Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2925–2929, May 1985
Immunology

Rearrangement of T-cell receptor β-chain genes during T-cell development

(thymocytes/ontogeny/receptor/β chain)

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Communicated by David W. Talmage, December 31, 1984

ABSTRACT **The kinetics and order of rearrangements in the gene complex encoding T-cell-receptor β chains were studied by Southern blot hybridization in a collection of hybridomas derived from fetal thymocytes at various stages of ontogeny (day 14 to day 17). Our results show a steady increase in the frequency of rearranged β complexes during this period and suggest that these rearrangements occur within the thymus. β-chain diversity region (Dβ) to β-chain joining region (Jβ) joining preceded other types of rearrangements. More complex hybridization patterns consistent with fully rearranged functional β-chain genes did not begin to accumulate until day 16, 1 day prior to significant surface expression of the receptor protein.**

The T-cell receptor for antigen is composed of two disulfide-bonded polypeptides, α and β (1–3). The genetic loci that encode the α and β chains have recently been identified (4–9), and comparison of β-chain cDNA and corresponding germ-line and genomic DNA clones have revealed variable (V), diversity (D), joining (J), and constant (C) gene segments similar to the corresponding gene segments of immunoglobulin chains (5, 6). Although the analysis of α-chain genes is not complete, it can be predicted from α-chain cDNA sequences and from the observation of T-cell-specific rearrangement of α-chain V region segments that this chain also is encoded in separate V, J, and C and, possibly, D gene segments (7–9).

The rearrangement of immunoglobulin heavy (H) chain V_H, D_H, and J_H segments to produce functional genes in B cells has been well studied (10–13). Two somatic recombination events are apparently required, mediated by characteristic conserved sequences flanking these gene segments. Developmental studies using Abelson murine leukemia virus-transformed B cells as a model for B-cell differentiation support an ordered sequence of events in which D_H to J_H rearrangements precede V_H to D_H rearrangements (14, 15).

Rearrangement of T-cell receptor Vβ, Dβ, and Jβ gene segments to produce a functional β chain is also well-documented (16–18). There are two β-chain C-region genes (*Cβ1* and *Cβ2*), each associated with a cluster of Jβ gene segments and a closely linked Dβ gene segment (16–20). In the present study, we generated a large collection of thymocyte hybridomas corresponding to various stages of mouse ontogeny and analyzed the configuration of their β-gene complexes by using Southern blot hybridization. We conclude that β-complex rearrangements probably occur after migration of T-cell precursors to the thymus and that DβJβ joining occurs prior to VβDβ joining.

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MATERIALS AND METHODS

Animals. BALB/cBy and C57BL/6 mice originally obtained from The Jackson Laboratory were bred in our own vivarium. The adult mice used in these studies were 8–12 weeks old. Timed pregnant BALB/cBy mice were derived from our own vivarium or purchased at The Jackson Laboratory. The day of finding a vaginal plug was designated as day 0 of embryonic development.

Collection of Hybridomas. Hybridomas were prepared as described (21), by fusion to the azaguanine-resistant AKR thymoma, BW5147.G.1.4 Oua^r.1. Samples of every stable hybrid cell line were frozen, and grown up to $\approx 10^8$ cells for genomic DNA preparations. Based on the proportion of wells in which hybridoma growth occurred in the initial plating, we estimated that <2% of wells positive for growth in any given fusion contained products of more than one fusion event.

Southern Hybridizations. DNA was digested with restriction enzymes and electrophoresed by standard methods. Southern blots were carried out by the procedure of Wahl *et al.* (22) except that the acid depurination step was omitted and the gel was irradiated with shortwave UV light for 5 min before the denaturation step. After hybridizations, the filters were washed in high salt buffer (2× NaCl/Cit/0.1% NaDodSO$_4$; 1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate) at 37°C and then in low salt buffer (0.1× NaCl/Cit/0.1% NaDodSO$_4$) at 55°C. Filters were exposed for at least 48 hr at −70°C using XAR film (Kodak) with an intensifying screen. Restriction analysis of β-chain gene configurations in the hybridomas was based on a map of the β-chain genes published by others (16–20) and confirmed in nearly all cases for C57BL/6 and BALB/c by us (see Fig. 1 and Table 2).

β Probe. The probe used in these experiments (pDOβ2) was isolated from a cDNA library derived from the T-cell hybridoma DO-11.10 (2) and was kindly provided by C. Coleclough (Roche Institute for Molecular Biology, Nutley, NJ). The structure of this probe was determined by sequence analysis and is shown in Fig. 1*a*.

RESULTS

Generation of Thymocyte Hybridomas. Table 1 summarizes the results of our fusions between fetal liver cells or adult or fetal thymocytes and the thymoma BW5147. From each fusion, between one and eight hybridomas were obtained for each 10^6 normal cells used in the experiment. There was no evidence from these numbers that cells from thymi of different stages preferentially fused to BW5147. In

Abbreviations: V, variable region; D, diversity region; J, joining region; C, constant region; H, heavy chain; kb, kilobase(s); bp, base pair(s).

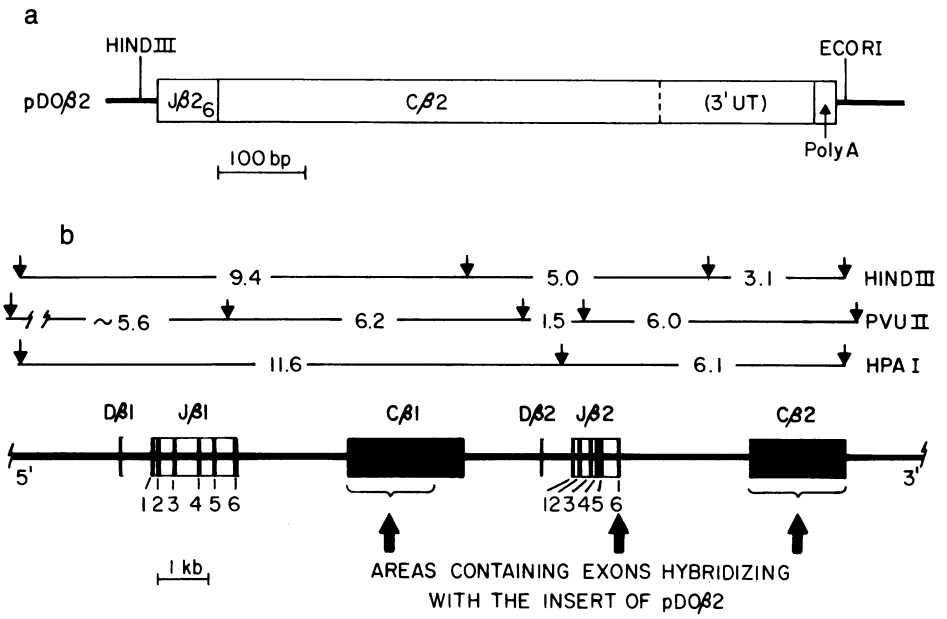

FIG. 1. (*a*) Structure of the insert of the cDNA clone, pDOB2. (*b*) Restriction enzyme map of the β complex. Structure of the β complex is shown with the positions of cleavage sites for *Hin*dIII, *Pvu* II, and *Hpa* I indicated above. Arrows denote segments hybridizing with the pDOβ2 insert. UT, untranslated region.

fact, fetal liver cells were as effective as thymocytes as fusion partners.

Analysis of β-Chain Gene Rearrangements. From the restriction enzyme map shown in Fig. 1*b*, certain predictions can be made about changes in restriction enzyme fragment lengths accompanying β-complex rearrangements using the insert of the cDNA clone pDOβ2 as a probe. The predictions involving D to J rearrangements are listed in Table 2. For example, by using the enzyme *Hin*dIII, three germ-line fragments would be seen, a band of 9.4 kilobases (kb), containing *Cβ1*, a faint band of 5.0 kb, containing *Jβ2*$_6$, and a band of 3.1 kb, containing *Cβ2*. A rearrangement of *Dβ1* to the *Jβ2* cluster, assuming that Dβ to Jβ gene rearrangements occur, as with immunoglobulin H-chain genes, primarily through "looping out and deletion" of intervening DNA (12), would yield a faint *Hin*dIII band of 4.7–3.6 kb, depending on the *Jβ2* gene involved in the rearrangement and a germ-line *Cβ2*-containing 3.1-kb band. The *Dβ1* to *Jβ2* rearrangement

Table 1. Summary of hybrids and their β-complex rearrangements

Normal cell origin	Number of hybrids: Total analyzed	With at least one normal cell-derived β complex (%)	With at least one unrearranged β complex (%)	With at least one rearranged β complex (%)	Total β-complex rearrangements	Rearrangement designations*
BALB/c						
Fetal liver Day 14	27	19 (100)	19 (100)	0 (0)	0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
Fetal thymus						
Day 14	23	20 (100)	19 (95)	3 (15)	4	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 01, 02, 12
Day 15	24	23 (100)	17 (74)	12 (52)	17	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 01, 02, 02, 02, 03, 11, 12, 12, 13, 23, 4, 0?
Day 16	31	29 (100)	13 (45)	21 (72)	38	0, 0, 0, 0, 0, 0, 0, 0, 1, 2, 01, 02, 03, 11, 11, 13, 13, 13, 13, 22, 013, 4, ?, 04, 14, 14, 134, 345, 1334
Day 17	27	21 (100)	6 (29)	19 (90)	28	0, 0, 1, 2, 2, 2, 2, 01, 01, 02, 11, 12, 013, 5, 5, 34, 44, 45, 45, 4?, 55
Adult thymus	30	23 (100)	1 (5)	23 (100)	37	1, 2, 2, 3, 13, 22, 123, 4, 4, 5, 5, 5, 5, 05, 45, 45, 45, 45, 55, 55, 5?, 5?, 344
C57BL/6						
Adult thymus	30	23 (100)	4 (17)	20 (87)	33	0, 0, 0, 2, 2, 11, 13, 4, 5, 5, 5, 5, 5, ?, 1?, 34, 45, 55, 55, 5?, 10?, 234, 234

*See text and Table 2 for explanation.

Table 2. Predicted restriction enzyme fragment lengths accompanying Dβ to Jβ rearrangements

Enzyme	Germ-line fragment length, kb (detected by pDOβ2)	Fragment length, kb (after β-complex rearrangements)		
		Class 1 (*Dβ1* to *Jβ1*)	Class 2 (*Dβ1* to *Jβ2*)	Class 3 (*Dβ2* to *Jβ2*)
*Hin*dIII	9.4	8.8 to 7.0		No change
	5.0	No change	4.7 to 3.6	4.4 to 3.3
	3.1	No change	No change	No change
Pvu II	6.2	No change for ($J\beta1_{1-5}$) 9.4 for ($J\beta1_6$)	Deleted	No change
	6.0	No change	No change for ($J\beta2_{1,2}$) ≈9.1 to 8.5 for ($J\beta2_{3-6}$)	No change for ($J\beta2_{1,2}$) 6.2 to 5.6 for ($J\beta2_{3-6}$)
Hpa I	11.6	11.0 to 9.2	7.9 to 6.8	17.1 to 16.0
	6.1	No change		

would be confirmed by examination of restriction enzyme fragments revealed after *Pvu* II and *Hpa* I digestions.

Thus, to analyze β-chain gene rearrangements, genomic DNA from each hybridoma was separately digested with *Hin*dIII, *Pvu* II, or *Hpa* I, electrophoresed, blotted, and probed with pDOβ2. The restriction enzyme fragments so revealed were examined and assigned to different classes depending on the types of rearrangements they indicated.

The various sorts of D to J joinings described in Table 2 were designated class 1 (*Dβ1* to *Jβ1*), class 2 (*Dβ1* to *Jβ2*), or class 3 (*Dβ2* to *Jβ2*) rearrangements. The presence of a chromosome(s) in the germ-line configuration was designated class 0. A rearrangement that did not fall into class 1, 2, or 3 was designated class 4 if it involved the *Jβ1* cluster and class 5 if it involved the *Jβ2* cluster. In the few cases in which the hybridization pattern was too complex to assign clearly all the rearrangements, a "?" designation was used. The designation of the hybrids in each group is listed in Table 1.

Fig. 2 shows examples of how some of these assignments were made for hybrids derived from day 15 fetal thymocytes. Hybrid 7DTE7 was given a class 0 designation, because in addition to the bands of BW5147, only a germ-line hybridization pattern was seen. Hybrid 8DTE7 was designated 01, because in addition to a germ-line pattern, a single rearrangement was seen. Since the rearrangement was detected in a *Hin*dIII digest (as a strongly hybridizing band), it involved the *Jβ1* cluster. Identical size changes in the rearranged *Hin*dIII and *Hpa* I bands [≈600 base pairs (bp)] suggested that the new gene configuration was the result of a *Dβ1* to *Jβ1* rearrangement, and the overall length of the rearranged restriction fragments (*Hin*dIII, 8.8 kb and *Hpa* I, 11.0 kb) together with the absence of rearranged bands in the *Pvu* II digest were compatible with a rearrangement of Dβ1 to the first or second J segment of the *Jβ1* cluster. Similarly, working from the predictions in Table 2, hybrid 8DTE1 contained a *Dβ1* to *Jβ2* rearrangement and was classified 02; hybrid 8DTE5 contained a *Dβ2* to *Jβ2* rearrangement and was classified 03.

Kinetics of β-Chain Rearrangements in T-Cell Ontogeny. Shown in Table 1 are the number and percentage of the hybrids in each group that contained β-complex rearrangements. Because some hybrids contained more than one β complex or, in some cases, multiple rearrangements in the same β complex, we have also shown in Table 1 the number of hybrids containing an unrearranged β complex and the total number of rearrangements found in the different groups. A summary of the data follows. No rearrangements were seen in any of the fetal liver hybrids. In the fetal thymocyte hybrids, very few rearrangements were seen at day 14. There was a dramatic increase in rearrangements on day 15, although many unrearranged β complexes were seen as well. By day 17, nearly all the hybrids had at least one rearrangement; however, 29% of the hybrids still contained at least one unrearranged β complex. Also, nearly all hybrids de-

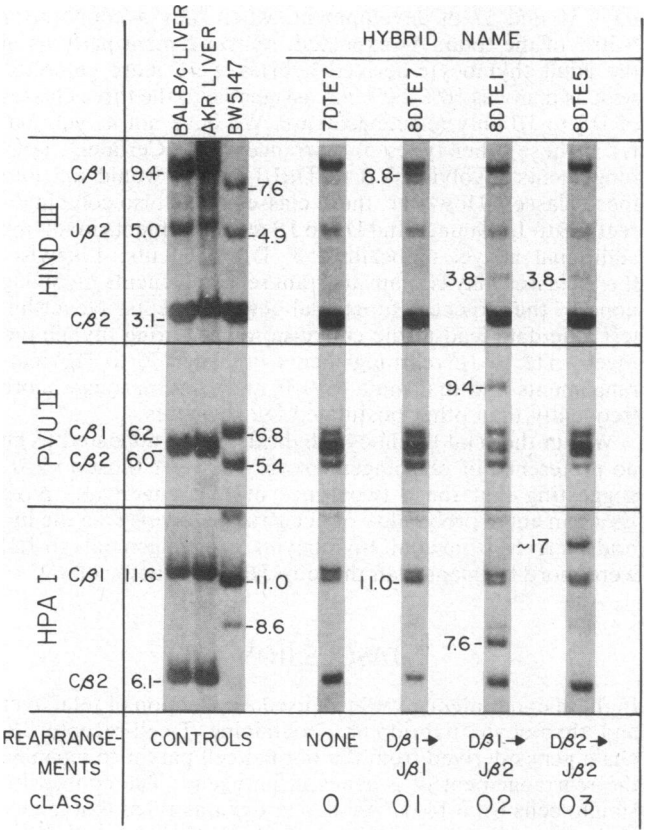

FIG. 2. Examples of β-chain gene rearrangements. Genomic DNAs of germ-line controls from BALB/c and AKR liver, from BW5147, and from BALB/c day 15 thymus hybridomas were digested with the restriction enzymes *Hin*dIII, *Pvu* II, or *Hpa* I and analyzed by agarose gel electrophoresis and Southern blotting. Sizes of rearranged restriction fragments (in kb) were estimated by comparison with an *Hin*dIII digest of λ phage DNA and known β-chain gene germ-line bands. All examples shown are derived from the same experiment. Rearrangements in hybridomas were assigned to different classes, indicated along the bottom of the figure, according to the criteria described in text and in Table 2.

Table 3. Ordered rearrangements of β complex in thymocyte development

Source of normal cells	Number (%) of rearrangements compatible with*						
	Class 1 (*Dβ1* to *Jβ1*)	Class 2 (*Dβ1* to *Jβ2*)	Class 3 (*Dβ2* to *Jβ2*)	Class 4 (other to *Jβ1*)	Class 5 (other to *Jβ2*)	?*	*Jβ2/Jβ1*†
14 day BALB/c	2 (50)	2 (50)	0	0	0	0	1.0
15 day BALB/c	6 (35)	6 (35)	3 (18)	1 (6)	0	1 (6)	1.3
16 day BALB/c	15 (39)	4 (11)	10 (26)	7 (18)	1 (3)	1 (3)	0.7
17 day BALB/c	7 (25)	6 (21)	2 (7)	6 (21)	6 (21)	1 (4)	1.0
Adult BALB/c	3 (8)	5 (14)	4 (11)	8 (22)	15 (41)	2 (5)	2.2
Adult C57BL/6	5 (15)	4 (12)	4 (12)	5 (15)	11 (34)	4 (12)	2.2

*For assignment of rearrangements see text and footnote to Table 1.
†Ratio of rearrangements involving *Jβ2* to rearrangements involving *Jβ1*.

rived from adult thymocytes carried rearrangements and, in fact, only rarely had an unrearranged complex.

Order of β-Chain Rearrangement in T-Cell Ontogeny. Shown in Table 3 is a summation of the various classes of rearrangement seen in the hybrids and the ratio of rearrangements involving *Jβ2* to those involving *Jβ1*. The clearest conclusion that arises from this analysis is that early in thymocyte ontogeny most of the β-chain gene rearrangements involve *Dβ1* or *Dβ2* to Jβ joining only, with perhaps some indication that rearrangements involving *Dβ1* are more frequent than those involving *Dβ2*. All rearrangements at day 14 and nearly all at day 15 were of this type. Other types of rearrangements did not appear in substantial numbers until days 16 and 17 of development, when they accounted for ≈30% of the total. As expected, rearrangement patterns in the adult thymocyte-derived hybrids were more complex, with as many as 50%–60% not assignable to the three classes of Dβ to Jβ only rearrangements. We have not as yet analyzed these other types of rearrangements. Certainly, rearrangements involving Vβ to DβJβ joining would fall into these classes. However, these classes could also contain direct Vβ to Jβ joinings and Dβ to Jβ rearrangements involving additional as yet unlocalized 5′ Dβ segments. Likewise, these classes may contain aberrant rearrangements involving none of the expected functional gene segments. Nevertheless, the data lead to the conclusion that during thymic ontogeny Dβ to Jβ rearrangements precede Vβ to Dβ rearrangements and that initially *Dβ1* and *Dβ2* rearrange more frequently than other postulated Dβ segments.

Within the fetal thymocyte hybrids, there appeared to be no preference of rearrangements to *Jβ2* over those to *Jβ1*, suggesting that these two types of rearrangements have about an equal probability of occurring. However, in the hybrids derived from adult thymocytes, rearrangements to *Jβ2* were more frequent than those to Jβ1 by a factor of ≈2.

DISCUSSION

In these experiments, we have used a collection of fetal liver and thymocyte hybridomas containing T-cell-receptor β-chain genes derived from the normal cell parent to examine the rearrangement of β genes in ontogeny. The control fusion to cells from 14-day fetal liver demonstrated that in heterokaryons the tumor cell parent, BW5147, did not itself induce rearrangements of the receptor β-chain genes of the normal cell partner in the fusion. This fusion also showed that fetal liver cells, a rich source of stem cells capable of populating the thymus (reviewed in ref. 23), contained few if any cells (<5%) with rearranged receptor β-chain genes. Fusion of BW5147 to fetal thymocytes demonstrated many cells with rearranged β-chain genes. There was a steady increase in the frequency of cells with rearranged β genes during the critical developmental period of day 14 to day 17, with the largest increase in their frequency between days 14 and 15. Fusions to adult thymocytes showed almost universal rearrangement of the β-chain genes in these cells. We conclude from these results that the earliest T-cell precursors in the thymus have very infrequent or no rearranged β-chain genes, and that rearrangements occur predominantly during the maturation of these cells in the thymus. An alternative, but perhaps less likely, hypothesis is that the earliest cells in the thymus do not have rearranged β-chain genes, but that succeeding waves of thymus immigrants contain more complex organizations of their β complex (see below), reflecting rearrangements that have occurred before the immigrants arrived in the thymus.

Our conclusions depend somewhat on the assumption that the hybridomas, particularly those derived from day 14 and 15 fetal thymi, were mainly the product of fusion of BW5147 to T-cell precursors rather than other cell types. Our confidence in this assumption is based primarily on the observation that nearly all cells in the fetal thymus from day 14 to 17 bear the *Thy-1* marker (28), which is present only on T-cell precursors in the thymus, and that there is no evidence that BW5147 preferentially hybridizes to non-T cells, because the frequency of hybrids produced by fusing BW5147 to fetal liver cells, for example, is about the same as that produced by fusing BW5147 to adult thymocytes. The activation of silent *Thy-1* genes by fusion of BW5147 to non-T cells (24) precluded our use of the allelic forms of this marker to demonstrate the T-cell origin of our hybrids; however, it seems likely that the majority were in fact derived from T cells.

Examination of the Jβ clusters involved in the rearrangements showed that early in thymocyte ontogeny rearrangements to either cluster occurred with equal frequency, but in hybridomas from adult thymocytes there was a predominance of events involving *Jβ2*. This may be consistent with the suggestion that functional T-cell lines more often use the *Cβ2* than the *Cβ1* locus (25). Progressive skewing of rearrangements to the *Jβ2* cluster may occur because unproductive rearrangements involving the *Jβ1* cluster still allow productive rearrangements to the *Jβ2* cluster with deletion of upstream *Jβ1* and *Cβ1* genes.

When the types of rearrangements found in each hybridoma were examined in more detail, we found that during ontogeny Dβ to Jβ joining precedes other types of rearrangements with perhaps some tendency of *Dβ1* to rearrange earlier and more frequently than *Dβ2*. The fact that rearrangements involving Dβ to Jβ occur before those involving Vβ regions, and that more proximal loci may be used more frequently early in development than those further away is, of course, strikingly similar to results previously reported for immunoglobulin genes (26).

We and others have studied the expression of T-cell-receptor protein in thymocyte development and have shown that receptors are not expressed significantly on the surfaces of these cells until day 17 of fetal life (27). Our observation that a substantial frequency of fully rearranged β-chain genes does not exist until at least day 16 of fetal life is cer-

tainly consistent with the subsequent expression of surface receptor protein.

We would like to thank Jim Leibson, Ella Kushnir, Janice White, Lee Niswander, and Virginia Barr for excellent technical assistance and Edna Squillante and Kelly Bakke for continued secretarial help. This work was supported by U.S. Public Health Service Grants AI-18785 and HD-17717 and American Chemical Society Research Grants IM-49 and IM-319. This work was done during the tenure of ACS Faculty Research Award 218 by J.K. and while J.Y. was on leave from the Servei d'Immunologia, Hospital Clinic (Barcelona, Spain), partially supported by a fellowship from Comissio Interdepartamental de Recerca i Innovacio Technologica, Generalitat de Catalunya, Spain.

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