The Human UTY Gene Encodes a Novel HLA-B8-Restricted H-Y Antigen[1]

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The mammalian Y chromosome encodes male-specific minor histocompatibility (H-Y) Ags that are recognized by female T cells in an MHC-restricted manner. Two human H-Y epitopes presented by HLA-A2 and HLA-B7, respectively, have been identified previously and both are derived from the SMCY gene. We previously isolated CD8$^+$ CTL clones that recognized a male-specific minor histocompatibility Ag presented by HLA-B8. In contrast to the SMCY-encoded H-Y epitopes, the B8/H-Y Ag was not presented by fibroblasts from male donors, suggesting that it was encoded by a novel gene. We now report that the HLA-B8-restricted H-Y epitope is defined by the octameric peptide LPHNHTDL corresponding to aa residues 566–573 of the human UTY protein. Transcription of the UTY gene is detected in a wide range of human tissues, but presentation of the UTY-derived H-Y epitope to CTL by cultured human cells shows significant cell-type specificity. Identification of this CTL-defined H-Y epitope should facilitate analysis of its contribution to graft/host interactions following sex-mismatched organ and bone marrow transplantation. *The Journal of Immunology,* **2000, 164: 2807–2814.**

The mammalian Y chromosome encodes histocompatibility determinants that trigger rejection of skin grafts from male mice by syngeneic females (1) and has been implicated in the rejection of human bone marrow grafts from MHC-compatible male donors by female recipients (2, 3). In both mice and humans, class I MHC-restricted CTL recognizing male-specific (H-Y)[4] Ags have been generated in vitro by secondary mixed lymphocyte culture from females sensitized in vivo with male cells (2, 4–6). Two genes on the murine Y chromosome have been shown to encode H-Y Ags. SMCY encodes H-2K^k- and H-2D^k-restricted H-Y epitopes (7, 8) and UTY encodes a H-2D^b-restricted H-Y epitope (9). Studies in humans have demonstrated that the human homologue of SMCY encodes two distinct human H-Y epitopes, presented by either HLA-A2 (10) or HLA-B7 (11). However, H-Y Ags encoded by genes other than SMCY have not been described in humans.

In a previous study (12), we isolated CD8$^+$ cytotoxic T cell clones from a male who had received an allogeneic hemopoietic cell transplant (HCT) from his MHC genotypically identical sister. These CTL were restricted by HLA-B8 and recognized a H-Y Ag presented by hemopoietic cells, but not fibroblasts obtained from HLA-B8$^+$ males (12). Because fibroblasts were recognized by the HLA-A2- and HLA-B7-restricted CTL specific for epitopes derived from SMCY (13), this suggested that a distinct Y chromosome gene might encode the HLA-B8-restricted H-Y Ag. We show in this study that aa 566–573 of the protein encoded by the human UTY gene comprise the HLA-B8-restricted H-Y epitope, thereby demonstrating that human H-Y Ags are encoded by more than one locus on the Y chromosome.

Materials and Methods

Cell culture

The CD8$^+$ HLA-B8-restricted, H-Y-specific CTL clones MRR-2, MRR-17, and MRR-24 used in these experiments were isolated and characterized, as previously described (12). The CTL were thawed, resuspended in RPMI-HEPES (4 mM) supplemented with 10% pooled, heat-inactivated human serum, 2 mM L-glutamine, and 1% penicillin/streptomycin (termed CTL medium), and stimulated in vitro every 14–21 days using anti-CD3 mAb (Ortho, Raritan, NJ), γ-irradiated (70 Gy) allogeneic EBV-transformed B cells (LCL), and γ-irradiated (35 Gy) allogeneic PBMC as feeder cells, and 50 U/ml IL-2, as previously described (14). CTL were used in cytotoxicity and epitope reconstitution assays on days 11–15 following stimulation.

EBV-LCL lines used in this study were derived from the bone marrow donor and recipient, normal male and female volunteers, and individuals known to have terminal deletions of the Y chromosome. The EBV-LCL lines derived from individuals with Y chromosome deletions had been analyzed and reported in previous publications (15–18), but for the purposes of this study, their identity was concealed and the lines were examined for B8/H-Y expression and Y chromosome content in a blinded fashion. After completion of the analysis, the identity of the lines was revealed; the six lines discussed in the text and depicted in Fig. 1 are referred to using the original designations from the publications in which they were previously reported. EBV-LCL were maintained in RPMI-HEPES (4 mM) supplemented with 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine, and 1% penicillin/streptomycin (termed LCL medium). Primary fibroblast lines from the bone marrow donor and recipient and from normal volunteers were grown from explants of skin biopsy specimens, as described (19). Bone marrow stromal cell lines were generated from bone marrow mononuclear cells obtained from healthy HLA-B8$^+$ male donors, as previously described (20, 21). COS-7 and WEHI 164 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in

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[4] Abbreviations used in this paper: H-Y, male-specific histocompatibility Ag; EBV-LCL, EBV-transformed lymphoblastoid cell line; GVHD, graft-vs-host disease; GVL, graft-vs-leukemia; HCT, hemopoietic cell transplant; STS, sequence-tagged site.

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Table I. *Oligonucleotide primer pairs*[a]

Gene	5′ Primer	Restriction Site	3′ Primer	Restriction Site
DFFRY	GGGGTACCGTGTCAAGTATGACAGCCATC	*Kpn*I	AATTCGAGCGGCCGCTTTTCACTGATCCTTCATCT	*Not*I
DBY	GGGGTACCAGGGATGAGTCATGTGGTG	*Kpn*I	GGAATTCTGCAAAGCAGATTCAGTTGC	*Eco*RI
UTY	GGGGTACCGTTTCCATGAAATCCTGCGC	*Kpn*I	GCTCTAGACCAGTGGTGCAGAAATTTCC	*Xba*I

[a] Oligonucleotide primer pairs used to amplify the entire coding sequence of the DFFRY (Genbank accession no. AF000986), DBY (Genbank AF000985), and UTY (Genbank AF000994) genes from first-strand cDNA prepared from EBV-LCL from a healthy HLA-B8$^+$ male donor. All sequences are written 5′ to 3′. Each primer sequence contains in its 5′ portion the recognition sequence (underlined) of a restriction enzyme which facilitated cloning of the PCR-amplified sequence into a cDNA expression vector.

DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin (termed complete medium).

Cytotoxicity assays

For cytotoxicity assays, 1–2 × 10^6 EBV-LCL, fibroblasts, or bone marrow stromal cells were labeled overnight at 37°C with 100 μl ^{51}Cr, washed twice, dispensed into triplicate cultures at 5 × 10^3 target cells/well in 96-well round-bottom microtiter plates, and incubated for 4 h with effector T cells at various E:T ratios in a total volume of 200 μl. In some experiments, ^{51}Cr-labeled target cells were pulsed with various concentrations of synthetic peptides for 30 min at 18°C before being used in the cytotoxicity assay. Percentage of specific lysis was calculated using the standard formula (12).

Transfection of COS-7 cells and cytokine release assays

COS-7 cells were transiently cotransfected with cDNA expression vectors (pcDNA3.1; Invitrogen, San Diego, CA) encoding a class I MHC allele and a selected gene from the human Y chromosome. COS-7 cells were plated the day before transfection at 7 × 10^3 cells/well into 96-well flat-bottom microtiter plates, washed once with PBS, and overlaid in duplicate or triplicate with 50 μl Opti-MEM I (Life Technologies, Gaithersburg, MD) containing 50 ng of plasmid DNA encoding the Y chromosome gene of interest, 25 ng of plasmid DNA encoding an MHC class I allele, and 0.35 μg Lipofectamine (Life Technologies). After 4–5 h at 37°C, 50 μl of DMEM containing 20% (v/v) FCS was added to each well. The medium in each well was replaced at 24 h after transfection with 200 μl complete medium, and again at 48 h after transfection with 200 μl of a cell suspension containing 2 × 10^4 H-Y-specific T cells in Iscove's medium supplemented with 10% human serum, 2 mM L-glutamine, 1% penicillin/streptomycin, and 5 U/ml IL-2.

Supernatants from COS-7/T cell cocultures were harvested after 24 h and assayed for the presence of TNF and IFN-γ. The TNF assay was performed by immediately transferring 100 μl of supernatant from COS-7/T cell cocultures to 96-well flat-bottom microtiter plates containing 3.5 × 10^4 TNF-sensitive WEHI 164 cells/well in 100 μl of complete medium supplemented with 4 μg/ml actinomycin D (Sigma, St. Louis, MO) and 50 mM LiCl (22). After 16 h at 37°C, 20 μl of Alamar blue (BioSource International, Camarillo, CA) was added to each well. Viability of the WEHI cells in each well was determined 24 h later by measuring the absorbance at 570 and 630 nm. In each assay, absorbance at 570/630 nm was also measured in identical wells to which standard dilutions of recombinant TNF (Genzyme, Cambridge, MA) had been added. Supernatants from COS-7/T cell cocultures were also assayed for the presence of IFN-γ by ELISA (Endogen, Woburn, MA).

Flow cytometry

Aliquots of 5 × 10^5 cells were washed once in PBS/2% FCS, stained with a FITC-conjugated HLA-B8-specific mAb (One Lambda, Los Angeles, CA) or a FITC-conjugated murine IgG2a isotype control (Becton Dickinson Immunocytometry Systems, Mountain View, CA), and analyzed in a FACSCalibur cytometer with Cellquest software (Becton Dickinson Immunocytometry Systems).

STS content mapping of Y chromosome deletion mutants

Genomic DNA was isolated from EBV-LCL using QIAamp spin columns (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. Oligonucleotide primer pairs specific for STSs previously mapped to the Y chromosome (18) were used to amplify via PCR the corresponding Y-chromosomal target sequences from genomic DNA of each cell line. Amplification of STSs was performed in 25-μl reactions containing dATP, dTTP, dCTP, and dGTP at 200 μM each, 1.6 mM $MgCl_2$, each primer at 1 μM, 1 U platinum *Taq* DNA polymerase (Life Technologies), and 500 ng genomic DNA template, using previously described thermal cycling protocols (18). Aliquots (8 μl) of each PCR reaction were separated electrophoretically on 2% agarose or 5% polyacrylamide gels, and cell lines were scored as positive or negative for each STS on the basis of the presence or absence, respectively, of a band of the appropriate size (18). Genomic DNA extracted from EBV-LCL derived from normal male and female donors served as positive and negative controls, respectively.

PCR cloning of Y chromosome genes

Pairs of oligonucleotide primers (Table I) containing selected 5′ restriction sites and complementary to sequences spanning the translation start and translation stop sites of the human DFFRY (GenBank accession no. AF000986), DBY (AF000985) and UTY (AF000994) genes were used to amplify the entire coding sequence of the corresponding genes from first strand cDNA prepared from EBV-LCL from a healthy HLA-B8$^+$ male donor. Briefly, total RNA was isolated from 1 × 10^7 EBV-transformed lymphoblastoid cells using STAT-60 (Tel-Test, Friendswood, TX), then reverse transcribed (Superscript II; Life Technologies) and amplified using the Clontech Advantage cDNA PCR kit (Clontech Laboratories, Palo Alto, CA), according to the manufacturer's instructions. The RT-PCR product was digested with the appropriate combination of restriction enzymes (Table I), ligated into the corresponding sites of pcDNA3.1 (Invitrogen), and transformed into DH10α *Escherichia coli*. Minipreps of plasmid DNA were made from five to six individual colonies from each ligation reaction using standard protocols.

Peptide synthesis

Peptides were synthesized on a model 432A Synergy Peptide Synthesizer (Perkin-Elmer Applied Biosystems, Norwalk, CT).

Northern blot analysis

A 1.3-kb *Pst*I-*Bam*HI fragment of the UTY gene (corresponding to nt 2570–3882 of GenBank accession no. AF000994) was labeled with ^{32}P using the Random Prime Labeling Kit (Boehringer Mannheim, Indianapolis, IN) and used to probe a dot-blot array of poly(A)$^+$ RNA extracted from 50 different human tissues (Human RNA Master Blot; Clontech) or Northern blots of poly(A)$^+$ RNA extracted from cultured human cells. Hybridization was performed overnight at 65°C in the presence of 100 μg/ml sheared salmon sperm DNA (Life Technologies) and 6 μg/ml human C_0t-1 DNA. RNA for the Northern blots was prepared by extracting total RNA from 50–100 × 10^6 EBV-LCL, fibroblasts, or bone marrow stromal cells derived from normal HLA-B8$^+$ male donors using RNeasy spin columns (Qiagen), then purifying the poly(A)$^+$ fraction using Oligotex (Qiagen) beads.

Results

The gene encoding the HLA-B8-restricted H-Y Ag maps to either the proximal short arm or proximal long arm of the Y chromosome

The CD8$^+$ CTL clones MRR-2, MRR-17, and MRR-24 recognized EBV-LCL lines from HLA-B8$^+$ male donors, but not HLA-B8$^+$ female donors, suggesting that expression of the B8-restricted Ag was controlled by a gene or genes on the Y chromosome (12). To determine the location of this Y chromosome gene, these three CTL clones were assayed for lytic activity against EBV-LCL derived from individuals known to carry terminal deletions of the Y chromosome. Forty-one such EBV-LCL lines were screened by flow cytometry for expression of HLA-B8. Nine lines that expressed HLA-B8 were identified, and these were then tested as

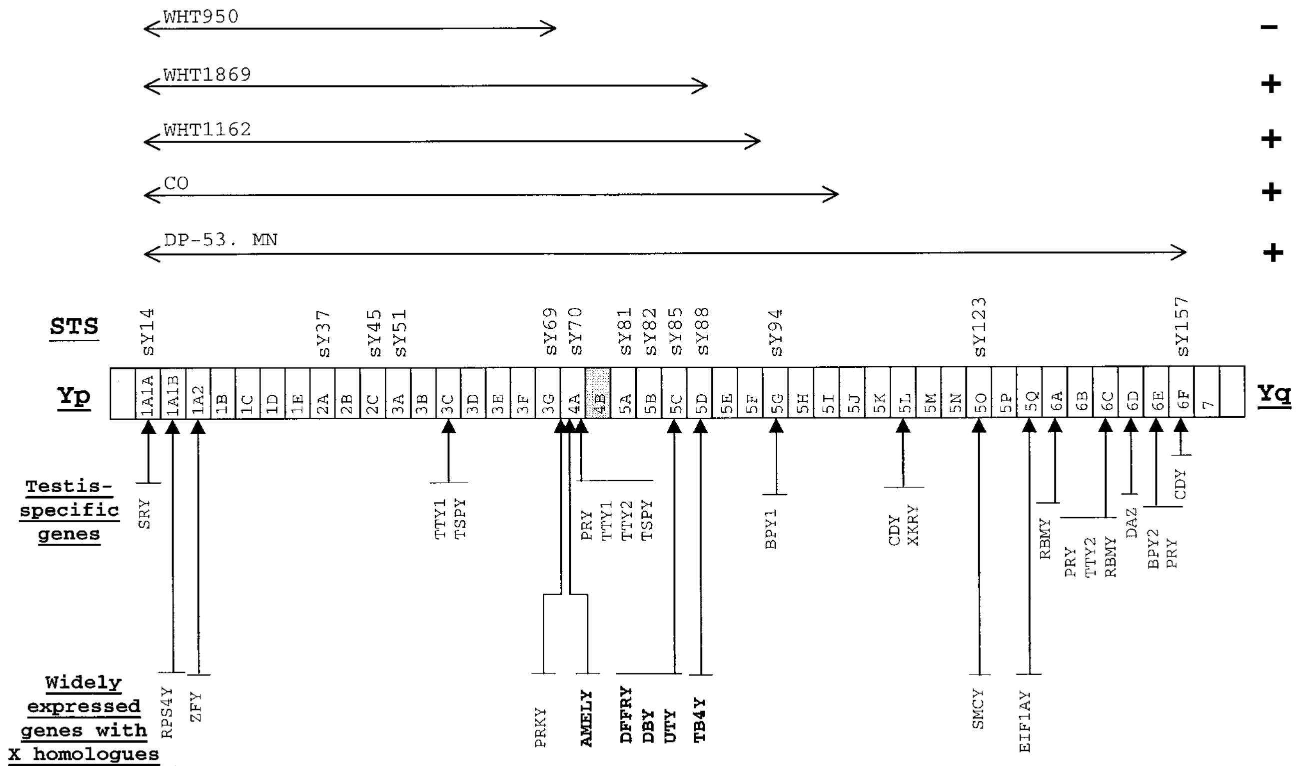

FIGURE 1. The gene encoding the HLA-B8-restricted H-Y epitope maps to the Y chromosome interval defined by the STSs sY69 and sY88. B8/H-Y Ag expression (indicated in the column at *upper right*) in EBV-LCL lines derived from HLA-B8$^+$ individuals known to carry terminal deletions of the Y chromosome was established by testing the lines in a 4-h ^{51}Cr release assay with three B8/H-Y-specific CTL clones, MRR-2, MRR-17, and MRR-24, at an E:T ratio of 10:1. +, Indicates specific lysis of >20%; −, indicates specific lysis of ≤2%, with all three clones. Y chromosome content was determined by testing genomic DNA extracted from each LCL line for the presence of 13 STSs previously mapped to the short (Yp; *left*) or long (Yq; *right*) arms of the Y chromosome (18). The location of each STS along the 43-interval deletion map of the Y chromosome is indicated; the centromere maps to interval 4B, which is shaded gray. Y chromosome content of six of the LCL lines is shown by a double-ended arrow that encompasses all of the STSs for which each line tested positive. Localization of the Yq breakpoint for lines WHT1162 and CO was further refined using published data (18). In the *lower half* of the figure, the deletion interval to which previously identified Y chromosome genes have been mapped is indicated (18, 23). The genes are organized into two groups, testis-specific genes and widely expressed genes with X homologues, as previously defined (23). The AMELY gene, whose expression appears limited to the developing tooth bud, is included in the latter category because it has an X homologue and is expressed outside the testes (24). Line WHT950 was previously reported (16), line WHT1162 was initially published as patient 5 (15), WHT1869 was previously reported (18), and lines CO and MN were previously reported (17).

targets for the three B8/H-Y-specific CTL clones in a 4-h ^{51}Cr release assay. Five of the LCL lines were lysed (specific lysis >20% at an E:T ratio of 10:1 by all three clones), while the remaining four were not (specific lysis ≤2% at an E:T ratio of 10:1).

Genomic DNA was harvested from each of the nine lines to determine their Y chromosome content by the technique of STS content mapping (18). Lines were scored for the presence or absence of six previously mapped STSs distributed over the short arm of the Y chromosome (Yp) and seven STSs distributed over the long arm (Yq) (18). All four LCL lines that failed to express B8/H-Y were positive for one or more STSs on Yp, but negative for all STSs on Yq. One of these lines, WHT950, was positive for the five most distal Yp STSs of the six that were evaluated, thereby excluding distal Yp as the locus for the B8/H-Y gene (Fig. 1). Of the five LCL lines that were lysed by B8/H-Y-specific CTL, two (DP-53, MN) were positive for all six STSs on Yp and all seven STSs on Yq; the remaining three lines were positive for all six of the Yp STSs tested, and for one or more of the STSs on proximal Yq. One of these lines, CO, appeared to have a Yq breakpoint distal to deletion interval 5G, but proximal to deletion interval 5O (Fig. 1). The other two lines, WHT1869 and WHT1162, appeared to have Yq breakpoints that mapped proximal to deletion interval 5G (Fig. 1). Significantly, all three lines were negative for the STS sY123, which has previously been mapped to deletion interval 5O on the Y chromosome that is known to encode the SMCY gene (23). Thus, the results of STS content mapping excluded SMCY as the gene encoding B8/H-Y and suggested that the gene or genes controlling expression of B8/H-Y mapped to that region of the Y chromosome defined by the STSs sY69 and sY88 and between deletion intervals 4A and 5D (Fig. 1).

The UTY gene encodes the B8/H-Y Ag

The region of the human Y chromosome lying between the markers defined by sY69 and sY88 has been shown in previous studies to contain at least nine distinct expressed genes (23). Four of these genes, PRY, TTY1, TTY2, and TSPY, are expressed exclusively in the testes (23), and another, AMELY, is expressed solely in the developing tooth bud (24). Thus, these five genes were thought

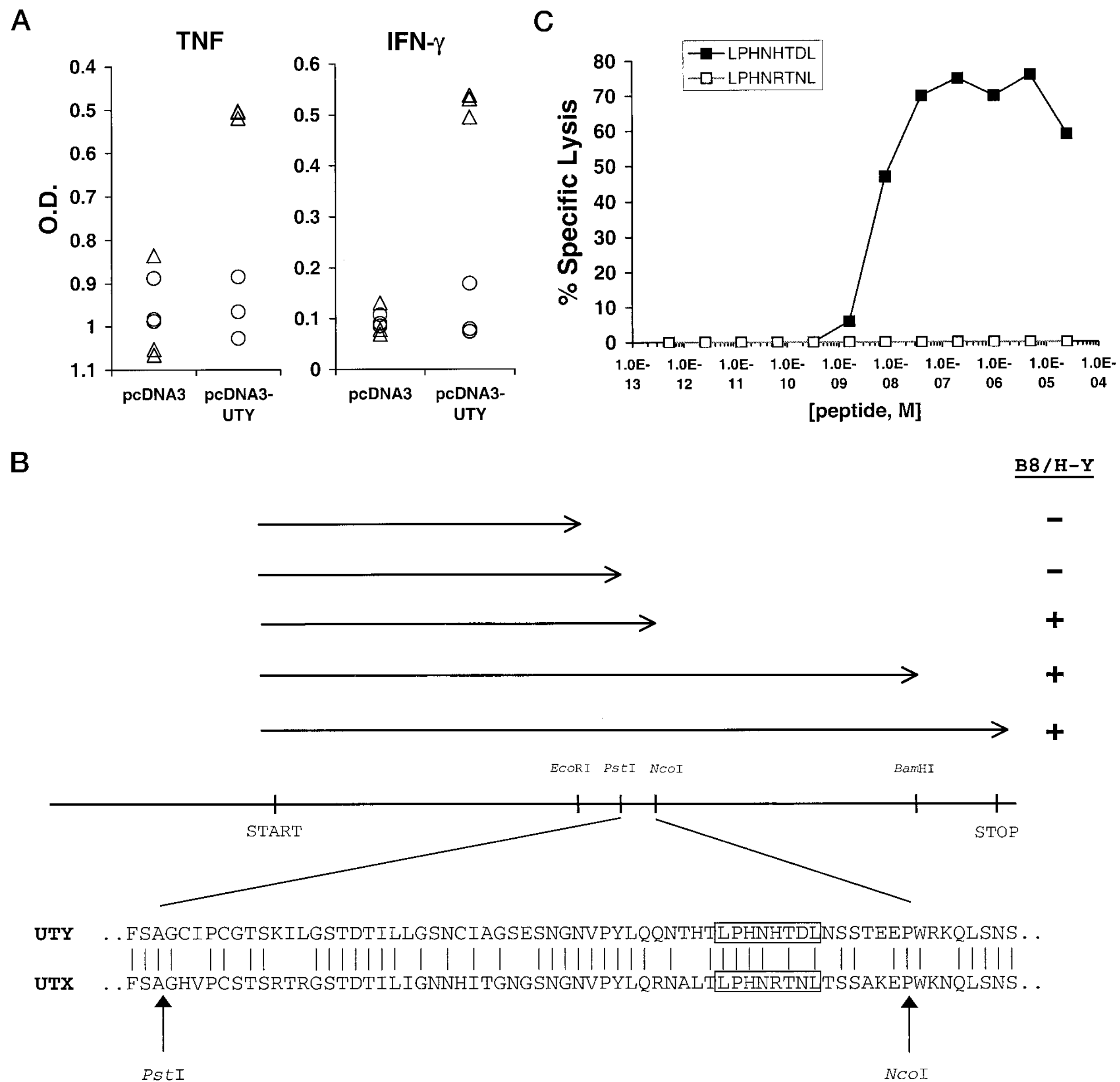

FIGURE 2. Amino acid residues 566–573 of the UTY gene comprise the HLA-B8-restricted H-Y Ag. *A*, Transient transfection of a UTY cDNA into COS-7 cells reconstitutes the B8/H-Y epitope and stimulates TNF and IFN-γ release from B8/H-Y-specific CTL clone MRR-24. MRR-24 CTL were cultured for 24 h in triplicate wells with COS-7 cells that had been transfected 48 h previously with vector pcDNA3 (Invitrogen) or pcDNA3 containing a UTY cDNA, in conjunction with either pcDNA3/HLA-A3 (○) or pcDNA3/HLA-B8 (△). Supernatants were harvested and assayed for the presence of TNF (*left*) or IFN-γ (*right*). The units on the ordinate of the TNF panel (*left*) were intentionally inverted so that, in both panels, increasing cytokine release would be indicated in the upward direction. *B*, The B8/H-Y epitope is encoded by a ~173-nt interval in the 5′ half of the UTY gene. B8/H-Y-specific CTL from clone MRR-24 were cultured with COS-7 cells transfected with a HLA-B8 cDNA and UTY cDNAs with nested 3′ deletions, and the supernatants were harvested after 24 h and assayed for TNF and IFN-γ release. The size of each UTY cDNA is indicated by an arrow above a map of the UTY gene (GenBank accession no. AF000994) on which the relative positions of the translation start and stop sites and several restriction sites are noted. cDNAs that stimulated HLA-B8-dependent TNF and IFN-γ release from MRR-24 CTL are indicated by + in the *right-hand column*. The amino acid sequence of the predicted UTY protein encoded by the interval between the *Pst*I and *Nco*I sites at nt 2570 and 2743, respectively, is aligned with the homologous sequence from the X chromosome-encoded UTX gene. Residues at which there is identity between the two sequences are indicated by a vertical bar. Boxes are drawn around the octameric sequence LPHNHTDL in UTY and the homologous octameric sequence LPHNRTNL of UTX. *C*, Evaluation of synthetic LPHNHTDL and LPHNRTNL peptides for B8/H-Y epitope-reconstituting activity. Female HLA-B8$^+$ EBV-LCL, derived from the hemopoietic cell donor from whom the B8/H-Y-specific CTL were generated, were labeled overnight with ^{51}Cr, then pulsed with various concentrations of each of the two peptides, and used as targets for B8/H-Y-specific CTL clone MRR-24 in a 4-h cytotoxicity assay. The E:T ratio was 5:1. Comparable results were obtained with B8/H-Y-specific CTL clones MRR-2 and MRR-17 (not shown), and the reproducibility of the results was demonstrated in three separate experiments.

unlikely to encode the B8/H-Y Ag. The other four, DFFRY, DBY, UTY, and TB4Y, are expressed in a wider range of tissues, including hemopoietic cells (23). All four genes also have functional homologues on the X chromosome that encode similar, but not identical, proteins. Thus, sequence divergence between these four Y chromosome genes and their X chromosome homologues could have created a male-specific T cell epitope. Because the short (44-aa) protein encoded by the TB4Y gene contained within it no peptide sequences predicted to bind to the HLA-B8 molecule (25), analysis of this gene was not pursued further.

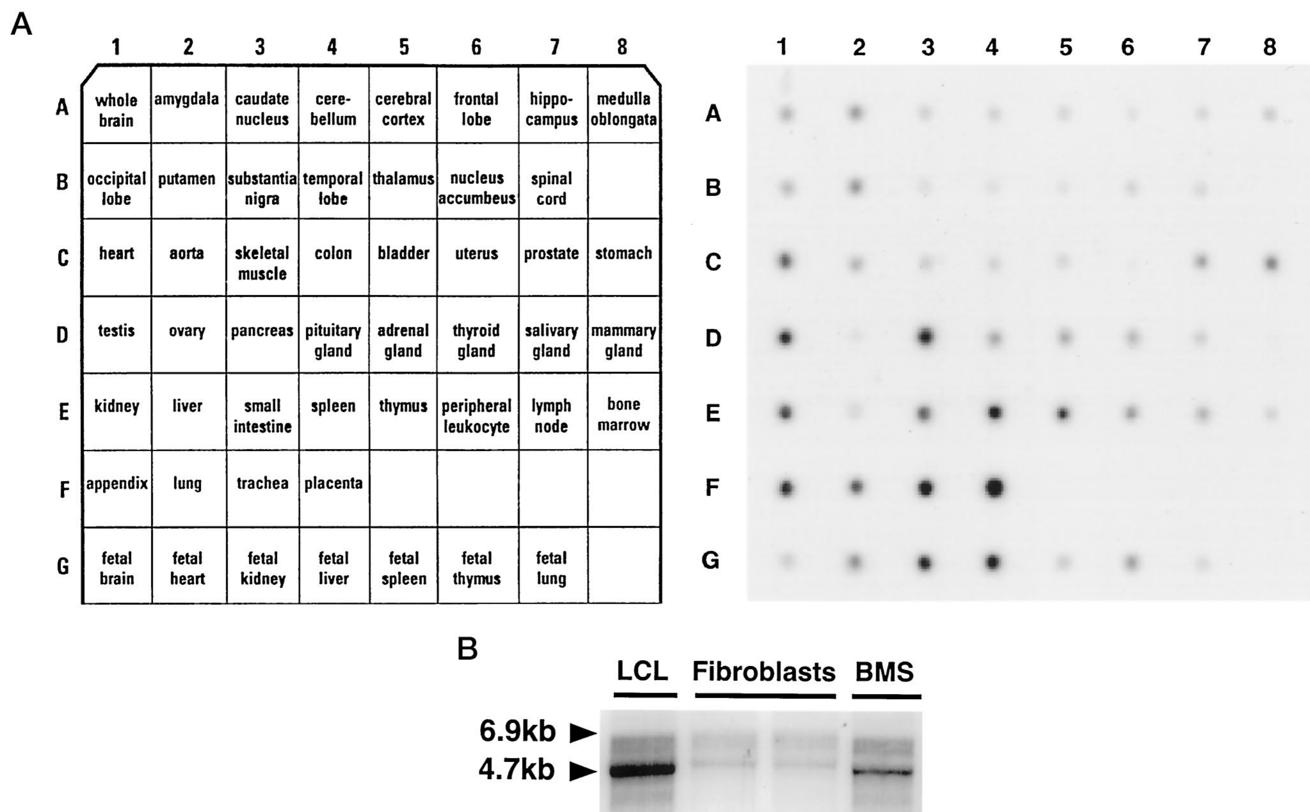

FIGURE 3. Analysis of UTY gene expression. *A*, RNA dot-blot analysis of UTY expression in 50 different human tissues. An array of poly(A)$^+$ RNA extracted from the human tissues shown in the legend (*left*) and adjusted to reflect a comparable level of transcriptional activity (RNA Master Blot; Clontech) was probed with a ^{32}P-labeled 1.3-kb UTY cDNA restriction fragment spanning the interval between nt 2570 and 3882. *B*, Northern analysis of UTY expression in cultured human cells. Poly(A)$^+$ RNA (1.5 μg/lane) from EBV-LCL (*lane 1*), dermal fibroblasts (*lanes 2* and *3*), and bone marrow stromal cells (*lane 4*) derived from two normal HLA-B8$^+$ males was size fractionated on a formaldehyde/1.2% agarose gel and probed with the same 1.3-kb UTY cDNA fragment as in *A*.

To determine whether DFFRY, DBY, or UTY encoded the B8/H-Y epitope, cDNAs for each of these genes were cloned into pcDNA3 and transiently cotransfected with a plasmid encoding HLA-B8 or a plasmid encoding HLA-A3 into COS-7 cells. The COS transfectants were then cultured with B8/H-Y-specific CTL and the supernatants assayed for the presence of TNF and IFN-γ. Expression of the cDNAs for DFFRY and DBY together with HLA-B8 did not stimulate either TNF or IFN-γ release from B8/H-Y-specific CTL (data not shown). However, expression of a UTY cDNA stimulated both TNF and IFN-γ release from B8/H-Y-specific CTL in a HLA-B8-dependent fashion (Fig. 2*A*). These data demonstrated that the B8/H-Y epitope is derived from UTY.

Identification of the B8/H-Y peptide

The location of the B8/H-Y epitope within the UTY gene was identified by generating a panel of nested deletions of the UTY cDNA lacking variable amounts of the 3′ portion of the UTY gene, and cotransfecting these with a plasmid encoding HLA-B8 into COS-7 cells. Three such UTY deletion mutants, terminating at the *Eco*RI, *Pst*I, and *Nco*I restriction sites located at nt 2417, 2570, and 2743 of the UTY gene (GenBank accession no. AF000994), respectively, were particularly informative. While transfection of the UTY gene fragment 5′ to the *Nco*I site along with HLA-B8 was able to reconstitute B8/H-Y epitope in COS-7 cells, transfection of either of the shorter 5′ fragments terminating at the *Eco*RI or *Pst*I sites, respectively, was not (Fig. 2*B*).

The region between the *Pst*I site and *Nco*I site encoded residues ~472 through ~581 of the corresponding UTY protein sequence (GenBank accession no. AF000994). This amino acid sequence was examined for octameric and nonameric peptides that (1) differed by at least 1 aa from the corresponding sequence found in the protein encoded by the homologous UTX gene carried on the X chromosome, and (2) was compatible with the described sequence motif for peptides binding to the HLA-B8 molecule (25). One such peptide, the octamer LPHNHTDL, comprising residues 566–573, satisfied both criteria. This peptide differs at two residues with the octameric sequence found in the corresponding region of the predicted UTX protein (LPHNHTDL vs LPHNRTNL) (Fig. 2*B*).

To determine whether the B8/H-Y epitope corresponded to the LPHNHTDL sequence from UTY, the peptide was synthesized and tested for its ability to sensitize female HLA-B8$^+$ target cells to lysis by B8/H-Y-specific CTL. The octameric peptide LPHNRTNL corresponding to residues 617–624 of the homologous UTX protein, which was also compatible with the HLA-B8 peptide-binding motif, was synthesized and tested as a control. Female HLA-B8$^+$ EBV-LCL and fibroblasts derived from the original BMT donor were labeled overnight with ^{51}Cr, incubated for 30 min at room temperature in medium containing 10-fold serial dilutions of the two peptides ranging from 1 mM to 10 fM, and used as target cells for B8/H-Y-specific CTL clones MRR-2, MRR-17, and MRR-24. Target cells pulsed with the LPHNRTNL peptide were not lysed at any of the peptide concentrations tested (Fig. 2*C*). However, cells pulsed with the LPHNHTDL peptide were lysed by all three CTL clones at peptide concentrations above 1 nM, with half-maximal lysis seen at ~7 nM (Fig. 2*C*). Thus, the

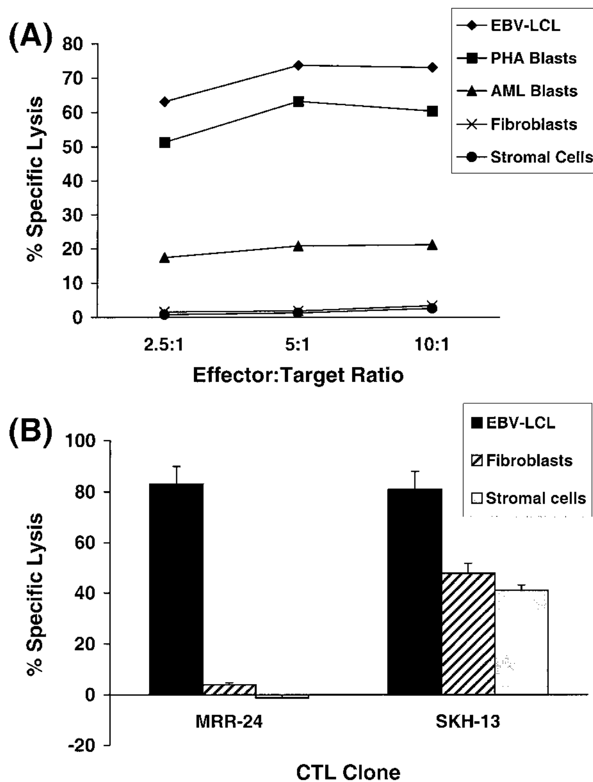

FIGURE 4. Expression of the B8/H-Y epitope in cultured cells. *A*, Cytolytic activity of representative B8/H-Y-specific CTL clone MRR-24 against EBV-transformed B cell lines (EBV-LCL), PHA-stimulated T cell blasts, acute myeloid leukemia (AML) blasts, fibroblasts, and bone marrow stromal cells derived from HLA-B8$^+$ males with cytogenetically normal Y chromosomes. *B*, Cytolytic activity of CD8$^+$ CTL clones MRR-24 and SKH-13, specific for B8/H-Y and a distinct minor H Ag presented by HLA-A2, respectively, against EBV-LCL, fibroblasts, and bone marrow stromal cells derived from a HLA-A2$^+$/HLA-B8$^+$ normal male donor, at an E:T ratio of 5:1. The mean and SD of triplicate determinations are indicated.

LPHNHTDL peptide sequence defines the HLA-B8-restricted H-Y epitope derived from UTY.

Expression of the UTY gene and of the B8/H-Y epitope in various tissues and cultured cell types

Previous studies have established that the human UTY gene is transcribed in spleen, thymus, peripheral blood leukocytes, prostate, testis, and small and large bowel (23). In an attempt to more comprehensively define the expression of the UTY gene in different tissues, Northern blot analysis was performed using a dot-blot array of poly(A)$^+$ RNA extracted from 50 different human tissues, in which the mass of RNA from each tissue has been adjusted to reflect comparable levels of transcriptional activity. Hybridization with a ^{32}P-labeled 1.3-kb cDNA derived from the 5′ portion of the UTY gene (spanning the region encoding the B8/H-Y epitope) confirmed the presence of UTY transcripts in a wide range of tissues (Fig. 3*A*). Significant UTY expression was found in many of the major organs tested, with the notable exception of the liver, which showed only a low level of UTY expression. When the blot in Fig. 3*A* was stripped and reprobed with a ^{32}P-labeled 1.4-kb cDNA derived from the human CD45 gene (spanning nt 1525–2963 of GenBank accession no. Y00638), CD45 transcripts were detected in several nonhemopoietic organs, including placenta, lung, appendix, small intestine, and stomach (data not shown). This suggested that UTY expression in these organs might be due at least in part to infiltration by hemopoietic cells expressing UTY. Very weak hybridization of the UTY probe with poly(A)$^+$ RNA extracted from ovary and uterus was most likely attributable to cross-hybridization with transcripts from the UTX gene (23). Northern analysis of poly(A)$^+$ RNA extracted from cultured human cell types demonstrated the presence of UTY transcripts in hemopoietic cells such as EBV-transformed B cell lines as well as nonhemopoietic cell types such as dermal fibroblasts and bone marrow stromal cells (Fig. 3*B*). In all three cell types, the *Pst*I-*Bam*HI UTY probe hybridized with at least three distinct transcripts measuring between 4.7 and 6.9 kb in size, similar to UTY RNA species identified in previous studies (23). This probe hybridized only very weakly with a single ~6.9-kb transcript in poly(A)$^+$ RNA derived from female EBV-LCL and female fibroblasts (data not shown), and this again was most likely attributable to cross-hybridization with transcripts from the UTX gene.

Despite the broad expression of the UTY gene as inferred from RNA analysis, expression of the B8/H-Y epitope as determined by in vitro cytotoxicity assays showed clear cell-type specificity (12). B8/H-Y-specific CTL clones demonstrated robust lytic activity against hemopoietic target cells such as cultured EBV-LCL, PHA-stimulated T cell blasts, and primary leukemic blasts, but not against cultured nonhemopoietic target cells such as dermal fibroblasts and bone marrow stromal cells derived from HLA-B8$^+$ male donors (Fig. 4*A*). The HLA-B8$^+$ male fibroblasts and bone marrow stromal cells were not intrinsically resistant to lysis by CD8$^+$ minor H Ag-specific CTL because they were lysed by a CD8$^+$ CTL clone specific for a HLA-A2-restricted minor H Ag (Fig. 4*B*). Moreover, fibroblasts from HLA-B8$^+$ males pulsed with synthetic LPHNHTDL peptide were recognized by B8/H-Y-specific CTL as efficiently as female HLA-B8$^+$ EBV-LCL pulsed with peptide, with comparable levels of lysis and an identical concentration required for half-maximal lysis (data not shown). Thus, the failure of B8/H-Y-specific CTL to recognize fibroblasts and bone marrow stromal cells from male HLA-B8$^+$ donors cannot be attributed to their inability to present Ag or be lysed by CTL, but is more likely due to insufficient levels or inefficient processing of UTY protein in these cell types.

Discussion

The results of this study demonstrate that the octameric peptide LPHNHTDL of the human UTY gene defines a novel H-Y Ag presented by HLA-B8. Using cell lines derived from HLA-B8$^+$ males with terminal deletions of the Y chromosome as target cells for cytotoxicity assays, the gene controlling expression of the B8/H-Y epitope was localized to the Y chromosome interval defined by the STSs sY69 and sY88, which includes the UTY locus. Expression of UTY, but not DFFRY or DBY, cDNAs in COS-7 cells stimulated TNF and IFN-γ release from B8/H-Y-specific CTL in a HLA-B8-dependent manner, and analysis of UTY cDNAs with nested 3′ deletions localized the epitope to a 173-nt interval in the 5′ half of the gene. The synthetic octameric peptide LPHNHTDL, corresponding to aa residues 566–573 of the UTY protein and encoded within the critical 173-nt interval, sensitized target cells from HLA-B8$^+$ females to lysis by B8/H-Y-specific CTL. The synthetic LPHNRTNL peptide corresponding to the homologous region of the X chromosome-encoded UTX protein showed no such sensitization.

The HLA-B8-restricted H-Y epitope is the third human H-Y Ag identified to date, and it is likely that additional H-Y epitopes remain to be identified (26). Each of the human H-Y epitopes found to date are encoded by either the SMCY (10, 11) or UTY genes, both of which belong to that class of Y chromosome genes that have homologues on the X chromosome and are transcribed widely in tissues outside the testes (23, 27). Although derived from

a common ancestor, the X and Y isoforms of these genes have diverged in sequence due to the lack of recombination between the X and Y chromosomes over the vast majority of their length (28). A second class of human Y chromosome genes characterized by transcription exclusively in the testes has also been identified (23, 27). Whether this class of Y chromosome genes with testes-specific expression can elicit responses from female T cells either in vivo or in vitro remains to be determined.

A role for H-Y Ags in graft-vs-host disease (GVHD) occurring after MHC-matched allogeneic HCT has been suggested by studies in animal models and in humans. Some clinical studies have shown that male recipients of female marrow are at an increased risk for the development of both GVHD (29) and therapeutic graft-vs-leukemia (GVL) (A. Gratwohl, unpublished data) when compared with other donor/recipient gender combinations. Other studies, however, have demonstrated that sex mismatch in either direction elevates GVHD risk (30–32), a finding that is not fully explained by a model in which female T cell responses against male H-Y Ags are responsible for the increased risk. Still other studies have failed to find any significant contribution of donor/recipient gender disparity to GVHD risk (33). There are several possible explanations for the failure to demonstrate a consistent effect of donor/recipient gender disparity on GVHD risk in MHC-compatible transplantation in the outbred human population. Possibilities supported by data from either animal or human studies include masking of the effects of Y-chromosome-encoded histocompatibility determinants by the large number of disparities at autosomally encoded histocompatibility loci (34), immunodominance of autosomal minor H Ags over H-Y Ags (35–38), and H-Y nonresponsiveness due to genotype of donor and recipient at the MHC (8). The identification of H-Y epitopes such as those encoded by SMCY (10, 11) and UTY and their MHC-restricting alleles should facilitate studies to evaluate the contribution of H-Y-specific T cell responses to GVHD in female→male transplant pairs. For example, MHC tetramers complexed with the relevant H-Y peptide could be used to detect and quantitate H-Y-specific T cell responses in blood and at tissue sites of GVHD (39, 40). Such techniques have recently been used to quantitate circulating $CD8^+$ CTL specific for the HLA-A2- and HLA-B7-restricted H-Y epitopes derived from SMCY in the blood of sex-mismatched (female→male) allogeneic HCT recipients (41).

Minor histocompatibility Ags that are restricted in their expression to hemopoietic cells or selected hemopoietic lineages have been suggested as targets for T cell therapy alter allogeneic HCT to induce graft-vs-leukemia activity without causing GVHD (12, 42–45). Several observations suggest that the B8/H-Y Ag encoded by UTY may be a suitable target for GVL therapy. First, the male patient from whom the B8/H-Y-specific CTL were isolated did not develop significant GVHD. Second, the B8/H-Y-specific CTL demonstrated robust lytic activity in vitro against normal recipient hemopoietic cells, including leukemic blasts, but not against skin fibroblasts. Finally, the B8/H-Y-specific CTL clones were effective in preventing engraftment of human acute myelogenous leukemia in nonobese diabetic/SCID mice, demonstrating that the Ag was expressed on leukemic stem cells (46). The finding that expression of UTY is detected in both hemopoietic and nonhemopoietic cells including fibroblasts does not preclude its use as a target for GVL, because many candidate tumor Ags including melanosome-associated proteins (47) and telomerase (48) are also expressed in normal cells. Additional studies to elucidate the potential role of UTY as a target for GVHD or GVL responses in humans are now in progress and should determine whether immune responses to B8/H-Y can be exploited to therapeutic advantage following female→male hemopoietic cell transplantation.

Note added in proof. Since submission of our manuscript, Pierce et al. have reported (*J. Immunol. 163:6360*) that the human DFFRY (USP9Y) gene encodes an HLA-A1-restricted H-Y Ag.

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