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Antibody Repertoire Development in Fetal and Neonatal Piglets. II. Characterization of Heavy Chain Complementarity-Determining Region 3 Diversity in the Developing Fetus[1,2]

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Since the actual combinatorial diversity in the V_H repertoire in fetal piglets represents <1% of the potential in mice and humans, we wondered whether 1) complementarity-determining region 3 (CDR3) diversity was also restricted; 2) CDR3 diversity changed with fetal age; and 3) to what extent CDR3 contributed to the preimmune VDJ repertoire. CDR3 spectratyping and sequence analyses of 213 CDR3s recovered from >30 fetal animals of different ages showed that >95% of VDJ diversity resulted from junctional diversity. Unlike sheep and cattle, somatic hypermutation does not contribute to the repertoire. These studies also revealed that 1) N region additions are as extensive in VDJ rearrangements recovered at 30 days as those in late term fetuses, suggesting that TdT is fully active at the onset of VDJ rearrangement; 2) nearly 90% of all rearrangement are in-frame until late gestation; 3) the oligoclonal CDR3 spectratype of 30-day fetal liver becomes polyclonal by 50 days, while this change occurs much later in spleen; 4) there is little evidence of individual variation in CDR3 spectratype or differences in spectratype among lymphoid tissues with the exception of the thymus; and 4) there is a tendency for usage of the most J_H proximal D_H segment (D_HB) to decrease in older fetuses and for the longer D_H segment to be trimmed to the same length as the shorter D_H when used in CDR3. These findings suggest that in the fetal piglet, highly restricted combinatorial diversity and the lack of somatic mutation are compensated by early onset of TdT activity and other mechanisms that contribute to CDR3 junctional diversity. ***The Journal of Immunology,*** **2000, 165: 6999–7010.**

Primary Ab repertoire development (VDJ rearrangement) occurs in the fetal liver of most mammals (1) and in the avian yolk sac (2). The repertoire of these B cells becomes further diversified after trafficking to lymphoid tissues such as the bursa of Fabricius in chickens (3), the rabbit appendix (4), the ileal Peyers patches in sheep (5), and other secondary lymphoid organs such as the spleen and lymph nodes in humans and mice. Diversification of the primary VDJ rearrangements at these secondary sites can result from untemplated mutation as in sheep, cattle, mice, and humans (6–8), both gene conversion and untemplated mutation in rabbits and cattle (9–11), or entirely by gene conversion as in the chicken bursa (12, 13). Before birth, this diversification proceeds independently of environmental Ag in chickens and sheep (8, 14). In mice, humans, and rabbits Ag-independent diversification at least by untemplated (point) mutation is seldom observed. In any case, these processes give rise to the so-called preimmune, intrinsic or natural Ab repertoire. This repertoire contains highly cross-reactive, low affinity autoantibodies often encoded by 3′ proximal V_H genes (15, 16). The exact size of this repertoire is unknown, and, as cited above, the exact source of its diversity may vary among species. After birth and/or in response to environmental Ag, this preimmune repertoire is further diversified by Ag selection in secondary lymphoid tissues as part of the adaptive immune response.

The V_H repertoire of swine is encoded by <20 V_H genes (17), a single J_H (18), and almost exclusively by two D_H segments (19–21). VDJ rearrangements are first recovered from the liver of 30-day-old fetuses, and in the remaining 84 days of gestation the monotonous usage V_HA, V_HB, V_HC, and V_HE (the fetal V_H genes) and two D_H segments account for approximately 80% of the preimmune VDJ repertoire in all major lymphoid tissues (20). If V_HF usage is included (Table I), >90% of total V_H usage can be accounted for by just five V_H genes. Since there is virtually no somatic mutation in fetal life (Ref. 19 and this report), the combinatorial heavy chain preimmune repertoire of the piglet is derived from 8–10 nonmutated combinations. Considering that all heavy chain V region gene segments in human and mice can be potentially used, the combinatorial repertoire of swine is conservatively <1% of that in humans and mice (22). While fetal rabbits also use a small number of V_H genes and a single V_H (V_H1) >80% of the time, these are combinatorially joined with eight D_H and two J_H segments (23, 24). Since fetal and newborn piglets appear capable of producing Abs to a broad range of Ags (25, 26), it raises the question as to how so few VDJ recombinations are able to encode such an apparently broad Ab repertoire. Theoretically, such a

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Table I. *Characteristics of CDR3s in clonal VDJs as a function of fetal age*

Fetal Age (days)	No. of Clones	Proportion of Duplicate Clones (%)	Proportion of In-Frame Clones (%)	D_H Reading (%)[a] Frame Usage			V_H Usage (%)								D_H Length[b]	
				RF1	RF2	RF3	V_HA	V_HB	V_HC	V_HD	V_HE	V_HF	Chimeras	Other	D_HA	D_HB
30[c]	74	35	88	9	38	53	13	29	10	2	13	2	25	7	21.2 ± 7.7	17.5 ± 6.3
40[c]	43	19	94	0	50	50	26	26	20	11	3	0	14	0	14.3 ± 9.6	21.2 ± 6.5
60	20	20	85	5	74	21	6	25	25	0	0	13	31	0	23.2 ± 7.3	24.1 ± 2.5
70	21	0	91	5	57	38	43	19	5	0	5	19	10	0	26.1 ± 3.2	21.7 ± 4.6
90	20	0	85	20	40	40	45	20	10	0	10	14	0	0	22.2 ± 5.5	21.7 ± 4.6
110	35	0	74	21	44	35	26	34	17	0	6	3	14	0	21.3 ± 8.4	19.4 ± 3.5
GF piglets (6 wk)	42	0	64	18	47	35	14	50	11	0	16	7	ND	2	21.3 ± 6.0	19.7 ± 5.8

[a] All RF1 rearrangements are nonproductive due to stop codons in D_HA and D_HB.
[b] Length differences are not statistically significant by Student's *t* test.
[c] Data from fetal liver; all other data are from spleen.

highly restricted combinatorial diversity could be offset by 1) variable light chain diversity, 2) junctional diversity in complementarity-determining region 3 (CDR3),[4] 3) gene conversion of the fetal V_H genes, 4) somatic hypermutations as in the sheep Vλ, or 5) a combination of these mechanisms. CDR3 has been shown to play a major role in the specificity of the Ab binding site (27), and experiments by Xu and Davis (28) have shown that a mouse with a single V_H transgene can make responses to all Ags provided full CDR3 diversity is still possible. Thus, we have chosen to initially address the second theoretical possibility by examining >200 CDR3 sequences recovered from fetal piglets ranging from 30–110 days of age. In addition, VDJs from various lymphoid tissues were analyzed for their CDR3 spectratype (length). Based on N region addition, our finding suggest that TdT is active from the time of first VDJ rearrangement, and that >90% of the sequences contain no somatic mutations. While fetal piglets tend to first use the shortest of two D_H segment, the longer D_H segment is more extensively trimmed so the segment length of both D_H segments in CDR3 is the same. Spectratypic and sequence analyses failed to show any significant increase in the length of CDR3 during fetal life.

Materials and Methods

Source of animal tissues and DNA

White crossbred (WC) gilts (one-quarter Yorkshire, one-quarter Chester White, one-quarter Large White, one-quarter Landrace) from the Roman L. Hruska U.S. Meat Animals Research Center and Yorkshire and Meishan F_1 crosses from Iowa State University were used in the study. Animals were hand-mated and scheduled for slaughter and collection of 23-, 26-,28-, 30-, 40-, 50-, 60-, 70-, 90-, and 110-day fetuses. Gestation in swine is 114 days. All gilts were healthy and normal at slaughter, and fetuses were immediately removed from the gravid uterus. Fetal liver was collected from 23-, 26-, 28-, 30-, and 40-day-old fetuses. Spleen samples and a variety of lymphoid tissues were collected from 40-day-old and older fetuses. We also include in our study a noninductive site of the mucosal immune system (parotid gland) that depends on B cell immigration (29) and a controversial B cell tissue, the thymus. The results summarize data for >30 fetuses of different ages.

Amplification and cloning of VDJs and CDR3

Since all swine V_H genes are members of a single, highly homologous family (17) and have a single J_H (18), VDJ rearrangement can be amplified from DNA or cDNA using an FR1 primer (recognizing a sequence shared by all swine V_H genes) and an anti-J_H primer. The approximately 500-bp product was then cloned into pBluescript and grown in XL-1 Blue cells, and individual recombinant clones were selected and transferred to a master filter as described previously (19, 20). These were then hybridized with a ^{32}P-labeled pan-V_H probe to confirm that each clone contains a VDJ insert and with gene-specific oligonucleotide probes to determine V_H gene usage (20). In animals older than 50 days DNA was prepared from whole tissues, whereas in younger fetuses leukocytes were first prepared as previously described (30), since cells containing rearranged VDJ are rare at 30 days (M. Sinkora et al., unpublished observations).

Length analysis (spectrotypic analysis) of CDR3

The CDR3 segments of the uncloned PCR products described above were amplified using a FR3-A^+ primer (gtttctttgagaaccgaagacacggc) and a ^{32}P-labeled anti-J_H primer (tgaggacacgacgacttcaa). Use of the FR3-A^+ primer yields PCR products of uniform length in >90% of the amplicons (31, 32), thus reducing shadow band formation during spectratyping. The products were separated on 6% polyacrylamide sequencing gels, the gels were dried and then scanned using a Hewlett-Packard Instant Imager (Palo Alto, CA), and the results were displayed directly (see Figs. 1, 2*A*, and 5). To confirm that the CDR3 spectratypes obtained were not artifacts of PCR, the CDR3 product of an initial porcine CDR3 amplification was repeatedly amplified. This procedure resulted in no PCR-dependent changes in CDR3 spectratype (Fig. 1*A*). We conclude that the CDR3 spectratypes seen on sequencing gels (Figs. 1, 2, and 5) are an accurate reflection of cell numbers and the distribution of CDR3s lengths in vivo.[5]

Quantitative spectratypic analysis

The distribution and relative concentration of CDR3 lengths were quantified by scanning (Fig. 2*B*) using the Hewlett-Packard Electronic Autoradiography Program (Packard Imager version 2.05 for Windows 95).

Sequence analysis of CDR3

The CDR3 region of 213 randomly selected VDJ clones from the fetal liver of 30- and 40-day-old fetuses and the spleen of 60-, 70-, 90-, and 110-day-old (essentially newborn) fetuses were sequenced using previously described methods (19). CDR3 sequences were compared as previously described (21) in terms of total length, P and N nucleotide additions both 5′ and 3′ of D_H, D_H usage, D_H length, and D_H reading frame usage (Fig. 3 and Table I). Data were compared with those obtained from 42 sequences from 6-wk-old germfree piglets (Table I).

Results

The preimmune CDR3 repertoire expands from an oligoclonal to a polyclonal profile first in fetal liver

Spectratypic analysis revealed a oligoclonal repertoire in the 30-day-old fetal liver with prominent CDR3 lengths of 42–45 bp in most animals (Fig. 1*B*), but also with prominent 36–39 lengths in some animals (Figs. 1*B* and 2). The oligoclonal spectratype of the 30-day-old liver was a common feature of all but one of nine 30-day-old fetal livers examined (Fig. 1*B*; animal 1 excepted). These spectratypic results are consistent with the mean CDR3

[4] Abbreviations used in this paper: CDR3, complementarity-determining region 3; RF1, reading frame 1; MLN, mesenteric lymph node; IPP, ileal Peyer's patches.

[5] Since a single band seen on a sequencing gel, e.g., 51 bp (Fig. 2B), may represent many different clones of equal CDR3′, the term oligoclonal is used here in the vernacular to denote only length distribution.

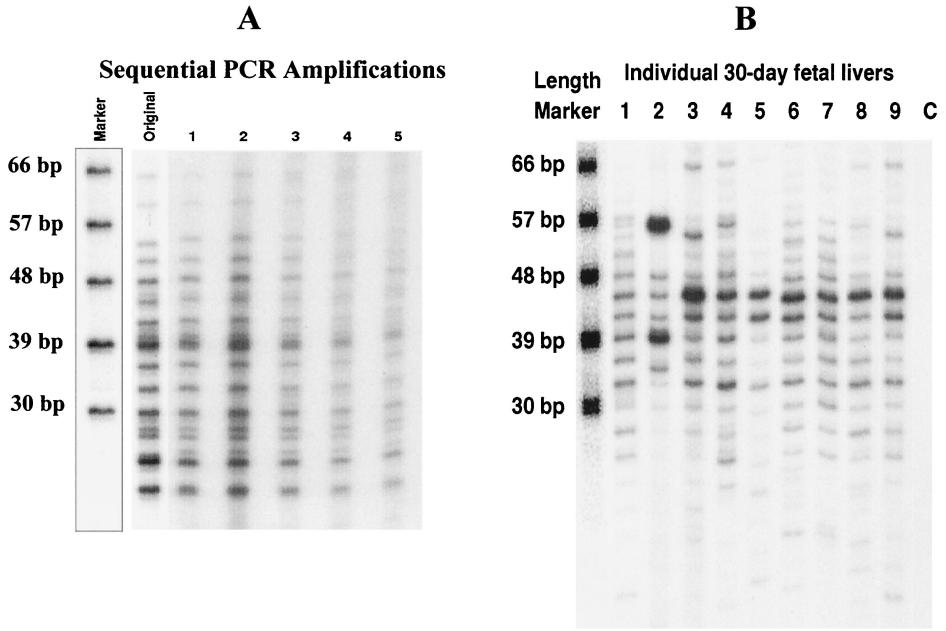

FIGURE 1. Fidelity and animal variation in spectratyping. *A*, Using five successive rounds (*lanes 1–5*) of PCR starting with the same original template, the spectratype generated remains constant. *B*, The CDR3 spectratype of VDJs from 30-day-old fetal liver in nine outbred fetuses. Marker polynucleotides of the indicated length show that most major polynucleotide bands represent productive rearrangements.

length determined by sequence analysis (Figs. 3 and 4*B*). However, CDR3s as long as 66 nucleotides and as short as 12 were also present at 30 days (Figs. 1*B* and 2). The polynucleotide spectrum had the appearance of a repertoire selected for the proliferation of B cells carrying a single in-frame rearrangement (only every third polynucleotide is present; Figs. 1*B* and 2*A*). This contrasts with a totally nonselected repertoire (see thymus; Fig. 5) or the spectratype from the spleen of older fetuses, e.g., 110 days, in which out-of-frame rearrangements are routinely observed (Fig. 2*A*). This observation is further described below. Evidence that the prominent CDR3 lengths comprising the oligoclonal spectratype in the day 30 fetal liver (Figs. 1*B* and 2*B*) represent expansion of certain clones was also consistent with data on the random recovery of duplicate clones (Table I). The random recovery of duplicate clones before 70 days indicated that the B cell compartment is small early in development.

At 40 days the spectratype in fetal liver remained oligoclonal, but with as many as seven prominent lengths, including one of only 12 bp (Fig. 2*B*). However, at 50 days the fetal liver was polyclonal and would have appeared nearly Gaussian in profile were it not for the short 12-bp CDR3, which was also prominent in spleen from 50- to 60-day-old animals (Fig. 2*B*) as well in the parotid gland of older animals (Fig. 5*B*).

The spleen in 40-day-old fetuses is poorly developed to the extent that it is difficult to recover in all fetuses. Although the spleen is morphologically well developed by day 50, lymphocyte cellularity is low (30), and the CDR3 spectratype of rearranged VDJs from 50- to 70-day-old fetal spleen remains oligoclonal in contrast to fetal liver, which is polyclonal by 50 days. Not until 90 days does the splenic CDR3 profile appear polyclonal and Gaussian. Coincident with the appearance of a polyclonal profile in older fetuses, the short 12-bp CDR3 is no longer prominent in any major lymphoid tissue tested, but can still be seen in the parotid gland (Figs. 2 and 5*B*).

N region additions were similar in number during fetal development, and point mutations were rare

The analysis of the CDR3 sequences presented in Fig. 3 is graphically summarized in Fig. 4. Results indicate that N region additions were of the same magnitude at 30 days as at 110 days (Fig. 4*D*). P nucleotide additions were seldom seen either 5′ or 3′ of D_H. We found no evidence for D-D rearrangements, for mini-D_H sequences, or that short homology segments had been inserted. There was no increase in the frequency of point mutation with age, and overall, >90% of all CDR3s were nonmutated (Fig. 4*A*).

D_HB is the most J_H-proximal D_H segment in swine (J. Sun et al., unpublished observations) (18). In germline configuration, D_HB is comprised of 28 nucleotides, whereas D_HA is comprised of 36 nucleotides. In 30-day-old fetal liver, nearly two-thirds of the B cells randomly sampled used D_HB, but its proportional usage decreased with fetal age (Fig. 4*C*). Although usage of the shorter D_H decreased gradually during fetal development, there was no age-dependent change in the length of CDR3 (Fig. 3). This constancy in length appeared to result from greater trimming of the longer D_HA, such that the actual lengths of D_HA and D_HB used in CDR3 were not significantly different (Table I). Usage of reading frame 1 (RF1) increased in late term fetuses and was also seen in 6-wk-old germfree piglets (Table I), although RF1 is nonproductive in both D_H segments due to a stop codon.

The proportion of in-frame rearrangements[6] decreased in late gestation

VDJ clones recovered from 30- to 70-day-old fetuses were nearly 90% in-frame compared with 65% in newborn germfree piglets and 74% in 110-day-old fetuses (Table I). The latter values straddle the expected value of approximately 71% if VDJ rearrangement and cell survival are merely random. This age-associated increase in nonproductive rearrangement was also apparent from spectratypic analyses (Fig. 2*A*). The heavy bands spaced at three-nucleotide intervals in Figs. 1, 2, and 5 are the in-frame sequences as determined by comparison with the CDR3 length standards provided. Spectratypes showing out-of-frame rearrangements are especially prominent in 110-day-old fetal spleen (Figs. 2*A* and 5*A*) in the mesenteric lymph nodes (MLN) of some animals (Fig. 5*A*) and in the 90- and 110-day-old parotid gland (Fig. 5*B*). Surprisingly, the bone marrow, which is regarded as a primarily B cell tissue in

[6] In some literature, the terms in-frame rearrangement and productive rearrangement are used synonymously. Since RF1 in swine contains stop codons in both D_HA and D_HB, use of the term in-frame rearrangement is more accurate.

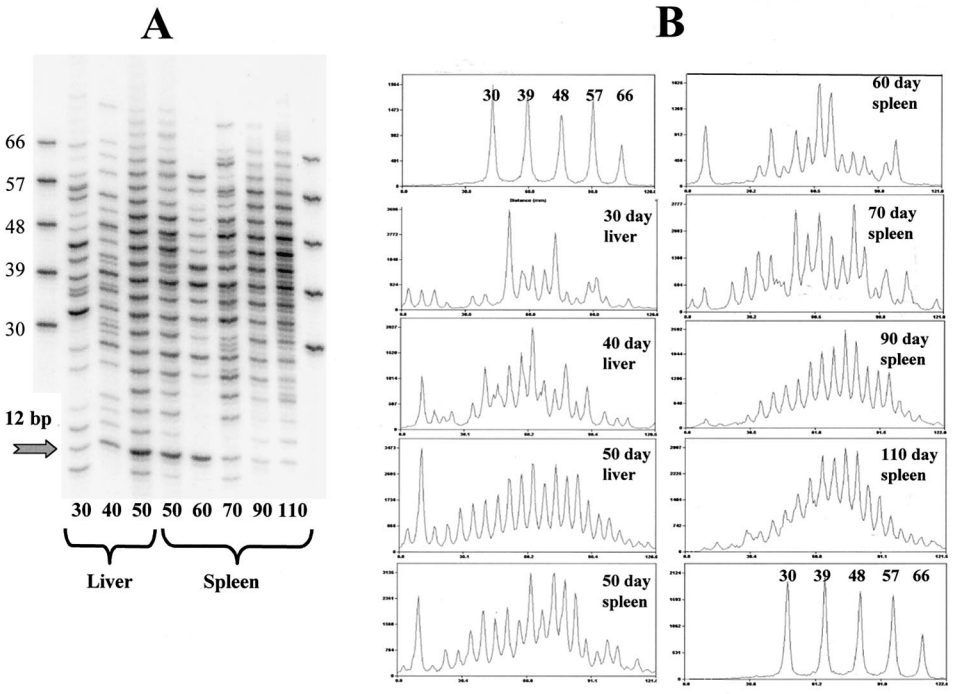

FIGURE 2. Qualitataive and quantitative analyses of the CDR3 spectratype in VDJs recovered from fetal liver and spleen of fetuses of different age. *A*, Direct image analysis of CDR3 spectratypes. *B*, Quantitative CDR3 profiles from fetal liver and spleen of fetuses of different ages. CDR3 size markers are indicated for both *A* and *B*.

mammals and where unselected pre-B cells should be found, shows few out-of-frame rearrangements (Fig. 5*A*). Scanning spectratypes generated on gels allowed to run longer allowed us to estimate that about 40% of all B cells in tissues from older fetuses carried such rearrangements (data not shown). Thus, both spectratypic and sequence analysis show that B cells with nonproductive rearrangements are rare before 70 days of fetal life (Fig. 2, *A* and *B*).

CDR3 diversity was highly conserved among diverse lymphoid tissues and among individuals

Figs. 1*B* and 2 show that the earliest VDJ rearrangements (30- to 40-day-old fetal liver) have CDR3s as diverse in length as older fetuses, but decidedly more oligoclonal. Clones bearing prominent CDR3 lengths of 36–45 bp in the youngest fetuses persisted throughout fetal development (Fig. 4*B*). When the CDR3 spectratype of VDJs from diverse lymphoid tissues of 90- and 110-day-old fetuses were compared, the same broad spectrum of CDR3 lengths was seen in all samples tested, including even the parotid gland, but excluding the thymus (Fig. 5). In typical secondary lymphoid tissues of 110-day-old animals, nonproductive rearrangements were universally present. While the spectratype of CDR3 in the bone marrow, spleen, IPP, and MLNs of 110-day-old fetuses differs little among four unrelated animals (Fig. 5*A*) the parotid gland of unrelated individuals exhibited animal differences (Fig. 5*B*). As indicated above, the frequency of nonproductive rearrangements was also quite conspicuous in the parotid glands from 90- and 110-day-old fetuses, but not in the parotid glands of three 6-wk-old animals (Fig. 5). Also prominent in the fetal parotid was the 12-bp CDR3.

The CDR3 spectratype from thymus indicated the absence of selection for in-frame rearrangements

The spectratype of CDR3 in the fetal porcine thymus was the only one resembling the pattern expected if VDJ rearrangement was random and no selective proliferation of B cells with productive rearrangements had occurred. This pattern also persisted into adulthood (Fig. 5*A*).

V_H usage favors VHA and VHB

Although the focus of the current study was on the diversity of fetal CDR3, sequence and hybridization analyses of VDJs were consistent with our earlier report in showing the V_HA and V_HB are the predominant V_H genes used during fetal life by piglets and generally account for about 50% of all V_H usage (20) (Table I). V_HD was rarely encountered and, as we earlier speculated, may be an allelic variant of V_HC (20). We also recovered chimeric VDJs, but with higher frequently in early fetal life when the B cell compartment was small (Table I). Evidence for the small size of the B cell compartment at this time is supported by the high frequency of duplicate clones that were recovered (Table I).

Discussion

The epitheliochorial placenta of the pig prevents the transfer of any maternal proteins into the fetal circulation, unlike the situation in rodents and primates (33). Therefore, developmental changes in the immune system observed during fetal life must be encoded in the genome of the developing fetus and cannot be a consequence of environmental Ags or maternal regulatory factors that can cross the placenta as observed in rodents and primates. Based on this premise, we have embarked on a series of studies of fetal B cell repertoire development in piglets. Previously we reported that fetal piglets display a rather monotonous pattern of variable heavy chain gene segment usage throughout 84 days of fetal life in all major tissues (20). Specifically, four or five V_H genes account for about 90% of V_H usage, and only D_HA and D_HB are used. Since swine have only a single J_H (18), combinatorial diversity provides only 8–10 possibilities. This pattern of V_H region segment usage does not fit the paradigm of diversity generated through combinatorial usage of many V region segments as described in immunology textbooks. Either the repertoire in piglets is highly restricted, or diversity is dependent on other mechanisms. Here we have investigated 1) the extent to which CDR3 diversity in the heavy chain variable region accounts for repertoire diversity in this species, 2)

	Clone name	FR3	p	n	DH A	p	n	JH	FR4
DHA									
30day	1/008	aaccgaagacacggccccgctattactgtgcaaga			ttgcgagaggttatggtggtagttgcta		ctccgtcc	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal	1/021	aaccgaagatacggccccgctattactgtgcaaga		gagt	attgctataactatggtgctagttgct		tggaatggagagtcgg	ttacgttgctatggatctc	tgggggcccaggcgttgaagtccgtcgtgtcctca
liver	1/028	aaccgaagacacggccccgctattactgtgcaaga		gtcg	atagctatggtgctagttactatggtga		gaggg	tggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/058	aaccgaagacacggccccgctattactgtgcaggg		gaa	aattgctatagctatggtgctagttgctata	t	gac	ttactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/020	aaccgaagatacggccccgctattactgtgcaaga	t	atgct	taactatggtgctagttgctgatggaga		gtcgg	ttacgttgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/034	aaccgaagacacggccccgctattactgtgcgatg		tggt	tagttgctatgatg	ca	ggcc	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/055	aaccgaagacacggccccgctattactgtgcgata		ggg	tgctatagctatggtgctagttgctat		ggcg	gctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/070	aaccgaagacacggccccgctattactgtgcaacg	c	ta	ggtgctagttgctatgatg		ggcg	ggatctc	tgggggcccaggcattgaagtcgtcgtgtcctca
	1/073	aaccgaagacacggccccgctattactg			ttgctatagctatggtcg		aga	actatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/086	aaccgaagacacggccccgctattactgtgcaagt		gga	atggtgctacttactattactac		ag	attactatctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/094	aaccgaagacacggccccgctattactgttgct			attgctatagctatggtg		aga	actatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	1/097	aaccgaagacacggccccgctattactgtgcaagg		ggtagctgggacaaaggca	gctttag			ttactatgctatggatctc	tgggggcctaggcgttgaagtcgtcgtgtcctca
	1/099	aaccgaagatacggccccgctattactgtgcaagg			attgctatagctatggtgctagttgctat		gacctcaaacgag	tatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	4/03	aaccgaagacacggccccgctattactgtgcaaca		ggc	tgctatagctatggtgctagttgcta			ctatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	4/01	aaccgaagacacggccccgctattactgtgcagga			tgctatagctatggtgctagttgctat		gatgccagat	attactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	4/13	aaccgaagacacggctcgctattactgtgcaaca			gctatggtgctagttgctat			ggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	4/02	aaccgaagacacggccccgctattactgtgcgata		gtgg	tagct		ggg	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	6/03	aaccgaagacacggccccgctattactgtgcaaga		gccgg	tgctatagctatggtgctagttgct		ggg	ggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	6/12	aaccgaagacacggccccgctattactgtgcaaca		attatctggg	tagctatggtgctagttgctat		gatg	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	7/05	aaccgaagacacggccccgctattactgtgcgata		ggctggg	ttgctatagctat	ata		c	tgggggcccaggcgttgaagtcgtcgtgtcctca
40day	1	aaccgaagacacggccccgctattactgtgcaaga		ggct	tagctatggtgctagttgctata		attggta	atgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal	2	aaccgaagacacggccccgctattactgt			tgctatagctatggtg		aga	actatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
liver	22	aaccgaagacacggccccgctattactgtgcaaca		ag	tatg		atagg	tgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	33	aaccgaagatacggccccgctattactgtgcaagt			tgctatagctatggtgctagttgcta		cgact	ctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	44	aaccgaagacacggccccggttattactgtgcgacc		cttggg	gctagtt		tagtcacacctgaaacccgaaaaaatgt	atctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	51	aactgaagacacggccagcgttattactgtgcaaga		ggcc	tagctatggtgctagttgcta		aaggatgttgggat		tgggggcctaggcgttgaagtcgtcgtgtcctca
60day	A2	aaccgaagacacggccccgctattactgtgcaaca		ggcct	ttgctatagctatggtgctagttgctatagcgac		ta	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal	A5	aaccgaagacacggccccgctattactgtgcaaga		g	attgctatagctatggtgctagttgc			tactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
spleen	A8	aaccgaagacacggccccgctattactgtgcaaca		ggcgggtgcaatagg	tatggtgctagttgct		t	ctatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	A11	aaccgaagacacggccccgctattactgtgcaaga		ggct	tagctatggtgctagttgctata		atggta	atgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	B10	aaccgaagacacggccccgctattactgtgcagca	tgc	g	atggtgctagttgctat		tatgac	gctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
69day	108	aaccgaagacacggccccgctattactgtgcaaga		g	tagctatggtgctagttgctat		c	atggatctc	tgggggcccaggagttgaagtcgtcgtgtcctca
fetal	113	aaccgaagacacggccccggttattactgtgcgata		ggctac	atagctatggtgctagttgctatgtgac		tattaagt	attactatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
spleen	114	aaccgaagacacggccccgctattactgtgcgata	t	tacct	aattgctatagctatggtgctagttgcta		cccct	ctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	116	aaccgaagacacggccccgctattactgtgcaaga		ggcta	ctatagctatggtgctagttgctatgtgac		tag	tatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	119	aacagaagacacggccccgctattactgtgcaggg			aattgctatagctatggtgctagttgc		ccgtaccgtggg	ctc	tgggggcccaggtgttgaagtcgtcgtgtcctca
	121	aaccgaagacacggccccgctattactgtgcaaca		gg	tagctatggtgctagttgctat		gatg	actatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	126	aaccgaagacacggccccgctattactgtgcaaga		ggcca	ctatagctatggtgctagttgctat		gggttgtca	tatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	127	aaccgaagacacggccccgctattactgtgcaagg		attc	attgctatagctatggtgctagttgctat		ttcgaca	actatgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
	130	aacagaagacacggccccgctattactgtgcaggc		cg	ttgctatagctatggtgctagttgc		cactgggggg	tggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca

FIGURE 3. The nucleotide sequence of unique CDR3s recovered from 30-, 40-, 60-, 70-, 90-, and 110-day-old fetuses. Sequences are grouped according to their usage of D_HA, D_HB, or unidentified D_H segments. Duplicate sequences (Table I) have been deleted.

93day	5	aacagaagacacggccgcattactgtgcagga	t	gcgg	tagctatggtgctagttgctata	ta		tactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
fetal	7	aacgaagacacggccgcattactgtgcaaca		ggtggag	tagctatggtgctagttgctat		gccata	tatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
spleen	8	aacgaagacacggccgcattactgtgcaaca		ggcc	tgctatagctatggtgctagt			tgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	9	aacgaagacacggccggtattactgtgcaaga			attgctatagctatggtgctagttgct		cccagta	atgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	12	aacgaagacacggccgcattactgtgcaaca		ggcttgg	attgctatagctatggtgctagttgctat		cccc	atgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	16	aacgaagacacggccgcattactgtgcaaga		ggctttt	attgctatagctatggtgctagttgctata	ta		gctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	20	aacgaagacacggccgcattactgtgcaaga		ataaaact	tatggtgctag		atat	ctatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	21	aacagaagacacggccgcattactgtgcagga	t	gcactg	gctatggtgctagttgctat		ttacgg	tgggggcccaggcgttgaagtcgtgtcctca
	22	aacgaagacacggccgcattactgtgcaaga			tatagctatggtgctagttg		gg	atgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	27	aacgaagacacggccgcattactgtgcaaca		ggc	agctatggtgctagttgct		tggcaattcg	ctatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
110day	C1	aacgaagacacggccgcattactgtgcgact			tgctatagctatggtgctagttgctat		gcggt	ctatgctatggttctc	tgggggcccaggcgttgaagtcgtgtcctca
fetal	C5	aacgaagacacggccgcattactgtgcgata		gcgggggg	tatagctatggtgc	g	gg	atgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
spleen	C8	aacgaagacacggccggtattactgtgcgata	t	gtggga	tatggt		ccc	attactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	C9	aacgaagacacggccgcattactgtgcaaca			ttgctatagctatggtgctagttgctat		cttggaga	tatggatctc	tgggggcccaggcgttgaagtcgtgtc
	D1	aacgaagacacggccgcatta		gga	gctatagctatggtgctagttgctatag	c	aggcggat	tatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	D3	aacgaagacacggccggtattactgtgcgatt		gctat	ctatagctatgg		ggg	tatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	D9	aacgaagacacggccgcattactgtgcaaca		ggccgtc	gctatagctatggtgctagttgc			tactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	D12	aacgaagacacggccggtattactgtgcaaga		gagtgctg	tagctatggtgctagttgctatgtagtgac	g	cgccgtgg	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	R1 4	aacgaagacacggccgcattactgtgcaaga			atagctatggtgctagttg		agg	attactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	R2 12	aacgaagacacggccgcattactgtgcaaag		ttacggaggggg	gctatggtgctagttgc		cccatgccag	tatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	R1 19	aacgaagacacggccgcattactgtgcaagt		gg	tagct		gg	tatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	Clone								
	name	FR3	p	n	DH B	p	n	JH	FR4
DHB									
30day	1/004	aacgaagacacggccgcattactgtgcaaga			atagcggttcctatagcgtttac	gt	tctatc	tggatctc	tgggggcccaggcgttgaagtcgtgtcctca
fetal	1/027	aatcgaagacacggccgcattactgtgcaaga		g	atagcggttcct		gggg	ggatctc	tgggggcccaggcgttgaagtcgtgtcctca
liver	1/030	aacgaagatacggccgcattactgtgcaaga	t		tatagcggttgctatagcggttac			tatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	1/046	aacgaagacacggccgcattactgtgcagga	t	gcgaaag	ctatagcggttgctatagcggttac			tatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	1/075	aacgaagacacggccggtattattgttcgatgg		g	ctatagcggttgctactgc		tatccgaa	actatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	1/077	aacgaagatacggccgcattactgtgcagga	t		gcggttgctatagcggttac			tatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	4/04	aacgaagacacggccgcattactgtgcaaga		ggcc	gcggttgctatagcggttac			gctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	4/05	aacgaagacacggccggtattactgtgcg			atagcggttgctatagcggttac		tgaa	gctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	4/06	aacgaaggcacggccggtattactgtgcgata		ggcaaagg	tagcggttgctatagcggt	a	tatgt		tgggggcccaggcgttgaagtcgtgtcctca
	4/09	aacgaagacacggccccggtattactgtgcaacg		tc	gactatagcggtcgctatagcggttac	g	ggaaaa	gctatggatctc	tgggggcccaggcattgaagtcgtgtcctca
	4/10	aacgaagacacggccgcattactgtgcaaca		ggttttt	tagcggttgctatagcggtt	cc	ggg	atggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/09	aacgaagacacggccgcattactgtgcaaga		ac	gactatagcggttgctatagcgg		ctt	tatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/01	aacgaagacacggccggtattactgtgcgata		g	taacggttgctatagcggttac		cg	tc	tgggggcccaggcgttgaagtcgtgtcctca
	6/05	aacagaagacacggccgcattactgtgcga			tgactatagcggttgctatagcggttac	gt	ccc	ctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/06	aacagaagacacggccgcattactgtgcagga	t	gga	cggttac	gt	t	tatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/07	aacgaagacacggccgcattactgtgcaagg		gg	agcggttgctatagcggt		cg	attactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/08	aacgaagacacggccgcattactgtgcaact	a	ta	cggttgctatagcggtta		actggcatc	tactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/10	aacgaagacacggccgcattactgtgcaaga		ac	gactatagcggttgctata		cggccctttat	ctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	6/11	aacgaagacacggccggtattactgtgcg			atagcggttgc		cgg	tatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	7/01	aacagaagacacggccgcattactgtgcagga		gcct	gttcctgtag		aagtggccgg	atggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	7/02	aacgaagacacggccgcattactgtgcaaga	t	tcttaca	ggttgc		ctaa		tgggggcccaggcgttgaagtcgtgtcctca
	7/03	aacgaagacacggccggtattactgtgcaact	a	tagcggtg	ctatagcggttgctatagcggttac	g	ggagggactt	ttactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	7/04	aacagaagacacggccgcattactgtgcagga	t	gcatata	cggttgctatagcggttac	gt	taaaac	ttactatgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	7/07	aacgaagacacggccgcattactgtgcgata		ggct	taacggttgctatagcggtta	ta		atggatctc	tgggggcccaggcgttgaagtcgtgtcctca
	7/09	aacagaagacacggccgcattactgtgcagga	t	gc	agcggttgctatagcggtta			tgctatggatctc	tgggggcccaggcgttgaagtcgtgtcctca

FIGURE 3—*Continued*.

	7/10	aacagaagacacggccccgctattactgtgcaaca		gg	ctatagcggttgctatagcggttac				tactatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	7/11	aaccgaagacacggccccgctattactgtgcaaga			gcggttgctatagcggttac		caat			tgggggccaggcgttgaagtcgtcgtgtcctca		
	7/13	aaccgaagacacggccccgctattactgtgcaaca			gcggttgctatagcggttac	g	gggatgggggcc		tggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
40day	4	aaccgaagacacggccccgctattactgtggtatt		ggcctagtgccagagtgctcgttac	ggttac				ggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
fetal	6	aaccgaagacacggccccgctattactgtgcaaca			gactatagcggttgctatagcgg		acccggcct		tatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	8	aaccgaagacacggccccgctattactgtgcaaga			gactatagcggttgctatagcggttac	g	act		tatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	9	aaccgaagacacggccccggtattactgtggcgaag			tatagcggttgctatagcggttac	gtaa	tacggtg		tgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	10	aaccgaagacacggccccgctattactgtgcaaga		gcc	atagcggttgctatagcggttac	g	ctgg		c	gggccaggcgttgaagtcgtcgtgtcctca		
	12	aaccgaagacacggccccgctattactgtgcaaca			gactatagcggttgctatagcggttac	g	aattacct		tatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
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	17	aaccgaagacacggccccgctattactgtgcaaca			gactatagcggttgctatagcgg		t		tc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	20	aaccgaagacacggccccgctattactgtggcgaag			tatagcggttgctatagcggttac	gtaa	tacgg		tatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
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	31	aaccgaagacacggccccgctattactgtgcaata			agcggttgctatagcggttac		tga		ggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	32	aaccgaagacacggccccggtattactgtgcgact			atagcggttgctatagcggt		gacgacgt		attactatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	34	aaccgaagacacggccccggtattactgtgcgaca		aaaaggtcta	ttgctatagc		tataggg		gctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	35	aaccgaagacacggccccgctattactgtgca			gactatagcggttgctatagcgg				ttactatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	36	aaccgaagacacggccccggtattactgtgcgatg			gttgctatagcggttac		agggggcgggtaaccggt		ctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	39	aacagaagacacggccccgctattactgtgcaaac		tta	gactatagcggttgctatagcggttac		aagt		tc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	41	aaccgaagacacggccccgctgttactgtgcgct			agcgg	cc	c		ttactatgctatggatctc	tgggggccaggcgttgaagtgtcgtgtccaa		
	43	aaccgaagacacggccccgctattactgtgcagga	t	g	tatagcggttgctatagcggt				tatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	50	aaccgaagacacggccccaactattactgtgcaaga		gagggg	gcggttgctat		tgggggga		ctatggatctc	tgggggccctggcgttgaagtcgtcgtgtcctca		
60day	A1	aaccgaagacacggccccgctattactgtgcgata		gggaac	actatagcggttgctatagcggt	g	gggtgtt		ctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
fetal	A3	aactgaagacacggcacgctattactgtgcaagc			gttgctatagcggttac				atgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
spleen	A4	aaccgaagacacggccccgctattactgtgcaaac		atggcc	atagcggttgctatagcggttac		ca		tatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	A6	aaccgaagacacggccccgctattactgtgcgata		ggccggtcctac	gactatagcggttgctatagcggt		gggtgtt		ctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	A7	aaccgaagacacggccccgctattactgtgcaact		cgtgggatg	tatagcggttgctatagcggttac	g	agggacctta		tatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctat		
	A9	aaccgaagacacggccccgctattactgtgcgata		gggaac	actatagcggttgctatagcggttac		ttt		tacggatctc	tgggggccaggcgttgaagtcgtcgtgtcctat		
	A12	aacgaagacacggccccgctattattgtgcagga	t	gccc	tgactatagcggttgctatagcggttac	gt	tg		actatgttatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctat		
	B1	aaccgaagacacggccccgctattactgtgcaact		cgtgggatg	tatagcggttgctatagcggttac	g	agggaccttatatggat		tccc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	B4	aaccgaagacacggccccgctattactgtggtacgg			tatagcggttgctatagcggttac	g	gtaatttg		tggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	B5	aaccgaagacacggccccgctattactgtgcaaca		gg	ctatagcggttgctatagcggttac	g			atgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
69day	104	aaccgaagacacggccccggtattactgtgcgaag			actatagcggttgctatagcggttac		actg		tactatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
fetal	105	aaccgaagacacggccccgctattactgtggcgata		gg	tatagcggttgctatagcggttac		ca		actatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
spleen	106	aaccgaagacacggccccgctattactgtgcaaca		ggcct	tagcggttgctatagcggttac	g	ggctcgggcc		ggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	111	aaccgaagacacggccccggtattactgtggcgata		gg	agcggttgctatagcggt				tatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	115	aaccgaagacacggccccgctattactgtgcaaca		gg	ctatagcggttgctatagcggtt		gggg		tctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	117	aaccgaagacacggccccgctattactgtgcaagg		agcgtg	gcggttgctatagcgg		gccg		c	tgggggccaggcgttgaagtcgtcgtgtcctca		
	118	aaccgaagacacggccccgctattactgtgcaggg			actatagcggttgctatagcggtta	t	ggcc		ctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	120	aaccgaagacacggccccggtattactgtgcaatg		gag	tagcggttgctatagcggttac	gt	tttaggg		tctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	128	aactgaagacacggcacgtattactgtgcaaga			gactatagcggttgctatagcgg		atgcctttatga		ggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	129	aacagaagacacggccccgctattactgtgagga			tgctatagcgg				ttactatgctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		
	134	aaccgaagacacggccccgctattactgtgcaaca		gg	ctatagcggttgctatagcggtt		cca		ctatggatctc	tgggggccaggcgttgaagtcgtcgtgtcctca		

FIGURE 3—*Continued*.

93day	1	aaccgaagacacggcccgctattactgtgcgata		a	tgactatagcggctactatagcggt		ccg		c tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal	3	aaccgaagacacggcccgctattactgtgcaaca		aa	tatagcggttgctatagcggttac		ttt		tatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
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	11	aaccgaagacacggcccggtattactgtgcgaca		gttg	tagcggttgct		c		attactatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	17	aaccgaagatacggcccgctattactgtgcaagg		gtgggg	tagcggttgctatagcggttac	g	accccgggggcgggg		gctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
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	24	aaccgaagacacggcccgctattactgtgcaaaa			tagcggttgctatagcggt				tatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	25	aaccgaagacacggcccgctattactgtgcaagt		ttc	gactatagcggttgctatagcggttac	g	gggt		tctc tgggggcccaggcgttgaagtcgtcgtgtcctca
110day	C2	aaccgaagacacggcccgctattactgtgcaact			agcggttgctatagcgg	cc	cat		attactatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal	C3	aaccgaagacacggcccgctattactgtgcaact		tgg	agcggttgctatagcggttac		tttgttggccgcgattta		atggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
spleen	C6	aaccgaagacacggcccggtattactgtgcaaga		gtgagg	gcggttgctatagcggttac	gt	tgaga		ctc tgggggcccaggcgttgaagtcgtcgtgtcct
	C7	aaccgaagacacggcccgctattactgtgcgcgg		gaa	agcggttgctatagcggttac				gctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	C11	aaccgaagacacggcccgctattactgtgcagga			agcggttgctata				ctatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	C12	aaccgaagacacggcccgctattactgtgcgaca		gaaa	atagcggttgctatagcggt		cgcc		atgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	D2	aaccgaagacacggcccgctattactgtgcgata	t	t	tatagcggttgctatagcggttac				tgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	L3 1	aacagaagacacggcccgctattactgtgcagga	t		tagcggttgctatagcggttac	gt	gga		ggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	L3 2	aacagaagacacggcccgctattactgtgcaaca			tagcggttgctatagcgg				ttactatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	L3 3	aaccgaagacacggcccgctattactgtgcgaca		ggcattgtggg	ctatagcggttgctatagcggttac				tatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R1 5	aaccgaagacacggcccggtattactgtgcggta		ggccttaag	tatagcggttgctatagcggttac		ttgaaggggggttaa		tatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R1 6	aaccgaagacacggcccgctattactgtgcgata		ggc	agcggttgctatagcggttac	g			actatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	L3 9	aacagaagacacggcccgctattactgtgcaaca			tagcggttgctatagcggt				tatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R2 10	aaccgaagacacggcccgctattactgtgcgata		ggccaggattcc	gactatagcggttgctatagcggt				tatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R2 11	aaccgaagacacggcccgctattactgtgcaaca		ctgat	tatagcggttgctatagcggttac		agtgtgaatttttttcctacta		tc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R3 13	aaccgaagacacggcccggtattactgtgcgata			gcggttgctatagcggtt		gagg		atgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R3 14	aaccgaagacacggcccgctatta			gcggttgctatagcggttac				ttactatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R3 16	aaccgaagacacggcccgctattactgtgcgata			atagcggttactatag		gcc		ctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	L3 17	aaccgaagacacggcccggtattactgtgcgata		gcct	ctatagcggttgc				ctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	L3 18	aactgaagacggcggcccgctattactgtgcaaca		ggcc	tagcggttgctatagcggttac		t		ttactatgctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R1 20	aacagaagacacggcccgctattactgtgcaggg			actatagcggttgctatagcggtt		gatagc		gctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	R2 22	aaccgaagacacggcccggtattactgtgcgata		gacaaaact	tatagcggttgctatagcggtt		ctgtc		tactatgctatggatctc tgggggcccaggcgttgaagtcgtcgtg
	R2 23	aacagaagacacggcccgctattactgtgcagga	t	gta	atagcggttgctatagcggttac	g	ac		gctatggatctc tgggggcccaggcgttgaagtcgtcgtgtcctca
	Clone								
DH unid'd	name	FR3	p	n	DH unidentified	p	n	JH	FR4
40day	5	aaccgaagacacggcccgctattactgtgcaaca			agtatgatagg			tgctatggatctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal	26	aaccgaagacacggcccgctattactgtg			accctcgtgggc			atctc	tgggggctaggcgttgaagtcgtcgtgtcctca
110day	R2 21	aaccgaagacacggcccgctattactgtgcaaga			ggcatgtgtct			tctc	tgggggcccaggcgttgaagtcgtcgtgtcctca
fetal									
spleen									

FIGURE 3—Continued.

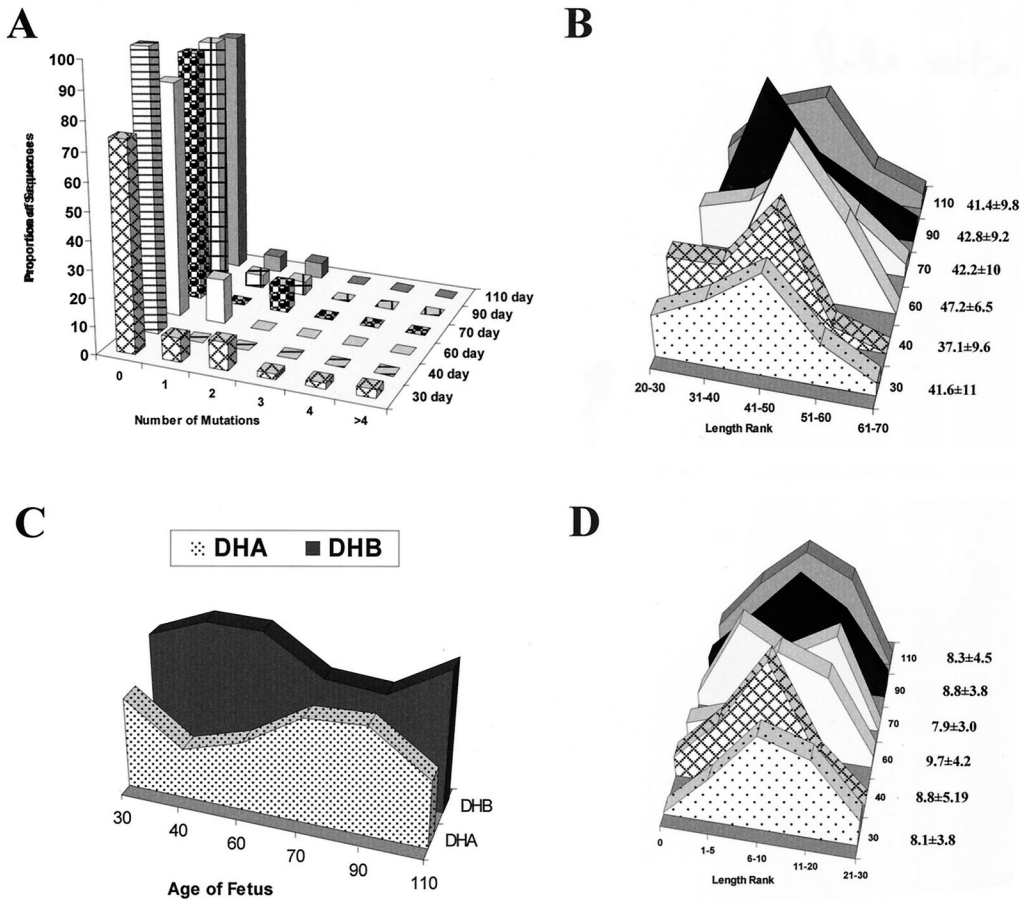

FIGURE 4. Characteristics of CDR3 during fetal development in piglets. Data obtained by analysis of the sequences presented in Fig. 3. *A*, The frequency of somatic point mutation in CDR3 in randomly cloned VDJs from fetal piglets of various ages. *B*, The distribution of CDR3 lengths during development in piglets. Sequences for 30 and 40 days were obtained from liver. All other sequences were recovered from spleen. Results are presented as the percentage of total usage according to the length of CDR3 ranked into the categories shown on the *x*-axis. The mean CDR3 lengths and their standard deviation (depicted as ±) are given to the *right* of the histogram in correspondence with the age of the fetus. *C*, The proportional usage of D_HB and D_HA in VDJ sequences recovered from fetal piglets of various ages. *D*, The distribution of N region additions in CDR3 during development. Data are presented as the mean ± SD for N region additions ranked into the categories indicated on the *x*-axis. The mean and SD for each age is given on the *right*.

whether diversity in the preimmune CDR3 repertoire changes during development, and 3) whether CDR3 diversity or its spectratype differs among lymphoid tissues.

Fig. 2 shows that the CDR3 spectratype is oligoclonal in the 30-day-old fetal liver. This was true in nearly all animals tested (Fig. 1*B*; animal 1 excepted) and is not particularly surprising, since the fetal liver at 30 days is the first site at which VDJ rearrangement is detected (20). This oligoclonal pattern suggests that the preimmune B cell compartment at 30 days is small, an observation supported by the high proportion of duplicate clones recovered (Table I).

Noteworthy is that while the CDR3 spectratype in fetal liver is polyclonal by day 50, that of fetal spleen remains oligoclonal to at least day 70. While the oligoclonality of early fetal liver may simply be a consequence of the size of the B cell compartment, the oligoclonal pattern in spleen may result from selection by some intrinsic ligand. We believe that this is likely, since after 50 days there is a marked expansion of the B cell (34) and peripheral T cell (29) compartments so that a polyclonal splenic spectratype would be expected if immigration and proliferation had been random.

While short CDR3s are the hallmark of early pre-B cells in humans (35, 36), we found no evidence that short CDR3s were predominant in early fetal life and progressively increased in length during development (Fig. 4). The increased length of CDR3 in adult vs fetal mice and humans has been ascribed to the absence or limited use of N region additions during fetal life (36–39) and the use of shorter D_H or J_H segments in fetal life (39, 40). In the fetal pig N region additions are the same length at 30 days as they are at 110 days (Fig. 4*D*). In fact, the mean CDR3 length at 30 days (Fig. 4*B*) is only one codon shorter than reported for adult humans (40) and is the same length as we have observed in both colonized isolator piglets and adults (21). Consistent with studies in mice (39) and humans (16, 36, 40), piglets also first use their shortest D_H segment (D_HB; Fig. 4*D*). The preferential usage of D_HB in the earliest VDJ rearrangements is consistent with the usage of a J_H-proximal D_H segment as reported in rabbits (24), humans (37, 37, 41, 42), and mice (43, 44). The preferential use of RF1 in the VDJ of fetal mice (44) and of RF3 in newborn rabbits (23) was not observed in swine. However, we previously reported that RF1 of both D_HA and D_HB contains a stop codon (19), so all arrangements using RF1 are nonproductive (Table I). The reason why we observed greater RF1 usage in late term and GF piglets than in very early gestation fetuses is unexplained. While a higher usage of RF1 could lead to a higher proportion of nonproductive rearrangements, it does not explain the lower proportion of in-frame rearrangements observed at this time. Since it has been shown by some (45), but not all (40), that TdT expression abrogates RF bias, our finding of random/nearly equal usage of RF2 and RF3 in fetal piglets

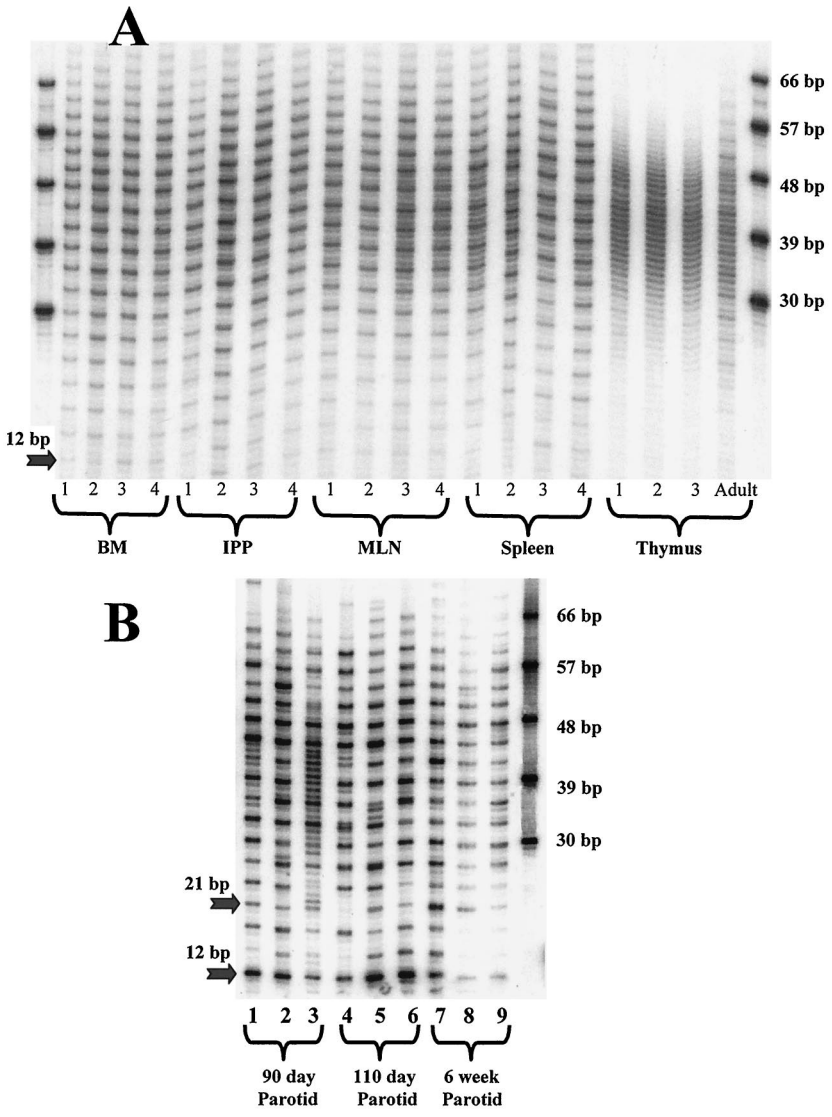

FIGURE 5. Spectratypic profiles of CDR3 in various tissues of different outbreed fetal piglets. *A*, The comparative CDR3 spectratypic in bone marrow, IPP, MLN, spleen, and thymus from four outbred 110-day-old fetal piglets. Polynucleotide size standards are shown on both *right* and *left*, with the locations of the 21- and 12-bp polynucleotides indicated by arrows. The faint bands between the major polynucleotides (those representing the productive rearrangements) in bone marrow, IPP, MLN, and spleen are CDR3s out-of-frame by having one too many or one too few nucleotides. Note that all polynucleotide bands are of equal intensity in fetal thymus, whereas some evidence of selection is seen in the thymus of a young pig provided for comparison. *B*, The CDR3 spectratype of B cells from the parotid gland of 90- and 110-day-old fetal piglets. The spectratype of B cells in the parotid gland of 6-wk-old isolator piglets is shown on the *extreme right*. Polynucleotide size is indicated as described in *A*.

(Table I) may not be surprising. Our failure to observe changes in CDR3 length or N region additions compared with mice and human is consistent with the view that TdT in fetal piglets is fully active at the earliest time at which VDJ rearrangement occurs during embryological development.

Although we found no progressive age-dependent change from short to longer CDR3 during development, we did find a 12-bp CDR3 that occurred in fetal liver, fetal spleen, and fetal parotid B cells, but was absent or no longer prominent in the CDR3 repertoire of lymphoid tissues/organs of older fetuses. It is possible that such very short CDR3s characterize early B cell lymphogenesis, but disappear from the spectratype due to lack of positive selection. Consistent with idea, we recovered triplicate clones from 40-day-old fetal liver that had D_HA truncated to four nucleotides (clone 3; Fig. 3). VDJs with highly truncated D_H segments are very often recovered from fetal thymus (J. Sun and M. Sinkora, unpublished observations). If the unselected CDR3 profile of thymus (Fig. 5*A*) reflects B cell lymphogenesis in this organ (see below), the occurrence of such short CDR3s may reflect unselected B cells that should be rare among B cells in older fetuses. Since it is generally believed that the parotid depends on immigration of B cells from especially secondary mucosal lymphoid tissues, e.g., Peyers patches, early immigrants may be unselected, thus explaining the 12-bp CDR3 found in VDJs from this organ even in late term fetuses. In fact, VDJs are difficult to recover from the parotid before day 90 and are highly oligoclonal (data not shown).

It has been shown in studies of mice and humans, that CDR3 length is regulated during rearrangement or by selection during development (40). This is also true in swine, in that 1) CDR3 lengths of 36–45 bp dominate the 30-day spectratype (Fig. 2*B*); 2) the mean CDR3 length does not change during 84 days of fetal development (Fig. 4*B*); and 3) the longer porcine D_HA is trimmed during usage in CDR3 to the same length as the shorter D_HB (Table I).

Although swine are also artiodactyls, the extremely long CDR3s reported for cattle (46, 47) are not seen in the preimmune repertoire of the piglet. Another phylogenetic discontinuity is our failure to find that somatic point mutation contributes to repertoire diversity in the fetal piglets (Fig. 4*A*), although this has been reported to be a major Ag-independent mechanism in the λ-chains of other artiodactyles, such as sheep and cattle (6, 8, 11). Apart from the idea that λ-chain diversification may be regulated differently from heavy chain diversity, it seems increasingly clear that phylogeny is not a reliable indicator of the pattern or mechanism of repertoire development among mammals (48).

The fact that maternal regulatory factors are unable to cross the swine placenta probably does not explain the different features of CDR3 in fetal piglets vs those in mice and humans. For example, it seems unlikely that suppression of TdT activity in fetal mice and in early gestation human fetuses is due to maternal factors, although we could not identify studies that directly tested this hypothesis. The apparent early intrinsic onset of TdT activity in fetal piglets may have evolved as a compensatory mechanism in a species with limited combinatorial diversity. On the other hand, reported differences in CDR3s between fetal and adult mice or humans is quite likely another matter, and some differences may be the result of extrinsic factors acting on the fetal immune system, since these cannot or have not been controlled in rodent and primate studies. In the piglet we have already shown that there are no differences in V_H usage, D_H usage, or the characteristics of CDR3 in piglets reared germfree for 6 wk in an isolator (21). On the other hand, both colonized isolator piglets and adults use D_HA two or three times more frequently than D_HB, suggesting that pronounced D_HA usage is a marker for diversification of the VDJ repertoire in swine as is increased CDR3 length due to N region additions 3′ of D_H (21). In fetal piglets we observed a trend to greater D_HA usage during development, but unlike colonized or conventional animals, no increase in N region additions was seen (Fig. 4*D*). Other differences between fetal and adult mice may be intrinsic. For example, the decline in V_H 81X usage (a D_H-proximal V_H gene) in adult mice occurs before surface Ig is expressed on pre-B cells (49). Interestingly, this decline parallels a decrease in the ratio of productive/nonproductive rearrangements (50). This decline is exactly what we observed in fetal piglets (Table I), whereas Tunyaplin and Knight (51) observed the opposite effect in rabbits. In piglets, this decline is also paralleled by the tendency to shift from usage of D_HB to D_HA. This decline in the frequency of in-frame rearrangements may suggest that 1) cells carrying both nonproductive and in-frame rearrangements are inhibited in proliferation; 2) rearrangement is nonstoichastic and differentially regulated during ontogeny in fetal liver vs bone marrow; 3) rearrangements in the swine variable heavy chain locus in fetal liver may follow a pattern reminiscent of the TCRα rearrangement, in which multiple attempts can be made on the same chromosome (52, 53); or 4) a mechanism may have evolved in swine that favors repair or rescue of an out-of-frame rearrangement before switching to the use of the second chromosome. In support of the second possibility, it is interesting that usage of nonproductive RF1 increases as the proportion of in-frame rearrangements decreases (Table I), suggesting more stringent control of early VDJ rearrangements. The third possibility is inconsistent with the known organization of the porcine V_H-D_H-J_H locus, because swine have only a single J_H (18) so that progressive rearrangement attempts in this locus, as described for TCRα, would be impossible. Various permutations of the last possibility have been recently discussed by Nemazee (22).

The thymus is not regarded as a B cell organ, although reports of the occurrence of B cells in the thymus date back 35 yr (54). Within this time span, the presence of thymic B cells, plasma cells, and/or their products has been shown in cattle (55), humans (56), swine (19, 57), and mice (58). Our observations on the nature of the VDJs recovered from the porcine thymus are consistent with the idea that cells expressing productive rearrangements have not been selected. This is especially surprising, since even in bone marrow, a known site of B cell lymphogenesis in the fetal piglet (J. Sinkora et al., unpublished observations) selection is apparent (Fig. 5). Therefore, such a spectratype can be interpreted to mean that 1) such cells represent an epiphenomenon in which B cell precursors that migrate to the thymus do not encounter selection pressures for those with in-frame rearrangements; 2) the observed spectratype is derived from thymocytes that have rearranged their heavy chain Ig segments and carry them in a nonfunctional state; or 3) the thymus is a true site of B cell lymphogenesis, and our studies have captured pro-B cells before selection and/or migration to other sites where selection occurs. Ig, specifically Ig DJ rearrangements, have been reported in human thymocytes (59, 60). To rule out the second possibility in swine, we bulk-sorted thymocytes from a late term fetus and T cells from spleen. We could not identify any developing peripheral T cells containing VDJ rearrangements (data not shown). Therefore, we conclude that the VDJ rearrangements in the thymus of fetal piglets are indeed associated with the B cell lineage, consistent with the recent report by Akashi et al. that B cell lymphogenesis occurs in the mouse thymus (61).

Gene conversion is an attractive mechanism for increasing VDJ diversity in species that use few V_H genes. However, experimental evidence to support this idea, beyond data obtained by PCR-independent studies in chickens and rabbits, is ambiguous. This is especially true since we show elsewhere that when homologous templates are amplified in the same PCR, a surprisingly high proportion of the resultant VDJs are in vitro chimeras resulting entirely from PCR (62).

Based on the recovery of $<$200 unique CDR3 sequences associated with only five nonmutated V_H genes, junctional diversity in CDR3 accounts for $>$95% of repertoire diversity during fetal life in piglets. This diversity is associated with no change in length and with only a gradual change in D_H usage. Since no maternal factors cross the swine placenta, the subtle changes we did observe in CDR3, such as the gradual shift from D_HB to D_HA usage and the decline in the proportion of in-frame rearrangements in late gestation, are intrinsic features of the developing preimmune repertoire in this species. The CDR3 length distribution before 50 days of fetal age is oligoclonal in fetal liver and is polyclonal in all lymphoid tissues of older fetuses as well as in the parotid. This may explain the ability of fetal piglets to make Abs to a broad spectrum of Ags during the second half of gestation (25, 26). Considering the limited combinatorial diversity and lack of somatic mutation in fetal piglets, our results are consistent with the views of Davies et al. regarding both Ab (28) and T cell repertoires (63), in that junctional diversity in CDR3 is responsible for most VDJ diversity and perhaps Ab specificity. Interestingly, in sharks, where VDJ rearrangement does not occur and variable regions are encoded by approximately 200 tandomly arrayed VDJs that are already rearranged in the germline, Ab diversity in these elasmobranches is predominately encoded in CDR3 (64). Thus, a species with very restricted combinatorial diversity and those lacking it altogether are still able to generate a diverse Ab repertoire using CDR3 alone.

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References

1. Osmond, G. D. 1990. B cell development in the bone marrow. *Semin. Immunol. 2:173.*
2. Reynaud, C. A., B. A. Imhof, V. Anques, and J. C. Weill. 1992. Emergence of committed B lymphoid progenitors in the developing chicken embryo. *EMBO J. 12:4349.*
3. Weill, J.-C., and C. A. Reynaud. 1987. The chicken B cell compartment. *Science 238:1094.*
4. Weinstein, P. D., A. O. Anderson, and R. G. Mage. 1995. Rabbit Igh sequences in appendix germinal centers: V_H diversification by conversion-like and hypermutation mechanism. *Immunity 1:647.*
5. Reynaud, C. A., C. R. MacKay, R. G. Müller, and J. C. Weill. 1991. Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyers patches. *Cell 64:995.*

6. Parng, C.-L., S. Hansal, R. A. Goldsby, and B. A. Osborne. 1996. Gene conversion generates immunoglobulin gene diversity in cattle. *J. Immunol. 157:547.*
7. Reynaud, C. A., C. Garcia, W. R. Hein, and J.-C Weil. 1995. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. *Cell 80:115.*
8. Wagner, S. D., and M. S. Neuberger. 1996. Somatic hypermutation of immunoglobulin genes. *Annu. Rev. Immunol. 14:441.*
9. Becker, R. S., and K. L. Knight. 1990. Somatic diversification of immunglobulin heavy chain VDJ genes: evidence for somatic gene conversion in rabbits. *Cell 6387.*
10. Short, J. A., P. Sethupathi, S. K. Zhai, and K. L. Knight. 1991. VDJ genes in V_Ha2 allotype-suppressed rabbits. *J. Immunol. 147:4014.*
11. Lucier, M. R., R. E. Thompson, J. Waire, A. W. Lin, B. A. Osborne, and R. A. Goldsby. 1988. Multiple sites of Vλ diversification in cattle. *J. Immunol. 161:5438.*
12. Reynaud, C. A., V. Anquez, H. Grimal, and J. C. Weill. 1987. A hyperconversion mechanism generates the chicken light chain pre-immune repertoire. *Cell 48:379.*
13. McCormick, W. T., and C. B. Thompson. 1990. Somatic diversification of the chicken immunoglobulin light-chain gene. *Adv. Immunol. 48:41.*
14. Lydyard, P. M., C. E. Grossi, and M. D. Cooper. 1976. Ontogeny of B-cells in the chicken. I. Sequential development of clonal diversity in the bursa. *J. Exp. Med. 144:79.*
15. Cairns, E., P. C. Kwong, V. Misener, P. Ip, D. A. Bell, and K. A. Siminovitch. 1989. Analysis of variable region genes encoding a human anti-DNA antibody of normal origin: implications for the molecular basis of human autoimmune responses. *J. Immunol. 143:685.*
16. Logtenberg, T., F. M. Young, Y. van Es, F. H. Gmelig-Meyling, J. E. Berman, and F. W. Alt. 1989. Frequency of V_H-gene utilization in human EBV-transformed B-cell lines; the most J_H-proximal V_H segments encode autoantibodies. *J. Autoimmun. 2(Suppl.):203.*
17. Sun, J., I. Kacskovics, W. R. Brown, and J. E. Butler. 1994. Expressed swine V_H genes belong to a small V_H gene family homologous to human V_H III. *J. Immunol. 153:5618.*
18. Butler, J. E., J. Sun, and P. Navarro. 1996. The swine immunoglobulin heavy chain locus has a single J_H and no identifiable IgD. *Int. Immunol. 8:1897.*
19. Sun, J., and J. E. Butler. 1996. Molecular characteristics of VDJ transcripts from a newborn piglet. *Immunology 88:331.*
20. Sun, J., C. Hayward, R. Shinde, R. Christenson, S. P. Ford, and J. E. Butler. 1998. Antibody repertoire development in fetal and neonatal piglets. I. Four V_H genes account for 80% of V_H usage during 84 days of fetal life. *J. Immunol. 161:5070.*
21. Butler, J. E., J. Sun, P. Weber, P. Navarro, and D. Francis. 2000. Antibody repertoire development in fetal and newborn piglets. III. Colonization of the gastrointestinal tract selectively diversifies the pre-immune repertoire in mucosal lymphoid tissues. *Immunology 100:119.*
22. Nemazee, D. 2000. Receptor selection in B and T lymphocytes. *Annu. Rev. Immunol. 18:19.*
23. Friedman, M. L., C. Tunyaplin, S. K. Zhai, and K. L. Knight. 1994. Neonatal V_H, D_H and J_H gene usage in rabbit B lineage cells. *J. Immunol. 152:632.*
24. Chen, H. T., C. B. Alexander, F. F. Chen, and R. G. Mage. 1996. Rabbit DQ52 and D_H gene expression in early B-cell development. *Mol. Immunol. 33:1313.*
25. Cukrowska, B., J. Sinkora, Z. Rehakova, M. Sinkora, I. Splichal, L. Tuckova, S. Avrameas, A. Saalmueler, R. Barot-Ciobaru, and H. Tlaskalova-Hogenova. 1996. Isotype and antibody specificity of spontaneously formed immunoglobulins in pig fetuses and germ-free piglets: production of $CD5^-$ B-cells. *Immunology 88:611.*
26. Schultz, R. D., J. T. Wang and H. W. Dunn. 1971. Development of the humoral immune response of the pig. *Am. J. Vet. Res. 32:331.*
27. Padlan, E. A. 1994. Anatomy of the antibody molecule. *Mol. Immunol. 31:169.*
28. Xu, J. L., and M. M. Davis. 2000. Diversity in CDR3 region of V_H is sufficient for most antibody specificities. *Immunity 13:37.*
29. Gleeson, M., A. W. Cripps, and R. L. Clancy. 1995. Modifiers of the human mucosal immune system. *Immunol. Cell Biol. 73:397.*
30. Sinkora, M., J. E. Butler, J. Sinkora, and Z. Rehakova. 2000. Development of γ/δ thymocytes during ontogeny of pigs: a flow cytometry study. *J. Immunol. 165:1832.*
31. Brownstein, M. J., J. D. Carpten, and J. R. Smith. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modification that facilitate genotyping. *BioTechniques 22:1004.*
32. Magnuson, V. L., D. S. Ally, S. J. Nylund, Z. E. Karanjawal, J. B. Bayman, J. I. Kmappa, A. L. Lowe, S. Ghosh, and F. S. Collins. 1996. Substrate nucleotide-determined non-templated additions of adenine by Taq polymerase: implications from the PCR-based genotyping and cloning. *BioTechniques 21:700.*
33. Butler, J. E. 1974. The immunoglobulins of the mammary sections. In *Lactation: A Comprehensive Treatise*, Vol. 3, Chapt. 5. B. L. Larson and V. Smith, eds. Academic Press, New York, pp. 217–255.
34. Sinkora, M., J. Sinkora, Z. Rehakova, I. Splichal, H. Yang, R.M.E. Parkhouse, and I. Trebichavsky. 1998. Prenatal ontogeny of peripheral lymphoid cells in miniature pigs. *Immunology 95:595.*
35. Tonnelle, C., A. M. Cuisinier, L. Gauthier, V. Guelpa-Fonlupt, M. Milili, C. Schiff, and M. Fougereau. 1995. Fetal versus adult pre-B or B cells: the human V_H repertoire. *Ann. NY Acad. Sci. 764:231.*
36. Milili, M., C. Schiff, M. Fougeueau, and C. Tonnelle. 1996. The VDJ repertoire expressed in human pre-B cells reflects the selection of bona fide heavy chains. *Eur. J. Immunol. 26:63.*
37. Raaphorst, F. M., E. Timmers, M. J. Kenter, M. J. van Tol, J. M. Vossen, and R. K. Shuurman. 1992. Restricted utilization of germ-live V_H3 genes and short diverse third complementarity-determining regions (CDR3) in human fetal B lymphocyte immunoglobulin heavy chain rearrangements. *Eur. J. Immunol. 22: 247.*
38. Feeney, A. J. 1992. Predominance of V_H-D-J_H junctions occurring at sites of short sequence homology results in limited junctional diversity in neonatal antibodies. *J. Immunol. 149:222.*
39. Bangs, L. A., I. E. Sanz, and J. M. Teale. 1991. Comparison of D_H, J_H and junctional diversity in the fetal, adult and aged B cell repertoire. *J. Immunol. 146:1996.*
40. Shiokawa, S., F. Mortari, J. O. Lima, C. Nunez, F. E. Bertrand III, P. M. Korkham, S. Zhu, A. P. Dasanayake, and H. W. Schroeder, Jr. 1999. IgM heavy chain complementary-determining region 3 diversity is constrained by genetic and somatic mechanisms until two months of age. *J. Immunol. 162:6060.*
41. Mortari, F., J. Y. Wang, and H. W. Schroeder, Jr. 1993. Human cord blood antibody repertoire: mixed population of V_H gene segments and CDR3 distribution in the expressed Cα and Cγ repertoire. *J. Immunol. 150:1348.*
42. Schroeder, H. W., Jr., and J. Y. Wang. 1990. Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. *Proc. Natl. Acad. Sci. USA 87:6146.*
43. Atkinson, M. J., Y. Chang, J. W. Celler, C. Huang, C. J. Paige, and G. E. Wu. 1994. Overuse of mouse D_H gene segment, DFL 16.1 is strain-dependent and determined by cis-acting elements. *Dev. Immunol. 3:283.*
44. Chang, Y., C. J. Paige, and G. E. Wu. 1992. Enumeration and characterization of DJH structures in mouse fetal liver. *EMBO J. 11:1891.*
45. Marshall, A. J., N. Doyen, L. A. Bentolila, C. J. Paige, and G. E. Wu. 1998. Terminal deoxynucleotide transferase expression during neonatal life alters D(H) reading frame usage and Ig-receptor-dependent selection of V regions. *J. Immunol. 161:665.*
46. Berens, S. J., D. E. Wylie, and O. J. Lopez. 1997. Use of a single V_H family and long CDR3 in the variable region of cattle Ig heavy chains. *Int. Immunol. 9:198.*
47. Saini, S. S., W. R. Hein, and A. Kaushik. 1997. A single predominately expressed polymorphic immunoglobulin V_H family related to mammalian group I/clan II, is identified in cattle. *Mol. Immunol. 34:641.*
48. Butler, J. E. 1997. Immunoglobulin gene organization and the mechanism of repertoire development. *Scand. J. Immunol. 45:455.*
49. Marshall, A. J., G. E. Wu, and G. J. Paige. 1996. Frequency of V_H81x usage during B-cell development: initial decline in usage is dependent on Ig heavy chain cell surface expression. *J. Immunol. 156:2077.*
50. Marshall, A. J., C. J. Paige, and G. E. Wu. 1997. V(H) repertoire maturation during B cell development in vitro: differential selection of Ig heavy chains by fetal and adult B-cell progenitors. *J. Immunol. 158:4282.*
51. Tunyaplin, C., and K. L. Knight. 1995. Fetal VDJ gene repertoire in rabbit: evidence for preferential rearrangement of V_H1. *Eur. J. Immunol. 25:2583.*
52. Petrie, H. T., F. Livak, D. G. Schatz, and A. Strasser. 1993. Multiple rearrangements in T-cell receptor α-chain genes maximize the production of useful thymocytes. *J. Exp. Med. 178:615.*
53. McCormick, W. T., M. Liu, C. Postema, C. B. Thompson, and L. A. Turka. 1993. Excision products of TCR Vα recombination contain in-frame rearrangements: evidence of continued V(D)J recombination in TCR^+ thymocytes. *Int. Immunol. 5:80.*
54. Saint-Marie, G. 1965. Plasmacytes in the thymus of the normal rat. *J. Immunol. 94:172.*
55. Butler, J. E., C. F. Maxwell, C. S. Pierce, M. B. Hylton, R. Asofsky, and C. A. Kiddy. 1972. Studies on the relative synthesis and distribution of IgA and IgG1 in various tissues and body fluids of the cow. *J. Immunol. 109:38.*
56. Henry, L., and G. Anderson. 1989. Immunoglobulin-producing cells in the human thymus. *Thymus 12:77.*
57. Cukrowska, B., J. Sinkora, L. Mandel, I. Splichal, A. T. Bianchi, F. Kovaru, and H. Tlaskalova-Hogenova. 1996. Thymic B cells of pig fetuses and germ-free pigs spontaneously produce IgM, IgG and IgA: detection by ELISPOT method. *Immunology 87:487.*
58. Miyana-Inaba, M., S.-I. Kuma, K. Inaba, H. Ogata, H. Iwai, P. Yasumizu, S. Muramaatsu, R. M. Steinman, and S. Ikehara. 1998. Universal phenotype of B-cells in the thymus of normal mice. *J. Exp. Med. 168:811.*
59. Berger, C. L., A. Eisenberg, L. Soper, J. Chow, J. Simone, Y. Gapas, B. Cacciapaglia, L. Bennett, R. L. Edelson, and D. Warbarton. 1988. Dual genotype in cutaneous T-cell lymphoma: immunoglobulin gene rearrangement in clonal T-cell malignancy. *J. Inv. Dermatol. 90:73.*
60. Schlissel, M., A. Vornova, and D. Baltimore. 1991. Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy chain gene transcription and rearrangement in a pre-T cell line. *Genes Dev. 5:1367.*
61. Akashi, K., L. I. Richie, T. Miyamoto, W. H. Carr, and I. L. Weisman. 2000. B lymphogenesis in the thymus. *J. Immunol. 164:5221.*
62. Sinkora, M., J. Sun, and J. E. Butler. 2001. Antibody repertoire development in fetal and neonatal piglets. V. V_H gene chimeras resembling gene conversion products are readily generated by PCR in vitro. *Mol. Immunol. In press.*
63. Davis, M. M., J. J. Boniface, Z. Reich, D. Lynons, J. Hampl, B. Arden, and Y.-H. Chien. 1998. Ligand recognition by α/β T cell receptors. *Annu. Rev. Immunol. 16:532.*
64. Hohman, V. S., D. H. Schuchman, S. F. Schluter, and J. J. Marchalonis. 1993. Genomic clone for sandbar shark λ light chain: generation of diversity in the absence of gene rearrangement. *Proc. Natl. Acad. Sci. USA 90:9882.*