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Genes for the tumor necrosis factors α and β are linked to the human major histocompatibility complex

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ABSTRACT **The human major histocompatibility complex (MHC) includes the closely linked genes for the tumor necrosis factors α and β. Their location is within the chromosomal segment between *HLA-DR* and *HLA-A* or centromeric of *HLA-DP*. This assignment is based on Southern blot analysis of a number of different MHC deletion mutants and is corroborated by chromosome *in situ* hybridization.**

The human major histocompatibility complex (MHC) is located within 3 centimorgans on the short arm of chromosome 6, between p21.1 and p21.3. It includes two highly polymorphic gene families involved in immune regulation (see Fig. 2 for arrangement of loci). Telomerad are the class I *HLA-A*, *-B*, and *-C* loci (*H-2K*, *H-2D*, and *H-2L* in the mouse), which determine ubiquitous cell surface glycoproteins associated with β_2-microglobulin (reviewed in ref. 1). Additional 10–14 class I-related loci have been detected in proximity to *HLA-A* and are presumed to be homologous to the genes expressing the murine Qa and TL differentiation antigens (2, 3). Centromerad is the class II (*HLA-D*) region containing multiple subsets of α (heavy) and β (light) chain genes. They specify the DP, DQ, and DR glycoproteins (I-A and I-E in the mouse), present as α–β heterodimers on B lymphocytes and cells of the myeloid lineage (reviewed in ref. 4). Class I and class II molecules constitute parts of antigenic structures recognized by cytolytic and helper T lymphocytes, respectively. Between the most distal class II locus, *HLA-DR*, and the most proximal class I locus, *HLA-B*, is the class III region coding for the complement components C2, factor B, and C4. The two C4 loci, *C4A* and *C4B*, are closely linked to two copies of the steroid 21-hydroxylase gene, *CA21HA* and *CA21HB* (5, 6).

To identify additional genes within the MHC is of interest, as some MHC haplotypes predispose to susceptibility to a number of diseases (reviewed in ref. 7). Recently, the genes for the tumor necrosis factors α and β (TNFα and -β genes) have been localized by Southern blot analysis of human–murine somatic cell hybrids between 6p23 and 6q12, a chromosomal segment that also encompasses the MHC (8). Both genes have been found within 6 kilobases in a λ-phage clone (9). These observations suggest that TNFα and -β could be encoded within the MHC. To address this possibility, a panel of MHC deletion mutants was investigated by genomic blot hybridization. The cell lines used lack different partially overlapping regions of their MHC in one or both haplotypes. The results obtained indicate that the TNFα and -β genes are included within the MHC, most likely telomeric of the class II region. This assignment was corroborated by an examination of metaphase chromosomes following *in situ* hybridization to a TNFα cDNA probe.

TNFα and TNFβ (synonymous with cachectin and lymphotoxin, respectively) are structurally related cytokines secreted in response to a variety of invasive stimuli by activated macrophages and mitogen-stimulated lymphocytes, respectively (10, 11). They exert multiple pleiotropic activities, including cytostatic and cytotoxic effects against certain tumor cells (12, 13). TNFα has also attracted much attention because of its role in the acute-phase response to inflammation, which eventually may result in a pathological perturbation of the host metabolism (cachexia) (reviewed in ref. 14). Specifically, TNFα has been shown to enhance lipid catabolism, to increase the level of several hepatic serum proteins, and to activate polymorphonuclear leukocytes (15–17). Interestingly, it has also been demonstrated to amplify strongly the transcription of class I genes (18). Like interferons, TNFα through this mechanism augments the surface expression of class I molecules in some tissues and, thus, may enhance a cytolytic immune response. With regard to these properties, the genetic linkage of TNFα (and TNFβ) to the MHC may have substantial biological significance.

MATERIALS AND METHODS

Cell Lines and Deletion Mutants. Priess and JY are lymphoblastoid *B*-cell lines with wild-type MHC genotypes. The mutants used in this study have been generated by γ-irradiation or chemical mutagenesis and subsequent immunoselection, resulting in different physical deletions that affect either one or both of their MHC haplotypes. The single-haplotype mutants 8.1.6, 9.28.6, 6.3.6, and 3.1.0 are derived from the lymphoblastoid B-cell line T5-1; their deletion breakpoints have been mapped by phenotyping, karyotyping, and Southern blotting (19, 36). Mutant 8.1.6 is the parent of 9.22.3, which has an additional deletion of the DRα gene alone in the second haplotype (unpublished data). The other double haplotype mutants, 721.82 and 721.180, are derived from different single-haplotype loss mutants, which originated independently from the lymphoblastoid B-cell line 721 (20–23). The 721.180 mutant cell line was not available; however, the DNA required was provided by R. DeMars (University of Wisconsin, Madison).

DNA Probes and Southern Blot Analysis. All DNA probes were of human origin. Class II heavy chain cDNA probes were the DRα 1.1-kilobase *Pst* I insert from α-15 (24), the HLA-DQ α 780-base-pair *Pst* I fragment from pDCH1 (25), and the HLA-DP α 1.044-kilobase *Pst* I insert from LB14 (26). The TNFα cDNA probe was the 650-base-pair *Pst* I fragment from phTNF5, including a major portion of the coding region and the 3′ untranslated region (27). The TNFβ probe was made from a 2.4-kilobase *Eco*RI subclone derived

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Abbreviations: TNFα and TNFβ genes, genes for tumor necrosis factors α and β; DRα, DQα, DPα, DXα, and SXα, HLA-DR, -DQ, -DP, -DX, and -SX α chains; MHC, major histocompatibility complex.

from a genomic λ-phage insert (9). The 380-base-pair *Pst* I/*Eco*RI fragment isolated contained part of the coding region and the 3′ untranslated region from exon IV. The β-globin probe was a 826-base-pair *Eco*RI/*Pst* I fragment obtained from pHβR3.1 (28). It extended from exon III into the 3′ untranslated region.

For Southern blotting and subsequent hybridization, total genomic DNAs (2.5 μg) were digested with either *Bam*HI or *Eco*RI. Fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters (29). DNA probes were labeled with [^{32}P]NTPs by nick-translation (29). Hybridizations were carried out in 6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate) containing 5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 10% (wt/vol) dextran sulfate, 0.1% $NaDodSO_4$, and sonicated salmon sperm DNA (100 μg/ml) for 16 hr at 65°C. Filters were washed in 0.1× NaCl/Cit containing 0.05% $NaDodSO_4$ at 65°C and exposed to Kodak XAR-5 film.

Chromosome *in Situ* Hybridization. Midmetaphase chromosomes were prepared from peripheral blood lymphocyte cultures established from two normal males as described (30). They were spread on slides and hybridized *in situ* to phTNF5 labeled with [^{3}H]NTPs by nick-translation as described (31). After autoradiography, the slides were stained with quinacrine mustard dihydrochloride. The distribution of grains on 164 random metaphases was recorded with reference to a metaphase chromosome ideogram at the 400-band stage (32).

RESULTS AND DISCUSSION

A general approach to explore genetic linkage is to determine whether a locus of interest resides within or outside the genomic breakpoints characteristic of a number of different deletion mutants. The deletion of a DNA sequence from a mutant haplotype is monitored by Southern blot analysis. Depending on whether the wild-type genome has a heterozygous restriction fragment length polymorphism or not, some fragments will be absent or all will be reduced to half intensity, respectively.

A series of Southern blots including *Bam*HI- and *Eco*RI-cleaved DNA from the cell lines Priess, JY, and the mutants 721.82, 9.28.6, and 9.22.3 was prepared under standardized conditions. In addition to the probe for the TNFα gene, probes for the β-globin gene and several class II α chain genes were used to provide controls and to ascertain the identity of the mutants (see *Materials and Methods*). After hybridization to the TNFα cDNA probe, the bands detected in 721.82 and 9.28.6 were found to be significantly reduced in intensity as compared to those in Priess, JY, and 9.22.3 (Fig. 1*c*). In contrast, the control β-globin gene fragments were equal in strength in JY, 721.82, 9.28.6, and 9.22.3 (Fig. 1*d*; both of the bands in the first Priess lane are half-intensity because of a heterozygous *Bam*HI restriction fragment length polymorphism). This disparity indicated that the TNFα gene mapped to a chromosomal segment commonly absent from one haplotype in both 721.82 and 9.28.6 (half-intensity bands) and was located outside the deletions in the mutant 9.22.3 (full-intensity band). This conclusion was experimentally verified several times. Analogous data were obtained using the TNFβ probe (see *Materials and Methods*; data not shown).

The deletion breakpoints in 9.28.6 have been reported to be centromeric of the class II HLA-SX and -DP α and β loci (excluding the glyoxalase locus) and telomeric of *HLA-A* (ref. 19; see Fig. 2 for arrangement of loci within the MHC). Accordingly, blot hybridization revealed half-intensity DRα gene bands in 9.28.6, confirming the absence of the DRα gene in the mutant haplotype (Fig. 1*a*). Centromeric of *HLA-DR*

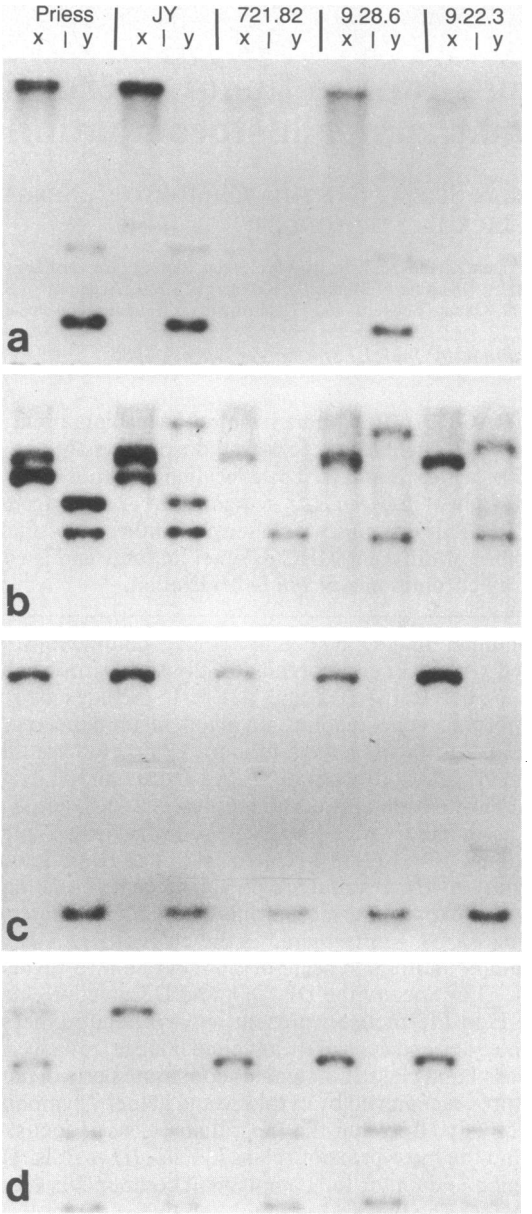

FIG. 1. Genomic blot analysis of the MHC deletions in the mutants 721.82, 9.28.6, and 9.22.3 and comparison to the intact MHC haplotypes in the cell lines Priess and JY. Total cell DNA samples were digested with *Bam*HI (lanes x) and *Eco*RI (lanes y), respectively. The probes used were DRα (*a*), DQα (*b*), TNFα (*c*), and β-globin (*d*) DNAs. The data show that the TNFα gene is included in the MHC deletions in the mutants 721.82 and 9.28.6.

is the *HLA-DQ* subregion encompassing the highly homologous DQα and DXα genes (Fig. 2). Using the DQα cDNA probe, the hybridizing DQα and DXα gene fragments were also found to be much lower in intensity in 9.28.6 relative to the standards Priess and JY (Fig. 1*b*). In addition, the results obtained for the DPα gene indicated that this locus resides inside the deletion in 9.28.6 (data not shown). Thus, the loci for TNFα and TNFβ were confined to a segment of nonoverlap between the deletions in the mutants 9.28.6 and 9.22.3, ranging from a breakpoint centromeric of the DPα gene to a location distal of *HLA-A* (Fig. 2).

The mutant 9.22.3 shares with its parent 8.1.6 a heterozygous deletion within the class II region comprising the genes for the HLA-DR, -DQ, and -DX α and β chains. In addition, 9.22.3 lacks the DRα gene in the second haplotype as well (Fig. 2). These features were in agreement with the Southern

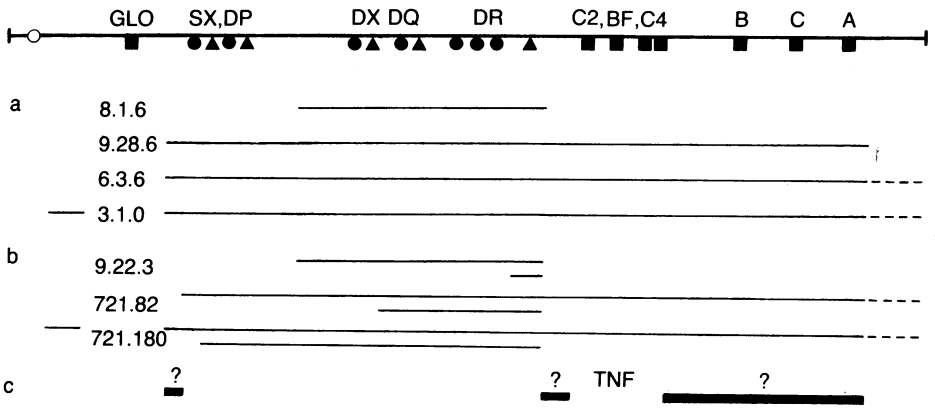

FIG. 2. Linear order of genetic loci in the MHC based on studies cited in the text. Class II α and β chain genes are shown as triangles and circles, respectively, and the glyoxalase gene as GLO. Lines underneath depict chromosomal segments that are absent from the MHC of various deletion mutants. (*a*) Single-haplotype deletion mutants. (*b*) Double-haplotype deletion mutants. (*c*) Presumptive location of the TNFα and TNFβ genes.

blot data. Fig. 1*a* documents the complete absence of bands hybridizing to the DRα cDNA probe. The DQα and DXα gene fragments in Fig. 1*b* show half intensity as compared to those in the controls Priess and JY (the band in the *Bam*HI lane of 9.22.3 represents a doublet of two comigrating fragments). The DPα gene was not affected by the deletions in 9.22.3 as inferred from the normal intensity of the bands hybridizing to the DPα cDNA probe (data not shown).

On the basis of these facts, it was concluded that TNFα and TNFβ were encoded within the chromosomal segment between the DRα gene (the most distal breakpoint of the deletions in 9.22.3) and *HLA-A* (the telomeric breakpoint in 9.28.6) or in proximity to the DPα gene distal of the centromeric breakpoint in 9.28.6 (Fig. 2). Further support was provided by the mutant 721.82 that has been derived independently from another parent cell line in a different laboratory as opposed to 9.28.6 and 9.22.3 (8.1.6). In this mutant, an entire MHC haplotype is lost. In addition, the genes for HLA-DR and -DQ α and β chains are also deleted in the other haplotype (Fig. 2). Fig. 1*a* confirms the absence of the DRα gene in 721.82. In Fig. 1*b*, the single half-intensity band in the *Bam*HI and *Eco*RI lanes represents the DXα gene, which was retained on one chromosome in 721.82, whereas both DQα alleles were deleted. Thus, the proximal breakpoint of the small deletion in 721.82 was between the DXα and DQα genes (Fig. 2). The presence of the TNFα and -β genes in only one haplotype in 721.82 and the extensive alignment of the deletions in 721.82 with those in 9.28.6 and 9.22.3 lent further support to the localization of these genes to the MHC telomeric of the DRα gene or centromeric of the DPα gene.

This result was reproduced in a separate experiment involving additional previously characterized deletion mutants. It included the mutants 8.1.6, 9.28.6, 6.3.6, and 3.1.0, all of which originated from the cell line T5-1 (19), and 721.180, which was derived from the lymphoblastoid B-cell line 721 (21, 22). As expected from the deletion breakpoints in these mutants (Fig. 2), Southern blot analysis demonstrated the absence of the TNFα gene from one haplotype only in 6.3.6, 3.1.0, and 721.180, in accordance with 9.28.6 (Fig. 3). The mutant 721.180 was particularly informative because of its loss of the entire class II region in both haplotypes [the centromeric breakpoint of the smaller deletion is between the SXα and SXβ gene pair (ref. 23; Fig. 2)]. This property substantiated the linkage of the TNFα and -β genes to the MHC telomeric of the class II region or centromeric of the SXβ gene.

This localization was corroborated by *in situ* hybridization of the phTNF5 cDNA to metaphase chromosomes (see *Materials and Methods*). From a total of 465 grains on 164 metaphases, 22 (4.7%) were found between 6p21.1 and 6p22, representing 48.9% of all grains on chromosome 6 (Fig. 4). This distribution was significantly above background and corresponded to the location of the MHC between 6p21.1 and 6p21.3.

In an attempt to establish a physical linkage to a specific MHC locus, the TNFα and TNFβ genes were cloned in a 42-kb cosmid (unpublished data). However, upon restriction site mapping, this cosmid did not share an overlap with other cosmids containing the class III region (6) and a number of different class I loci. The collection of 89 human class I cosmids containing 17 class I genes was also probed with terminal fragments derived from the isolated cosmid with negative results. In addition, the TNFα gene was not found by probing a series of cosmids including the murine *H-2K, H-2I, H-2D, Qa*, and *Tla* regions [ref. 34; kindly provided by R. Flavell (Biogen, Cambridge, MA)] but excluding the *H-2S* region, which is homologous to the human class III region; the mouse and human TNFα genes are highly homologous (27). Thus, the most likely locations of the TNFα and TNFβ genes are at either side of the class III region centromeric of *HLA-B* or else in an uncloned region of the class I genes. This assumption can be tested by pulse-field gradient electrophoresis (35), a technique suitable to overcome the large distances separating most MHC loci.

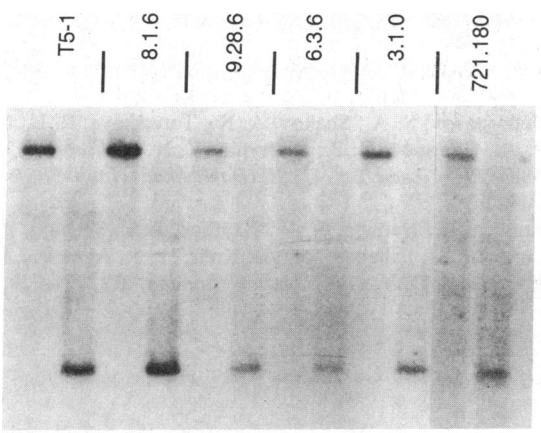

FIG. 3. Genomic blot analysis of a panel of MHC deletion mutants using the TNFα cDNA probe. Total cell DNA preparations were digested with *Bam*HI (each first lane) and *Eco*RI (each second lane), respectively. The gene for TNFα is absent from one haplotype in the mutants 9.28.6, 6.3.6, 3.1.0, and 721.180, as indicated by the approximately half intensity of the hybridizing bands.

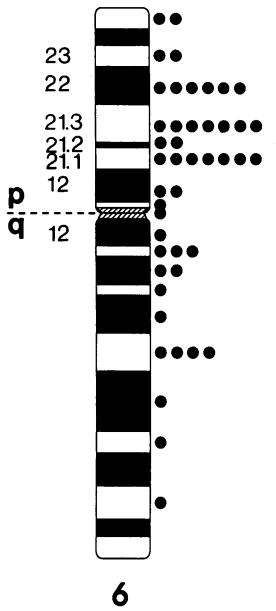

FIG. 4. Graph showing the distribution of grains on chromosome 6 after *in situ* hybridization to the TNFα cDNA probe. The MHC maps between p21.1 and p21.3 (33). The previous localization of the TNFα and TNFβ genes by Southern blot analysis of human–murine somatic cell hybrids was between q12 and p23 (8).

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