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A transgenic mouse strain expressing four drug-selectable marker genes

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ABSTRACT

Murine embryonic stem (ES) cells are commonly cultured on feeder layers of primary murine embryonic fibroblasts (MEFs). Because gene targeting experiments often involve sequential selection for multiple-drug resistance in single ES cell lines, we have developed a new mouse strain which represents an economical donor for the production of multiple-drug resistant MEFs. MEFs prepared from the DR-4 mouse strain displayed resistance to concentrations of the drugs G418, 6-thioguanine, puromycin and hygromycin well above those used normally for the selection of drug-resistant ES cells.

Murine embryonic stem (ES) cells are commonly cultured on feeder layers of primary murine embryonic fibroblasts (MEFs). MEFs provide both a substrate for ES cells to proliferate and also produce leukemia inhibitory factor, which inhibits ES cell differentiation (1). MEFs are useful not only for routine maintenance of ES cells but also in gene targeting experiments which involve the sequential cultivation of transfected cells in media with different drugs for selection of drug-resistant clones. Growth in selection media necessitates cultivation of feeder cells which are resistant to the respective drug employed. Individual mouse strains bearing resistance markers for neo^R (2), hyg^R (3) and $puro^R$ (4) have been reported. However, MEFs must be prepared separately from the appropriate strain for each type of drug selection, and the different mouse strains have to be maintained at considerable cost. Many experiments require sequential selection for different markers and this would be facilitated by the availability of feeders with multiple-drug-resistance markers. In this paper we describe the derivation of a mouse strain which carries four different drug resistance markers and can be used for preparing MEFS.

The DR-4 strain of mice was prepared by the intercrossing of three different strains, one bearing resistance genes neo^R and $puro^R$ (4), a second bearing the resistance gene hyg^R (3), and a third bearing a natural deletion encompassing the *Hprt* gene. The neo^R and $puro^R$ genes, which confer resistance to G418 and puromycin, respectively, were introduced by homologous recombination in the *Dnmt* gene, which encodes the mammalian cytosine methyltransferase (4). The hyg^R gene confers resistance to hygromycin and was introduced into C57BL/6 mice by pronuclear injection (3). All three marker genes are controlled by the promoter and polyadenylation signal of the murine 3-phosphoglycerate kinase (*Pgk-1*) gene, which ensures high levels of expression in both ES cells and MEFs (5). Finally, an *in vitro*-selected 10 kb deletion within the X-linked *Hprt* gene confers resistance to the drug 6-thioguanine (6TG) (6).

Mice homozygous for all four genes were generated as follows. Firstly, animals homozygous for the linked neo^R–$puro^R$ constructs were generated. As described previously, a $puro^R$-containing cassette was transfected into ES cells homozygous for the neo^R-bearing $Dnmt^s$ gene-knockout construct, which resulted in wild-type *Dnmt* gene expression and the linkage of the $puro^R$ and neo^R markers (4). These ES cells were used to derive chimeras which were mated with BALB/c females, and progeny containing the neo^R–$puro^R$ transgene were intercrossed to produce mice homozygous for the insert.

A second drug-resistant strain was derived from blastocyst injection of the ES cell line E14TG2a. This male cell line was generated by *in vitro* selection of wild-type E14 ES cells with 6TG, selecting for loss of HPRT function, which is X-chromosome linked (6). The 6TG-resistant derivative E14TG2a bears a 10 kb deletion spanning the promoter and the first two exons of *Hprt* ($hprt^{b–m3}$) (7). E14TG2a ES cells were injected into C57BL/6 blastocysts, and male chimeras were crossed to C57BL/6 females. Female progeny carrying the $hprt^{b–m3}$ allele were used to generate homozygous animals in two generations by crossing to 129/SvJae males and intercrossing the progeny.

To generate animals heterozygous for all three loci, homozygous neo^R–$puro^R$ animals were mated to F1 progeny of the two strains $hprt^{b–m3}$ and C57BL/6J-TgN(pPWL512hyg)1Ems, the latter of which is homozygous for the hyg^R transgene (3). Triple heterozygotes were crossed, using males bearing the $hprt^{b–m3}$ allele, and triple homozygotes were obtained at the expected frequency of 1/32. The genotypes of all mice were ascertained by Southern blot assays described previously (3,7,8). Homozygous animals were intercrossed to produce the strain DR-4 (of mixed 129/SvJae, 129/OlaHsd, BALB/c and C57BL/6 backgrounds, notation as in ref. 9).

MEFs were prepared by crossing homozygous male DR-4 mice to wild-type BALB/c or C57BL/6 females. Embryos were removed at 14.5 days after conception, and MEFs were prepared from their decapitated, eviscerated carcasses. Carcasses were

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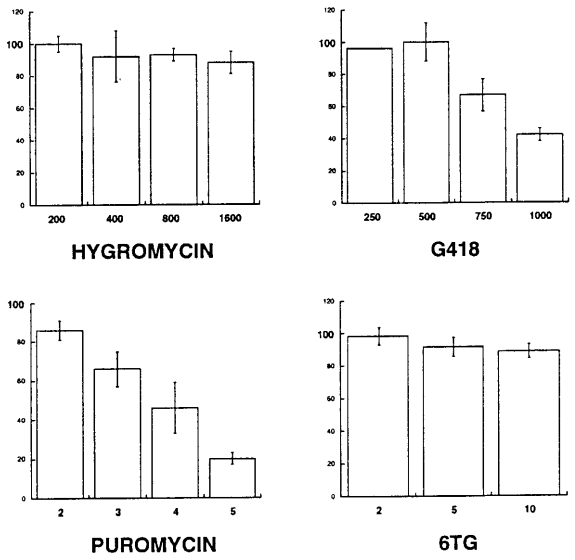

Figure 1. Drug resistance of irradiated MEFs prepared from strain DR-4 mice. MEFs (5×10^5) were plated on 10 cm^2 wells in triplicate and their survival in media tested with four separate drugs commonly used in ES cell transfections. Cells were fed daily and counted after 9 days of selection. Survival is compared to similarly-plated cells without drug selection and expressed as a percentage, with standard deviation indicated. Fibroblasts prepared from a wild-type 129/SvJae mouse were completely killed at the lowest concentration of each drug used. All drug concentrations in the Figure are in μg/ml.

minced in 0.25% trypsin (BRL), incubated for 30 min, and vigorously pipetted to aid in suspension before plating out. MEFs were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acids (BRL), and antibiotics. These primary cultures were frozen in aliquots with 10% DMSO after 24 h in culture. The drug resistance of the MEFs was tested after expansion and γ-irradiation with 3000 RADs in a Gammacell 40 (Atomic Energy of Canada).

Drug resistance was assayed by plating 5×10^5 irradiated MEFs in 10 cm^2 wells and feeding 24 h later with 2.5 ml drug-containing media once a day. Cells were washed, trypsinized, and counted after 9 days of drug selection. Assays were performed in triplicate and survival compared to untreated cells (Fig. 1). DR-4-derived MEFs were found to be fully resistant to G418 (BRL) and hygromycin (Boehringer Mannheim) at concentrations well above those normally used for ES cell transfections. Common concentrations employed are 250 μg/ml (active) for G418 (10,11), 110 μg/ml for hygromycin (12,13), and 2 μg/ml for puromycin (Sigma) (4). The MEFs did show some sensitivity to puromycin at 2 μg/ml. However, when the MEFs were plated at a proportionately higher density, subsequent selection left behind a layer of MEFs which was suitable as a feeder layer (data not shown).

The DR-4 strain was crossed in timed matings to *hprt*$^{b\text{-}m3}$ homozygotes and MEFs prepared from embryos, as described above. MEFs were tested for their sensitivity to 6TG (Sigma) (Fig. 1) and found to be >80% resistant in a range of concentrations used for ES cell selection (6,10).

MEFs prepared from the DR-4 mouse strain displayed resistance to concentrations of the drugs G418, 6TG, puromycin and hygromycin normally used for the selection of drug-resistant ES cells. Because gene targeting experiments often involve sequential selection for multiple-drug resistance in single ES cell lines, the DR-4 strain represents a suitable and economical donor for the production of multiple-drug resistant MEFs.

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