The DNA template was then digested with ribonuclease-free deoxyribonuclease I. The RNA transcripts were translated in micrococcal nuclease-treated rabbit reticulocyte lysates (Promega Biotec) in the presence of ^{35}S-labeled methionine under conditions suggested by the manufacturer. No differences were detected when capped or uncapped RNAs were used. Proteins were separated on a 15% SDS-polyacrylamide gel.

17. Hormone-binding assay. Proteins were synthesized in vitro essentially as described above except that unlabeled methionine was added. Protein extracts were eightfold diluted in T_3 buffer (20 m*M* tris, *p*H 7.5, 10% glycerol, 50 m*M* NaCl, 1 m*M* $MgCl_2$, 5 m*M* mercaptoethanol) and incubated for 1 hour at 28°C in the presence of ^{125}I-labeled hormone. Bound T_3 was determined by a filter-binding assay [A. Inoue, J. Yamakawa, M. Yakoika, S. Morisawa, *Anal. Biochem.* **134**, 176 (1983)]. The incubation mixtures were resuspended in 800 μl of ice-cold T_3 buffer and filtered over Millipore filters, type HAWPO2500, and washed four times with 800 μl of cold T_3 buffer. Bound radioactivity was determined in a liquid scintillation counter. To determine the amount of specifically bound ^{125}I-T_3, incubation mixtures were also incubated in the presence of a 1000-fold excess of unlabeled hormone over labeled hormone; as a control, reticulocyte lysate was incubated with labeled hormone and a mixture of labeled and unlabeled hormone.
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Early Restriction of the Human Antibody Repertoire

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Diversification of the antibody repertoire in mammals results from a series of apparently random somatically propagated gene rearrangement and mutational events. Nevertheless, it is well known that the adult repertoire of antibody specificities is acquired in a developmentally programmed fashion. As previously shown, rearrangement of the gene segments encoding the heavy-chain variable regions (V_H) of mouse antibodies is also developmentally ordered: the number of V_H gene segments rearranged in B lymphocytes of fetal mice is small but increases progressively after birth. In this report, human fetal B-lineage cells were also shown to rearrange a highly restricted set of V_H gene segments. In a sample of heavy-chain transcripts from a 130-day human fetus the most frequently expressed human V_H element proved to be closely related to the V_H element most frequently expressed in murine fetal B-lineage cells. These observations are important in understanding the development of immunocompetence.

ANTIBODY GENES ARE ASSEMBLED from discontinuous germline gene segments that are juxtaposed during B lymphocyte development (*1, 2*). In human heavy chains, one of several diversity (D_H) gene segments is first joined to one of six joining (J_H) gene segments that are clustered several kilobases on the 5′ side of the element encoding the constant region (Cμ) of antibodies of the immunoglobulin M (IgM) class (*3*). Subsequent joining of one of a large number of variable (V_H) gene segments (several hundred have been identified in mouse DNA) to the D_H-J_H element generates a complete transcriptional unit. Cells bearing this rearrangement and producing a cytoplasmic μ heavy chain are referred to as pre-B cells. Juxtaposition of the V_L and J_L gene segments of light chains occurs next and results in the synthesis of the complete IgM molecule that is displayed on the surface of the B lymphocyte.

Considerable evidence indicates that the pool of functional antibody gene rearrangements is generated randomly in the adult mouse (*4*). Nevertheless, recent studies suggest that the murine fetal V_H repertoire preferentially includes members of the 7183 V_H gene family, particularly the V_H81X gene segment (*5, 6*). This preference has been observed in both B cells and pre-B cells, which, lacking surface antibody, cannot be targets for antigen-driven selection.

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With these observations in mind, we have used molecular cloning strategies to examine the development of the human V_H repertoire at 130 days of gestation. In humans, fetal B lymphopoiesis begins in the liver, with pre-B cells first becoming detectable by about 8 weeks of life (*7*). We therefore isolated fetal liver mononuclear cells, which are rich in B cell precursors (*8*), and used RNA from these cells to obtain complementary DNA (cDNA) clones containing human fetal heavy-chain sequences. These Cμ-containing clones proved to be typical of those found in immature B-lineage cells in that one-third (9 of 27) were derived from "sterile" transcripts that had initiated within the Cμ intron. A high level of such transcripts is characteristic of early pre-B cells (*9*). Similarly, the library contains three times as many Cμ-containing recombinants as light-chain recombinants.

We determined the complete nucleotide sequences of the first 15 J_H- and V_H-containing clones selected only on the basis of the order in which they were isolated. One of these clones (64P1) ended within the J_H region; each of the others resulted from a different V_H-D_H-J_H joining event (Figs. 1A and 2). Thus, each cDNA clone defines an independent gene rearrangement selected from the total repertoire of such rearrangements that are capable of generating transcripts in these cells. All but five of these clones include a complete V_H coding sequence. Two (13P1 and 60P1) end within the second hypervariable region, whereas two others (37P1 and 20P1) include more than two-thirds of the V_H segment. We assumed that the ascertainment of sequences in our sample was unbiased with respect to the transcriptional efficiency of different V_H elements, an assumption supported by data obtained in murine lymphocytes (*10*). However, it is noteworthy that all but one (20P3) of the variable region sequences resulted from a productive V_H-D_H-J_H joining event, perhaps as a result of decreased stability of nonproductive heavy-chain messenger RNAs (mRNAs), which should otherwise comprise most of the Cμ transcripts (*1*). Thus, the sequences presented here sample mainly the translatable repertoire of fetal heavy chains.

Analysis of these variable region sequences reveals that certain antibody gene segments are preferentially rearranged in human fetal B cells. For example, although there are six functional human J_H gene segments, only J_H3 (seven sequences), J_H4 (six sequences), and J_H5 (two sequences) are represented in these early fetal cDNAs (Fig. 2). Two allelic forms of the J_H3 sequence were observed (compare 37P1 with 51P1 in Fig. 2), indicating that readout of the J_H

repertoire is biased on both chromosomes in these fetal cells. Preferential utilization of the J_K1 and J_K2 gene segments has been reported in the mouse (*11*), and it is possible that the adult human J_H repertoire may include a relative excess of J_H3 and J_H4 gene segments (*12*). Interestingly, all of the J_H3 and J_H5 rearrangements have recombined at the 3′ end of the heptamer recognition element in the germline sequence, whereas three of six J_H4 rearrangements truncate five to eight bases of the germline J_H4 sequence (*12*). This distribution of J_H breakpoints is consistent with the view that the antibody gene recombinase preferentially cleaves target sequences adjacent to a TpG dinucleotide (*13*). A TpG sequence or its complement is found at the 5′ border of both the human J_H3 and J_H5 gene segments, but not until position 8 of the J_H4 segment (*12*).

By comparison with the J_H regions, the fetal D_H sequences are quite heterogeneous (Fig. 2). Nevertheless, eight of these sequences share short regions of homology (between five and nine nucleotides) with the human D_HQ52 sequence. This homology depends, however, on the tetranucleotide CTGG, which is also found in other unrelated D segments (*14*). Five of the remaining clones (56P1, 13P1, 15P1, 38P1, and 60P2) contain D_H sequences that cannot easily be assigned to previously described human germline D_H gene segments (*12, 14*), and the last (20P3) probably contains a member of a human D_HSP2-like family (*14*). The combined D_H and presumed N-region sequences [products of random nucleotide addition at the sites of V_H-D_H-J_H joining (*15*)] vary in length from 12 (20P1) to 34 (37P1) nucleotides. This heterogeneity produces considerable variation in the sequences of the third hypervariable regions of the fetal antibody heavy chains.

The 14 sequenced heavy-chain variable regions (Fig. 1, A and B) are apparently encoded by a limited set of V_H gene segments, one of which was used three times and three of which were used twice. This interpretation is based on the assumption that clones 13P1 and 60P1, though incomplete, would prove to be identical to 56P1 and 51P1, respectively, if the entire V_H regions of each were available for comparison. Although not directly verifiable, this assumption is supported by the complete identity of the nearly 100 nucleotides analyzed for each of these pairs of sequences and the presence of other completely identical sequence pairs (2P1 and 56P1, 60P2 and 63P1) that include the entire V_H coding region (Fig. 1A). The total size of the fetal V_H repertoire estimated from these data by established statistical techniques (*16*) is between 9 and 39 elements at 95% confidence. This analysis includes one pair (51P1 and 60P1) that have identical–although partial (107 nucleotides)—V_H sequences joined to allelic J_H3 gene segments, thus indicating that recruitment of V_H elements is likely restricted on both chromosomes in this fetal sample (Figs. 1A and 2). If we include only those V_H sequences that extend through the

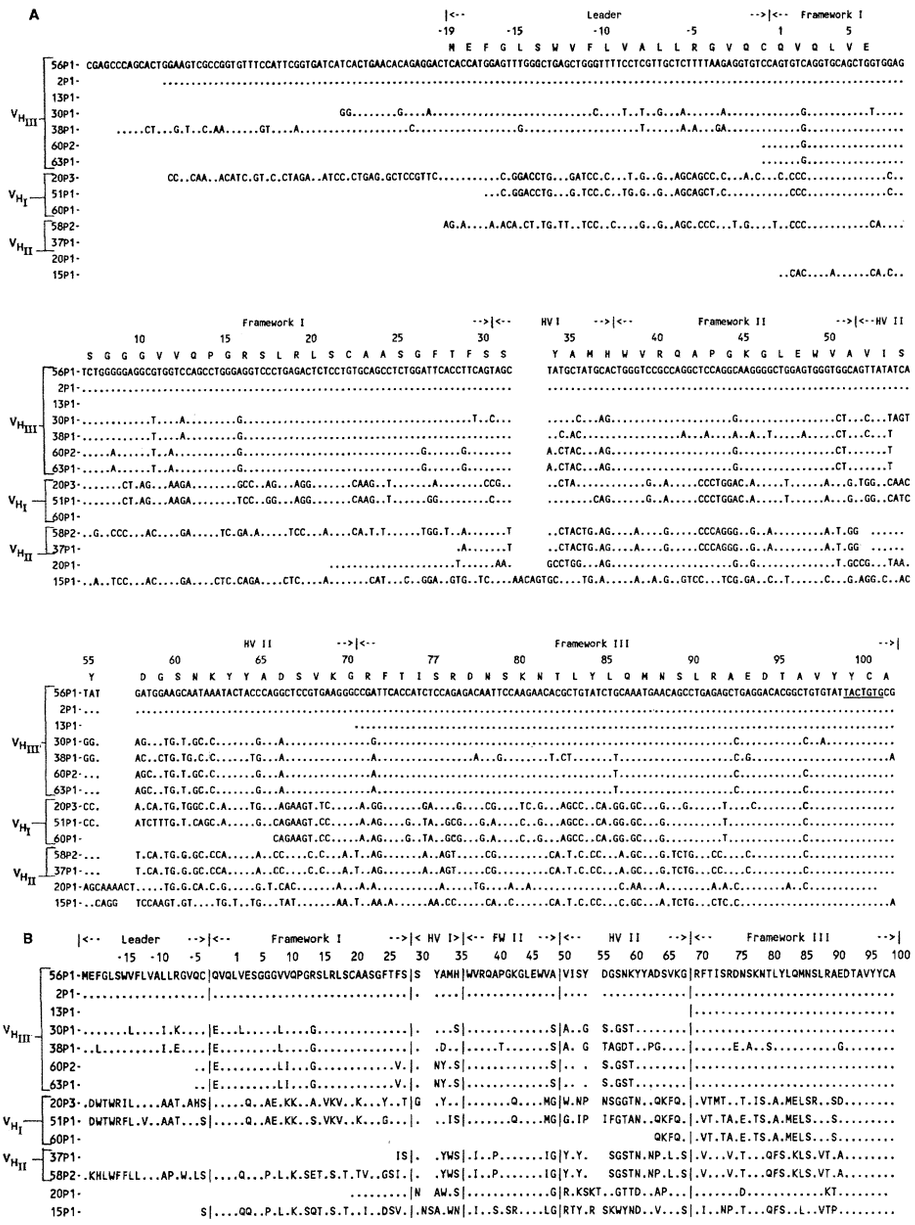

Fig. 1. (**A**) Nucleotide sequences of 14 human fetal V_H regions. Fetal liver from a karyotypically normal 130-day anencephalic abortus, provided by T. Shepard, was disaggregated and mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (*23*). A λgt10 cDNA library of 4×10^6 recombinants was prepared from 2 μg of polyadenylated RNA by conventional techniques (*24*). Clones were sequentially screened by hybridization to a 3′ Cμ cDNA probe and to a 5′ Cμ oligonucleotide (5′-AGTGCATCCGCCCCAACCCTTTTCCCCCTC-3′). Sterile transcripts were initially identified by direct sequencing and later by hybridization to a sterile-specific oligonucleotide probe (5′-GCCCA-GACTGTCATGGCTATCA-3′). Sequences of V_H-containing clones were determined on both strands by the method of Sanger *et al.* (*25*) with specific oligonucleotide primers (*26*). All are compared to clone 56P1, with a dot denoting nucleotide identity. Codons of 56P1 are numbered sequentially with the conceptual translation product presented in single letter code above the nucleotide sequence. The 3′ terminus of each V_H is arbitrarily defined at codon 101. V_H family assignments, defined by sequence homology (*12*), are at the left. V_H15P1 and V_H20P1 are probably derived from previously undescribed V_H gene families. A potential heptamer recognition element (*1*) is underlined. (**B**) Amino acid sequence homologies among the 14 human fetal V_H clones. The translation products of each cDNA clone are presented in single letter code, aligned with clone 56P1 as in (A). The positions of conventionally defined leader, framework, and hypervariable regions are noted above the sequences in both (A) and (B).

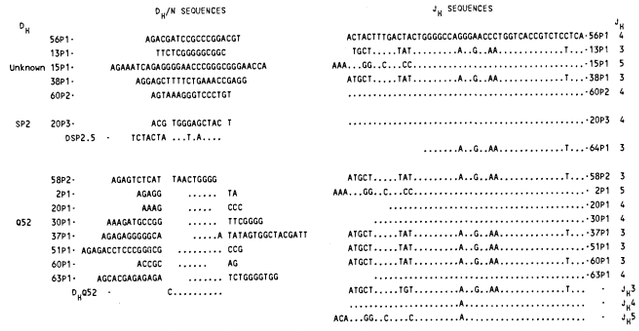

Fig. 2. Sequences of D_H and J_H regions from human fetal Cμ cDNA clones. Nucleotide sequences were determined for 15 Cμ-containing cDNA clones as described in the legend to Fig. 1 and aligned with human J_H3, J_H4, J_H5 (*12*), D_HQ52 (*14*), and murine DSP2.5 (*14*) sequences. Clone 64P1 ends within the J_H region. The J_H4 sequence includes a single G to A transition (compared with published J_H4 sequences) that probably reflects polymorphism in the human population (*12*). Similarly, the J_H5 sequence differs at three positions from that previously published but encodes a known J_H5 peptide and hence most likely represents a natural polymorphism (*12*).

first hypervariable region, the 95% confidence interval extends to 117 elements; however, the probability distribution reaches a maximum at just 18 V_H gene segments. Athough the true number of functional V_H elements has not been determined in humans, crude estimates based on genomic blot complexity (*10, 17*) and comparisons with other mammalian species (*18*) suggest that the human V_H repertoire probably includes several hundred distinct sequences. The very low number calculated for "equally accessible" V_H segments in our population indicates that the fetal repertoire is highly skewed toward the use of a small subset of the entire adult repertoire.

Remarkably, the 56P1 V_H gene segment that occurs in 3 of the 14 human fetal heavy chains is closely related to the murine V_H81X gene segment, which participates in most of the fetal heavy-chain gene rearrangements in mouse B cells (*5, 6*). Homology between the 56P1 human V_H gene segment and the V_H81X murine gene segment extends into the leader and 5′ untranslated regions, which are 80 and 69% identical, respectively. These structural comparisons support our view that the 56P1 human V_H gene segment defines a set of elements functionally equivalent to the 7183 V_H gene family in the mouse. The analogy between human and mouse V_H repertoire development must be viewed with some caution, since it is based on analysis of a single human fetus at 130 days of gestation; nevertheless, these results are consistent with the hypothesis that a restricted early V_H repertoire limits immunocompetence in fetal and neonatal life (*5*).

One possible explanation for the developmentally programmed appearance of specific V_H gene segments in the mouse derives from the C_H proximal position of the V_H81X element (*6*). If V_H rearrangements follow a developmental program imposed by proximity to the C_H locus (*19*), we would expect 56P1 and other V_H gene segments frequently occurring in the fetal repertoire [including members of the V_HI (51P1), V_HII (58P2), and V_HIII (56P1) gene families, as shown in Fig. 1A] to be positioned nearest the constant region genes in the human genome. This prediction remains to be tested but is consistent with the demonstration of interspersion of V_HI, V_HII, and V_HIII gene segments in humans (*17*). It is also noteworthy that the D_HQ52 gene segment, which may be used in 8 of 14 of these fetal sequences (Fig. 2), is the most C_H proximal of the human D elements (*14*).

Developmentally programmed rearrangements of antibody V_H gene segments have been invoked as a possible explanation for the regulated appearance of certain antibody specificities during fetal and neonatal life (*5, 20*). This type of regulation might predispose the early repertoire to rearrangements of high utility (for example, antibodies directed against certain pathogens) or might preclude the expression of potentially injurious antibodies with anti-self specificities before more complex T cell–mediated regulatory mechanisms have developed. However, at 130 days of gestation, the repertoire of distinct heavy-chain sequences generated through combinatorial joining was already quite large in our fetal sample.

Preferential rearrangement of certain V_H elements during fetal life might instead provide an especially fertile substrate for subsequent V region replacement events (*21*). This model could also explain the comparative abundance of particular V_H sequences in transcripts derived from adult B cell populations (*6, 22*). All of the V_H sequences in our fetal sample, with the exception of 20P1, contained heptamer elements at their 3′ ends (Fig. 1A) that could serve as recognition sequences for replacement of a rearranged and expressed V_H gene segment with another V_H element.

We have demonstrated that generation of the human antibody heavy-chain repertoire follows a developmental program that closely resembles the hierarchical pattern of V_H rearrangements previously observed in the mouse (*5, 6*). Structural similarities among the V_H elements that are expressed first in both species suggest that this early restriction in the antibody repertoire plays an important role in the development of immunocompetence. By extension, it is conceivable that abnormalities in V_H repertoire development may contribute to the pathogenesis of dysgammaglobulinemia and immunodeficiency in humans (*19*).

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