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Gene Modification via "Plug and Socket" Gene Targeting

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Conventional gene targeting experiments in embryonic stem (ES)¹ cells frequently involve the disruption of endogenous sequences with a positively selectable marker such as neomycin phosphotransferase (*neo*) or hypoxanthine phosphoribosyltransferase (*HPRT*), often in conjunction with a negative selectable marker such as herpes simplex thymidine kinase (1). ES cells which have incorporated the positive marker and have lost the negative marker, as determined by selection with the appropriate drugs, can be further analyzed by Southern hybridization or PCR to identify cell lines that have been correctly targeted.

Repeatedly altering a chosen target gene, in order, for example, to study the effects of a series of related but not identical changes, can, however, be very time consuming unless the target gene is easily targeted. Strategies have therefore been devised to allow easier isolation of targeted cells when such repeated modifications are desired. Two-step gene targeting is such a strategy. The first targeting step introduces a selectable marker into the genome of the ES cells by conventional gene targeting. The second targeting step in several versions of the two-step strategy has used a series of different constructs to obtain the desired modifications, while at the same time deleting the marker which was inserted in the first step. This second event is therefore detected by selecting against the initial marker gene (2–4). Deletion is not, however, the only mechanism by which a marker gene function can be lost. For example, spontaneous mutations can produce a nonfunctional marker gene. Such mutations therefore give rise to a "background" of nonrecombinant colonies, and force additional screening to eliminate colonies which should have been ex-

cluded during the second selection. Although the spontaneous mutational event may itself be infrequent, if it occurs in culture before the targeted loss of the marker gene, the background of nontargeted clones may be considerable. Termed the "fluctuation phenomenon" because its magnitude fluctuates with the time of its occurrence (5), the problem can be eliminated by using positive selection for both the first and second gene targeting events.

We have devised a version of the two-step targeting strategy, termed the "plug and socket" method, which can be used repeatedly to make subtle mutations or to replace entire genes while avoiding the problem of background colonies by using only positive selection. This method in its present form can be used to target any murine gene in *HPRT* deficient ES cells. The first targeting step inserts the "socket" downstream (or upstream) of the target gene; the socket is a functional *neo* gene linked to a nonfunctional *HPRT* minigene (Δ HPRT) lacking a promoter and exon 1. Since this step is a simple insertion via homologous recombination, the integration frequency is similar to that of other such targeting events (6). Cells which have the socket correctly integrated, as judged by PCR and Southern hybridization, can then be used repeatedly for different plug targeting events. It is important to confirm the euploidy of the socket ES cells by karyotypic analysis; and the socket ES cells should also be tested for germline competency after injection into blastocysts to create chimeras. If the chimeras fail to transmit the modified socket to their offspring, another socket cell line which is both euploid and germline competent should be used.

In the second targeting step, repeated alterations can be made to the target gene by using homologous recombination to insert different plugs into the socket. A "plug" contains sequences that are homologous to the mouse gene upstream of the target gene as well as a nonfunctional human *HPRT* gene (*HPRT* Δ) that is complementary to the Δ HPRT in the socket. The nonfunctional *HPRT* Δ minigene includes the promoter and exon 1 of the *HPRT* gene and has overlapping homology with Δ HPRT. When recombination occurs between *HPRT* Δ and Δ HPRT, a functional *HPRT* gene is created. The modifying sequences in the plug are chosen so that they alter the target gene in the desired manner. For example, if the target region is to be replaced, the replacement sequences will be placed between the upstream homology and the *HPRT* Δ . If deletion of the target area is the desired event, the plug will lack the region to be deleted.

We have used this method to repeatedly alter the murine beta globin locus. The socket construct contained 6 kb of ho-

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1. Abbreviations used in this paper: ES, embryonic stem; *HPRT*, hypoxanthine phosphoribosyltransferase; *neo*, neomycin phosphotransferase.

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Figure 1. Socket insertion step. Endogenous locus, socket-containing construct and the correctly targeted chromosome are represented. Thick lines and black boxes indicate *HPRT* introns and exons, respectively. Dashed lines indicate the alignment of the regions of homology. Figure adapted from reference 7.

Figure 2. Plug targeting step. The socket-containing chromosome, the linearized plug targeting construct for replacing murine beta with a human beta globin gene, and the chromosome obtained after insertion are shown. Representations are as in Fig. 1. Figure adapted from reference 7.

Figure 3. Socket-lox site insertion step. Endogenous chromosome, socket construct-containing *lox* site, and socket-containing chromosome are shown. Representations are as in Fig. 1, with the addition of the stippled arrow as the *lox* site.

mology to the murine beta globin locus, including the mouse β^{minor} gene. This homology was joined to the *neo* gene, $\Delta HPRT$, and 2 kb of additional downstream homology. (Fig. 1) We made three plugs for insertion into the socket, each able to replace the adult murine beta globin genes with a specific human beta globin gene. All of these plugs contain sequences homologous to DNA 5' to the mouse β^{major} gene, and all have the complementary $HPRT\Delta$. Recombination in the 5' region of homology and in the $HPRT$ region forms a functional *HPRT* gene and replaces both adult beta globin genes with the desired human sequence while at the same time deleting the *neo* gene. (Fig. 2) The three plugs differ in the human sequences

that replace the mouse beta globin genes. One plug contains the β^S globin gene; the second contains the β^A gene; the third plug contains the human beta globin gene containing a C→T point mutation in $\beta^{IVS2-654}$ (6). A fourth plug was also made that deletes both the mouse β^{major} and β^{minor} genes.

We are now using the plug and socket method to repeatedly target the murine alpha globin locus in a similar fashion. The mouse-derived sequences used in making both the socket and the plug targeting constructs were derived from DNA isolated from the ES cells which were to be modified. The murine alpha globin socket consisted of a 10.6-kb region of 5' homology and a 1.4-kb region of 3' homology. The murine

Figure 4. Plug-lox site targeting step. Chromosome containing correctly targeting socket, linearized plug containing second lox site, and chromosome containing plug modification are shown. After the plug modification, Cre is introduced exogenously to remove the *HPRT* marker. All other representations are as in Figs. 1 and 3.

sequences were interrupted by *neo* and Δ HPRT, as in the beta globin socket. The first round of targeting yielded 1 in 16 G418^R colonies, which had the socket correctly integrated downstream of the alpha globin locus, a frequency which is comparable to that with the beta globin locus. Two plug constructs have been built. The first plug will simply delete both adult murine alpha globin genes. The second plug will replace the two mouse adult alpha globin genes with the two human alpha globin genes.

The plug and socket method is designed to make it easier to achieve related targeting events which might otherwise be tedious to execute. For example, the plug and socket technique could be used to repeatedly modify a locus control region, as in the beta globin locus, in order to determine which areas are important for in vivo expression of each gene. Or, the promoter of a target gene could be replaced with modified promoters to test their functions in vivo. The method also could be used to replace or delete various sections of a gene to identify protein and DNA binding domains.

Although the two-step plug and socket procedure normally leaves the *HPRT* gene in the targeted region, this can be eliminated if necessary. A third targeting construct can be created which contains homologies just upstream and downstream of the *HPRT* gene. This construct then can be used in association with selection for the loss of the *HPRT* gene. Alternatively, the plug and socket method could incorporate the Cre-lox recombinase system. To do this, the socket construct would need to contain a lox site downstream of the Δ HPRT. (Fig. 3). The plug would contain a second lox site between the upstream homology and HPRT Δ . The *HPRT* gene subsequently would be

removed by introduction of Cre. (Fig. 4). The plug and socket method is not restricted to use with the *neo* and *HPRT* genes. Other pairs of positively selectable markers could be used. For example, the socket could be introduced with the help of *hyg* (hygromycin resistance), and the plug could be inserted with the help of recombination between *neo* Δ and *Δneo* (7).

In summary, the plug and socket method of gene targeting is a versatile procedure which is useful when a series of systematic changes are to be made at a specific target locus.

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