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


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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\textsuperscript{1,2}

J. E. Butler,\textsuperscript{*} P. Weber,\textsuperscript{*} M. Sinkora,\textsuperscript{*} J. Sun,\textsuperscript{*} S. J. Ford,\textsuperscript{†} and R. K. Christenson\textsuperscript{‡}

Since the actual combinatorial diversity in the V\textsubscript{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\textsubscript{H} proximal D\textsubscript{H} segment (D\textsubscript{H}B) to decrease in older fetuses and for the longer D\textsubscript{H} segment to be trimmed to the same length as the shorter D\textsubscript{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 Peyer's 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\textsubscript{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\textsubscript{H} repertoire of swine is encoded by <20 V\textsubscript{H} genes (17), a single J\textsubscript{H} (18), and almost exclusively by two D\textsubscript{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\textsubscript{H}A, V\textsubscript{H}B, V\textsubscript{H}C, and V\textsubscript{H}E (the fetal V\textsubscript{H} genes) and two D\textsubscript{H} segments account for approximately 80\% of the preimmune VDJ repertoire in all major lymphoid tissues (20). If V\textsubscript{H}\textsubscript{F} usage is included (Table I), >90\% of total V\textsubscript{H} usage can be accounted for by just five V\textsubscript{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\textsubscript{H} genes and a single V\textsubscript{H} (V\textsubscript{H}1) >80\% of the time, these are combinatorially joined with eight D\textsubscript{H} and two J\textsubscript{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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highly restricted combinatorial diversity could be offset by 1) variable light chain diversity, 2) junctional diversity in complementarity-determining region 3 (CDR3), 3) gene conversion of the fetal VH genes, 4) somatic hypermutations as in the sheep Vα, 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 a combination of these mechanisms. CDR3 has been shown to play a major role in the specificity of the Ab binding site (27), and a combination of these mechanisms.

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 F1 crosses from Iowa State University were used in the study. Animals were hand-fed and mated 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 noninvasive study 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 VDJCs and CDR3

Since all swine VH genes are members of a single, highly homologous family (17) and have a single JH (18), VDJ rearrangement can be amplified from DNA or cDNA using an FR1 primer (recognizing a sequence shared by all swine VH genes) and an anti-JH 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 32P-labeled pan-VH probe to confirm that each clone contains a VDJ insert and with gene-specific oligonucleotide probes to determine VH 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 (gtctccagaaagcagacagc) and a 32P-labeled anti-JH primer (tgagacgccagcactaa). Use of the FR3-A' primer yields PCR products of uniform length in >90% of the amplions (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, 2A, 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. 1A). 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 CDR3 lengths in vivo.

Quantitative spectrotypic analysis

The distribution and relative concentration of CDR3 lengths were quantified by scanning (Fig. 2B) 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<sub>H</sub>, D<sub>H</sub> usage, D<sub>H</sub> length, and D<sub>H</sub> 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 an oligoclonal repertoire in the 30-day-old fetal liver with prominent CDR3 lengths of 42–45 bp in most animals (Fig. 1B), but also with prominent 36–39 lengths in some animals (Figs. 1B 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 liver examined (Fig. 1B; animal 1 excepted). These spectratypic results are consistent with the mean CDR3

Table 1. Characteristics of CDR3s in clonal VDJJs as a function of fetal age

<table>
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<tr>
<th>Fetal Age (days)</th>
<th>No. of Clones</th>
<th>Proportion of Duplicate Clones (%)</th>
<th>Proportion of In-Frame Clones (%)</th>
<th>D&lt;sub&gt;H&lt;/sub&gt; Reading (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>V&lt;sub&gt;14&lt;/sub&gt; Usage (%)</th>
<th>V&lt;sub&gt;14&lt;/sub&gt; Frame Usage</th>
<th>V&lt;sub&gt;14&lt;/sub&gt; Frame Usage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Other Clones&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>18 47 35</td>
<td>14</td>
<td>50 11 0 16 7 ND</td>
<td>2 21.3 ± 6.0</td>
<td>19.7 ± 5.8</td>
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</table>

<sup>a</sup> All RF1 rearrangements are nonproductive due to stop codons in D<sub>H</sub>a and D<sub>H</sub>b.

<sup>b</sup> Length differences are not statistically significant by Student's t test.

<sup>c</sup> Data from fetal liver; all other data are from spleen.

<sup>d</sup> Sequence from fetal liver; all other data are from spleen.

4 CDR3 diversity is still possible. Thus, we have chosen to initially filter as described previously (19, 20). These were then hybridized with an individual recombinant clone was selected and transferred to a master filter as described previously (19, 20). These were then hybridized with a 32P-labeled pan-VH probe to confirm that each clone contains a VDJ insert and with gene-specific oligonucleotide probes to determine VH 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).

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 verbatim to denote only length distribution.
length determined by sequence analysis (Figs. 3 and 4B). However, CDR3s as long as 66 nucleotides and as short as 12 were also present at 30 days (Figs. 1B 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. 1B and 2A). 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. 2A). This observation is further described below. Evidence that the prominent CDR3 lengths comprising the oligoclonal spectratype in the day 30 fetal liver (Figs. 1B and 2B) 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. 2B). 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. 2B) as well as the parotid gland of older animals (Fig. 5B).

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 VDJ 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 5B).

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. 4D). P nucleotide additions were seldom seen either 5’ or 3’ of Dµ. We found no evidence for D-D rearrangements, for mini-Dµ 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. 4A).

DµB is the most JH-proximal Dµ segment in swine (J. Sun et al., unpublished observations) (18). In germline configuration, DµB is comprised of 28 nucleotides, whereas DµA is comprised of 36 nucleotides. In 30-day-old fetal liver, nearly two-thirds of the B cells randomly sampled used DµB, but its proportional usage decreased with fetal age (Fig. 4C). Although usage of the shorter Dµ 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 DµA, such that the actual lengths of DµA and DµB 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 DµA segments due to a stop codon.

The proportion of in-frame rearrangements increased 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. 2A). 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. 2A and 5A) in the mesenteric lymph nodes (MLN) of some animals (Fig. 5A) and in the 90- and 110-day-old parotid gland (Fig. 5B). 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 DµA and DµB, use of the term in-frame rearrangement is more accurate.
mammals and where unselected pre-B cells should be found, shows few out-of-frame rearrangements (Fig. 5A). 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. 1B 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. 4B). 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. 5A), the parotid gland of unrelated individuals exhibited animal differences (Fig. 5B). 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. 5A).

**VH 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 VHA and VHB are the predominant VH genes used during fetal life by piglets and generally account for about 50% of all VH usage (20) (Table I). VH D was rarely encountered and, as we earlier speculated, may be an allelic variant of VH C (20). We also recovered chimeric VDJ, but with higher frequency 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 (20). Specifically, four or five VH genes account for about 90% of VH usage, and only DHA and DHB are used. Since swine have only a single JH (18), combinatorial diversity provides only 8–10 possibilities. This pattern of VH 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 VH diversity in the heavy chain variable region accounts for repertoire diversity in this species, 2)
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*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 H A, D H B, or unidentified D H segments. Duplicate sequences (Table I) have been deleted.
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<tr>
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FIGURE 3—Continued.
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. 1B; 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. 4D). In fact, the mean CDR3 length at 30 days (Fig. 4B) 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$_{H}$B; Fig. 4D). The preferential usage of D$_{H}$B 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$_{H}$A and D$_{H}$B 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 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$_{H}$B and D$_{H}$A 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.
(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 DH A truncated to four nucleotides (clone 3; Fig. 3). VDJs with highly truncated DH segments are very often recovered from fetal thymus (J. Sun and M. Sinkora, unpublished observations). If the unselected CDR3 profile of thymus (Fig. 5A) 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. 2B); 2) the mean CDR3 length does not change during 84 days of fetal development (Fig. 4B); and 3) the longer porcine DH A is trimmed during usage in CDR3 to the same length as the shorter DH B (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. 4A), although this has been reported to be a major Ag-independent mechanism in the \( \gamma \)-chains of other artiodactyles, such as sheep and cattle (6, 8, 11). Apart from the idea that \( \gamma \)-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 \( D_{H} A \) two or three times more frequently than \( D_{H} B \), suggesting that pronounced \( V_{H} -D_{H} -J_{H} \) locus, because swine have only a single \( J_{H} \) (18) so that portion of in-frame rearrangements decreases (Table I), suggesting interesting that usage of nonproductive RF1 increases as the pro-

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). 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 \( D_{H} A \) to \( D_{H} A \). 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 nonstochastic 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 TCR \( \alpha \) 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 TCRs, 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 \( D_{H} \) to \( D_{H} \) 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 tandemly 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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