Targeted disruption of a human interferon-inducible gene detected by secretion of human growth hormone

Jane E.Itzhaki and Andrew C.G.Porter*

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Received May 17, 1991; Revised and Accepted July 1, 1991

ABSTRACT

A new method is described for the sib-selection of 'targeted' mammalian cells that have undergone homologous recombination (HR) with a transfected DNA construct. This method has been used to disrupt the 6-16 gene, an interferon (IFN)-inducible gene of unknown function, in two different human cell lines. Disruption was caused by integration of a targeting construct containing a promoterless gene for human growth hormone (hGH) which was expressed after HR with the 6-16 gene. Homologous recombinants were detected in pools of non-homologous recombinants by the appearance of hGH in the growth medium after the addition of IFN. Secondary and tertiary rounds of hGH assays were used to sib-select 9 homologous recombinants that were shown to have 1, 2 or 3 copies of the targeting construct integrated at the 6-16 locus. The method, which should be applicable to other transcribed targets, provides an alternative to selection methods, and offers advantages over other screening methods in being simple, rapid, sensitive and reliable.

INTRODUCTION

The isolation of rare mammalian cells that have incorporated transfected DNA into their genomes via homologous recombination (HR) has now been achieved by a variety of methods. Such 'targeting' experiments and their potential in genome manipulation, gene therapy, the analysis of gene function and the generation of animal models for human disease have been reviewed (1,2). A variety of methods is desirable since none is perfect, and different methods suit different situations in ways that are not always predictable.

All targeting methods require the transfected DNA (targeting construct) to have a stretch of homology to the target locus. However, targeting constructs must also include some feature that will permit the detection and/or selection of homologous recombinants in the presence of what is likely to be a large excess of other cells i.e. non-homologous recombinants (cells that have incorporated the targeting construct at 'random' loci) and sometimes untransfected cells.

To date, screening/sib-selection methods have used bacteriophage rescue, Southern analysis or the polymerase chain

reaction (PCR) to detect the predicted structural changes at the target locus. These DNA-based assays require colonies, or pools of colonies, of candidate homologous recombinants to be split into two portions: one for assay and one for further growth and analysis. Bacteriophage rescue is an extremely sensitive assay that was used in the first demonstration of targeting to an endogenous mammalian gene (3), but it is time consuming and no longer used. Southern analysis is appropriate for the screening of individual clones but requires large numbers of cells and is insufficiently sensitive for pools of any size. PCR is exquisitely sensitive and in theory may be used to screen large pools (4). In practice it has been found, especially with large pools, to generate 'false positives' that are thought to arise, not by contamination, but by the interaction of incompletely extended primers (5). Despite these drawbacks, for targeting events that occur at particularly high frequencies, PCR and Southern analyses can be used successfully to sib-select homologous recombinants without a prior selection step for their enrichment (6–9). PCR and Southern analyses are also used to screen colonies that survive selection methods designed to enrich for homologous recombinants.

In the positive/negative selection (PNS) method (10) the targeting construct contains a positively selectable gene within the region of homology to the target and one or two negatively selectable genes outside this region; double selection (for the former and against the latter) enriches for homologous recombinants that have undergone two crossovers (one on each side of the positively selectable gene) at the expense of both nonhomologous recombinants and homologous recombinants resulting from a single crossover. A great advantage of this selection method compared to those described below is that the target locus need not be transcribed. However, for reasons that are not clear, the efficiency of enrichment is variable and not always good. Thus, in recent applications of this method, 0.25% (11), 0.36% (12), 2.6% (13), 4.9% (10) and 5–10% (14) of doubly selected colonies were shown, by sib-selection using Southern or PCR analyses, to be homologous recombinants.

Another approach, for targets that are transcribed genes, is to include a promoterless marker gene in the targeting construct in such a way that it becomes activated upon HR, involving one or two crossovers, between target and construct. In this way marker genes such as *gpt* (15), *neo* (16–21) and hygromycin

* To whom correspondence should be addressed

(21) confer a selective advantage (drug-resistance) specifically on the homologous recombinants. Alternatively, the marker gene may encode a cell surface antigen not normally expressed by the cells to be used so that homologous recombinants may be isolated by flow cytometry (22). Enrichment is sometimes very good, but can be poor, presumably because of marker gene activation caused by random integration into active genes. Thus, in recent applications of these methods 1% (18), 7% (20), 20% (19), 70% (22) and 55–85% (21) of clones isolated on the basis of marker gene activation were shown, by sib-selection using PCR and/or Southern analyses, to be homologous recombinants.

Extension of this approach to include a marker gene that encodes a secreted antigen has so far been restricted to experiments that involve immunoglobulin (Ig) genes (23, 24). In these experiments, HR between defective chromosomal and transfected IgM genes restored secretion of a functional IgM which could be detected by complement mediated plaque formation. Use of IgM heavy (or light) chain genes as markers for the targeting of genes other than IgM genes may be complicated by a requirement for coexpression of the corresponding light (or heavy) chain gene for efficient plaque formation.

In theory, activation of any marker gene that encodes a secreted antigen for which there is a sensitive assay can be used as a basis for the sib-selection of homologous recombinants, provided that the antigen is not normally secreted by the cells to be used. In the present study we show that the gene for human growth hormone (hGH) may be used in this way. Pools of stable transfectants are screened for the presence of homologous recombinants, simply and rapidly, by the removal and assay of samples of culture supernatant. In contrast to DNA-based screening procedures described above, manipulation and lysis of cells is not required so that screening and sib-selection is relatively simple. Using this method we have disrupted the human gene 6-16 whose structure (25) and transcriptional regulation (26) by interferon (IFN) have been characterised, but whose function is unknown.

METHODS

Construction of plasmids

The control and targeting constructs (p6-16/hGHC and p6-16/hGHX respectively) were made from another control plasmid (p06-16/hGHC) which lacks the *neo* gene. This plasmid was made by the ligation of three DNA fragments at their complementary ends: i) a 4.5 kilobase-pair (kb) *AatII/BamHI* fragment from pOGH (27) carrying most of the hGH gene and additional vector (pUC12; ref 28) sequences; ii) a 3.5 kb fragment from the 6-16 gene stretching from the *BglIII* site 604 base-pair (bp) 5' of exon 1 to the *BssHIII* site 13 bp upstream of the 6-16 initiation codon in exon 2; iii) a synthetic oligonucleotide *BssHIII/AatII* linker:

5'-CGCGCGCGCCACCATGGCTACAGGCTCCCGGACGT-3'
3'-GCGCGGTGGTACCGATGTCCGAGGGCC-5'

which was designed to include the 6-16 Kozak consensus sequence (25) and replace the 22 bp of hGH coding sequence that were lost on cutting pOGH at the *AatII* site in hGH exon 2 (29).

The control and targeting constructs were derived from pO6-16/hGH as follows. A 1.7 kb *NdeI* fragment containing all of the 6-16 DNA 5' of an *NdeI* site in 6-16 intron 1, and a small

portion of vector DNA, was removed from pO6-16/hGH and replaced by a 3.5 kb *NdeI/EcoRI* fragment from pSV2*neo* (30) and an *EcoRI/NdeI* fragment of 6-16 DNA isolated from previously described 6-16 constructs (26) in which various amounts of DNA upstream of exon 1 had been deleted placing an *EcoRI* site at the end of the deletion. The latter fragment, which was 1.5 kb (6-16/hGHC) or 1.1 kb (6-16/hGHX), stretched from a position 408 bp (6-16/hGHC) or 6 bp (6-16/hGHX) upstream of exon 1 to the *NdeI* site in intron 1 and was ligated so as to restore intron 1.

hGH assays

A radioimmune assay kit with solid support-linked first antibody and ¹²⁵I-labelled second antibody was used as recommended by the suppliers (Nichols Institute Diagnostics). Briefly, samples (100 µl) of conditioned medium (stored at -20°C) were shaken with the antibodies at room temperature for 1 h. The solid supports were washed and counted in a Clinigamma counter (LKB). Where necessary, conditioned medium was diluted with serum to bring it within the linear range of the assay (0.2–50 ng/ml).

Growth, transfection and selection of cells

HeLa (epitheloid carcinoma) and HPRT⁻ HT1080 (fibrosarcoma) cells were grown in Dulbecco's Modified Eagles Medium (Gibco/BRL) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Imperial Laboratories) and antibiotics. Treatment with a mixture of α-IFNs (300 IU/ml) was as described (26). Cells for electroporation were trypsinised at ~50% confluence, washed and resuspended in phosphate buffered saline at 7.5–10 × 10⁶ cells/ml. Plasmid DNA (10 µg), linearised with either *XmnI* (control construct) or *Asp718* (targeting construct), was mixed with cells (0.8 ml; 6–8 × 10⁶ per electroporation) in a cuvette (Biorad, 4mm gap) and placed on ice for 5 min. A gene pulser (Biorad) with capacitance extender was used for electroporation at 250 µF and 400 V. Cells were placed on ice for a further 5 min and then distributed into 90 mm plates (5 × 10⁵/plate) or, for the purposes of screening, into 12-well plates (25 mm wells) to give 150–180 wells per experiment (~0.7–1.5 × 10⁵ per well). G418 (Sigma) was added 24 h later at 400 µg/ml (HeLa) or 200 µg/ml (HT1080). Colonies appeared 9 to 11 (HT1080) or 11 to 13 days (HeLa) after electroporation. Once obtained, stable transfectants of both cell types were maintained in G418 at 200 µg/ml.

Screening procedure

For the first screen, G418-resistant colonies appearing 9–13 days after electroporation were given fresh medium (0.8 ml/well). After incubation for 36–48 h the conditioned medium of each well was removed and kept for later analysis (–IFN samples). The cells were then fed an equal volume of fresh medium containing IFN and, after incubation for the same period as for the –IFN samples, conditioned medium was again removed from each well (+IFN samples). The +IFN samples were assayed first, pooling equal volumes from three different wells for each assay. For a selection of these pools, corresponding pools of –IFN samples were assayed.

In the second screen, –IFN or +IFN samples representing individual wells from pools chosen after the first screen were assayed separately so that individual IFN-responsive wells could be identified. In the third and last screen, each colony from such wells was transferred to a separate well of a 24-well plate (15mm

wells). After growth for 3–7 days such plates were split to form duplicate plates, one of which was treated with IFN. After growth for a further 36 h, pools of conditioned medium corresponding to the rows and columns of such plates were assayed for hGH. Thus IFN-responsive clones could be identified and expanded for further analysis.

Timecourse of hGH secretion

Cells (2×10^4) in 0.8 ml medium were placed into each of 8 wells of a 12-well plate. After incubation for 24 h, half of the wells received IFN and all were incubated for a further 72 h. Samples of conditioned medium were removed for hGH assay at the times indicated.

Electrophoretic and Southern analyses

Genomic DNA for conventional gel electrophoresis was prepared from IFN-responsive clones and digested with restriction enzymes by standard procedures (31). Digested DNA ($10 \mu\text{g}/\text{lane}$) was electrophoresed through 0.65% agarose. Klenow fragment was used to end-label markers (1 kb ladder; Gibco BRL) by standard procedures (31). Samples for analysis by pulsed-field gel electrophoresis were prepared as described (32) except that the final cell concentration in agarose plugs was $2 \times 10^7/\text{ml}$. Plug slices containing $\sim 8 \times 10^5$ cells were digested as described (33) and electrophoresed in a 'Walzer' rotating gel apparatus (34) for 52 h at 17.5°C and 150V with a pulse time of 3 s. Pulsed-field gel markers were *Saccharomyces cerevisiae* (strain YP148) chromosomes prepared as described (32), two of which (90 kb and unresolved) hybridised weakly to probe a.

Gels were transferred to a Genescreen (Dupont) membrane. Probes were labelled with α - ^{32}P dCTP by 'random priming' (35). Hybridisations were carried out as described (36) and washes to a final stringency of $0.2 \times \text{SSC}$ and 0.5% SDS at 65°C . Probe a was a 240 bp *Hae*III/*Bam*HI fragment from the IFN regulatory region immediately 5' of 6-16 (26). Probe b was a 300 bp *Pst*II fragment spanning 6-16 intron 3. Probe c was a 1 kb *Bgl*III/*Sma*I fragment from pSV2neo.

Figure 1. Experimental design. **A.** The targeting construct (a), the 6-16 gene (b), and the expected product of HR between them (c). **B.** The control construct. DNA is represented to scale as follows: thick black lines (6-16 DNA), black boxes (6-16 exons), white ellipses (6-16 IFN-response region), dark stippled boxes (hGH gene), light stippled lines (pSV2neo DNA) and thin black lines (pUC12 DNA). Constructs are shown linearised at unique sites for *Asp*718 (targeting construct) or *Xmn*I (control construct).

RESULTS

Experimental design

The targeting construct, the 6-16 gene and the predicted outcome of HR between them are shown in Figure 1A. The targeting construct includes a promoterless portion (3 kb) of the 6-16 gene starting 6 bp upstream of exon 1 and ending at the initiation codon in exon 2. This is fused to the hGH gene as described (Methods) to create a promoterless 6-16/hGH fusion gene encoding the full hGH protein including the secretion signal sequence. The targeting construct also has a unique site within the 6-16 sequence for the restriction enzyme *Asp*718 and a constitutively expressed *neo* gene conferring resistance to the drug G418.

Insertion of the targeting construct into the host genome via HR is expected to separate the host 6-16 gene from its promoter and upstream regulatory sequences i.e. to disrupt and silence the target gene (Figure 1A). Simultaneously, the same promoter and upstream regulatory sequences are positioned upstream of the 6-16/hGH fusion gene making it transcriptionally inducible by IFN. Homologous recombinants should therefore secrete hGH in response to IFN. Non-HR integration events should not cause hGH-secretion except for rare integrations within an expressed gene. In such cases, hGH secretion would not be IFN-inducible (unless the promoter happened to be from another IFN-inducible gene). Provided that the fusion gene is activated appropriately by HR, and the resulting transcript correctly processed and translated, it should be possible to identify pools of stable (G418-resistant) transfectants that contain homologous recombinants as those pools that secrete hGH in response to IFN.

Inducible hGH expression from a control construct

A control construct was made to mimic the activated form of the 6-16/hGH fusion gene expected from HR between the targeting construct and the 6-16 gene (Figure 1B). This construct is identical to the targeting construct except that its 6-16 DNA stretches 402 bp further upstream to include the 6-16 promoter and IFN response sequences (26). Populations of G418-resistant clones were obtained after electroporation of cells in the presence of the control construct and tested for secretion of hGH in the presence or absence of IFN over a period of 72 h (Methods).

Both HeLa and HT1080-derived populations secreted hGH in response to IFN, confirming that the fusion gene is capable of expressing hGH from the 6-16 promoter. The results for HT1080 cells are shown in Figure 2A. Expression in the absence of IFN

Figure 2. Time courses of hGH secretion. Growth of cells in the presence (open symbols) or absence (closed symbols) of IFN and assay of conditioned medium from is described (Methods). **A.** A population (200 clones) of G418-resistant HT1080 cells stably transfected with the control construct. **B.** IFN-inducible clone 3-17. **C.** IFN-inducible clone 6-42.

Figure 3. Summary of initial hGH screens of G418 resistant colonies obtained from six electroporation experiments involving HeLa (experiments 1–3) or HT1080 (experiments 4–6) cells. For each experiment, the total number of colonies assayed (T) and the average number of colonies per pool (A) are shown. Each vertical line represents the hGH secreted by a pool of 30–60 colonies after treatment with IFN (Methods). Horizontal bars represent hGH secreted by the same pools in the absence of IFN (Methods). Circles mark pools selected for further analysis. Grey and black circles indicate pools for which a second screen yielded a strongly IFN-inducible well. Black circles indicate pools from which IFN-inducible clones were isolated.

was high (~50 ng/ml at 72 h), partly accounting for the poor induction by IFN (4 to 5-fold at 72 h). This was disappointing given the larger (20 to 100-fold) inductions seen for similar transfection experiments involving the 6-16 gene itself or related 6-16 constructs with different reporter genes (25,26). Such constitutive expression may be explained, at least in part (see discussion), by the presence in the cell population of clones for which the control construct has integrated close to genomic *cis*-acting sequences that enhance expression from the 6-16 promoter. The targeting construct, lacking this promoter, was expected to be less susceptible to such position effects.

Screening for homologous recombinants

Six electroporations were carried out, three of HeLa cells and three of HT1080 cells. In each case, approximately 8×10^6 cells were electroporated in the presence of linearised targeting construct, distributed amongst 150–180 wells and incubated in the presence of G418 until colonies appeared (10–20 colonies per well; see Methods). Conditioned medium was collected from each well before and after treatment with IFN (Methods). In the initial screen, conditioned medium from IFN-treated cells was assayed for hGH, each assay representing a pool of 30–60 colonies (i.e. three wells). The results for each experiment are summarised in Figure 3. A selection of pools, including those showing high levels of hGH, was reassayed using conditioned medium obtained before IFN treatment (horizontal bars, Figure 3). A minimum apparent induction (~2-fold) was observed reflecting the cell growth that occurred between the harvesting of –IFN and +IFN samples (see Methods). However, several pools showed a strong induction. These, and other pools showing intermediate induction, or poor induction but particularly high hGH expression, were chosen for further analysis (circles, Figure 3).

Figure 4. Examples of the second hGH screen. Each histogram shows the hGH in the conditioned medium obtained, before (solid bars) and after (hatched bars) IFN treatment, from the three wells making up one of the pools assayed in the initial screen (Figure 3) and chosen for further analysis. The pools, numbered according to the experiment and pool numbers of Figure 3, were 3-9 (A), 3-17 (B), 4-7 (C) and 4-12 (D). Of the wells shown, only well 1 of pool 3-17 and well 3 of pool 4-7 were chosen for the third screen.

In a second screen, both types of conditioned medium from each of the wells making up the pools of interest were assayed so that individual wells containing an IFN-responsive clone could be identified. As an example, the results for four IFN-responsive pools are shown in Figure 4. Pools for which no well showed a strong IFN response (white circles, Figure 3) were discarded. Sixteen wells showing a clear IFN response were identified in this way from the pools indicated (grey and black circles, Figure 3).

In a final screen, individual colonies were picked from such IFN-responsive wells, expanded and assayed for IFN-induced hGH secretion (Methods). Ten IFN-responsive clones obtained

Figure 5. Structure and restriction fragments expected of the 6-16 locus with no (A) one (B) or two (C) targeting constructs integrated by HR. DNA is represented as in Figure 1. Sites for restriction enzymes *Bgl*III (B), *Sma*I (St) and *Sph*I (S) are shown. Probes a, b and c are shown in alignment with regions of chromosomal homology.

in this way from the pools indicated (black circles, Figure 3) are numbered according to the experiment and pool in which they were originally identified (2-23, 3-17, 4-7, 5-14, 5-27, 6-27, 6-32, 6-39, 6-42, 6-59). For each clone the IFN-responsive secretion of hGH was measured over a 72 h period (Methods). Secretion of hGH by all of the clones was similar in quantity, kinetics and inducibility (10 to 15-fold at 72 hrs). Results from one HeLa-derived clone (3.17) and one HT1080-derived (6-42) clone are shown in Figure 2B and C.

Short-range Southern analysis of inducible clones

Three probes were used to analyse the genomes of the inducible clones. Two were fragments from the 6-16 locus, 5' (probe a) and 3' (probe b) of the region of homology with the targeting construct. The third (probe c) was a *neo* probe. These probes and the fragments of parental or recombinant genomes they are expected to detect are shown in Figure 5. The results for the two HeLa-derived clones and three representative HT1080-derived clones are shown in Figure 6.

Probe a detected parental and recombinant bands of the expected sizes in all clones (Figure 6A). For most clones, these were of equal intensity, but for the HeLa derived clones (Figure 6A, lanes 2, 3, 5, 6, 8 and 9) and clone 4.7 (not shown), the recombinant band was 2 to 3-fold less intense than the parental band. These clones therefore appear to have 3 or 4 copies of the 6-16 gene which we assume to reflect the aneuploidy known to be a feature of HeLa and, to a small extent, HT1080 cells.

Probe b detected recombinant and parental fragments in all of the clones except 2-23 (Figure 6B). The sizes of the recombinant fragments were consistent with a single (Figure 5B) or double (Figure 5C) integration event. As with probe a, the relative intensities of recombinant and parental bands detected by probe b suggests that clones 3-17 (Figure 6B, lanes 2, 5 and 8) and 4-7 (not shown) are aneuploid. Only the parental band was detected by probe b in clone 2-23 (Figure 6B, lanes 3, 6 and 9) suggesting, in contrast to the result for probe a, that this is not a homologous recombinant (see discussion).

Table 1.

Clone	2-23	3-17	4-7	5-14	5-27	6-27	6-32	6-39	6-42	6-59
Cell Type ^a	He	He	HT	HT	HT	HT	HT	HT	HT	HT
Number of insertions in target	0	2	1	2	3	1	1	2	1	2
Additional Feature ^b	P	P	P	—	—	—	—	X	—	—

^aHe = HeLa, HT = HT1080

^bP = polyploid, X = extra insertion at random site

For all clones, except 2-23 and 6-39 (see below), probe c detected bands whose sizes and number (one or two) were as predicted for single or double integration events and in agreement with the results for probe b (Figure 6C). However, in the case of clone 5-27, for which two bands were detected, the intensity of the band detected in common with probe b was approximately half of that for the band uniquely detected by probe c (Figure 6B, lanes 7 and 10). This suggests that clone 5-27 has three copies of the targeting construct integrated at the target locus.

The sizes of the bands detected by probe c in clone 2-23 (Figure 6C lanes 3 and 4) cannot be explained except by random integration of the targeting construct (see discussion). For clone 6-39, three bands of equal intensity were detected by probe c (not shown), two of which were of sizes expected for a double integration event (Figure 5C). It therefore appears that in clone 6-39, double integration at the target locus has been accompanied by a third, random integration.

A summary of the conclusions of these analyses for all ten inducible clones is given in Table 1.

Long range Southern analysis of inducible clones

Southern analyses of pulsed field gels support the conclusion that zero, one, two or three copies of the (~ 10 kb) targeting plasmid have integrated into the 6-16 loci of the inducible clones. Results for the two HeLa-derived clones (3-17 and 2-32) and three

Figure 6. Short range Southern analysis of a selection of inducible clones derived from HeLa (clones 3-17 and 2-32) or HT1080 (clones 5-14, 5-27, 6-42) cells. DNA from clones or from parental HT1080 (HT) or HeLa (He) cells was prepared, digested with the restriction enzymes indicated, fractionated with labelled markers (M), blotted and probed as described (Methods). A. Probe a. B. Probe b. C. Probe c.

representative HT1080-derived clones are shown in Figure 7. Thus, in addition to the wild-type 80 kb (*EcoRV*) and 39 kb (*BstEII*) fragments detected by probe a, the homologous recombinants also have recombinant fragments that are

Figure 7. Pulsed-field gel analysis of a selection of inducible clones derived from HeLa (clones 3-17 and 2-32) or HT1080 (clones 5-14, 5-27, 6-42) cells. Clonal or parental HT1080 (HT) DNA was prepared, digested with restriction enzymes indicated, fractionated with markers (M), blotted and probed with probe a (Figure 5) as described (Methods).

approximately 10 kb (clone 6-42), 20kb (clones 3-17 and 5-14) or 30kb (clone 5-27) larger (Figure 7). Only the recombinant fragments were detected when such blots were probed with probe c (not shown). Clone 2-23 did not produce such predictable recombinant fragments but rather a recombinant *EcoRV* fragment of approximately 39 kb (Figure 7, lane 6) and an undetectable recombinant *BstEII* fragment (Figure 7, lane 12) which comigrated with the parental *BstEII* fragment.

DISCUSSION

Despite many successful reports of gene targeting, the application of targeting methods to a given target is not always straightforward. Selection procedures work with unpredictable efficiencies and often need to be followed by screening and sib-selection methods (i.e. Southern analyses or PCR) which can themselves be either unduly labour intensive or misleading. A choice of targeting methods is therefore important and the method described here widens this choice.

The method we have described is an extension of previously described methods in which a promoterless marker gene is activated by HR with the target gene; it therefore shares the advantages and disadvantages of these methods. Thus, although it can be applied only to transcribed genes, it can be used for targeting by either single crossover (insertion) or double crossover (replacement) events (in contrast to the PNS method which requires two crossovers), allowing for the design of two-step insertion/excision protocols (9,37,38) for the generation of subtle mutations. Promoter activation methods, together with the PNS method, provide a means of enriching for homologous recombinants so that fewer transfectants need to be screened by PCR or Southern analyses. Reports of successful targeting experiments show the efficiencies of enrichment by the promoter activation method to compare well with those for the PNS method (see introduction), and in some situations it may be that a promoter activation method is more successful than the PNS method. This was the case for the 6-16 gene of HT1080 and HeLa cells: the number of stable transfectants obtained after Ganciclovir/G418 selection for a 6-16 targeting construct expressing the *neo* and herpes simplex viral thymidine kinase

genes was only 50% less than the number obtained by the same selection of a construct expressing the *neo* gene alone (ACGP, unpublished results).

A choice of promoterless marker genes is valuable since it can be difficult to make targeting constructs that are capable of expressing the marker gene after HR. For example, when *neo* or *gpt* genes were placed in exon 2 of the 6-16 gene to form control constructs analogous to that of Figure 1, neither gene was expressed, presumably because of unpredictable and adverse effects on transcript processing (ACGP, unpublished results). Furthermore, experiments involving the sequential targeting in the same cell of both alleles of a gene, or of different loci, will benefit from a choice of promoterless markers since, once used, a given marker cannot be used again.

In its practical application the method differs substantially from the previous use of promoterless marker genes. Because the product of the marker gene (hGH) is secreted and assayed in conditioned medium, the method becomes a screening (rather than a selection or sorting) procedure. Compared to other screening procedures i.e. PCR and Southern analyses, it offers certain advantages. It is sensitive, so that it can be used on large pools (e.g. 60 colonies), and is not prone to giving false positives. It is also rapid, simple to perform and does not require cells to be grown in large numbers or to be manipulated or lysed. Thus, the approximately 14,000 stable transfectants screened in the experiments we have described could not easily have been screened by Southern analyses. Furthermore, in a related 6-16 targeting experiment, when pools of a similar size to those we have screened comfortably by hGH assay were screened by PCR, they gave rise to false positives that were not the result of contamination (ACGP, unpublished results).

Provided that the target gene is well expressed in the cells to be used, this method should be applicable to other target genes. The inducibility of the target gene in our experiments was helpful in the elimination of pools for which constitutive hGH secretion indicated a non-homologous integration event in a constitutively expressed gene. For constitutively expressed target genes this would not be possible, and clones isolated on the basis of hGH secretion alone, while enriched for homologous recombinants, would still include a proportion of non-homologous recombinants; as with other methods involving promoterless marker genes, these could be eliminated by PCR or Southern analyses. In the case of cell types known to be affected by hGH, the method could be adapted for use with a different secreted antigen. This may be necessary if ES cells are to be used with a view to obtaining targeted mice since altered phenotypes have been noted in transgenic mice expressing the hGH gene (39,40,41).

We have shown the method to be successful for the targeted disruption of the human IFN-inducible gene 6-16 in two different human cell lines. Of the 10 independent clones isolated that secrete hGH in response to IFN, 9 were shown to have resulted from HR between the targeting construct and target locus. Since a total of ~14,000 G418-resistant cells were screened, a rough estimate of targeting frequency of 1/1550 for the combined cell types can be made. This is a minimum estimate since screening was not exhaustive. Thus clones were not obtained from 6 strongly IFN-inducible pools (grey circles, Figure 3) for which inducible wells were identified in the second screen. Furthermore, some weakly inducible pools not analysed beyond the initial screen (e.g. pool 28, experiment 3) may have also contained inducible clones masked by constitutive hGH secreting clones, as was the case for pool 17 of experiment 3. For these reasons

also, the apparent difference in targeting frequency between the six targeting experiments is not meaningful.

One of the inducible clones (2-23) does not appear to be a homologous recombinant. Although probe a detects the fragment sizes predicted for a homologous recombinant (Figure 6A, lanes 3, 6 and 9), probe b does not (Figure 6B, lanes 3, 6 and 9), and probe c detects bands of unpredictable sizes (Figure 6C, lanes 3 and 4). We therefore believe that the targeting construct must have interacted with the target locus so that its region of homology was extended, by DNA synthesis using the chromosomal DNA as template, beyond exon 1 to include the promoter and regulatory region of 6-16. Following such an event, we suggest that the extended targeting construct disengaged from the 6-16 locus and integrated elsewhere in the genome by non-HR. The results of the long range Southern analysis (Figure 7, lanes 6 and 12) are consistent with this suggestion. Similar results and mechanisms have been described for other targets (21, 42).

An initially surprising observation was the greater average level of hGH secreted by HT1080 cells compared to HeLa cells during the screening process (Figures 3 and 4). This can best be explained by variations in average colony size at the time of harvesting the conditioned medium which was always much greater for HT1080 cells than for HeLa cells. When analysed at comparable cell densities, secretion of hGH by the two cell types was very similar (Figure 2B and C).

The inducibility of the homologous recombinants is greater than the inducibility of the population of cells transfected with the control construct (Figure 2) reflecting both a lower level of constitutive secretion and a higher level of induced secretion by the clones relative to the population. Both of these effects suggest that the 6-16/hGH fusion gene is less susceptible to position effects at the 6-16 locus than at an 'average' locus elsewhere in the genome. Nevertheless, inducibility (10 to 15-fold) of the homologous recombinant clones is still less than the inducibility (~100-fold) of the endogenous 6-16 transcript (25) reflecting the still appreciable secretion of hGH by the clones in the absence of IFN. We assume this to be a locus-independent effect on the 6-16 promoter of some (unknown) *cis*-acting sequences common to the control and targeting constructs; this would also have contributed to the constitutive expression of the population of cells transfected with the control construct.

The integration of more than one targeting construct at the target locus is unusual but not unprecedented (8, 20, 43). Such structures could be generated by the ligation of targeting constructs before integration, by the sequential integration of targeting constructs or by integration of a single construct followed by unequal sister chromatid exchange. We do not know which of these mechanisms occurred. As expected, similar amounts of hGH were secreted by homologous recombinants regardless of the number of integrated constructs. Screening for large amounts of hGH secretion cannot therefore explain the high frequency of multiple insertions. However, the initial selection in G418 could have favoured the survival of clones with more than one insertion.

A study of the effects of disruption on the expression of 6-16 mRNA and on the phenotype of cultured cells awaits disruption of the second 6-16 allele.

ACKNOWLEDGEMENTS

We thank the Royal Society and the Medical Research Council for their support.

REFERENCES

1. Capecchi, M.R. (1989) *Science*, **244**, 1288–1292.
2. Porter, A.C.G. (1989) *Technique*, **1**, 53–65.
3. Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A. and Kucherlapati, R.S. (1985) *Nature*, **317**, 230–234.
4. Kim, H.-S. and Smithies, O. (1988) *Nucleic Acids Res.*, **16**, 8887–8903.
5. Frohman, M.A. and Martin, G.R. (1990) In Innes, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds.), *PCR Protocols-A Guide To Methods And Applications*. Academic Press, Inc, San Diego.
6. Zimmer, A. and Gruss, P. (1989) *Nature*, **338**, 150–153.
7. Joyner, A.L., Skarnes, W.C. and Rossant, J. (1989) *Nature*, **338**, 153–156.
8. Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) *Cell*, **64**, 693–702.
9. Hastly, P., Ramirez-Solis, R., Krumlauf, R. and Bradley, A. (1991) *Nature*, **350**, 243–246.
10. Mansour, S.L., Thomas, K.R. and Capecchi, M.R. (1988) *Nature*, **336**, 348–352.
11. Thomas, K.R. and Capecchi, M.R. (1990) *Nature*, **346**, 847–850.
12. McMahon, A.P. and Bradley, A. (1990) *Cell*, **62**, 1073–1085.
13. De Chiara, T.M., Efstratiadis, A. and Robertson, E.J. (1990) *Nature*, **345**, 78–80.
14. Johnson, R.S., Sheng, M., Greenberg, M.E., Kolodner, R.D., Papaionnou, V.E. and Spiegelman, B.M. (1989) *Science*, **245**, 1234–1236.
15. Jasin, M. and Berg, P. (1988) *Genes & Development*, **2**, 1353–1363.
16. Doetschman, T., Maeda, N. and Smithies, O. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8583–8587.
17. Dorin, J.R., Inglis, J.D. and Porteous, D.J. (1989) *Science*, **243**, 1357–1360.
18. Sedivy, J.M. and Sharp, P.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 227–231.
19. Charron, J., Malynn, B.A., Robertson, E.J., Goff, S.P. and Alt, F.W. (1990) *Mol. Cell Biol.*, **10**, 1799–1804.
20. Schwartzberg, P.L., Robertson, E.J. and Goff, S.P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3210–3214.
21. Reilete, H., Maandag, E.R., Clarke, A., Hooper, M. and Berns, A. (1990) *Nature*, **348**, 649–651.
22. Jasin, M., Elledge, S.J., Davis, R.W. and Berg, P. (1990) *Genes & Development*, **4**, 157–166.
23. Baker, M.D., Pennell, N., Bosnoyan, L. and Shulman, M.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6432–6436.
24. Smith, A.J.H. and Kalogerakis, B. (1990) *J. Mol. Biol.*, **213**, 415–435.
25. Kelly, J.M., Porter, A.C.G., Chernajovsky, Y., Gilbert, C.S., Stark, G.R. and Kerr, I.M. (1988) *EMBO J.*, **5**, 1601–1606.
26. Porter, A.C.G., Chernajovsky, Y., Dale, T.C., Gilbert, C.S., Stark, G.R. and Kerr, I.M. (1988) *EMBO J.*, **7**, 85–92.
27. Selden, R.F., Howie, K.B., Rowe, M.E., Goodman, H.M. and Moore, D.D. (1986) *Mol. Cell Biol.*, **6**, 3173–3179.
28. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, **33**, 103–119.
29. DeNoto, F.M., Moore, D.D. and Goodman, H.M. (1981) *Nucleic Acids Res.*, **9**, 3719–3730.
30. Southern, P.J. and Berg, P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.
31. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor.
32. Tyler-Smith, C., Taylor, L. and Muller, U. (1988) *J. Mol. Biol.*, **203**, 837–848.
33. Anand, R. (1986) *Trends Genet.*, **2**, 278–283.
34. Southern, E.M., Anand, R., Brown, W.R.A. and Fletcher, D.S. (1987) *Nucleic Acids Res.*, **15**, 5925–5943.
35. Hodgson, C.P. and Fisk, R.Z. (1987) *Nucleic Acids Res.*, **15**, 6295.
36. Church, G.M. and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
37. Maniatis, T. (1985) *Nature*, **317**, 205–206.
38. Reid, L.H., Gregg, R., Smithies, O. and Koller, B.H. (1990) *Proc. Natl. Acad. Sci.*, **87**, 4299–4303.
39. Palmiter, R.D., Norstedt, G., Gelin, R.E., Hammer, R.E. and Brinster, R.L. (1983) *Science*, **222**, 809–814.
40. Brem, G., Wanke, E., Wolf, T., Buchmuller, M., Brenig, B. and Hermanns, W. (1989) *Mol. Biol. Med.*, **6**, 531–547.
41. Bchini, O., Andres, A.C., Schubaur, B., Mehtali, M., LeMeur, M., Lathe, R. and Gerlinger, P. (1991) *Endocrinology*, **128**, 539–546.
42. Adair, G.M., Nairn, R.S., Wilson, J.H., Seidman, M.M., Brothman, K.A., MacKinnon, C. and Scheerer, J.B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4574–4578.
43. Thompson, S., Clarke, A.R., Pow, A.M., Hooper, M.L. and Melton, D.W. (1989) *Cell*, **56**, 313–321.