DEVELOPMENTAL DYNAMICS 209:85–91 (1997)

Trisomy Eight in ES Cells Is a Common Potential Problem in Gene Targeting and Interferes With Germ Line Transmission

XIN LIU,[1] HONG WU,[1] JANET LORING,[1] SHERIAR HORMUZDI,[3] CHRISTINE M. DISTECHE,[4] PAUL BORNSTEIN,[3] AND RUDOLF JAENISCH[1,2]*

[1]*The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts*
[2]*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts*
[3]*Department of Biochemistry, University of Washington, Seattle, Washington*
[4]*Department of Pathology, University of Washington, Seattle, Washington*

ABSTRACT **The ability to contribute to the germ line is the most important experimental feature of embryonic stem (ES) cells. Using ES cells, it is possible to introduce targeted mutations into any gene and to derive the corresponding mutant mice. A common problem with this technology is that the ES cells often lack or have only a low efficiency of germ line transmission. To address this issue, we examined the relationship between the growth rate and karyotype of ES cells, and their ability to contribute to the germ line. We found that chromosomal abnormalities occurred rather frequently in ES cells. Cells having an abnormal number of chromosomes, in particular trisomy 8, were found in three independently derived ES cell lines, and this abnormality conferred a selective growth advantage on these cells. Selection of abnormal cells led to depletion and eventual loss of normal ES cells during consecutive passages. In comparison with parental ES cells, ES cells with trisomy 8 contributed rarely to the germ line. This realization allowed us to select, based upon ES cell clone morphology, those clones with the highest probability of contributing to the germ line. This insight is of practical value for any given gene targeting experiment as it permits optimization of the rate of success without having to rely on more elaborate tests such as karyotyping individual clones prior to blastocyst injection.** ***Dev. Dyn. 209:85–91, 1997.*** **© 1997 Wiley-Liss, Inc.**

Key words: ES cells; germ line transmission; gene targeting

INTRODUCTION

Murine ES cells are permanent cell lines that are derived from the inner cell mass of a preimplantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981). The pluripotent state of ES cells can be maintained in culture and, upon return to the embryonic environment, these cells can contribute to all cell lineages, including the germ line of resulting chimeric mice (Bradley et al., 1984). The ability of ES cells to contribute to the germ line provided an opportunity to introduce targeted mutations in cell culture and to derive mouse strains carrying these mutations (Capecchi, 1994; Hooper et al., 1987; Soriano, 1995; Thomas et al., 1986; Zijlstra et al., 1989). Since the first reports in 1990 on the phenotypic consequences of using this approach to generate mutant mice, (Koller et al., 1990; Zijlstra et al., 1990), there has been an explosion of efforts to achieve gene targeting in many laboratories. Over 300 different mutant strains carrying targeted mutations have been reported, and attempts to derive many more mutant strains are in progress. However, despite recent successes, inefficient germ line transmission of mutant genes by chimeric mice remains a major problem.

Previous studies showed that leukemia inhibitory factor (LIF) was a key supplement in the culture medium that preserved the ability of ES cells to contribute to the germ line (Nichols et al., 1990; Williams et al., 1988), and that the efficiency of germ line transmission decreased when the passage number of ES cells increased (Nagy et al., 1990). However, what caused the decrease in the ability of ES cells to contribute to the germ line when they were cultured in standard ES cell culture medium was not known. It often seemed that some clones were more successful in targeting experiments than others, leading to the suspicion that in vitro cultivation of ES cells may accumulate genetic alterations, which could interfere with germ line competence. Recently, more sophisticated mutations, including point mutations and mutations that permit conditional knockouts, have been introduced into ES cells and these procedures require that ES cells undergo more passages in culture before they are injected into blastocysts (Gu et al., 1993; Hanks et al., 1995; Hasty et al., 1991; Liu et al., 1995; Wang et al., 1996). Therefore, it would be desirable to establish easy-to-use criteria which would allow the selection of those clones with the highest germ line competence. To address this

*Correspondence to: Dr. Rudolf Jaenisch, The Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA 02142.
Received 24 December 1996; Accepted 6 February 1997

TABLE 1. Summary of the Contributions of Different ES Cell Clones to Chimeric and Germ Line Mice[a]

			Chimeric mice			
Locus	Clone no.	Colony size	Fraction of total offspring	% Agouti coat color	G producing/ total bred	G/total offspring
Msx1	12	L	4/10	10–40	0/4	0/53
	82	L	19/24	<50 × 16 60–80 × 3	0/19	0/461
TSP2		L		<50 × 12 60–80 × 2	0/14	0/315
NT4	2	L	7/11	10–50	0/7	0/182
	25	M	14/19	60–90 10–50	1/14	2/417
	26	L	2/5	30, 40	0/2	0/61
	33	L	15/19	15–60	0/15	0/72
	34	S	4/5	100	1/1	14/14
EpoR	15	L	4/10	40–50	0/4	0/207
	23	S	13/20	70 × 3, 100 × 5	5/8	29/64
	32	M	9/15	60–80	3/9	19/409
	37	L	10/20	30–70	0/10	0/443
	78	L	6/10	20–40	0/6	0/150
	96	S	14/18	100 × 12		
Epo	6	S	9/14	60–80	4/6	63/166
	52	S	9/13	All 100	2/2	24/27
	119	S	6/9	50–60	0/4	0/67
	132	S	18/24	70–90 × 9 100 × 8	4/15	48/220
	189	S	15/20	40–90	5/10	101/142

[a]All clones are derived from the J1 cell line. The results are based on a single injection of each clone. G, germ line animal based on coat color; L, large; M, medium; S, small; TSP2, thrombospondin 2.

issue, we examined the growth rate, karyotype, and the efficiency of germ line transmission of several lines of ES cells. In this study, we show that trisomy 8 is associated with a selective growth advantage in vitro and represents a common cause for the failure of ES cells to contribute to the germ line. Use of small, slower growing clones rather than vigorously growing, large clones for blastocyst injection is a simple means of substantially improving the success of germ line transmission.

RESULTS

Inverse Correlation Exists Between the Efficiency of Germ Line Transmission and the Growth Rate of Different ES Cell Clones

We first encountered the problem of low efficiency of germ line transmission when we injected ES cells, carrying targeted mutations in Msx1 or thrombospondin 2 (TSP2) genes, into normal blastocysts. These cells did not contribute to the germ line of resulting mice, although they did give rise to many chimeras, including some high percentage coat color chimeras (Table 1, top). Since previous studies of ES cells showed a correlation between higher passage number and lower efficiency of germ line transmission, we used an early passage of J1 ES cells (passage 6) for the next three experiments in which targeted mutations of the neurotropin 4 (NT4), erythropoietin receptor (EpoR), and erythropoietin (Epo) genes were performed. While deriving mutant mouse strains for NT4, we found that there was an inverse correlation between the efficiency of germ line transmission and the growth rate of ES cell clones. Slow-growing ES cell clones generated chimeras with a high percentage of agouti coat color and generally contributed to the germ line (Table 1, middle). We define slow-growing ES cell clones as those with a small colony size (Fig. 1, left) after eight or nine days in culture following electroporation, and for which a longer time is needed for clones to grow to confluence during expansion. In contrast, many large colonies (Fig. 1, right), isolated at the same time, reached confluence earlier during expansion and did not contribute to the germ line after injection into blastocysts.

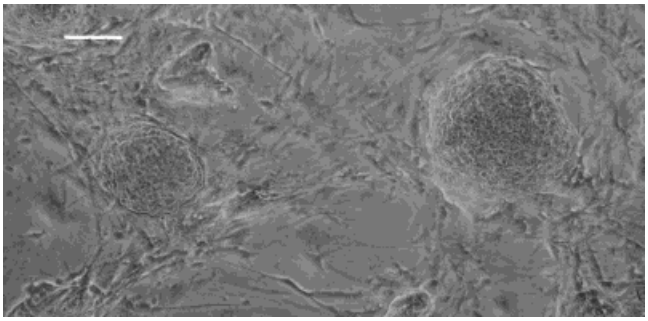

Fig. 1. The morphology of ES cell colonies grown in ES cell medium plus 400 μg/ml G418 for 9 days after electroporation. Examples of a small, slowly growing clone (left) and a large, more rapidly growing clone (right) are shown. Bar = 150 μm.

To test more systematically for a potential effect of colony size (a function of growth rate) on germ line

transmission, we injected either fast- or slow-growing clones of EpoR mutant ES cells into blastocysts to compare efficiencies of generating chimeras and contribution to the germ line. The results confirmed the earlier observation: the slow-growing ES cell clones gave rise to a high percentage of chimeras and demonstrated a high efficiency of transmission to the germ line (Table 1, middle). In subsequent experiments with mutant ES cells for Epo, we injected only slow-growing ES cell clones. As predicted, almost all of these clones gave rise to chimeras, which subsequently showed a high efficiency of transmission of the mutation (Table 1, bottom).

Trisomy 8 Is Correlated With a Low Efficiency of ES Cell Germ Line Transmission

To enable a better understanding of factors affecting the ability of ES cells to transmit mutations to the germ line, we analyzed the karyotypes of ES cell clones and found that all of the TSP2-targeted ES cells carried trisomy 8 (data not shown). The surprisingly high rate of this chromosomal abnormality in ES cells from targeted clones led us to examine the parental ES cells. To our surprise, 50% of passage six J1 cells and all passage eight J1 cells were trisomic for chromosome 8 (data not shown). Since about half of the passage six J1 cells carried trisomy 8, we karyotyped ES cells from NT4 and EpoR targeting experiments. The upper half of Table 2 shows that the karyotypes of all of the cells from the rapidly growing clones (nos. 2, 33, and 37) were trisomy 8, either as free trisomy 8 or in the form of an isochromosome 8. In contrast, most of the cells from the more slowly growing ES cell clones (nos. 23, 34, and 96) displayed a normal karyotype. The data in Table 1 correlate well with the karyotype analysis in Table 2; most ES cell clones with a normal karyotype gave rise to a high percentage of chimeras and contributed to the germ line whereas none of the ES cell clones with trisomy 8 produced chimeras that transmitted the mutation to the germ line.

TABLE 2. Karyotypes of Different ES Cell Clones[a]

Cell line (locus)	Clone no.	Abnormality	% of total cells tested
J1 (NT4)	2	Trisomy 8 [i(8)][b]	100 (22/22)
	25	Trisomy 8	30 (4/14)
		Translocation of Chromosome 12 (band C1 → tel) to tel of Y	70 (10/14)
	33	Trisomy 8	100 (20/20)
	34	Normal	95 (19/20)
		41 chromosomes	5 (1/20)
J1 (EpoR)	23	Normal	80 (16/20)
		Trisomy 8	15 (3/20)
		Trisomy 11	5 (1/20)
	32	Loss of Y (XO)	100 (20/20)
	37	Trisomy 8 [i(8)][b]	100 (16/16)
	96	Normal	100 (20/20)
J1	L4	Trisomy 8 [i(8)][b]	100 (20/20)
	L5	Trisomy 6, 7, 8, 12, 15	100 (20/20)
	M3	Normal	100 (20/20)
	S5	Normal	100 (20/20)
	S7	Normal	100 (20/20)
REK2	p9	Normal	95 (19/20)
		Trisomy 11	5 (1/20)
	p11	Normal	80 (16/20)
		Trisomy 8	15 (3/20)
		Trisomy 8 and 11	5 (1/20)
REK2 (TSP2)	86	Trisomy 8	95 (18/19)
		Normal	5 (1/19)
	112	Trisomy 8	80 (16/20)
		Trisomy 8 and marker	20 (4/20)
REK2 (Col)	54	Trisomy 8	95 (19/20)
	56	Trisomy 8	70 (7/10)
		Normal	20 (2/10)
		Trisomy 8 and 15	10 (1/10)
	64	Trisomy 8	100 (20/20)
	118	Trisomy 8	100 (19/19)
RW4	p9	Normal[c]	100 (20/20)
	p11	Normal	60 (12/20)
		Trisomy 8	20 (4/20)
		Various abnormalities	20 (4/20)
RW4 (TSP3)	9	Various abnormalities	100 (20/20)
	89	Trisomy 8	100 (20/20)
	239	Normal	100 (20/20)
RW4 (Met)	6	Trisomy 8	100 (20/20)
	10	Normal	100 (20/20)
	32	Trisomy 8 [1(8)][b] Inversion of chromosome 3	100 (20/20)

[a]L, large; M, medium; S, small; p, passage.
[b]An isochromosome of chromosome 8 resulting in trisomy for this chromosome.
[c]Normal complement of 40 chromosomes, but banding was not performed.

ES Cells Carrying Trisomy 8 Have a Higher Growth Rate Than That of Normal Cells

To study further the correlation between the karyotype and growth rate of different ES cell clones, we undertook two experiments. First, we grew passage six J1 cells from a cell stock (in which the majority of cells were shown to have a diploid karyotype) for eight days in normal ES cell medium. The resulting colonies were isolated and categorized into three classes: large, medium, and small, and were subsequently expanded and karyotyped. All the cells examined from large colonies (Table 2, J1: L4, L5) displayed chromosomal abnormalities, mostly trisomy 8 (Fig. 2B), while all the cells from small colonies (Table 2, J1: S5, S7) were normal (Fig. 2A). To demonstrate directly that ES cells carrying trisomy 8 have a selective growth advantage over normal cells, we mixed and plated equal numbers of cells from clone 2 (a 100% trisomy 8 ES cell line derived from cells carrying targeted mutations of the NT4 gene) and clone 96 (an ES cell line with 100% normal karyotype derived from cells carrying targeted mutations of the EpoR gene). To control for the possibility that a property of NT4-targeted ES cells other than trisomy 8 was responsible for an increased growth rate, equal numbers of cells from clone 34 (an ES cell line derived from NT4-targeted cells with 85% normal karyotype) with clone 37 (an ES cell line derived from EpoR-targeted cells with 100% trisomy 8) were mixed and cultivated. The mixed clones were cultured

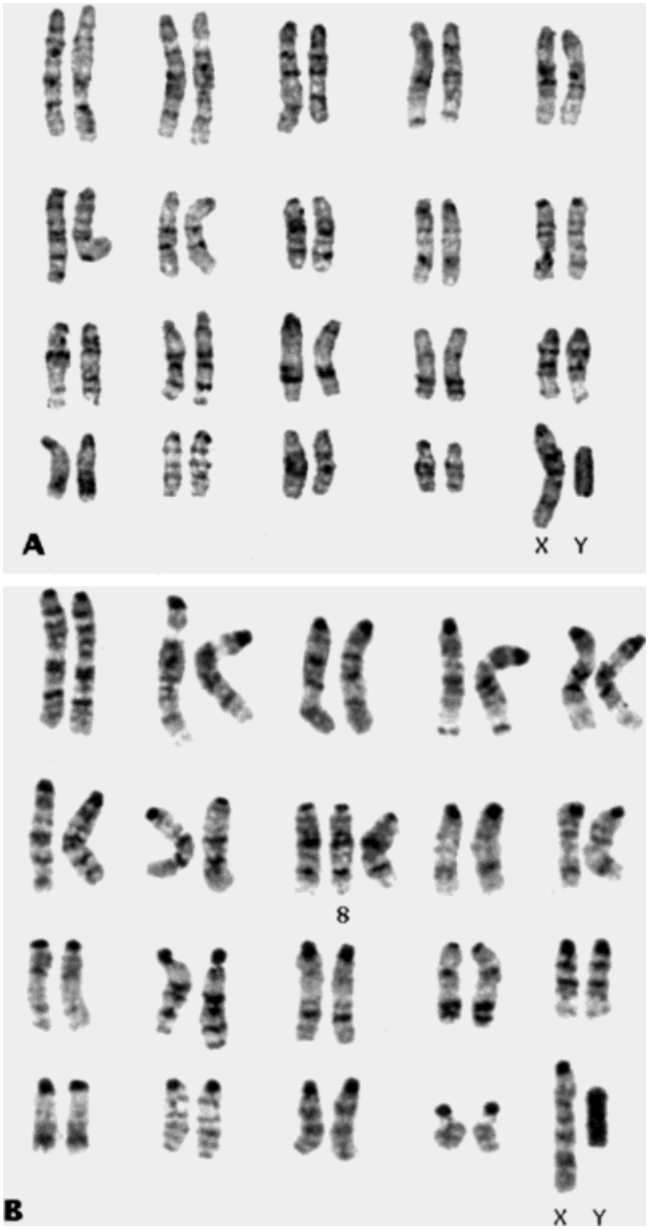

Fig. 2. Karyotypes of (**A**) an S7 ES cell and (**B**) an NT4, clone 33 ES cell. G-banding was performed. Note trisomy eight in B.

in normal ES medium and passaged every third day. After each passage, some of the cells were harvested and their genomic DNA was prepared and used for Southern blot analysis. Figure 3 shows that, from passage one to five, the ratios between the intensity of the bands representing ES cells carrying trisomy 8, and that of bands from cells with normal karyotype, increased considerably, indicating that the percentage of ES cells carrying trisomy 8 in the whole population increased with increasing passage number. These experiments show that cells with trisomy 8 outgrew the diploid ES cells during growth in culture.

Trisomy Eight May Represent a Common Problem That Leads to a Low Efficiency of Contribution of ES Cells to the Germ Line

To address the question of whether chromosomal abnormalities, especially trisomy 8, are phenomena that are associated with J1 cells in particular, or if they represent a more common problem, we analyzed REK2 and RW4 ES cells, which are two independently derived ES cell lines. We found that a small portion of REK2 cells had chromosomal abnormalities at passage nine (Table 2). Interestingly, we observed that two passages later (p11), the percentage of ES cells carrying trisomy 8 became more obvious (Table 2). More importantly, after one round of targeting experiments, which entails an additional six passages, the majority of the cells were trisomic for chromosome 8 (Table 2). Similar findings were observed with RW4 (Table 2, bottom) a widely used cell line that is available commercially. We speculate that the increase in the proportion of abnormal cells in these experiments was due not only to the selective growth advantage of cells carrying trisomy 8, but also to the tendency of the experimenter to isolate large, more vigorously growing colonies after electroporation. We conclude that such colonies are more likely to contain trisomy 8 (or other chromosomal abnormalities) as a result of the increased growth rate of these karyotypically abnormal cells.

DISCUSSION

In this study, we found a strong correlation between the growth rate, karyotype, and the efficiency with which ES cells contribute to the germ line of chimeric mice. Nine fast-growing ES cell lines from different gene targeting experiments were injected, 81 chimeras derived from these injections were bred, and none of the 1,844 offspring from these breedings contributed to the germ line (Table 1). Karyotype analyses were performed on 4 of these 9 cell lines and the cells were found to be trisomic for chromosome 8 (Table 2). These results indicate that ES cells carrying trisomy 8 can contribute to somatic tissues, but lose the ability to contribute to the germ line. In contrast, all of these slow-growing clones, except Epo 119 (Table 1), contributed to the germ line efficiently and the karyotypes of most of the cells from the slow-growing clones were normal (Table 2). Moreover, the correlation between growth rate and karyotype was easily reproduced by plating wild-type J1 ES cells, isolating colonies based on their size, and karyotyping the cells from these colonies. Among these recloned J1 ES cell lines, we have injected cells from line J1 M3, and these cells contributed to the germ line (Table 2). These data strongly suggest that ES cells carrying trisomy 8 have a higher growth rate and a diminished efficiency of contribution to the germ line, compared to cells with a normal karyotype. Our data emphasize a particularly important and counterintuitive point in gene targeting experiments: when selecting ES cell clones for injection into blastocysts, the smaller and slower growing colonies should be

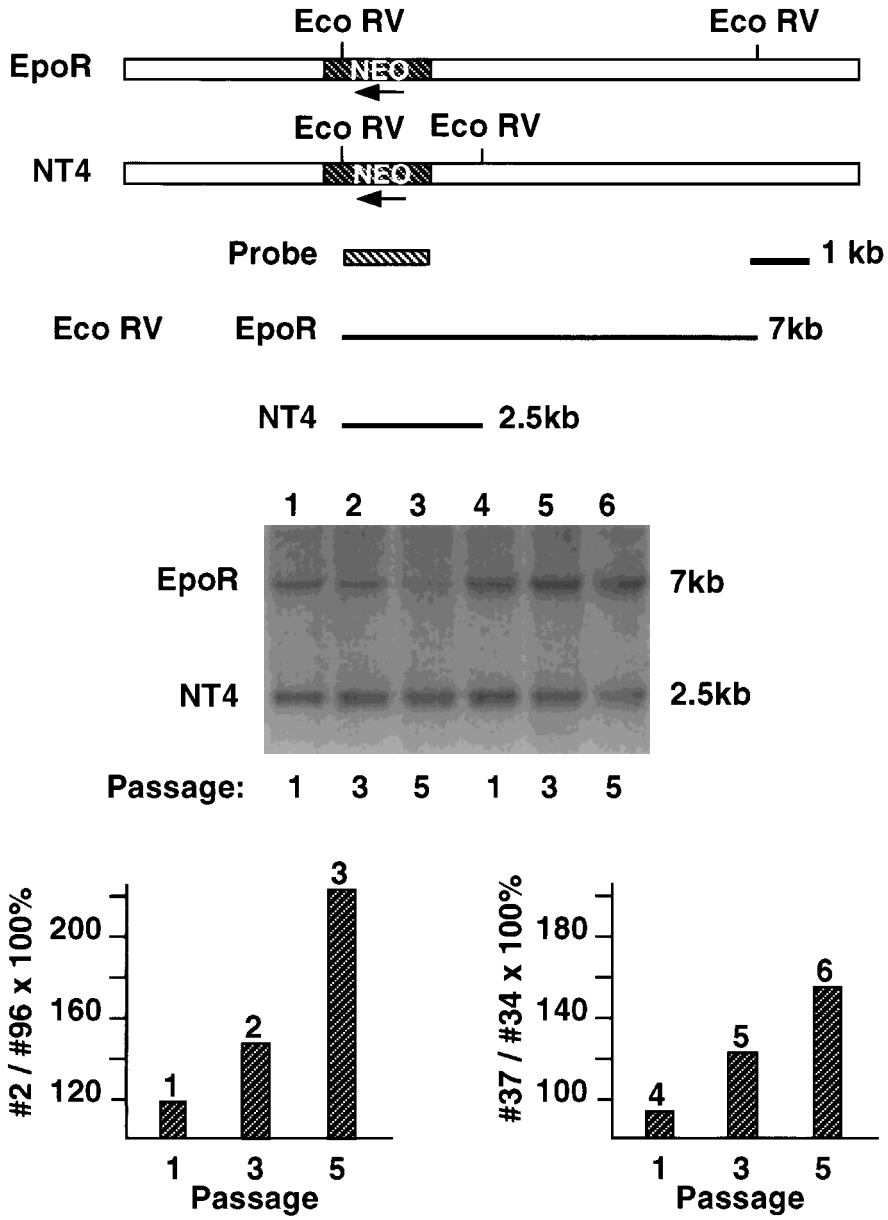

Fig. 3. Relative growth rates of ES cells carrying trisomy 8 and cells with a normal karyotype, as determined by Southern blot analysis. **Top:** Restriction map of the targeted alleles of EpoR and NT4 genomic DNA, the PGK NEO probe used for the hybridization, and the expected sizes of the Eco RV fragments of the targeted alleles of EpoR and NT4 genomic DNA. **Middle:** Southern blot. **Lanes 1, 2,** and **3:** DNA from the mixed cultures of ES cells from clone 2 with a targeted NT4 allele (100% trisomy 8) and clone 96 with a targeted EpoR allele (100% normal karyotype) of passage number 1, 3, and 5, respectively. **Lanes 4, 5,** and **6:** DNA from the mixed cultures of cells from clone 34 with a targeted NT4 allele (85% normal karyotype) and clone 37 with a targeted EpoR allele (100% trisomy 8) of passage number 1, 3, and 5, respectively. **Bottom:** On the left is the relative density of the NT4 band from 2 to the EpoR band from 96, when the 96 band in each lane is counted as 100%. On the right is the relative density of the EpoR band from 37 to the NT4 band from 34, when the 34 band in each lane is counted as 100%.

selected rather than the larger more vigorous-looking clones.

It is known that long-term tissue culture will select for abnormal cells within a population. It is not clear, however, how well the ability of ES cells to contribute to germ line transmission is retained in long-term culture. Previous studies showed that ES cells could still contribute to the germ line after 15 passages (Bradley et al., 1984; Evans et al., 1985). Another study found that efficiency of contribution to the germ line declined as the number of ES cell passages increased (Nagy et al., 1993). We confirm here that chromosomal abnormalities can occur relatively frequently in ES cells and that, among the cells carrying abnormal karyotypes, ES cells carrying trisomy 8 are capable of overgrowing the culture in a rather short period of time.

The higher growth rate of trisomic cells very likely contributes to the enrichment of abnormal cells, since ES cells carrying trisomy 8 overgrew normal cells in 5 passages in co-culture experiments (Fig. 3).

Although our data show that an extra copy of chromosome 8 in ES cells confers on these cells a growth advantage and diminishes their ability to contribute to the germ line, concomitant cellular and molecular changes were not evident. Under the light microscope, there was no detectable difference between ES cells carrying trisomy 8 and cells with a normal karyotype, except that the colonies of cells carrying trisomy 8 were relatively uniform whereas the plating of normal cells usually gave rise to colonies that were heterogeneous in size. Trisomy 8 appears to be of particular growth advantage because it arose independently and was selected for in three different ES cell lines. Chromosome 8 and the syntenic human chromosomes 8 and 16 carry oncogenes such as Jun b and d, Lyl-1, and Ras 15-2, 6 (Ceci et al., 1990; Drivas et al., 1991; Howard et al., 1991; Kuo et al., 1991; Mattei et al., 1990). Furthermore, trisomy 8 or 16 in humans has been associated with acute lymphoblastic leukemia (Helm and Mitelman, 1992; Pettenati et al., 1994). Future studies may reveal whether these, or other determinants, contribute to the altered phenotype of ES cells with trisomy 8.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture

The J1 ES cell line was derived from a male agouti 129/terSv mouse embryo (Li et al., 1992). The J1 (NT4) cell lines are J1 cells carrying a targeted mutation such that the coding region in one allele of the neurotropin 4 gene was replaced with a PGK NEO gene (Liu et al., 1995). J1 (EpoR) cell lines are J1 cells carrying a mutation that partially deleted the coding region in one allele of the erythropoetin receptor gene and replaced it with a PGK NEO gene (Wu et al., 1995). The REK2 ES cell line was also derived from a male agouti 129/SvJ embryo (Brandon et al., 1995). REK2 (TSP2) and REK2 (Col) are REK2 cells carrying targeted mutations in thrombospondin 2 and α1(I) collagen genes, respectively (unpublished data). RW4 cells were derived from a male agouti 129/SvJ embryo and were purchased from Genome Systems, St. Louis, MO, RW4 (TSP3) and RW4 (Met) are RW4 cells carrying targeted mutations in the thrombospondin 3 and metaxin genes, respectively (unpublished data).

ES cells were grown in an ES cell culture medium, which is HEPES-buffered (20 mM, pH 7.3) Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), 0.1 mM nonessential amino acids (Gibco, Grand Island, NY), 0.1 mM β-mercaptoethanol, antibiotics, and 500 U/ml leukemia inhibitory factor. ES cell clones which carried targeted mutations were isolated after growth in ES cell medium plus 400 μg/ml G418 (Gibco) for 8 to 9 days following electroporation.

The comparison of growth rate between cells with trisomy 8 and cells with a normal karyotype was performed as follows: 1×10^5 cells of clone 2 [J1 (NT4), 100% trisomy 8] were mixed with 1×10^5 cells of clone 96 [J1 (EpoR), normal karyotype] and grown in ES culture medium in one well of a six well plate. Three days after plating, the cells were trypsinized (passage one) and 1/10 of the suspension was plated in one well of a six well plate. The rest of the cell suspension was used for preparation of DNA for Southern blot analysis. The same procedure was repeated until DNA from passage five was collected. Cells from clone 34 and 37 were mixed and treated in a similar fashion.

All the ES cell cultures were performed on layers of γ-irradiated embryonic mouse fibroblasts (EF) obtained from day 14 embryos that carried a targeted mutation in which a portion of the β2-microglobulin gene was replaced by PGK-NEO (Zijlstra et al., 1989).

Blastocyst Injection and Breeding of Chimeric Mice

Manipulation of mouse embryos was carried out as described (Bradley, 1987). ES cells were trypsinized and washed once in EF cell medium (DMEM supplemented with 10% FCS and antibiotics). Each of the BALB/c or C57BL/6J blastocysts was injected with about 20 ES cells in M2 medium and the blastocysts were then transferred to the uterine horns of pseudopregnant females (10–14 embryos/mouse). Chimeric mice were identified by coat color, and chimeric mice derived from injected BALB/c or C57BL/6J blastocysts were bred with BALB/c or C57BL/6J mice, respectively.

Karyotype Analysis

Karyotype analysis was performed on ES cells using the previously described methodology (Robertson, 1987) with minor modifications. G-banding was carried out to identify chromosomes. Approximately 20 cells were examined for each line and at least two complete haplotypes were cut.

Southern Blot Analysis

DNA was prepared as described (Laird et al., 1991), digested with Eco RV, fractionated by electrophoresis through a 0.7% agarose gel, blotted with 10 × SSC onto Hybond N^+ (Amersham, Arlington Heights, IL), and hybridized with a 1.6 kb PGK NEO probe. The Southern blot was exposed to a FUJI imaging plate, which was then scanned in a FUJIX BAS 2000 scanner. The image and the relative density were produced using the MacBas V2.2 program.

ACKNOWLEDGMENTS

We thank Dr. Rejean Idzerda for providing us with the REK2 ES cell line, and Doug Chapman and Ann Stampalia for excellent technical assistance. This research was supported by NIH/NCI grant R35-CA44339 to R.J. and NIH grants AR11248 and DE08229 to P.B. X.L. was supported by a fellowship from the Leukemia

Society, and S.H. by a fellowship from the Arthritis Foundation.

REFERENCES

Bradley, A. (1987) Production and analysis of chimeric mice. In: "Teratocarcinomas and Embryonic Stem Cells." Robertson, E.J. (ed). Oxford: IRL Press, pp. 113–52.

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309:255–256.

Brandon, E.P., Gerhold, K.A., Qi, M., McKnight, G.S., and Idzerda, R.L. (1995) Derivation of novel embryonic stem cell lines and targeting of cyclic AMP-dependent protein kinase genes. Recent Prog. Horm. Res. 50:403–408.

Capecchi, M.R. (1994) Targeted gene replacement. Sci. Am. 270:52–59.

Ceci, J.D., Justice, M.J., Lock, L.F., Jenkins, N.A., and Copeland, N.G. (1990) An interspecific backcross linkage map of mouse chromosome 8. Genomics 6:72–79.

Drivas, G., Massey, R., Chang, H.Y., Rush, M.G., and D'Eustachio, P. (1991) Ras-like genes and gene families in the mouse. Mamm. Genome 1:112–117.

Evans, M., Bradley, A., and Robertson, E.J. (1985) Genetic manipulation of the early mammalian embryo. p. 93. In: "Banbury Report." Jaenisch, R., and Constantini, F. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Evans, M.J., and Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156.

Gu, H., Zou, Y.R., and Rajewsky, K. (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell 73:1155–1164.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A.B., and Joyner A.L. (1995) Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. Science 269:679–682.

Hasty, P., Ramirez-Solis, R., Krumlauf, R., and Bradley, A. (1991) Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. Nature 350:243–6 [published erratum appears in Nature (1991) Sept. 5, 353:94].

Heim, S., and Mitelman, F. (1992) Cytogenetic analysis in the diagnosis of acute leukemia. Cancer 70:1701–1709.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germ line colonization by cultured cells. Nature 326:292–295.

Howard, T.A., Rochelle, J.M., Saunders, A.M., and Seldin, M.F. (1991) A linkage map of mouse chromosome 8: Further definition of homologous linkage relationships between mouse chromosome 8 and human chromosomes 8, 16, and 19. Genomics 10:207–213.

Koller, B.H., Marrack, P., Kappler, J.W., and Smithies, O. (1990) Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. Science 248:1227–1230.

Kuo, S.S., Mellentin, J.D., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Cleary, M.L. (1991) Structure, chromosome mapping, and expression of the mouse Lyl-1 gene. Oncogene 6:961–968.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R., and Berns, A. (1991) Simplified mammalian DNA isolation procedure. Nucleic Acids Res. 19:4293.

Li, E., Bestor, T.H., and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926.

Liu, X., Ernfors, P., Wu, H., and Jaenisch, R. (1995) Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. Nature 375:238–241.

Liu, X., Wu, H., Byrne, M., Jeffrey, J., Krane, S., and Jaenisch, R. (1995) A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. J. Cell Biol. 130:227–237.

Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U.S.A. 78:7634–7638.

Mattei, M.G., Simon-Chazottes, D., Hirai, S., Ryseck, R.P., Galcheva-Gargova, Z., Guenet, J.L., Mattei, J.F., Bravo, R., and Yaniv, M. (1990) Chromosomal localization of the three members of the jun proto-oncogene family in mouse and man. Oncogene 5:151–156.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. 90:8424–8428.

Nichols, J., Evans, E.P., and Smith, A.G. (1990) Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. Development 110:1341–1348.

Pettenati, M.J., Rao, N., Wofford, M., Shuster, J.J., Pullen, D.J., Ling, M.P., Steuber, C.P., and Carroll, A.J. (1994) Presenting characteristics of trisomy 8 as the primary cytogenetic abnormality associated with childhood acute lymphoblastic leukemia. A Pediatric Oncology Group (POG) Study (8600/8493). Cancer Genet. Cytogenet. 75:6–10.

Robertson, E.J. (1987) Embryo-derived stem cell lines. In: "Teratocarcinomas and Embryonic Stem Cells." Robertson, E.J. (ed). Oxford: IRL Press, pp. 71–112.

Soriano, P. (1995) Gene targeting in ES cells. Annu. Rev. Neurosci. 18:1–18.

Thomas, K.R., Folger, K.R., and Capecchi, M.R. (1986) High frequency targeting of genes to specific sites in the mammalian genome. Cell 44:419–428.

Wang, Y., Schnegelsberg, P.N., Dausman, J., and Jaenisch, R. (1996) Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. Nature 379:823–825.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336:684–687.

Wu, H., Liu, X., Jaenisch, R., and Lodish, H.F. (1995) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 83:59–67.

Zijlstra, M., Bix, M., Simister, N.E., Loring, J.M., Raulet, D.H., and Jaenisch, R. (1990) Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. Nature 344:742–746.

Zijlstra, M., Li, E., Sajjadi, F., Subramani, S., and Jaenisch, R. (1989) Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. Nature 342:435–438.