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19 February 1988; accepted 12 May 1988

Mitochondrial Transformation in Yeast by Bombardment with Microprojectiles

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The genetic transformation of mitochrondria and chloroplasts has been an intractable problem. The newly developed "biolistic" (biological ballistic) process was used to deliver DNA into yeast cells to stably transform their mitochondria. A nonreverting strain, which is respiratory deficient because of a deletion in the mitochondrial *oxi3* gene, was bombarded with tungsten microprojectiles coated with DNA bearing sequences that could correct the *oxi3* deletion. Respiratory-competent transformants were obtained in which the introduced *oxi3* DNA is integrated at the homologous site in the mitochondrial genome. Organelle genomes can now be manipulated by molecular genetic techniques in the same way as nuclear genomes.

THE ABILITY TO TRANSFORM CELLS with foreign DNA has proved to be an invaluable tool of molecular genetic analysis. However, many important questions in mitochondrial molecular biology remain unapproachable because of the lack of a transformation system. This inability has, for example, stymied systematic mutational studies of cis-acting sequences in the mitochondrial genome and of mitochondria-encoded proteins. Previous attempts at mitochondrial transformation, both published (*1*) and unpublished, have been unsuccessful, nonreproducible, or have lacked unambiguous molecular proof. One presumed difficulty has been in introducing DNA across the outer and inner mitochondrial membranes. The invention of the biolistic process by Sanford and co-workers (*2*) suggested a new approach to mitochondrial transformation. The procedure entails coating small (1-μm) tungsten projectiles with the nucleic acid of interest. These microprojectiles are accelerated in a special device to velocities sufficient to penetrate cells (*2*), as has recently been demonstrated by the introduction of biologically active DNA and RNA into various plant cells (*3*). We reasoned that this process might deliver DNA directly into the mitochondria by physically disrupting the mitochondrial membranes. We chose to test the applicability of this procedure to mitochondrial transformation by using the yeast *Saccharomyces cerevisiae*. Yeast are well suited for this effort since they do not require functional mitochondria for growth. In addition, the yeast mitochondrial genome is highly recombinogenic and has been extensively characterized at the genetic and molecular levels. Moreover, a wide variety of mitochondrial mutations are available [reviewed in (*4*)]. We developed a method for high-efficiency transformation of yeast nuclear genes by the biolistic process (*5*); our method proved to be a prerequisite for successful mitochondrial transformation.

Our approach to mitochondrial transformation was to attempt to correct a molecularly defined deletion in a mitochondrial gene with an identifiable foreign mitochondrial DNA (mtDNA) sequence. By using a mitochondrial lesion which makes the strain unable to respire (that is, unable to grow on a nonfermentable carbon source such as glycerol), we could select for restoration of respiratory competence. This approach allowed us to preclude any apparent transformants that might arise from contamination or any unforeseen suppression events. Use of a deletion strain also obviated any concern about interference from a defective protein. Co-transformation with a nuclear plasmid was used to increase the probability of recovering stable transformants. We reasoned that initially only one or a few of the approximately 50 mitochondrial chromosomes per yeast cell are likely to be corrected in the transformant. Therefore, by first selecting for a nuclear marker, we hoped to allow for the production of segregants with high copy numbers of transformed mitochondria prior to selection for restoration of mitochondrial function. Co-transformation also provided a measure of mitochondrial versus nuclear transformation frequencies.

We used a respiratory-deficient (mit^-) strain, 947/PA5, as the recipient in mitochondrial transformation. This strain harbors a 2547-bp deletion within the "long" form of the mitochondrial *oxi3* gene, which encodes the largest subunit of cytochrome oxidase (COXI) (Fig. 1) (*6, 7*). Briefly, the mitochondrial mutation in this strain was originally isolated as a spontneous suppressor of the respiratory-deficient *oxi3* splicing–defective mutant C1036. The strain C1036 does not express COXI because it contains a termination codon within the open reading frame (ORF) of the *oxi3* intron 1 (aI1) (Fig. 1). Like a number of other introns within the yeast mitochondrial genome, the aI1 ORF is in-frame with its upstream exon, and the fusion gene product is a trans-acting splicing factor (maturase) required for splicing of that intron (*8*). The mitochondrial lesion designated PA5 arose as a result of a crossover that joined the coding sequence from position 967 of aI1 to position 3514 of aI2 (*9*) (Fig. 1). This recombination

Table 1. Cytochrome oxidase activities (nanomoles of O_2 per minute per milligram of protein) in wild-type and transformed strains. The wild-type strain, (COP161, ρ^+), the mit^- recipient (947/PA5), and the six transformed strains were grown in medium containing 2% raffinose as the carbon source to a cell density of 1×10^7 to 3×10^7 cells per milliliter. The cells were pelleted and washed with distilled water and protein extracted as previously described (*21*). Cytochrome oxidase assays (*22*) and protein determination (*23*) were performed on the supernatant. Activities are expressed as means ± 2 SD and are based on three repetitions for each determination.

Strain	Cytochrome oxidase activity (nmol/min-mg)
COP161	331 ± 92
947/PA5	0
947/PA5-1	210 ± 6.5
947/PA5-3	210 ± 13
947/PA5-8	257 ± 17
947/PA5-9	269 ± 29
947/PA5-10	217 ± 24
947/PA5-13	204 ± 9.0

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eliminated both the C1036 mutation and exon 2 (E2), creating an in-frame fusion of the aI1 and aI2 coding sequences. Unlike C1036, the mit$^-$ strain 947/PA5 does not revert to glycerol growth. Because of the deletion, the *oxi3* gene of PA5 exhibits a unique, and thus diagnostic, restriction enzyme pattern.

The transforming DNA was derived from the mtDNA of a respiratory competent strain, AE1-B, which contains a mitochondrial genome harboring a functional but novel form of the *oxi3* gene lacking introns 1 and 2. A DNA fragment isolated from the mitochondrial genome of AE1-B, containing *oxi3* sequences from a Hpa II site 270 bp upstream from the ATG of *oxi3* E1 to a Hind III site located within I4, was cloned into the vector pEMBL-18 (*10*). The structure of this partially processed gene, in particular the presence of E2, was verified by subcloning a 1476-bp Rsa I fragment, extending from E2 to aI3, into the Sma I site of M13mp18 and sequencing from the Rsa I site in E1 across the spliced exons (*11*). This plasmid, designated pQAoxi3 (Fig. 1), was used for bombardment of strain 947/PA5.

Prior to bombardment of the cells, a 1:1 molar mixture of two uncut plasmids, YEp352 and pQAoxi3, was precipitated (*3*) onto the surface of the 1-μm tungsten microprojectiles. YEp352 is a multicopy plasmid bearing the wild-type nuclear gene *URA*3 (*12*). Details of the transformation of yeast nuclear genes by the biolistic process are described elsewhere (*5*). Briefly, strain 947/PA5 was grown to stationary phase in nonselective medium. The cells were then pelleted and washed, and 5×10^7 cells were spread on agar plates containing 2% glucose and 1*M* sorbitol (as osmoticum) and lacking uracil; strain 947/PA5 contains a lesion in the nuclear *ura*3 gene that makes it unable to grow on this medium.

These plates were then bombarded with the DNA-coated microprojectiles in a near vacuum by use of a biolistic device similar to the one described earlier (*3*). The plates were incubated at 30°C after bombardment. Two days later 200 to 1200 Ura$^+$ nuclear transformants appeared on each plate. These were replica-plated to medium containing glycerol as the sole carbon source. From approximately 6300 Ura$^+$ nuclear transformants, six were able to grow on glycerol medium (Gly$^+$). No Gly$^+$ colonies were obtained from roughly 3600 Ura$^+$ colonies produced from bombardments with the YEp352 plasmid alone.

The six putative mitochondrial transformants were extensively characterized. Cytochrome oxidase activities were determined for each (Table 1). Although no cytochrome oxidase activity was detectable in the recipient strain, each transformant had activity comparable to wild-type levels. The slightly lower cytochrome oxidase levels in the transformants relative to the wild-type control are likely due to strain differences.

A genetic analysis of each Ura$^+$ Gly$^+$ transformant indicated that the acquisition of the Gly$^+$ phenotype was due to the transformation of the mitochondria. First, loss of the YEp352 vector had no effect on the respiratory competence of the transformed strains. Second, when each transformant was crossed to a ρ° strain (which lacks mtDNA) and sporulated, the segregation pattern of the respiratory competent phenotype was 4:0 (ratio of Gly$^+$ to Gly$^-$) in contrast to the nuclear markers, which segregated 2:2.

To provide molecular evidence for mitochondrial transformation, purified mtDNA from two Gly$^+$ transformants, 947/PA5-9 and 947/PA5-10, was analyzed with diagnostic restriction enzymes. A comparison of the ethidium bromide–stained fragments generated by digestion of the mtDNAs from the wild-type strain COP161, from 947/PA5, and from the two transformants with Hha I and Bgl II (Fig. 2A) shows identical patterns, with the exception of the expected strain-specific fragments (see below); thus, there is no indication that plasmid sequences have integrated into the mitochondrial genome. A Southern hybridization analysis (Fig. 2B) of this Hha I plus Bgl II digest, using an *oxi3* probe, shows that mtDNA from the parental wild-type strain COP161 (Fig. 2B, lane 2) generates four *oxi3* DNA fragments: a 6.7-kb Hha I fragment (extending from the 15*S* rRNA gene to *oxi3* I1), a 3.6-kb Bgl II fragment (extending from I2 to E5), a 2.5-kb Hha I fragment (extending from aI1 to aI2), and a 1.9-kb Hha I–Bgl II fragment that is internal to aI2 (see Fig. 1). The same digest of mtDNA from the recipient strain 947/PA5 yields the parental 6.7-kb Hha I and the 3.6-kb Bgl II fragments, but since the mit$^-$ deletion removed the aI2 Hha I site (see Fig. 1), both the 2.5- and 1.9-kb fragments have been replaced with a 2.0-kb deletion-specific fragment (Fig. 2B, lane 3). An *oxi3* gene containing the E1-E2-E3 fusion sequence will lack all three sites, and thus a Hha I plus Bgl II double digest of this mtDNA will generate just a 9.8-kb fragment, which extends from the 15*S* rRNA gene to E5 (Fig. 1). This is exactly what is observed for transformants 947/PA5-9 and 947/PA5-10 (Fig. 2B, lanes 4 and 5). As a control, mtDNA from a Gly$^-$ Ura$^+$ transformant was also examined (Fig. 2B, lane 6). The pattern of this DNA is identical to that of the recipient 947/PA5.

Further demonstration that the Gly$^+$ transformants have acquired the anticipated novel *oxi3* gene was obtained from a Bam HI plus Xba I digest. The *oxi3* gene in

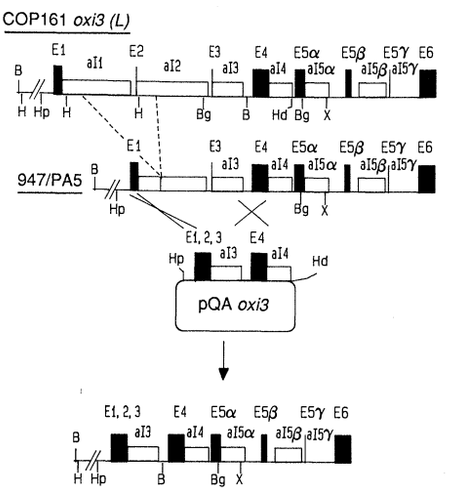

Fig. 1. Different structures of the mitochondrial *oxi3* gene of yeast and the 5′ half of a novel form of the *oxi3* gene derived from strain AE1-B and cloned in pEMBL-18. (i) The long form of the *oxi3* gene of the wild-type strain COP161 contains six exons, E1 to E6, indicated by the solid boxes, and seven introns I1 to I4 and I5α to I5γ (*6–8*), five of which have open reading frames (open boxes) in frame with the upstream exons; noncoding sequences are represented by the horizontal lines. Relevant restriction sites in this study are: B, Bam HI; Bg, Bgl II; H, Hha I; Hd, Hind III; Hp, Hpa II; and X, Xho I. All of these sites are shown for COP161 *oxi3*, but some have been omitted below for clarity. (ii) Strain 947/PA5, a mit$^-$ derivitive of COP161, was used as the recipient strain for mitochondrial transformation. This strain is a nonreverting respiratory-deficient strain containing a deletion within the *oxi3* gene, schematically shown by the dotted lines. The deletion in 947/PA5 extends from position 967 of aI1 to position 3514 of aI2 and was determined by sequencing upstream from an Eco RI site in aI2, located at position 3855 [numbered as in (*6*)]. (iii) The recombinant plasmid pQAoxi3 contains a 3200-bp Hpa II–Hind III *oxi3* fragment from the mit$^+$ strain AE1-B. The *oxi3* gene of AE1-B is a partially processed form of the long form of *oxi3*, lacking aI1 and aI2. The mtDNA containing the *oxi3* gene in pQAoxi3 was obtained by isolating mtDNA from AE1-B and purifying it on CsCl-bisbenzimide gradients as described in Fig. 2. Dialyzed AE1-B mtDNA was digested with Hpa II, and the largest mtDNA Hpa II fragment was isolated, cut back with Hind III, and subcloned into the Acc I and Hind III sites of pEMBL-18 (*10*). (iv) Potential points of recombination between recipient strain 947/PA5 and the transforming recombinant plasmid pEMBLoxi3 are shown as solid crosses. This type of double-crossover event would replace the PA5oxi3 sequence with that on pQAoxi3. The area of identity between the PA5oxi3 sequences and the pQAoxi3 plasmid extends for about 440 bp upstream of the 5′ splice junction of the aI1/aI2 hybrid intron and 2975 bp downstream of its 3′ splice junction, including sequences from E3 into aI4.

COP161 and 947/PA5 contains a single Bam HI site located at the end of aI3 (Fig. 1). Digestion of COP161 mtDNA with Bam HI generates a 14-kb fragment that contains the upstream half of *oxi3*, intergenic sequences, and the downstream half of the 15*S* rRNA gene (Fig. 2C, lane 1). Because of the deletion in the *oxi3* gene of 947/PA5, that fragment is shortened to 11.5 kb (Fig. 1 and lane 2 of Fig. 2C). Replacement of the *oxi3* sequences in 947/PA5 by homologous recombination with *oxi3* sequences in pQAoxi3 would result in a new 9-kb Bam HI fragment, since both aI1 and aI2 would be deleted, having been replaced with the E1-E2-E3 fusion. Inspection of the Southern blot in Fig. 2C, lanes 3 and 4, shows that the mtDNA of both 947/PA5-9 and 947/PA5-10 have acquired the 9-kb Bam HI fragment. From these data we conclude that the mitochondrial genomes of the Gly^+ transformants result from the replacement of the resident *oxi3* gene with the mitochondrial sequences on the pQAoxi3 plasmid.

By the criteria outlined above, it is clear that the mitochondria of strain 947/PA5 have been stably transformed with exogenous mtDNA sequences. Repetitions of this experimental protocol have produced one mitochondrial transformant per 1000 to 2000 nuclear transformants. Efforts to select directly for cells bearing the mitochondrial transformants, bypassing the need for cotransformation, have not been successful to date. This negative result can be explained if initially, after transformation, only one or a few mitochondrial chromosomes contain a functional *oxi3* gene. In this case the levels of cytochrome oxidase activity would probably be insufficient to support glycerol growth until corrected genomes segregated and were amplified. We have found that by including an *ade1* nuclear lesion in the recipient strain, we can visually screen the nuclear transformants growing on glucose medium for mitochondrial transformants, since an *ade1* mit^+ strain is red, whereas an *ade1* mit^- strain is white. This facilitates the screening process by eliminating the need for replica plating.

There are two striking features of the transformation process we describe. The first is that it works, despite the fact that the mean diameter of the microprojectiles (1 μm) is approximately the same as the cross-sectional diameter of the mitochondria in yeast (*13*). Preliminary efforts at separating "hit" from "not-hit" yeast cells after bombardment look promising and may provide a method for gaining some insight into the interaction between the microprojectiles and cells. The second is that all the transformants extensively analyzed to date (six) have occurred by homologous replacement of the *oxi3* gene, without any indication of integration of other plasmid sequences. This is also the predominant form of transformation in cyanobacteria (*14*) and in the chloroplasts of *Chlamydomonas* (*15*).

In the transformants analyzed, restoration occurred by homologous recombination of the transforming DNA into the mitochondrial chromosome. This strategy should prove useful as a general method for mitochondrial transformation in yeast. Situations will undoubtedly arise, however, in which homologous integration of the DNA sequences under examination will not restore mitochondrial function or is not desired. Fortunately, it is now well established that complementing, but otherwise defective, mitochondrial genomes can coexist as heteroplasms in the same cell (*16*). Therefore, it should be possible to introduce DNAs of interest into yeast cells and maintain them through selection by their complementation in trans of some particular defect in the mitochondrial genome. Furthermore, such a mitochondrial defect need not be related to the sequences or region of the genome under study as long as the complementing sequences are in vectors that can replicate autonomously. For this latter purpose, it may not even be necessary to have an authentic mitochondrial origin of replication sequence (*17*) in cis, since there does not appear to be a stringent requirement for these sequences for the maintenance of mtDNAs in the cell. Such vectors would be maintained as long as the appropriate selection pressure—for example, glycerol prototrophy—is applied. It may also be possible to engineer useful autonomously replicating mitochondrial plasmids in *Saccharomyces cerevisiae* similar to those occurring naturally in other fungi [reviewed in (*18*)] and plants [reviewed in (*19*)].

Recently it has been demonstrated that mitochondrial genes can be modified to be expressed in the nucleus, translated in the cytosol, and the products directed to the mitochondria (*20*). Although this approach requires laborious recoding of the mitochondrial gene, it may be useful for directed mutational studies of some mitochondrial

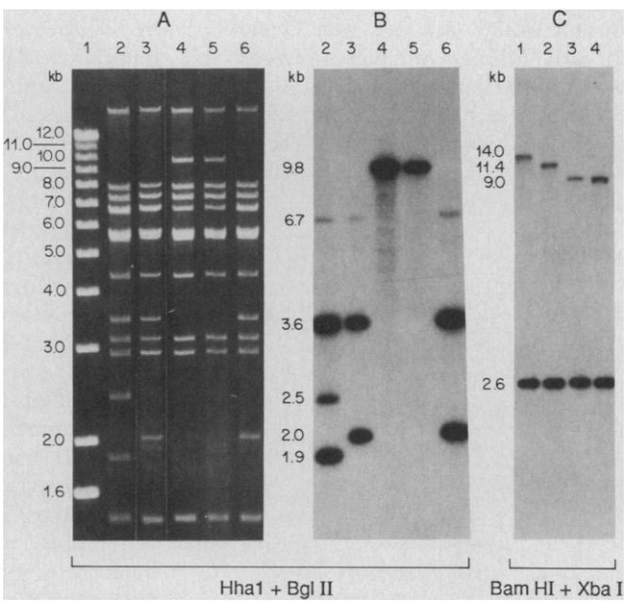

Fig. 2. Physical analysis of the mitochondrial DNA (mtDNA) from the transformant and control strains. (**A**) An ethidium bromide–stained pattern of mtDNA digested with the restriction enzymes Hha I plus Bgl II. (Lane 1) 1-kb markers (BRL); (lane 2) mtDNA from wild-type parental strain COP161; (lane 3) mtDNA from the recipient, mit^- deletion strain 947/PA5; (lanes 4 and 5) mtDNA from two Gly^+ transformants, 947/PA5-9 and 947/PA5-10, respectively; (lane 6) mtDNA from a Gly^- Ura^+ (nuclear) transformant from the actual transformation. Overall patterns of the mtDNAs are similar except for the differences in strain-specific Hha I and Bgl II fragments (see text). Mitochondrial DNA was isolated as described by Hudspeth *et al.* (*24*). The digested DNAs were separated on a 0.7% agarose gel. (**B**) Southern analysis of a Hha I plus Bgl II restriction digest of mtDNA from transformants and control strains. The probe used for this analysis contains the first half of the *oxi3* gene from the Hpa II site 5′ to *oxi3* to the Hind III site of aI4 (Fig. 1); this DNA fragment was used to show all strain-specific fragments. (Lane 2) Mitochondrial DNA from wild-type parental strain COP161; (lane 3) mtDNA from transformation recipient 947/PA5; (lanes 4 and 5) mtDNA from transformants 947/PA5-9 and 947/PA5-10, respectively; (lane 6) mtDNA from a Gly^- Ura^+ isolate from the same transformation. See text for details. The probe was labeled and Southern analysis was carried out by standard procedures. Hybridization analyses of total yeast DNA with *oxi3* sequences gave no indications of pQAoxi3 recombinant specific fragments in the Gly^+ transformants. (**C**) Southern analysis of a Bam HI-plus-Xba I restriction digest of mtDNA from transformants and control strains. The probe was the same as used in (B). (Lane 1) Mitochondrial DNA from COP161, the wild-type parent; (lane 2) mtDNA from transformation recipient 947/PA5; (lanes 3 and 4) mtDNA from transformants 947/PA5-9 and 947/PA5-10. See text for details of strain-specific Bam HI fragments. The restriction enzyme Xba I was included in this analysis to generate a 2.6-kb parental Bam HI (*oxi3*aI3)–Xba I (*oxi3*aI5α) restriction fragment in order to show that there has been no rearrangement of downstream *oxi3* sequences. This fragment extends past the region of homology contained within pQAoxi3.

proteins. Mitochondrial transformation, however, should provide a generally applicable technique for mutational studies of mitochondria-encoded proteins as well as permit many studies of the function and evolution of mtDNA sequences that could not be done with the techniques previously available.

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25. We thank D. Berthold for technical assistance and A. Brock for manuscript preparation. This research was supported by NSF grant DCB-8502626 and a North Carolina Biotechnology Center grant (to S.A.J.), by PHS grant GM 22525, and a grant from the Robert A. Welch Foundation (to R.A.B.) and a grant from AMOCO (to J.C.S.). Biolistics supplied the biolistic apparatus.

19 February 1988; accepted 12 May 1988

Extensive Junctional Diversity of Rearranged Human T Cell Receptor δ Genes

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The human T cell receptor δ (TCR δ) gene encodes one component of the TCR γδ-CD3 complex found on subsets of peripheral blood and thymic T cells. Human TCR δ diversity was estimated by characterizing rearrangements in TCR γδ cell lines and determining the structures of complementary DNA clones representing functional and nonfunctional transcripts in these cell lines. One Vδ segment and one Jδ segment were identified in all functional transcripts, although a distinct Jδ segment was identified in a truncated transcript. Further, one Dδ element was identified, and evidence for the use of an additional Dδ element was obtained. Thus human TCR δ genes appear to use a limited number of germline elements. However, the apparent use of two Dδ elements in tandem coupled with imprecise joining and extensive incorporation of N nucleotides generates unprecedented variability in the junctional region.

TWO DISTINCT CD3-ASSOCIATED T cell receptor (TCR) structures have been identified on the surface of mature, peripheral blood T lymphocytes. The first receptor structure, TCR αβ (*1*), is expressed on most peripheral blood T lymphocytes. These cells, which are typically either $CD4^+CD8^-$ or $CD4^-CD8^+$, are involved in antigen-specific, major histocompatibility complex–restricted recognition and display either helper or cytotoxic activity (*2*). The second receptor structure, TCR γδ (*3, 4*), is expressed on a much smaller subset of peripheral T lymphocytes (0.5% to 12%), as well as on thymocytes and other cells (*5–8*). These cells are either $CD4^-CD8^-$ or $CD4^-CD8^+$ and have cytotoxic activity, but the nature of the antigens and restricting elements recognized by these lymphocytes is unknown.

All four receptor chains are encoded by immunoglobulin-like gene segments that rearrange to form a functional gene during T cell differentiation (*9–14*). The human TCR α locus is composed of approximately 50 variable (V) segments, 50 joining (J) segments, and a single constant (C) segment, whereas the human TCR β locus is composed of approximately 70 V segments, two diversity (D) segments, 13 J segments, and two C segments (*15*). Germline diversity, combinatorial and imprecise V-(D)-J joining, the insertion of template-independent N-region junctional nucleotides, and combinatorial association of α and β polypeptides generate an extensive TCR αβ repertoire (*15, 16*).

Characterization of the human TCR γ locus (*17, 18*) and the isolation of cDNA clones encoding human TCR δ (*11–13*) allow a similar assessment of the human TCR γδ repertoire. In contrast to the TCR α and β loci, the human TCR γ locus is composed of only six or seven functional V segments, five J segments, and two C segments (*17, 18*). Furthermore, the two most commonly used J segments are identical in amino acid sequence. Thus germline diversity is limited, and imprecise joining and N nucleotides play important roles in generating variability in TCR γ (*17*).

In order to investigate the variability of TCR δ, we obtained and analyzed cDNA clones representing seven distinct TCR δ transcripts from four TCR γδ cell lines. These clones display use of a single TCR δ V segment and a predominant TCR δ J segment, suggesting limited germline diversity. However they show unprecedented variability in the V-J junctional region.

We used a V-specific probe derived from the IDP2 TCR δ cDNA clone 0-240/47 (*11*) to examine TCR δ rearrangements in Xba I digests of genomic DNA from five different TCR γδ cell lines (Fig. 1A). This probe detects a single germline fragment of 7.5 kb that is rearranged to a 6.2-kb fragment on one chromosome in Molt-13 (*12, 19*), IDP2 (*3*), PEER (*5–7*), PBL L1 (*6*) (Fig. 1A) and PBL C1 (*6*). We also subcloned a genomic fragment encoding the Jδ segment present in the IDP2 TCR δ cDNA clone (Jδ1) (*20*). This probe detects a single 1.7-kb germline fragment that is rearranged on both chromosomes in all TCR γδ cell lines examined (Fig. 1B). One rearranged band, of 6.2 kb, corresponds exactly to the rearranged fragment detected by the V-specific probe, indicating the rearrangement

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