This is a copy of the free text from BioMed Centrals Repository (PMC)
PMCID: PMC145857
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC145857/
published in

Karreman, S., Hauser, H., Karreman, C.
On the use of double FLP recognition targets (FRTs) in the LTR of retroviruses for the construction of high producer cell lines
(1996) Nucleic Acids Research, 24 (9), pp. 1616-1624.

1616–1624 *Nucleic Acids Research, 1996, Vol. 24, No. 9* © *1996 Oxford University Press*

On the use of double FLP recognition targets (FRTs) in the LTR of retroviruses for the construction of high producer cell lines

Simone Karreman, Hansjörg Hauser and Christiaan Karreman*

Gesellschaft für Biotechnologische Forschung (GBF), Department of Gene Regulation and Differentiation, Mascheroder Weg 1, 38124 Braunschweig, Germany

Received February 7, 1996; Revised and Accepted March 22, 1996

ABSTRACT

A pilot experiment for the construction of a hamster derived, high producer cell line using site specific recombination is described. In the experiment chromosomal loci with intrinsic high expression characteristics were sought via infection with a retroviral construct, containing double FRT sites and subsequent screening for overproduction of an encoded markergene. These sites were then targeted with a second vector, that recombined via the FLP/FRT system from *Saccharomyces cerevisiae* yielding cells that had the second construct at exactly the same position as the first. By using retroviral vectors with double and single FRT sites, respectively, stable clones can be created that can no longer be excised with FLP.

INTRODUCTION

The chromosomal locus of an artificially introduced expression cassette is one of the most important parameters of the level of its expression and the stability thereof. Since the integration after transfection or infection is a random process the creation of high producer cell lines requires long periods of screening of a large number of cell clones for the appropriate characteristics.

An alternative would be a two-step method, wherein the transfection and selection for a high producer would be necessary only once, on an initially constructed cell line using a reportergene. This cell line could then be used repeatedly for the construction of all kinds of producers by exchanging the original reporter-construct with an expression cassette of choice. The construction of a producer cell line would be reduced to this replacement step without further screening, since the chromosomal surrounding would be a constant instead of a variable.

The use of heterologous recombination systems, like the FLP/FRT system from the yeast *Saccharomyces cerevisiae* allows just such an exchange of cassettes (1). This leaves another practical problem, namely that of multiple integrations of the first, reporter-gene containing, construct. Multiple integrations will result in high expression by copy number rather then by expression level of a single construct.

The use of retroviruses at low mois is a most elegant way of overcoming the problem of multiple integrations. Especially since accepted producer cell lines can be infected with ecotropic, low risk retroviruses (2). The exchange of the cassette giving a stable endproduct demands the use of two FRTs with different sequences (3). These FRTs will recombine with another FRT with exactly the same sequence, but they cannot recombine with each other. The reproductive cycle of retroviruses will not allow for the use of two different FRT sites inserted into the LTRs. Such a construct would be reduced to a provirus with only one kind of FRT and with its intrinsic instability towards FLP (upper part of Fig. 1A). If the two FRTs are placed inside the LTRs the insertion of the second pair of genes would lead to a stable endproduct. However, this strategy would also result in leaving large parts of the retrovirus in the direct surroundings of the expression cassette (lower part of Fig. 1A). For this reason a new approach was chosen with double FRTs, one wild type and the second a mutant, tandemly integrated in the 3′-LTR. This leads to stable recombinants with a minimum of left residues after the replacement step (Fig. 1B).

In this report we describe the use of such double FRT sites in the LTR of Myoloproliferative sarcoma virus (MPSV) derived ecotropic retroviral constructs for the infection of the BHK-A cell line and the exchange of this integrate with a second expression cassette.

MATERIALS AND METHODS

Plasmids

pAPTKNEF (Fig. 2): the primary retroviral targeting vector. The two LTRs and packaging signal (ψ) are derived from MPSV. In the 3′-LTR a double FRT (oligo FRT1FRT2) was inserted into the *Nhe*I site.

Upon packaging and infection the retroviral construct expresses two genes. In order to prevent the problem of promoter occlusion, both will be driven from the 5′-LTR via a bicistronic mRNA. This was accomplished through inserting the 5′ untranslated region of polio virus between the two genes (IRES, internal ribosomal entry site). All vector construction elements, including the IRES are

* To whom correspondence should be addressed at present address: Forschungszentrum für Umwelt und Gesundheit (GSF), Department of Clinical Molecular Biology and Tumorgenetics, Marchioninistraße 25, 81377 Munich, Germany

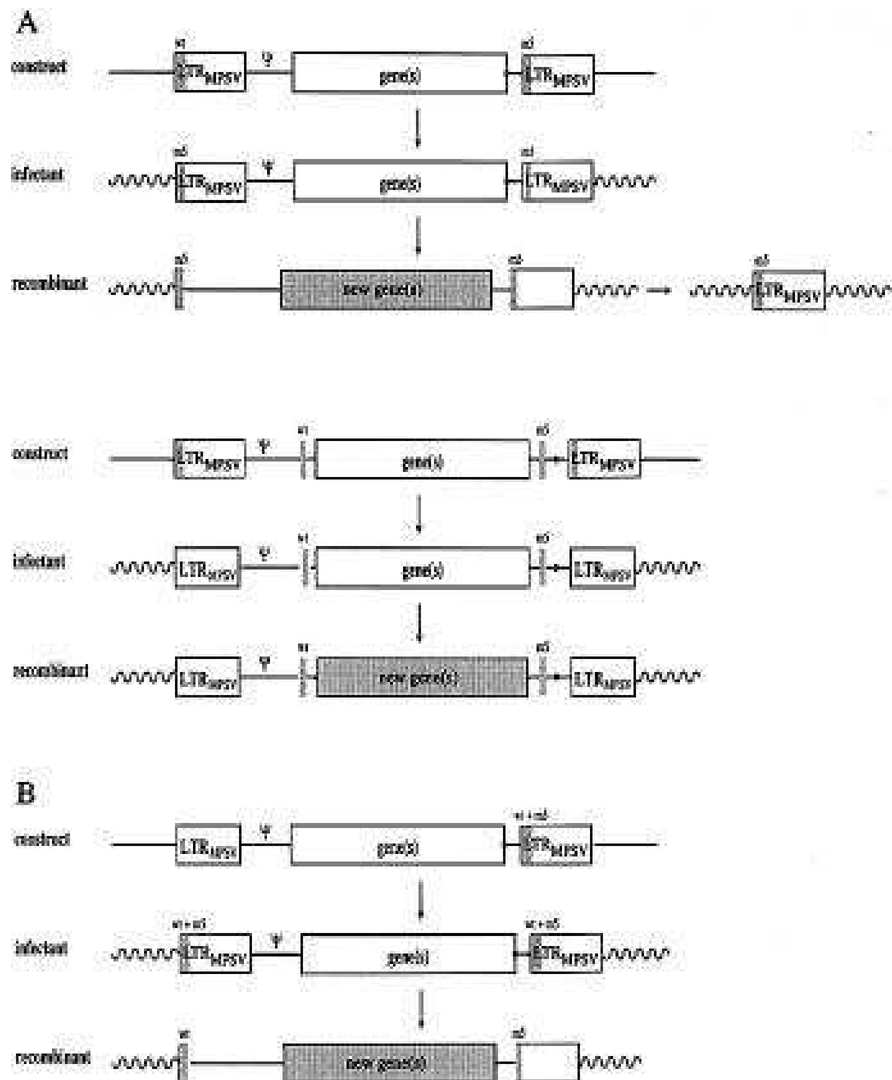

Figure 1. Schematic representation of FRT sequences in retroviral vectors and their respective proviral forms. ψ is the retroviral packaging sequence, wt is an FRT with wildtype sequence, m5 is an FRT with the mutant 5 sequence. Chromosomal DNA is depicted as wavy lines. All constructs are depicted as vector (top), as provirus after packaging and infection of a target cell (middle) and after successful replacement with the second construct containing the new gene(s) (bottom). (**A**) The insertion of two different FRT sites in the LTR of a retroviral construct will result in an infectant where the FRT of the 3′-LTR will have been duplicated and be present in both LTRs. Recombinational integration is possible but the resulting configuration is unstable in the presence of FLP recombinase. Lingering expression of this protein would lead towards recombination of the two FRTs and deletion of the inserted regions. The use of two different FRTs between the LTRs, as depicted in the lower part of (A), will give rise to stable integrates, but all sequences outside the FRTs will remain after the recombination. This might cause problems for the expression of the targeting construct (e.g. promoter occlusion). (**B**) Both problems are overcome by the use of double FRTs in the 3′-LTR. After integration the FRTs will be duplicated faithfully into both LTRs and after recombination the product will be stable in the presence of FLP activity.

based upon a previously published vector family (4). The two genes encoded are a selectable gene and a reporter gene for assaying the expression level of the provirus carrying cell clones. As selectable gene, in second position behind the IRES element, a fusion between the thymidine kinase of herplex simplex virus (HSV) and the neomycin resistance gene from Tn5 was chosen. Cells expressing this fusion construct can be selected positively as well as negatively. Positive selection makes use of the drug neomycin, negative selection on the drug gancyclovir (GANC). The reporter gene encodes SEAP (secreted alkaline phophatase) (5). The expression of this gene can be assayed for a large number of cell clones in an agar overlay assay (6,7).

pGETHEAD (Fig. 2): the secondary targeting vector. This vector has a retroviral backbone identical to that of pAPTKNEF. It is not used, however, as a retrovirus. The use of the same basic construct was chosen to minimise the risk of promoter-influences on the expression of the recombinational product. In this construct both LTRs have a single FRT site, the 5′-LTR carrying the wild type sequence, the 3′-LTR carrying the mutant 5 (m5) (see oligos FRT1UPNH and FRT2UPNH for their sequences). Since this vector will not undergo retroviral replication both LTRs will stay intact during transfection. The genes in this construct encode PAC (puromycin *N*-acetyltransferase) as selectable marker and the firefly luciferase as reporter. In the *Ppu*MI site of the 3′-LTR an

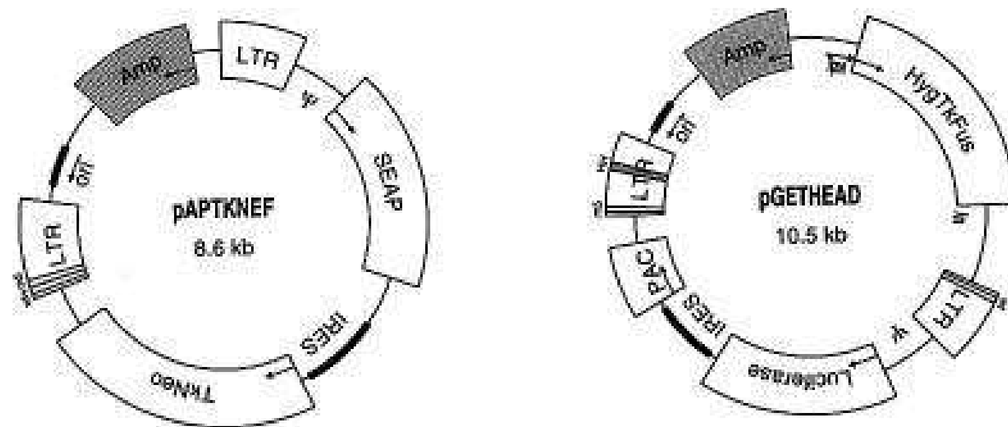

Figure 2. Circular representation of the screening vector and the targeting construct. Amp, prokaryotic ampicylin resistance gene; Ori, origin of replication; LTR, long terminal repeat of MPSV; ψ, retroviral packaging signal; SEAP, secreted alkaline phophatase; IRES, internal ribosomal entry site; TkNeo, gene encoding a fusion protein of the thymidine kinase (TK) of herplex simplex virus (HSV) and the neomycin resistance gene from Tn5; PAC, puromycin acetyl transferase; Tkprom, promoter of the TK gene of HSV; HygTkFus, gene encoding a fusion protein of the hygromycin resistance gene and the TK gene of HSV; An, poly-A signal of the TK gene of HSV. FRTs are depicted as open boxes in the LTRs. The hammerhead structure is represented by a shaded box in the 3′-LTR of pGETHEAD.

oligo (oligo SHARK) was inserted. Upon integration and transcription this oligonucleotide will form a ribozyme on mRNA level. This moiety, a hammerhead structure (HH), should recognise the PAC part of its own RNA and cut it, resulting in a reduction of the puromycin resistance. For an analogue usage of ribozymes see (8). This hammerhead should therefore be able to allow selection for recombination and against ‘normal’ integration of the vector. Normal integration of the vector would result in a cell with a puromycin-sensitive phenotype and site-specific recombination would lead to a puromycin-resistant phenotype due to the loss of the hammerhead structure (see Figure 6A, middle and bottom drawings for the differences between a ‘normal’ transfectant and a recombinant). The hammerhead ribozyme should also function *in trans* and thereby reduce the problem of selecting cell clones showing both recombination and integration(s).

Another negative selectable gene was placed outside the LTRs, it encodes a fusion between the thymidine kinase from HSV and a hygromycin resistance inducing protein (created analogue to 9). This to select against normal integrations of the whole construct, but rather select for a replacement of the cassette, without the rest of the plasmid integrating.

pGET: control plasmid. It is identical to pGETHEAD only without the hammerhead structure.

pOG44. Plasmid encoding the FLP protein (1).

pIC20R. Cloning vector (10).

Oligonucleotides. For the double FRT in the 3′-LTR of pAPTKNEF:
FRT1FRT2: 5′-CTAGAAGTTCCTATTCCGAAGTTCCTATTC-TCTAGAAAGTATAGGAACTTCGAAGTTCCTATTCCGAA-GTTCCTATTCTTCAAAAGGTATAGGAACTT-3′
2TRF1TRF: 5′-CTAGAAGTTCCTATACCTTTTGAAGAATA-GGAACTTCGGAATAGGAACTTCGAAGTTCCTATACTTTC-TAGAGAATAGGAACTTCGGAATAGGAACTT-3′

Single FRTs in pGETHEAD. For wild type FRT:
FRT1UPNH: 5′-CTAGAAGTTCCTATTCCGAAGTTCCTATT-CTCTAGAAAGTATAGGAACTT-3′
FRT1UNNH: 5′-CTAGAAGTTCCTATACTTTCTAGAGAATA-GGAACTTCGGAATAGGAACTT-3′

For mutant m5:
FRT2UPNH: 5′-CTAGAAGTTCCTATTCCGAAGTTCCTATT-CTTCAAAAGGTATAGGAACTT-3′
FRT2UNNH: 5′-CTAGAAGTTCCTATACCTTTTGAAGAATA-GGAACTTCGGAATAGGAACTT-3′

The hammerhead in pGETHEAD.
SHARK: 5′-GACGCTAGCAGATCTGATGTCGAGCCCCTG-ATGAGTCCGAGAGGACGAAACGCGCGTGAGCTAGC-3′
KRAHS: 5′-GTCGCTAGCTCACGCGCGTTTCGTCCTCTC-GGACTCATCAGGGGCTCGACATCAGATCTGCTAGC-3′

Isolation of bordering fragment via inverse nested PCR. External primers:
INVPCR2: 5′-GCCATACGCGCTTCTACAAGG-3′
GA0930: 5′-GTTCCTTGGGAGGGTCTCC-3′
Internal primers:
INVPCR1: 5′-GGGAAGCTTAGTTTCACGCCACCAAGATC-3′
INV8C: 5′-GGGAAGCTTCTGAGTGATTGACTACCCG-3′

Cells and media

ψ2 was used as the ecotropic packaging cell line (11). The Syrian hamster cell line BHK-A was described before (2). It represents a subclone of BHK-21 (ATCC CLL10) which was selected for infectibility with ecotropic murine retroviruses. NIH 3T3 is a murine fibroblastoid cell line, ATCC CRL 1658 (12). E25B2 is a derivative of the CV1 African green monkey kidney cell line (1).

DMEM, Dulbeco’s modified Eagles medium (Sigma Chemie GmbH, Deisenhofen, Germany), complemented with 10% foetal calf serum, 20 mM glutamine, 60 µg/ml penicillin and 100 µg/ml streptomycin. For selection on neomycin resistance DMEM was supplemented with 1 mg/ml geneticin (Sigma Chemie GmbH, Deisenhofen, Germany). For hygromycin resistance the DMEM was supplemented with 1000 U/ml of hygromycin B (Sigma Chemie GmbH, Deisenhofen, Germany). Puromycin medium consisted of DMEM with puromycin (Sigma Chemie GmbH, Deisenhofen, Germany) added to a final concentration of 2.5 µg/ml. To test for the loss of the thymidine kinase gene cells were grown in DMEM, 16µM GANC (Syntex Arzneimittel GmbH, Aachen, Germany).

All cells were grown at 37°C, under 5% CO_2 and 95% humidity.

Transfections and infections

The packaging cells were transfected with the retroviral vector constructs using the $CaPO_4$ method originally described by Graham and van der Eb (13). Stably transfected packaging cell lines confluently grown were put on half of their usual amounts of medium for 24 h. After this period of time, supernatants were harvested, passed through filters (0.45 µm, Sartorius, Göttingen, Germany) and used directly to infect the target cells. Infections were incubated for 24 h with virus and 8 µg/ml Polybrene (Sigma Chemie GmbH, Deisenhofen, Germany). After 24 h the cells were trypsinized and divided, in various dilutions, into selective media. Simultaneously, all virus stocks were titrated on NIH 3T3 cells.

Southern blotting

Blotting was performed on Zeta-probe (Bio Rad, Richmond, USA), using the standard protocol as recommended by the manufacturer.

FRT checking

The FRTs were tested on their functionality by *in vivo* recombination. For the FRT with wild type sequences this was accomplished by co-transfection of the FLP encoding plasmid pOG44 and the FRT containing constructs into E25B2 cells and screening for white colonies (1). These cells contain a single copy of the β-galactosidase gene bearing a single FRT site in its coding region. This mutated β-galactosidase gene is still capable of expressing functional enzyme. Upon site specific recombination with a construct also containing a FRT site the open reading frame of the β-galactosidase gene is interrupted and no longer capable of directing the synthesis of functional enzyme. Scoring is done by counting the resulting white colonies after X-gal staining. The FRT constructs with the mutant 5 sequence were tested by co-transfecting pOG44 and two plasmids, both with FRT-m5, isolating total DNA after 2 days and assaying the resulting extrachromosomal DNA. PCR was used to detect recombined DNA molecules. Transfections without pOG44 were performed as controls.

Ribozyme test

To test whether the ribozyme had its desired function, pGET and pGETHEAD were transfected into BHK-A cells and after 2 days cells were split and diluted into selective media. The ratio between clones able to grow in hygromycin and those able to grow in puromycin containing medium was scored. Repeatedly those cells that were transfected with pGETHEAD had a lower ratio in this test, i.e. less cell clones survived puromycin than should have when compared to pGET clones. Ratios were dependent on the concentration of puromycin, higher concentrations showed the influence of the ribozyme more strongly. Under standard selection-conditions the difference was at least a factor of two. Therefore we conclude that in the described transfections, the level of PAC is reduced under the level of resistance in 50% of the cells.

SEAP agar overlay test

The SEAP overlay test was carried out as described previously (6,7). Cell clones were covered with an overlay of 0.45% agarose. PVDF membrane (Millipore IPVH 00010, 0.45 µm) was placed on top of the agarose and incubated overnight. During this time the secreted enzyme will diffuse and bind to the membrane. After the incubation period the membrane was taken off and washed with water. The enzyme was visualised by incubation of the filter in buffer containing *p*-nitro blue tetrazolium chloride and bromo-4-chloro-3-indolyl phosphate. The enzyme activity was visible as blue spots. The highest producers gave the most intensive blue spots in the shortest period of time. High producers were defined as clones giving intense spots in 30 min, low producers were those clones where the spots took 5 h to become visible. Cell clones were identified by comparing an acetate copy of the filters with the original cell culture plates and subsequently isolated.

Inverse PCR

Total chromosomal DNA was isolated from infected cells. DNA, 15 µg, was cut with *Bgl*II, phenolized, and alcohol precipitated. DNA was taken up in 100 µl ligation mix and ligated overnight at 12°C. On the thus formed circular DNA molecules nested PCR was performed. Fragments were run on agarose gels, isolated, digested with *Hin*dIII and used for cloning.

RESULTS

Infection and expression screening

We constructed a MPSV based retroviral vector (pAPTKNEF see Fig. 2) and used infectable BHK-A cells (2). As target we infected 10^7 cells with an moi of 2×10^{-4}. Two thousand of the resulting neomycin resistant cell clones were assayed for SEAP expression using the agar overlay screening method. About 50% of the clones which express the neomycin resistance gene via the bicistronic mRNA gave rise to observable blue colouring in this assay (see also the measured SEAP levels in single clones and that in the cell pool as shown in Figure 3). Of those clones that expressed SEAP at visible levels, clones were picked with low SEAP levels, with intermediate levels and with high levels. Furthermore, random clones were picked as control. As can be seen in Figure 3 some of the cell clones did not, when measured directly on cell supernatants, conform to the original division into high, medium and low anymore. Nevertheless, the original naming of the cell clones, beginning with 'H', 'M', 'L' and 'ΔA' was retained.

Proviral copy number and integrity

After studying the picked clones for stability of expression, eight clones were selected for further study: three high (H51, H32, H52), three middle (H8, M1, H22) and two low (L9, ΔAV) clones. The chromosomal DNA of these clones was subjected to Southern blot analysis to identify those cell clones which contain one fully integer recombinant provirus (results not shown). Even after the extreme low moi used, two clones (H52 and L9) contained at least two proviruses, ΔAV and one of the two retroviral integrates of L9 had a deletion in their proviral inserts. The bordering fragment of H8 was almost too small to be really outside the retroviral integrate as calculated. To prevent unpleasant surprises none of these clones were used in further experiments.

Functionality of the proviral FRTs

To confirm the functionality of the FRT sites after the proviral establishment an excision experiment was performed. For this the

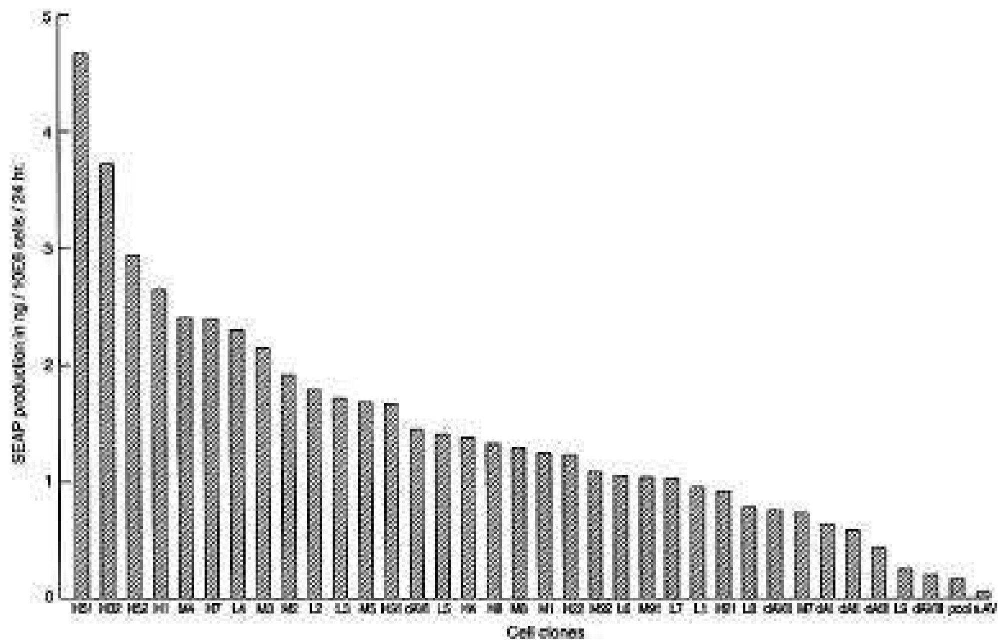

Figure 3. SEAP production of the various cell clones picked using the SEAP-overlay test. The original division in high, medium and low producers from data derived in the overlay test is expressed in the names of the cell clones: H for high, M for medium and L for low. The control clones, which were not tested with the overlay test, are named starting with ΔA. Although the original division into high, medium and low is not as strict as expected, the spread of the various clone types is not random. SEAP production of the cell pool is significantly lower than that of all clones picked after the SEAP-overlay test, even lower than that of almost all randomly picked clones.

remaining cell clones with full length, single copy proviruses were transfected with pOG44, an FLP expressing plasmid, and selected subsequently with GANC for the loss of the TkNeo moiety. As controls transfections with an empty cloning vector (pIC20R) and mock transfections were performed. The results are shown in Table 1. It is obvious that the efficiency of excision differs greatly between the cell clones. This could be due to undetected proviral mutations or to conformational differences of the DNA linked to expression levels of the integration sites (14). Due to its low recombinational activities, clone H32 was not used in further experiments.

Table 1. Frequency of intraviral recombination

	M1	H22	H32	H51
mock	24	26	10	32
pIC20R	61	80	15	18
pOG44	789	273	23	235

Cell lines were transfected using the $CaPO_4$ method. The precipitation contained plasmid pOG44, an equimolar amount of plasmid pIC20R or no DNA at all. pOG44 encodes the FLP protein capable of recombining FRT sites.

From one clone, M1, the 3′ neighbouring chromosomal fragment of the provirus was isolated by inverse PCR (15,16) and this fragment was used to probe the process of excision. In total six GANC resistant cell clones (see above) were picked and expanded. Five had been transfected with pOG44, one with pIC20R. Total chromosomal DNA was isolated and assayed in a Southern blot. The results are shown in Figure 4; the retroviral construct has inserted almost exactly in the middle of an originally 1200 bp long *Bgl*II fragment of BHK-A. The integrated provirus, as depicted in the middle of Figure 4, is represented by a band of 3.5 kb on the blot. After FLP expression the part of the provirus between the FRTs was removed, leaving ~700 bp of the vector behind. All assayed clones gave the same, predicted, 1900 bp band. This fact together with the high number of GANC resistant cell clones after pOG44 transfection confirms that intraviral excision is a rather efficient process in M1.

The clone transfected with pIC20R and subsequently selected with GANC has lost the original 3.5 kb band entirely. Here a larger deletion has taken place which is probably not due to FLP activity. This deletion makes this allele no longer detectable with the M1 specific chromosomal DNA fragment.

Targeting the proviral locus

The targeting construct (pGETHEAD; Fig. 2) was introduced both by electroporation and by $CaPO_4$ transfection. In case of transfection, the FLP protein, necessary for the recombination, was delivered by co-transfection of the pOG44 plasmid. For electroporation the pOG44 plasmid was transfected into the various cell lines 2 days prior to electroporation. Different ratios of pGETHEAD and pOG44 were tested, indicated in the names of the resulting clones; 2/10 indicates a ratio of 2 µg pOG44 on 10 µg pGETHEAD per 2× 10 cm Petri dishes. The roman figures in the names of individual cell clones are clone numbers.

Three selectional criteria were used to test integration of the second construct. Firstly, puromycin resistance, for the insertion of the construct. Secondly, on the function of the ribozyme, to ensure that the number of 'normal' transfectants, based on random integration, would be very low. Thirdly, on the selection

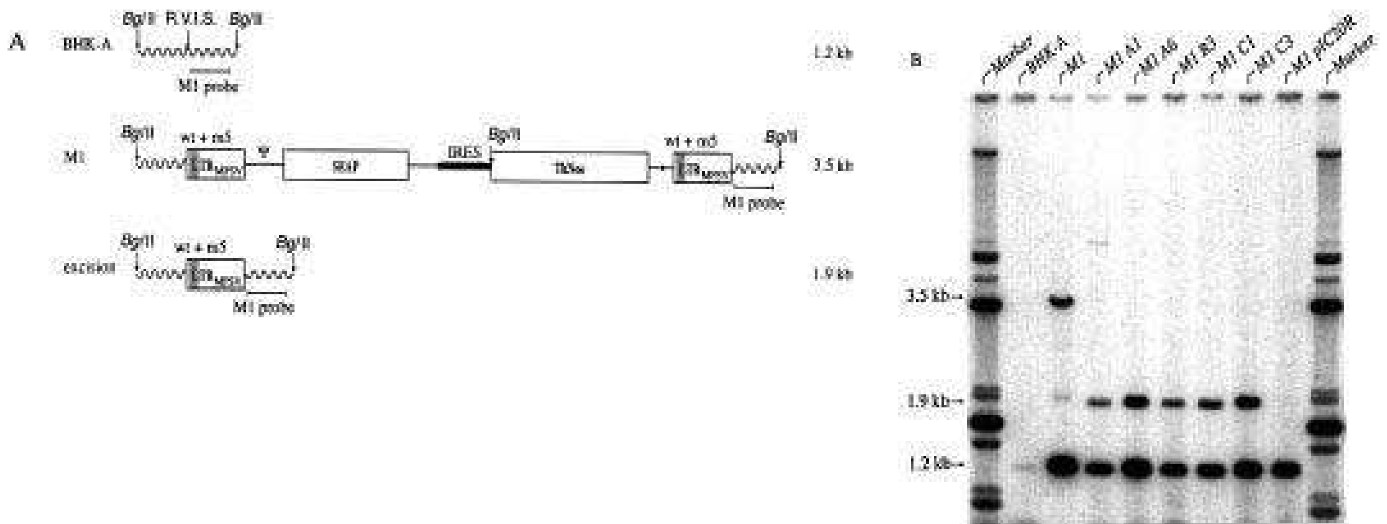

Figure 4. Southern blot of the clones generated by pOG44 induced intraviral excision. (**A**) Schematic representation of the various chromosomal structures of the cell lines. DNA was cut with the restriction enzyme *Bgl*II. In BHK-A cells all alleles are recognised by the probe and give rise to a 1.2 kb band [see also (B), lane 2]. The insertion of the retroviral construct at the site indicated by the arrow (RVIS, retroviral integration site) gives rise to a 3.5 kb band. After recombination (using either the wt-FRT or the m5-FRT) a band of 1.9 kb is predicted. (**B**) Southern blot. Five independent GANC resistant clones, that arose after transfection of clone M1 with the FLP encoding plasmid pOG44 are assayed on this blot. The sixth, M1 pIC20R, is a clone spontaneously becoming $GANC^R$ after transfection with pIC20R.

with GANC to select against the integration of the HygTk moiety. The GANC selection also serves as selection for the removal of the TkNeo gene of the inserted provirus.

Two days after the targeting, cells were split and put on medium containing puromycin. GANC was not added to the media at this time. This was done because of the possibility that correctly targeted cells could still be sensitive toward GANC because of lingering TkNeo fusion protein. Half of the cells were put on medium containing puromycin and GANC 2 days after the start of puromycin selection, the other half after 7 days. This is also indicated in the names of the resulting clones: those that had the late addition of GANC all start with 'P'.

Electroporation, a method which is famous for the high ratio of single copy integrates, gave only one stable cell clone (EP H51 42) from ~100 original survivors.

Transfection gave a lot more stable clones, albeit only for two of the primary infectants: only M1 and H51 could be converted to the puromycin/GANC-resistant phenotype at all. Most clones were found in experiments with the lower ratios of pOG44 versus pGETHEAD. However, most of these cell clones proved to be unstable under prolonged culture conditions and died. In the end most remaining clones were derived from transfection with high ratios of pOG44 versus pGETHEAD.

From the cell lines M1 and H51 a total of 62 puromycin/GANC-resistant clones were obtained after transfection. Since the selection on puromycin in BHK-A cells is not optimal (see Discussion) these cells clones were subsequently tested for the production of luciferase. Only 11 produced any detectable amounts of this enzyme. These 11 clones were assayed for all markers, and were neo^-, $SEAP^-$, hygromycin-sensitive, GANC-resistant, puromycin-resistant and $luciferase^+$. This means that they all showed the expected phenotype. The only electroporant tested $luciferase^-$ and was used as control in the further experiments.

Southern blot analysis of recombinants

The probe for the 3′ bordering host sequence from M1 was used to test the M1 derivatives by Southern blotting. In Figure 5A the theoretical pattern of such an experiment is depicted and in Figure 5B the Southern blot of the actual DNA is shown. In all cases only excision of the retroviral construct had taken place (compare with Fig. 4). Using the sequence of PAC for reprobing this blot a strong signal was found in all lanes with M1 derivatives (results not shown), indicating that many copies of pGETHEAD are present in these cells.

The H51 series could not be analysed in the same way since the H51 neighbouring sequences were resistant to full length inverse-PCR cloning. Therefore, we assayed these clones with a neomycin and puromycin probe, respectively. As can be seen in Figure 6A, when H51 DNA gives rise to a 5 kb fragment after digestion with *Bgl*II and *Xho*I then H51 derivatives with specific recombination should harbour a 4.3 kb band. Figure 6B shows that the subclone H51 2/10I carries the expected fragment of 4.3 kb. Again, it is obvious that there have been multiple integrations in this and various other cell clones.

Three of the derivative cell clones did not show hybridisation with the PAC probe. Although they are puromycin resistant and produce luciferase (except EP H51 42). The difficulties of PAC selection with BHK-A cells (also see Discussion) are probably responsible for these fake puromycin resistant cells. Since these cells produce luciferase and show no hygromycin resistance, unusual rearrangements must have taken place.

To further investigate whether H51 2/10I is a true site specific recombinant, we assayed this clone by a restriction enzyme panel. The DNA of both of the original H51 and its derivative cell line were restricted with *Bgl*II and *Xho*I and a third enzyme (Fig. 7). As the various length of the *Bgl*II–third enzyme fragment are

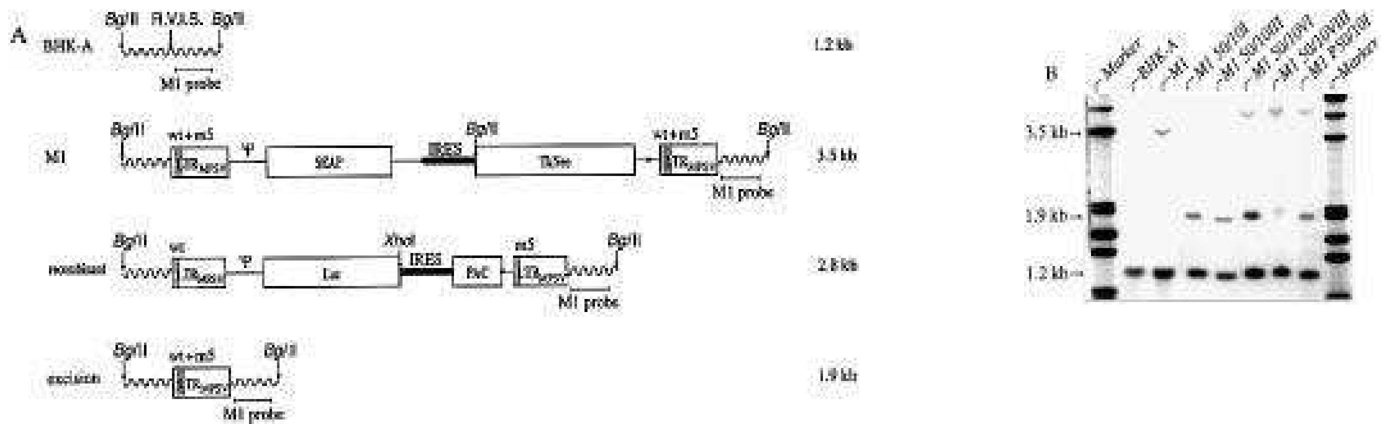

Figure 5. Southern analysis of M1 derived clones. (**A**) Schematic representation of the expected fragments resulting from restriction of chromosomal DNA with *Bgl*II and *Xho*I and subsequent probing with the 3′ bordering sequence specific fragment (M1 probe). (**B**) Corresponding Southern blot of all M1 derivatives.

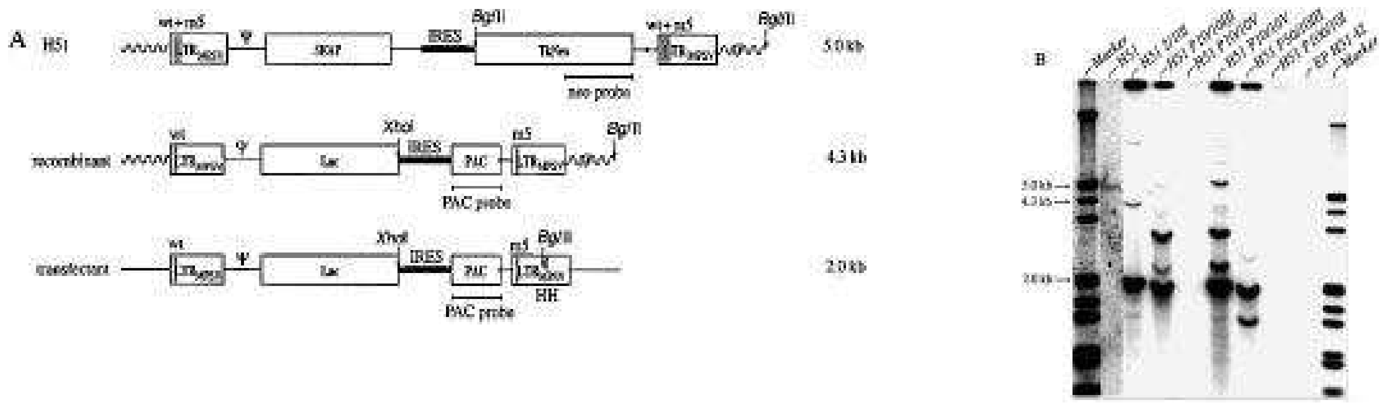

Figure 6. Southern analysis of H51 derivatives. (**A**) Schematic drawing of H51 and its possible derivatives induced by recombination and transfection. (**B**) Southern blot. H51 was probed with the indicated neomycin probe, the others with a PAC specific probe. All incubations were done with *Bgl*II and *Xho*I. In lanes 3–9 the H51 derivatives are shown, the only cell line that gives the expected 4.3 kb band is H51 2/10I. Four cell lines, including H51 2/10I show multiple integrations of pGETHEAD. Three others, including the single clone resulting from electroporation (EP H51 42), do not carry a detectable PAC gene.

determined for H51, the corresponding band for H51 2/10I can be calculated and compared. As can be seen in Figure 7B all bands in H51 have a corresponding band in H51 2/10I. From Figure 7B it is again obvious that also in the case of H51 2/10I there have been multiple normal integrations along with the site specific recombination.

DISCUSSION

A prerequisite for retroviral manipulation of cells is that they can be infected efficiently. We have shown earlier that a substrain of BHK-21 cells (BHK-A) is infectable with ecotropic murine retroviruses (2). Since the BHK cell lines are frequently chosen for overexpression of transferred genes, have a highly specific glycosylation pattern (17) and can be grown in tissue culture and fermentors (18) even in the absence of serum, we regard it as a highly versatile object for scientific and technological purposes which now include retroviral gene targeting.

The concept of using double FRT sites in retroviruses and its intrinsic advantage with respect to stability in the presence of FLP (Fig. 1) is proven to be feasible by our work. The synthetic double FRT structure inserted into the LTR is stable upon expansion in *Escherichia coli* and later during the replication cycle of the retrovirus. It was transcribed faithfully from the plasmid template, via RNA, into the proviral integrates of the target cells. As demonstrated by excision and integration, the double FRT is fully functional.

The agar-overlay assay has enabled us to screen thousands of individual clones for high expression of the proviral DNA in a very short time. The rough expression data of the clones has proven to be fairly consistent with the data we gathered of these clones in the follow-up experiments. The experiments also demonstrate that the number of high producers is rather small. Only ~50% of the obtained cell clones showed any colouring in the agar-overlay test and the SEAP production levels of the cell pool is ~25-fold below that of cell clones like H51 and H32 (Fig. 3).

The influence of the proviral integration site on the intraviral excision frequency is a new fact that might be linked to the expression level of both the provirus and its immediate surroundings (14). However, there is no direct correlation between the SEAP-expression from the provirus and the frequency of recombination. This becomes obvious when the expression levels

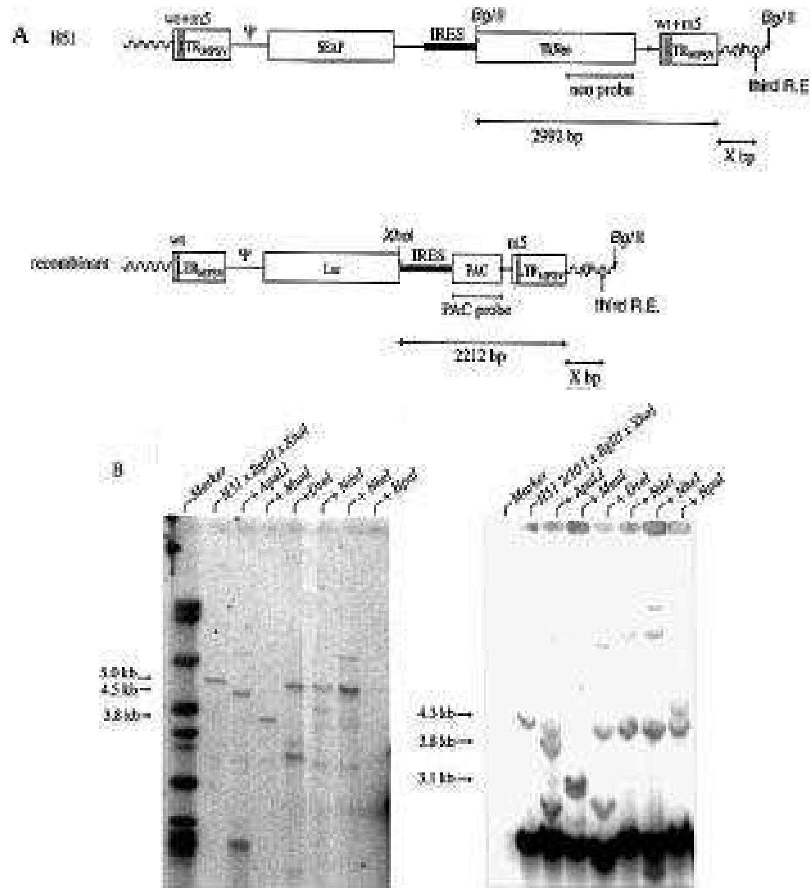

Figure 7. Analysis of H51 derived clones via a restriction enzyme panel. (**A**) Schematic representation of H51 cut with *Bgl*II and *Xho*I and a third enzyme and the corresponding experiment with H51 2/10I. H51 was probed with a neomycin fragment, and its derivative with a PAC probe. (**B**) Southern blot. The various 'third' enzymes are indicated above the corresponding lanes. *Apa*LI and *Mun*I are the only restriction enzymes that have a site in the 3′ bordering fragment of the retrovirus integrated in H51. The same enzymes give also smaller bands (700 bp shifted) in the H51 2/10I blot, as indicated with arrows.

of SEAP (Fig. 3) and the excision frequencies (Table 1) are compared. The cell clones with the highest expression (H51 and H32) have very different excision frequencies. Furthermore, the excision frequencies of both clones are below that of M1, a clone expressing SEAP at a medium level. The fact that only the FRT-sequences in the 3′-LTR are transcribed directly by the provirus and that the 5′-FRT transcription depends on the upstream cellular sequences could be responsible for different accessibility of FRTs. Furthermore, the influence of the overall chromatin structure of a particular proviral integration site might differentially influence transcription and recombination events. This variance in recombination level will make the process of screening for a high-producer more complicated, since cell clones that show high proviral expression must also be tested for their recombinational potential. Assuming that the excision frequency parallels insertional events, the test would consist of transfecting a replica culture of the cell clone with an FLP-expressing plasmid (pOG44) and subsequent selection against TK expression. The number of GANC-resistant cell clones would reflect FRT-accessibility.

The main problem we encountered was the appearance of multi-copy integrates of the second targeting construct. The choice of PAC as selection marker may have been the main reason for this phenomenon: a small subpopulation of BHK-A cells shows a high natural tolerance towards puromycin. In order to reduce the background in a transfection, concentrations of puromycin have to be used that the majority of BHK-A cells will only survive if they show a strong expression of PAC. This is accomplished in most of the cases by cells with multi-copy integrates. For the electroporation we used an experimental protocol that gives a high percentage of single-copy integrates, this would explain the fact that after electroporation of the various cell lines with the targeting vector we did not find any clones with the desired phenotype. Single-copy replacements without normal integrations that may have been formed after transfection or electroporation were most likely killed by the puromycin and did not give rise to any colonies.

Since spontaneous GANC-resistant cells are rare (Table 1) most of the cell clones found after combined puromycin/GANC selection had transiently expressed FLP (from pOG44). These cells have lost the TK expression capacity of the provirus. However, these cells do not necessarily harbour a complete copy of pGETHEAD since some cells are naturally resistant to puromycin and do not need the expression of the PAC gene. As our selection for targeting included the selection against HygTK, cell clones completely without pGETHEAD are overrepresented in the final population of cell clones (51 of 62 clones). Two of the remaining cell clones (H51 P100/10I and H51 P10/10IV) have,

at least, parts of pGETHEAD integrated, the deletions and rearrangements were probably the result of the loss of the (Hyg)Tk moiety. Only a few cell clones (9 of 62 clones) are descendants of the BHK-A phenotype with the lower tolerance towards puromycin. In all these cases multi-copy integrations were found, even though the ribozyme should select against random integration. At the used concentration of puromycin the effectivity of the hammerhead is only a factor 2. The marked need of BHK-A for multi-copy integrates to become puromycin-resistant has covered the effect of the ribozyme. We think the ribozyme can be a promising tool for selecting single copy replacements in other cells with a normal resistance pattern for puromycin. Preliminary experiments with the same constructs in NIH 3T3 cells showed a more explicit effect on the puromycin resistance than we ever saw in the BHK-A cell line.

With this work we have shown that a procedure for targeting single copy proviral sites is feasible. We have further defined the main problems for selection of these infrequent events. The problem can be overcome either by using another cell line or with a better selection method. It might also be possible to eliminate puromycin-tolerant BHK-A cells by subcloning. We still believe that this cell line, with its many advantages, would be an excellent tool for our approach.

The fundamental question which we have attempted to answer with the site-specific recombination system remains: are certain chromosomal sites (e.g. those which have tagged with our screening procedure) indeed determing the strength and stability of expression of different genes when inserted? To investigate this, a range of genes, probably even controlled by various promoters, have to be targeted into various loci. The results will also answer questions concerning the influence of chromosomal loci versus transferred promoters and enhancers. The establishment of a whole series of cell clones to be targeted, ranging in their expression from high to almost nothing (Fig. 3), will make these experiments statistically meaningful. Apart from these scientific questions we hope to develop the process as outlined in this work until it becomes a reliable and fast routine method for the generation of biotechnologically interesting cell lines.

ACKNOWLEDGEMENTS

This work was supported by an EC grant, PL 133069. The authors wish to express their thank to Maria Höxter for excellent help in cell cultivation and to T. Beckers (Asta Medica) for providing us with the gene encoding the TkNeo fusion protein.

REFERENCES

1 O'Gorman,S., Fox,D.T. and Wahl,G.M. (1991) *Science,* **251**, 1351–1355.
2 Karreman,C., Karreman,S. and Hauser,H. submitted for publication.
3 Schlake,T. and Bode,J. (1994) *Biochemistry,* **33**, 12746–12751.
4 Dirks, W., Wirth, M. and Hauser, H. (1993) *Gene*, **128**, 247–249.
5 Berger, J., Hauber,J., Hauber,R., Geiger,R. and Cullen,B.R. (1988) *Gene,* **66**, 1–10.
6 Dirks,W., Mielke,C., Karreman,S., Haase,B., Wirth,M., Lindenmaier,W. and Hauser,H. (1994) In Fusenig,F.E. and Graf,H. (eds), *Cell Culture in Pharmaceutical Research.* Springer Verlag, Berlin, Heidelberg, New York, pp. 239–265.
7 Kirchhoff,S., Köster,M., Wirth,M., Schaper,F., Gossen,M., Bujard,H. and Hauser,H. (1995) *Trends Genet.,* **11**, 219–220.
8 Zhao,J.J. and Pick,L. (1993) *Nature* (*London*), **365**, 448–451.
9 Lupton, S.D., Brunton, L.L., Kalberg, V.A. and Overell, R.W. (1991) *Mol. Cell. Biol.,* **11**, 3374–3378.
10 Marsh, J.L., Erfle, M. and Wykes, E.J. (1984) *Gene,* **32**, 481–485.
11 Mann,R., Mulligan, R.C. and Baltimore.D. (1983) *Cell,* **33,** 153–159.
12 Jainchill,J.L., Aaronson,S.A. and Todaro,G.J. (1969) *J. Virol.* **4**, 549–553.
13 Graham, F.L. and van der Eb,A.J. (1973) *Virology,* **52**, 456–467.
14 Thyagarajan,B., Johnson,B.L. and Campbell,C. (1995) *Nucleic Acids Res.*, **23**, 2784–2790.
15 Triglia, T., Peterson, M.G. and Kemp, D.J. (1988) *Nucleic Acids Res.*, **16**, 8186.
16 Silver, J. and Keerikatte, V. (1989) *J. Virol.*, **63**, 1924–1928.
17 Grabenhorst, E., Hoffmann, A., Nimtz, M., Zettlmeisl, G. and Conradt, H. S. (1995) *Eur. J. Biochem*., **232,** 718–725.
18 Racher, A.J., Moreira, J.L., Alves, P.M., Wirth, M., Weidle, U.H., Hauser, H., Carrondo, M.J.T. and Griffiths, J.B. (1994) *Appl. Microbiol. Biotechnol.,* **40**, 851–856.