Table 1. Assays in vitro of wild-type and mutant Klenow fragments. Assays of the mutant proteins were carried out on peak fractions from the final Sephacryl S-300 column. Polymerase activity was measured by the standard assay (*16*), with the use of poly[d(AT)]. Protein concentration was measured by enzyme-linked immunosorbent assay (ELISA) (*17*), with rabbit antiserum to Klenow fragment. Exonuclease activity was measured as described (*9*). The rate of solubilization of ^{32}P from 3′ end-labeled DNA was divided by the number of polymerase activity units present in the reaction mixture, to give the ratio of exonuclease to polymerase. The 3′ end-labeled DNA substrate was first treated with pyrophosphatase (1.4 unit/ml) for 15 minutes at 37°C to eliminate solubilization due to pyrophosphorolysis. The values reported are the mean (±SEM) of at least four independent determinations.

Protein	Polymerase specific activity (unit/mg)	Ratio of exonuclease to polymerase (arbitrary units)
Wild type	$(1.07 \pm 0.11) \times 10^4$	1 ± 0.3
D424A	$(0.93 \pm 0.16) \times 10^4$	$(1.3 \pm 0.6) \times 10^{-5}$
D355A,E357A	$(1.11 \pm 0.34) \times 10^4$	$(1.4 \pm 0.5) \times 10^{-5}$

clease active site, then the crystallographic studies suggest that the structure of the enzyme-substrate complex is the same for both the D424A and wild-type proteins. Therefore the loss of exonuclease activity in the D424A protein indicates a direct involvement in catalysis of one or both of the components missing in its structure, namely the metal ion at site B and the carboxylate of Asp^{424}. An additional contribution of metal B to the strength of substrate binding is not ruled out by the crystallographic experiment since only a single concentration of dTMP was used.

The failure of the D355A,E357A mutant protein to bind either dTMP or metal ions indicates a role for metal site A in substrate binding. The inability of the D355A,E357A enzyme to bind dTMP was not surprising because previous binding studies in solution (*12*) and in the crystal (*13*) had shown that removal of divalent metal ions with EDTA abolished dNMP binding (*14*). Since the D424A protein binds dTMP in the absence of metal B, then metal site A must be the more important in substrate binding. Additional contacts are important when binding a DNA substrate (as opposed to the inhibitor dNMP), as shown by recent studies on Klenow fragment–DNA crystals in which DNA can bind to the exonuclease active site even in the absence of divalent metal ions (*15*).

The mutagenesis studies described above establish, at the minimum, a role for metal A in substrate binding and for metal B in catalysis of the 3′,5′-exonuclease reaction. They do not exclude additional functions of metal A in catalysis and metal B in substrate binding.

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A Previously Undetected MHC Gene with an Unusual Periodic Structure

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The major histocompatibility complex is a chromosomal segment embodying several gene clusters among which those with immune functions are the best characterized. This region is suspected to host other as yet undetected genes whose characterization may shed light on the population genetics and evolution of the whole gene complex and thus on its unexplained character of marker locus for a number of diseases of nonimmune or unknown pathogenesis. A novel gene was identified that is transcribed in all tissues tested and is located in mouse and man between the *C4* and *Bf* genes of the H-2 and HLA complexes, respectively. From the nucleotide sequence, derived from liver complementary DNA clones, it is predicted that this novel single-copy gene encodes a 42-kilodalton polypeptide that bears no recognizable relation to the protein families known so far, but it displays striking hallmarks of natural selection.

IN ORDER TO SEEK OUT NEW GENES IN the major histocompatibility complex (MHC) (*1*), we set up a collection of genomic DNA probes derived from the mouse H-2S subregion which, on account of its relative length (*2*), is likely to enclose a large number of such undetected transcribed sequences. Because protein coding sequences are subject to stronger evolutionary constraints than noncoding ones, murine DNA segments were selected for their ability to hybridize at high stringency on rat DNA. This strategy, which we have previ-

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ously described (*3*), enabled us to isolate a DNA fragment designated WL10S from the extremity of cosmid 2.1 (*4*) that falls between the *C4-Slp* and *Bf* genes of the H-2^d haplotype. This genomic probe, which also hybridized with a polyadenylated [poly(A)] RNA species slightly smaller than the 18*S* ribosomal marker, was used to screen a mouse liver complementary DNA (cDNA) library constructed in the λgt10 vector. Only two clones, designated WL623 and WL691, were detected among 1.5×10^6 recombinant phages screened with the WL10S probe. However, ten additional clones were detected when the WL623 cDNA was used as a probe on the same library.

Although from DNA blot experiments it appeared that WL10S identifies a single-copy element, we first compared its sequence to that of the cDNA clone WL623 in order to ascertain the H-2S origin of the corresponding RNA. The 507-bp genomic fragment of WL10S, shown in Fig. 1A, contains two nonadjoining blocks of nucleotides that are identical to those found contiguously in the first 187 positions of the WL623 cDNA (Fig. 1B). It is evident that these 187 bp belong to two exons separated by a 320-bp intron flanked by the GT/AG consensus dinucleotides typical of splice junction sites (*5*). This finding demonstrates that a previously undetected transcribed sequence lies between the *C4-Slp* and *Bf* genes of the H-2S region.

Figure 1B shows the sequence of the 1513-bp insert of the WL623 cDNA clone, which has a poly(A) stretch defining the 3′ extremity of the coding strand. The size of WL623 suggests that this clone covers essentially the entire length of the natural messenger RNA, and thus little or no information is missing on its 5′ end. The longest of the open reading frames sought on both strands extends from the beginning of the WL623 sequence up to nucleotide 1264. The 5′-most ATG codon (position 140) is preceded by five nucleotides (CCAAC) in satisfactory agreement with the consensus sequence for translation initiation sites (*6*). Translation starting at this codon generates a 375-residue-long polypeptide with a molecular mass of 42 kD. As already mentioned, information on the WL623 transcripts is not sufficient to rule out a more distal in-phase initiation site located a little farther upstream.

The WL623 sequence is characterized by a very peculiar core region (centered on, but not limited to, nucleotides 725 to 880) containing about eight imperfect repeats of approximately 15 nucleotides. This part of the messenger RNA encodes, in its most likely translation frame, a stretch of 52 amino acids composed exclusively by the perfect and monotonous reiteration of a dipeptide made of a basic residue (arginine or lysine) next to an acidic one (aspartic acid or glutamic acid). Although such periodic structure is a clear if exaggerated example of gene elongation (*7*), certain features are worth commenting on. The strong conservation of

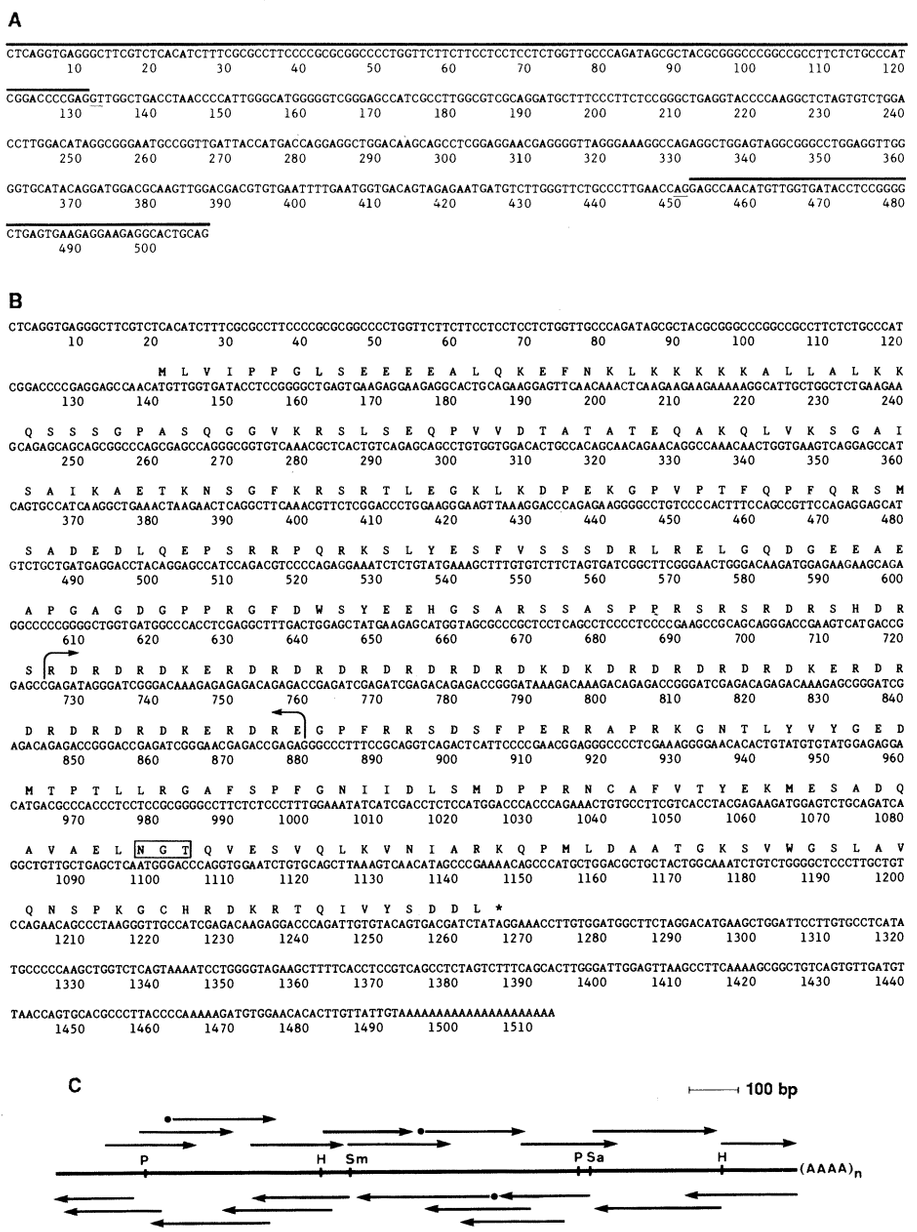

Fig. 1. (**A**) Nucleotide sequence (*14*) of the WL10S (H-2^d) genomic fragment. The two blocks of nucleotides marked by a bar correspond to the exon segments identified within the cDNA sequence shown below. The GT-AG consensus dinucleotides typical (*5*) of splice junction sites are underlined. (**B**) Nucleotide (*14*) and predicted amino acid sequence of the B10.HTT (H-2S^k) WL623 liver cDNA. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The first 187 positions correspond exactly to the two blocks of nucleotides marked in (A). The two sequences are identical in spite of their origin from mouse strains of different genotype (WL10S, H-2S^d; WL623, H-2S^k). (**C**) Scheme outlining the sequencing strategy and the restriction map of the insert (P, Pst I; H, Hind III; Sm, Sma I; Sa, Sac I). The arrows preceded by a dot denote the sequence reactions performed with synthetic oligodeoxynucleotide primers. In (B) the bracketed region (nucleotides 725 to 880) encodes a perfect repeat of a dipeptide motif resulting in the strict alternation of a basic amino acid [arginine (R) or lysine (K)] with an acidic one [aspartic acid (D) or glutamic acid (E)]. A perfect duplication of a nucleotidic stretch is recognizable in this region at positions 809 to 826 and 833 to 850. A loosely conserved arginine-containing dipeptide is repeated six times upstream from the bracketed region. A potential *N*-linked glycosylation site is boxed. A typical poly(A) site is lacking (*15*). Double-stranded cDNA was synthetized from liver RNA according to a protocol modified from that of Land *et al.* (*16*) and was inserted in the λgt10 phage vector with Eco RI linkers.

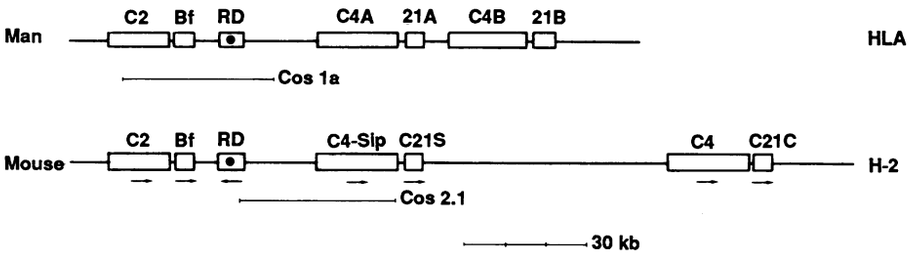

Fig. 2. Schematic representation of the class III region genes of the human and murine major histocompatibility complex showing the localization, the guessed size, and the transcriptional orientation of the new gene marked by a dot and denoted, for want of a better name, as RD (that is, by the acronym of the most common dipeptide repeat). The data used to construct these maps were collected from relevant references in a published monograph (*17*). The positions of the HLA cosmid 1a (Cos 1a) (*10*) and of the H-2 cosmid 2.1 (Cos 2.1) (*4*) are indicated.

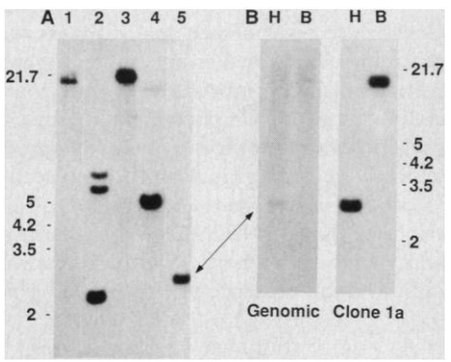

Fig. 3. DNA blot hybridization experiments with the WL623 insert used as a probe. Numbers on the sides of the figure refer to DNA size markers in kilobase pairs. (**A**) Interspecies hybridizations: Hind III digestions of 15 μg of DNA. (Lane 1) Hamster; (lane 2) rat (Lou strain); (lane 3) mouse (BALB/c; H-2^d); (lane 4) mouse (B10.BUA1; H-2^{w16}); and (lane 5) man. (**B**) Assignment of the WL623 hybridizing human DNA fragments to the cosmid clone 1a (*10*). Hind III (H) or Bam HI (B) digestions of 15 μg of genomic DNA or 1 μg of DNA of the HLA clone 1a. The arrow points to the same Hind III human DNA fragment hybridized under different stringency conditions. DNA samples were separated by electrophoresis (*18*) in (A) 0.8% or (B) 1% agarose gel. The WL623 insert was radiolabeled to a specific activity of 10^9 cpm/μg by using the random-priming procedure (*19*) and was used in the hybridization mixture (*18*) at a concentration of 10^6 cpm/ml. Filters were subsequently washed extensively at room temperature and finally four times for 20 minutes each time at 65°C in the following solution: 16 m*M* sodium phosphate, *p*H 7.5; 1 m*M* sodium pyrophosphate; 0.1% SDS and (A) 0.1× SSC or (B) 0.5× SSC (1× SSC is 150 m*M* sodium chloride and 15 m*M* sodium citrate).

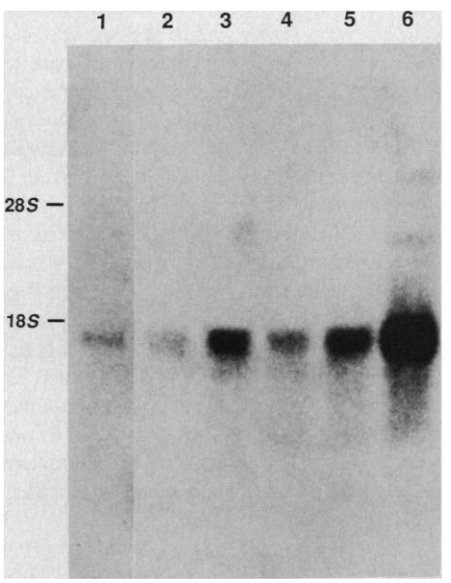

Fig. 4. RNA blot hybridization experiment with the WL623 insert as a probe. (Lane 1) Twenty micrograms of total RNA from the human hepatoma cell line HepG2; (lane 2) 20 μg of total murine liver RNA; (lane 3) 20 μg of total murine LPS-blasts RNA; (lane 4) 20 μg of total murine spleen RNA; (lane 5) 20 μg of total RNA from the murine hepatoma BW1 (*20*); and (lane 6) 10 μg of murine liver poly(A)$^+$ RNA. Samples were separated by electrophoresis on a 1.2% agarose gel containing 3% formaldehyde (*18*) and were transferred to GeneScreen membranes (New England Nuclear). Hybridization and washing of the filters were performed under the same conditions as for filter A of Fig. 3. The filter was exposed for 8 days at −80°C with an intensifying screen.

the dipeptide residues stands in sharp contrast to the poor conservation of their codons, thus implying that the periodicity cannot be entirely accounted for by trivial rounds of recent duplications. Indeed, the multiplicity of codons specifying the most common dipeptide Arg-Asp as well as the presence of other combinations of amino acids such as Arg-Glu, Lys-Glu, and Lys-Asp imply the accumulation of multiple mutations which, even when occurring in the first base of the triplets, rigorously respect the charge periodicity of the structure by specifying the same or an isofunctional amino acid. These observations, together with the absence of any kind of imperfection in the periodic structure, indicate the existence of a strong selective pressure responsible for the conservation of the unusual repeated motif.

Since cosmid clones may contain DNA fragment unlinked in the "natural" genome but artificially joined during the cloning procedure, we sought a confirmation of the genomic location of this new gene and determined its transcriptional orientation by using another murine cosmid library constructed from DNA of the B10.W7R mouse. This mouse strain, which carries a quadruplication of the DNA segment surrounding the *C4-Slp* gene (*8*), has a single copy of the WL623 sequence, which, as in BALB/c, is located between its single *Bf* gene and one of the four *C4-Slp* genes. By cDNA hybridization on cosmid clones the 5′ part of the gene was found to lie on a clone (W794) containing a *C4-Slp* gene copy while its 3′ part was found on another cosmid clone (WBf7) carrying the *Bf* gene of B10.W7R. Thus the transcriptional orientation of this new gene is opposite to that of all the neighboring class III genes (Fig. 2). Figure 3A shows that the rat, hamster, and human genomes also carry a sequence related to WL623. Taking into account the very stringent hybridization conditions used in the DNA blot experiment, we conclude that this gene displays a pronounced evolutionary conservation among mammals. In comparison, under the same stringency conditions a murine C4 cDNA probe (*9*) gives only a faint hybridization signal on human DNA.

Using HLA cosmid clones (*10*) corresponding to the complement gene region, we tried to map the human WL623-related sequence. Figure 3B shows that the restriction fragments detected in the genomic DNA are entirely contained in the cosmid clone designated 1a (*10*), thus demonstrating that this single-copy sequence occupies, in the human MHC, a position corresponding to that established in the mouse, namely between the *C4A* and *Bf* genes.

In order to ascertain whether also the human homolog of the murine gene is transcribed, and to get information concerning the tissue distribution of the expression of this gene, we performed a RNA blot experiment. As in the mouse, the transcription product of this new gene has a size slightly smaller than that of the 18*S* ribosomal RNA marker (Fig. 4). This experiment together with data on RNAs from human testis, mouse L cells, and B and T murine cell lines show that the gene is expressed at very low level in a number of different cell types, thereby indicating that it may encode a

housekeeping protein. The absence of a typical leader peptide from the deduced amino acid sequence (*11*) may suggest that the protein is not destined to penetrate the endoplasmic reticulum and therefore its potential *N*-linked glycosylation site (Fig. 1) may not be used. The strong interspecies conservation of the WL623 nucleotide sequence, and the even more rigidly preserved dipeptide repeat element as well as the widespread expression of the gene, provide strong evidence that the protein subserves a fundamental and probably phylogenetically ancient function. The most striking feature of this previously undetected gene resides in its central repeat element for which we have failed to find a significant match in the protein sequences available from different databases. Moreover, our inability to relate any segment of the remaining sequence to that of other proteins (*12*) indicates that WL623 identifies a novel class of proteins. Finally our finding adds a new element in support of the genetic heterogeneity of the class III region of the MHC, a concept that already emerged with the cloning of the cytochrome P450 gene encoding the steroid 21 hydroxylase isozyme (*13*).

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Genetically Transformed Maize Plants from Protoplasts

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Genetically transformed maize plants were obtained from protoplasts treated with recombinant DNA. Protoplasts that were digested from embryogenic cell suspension cultures of maize inbred A188 were combined with plasmid DNA containing a gene coding for neomycin phosphotransferase (NPT II) next to the 35*S* promoter region of cauliflower mosaic virus. A high voltage electrical pulse was applied to the protoplasts, which were then grown on filters placed over feeder layers of maize suspension cells (Black Mexican Sweet) and selected for growth in the presence of kanamycin. Selected cell lines showed NPT II activity. Plants were regenerated from transformed cell lines and grown to maturity. Southern analysis of DNA extracted from callus and plants indicated the presence of the NPT II gene.

AGRICULTURALLY IMPORTANT CEREal crops, including maize, have been difficult to engineer genetically by current techniques for gene insertion. With few exceptions (*1, 2*), most of the graminaceous crops are not readily susceptible to infection by *Agrobacterium tumefaciens*, which is a vector for gene transfer commonly used with many dicot species (*3, 4*). Genes can be transferred directly into protoplasts, without an *Agrobacterium* vector, by methods that permit DNA to cross the plasmalemma (*5–8*). Stable transformation of maize cells has been achieved through direct uptake of DNA into protoplasts that had been permeabilized by electroporation (*8, 9*), but until recently (*10*) no plants had been recovered from maize protoplasts. We now describe regeneration of maize plants derived from protoplasts into which a gene encoding neomycin phosphotransferase II (NPT II) was introduced via electroporation. NPT II permits plant cells to grow on inhibitory levels of the antibiotic kanamycin (*3, 8*) and can be used as a dominant marker to select for transformed cells.

Protoplasts were isolated from an embryogenic cell suspension culture of maize inbred A188 (*10*). The cell culture was initiated as callus from immature embryos 18 months before these experiments were begun and had been grown as a suspension culture in liquid N6ap medium (*10*) for approximately 1 year. Plants were then easily regenerated by transferring callus to N6ap or MS (*11*) medium without auxin.

Freshly prepared protoplasts were suspended at densities of 3×10^6 to 6×10^6 per milliliter of N6ap medium (450 mos*M*,

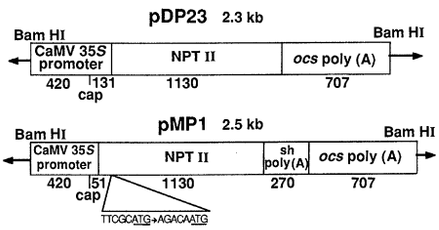

Fig. 1. Diagram of pDP23 and pMP1. The 35*S* promoter fragment in pDP23 (*9*) extends from the Bam HI site at −420 bp to the Dde I site at +131 bp with respect to the start of RNA transcription (*12*). The fragment carrying the NPT II gene includes 15 bp of 5′ noncoding region and 343 bp of 3′ noncoding region extending to the Sal I site in Tn5. The false start ATG 16 bp upstream from the original Tn5 version has been deleted. The 3′ region of the *ocs* gene was recovered as a 707-bp Pvu II fragment (*13*). The cassette in pMP1 contains a version of the 35*S* promoter that includes 51 bp of CaMV sequence downstream from the start of RNA transcription (*9*). For pMP1, the sequence in the 5′ noncoding region of NPT II in pDP23 was changed from 5′-TTCGCATG-3′ to 5′-AGACA ATG-3′. The 3′ region of pMP1 includes a 270-bp Rsa I fragment from the 3′ region of the sucrose synthase (sh) gene (*19*) fused at its 3′ end to the Pvu II fragment of the *ocs* polyadenylation region. The NPT II cassettes in pDP23 and pMP1 are bordered by Bam HI sites and were inserted into the Bam HI site of pUC19.

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