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Expression of a microinjected immunoglobulin gene in the spleen of transgenic mice

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Abstract

Transgenic mice were produced by microinjection of a rearranged, functional immunoglobulin κ gene into fertilized mouse eggs and implantation of the microinjected embryos into foster mothers. Mice that integrated the injected gene were mated and the DNA, RNA and serum κ chains of their offspring were analysed. The data from offspring of three different transgenic mice indicate that the microinjected gene is expressed in the spleen, but not the liver of mice which inherited the injected gene.

Immunoglobulin variable (V) and constant (C) region genes must be rearranged into close proximity before expression into a functional immunoglobulin protein can occur^{1,2}. The functional rearrangement process takes place only in the B-lymphocyte lineage. To investigate the control of immuno-globulin gene expression we have produced transgenic mice by microinjection³ of an immunoglobulin gene into fertilized mouse eggs. Microinjection of a gene into an embryo introduces that gene into every cell of the resultant mouse and often leads to efficient expression of the injected gene^{4–6}. The presence of the injected gene in germ cells allows offspring of transgenic mice to inherit the injected gene. Compared with cell transfection experiments, this approach has the advantages of avoiding potential problems due to the transformed phenotype of cultured cells and allowing direct comparison of expression in different tissues that have the injected gene at the same location in the genome.

In our experiments the injected gene is the functional κ gene of the myeloma MOPC-21 (ref. 7). Its variable region, V_{κ} M.21, is rearranged next to the joining gene segment $J_{\kappa}2$ (ref. 8) and is contained in the plasmid pB1–14 (Fig. 1). The V_{κ} M.21 portion of the microinjected gene provides a means to distinguish the expression of the introduced gene from that of the endogenous κ genes, both on the RNA and protein levels. The V_{κ} M.21 gene is a member of the $V_{\kappa}15$ family of V_{κ} genes^{9,10}. This gene family has approximately ten members which are sufficiently homologous to cross-hybridize using a V_{κ} M.21 probe¹¹. V_{κ} genes of this family are probably rearranged and expressed in some B lymphocytes in normal mice, and transcripts from endogenous genes might be expected to confound the detection of transcripts from the injected gene. However, transcription of homologous endogenous $V_{\kappa}15$

genes is sufficiently infrequent to be virtually undetectable, as shown below. RNA encoded by the microinjected gene would therefore be uniquely detected by a V_{κ} M.21 probe if it were expressed in a tissue at a normal rate in most cells or at a high rate in a subpopulation of cells. The MOPC-21 κ protein is also sufficiently distinct in molecular weight¹² and isoelectric point (see below) that it can be distinguished from other κ chains in mouse serum, provided it is produced and secreted in some quantity. Analysis of transgenic mice containing the pB1–14 gene shows that expression of the microinjected κ gene is apparent on both the RNA and protein levels.

Production of transgenic mice

DNA for injection was prepared as described in Fig. 1. Eggs from (C57BL/6 $\times$ SJL)F₁ mice were fertilized with F₁ sperm. The male pronuclei of 300 fertilized eggs were microinjected with 2 μ l containing about 440 copies of the pB1–14 fragment and 192 eggs were inserted into the reproductive tract of random bred Swiss foster mothers⁴. Eleven animals developed from these eggs.

After the mice were weaned, total nucleic acids were extracted from a piece of their tail, and the presence of pBR322 DNA was detected by dot hybridization. Six of the 11 mice had integrated the injected gene. The six transgenic mice, all males, were then mated with C57BL/6 females and the offspring were tested for the presence of the pB1–14 gene. Consistent with mendelian inheritance of a single integration site, approximately 50% of the offspring also contained pB1–14 sequences in amounts relatively equal to their respective fathers. We are reporting here the analysis of first generation offspring from three different transgenic fathers (A, B and C).

Characterization of the microinjected DNA

The integrated genes were characterized with respect to copy number and mode of integration. Figure 2a shows an autoradiogram for an approximate quantitation of the copy number of the integrated gene. Kidney DNA from the offspring of transgenic mice was analysed by dot hybridization with pBR322. The pBR322 sequences were quantitated by cutting the dots out of the nitrocellulose filter and counting them in a liquid scintillation counter. Offspring that inherited the injected gene are shown in lanes 4–8, while littermates that did not inherit the gene are in lanes 9–11. Compared with the DNA standards (lanes 1–3) the two positive offspring of father A contain about 50 copies, the positive offspring of father B contains about 20 copies, while the two offspring of father C differ in their copy number. One C offspring contains about 95 copies, the other about 40 copies. If in father C the injected gene is integrated in two different locations in the genome, one would expect those two loci to be inherited separately. To distinguish multiple from single integration sites, the kidney DNA of the offspring was analysed by restriction enzyme digestion and Southern blotting. Figure 2b shows a representative autoradiogram. A *Bam*HI restriction site polymorphism between C57BL/6 and SJL causes the endogenous C_{κ} genes to appear as two bands, E₁ and E₂. The three darkest bands are those expected from a *Bam*HI digestion of concatenated pB1–14 in head to tail integration. Thus in analogy to other transgenic mice^{4–6} the majority of the multiple copies appear to be integrated in tandem. This is supported by

the Southern blot (Fig. 2c) using *EcoRI* which cuts only once in the linearized pB1–14 gene that was microinjected (site *EcoRI***). An amplified hybridization band of 14.8 kilobases (kb) is seen in the DNA of all five mice. The length of 14.8 kb is that of the linearized pB1–14. The other bands in Fig. 2b and c probably represent junction fragments between pB1–14 and endogenous DNA, as well as some pB1–14 DNA which has suffered a partial deletion (see especially the 1.8 kb band in one offspring (lane 4) of father C). The two mice of line C, besides sharing the major hybridization bands, differ in the respective minor bands produced by *Bam*HI or *Eco*RI digestion. This suggests that there may be at least two different integration sites in the transgenic father C. This seems to be supported by the finding that father C has an approximately additive copy number (not shown). However, details of the integration mode in all the transgenic mice containing the pB1–14 gene need to be further studied.

Expression of microinjected κ gene into RNA

We determined whether the injected κ gene was transcribed by analysing the poly(A)⁺ RNAs of spleen and liver of positive mice and of their normal littermates. Figure 3a shows that the spleens of both positive offspring and their negative littermates contain similar quantities of C_{κ} containing RNA. However, only the spleens of the positive mice contain detectable RNA having the V_{κ} region of the injected gene (Fig. 3b). The livers of the positive mice do not contain appreciable amounts of κ RNA (Fig. 3a); only faint hybridization of the C_{κ} probe with liver RNA was detected after long exposure of the X-ray film. When measured by scintillation counting this represents only 0.6–0.9% of the C_{κ} RNA found in spleen and could be due to circulating lymphocytes present in the highly vascularized liver tissue (see below). As a control for the quality of the liver RNA, the same RNAs were also hybridized with cDNA prepared from total liver RNA (Fig. 3d): the prevalent liver specific sequences hybridize strongly with liver RNA and weakly with spleen RNA as expected. There are no transcripts of pBR322 sequences in the poly(A)⁺ RNAs of either spleen or liver (Fig. 3c). Poly(A)[–] RNAs are also negative for pBR322 (not shown). This result argues against expression via pBR322 sequences.

To evaluate whether the small amounts of κ RNA seen in the livers of transgenic mice could be due to a low level of transcription from the transferred κ gene, comparisons with normal liver RNA were made. It was found that livers from normal mice also contain small amounts of κ RNA (not shown) probably due to circulating lymphocytes. It is therefore apparent that the microinjected κ gene is not transcribed in the liver of transgenic mice at a detectable level.

To determine whether κ RNAs in the spleens of transgenic mice were of correct size, Northern blots were performed with total spleen RNAs (Fig. 4). Both V_{κ} M.21 and C_{κ} sequences of transgenic spleens were found to be in RNA of mature size, the same as that of κ mRNA of normal spleen (Fig. 4 and ref. 13). In analogy to the results with dot hybridization of poly(A)⁺ RNAs (Fig. 3), the total spleen RNA of normal mice does not show detectable levels of V_{κ} M.21 RNA (even after extensive exposure of the X-ray film, not shown). These data favour accurate initiation and termination of transcription from the microinjected pB1–14 gene.

A comparison was made of the relative expression of the microinjected gene and endogenous κ genes (Fig. 5). The results of approximate quantitations using dot hybridizations are shown in Fig. 5. These data are only approximate because the $V_{\kappa}M.21$ probe is only about 100 nucleotides long (one-fifth the length of the C_{κ} probe). For this reason, a larger quantity of spleen RNA and standard DNA was used for the $V_{\kappa}M.21$ hybridization than for the C_{κ} hybridization. Thus the hybridization conditions are not identical. After exposure of the dot hybridizations to X-ray film, the dots were cut out and counted in a liquid scintillation counter. When RNA quantities were normalized by comparison with the DNA standards, it was found that the $V_{\kappa}M.21$ -containing RNA represents about 50% of the total κ RNA in the spleens of positive mice, but less than 4% of total κ RNA from normal littermates. In addition we found that spleens of the transgenic mice contain 1.5 to 2 times as much κ RNA in proportion to total poly(A)⁺ RNA as spleens of the normal littermates. Since in the normal spleen approximately 6,000 κ RNA molecules are produced per average cell¹⁴ spleen cells of the transgenic mice may synthesize as many as 3,000 pB1-14 encoded κ RNA molecules per cell. These relatively high levels of expression are similar to the levels of growth hormone RNA in the most actively transcribing transgenic mice which contain the metallothionein-growth hormone fusion gene⁶.

Serum κ chains encoded by transferred gene

The MOPC-21 κ gene codes for a L chain of relatively high molecular weight¹², and because of the unique $V_{\kappa}M.21$ region the L chain has a distinct isoelectric point. The protein is apparently glycosylated and produces two spots of characteristic size, shape and location in two-dimensional (2-D) protein gels¹⁵. Figure 6a shows the 2-D gel pattern of purified MOPC-21 immunoglobulin. The light chain appears as two spots at about 29,000 molecular weight (29K) (just below the 30K marker). Figure 6b-d shows 2-D gels of the sera of transgenic mice and normal littermates. Differences in intensities and location of many of the spots may be due to allelic differences in serum proteins in these backcrossed mice. This makes a one-to-one correlation difficult.

However, in keeping with their apparent monoclonal origin, the characteristic two MOPC-21 protein-like spots seem to be relatively invariable, where they occur. Figure 6b shows the characteristic spots in the serum from positive offspring number 1 of transgenic mouse A. Figure 6c shows the absence of such spots in a littermate negative for pB1-14 DNA. We have tested the sera of a total of 13 transgenic mice and of 11 normal littermates (some additional examples are shown in Fig. 6d). Although normal mice probably express $V_{\kappa}M.21$ genes at a low rate, we have not seen the two characteristic spots in our conditions in normal sera, but consistently find them in the sera of transgenic mice. Because these gels are run in reducing conditions, it is not yet determined whether the injected light-chain gene is expressed in the serum in association with heavy chain, or as free light chain, or both.

Discussion

These results from mice that are transgenic for a functionally rearranged κ gene have several implications concerning control of expression of the injected gene with respect to amount, tissue specificity, B-cell ontogeny and post-transcriptional events. In contrast to

immunoglobulin genes used in transfection experiments, our injected gene contains no attached viral sequences which could potentially enhance transcription. Furthermore, pBR322 sequences appear not to be involved in transcription of the injected κ gene. Also, since the injected gene is equivalently expressed in different transgenic mice the exact mode of integration and copy number appear to have little bearing on expression. So far, the behaviour of the injected gene seems to reflect the function of a normal functionally rearranged immunoglobulin light-chain gene.

Expression of the injected κ gene at a relatively high rate in all transgenic mice so far examined was an unexpected finding. In transgenic mice obtained by others with several different genes, expression was low or absent^{16–18}. Consistently successful expression was only obtained with fusion genes of the metallothionein (*MT*) promoter and either the herpes simplex thymidine kinase (*tk*) gene or the rat growth hormone gene^{4–6}. However, in the *MT-tk* system the expression was variable in offspring which apparently contained the same number and arrangement of the microinjected genes as their transgenic parents. Perhaps the expression signals of immunoglobulin genes are particularly strong. Examination of more transgenic mice is under way and may reveal an as yet undetected variability of expression.

While only spleen and liver have been studied so far, it appears that the microinjected pB1–14 gene may be expressed in a tissue-specific fashion. We presume that the cells in the spleen which express this gene are B lymphocytes, although this has not yet been shown directly. Since immunoglobulin genes are not rearranged in liver cells, for the first time in these transgenic mice liver cells contain a functionally rearranged and potentially expressible immunoglobulin gene. Hepatocytes, however, do not express the injected gene. This indicates that immunoglobulin gene rearrangement is not the sole required event for the tissue-specific expression of the genes by B cells. Rather, signals necessary for tissue-specific expression must be contained within the 14.8 kb of injected pB1–14 DNA. Other studies have suggested a possible immunoglobulin gene control region. The observations that unrearranged C_{κ} genes, but not generally V_{κ} genes, are DNase I sensitive¹⁹, undermethylated²⁰ and transcribed²¹ in B-lymphoid cell lines indicate that some control region other than the 5' flanking region of the rearranged gene is probably associated with C_{κ} . A short region within the $J_{\kappa}5-C_{\kappa}$ intron, which is highly conserved between mouse and man²² and which is DNase I hypersensitive in activated B lymphocytes²³ may be the tissue-specific control sequence. Also, this portion of the $J_{\kappa}-C_{\kappa}$ intron seems to be important for expression of κ genes transfected into lymphoid cells^{24–26}. An analogous sequence is present in immunoglobulin heavy-chain genes^{27–29}. We are presently determining if the sequence within the $J_{\kappa}-C_{\kappa}$ intron is responsible for tissue-specific expression of κ genes in transgenic mice.

B cells rearrange their heavy-chain locus before their light-chain loci³⁰. For the first time in these transgenic mice, developing B cells have a functionally rearranged light-chain gene present before heavy chain genes are rearranged. It will be interesting to investigate the possible effects of this situation on B-cell ontogeny, with respect to the rearrangement and regulation of endogenous genes.

Because the migration of the protein from the injected gene is identical on 2-D gels to the purified protein from MOPC-21, it can be inferred that the coding and control sequences have remained intact and that the protein is properly translated, glycosylated and secreted in transgenic mice.

The technology of microinjection of cloned genes into fertilized mouse eggs has been proven to be a powerful tool for the introduction of fusion genes with the ubiquitously expressed metallothionein promoter. The immunoglobulin κ gene is a clear example where a natural gene and one which is normally expressed in a highly tissue-specific manner apparently retains this property in transgenic mice. The details of the control of κ gene expression can now be studied in B-cell hybridomas obtained from these transgenic mice.

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Fig. 1.

Map of plasmid DNA pB₁-14. The functional MOPC-21 κ gene was initially cloned from genomic DNA into Charon 4A (M21B1)⁷. The V _{κ} M.21-C _{κ} gene insert was excised at the *Eco*RI sites and cloned into the *Eco*RI site of pBR322. Because injection of linear DNA with heterologous ends seems to increase the likelihood of integration (R. L. B. and R. Palmiter, unpublished), such a fragment was restricted from plasmid pB₁-14 using *Bgl*I and *Xho*I. The large *Bgl*I*-*Xho*I* fragment (14.8 kb) was separated from the smaller *Bgl*I-*Bgl*I and *Bgl*I-*Xho*I fragments by sucrose gradient centrifugation, ethanol precipitated, redissolved in

10 mM Tris pH 7.5, 0.25 mM EDTA and injected. V_L , leader sequence; VMOPC-21, variable region; J_2 to J_5 , joining segments; C_K , constant region. Probes for $V_{K.M.21}$ (pES205) and C_K (M13- C_K) are indicated. $EcoRI^{**}$, the unique *EcoRI* site present in the microinjected pB1-14 gene. The 5.3 kb, 2.0 kb and 2.9 kb *Bam*HI restriction fragments obtained by hybridization with pBR322 and C_K (see Fig. 2*b*) are indicated; the broken line denotes the portion (5.9 kb) which was eliminated from the plasmid DNA before microinjection. The fact that a 2.9-kb long pBR322 positive *Bam*HI restriction fragment was obtained in DNA of transgenic mice (Fig. 2*b*) suggests that the free ends of the linear plasmid DNA are joined in the integrated, amplified pB1-14 DNA.

Fig. 2.

DNA analysis of transgenic mice (same mice as in RNA and protein analyses in the following figures), *a*, Quantitation of the microinjected gene. The dot hybridizations were probed with pBR322. Each row (top to bottom) contains 0.2, 1.0, 5.0 µg DNA. 1–3, Normal mouse DNA with 1.5, 7.3 or 29.3 copies, respectively, of 1,000 base pairs (bp) of pBR322 added per cell; 4–8, kidney DNA from transgenic mice: offspring of father A (4, 5), B (6), C (7, 8); 9–11, kidney DNA from normal littermates A, B, C, respectively, *b*, Southern³¹ blot. Each lane contains 30 µg of kidney DNA digested with BamHI. The blot was probed with C_κ and pBR322. 1, 2, Positive mice of transgenic line A; 3, line B; 4, 5, line C. E₁ and E₂, endogenous κ genes of C57BL/6 and 5JL mice respectively. →, Bands expected from

*Bam*HI digestion of plasmid pB1–14 when tandemly integrated in head to tail fashion (see Fig. 1). When the same blot was probed with pBR322 alone, only the 2.9 and 2.0 kb bands were seen (not shown), *c*, Southern blot. Each lane contains 30 µg of kidney DNA (except lane 5 which contains 30 µg of tail DNA), digested with *Eco*RI. The blot was probed with pBR322. 1–5, As in *b*.

Fig. 3.

RNA blots of transgenic mice and normal littermates. Poly(A)⁺ RNA was prepared from spleens (S) and livers (L) and 0.5 µg (a) or 1 µg (b, c, d) after denaturation with gly-oxal³² were spotted onto nitrocellulose³³. The spots were hybridized with the following probes: M13 C_κ (contains about 500 bp of C_κ and 3' untranslocated region subcloned into M13) (a); pES205 (contains 103 bp of the V_κ region of MOPC-21 subcloned into pBR322) (b); pBR-322 (c); cDNA prepared from total liver poly(A)⁺ RNA (d). Hybridization conditions were 0.45 M NaCl, 0.045 M Na citrate 5× Denhardt's solution³⁴, 0.1% SDS, 50 µg ml⁻¹ yeast tRNA (Sigma), 2.5 µg ml⁻¹ poly(A), 50 µg ml⁻¹ heat-denatured salmon testis DNA, 25 ng ml⁻¹ ³²P-labelled, nick translated, heat-denatured probe, 10 ml total volume per nitrocellulose filter, 67 °C, 24 h. The specific activity of the probes was about 2 × 10⁸ c.p.m. µg⁻¹. After hybridization the filters were washed exhaustively in 15 mM NaCl, 1.5 mM Na citrate, 0.05% SDS at 65 °C. A, B, C represent three different transgenic mouse lines. Each subscript number represents an individual mouse. +, Transgenic mice, -, negative littermates. X, spot of probe DNA. Exposures to Kodak X-omat film were for 4 days (a), 2 days (b), 5 days (c), 12 h (d).

Fig. 4. Northern blot³³ of total glyoxal treated³² spleen RNAs (30 µg per lane) of a pool of five normal mice (1, 5 and 9) and pools of three, five and three positive offspring from three different transgenic mice respectively (2, 6 and 10; 3, 7, and 11; 4, 8 and 12). Hybridization conditions were as in Fig. 3; probes were M13 C_K (1–4) and pES205 (V_K M.21) (5–12). Sizes of 28S and 18S ribosomal RNAs were determined from total mouse myeloma RNA electrophoresed in the same gel and then stained with ethidium bromide. After hybridization with C_K the blot of 1–4 was treated in 90% formamide at 65 °C for 3 h to remove most of the probe and later rehybridized with V_K M.21 (tracks 5–8). The small amount of radioactivity in lane 5 is from some remaining C_K probe which gave the same signal after reexposure to X-ray film before hybridization with V_K M.21 (not shown). The RNAs of tracks 9–12 were electrophoresed on a different gel and hybridized only with V_K M.21.

Fig. 5.

Relative quantities of C_{κ} and $V_{\kappa}M.21$ RNAs in spleens of transgenic mice and normal littermates. *a*, Probed with M13 C_{κ} . The spleen RNAs were 200 ng, 400 ng and 800 ng. *b*, Probed with pES205. The spleen RNAs were 0.5 μ g, 1 μ g and 2 μ g. **Methods:** Spleen poly(A)⁺ RNAs of the two positive offspring A were pooled, as were the poly(A)⁺ RNAs of offspring C. As controls, spleen RNA of negative A and C offspring were pooled together. Dot hybridizations of these RNAs included as a standard the DNA of the M21B₁ clone, and were hybridized with a C_{κ} or $V_{\kappa}M.21$ probe. A⁺: pool of spleen poly(A)⁺ RNAs of transgenic mice 1 and 2 (see Fig. 3); C⁺: pooled spleen poly(A)⁺ RNAs of transgenic mice 6 and 9. AC⁻: pooled spleen poly(A)⁺ RNAs of negative littermates 3, 7 and 8. PH, DNA of phage clone M21B₁ (see Fig. 1), 1, 2 and 4 ng (in *a*), 10, 20 and 40 ng (in *b*). Exposure to X-ray film: 14 h.

Fig. 6.

Two-dimensional (2-D) gels of serum proteins, *a*, Purified MOPC-21 immunoglobulin (Litton Bionetics). *b* and *c*, 25 μ l of serum of + and - offspring A, respectively, *d*, Additional examples of the characteristic 2-D gel regions of serum from one transgenic mouse (+) and five normal littermates (-). The sera were precipitated with 40% ammonium sulphate, the precipitate redissolved in glass distilled water and run on 2-D gels after O'Farrel gels which

were silver stained³⁵. Molecular weights (MW) of marker proteins run in the second dimension of the same gel (*a*) or of a control gel (*b*, *c*) are indicated.

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