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Genetics

Inactivating the β_2-microglobulin locus in mouse embryonic stem cells by homologous recombination

(class I antigens/gene targeting)

BEVERLY H. KOLLER AND OLIVER SMITHIES

Department of Pathology, University of North Carolina, Chapel Hill, NC 27599-7525

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ABSTRACT **We have inactivated, by gene targeting, the endogenous β_2-microglobulin gene in a mouse embryonic stem cell line. A cloned fragment of the β_2-microglobulin gene with the coding sequence disrupted by the insertion of the neomycin-resistance gene was used to transfect the embryonic stem cells. G418-resistant colonies were selected and then screened using the polymerase chain reaction to identify those in which the incoming DNA had integrated into the embryonic stem cell genome by homologous recombination. Of a total of 234 G418-resistant colonies screened, 2 correctly targeted colonies were identified. Chimeric mice carrying the inactivated β_2-microglobulin gene have been obtained from both of these targeted embryonic cell lines. Breeding of offspring from such animals will allow investigation of the effects of homozygous loss of β_2-microglobulin.**

β_2-Microglobulin (β_2m) is a 99-amino acid polypeptide necessary for the assembly and expression at the cell surface of the many varieties of histocompatibility class I proteins, including the highly polymorphic H2-K and H2-L/D proteins. These proteins form complexes with viral, tumor, and normal self antigens and display them to potentially responsive cells (1, 2). Although the role of this presentation for inducing and suppressing immune responses has been well documented, its importance to the integrity of the complete organism is not well understood. In addition, the complex patterns of expression of various members of the class I family have led to suggestions that they serve as differentiation antigens critical for normal development, particularly but perhaps not solely of cells in the immune system. β_2m is also required for expression of the products of the *Qa/Tla* genes at the cell surface. These genes have a high level of sequence homology with the classical class I antigens, but they differ from them in not being polymorphic. It has not been possible to assign a function to any of the Qa/Tla antigens.

Low levels of H2-K and H2-D/L expression are first detected during the midsomite stage of embryogenesis (day 9) when embryos have developed beyond primordial organogenesis and blood circulation has commenced. The level of transcription of these genes remains low until day 13 of gestation (3). Although class I antigens continue to be detectable on virtually all somatic cells, the level of their expression can vary 100-fold. A dramatic variation in expression occurs during maturation of T cells, suggesting that class I antigens play a role in this process. Although the classical class I antigens do not seem to be expressed until the midsomite stage of embryogenesis, β_2m has been detected in two- to eight-cell embryos (4). This β_2m may be associated with the products of the *Qa/TI* region, since the Qa-2 antigen has been detected in the two-cell to blastocyst stage of mouse embryos (5).

The ability to create specific genetic mutations in embryonic stem cells (ES cells) (6) using homologous recombination (7, 8) and to transfer these mutations to the germ line (9, 10) provides an approach to many developmental questions. As a first step toward better understanding the function of β_2m-associated antigens during development, we have disrupted the β_2m gene in a mouse ES cell line using homologous recombination. Two independent targeted ES cell lines containing the disrupted β_2m gene were identified using the polymerase chain reaction (PCR) to screen for homologous recombination events (11, 12). Chimeric mice have been produced with both of these lines.

MATERIALS AND METHODS

Construction of the Targeting Plasmid. Plasmid pKCβ_2B contains the entire β_2m gene within an 8.4-kilobase-pair (kbp) *Xho* I fragment (13, 14). The 5′ *Xho* I–*Bam*HI fragment of this gene was subcloned into pUC19. Two *Kpn* I restriction enzyme sites, one in the 5′ flanking DNA and the other within the first intron, were removed by digestion with *Kpn* I followed by treatment with T4 polymerase and religation. A unique *Cla* I site was created in exon 2 by partial digestion with *Eco*RI followed by treatment with the Klenow fragment of DNA polymerase I and ligation with *Cla* I linkers. The 1150-bp *Xho* I–*Bam*HI fragment of the plasmid pMC1Neo (8), containing a neomycin-resistance gene driven by the Herpes simplex virus thymidine kinase gene (HSV-tk) promoter and a polyoma enhancer, was inserted into this *Cla* I site by using linkers. Two plasmids, C65.2.3 and C65.5.9, were obtained that differed in the transcriptional orientation of the inserted fragment with respect to that of the β_2m gene. The 5′ *Xho* I –*Kpn* I fragment of each of these was cloned into pUC19 to obtain the targeting vectors used in our experiments. In plasmid C84.4B the 5′ to 3′ orientation of the neomycin and β_2m promoters is identical (see Fig. 1). The opposite configuration occurs in plasmid C84.2D.

Culturing, Electroporation, and Selection of ES Cells. The ES cell line E14TG2a (15) was cultured on mitomycin-treated primary embryonic fibroblast-feeder layers essentially as described (16). The embryonic fibroblasts were prepared from embryos from C57BL/6 females that had mated 14–17 days earlier with a male homozygous for a neomycin-resistance transgene (17); these cells are capable of growth in medium containing G418. Electroporation conditions were similar to those that have been described (18). ES cells were trypsinized, resuspended in culture medium at 4×10^7 cells per ml, and electroporated in the presence of the targeting DNA at 12 nM in the first experiment and 5 nM DNA in the second. A voltage of 300 V with a capacitance of 150–250 μF was found optimal with an electroporation cell of 5 mm length

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Abbreviations: β_2m, β_2-microglobulin; ES cell, embryonic stem cell; PCR, polymerase chain reaction.

and 100 mm^2 cross-section (18). Approximately 5×10^6 electroporated cells were plated onto mitomycin-treated fibroblasts in 100-mm dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (vol/vol) fetal bovine serum and 0.1 mM 2-mercaptoethanol. The medium was replaced 24 hr after electroporation with medium containing G418 (200 μg/ml).

Analysis of G418-Resistant ES Cell Colonies. ES colonies visible 10–14 days after electroporation were picked with drawn-out capillary pipettes for analysis using the PCR. Half of each picked colony was saved in 24-well plates already seeded with mitomycin-treated feeder cells. The other halves, combined in pools of three or four colonies, were transferred to Eppendorf tubes containing ≈0.5 ml of isotonic phosphate-buffered saline (PBS) and analyzed for homologous recombination by PCR. Conditions for PCR reactions were essentially as described (11). The ES cells were pelleted, resuspended in 5 μl of PBS, and lysed by the addition of 55 μl of H_2O to each tube. DNases were inactivated by heating each tube at 95°C for 10 min. After treatment with proteinase K at 55°C for 30 min, 30 μl of each lysate was transferred to a tube containing 20 μl of a reaction mixture including PCR buffer, each primer at 1.5 μg, 3 units of *Thermus aquaticus* polymerase, 10% (vol/vol) dimethyl sulfoxide, and 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, and 0.2 mM dTTP. PCR was carried out for 55 cycles using a thermocycler modeled after one described (8), with a 65-sec melt incubation at 92°C and a 10-min annealing and extension incubation at 65°C. The two priming oligonucleotides, TGGCGGACCGCTATCCCCCAGGAC and GATGCTGATCACATGTCTCG, correspond, respectively, to sequences located 650 bases to the 3′ side of the start codon of the neomycin gene and sequences located in exon 3 of the β_2m gene. The reaction mixture (20 μl) was electrophoresed on agarose gels and transferred to nylon membranes (Zeta Bind). Filters were probed with a ^{32}P-labeled 450-bp *Eco*RI–*Kpn* I fragment of the β_2m gene indicated in Fig. 1 (see below).

Preparation and Restriction Enzyme Analysis of Genomic DNA. Genomic DNA was prepared from ES cells, whole new-born mice, and mouse tails by conventional methods. DNA was digested with restriction enzymes as directed by the manufacturers, and fragments were separated on 0.7% agarose gels. DNA was transferred to nylon membranes and probed with the ^{32}P-labeled fragment described above.

Embryo Manipulation and Blastocyst Injection. Mice were purchased from either The Jackson Laboratory or Charles River Breeding Laboratories. C57BL/6 blastocysts were obtained from 3- to 4-week-old superovulated females. Uteri were flushed with M2 medium (19) 3.5 days after ovulation. Blastocysts were collected, washed several times in fresh M2 medium, and placed in a 100-μl droplet of M2 under paraffin oil. ES cells were trypsinized, washed once with fresh DMEM, and diluted to ≈2×10^6 cells per ml. Cells (5 μl) were added to the droplet containing the blastocysts. Between 10 and 15 ES cells were injected into the blastocoel of each blastocyst. After injection, six to nine blastocysts were returned to each uterine horn of pseudopregnant females mated 2.5 days previously with vasectomized males. Both C57BL/6 × DBA F_1 and C57BL/6 × CBA F_1 mice proved to be excellent foster mothers that had a pregnancy rate close to 100% and could raise small litters.

RESULTS

Strategy. The strategy used to inactivate the β_2m gene by homologous recombination with exogenous DNA is illustrated in Fig. 1. The incoming DNA had ≈5 kbp of sequence in common with the native gene, and it was this common sequence, extending from the 5′ flanking region to the second intron, that mediated the homologous recombination leading to the modification of the target gene. After the recombination had occurred, the reading frame of the modified β_2m gene was disrupted by the neomycin-resistance gene inserted into the second exon. Two *Kpn* I sites, one in the 5′ flanking DNA and one in the first intron, were removed in constructing the targeting plasmid. A third site remained in the second intron; it allowed the targeting plasmid DNA to be cut to provide recombinogenic ends in the incoming DNA and facilitated the use of Southern blot analysis to locate the 5′ crossover site in the homologous recombinants.

A mutant polyoma enhancer and a thymidine kinase promoter were used to drive the neomycin gene in our construct. They have been shown to be active in both EC and ES cells (8, 20, 21), and we therefore expected that G418 could be used to provide an efficient selection for cells that stably integrated the targeting DNA into their genomes after electroporation. Some initial experiments were carried out with DNA prepared from the plasmid C84.2D in which the promoters of the neomycin gene and the β_2m gene were oriented in opposite directions. Surprisingly, electroporation of ES cells in the presence of this DNA failed to yield any G418-resistant colonies. Resistant colonies were nevertheless obtained when the neomycin gene was released from the β_2m DNA by digestion with the *Cla* I prior to electroporation. This shows that the neomycin sequences in the plasmid were functional and suggests that the β_2m sequences inhibited transcription initiating from the oppositely oriented neomycin promoter. This effect was not seen with the plasmid C84.4B, in which the neomycin gene and the β_2m promoter were in the same orientation. C84.4B was therefore used in all the experiments described here.

Homologous integrants were distinguished from random integrants by a PCR assay (11). This assay exploits the fact that homologous recombination joins the incoming DNA to chromosomal DNA in a predictable way. Two primers were chosen so that their binding sites would be juxtaposed only

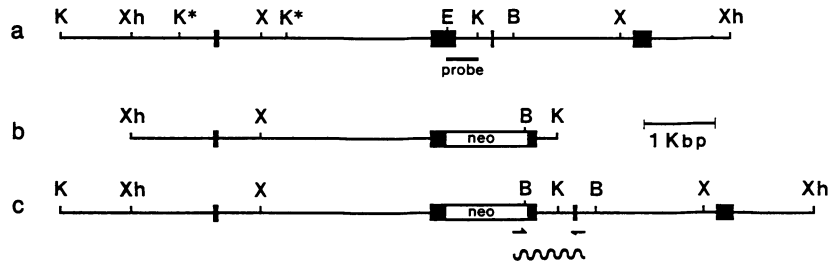

FIG. 1. Planned disruption of the β_2m gene. (*a*) Native β_2m locus of mouse. (*b*) Targeting DNA isolated from plasmid C84.4B. (*c*) Expected structure of the locus after gene targeting by homologous recombination. Thin line, introns and flanking DNA; solid boxes, exons; open box, neomycin-resistance (neo) gene. Restriction sites present in the native locus but removed from the targeting DNA are marked by an asterisk. B, *Bam*HI; E, *Eco*RI; K, *Kpn* I; X, *Xba* I; Xh, *Xho* I. The primers used for PCR are shown as arrows, and the 910-bp fragment that will be amplified in the recombinants is indicated by a wavy line. The DNA fragment used as a probe in the analysis of Southern blots generated during screening by the PCR assay and for the analysis of DNA from positive clones is shown as a thick line below the native locus.

after homologous integration of the incoming plasmid into the β_2m gene. The inserted neomycin gene provided one primer binding site. The second primer bound to a region of the β_2m gene that was not included in the incoming DNA but would be located downstream of the neomycin gene after the homologous recombination event. Amplification of cellular DNA by PCR with these two primers was expected to yield a fragment of 910 bp only when DNA from a successfully targeted cell was present.

Isolation and Characterization of Targeted ES Cells. Two independent targeting experiments were carried out. In each, the ES cells were electroporated in the presence of the incoming DNA and were then cultured in medium containing G418. After about 2 weeks, several hundred G418-resistant colonies were apparent. A portion of each of about 100 colonies was then transferred to an individual well of a 24-well plate, while the remaining portion was pooled with portions from two to four other colonies for PCR analysis. In the first experiment (Table 1), one pool gave a positive PCR signal out of 32 pools analyzed that included a total of 100 G418-resistant colonies. The three individual colonies that had contributed to this positive pool were analyzed individually by PCR, and a positive clone, ES39B, was identified. Similar analysis of 134 G418-resistant colonies sampled in the second experiment also yielded a clone, ES22A, which generated the 910-bp DNA fragment, indicating successful targeting when subjected to PCR.

To verify the targeted disruption of one copy of the β_2m gene, (the gene is autosomal and present in two copies), the two PCR-positive clones, ES39B and ES22A, were expanded, and their DNA was isolated and then analyzed by Southern blotting using a probe that detects sequences from the second exon and part of the first intron of the β_2m gene (Fig. 2). Patterns obtained with *Xba* I, *Bam*HI, and *Kpn* I matched those expected if one of the two copies of the β_2m gene had been disrupted in the planned manner (see Fig. 1) in the PCR-positive clones. That is, one DNA fragment identical in size to that present in untreated cells, but of decreased intensity in the PCR-positive clones, was present with all three enzymes. An additional fragment of the size predicted for a homologous recombination event was present only in the PCR-positive clones. The insertion of the neomycin gene in the second exon by the recombination resulted in an *Xba* I fragment detectable with the β_2m-specific probe that is ≈1 kilobase (kb) longer than the equivalent fragment in the native locus. A new *Bam*HI site was introduced into the locus by the targeting DNA, reducing the size of the *Bam*HI fragment detected by the β_2m probe from 10.6 kbp to 900 bp. A new fragment was also seen after *Kpn* I digestion. In ES39B, the *Kpn* I fragment was 7 kb long, as predicted by a crossover between the 5′ end of the targeting plasmid and the native locus. In ES22A this new *Kpn* I fragment was 4.0 kb long, which shows that the deleted *Kpn* I sites were not incorporated into the locus. This observation indicates that one of the crossovers in cell line ES22A resolved between the third *Kpn* I site of the native locus and the inserted neomycin gene of the incoming DNA, presumably after branch migration of a crossover intermediate. Although the 5′ crossover sites differ, both modified cell lines now contain a β_2m gene disrupted in the planned way by insertion of a neomycin gene

Table 1. Homologous recombination into the mouse β_2m locus

Exp.	E14 cells treated, no. × 10^{-7}	G418^r colonies obtained, no.	G418^r colonies analyzed by PCR, no.	ES cells correctly targeted, no.
1	12	602	100	1
2	3.5	244	134	1

G418^r, G418 resistant.

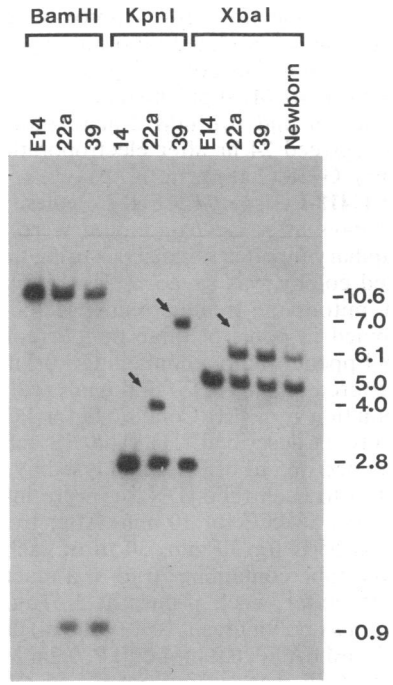

FIG. 2. Southern blot analysis of the β_2m locus. DNA samples are from the parental cell line E14 (E14TG2a), two targeted cell lines [ES22A (22a) and ES39B (39)], and from a newborn pup of a mouse who had received blastocysts injected with ES22A cells. Genomic DNA (10 μg) was digested with the restriction endonuclease indicated and probed with the fragment shown in Fig. 1. Arrows indicate bands that hybridized when the filter was reprobed with a probe specific for the neomycin gene.

in exon 2. Rehybridization of the filter used for the autoradiograph illustrated in Fig. 2 with a probe for the neomycin gene showed that the only bands that hybridized were those predicted by the structure shown in Fig. 1. They are marked by small arrows in Fig. 2.

Chimeric Offspring of Targeted ES Cells. ES cell lines carrying inactivated β_2m genes should allow the introduction of this mutation into the mouse germ line. Toward this end, we injected 10–15 cells from each of the targeted ES cell lines into C57BL/6 blastocysts. Embryos were reimplanted into pseudopregnant females. Because the initial ES cell line E14TG2a was isolated from strain 129/Ola embryos, it and all cell lines derived from it carry the coat color markers characteristic of this mouse strain. These include the dominant A^w allele at the agouti locus, the recessive chinchilla allele at the *c* locus, and the recessive *p* allele (pink-eyed dilution) at the *p* locus (9). Contribution of ES cells to the mesoderm-derived portions of hair follicles results in an agouti coat. Hair follicles to which melanocytes of ES cell origin (and therefore carrying the *p* and c^{ch} mutations) have migrated produce cream colored hairs. Both of these coat colors are easily distinguished from the solid black coat seen in pups derived from nonagouti C57BL/6 host blastocysts.

The efficiency with which blastocysts injected with the two targeted cell lines produced coat color chimeras is shown in Table 2. More than 70% of surviving pups were chimeras. DNA isolated from a newborn pup of a female implanted with blastocysts injected with ES22A is shown in the farthest lane

Table 2. Chimera production from β_2m-targeted ES cell lines

Cell line	Progeny, no.	Chimeras, no.	% chimeras	Male/female ratio
E1439B	23	17	74	10/7
E1422A	26	21	81	6/15

to the right in Fig. 2. The intensity of the 6.1-kb *Xba* I band diagnostic of the targeted β_2m locus shows that the modified ES cells contributed extensively to the tissue of this animal.

DISCUSSION

In a total of 234 G418-resistant colonies screened for homologous recombination, two targeting events at the β_2m locus have been identified. Comparisons of this frequency to those obtained for other loci are complicated by a number of factors. If a comparison is made on the basis of cells electroporated, it is necessary to take into account variables such as cell death during electroporation and the plating efficiency of the ES cells. When comparison is made on the basis of the number of cells transformed by the incoming DNA, the ratio of targeted to G418-resistant colonies in experiments carried out by various investigators is affected by the strength of the specific promoter and enhancer used to drive the neomycin gene, for this affects its ability to function in sites of random versus targeted integration. Although strong promoters may be desirable to assure expression of the neomycin gene when it is inserted into a target locus that is unexpressed or poorly expressed, they increase substantially the number of sites at which random integration will confer G418 resistance on the resulting cells (8). This in turn will increase the number of colonies that must be screened to identify a targeted cell. Comparisons of frequencies are further complicated by the fact that promoterless neomycin genes lacking poly(A) sites are often used to enrich for targeting. We have also found that the strength of a promoter can vary in constructs, depending on the DNA in which it is embedded and even on its orientation. For example, no G418-resistant colonies were obtained with our present construction when the neomycin gene was in the reverse orientation relative to the β_2m gene. With these difficulties in mind, the following comparisons are presented of targeting events/G418-resistant colonies: 1/117 for the β_2m gene (this work), 1/300 for *en-2* (12), 1/950 for *hprt* (8), and 1/400,000 for *int-2* (22).

The high frequency with which the β_2m locus was targeted is somewhat surprising, for it has been suggested that the level of expression of a gene may be one factor influencing the frequency of targeting (22). For example, the low frequency with which *int-2* was targeted was suggested to be a reflection of an expression level of only 0.5 copy per cell. No β_2m protein can be seen by fluorescent antibody techniques in the cell surface of ES cells (unpublished observations), and, although no data are specifically available for ES cells, the closely related EC cell lines do not show detectable β_2m mRNA (23). Thus our data suggest that gene targeting with DNA containing the neomycin gene may be feasible for nonexpressed genes. Certainly the frequency of targeting is not easily correlated with the level of expression of the gene being targeted. This lack of correlation is supported by our lack of success in targeting the β_2m gene in mouse L cells (unpublished data), which clearly express this gene. When frequency comparisons are made on the basis of targeting events per starting cell, the following frequencies per 10^7 cells are obtained: for the β_2m gene, 0.5; for *en-2*, 0.7; for *hprt*, 1.3; and for *int-2*, 1.3. Thus all four loci are essentially the same when compared in this way.

Both of the targeted cell lines that we have isolated in which the β_2m gene has been inactivated have been introduced into blastocysts, and chimeric animals have been obtained at high frequencies. Obtaining these chimeras establishes, not surprisingly, that cells heterozygous for an inactive β_2m gene are viable in the whole animal. We can therefore expect that animals fully heterozygous for that genotype will survive. Such animals should be obtained when the present crop of chimeras (or others comparable to them) are old enough to transmit the inactivated β_2m gene to their progeny. The most dramatic and revealing observations on the function of β_2m and its associated proteins during development are likely to come when these progeny are interbred to obtain the inactivation in a homozygous form. An alternative approach to studying homozygosity is to make chimeras by injecting normal blastocysts with ES cells that have both β_2m genes inactivated. Accordingly we intend to inactivate the second gene in our ES cells once they have been proven germ-line competent. In this way the effects of absence of β_2m and β_2m-dependent proteins in a fraction of the cells of a developing chimeric embryo can be studied.

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