defective gene in RMA-S and the human class I mutants may be identical to the *cim* gene.

The $A_{\beta 3}$ to $A_{\beta 2}$ region of the murine MHC contains at least seven previously undetected genes (*8*). Three of these genes are

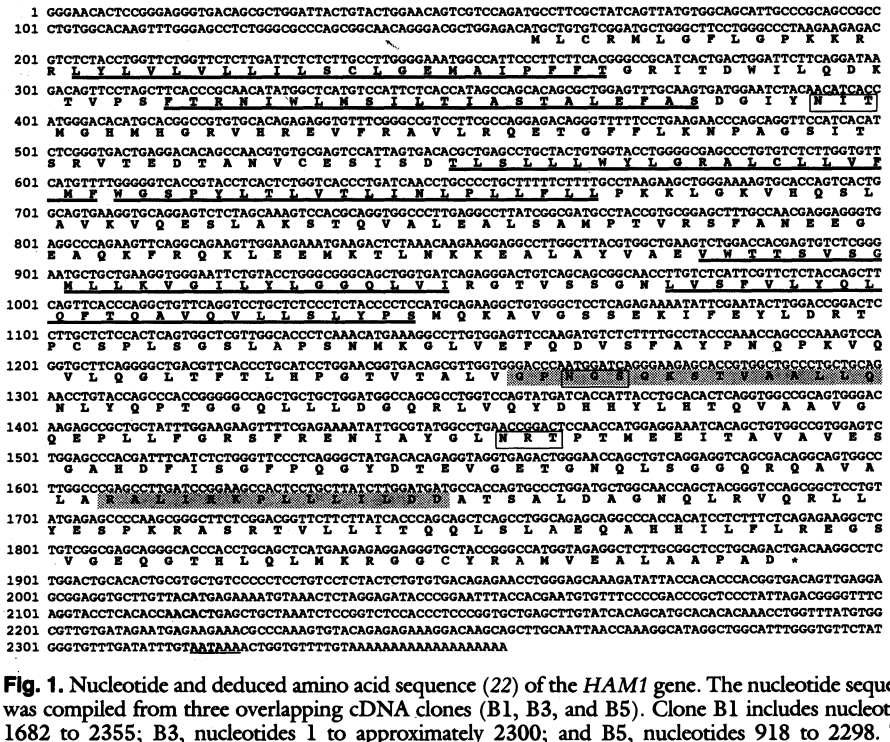

Fig. 1. Nucleotide and deduced amino acid sequence (*22*) of the *HAM1* gene. The nucleotide sequence was compiled from three overlapping cDNA clones (B1, B3, and B5). Clone B1 includes nucleotides 1682 to 2355; B3, nucleotides 1 to approximately 2300; and B5, nucleotides 918 to 2298. The nucleotide sequence of overlapping portions of the three clones that were sequenced are identical except for the G residues at positions 2239 and 2241 in the 3′ untranslated region, which are both T residues in B5. The assignment of these nucleotides as G was confirmed by sequencing cloned BALB/c genomic DNA. In the nucleotide sequence, the polyadenylation signal sequence is underlined and the stop codon identified with an asterisk. In the protein sequence, putative membrane-spanning regions are underlined, potential N-linked glycosylation sites are boxed, and the two regions putatively involved in ATP binding are shaded. Numbering on the left refers to nucleotide positions, and numbering on the right refers to amino acid positions. The GenBank accession number for the nucleotide sequence is M55637.

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