As our goal was to use bacterial β-galactosidase activity as an indicator of the phagolysosomal calcium concentration, it was important to distinguish between the β-galactosidase activity of the macrophage and that of *Y. pestis*; we therefore examined the *p*H optima for expression of macrophage and mutant *Y. pestis* β-galactosidase activity[7] (Fig. 3). β-Galactosidase from disrupted macrophages was almost undetectable at *p*H 8.0, but fully active at *p*H 4.0. In contrast, β-galactosidase produced by the mutant *Y. pestis* grown in a low-calcium (1.0 μM) environment was highly active at *p*H 8.0; at *p*H 4.0, less than 10% of the microbial β-galactosidase activity was detected.

Figure 4 compares β-galactosidase activity of extracellular mutant *Y. pestis* with that of organisms contained within human macrophages. In media containing 1 mM calcium, extracellular *Y. pestis* which had never been exposed to the macrophages produced little β-galactosidase. Similarly, extracellular *Y. pestis* recovered from the supernatant of dishes containing infected macrophages also produced little β-galactosidase. In contrast, the mutant *Y. pestis* contained within the human macrophages produced large quantities of β-galactosidase.

These studies, in which we used a genetically engineered intracellular pathogen to elucidate the environmental conditions in which it grows and multiplies *in vivo*, demonstrate that the intraphagolysosomal calcium concentration surrounding *Y. pestis* is sufficiently low to promote expression of calcium-sensitive virulence genes. We hypothesize that those genes encoding V antigen and outer membrane proteins are expressed during intraphagolysosomal growth of this pathogen; this was not expected, because it has been assumed that the *Y. pestis*-containing phagolysosome contains millimolar levels of free Ca^{2+} (ref. 1). Based on our data (Fig. 2), we predict that the intraphagolysosomal calcium concentration is <100 μM. This is a surprising result in view of recent descriptions of ATP-dependent Ca^{2+} translocating pumps located in the plasma membrane of macrophages[8] and neutrophils[9] as well as a similar Ca^{2+} uptake pump in neutrophil lysosomes[10]. If the portion of the phagocyte plasma membrane which formed the phagocytic vacuole contained Ca^{2+} pumping activity and fused with lysosomes containing a similar Ca^{2+} pump, both of these ion translocating pumps would tend to raise the calcium concentration within this compartment. The consequences to intracellular *Y. pestis* would be the inability to express certain virulence genes and the organism would then be eradicated. It remains to be determined whether the calcium concentration is generally low in this intracellular compartment or whether *Y. pestis*, like *Toxoplasma gondii*[11], can modify its ambient growth environment to its own advantage.

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Diversity of murine gamma genes and expression in fetal and adult T lymphocytes

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The search for the genes encoding the T-cell receptor α and β chains revealed a third gene, Tγ (ref. 1), which shares with the Tα (refs 2–7) and Tβ (refs 8–15) genes a number of structural features, including somatic rearrangement during T-cell development. Tγ gene expression appears to be unnecessary in some mature T cells[16,17] and is at its greatest in fetal thymocytes[18,19], encouraging speculation that Tγ has a role in T-cell development and may be involved in the recognition of polymorphic major histocompatibility complex (MHC) products during thymic education[20,21]. One argument against the participation of Tγ in such a process has been its apparently limited diversity, due to the small number of gene segments available for rearrangement[1,22]. We here describe the identification of additional Tγ V-gene segments and demonstrate that they can be rearranged to previously identified J- and C-gene segments and are expressed in fetal thymocytes. In addition we describe a variety of patterns of Tγ mRNA processing which may be significant for Tγ gene regulation.

Previous studies[1,22] identified three cross-hybridizing mouse V_γ-gene segments, V_1, V_2 and V_3, and three sets of cross-hybridizing J_γ- and C_γ-gene segments, J_1-C_1, J_2-C_2 and J_3-C_3 (see ref. 23 for nomenclature). Recently Iwamoto and co-workers[24] have reported a fourth set of *J* and *C* segments, J_4-C_4, which does not cross-hybridize with the other three (see Fig. 3). Although none of these germline gene segments have (with possible exception of C_3) obvious structural defects only the V_2-J_2-C_2 combination has been found as a complete rearranged gene[1,16]. However, the existence of additional germline V_γ-gene segments, which do not cross-hybridize to the first three *V*-gene segments and are also rearranged in T cells, is suggested by two observations. First, many T-cell clones and splenic and peripheral T cells carry, in addition to a joined V_2-J_2-C_2 gene, an *Eco*RI DNA fragment containing a rearranged J_1-C_1 segment[1,16,17,22]. Second, fetal thymocytes seem to contain more transcripts hybridizing to a C_γ-probe which detects C_1, C_2 and C_3 sequences than to a V_γ-probe which detects V_1, V_2 and V_3 sequences[18].

To confirm this suspicion we constructed a cDNA library from fetal thymocytes and isolated cDNA clones which hybridize to a C_γ-probe but not to a V_γ-probe. Sequence analysis of one of these clones (FT2) revealed a new V_γ-gene segment (V_4) which shares 50% amino acid sequence homology with V_2 and is rearranged to the J_1-C_1 gene segment (Fig. 1). As is often the case with Tγ-gene transcripts[1,16,23,25] the V_4-J_1 joint in FT2 results in a frame shift such that a translation product would terminate in the *J* region. Another clone, FT6, contains part of the sequence of another V_γ-gene segment (V_5) which is also rearranged to J_1-C_1 (Fig. 1). Although the complete sequence of V_5 was not contained in this clone, sufficient sequence was obtained to distinguish it from other *V*-gene segments. The V_5-J_1 joint in FT6 would also result in premature termination of translation. Nevertheless these new V_γ-gene segments indicate the potential of additional variability in the γ-gene family.

Figure 2 shows the result of a Southern blot analysis[26] in which DNA from a variety of T-cell clones and a 17-day-old fetal thymocyte population was analysed using C_2-, V_2-, V_4- and V_5-probes in panels *a*, *b*, *c* and *d*, respectively. The C_2-probe detects C_1, C_2 and C_3; the V_2-probe V_1, V_2 and V_3. The characteristic pattern of double rearrangement with C_2 and

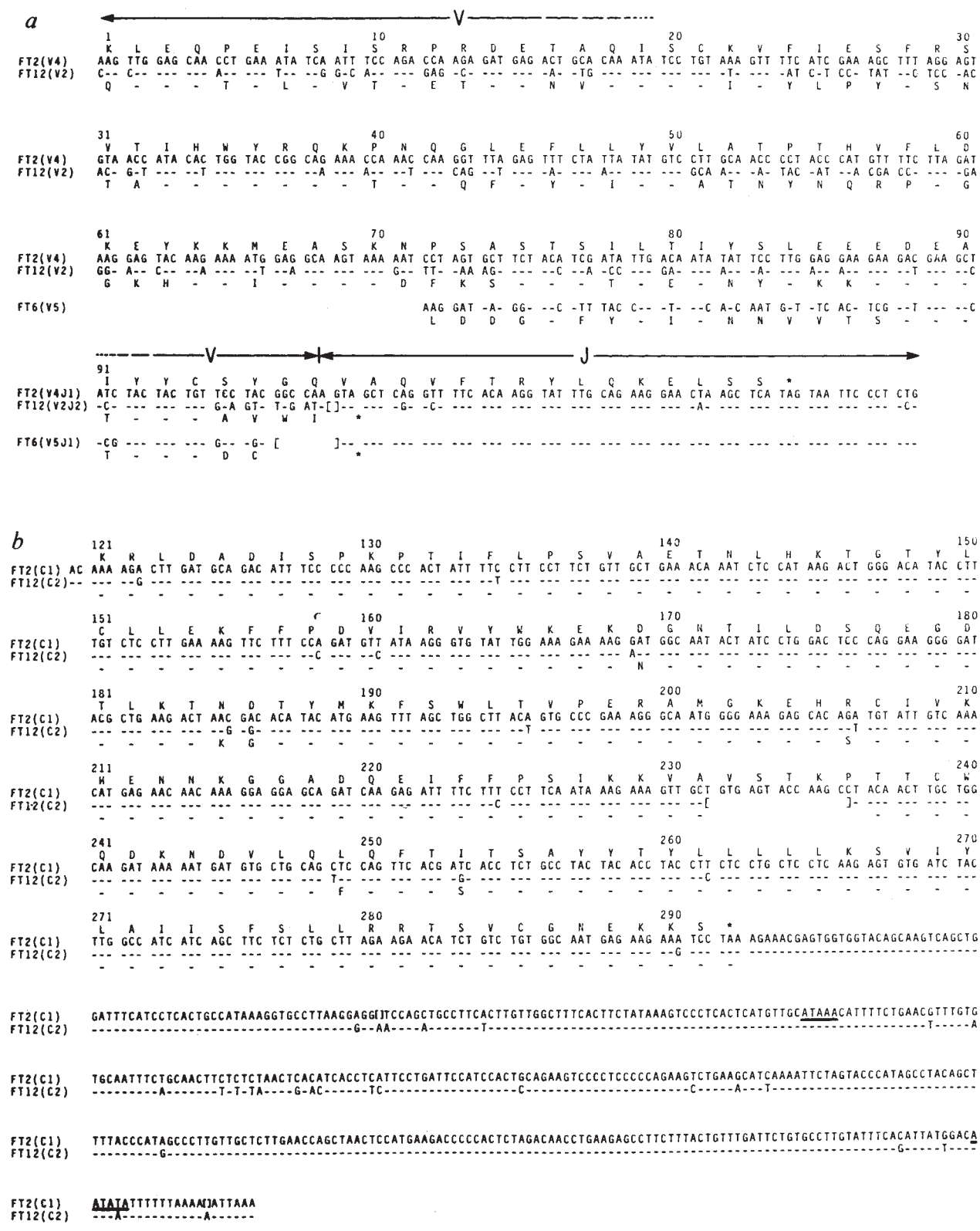

Fig. 1 The nucleotide sequences of Tγ-cDNA clones. *a*, the *V*- and *J*-region sequences of three cDNA clones. FT2 (V_4-J_1-C_1), FT12 (V_2-J_2-C_2) and FT6 (V_5-J_1-C_1). *b*, C_1 sequence, determined by analysing clone FT2, compared with C_2 sequence from FT12. The predicted amino acid sequences are indicated by the single-letter code. Dashes indicate sequence identity to that of FT2; parentheses enclose gaps necessary to align the sequences. The underlined regions in *b* indicate polyadenylation signals.

Methods. Pregnant BALB/cByJ mice at day 17 of gestation were killed and the fetuses removed. Thymuses of 82 fetuses were pooled and disrupted by gentle homogenization in a ground-glass tissue grinder. The thymocytes were collected and washed twice in cold phosphate buffered saline (PBS) and RNA was prepared by the guanidinium/CsCl method[34]. Poly(A)$^+$ RNA was isolated[35] and used as a template for cDNA synthesis[36] and the resulting double-stranded DNA was cloned into λgt10 by standard procedures[37]. All sequences were determined by the method of Maxam and Gilbert[38].

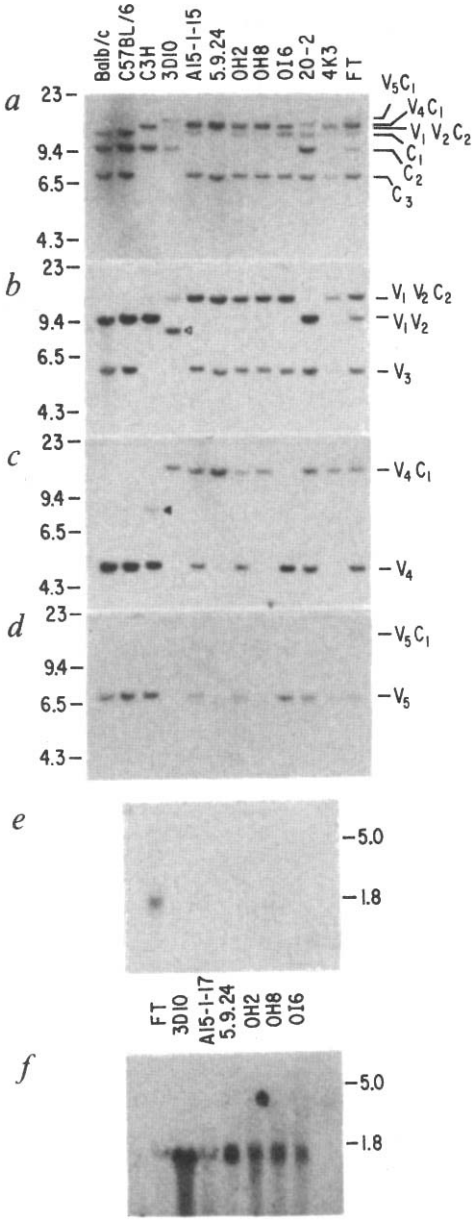

Fig. 2 Southern[26] and Northern[39] blot analyses of T-cell lines and fetal thymocytes. Panels *a–d* show Southern blot data while panels *e* and *f* show Northern blot data. In the Southern blots, BALB/c, C57BL/6 and C3H indicate kidney DNA of the designated strains; FT, DNA or RNA from 17-day-old BALB/c fetal thymocytes; 3D10, a KLH-specific suppressor T-cell line from C3H[40]; A15-1-17, an alloreactive anti-I-A^k cytolytic T-cell line from A.TH[41]; 5-9-24, an I-A^d- ovalbumin-specific cytolytic/helper T-cell line from BALB/c (gift of B. Jones and C. Janeway); OH-2 and OH-8, D^b- H-Y-specific cytolytic T-cell lines from C57B/6 (gift of O. Kanagawa); OI-6, an I-A^b H-Y- specific helper T-cell line from C57BL/6 (gift of O. Kanagawa); 20.2, an anti-H-2^b alloreactive helper T-cells line from BALB/c[16]; 4K3, an anti-L^d alloreactive cytolytic T-cell line from BALB.K[23]. The unrearranged and rearranged gene segments are identified on the right hand side of panels *a–d*. The arrow in *b* indicates a V_2-cross-hybridizing band rearranged to unidentified sequence; probably an example of V_1-J_4-C_4 rearrangement (see Fig. 3 and ref. 24). The arrow in *c* indicates a V_4 cross-hybridizing band which may indicate partial digestion of the C3H kidney DNA.

Methods. In the Southern blot analysis, DNA samples were digested with *Eco*RI. Approximately 2 μg of each sample was electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose and hybridized by standard procedures[42]. For the Northern blot analysis, RNA was prepared by the guanidinium/CsCl method[34] and approximately 12 μg of each sample was electrophoresed, transferred to nitrocellulose and hybridized as previously described[17,39,42]. Probes used are: (*a*), the DNA encoding nearly the entire C_2 region depicted in Fig. 1*b* (detects C_1, C_2 and C_3); *b*, *f*, the region encoding the first 100 amino acids of V_2 (Fig. 1*a*) (detects V_1, V_2 and V_3); *c*, *e*, the region encoding the first 78 amino acids of V_4 (Fig. 1*a*); *d*, V_5 as described below. C_2, V_2 and V_4 probes were labelled by nick translation[43]. Filters hybridized with these probes were washed in 0.4×SSC/0.1% SDS at 65 °C. The V_5 probe was prepared and used as follows. A synthetic oligonucleotide corresponding to the 76 nucleotides of V_5 (Fig. 1*a*) was prepared and purified as described[44]. A sesquidecamer complementary to the 3′ end of this sequence (5′ GCAGGCACAGTAGTA 3′) was also synthesized. The probe was radiolabelled by a modification of the primer extension method[45]. Hybridization was carried out at 55 °C. Filters were washed in 2×SSC/0.1% SDS at 55 °C and exposed to film.

C_1 previously reported[1,16] can be seen in most of these cells. In 20-2 however only C_1 rearrangement has occurred and in OI-6 only C_2 rearrangement. In cells containing a rearranged C_1 segment, the V_4 segment always rearranged to the same *Eco*RI fragment (Fig. 2*a*, *c*). The same correlation applies to C_2- and V_2-segments (Fig. 2*a*, *b*). By contrast, despite its rearrangement to the J_1-C_1 segment in the 17-day-old thymocytes, as evidenced by the Southern blots (Fig. 2*a*, *d*) and the isolation of the FT6 cDNA clone (Fig. 1), V_5 is not rearranged in any of the T-cell clones studied. Instead, V_5 is present only in those T-cell clones that retain at least one copy of unrearranged V_4- and C_1-segments, suggesting that rearrangement of V_4 to J_1-C_1 results in deletion of V_5. When these DNA samples are hybridized with a probe detecting a part of the C_4 gene, rearrangement is seen only in 3D10 DNA (data not shown, but apparently the same band as the one indicated by the arrow in Fig. 2*b*) which suggests that the $V_1J_4C_4$ rearrangement reported by Iwamoto and coworkers[24] is not very frequent among T-cell clones. The most probable organization of the various γ-gene segments, on the basis of all available data, is shown in Fig. 3.

The level of RNA containing V_2- or V_4-sequences was examined in 17-day-old fetal thymocytes as well as in a variety of T-cell clones (Fig. 2*e*, *f*). All cells examined contained V_2 RNA. By contrast only fetal thymocytes contained V_4 RNA. Further analysis by cDNA cloning and sequencing revealed that most of the γ RNA in 17-day-old fetal thymocytes, whether of the V_2-J_2-C_2, V_4-J_1-C_1 or V_5-J_1-C_1 type, is apparently nonfunctional because of out-of-frame *V–J* junctions. Of eight cDNA clones sequenced which contain a rearranged V_2-, V_4- or V_5-segment, all had out-of-frame *V–J* junctions (see Fig. 1*a*, clones FT2, FT12 and FT6). Furthermore, many of these cDNA clones use splice sites other than those used in generating normal γ mRNA. Thus, as shown in Fig. 4, clone FT11 uses a splice acceptor in what is normally the V_2 coding region, clone FT6 employs a splice acceptor in what is normally the J_1-C_1 intron and clone FT5 uses the same acceptor and a splice donor also in the J_1-C_1 intron. In addition, clone FT10 presumably resulted from transcription initiating upstream of an unrearranged J_2-gene segment and clone FT13 uses an alternate polyadenylation site.

This study has added two V_γ- gene segments, V_4 and V_5, to the previously recognized sets of three *V*- and four *J–C*-gene segments. We now have evidence for rearrangement and expression at the RNA level of at least three types of Tγ genes,

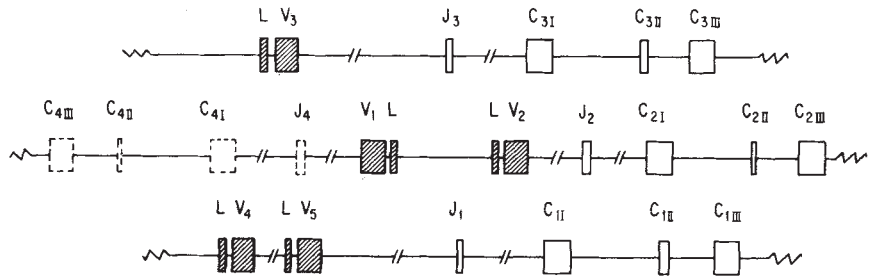

Fig. 3 The arrangement of the γ genes, inferred from all available data. The orientation of the three gene clusters with respect to one another has not yet been determined. The broken line indicates the J_4-C_4 cluster described elsewhere[24].

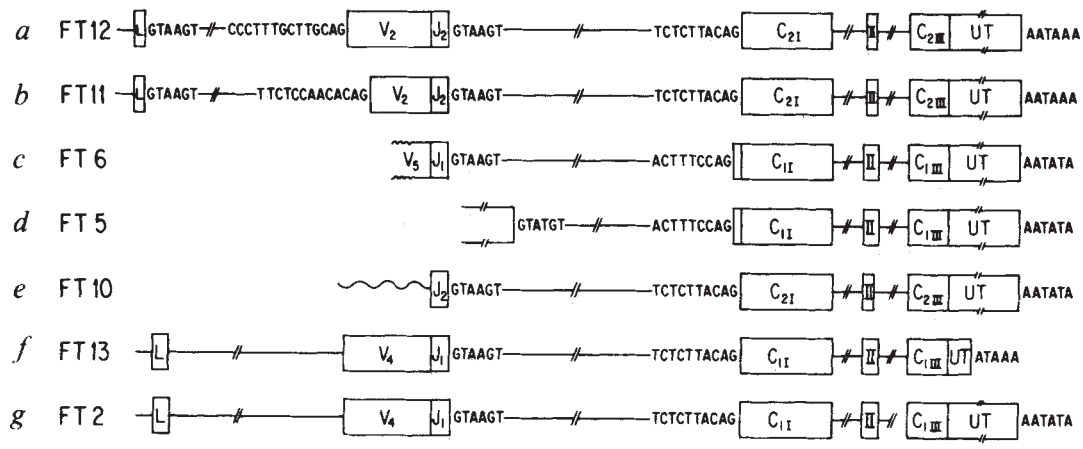

Fig. 4 Alternate splice patterns of γ-gene transcripts. Several types of mRNA generated by use of different splice signals are shown schematically. The boxed regions indicate exons and are labelled when the identity is known. The leader region is indicated by L; the first, second and third exons of the C regions by I, II and III respectively; the 3′ untranslated regions by UT. Straight lines represent introns and are flanked by the nucleotide sequences of the splice signals. Only the sequences of relevant splice signals are indicated. In clone FT10 the wavy line indicates chromosomal sequence 5′ of J_2. The nucleotide sequence to the right of the UT region is the polyadenylation signal[46] presumed to be used by that clone. The normal splice patterns are depicted by clones FT12 and FT2. In clone FT6 the novel splice acceptor is 13 nucleotides upstream of the C_{1I} exon. In clone FT5 a splice donor in the J_1C_1 intron 248 nucleotides 5′ of C_{1I} is used, as the acceptor described for clone FT6. The polyadenylation signals are underlined in Fig. 1*b*. We sequenced ten cDNA clones, two examples each of types *a*, *b* and *g* and one each of *c*, *d*, *e* and *f*. In each of the eight cDNA clones containing a *V-J* junction (FT5 and FT10 do not) the sequence at the *V-J* junction was different and resulted in frame shift. Splice signals were deduced by comparison to the consensus sequences suggested in ref. 47.

V_2-J_2-C_2, V_4-J_1-C_1 and V_5-J_1-C_1. Within each type there exists sequence variability in the V-J junction (this study and refs 16, 17, 23), indicating that the structural diversity of the Tγ-gene products is not as limited as previously thought. Additional Tγ-gene segments may exist which do not hybridize with the available γ probes. In humans, at least two C_γ- and six V_γ-gene segments have been identified[27,28]. The expanded Tγ-gene diversity and the structural similarity of this gene to the T_α and T_β genes support the idea that the role of the Tγ gene is in the recognition of polymorphic determinants on target cells. RNA transcripts detected by a C_2 or V_2 probe accumulate in the fetal thymocyte population, which is relatively rich in immature T cells, and are barely detectable in the resting T-cell population obtained from adult lymph nodes[18,19], suggesting that the $V_2J_2C_2$ gene product is involved in the early development of T cells, perhaps in the interaction with the polymophic MHC determinants presented to immature T cells by thymic epithelial cells[29,30]. The present finding that V_4-J_1-C_1 and V_5-J_1-C_1 transcripts are present in fetal thymocytes but not in a variety of T-cell clones supports this hypothesis. However, the fourth Tγ gene, V_1-J_4-C_4, does not appear to be used abundantly in 17 day-old fetal thymocytes preferentially because it is not represented in our cDNA library, as shown by the fact that all clones hybridizing to a V_2- probe also hybridize to a C_1 probe (V_2 and V_1 cross hybridize).

The present study shows that most of the Tγ RNA present in 17-day-old fetal thymocytes is defective due to out-of-frame *V-J* joining and abnormal splicing. Examples of out-of-frame joining and abnormal splicing of T-cell-receptor genes have been reported[4,17,25]. However, the strong bias in favour of presumably nonfunctional mRNA for Tγ is striking. Since it is difficult to imagine that in-frame *V-J* joining is specifically suppressed, the high incidence of out-of-frame cDNA may reflect an aspect of regulation of Tγ-gene expression. It is possible that the γ protein is required only during an early phase of T-cell development and its continued presence is detrimental to the cell. If so, T cells will be more tolerant to continued transcription of an out-of-frame T_γ gene than an in-frame gene. The implication of this view is that the 17-day-old fetal thymocyte population is dominated by immature T cells that have already passed through the stage in which the Tγ gene plays a critical role. Alternatively, the functional expression of the T_γ gene is restricted to a small fraction of thymocytes destined to become a subset of T-cells as yet unidentified. The significance of the high incidence of apparently abnormal RNA splicing is unclear but may also reflect the need to eliminate in-frame Tγ gene products once the cell has passed a critical stage in development. Precedents exist in the literature for the role of alternative splicing in the inactivation of a gene function during viral and cellular development[31-33].

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Note added in proof: After submission of this paper other groups have reported the identification and sequencing of additional V_γ-gene segments: V_4 in refs 48, 49; V_4, V_5, V_6 in ref. 50

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A novel V_H to V_HDJ_H joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production

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During B-cell development, the V_H genes of immunoglobulin heavy (H) chains are assembled from three different germline components: the variable (V_H) segment, the diversity (D) segment and the joining (J_H) segment[1,2]. The joining between two segments involves the recognition of conserved nonamer–heptamer sequences bordering each segment, double-stranded cuts at the heptamer–segment border, and the re-ligation of the two segment ends which have frequently been modified by the deletion and addition of nucleotides[3–6]. The flexibility of the joint increases V_HDJ_H variability. However, it also results in many pre-B cells which do not produce immunoglobulin H chains and have non-functional V_HDJ_H complexes carrying the V_H and J_H coding sequences in different reading frames[7]. We show here that such 'null cells' are not dead-end products of the B-cell developmental pathway but can perform a novel V_H to V_HDJ_H joining using a 5′ V_H segment to replace the V_H sequence of the $V_HDJ_H^-$ complex. This process can result in the generation of a $V_HDJ_H^+$ complex and the subsequent expression of an immunoglobulin heavy chain.

In the past we have studied the order and control of immunoglobulin gene rearrangements in the Abelson pre-B-cell line 300-19 (refs 8–11) derived from the bone marrow of an outbreed NIH/Swiss mouse[12]. 300-19 cells which originally carry a DJ_H3 complex on each J_H allele[8] undergo D to J_H and and V_H to DJ_H assembly while growing in culture[9]. Approximately 30% of the V_H to DJ_H joints of 300-19 place the V_H and J_H coding sequence in the same reading frame thus resulting in the production of a μ-chain[9], the first H chain class expressed in pre-B cells. Many of the 300-19 subclones, however, contain $V_HDJ_H^-$ complexes and fail to produce a μ-chain. To study the fate of such null cells, we analysed P17 and Pa2-5, two isolates of 300-19 carrying unproductive V_HDJ_H complexes.

Aberrant V_H to DJ_H joints had obviously occurred on both J_H alleles of P17 because we could not detect any μ-chains nor 5′ D sequences in P17 (data not shown). Indeed, all 5′ D sequences are generally deleted during the V_H to DJ_H joint of a J_H allele[3]. To our surprise, P17 became more and more μ-positive over a 3–4 week culture period. After 4 weeks of culture we subcloned P17 by limiting dilution and analysed μ production and J_H rearrangement of isolated subclones. Of 55 P17 subclones analysed, 8 (15%) were found strongly μ-positive in a Western dot assay (data not shown). On a Southern blot the two $V_HDJ_H^-$ alleles of P17 (A and B) are visible as J_H-positive *Eco*RI fragments of 2 and 2.2 kilobases (kb) respectively (Fig. 1, lane P). In the μ-positive subclones P17-17 and P17-32, the 2-kb *Eco*RI fragment of the *P17-A* allele had undergone rearrangement and appeared in a new J_H-positive *Eco*RI fragment of 3.2 kb (Fig. 1, lanes 7, 8). Three of the six randomly picked subclones had deleted the 2-kb *Eco*RI fragment (Fig. 1, lanes 2–4). Another of these subclones (P17-5) with weak μ expression (data not shown) showed a faint band of 3.2 kb (Fig. 1, lane 5), suggesting that part of the P17-5 culture was undergoing the same rearrangement event as that occcurring in P17-17 and P17-32. The 2.2-kb *Eco*RI fragment of the *P17-B* allele stayed unchanged in all P17 subclones. Thus only the V_HDJ_H complex of the *A* allele of P17 had undergone genetic alterations. These were either its complete deletion or a further rearrangement correlated with μ production.

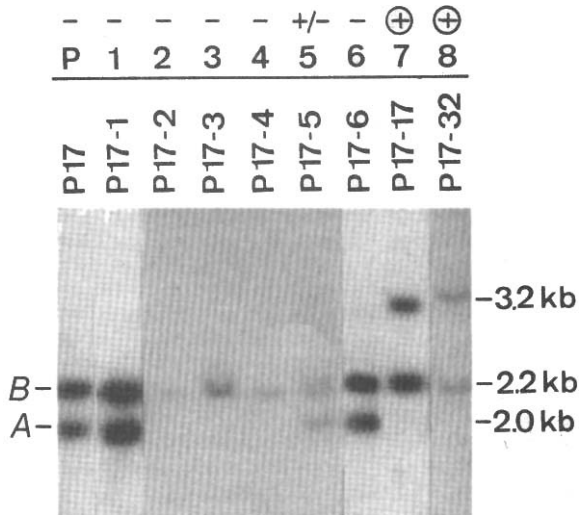

Fig. 1 J_H rearrangement in subclones of P17. The two parental $V_HDJ_H^-$ alleles of P17 are labelled *A* and *B*. Approximately 10 μg of genomic DNA of the different subclones was digested with *Eco*RI, electrophoresed through 1% agarose, blotted onto nitrocellulose and assayed for hybridization to a ^{32}P-labelled J_H probe as described previously[3,8]. μ Production in the subclones was determined by Western blotting (see Fig. 2) and is indicated by + or −.