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Immunology

# A repertoire of monoclonal antibodies with human heavy chains from transgenic mice

**(human immunoglobulin/DNA rearrangement/antibody engineering)**

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**ABSTRACT** **The introduction of human immunoglobulin gene segments in their unrearranged configuration into the germ line of mice might allow the production of a repertoire of human antibodies. Such transgenic mice could be used for the production of human monoclonal antibodies against human antigens. To test the feasibility of this approach, mice were created that carry a human heavy-chain minilocus comprising unrearranged immunoglobulin variable, diversity, and joining elements linked to a human $\mu$-chain gene. The gene segments of this minilocus are rearranged in a large proportion of cells in thymus and spleen but not in nonlymphoid tissue. Some 4% of the B lymphocytes synthesize human $\mu$ chains resulting in a serum titer of about 50 $\mu$g of transgenic IgM antibody per ml. Hybridomas were established from the transgenic mice that stably secreted several micrograms of antibodies containing human $\mu$ heavy chains per milliliter.**

The preparation of monoclonal antibodies of rodent origin is an established and widely used procedure (1). Their therapeutic application would be greatly assisted by the availability of monoclonal antibodies of human origin as these are likely to exhibit greatly reduced immunogenicity in humans. However, two types of problem beset the preparation of such antibodies (2). (*i*) Most immortalized human cell lines or hybridomas do not stably express large amounts of antibody. (*ii*) *In vivo* immunization of humans is not feasible for many antigens; *in vitro* priming is usually inefficient.

Antibodies are composed of variable (V) and constant (C) domains with the V domains defining the antigen specificity. Recently, a considerable advance has been achieved by making chimeric antibodies composed of mouse V domains linked to human C domains; these retain the antigen-binding specificity of the original mouse antibody but over two-thirds of the sequence of the human chimeric antibody is of human origin (3–5). Further improvements have been made by exploiting the fact that the hypervariable regions of the V domains are—at least in several cases—sufficient to determine antigen specificity; antibodies have been prepared in which only the hypervariable regions are of rodent origin (6, 7).

The genes encoding the V domains of the immunoglobulin heavy (IgH) chain are assembled during lymphoid differentiation by rearrangement of constituent $V_H$, diversity (D), and joining heavy-chain ($J_H$) segments (8). In this work, we create transgenic mice that carry a human IgH minilocus containing $V_H$, D, and $J_H$ segments linked to a $C_\mu$ gene. The mice rearrange this minilocus in their lymphoid tissue and contain B lymphocytes that make IgM molecules composed of human $\mu$ chains. This approach may allow the preparation of a repertoire of entirely human monoclonal antibodies that lack any sequences of rodent origin.

## MATERIALS AND METHODS

**Construction of the Minilocus and of Transgenic Mice.** The minilocus was assembled in a pUC12 derivative that contained an *Sph* I linker in the *Nar* I site. The mouse $C_\mu$ membrane exons were inserted as a *Hin*dIII–*Sph* I fragment and the *Sph* I site then converted to a *Bgl* II site by linker insertion. The $C_\mu$ region (9) was completed by inserting the human $S_\mu$ and $C_\mu$ 1–4 exons as an *Xba* I fragment into the polylinker. For the V domain, an *Eco*RI fragment containing the human $V_H26$ segment (10) was cloned into a pUC18 derivative that included a *Sac* I–*Kpn* I fragment that spans the mouse $V_H186$-2 gene (11). A separate pUC12 derivative was created with the mouse IgH enhancer in the *Xba* I site and a *Bgl* II fragment that spans the human $J_H$ locus in the *Bam*HI site. Following the use of linkers, the fragment containing the $V_H$ segments and the fragment containing the enhancers and $J_H$ segments were both introduced into the pUC12 derivative that included $C_\mu$. For the creation of the D segments, an M13tg131 clone that contained the 221-base-pair (bp) *Xho* I–*Sac* I mouse D-Q52 fragment was mutagenized using oligonucleotides to create D segments (TTTATTACTACGGTAGCAGCTAC and TCTACTATGGTTACGAC) that we designate D′-FL16 and D′-SP2. The mutagenized D segments were excised as *Bam*HI–*Bgl* II fragments from the M13tg131 polylinker, joined in tandem with the mouse D-Q52 and inserted into the unique *Bam*HI site of the pUC12 derivative containing the $V_H$, $J_H$, and $C_\mu$ genes to yield the final minilocus (Fig. 1). This plasmid containing the minilocus was linearized with *Bgl* II and introduced into the pronucleus of fertilized (C57BL/6 × CBA)$F_1$ mice as described (12).

**Analysis of DNA and RNA.** DNA from cell lines, from tissues, and from spleen cells that had been cultured for 3 days in medium containing 50 $\mu$g of bacterial lipopolysaccharide (LPS) per ml was extracted by phenol extraction following proteinase K digestion. Southern blots were hybridized with probes made by random oligonucleotide priming (13). Cytoplasmic RNA was prepared by phenol extraction of lysates made by use of Nonidet P-40 and analyzed by using glyoxal gels and the Northern blot procedure of Thomas (14).

**Preparation of Hybridomas and Analysis of Immunoglobulin.** Hybridomas were made by fusion of spleen cells with the NS0 plasmacytoma. In some fusions, LPS was included in the selective medium to stimulate class-switching of the endogenous IgH locus. The enzyme-linked immunosorbent assay (ELISA) for human $\mu$ chain was carried out by incu-

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Abbreviations: V, C, D, and J regions, variable, constant, diversity, and joining regions of immunoglobulin; IgH, immunoglobulin heavy chain; LPS, bacterial lipopolysaccharide.

bating samples in wells of a microtiter plate to which had been bound a monoclonal anti-human $\mu$ antibody (Binding Site, Birmingham, UK). Human $\mu$ determinants retained in the well were then detected by using a biotinylated goat anti-human $\mu$ antiserum (Amersham) and peroxidase-conjugated streptavidin. The assay was calibrated by using a human chimeric IgM antibody (15). Immunofluorescence as well as immunoprecipitations of biosynthetically labeled samples were carried out by using the biotinylated anti-human $\mu$ antiserum in the presence of an unlabeled mouse IgM antibody as competitor essentially as described (5, 15).

## RESULTS

**Transgenic Mice Carrying the Human Mini-IgH Locus.** A plasmid (Fig. 1) was assembled that contains the human $J_H$ cluster and $\mu$ C region ($C_\mu$) linked to two germ-line $V_H$ gene segments and four D segments. For the $V_H$ segments, we chose the human $V_H26$ and the mouse $V_H186$-2 genes (10, 11). The mouse $V_H$ gene was included as it is known to be functional, whereas we were not initially sure that the same was true of the human $V_H26$. Two of the D segments (D'-SP2 and D'-FL16) were generated by site-directed mutagenesis of the mouse D-Q52 element, since sequences corresponding to these D segments have been described in human heavy chains (16). The minilocus also included mouse and human D-Q52 elements. Since our human $C_\mu$ clone did not extend sufficiently far downstream of $C_\mu 4$, we used $\mu$ membrane exons of mouse origin. The minilocus includes a 1-kilobase (kb) *Xba* I fragment containing the mouse IgH enhancer as well as the 5' end of the human IgH enhancer.

The linearized minilocus plasmid was injected into the pronucleus of fertilized mouse eggs. Of 32 mice born, 12 carried the minilocus transgene as judged by Southern blot analysis of tail DNA. Most of the work described here was carried out on descendants of founders F17, F19, and F29, which have 3–5 copies of the transgene, as well as offspring of founder F23, which transmits about 50 copies.

**The Minilocus Rearranges in Spleen and Thymus.** To test whether the gene segments in the transgenic minilocus were able to undergo rearrangement, DNA was prepared from different organs and, following digestion with *Eco*RI, analyzed by Southern blot hybridization. The results (Fig. 2*A*) reveal that the locus has rearranged at high frequency in spleen and thymus of the low-copy-number F17 transgenic mice but not in the nonlymphoid tissues. Comparison of spleen and thymus DNAs from transgenic offspring of founders F17 and F24 reveals a striking similarity in their pattern of rearrangements (Fig. 2*B*). In contrast to the mice bearing a low transgene copy number, offspring of founder F23 (the high-copy-number founder) show a greatly reduced frequency of rearrangement of the minilocus.

A detailed analysis of the different types of rearrangement must await molecular cloning, although some information can be obtained by Southern blot analysis. Any rearrangement of a D element to a $J_H$ segment occurring by a looping-out mechanism will delete the *Bam*HI site. Analysis of DNA hybridized with a vector probe (Fig. 2*C*) suggests that a substantial proportion of the rearrangements involves D-$J_H$ integration as loss of the *Bam*HI site leads to an increase in the size of the hybridizing fragment in an *Fsp* I + *Bam*HI digest. Similarly, if the minilocus rearrangement involves one of the two $V_H$ segments, then there should be an alteration in the size of the *Sca* I fragment that hybridizes with the vector probe. The results (Fig. 2*D*) suggest that only a minority of the rearrangements involves the $V_H$ genes as most of the hybridizing DNA in the *Sca* I digest is unrearranged. Comparison of the results obtained with DNA from thymus and from LPS-activated spleen suggests that the pattern of rearrangements in B and T lymphocytes is similar.

**Mouse B Cells Making Human $\mu$ Chains.** To ascertain whether the DNA rearrangements of the minilocus could lead to the synthesis of antibodies comprising human $\mu$ heavy chains, serum immunoglobulin was analyzed in an ELISA assay. Analysis of sera from the 12 founder mice revealed that 7 were positive for human $\mu$ chains and had transgenic IgM concentrations in the range 1–100 $\mu$g/ml. It was notable that the high-copy-number founder F23 does contain antibodies with human $\mu$ chains in its serum (about 5 $\mu$g/ml) despite the fact that most of the copies of the transgene do not rearrange. Immunofluorescence analysis was performed to discover the proportion of B lymphocytes making human $\mu$ chains. The results (Fig. 3) show that about 4% of mitogen-activated B cells from an F17 offspring stain in the cytoplasm for human $\mu$ chain; this is similar to the proportion of unstimulated B cells that stain for human $\mu$ chain on the cell surface (not shown).

**Hybridomas from the Transgenic Mice.** Splenic hybridomas were prepared from the transgenic mice to allow a more detailed analysis of the expressed immunoglobulin. After fusion, cells were incubated in selective medium under conditions of limiting dilution. ELISA analysis of culture supernatants indicated that 3% of the hybrids secreted an antibody containing human $\mu$ chains. Following cloning by limiting dilution, the concentration of human IgM antibody in the culture supernatant was estimated as 0.5–5 $\mu$g/ml. Northern blot analysis of RNA from several cloned hybrids (Fig. 4*A*) revealed the presence of human $\mu$-chain mRNA and evidence that the human $V_H26$ and the mouse $V_H186$-2 were being utilized with an apparent preference for $V_H186$-2 although one hybrid (NW497/16.20) appears to express both $V_H$ genes. Short $V_H186$-2 transcripts were observed in several of the hybrids that made human $\mu$ chains; these short transcripts may be analogous to the germ-line $V_H$ transcripts

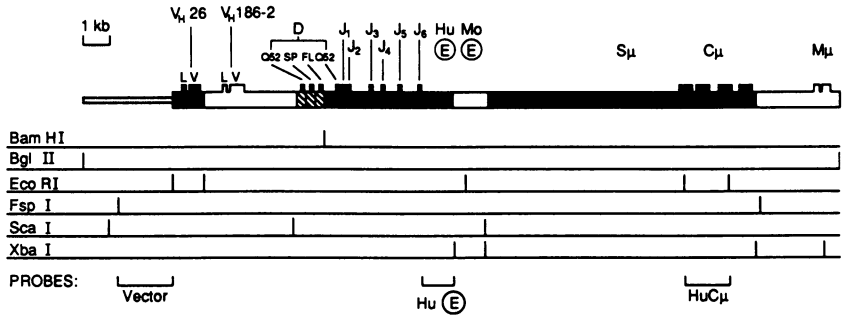


FIG. 1. Minilocus. Human sequences are depicted with thick filled lines, vector sequences with thin lines, and mouse sequences with unfilled lines. The D elements that were created by site-directed mutagenesis of mouse D-Q52 are hatched. Locations of exons, $\mu$ switch region ($S_\mu$), and the mouse and 5' end of the human enhancer elements (E) but not the human pseudo-$J_H$ segments are indicated. The derivation of probes used for hybridization is also shown.

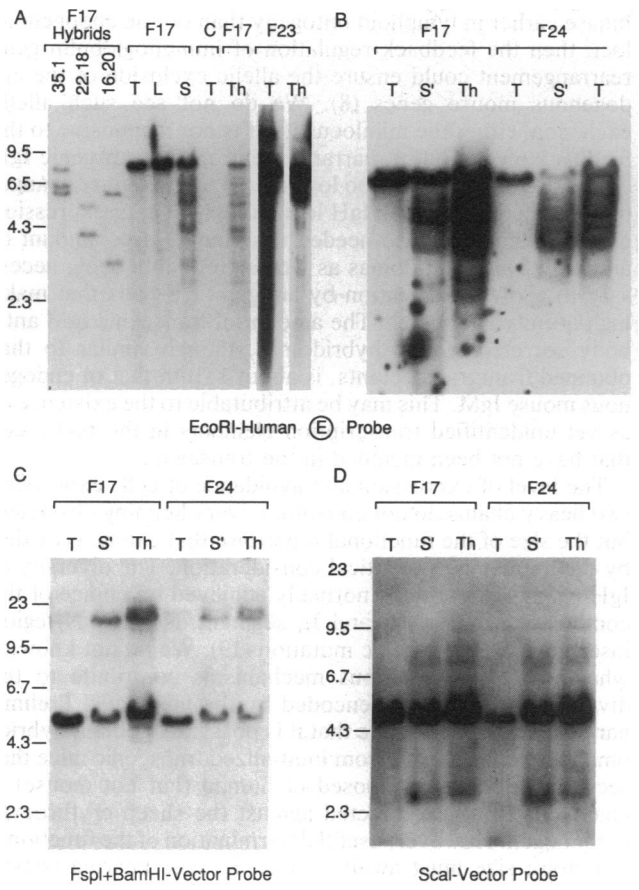


FIG. 2. Southern blot analysis of rearrangements. (*A*) DNA from tail (T), liver (L), spleen (S), and thymus (Th) of a transgenic daughter of F17 as well as tissues from a transgenic and a nontransgenic control (C) offspring of F23 was digested with *Eco*RI and hybridized with 1-kb *Bal* I–*Bgl* II probe from the 5′ end of the human IgH enhancer (see Fig. 1). Also included is DNA from cloned human $\mu$-expressing hybridomas NW499/35.1, NW499/22.18, and NW499/16.20 that were derived from another transgenic F17 offspring. (*B*) Comparison of minilocus rearrangements in tail (T), thymus (Th), and LPS-activated spleen cells (S′) from a daughter of F24 and from a granddaughter of F17; spleen cells were cultured for 3 days in the presence of LPS prior to preparing DNA from viable cells. The DNA was digested and probed as described for *A*. (*C*) Blot to detect rearrangements involving the D segments. DNA from tissues was digested with *Fsp* I + *Bam*HI and hybridized with a vector probe (pUC18 *Fsp* I + *Eco*RI; see Fig. 1). (*D*) Blot to detect rearrangements involving the $V_H$ segments. DNA was digested with *Sca* I and hybridized with the vector probe. Size markers (kb) are provided by a *Hin*dIII digest of $\lambda$ DNA.

observed in pre-B cells (8). The abundance of human $\mu$-chain mRNA in the hyridomas from the transgenic mice is similar if not greater than that found in plasmacytoma transfectants that secrete about 2 mg of chimeric human IgM per liter (Fig. 4*B*). ELISA of culture supernatants from the cloned hybrids that were positive for human $\mu$ chain and that had been obtained in the absence of LPS revealed that about 80% were also positive for mouse $\mu$ chain.

Biosynthetic labeling was carried out to confirm that the hybrids did indeed secrete an antibody with human $\mu$ chains of the expected size; immunoprecipitation was carried out by using an anti-human $\mu$ antiserum (Fig. 5). No band was obtained by using a control cell line transfectant (JW377) that secreted mouse but not human IgM. Four of the transgenic hybrids (lines NW499/16.20, NW499/31.10, NW500/24.2, and NW478/35.5) simultaneously express mouse and human $\mu$ chains, and the immunoprecipitates are likely to contain mixed IgM molecules composed of $\mu$ chains from both

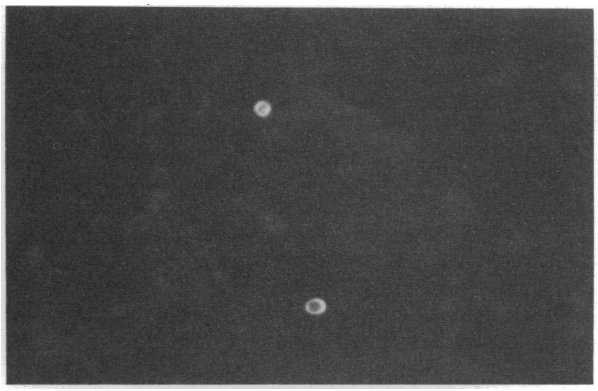

FIG. 3. Cytoplasmic immunofluorescence analysis of spleen cells from an F17 offspring that have been cultured in the presence of LPS for 3 days prior to staining with anti-human $\mu$ antiserum.

species. However, the other three hybrids (NW499/23.18, NW499/35.14, and NW497/21.1) do not make mouse IgM and the heavy-chain band is solely due to human $\mu$ chains.

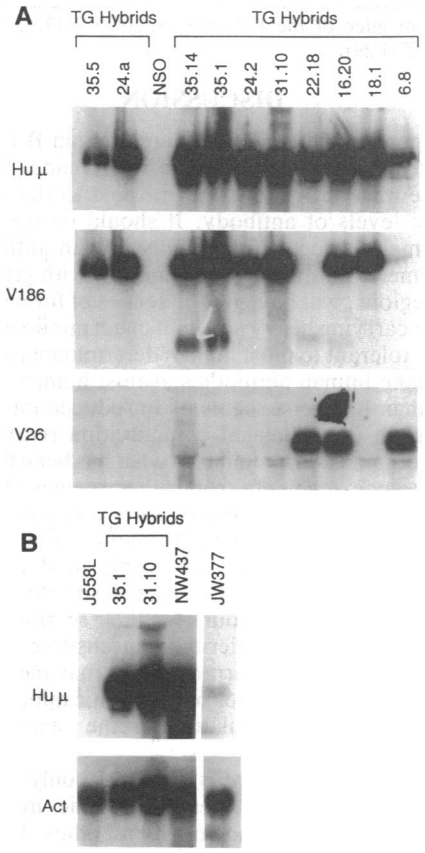


FIG. 4. Northern blot analysis of total cytoplasmic RNA from hybridomas. (*A*) Comparison of $V_H$ usage by different hybrids. The blot was sequentially hybridized to probes specific for human $C_\mu$ (Hu $\mu$), $V_H$186-2, and $V_H$26. All of the hybridomas secreted antibodies containing human $\mu$ chains. Hybridomas NW478/35.5 and NW475/24a were from an F29 descendant, NW497/16.20, NW497/18.1, and NW497/6.8 were from an F19 offspring, and all other hybrids were obtained from the F17 line. (*B*) Comparison of the abundance of human $\mu$-chain mRNA in F17 transgenic hybrids NW499/35.1 and NW499/31.10 with that in a transfectant (NW437) of plasmacytoma NS0 that secretes 2 mg of a chimeric human IgM antibody per liter; plasmacytoma J558L that expresses no IgH chain and its transfectant JW377 that secretes a mouse IgM antibody provide controls. The blot was rehybridized with a mouse $\beta$-actin probe (Act).

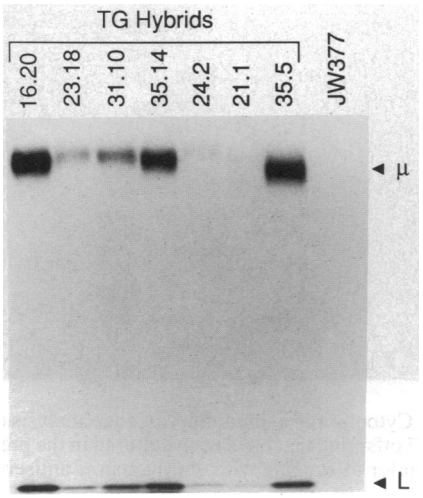


FIG. 5. Analysis by SDS/polyacrylamide gel electrophoresis of biosynthetically labeled antibodies secreted by hybridomas from the transgenic mice; positions of $\mu$ and light (L) chains are marked. Antibodies were purified by immunoprecipitation using anti-human $\mu$ antiserum. The transfectant JW377, which secretes a mouse IgM antibody, provides a control. All of the transgenic hybrids were derived from mice of the F17 line except NW497/21.1 (F19) and NW478/35.5 (F29).

## DISCUSSION

The transgenic mice described here contain B lymphocytes that make antibodies with human IgH chains. Hybridomas from these mice are stable with respect to the secretion of reasonable levels of antibody. It should be possible in the future to make a repertoire of fully human antibodies that, unlike chimeric antibodies or antibodies with grafted hypervariable regions, will have no sequences of foreign origin, by using mice carrying heavy and light chain miniloci. Such mice will not be tolerant to most human determinants and could be used to make human antibodies against human antigens.

The human IgH gene segments introduced into the mouse genome in an unrearranged configuration rearrange in the lymphoid organs; this is similar to what has been found for the light-chain gene segments from other species (17, 18). The pattern of minilocus rearrangements is very similar in spleen and thymus; this contrasts with the endogenous locus, where $V_H$ to $DJ_H$ rearrangements occur only in B cells (8). The proximity of the germ-line $V_H$ segments in the minilocus to the IgH enhancer may account for their rearrangement in T cells (12) and may also render them insensitive to the normal feedback regulation of rearrangement that mediates allelic exclusion (8). Further studies of transgenic miniloci are likely to shed light on the regulation of the rearrangement of V-region gene segments.

The work described here is obviously only a step in the direction of creating the ideal transgenic mouse for the preparation of human monoclonal antibodies. We have concentrated on an IgH locus that includes only a $\mu$-chain C region. The major remaining problems are focused on (*i*) the avoidance of B cells that express transgenic and endogenous IgH chains, (*ii*) the levels of transgene expression, and (*iii*) the effective functional repertoire size.

It would be useful to have transgenic mice that have nonfunctional endogenous immunoglobulin gene loci so that they can only make human antibodies. However, other strategies can also be used to obtain hybridomas that express a transgenic human IgH chain but not an endogenous mouse chain. One could select for loss variants of transgenic hybridomas that no longer retain a functional mouse IgH allele but still express an IgH chain encoded by the minilocus. Alternatively, if the minilocus becomes accessible to recombinase earlier in lymphoid ontogeny than do the endogenous loci, then the feedback regulation of immunoglobulin gene rearrangement could ensure the allelic exclusion of the endogenous mouse genes (8). We do not see such allelic exclusion; either the minilocus itself is not responsive to the feedback regulation of rearrangement or the transgenic IgH chains are expressed at too low a level to prevent rearrangement of the endogenous IgH loci. A high level of expression of the minilocus is also needed to obtain a large amount of antibody from hybridomas as well as probably being necessary to allow the selection by antigen of B cells that make high-affinity antibodies. The amount of transgenic IgM antibody secreted by the hybridomas, though similar to that obtained from transfectants, is about 1/10th that of endogenous mouse IgM. This may be attributable to the existence of as yet unidentified transcription elements in the IgH locus that have not been included in the transgene.

The level of expression and avoidance of cells expressing two heavy chains do not constitute overwhelming obstacles, but the size of the functional repertoire that can be provided by the minilocus is a critical consideration. The diversity of IgH chain expression is normally achieved by choice of the combination of $V_H$, D, and $J_H$ segment used, by N-region insertion, and by somatic mutation (19). We do not know to what extent these various mechanisms contribute to the diversity of IgH chains encoded by the minilocus. Preliminary experiments indicate that it is possible to obtain hybridomas at low frequency from immunized transgenic mice that secrete antibodies composed of human (but not mouse) $\mu$ chains and that are directed against the sheep erythrocyte immunogen. However, useful determination of the functional repertoire size must await transgenic mice bearing heavy-chain and light-chain miniloci.

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