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Stable Production of Mutant Mice from Double Gene Converted ES Cells with Puromycin and Neomycin

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SUMMARY: The antibiotic puromycin is an effective inhibitor of protein synthesis and puromycin N-acetyl transferase gene could be used as a dominant selection marker. We report the effective production of mutant mice from double gene-converted ES cells by selection with G418 and puromycin. We confirmed that (i) puromycin efficiently inhibited the growth of ES cells at a low-dose (0.1 μg/ml) and for a short time (2 days), independent of G418 selection; (ii) when these selected ES cells were injected into eight-cell stage embryos, the cells produced chimeras with high levels of chimerism; (iii) these chimeric males were fertile and exclusively yielded ES cell-derived offspring; and (iv) each offspring contained both neomycin transferase and puromycin N-acetyl transferase genes. © 1995 Academic Press, Inc.

Mutant mice production by gene targeting or gene trapping in ES cells has developed greatly in mammalian genetics. Efficient colonization into the germline of gene converted ES cells has primarily been by a *neo* gene and G418 selection (1-3). Efforts are being made with other dominant selection drugs, because double gene-converted methods are useful for the complete disruption of two alleles of a certain gene in ES cells, the both disruption of two closely linked genes, or to reverse abnormalities of gene-disrupted mice using ectopic expression of a gene. Only double dominant selection of G418 and hyg have to date been reported to completely disrupt a gene in cultured ES cells. All the phenotypes in these experiments have been analyzed using ES cells (4) or chimeric mice (5). Gene disrupted bone marrow cells of the chimeric mice are particularly advantageous

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Abbreviations: hyg: hygromycin B; neo: neomycin transferase; pac: puromycin N-acetyl transferase; ES cell: embryonic stem cell; PCR: polymerase chain reaction; NTR: 5' non-coding region of encephalomyocarditis; MBP: Myelin basic protein.

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for immunological studies, when the cells are transplanted into immune disrupted mice by X-ray irradiation (6). Since the double gene converted experiments have been limited to the stage of chimeric mice, no evidence has been reported of germline differentiating potency in ES cells doubly selected by G418 and hyg. To enrich homologous recombinants by positive-negative selection, however, double-drug selections with the *neo* gene and thymidine kinase (7) or hypoxiantine phosphoribosyltransferase genes (8) can be employed; these are less inhibitory of the colonization of the selected ES cells into each tissue in chimeric mice including the germline. In this type of selection, the negative selection marker genes were removed at the step of homologous integration and only one dominant selection marker remained (7).

Fyn-deficient mice appeared to have several types of neurological dysfunction: abnormalities in suckling (9), spatial learning (10), and emotional behavior (11) and susceptibility to audiogenic seizure (12). Since Fyn is widely expressed in the central nervous system through the developmental stage (13), it is not clear whether these abnormalities are derived from any brain region or at any developmental stage. To determine this, in addition to G418 and *neo* gene, improvement of a secondary dominant drug which does not inhibit the germline differentiating potency of the ES cells would be advantageous. Gene rescue experiments were individually performed on gene disrupted mice. Knockout mice were crossed with transgenic mice which had been independently produced. In this case, F2 offspring from chimeric or transgenic mice were the first generation in which the reverse phenotypes could be analyzed in the absence of a gene. If, however, the transgene could be integrated in the heterozygously gene disrupted ES cells (which are almost totally resistant to G418), we would be able to analyze them in the F1 generation by crossing the chimeric male and the gene knockout female. This F1 analysis would make possible systematic rescue analyses of, for instance, promoter trapping in many chimeric lines. Improvement of a secondary dominant drug will allow us to understand in what region and developmental stage Fyn is necessary to accomplish normal neurological function.

An antibiotic of puromycin inhibits growth of animal cells by blocking protein synthesis by 80S ribosomes. The puromycin resistant gene was isolated from *Streptomyces aboniger*, and it encoded *pac* gene (14). If expressed in animal cells, this *pac* gene rescued the growth and the protein synthesis in puromycin (15). Here, we provide the first evidence that, in addition to G418 and *neo* gene, puromycin efficiently killed the ES cells, the *pac* gene functioned as dominant selection marker for the cells and these selected ES cells possessed germline differentiating potency and could be used in the stable production of double gene-converted mice.

Materials and Methods

Construction of vectors: The vector, pGKPuro, the gift of Peter W. Laird, was the *pac* (puromycin resistant) gene and was inserted between the phosphoglycerate kinase-1 promoter and the polyadenylation signal (16). The co-electroporated vector, pAct-hfyn/myc or pMBP-hfyn/myc had the following fragments from 5' to 3' (in order):

XhoI-NcoI 1.3-kb chicken β-actin promoter from pactβgal' (17) or BglII-SalI 1.2-kb MBP promoter from pUC18 MBP promoter (18), XbaI-BamHI 0.6-kb NTR from pNTR-lacZ (19), 1.6-kb human *fyn* and myc epitope cDNA which was amplified by PCR between FD52 (5'-GGT CGA CAA TGG GCT GTG TGC AAT GTA AGG A-3') and FT34 containing myc epitope sequence (5'-GTC GAC ACG CGT TCA GTT GAG GTC CTC CTC GGA AAT AAG TTT CTG CTC CAT CGA CAG GTT TTC ACC AGG TTG GTA-3') from human *fyn* cDNA (20), MluI-XbaI 1.1-kb SV40 polyadenylation signal from pMT2-T-Z in EcoRV and SpeI sites of pBluescripts.

Cell culture and electroporation and screening of recombinant ES cells: The ES cell line, Fz45 in which one copy of *neo* gene was inserted into *fyn* gene locus, was cultured on embryonic fibroblast feeder layers that had been treated with Mytomycin C as described (2). Electroporation conditions were similar to those that have been described (3). The Fz45 cells were freshly thawed from frozen stock and subjected to electroporation after one passage. For electroporation, one microgram of linearized pGKPuro vector DNA by XbaI and/or 10 μg of linearized pAct-hfyn/myc or pMBP-hfyn/myc by SalI were used. The electroporated cells were selected at both 0.1 μg/ml of puromycin (Sigma) and 150 μg/ml of G418 (GIBCO-BRL) for 8 days. After G418 and puromycin selection, these drug resistant colonies were screened by PCR (see below). We chose 3 clones, FzP1, FzAF1 (co-electroporated with pAct-hfyn/myc) and FzMF1 (co-electroporated with pMBP-hfyn/myc) and confirmed them to be *pac* and *neo* and/or human *fyn/myc* gene positive clones.

Production of chimeric mice: Microinjection procedures were also similar to those described (2). The chimeras were bred with CD-1 females or checked for contribution of ES cells to the germline. Genomic DNA of F1 generation was also purified and analyzed by PCR.

PCR analysis: DNA was isolated as described (3), and 0.1 μg/ml DNA was dissolved in a final 25-μl vol of 1X PCR buffer (10 mM Tris-HCl, pH9.0, 50 mM KCl, 1.5 μM $MgCl_2$, 1% Triton X-100), containing 0.2 mM dNTPs, 0.2 mM primers, and 50 U/ml Taq polymerase. For the *pac* gene, 4% glycerol was also mixed with this reaction buffer and PCR was performed at 30 cycles of reaction at 96°C for 45 sec, at 58 °C for 25 sec, and at 72 °C for 3 min. Primers used were 5'Pac (5'-ATG ACC GAG TAC AAG CCA C-3') and 3'Pac (5'-TTA CGG GTC ATG CAC CAG GA-3'). For *neo* gene inserted *fyn* locus or the co-electroporated human *fyn* gene, PCRs were performed with each primer, FAP0 (5'-TGC ACA CTT AAG TAG GCT-5') and AGN0 (3) or FKS01 (5'-TGC AGA ATC CCT GCA GTT GA-3') and FKS02 (5'-GAG CTC TTC CTT CTC CAT CT-3'), respectively. The products were analyzed by 2% agarose gel electrophoresis.

Results

Puromycin resistance of ES cells. To determine the most appropriate concentration of puromycin for the selection of ES cells, drug resistance was confirmed by culturing the cells in various concentrations of the drug. ES cell lines, wild type TT-2, E14 and G418-resistant Fz45 cells were cultured in concentrations of puromycin of from 0.05 to 0.5 μg/ml on gelatin coated dishes for ten days. In these cell lines, no colony was obtained in more than 0.1 μg/ml puromycin (Fig.1A) and, at that concentration, the cells died of culturing after more than two days selection (Fig. 1B). This suggested that recombinants could be obtained by selection in 0.1 μg/ml for two days, and that puromycin could independently select against *neo* gene. We also sought to determine the optimal dose of puromycin for feeder cells produced from primary fibroblasts of 14-day CD-1 embryos; most feeder cells survived at 0.1 μg/ml concentration but died at a dose

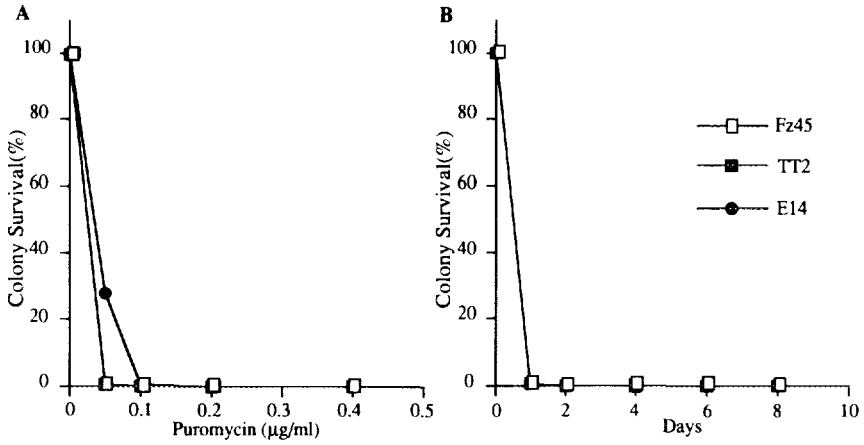

Figure 1. Resistance to puromycin. (A) Puromycin dose response of each ES cell line. (B) Selection period of each ES cell line with puromycin at a concentration of 0.1 μg/ml, Fz45 (G418 resistant), TT-2 (wild type), E14 (wild type).

of more than 1.0 μg/ml. We therefore used this 0.1 μg/ml concentration for selection of recombinant ES cells on the feeder cells which did not contain *pac* gene.

Isolation of puromycin and G418 resistant ES cells. Undifferentiated, highly pluripotent G418-resistant ES cell line Fz45 (9) was electroporated with pGKPuro or co-electroporated with pGKPuro and pAct-hfyn/myc or pMBP-hfyn/myc (Fig. 2). After electroporations were performed at 5 X 10^7 Fz45 cells with pGKPuro, these cells were subjected to selection with the 0.1 μg/ml puromycin and 200 μg/ml G418 for eight days on wild-type feeder cells, and 5, 3 and 5 colonies which were puromycin resistant were obtained with the plasmids of pGKPuro, pGKPuro and pMBP-hfyn/myc or pGKPuro and pAct-hfyn/myc, respectively. Surviving colonies were isolated and expanded, and their DNA was isolated from individual clones to identify recombinant directly by PCR on the *pac* gene and/or human *fyn*/myc gene (data not shown). *pac* gene and *neo* gene were inserted to all cells. However, low frequency was obtained by puromycin selection only on G418 resistant primary feeder cells. When puromycin-resistant STO cells were used as feeder cells, the frequency was approximately 200 / 2 X 10^7 electroporated ES cells (data not shown). These had been produced by inserting the pGKPuro to an NHL-7 cell line given us by H. Kondo (5). These results indicated that a high frequency for gene integrated cells was induced using the feeder cells containing *pac* gene.

In transformants of pAct-hfyn/myc, human Fyn with tagged myc epitope was detectable by Western blot analysis as a 60 kdal band with three kinds of anti-Fyn (MbF1) and an anti-c-Myc (Ab-1) antibody (data not shown). Transformants stably maintained the transgenes not only on the *pac* gene but also on the human Fyn cDNA, so that the NTR sequence functioned in TT2 cells.

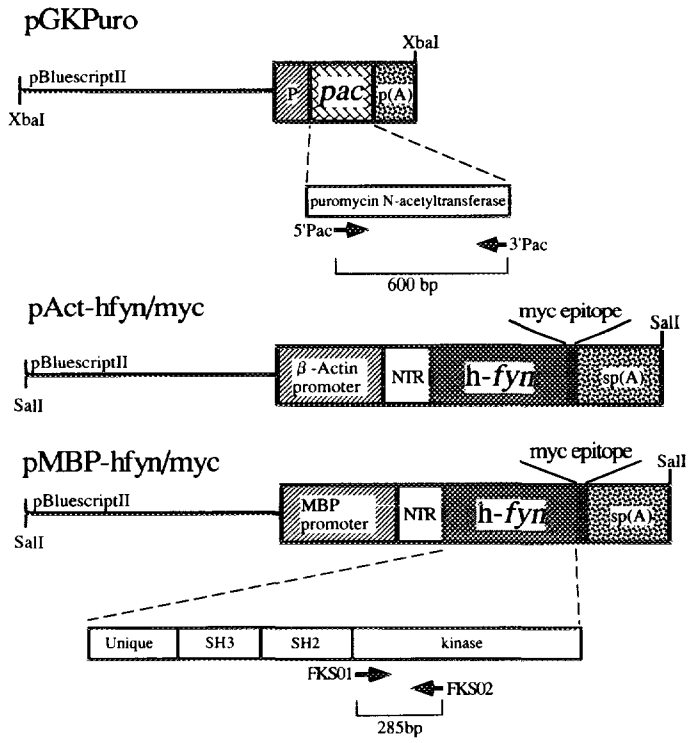

Figure 2. Schematic showing the transgenes used to derive the expression of puromycin resistant gene and human *fyn* cDNA. pGKPuro vector for expression of puromycin resistant gene, P, PGK1 promoter; p(A), poly A signal of PGK1 gene. Insertions of vectors were detected by PCR with 5' and 3' primers. pAct-hfyn/myc and pMBP-hfyn/myc are vectors for expression of human *fyn* cDNA driven by β-actin or MBP promoters, h-*fyn*, human *fyn* cDNA; myc epitope, coding sequences of epitope for anti-myc monoclonal antibodies; sP(A), poly A signal of SV40. Insertions were detected by PCR with FKS01 and FKS02 primers in both cases.

Efficient colonization of somatic tissues and germline. We tested the totipotency of the 4 ES cell lines, Fz45, FzP1, FzMF1 and FzAF1 by producing chimeric mice. The multipotency of the Fz45 cell lines (agouti coat color and black eyes) was judged by the coat color of pups after cells were injected into eight-cell stage embryos (Table 1). In control experiments, 71% of chimeric mice derived from Fz45 of parent cells had more than 50% of the agouti coat color. In puromycin and G418 resistant cells, FzP1, FzMF1 and FzAF1 also yielded over-half agouti chimeras in 91, 42 and 35%, respectively. One hundred percent chimeras in which ES cells were nearly dominant were obtained from double drug-resistant ES cells. Germline-differentiating potency of these cell lines was tested on these male chimeras by mating with albino CD-1 females. Eleven, 2 and 3 fertile chimeric males from FzP1, FzMF1 and FzAF1 produced ES-derived F1 offspring exclusively. Thus, double drug-selected TT2 cells had similar potency to TT2 cells (17); puromycin and G418 therefore little influenced the stability of totipotency and germline differentiating potency of TT2 cells in culture.

Table 1. Germ-line differentiating potency of G418 and puromycin resistant ES cells

Clone name	Transgene	No.embryo transplanted	No.pups born		No.males with ES contribution in coat at:		No.males mated and fertile	No. males that generated ES cell-derived offspring at:		
			Total (%)[a]	Male (%)[b]	100%	>50%		100%	100> >0%	0%
neo and *pac*										
FzP1	pGKPuro	80	26 (33)	24 (92)	10	10	10	10	0	0
FzAF1	pGKPuro and pAct-hfyn/myc	260	63 (24)	36 (57)	3	4	7	7	0	0
FzMF2	pGKPuro and pMBP-hfyn/myc	120	29 (24)	19 (66)	0	3	3	2	0	1
Total		460	118 (26)	79 (67)	13	17	20	19	0	1
neo										
Fz45	pGK2Neo	180	36 (20)	32 (89)	18	21	8	8	0	0

[a]% of embryos transplanted, [b]% of total pups born.

We also confirmed that F1 offspring derived from double drug-resistant ES cells had both *neo* and *pac* genes in the context of Mendelian ratio by PCR (Fig. 3A and C). The co-electroporated cDNA of human *fyn/myc* was inserted in multiple loci, because in 2, 3, 5, 6, 8 and 11 F1 offsprings, both *pac* and human*fyn/myc* genes were integrated, while in 13 and 14 only human *fyn/myc* was transmitted (Fig. 3B). The length of each PCR unit is shown in Fig 2. These results conclusively show that the ES cells can stably transmit *pac* and transgenes through the germline.

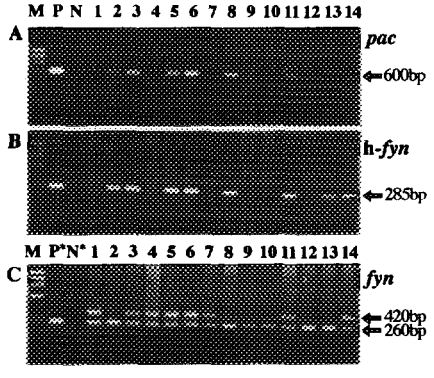

Figure 3. PCR analysis of tail DNA from fourteen F1 mice between FA+1 chimera-11 and CD-1 female (lanes 1-14). (A) *pac* gene. (B) human *fyn* cDNA. Lane P, positive control (10pg each of pGKPuro and pAct-hfyn/myc vectors) and lane N, negative control (100ng of genomic DNA from C57BL/6). (C)Targetted *fyn* locus. Lane P*, positive control (100ng of genomic DNA from C57BL/6) and N*, negative control (10pg each of pGKPuro and pAct-hfyn/myc vectors).

Discussion

These experiments have demonstrated that the antibiotic puromycin is effective in the selection of ES cells with high germline differentiating potency. It works at lower concentration of 0.1 μg/ml for the ES cells than the 1.0 μg/ml required for feeder cells, and might more completely block the growth of higher growth rate strains such as the ES cells. In comparion with other conventional selection drugs, G418 and hyg, the period of selection with puromycin is two days, much less than any other selection drug (six to ten days). Since the selection period of ES cells influence their germline differentiating potency, it is possible that puromycin is better as a dominant selection marker than G418 or hyg.

These results also indicated that puromycin could be used with G418. As described in the Introduction, a secondary drug against G418 with stable germline differentiating potency will be useful for our systematic phenotypic rescue experiments. Puromycin is one of the best candidates for our future analyses of the Fyn working region or developmental stage regulating suckling, learning or emotional behavior or forming ordered cell layers of the hippocampus. The drug might also be useful for secondary gene targeting for another genetically linked locus such as the gene of a Hox cluster. Double drug selections have usually been used for the complete disruption of two alleles of a certain gene in ES cells. However, more precise analysis of chimerism, such as GPI assay, should be performed using several organs of the chimeric mice. We obtained 100% chimeric mice from several ES cell lines selected by puromycin as judged by hair and eye color, male distortion and body size; CD-1 and chimeric mice with less than 100% chimerism were larger than the F1 between C57BL/6 and CBA mice (data not shown). It is thus possible the drug could be used for analyses of chimeric mice by gene knockout in ES cells. Using puromycin and *pac* gene, mutant mice production could be greatly improved for mammalian genetic studies.

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References

1. Gossler, A., Doetschman, T., Korn, R., Serfling, E. and Kemler, R. (1986) Proc. Natl. Acad. Sci. USA, 83,9065-9069.
2. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y. and Aizawa, S.. (1993) Analytical Biochem., 214, 70-76.
3. Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y. and Aizawa, S.. (1993)Analytical Biochem., 214, 77-86.
4. te Riele, H., Maandag, E. R., Clarke, A., Hooper, M. and Berns, A. (1990) Nature, 348, 649-651.

5. Sawai, S., Shimono, A., Hanaoka, K. and Kondoh, H. (1991) The New Biologist, 3, 861-869.
6. Jakobovits, A., Vergara, G.J., Kennedy, J.L., Hales, J.F., McGuinness, R.P., Casentini-Borocz, D.E., Brenner, D.G. and Otten, G.R. (1993) Proc. Natl. Acad. Sci. USA, 83,9065-9069.
7. Mansour, S.L., Thomas, K.R., and Capecchi, M.R. (1988) Nature, 336, 348-352.
8. Reid, L.H., Gregg, R.G., Smithies, O. and Koller, B.H. (1990) Proc. Natl. Acad. Sci. USA, 87, 4299-4303.
9. Yagi, T., Aizawa, S., Tokunaga, T.,Shigetani, Y., Takeda, N., and Ikawa, Y. (1993) Nature, 366, 742-745.
10. Grant, S.G.N., O'Dell, T.J., Karl, K.A., Stein, P.L., Soriano, P. and Kandel, E.R. (1992) Science, 258, 1903-1910.
11. Miyakawa, T., Yagi, T., Watanabe, S. and Niki, H. (1994) Mol. Brain Res., 27, 179-182.
12. Miyakawa, T., Yagi, T., Taniguchi, M. and Niki, H. (1995) Mol. Brain Res., in press.
13. Yagi, T. (1994) Develop. Growth & Differ. 36, 543-550.
14. Vara, J., Malpartida, F., Hopwood, D.A. and Jimenez, A. (1985) Gene, 33,197-206.
15. Vara, J.A., Portela, A., Ortin, J. and Jimenez, A. (1986) Nucleic Acids Res., 14, 4617-4624.
16. Boer, H.P., Potten, H., Adra, N.C., Jardine, K., Mullhofer, G., and McBurney, M.W. (1990) Biochem. Genet., 28, 299-307.
17. Yagi, T., Shigetani, Y., Okado, N., Tokunaga, T., Ikawa, Y., and Aizawa, S. (1993) Oncogene, 8, 3343-3351.
18. Miura, M, Tamura, T., Aoyama, A. and Mikoshiba, K. (1989) Gene, 75, 31-38.
19. Jang, S.K., Krausslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C. and Wimmer, E. (1988), J. Virol., 62, 2636-2643.
20. Semba,K., Nishizawa, M., Yoshida, M.C., Sukegawa, J., Yamanashi, Y.,Sasaki, M., Yamamoto, T. and Toyoshima, K. (1986) Proc. Natl. Acad. Sci. USA, 83, 5459-5463.