Brief Definitive Report

A POLYMORPHIC MICROSATELLITE IN THE TUMOR NECROSIS FACTOR α PROMOTER IDENTIFIES AN ALLELE UNIQUE TO THE NZW MOUSE STRAIN

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The TNF locus comprises the closely linked genes coding for TNF-α (TNF, cachectin) and TNF-β (lymphotoxin) (1, 2). TNF-α is one of the first products synthesized by macrophages in response to bacterial or viral infections, and plays a major role in the generation of inflammatory responses (3). In both mouse and man, the TNF locus is contained within the MHC, at the boundary of the class III and class I regions. TNF maps 70 kb centromeric to H-2D (4), and ~350 kb centromeric to HLA-B (5, 6). This fact has raised the possibility that some diseases whose incidence is MHC associated could be elicited by genetic defects in the TNF locus. This hypothesis would be consistent with the observation that a number of MHC-linked autoimmune diseases are accompanied by altered cytokine production (7, 8). Unfortunately, a thorough study of potential disease linkages to TNF has been hampered by a lack of polymorphic markers in this region, which is very conserved at the RFLP level (4, 8, 9).

Several groups have recently demonstrated the utility of simple repeated sequences as polymorphic markers (10–12). Eukaryotic genomes contain many short tandem repeats of very simple motifs (usually dinucleotides), among which, $(CA)_n:(GT)_n$ seems to be the most abundant. These so-called microsatellites are more or less evenly distributed throughout the genome, and are commonly found within the noncoding parts of genes. The length of a given microsatellite is variable in the population, but is inherited as a stable allele (10–12). In human populations, randomly chosen microsatellites are highly polymorphic (four or more observable alleles in all reported cases), with a heterozygosity ranging from 34 to 80% (11).

As a model of a potential linkage of TNF to an autoimmune disease, we chose to analyze the TNF allele of the NZW strain. In contrast to their parental strains, (NZB × NZW)F₁ mice develop a lupus-like glomerulonephritis with a very high frequency (13). The contribution of the NZW parent to the generation of the disease has been traced to a single dominant allele mapping within or very close to the MHC

(13, 14). Interestingly, peritoneal macrophages from NZW mice are defective in their ability to secrete TNF in response to LPS, and treatment with recombinant TNF-α can prevent the appearance of the disease in (NZB × NZW)F₁ mice (9). The TNF loci of NZB and NZW strains can be distinguished by a Bam HI RFLP, but this RFLP does not define an allele unique to the NZW strain, or preferentially found in strains that develop autoimmune diseases (4, 8, 9). In the present study, we have used a microsatellite within the mouse TNF-α promoter as a polymorphic marker, and have found that NZW mice do indeed possess a unique TNF allele.

Materials and Methods

Liver DNA was extracted from mice of different strains using standard protocols. For the polymerase chain reactions (PCRs) (15), ∼100 ng of total genomic DNA was added to a mixture containing 100 ng of cold primer 1 (Fig. 1) and 1 ng of 5′ end-labeled primer 2 in a total volume of 25 μl of 66 mM Tris-HCl, pH 8.8, 17.5 mM (NH₄)₂SO₄, 6.6 mM MgCl₂, 10 mM β-mercaptoethanol, 170 μg/ml BSA, and 0.4 mM each dATP, dCTP, dGTP, and dTTP. The template DNA was denatured for 8 min at 98°C, the reaction mixtures were cooled to 74°C, and 2 U of Taq DNA polymerase (Biofinex, Praroman, Switzerland) was added to each reaction. The fragment bounded by the two oligonucleotides was amplified through 25 cycles of denaturation at 93°C (1.5 min), annealing at 60°C (2 min), and elongation at 74°C (2 min). An aliquot of the amplified fragments was analyzed on a denaturing 6% acrylamide gel ("sequencing gel").

Results and Discussion

As a potential polymorphic marker for the TNF locus, we chose a (CA)ₙ repeat located 260 nt upstream of the mRNA 5′ end in the mouse TNF-α promoter. We suspected that this microsatellite might be polymorphic, as our sequence of the TNF-α gene (from strain C57Bl/6, reference 16; the original paper by Semon et al. [17] erroneously reported a poly(A) stretch at this location) differed from that published by Shirai et al. (from an unidentified mouse strain; reference 18) in the number of CA repeats (20 vs. 14). We synthesized two unique sequence oligonucleotides flanking the microsatellite (Fig. 1) and used them as primers in PCRs. To ensure that the resulting labeled fragments would have unique ends, one primer was 5′ end labeled, and in some experiments, the amplified DNA was digested with a restriction endonuclease (Dde I) that cuts distally from the labeled end (Fig. 1). Fig. 2 shows the fragments obtained by amplifying the microsatellite from a number of mouse strains of known H-2 haplotypes. The results can be summarized as follows.

The Amplified DNA Is Heterogeneous in Length. In spite of the precautions taken to eliminate terminal heterogeneity (a well-known artifact of PCR), we never obtained a perfectly homogeneous final population (Fig. 2). The slowest migrating band
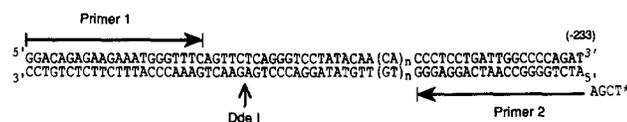

FIGURE 1. Nucleotide sequence of the region surrounding the mouse TNF-α promoter microsatellite, and of the PCR primers. Primer 2 overlaps an MHC class II–like "Y box," and contains four mismatched nucleotides at its 5′ end. The Dde I site used for secondary digestions is marked by an arrow. These sequence data are stored in the EMBL/GenBank Data Libraries under accession numbers Y00467 and M20155.

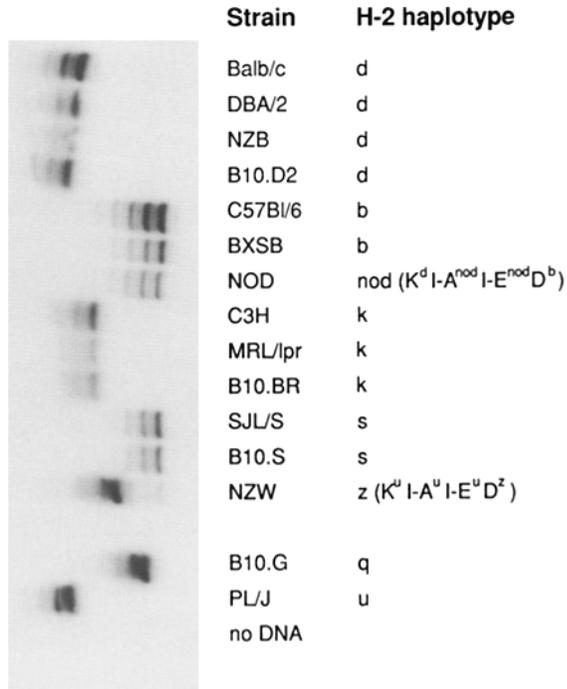| Strain	H-2 haplotype
Balb/c	d
DBA/2	d
NZB	d
B10.D2	d
C57Bl/6	b
BXSB	b
NOD	nod (K^d I-A^{nod} I-$E^{nod}D^b$)
C3H	k
MRL/lpr	k
B10.BR	k
SJL/S	s
B10.S	s
NZW	z (K^d I-A^u I-E^uD^z)
B10.G	q
PL/J	u
no DNA	

FIGURE 2. Amplified fragments generated from a panel of inbred mouse strains using the primers depicted in Fig. 1. Liver DNA from a single mouse of each strain was used as a template for PCR, as described in Materials and Methods. The resulting fragments were resolved on a denaturing polyacrylamide gel, and revealed by autoradiography.

of the pattern obtained with C57Bl/6 DNA corresponded to the expected amplified fragment (103 nt), when compared with a sequencing pattern obtained on cloned DNA using end-labeled primer 2. The same heterogeneity was observed when using a cloned C57Bl/6 TNF-α gene as a template (data not shown). In fact, secondary cutting with Dde I, which should eliminate terminal heterogeneity from the left end, had no effect on the observed banding pattern (data not shown). Since we consistently observed a pattern of fragments differing by multiples of two nucleotides (Fig. 2), it can be assumed that the heterogeneity is due either to polymerase slippage during copying of the $(CA)_n$ repeat, or to out-of-frame priming within the repeat. Similar observations were made by other groups (10–12).

Five Distinct Alleles of TNF Segregate with H-2 Haplotypes.   Among the 15 strains shown in Fig. 2, five alleles could be clearly distinguished on the basis of the length distribution of the amplified fragments (from 14 to 20 copies of the CA motif for the largest fragment in each pattern). Mouse strains sharing H-2 haplotypes also shared TNF microsatellite alleles; in particular, the B10.D2, B10.BR, and B10.S congenic strains all carried the TNF alleles predicted from their H-2 haplotypes, and not that of the C57Bl/10 (H-2^b) strain. The NOD strain, with the haplotype K^d, I-A^{nod}, I-E^{nod}, D^b (19), has the TNF allele of H-2^b, consistent with its close physical linkage to H-2D. The TNF alleles found in H-2^b and H-2^s, as well as in H-2^d and H-2^u, carry microsatellites of the same length; therefore, not all H-2 haplotypes can be distinguished on the basis of the TNF microsatellites that they carry. We also tested a number of outbred strains and other species of the *Mus* genus; among four strains where an amplified pattern could be obtained (from MOLF/E1, *M. spretus*,

M. caroli, and *M. p saxicola*; see reference 8 for details), three carried alleles not found among the inbred strains (data not shown).

The NZW Strain Carries a Unique TNF Allele.   Serologically, NZW mice were typed as K^u, $I-A^u$, $I-E^u$, D^z (haplotype H-2^z) (20). It was recently shown that the nucleotide sequences of the hypervariable regions of the NZW MHC class II genes differ from those of H-2^u by a single nucleotide in the Eβ chain (21). The region of divergence between H-2^z and H-2^u has not yet been precisely defined. The TNF allele of NZW is distinct from those of all of the other strains that we tested, including PL/J (H-2^u) and the outbred strains. Therefore, as seen for the NOD strain, TNF segregates with H-2D, and there is a unique allele associated with D^z.

There Is No TNF Allele Uniquely Found in Autoimmune Strains.   Our collection of mouse strains included five that are susceptible to the development of autoimmune diseases: NOD, BXSB, MRL/lpr, NZB, and PL/J. Each one of these strains carried a different TNF allele, making it very unlikely that an identical defect in the TNF gene could underlie or facilitate the very different manifestations of autoimmunity in these strains. We have consistently observed a low yield of amplified TNF promoter microsatellite from NZW and BXSB DNA, and a more heterogenous population of amplified fragments than in other strains. This could reflect the existence of mismatches between the oligonucleotide primers and the genomic DNA, and therefore, additional differences between the NZW and BXSB alleles and those of "normal" strains. To test this possibility, we are in the process of cloning the NZW and BXSB TNF genes.

Our data are relevant to the genetics of autoimmune diseases in several ways. First, they show that one can easily obtain polymorphic markers in genes that were considered "highly conserved" on the basis of limited RFLPs. This opens the way to a systematic study of possible disease linkages for many genes (e.g., those coding for other cytokines), provided a microsatellite can be detected nearby. Second, they document the existence of a highly polymorphic marker in a region of the mouse MHC where such markers were scarce. Third, they imply that NZW mice may in fact carry a unique TNF allele, and therefore revive the idea that this allele may be associated with a regulatory or structural defect.

Summary

We have amplified a $(CA)_n:(GT)_n$ microsatellite from the TNF promoters of a panel of mouse strains using the polymerase chain reaction. The length of the microsatellites was polymorphic, with eight alleles observed among 15 inbred strains bearing seven distinct H-2 haplotypes, and four outbred strains. In B10 congenic strains, the TNF allele detected by microsatellite polymorphism segregated with the MHC, and in recombinant haplotypes (NOD, NZW), it segregated with H-2D. The TNF allele found in the NZW strain (H-2^z) was distinct from those of all other haplotypes, consistent with the hypothesis that this strain may carry a genetic defect in TNF production.

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