Nucleic Acids Research, 2005, Vol. 33, No. 9 ***e85***
doi:10.1093/nar/gni083

A novel transgenic chimaeric mouse system for the rapid functional evaluation of genes encoding secreted proteins

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Received February 12, 2005; Revised and Accepted May 2, 2005

ABSTRACT

A major challenge of the post-genomic era is the functional characterization of anonymous open reading frames (ORFs) identified by the Human Genome Project. In this context, there is a strong requirement for the development of technologies that enhance our ability to analyze gene functions at the level of the whole organism. Here, we describe a rapid and efficient procedure to generate transgenic chimaeric mice that continuously secrete a foreign protein into the systemic circulation. The transgene units were inserted into the genomic site adjacent to the endogenous immunoglobulin (Ig) κ locus by homologous recombination, using a modified mouse embryonic stem (ES) cell line that exhibits a high frequency of homologous recombination at the Igκ region. The resultant ES clones were injected into embryos derived from a B-cell-deficient host strain, thus producing chimaerism-independent, B-cell-specific transgene expression. This feature of the system eliminates the time-consuming breeding typically implemented in standard transgenic strategies and allows for evaluating the effect of ectopic transgene expression directly in the resulting chimaeric mice. To demonstrate the utility of this system we showed high-level protein expression in the sera and severe phenotypes in human EPO (hEPO) and murine thrombopoietin (mTPO) transgenic chimaeras.

INTRODUCTION

The completion of human genome sequencing (1) has identified thousands of open reading frames (ORFs) that have not yet been assigned significant annotation. Exploring the functions of each of these ORFs is clearly an important step in biomedical research, and may lead to the development of novel pharmaceuticals and strategies to treat human diseases. In this context, much effort has been made to develop methodologies that can define gene functions in a variety of model organisms, thereby determining whether the candidate gene is 'drugable' or not. Secreted protein-coding ORFs, which are estimated to account for about one-fifth of all human protein-coding genes (2,3), bear particular attention in this regard since many of the most important biotherapeutics have resulted from the application of secreted proteins, including hormones, growth factors and cytokines. These can also be developed as potential targets for small molecule and antibody therapeutics.

Among various approaches for defining orphan gene function, transgenics and knockouts have been recognized as direct means for elucidating *in vivo* gene functions and have been widely employed (4). Generating an overexpressing transgenic by pronuclear injection can be informative; Simonet *et al*. (5) generated transgenic mice that expressed cDNAs from a liver-specific apolipoprotein E (ApoE) gene promoter. This study

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identified a novel protein, osteoprotegerin (OPG), whose expression led to a significant increase in bone mass in the resulting transgenic mice. Erythropoietin (EPO) is another example of a protein that, when overexpressed in the mouse, results in a phenotype that mimics the action of a therapeutic protein in humans (6).

The standard methods used to produce transgenic mice are inherently expensive and low throughput. Random integration of a transgene into an undefined region of the host genome often causes difficulty in obtaining lines with the intended transgene expression. Embryonic lethality caused by transgene expression is a common impediment to the functional assessment of therapeutic candidates. Single-copy transgenic mice (7), generated from embryonic stem (ES) cells carrying a targeted integration of transgene into a predetermined genomic site, could confer more controlled expression. However, both labor-intensive homologous recombination in ES cells and low efficiencies in obtaining germline transmission have hampered the application of this strategy to large-scale screening.

We describe here a novel approach that enables the rapid and efficient evaluation of the *in vivo* functions of ORFs encoding secreted proteins. Candidate cDNAs flanked by immunoglobulin kappa (Igκ) light-chain gene promoter are integrated into a site adjacent to the endogenous Igκ gene by homologous recombination, thus directing the transgene expression to circulating B-lymphocytes. The use of a modified ES cell line exhibiting improved rates of homologous recombination at the Igκ locus facilitates the production of an ES line carrying a targeted transgene. Large numbers of chimaeras that show a uniform level of transgene expression without chimaerism dependency can be readily produced by blastocyst complementation using B-cell-deficient host embryos. Phenotypic assessments can be performed immediately in the resultant chimaeras, thus eliminating the need to establish germline transmission of the transgene. To demonstrate the utility of this system we showed high-level protein expression in the sera and severe phenotypes in human EPO (hEPO) and murine thrombopoietin (mTPO) transgenic chimaeras.

MATERIALS AND METHODS

Generation of ΔRS-ES cell line

A C57BL/6 strain-derived bacterial artificial chromosome (BAC, RP23-435I4) including the recombining sequence (RS) was used for the PCR amplification of 5′ (5.2 kb) and 3′ (2.2 kb) homologous arms to construct a targeting vector for generating the ΔRS-ES cell line. Both PCR products were subcloned into pBluescript II KS (-) vector containing the DT-A cassette (8) as a negative selection marker, and subsequently the RS was replaced by pSTneoB (9) (RS-KO vector). Linearized RS-KO vector was electroporated to TT2F ES cells (39,XO) (9) and the resultant G418-resistant clones were analyzed by Southern blotting to identify homologous recombinants. Details of the generation of ΔRS-ES cell lines will be described elsewhere (M. Kakitani and K. Tomizuka, manuscript in preparation).

Targeting vector construction

A C57BL/6 strain-derived genomic DNA fragment (13.5 kb, EcoRI–XhoI) including the Jκ–Cκ region and the DT-A cassette (8) for negative selection were subcloned into pBluescript II KS (-) vector (pIgκDTA). The Igκ expression unit for introducing the exogenous cDNA was constructed as follows. The 0.21 kb Vκ promoter fragment (VκP) and 0.31 kb fragment (CκpA) including the Cκ polyA site were amplified by PCR using C57BL/6 mouse genomic DNA as a template and the following primer pairs: VκP-forward, CCCAAGCTTTGGTGATTATTCAGAGTAGTTTTAGATGAGTGCAT; VκP-reverse, ACGCGTCGACTTTGTCTTTGAACTTTGGTCCCTAGCTAATTACTA; CκpA-forward, ACGCGTCGACGCGGCCGGCCGCGCTAGCAGACAAAGGTCCTGAGACGCCACCACCAGCTCCC (including SalI, NaeI, FseI and NheI sites at the 5′ end); CκpA-reverse, GAAGATCTCAAGTGCAAAGACTCACTTTATTGAATATTTTCTG (including the BglII site at the 5′ end). The VκP and CκpA fragments were subcloned and the multi cloning site (MCS: SalI–NaeI–FseI–NheI) for cDNA insertion was artificially generated between these two fragments. The VκP–MCS–CκpA unit (Igκ expression unit) and puro^r marker cassette (2.4 kb, pPGKpuro) (9) were inserted into the site 0.25 kb downstream from the native Cκ polyA site on the pIgκDTA vector (pKIuniv). The orientation for transcription of the Igκ expression unit and puro^r cassette is the same as that of endogenous Igκ gene.

The following primer pair was used for amplification of a human EPO cDNA fragment from human kidney first-strand cDNA (Stratagene): hEPO, forward, CCGCTCGAGCGGCCACCATGGGGGTGCACGAATGTCCTG (including the XhoI site and Kozak sequence at the 5′ end) and reverse, CCGCTCGAGCGGTCATCTGTCCCCTGTCCTGCA (including the XhoI site at the 5′ end). The XhoI-digested PCR product was inserted into the SalI site within the MCS of the pKIuniv vector. A cDNA fragment of murine TPO was also prepared from mouse liver first-strand cDNA (Stratagene) by using a following primer pair and subsequently inserted into the SalI–FseI site of the pKIuniv vector: mTPO, forward, CCGCTCGAGCGGCCACCATGGAGCTGACTGATTTGCTCCTG (including the XhoI site and Kozak sequence at the 5′ end) and reverse, CCGGCCGGCCGCAAATCTATGTTTCCTGAGACAAATTCCTGGG (including the Fse I site at the 5′ end). Targeting vectors containing the other transgenes were also constructed as described above and details will be given elsewhere (M. Kakitani and K. Tomizuka, manuscript in preparation).

Generation of targeted ES cells

NotI-digested, linearized targeting vector was transferred by electroporation into wild-type (WT)-ES or ΔRS-ES cells. Genomic DNA samples prepared from puromycin-resistant ES clones were analyzed by Southern blotting to identify homologous recombinants. ES clones with normal karyotype were selected for subsequent chimaera production.

Chimaera production

The targeted ES cells were injected into eight-cell to morula stage embryos prepared from the immunoglobulin μ heavy-chain-KO (Δμ) homozygous mouse strain (9). After development of embryos to blastocysts, approximately 10 injected embryos were transplanted into a pseudopregnant MCH(ICR) mouse (Clea Japan, Inc.). Chimaerism in the offspring was

determined by the extent of ES cell-derived agouti coat color (dark brown) in the host embryo MCH(ICR)-derived albino coat color (white). Mice were kept under a 12/12-h dark/light cycle (lights on at 8:00 a.m.) and received 5 μm filtered water and CE-2 food (Clea Japan, Inc.) *ad libitum*. Male mice were housed individually after weaning.

Expression analysis of transgenes

First-strand cDNA was synthesized with Superscript III (Invitrogen) using random hexamers and 250 ng of total RNA extracted from 4-week-old hEPO/ΔRS and ΔRS control chimaeric tissues. Semi-quantitative RT–PCR analysis was carried out using the cDNA at specific annealing temperatures for each primer pair. PCR products were electrophoresed on 2% agar gels and stained with ethidium bromide. The integrity of RNA was controlled by the amplification of cDNA generated by the murine GAPDH. Primer pairs were hEPO-forward, GGCCAGGCCCTGTTGGTCAACTCTTC; hEPO-reverse, CGCTTGTGGGGAAGCCTCCAAGACC; Cκ-forward, CAACTGTATCCATCTTCCCACCATC; Cκ-reverse, ATGTCGTTCATACTCGTCCTTGGTC; GAPDH-forward, CACCATGGAGAAGGCCGGGGCCCAC; GAPDH-reverse, ATCATACTTGGCAGGTTTCTCCAGG.

Serum concentrations of mouse TPO and human EPO were measured by ELISA (R & D Systems). Mouse Ig μ (mμ) and γ (mγ) heavy chains were assayed as described previously (9).

Phenotypic analysis of transgenic chimaeras

All procedures were approved by the Institutional Animal Care and Use Committee at the Pharmaceutical Research Laboratories, Kirin Brewery Co. Ltd. (Takasaki, Japan). Peripheral blood samples used for measurement of B- and T-cell count, ELISA for serum immunoglobulins, and blood cell analysis (red blood cell, hemoglobin, platelet, reticulocyte, white blood cell, lymphocyte, bosophil, eosinophil, neutrophil, monocyte, large unstained cell, lobularity index) were obtained from retro-orbital bleeds of chimaeric animals. Serum samples for blood chemical analysis (albumin, total protein, alkali phosphatase, blood urea nitrogen, calcium, creatinene kinase, chloride, creatinine, total bilirubin, direct bilirubin, free cholesterol, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, phospholipid, triglyceride, non-esterified fatty acid, fructosamine, glycated albumin, glucose, amylase, uric acid, total ketone body, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, inorganic phosphorus, potassium, lactate dehydrogenase, leucine aminopeptidase, magnesium, sodium, n-acetyl-beta-d-glucosaminidase, iron and unsaturated iron-binding capacity) were prepared from the abdominal vein of sacrificed chimaeras and analyzed using a 7180 Automatic Analyzer (Hitachi High-Technologies Corp., Tokyo, Japan). B- and T-cell counts of mice in peripheral blood samples were measured by FACS analysis. IgM and IgG concentration in serum samples were measured by ELISA. An ADVIA 120 hematology system (Bayer Medical Ltd.) was used for blood cell analysis. Chimaeric animals were sacrificed under ether anesthesia for weight measurement and histological assessment of organs (brain, heart, lung, thymus, mesenteric lymph nodes, femur, ovary, testis, pancreas, liver, adrenal, kidney and spleen). Hematoxylin-eosin staining was carried out for histological analysis.

RESULTS

Generation of targeted ES clones

Our procedure is outlined in Figure 1. A targeting vector was designed to optimize the expression of exogenous cDNAs in B-lymphocytes (Figures 1 and 2A). The cDNA fragment has been inserted into a cloning site within an expression unit (Figures 1 and 2A) comprising the mouse Igκ promoter (0.21 kb) (10) and Cκ polyA addition site (0.31 kb). This expression unit is flanked by a puro^r marker located at a downstream site of the Cκ exon (Figure 2A). Targeted integration of this expression unit by homologous recombination places the expression of the transgene by the mouse Igκ promoter; *cis*-acting elements for this promoter include intronic and 3′ Igκ enhancers (11,12). We constructed vectors encoding the cDNA of human EPO (hEPO) and murine TPO (mTPO) as model genes to evaluate the potential of this system since these proteins have clear biological activities and have been used in studies utilizing conventional trangenic techniques (6,13,14).

The recombining sequence (RS) is located 25 kb downstream of the Cκ exon and undergoes V(D)J recombinase-dependent rearrangement that inactivates the Igκ locus by deletional rearrangements *in cis* (15) (Figure 2A). Although the involvement of RS rearrangements in receptor editing during B-cell development has been suggested (16), the functional significance of this phenomenon remains unclear. To examine the effects of deleting the RS on the expression of

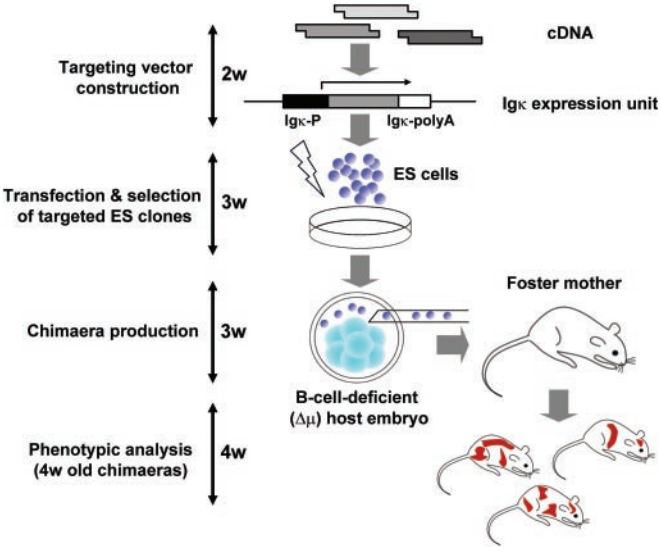

Figure 1. Schematic diagram showing the generation of transgenic chimaeric mice expressing secreted protein-coding ORFs in B-lymphocytes. The vector construction, isolation of targeted ES clones and chimaera production usually take 2, 3 and 3 weeks, respectively. Therefore, adult (4-week-old) chimaeras can be obtained within 3 months from the starting point of vector construction. The Igκ expression unit is located at a site downstream of the C exon on the targeting vector (see Figure 2A). The targeted ES clones were injected into eight-cell to morula stage embryos prepared from a mouse strain homozygous for Igκ μ heavy-chain-KO (Δμ) (9), in which mature B-lymphocytes and immunoglobulin production are absent. Igκ-P: Igκ promoter.

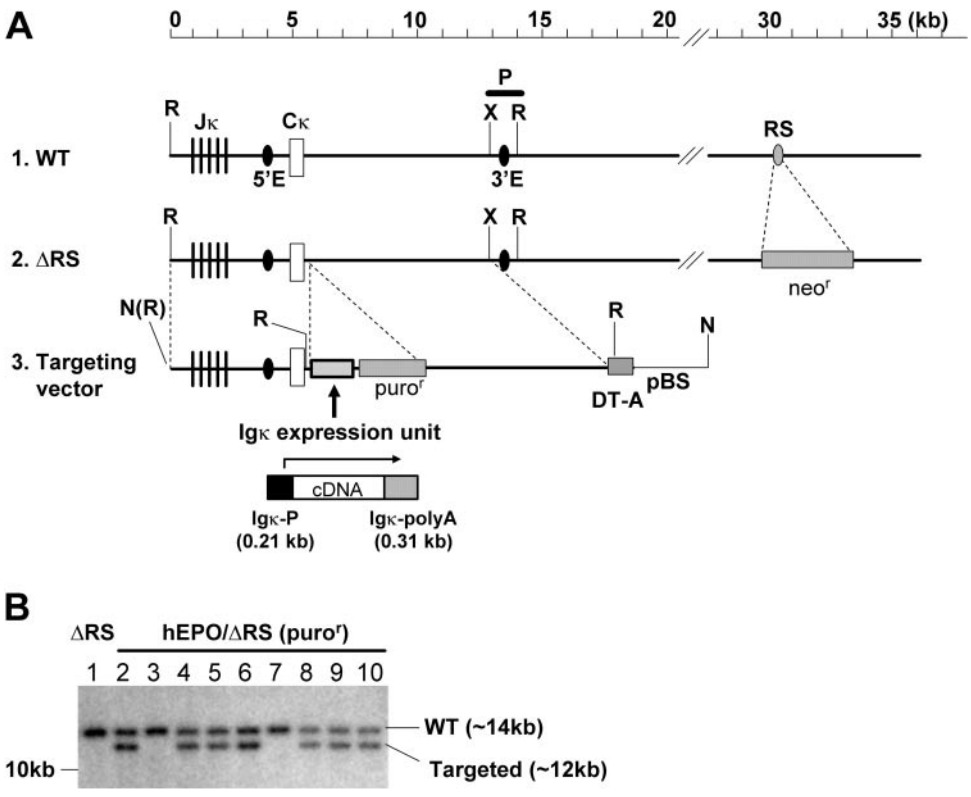

Figure 2. Generation of targeted ES cell lines. (**A**) Targeting strategy. (1) Wild-type (WT) endogenous Igκ locus. A closed rectangle above represents the probe used in Southern blot analysis (P, 1.28 kb XhoI–EcoRI fragment). (2) ΔRS locus with the G418-resistant (neo^r) cassette replacing the RS site of the WT locus. (3) Targeting construct. The Igκ expression unit flanked by a puromycin-resistant (puro^r) marker locates at a downstream site of the Cκ exon. 5′E, Igκ intronic enhancer; 3′E, Igκ 3′-enhancer; DT-A, diphtheria toxin A-fragment gene; pBS, pBluescript. Restriction enzyme sites are as follows: E, EcoRI; N, NotI; X, XhoI. (**B**) Southern blot analysis of genomic EcoRI-digested DNA prepared from nine randomly selected puro^r ES clones hybridized to the probe showed in (A). Lane 1, ΔRS-ES cells. Lane 2–10, puromycin-resistant ΔRS-ES clones obtained by transfection with hEPO targeting vector. Size markers, restriction fragments that represent WT allele (~14 kb) and targeted allele (~12 kb) are indicated.

inserted transgenes, we constructed a ΔRS-ES cell line in which RS was replaced with neomycin-resistant (Neo^r) marker cassette (Figure 2A). We used a karyotypically normal ΔRS line in addition to a WT-ES cell line to establish targeted ES clones containing various cDNA fragments.

Targeting vectors containing the hEPO and mTPO cDNA were transfected into both WT-ES (data not shown) and ΔRS-ES (Figure 2B) cells and the resultant puro^r clones were subjected to Southern analysis to identify homologous recombinants. In the WT-ES line studies, the ratio of homologous recombinants to total puro^r clones (targeted/random: TR ratio) was 16% and 3% in the hEPO and mTPO vectors, respectively. Using the ΔRS-ES line, we observed a remarkable increase in the TR ratio (hEPO: 65%, mTPO: 33%). Further examinations using targeting vectors of 20 independent cDNA fragments (0.5–1.6 kb) showed that the averaged TR ratio is 15 times higher in the ΔRS-ES line (46%) than in the WT-ES line (3%), indicating that the effect of the ΔRS-ES is independent of the sequence of inserted cDNA fragments (data not shown) and is not the simple result of clonal variation.

Production of chimaeric mice

To circumvent the typical chimaerism-dependent variation seen in the expression levels of transgenes, we utilized a blastocyst complementation strategy that was developed to evaluate gene function in lymphocytes (17). Injection of normal ES cells into blastocysts from RAG-2 (recombination-activating gene 2) gene-deficient blastocysts leads to the generation of somatic chimaeras with mature B- and T-cells, all of which derive from the injected ES cells (17). In an analogous process intended to produce B-cell-restricted expression, we used embryos prepared from female mice with B-cell deficiency caused by the homozygous deletion of the Ig μ gene (Δμ) (9). It should be noted that Δμ animals have no obvious defects other than the absence of mature B-lymphocytes and immunoglobulin production.

Eight-cell to morula Δμ (albino) embryos were injected with WT-ES and ΔRS-ES cells (agouti) and then implanted into foster mothers. As with normal embryos, ~20% of injected Δμ embryos were able to develop to viable chimaeras as judged from coat colors. The B-cell population in peripheral blood mononuclear cells of the resulting chimaeric pups was assayed 13 weeks after birth. Approximately 20–30% of the CD45-positive cells were B220-positive in both WT-ES and ΔRS-ES cell-derived chimaeric mice, a similar percentage to that seen in normal MCH(ICR) mice (data not shown). Serum levels of Igμ and Igγ in both types of chimaeras are comparable to those in normal MCH(ICR) mice (data not shown), also demonstrating the reconstitution of mature B-cell populations derived

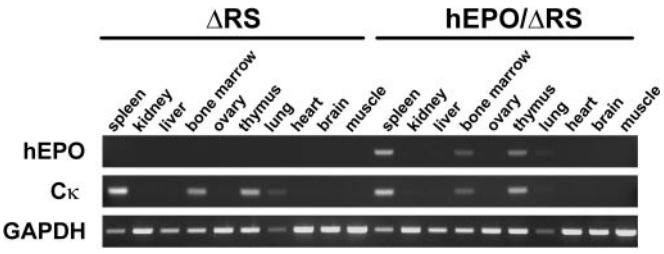

Figure 3. Total RNA samples prepared from 10 different tissues of 4-week-old hEPO/ΔRS and control ΔRS chimaeras were subjected to RT–PCR analysis using hEPO (35 cycles) and Cκ (35 cycles) specific primer pairs. The sizes of resulting PCR products in all experiments were consistent with the expected sizes predicted from reported cDNA sequences. Murine GAPDH (25 cycles) was used as a control.

from the injected ES cells. Furthermore, there was no correlation between coat color chimaerism and serum Igμ and Igγ levels (data not shown). We did not observe an unusual rate of physical abnormalities in the chimaeras derived from WT-ES and ΔRS-ES cells, nor any apparent changes in blood cell analysis, serum chemical analysis, urinalysis and tissue weight measurement (data not shown). We obtained 17 and 47 chimaeras derived from mTPO/WT-ES and hEPO/ΔRS-ES lines, respectively.

Expression of transgenes in chimaeric mice

Total RNA samples were prepared from various tissues of 4-week-old hEPO/ΔRS chimaeras and were subjected to semi-quantitative RT–PCR analysis to examine tissue-specificity of transgene expression (Figure 3). hEPO transcripts were prominent in the spleen, thymus and bone marrow, whereas a weak but detectable signal was observed in lung and kidney sections. This expression pattern is consistent with that of the Cκ transcript (Figure 3) and represents the distribution of B-lymphocytes in various adult tissues. These results indicate that the Igκ-driven transgene is properly regulated and the transcript is predominantly detected in tissues abundant in B-lymphocytes.

Serum protein levels produced from the transgenes were also determined by enzyme-linked immunosorbent assays (ELISAs) in hEPO/ΔRS and mTPO/WT chimaeras (Figure 4A and B). The hEPO protein level was low but detectable at 9 days of age and reached a plateau at 8 weeks (Figure 4A). A similar expression profile was observed for Igμ protein (Figure 4A), also implying that the major site of transgene expression is B-lymphocytes. In addition, the average serum concentration of hEPO protein in 6-week-old hEPO/WT chimaeras (4.8 ± 2.1 ng/ml, $n = 6$) was comparable to that in hEPO/ΔRS chimaeras (4.3 ± 1.3 ng/ml, $n = 47$, Figure 4A), which indicates that the insertion of neo^r marker cassette at the RS site did not significantly affect the transgene expression. In other studies, the levels of mTPO in the sera of mTPO/WT chimaeras (4.3 ± 1.6 ng/ml, $n = 2$) were indistinguishable from those in a control WT chimaera (4.5 ng/ml, $n = 1$) at 3 weeks of age (the first time point evaluated), whereas the increase became evident at 4 weeks (11.1 ± 3.5 ng/ml, $n = 12$). At 8 weeks, expression had

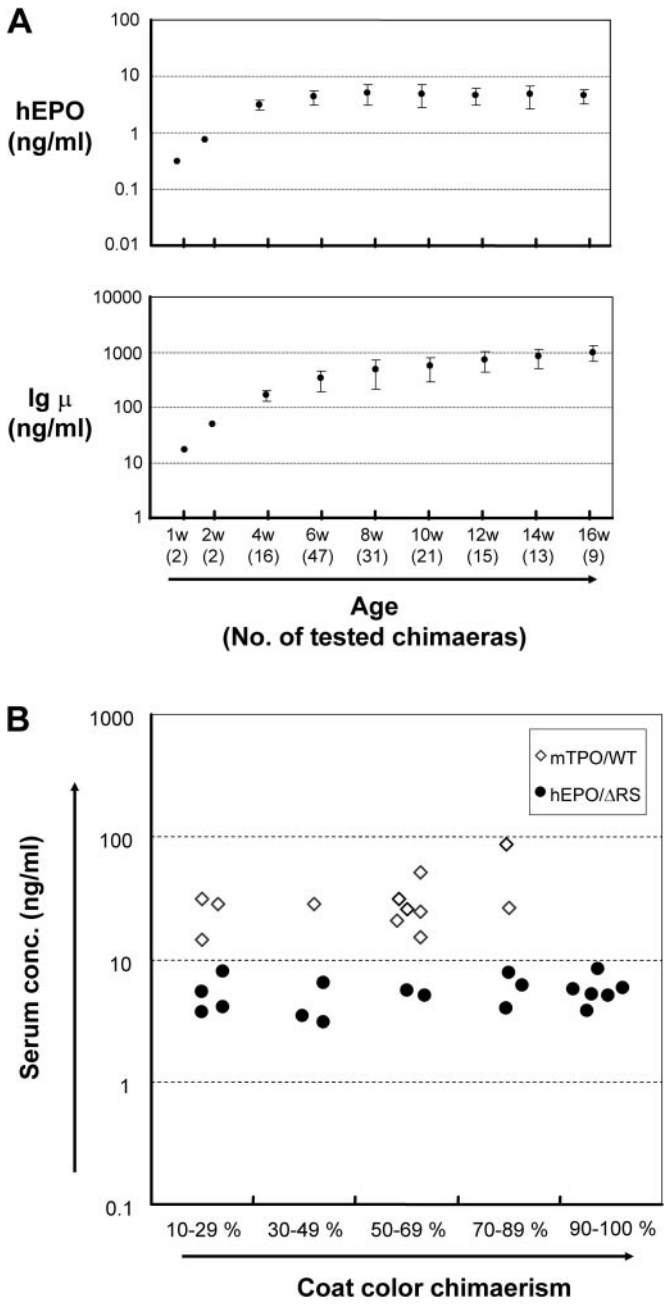

Figure 4. Serum expression of hEPO and mTPO in transgenic chimaeras. (**A**) Time course of hEPO and Ig μ levels in the sera of hEPO/ΔRS chimaeras. The number of tested individuals at each time point is indicated below ($n = 2$–47). Data are mean (1- to 16-week-old) and standard deviation (±SD, 4- to 16-week-old). (**B**) Serum hEPO and mTPO protein levels in hEPO/ΔRS ($n = 18$) and mTPO/WT ($n = 12$) chimaeras. Serum samples were prepared from 8-week-old animals with a range of coat color chimaerism. In the sera of control WT and ΔRS chimaeras, the hEPO protein was not detectable (<0.1 ng/ml) and mTPO level was 4.1 ± 1.5 ng/ml ($n = 4$). No mTPO/WT chimaeras with 90–100% chimaerism were examined.

reached a plateau (36 ± 20 ng/ml, $n = 12$, Figure 4B), nine times higher than the level of the controls (4.1 ± 1.5 ng/ml, $n = 4$). Both hEPO and mTPO levels in all the tested chimaeras persisted for at least 16 weeks. There was no apparent correlation between coat color chimaerism and

serum hEPO and mTPO levels at 8 weeks of age (Figure 4B), thus demonstrating the achievement of transgene expression that was independent of chimaerism by B-cell complementation.

Phenotypic analysis of hEPO and mTPO transgenic chimaeras

Severe erythrocytosis in hEPO/ΔRS chimaeras was evidenced by a significant increase in red blood cell (RBC), hematocrit (HCT) and reticulocyte (Retic) counts (Table 1). We also observed a significant decrease in platelet (PLT) counts (Table 1). Visual inspection of hEPO/ΔRS mice at necropsy revealed an enlarged spleen, suggesting enhanced extramedullar erythropoiesis. The wet weight of hEPO/ΔRS chimaera spleens (0.98 ± 0.62 g, $n = 3$, 16 weeks) was ~10-fold greater than that of the ΔRS controls (0.097 ± 0.012 g, $n = 7$, 16 weeks). Histological examination confirmed extramedullar erythropoiesis in the spleen (data not shown). In addition, the life span was markedly reduced in hEPO/ΔRS chimaeras; mean survival of hEPO/ΔRS chimaeras was 14 weeks ($n = 27$).

The mTPO/WT chimaeras exhibited thrombocytosis with a 5- to 8-fold increase in circulating PLT counts (Table 2). To evaluate the effects of mTPO overexpression on other blood cell parameters, we measured the levels of white blood cells (WBC), neutrophil (NEUT), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), RBC and hematocrit HCT (Table 2). We observed >10-fold increases in the circulating levels of myeloid and lymphoid cells, which contrasted with a 30–40% decrease in the HCT and RBC. Extramedullary hematopoiesis was observed in the liver, kidney, lymph nodes and lung.

DISCUSSION

Secreted protein encoding sequences can be identified using various bioinfomatic algorithms that predict the presence of N-terminal secretory signal peptides (18,19). In addition, soluble versions of single-pass membrane proteins can be produced that lack trasmembrane and cytoplasmic domains, yet retain their ability to bind cognate ligands. Both of these approaches have found considerable success in developing candidate biotherapeutic proteins. Our system has been optimized for screening large numbers of such cDNAs to ascertain their role in physiological processes. The procedure is rapid, simple and reproducible. Following vector construction by single-step insertion of a PCR-amplified coding sequence, we obtain drug-resistant ES cell transformants using standard electroporation methods. The isolation and analysis of only 12 ES clones is sufficient to establish multiple ES lines carrying a transgene insertion at the genomic site adjacent to the Igκ gene. Subsequently, large numbers of chimaeras can be produced by injecting the resultant ES clones into B-cell-deficient host embryos. We have routinely produced >20 chimaeras in a single-day injection (200 embryos) experiment. The primary phenotypic assessments using adult (4-week-old) chimaeras can be started within 3 months of the starting point of vector construction (see Figure 1). Furthermore, in the case that the transgene expression does not affect fertility in the chimaeras, germline transmission can be obtained for more detailed studies.

The B-cell complementation strategy allows for the generation of high-expressing transgenic mice in a single step without the requirement of time-consuming breeding techniques. The data presented demonstrate that the chimaeras display uniform transgene expression, irrespective of their coat color chimaerism; thus the functional assessment of transgenes

Table 1. Erythropoietic parameters of non-transgenic ΔRS and hEPO/ΔRS chimaeras

	ΔRS control		hEPO/ΔRS	
	Female ($n = 5$)	Male ($n = 4$)	Female ($n = 10$)	Male ($n = 8$)
RBC ($\times 10^6$ cells/μL)	10.8 ± 0.32	10.2 ± 0.71	19.1 ± 1.48*	18.2 ± 1.38*
HCT (%)	60.4 ± 3.03	59.5 ± 3.3	94.4 ± 2.65*	94.6 ± 2.56*
Retic ($\times 10^3$ cells/μL)	354 ± 58	429 ± 206	2674 ± 373*	2248 ± 304*
PLT ($\times 10^3$ cells/μL)	1290 ± 201	1353 ± 212	747 ± 198*	621 ± 241*

Data are presented as mean values ± SD for animals of 8 weeks of age.
*$P < 0.001$.

Table 2. Hematologic analysis of non-transgenic WT and mTPO/WT chimaeras

	WT control		mTPO/WT	
	Female ($n = 79$)	Male ($n = 23$)	Female ($n = 13$)	Male ($n = 4$)
RBC ($\times 10^6$ cells/μL)	10.8 ± 0.5	10.8 ± 0.5	6.4 ± 1.5*	7.2 ± 1.8*
HCT (%)	59 ± 2.9	62 ± 2.7	38 ± 8.2*	43 ± 10.2*
PLT ($\times 10^3$ cells/μL)	1213 ± 158	1320 ± 167	10 726 ± 3987*	6930 ± 4840*
WBC ($\times 10^3$ cells/μL)	5.3 ± 1.8	4.0 ± 1.2	81.9 ± 39.4*	83.8 ± 68.8*
NEUT (cells/μL)	576 ± 230	548 ± 191	33 764 ± 20 917*	34 500 ± 29 683*
MONO (cells/μL)	76 ± 35	62 ± 26	2494 ± 1896*	2786 ± 2337*
LYM (cells/μL)	4464 ± 1697	3067 ± 942	40 259 ± 16 938*	40 625 ± 32 552*
EOS (cells/μL)	127 ± 56	89 ± 23	918 ± 432*	767 ± 580*
BASO (cells/μL)	17 ± 14	12 ± 9.0	337 ± 159*	371 ± 312*

Data are presented as mean values ± SD for animals of 8 weeks of age.
*$P < 0.001$.

should be comparable to that of established transgenic lines. Moreover, the B-cell-restricted expression of these chimaeras may have significant advantage over conventional transgenic strategies. The postnatal onset of B-cell development, migration and directed expression can prevent developmental effects often seen in transgenic lines. One might presume that genomic lesions that can arise in cultured ES cells could result in phenotypes unrelated to transgene expression, especially when the tissues in the chimaeras are derived completely from the ES cells. In this regard, the B-cell complementation system, in which only B-lymphocytes are derived from the injected ES cells, is 'moderate' when compared with other complementation systems [tetraploid (20); RAG-2 (17)]. In addition, producing the chimaeras from multiple independent, karyotypically normal ES clones may also be helpful to avoid the confusion in identifying and characterizing subtle phenotypic changes. The following are possible limitations of our system, which relies on B-cell-specific transgene expression. First, the blood–brain barrier should prevent most circulating transgene products from penetrating the brain. This may cause difficulties in functional evaluation of transgenes in the central nervous system. Second, some proteins that require processing by cell-type-specific enzyme(s) to generate active forms may not be amenable to B-cell expression.

The introduction of a single-copy transgene into a predetermined site can overcome problems associated with integration of variable copy numbers of transgenes into random genomic loci (7). Although the rate of homologous recombination in mouse ES cells could be an impediment to the application of this strategy, there are now several approaches for efficient isolation of ES clones carrying the transgene at the chosen sites either by site-specific recombination (21) or by direct selection of transgene insertion into the Hprt locus (7). In the present study, we found a greatly improved TR ratio at the Igκ locus in the ΔRS-ES cell line, which allows for the efficient isolation of homologous recombinant ES clones using a standard positive–negative selection vector. Although this feature of the ΔRS-ES cell line should benefit our procedure, further investigations will be required to unravel the molecular basis for this phenomenon.

As expected, expression studies of our transgenic system indicate that the transgene is expressed mainly in B-lymphocytes, which may rely on the transcriptional regulation from the Igκ promoter under the control of surrounding *cis*-acting elements. This is supported by successful production of hybridomas secreting the mTPO protein using splenocytes prepared from mTPO/WT chimaeras (data not shown). Although B-cell precursors have been identified in developing fetal liver as early as day 12 of fetal life, the population shift from pre-B-cell to a B-cell capable of producing Ig is known to occur between days 17 and 18 (22). Furthermore, the development of various lymphoid organs after birth is accompanied by dramatic increase in the number of B-cells and the synthesis of Igs. We therefore suspected that the B-cell-specific transgene expression facilitates the analysis of genes whose overexpression with a strong, constitutive promoter (23) could cause embryonic lethality. Although the effect of transgene insertion on the expression of the neighboring endogenous Igκ allele has not been addressed, it may not circumvent the phenotypic analysis; the serum concentration of total Igs and κ/λ ratio were within normal range in transgenic chimaeras (data not shown).

We observed serum hEPO (5.4 ± 1.6 ng/ml, $n = 18$) and mTPO (36 ± 20 ng/ml, $n = 12$) protein levels in the chimaeras (8 weeks of age, Figure 4B) that were elevated compared with those observed in transgenic animals described previously [hEPO: 0.1–0.15 ng/ml (6), human TPO: ∼3 ng/ml (14)]. These results are consistent with the relatively severe phenotypes observed in the chimaeras. For example, splenomegaly associated with extramedurally erythropoiesis and doubled RBC counts were phenotypes described only in a recently established transgenic with high-level expression of hEPO (∼1.25 ng/ml) (13) and were not commented on in an earlier report (6). Among a number of previous studies of disregulated TPO production in mice, either by retrovirus (24–26), adenovirus (27,28), repeated TPO injections (29) or transgenesis (14), only two studies (26,28) reported a large increase in WBC. Villeval *et al*. (26) attributed the WBC elevation in their study to the high-level expression of virally transduced TPO. It should be noted that, in a traditional transgenic procedure, at least several lines may generally need to be analyzedto obtain a single line exhibiting a significant level of transgene expression. We therefore believe that our system has an advantage in terms of speed and efficiency in generating animals that continuously secrete a foreign protein into the systemic circulation at the levels of tens of nanograms per milliliter.

As an alternative approach, adenoviral vectors have been utilized as vehicles for *in vivo* gene delivery. The latest versions of Ad vectors exhibit improved duration of transgene expression and reduced systemic toxicity (30); however, their efficacy may depend on the quality of vector preparation and the route of administration in immuno-competent animals (30). In addition, potential immune responses against human proteins in transduced animals could circumvent the effective evaluation of human genes. The current data have demonstrated the utility of this procedure as a complementary approach to viral gene delivery, which should be well suited for studying chronic, long-term effects of the systemic expression of human proteins.

Recent technical advances in forward and reverse genetics in model organisms have facilitated the analysis of *in vivo* functions of numerous genes recorded by the Human Genome Project (31,32). The procedure we described here should be an important addition to the currently available repertoire of techniques. Systematic and careful phenotypic analysis of transgenic chimaeric animals should provide clues to the function of the newly identified secreted protein-coding cDNAs, which may lead to the identification of factors that modulate complex biological processes.

ACKNOWLEDGEMENTS

We thank M. Hiratsuka, T. Mikayama, J. Koumegawa, T. Sudoh and M. Nishikawa for valuable discussions, and W. Funk and M. Binnerts for comments on the manuscript. Funding to pay the Open Access publication charges for this article was provided by Kirin Brewery, Co., Ltd.

Conflict of interest statement. None declared.

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