4. See H. Teitelbaum [*J. Comp. Physiol. Psychol.* **76**, 51 (1971)] for experiments with adult rats testing the location of olfactory memories.
5. The subjects were six litters of 6-day-old offspring of Charles River CD strain rats mated in our laboratory (n = 6). Pregnant females were housed in individual cages (45 by 24 by 20 cm) containing wood-chip bedding. Water and Purina Lab Chow pellets were continuously available. Pups were removed from their mothers 24 hours before training and testing. During the deprivation period, they were housed individually in a warm (32.5° ± 0.5°C), moist (70 to 90% relative humidity) incubator.
6. Just before training, we emptied the pups' bladders by gently stroking the anogenital region with a soft, sable brush. Then pups were placed in a clear, plastic container (11 by 6 by 11 cm) in a warm test incubator (32° to 34°C). The incubator was constructed from a 57-liter glass aquarium covered with a roof of hinged Plexiglas panels (*7*). To deliver the cedar odor stimulus to a pup, we pumped a light stream of air through plastic tubing into a sealed 25-ml flask containing 1 ml of cedar wood oil. A piece of plastic tubing exiting from the flask extended into the pup's test container. Activating the pump immediately introduced an air stream bearing cedar odor into the pup's environment. A blower in the test container rapidly removed scented air.
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8. The oral cannulas were installed at least 30 minutes prior to training (*7*). This required less than 10 seconds, and pups appeared to habituate rapidly to the presence of the cannula.
9. The plug was made from a 0.6- to 0.7-cm piece of Silastic tubing (Dow Corning; 0.067-cm outside diameter for 6-day-olds; 0.094-cm outside diameter for 12-day-olds) with a thread knotted and inserted into the lumen to block air flow and as an aid in removal. The plug was installed after coating the tip with petroleum jelly. Installation could be accomplished in a few seconds. Pups appeared to adapt to the presence of the plug quickly and paid it little attention. The naris that was occluded during training was counterbalanced between litters. The nose plug was installed at least 30 minutes prior to training or testing.
10. For testing, pups were placed in a container that had fresh bedding on one side and fresh bedding scented with cedar odor on the other side (approximately 0.5 ml of cedar wood oil was added to 85 g of bedding). Each preference test consisted of five 30-second trials. For each trial, the pup was placed lengthwise in the middle of a start zone (direction of placement was alternated from trial to trial), and the cumulative amount of time over cedar was recorded. The pup was scored as being over cedar if its snout was out of the start zone and in the cedar shavings area. The subjects were completely counterbalanced in each litter for order of testing (trained or untrained naris open during first test) and direction of first placement in the testing apparatus. No more than one pup from a litter was assigned to a counterbalanced condition; however, as there were no differences for order of testing (that is, animals spent an equivalent amount of time over cedar when tested with the trained naris open regardless of whether the test occurred before or after the test with the untrained naris open; animals spent an equivalent amount of time over cedar when tested with the untrained naris open regardless of whether the test occurred before or after the test with the trained naris open), the littermates' scores were averaged for these conditions to create a single litter mean for analysis. Thus, we treated litters as the experimental unit throughout these experiments.
11. To transect the anterior commissure (either 1 hour after conditioning at 6 days or 4 hours before retention testing at 12 days) we anesthetized the pups with methoxyflurane (Metofane). The skull was exposed and a burr hole made just lateral to the midsagittal suture and just posterior to bregma. The transection was made with a 30-gauge hypodermic needle (0.9 mm long), rounded at the tip and inserted through the hole perpendicular to the animal's skull. The needle was swung in the sagittal plane forward through 30° of arc. The needle was withdrawn, and the wound was closed and dressed. Side of needle insertion (just right or just left of the suture) was counterbalanced between litters. After the test, animals were killed, and the brains were removed and cut horizontally in 20-μm sections. Damage to the anterior commissure was investigated in Nissl stained sections. Area of damage was traced onto atlas figures [G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, 1982)]. For the sham-operated pups, the needle was inserted only into the cortex. Littermates' scores for a particular treatment group were averaged so that the number of litters remained the unit of analysis.
12. Control values were obtained from previous studies. These studies replicate the general finding that 12-day-old pups exhibit conditioning when tested with the trained or untrained naris open. Conditioned pups spent substantially more time over cedar than naïve controls, and the values obtained for conditioned pups were nearly identical to those reported in our present experiment. The preference test scores obtained in the present experiment for animals tested with the trained or untrained naris open are shown in Fig. 2B with the data further partitioned according to the time when separate groups received sham surgery for comparison with pups that received transections of the anterior commissure.
13. In our control studies we have trained rats at 6 days of age and tested them at 12 days of age for unilateral conditioning, but without any previous testing at 6 days of age. These animals responded bilaterally when tested at 12 days of age, precluding the argument that the testing session administered at 6 days itself contributed to the "transfer" effect when pups were retested for 6-day retention. Previous investigations have also included various other conditioning controls (including backward-conditioning groups), and no differences have been found between any of these groups with this conditioning protocol, although differences were detected between each of the control conditions and the experimental condition. Finally, conditioning has also been found to be specific to the odor that was paired with milk reward.
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A Novel Thyroid Hormone Receptor Encoded by a cDNA Clone from a Human Testis Library

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The c-*erb*A gene belongs to a multigene family that encodes transcriptional regulatory proteins including the v-*erb*A oncogene product, steroid hormone receptors, and the vitamin D_3 receptor. A v-*erb*A DNA probe encoding the DNA-binding region of the v-*erb*A protein was used to screen a human complementary DNA testis library. One of the clones isolated, *erb*A-T-1, was found to encode a 490–amino acid protein (*erb*A-T). The *erb*A-T polypeptide shows high homology with the proteins encoded by both the chicken c-*erb*A and the human c-*erb*A-β genes but is most closely related to the chicken gene. The chicken c-*erb*A and the human c-*erb*A-β genes encode high-affinity receptors for thyroid hormone, and here it is shown that the *erb*A-T protein binds specifically to 3,5,3′-triiodo-L-thyronine with a dissociation constant of $3.8 \pm 0.2 \times 10^{-10} M$. These data imply that more than one thyroid hormone receptor exists in humans and that these receptors might have different tissue- and gene-activating specificities.

THE *erb*A GENES ENCODE A CYSTEINE-RICH domain that shows high homology with the putative DNA-binding domain of steroid hormone receptors (*1–8*). The amino acid sequence of this domain is almost fully conserved for the glucocorticoid receptors of the mouse, rat, and human (*9, 10*) and for the estrogen receptors of the chicken and the human (*11*). The chicken (*7*) and human *erb*A (*8*) proteins, which are both thyroid hormone receptors (*7, 8*), share only 90% homology in this region and may therefore correspond to different genes encoding proteins with similar but not identical functions. To isolate further members of the *erb*A gene family, we used a 600-bp Ava I–Pst I DNA fragment from v-*erb*A (*12*) encoding the cysteine-rich domain to screen human complementary DNA (cDNA) libraries. Two cDNA clones, which hybridized strongly with the ^{32}P-labeled v-*erb*A probe, were isolated from a testis library (*13*). Both clones also hybridized with a probe from the 3′ end of v-*erb*A. We subcloned and sequenced two Eco RI inserts of the larger of these clones (*erb*A-T-1) (*14*). The 2112-bp-long sequence of *erb*A-T-1 contains a long open reading frame of 490 amino acids with an initiator methionine codon at nucleotide 334 and a terminator codon at nucleotide 1804 (*15*).

The predicted polypeptide encoded in the

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*erb*A-T-1 clone has a molecular mass of 54,753 (M_r, 55,000), is larger than the polypeptides encoded by the cc-*erb*A and the hc-*erb*A-β clones, but shows high homology with both these proteins and the v-*erb*A oncogene product (Fig. 1). The protein also contains the domains typical for steroid (*1–6*) and thyroid hormone receptors (*7, 8*) (Fig. 1b).

The *erb*A-T protein is 71% homologous with cc-*erb*A in the amino-terminal domain but shows no homology in most of this region with hc-*erb*A-β; the presumed DNA-binding domain is 96% homologous to the chicken protein and 87% homologous to the other human protein (Fig. 1). Similarly high homologies are observed in the hormone-binding domain, 94 and 84% for the chicken and human proteins, respectively (Fig. 1). Domain D shows decreased homology (85% with the chicken and 71% with the other human receptor) in comparison with its adjacent domains with known functions. The carboxyl-terminal portion of the *erb*A-T protein shows no similarity to the other two *erb*A proteins (which are 97% homologous in this region) and is much longer. The *erb*A-T protein is overall more closely related to the chicken protein than to the other human protein; however, all three cellular genes and the v-*erb*A gene encode three colinear highly homologous domains of identical sizes (Fig. 1b).

The chicken c-*erb*A and the human c-*erb*A-β encode high-affinity receptors for the thyroid hormone 3,5,3′-triiodo-L-thyronine (T_3) (*7, 8*), whereas the v-*erb*A oncogene product does not bind T_3 and appears to be a constitutively active molecule (*7*). Because of the possibility that our new *erb*A gene product was also a thyroid hormone receptor, we used in vitro transcription and translation systems (*16*) to synthesize the protein encoded by the *erb*A-T clone and tested its affinity for thyroid hormone in vitro (*17*). As a control gene, we used the human estrogen receptor (*2*). RNA from *erb*A-T clones containing the complete or a partially deleted 5′ flanking region was poorly trans-

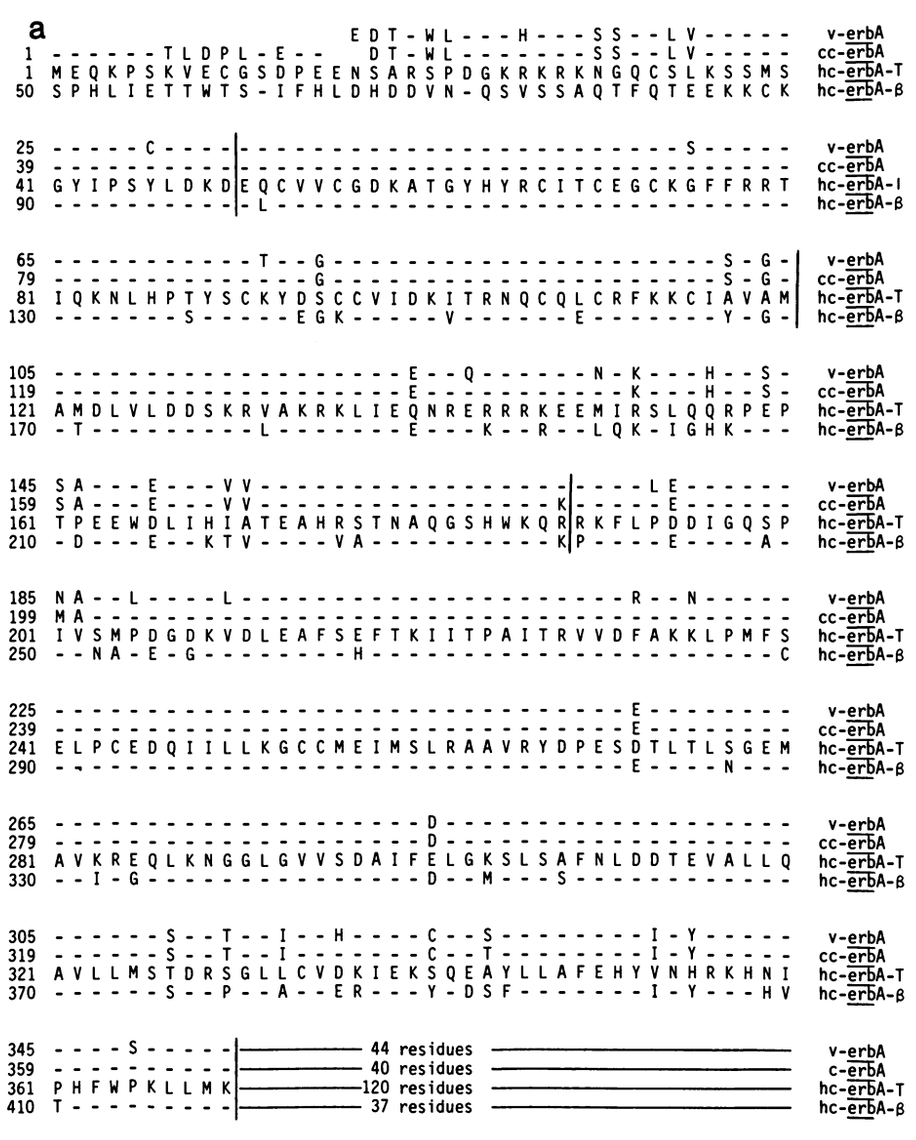

Fig. 1. Comparison of amino acid sequences encoded by *erb*A genes. (**a**) Alignment of amino acid residues encoded by hc-*erb*A-T with the predicted amino acid sequences of v-*erb*A (*12*), cc-*erb*A (*7*), and hc-*erb*A-β (*8*) proteins. Only amino acid residues that are different from the *erb*A-T sequence are shown. Identical residues are indicated by hyphens, two gaps were inserted in the amino-terminal region of cc-*erb*A to maximize the number of matches. Vertical lines indicate borders of domains. (**b**) Schematic presentation of homologies among three cellular *erb*A proteins. Domains were designated in accordance with Krust *et al.* (*28*). The cysteine-rich DNA-binding domain (C) and the hormone-binding domain (E) are connected by a possible hinge region (D). Domains C and E have been defined by mutations for both the glucocorticoid (*9, 29, 30*) and the estrogen receptor (*11, 31*), whereas direct functions for the two terminal domains, B and F, have not yet been determined. Percent homologies between the *erb*A-T protein and the chicken and human β proteins are indicated. In one case, the percent homology between the human β and the chicken protein is also indicated in parentheses.

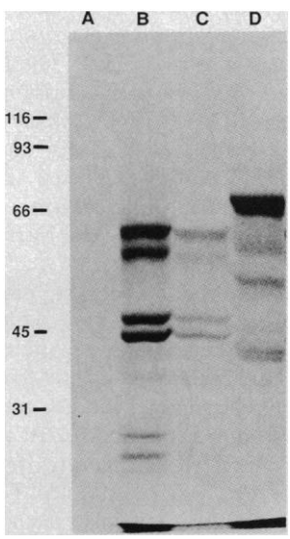

Fig. 2. In vitro synthesis of the hc-*erb*A-T protein. RNA synthesized in vitro (*16*) using T_7 or T_3 RNA polymerase was translated in rabbit reticulocyte lysates. Lane A, lysate without RNA; lane B, translation of RNA from clone T-1-4 (which has 296 bp deleted from the 5′ end); lane C, translation of RNA from clone T-1-222 (which has 22 bp deleted from the 5′ end); lane D, translation of RNA from a construct that contains the human estrogen receptor cDNA clone (*2*).

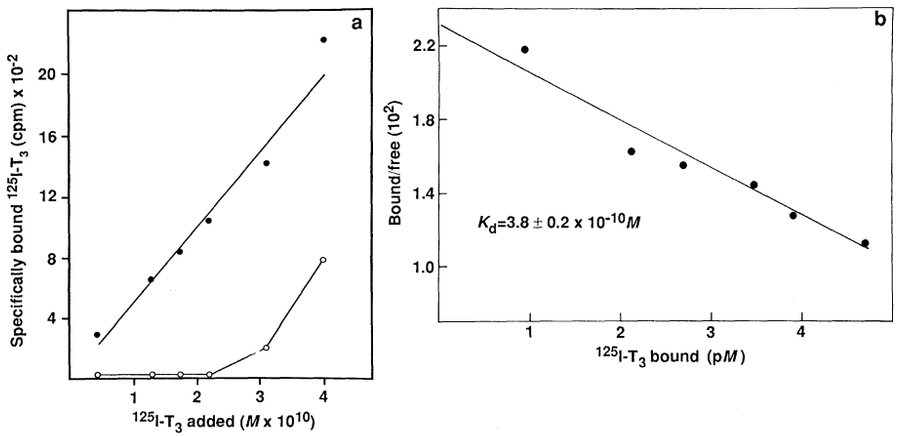

Fig. 3. Specific T_3-binding of the *erb*A-T translation product. (**a**) In vitro translated protein from clone T-1-4 (●) was incubated with various concentrations of ^{125}I-T_3 (New England Nuclear) in the presence and absence of unlabeled T_3. Specifically bound counts were determined as described (*17*). Specific binding to reticulocyte lysate which had not been incubated with RNA is also shown (○). (**b**) Scatchard plot of binding data. For these calculations, the radioactivity bound (expressed in counts per minute) in the control extract were substracted from the specifically bound radioactivity in the T-1-4 extract.

lated (Fig. 2, lane C), whereas RNA from a clone (T-1-4) in which we deleted all but 37 bp of the 333-bp 5′ flanking region (Fig. 2, lane B) was translated as efficiently as the estrogen control gene (Fig. 2, lane D). Four major protein bands were detected when the T-1-4 clone was transcribed in the sense direction and translated (Fig. 2, lane B). The largest protein (about M_r 56,000) probably represents the complete polypeptide encoded in the *erb*A-T clone. The smaller proteins may represent translation products initiating at methionines 39, 120, 122, or 150, or they could be incomplete translation or proteolytic breakdown products. In the case of the ER clone, one major product of 66,000 was observed (Fig. 2, lane D). It is of interest that the ER translation start sequence is GGACCATGA, which is closely related to the high-efficiency translation start sequence CCpuCCATGpu (*18, 19*), while the *erb*A-T clone sequence (AGTGAATGG) differs considerably from the high-efficiency start sequence.

To measure specific hormone binding of the translation products, we added ^{125}I-labeled T_3 to the translation reactions in the presence and absence of a 1000-fold excess of unlabeled hormone, the ER translation product serving as one control. As a further control, untreated reticulocyte lysate was also incubated with ^{125}I-labeled T_3 in the presence and absence of unlabeled hormone.

Figure 3 shows specific T_3 binding of the *erb*A-T protein when incubated with increasing concentrations of the labeled hormone. At high concentrations of the hormone, some specific T_3 binding was also detected in the control extract (Fig. 3a). Scatchard analysis (*20*) of T_3 binding (Fig. 3b) revealed a dissociation constant (K_d) of $3.8 \pm 0.2 \times 10^{-10}M$, which is similar to that observed for the chicken thyroid hormone receptor (2.1×10^{-10} to $3.3 \times 10^{-10}M$) (*7*), but larger than that observed for the human *erb*A-β receptor ($5 \times 10^{-11}M$) (*8*).

The data we present show that we have isolated a cDNA clone that encodes a novel human thyroid hormone receptor. The existence of different thyroid hormone receptors has not been predicted by physiological studies although thyroid hormone receptors of different molecular weights have been described (*21–23*). Multiple human homologs to v-*erb*A have also been described (*24*), and the homolog most closely related to v-*erb*A (called hc-*erb*A1) has been assigned to chromosome 17 and is located between 17p11 and 17q21 (*25*). From the high homologies observed between the v-*erb*A protein and the *erb*A-T protein (Fig. 1a), we consider it likely that the hc-*erb*A1 gene on chromosome 17 encodes the *erb*A-T protein, whereas the hc-*erb*A-β gene is located on chromosome 3 (*8*). For other members of the *erb*A-steroid hormone receptor gene family, multiple functional receptors have not yet been described. The existence of multiple thyroid hormone receptors allows the assumption that these different receptors fulfill different functions in that they are either tissue-specific, have different ligand affinities, or are able to activate different genes.

Recently, a T_3 derivative that stimulates liver function but has very little effect on cardiac function has been described (*26*). Different receptors expressed in a tissue-specific manner may account for these results. The novel human T_3 receptor encoded by the *erb*A-T gene is likely to be expressed at least in some cell types of the testis and has a lower affinity for T_3 than the previously described *erb*A-β receptor for which the cDNA clone was isolated from a human placental cDNA library (*8*). The *erb*A-T receptor also binds thyroxine (T_4) but with a more than tenfold reduced affinity (*27*). The incomplete homology (87%) of the two human receptors in the putative DNA-binding domain also suggests that these receptors may exhibit different target gene specificities. These target gene specificities and the tissue-specific expression of the T_3 receptors can now be examined.

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14. For DNA sequencing, the Eco RI inserts from the λgt_{11} clone, *erb*A-T-1, were cloned into the Eco RI site of the sequencing vector, Bluescript (Stratagene). Sequential deletions extending from both sides into the Eco RI insert were created by means of the Exo III deletion method [S. Henikoff, *Gene* **28**, 351 (1984)]. The dideoxy sequencing method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)] was used with double-stranded DNA [E. Y. Chen and P. H. Seeburg, *DNA* **4**, 165 (1985)] to obtain the complete sequence from both strands.
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16. For in vitro transcription and translation, we used the Bluescript sequencing vector (Stratagene). This vector contains T_7 and T_3 promoters flanking the multiple cloning box. The 5′ deletions created for DNA sequencing were combined with the 3′ Eco RI fragment of clone *erb*A-T-1. DNAs linearized at a unique restriction enzyme site in the vector cloning box at the 3′ end of the inset were transcribed with T_7 or T_3 polymerase at 37°C for 30 minutes. Transcription mixtures (25 μl) contained 1 μg of restricted DNA templates, 25 units of ribonuclease-block (Stratagene), 10 units of T_3 or T_7 polymerase (Stratagene) in transcription buffer (40 mM tris, pH 8, 8 mM $MgCl_2$, 2 mM spermidine, 30 mM dithiothreitol, and 0.4 mM of all four ribonucleotides).

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