Expression Screening of a Yeast Artificial Chromosome Contig Refines the Location of the Mouse H3a Minor Histocompatibility Antigen Gene

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The H3 complex, on mouse Chromosome 2, is an important model locus for understanding mechanisms underlying non-self Ag recognition during tissue transplantation rejection between MHC-matched mouse strains. H3a is a minor histocompatibility Ag gene, located within H3, that encodes a polymorphic peptide alloantigen recognized by cytolytic T cells. Other genes within the complex include β2-microglobulin and H3b. A yeast artificial chromosome (YAC) contig is described that spans the interval between D2Mit444 and D2Mit17, a region known to contain H3a. This contig refines the position of many genes and anonymous loci. In addition, 23 new sequence-tagged sites are described that further increase the genetic resolution surrounding H3a. A novel assay was developed to determine the location of H3a within the contig. Representative YACs were modified by retrofitting with a mammalian selectable marker, and then introduced by spheroplast fusion into mouse L cells. YAC-containing L cells were screened for the expression of the YAC-encoded H3a Ag by using them as targets in a cell-mediated lympholysis assay with H3a-specific CTLs. A single YAC carrying H3a was identified. Based on the location of this YAC within the contig, many candidate genes can be eliminated. The data position H3a between Tyro3 and Epb4.2, in close proximity to Capn3. These studies illustrate how genetic and genomic information can be exploited toward identifying genes encoding not only histocompatibility Ags, but also any autoantigen recognized by T cells. The Journal of Immunology, 1998, 161: 821–828.

T he identification of non-self peptides that are presented to T cells by MHC molecules represents an important first step toward elucidation of the molecular and cellular processes responsible for rejection of donor grafts by MHC-matched recipients. These polymorphic peptide alloantigens, collectively termed minor histocompatibility Ags (mHA), also play a crucial role in graft vs host disease following bone marrow transplantation. An understanding of these genes encoding these peptide Ags would lead to improved and more directed strategies toward the matching of donor and recipient tissues, more specific attempts at generating immunologic tolerance before transplantation, and maximizing the effectiveness of donor bone marrow antileukemia immunologic responses. Estimates of exact numbers of mHA genes vary greatly. More than 50 loci have been mapped throughout the mouse genome (http://www.informatics.jax.org), and estimates of up to several hundred additional genes exist.

Several strategies have been used to attempt to characterize mHA and to clone the corresponding genes. This includes purification and sequencing of natural mHA peptides (1–5), screening of random peptide libraries (6, 7), and expression cloning (8–10). Positional cloning approaches have been limited to situations in which the genome from which the Ag is derived is smaller, as in the case of mitochondrial-encoded Ags (11), or when the region of the chromosome containing the mHA gene is well studied, as in the case of Y chromosome-encoded H-Y Ag genes (2, 12, 13). However, the systematic molecular characterization of the more numerous autosomal mHA genes remains elusive.

Of the autosomal mHA loci, the H3 locus on mouse Chromosome 2 is of historical significance (14). This locus is complex, in that it contains at least three genes that can elicit an immune response (15–18). H3 spans 10 to 12 cM, and additional mHA genes have been reported to map to the complex (19–21). The H3a gene within H3 is of particular interest, as genetic differences in this gene can elicit cytotoxic immune responses after transplantation or immunization between H3-congenic mouse strains. These CTLs are directed against a peptide, encoded by H3a, presented on the H-2Dβ class I MHC molecule.

We have taken a positional cloning approach to identify H3a. High resolution genetic linkage studies have mapped H3a between D2Mit444 and B2m (17, 18), an estimated genetic distance of 1 to 2 cM. We report the construction of a physical map of YAC clones spanning this region. The use of YACs was advantageous for two reasons. One was to minimize the number of clones required to cover the relatively large region, and the second was to utilize established molecular genetics methods for modifying YACs in yeast to incorporate an expression screen for H3a. This screen exploits the fact that the H3a Ag can be assayed in mouse cell lines. Transfer of retrofitted YACs into mammalian cells can be used to identify a YAC clone that contains H3a.

Materials and Methods

Mouse YAC libraries

The primary YAC library screened was the B6-derived Whitehead/MIT library (22), purchased from Research Genetics (Huntsville, AL). Other libraries were screened with specific markers to fill gaps or to provide
additional coverage. One was the B6-derived library constructed at Princeton University (Princeton, NJ) (23). The other library is a composite of a (C3H × B6)F1 library (24), and a B10 library (25). These latter two libraries have been pooled and are distributed by the mouse YAC screening service at Baylor College of Medicine (Houston, TX). YACs M3H6, M3H10, and MSGT have been described previously (26). Libraries were screened by successive rounds of PCR to identify YACs that carry desired sequences. Primers designed to other genes screened in this study are listed in Table I. These techniques were performed as described previously (18).

YAC transduction

The method of Spencer et al. (32) was used to transfer YACs from the host strain AB1380 into the strain YPH925, to allow His\(^*\) selection in subsequent retrofitting experiments (see below). Individual YAC transductants were screened for all markers previously determined to be present on the YAC to ensure that YACs had been transferred intact.

YAC retrofitting

Selected YACs, containing either B6- or B10-derived DNAs, were modified by retrofitting to introduce the mammalian selectable Neo\(^*\) gene into one of the two YAC arms, thus permitting selection for YAC transfer into mammalian cells for functional studies. In this context, retrofitting refers to process whereby existing YAC clones are modified, by using the efficient homologous recombination mechanisms of yeast cells, to insert the Neo gene into the YAC arm. One of two plasmids was used to retrofit YACs. Plasmid pRV1 (33) contains homology to the ura3 gene present on the YAC arm, and carries the yeast lys2 gene and Neo\(^*\) gene. Retrofitting of YACs with pRV1 was performed as described (33). YAC-containing AB1380 yeast Lys\(^+\) transformants were screened for a Ura\(^*\) phenotype, as these were likely to have arisen as a result of specific integration of the retrofitting plasmid into the YAC arm. Because the lys2 mutation present within AB1380 reverts high frequency, some YAC-bearing AB1380 strains were already Lys\(^+\) (34). In these cases, a second retrofitting plasmid (obtained from Bruce Lamb, Case Western University, Cleveland, OH) was used. Plasmid pl33PYF101neo (a derivation of pPol2sneobpA; Ref. 34) was used to transform YAC-containing YPH925 clones to His\(^*\). YPH925 carries a chromosomal deletion in the his3 gene, and because the plasmid carries the intact his3 gene, as well as homology to the \(\beta\)-lactamase gene present in the \(tpr\) arm of the YAC, all transformants arose through integration of the \(Sac\)I-linearized plasmid into the YAC arm. Plasmids were introduced into yeast cells by electroporation using a Bio-Rad Gene Pulser and Pulse Controller. The manufacturer’s suggested procedures were followed. The presence of the retrofitting plasmid-derived Neo gene in yeast transformants was confirmed by PCR with gene-specific primers (Table I).

Transfer of YACs into mammalian cells by spheroplast fusion

Yeast strains containing retrofitted YACs were grown in media selective for both the YAC-encoded \(ara\) and \(trp\) genes (in the case of YACs retrofitted with pl33PYF101neoA, or for the \(trp\) gene only (in the case of pRV1-retrofitted YACs). Cells were spheroplasted and fused to D\(^-\)L cells (obtained from Larry Pease, Rochester, MN), as previously described (35). YAC-containing L cells were selected in DMEM media containing 500 \(\mu\)g/ml G418 (Life Technologies, Gaithersburg, MD). Typically, 10 to 100 clones were generated after 2 wk in culture. Usually three independent clones of each transfection were screened for expression of the YAC-encoded H3\(^a\) Ag.

\textbf{CTL and CML assay}

G418\(^*\) D\(^-\)L cells or Con A-stimulated splenocytes were used as target cells in a standard CML assay (36). Effector cells were the H3\(^a\)-specific CTL clone C1 (15), and either the H4\(^7\)-specific clone CML3 (37) or the H3\(^a\)-specific B/L line derived from immunization of B10 mice with
B10.L-P-H3b splenocytes (Ref. 17; this manuscript). L cells express the H47b and H3a Ags; therefore, cytolysis by either CTL3 or B/L provided a positive control. Percent specific lysis of CML assays was calculated as (51 Cr release in the presence of effector - spontaneous chromium release) divided by (maximal chromium release caused by SDS addition - spontaneous release).

Flow cytometry

Ab staining of cells was performed as described (38). Relative immunofluorescence was analyzed on a Becton Dickinson flow cytometer FACSscan (Sunnyvale, CA). Mononuclear Abs specific for B2m b (S19.8; Ref. 39) and H-2D b (28-13-3; Ref. 40) were supplied from Flow Cytometry Service of The Jackson Laboratory (Bar Harbor, ME).

Database accession numbers

The nucleotide sequences of STS and YAC ends reported in this manuscript have been deposited with GenBank under the accession numbers AF037327, AF037453, and G36380-G36415.

Results

Development of a physical map from D2Mit444 to D2Mit17

Figure 1 shows the relative positions of YAC clones that span the interval between D2Mit444 and D2Mit17. Yeast clones containing desired YACs were identified after screening of YAC libraries with gene-specific primers (Table I) and D2Mit loci shown to map within this region (18). Thirty-three YAC clones were recovered (Table II). An additional three YACs (M3H6, M5G7, and M2H10), previously reported by Richard and Beckmann (26) to contain the Capn3 gene (encodes the calpain 3 protease subunit) and to be derived from this region of the genome, were included in this study.

The YACs were assembled in the order previously established from high resolution genetic linkage maps (18). YAC clones sharing the presence of a particular STS were deduced to overlap at that locus. The contig was completed by linking together YACs with STS markers generated from the ends of YAC inserts (Table III). In some cases, the libraries were rescreened to identify overlapping clones positive for a specific STS.

To identify possible chimerism, YAC ends were screened to determine whether they mapped to Chromosome 2. Of 39 ends screened, 15 were chimeric (Table III; Fig. 1). The nucleotide sequence of 14 of these ends demonstrated no homology to any known gene or EST. For one end, rescued from the left arm of YAC 12, we observed a 39-bp region of sequence identity with the mouse Nnt NADP transhydrogenase gene (EMBL accession number Z49204). The sequence of STS 12L most likely identifies an exon-intron boundary within this gene. Nnt maps to chromosome 13 (41), and as YAC 12 also contains B2m, this chimeric YAC contains DNA derived from Chromosomes 2 and 13.

YAC 6 is also chimeric. The 471-bp cloned DNA fragment from the right arm of YAC 6 (GenBank accession No. AF037453) was mapped by Southern hybridization using a BglII restriction fragment-length polymorphism, on a panel of (B10 × 129)F1 somatic cell variants that demonstrate LOH on Chromosome 2 (18). All variants remain heterozygous at the STS 6HR locus, signifying that it probably maps to a chromosome other than Chromosome 2 (data not shown). The other end of YAC 6 was derived from the B2m gene. The sequence of the cloned 120-bp DNA fragment was identical to nucleotides 268 to 388 of intron 1 of B2m (GenBank accession No. M15535). YAC 6 was identified because it contains exon 3 of B2m, and subsequently the YAC was found to contain Trnah, a gene that is distal to B2m on the physical map. These data suggest that the genomic orientation of B2m is the same as the orientation of the chromosome, with transcription proceeding from centromere to telomere.

Of the 36 YACs recovered, only five nonchimeric YACs (1, 23, 33, 40, and 45) have been positively identified. This number is likely an underrepresentation of the actual number of nonchimeric YACs. Seven YAC ends contained repetitive sequences that precluded the determination of chimerism because they could not be

FIGURE 1. A physical map around H3a. The alignment of YAC clones from the H3a region between D2Mit444 and D2Mit17 is shown. The map is not drawn to scale. Relative positions of known genes and D2 Mit loci are shown above the map. The positions of the Chromosome 2 centromere and telomere would be on the left and right, respectively. Positions of new STS markers are shown below the map. The specific YAC end origin of each STS can be deduced from Table III. The extents of genomic DNA from this region present in individual YACs are indicated. YACs are identified by number (1-36). Yeast clones containing STS markers expected to be present, and identify an internal deletion within the YAC. Ends of YACs without symbols indicate that their origin was not determined.

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mapped, and some YAC ends could not be isolated. Therefore, for some YACs shown in Figure 1, only one end has been characterized. If this end was deduced to be chimeric, then the orientation of this YAC with respect to the other YACs in the contig is unknown, and their alignment is thus ambiguous. In total, 23 new STS markers from the H3a region have been generated (Table III). These new markers identify unique loci in that they do not amplify DNA from a known gene or EST. These STS loci increase the genetic resolution surrounding the size of the cloned DNA fragment within YACs. Whereas most yeast colonies carried only a single YAC, two yeast clones, 131E11 and 183G8, harbored two different sized YACs within the same cell (Table II). As 131E11 was identified after screening for B2m-positive YACs, a Southern hybridization was sufficient to establish that the smaller 150-kb YAC (designated YAC 2) contained the B2m gene (data not shown). Yeast 183G8 was isolated after screening for YACs carrying D2Mit190. Two YACs of 450 and 570 kb were found within a single yeast clone. These YACs were segregated from each other after yeast 183G8 was mated with YPH295. D2Mit190-positive transductants contained only the smaller 450-kb YAC (designated YAC 16). The fact that many of the YAC clones were either chimeric or contained internal deletions, limits estimates of physical distances between STS markers. Based on the sizes of nonchimeric YACs 1 and 23, we can only effectively state that D2Dcr11 must be at least 685 kb from D2Dcr3.

Table II. *Origin of YAC clones*

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<th>Size (kb)</th>
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<td>B2m</td>
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<td>B2m</td>
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<td>B</td>
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<td>Ccap3 (ex 1)</td>
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</table>

* Identifies the specific STS used to recover the YAC clone from the library. Trnah is identified by D2Mit50.

YAC retrofitting and expression screening for the H3aα Ag

Transfer of YACs into mammalian cells allows one to rapidly screen for genes that confer a recognizable phenotype (42–45). To identify which YAC carried H3aα, we reasoned that transferring selected YACs into an H3aα Ag-negative mammalian cell line, and screening for the presence of the B6- or B10-encoded H3aα Ag would be a successful strategy.

L cells are H3aα-expressing C3H-derived fibroblasts. H3aα Ag expression has been observed in a large variety of cell types, including fibroblasts, and so L cells would likely be able to express this Ag when fused to YACs containing the genomic H3aα gene. However, since CTL recognition of the H3aα Ag requires that the cells coexpress the H2-Dβ class I MHC molecule, a H2-Dβ-transfected L cell line was used as our host cell for YAC transfer.

As some of the YACs in the contig could be C3H derived, and these would not be appropriate for an H3aα Ag expression screen in L cells, YACs derived from the Baylor library, and the three isolated by Richard and Beckmann (26), were typed with DNA markers that would distinguish between B6 (or B10) and C3H. YAC M3H6 is B6 (or B10) derived. The other YACs, M5G7, M2H10, 44, 45, and 47, are derived from the C3H mouse (data not shown).

To ensure YAC transfer and expression of a YAC-encoded gene by L cells, we took advantage of the allele-specific Ab directed against the B2mβ protein expressed from B6-derived DNA, but not from the host L cell line (genotype: B2mα). YAC 1 carries B2mβ. It was retrofitted and transferred into Dβ-L cells. Three independent clones were screened by Ab staining for levels of B2mβ expression (Fig. 2). All three clones expressed B2mβ on the cell surface ranging from 2.5- to 6-fold times the parental Dβ-L cell control. The normal distribution pattern of fluorescence in all three clones suggests that, once integrated, the expression of YAC-encoded B2mβ is stable. Thus, we established that YAC transfer and expression of YAC-encoded genes occurred efficiently.

YAC 1 was also screened for expression of H3aα, by using all three clones as targets in a CML assay. No lysis was observed (data not shown), indicating that YAC 1 does not contain H3aα. Figure 3 identifies other B6- or B10-derived YACs that were retrofitted, transferred into Dβ-L cells, and assayed for H3aα expression. Only YAC M3H6 conferred antigenic activity. Two independent clones arose as a result of M3H6 transfer into the Dβ-L cells, and both were lysed by the H3aα-specific CTL clone C1 at levels approaching mitogen-stimulated lymphoblast target cells (Fig. 4). The parental Dβ-L cells are not lysed by C1, but are lysed by the H3aα-specific CTL line B/L, as expected.

The STS content of the H3aα-containing YAC M3H6, and overlapping YACs, was examined in more detail. M3H6 is chimeric. The STS derived from its right arm does not map to Chromosome...
2, but D2Decr24, derived from the nonchimeric left arm, is present on YACs 13, 14, 15, 16, and 28, but not on YAC 23 (Fig. 5). Because M3H6 does not contain D2Mit190, D2Decr24 most likely maps between Epb4.2 and D2Mit190. Based on this data, it was expected that M3H6 should also contain Epb4.2. However, no amplification was detected with Epb4.2-specific PCR primers. Thus, M3H6 also contains an internal deletion. The extent of this deletion is not known. Nevertheless, the expression data from a retrofitted M3H6 clone suggest that this YAC does carry an intact Epb4.2 gene, or at least enough of the gene to express the peptide Ag. The regions proceeding from centromere to telomere. YAC 45 also contains overlapping YACs was determined. M3H6 does not contain either the ends of this gene (Fig. 5). Thus, YAC 45, despite being a nonchimeric clone, also contains an internal deletion of unknown extent.

The extent of tyrosine kinase 3 (Tyro3) within M3H6 and overlapping YACs was determined. M3H6 does not contain either the 5’ or 3’ ends of this gene (Fig. 5). Thus, Tyro3 can be eliminated as a candidate for H3a. YAC 50, M2H10, and YAC 45 contain the 5’ and 3’ ends of Tyro3, and are likely to carry the whole gene. M5G7 carries only the 3’ end. This YAC also carries Capn3. These data, and those deduced from the STS content of YAC 45 and M2H10, suggest that Tyro3 and Capn3 are oriented similarly on the chromosome with the direction of transcription of both genes proceeding from centromere to telomere. YAC 45 also contains Ltk, M2Mit195 (Fig. 1). It does not, however, contain D2Decr23 (Fig. 5). Thus, YAC 45, despite being a nonchimeric clone, also contains an internal deletion of unknown extent.

### Genetic confirmation of the location of H3a

H3a Ag-loss variants have been generated after in vitro immunoselection with H3a Ag-specific CTL (17). The parental line from which these variants were derived was a (B10 × 129)F1, pre-B cell line that expresses the H3a Ag heterozygously. Many of these variants demonstrate LOH at loci surrounding H3a, and this has provided useful information on the fine mapping of H3a with respect to flanking DNA markers (17, 18). Variant 2A12, however, did not demonstrate LOH for any marker tested. We
reasoned that perhaps 2A12 contained a much smaller region of LOH, and the genetic resolution with the markers tested was not sufficient to identify this region. Therefore, we analyzed this variant in greater detail.

The analysis of YAC M3H6 suggested \( H3a \) maps in close proximity to \( Capn3 \) (Figs. 4 and 5). To determine whether 2A12 carried a LOH in this region, we typed 2A12 at \( Epb4.2 \), \( Capn3 \), and \( Ltk \). No polymorphism was observed between B10 and 129 for \( Tyro3 \) and, hence, this locus could not be typed. Variant 2A12 demonstrated a LOH at \( Capn3 \), but not at any other marker (Fig. 6). Because this LOH is causally related to the lack of expression of the H3aa Ag (17), the LOH provides genetic proof that \( H3a \) also maps in close proximity to \( Capn3 \).

**Discussion**

We describe a YAC contig, consisting of 36 YAC clones, that spans the interval between \( D2Mit444 \) and \( D2Mit17 \) on mouse Chromosome 2. A total of 23 new STS markers has been generated as a result of this study and placed on this map. In addition, several genes and other markers, previously mapped to this 1- to 2-cM genetic region, have been ordered with respect to each other. The region covered is either a single contiguous sequence, or two adjacent contigs, depending upon whether YAC 34 overlaps with YAC 47. We cannot ascertain this for certain because YAC 34 contains repetitive DNA at both ends, and YAC 47 is chimeric. YAC chimerism also limits our ability to determine the physical distances between markers within the contig. In spite of these constraints, the contig provides a starting point for the genomic characterization of existing genes, and for the identification of new
Figure 5. STS content of YAC M3H6 and overlapping YAC clones. A physical map of the region between D2Dcr23 and D2Dcr11 is shown. The map is not drawn to scale. This region includes the genes for Tyro3, Capn3, and Epb4.2. Extent and identity of YAC clones are shown below the map. Open triangles (Δ) signify that the STS screened is present in the YAC clone. Black boxed areas signify the absence of an expected STS. Boxed YAC ends signify chimerism, and open circles identify repetitive sequences.

We developed a novel assay whereby retrofitted YACs were transferred into mouse D+L cells, and YAC-containing mouse cell lines were then screened for lysis after coincubation with H3a-specific CTLs. The technique was valuable in that it allowed the rapid mapping of H3a from a large genomic region, without the necessity of generating and analyzing higher resolution genetic linkage maps. By systematically retrofitting and expression screening 14 YACs that covered the majority of the H3a region, we identified a single YAC clone, M3H6, that carries H3a. Others have reported the use of an expression screen in refining the location of desired genes within larger YAC contigs (43–45). We show that this approach can be extended to include the screening of genes that encode Ags recognized by T cells, and that considerable physical distances can be covered. The localization of H3a to the region of DNA covered by YAC M3H6 has been confirmed genetically, because of the discrete LOH observed at Capn3 in the H3aAg loss immunoselected variant 2A12.

The gene order determined from this region of mouse Chromosome 2 is in perfect agreement with that determined from a physical map of the syntenic region on human Chromosome 15q (46, 47), and the placement of the H3a-containing YAC M3H6 within the physical map eliminates many genes as H3a candidates. H3a maps in close proximity to Capn3, a gene that encodes a member of the calpain protease family. The close proximity of these genes suggests that they could be the same. However, published reports on the expression pattern of Capn3 suggest that it is muscle specific (48, 49, 26). In contrast, expression of H3a is observed in a wide variety of tissues, including skin, lymphoid-derived T and B cells, myoblasts, and fibroblasts. These data suggest that Capn3 is unlikely to encode the H3aAg, and that H3a is an unknown gene contained within the Tyro3 to Epb4.2 region.

Additional genes from the human region have been identified, primarily as ESTs expressed in human muscle, and subsequent coexpression of a subset of these genes in other tissues (47). Some of these ESTs map in close proximity to Capn3. Examination of the human transcript map for 15q21 (http://www.ncbi.nlm.nih.gov) indicates that, to date, 185 cDNA transcripts have been mapped in close vicinity to CAPN3. The majority of these are “unidentified” in that they are not homologous to any known gene. Although some of these cDNA fragments are likely to be derived from the same gene, the abundance of ESTs mapped to this small region of the chromosome suggests a moderate to high gene density surrounding H3a.

The ability to retrofit and routinely transfer YAC clones, identified as a result of completion of the human and mouse physical maps, into mammalian cells should allow one to efficiently bridge the gap from physical map to gene, and could be applied to the positional cloning of any gene whose expression can be assayed in cell lines. YACs have been transferred into a number of different human and mouse cell types in addition to murine L cells. These include embryonic stem cells, embryonic carcinoma cells, renal carcinoma cells, and Chinese hamster ovary cell lines (35, 45, 50–52). There are conflicting reports on the effects of YAC size on transfer into mammalian cells. Gobin et al. (53) suggest that YAC size is not limiting for transfers involving spheroplast fusion. However, Blunt et al. (43) find that smaller YACs undergo fewer rearrangements as a result of YAC transfer than larger ones. Nevertheless, we found the fusion approach to be reliable in that YACs carrying B2m or H3a reproducibly conferred strong and stable Ag expression in mouse L cells. We never observed Ag expression from YACs that were derived from other regions of the contig. In light of the real possibility of rearrangements and internal YAC deletions, a prudent approach in expression screening of this type would require testing multiple independent and overlapping YACs if at all possible. The use of CTLs specific for mHA in expression screening of retrofitted YAC clones toward the positional cloning of clinically significant human and mouse Ag genes is just one application for this technology. T cell clones can be generated to autoantigens that are responsible for, or contribute to, an array of autoimmune diseases, including autoimmune diabetes and multiple sclerosis, thus making the genes encoding these Ags amenable to a positional cloning approach.

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