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A mutational hot-spot within an intron of the mouse β_2-microglobulin gene

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β_2-Microglobulin is the smaller, relatively non-polymorphic chain of class I major histocompatibility complex proteins. We have previously described a mutant mouse cell line which had been selected for loss of the class I thymus leukemia (TL) antigen and had concomitantly lost surface expression of H-2^k antigens. Expression of class I antigens on the cell surface was restored by fusion to an antigenically distinct mouse lymphoma line, and the defect in the mutant was shown to be the loss of a functional β_2-microglobulin gene. We now describe three additional mutants with the same phenotype, all selected for loss of TL but after different types of mutagenesis. All of these mutants have genomic rearrangements resulting in the absence of a functional β_2-microglobulin gene. These data provide strong evidence for the requirement of β_2-microglobulin for cell surface expression of the heavy chain of class I major histocompatibility complex proteins. We further show that the defects in at least one β_2-microglobulin gene in each mutant cell line map to the same small DNA segment within the first intron. The breakpoints of these mutations define a hypermutable site within the mouse β_2-microglobulin gene.

Key words: β_2-microglobulin/hypermutable/MHC/B1 repeat/transplantation antigen

Introduction

Class I major histocompatibility complex (MHC) proteins are heterodimeric structures expressed on the surface of most mammalian cells and serve as restriction elements for the recognition of virally infected, chemically altered or tumor cells by cytotoxic T lymphocytes (Klein, 1975, 1979; Snell *et al.*, 1976; Vitetta and Capra, 1978; Ploegh *et al.*, 1981; Zinkernagel and Doherty, 1980). In the mouse these proteins include the classical transplantation antigens (H-2K, D and L), the thymus leukemia antigen (TL), Qa antigens and perhaps other as yet unidentified, proteins which are encoded by a large multi-gene family on chromosome 17. The class I proteins consist of a polymorphic, MHC-encoded transmembrane glycoprotein of ~45 kd which is tightly but non-covalently associated with the relatively invariant 12-kd polypeptide β_2-microglobulin. β_2-Microglobulin, which is not encoded within the MHC, does not itself span the plasma membrane, but rather is found on the cell surface only by virtue of its association with class I heavy chains (reviewed in Klein, 1975; Snell *et al.*, 1976; Vitetta and Capra, 1978; Ploegh *et al.*, 1981).

Data from both human and mouse systems have suggested that class I MHC molecules are not expressed on the cell surface in the absence of β_2-microglobulin, despite the fact that both chains have their own signal peptides (Dobberstein *et al.*, 1979; Steinmetz *et al.*, 1981; Parnes and Seidman, 1982). The human Daudi cell line (established from a Burkitt lymphoma) has a single defective β_2-microglobulin gene with a point mutation which creates an early termination codon, thereby preventing translation of β_2-microglobulin mRNA (Rosa *et al.*, 1983; de Preval and Mach, 1983). This line is surface HLA-A and -B negative, but HLA heavy chains are present within the cell, and fusions with β_2-microglobulin-positive human or mouse cells result in rescue of surface expression of the Daudi HLA-A and -B antigens (Arce-Gomez *et al.*, 1977; Ploegh *et al.*, 1979). In the mouse system we found that a mutant cell line selected *in vitro* for loss of expression of TL after chemical mutagenesis had lost surface expression not only of this class I antigen, but also of H-2 antigens (Hyman and Stallings, 1976). As in the case of the Daudi cell, surface expression of class I antigens of the parental type could be rescued by fusion to an antigenically distinct cell

Table I. Cell surface expression of class I antigens by mutants and somatic cell hybrids

Cell line	Relative DNA content/cell	Relative antigen expression/cell	
		TL	H-2^k
Experiment 1			
R1.1	ND	1.0	1.0
EL4	1.00	<0.05	<0.02
Mutant 2	1.07	<0.03	<0.02
Mutant 2 × EL4 hybrids			
F1570.1	2.08	0.8	0.4
F1572.1	1.98	1.0	0.3
F1573.1	1.93	0.6	0.8
Experiment 2			
R1.1	ND	1.0	1.0
EL4	1.00	<0.03	<0.02
Mutant 3	2.03	<0.05	<0.04
Mutant 3 × EL4 hybrids			
F3671.1	2.79	1.0	0.7
F3673.1	2.98	1.9	0.3
Experiment 3			
R1.1	ND	1.0	1.0
EL4	1.00	<0.01	<0.02
Mutant 4	1.07	<0.005	0.01
Mutant 4 × EL4 hybrids			
F3740.2	1.93	0.6	0.09
F3741.1	1.95	0.6	0.09
F3743.1	2.16	0.6	0.1
F3745.2	1.91	0.8	0.3

Cell lines and generation of somatic cell hybrids are described in Materials and methods. Relative DNA content/cell was determined by mithramycin staining. Levels of surface antigen expression were determined by quantitative cytotoxic absorption as previously described (Hyman and Stallings, 1974, 1976). ND, not done.

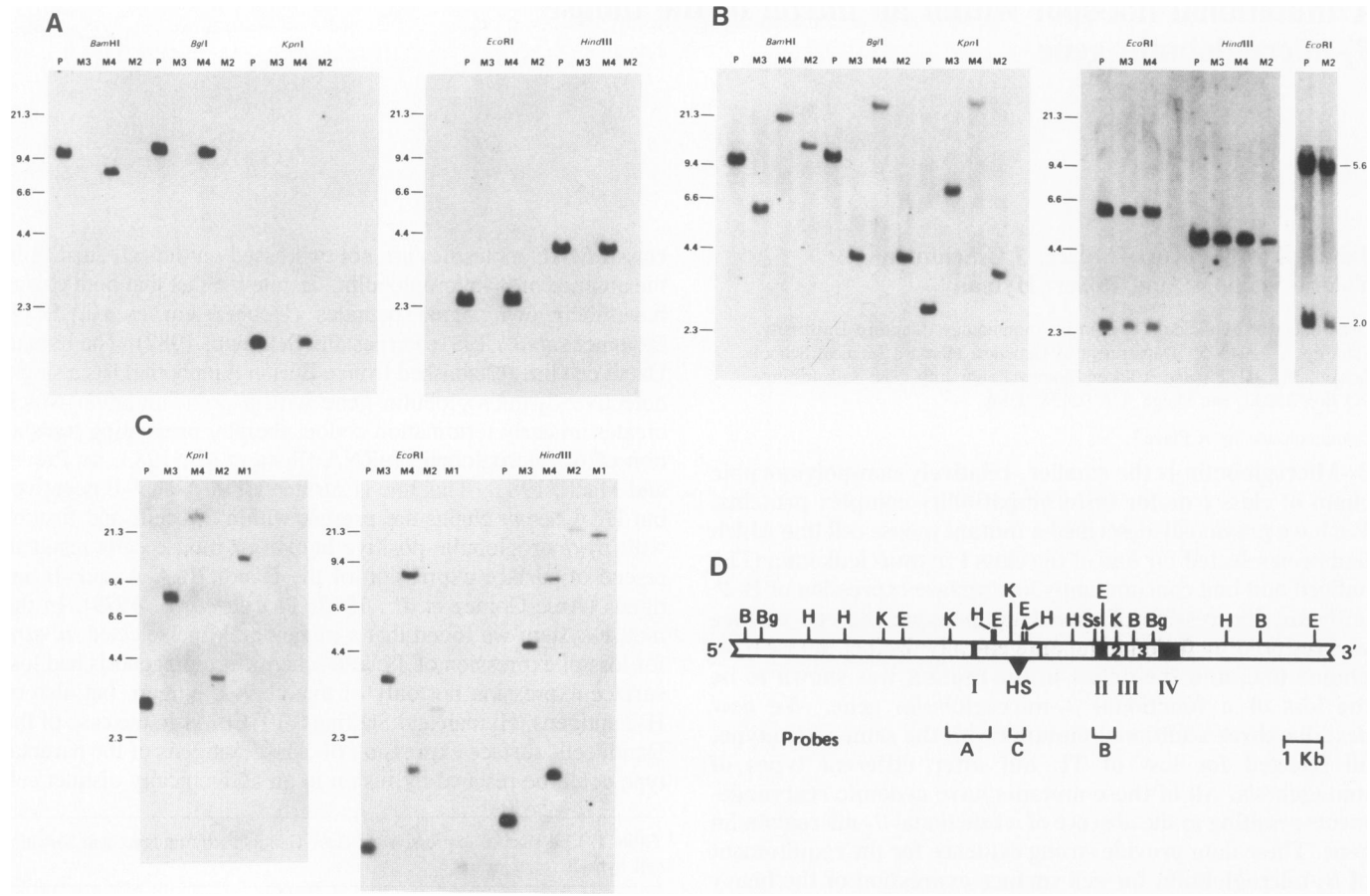

Fig. 1. Southern blot analysis of DNA from parent and mutant cell lines. **A, B** and **C**: 8 μg of DNA from the parental R1.1 cell line (P) or mutants 1, 2, 3 or 4 (M1, M2, M3, M4, respectively) were digested with the indicated restriction endonuclease, electrophoresed on 0.8% agarose gels and transferred to nitrocellulose. Blots were hybridized with the nick-translated probes shown in **panel D**: probe A (used in **panel A**) was a 1.1-kb *Kpn*I-*Hind*III fragment containing 5′-flanking sequence, exon I and part of the first intron; probe B (used in **panel B**) was a 600-bp *Sst*I-*Kpn*I fragment containing exon II (the major protein-coding exon) and parts of the flanking introns; probe C (used in **panel C**) was a 500-bp *Kpn*I-*Hpa*II fragment which includes from *Kpn*I (5′) to *Hind*III (3′) in the first intron (spanning the entire mutational hot-spot) and extends from *Hind*III to a *Hpa*II site in pBR322. Autoradiographs of the hybridized blots are shown. Restriction enzyme sites are indicated: B (*Bam*HI), Bg (*Bgl*I), H (*Hind*III), K (*Kpn*I), E (*Eco*RI), Ss (*Sst*I). Exons are indicated by shaded bars and are numbered below with Roman numerals. Introns and flanking sequences are indicated by open bars; the introns are numbered with Arabic numerals. The mutational hot-spot (HS) is indicated (▼). Size markers are indicated in kb and were determined by electrophoresis of a *Hind*III digest of phage λ DNA run in parallel and stained with ethidium bromide. **D**: a map of the β_2-microglobulin gene (*a* allele) containing the relevant restriction enzyme sites for interpreting the Southern blots is shown.

line (Hyman and Stallings, 1977). Analysis of this mutant line indicated that the defect was in both copies of the β_2-microglobulin gene (Parnes and Seidman, 1982). Two models were proposed which could explain the genetic defects in that cell line: (i) one chromosomal copy of the β_2-microglobulin gene could be totally deleted while the other had a small deletion (500 – 1200 bp) and a large insertion or inversion within the first intron, or (ii) the 3′ portion of the gene could be deleted on one chromosomal copy and the 5′ portion deleted on the other (Parnes and Seidman, 1982). We have now generated and analyzed three additional mutants of the parental R1.1 cell line, all selected for loss of TL expression after either chemical or radiation mutagenesis. We find that all three mutants have lost surface expression of H-2 antigens as well and all three have mutations which result in the absence of a functional β_2-microglobulin gene. These mutations all involve major deletions or inversions and, in each mutant, at least one of the breakpoints lies within the same 400-bp region in the first intron of this gene. These data not only provide strong evidence of the requirement for β_2-microglobulin for surface expression of class I antigens, but also demonstrate the existence of a mutational 'hot-spot' within the β_2-microglobulin gene.

Results

Selection for loss of TL results in concomitant loss of H-2 expression

Mutant 2 [R1.1N(TL$^-$)7 × .1] was selected for loss of surface expression of the TL antigen after nitrosoguanidine mutagenesis as described in Materials and methods. As shown in Table I, this mutant is phenotypically similar to the original R1(TL$^-$) mutant [R1E[TL$^-$]8 × .1, referred to herein as mutant 1] in that it also lacks surface expression of H-2^k antigens as determined by quantitative cytotoxic absorption. It does, however, retain expression of Thy 1.2. Similarly, mutants 3 (R1.1R/TLIII 7 × .6) and 4 (R1.1.1R/TL 3 × .16) have also lost expression of H-2^k despite selection only for loss of TL following cobalt irradiation (Table I). To characterize further the defects in these mutants, somatic cell hybrids were generated between each of them and the TL$^-$, H-2^b lymphoma EL4. Two to four independent hybrids

from each of these fusions were characterized for their surface antigen expression by quantitative cytotoxic absorption. As indicated in Table I, the hybrids from each of these mutants re-express H-2^k and TL antigens. These results are identical to those previously found for mutant 1 and imply that the defects in mutants 2, 3 and 4 are recessive. These data are consistent with (but not proof of) a model in which the TL^-, $H\text{-}2^-$ phenotype of the mutants results from lack of expression of a gene product necessary for expression of both the TL and H-2 antigen heavy chains on the cell surface. The obvious candidate for such a gene product is β_2-microglobulin.

Mutants 2, 3 and 4 have defective β_2-microglobulin genes

Since we had previously found that the defect in mutant 1 was the lack of a functional β_2-microglobulin gene, we examined the organization of this gene in mutants 2, 3 and 4 by genomic Southern blot analysis. Figure 1 shows autoradiographs of Southern blots of genomic DNA from the parental line R1.1 and the three new mutants hybridized to radiolabeled probes of different segments of the β_2-microglobulin gene. In panel A the probe was a 5′ 1.1-kb *Kpn*I-*Hind*III fragment which contains the promoter and leader segments and ends within the first intron (probe A). No hybridizing bands were seen with this probe in mutants 2 or 3 with any of the five restriction enzyme digests. These two mutants therefore contain deletions which span this 1.1-kb segment on both chromosomal copies of their β_2-microglobulin gene. In contrast, the bands which hybridized in mutant 4 with *Eco*RI, *Hind*III and *Kpn*I were all identical in size to the parental bands. However, with enzymes which yield fragments extending more 5′ and 3′ (*Bam*HI and *Bgl*I) the hybridizing bands in mutant 4 were smaller than in the parent, and only one (abnormal) hybridizing band was present in each case. The relative sizes of these mutant bands were not consistent with a simple deletion. From this data one can predict that there must be a breakpoint in this mutant either 5′ of the first *Hind*III site upstream (~3.2 kb) of exon I or 3′ of the *Kpn*I site in the first intron (see map, Figure 1, panel D). Additional mapping and sequence data using the cloned gene from this mutant (see below) show that the latter is the case.

Panel B shows the results using a 3′ probe (600-bp *Sst*I-*Kpn*I genomic fragment) which contains most of the protein coding sequence of the β_2-microglobulin gene (exon II) and some intervening sequence (probe B). With this probe hybridizing bands were found in all of the mutants. In all three cases hybridizing fragments were identical in size to those in the parental DNA with *Eco*RI and *Hind*III. In contrast, the hybridizing bands with *Bam*HI, *Bgl*I and *Kpn*I were all different from those in the parental DNA and unique for each independent mutant. Furthermore, only a single hybridizing band was present in each mutant with any of the enzymes which produced abnormal sized bands. Probes and enzymes which look at more 3′ segments of the gene gave normal patterns for all of the mutants (data not shown). These results indicate that there is only a single chromosomal copy of the main protein coding exon of this gene in each of the mutants. Furthermore, in all three cases there must be breakpoint in the gene somewhere 5′ of the most 3′ *Eco*RI site in the first intron (see map, Figure 1, panel D).

To examine the region of these breakpoints in the first intron more closely, we used a probe containing the 400-bp *Kpn*I-*Hind*III fragment from this intron (probe C). As shown in Figure 1, panel C, a single band hybridized to this probe in *Eco*RI, *Hind*III or *Kpn*I digested DNA from mutants 1, 2 and 3, as well as in DNA from the parent. In contrast, two abnormal bands hybridized in mutant 4 DNA with *Eco*RI and *Hind*III, indicating that both the 5′ and 3′ breakpoints are contained within the probe fragment. In all cases the mutant bands differed from the parental and from each other. It is noteworthy that only a single band was detectable in the *Kpn*I digest of mutant 4 using the breakpoint probe. We believe this to be doublet since mapping data with isolated genomic clones (see below) indicates that the *Kpn*I fragments at each breakpoint would have been large enough to be detected on the Southern blot. Although the single *Kpn*I band detected could correspond to a large *Kpn*I fragment representing an insertion in mutant 4, this interpretation is not consistent with data below showing that mutant 4 has an inversion rather than an insertion.

We can draw several conclusions from these data. First, all three new mutants are different from one another and from the previously described mutant. Second, mutants 2 and 3 each have only one chromosomal copy of the β_2-microglobulin gene, and that single copy has deleted the 5′ end of the gene from a point somewhere within the first intron. In both cases the breakpoint must lie between the *Kpn*I site in the first intron and the *Eco*RI site 400 bp 3′ of it (i.e., the most 3′ *Eco*RI site in the first intron) (Figure 1, panel D). This is the same location as we had previously found for the 3′ breakpoint of mutant 1. Although the breakpoints within the β_2-microglobulin gene could be identical for mutants 2 and 3 (and for one of the breakpoints of mutant 1), the new DNA that is now 5′ cannot be, since all of the abnormal sized bands seen with the 3′ and intron probes were very different for these mutants. We cannot say whether the new 5′-flanking sequences are normally present upstream of the gene, or whether they were inserted from elsewhere on the chromosome or in the genome. Finally, mutant 4 is more complicated than mutants 2 and 3 and appears more like mutant 1. It has one 5′ and one 3′ segment of the β_2-microglobulin gene, but they are not juxtaposed with each other normally. These data do not allow us to distinguish whether the 5′ and 3′ ends are from the same or homologous chromosomes. The 3′ breakpoint can be placed between the *Kpn*I site in the first intron and the *Eco*RI site 400 bp 3′ of it, i.e., within the same 400-bp fragment as for mutants 1, 2 and 3. The 5′ breakpoint is further discussed below.

Nucleotide sequence of a hypermutable segment within the first intron

Since at least one breakpoint in each of the four independent mutants was within a short segment of DNA, we determined the nucleotide sequence of the normal β_2-microglobulin gene in this region to see whether there were any unusual features which could account for this phenomenon. The sequence of an ~900-bp segment spanning this region is shown in Figure 2. The only noteworthy finding is the presence of a BI repetitive element (mouse Alu-type repeat sequence) near the 5′ end, 311 bp 5′ of the segment (*Kpn*I-*Eco*RI) which contains at least one breakpoint for each of the four mutants. There are no striking internal repeats or inverted repeats, and no homologies to other sequences in the GenBank™ Genetic Sequence Data Bank. We cannot easily account for the mutations in this region based upon the normal sequence alone unless the B1 sequence to the 5′ side is involved. It should be noted that the sequence shown is for the C57B1/6 gene rather than for the parental cell line R1.1 (from a C58 mouse). However, detailed restriction mapping shows no difference between R1.1 and C57B1/6 throughout this region, and data for mutant 4 below show identity of its nucleotide sequence in this region to that of C57B1/6 up to the mutational breakpoints (see below).

Fig. 2. Nucleotide sequence of the hypermutable region of the β_2-microglobulin gene. The nucleotide sequence of a 900-bp *Hind*III fragment spanning the hypermutable region is shown. The sequence was determined by the chemical degradation method of Maxam and Gilbert (1980). The hypermutable segment, as defined by Southern blot analysis, extends from the *Kpn*I site to the 3′ most *Eco*RI site and is underlined. A complete B1 repeat element 5′ of the hypermutable region is bracketed and the 8-bp direct repeats flanking the repeat unit are marked above by asterisks.

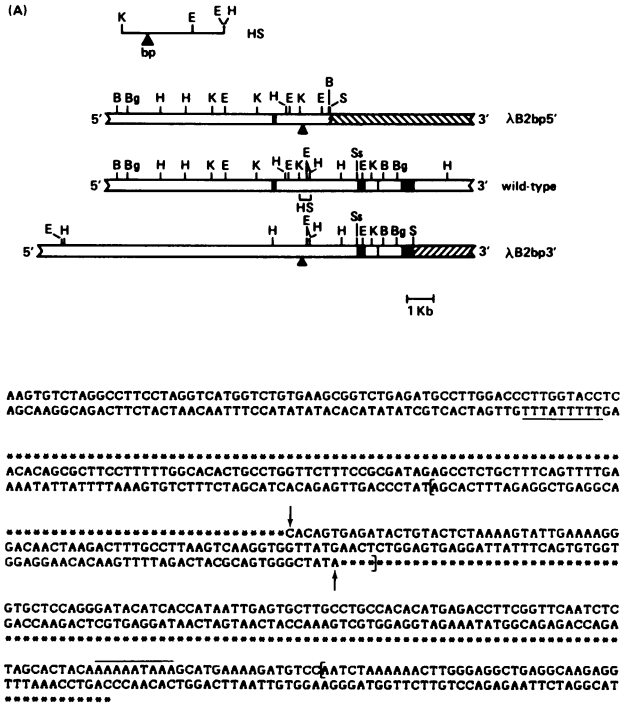

Fig. 3. Restriction maps of the 5′ and 3′ breakpoint clones of mutant 4 and nucleotide sequence of the breakpoints. The isolation of 5′ (λB2bp5′) and 3′ (λB2bp3′) breakpoint clones from mutant 4 genomic DNA is described in Materials and methods. (**A**) Restriction maps are shown in comparison with that for the wild-type gene (*a* allele). A blow-up of the mutational hot-spot (HS) is shown above. The breakpoint (bp) in mutant 4 is indicated (▲) in the hot-spot blow-up and in the maps of the breakpoint clones. Restriction enzyme sites are labeled as in the legend to Figure 1 with the addition of S (*Sal*I). Phage arms are indicated by hatched bars. Exons are indicated by shaded bars. (**B**) The nucleotide sequence of the breakpoints of the 5′ (5′ BP) and 3′ (3′ BP) clones are shown aligned with the wild-type (WT) sequence. Asterisks are used where the mutant sequence is identical to the wild-type up to the breakpoint, after which the mutant sequence is shown in full. The wild-type sequence shown corresponds to nucleotides 438 – 777 in Figure 2. The breakpoints are indicated by vertical arrows. Sequences homologous to Alu-family repeats are bracketed. An A- (or T-) rich inverted repeat is underlined in the 3′ breakpoint clone and overlined in the 5′ breakpoint clone.

Isolation of the 5′ and 3′ breakpoints of mutant 4

Since the normal sequence did not itself reveal the reason for the localization of the mutations to this region, we decided to examine the precise breakpoints in one of the mutants by isolating genomic clones containing them. We chose mutant 4 since it appeared to have two breakpoints, and since we were interested in whether the 5′ and 3′ ends were from the same or homologous chromosomes. A *Mbo*I partial genomic library was constructed from this mutant in the lambda phage vector EMBL3 as described in Materials and methods. The library was screened with a combination of 5′ and 3′ probes (probes A and B in Figure 1). Three plaques were isolated which re-tested positive. Of these, two hybridized with the 3′ probe and one with the 5′ probe. The inserts in the two 3′ clones appeared to be equivalent, so only one will be discussed further. The 5′ and 3′ clones were analyzed by restriction mapping, hybridization to β_2-microglobulin probes and comparison with the genomic Southern blotting results. The 5′ clone (λB2bp5′) has an insert of ~11.5 kb which extends ~1.5 kb 3′ of the *Kpn*I site in intron 1. The restriction map of this clone is identical to that of the normal β_2-microglobulin gene 5′ of this *Kpn*I site, but differs to the 3′ side (Figure 3, panel A). The 3′ clone has an insert of ~18.7 kb and extends ~12.5 kb 5′ of the 120-bp *Eco*RI fragment within the first intron. This clone is identical in restriction map to the wild-type gene to the 3′ side of this small *Eco*RI fragment, but differs to the 5′ side (Figure 3, panel A). The nucleotide sequences of the breakpoints of both of these clones are shown in Figure 3 (panel B) in comparison with the normal gene sequence. The breakpoints of the two clones are separated by 6 bp which are not present in either clone. As indicated in Figure 3, a segment of DNA spanning the 3′ breakpoint (including 4 bp from the normal gene) is homologus to Alu-type repeat segments (74% homologous to a Chinese hamster Alu-equivalent repeat over 62 bp). There is another segment of the new DNA in the 3′ breakpoint clone which is also homologous to Alu-family repeats located 108 – 144 bp 5′ of the breakpoint. The 5′ breakpoint clone also contains a portion of an Alu-family repeat, but in this case it begins farther (141 bp) from the breakpoint. There is also an A- or T-rich inverted repeat (AAAAATAAA or its reverse complement) found in the new DNA 116 bp from the 5′ breakpoint and 108 bp from the 3′ breakpoint. The relationship, if any, of these sequences to the mutational events is not clear. The new DNA at each breakpoint is not homologous to any other sequences in the GenBank™ Genetic Sequence Data Bank except for the segments discussed above which are homologous to Alu-type repetitive elements.

Mutant 4 represents an inversion

The fact that the 5′ and 3′ breakpoint clones of mutant 4 contain no overlapping sequence from the β_2-microglobulin gene is consistent with the two clones originating either from the same chromosome (in which case an insertion or inversion must have separated the 5′ and 3′ ends) or from homologous chromosomes (in which case large deletions occurred beginning at almost identical breakpoints but extending in opposite directions). To discriminate between these possibilities, we hybridized radiolabel-

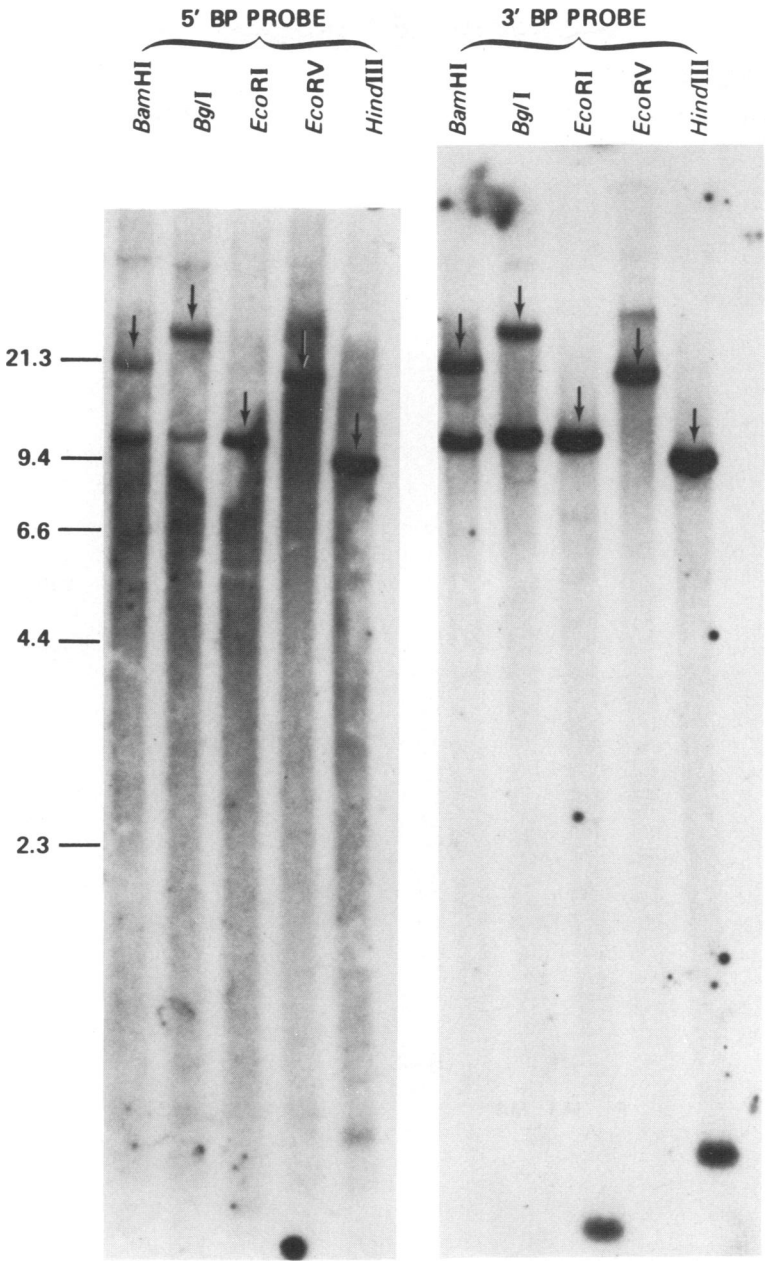

Fig. 4. Southern blots indicating that mutant 4 has an inversion in the β_2-microglobulin gene. R1.1 genomic DNA (8 μg) was digested with the indicated restriction enzymes, electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose. One half of the blot was hybridized to a nick-translated probe spanning the 5′ breakpoint from clone λB2bp5′ (1.2-kb *Kpn*I-*Eco*RI fragment) and the other duplicate half to a nick-translated probe spanning the 3′ breakpoint from clone λB2bp3′ (560-bp *Hpa*I-*Hind*III fragment). Two bands are visible in each lane because the probes contain a small portion of the normal β_2-microglobulin gene as well as the new DNA. The bands which hybridize to the latter are indicated by arrows.

ed probes of the new DNA at each breakpoint to Southern blots of genomic DNA from the parental line R1.1. As shown in Figure 4, identical bands hybridized with both the 5′ and 3′ breakpoint probes in digests with five restriction enzymes. These results are strong evidence for the mutation in mutant 4 being an inversion. An inversion would imply that the new DNA at the 5′ and 3′ breakpoints would have originally been adjacent to each other in the parental genome, so fragments from each would be different from each other yet hybridize to the same restriction fragments in parental DNA. We have also used the new DNA at the 5′ and 3′ breakpoints as probes to hybridize to genomic DNA from the other three mutants and found it to be in the parental configuration in each case (data not shown). This implies that the new DNA that is brought into the β_2-microglobulin gene is not identical in all of the mutants.

The mutant cell lines contain class I but not β_2-microglobulin mRNA

The data shown above indicates that all four mutants have defects in both copies of their β_2-microglobulin gene. However, it is important to demonstrate that these defects result in a lack of β_2-microglobulin mRNA (and hence protein) if one is to attribute the lack of surface class I expression to these defects. We have examined the presence of β_2-microglobulin mRNA in all four mutants and in the parental line by Northern blot analysis using probes from 5′ (leader) and 3′ (main protein coding exon)

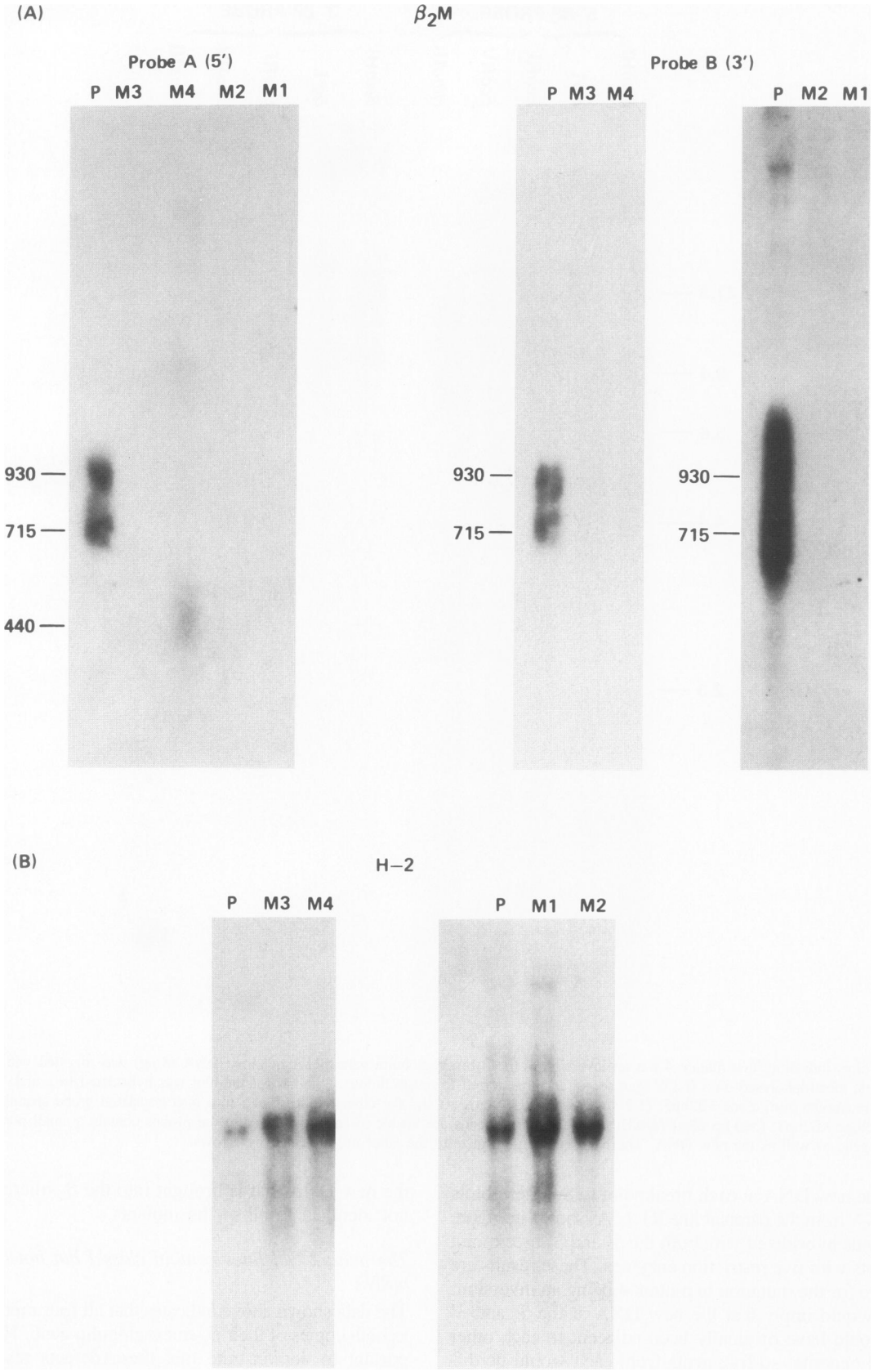

Fig. 5. Northern blot analysis of RNA from the mutant cell lines. Total cellular RNA (10 μg) from the parent R1.1 cell line (P) and from mutants 1, 2, 3 and 4 (M1, M2, M3, M4, respectively) was electrophoresed on a 1.5% agarose-formaldehyde gel and transferred to nitrocellulose. The blots were hybridized with nick-translated probes for β_2-microglobulin (**A**) or H-2 (**B**). β_2-microglobulin probes were: 5′ probe, probe A in Figure 1, panel D; and 3′ probe, probe B in Figure 1, panel D. The H-2 probe was a 2250-bp *Eco*RI-*Pvu*II fragment isolated from cDNA clone pMHC1 and contains the coding sequence for amino acids 50–87 of H-2L^d as well as some pBR322 sequences; it hybridizes to RNA for all class I molecules (Croce *et al.*, 1981). Autoradiographs of the hybridized blots are shown.

segments of this gene (probes A and B, respectively, in Figure 1, panel D). As shown in Figure 5 (panel A), no hybridizing mRNA was detected in any of the mutants with the 3′ probe (containing almost all of the protein coding sequence), implying that a β_2-microglobulin protein cannot be produced by these cells. With the 5′ probe no mRNA was seen with mutants 1, 2 or 3. However, with mutant 4 a small amount of a much smaller RNA species (440 bases) hybridized to the probe, suggesting that the promoter is still functional and that transcription occurs through the 5′ breakpoint to an abnormal termination site. It is not known whether this RNA is polyadenylated. The small quantity detected suggests that it is unstable and degraded more rapidly than the normal β_2-microglobulin mRNA. Based on our conclusions concerning the nature of the mutation in mutant 1, it is also possible that this mutant could transcribe an abnormal mRNA species. However, we have not detected any hybridizing RNA in this mutant, and it is possible that no promoter segment is present on either chromosome.

Although the β_2-microglobulin gene defects preclude production of this protein, there could be an additional defect(s) in these mutants in the regulatory mechanism for control of class I MHC gene transcription. The data illustrated in Figure 5 (panel B) show that this is not the case. The level of class I mRNA expression in all four mutants was at least that of the parental line. Although we cannot exclude the possibility of a defect preventing translation of all class I mRNA species, this does not seem likely.

Discussion

We have previously characterized a mutant cell line, R1 (TL^-) or R1($\beta2^-$), generated by ethylmethane sulfonate mutagenesis of the C58 mouse thymoma cell line R1.1, followed by cytotoxic immunoselection against expression of the TL antigen (Hyman and Stallings, 1976). This mutant was found to lack surface expression of H-2 antigens as well, but expression of both TL and H-2 of the parental haplotype could be rescued by fusion to the TL^- lymphoma line EL4 (Hyman and Stallings, 1977). We demonstrated that the mutant made no immunoprecipitable β_2-microglobulin protein and had defects in both copies of its β_2-microglobulin gene (Parnes and Seidman, 1982). Together with data from the human Daudi cell line (Arce-Gomez *et al.*, 1977; Ploegh *et al.*, 1979), these results provided evidence for the necessity of β_2-microglobulin for cell surface expression of class I MHC molecules. We have now generated three additional mutants from the R1.1 cell line. One of these (mutant 2) was also found after chemical mutagenesis, but with a different agent (nitrosoguanidine). The other two (mutants 3 and 4) were isolated after γ-irradiation. All were selected by cytotoxic immunoselection against surface TL antigen expression, and all were found to have concomitantly lost surface expression of H-2. As in mutant 1, expression of both TL and H-2 could be restored by fusion to EL4. All of the mutants produce at least normal amounts of H-2 mRNA but no detectable mRNA which could encode β_2-microglobulin. Finally all of the mutants have major deletions and/or rearrangements of both chromosomal copies of their β_2-microglobulin gene, precluding transcription of functional β_2-microglobulin mRNA. These data argue strongly in favor of the hypothesis that association of class I molecules with β_2-microglobulin is required for transport of the class I heavy chain to the cell surface. It is not clear why this should be the case since each of the two chains has a signal peptide. However, recent studies of human class I antigen transport in *Xenopus laevis* oocytes have shown that the heavy chain is retained in the endoplasmic reticulum if β_2-microglobulin is not present (Severinsson and Peterson, 1984). The addition of β_2-microglobulin mRNA and its translation results in protein chain association and transport of the heterodimeric class I molecule to the Golgi. In contrast, β_2-microglobulin can be translated and secreted by oocytes in the absence of class I heavy chains (Severinsson and Peterson, 1984). It was suggested that association with β_2-microglobulin might induce a conformational change in the heavy chain which allows its further transport, or that β_2-microglobulin itself might have specific features which promote its transport whether as a free chain or as a complex with a class I heavy chain (Severinsson and Peterson, 1984). There is indeed evidence that the conformation of human class I heavy chains is quite different when the denatured chain is allowed to re-fold in the absence as compared with the presence of β_2-microglobulin (Lancet *et al.*, 1979).

Recent studies by Potter *et al.* (1984, 1985) have suggested a possible exception to the normal requirement of β_2-microglobulin for class I MHC antigen expression. They have described a variant of the T cell lymphoma EL4 with defective synthesis of H-2K^b. Although H-2D^b was still expressed on the cell surface, no surface β_2-microglobulin could be detected (Potter *et al.*, 1984). This variant (EL4/Mar) did synthesize normal amounts of β_2-microglobulin (*b* allele) which could be detected within the cytoplasm, and surface expression could be rescued by fusion to spleen cells of mice expressing the *a* allele of β_2-microglobulin (Potter *et al.*, 1985). These results suggested that H-2D^b, in contast to other class I antigens, might be inserted into the plasma membrane in the absence of β_2-microglobulin. It was hypothesized that this might be because of an additional glycosylation site in the $\alpha3$ domain of H-2D^b (Potter *et al.*, 1985). Since this is the domain of H-2 molecules which has been shown to bind β_2-microglobulin, it is possible that this glycosylation replaces β_2-microglobulin. However, the data could not rule out an intracellular association between β_2-microglobulin and H-2D^b which allows transport to the cell surface, followed by dissociation of the complex and shedding of β_2-microglobulin into the culture medium. Whatever the explanation for the findings in this variant, the data suggest strongly that β_2-microglobulin is required for surface expression of at least most class I MHC molecules.

The demonstration of a 'hot-spot' for mutation within the β_2-microglobulin gene is the most intriguing finding of the current study. Despite the fact that all four mutants were selected for loss of TL expression, all four lost that expression because of defects in the structural gene for β_2-microglobulin rather than because of defects in the TL gene(s). These mutants were found with a frequency ($\sim 10^{-6}$ M) that is much higher than expected for loss of expression of diploid genes. Two possible explanations can be put forward. Either the β_2-microglobulin gene is much more susceptible to mutagenesis than the TL gene, or perhaps one chromosomal copy of the β_2-microglobulin gene is already deleted prior to the mutagenesis. One entire copy of the β_2-microglobulin gene is indeed deleted in mutants 2, 3 and 4, and may well be in mutant 1, so that the latter possibility is feasible. Karyotype analysis showed two normal-appearing copies of chromosome 2 in the parent and in mutant 1 (Parnes and Seidman, 1982), but these data cannot exclude the possibility of a deletion of a small chromosomal region including the β_2-microglobulin locus on one chromosome. Comparative quantitative Southern blots using a probe for a marker on a different chromosome (kappa immunoglobulin light chain gene) together with a β_2-microglobulin probe do not suggest that the parental

line is haploid for β_2-microglobulin, but such studies are inconclusive. It is also possible that a small portion of chromosome 2 is lost from the parental line at a low frequency, and that such variants are the substrate for the further mutational events.

Even if the absolute frequency of mutations in the β_2-microglobulin gene is not high, the most striking finding is the localization of the mutational events to within the same 400-bp segment of the gene. Although it might not be surprising if all the mutations were within a short region if that region included the promoter or other upstream control elements, it is remarkable that this 'hot-spot' is within an intron. Three distinct methods of mutagenesis were used to generate these mutations, so the susceptibility is not linked to a specific agent. In fact, two of the mutations were generated by chemical mutagenesis (mutants 1 and 2) and both involve major DNA rearrangements rather than point mutations. It is noteworthy that mutants 1 and 4 are similar in their defects, as are mutants 2 and 3, and each of these two groups of mutational types contains one chemical- and one radiation-induced mutant. It is possible that the mutations at this site are the result of recombinational events taking place during the repair process after mutagenesis and that the 'hot-spot' is a region that is for some reason highly susceptible to recombination events. There is no obvious explanation for such a susceptibility based upon the DNA sequence of the normal gene in this region or from analysis of the DNA surrounding the breakpoints of mutant 4. It is intriguing that there are B1 or Alu-like repeat sequences near the breakpoints in both the β_2-microglobulin gene and the new DNA that is brought in by the inversion event. However, the β_2-microglobulin B1 sequence (which is a complete B1 repeat unit) is 311 bp to the 5′ side of the breakpoint. The partial Alu-like sequences in the new DNA are closer, and one actually spans the breakpoint. Nearby Alu sequences have been associated with mutational events in globin genes (Orkin and Michelson, 1980; Jagadeeswaran *et al.*, 1982; Ottolenghi and Giglioni, 1982; Vanin *et al.*, 1983) and, recently, a deletion between two Alu sequences oriented in opposite directions has been found to be responsible for a patient's mutant low density lipoprotein (LDL) receptor gene which produces a truncated LDL receptor protein (Lehrman *et al.*, 1985). These short interspersed repeats (300 bp for human Alu, 130 bp for mouse B1) could promote mutations by intra- or inter-chromosomal recombination events which could lead to deletions or inversions. Such events might occur more frequently during DNA repair after mutagenesis. We cannot say whether the B1 sequence within the first intron of the β_2-microglobulin gene is related to the hypersusceptibility to mutation or whether perhaps some as yet unidentified feature of the sequence or structural conformation is responsible. In regard to the latter possibility, preliminary data (L.Shiue and J.R.Parnes) indicate that the hypermutable region is in fact a DNase-hypersensitive site.

Materials and methods

Cell lines and isolation of mutants

The generation and isolation of the original R1 (TL^-) mutant cell line, R1E (TL^-)8 × .1, after ethylmethane sulfonate mutagenesis of the R1.1 ($H\text{-}2^k$) C58 mouse thymoma cell line has been described (Hyman and Stallings, 1976). This mutant is referred to as mutant 1 in the current study. Mutant 2, R1.1N(TL^-)7 × .1 was isolated from an independent clone of the R1 parental line from that used to obtain mutant 1. R1.1 cells (5×10^6) were mutagenized with 0.5 μg/ml N-methyl-N-nitro-N-nitrosoguanidine at 37°C for 30 min. Two weeks later the mutagenized cells were subjected to cytotoxic immunoselection against surface TL antigen expression by growth in the presence of anti-TL antiserum and complement as previously described (Hyman and Stallings, 1976). Survival was <0.4%. The surviving cells were grown up and re-selected. At the seventh cycle of selection, 37% survival was seen after selection. These surviving cells were cloned; 5/5 clones were negative for TL by qualitative cytotoxic absorption. One clone, termed R1.1N(TL^-)7 × .1, was chosen for further study, and is herein referred to as mutant 2. Mutants 3 (R1.1R/TLIII 7 × .6) and 4 (R1.1.1R/TL 3 × .16) were also derived by cytotoxic immunoselection as above, but after mutagenesis of R1.1 cells with 700 or 600 rads, respectively, of ^{60}Co γ-irradiation. Quantitative cytotoxic absorption studies were carried out as previously described (Hyman and Stallings, 1974, 1976).

Somatic cell hybridization

Somatic cell hybridization of the mutant cell lines was carried out using 40% polyethylene glycol 1500 as described (Hyman *et al.*, 1980). DNA content/cell was determined by staining with mithramycin (Hyman and Stallings, 1978). Of note, mutant 3 appears to be tetraploid, while mutants 1, 2 and 4 are diploid (see Table I).

DNA isolation and Southern blots

Genomic DNA was isolated from the parental and mutant cell lines using the citric acid procedure (Hieter *et al.*, 1981). For Southern blots, DNAs (8 μg) were digested with the indicated restriction enzymes (New England Biolabs) for 3 h and electrophoresed on 0.8% agarose gels. The DNA was transferred to nitrocellulose filters by the procedure of Southern (1975). The blots were hybridized to DNA fragments labeled with [α-^{32}P]dCTP by nick-translation to a specific activity of 200–400 c.p.m./pg. DNA fragments used as probes were isolated from plasmid subclones of genomic clones and were electrophoresed twice through low melting point agarose gels prior to extraction. Blot hybridization and wash conditions were as described (Sukhatme *et al.*, 1985). Filters were exposed overnight at −70°C to XAR-5 film with an intensitying screen.

Isolation of breakpoint genomic clones from mutant 4

DNA from mutant 4 was partially digested with *Mbo*I to yield a predominance of fragments in the range of 15–20 kb and then treated with calf intestinal alkaline phosphatase. DNA from the lambda phage vector EMBL3 (Frischauf *et al.*, 1983) was digested with *Bam*HI to yield arms and with *Eco*RI to inactivate the stuffer fragment. The phage DNA was then extracted and isopropanol precipitated. The genomic DNA partial digest was ligated to the *Bam*HI phage arms and the ligated DNA packaged *in vitro*. The resultant phage particles were plated on *Escherichia coli* strain KM392. 4×10^5 independent recombinant phage were obtained and screened without further amplification. The plaques were transferred to nitrocellulose filters according to Benton and Davis (1977). The filters were hybridized to a mixture of two nick-translated probes: a 5′ *Kpn*I-*Hind*III fragment and a 3′ *Sst*I-*Kpn*I fragment (probes A and B, respectively in Figure 1, panel D) from the β_2-microglobulin gene. Hybridizing plaques were further purified and re-tested separately with the 5′ and 3′ probes. Of the three positive plaques isolated, one hybridized to the 3′ probe (λB2bp3′) and two to the 5′ probe. Since the latter two were essentially identical in restriction maps, only one is discussed further (λB2bp5′). DNA was isolated from CsCl banded preparations of the 5′ and 3′ breakpoint clones.

DNA sequencing

The DNA sequence of the ~900 bp *Hind*III–*Hind*III segment of the C57B1/6 β_2-microglobulin gene was determined using the chemical degradation procedure of Maxam and Gilbert (1980) on multiple overlapping 5′ and 3′ end-labeled restriction fragments isolated from plasmid subclones. The DNA sequences of the breakpoint fragments in mutant 4 clones λB2bp5′ (*Kpn*I-*Eco*RI) and λB2bp3′ (*Hind*III-*Eco*RI) were determined by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) using M13 vectors mp 18 and mp 19 (Messing, 1983).

Northern blots

Total RNA was isolated from the parental R1.1 and mutant cell lines using the guanidine thiocyanate procedure of Chirgwin *et al.*, (1979), pelleting the RNA through a CsCl cushion. 10 μg of each RNA were electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde (Lehrach *et al.*, 1977; Goldberg, 1980). The RNAs were transferred to nitrocellulose according to Thomas (1980) and hybridized to ^{32}P-labeled nick-translated probes. Hybridization and wash conditions were as above for Southern blots (Sukhatme *et al.*, 1985). The filters were exposed to XAR-5 film overnight or up to 1 week at −70°C with an intensifying screen.

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