A mis padres, por su confianza y apoyo

y para Xiomara, por su paciencia

Manipulation of Gene Targeting Frequency in Mammalian Cells

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Declaration

I declare that I have written this thesis based on my own work. The contribution of others has been clearly indicated.

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Abbreviations

A.T.F.: Absolute targeting frequency

AAV: Adeno-associated virus

BSA: Bovine serum albumin

CMV: Cytomegalovirus

DAPI: 4'6-diamindino-2-phenylindole

DSB: Double-strand break

dsDNA, RNA: Double-stranded DNA, RNA

E.T.F.: Effective targeting frequency

EC: Embryonic carcinoma

EDTA: Ethylenediamintetraacetic acid

EGTA: Ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid

ES: Embryonic stem

GFP: Green fluorescent protein

GPT: Guanine phosphoribosyl transferase

HAT: Hypoxanthine-aminopterin-thymidine

HPRT: Hypoxanthine phosphoribosyl transferase

HR: Homologous recombination

HRP: Horseradish peroxidase

ICM: Inner cell mass

NHEJ: Non-homologous end-joining

NHR: Non-homologous recombination

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PHA: Phytohemagglutinin

PRPP: 5-phosphoribosyl-alpha-1-pyrophosphate

RT: Retrotranscriptase

SDS: Sodium dodecyl sulphate

ssDNA, RNA: Single-stranded DNA, RNA

6-TG: 6-thioguanine

TSA: Trichostatin A

Abstract

Gene targeting is a technique for the manipulation of the genome through homologous recombination with exogenous DNA fragments (targeting vectors). In conjunction with embryonic stem (ES) cell technology, this technique has become one of the most powerful tools in molecular biology for the analysis of gene expression and regulation in mice. With the development of nuclear transfer from somatic cells in several species, gene targeting can now be utilised for the design of more accurate animal models for human diseases and the generation of genetically modified livestock. However, its use is limited by the low frequency of homologous recombination in somatic cells. Future applications of gene targeting, such as the development of human gene therapies, will also require dramatic improvements in the efficiency of homologous recombination.

The aim of this work has been to devise strategies for the stimulation of gene targeting efficiency *in vitro*. Using a very sensitive test system based on the directly selectable knockout of the *HPRT* gene in ES cells *in vitro*, a variety of experimental approaches were assessed for their ability to enhance effective targeting frequency – measured as the ratio of homologous to total integrants. These can be grouped into three main subcategories: (1) Modifications of the targeting vector (nuclear localisation signals, dsRNA vectors); (2) Alteration of the target conditions (methylation status, chromatin configuration); and (3) Manipulation of the expression of recombination-related genes (down-regulation of homologous recombination repressors and overexpression of recombinases). Loss of p53, Ku80 or DNA-PK_{cs} function did not result in enhanced targeting efficiency in ES cells. In contrast, constitutive overexpression of the eukaryotic recombinase Rad51 yielded a 4-fold increase in effective targeting frequency compared to wild-type control cells. Significant increases were also observed in *Dnmt1* -/- and poly(ADP-ribosyl)polymerase (PARP) -defective cells, as well as in cells treated with chemical inhibitors of PARP activity. These results contribute to the knowledge of the mechanisms underlying homologous recombination in mammalian cells, and suggest possible avenues of research to overcome the practical limitations of gene targeting.

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i. project overview

Gene targeting is a technique which allows the precise alteration of endogenous genes through homologous recombination with introduced DNA fragments. Following the isolation and characterisation of murine embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981), it has been possible to introduce targeted ES cells into mouse blastocysts, where they can efficiently contribute to the germ line of the resultant chimaeras (Bradley *et al.*, 1984). As a result of the application of this technology, several hundred mutants (Brandon *et al.*, 1995) have been generated in the decade since gene targeting in ES cells was first reported (Thomas *et al.*, 1986). Most of these targeting experiments have been designed to inactivate genes ('knockout'), thus enabling the study of their function and regulation.

The most striking advantage of this strategy is that genes can be manipulated in their natural chromosomal environment, whereas conventional methods for introducing DNA sequences into the germ line (rev. by Jaenish, 1988) do not allow any control over the site of integration or the number of copies introduced. Although the random integration of a transgenic insertion may have mutational consequences, transgenic animals created by pronuclear injection of DNA are usually considered as 'dominant' systems, for this technology only allows the introduction of additional genetic information (Clark *et al.*, 1992). In contrast, gene targeting has made it possible to precisely knockout defined genes, creating animal models for human genetic diseases (rev. by Clark and McWhir, 1996); to analyse the function of housekeeping and developmental genes (rev. by Melton, 1994; Shastry, 1998); to study gene regulation by targeting changes to control sequences (rev. by Porter, 1998); and to ensure both the repeatability and pattern of transgene expression by directing transgenes to chosen sites in the genome (Stacey *et al.*, 1994; Kolb *et al.*, 1999; Wallace *et al.*, 2000).

An even greater flexibility in gene modification has been achieved in recent years through conditional/ inducible gene silencing techniques (page 36). These approaches have been devised to overcome the problems associated with the study of genes that are

essential for cell viability, such as those involved in cell cycle or body development. Constitutive inactivation of such genes by conventional gene targeting may be entirely uninformative, since targeted cells are not always capable of generating viable animals. Thus, the development of conditional gene silencing methods allowing the precise control of the expression of these genes has opened up key areas of cell and animal biology to genetic analysis (rev. by Porter, 1998).

Basic research on gene function and regulation is not the only area in which gene targeting can be useful. The ability to correct mutant genes offers enormous possibilities in medical research as an alternative to current methods of gene therapy, which so far are exclusively based on the addition of genetic material. While gene targeting has been achieved in several human cell lines, its low efficiency has been a major limitation to its therapeutical potential. Gene therapy by *in vivo* gene targeting is therefore impractical without a dramatic enhancement in its overall efficiency. *Ex vivo* approaches might be more realistically considered, though improvements in gene targeting efficiency would also represent an advantage.

Application of this technology in livestock species to increase output or produce therapeutic proteins (Clark *et al.*, 1992; Wilmut and Whitelaw, 1994; Houdebine, 1994, 1995) would undoubtedly be of commercial value. Until very recently, the use of gene targeting to create modified animals has been limited to the mouse, since this is the only species from which ES-like cells have shown the ability to contribute to the germ line. However, combined progress in enhancing targeting efficiency in somatic cells and the new nuclear transfer technologies (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Schnieke *et al.*, 1997; Cibelli *et al.*,1998a, 1998b; Solter, 1998; Wakayama *et al.*, 1998; Polejaeva *et al.*, 2000; Polejaeva and Campbell, 2000; Revel, 2000) have already led to the generation of targeted sheep (McCreath *et al.*, 2000) and should allow targeting in other species in the short term.

Practical applications of gene targeting are usually hindered by its low efficiency, which can be measured as **absolute, effective** and **enriched frequencies. Absolute targeting frequency (A.T.F.)** is the ratio between the number of homologous (targeted) recombinants and the total number of cells exposed to the transfection

procedure. In *ex vivo* systems, or where cell number is limiting *in vitro*, A.T.F. is critical. It depends on transfection efficiency and **effective targeting frequency (E.T.F.)**, which can be defined as the ratio of homologous recombinants to the total number of integrants (homologous and non-homologous). Finally, **enriched frequency** is expressed as the ratio of homologous to total integrants observed after enrichment procedures, which reduce the background of random integrants *in vitro*. Several factors may affect effective frequency and account for an extensively documented locus-to-locus variability. Non-homologous recombination has been consistently observed to be 2 to 5 orders of magnitude more frequent than homologous recombination (Hanson and Sedivy, 1995). Although enrichment systems have been designed in order to eliminate clones derived from non-homologous recombination events (page 29), there has been a relative lack of success in significantly enhancing the absolute incidence of homologous recombination.

As the majority of targeting experiments have been done in mouse ES cells, the conventional wisdom is that ES cells are more proficient at gene targeting than other cell lines. However, few studies have compared gene targeting frequencies in ES cells with those in other mouse cell lineages using the same targeting constructs. Some of such studies (Charron *et al.*, 1990; Coll *et al.*, 1995; Arbonés *et al.*, 1994) demonstrate that several murine cell lines give an absolute targeting frequency comparable to that observed in ES cells. More interestingly, human cell lines can also undergo targeted modifications with enriched gene targeting frequencies between 1:40 and 1:6550, and absolute targeting frequencies (1.2×10^{-8} - 2.7×10^{-5}) similar to those described for murine cells (Yáñez and Porter, 1998). Therefore, it appears that gene targeting can be performed with similar efficiencies in a variety of cell types from different species. Nevertheless, it is clear that future applications of gene targeting, such as the design of more accurate models for human diseases, the generation of targeted livestock or the efficient application of gene therapies *in vivo*, would greatly benefit from an improvement of effective targeting frequency in somatic cell lines derived from species other than the mouse.

The aim of this Ph.D. project is to explore experimental approaches to manipulate effective targeting frequency in ES and somatic cells. A thorough

understanding of the molecular mechanisms underlying gene targeting is essential to this purpose. This introduction reviews the current knowledge about molecular homologous recombination and gene targeting techniques. ES cell-based technologies are also briefly outlined in a two-fold context: the classic targeting approaches for the production of mice with defined genetic modifications and the *in vitro* study of homologous recombination. Finally, the most relevant strategies to enhance overall gene targeting efficiency are discussed, bearing in mind the benefits of such enhancement in the development of efficient gene therapy and other somatic targeting protocols. The general objectives of this project are examined within this framework.

ii. homologous recombination

a.introduction
b.models of homologous recombination
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d.homologous recombination in eukaryotes

a.introduction

Homologous recombination is one of the two known repair pathways for double-strand breaks (DSBs) in eukaryotes. It involves interaction of the damaged molecule with a homologous recombinant partner elsewhere in the genome (e.g., a homologous chromosome or a sister chromatid). Gene targeting occurs when artificially introduced DNA vectors are integrated in the genome via the endogenous homologous recombination pathway.

In contrast with yeast, where homologous recombination is the primary mechanism of DSB repair, mammalian cells favour an alternative route termed illegitimate recombination or non-homologous end-joining (NHEJ). This pathway joins the two ends of a DSB without regard to sequence homology. Although this process is more prone to error than homologous recombination, it might have evolved to prevent hyperrecombination in the mammalian genome, rich in DNA repeats.

Gene targeting is inefficient in mammalian cells because targeting vectors are preferentially integrated in a non-homologous fashion. Understanding the interrelationship between homologous and non-homologous recombination is essential to experimentally manipulate it in a directed manner. This and the next chapter will review the molecular basis of these two processes, emphasising those aspects that may be relevant to the design of effective targeting strategies in higher organisms.

b. models of homologous recombination

In eukaryotic organisms, homologous recombination takes place during meiosis, mitotic cell growth and DNA repair. Although overlapping, each one of these processes may have different biochemical routes. Even in *E. coli*, there are three recombination pathways, which may be related to diverse types of recombination in eukaryotes (see next section).

The current models of homologous recombination have been developed from observations made in yeast and other fungi. All of them feature a sequence of reactions that can be divided into three phases: initiation, repair and resolution (Leach, 1996). **Initiation** involves alignment of homologous sequences, generation of cross-overs and branch migration. **Repair** happens after any change or loss of information occurred prior to or during initiation, and involves mismatch repair or DNA synthesis. Finally, **resolution** is the excision of the cross-overs and separation of the two recombinant molecules.

In the **Holliday** model (Holliday, 1964), the phase of initiation starts when two aligned homologous chromosomes simultaneously acquire a single-strand nick (figure 1). Generation of cross-overs (Holliday junctions) are followed by branch migration and resolution in either of the two senses observed in nature: Cross-overs (splices) or patches (recombinant regions that have not exchanged flanking markers). A modification of Holliday's model (Meselson and Radding, 1975) suggests that only one of the two molecules initiates recombination. The initiator is nicked in one of the strands, thus generating a 3' end for DNA synthesis. This reaction displaces the 5' end, which then invades a homologue. This results in the formation of a Holliday junction that is resolved as in Holliday's model, to form splices or patches.

The model of **Szostak** *et al.* (1983) was proposed to account for the observation that in many organisms, DNA double-strand breaks (DSBs) enhance recombination locally. Earlier studies on plasmid-chromosome recombination in yeast (Orr-Weaver *et*

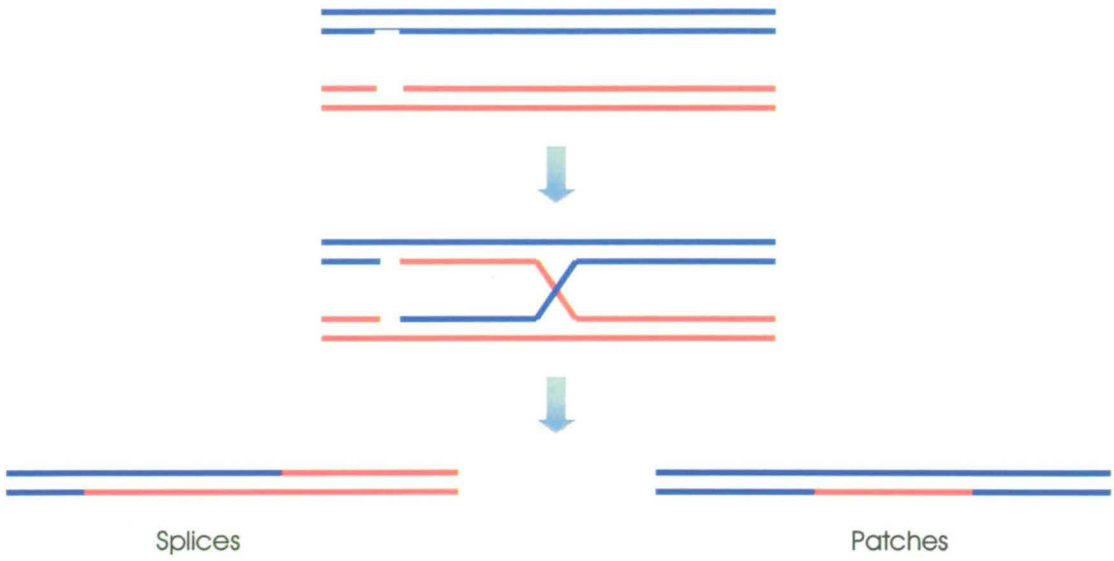

Figure 1. Holliday model of homologous recombination (details in text).

al., 1981, 1983) established the recombinogenic nature of DSBs and demonstrated the occurrence of gene conversion by double-strand-gap repair.

According to this model, homologous recombination is initiated from a DSB in the 'receptor' molecule. 5'-3' exonucleases selectively digest one of the strands at both ends of the cut, generating a gap with 3' overhangs (see figure 2). One of these invades a homologue, creating a heteroduplex and displacing the complementary strand of the 'donor' molecule. This generates a loop, which is subsequently expanded through DNA synthesis from the 3' end of the invading strand. Eventually, the loop will be large enough to base-pair with the 3' end of the receptor, which then could prime new DNA synthesis to reconstitute the missing strand from the donor template. Migration of the invading 3' end creates a molecule with two cross-overs, that can be resolved in either way (splices or patches).

It now seems likely that, although admitting several variations, both models (initiation by single-strand or by double-strand breaks) are valid, and are associated with different pathways. Indeed, some mutants of *Drosophila* (Carpenter, 1982) and yeast (Engebrecht *et al.*, 1990) show normal levels of meiotic gene conversion (the correction

of one strand of a heteroduplex DNA to make it complementary with the other at mismatch positions) but reduced levels of crossing over, suggesting that not all gene conversion events are resolved as cross-overs. It is possible that homologous recombination mechanisms involve several overlapping pathways that cannot be explained by a single model. In any case, the details of the enzymology and the genes encoding recombination enzymes are better known in *E. coli* than in yeast and mammals. As in so many other cellular processes, a thorough knowledge of the molecular basis of homologous recombination in prokaryotes may help us to better understand the equivalent pathways in higher organisms.

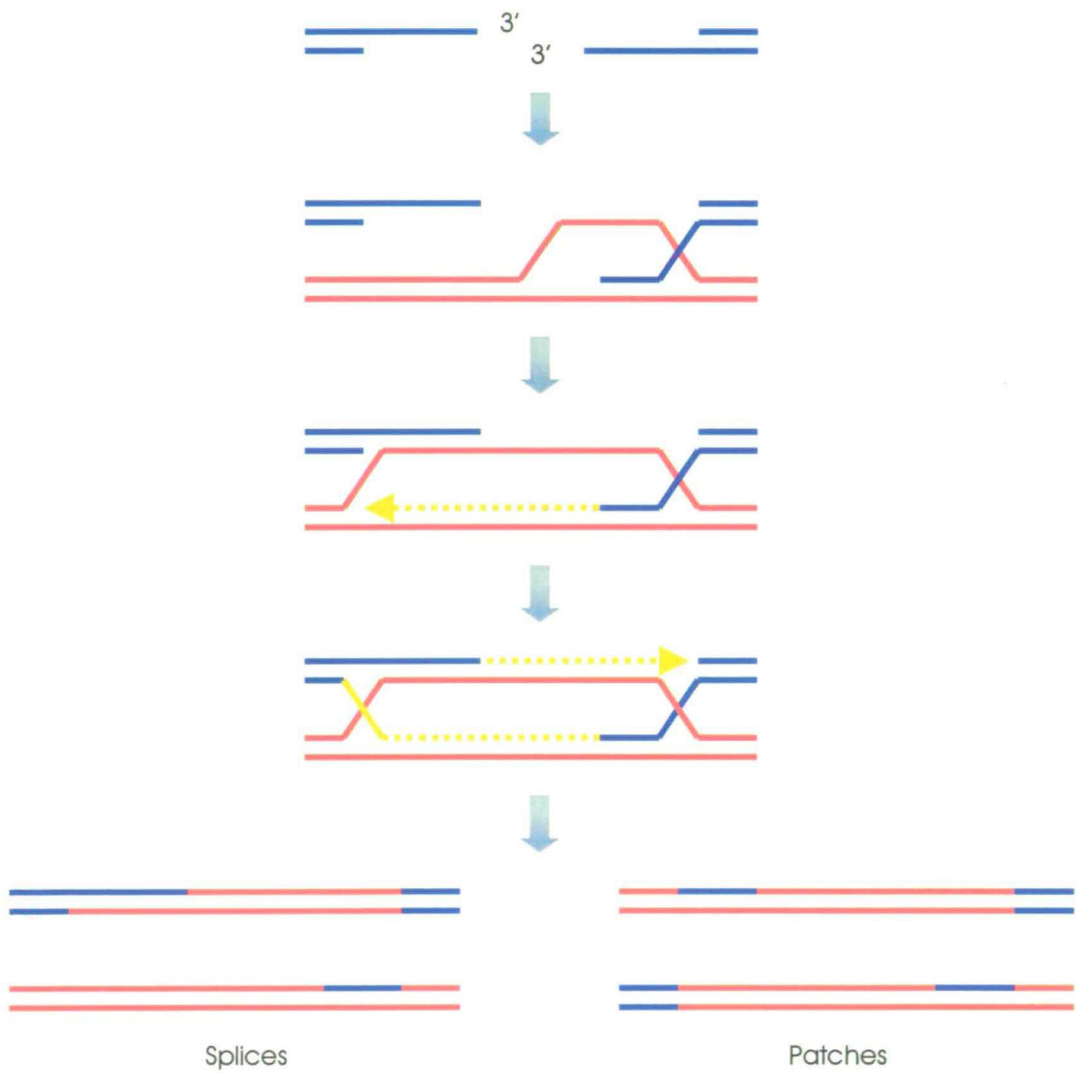

Figure 2. Szostak model of homologous recombination (details in text)

c.homologous recombination in *E. coli*

The **RecA** protein is required for the three pathways of recombination that have been demonstrated in *E. coli* (RecBCD, RecE and RecF) (Smith, 1988; 1989). This enzyme coats single-stranded DNA in large clusters, promotes formation of joint DNA molecules with a homologous duplex and aids strand-exchange within the joint molecule (Radding, 1989; Eggleston and Kowalczykowski, 1991; Stasiak *et al.*, 1991). ATP enhances both protein-protein interactions and DNA binding (Radding, 1989), and its hydrolysis is an absolute requirement for the strand-exchange activity of the enzyme.

The RecBCD pathway is the principal mechanism of recombination following conjugation and transduction. It has been named after the **RecBCD** enzyme, a heteromultimer with subunits encoded by the *recB*, *C* and *D* genes (Finch *et al.*,1986a; 1986b; 1986c). Originally designated ExoV, the enzyme has two apparently contradictory functions. First, it is a powerful double-strand exonuclease, which binds to linear dsDNA ends and degrades the molecule to small oligonucleotides in an ATP-dependent manner (Rosamond *et al.*, 1979; Telander-Muskavitch and Linn, 1981). This degradation is asymmetric, since the 3'-terminal strand at the entry site for the enzyme is degraded much more extensively than the 5'-terminal strand (Dixon and Kowalczykowski, 1993). Second, it is a helicase-recombinase that moves rapidly along the DNA, unwinding it and producing single-stranded loops (Taylor and Smith, 1980; 1985). This unwinding is also asymmetric and generates loop-tail structures in the presence of single-stranded DNA-binding proteins (SSBs) (Braedt and Smith, 1989).

RecBCD plays a central role in homologous recombination, recovery from DNA damage, maintenance of cell viability and destruction of damaged DNA (Telander-Muskavitch and Linn, 1981). *In vitro* and *in vivo* studies have indicated that RecBCD-mediated recombination acts preferentially in the presence of (chi) sites (Stahl *et al.*, 1975; Smith *et al.*, 1981). The chi site (5'GCTGGTGG) stimulates recombination up to two orders of magnitude in its vicinity (Dabert and Smith, 1997; Friedman-Ohana *et al.*, 1998), and this stimulation is detectable up to 10 kb downstream of the chi site (Stahl *et al.*, 1980; Ennis *et al.*, 1987; Cheng and Smith, 1989; Myers *et al.*, 1995b; Eggleston and

West, 1997). RecBCD-mediated cutting of the DNA molecule occurs with a high frequency at chi sites (Ponticelli *et al.*, 1985; Taylor *et al.*, 1985), and it has been proposed that the combined chi- dependent DNA cutting and unwinding activities generate a ssDNA tail that is efficiently paired with homologous dsDNA by RecA and SSB proteins (Smith *et al.*, 1984; Roman *et al.*, 1991; Rosenberg and Hastings, 1991; Dixon and Kowalczykowski, 1991, 1993; Taylor and Smith, 1992; Anderson and Kowalcykowski, 1997).

A model incorporating most of the known biochemical and genetic properties of the components participating in the RecBCD recombination pathway has been recently described by Kowalczykowski (Anderson and Kowalczykowski, 1997). According to this model, the enzyme begins unwinding dsDNA from a double strand break, generating a loop. Prior to its interaction with a chi site, RecBCD also displays a high asymmetric nuclease activity, resulting in extensive degradation of the 3' terminal strand (see figure 3). The 5' terminal strand remains relatively intact, and can be preferentially bound by SSB proteins. Upon encountering a chi site, RecBCD pauses and cleaves the DNA molecule. This cleavage has been proposed to be a manifestation of the non-specific nuclease activity of RecBCD (Dixon and Kowalczykowski, 1993), as long as any pause results in an increased probability of a nucleolytic event at the site of pausing. Interaction with chi changes the conformation of the enzyme and reverses the polarity of the nuclease activity. Thus, when unwinding resumes, the 3' terminal strand is released intact, while the 5' terminal strand is degraded (even if at a lower rate). It has been proposed that this change of conformation follows the ejection of the recD subunit, apparently responsible for the 3'-5' exonuclease activity of the enzyme (Myers *et al.*, 1995a). Although recent studies suggest that the alteration induced by chi is not simply the loss of the RecD subunit (Anderson *et al.*, 1997), but a subtle displacement to the other strand (Yu *et al.*, 1998), the fact is that RecBC mutant enzymes (i.e., in which the recD subunit is lost or not operative) are recombination-proficient and behave as constitutively chi-activated RecBCD proteins even in the absence of such recognition sequences (Myers *et al.*, 1995a). This indicates that the 5'-3' exonuclease activity of RecD after chi-recognition is not as essential to recombination as its loss of 3'-5' exonuclease activity.

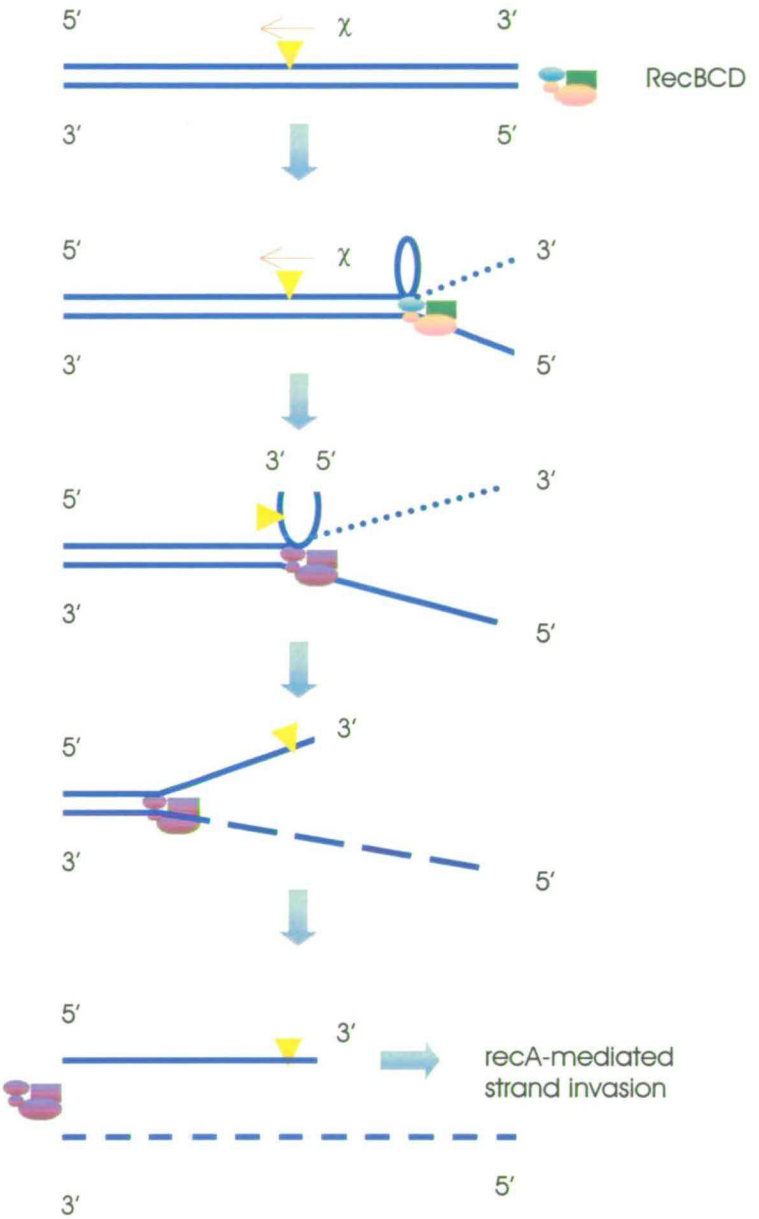

Figure 3. Model of Chi-mediated recombination in *E. coli* (based on Anderson and Kowalczykowski, 1997) Details in text.

The generation of a 3' terminal single strand upon activation of RecBCD by chi is consistent with the models proposed by Holliday and Szostak to explain homologous recombination in eukaryotes. This biochemical step is likely to represent the initial *in vivo* event needed by RecA to promote strand invasion. A recent study suggests that chi-activated RecBCD acts as a nucleator to coordinate the preferential loading of RecA

onto this resultant single-stranded DNA molecule (Anderson and Kowalczykowski, 1997). This nucleation begins at the 5' end of the loop created by the enzyme, displacing the resident SSB protein. Coated 3'-overhanging ssDNA molecules are subsequently able to invade a homologous DNA substrate, forming a joint molecule that is further processed into recombinant products.

In the absence of RecBCD enzyme, there is a residual level of recombination through the **RecE** and **RecF** pathways (Smith, 1989). RecE, also called Exonuclease VIII, digests linear dsDNA in the 5'-3' direction, generating dsDNA with 3'- ssDNA tails. The RecF pathway (which also involves the products of several additional genes such as *recJ, recN, recO, recQ* and *ruv*) also promotes plasmid recombination in wild-type cells. Both routes overlap in the requirement of RecA, RecF, RecJ, RecO and RecQ gene products, and seem to be activated following mutations in the major pathway for conjugational recombination, RecBCD. None of them requires chi sites.

Parallels have been drawn between the *E. coli* pathways and different types of recombination in eukaryotes. The RecF pathway in *E. coli*, mitotic recombination in eukaryotes and recombinational repair in all cell types seem to be initiated by single-strand DNA breaks and respond to DNA damage. In contrast, the RecBCD pathway and meiotic recombination are postulated to start from double-strand breaks and are not inducible by DNA damage (Smith, 1989). Indeed, chi-like hotspots of homologous recombination have been found in eukaryotic cells, sometimes widespread throughout the genome (Rüdiger *et al.*, 1995). This suggests that higher organisms may have recombination routes functionally similar to the RecBCD pathway.

d.eukaryotic homologous recombination

The majority of the studies about eukaryotic homologous recombination and DNA repair have been done in yeast. In *S. cerevisiae*, these processes seem to be mediated by the genes of the *Rad52* epistasis group, including *Rad50-57*, *MRE11* and *XRS2*. Although mutants of these genes exhibit similarity in general phenotypes, they display different responses to ionising radiation and particular deficiencies in defined steps of recombination. Among these mutants, *Rad51*, *Rad52* and *Rad54* have the most severe effects, which suggests that such genes play fundamental roles in recombination and DNA repair. **Rad51** shares both sequence and functional homology with *E.coli* recA (Aboussekhra *et al.*, 1992; Shinohara *et al.*, 1992). Like its bacterial counterpart, human Rad51 shows in vitro ATP-dependent filament formation and strand exchange activity on DNA substrates (Ogawa *et al.*, 1993; Sung, 1994; Baumann *et al.*, 1996; Gupta *et al.*, 1997; Namsaraev *et al.*, 1997; Benson *et al.*, 1998). Both in yeast and humans, this reaction is enhanced by the addition of Rad52, Rad55, Rad57 and recombination protein A (RP-A) (New *et al.*, 1998; Baumann and West, 1997; Shinohara and Ogawa, 1998; Benson *et al.*, 1998). A physical association has been demonstrated between Rad51 and the recA/Rad51 family members XRCC2 and XRCC3 (Thacker, 1999) and the repressor ubiquitin-like protein UBL-1 (Li *et al.*, 2000). In addition, Rad51 interacts with the products of the tumour suppressor genes p53 (Stürzbecher *et al.*, 1996; Buchhop *et al.*, 1997), BRCA1 (Scully *et al.*, 1997) and BRCA2 (Mizuta *et al.*, 1997).

In contrast with recA, the preferred DNA substrate for Rad51 is not ssDNA but dsDNA with ssDNA tails. This allows Rad51 to promote DNA strand invasion of both 3'- and 5'- ends with similar efficiencies (Mazin *et al.*, 2000). *In vivo*, vertebrate Rad51 is essential for DSB repair occuring during mitotic and meiotic recombination, or following treatment with DNA-damaging agents (Shinohara *et al.*, 1992). Like recA, Rad51 expression is up-regulated after genotoxic treatment (Abboussekhra *et al.*, 1992). Cell viability is also strongly dependent on Rad51, as shown in experiments where repression of an inducible Rad51 transgene in *Rad51* -/- chicken DT40 cells leads to accumulation of chromosomal abnormalities and cell death (Sonoda *et al.*, 1998). In addition, Rad51 is critical for mouse embryonic development and cell proliferation (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996), which clearly indicates a gain of function of the

mammalian protein compared to its yeast and bacterial homologues. Surprisingly, mutant Rad51 protein incapable of ATP hydrolysis still permits *in vivo* repair of radiation-induced DNA damage, as well as cell viability and growth (Baumann and West, 1998; Morrison *et al.*, 1999). This observation is consistent with *in vitro* studies showing that rates of ATP hydrolysis, homologous pairing and strand exchange are less than 1/10 those of recA (Gupta *et al.*, 1997). With the exception of meiosis, homologous recombination is usually tightly down-regulated in vertebrates in order to prevent uncontrolled recombination between the highly abundant genomic DNA repeats. In this context, it has been proposed that the reported decrease in Rad51 general activity compared to that of recA may reflect the avoidance of homologous recombination as preferred DNA-repair mechanism in higher organisms, in favour of non-homologous end-joining.

Rad52 is proficient in promoting heteroduplex formation between homologous ssDNA molecules and strand transfer between homologous ss- and dsDNA substrates (Mortensen *et al.*, 1996; Reddy *et al.*, 1997). Several studies conducted in yeast show that Rad52 may not be required for the initiation of recombination, but is essential for an intermediate stage following the generation of DSBs (Shinohara *et al.*, 1992). More recent evidence demonstrating physical association between Rad52 and Rad51 indicates that one of the functions of the former may be the modulation of recombinatorial/ DSBs repair processes by directly interacting with other factors (Shinohara *et al.*, 1992; Milne and Weaver, 1993) and/or stimulating Rad51 binding to ssDNA (Benson *et al.*, 1998; Baumann and West, 1998). The role of Rad52 in mammalian cells still remains unclear. Homologous recombination, but not DNA repair, is reduced in Rad52-deficient chicken DT40 (Yamaguchi-Iwai *et al.*, 1998) and murine ES cells (Rijkers *et al.*, 1998). In fact, Rad52 knockout mice are viable and fertile (Rijkers *et al.*, 1998). This result contrasts with early work in yeast, where Rad52-defective mutants display high sensitivity to ionising radiation (Orr-Weaver *et al.*, 1981), suggesting that there may have been an evolutionary loss of function of the mammalian protein in favour of other recombinases.

The **Rad54** protein belongs to the SNF2/ SWI2 family of DNA-dependent ATPases (Eisen *et al.*, 1995). Its members have been implicated in the remodelling of chromatin structure in the context of many aspects of DNA metabolism, such as transcription, recombination and repair (Kingston *et* al., 1996). As observed between Rad52 and Rad51, genetic and physical interactions between the latter and Rad54 have been reported (Jiang *et* al., 1996). *In vitro*, murine Rad54-knockout ES cells are hypersensitive to DSB-inducing agents, and exhibit reduced gene targeting frequencies compared with wild-type cells (Essers *et al.*, 1997). Unlike Rad51, however, Rad54 is not necessary for embryonic or neonatal development in the mouse, as shown by the viability of Rad54-deficient mice (Essers *et al.*, 1997). The fact that these mice are fertile seems to demonstrate that Rad54 has no essential function in meiosis, although more subtle meiotic defects have yet to be analysed in more detail.

iii. non-homologous recombination

a.introduction
b.non-homologous recombination in eukaryotes

a.introduction

The hypothesis that non-homologous (NHR) and homologous recombination (HR) are catalysed through distinct routes has been confirmed by several studies in yeast and mammalian cells (Hooper, 1992). For instance, HR frequency peaks in early to mid-S phase (Wong and Capecchi, 1987), whereas illegitimate recombination peaks in G_2/M phase (Yorifuji *et al.*, 1989). Early gene targeting experiments also indicate that both pathways operate separately and in response to specific circumstances. Thus, addition of dideoxynucleotides at the 3' end of the input DNA decreases the proportion of NHR to HR by 6-fold (Chang and Wilson, 1987). Furthermore, analysis of cells co-electroporated with a targeting construct and a non-homologous positive selectable marker (*neo*) showed that 75% of the cells which non-homologously integrated the targeting vector also integrated *neo*, but only 4% of the homologous integrants did so (Reid *et al.*, 1991). This result strongly points to the existence of discrete enzymatic pathways for homologous and illegitimate recombination. The lack of a prokaryotic NHR model, due to the fact that this process is very uncommon in bacteria, has somewhat delayed its study in eukaryotes. Although our understanding of NHR (also called non-homologous end-joining, or NHEJ) has benefited from a number of advances in recent years, many aspects (such as the details of its regulation and relation with HR) still remain obscure and need further investigation. The next section reviews the current knowledge about this process in yeast and mammals.

b. non-homologous recombination (NHR) in eukaryotes

DNA double-strand breaks (DSBs) result from a variety of DNA-damaging agents, both exogenous (ionising radiation or chemotherapeutic drugs) and endogenous, such as the metabolic release of free radicals. They are also produced as normal intermediates in V(D)J recombination, the process by which immunoglobulin genes are rearranged to produce the repertoire of antibodies and T-cell receptors during development (rev. by Roth and DeFranco, 1995; Jeggo *et al.*, 1995). DSBs are repaired either by homologous recombination (HR) or by non-homologous end-joining (NHEJ). This pathway, unlike HR, requires no homology with another strand of DNA, and very little homology, if any, between the two ends of a DSB. The principal components of this pathway are encoded by the genes *XRCC4* (XRCC4 protein), *XRCC5* (Ku80), *XRCC6* (Ku70) and *XRCC7* (DNA-PK_{cs}). XRCC4 protein associates with DNA ligase IV in mammals, and may stimulate its activity (Grawunder *et al.*, 1994). Mutations of *XRCC4* or ligase IV result in embryonic lethality in mice and may be the cause of predisposition to cancer and extreme radiosensitivity in humans (Featherstone and Jackson, 1999a). Ku70/80 heterodimers are known to bind to the ends of broken DNA (Mimori and Hardin, 1986) and recruit other proteins to the site of the break, amongst them the catalytic subunit of the DNA-protein kinase (DNA-PK_{cs}). This large protein (~465 kDa) becomes activated in the presence of Ku and DSBs. It phosphorylates other proteins around the lesion and seems to be involved in signal transduction (Weaver, 1995). DNA-PK_{cs} may also act as a molecular scaffold both to stabilise the break and to assemble the rest of the proteins of the repair complex (Featherstone and Jackson, 1999b). Mutations of Ku and/or DNA-PK_{cs} result in radiosensitivity and severe immunodeficiency (SCID) in mice, due to general NHEJ impairment and, particularly, V(D)J recombination defects. In addition, Ku knockout mice are much smaller than their heterozygous littermates (Nussenzweig *et al.*, 1996), and cultured cells show premature senescence and loss of proliferation (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). Two explanations have been proposed to account for these observations. First, that a deficient DSB repair mechanism diminishes the chances of the cell to resume progress through the cell cycle after DNA damage (Featherstone and Jackson, 1999b).

The small size of Ku-knockout mice would therefore be a direct consequence of a slower cell proliferation during development. A second possibility is that telomeres become unstable upon loss of Ku function: work in yeast (Gravel *et al.*, 1998; Laroche *et al.*, 1998; Polotnianka *et al.*, 1998; Martin *et al.*, 1999) and mammalian cells (Hsu *et al.*, 1999) demonstrate telomere instability associated with the absence of Ku, although the molecular mechanism remains unclear.

Double strand breaks can be repaired either by homologous recombination or NHEJ. Both pathways operate in all eukaryotic cells, but whereas yeast normally choose the former, mammals prefer the latter. It has been suggested that the abundance of repetitive sequences in the mammalian genome would increase the incidence of recombination-related chromosomal abnormalities if HR events were frequent (Featherstone and Jackson, 1999a). Therefore, NHEJ may be considered the 'default' mechanism by which DNA damage is repaired in higher organisms.

In this context, the activity of poly(ADP-ribose) polymerase (PARP) seems to be essential for the choice of the NHEJ pathway in response to DNA damage. PARP is a highly conserved nuclear enzyme thought to be involved in DNA repair and other cellular responses to DNA damage, including apoptosis and necrosis (reviewed by Boulikas, 1993; de Murcia *et al*, 1994; Lindahl *et al.*, 1995). The PARP protein has two distinct regions: The 46 kDa N-terminal domain contains two zinc fingers and a nuclear localisation signal. Footprinting experiments show that it binds preferentially to DNA double-strand breaks, stabilising a V-shaped DNA conformation (Gradwohl *et al.*, 1990). The C-terminal catalytic domain (54 kDa) is strongly activated upon binding of the protein to DNA breaks. Using NAD and ATP as substrates, it catalyses the poly(ADP-ribosyl)ation of several nuclear proteins, including histones, topoisomerases, replication factors and PARP itself (Ueda and Hayaishi, 1985; Oliver, 1998). This reaction is sensitive to a variety of competitive inhibitors, such as theophylline (Schraufstatter *et al.*, 1986) benzamide- (Ueda and Hayaishi, 1985) and isoquinoline-derivatives (Banasik *et al.*, 1992).

Automodification of PARP leads to the generation of very long poly (ADP-ribose) (PAR) chains, which are negatively charged. Eventually, the electrostatic

repulsion between the DNA strands and the activated protein releases it from the DSB (Ferro and Olivera, 1982). Poly(ADP-ribose) glycohydrolase rapidly degrades PAR chains, enabling PARP to bind to DNA breaks again (Lindahl *et al.*, 1995).

It is generally believed that one of PARP's functions might be the preservation of genomic integrity by (a) favouring DNA repair, and (b) minimising unwanted recombination events at sites of DNA breaks (Lindahl *et al.*, 1995). Although PARP -/- mice are viable, they show hypersensitivity to genotoxic agents, elevated rates of sister chromatid exchanges (SCE) and general genomic instability (de Murcia *et al.*, 1997; Wang *et al.*, 1997). PARP inhibition decreases the frequency of illegitimate recombination *in vitro* (Farzaneh *et al.*, 1988; Waldman and Waldman, 1990; Semionov *et al.*, 1999) and increases intrachromosomal homologous recombination (Waldman and Waldman, 1991). It has been proposed that DNA injury activates PARP, which catalyses the transfer of PAR to the enzyme itself and subsequently to other nuclear proteins, such as histones (Ueda and Hayaishi, 1985; Carson *et al.*, 1986). Although PARP does not appear to have a direct participation in DNA-repair, extreme poly(ADP-ribosyl)ation of histones may alter their charge and structural conformation, relaxing the nucleosome and allowing the access of other enzymes to the lesion. The negative charge around the DSB may also keep other DNA molecules away, preventing homologous recombination (Smulson *et al*, 1994; Chatterjee and Berger, 1994). However, extrachromosomal homologous recombination is also enhanced after PARP inhibition (Semionov *et al.*, 1999). This and the fact that PARP is present in dinoflagellates and other primitive eukaryotes that do not have histones (Werner, 1984) demonstrate that PARP is able to function in a chromatin-free environment.

It is unclear how PARP may help maintain genomic stability, but poly(ADP-ribosyl)ation seems to be essential for its function. Overexpression of the DNA-binding domain results in inhibition of endogenous PARP and recreation of the PARP -/- phenotype (Schreiber *et al.*, 1995). Enzymes involved in non-homologous end-joining, such as Ku70/80 and DNA-PK, are known to interact and compete with PARP for binding to DNA ends (Morrison *et al.*, 1997). Perhaps the primary function of PARP is to prevent homologous recombination after a chromosomal break. Due to the abundance of DNA repeats in the mammalian genome, the use of homologous

recombination to repair DNA breaks must be kept to a minimum in order to avoid undesirable genomic rearrangements. PARP would help this by creating a negatively charged 'exclusion zone' around the lesion, hindering the access of other recombinogenic DNA molecules. The eventual separation of PARP from the break (forced by its increasingly higher negative charge) may create a transient window in which Ku70/80, DNA-PK and other NHEJ enzymes can attach to the break and repair it.

The role of PARP in homologous recombination has been studied in depth only in recent years. Traditionally, PARP has been the object of scientific interest due to its involvement in apoptosis. Caspase-3-mediated cleavage of PARP in two 89- and 24 kDa fragments inactivates its catalytic activity, but does not affect its DNA-binding properties. This is generally considered one of the initial events in the activation of the apoptotic cascade after extensive DNA damage (Kaufmann *et al.*, 1993; Shah *et al.*, 1995; Oliver *et al.*, 1998; Boulares *et al.*, 1999; Simbulan-Rosenthal *et al.*, 1999). However, despite reports suggesting that PARP-mediated depletion of NAD and ATP compromises the energy-dependent process of apoptosis (Ha and Snyder, 1999), there is no conclusive proof that this cleavage is essential for its completion (Oliver *et al.*, 1998; Herceg and Wang, 1999; Jones *et al*, 1999). The role of PARP in apoptosis remains a matter of controversy, but increasing evidence (Latour *et al.*, 2000; Li and Darzynkiewicz, 2000) suggests that PARP cleavage may be a downstream effect of apoptosis rather than a part of it. Alternatively, it has been proposed that the irreversible binding of cleaved PARP to DSB may indirectly contribute to apoptosis by blocking the access of DNA repair enzymes (Smulson *et al.*, 1998). This would explain why expression of the 46 kDa DNA-binding domain of PARP (which leads to dominant inhibition of resident PARP) increases the apoptotic response after DNA damage in human cells (Schreiber *et al.*, 1995; Kim *et al.*, 2000), but not why uncleavable PARP seems to enhance apoptosis (Boulares *et al.*, 1999; Herzeg and Wang, 1999). Explanations for the latter observation are counter-intuitive, since PARP-cleavage is a naturally occurring event during apoptosis. Further work with engineered- (Oliver *et al.*, 1998) and naturally occurring- (Latour *et al.*, 2000) uncleavable PARP show no effect on (or even retardation of) apoptosis. In this context, the frequent contradictions reported in the apoptotic response to manipulations of PARP activity, expression or resistance to

cleavage could be interpreted as a consequence of the variability among the different biological systems studied.

A model reasonably consistent with most of the observations (figure 4) predicts that PARP's primary function is to ensure that DSBs are repaired by NHEJ and not by homologous recombination. If the damage is more severe (but not as much as to trigger apoptosis), PARP overstimulation may deplete the cell of NAD and ATP, leading to necrosis. Only extensive DNA damage activates the apoptotic programme, and PARP is cleaved in order to stop unnecesary DNA repair and save energy for the completion of cell 'suicide'.

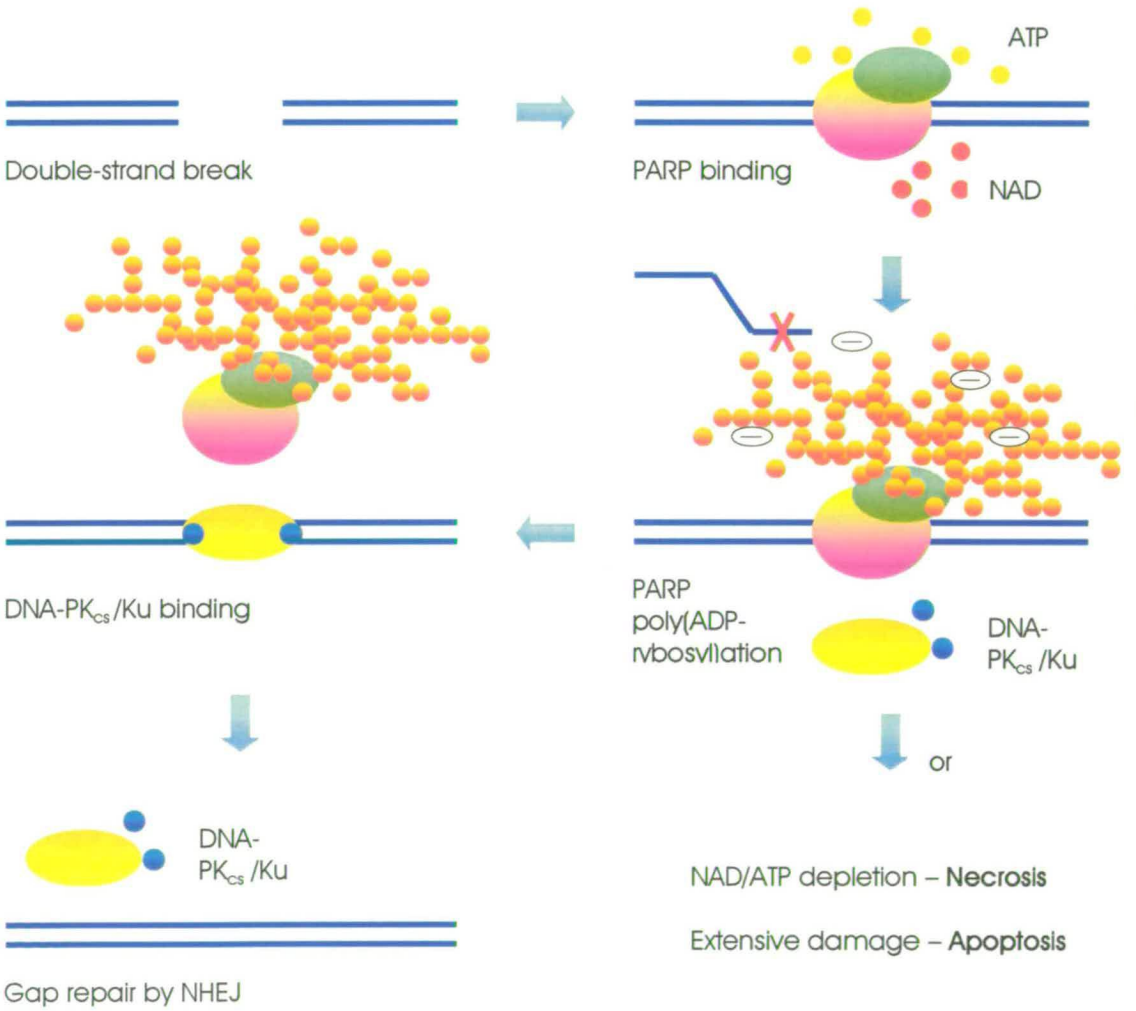

Figure 4. Model for PARP cycling and DNA repair by non-homologous recombination (details in text)

iv. gene targeting

a.introduction
b.embryonic-stem cell technology
c.gene targeting techniques

a.introduction

Over the last fifteen years, the application of gene targeting techniques to embryonic stem (ES) cells has become a routine procedure to generate genetically modified mice. The availability of large populations of these immortal cells makes it feasible to target specific genes, despite the low frequency of homologous recombination in mammalian cells. Targeted clones can be easily selected *in vitro* and used to generate chimaeric mice by aggregation or injection into blastocysts. If the host blastocyst and the donor ES cells belong to different strains of mice, chimaerism can be visually assessed by the mixed coat colour of the resulting animals. Typically, up to 70 % of injected blastocysts are overtly chimaeric (Jim McWhir, personal communication). The degree of chimaerism varies widely from barely detectable to complete ES coloration. Since ES cells retain the potential to contribute to all embryonic lineages, some of them may partially colonise the germ line. Because of the fine-grained nature of ES cell chimaerism, the germ line is usually a mixed population of donor- and host-derived cells. In order to obtain the highest possible number of ES-derived gametes, ES cells for blastocyst injection are of male genotype. The introduction of male ES cells into female host blastocysts normally results in the generation of fertile intersex animals, which only transmit the ES genotype (Evans *et al.*, 1985). This is a consequence of the expression of the Y chromosome-linked *sry* gene, which controls mammalian sex determination (rev. by Jiménez and Burgos, 1998). In these cases, backcrossing chimaeras with the strain from which the ES cells were originally derived renders animals with the original genetic background, with an ideal 50 % of the offspring carrying the modified allele (figure 5). A 25 % germ line transmission (1 out of the first

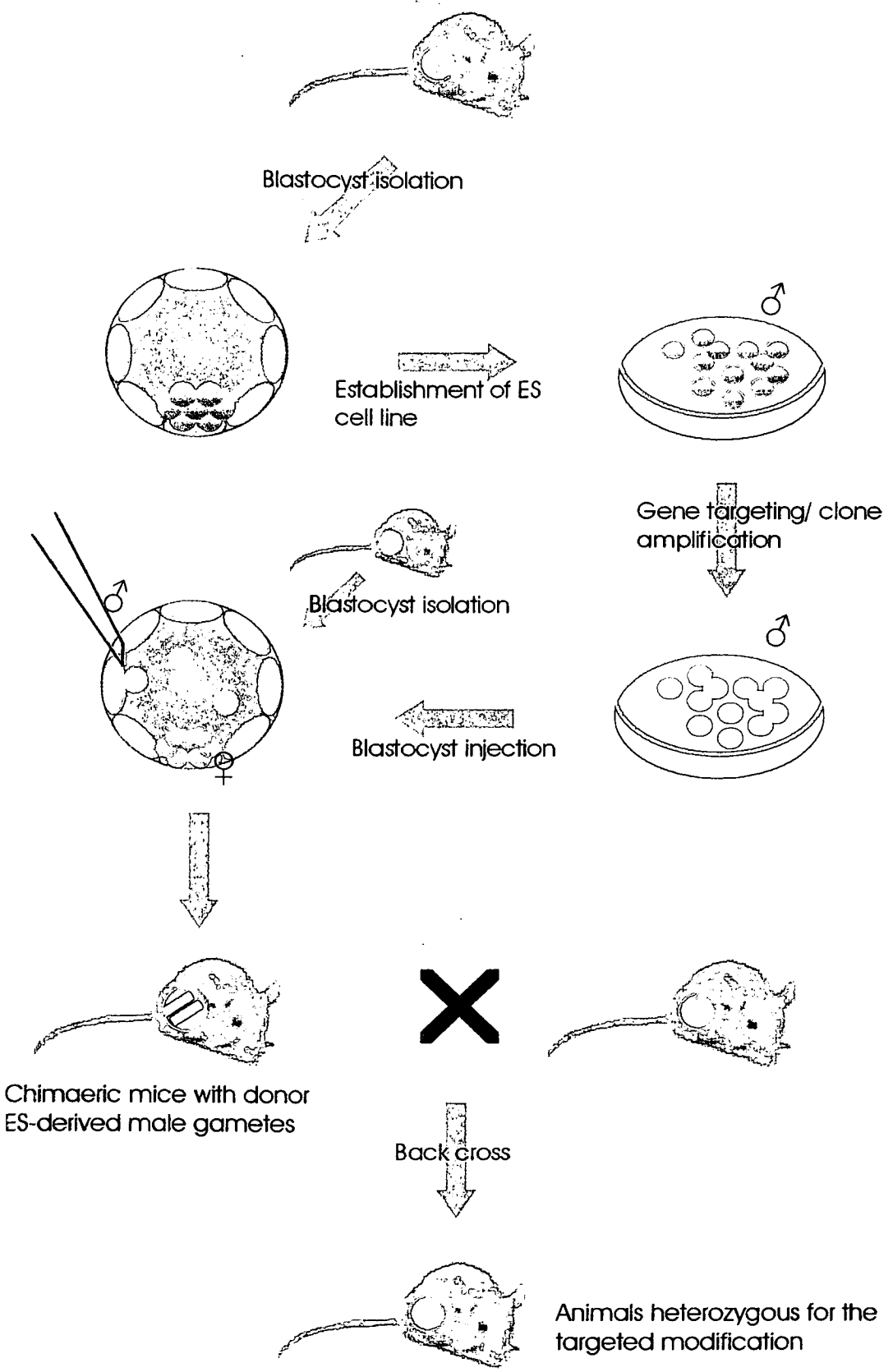

Figure 5. Gene targeting/ES cell technology (details in text). Arrows describe the desired outcome of each step.

4 male chimaeras tested) is not unusual, although this percentage varies from clone to clone.

Until very recently, the generation of targeted animals by gene targeting has been limited to the mouse. Although ES-like cells can be isolated from other species, their ability to contribute to the germ line has not been demonstrated. This problem has been recently circumvented with the development of nuclear transfer techniques, which allow the creation of genetically modified animals in one step following gene targeting of somatic cells *in vitro* (McCreath *et al.*, 2000). However, since targeting of somatic cells is usually hindered by a number of technical difficulties, including premature senescence and low targeting frequencies, the quest for non-murine ES cells is still ongoing. This section reviews the state of the art in gene targeting techniques in the context of their application in ES cells to create targeted animals.

b.embryonic-stem cell technology

Most gene targeting work in mammals has been conducted in embryonic stem (ES) cell lines, because these cells offer a route to germline transgenesis. Pluripotential cells are present in the mouse embryo until at least early post-implantation, as shown by the ability of some cell lines derived from this stage to take part in the generation of chimaeric animals and to form teratocarcinomas (Stevens, 1970). Embryonal carcinoma (EC) cell lines, established from the malignant stem cells of teratocarcinomas, have been for a long time an important tool to study developmental processes of early embryonic cell commitment and differentiation. They have been widely used to create chimaeric mice by microinjection into the cavity of a host blastocysts or aggregation with morulae (rev. by Bradley, 1987). However, EC cells contribution to chimaeras is very variable and tends to be poor following long-term culture (Mintz, 1981). Development of teratocarcinomas and other tumours is a common side effect when groups of cells from some EC cell lines are used (Robertson, 1987; Hooper, 1992). In addition, germline

colonisation has been reported only once for a single EC cell line (Stewart and Mintz, 1982).

In order to overcome such deficiencies, many groups sought to obtain similar cell lines without going through a teratocarcinoma intermediate stage. This led to the isolation and establishment of embryonic stem (ES) cell lines from peri-implantation mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). The contribution of ES cells to chimaeras is usually extensive and much less variable than is the case with EC cells. Unlike the latter, ES cells tend to be karyotypically normal and tumours rarely develop in ES cell-derived chimaeras (Hardy *et al.*, 1990). Most importantly, the confirmation of their capacity to colonise the germ-cell lineage of chimaeras (Bradley *et al.*, 1984) raised the possibility of transferring a wide variety of genetic modifications into the germ line, as depicted in figure 5.

Following fertilisation, the egg undergoes successive cleavages within the *zona pellucida* to produce the morula. At the 8- to 16- cell stage, the cells (blastomeres) become polarised, developing distinct apical and basal membrane and cytoplasmic domains. Cells on the outside of the embryo (derived from the apical domains of their polarised precursor cells) give rise to trophectoderm, while cells on the inside (derived from the basal domains) develop into the inner cell mass (ICM). The embryo becomes a blastocyst upon formation of a fluid-filled cavity (blastocœl), separated from the outside by a monolayer of trophectoderm cells (rev. by Hooper, 1992). These pre-implantation blastocysts (day 3.5 *post coitum*) are the starting material of choice for the isolation of embryonic stem cells. Under standard conditions of culture (rev. by Robertson, 1987), explanted embryos normally acquire a suitable morphology after 5 days of culture. ICM clumps are microsurgically removed, disaggregated with trypsin-EDTA and transferred into fresh feeder wells. Cultures are left for an initial 7-day period to allow the colonies to become established. Following identification of putative ES colonies, they are disaggregated and re-transferred to a new feeder well. After 4-5 days, cultures are passaged again.

ES cells can be manipulated *in vitro* and still maintain their totipotency. They can be aggregated to morulae or microinjected into blastocysts (rev. by Bradley, 1987),

leading to the formation of chimaeras and efficiently contributing to the germ line. The generation of animals with precise genetic modifications via gene targeting is based upon these properties of ES cells and the application of cell engineering.

Almost all ES cell lines currently utilised have been obtained from the mouse strains 129 and, less commonly, C57 BL/6. Some mouse strains are not permissive for ES cell isolation following the standard procedure described above. It has been suggested that genetic background strongly affects the efficiency of ES cell isolation, a hypothesis that could also account for the lack of success in isolating ES cell lines from other species. Two recent reports on the establishment of ES cell lines from non-permissive CBA strains may offer a new insight into this affirmation. In a transgenic approach (McWhir *et al.*, 1996), CBA mouse lines carrying a stable integration of the Oct-neo transgene (which is preferentially expressed on undifferentiated cells) allowed ES cell isolation following selective ablation of non-ES cell lineages in G418 selection of the explants. Similar results were reported in a microsurgical approach (Brook and Gardner, 1997) based on the unequivocal identification of ES progenitors in the mouse embryo, the epiblast or primitive ectoderm. Selective explantation of early epiblasts, instead of entire ICMs, yielded consistently high frequencies of isolation of ES cell lines even from non-permissive murine lines. These results suggest that the barrier to ES cell isolation in other animals may not be absolute, re-opening the possibility of applying conventional gene targeting techniques in ES cells to precisely alter the genome of larger mammals.

c.gene targeting techniques

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c.1.transfection

Both transient and stable integration of DNA vectors into ES cells have been accomplished by a variety of techniques. **Calcium phosphate/DNA precipitation** was the first to be successfully used in mammalian cells. It is simple but very inefficient in several cell lines (10^{-5}- 10^{-6}). **Microinjection** is very efficient (10^{-2}- 1), but is technically difficult, requires sophisticated apparatus and is labour intensive. **Lipofection** is based on the inclusion of the DNA in lipid vesicles which readily fuse to cell membranes, and has been employed to introduce DNA with a high efficiency in a wide variety of cells. **Viral vectors** (SV40, polyoma, bovine papillomavirus, retrovirus and adenovirus) usually offer the advantage of a 100 % efficiency in the delivery of the DNA to a cell population, but there are disadvantages associated with every particular type, among them the requirement of helper viruses, the limited size of the insert or the risk of recombination with wild-type viruses. Interestingly, 'retroadenoviral vectors' (Zheng *et al.*, 2000) have been recently designed to combine the versatility of adenoviral

vectors with the long-term expression and integration of retroviral vectors. Also, vectors based on adeno-associated viruses (AAV) have been reported to work at a high frequency to specifically target defined loci in human cells (Rusell and Hirata, 1998), a result that could have immediate applications in therapeutic gene targeting.

The most common technique for introduction of DNA into ES cells is **electroporation**. Although it is not particularly efficient (10^{-4}- 10^{-5}) and requires some apparatus, the procedure is simple, fast and useful for most cell lines. The technique involves the exposure of cells to a pulsed electric field generated by an electroporator. This shock creates pores in the membrane of the cell, thus allowing dissolved DNA to be uptaken.

c.2.targeting vectors and enrichment strategies

c.2.i.introduction

Targeting constructs carry a segment of DNA homologous to the target, including a selectable marker and, where desired, a genetic alteration to be introduced into the genome. **Insertion vectors** (O type) are linearised within the region of homology. Homologous recombination between these vectors and the chromosomal target involve a single reciprocal exchange, resulting in the insertion of the entire construct into the target locus and duplication of the region of homology.

In contrast, replacement vectors (Ω type) are linearised outside the region of homology, and their homologous integration results in replacement of a stretch of the target with a corresponding stretch from the targeting construct.

Positive selection markers are essential components of both types of targeting vectors, since they allow the isolation of rare transfected cells from a predominantly

untransfected population. A second level of selection in a targeting experiment involves the selection of targeted clones in preference to the more frequent random integrants. Several approaches have been considered to manipulate the ratio of homologous to illegitimate recombinants (**effective targeting frequency**) in order to increase the representation of targeted clones within a transfected population (see next section). *In vitro* **enrichment** methods allow a significant reduction of random integrants in targeting experiments, saving time and effort otherwise dedicated to exhaustively screen clones arising mostly from non-homologous recombination events.

c.2.ii.positive-negative selection

The original enrichment strategy was to place a positively selectable marker within the region of homology and a negatively selectable marker on the flank of the targeting vector (Thomas and Capecchi, 1987). The negative marker is lost upon recombination with the homologous target, whereas clones in which the vector has integrated elsewhere frequently incorporate it. Thus, while selection for the positive marker allows the survival of both random and homologous recombinants, selection against the negative marker specifically kills colonies derived from non-homologous events, producing an overall enrichment in targeted clones (see figure 6).

However, using a variety of targeting vectors featuring several positive (*neo*, *hyg*, *his*) and negative (*gpt*, *his*) selectable markers, this positive-negative selection system has been reported to produce only modest enrichments, typically ranging from 2- to 10-fold (Hanson and Sedivy, 1995). Both the spontaneous loss of the negative marker (or loss of its activity due to modifications such as methylation) in non-targeted clones, and metabolic exchange between non targeted and targeted cells may account for these results.

Figure 6. Positive/negative selection (see text for details)

c.2.ii.promoter-trap enrichment

The promoterless strategy uses vectors in which the positively selectable marker lacks its own promoter. Its expression is activated from the target gene promoter following homologous integration (see figure 7). Vectors of this type are therefore restricted to genes which are transcriptionally active in the target cells. The positively selectable cassette is usually cloned in frame with the endogenous translated product, creating a fusion gene. Alternatively, it can be positioned upstream the transcriptional initiation sequence of the target gene. Promoter trap selection yields average enrichments of about 100-fold, and works both in replacement and insertion vectors (Schwartzberg *et al.*, 1990). A variation of this method is known as enhancer-trapping, and is based on vectors whose selective markers have a weak position-dependent promoter. Activation of this promoter occurs upon homologous integration of the construct in the vicinity of a transcriptional enhancer element. Internal Ribosomal Entry Sites (IRES) have also been utilised in promoterless vectors. The IRES is a sequence of around 500 bp which acts as a ribosome binding site and permits the

effective internal initiation of translation in mammalian cells. The advantage of incorporating IRES sites to these vectors is that when they are integrated into a transcriptionally active gene, production of the marker is expected to be independent of context in the fusion transcript. In this respect, it has been reported that IRES-mediated transcription is significantly more efficient than strategies reliant upon the production of an active fusion protein (Mountford *et al.*, 1994).

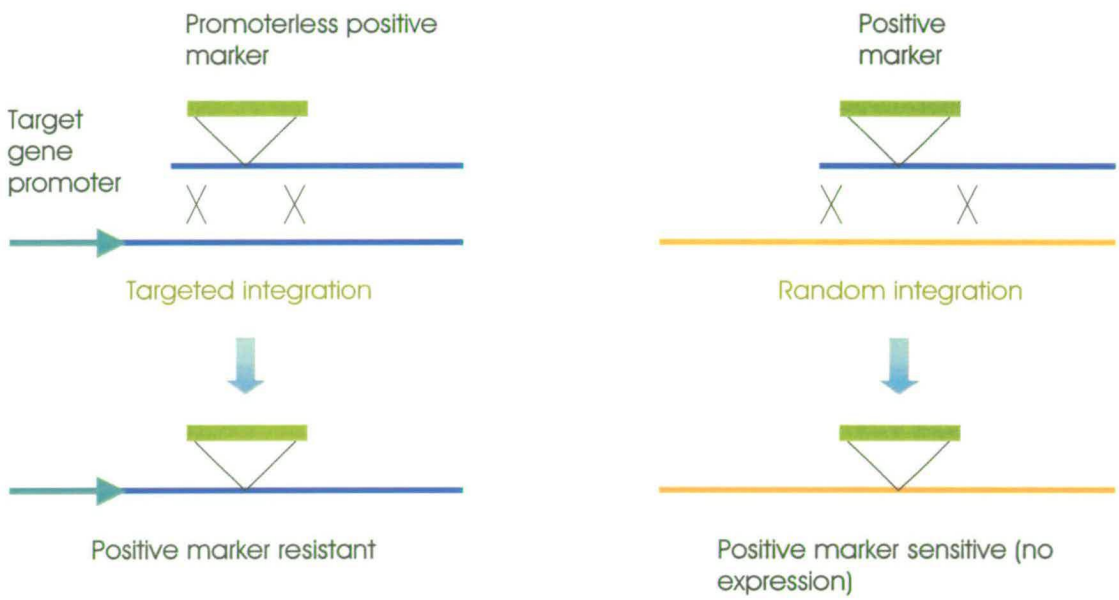

Figure 7. Promoter trap strategy (see text for details).

c.2.iii. polyadenylation-trap enrichment

Polyadenylation trap targeting vectors are designed to use the transcription termination/ polyadenylation signals of the target gene to generate a stable hybrid transcript, consisting of elements from both the target gene and the positive selection cassette. Unlike the previous approach, this type of positive selection should work for most genes, irrespective of their level of expression in the transfected cells. Typical enrichment factors obtained with such vectors range between 5- and 50-fold (Donehower *et al.*, 1992).

c.2.iv.HPRT selection

Several studies in gene targeting are based on the manipulation of the *HPRT* locus. The product of this gene is the hypoxanthine-guanine phosphoribosyl transferase, an enzyme involved in the purine salvage pathway. HPRT catalyses the addition of purines to the sugar intermediate 5-phosphoribosyl-alpha-1-pyrophosphate (PRPP) in wild-type cells. Either in the absence of purines in the medium or when HPRT is not functional, the cells are forced to follow an alternative *de novo* synthesis pathway. One of the components of the HAT medium (aminopterin) inhibits the enzyme dihydrofolate reductase, which is essential for *de novo* synthesis. Wild-type cells are able to use the salvage pathway, and are basically unaffected by the presence of aminopterin. However, *HPRT*-defective cells have both purine ribonucleotide biosynthesis routes blocked, and die. It is also possible to select against HPRT function in wild-type cells. The enzyme can efficiently catalyse the addition of 6-thioguanine (a toxic purine analogue) to PRPP, resulting in cell death. In contrast, cells deficient in HPRT do not show any alteration in their metabolism in the presence of 6-TG.

c.3.introduction of subtle mutations

c.3.i.tag-and-exchange (double replacement)

Comprehensive investigation of gene function sometimes requires the introduction of multiple, distinct and precise mutations into the locus of interest. One of the strategies devised for the introduction of subtle mutations into the mammalian genome is known as tag-and-exchange (Askew *et al.*, 1993) or double-replacement (Wu *et al.*, 1994). It is based on tag selection cassettes, such as *HPRT*, for which both positive and negative selection can be applied in cell cultures. Homologous integration of this cassette into the target locus is positively selected (tag step). In a second round of gene targeting (exchange step), a precise alteration is introduced into

the tagged locus, resulting in displacement of HPRT. Selection for loss of HPRT results in the selective elimination of illegitimate recombinants. This technique has been successfully employed in replacing the murine β-lactalbumin gene with its human counterpart (Stacey *et al.*, 1994), and has raised the possibility of generating multiple gene replacements at a single locus. Unless enrichment strategies (such as the use of promoterless HPRT) are used in the tag step, a problem of this strategy is the necessity to screen a large number of clones in the first round of gene targeting. In addition, the efficiency of the exchange step is often compromised by a high background of non-targeted lines, which survive selection due to the spontaneous loss (or loss of activity, for instance due to methylation) of the tag cassette (Whyatt and Rathjen, 1997). Thus, although double-replacement targeting allows precise alterations of the genome, the low frequencies of homologous recombinants surviving secondary selection greatly hinders the repeated introduction of independent mutations. A modification of this strategy, which involves the incorporation of an additional positive selection marker for increased stability, has been recently reported to improve the efficiency of the exchange step by 4-fold (Whyatt and Rathjen, 1997). A disadvantage of this approach is that the second marker would not be removed after the exchange step.

c.3.ii.plug and socket

An alternative two-step targeting system, termed plug and socket (Detloff *et al.*, 1994) has also been proposed as a way to alter any mouse locus efficiently and repeatedly. Using conventional targeting for the first step, a functional *neo* and a partial *HPRT* minigene ('the socket') are targeted into the genome of *HPRT*-deficient ES cells, close to the locus of interest. G418 resistant clones are screened for the presence of the socket at the target locus. The second step involves the use of a targeting vector ('the plug') that supplies the remaining portion of the *HPRT* minigene and generates a functional cassette upon homologous recombination with the integrated socket. This event can be selected in the presence of HAT medium. At the same time, the plug provides DNA sequences that recombine homologously with sequences in the target locus and modifies them in the desired manner. Using this method, the murine β-

globin gene was replaced by its human equivalent (Detloff *et al.*, 1994). As in double-replacement, the plug and socket strategy seems to be complicated by a high background of incorrectly targeted lines. In addition (unlike tag-and-exchange), it has the added disadvantage of leaving the *HPRT* marker at the target locus once the second round of targeting has been completed.

c.3.iv.hit and run (in & out)

'Hit and run' ('in & out') vectors (Hasty *et al.*, 1991b; Valancius and Smithies, 1991) are modified insertion constructs containing the desired mutation in the homologous sequence and both positive and negative selection cassettes in the vector backbone (alternatively, a single cassette comprising both selectable markers in one gene, such as *HPRT* or *gpt*, can be used in *HPRT*-defective cell lines). Positively-selected homologous recombination between this integration vector and the genome yields a duplication, in which one of the repeats carries both markers. Negatively-selected intrachromosomal recombination between them leads to the correction of the target and the loss of one of the repeats, together with the negative marker, if the crossover occurs downstream of the modification in the vector-derived repeat (green crossover in figure 8). However, if the crossover occurs upstream of the modification (in red, figure 8), a high background of incorrectly targeted clones will be observed. This can be minimised by increasing the length of homology downstream of the

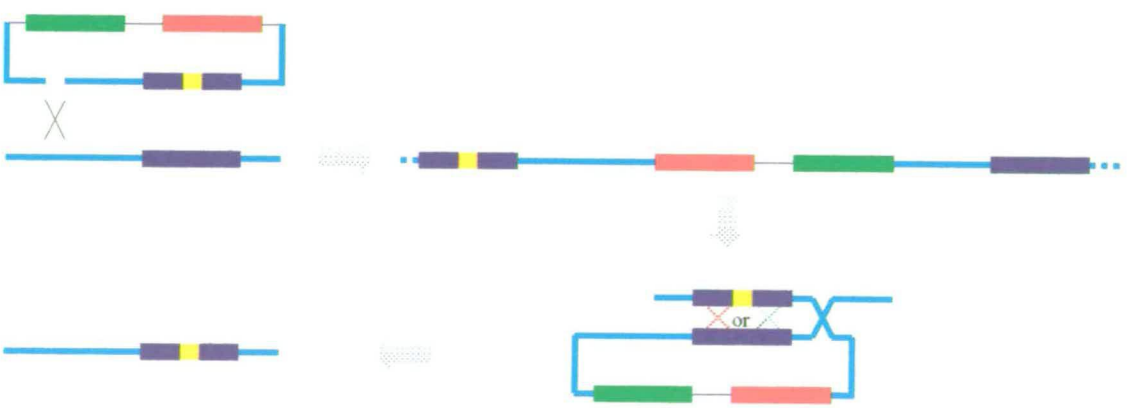

Figure 8. 'Hit and run' targeting strategy. Red line: negative marker. Green line: positive marker. See text for details.

desired mutation in the targeting vectors. As in other systems using negatively selectable genes, another limitation of this procedure is the background of non-targeted events due to the spontaneous loss (or loss of activity) of the negative marker.

c.3.v.Cre-*loxP*

Site-specific DNA recombinases from bacteriophage and yeasts have been used in recent years for genome engineering both in prokaryotes and eukaryotes. The 38kDa Cre protein efficiently produces both inter- and intramolecular recombination between specific 34bp sites called loxP (rev. by Kühn and Schwenk, 1997). The general strategy involves two steps: First, a genetic modification including flanking bacteriophage *loxP* sites is introduced into the genome by homologous recombination. Second, transient expression of the Cre recombinase (which excises *loxP*-flanked DNA) generates the final structure of the target locus. Specifically, this technique has been used (a) to remove the selection marker after conventional gene targeting in order to avoid interference with the expression of the targeted gene (Wang *et al.*, 1999; Delneri *et al.*, 2000); and (b) to efficiently mediate transgene integration in specific sites of the genome (Kolb *et al.*, 1999). This application involves two steps: First, a *loxP* site is integrated by homologous recombination next to the promoter region of a highly transcribed gene, replacing the translational initiation signal. Subsequently, co-transfection of a *loxP*-flanked transgene and a Cre-expression vector results in site-directed recombination with the genomic *loxP* sequence. The efficiency of this procedure can be increased by using incompatible *loxP* sites in the transgene. This would prevent the frequent excision of the transgene that is thought to occur through interaction between the flanking *loxP* sites after integration (Araki *et al.*, 1997; Lee and Saito, 1998).

c.4.conditional and inducible gene targeting

Conditional gene targeting involves a gene modification restricted to certain cell types or developmental stages of the mouse. This temporal/ spatial

specificity can be used to test the function of widely expressed genes in a particular cell lineage and to investigate postnatal gene function in those cases where conventional knockout leads to a severe or lethal phenotype during embryonic development. Also, it can be useful to analyse gene function in adults, because it allows gene modification after the normal establishment of adaptational responses of cellular systems.

The most widely adopted conditional targeting approach is based on the cre/*loxP* system (rev. by Kühn and Schwenk, 1997; Porter, 1998). This requires the generation of both a mouse strain in which the target gene is appropriately flanked by *loxP* sites, and a second strain expressing the Cre recombinase constitutively or upon induction in specific tissues. A conditional mutant is generated by crossing these two strains, so that the modification of the target gene is restricted to particular cell types and/ or ontogenic stages according to the pattern of Cre expression in the particular strain used. This system can also be used to activate transgenes in a tissue-specific manner, by removing loxP-flanked blocking fragments (Lewandoski *et al.*, 1997). As an alternative to the cross with Cre-expressing mouse strains, this recombinase can also be delivered to somatic tisues via infection of mice with adenoviral Cre expression vectors (Rohlmann *et al.*, 1996; Wang *et al.*, 1996; Sato *et al.*, 1998).

Two characteristics of the Cre-based approach are its binary nature (the target gene is either active or inactive, with no intermediate stage) and its irreversibility (once inactivated, the target gene cannot be reactivated by reversing the switch). **Inducible gene targeting** techniques have been recently devised to overcome these limitations, allowing the study of gene-dosage effects and the consequences of temporary gene silencing. Typically, gene targeting is used to inactivate one copy of the target gene, while the other is placed under the control of a regulatory system such as *Lac*, *Tet* or ecdysone (rev. by Porter, 1998).

A third approach is based on the use of gene targeting to disrupt both endogenous alleles. Viability depends on the ectopic expression of a minigene derived from the target gene, under the inducible control of exogenous transcriptional-regulatory sequences. This method has been successfully used in avian (Wang *et al.*, 1996) and mammalian cells (Legname *et al.*, 2000).

v. manipulation of gene targeting frequency

a.introduction
b.design of the targeting vector
c.manipulation of cell/target conditions
d.changing expression of recombination-related genes

a.introduction

Increasing absolute targeting frequency (number of targeted clones/ number of cells exposed to the transfection procedure) is an essential requirement for some future applications of this technology, like gene therapy *in vivo* (see Introduction). Such increase can be attained by improving both transfection efficiency and **effective targeting frequency**. This parameter has been defined here as the ratio between homologous recombinants and total (homologous and non-homologous) integrants, and typically ranges from 10^{-5} to 10^{-2} in most cell lines. Strategies aiming at enhancing effective targeting frequency can be grouped into three main categories: *Design of the vector, control of the target gene status* and *manipulation of the expression of recombination-related genes.* This section reviews the most recent advances in each one of these approaches.

b.design of the targeting vector

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b.1.introduction

Gene targeting is usually defined as the directed alteration of endogenous genes through homologous recombination with transfected DNA fragments. In a broader sense, any technique or procedure allowing directed modification of an endogenous gene's sequence or pattern of expression should also be considered gene targeting, even if it does not involve homologous recombination. This wider definition would include novel techniques such as ribozyme and antisense oligonucleotide downregulation, RNA interference (RNAi) and others. With the only exception of RNAi -a technology whose mechanism was unknown at the time we

started working on it-, this work is mainly focused on homologous recombination-based techniques, and any subsequent references to gene targeting in the experimental section must be understood in this context (see 'conventional targeting vectors').

b.2.conventional targeting vectors

b.2.i.vector types

The first step in undertaking a gene targeting experiment is to design a vector such that the desired mutation can be delivered to the target locus. The most widely used vectors are those involving replacement events. Their acceptance is based upon an early study (Thomas and Cappechi, 1987) reporting that recombination frequencies were similar to those obtained with insertion constructs, and that positive-negative selection is applicable to this vector type. However, a later report (Hasty *et al.*, 1991) suggested that insertion vectors target up to nine-fold more frequently than replacement vectors with the same length of homologous sequence. Two explanations were proposed to support these results: First, the fact that insertion vectors need only one cross-over to get integrated; second, the observation that double-strand breaks in the regions of homology –characteristic in the design of insertion vectors- seem to increase homologous recombination in several organisms (page 51).

b.2.ii.length of homology

The length of homology seems to be the most important factor affecting targeting frequency. It has been demonstrated that there is an exponential dependence of targeting efficiency on the extent of homology between the targeting vector and the target locus, up to 14 kb (Deng and Capecchi, 1992). In this study, based on the analysis of more than twenty targeting vectors, sequence replacement and sequence insertion vectors behaved equivalently with respect to the targeting efficiency. This result contradicts the observations reported by Hasty *et al.* (1991) in a less

comprehensive study. This relationship between recombination frequency and length of homology seems to be biphasic, suggesting that different mechanisms predominate above and below a transition point in the range of 150-400 bp (Rubnitz and Subramani, 1984; Ayares *et al.*, 1986).

b.2.iii.isogenicity

The use of isogenic or non-isogenic constructs is another important determinant of gene targeting efficiency. An evenly dispersed 19% mismatch reduces the frequency of extrachromosomal recombination up to 15-fold (Waldman and Liskay, 1987). Base-pair mismatches have been shown to strongly affect the frequency of homologous recombination in bacteria (Rayssiguier *et al.*, 1989), of intrachromosomal recombination in mammalian cells (Bollag *et al.*, 1989) and of gene targeting in embryonic stem cells (te Riele *et al.*, 1992). Recent work in yeast indicates, however, that mismatched sequences inhibit recombination between DNA fragments and the genome only when they are close to the edge of the fragment (Negritto *et al.*, 1997), suggesting that the effect of mismatches sequences may not be an absolute determinant on the frequency of homologous recombination, but rather dependent on the context.

b.2.iv.modification of DNA ends

Modifying DNA ends in the transfected molecules might also be a way to reduce illegitimate recombination and even to improve homologous recombination. Free DNA ends are thought to stimulate targeted recombination by allowing exposure of single strands, which then can invade homologous chromosomal sequences (Szostak *et al.*, 1983). Free DNA ends also appear to stimulate random integration by permitting direct joining of input DNA ends to genomic DNA, perhaps at transient chromosomal breaks. Analysis of randomly integrated DNA molecules suggests that the integration event requires very little, if any, nucleotide sequence homology (Kato *et al.*, 1986). Thus, modification of the ends of targeting constructs to block end joining might have a twofold utility: First, to block the illegitimate joining of

input DNA to chromosomes, therefore decreasing the frequency of random integration directly. Second, to prevent the joining of input DNA molecules to one another. This would maintain the concentration of free ends at a maximum, which might promote an increase in the absolute frequency of homologous recombination. This hypothesis has been tested by adding dideoxynucleotides to the 3'-ends of a linear plasmid transfected into monkey COS-1 cells. This treatment increased the homologous/ non-homologous recombination ratio about five-fold (Chang and Wilson, 1987). Other reports state that ligation of linear targeting constructs to self-complementary oligonucleotides to seal DNA ends, enhances stability of the vectors and increases the frequency of gene targeting (rev. in Yáñez and Porter, 1998) despite the loss of 3' recombinogenic ends. Different modifications of the ends already assayed in antisense oligonucleotides, such as C5-propyne, may prove to be useful for this purpose.

b.2.vi.nuclear localisation signals (NLSs)

It is generally acknowledged that transport of DNA molecules across the nuclear barrier could be one of the most important limitations in gene delivery (Shen, 1997). The nuclear membrane of eukaryotic cells is freely permeable to particles of up to 9-10 nm (such as 40-60 kD proteins), but larger molecules require energy-dependent active transport through the nuclear pore complex (NPC) (Goldberg and Allen, 1995; Aronsohn *et al.*, 1997; Ludtke *et al.*, 1999). NPCs are supramolecular aggregates that preferentially bind proteins carrying nuclear localisation signals (NLSs) in a process that also involves cytosolic factors (importins α and β) and the translocator protein GTPase-Ran (Adam and Gerace, 1991; Nigg, 1997; Ohno *et al.*, 1998; Melchior and Gerace, 1998). In recent years, several groups have reported enhanced DNA transport to the nucleus by complexing NLSs (normally the SV40 large T antigen PKKKRKV sequence) to plasmids, either by electrostatic interaction (Kaneda *et al.*, 1989; Remy *et al.*, 1995; Collas *et al.*, 1996; Fritz *et al.*, 1996; Collas and Alleström, 1997; Aronsohn and Hughes, 1997; Subramanian *et al.*, 1999) or covalent irreversible binding (Zanta *et al.*, 1999). Most interestingly, improved DNA transport through the nuclear membrane has been consistently associated to higher frequency of transgene expression, which indicates that NLS peptides may constitute a valuable tool to improve the

efficiency not only of gene targeting, but also of transgenesis (Collas and Alleström, 1997).

b.3.other vectors that target DNA

b.3.i.single-stranded DNA vectors

Small single-stranded fragments of DNA have been used to generate potent targeting constructs (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998). In these studies, a 491 bp PCR product derived from a functional CFTR gene was first denatured and then used to target and repair the 3 bp deletion (ΔF508) of the CFTR gene in transformed lung epithelial cells from a patient with cystic fibrosis. A high absolute targeting frequency (10^{-2}) was repeatedly observed, regardless of the method of transfection. Although an early study reported enhanced levels of homologous pairing following RecA coating of ssDNA molecules (Ferrin and Camerini-Otero, 1991) such treatment of the targeting fragments did not seem to produce detectable changes in the overall CFTR targeting frequency. The high concentration of targeting molecules/ exposed cell ($\sim 10^6$) and the absence of non-homologous sequences in the vectors were proposed to account for these unusually high levels of homologous recombination.

b.3.ii.triple helix-forming oligonucleotides (TFOs)

Triple helix-forming oligonucleotides (TFOs) can bind in the major groove of duplex DNA containing polypurine/polypyrimidine-rich regions in a sequence-specific manner (Moser and Dervan, 1987; Francois *et al.*, 1988). Originally used to block mRNA synthesis by preventing the binding of transcription factors to promoter sites (Cooney *et al.*, 1988; Blume *et al.*, 1992; Duval-Valentin *et al.*, 1992), this powerful and versatile technology has also been successfully employed to: (1) activate transcription from a target gene by coupling promoter sequence-specific TFOs to the activation domain of protein VP16 (Kuznetsova *et al.*, 1999); (2) target DSB-inducing

mutagens to selected loci (Wang *et al.*, 1995a, 1996; Faruqi *et al.*, 1996); (3) induce gene conversion via nucleotide-excision repair between tandem repeats in episomal substrates (Faruqi *et al.*, 2000); and (4) increase gene targeting frequency by linking targeting vectors to 'priming' TFOs (figure 9) (Chan *et al.*, 1999).

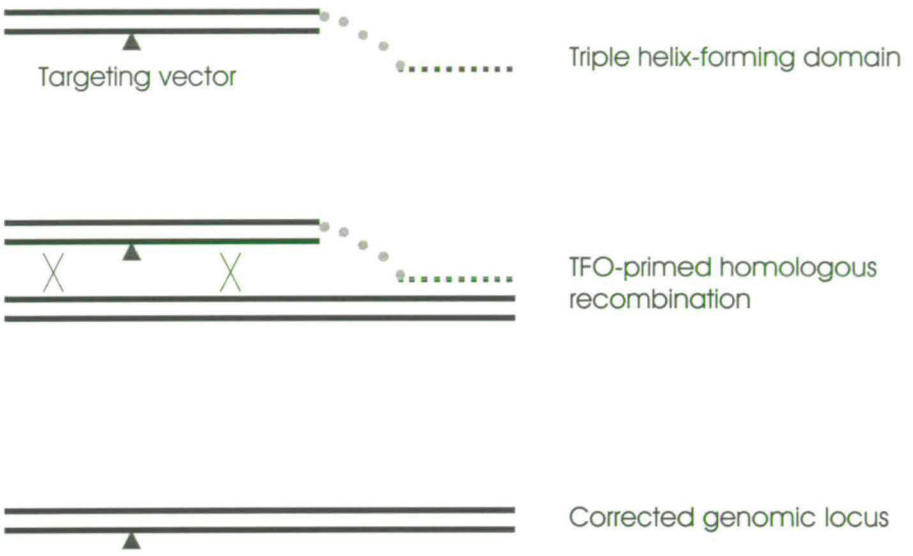

Figure 9. TFO-primed gene targeting. A targeting vector is engineered to contain a triple helix-forming domain homologous to a region adjacent to the locus to be modified. Homologous recombination occurs upon triple helix formation, favoured by the proximity of the recombinant molecules (Chan *et al.*, 1999)

b.3.iii.RNA/DNA oligonucleotides (RDOs)

Strategies involving the use of RNA/DNA oligonucleotides (RDOs or chimaeraplasts) have been devised over the past five years to specifically correct point mutations in bacteria and mammalian cells. The design of RDO vectors was inspired by *in vitro* studies of homologous recombination in *U. maydis*, which revealed that RNA/DNA hybrids were more active in homologous pairing than corresponding DNA duplices (Kmiec *et al.*, 1994). It has been suggested that homologous pairing is the rate-limiting step in homologous recombination (Wilson *et al.*, 1994), which led to the hypothesis that small synthetic RDOs would enhance this

process by (a) facilitating the access of vector to the nucleus in stoichiometric excess over the target (which is difficult when using larger vectors); and (b) increasing the binding affinity of the vector to the target DNA (Yoon *et al.*, 1996). RDO vectors are single oligonucleotides (typically 68-mers for a 25 bp region of homology) designed to fold in such a way that one strand of the duplex comprises all DNA residues, and the complementary strand contains alternating blocks of DNA and 2'-0-methyl RNA residues. Mismatches are introduced in the central DNA block of the RNA/DNA strand. 2'-0-methylation renders the molecule resistant to RNAse H, whereas thymidine hairpin caps enhance resistance to DNA-nucleases and prevent end-to-end ligation (Cole-Strauss *et al.*, 1996: Yoon *et al.*, 1996; Kren *et al.*, 1999).

RDO vectors have been successfully employed to correct single point mutations both in episomal (Yoon *et al.*, 1996) and genomic (Cole-Strauss *et al.*, 1996; Alexeev and Yoon, 1998) targets, in a variety of prokaryotic (Kren *et al.*, 1999), mammalian (Cole-Strauss *et al.*, 1996; Alexeev and Yoon, 1998) and plant (Zhu *et al.*, 1999; Beetham *et al.*, 1999) cells, as well as in cell-free extracts (Cole-Strauss *et al.*, 1999). Targeting efficiencies using RDOs are consistently higher than those obtained by conventional gene targeting, ranging from 0.01 % (Alexeev and Yoon, 1998) to 50% (Cole-Strauss, 1996) depending on the biological system, the design of the vector and the nature of the target. *MutS* and its eukaryotic counterpart, *Msh2* are essential for RDO-mediated gene targeting, as shown in studies conducted in bacteria (Kotani and Kmiec, 1994) and mammalian cell-free extracts (Cole-Strauss *et al.*, 1999), respectively. This suggests that the mechanism of gene modification by RDOs involves gene conversion by mismatch repair rather than strand exchange.

The advantage of RDO- over homologous recombination- mediated gene targeting is two-fold: (1) A higher targeting efficiency; and (2) The fact that RDOs are small synthetic molecules which can be manufactured like a drug. Therefore, this technology is a potentially powerful alternative to gene targeting for the therapeutic correction of point mutations in diseases such as sickle cell anemia, cystic fibrosis or hemophilia. However, due to the impossibility of engineering selectable markers into RDOs, selection of RDO-targeted clones *in vitro* would be possible only if there is a

phenotype associated to the mutation (such as 6-TG resistance when inactivating HPRT).

b.4.vectors that target RNA

b.4.i.antisense oligonucleotides (ODNs)

Antisense oligodeoxynucleotides (ODNs) are designed to base-pair to specific regions of target mRNA transcripts. This hybridisation results either in (a) inhibition of protein synthesis, or (b) RNAse H-directed destruction of the target mRNA. Despite its theoretical simplicity and both scientific and therapeutic potential, widespread application of antisense technology is hindered by a number of practical obstacles, such as: (1) the inherent instability of antisense molecules within the cellular environment; (2) the inefficient cellular uptake; (3) the frequent incidence of non-antisense effects; and (4) the necessity of screening large numbers of candidate molecules for effective and specific *in vivo* binding to the target (rev. by Stein and Cheng, 1993; Branch *et al.*, 1998).

b.4.ii.ribozymes

The ability of some RNA molecules to catalyse RNA cleavage and joining through consecutive transesterification reactions (rev. by Cech, 1987) has led to the development of a powerful technology for the selective down-regulation of gene expression. Ribozymes are catalytic RNA molecules intended to bind to and cleave target mRNA molecules. Upon cleavage, the transcript is destabilised and translation is suppressed. Ribozymes expressed from gene therapy vectors have been successfully used to specifically inhibit the expression of a variety of targets from oncogenes to growth factors, and ribozyme-based anti-HIV therapeutic protocols are currently moving into clinical trials in humans (rev. by Couture and Stinchcomb, 1996). Some

taking advantage of the splicing ability of ribozymes to correct defective transcripts, rather than prevent gene expression. This strategy is based on the replacement of a mutated portion of RNA with a functional sequence by targeted trans-splicing (Sullenger and Cech, 1994; Hagen and Cech, 1999).

Ribozyme technology clearly offers novel opportunities for basic studies of gene function and regulation, as well as therapeutic uses in humans. In theory, ribozymes can be engineered to target any RNA species in a site-specific manner. However, their practical applications often face many of the problems previously described for antisense oligonucleotides.

b.4.iii.protein-nucleic acids (PNAs)

Protein-nucleic acids (PNAs) are synthetic nucleic acid analogues in which the phosphate sugar polynucleotide backbone has been replaced by achiral and uncharged N- (2-aminoethyl)glycine polymers (Nielsen *et al.*, 1991; Good and Nielsen, 1998). These chimaeric molecules work by hybridising to complementary sequences of RNA and sterically blocking initiation of translation, rather than by targeting PNA-RNA complexes for RNAse degradation (Knudsen and Nielsen, 1996; Bonham *et al.*, 1995). PNAs were originally devised as a way to overcome the limitations of conventional antisense technology: compared to ODNs, PNAs ensure superior hybridisation properties, increased resistance to enzymatic degradation and higher accessibility for chemical engineering (Good and Nielsen, 1998). Cellular and nuclear uptake is enhanced when PNAs are coupled to protein transporters (Pooga *et al.*, 1998) and nuclear localisation signals (NLS) (Cutrona *et al.*, 2000), respectively. Even more interestingly, PNA-NLS can be hybridised to plasmids for efficient nuclear transport following transfection, without noticeable alteration of their pattern of expression (Brandén *et al.*, 1999) Therefore, this approach is potentially useful in assays and therapies involving transient transfections, as well as for improving accessibility of targeting vectors to the nucleus.

b.4.iv.double-stranded RNA

The recent discovery that microinjection of double-stranded RNA molecules can produce long-term, highly efficient *in vivo* inactivation of a variety of genes in C. elegans (Fire *et al.*, 1998) and other organisms (Kennerdell and Carthew, 1998, 2000; Sánchez-Alvarado and Newmark, 1999; Lohmann *et al.*, 1999; Wargelius *et al.*, 1999; Wianny and Zernicka-Goetz, 2000) has raised the possibility of using these molecules as knockout vectors. Although the fact that only a few molecules per cell can produce specific inhibition seemed to argue in favour of a DNA target (Wagner and Sun, 1998), several lines of evidence (Montgomery *et al.*, 1998) discard this hypothesis: (1) dsRNA effects (also called RNA interference, or RNAi) are not generally heritable beyond the first generation in *C. elegans*; (2) Sequencing of dsRNA 'targeted' loci does not reveal any mutation; (3) RNAi is efective when the vectors have regions present in mature RNA transcripts, but not intronic or promoter sequences; (4) *In situ* hybridisation shows that RNAi dramatically decreases the levels of nascent transcripts in the nucleus, and virtually abrogates their cytoplasmic accumulation. Taking all these observations together, it was concluded that specific mRNA transcripts are the primary target of dsRNA vectors through a mechanism involving degradation of mRNA before translation takes place.

Does RNAi have a biological role? *C. elegans* mutants resistant to RNAi (*rde-1*, *ego-1*, *mut-7*) are viable and healthy, indicating that this process is not essential under normal laboratory conditions (Tabara *et al.*, 1999; Ketting *et al.*, 1999; Hunter, 2000). Inactivation of some of these genes leads to transposon mobilisation, which suggests that one physiological role of RNAi might be the silencing of multicopy sequences such as viral and transposable pathogens (Lin and Avery, 1999). This hypothesis could also explain some cases of transgene silencing by co-suppression: repetitive sequences randomly integrated over the genome, as well as head-to-tail tandems, are likely to be transcribed from both strands by readthrough from external initiation sites. Base-pairing of the resulting transcripts would result in dsRNA formation and subsequent silencing (Montgomery and Fire, 1998; Ketting and Plasterk, 2000; Plasterk and Ketting, 2000).

One proposed model for the molecular mechanism of RNAi (Ketting *et al.*, 1999) involves recognition of dsRNA molecules by a protein complex that includes mut-7, a protein homologous to Werner Syndrome helicase and RNAse D. The complex thus activated then targets homologous mRNA transcripts for degradation in a catalytic process where the dsRNA is not affected. Consistent with this model is the observation that extracts of dsRNA-transfected *drosophila* cells contain a ribonuclease activity that specifically degrades exogenous transcripts homologous to the dsRNA vector (Hammond *et al.*, 2000).

Only two years after it was originally reported, RNAi has become a routine procedure in basic developmental studies in *C. elegans* and other species. The recent demonstration of the feasibility of this approach in the mouse embryo (Wianny and Zernicka-Goetz, 2000) offers new opportunities not only to study developmental phenotypes associated with the down-regulation of any single mammalian gene (or combination of genes), but also to design more effective gene therapy protocols to treat diseases such as cancer or virus/parasite infections. It remains to be seen, however, whether RNAi is effective in adult mammals and cells in culture.

c.manipulation of the cell/target conditions

c.1.transcription and homologous recombination

c.2.introduction of double-strand breaks

c.2.i.induction of DNA damage

c.2.ii.use of rare-cutting enzymes

c.3.cell cycle rates

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c.1.transcription and homologous recombination

Locus-to-locus intrinsic variability has been extensively documented to have dramatic effects in the overall targeting frequency. The level of expression of the target gene may account for some of these observations. Early work in *E. coli* showed that homologous recombination is highly enhanced by transcription (Ikeda and Matsumoto, 1979), an observation that led to the proposition that the strand displaced during transcription might invade a homologous duplex, promoting strand-exchange. These results have been reproduced in eukaryotes (Alt *et al.*, 1986; Blackwell *et al.*, 1986; Thomas and Rothstein, 1989; Nickoloff and Reynolds, 1990; Nickoloff, 1992; Prado *et al.*, 1997), suggesting either that the increased accessibility of the chromosome during transcription facilitates the activity of the recombination machinery (Blackwell *et al.*, 1986), or that there is an evolutionary link between the processes of transcription and recombination (Kassavetis and Geiduschek, 1993). In this respect, it has been proposed that methylation (which is a gene silencing mechanism) in higher organisms may contribute to stabilising their genomes by preventing homologous recombination between dispersed DNA repeats. This would provide biological significance to the fact that in many eukaryotes methylation occurs preferentially in repeated DNA sequences (Assaad and Signer, 1992; Rossignol and Faugeron, 1994). Experiments were conducted in cultured human fibrosarcoma cells to test whether these findings are applicable to gene targeting systems (Thyagarajan *et al.*, 1995). It was found that gene targeting was

significantly enhanced (3-fold to >20-fold) in the presence of an agent that stimulated target site transcription. Taken together, these results raise the possibility of developing more efficient gene targeting protocols in those cases for which the level of transcription of the target locus can be manipulated.

c.2.introduction of double-strand breaks

c.2.i.induction of DNA damage

Several types of DNA damage have a remarkable effect at increasing homologous recombination in eukaryotic cells. These include lesions caused by chemical carcinogens (Wang *et al.*, 1988), UV irradiation (Tsujimura *et al.*, 1990), gamma irradiation (Mudgett and Taylor, 1990) and photoreactive molecules (Saffran *et al.*, 1992). All these agents cause random DNA damage, thus activating the recombinatorial repair mechanisms of the cell (p53, Ku, PARP, Rad51). Of course, such a non-specific outcome is not desirable in gene targeting procedures, particularly in those for therapeutic correction of mutated genes. Nevertheless, it has been recently demonstrated that triplex-forming oligonucleotides, either bound to photoreactive molecules (Faruqi *et al.*, 1996) or by themselves (Faruqi *et al.*, 2000) can cause sequence-specific DNA damage around the target, thus stimulating the recombination machinery locally. This could be a way to direct homologous recombination enhancement to the desired DNA region in gene targeting experiments.

c.2.i.use of rare-cutting enzymes

The use of rare cutting enzymes to generate double-strand breaks (DSBs) in the chromosome has also been reported to improve intrachromosomal recombination (Brenneman *et al.*, 1995) and gene targeting frequencies (Choulika *et al.*, 1995; Smih *et al.*, 1995; Donoho *et al.*, 1998) in a variety of organisms. Studies in

mammalian cells show that repair of DSBs involves both homologous recombination and non-homologous DNA-end joining mechanisms (Roth and Wilson, 1988; Rouet *et al.*, 1994), results that are consistent with the prevailing models of recombination (pages 7-16). Introduction of I-*Sce*I sites (whose long recognition sequence makes them very rare in genomic DNA) into defined loci and subsequent cleavage of the target by expression of or electroporation with I-*Sce*I has been shown to increase gene targeting 50- to 10^4-fold in several mouse cell lines. However, practical applications of this strategy are hindered by the necessity of 'targeting the target' beforehand. The use of triplex-forming oligonucleotides linked to DSB-inducing agents has been proposed to overcome this limitation, by inducing cleavage of specific sequences in the target locus in one step (page 43).

c.3.cell cycle rates

Finally, it has been recently demonstrated that cell cycle rates may have a drastic effect on targeting efficiency. Targeting frequencies in mouse ES cells seem to keep an inverse linear correlation with the time of cell doubling, a parameter that depends both on the intrinsic characteristics of each cell line and the growth conditions (Udy *et al.*, 1997). The latter factor appears to be particularly important, since it was found that even a non-isogenic line, given optimised conditions for cell growth (i.e, high serum concentration), can support homologous recombination events up to 21-fold more frequently than an isogenic line grown in sub-optimal conditions. These results provide an explanation for variations in gene targeting frequencies detected between experiments. It is clear that maintaining ES cells under optimal growth conditions is essential to ensure reliably efficient gene targeting. Deviations from these conditions (resulting from factors such as rate of passage, cell density at passage or electroporation, time from last passage, cell overgrowth or variations in media or serum composition) can strongly affect its frequency.

d. expression of recombination genes

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d.1.rad genes

Recombination is enhanced following overexpression of genes directly involved in this process. In a recent report, a 2-3-fold overexpression of **Rad51** in CHO cells was found both to stimulate homologous recombination between integrated genes (20-fold) and to increase resistance to ionising radiation (Vispé *et al.*, 1998). Gene targeting frequency was also increased 2-fold in human cells with an extra copy of Rad51 (Yáñez and Porter, 1999) and 10-fold in mouse F9 teratocarcinoma cells transfected with its bacterial counterpart, recA (Shcherbakova, 2000). Comparable results were obtained in monkey cells when overexpressing **Rad52**, another component of the RAD52 epistasis group participating in DSB repair (Park, 1995). The use of this strategy to test the recombinagenic activity of other candidate proteins, such as **RecBC/RecBCD** could be useful not only to understand the molecular mechanisms underlying homologous recombination, but also to develop more efficient targeting protocols.

d.2.mismatch-repair genes

Down-regulation of genes not directly involved in recombination may also prove to be most informative. For instance, the mismatch repair (MMR)-related

genes ***Msh2***, ***Mlh1***, ***Pms1*** and ***Pms2*** represent a considerable barrier to homologous recombination in many organisms (Bailis and Rothstein, 1990; Mezard *et al.*, 1992; Selva *et al.*, 1995). Several groups have observed that defects in the mismatch repair machinery both in prokaryotes and eukaryotes greatly reduce the inhibitory effect of mismatches. It has been proposed that non-identical sequences are prevented from recombining due to the recognition of the mismatches by the MMR enzymes, after which the forming heteroduplex DNAs are unwound (Datta *et al.*, 1996). Alternatively, MMR may inhibit the formation of mismatched heteroduplices, as suggested by studies in the bacterial equivalent ***mutSL*** pathway (Worth *et al.*, 1994). In any case, the effect of the mismatches in inhibiting non-isogenic pairing seems to be position-dependent, since the presence of mismatched sequences away from the ends of a recombining partner does not prevent heteroduplex formation (Negritto *et al.*, 1997). This observation confirms that initiation is the critical, rate-limiting step of homologous recombination.

Inactivation of the MMR ***Msh2*** gene in murine cells results in a hyperrecombinatorial phenotype, according to a report by de Wind *et al.* (1995). Further research in *S. cerevisiae* has demonstrated that mutations in this gene stimulate the frequency of recombination between mismatched sequences over 1000-fold relative to wild-type cells (Negritto *et al.*, 1997). Indeed, *Msh2*-defective cells supported isogenic recombination 40-fold more frequently than wild-type cells transfected with the same constructs, unexpectedly suggesting that Msh2 represses not only mismatched but also isogenic recombination through a mechanism yet to be elucidated. A strategy based on the transient down regulation of this gene by antisense oligonucleotides could be useful to improve gene targeting efficiency.

d.3.oncogenes

d.3.i.p53

The tumour suppressor ***p53*** gene has also been directly related to DNA recombination. Its inactivation is an almost universal step in the development

cancer, and loss of its wild-type function is associated with hyperrecombination and karyotypic abnormalities such as amplifications, deletions, inversions and translocations (Nelson and Kastan, 1994). p53 is a potent transcriptional activator with the ability of sequence-specifically binding to DNA (Lane 1992). It is thought to function in the maintenance of genomic stability by modulating transcription and interacting with cellular proteins to influence the cell cycle, DNA repair and apoptosis. Following DNA damage, wild-type p53 upregulates the expression of the $p21^{Waf1}$ protein to effect a cell cycle arrest in order to prevent the replication of damaged DNA (Hartwell, 1992. If both cell cycle arrest and DNA repair functions fail to restore the integrity of the genome following DNA damage, p53 may also trigger the elimination of the abnormal cell via apoptosis (Yonish-Rouach *et al.*, 1991; Hermeking and Eick, 1994).

Even though it has been very recently reported that the frequency of meiotic recombination is normal in p53-deficient mice (Gersten and Kemp, 1997), several studies suggest that p53 directly affects the rate of somatic homologous recombination. Some of the chromosomal lesions arising from the loss of p53 function are based on molecular processes that involve recombinatorial events. Biochemical data show that p53 exhibits both exonuclease (Mummenbrauer *et al.*, 1996) and strand transfer activities (Oberosler *et al.*, 1993). Gene amplification, which may also require recombination, is significantly increased in p53-deficient cell lines (Livingstone *et al.*, 1992). Functional p53 tightly binds to Holliday junctions and facilitates cross-over resolution (Lee *et al.*, 1997). The rate of spontaneous intrachromosomal homologous recombination is increased up to 100-fold in a variety of *p53*-deficient cell types (Bertrand *et al.*, 1997; Meyn *et al.*, 1994; Mekeel *et al.*, 1997; Willers *et al.*, 2000a). Finally, it has been unequivocally demonstrated that p53 is directly linked to homologous recombination processes via Rad51/RecA protein interaction (Stürzbecher *et al.*, 1996; Buchhop *et al.*, 1997) and specifically recognising mismatches in heteroduplex intermediates (Dudenhöffer *et al.*, 1998).

d.3.ii.BRCA1 and BRCA2

Although BRCA1 and BRCA2 have been specifically associated with breast and ovarian tumourigenesis (Miki *et al.*, 1994; Wooster *et al.*, 1995), they are

ubiquitously expressed (Miki *et al.*, 1994) and appear to be essential in more widespread processes, such as embryonic proliferation (Hakem *et al.*, 1996; Suzuki *et al.*, 1997), regulation of transcription (Chapman and Verma, 1996; Milner *et al.*, 1997; Anderson *et al.*, 1998) and maintenance of genomic integrity through homologous recombination and DSB repair (reviewed by Welcsh *et al.*, 2000). Despite the lack of a convincing model to explain the role of these proteins in homologous recombination, their involvement in this process has been suggested by the interaction of BRCA1 and BRCA2 with Rad50 (Zhong *et al.*, 1999) and Rad51 (Scully *et al.*, 1997; Chen *et al.*, 1998), as well as the ATM-mediated phosphorylation of BRCA1 following DNA damage. BRCA1-deficient ES cells show decreased homologous recombination and increased non-homologous recombination frequencies compared to that of wild-type cells (Snouwaert *et al.*, 1999). This observation suggests that overexpression of BRCA genes may enhance gene targeting frequency, provided that strategies to overcome the consistently low levels of expression observed from BRCA1 transgenes are devised.

d.3.iii.BLM

Mutations in ***BLM*** cause Bloom's syndrome (BS), a recessive human genetic disorder characterised by dwarfism, immunodeficiency and cancer predisposition, among other abnormalities (rev. by German *et al.*, 1997). Stage-specific apoptosis, developmental delay and, ultimately, embryonic lethality are observed in *BLM* knockout mice (Chester *et al.*, 1998). *BLM* belongs to the RecQ subfamily of DNA helicases (Ellis *et al.*, 1995), which also includes *S. cerevisiae Sgs1p* and the human Werner syndrome gene *WRN* (Yu *et al.*, 1996; Stewart *et al.*, 1997). The products of these three genes are active helicases with 3'-5' DNA unwinding activity. Like in *WRN* knockouts, *BLM* -/- cells display a number of chromosomal aberrations in culture (Tsuji *et al.*, 1988), although the highly increased rate of sister chromatid exchange and error-prone homologous recombination are unique to BS cells. Immunofluorescence shows that *BLM* colocalises with Rad51 in meiotic recombinational hotspots, which suggests a role of this protein in meiotic homologous recombination (Moens *et al.*, 2000).

d.4.genes involved in illegitimate recombination

ATM is a member of the phosphatidylinositol 3-kinase family, which also includes the catalytic subunit of the **DNA-PKcs** (page 18). The ATM protein is involved in DSB recognition and subsequent p53 phosphorylation. Phosphorylated p53 then initiates either apoptosis or cell cycle arrest (see above). Ataxia telangiectasia (AT) patients have inactivating mutations in both copies of the *ATM* gene, which results in chromosomal instability and high propensity to cancer, among many other severe symptoms including dilated blood vessels, premature ageing and cerebellar degeneration (rev. by Rotman and Shiloh, 1998). Using a mouse model that facilitates *in vivo* detection of recombination events between a genomic tandem duplication, it has been recently reported that ATM deficiency increases the rate of intrachromosomal homologous recombination (Bishop *et al.*, 2000).

Although NHEJ (page 17) seems to be the default DNA-repair mechanism in vertebrates (in contrast with yeast), there is one biological stage where HR predominates in mammalian germ cells. A series of experiments conducted in both mitotic and meiotic murine cells (Goedecke *et al.*, 1999) show that meiotic recombination is essentially independent of NHEJ. Remarkably, **Ku70/80** levels are undetectable from preleptotene to zygotene, phases normally associated with the generation and processing of DSB. This observation led to the hypothesis that Ku relative abundance may determine the choice of one or another route. If this is the case, controlled down-regulation of Ku and other enzymes involved in NHEJ (such as **DNA-PKcs or PARP**) could be used to shift the balance between NHEJ and HR towards the latter pathway in order to increase gene targeting frequency.

vi. objectives

The general aim of this Ph.D. project is to explore different experimental approaches to manipulate effective targeting frequency in ES and somatic cells. The specific objectives are summarised as follows:

a) To develop a sensitive *ES cell-based test system* to monitor response in effective targeting frequency (E.T.F.) to several treatments **(Chapter III)**.

a) To manipulate E.T.F. by

i.changing the *design of the vector* **(Chapter IV)**.

ii.altering the *expression of recombination-related genes* **(Chapter V)**.

iii.modifying *cell/ target conditions* **(Chapter VI)**.

Chapter II. Materials and Methods

i.manipulation and analysis of DNA
ii.manipulation and analysis of RNA
iii.manipulation and analysis of proteins
iv.plasmids
v.cell culture

i. manipulation and analysis of DNA

a.DNA extraction and quantitation
b.DNA analysis
c.plasmid construction

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a.DNA extraction and quantitation

a.1.extraction of mammalian genomic DNA

a.2.extraction of plasmid DNA from bacteria

a.2.i.minipreps

a.2.ii.maxipreps and gigapreps

a.3.DNA purification

a.3.i phenol-chloroform extraction

a.3.ii.ethanol precipitation

a.4.DNA quantitation

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a.1.extraction of mammalian genomic DNA

The following procedure for mammalian DNA isolation has been adapted from Laird *et al.*(1991). Lysis buffer was prepared by mixing 100 mM Tris HCl pH 8.5 (50 ml from 1M Tris pH 8.5 stock), 5 mM EDTA pH 8.4 (5 ml from 0.5 M EDTA stock) and 200 mM NaCl (33.3 ml from 3M NaCl stock), for a total volume of 490 ml in distilled water. This solution was autoclaved and then supplemented with 10 ml of 10 % SDS (final concentration 0.2 %) and proteinase K (100 μg/ml) just before use. Cell cultures were rinsed onced with PBS before addition of lysis buffer (3 ml for 25 cm^2 flasks, 10 ml for 75 cm^2 flasks). Following overnight incubation at 37 ^{0}C with gentle shaking, one volume of isopropanol was added to the lysate. Samples were mixed or

swirled for several hours until a white DNA precipitate was visible. Using a Pasteur pipette, the DNA was lifted and placed in an eppendorf. Excess liquid was eliminated and the DNA was dissolved in 500 µl of Tris HCl buffer pH 8.5. Vials were incubated at 70 ^{0}C for several hours.

a.2.extraction of plasmid DNA from bacteria

a.2.i.minipreps

For routine plasmid DNA extraction from bacteria, individually picked colonies were incubated overnight at 37 ^{0}C in Falcon 15 ml tubes containing 5-7 ml of LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 litre of distilled water, pH 7.5; autoclaved) supplemented with 100 µg/ml of ampicillin. Following centrifugation of 1,5 ml cultures (5 minutes; 10,000 rpm) supernatants were discarded and pellets resuspended in 250 µl of solution 1 (50 mM Tris-HCl pH 8.0, 10 nM EDTA, 100 µg/ml RNase A). Lysis buffer (250 µl of 200 mM NaOH, 1 % SDS) was subsequently added to this solution, which was gently mixed and incubated for 5 minutes at room temperature. The lysis reaction was stopped by adding 350 µl of neutralising solution (3.0 M potassium acetate pH 5.5). Immediately after centrifugation (10 minutes at 13,000 rpm) supernatant was recovered and taken to a fresh tube, where it was mixed with an equal volume of isopropanol and centrifuged again (10 minutes at 13,000 rpm). Pellets were air-dried for 5 minutes and then resuspended in a suitable volume of Tris-HCl buffer pH 8.5.

For those applications requiring high purity DNA (such as subcloning), the Qiagen miniprep kit was used.

a.2.i.maxipreps and gigapreps

Purification of large amounts of plasmid DNA for electroporation was done using the Qiagen Maxiprep and Gigaprep kits. These procedures yield an average of 500-1000 μg of DNA from 500 ml cultures (Maxiprep) and 5-10 mg from 2,5 l cultures (Gigaprep). Large plasmids (i.e. >10 kb) are less efficiently purified.

a.3.DNA purification

Phenol/chloroform is used to extract and purify nucleic acids from a variety of sources (Sambrook *et al.*, 1989). An equal volume of 1:1 phenol:chloroform (Life Technologies) added to an aqueous DNA solution causes the denaturation and dissociation of proteins from DNA. After inverting several times the tube containing the mixture, centrifugation (13,000 rpm, 20 minutes) yields two distinct phases: a lower organic phenol:chloroform phase contaning the protein (mostly in the white, flocculent interphase) and the lighter aqueous phase with the DNA.

DNA was recovered from the aqueous solution by adding 1/10 volume of 3M sodium acetate pH 5.5 and 2 volumes of 100 % ethanol (alternatively, 1 volume of isopropanol can be used). The tube was mixed and then chilled for at least 30 minutes at –80 ^{0}C or 2 hours at –20^{0}C. This ensures a 75 % recovery for volumes <1ml containing at least 10 μg of DNA. For very low DNA concentrations, 2 μl of seeDNA (Amersham) were added to each tube, regardless of the volume. This pink-coloured reagent co-precipitates with the DNA and makes identification of the pellet easier. The tube was subsequently centrifuged for 30 minutes (13,000 rpm, 4 ^{0}C), supernatant removed, and the pellet rinsed once with 500 μl 70 % ethanol. This step is essential for getting rid of the excess salt, and for very sensitive applications (such as blunt-end ligation) it was done twice. The tube was centrifuged as before for 5 minutes, and the pellet allowed to air-dry for 5 minutes after removal of excess ethanol. DNA was resuspended in an

appropriate volume of distilled water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5).

a.4.DNA quantitation

Quantitation of diluted DNA (typically 1:100 in distilled water) was performed in an UNICAM 5625 UV/VIS spectrophotometer at a λ = 260 nm. Since an OD of 1 corresponds approximately to 50 μg/ml of double-stranded DNA, DNA concentration can be calculated in the following manner:

μg DNA/μl = O.D. x dilution factor x 50

When there was not sufficient DNA for spectrophotometric assay, fluorescent yields of 1 μl aliquots were compared to a series of standards of the same size in ethidium bromide-supplemented agarose gels.

b.DNA analysis

b.1.restriction analysis

b.2.southern blotting

b.3.polymerase chain reaction (PCR)

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b.1.restriction analysis

Genomic and plasmid DNA was digested using restriction enzymes and buffers (Roche) according to manufacturer's instructions. For DNA analysis, a 10-fold excess of enzyme was normally used. The following examples describe the typical proportions of reagents in a standard linearisation of plasmid DNA for electroporation (1) and restriction analysis (2), respectively.

(1)	Plasmid DNA	200 µg (200 µl)
	Buffer H	50µl
	Xho I	30 µl (300 units; 1 unit digests 1 µg of DNA in 1 hour)
	Distilled water	220 µl
	Total volume:	500 µl
	Incubation:	2-4 hours at 37 ^{0}C
(2)	Plasmid DNA	1 µg (1 µl)
	Buffer A	2 µl
	*Sma*I	1 µl (10 units)
	Distilled water	16 µl
	Total volume	20 µl
	Incubation:	1-5 hours at 25 ^{0}C

After digestion, and following addition of 6X loading buffer, samples were loaded in 0.8 % agarose gels: 2 g SeaKem agarose molecular biology grade; 250 ml TAE buffer and 3 µl of a 10mM ethidium bromide solution. Agarose gels were run in TAE buffer either for 2h (100-120 V) or overnight (25-30 V). A UV transilluminator was used to photograph the gels.

b.2.southern blotting

Southern blotting (Southern, 1975) allows DNA fragments corresponding to a particular probe to be identified directly from a restiction digest of genomic DNA. Since DNA fragments cannot be handled directly in an agarose gel, it is necessary to transfer them to membrane (nitrocellulose or nylon) which provides a suitable support. After immobilisation, the DNA can be subjected to hybridisation analysis, enabling bands with sequence similarity to a labelled probe to be identified. The following protocol was adapted from Sambrook *et al.* (1989).

Genomic DNA digests (10 µg/sample) were loaded in a 0.8 % agarose gel and run overnight at 30-40 V. A UV photograph was taken before proceeding to denaturation, an essential step for subsequent hybridisation of ssDNA probes. Denaturing buffers were prepared by dissolving 87.66 g of NaCl (1.5 M final concentration) and 20 g of NaOH (0.5 M f.c.) in 1 litre of distilled water (solution A); and 87.66g of NaCl (1.5 M f.c.), 500 ml of 1 M Tris HCl pH 7.5 (0.5 M f.c.) and 2 ml of 0.5 M EDTA pH (1 mM f.c.) in one litre of distilled water (solution B). DNA was denatured washing the gel 2 x 15 minutes in solution A and 2 x 15 minutes in solution B. The blotting apparatus was assembled as follows: a glass plate was placed on top of a large baking dish filled with 20x SSC buffer (175.3 g NaCl and 88.2 g tri-sodium citrate in 1 litre of distilled water), which is drawn from the reservoir by capillary transfer through four 3MM Whatman papers (two in direct contact with the buffer and two gel-sized papers on top). The gel was laid over the papers in an inverted position, avoiding the formation of bubbles. A nitrocellulose filter cut to the size of the gel (Hybond) was

carefully rinsed in distilled water and then immersed in 20x SSC for 5 minutes before placing it over the gel, smoothing out bubbles with a pipette. The apparatus was completed by successively laying two pieces of 3MM paper (same size of the gel) soaked in 2x SSC, a stack of paper towels and a 500g- 1kg weight. Filters were recovered the next day, rinsed and wrapped in Saran paper. DNA was fixed to the membrane by UV-crosslinking. After this treatment, filters were stored at room temperature.

Prehybridisation solution was prepared with 50 ml of 1 M sodium phosphate (0.5 M final concentration), 35 ml of 20 % SDS (7% f.c.), 200 µl of 0.5 M EDTA (1 mM f.c.) and 15 ml of distilled water, for a total volume of 100 ml. Filters were incubated for 1-2 hours at 65 ^{0}C in a hybridisation tube. DNA probes for hybridisation (~25 ng) were dissolved in a total volume of 11 µl, and then boiled for ten minutes and cooled down in ice. 4 µl of High Prime solution (Roche) were added for random priming. This reaction mixture contains random oligonucleotides, Klenow polymerase, dATP, dGTP and dTTP. Denatured DNA was radioactively labelled upon addition of 5 µl of ^{32}P-dCTP (incubation for 10-20 minutes at 37 ^{0}C). Addition of 12 µl of 2M NaOH stops the reaction and denatures the DNA. Incorporation of ^{32}P-dCTP was measured using a scintillation counter.

The probe was added to the tube containing the filter in prehybridisation solution, and then incubated at 65 ^{0}C overnight. The following day the filter was removed and washed at 65 ^{0}C 2x 15 minutes in solution 1 (2x SSC, 0.1 % SDS) and 2x 15 minutes in solution 2 (0.2x SSC, 0.1 % SDS). Without drying it out, the membrane was then carefully wrapped in Saran paper for exposure to X-ray film (1 week, -80 ^{0}C). The film was developed in an autoradiograph.

b.3.polymerase chain reaction (PCR)

Polymerase chain reaction was performed using the Expand High Fidelity PCR system (Roche). This kit contains an enzyme mix of thermostable Taq DNA and two

Pwo DNA polymerases for proof-reading activity (Barnes, 1994), ensuring high fidelity amplification of genomic DNA. Although PCR conditions had to be adjusted for every individual experiment, a normal setting for DNA amplification of fragments of up to 10 kb can be summarised as follows:

Master mix A:10 mM dNTP mix (Roche)	1 µl (0.2 mM final concentration)
Downstream primer 0.1 µg/µl (Genosys)	1 µl (300 nM ")
Upstream primer 0.1 µg/µl (Genosys)	1 µl (300 nM ")
Template genomic DNA	1 µl (100-350 ng)

Up to 25 µl in autoclaved, distilled water

Master mix B: 10X Expand HF buffer (with 15 mM $MgCl_2$)	5µl
Expand High Fidelity PCR enzyme mix	0.75 µl (2.6 units)

Up to 25 µl in autoclaved, distilled water

The preparation of two separate master mixes helps preventing degradation of primers and/or template by interaction with the polymerases in the absence of dNTPs. 25 µl of each tube were mixed together in a thin-walled PCR eppendorf, and the solution was covered with two drops of mineral oil to avoid evaporation during amplification. Samples were placed in a Hybaid Omnigene Thermocycler, which was typically set in the following manner:

1X denaturing step	94 ^{0}C for 4 minutes
10X cycle 1	Denaturation, 94 ^{0}C for 15 seconds; annealing, 45-65 ^{0}C for 30 seconds; elongation, 68 ^{0}C for 5 minutes
25X cycle 2	Denaturation, 94 ^{0}C for 15 seconds; annealing, 45-65 ^{0}C for 30 seconds; elongation, 68 ^{0}C for 5 minutes (+20 seconds extra/cycle to ensure proper elongation as the concentration of amplified DNA grows higher).

The above settings were used for long range PCR. For fragments <1 kb, 35x cycle 1 was sufficient to get efficient amplification. The annealing temperature was usually set 5 ^{0}C below that of the melting point of the primers, but it was increased in some cases to avoid amplification of unspecific products. A simple way to roughly estimate the melting point of the oligonucleotides is adding up 4 ^{0}C for every G+C and 2 ^{0}C for every A+T base pair.

For the PCR analysis described in page 141 of this work, 2 µl of each PCR reaction (50 µl) were taken after 5 cycles and digested as indicated in a total volume of 20 µl (restriction mix). Restriction enzymes were inactivated by heating at 70 ^{0}C for 15 minutes. A new PCR reaction was prepared, using 2 µl of the inactivated restriction mix as template in master mix A (see also page 74 for RT-PCR experimental details).

c.plasmid construction

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c.1.extraction of DNA bands from agarose gels

Small sized DNA bands can be easily extracted from from low melting point 0.6-0.8% agarose gels following protocols based on retention of the fragment in positively charged silica-gel minicolumns. The QIAquick Gel Extraction kit (Qiagen) was normally used for small bands with excellent results. However, it was repeatedly observed that the quality of DNA bands >10 kb isolated with this procedure was not as good, particularly for subcloning purposes. For the isolation of large DNA bands, 10 cm strips of dialysis tubing were boiled in distilled water for 5 minutes, rinsed in distilled water and secured at one end with a plastic clip. DNA bands were cut from gel and carefully put into the tubing, which was then filled with 500 µl of TAE buffer supplemented with 1 µl of 10 mM ethidium bromide. After sealing the other end with another clip, the device was placed on a gel tank and run at 100 V for 2 hours. Current was then reversed for 1 minute in order to detach DNA from the tubing. Liquid was gently removed from tubing into an eppendorff and DNA was phenol/chloroform extracted (optional) and ethanol precipitated.

c.2.ligation

Ligation of DNA fragments was usually performed using the Rapid DNA Ligation kit (Roche). This system enables sticky-end or blunt-end ligations in 5-10 minutes at room temperature. Ligation reactions contained no more than 200 ng of total DNA (insert + vector) in a total volume of 21 µl. The molar ratio of vector to insert was normally 1: 4, although 1: 5 and 1: 6 ratios were also ocassionally employed. Prior to

ligation, vectors were incubated (1 hour, 37 ^{0}C) with shrimp alkaline phosphatase (SAP) (Amersham) in order to de-phosphorylate the ends and prevent self-ligation. SAP was inactivated by heating the sample at 65 ^{0}C for 15 minutes.

Where required, blunt ends were generated by adding 1-5 units of the Klenow enzyme and 1µl of 1mM each dNTP to the restriction digest mixture (½ hour at room temperature). The Klenow fragment consists of the C-terminal domain of *E. coli* DNA polymerase I, which lacks the 5'-3' exonuclease activity while retaining the DNA polymerase and 3'-5' exonuclease activities (Joyce and Grindley, 1983). Repair of 5' overhanging ends is carried out by 3'-5' polymerase activity, whereas repair of 3' extensions is carried out by 3'-5' exonuclease activity. This latter process is not as effective using this enzyme, and that is why T4 DNA polymerase is recommended to repair overhanging 3' ends.

Subcloning of some DNA fragments may also require the introduction of specific restriction endonuclease recognition sequences. Linkers are short, self-complementary oligonucleotides which form blunt end duplexes containing the desired sites. For ligations involving phosphorylated linkers (annealed by heating at 95 ^{0}C for 5 minutes and overnight cooling down), a 100- to 1000- fold molar excess of linker (normally 0.1 µg) improved substantially the efficiency of the reaction.

The following example describes a typical ligation reaction with a 1:4 vector to insert ratio: 100 ng of DNA vector (10 kb) and 40 ng of DNA insert (1 kb) were dissolved with 2 µl of 5x DNA Rapid Ligation buffer (Roche) in a total volume of 10 µl (distilled water added as needed). After thorough mixing, 10 µl of 2x DNA Rapid Ligation buffer (Roche) were pipetted into the vial. The solution was mixed again before the final addition of 5 units (1 µl) of T4 DNA ligase (Roche). This ligation mixture was incubated for 5-10 minutes at room temperature.

c.3.transformation

For routine circular plasmid transformation, Subcloning Efficiency DH5α competent cells (Life Technologies) were used. Cells (50 μl) were thawed on ice and incubated with 50-500 ng of plasmid DNA for 30 minutes. After a 20 second heat-shock at 37 ^{0}C, vials were placed on ice for two minutes. Following 1 hour of incubation in 900 μl of LB medium (220 rpm, 37 ^{0}C), cultures were diluted as necessary, plated in 9 cm agar dishes and incubated overnight at 37 ^{0}C. For ligations of large DNA fragments and blunt-end ligations, Maximum Efficiency DH5α supercompetent cells (Life Technologies) and Gold Ultracompetent cells (Stratagene) were used, following manufacturer's transformation protocols.

ii. manipulation and analysis of RNA

a.mammalian RNA extraction and quantitation
b.RNA analysis

a.RNA extraction and quantitation

RNA was extracted from mammalian cell cultures using the TRIzol reagent (Life Technologies), according to manufacturer's instructions. This product is a mono-phasic solution of phenol and guanidine isothiocyanate which maintains the integrity of total RNA while lysing and dissolving cell components. Addition of chloroform and centrifugation separates the solution into an organic organic phase (bottom) and an aqueous phase (top), from which RNA can be isolated by isopropanol precipitation.

Quantitation was performed as described for DNA (page 63). 1 OD at 260 nm corresponds to ~40 μg/ml of single-stranded RNA.

b.RNA analysis

b.1.northern blotting

b.2.RT-PCR

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b.1.northern blotting

The rationale behind this technique is very similar to that previously described for DNA blotting (page 65). Fractionated RNA is transferred from an agarose gel to a suitable support for subsequent hybridisation analysis with DNA or RNA probes. The main difference with Southern blotting is that RNA has to be maintained at denaturing conditions at all times in order to avoid the formation of secondary structures by intramolecular base pairing. This was achieved by treating agarose gels with formaldehyde. In order to prevent degradation of the samples, RNase free solutions and glassware were used. Autoclaved water was treated with diethyl pyrocarbonate (DEPC, 0.1 %) and working surfaces were throughly cleaned with RNaseZAP (Sigma). The following protocol has been adapted from that of Sambrook *et al.* (1989): 10x running buffer (RB) was prepared by dissolving 4.18g of MOPS (200 mM), 0.372g of EDTA (10 mM) and 0.410g of sodium acetate (50 mM) in 100 ml of DEPC-treated distilled water. pH was adjusted to 7.0 with 5M NaOH. Formaldehyde agarose gels (0.8-1.2 %) were prepared by melting the appropriate amount of agarose in 10 ml RB (1x), 72.1 ml DEPC-water and 17.9 ml of 38 % formaldehyde. Gels were poured and set inside a fume hood. RNA samples (max. 40 μg) were dissolved in a total volume of 20 μl of sample buffer (200 μl of 10x RB, 1 ml of deionised formamide and 356 μl of 38 % formaldehyde) and then heated to 65 ^{0}C for 5 minutes. 8 μl of dye solution (7.5 % Ficoll 400 and bromophenol blue) were added to each sample before loading. After overnight running in 1x RB (20 mA), gels were stained in 1x RB containing 5-10 mg of ethidium bromide and photographed under UV transillumination. The absence of RNA degradation was verified and gels were destained again (1x RB, 30 minutes). A corner of the gel was cut off in order to determine the orientation. The transfer apparatus was

assembled as for Southern blot (page 65). Transfer of RNA to the nitrocellulose membrane was allowed to proceed overnight. Next day, the filter was recovered and rinsed briefly in 6x SSC to remove traces of agarose. Cross-linking and hybridisation were also done as before.

b.2.RT-PCR

RT-PCR was performed using the SUPERSCRIPT preamplification kit (Gibco BRL – Life technologies). This system is designed to synthesise first strand cDNA from purified total RNA, making use of oligo (dT) primers for hybridisation with the 3' poly-A tails found in eukaryotic mRNAs. The first strand cDNA synthesis reaction is catalysed by the enzyme M-MLV RNase H^- reverse transcriptase (RT), which retains the DNA polymerase activity of RNase H while lacking the capacity to degrade RNA. PCR amplification of single stranded-cDNAs obtained by mRNA retrotranscription was done as described (page 66). The reaction mix would contain:

10x PCR buffer	5 µl
25 mM $MgCl_2$	3 µl
10 mM dNTP mix	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (5 units)	1 µl
cDNA template from first strand reaction (as obtained using the standard conditions described by the manufacturer)	2 µl
Up to 50 µl	

To determine the effect of PCR amplification of contaminant genomic DNA in the RNA samples, it is recommended to set a control PCR reaction from the RNA extracts untreated with RT (not done in this work). For details about the experiment described in figure 39, see page 68.

iii. manipulation and analysis of proteins

a.protein extraction and quantitation
d.protein analysis

a.protein extraction and quantitation

Cells in culture were washed twice with ice-cold PBS buffer and then incubated on ice for 30 minutes with 1 ml of ice-cold lysis buffer (LB). LB was freshly prepared every time by mixing 250 µl of 1 M Tris HCl pH7.5 (final concentration 25 mM), 300 µl of 5 M NaCl (f.c., 150 mM), 1 ml of 10 % Nonidet P40 (f.c., 1 %), 20 µl of 0.5 M EDTA (f.c., 1 mM), 40 µl of 0.5 M EGTA (f.c., 2 mM), 100 µl of 0.1 M dithiothreitol (DTT) (f.c., 1 mM), 10 µl of 50 mM phenylmethylsulfonyl fluoride (PMSF) (f.c., 50 µM) and 8,28 ml of distilled water for a total volume of 10 ml. After incubation, cell lysate was scraped and transferred into a 1.5 ml eppendorf tube. Following centrifugation at 13,000 rpm (4 ^{0}C, 5 minutes), supernatant was aliquoted in 0.5 ml eppendorf tubes (100 µl each) for long-term storage at –70 ^{0}C. Protein extracts were not be refrozen once thawed.

Samples were taken to measure protein concentration with the BCA Protein Assay Kit (Pierce). This assay is based on the colourimetric detection of Cu^{+1} cations resulting from reduction of Cu^{+2} by protein in alkaline medium (the biuret reaction), in the presence of bicinchonic acid (BCA). The purple reaction product is formed by the chelation of of two molecules of BCA with one cuprous ion. Absorbance of this product at 562 nm is in linear correlation with increasing protein concentrations. For every quantitation, diluted bovine serum albumin (BSA) standards were prepared (25 µg/ml – 2000 µg/ml) and used to determine response curves after incubation at 37 ^{0}C (30 minutes). Samples were incubated similarly and 562 nm O.D. values compared

against the standard curve. BCA reagents were dispensed as indicated by manufacturer's protocols.

b.protein analysis

b.1.western blotting

b.2.immunostaining

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b.1.western blotting

Western blotting allows the immunological detection of specific proteins transferred to a membrane after electrophoresis of a cell protein extract. Samples (15-30 μg) were thawed on ice, mixed in a 1:1 proportion with 2x loading buffer (4 % SDS, 20 % Glycerol, 0.2 % bromophenol blue, 100 mM Tris HCl pH 6.8 and 2 % β-mercaptoethanol to be added freshly every time) and boiled for 5 minutes just immediately before loading. Polyacrylamide gels were prepared by pouring 10 % resolving gel [30 ml of 30 % 37:5:1 acrylamide/bisacrylamide (Gibco), 36.6 ml of autoclaved distilled water, 22.5 ml of 1.5 M Tris HCl pH 8.8, 450 μl of 20 % SDS, 450 μl of 10 % SDS and 45 μl of TEMED, added in this order] onto a BioRad Protean II xi 1x 20 cm glass plate sandwich (¾ of its total volume capacity). The interphase was gently covered with distilled water to avoid delayed polymerisation induced by air contact. 1-2 hours later, the water was removed, the cones placed between the plates and 4% stacking gel (3.25 ml of 30 % 37:5:1 acrylamide/bisacrylamide (Gibco), 15.25 ml of autoclaved distilled water, 6.25 ml of 0.5 M Tris HCl pH 6.8, 250 μl of 20 % SDS, 250 μl of 10 % SDS and 55 μl of TEMED) poured onto the resolving gel. Polymerisation was allowed to proceed for 30 minutes – 1 hour. The gel was assembled to the Protean II xi electrophoresis cell core, and the apparatus loaded with running buffer (25 mM Tris HCl, 0.2 M glycine and 0.1 % SDS in a total volume of 5 litres, pH 8.3). Samples were loaded alongside a protein size marker (Rainbow, Amersham), using sequencing tips. After overnight running (W = 03; V = 050; mA = 25), the gel was carefully extracted and placed on a glass plate. The semi-dry transfer apparatus was prepared placing, from bottom (anode) to top (cathode), the following: 2x 3MM Whatman paper sheets soaked in anode I buffer (0.3 M Tris HCl and 20 % methanol in distilled water, pH 10.4); 1x 3MM Whatman paper sheet soaked in anode II buffer (25 mM Tris HCl, 20 %

methanol, pH 10.4); 1x nitrocellulose filter (Hybond- ECL, Amersham) soaked in distilled water; the polyacrylamide gel; and 2x 3MM Whatman paper sheets soaked in cathode buffer (25 mM Tris HCl, 20 % methanol and 40 mM of hexanoic acid in distilled water, pH 9.4). Transfer was performed at 200-300 mA (V < 22 V) for 1 hour. The membrane was taken out and blocked in TBS-T buffer (50 ml of 1 M Tris HCl pH 7.5, 50 ml of 3 M NaCl and 1 ml of Tween 20 and distilled water up to 1 litre) containing 2 % bovine serum albumin (BSA). Two hours later, this solution was replaced by TBS-T containing 1 % BSA and the primary antibody (concentration adjusted to manufacturer's indications). Incubation was allowed to proceed for 2 hours at room temperature. The membrane was washed 6x 5 minutes in TBS-T before applying the secondary antibody diluted in TBS-T (1 % BSA). After incubation (1 hour, room temperature), the membrane was washed as above. Secondary antibodies used in this work are conjugated to horse radish peroxidase (HRP), which catalyses the oxidation of luminol in the ECL reaction mix (Amersham). This results in emission of light and detection after quick exposures of X-ray films.

b.2.immunostaining

For immunostaining, medium was removed and cultures were rinsed three times with PBS. Cells were fixed first with 3.7 % paraformaldehyde (in PBS) for 10 minutes at room temperature and then with methanol/4'6-diamindino-2-phenylindole (DAPI, 1μg/ml; Sigma) for 6 minutes at –20 °C. DAPI binds to DNA and is routinely used as a nuclear counter-staining. Following three washes with PBS, microchamber slides were incubated with 35 μl of primary antibody in a humidified atmosphere (1 hour, 37 °C). Cells were rinsed again as above (10 minutes) and then incubated with the secondary antibody (1 hour, 37 °C). Slides were washed three times with PBS, briefly rinsed in distilled water and then mounted using DBS mounting solution. Cover slips were sealed with clear nail polish.

iv. plasmids

a.plasmid material
b.plasmid construction

a.plasmid material

The following **commercial** plasmids were used in this project: pcDNA3 (Invitrogen); pPUR (Clontech); pUCSV-BSD (Funakoshi); pIREShyg (Clontech); pCMV/Zeo (Invitrogen); pEM7/Zeo (Invitrogen); pTracer (Invitrogen); pVP22-*myc*-His (Invitrogen); pCR II (Invitrogen); pGEM-T (Promega); pBlueScript II SK +/- (Stratagene).

The plasmids listed below were obtained from **non-commercial** sources: pM44 (given by Andrew Smith, Centre for Genome Research, Edinburgh, UK) (figure 10); p2016 and p2017 (given by Hellen Wallace, CGR, Edinburgh); pSVZeo/Rad51 (Vispé *et al.*, 1998); pHis-dMTase (Battacharya *et al.*, 1999); pBS-PGK-neo (given by E. Gallagher, Roslin Institute, Edinburgh); pOct-Neo1 (McWhir *et al.*, 1996).

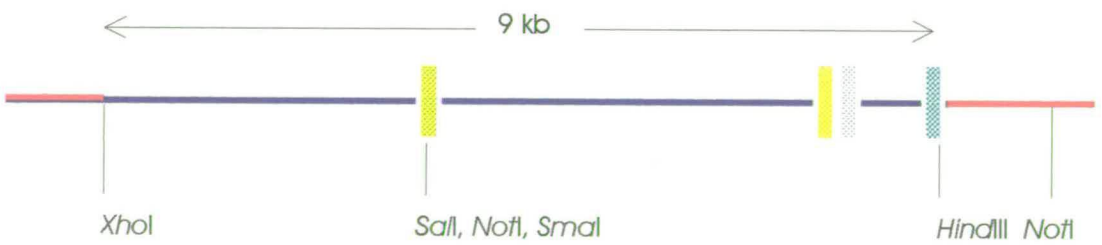

Figure 10. pM44 (given by A. Smith, CGR, Edinburgh, UK) is a vector designed to target the HPRT region encompassing intron 5 to exon 9. The total length of homology (isogenic with the 129 line, blue line and boxes) is 9 kb. Coloured boxes (from left to right): exons 6, 7, 8 and 9. Red lines, backbone vector (pBluescript). A multicloning site (*Sal*I, *Not*I and *Sma*I) has been engineered into the region corresponding to exon 6 for subsequent insertion of selectable markers. *Xho*I was normally used for linearisation.

b.plasmid construction

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b.1.p129-ΔHPRT

p129-ΔHPRT was constructed in collaboration with E. Gallagher. A 4 kb fragment (exon 6 - exon 7) of the *HPRT* locus (Melton *et al.*, 1984) was amplified by PCR (primer sequences: 5'A C G C G T C G A C G C T T T C C C T G G T T A A G C A G T A C A G and 5'A C G G G G T A C C C T G T A T C C A A C A C T T C G A G A G G T C) from murine strain 129-derived genomic DNA, and cloned into the 3'-T overhangs at the PCR insertion site of the plasmid pGEM-T (Promega). The region from exon 2 to exon 3 of the *HPRT* locus was similarly obtained (primer sequences: 5'-T A T G C G G C C G C G C G A T G A T G A A C C A G G T T A T G A C C-3' and 5'-T A T G C G G C C G C C T C C C A T C T C C T T C A T G A C A T C T C-3') and cloned into pGEM-T. A 3 kb *Not*I fragment of the latter plasmid containing the above genomic region was subcloned in the proper orientation into a *Not*I site of the former, to give p129-ΔHPRT. The *Sal*I site located immediately upstream exon 2 was then removed by partial digestion of p129-ΔHPRT with *Sal*I, electroelution of the resultant 10 kb band, filling-in recessed ends with DNA polymerase I-Klenow fragment (Boehringer) and blunt religation. This

plasmid, termed p129–ΔHPRT* (figure 11), has a unique *Sal*I site between the two regions of homology to *HPRT*, which was used in subsequent subcloning steps.

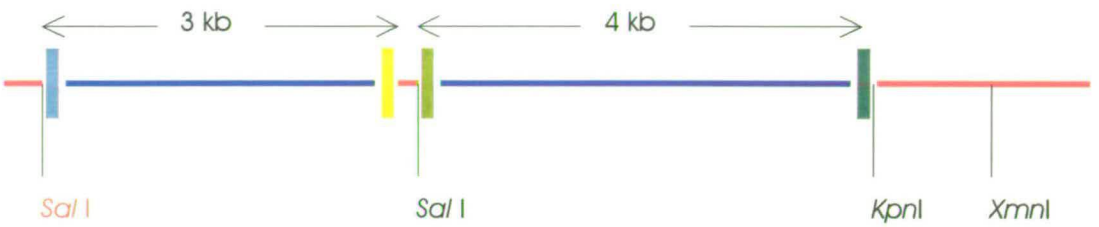

Figure 11. p129-ΔHPRT*. Coloured boxes (from left to right): *HPRT* exons 2, 3, 6 and 7. Blue lines represent regions of homology (7 kb in total). Red lines, backbone vector (pGEM-T). The *Sal*I site upstream exon 2 (red, dotted line) was eliminated as described in text.

b.2.p129-Zeo

A 1.2 kb *Xho*I/*Sal*I fragment of the plasmid pCMV/Zeo (Invitrogen) comprising the resistance factor to the antibiotic zeocin and the cytomegalovirus (CMV) promoter, was subsequently inserted into the *Sal*I site of p129-ΔHPRT* in both orientations.

b.3.p129/Oct-Zeo

A *Sal*I fragment containing 1.9 kb of the Oct3/4 promoter was purified from pOct/Neo1 (McWhir *et al.*, 1996) and subcloned into the *Xho*I site of pEM7/Zeo (Invitrogen), to give pOct/Zeo. This plasmid was digested with *Sal*I/*Bgl*II and a 2.4 kb fragment inserted into a *Sal*I/*BamH*I-digested pPolyIII shuttle vector, in order to place a suitable 5' *Xho*I site upstream the Oct/Zeo cassette. A 2.4 kb *Xho*I/*Sal*I fragment of the

resultant plasmid (pPolyIII/Oct-Zeo) was then subcloned into the *Sal*I-linearised p129-ΔHPRT* targeting vector, also in both orientations.

b.4.pCBA-Zeo and /Oct-Zeo

The CBA - derived constructs pCBA-Zeo and pCBA/Oct-Zeo feature the same regions of homology to the *HPRT* locus as their previously described 129-derived counterparts. Using the two sets of primers detailed above, two DNA fragments of 3 kb (exons 2-3) and 4 kb (exons 6-7) were PCR-amplified from CBA genomic DNA extracts and inserted into the PCR cloning sites of pGEM-T (Promega) and pCRII (Invitrogen), respectively. The *HPRT* region comprising exons 2-3 was then removed from pGEM-T as a 3 kb *Not*I fragment, and subcloned into a *Not*I site of the pCRII plasmid carrying exons 6-7. The digestion of this plasmid (pCRII-ΔCBA) with *Kpn*I and *Sal*I gave a 4 kb fragment (exons 6-7) which was then purified and ligated to a *Kpn*I/*Sal*I digest of pBlueScript II SK +/- (Stratagene). Following linearisation with *Not*I, the resultant plasmid was ligated to a 3 kb (exons 2-3) *Not*I fragment obtained from pCRII-ΔCBA, to yield pCBA-ΔHPRT (figure 12). pCBA-Zeo was made by inserting a 1.2 kb *Bgl*II/*Bam*HI fragment from pCMV/Zeo into the unique *Bam*HI site of pCBA-ΔHPRT, between the two *HPRT* regions. Similarly, a 2.4 kb *Sal*I/*Bgl*II insert from pOct-Zeo was directionally ligated to a 10 kb fragment resulting from the digestion of pCBA-ΔHPRT with *Sal*I and *Bam*HI, to yield pCBA/Oct-Zeo.

b.5.p129-Neo

p129-Neo was made by subcloning a 2 kb *Xho*I PGK-neo cassette (from pBS-PGK-neo, which contains a *neo* expression cassette under the control of the PGK promoter) into *Sal*I-linearised p129–ΔHPRT*.

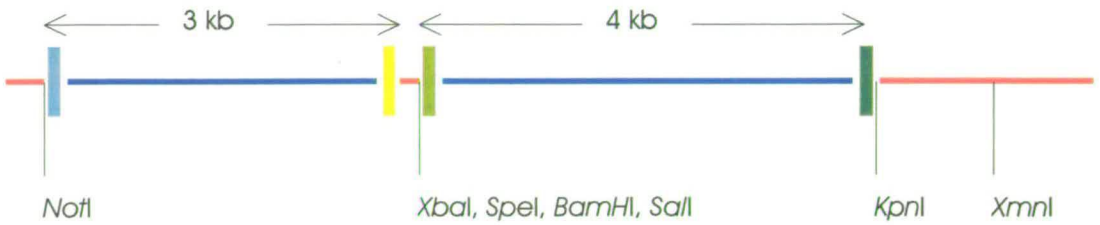

Figure 12. pCBA-ΔHPRT. Coloured boxes (from left to right): *HPRT* exons 2, 3, 6 and 7. Blue lines represent regions of homology (7 kb in total). Red lines, backbone vector (pBS-II SK +/-).

b.6.p129-Hyg

p129-Hyg was generated by subcloning a 2.1 kb *Acc*I-*Xho*I fragment of pIREShyg (Clontech) comprising a CMV-driven hygromycin resistance gene into *Acc*I-*Xho*I-linearised pBS-II (Stratagene). This intermediate step provides a *Sal*I site upstream the insert for subsequent subcloning of a *Sal*I-*Xho*I fragment into *Sal*I-linearised p129–ΔHPRT*.

b.7.p2000

p2000 was made by inserting a blasticidin cassette into pM44. A 1.6 kb *Bam*HI fragment from pUCSV-BSD (Funakoshi) comprising the SV40 promoter, the BSD gene and SV40 polyadenylation sequence was blunted with DNA polymerase I-Klenow fragment (Boehringer) and then subcloned into pM44 linearised with *Sma*I.

b.8.p2017/51

This vector was derived from p2017 (pM44 with a CMV puromycin selectable marker) by inserting a Rad51 expression cassette outside the region of *HPRT* homology. A 1.9 kb *Eag*I restriction fragment from pSVZeo/Rad51 (given by M.

Defais) encompassing the SV40 promoter, the hamster Rad51 ORF and SV40 polyadenylation tail was subcloned into p2017 linearised with *Not*I (figure 13). The total size of this vector is 16.5 kb.

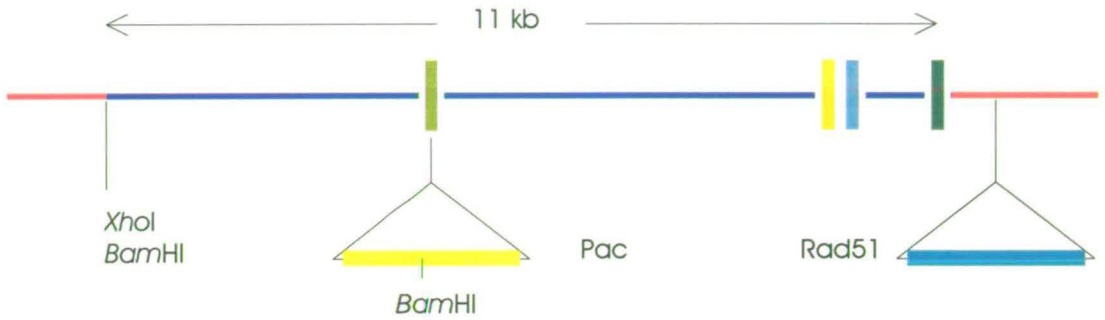

Figure 13. p2017/Rad51 (~16 kb) was derived from p2017 after insertion of a Rad51 expression cassette in the backbone vector. Coloured boxes (from left to right): exons 6, 7, 8 and 9. Red lines, backbone vector (pBluescript). Pac, puromycin expression cassette. Rad51, Rad51 expression cassette.

b.9. pVP22/Rad51

*Sty*I digestion of pSV-Zeo/Rad51 (kindly given by M. Defais) yielded a 1 kb fragment comprising the CgRad51 ORF from codons 1 to 340. This fragment was blunted, digested with *Kpn*I and subcloned into a *Kpn*I/*Eco*RV pVP22/His-*myc*

Figure 14. pVP22/Rad51 was made by fusing the hamster rad51 ORF in frame with the viral protein VP22. From left to right, CMV promoter (light blue); VP22 (yellow); Rad51 (green); *myc* epitope (red); 6xHis epitope (pink); BGH polyadenylation sequence (blue); Neomycin cassette (violet) and Ampicillin resistance gene (brown).

(Invitrogen) digest, in frame with VP22 (figure 14). Codon 341 was restored using a *Aat*II linker in the ligation mixture. Using Invitrogen pVP22/His-*myc* specific primers, automated sequencing of the VP22-Rad51 3' and 5' junctions confirmed that both protein domains were in frame (data not shown).

v. cell culture

a.routine culture conditions
b.transfection
c.analysis
d.cell lines

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a.routine culture conditions

a.1.ES cells

a.1.i.culture in LIF-supplemented medium

a.1.ii.culture in feeder layers

a.2.fibroblasts

a.3.trypsinisation

a.4.freezing down cells

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a.1.ES cells

a.1.i.culture in LIF-supplemented medium

Cells were grown at 37 ^{0}C (5 % CO_2) on 0.1 % gelatin-treated flasks (Iwaki) and fed daily with Glasgow modified Eagle's medium (GMEM, GIBCO/BRL-Life Technologies) supplemented with 0.1 mM MEM non-essential amino acids (GIBCO/BRL-Life Technologies), sodium pyruvate, 5 % (v/v) new-born bovine serum, 5 % (v/v) foetal calf serum, 400-1000 U/ml recombinant murine Leukaemia Inhibitory Factor (ESGRO-LIF, GIBCO/BRL-Life Technologies), 0.1 mM

2-mercaptoethanol, penicillin (100 U/ml)/streptomycin (100 μg/ml) and L-glutamine (250 μM) (GIBCO/BRL-Life Technologies).

a.1.ii.culture in feeder layers

STO fibroblasts were mitotically inactivated using mitomycin C (MMC, Sigma). 50 x MMC stocks (500 μg/ml) were prepared in PBS and stored in the dark at 4 ^{0}C for no longer than 30 days. Cells were incubated at 37 ^{0}C in freshly made MMC medium (1x) for 3-4 hours, and then washed three times with PBS, trypsinised (page 87), aliquoted and frozen down (page 88). A confluent flask provided enough feeder cells to cover 5x the confluent area. ES cells were subsequently plated out on feeder cell cultures.

a.2.fibroblasts

Fibroblasts were grown at 37 ^{0}C (5 % CO_2) on tissue culture plastic flasks (Iwaki) and fed daily with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL-Life Technologies) supplemented with 0.1 mM MEM non-essential amino acids (GIBCO/BRL-Life Technologies), 5 % (v/v) new-born bovine serum, 5 % (v/v) foetal calf serum, penicillin (100 U/ml)/streptomycin (100 μg/ml) and L-glutamine (250 μM) (GIBCO/BRL-Life Technologies).

a.3.trypsinisation

To trypsinise cells, medium was removed and cells washed once with PBS. Cultures were then incubated with 1-3 ml of TEG (NaCl, 3.15 g; Na_2HPO_4, 0.06 g; KH_2PO_4, 0.108 g; KCl , 0.166 g; D-Glucose, 0.45 g; Tris, 1.35 g; Phenol Red 1 %, 0.45 ml; add up to 400 ml in distilled water; 50 ml of 10 x trypsin 2.5 %; EGTA, 0.2 g; PVA, 0.05 g; adjust pH up to 7.6 and add distilled water up to 1000 ml) at 37 ^{0}C for 2-3

minutes. After incubation, flasks were shaken vigorously to detach the cells from the surface. 5 ml of medium were added and pipetted up and down several times until a single-cell suspension was obtained. Cells were then transferred to 15 ml falcon tubes and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and pellets resuspended in fresh medium and plated out as required.

a.4.freezing down cells

2X freezing mix was made by mixing 15 ml of ES cell culture medium, 5 ml dimethyl sulfoxide (DMSO, Sigma) and 5 ml of foetal calf serum. This solution was filtered and stored at –20 ^{0}C. Cells to be frozen down were trypsinised, resuspended in an appropriate amount of medium and placed on ice. One volume of ice-cold freezing mix was added and the suspension was gently mixed and aliquoted into pre-cooled freezing vials. Vials were stored at –80 ^{0}C for two or three days and then into liquid nitrogen. Cells were thawed by introducing the vials in lukewarm water. 5 ml of complete medium were added to the contents of a vial in a 15 ml Falcon tube, and then centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and pellets resuspended in medium for plating out.

b.transfection

b.1.electroporation

b.2.lipofection

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b.1.electroporation

Exponentially growing cells were fed two hours before electroporation. Cells were trypsinised and counted using a Neubauer Improved hemocytometer (Weber England). 10^7 to 3 x 10^7 cells were resuspended in 700 µl of HBS buffer (20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM Kcl, 0.7 mM Na_2HPO_4, 6 mM D-glucose) for each electroporation. Linearised plasmid DNA (25-500 µg) was also diluted in HBS (170 µl) and mixed with the cells in a 0.4 cm electrode Gene Pulser Cuvette (BioRad). A BioRad electroporator was used to give a single pulse of 800 V (3.0 µF). Cells were plated 20 minutes after electroporation at different densities on gelatin-treated 90 mm or 150 mm dishes (Iwaki).

b.2.lipofection

Lipofection was performed using the Effectene kit (Giagen). For transfection of 40-80 % confluent cell cultures in 60 mm dishes, best results were observed using the following proportion of reaction components: 2 µg of DNA in up to 150 µl of EC buffer; 16 µl of Enhancer solution and 20 µl of Effectene.

c.analysis

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c.1.Giemsa staining

Colonies to be counted were washed with PBS, fixed for 10 minutes in methanol and stained for 10-15 minutes in a 10% Giemsa R-66 solution. After staining, plates were gently washed with water and air-dried.

c.2.mitotic spreads

50 % confluent cultures were trypsinised and gently resuspended first in 1 ml of hypotonic solution (0.56 % w/v KCl) and then up to 5 ml. Tubes were incubated at room temperature for 10 minutes, and then centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and tubes flicked to ensure cell disruption. Cells were resuspended in 1 ml of ice-cold fixative (18 ml methanol, 6 ml glacial acetic acid) and then up to 6 ml. After 5 minutes at room temperature, tubes were centrifuged at 500 rpm for 5 minutes. Supernatant was removed and cells were washed with fixative twice more as above. Pellets were then resuspended in 1 ml of fixative. Using a Pasteur pipette, single drops of this suspension were released from approximately 30 cm above 70 % ethanol-treated slides. After evaporation of the fixative, slides were stained in 3 % Giemsa R-66 for 10-15 minutes for chromosome counting.

d.cell lines

All ES cell lines used in this work (see list below), with the exception of the CBAs, have a 129 genetic background. The background of MEF Ku80 and p53 knockouts is undefined.

Cell line	Genotype	Given by / reference
129/5.4 (ES)	Wild type	Ed Gallagher, Roslin Institute (UK)
RI (ES)	Wild type	Nagy *et al.*, 1993
CBAs (ES)	Oct-neo transgenic	Jim McWhir, Roslin Institute (UK)
J1 (ES)	Wild type	A. Bird, ICMB, Edinburgh University (UK)
E14 (ES)	Wild type	A. Clarke, Department of Pathology, Edinburgh University (UK)
TC-1 (ES)	Wild type	F. Alt, Center for Blood Research, Harvard Medical School (USA)
#2-35-2 (ES)	DNA-PK -/-	Gao *et al.*, 1998
#2	DNA-PK +/-	Gao *et al.*, 1998
HG-287 (ES)	p53 -/-	Prost *et al.*, 1998
R72 (ES)	p53 +/-	Prost *et al.*, 1998
210 (ES)	PARP -/-	M. Matsutani, National Cancer Centre Research Institute (Japan)
226 (ES)	PARP +/-	M. Matsutani, National Cancer Centre Research Institute (Japan)
B (ES)	Dnmt1 -/-	Chen *et al.*, 1998
Ku80 (MEF)	+/+, +/-, -/-	Nussenzweig *et al.*, 1997
p53 $^{-/-}$ (MEF)	p53 -/-	D. W. Melton, ICMB, Edinburgh University (UK)
STO	HPRT nullizygous	Jim McWhir, Roslin Institute (UK)

Chapter III. Development of a test system sensitive to isogenicity

i.description of the system
ii.selectable markers
iii.targeting vectors

i.description of the system

The priority of this project was originally to develop an ES cell-based test system sensitive to changes in targeting frequencies, both in isogenic and non-isogenic conditions. The *HPRT* locus was chosen as a target gene for several reasons: The structure of the gene is well characterised (Melton *et al.*, 1984); it is hemizygous in male cells; and cells in culture can be selected either for (HAT medium) or against (6-TG) its activity.

Replacement targeting vectors were used to inactivate the *HPRT* locus, either by creating a deletion between exons 3 and 6 or by inserting a selectable marker in exon 6. The presence of a positive selectable marker between the two regions of homology allows selection of both homologous and non homologous integrants. However, only targeted clones, in which the *HPRT* locus has been inactivated, survive further selection on 6-TG.

This chapter describes the assessment of selectable markers and targeting vectors subsequently used in different experimental conditions (chapters IV-VI).

ii.selectable markers

a.introduction

Many mutant cell lines used in this work were generated by disrupting endogenous genes with selectable markers, amongst them neomycin, puromycin and hygromycin. Hence, the same selective agent cannot be applied when using vectors that carry markers already present in the cells. For this reason, different versions of the targeting vectors were engineered to contain a variety of selectable markers, to be used where appropriate. When assessing a candidate selectable marker, it is necessary to determine the minimum concentration of the drug that kills all cells in a reasonable period of time (normally ten days). This 'kill curve' has to be done for every individual cell line, since different genetic backgrounds may have variable drug requirements to achieve the same effect. In this work, two kill curves were calculated for every cell line. One of them was done by applying increasing concentrations of the relevant drug in untransfected cells. The second was done in cells transfected with a marker that confers resistance to the drug. The minimum dose that kills all the cells in the untransfected control within ten days and yields colonies in the transfected culture was thus determined for each marker. A more precise way to determine the effect of each drug on every individual cell line would have been the calculation of survival curves. Survival curves are represented as the percentage of surviving cells after exposure to several concentrations of the drug for a given period of time, compared to untreated cells. In contrast with the kill curves described in this work (where the number of resistant colonies may reflect not only the intrinsic resistance to the drug, but also factors such as

line-specific plating efficiency or colony formation efficiency), survival curves would specifically measure the response of every cell line to the selective agent. However, since we aimed at determining the *proportion* of targeted to total integrants (effective targeting frequency), rather than transfection efficiency, kill curves were found to serve to this purpose.

b.zeocin

Zeocin shows strong toxicity against bacteria, fungi, plants and mammalian cells. It belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Although it is produced as an inactive copper/glycopeptide complex, the copper cation is reduced and removed by cytoplasmic sulfhydryl compounds upon entering the cell. This activates the molecule, which binds to and cleaves DNA, causing cell death (Berdy, 1980). The zeocin resistance factor is a 13 kD protein encoded by the gene *Sh Ble* (*Streptoalloteichus hindustanus* bleomycin gene). This protein binds zeocin, inhibiting its DNA strand cleavage activity (Drocourt *et al.*, 1990). Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to zeocin. In order to use this selectable marker in ES cells, a kill curve was calculated using untransfected E14 cells as control. The results are shown in table 1 and figure 15.

Cell line	Number of cells/ plating density	Plasmid DNA/ µg	Number of colonies recovered/ Zeocin cocentration (µg/ml)										
			0	5	7	10	12	20	25	40	50	70	100
E14	10^7/1,5 x 10^4 cells/ cm^2	NO DNA	*	*	*	10	0	0	0	0	0	0	0
E14	10^7/1,5 x 10^4 cells/ cm^2	pCMVZeo /100	*	*	*	30	0	0	0	0	0	0	0

Table 1. Zeocin kill curve. (*) Selection level too low to allow colony selection (confluency).

The difficulties associated with the use of this antibiotic in ES cells are discussed in detail in page 102. From the data presented above, it can be concluded that the window of zeocin selection is very narrow. 10 µg/ml of the antibiotic seem to be

effective at selecting zeo-resistant clones, but a significant number of clones turn out to be false positives using this level of selection. Slightly higher zeocin concentrations kill transfected and untransfected cells alike. For these reasons, although zeocin selection

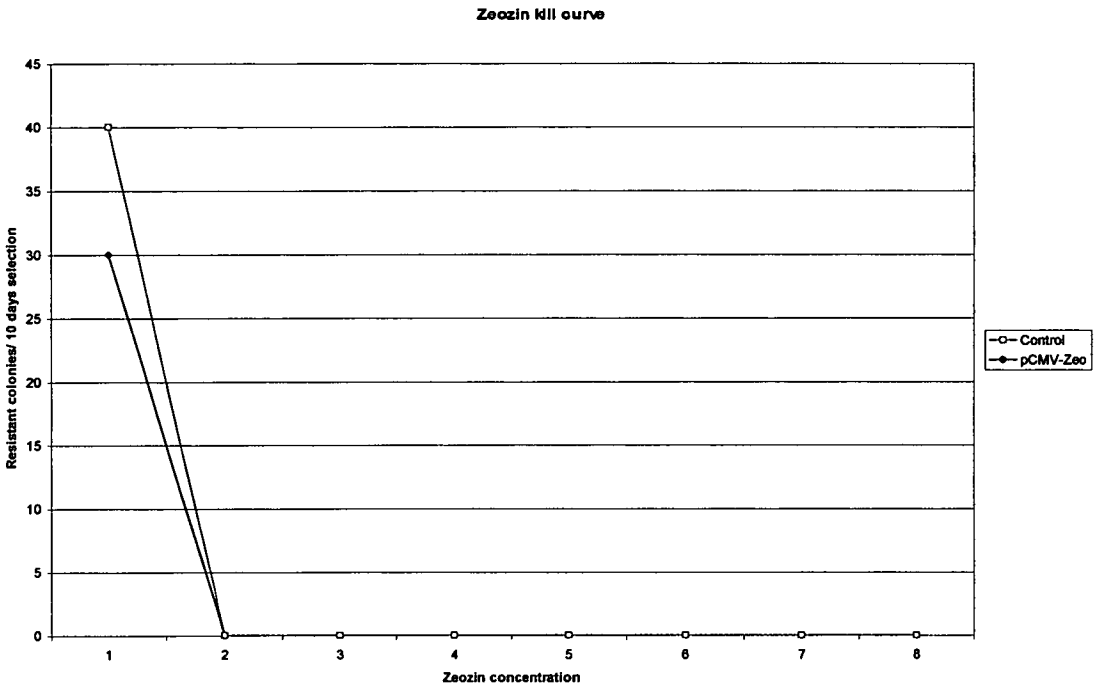

Figure. 15 Zeocin kill curve.

(10 μg/ml) was eventually utilised to isolate Rad51 transgenic clones (page 133), its routine use in targeting experiments was discarded.

c.neomycin / G418

G418 is an aminoglycosidic antibiotic commonly used for selection in eukaryotic cells transfected with the *neo* gene. Unlike related antibiotics such as gentamycin or kanamycin, G418 blocks protein translation by interfering with the 80S ribosome subunit (Daniels *et al.*, 1973). The product of the neomycin-resistance gene allows selection in G418 by phosphorylating the antibiotic, thus blocking its action (Southern and Berg, 1982). *Neo*-resistant ES cells are selected at 300 μg/ml, although in

specific applications it can be raised up to 1000 μg/ml. Because of its routine use in this laboratory, no kill curves were required.

d.hygromycin

Hygromycin B is used as a selective agent in eukaryotic transfection experiments. Produced by *Streptomyces hygroscopicus*, this aminoglycoside antibiotic is highly effective at killing bacteria, fungi and higher eukaryotic cells. It interferes with protein synthesis by hindering translocation and causing mistranslation. Resistance to hygromycin is conferred by the gene *hph*.

A kill curve (table 2 and figure 16) shows that hygromycin resistant colonies can be selected at 50-55 μg/ml. However, this selection was not routinely used due to the delayed response of ES cells to the antibiotic at normal working concentrations (differences between transfected and untransfected plates were not evident until the second week of selection). Although the use of hygromycin in targeting experiments has been extensively reported, our test system required a faster primary selection in order to avoid the problem of metabolite exchange.

Cell line	Number of cells/ plating density	Plasmid DNA/ μg	Number of colonies recovered/ Hygromycin concentration (μg/ml)										
			0	10	20	30	40	50	60	70	80	90	100
129/ 5.4	10^7/1,5 x 10^4 cells/ cm^2	NO DNA	*	*	*	*	*	5	3	0	0	0	0
129/ 5.4	10^7/1,5 x 10^4 cells/ cm^2	IRES-Hyg/100	*	*	*	*	*	100	23	2	3	0	0

Table 2. Hygromycin B kill curve. (*) Selection level too low to allow colony selection.

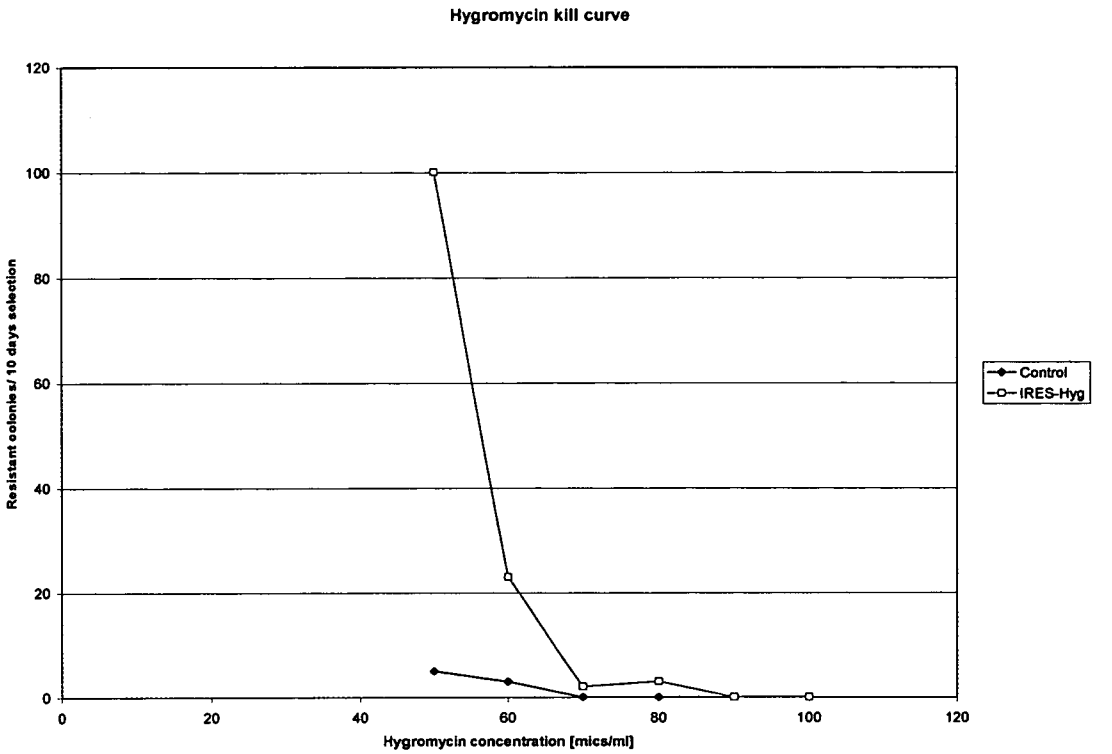

Figure 16. Hygromycin kill curve

e.puromycin

Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger*, which specifically inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes. Expression of the gene *pac* (puromycin N-acetyl-tranferase) confers resistance to the antibiotic in mammalian cells. A kill curve with *pac*-transfected (p2017) and untransfected control ES cells shows efficient selection at concentrations as low as 1 μg/ml (table 3 and figure 17) and 3 μg/ml for mouse embryonic fibroblasts (data not shown).

This antibiotic kills non-transfected cells very quickly, yielding clean cultures and healthy resistant colonies in a matter of days. For this reason, puromycin has been the antibiotic of choice in most of the targeting experiments discussed in this work.

87.5 % CBA ES cell lines (McWhir *et al.*, 1996) can also be selected in puromycin, although resistant colonies are usually highly differentiated. This is a particular feature of CBA ES cells in low plating density cultures, which cannot be attributed to puromycin selection.

Cell line	Number of cells/ plating density	Plasmid DNA/ µg	Number of colonies recovered/ puromycin concentration (µg/ml)										
			0	0,5	1	1,5	2	2,5	3	3,5	4	5	10
RI	10^7/1,5 x 10^4 cells/ cm^2	NO DNA	*	*	0	0	0	0	0	0	0	0	0
RI	10^7/1,5 x 10^4 cells/ cm^2	p2017 – 70µg	*	*	64	34	25	7	11	10	5	0	0
RI	10^7/1,5 x 10^4 cells/ cm^2	p2017 – 300µg	*	*	101	53	48	25	17	9	8	0	0
CBA	10^7/1,5 x 10^4 cells/ cm^2	NO DNA	*	*	0	0	0	0	0	0	0	0	0
CBA	10^7/1,5 x 10^4 cells/ cm^2	p2017 – 100µg	*	*	60	60	46	35	24	2	1	0	0

Table 3. Puromycin kill curve. (*) Selection level too low to enable colony selection. p2017 is an *HPRT* targeting vector containing a *pac* expression cassette. Although CBA-derived colonies are very differentiated (see text), genuine puromycin-resistance was demonstrated by the absence of surviving cells in *pac*-untransfected CBA cells.

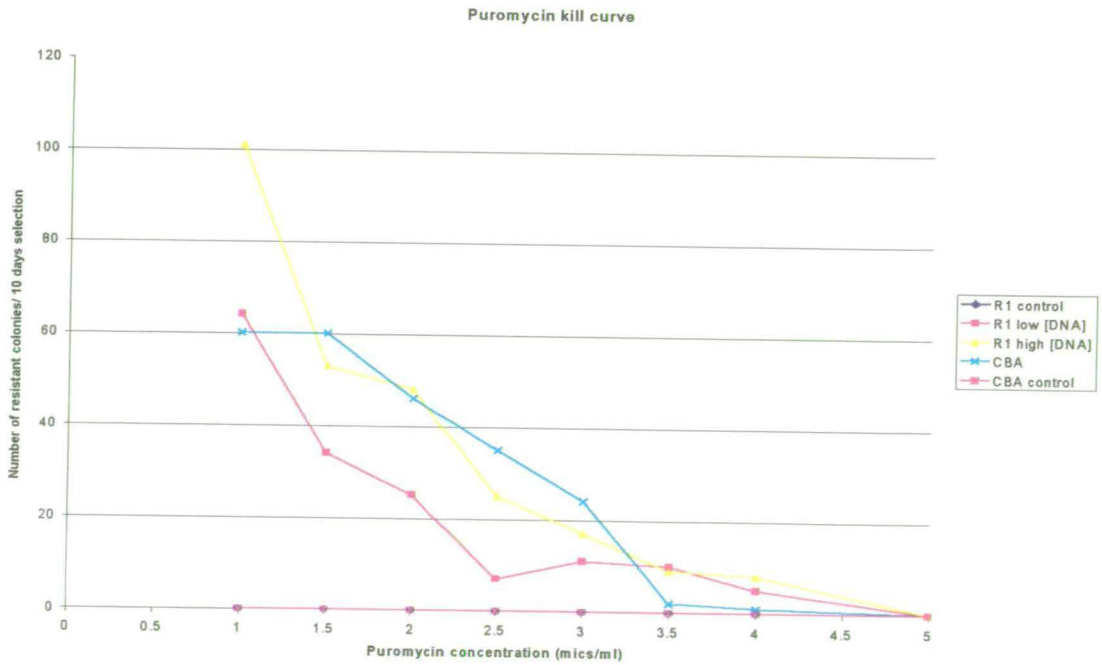

Figure 17. Puromycin kill curve.

f.blasticidin

Blasticidin S HCl is a nucleoside antibiotic that interferes with protein synthesis in prokaryotic and eukaryotic cells (Yamaguchi *et al.*, 1965). Expression of the blasticidin S deaminase gene *BSD* from *Aspergillus terreus* confers resistance by converting the antibiotic to an inactive deaminohydroxy derivative compound (Izumi *et al.*, 1991). A kill curve was determined for blasticidin selection in ES cells. As shown in table 4 and figure 18, any concentration between 4 and 11 μg/ml yields a similar number of resistant clones in pBSD-transfected ES cells, while completely killing untransfected controls. Thus, it was decided to use 5 μg/ml of the antibiotic in subsequent targeting experiments.

Cell line	Number of cells/ plating density	Plasmid DNA/ μg	Number of colonies recovered/ Blasticidin concentration (μg/ml)										
			0	1	2	3	4	5	7	9	11	13	15
210	10^7/1,5 x 10^4 cells/ cm^2	NO DNA	*	*	*	7	0	0	0	0	0	0	0
210	10^7/1,5 x 10^4 cells/ cm^2	pBSD/100	*	*	*	29	27	32	21	12	23	11	4

Table 4. Blasticidin kill curve. (*) Selection level too low to allow colony selection.

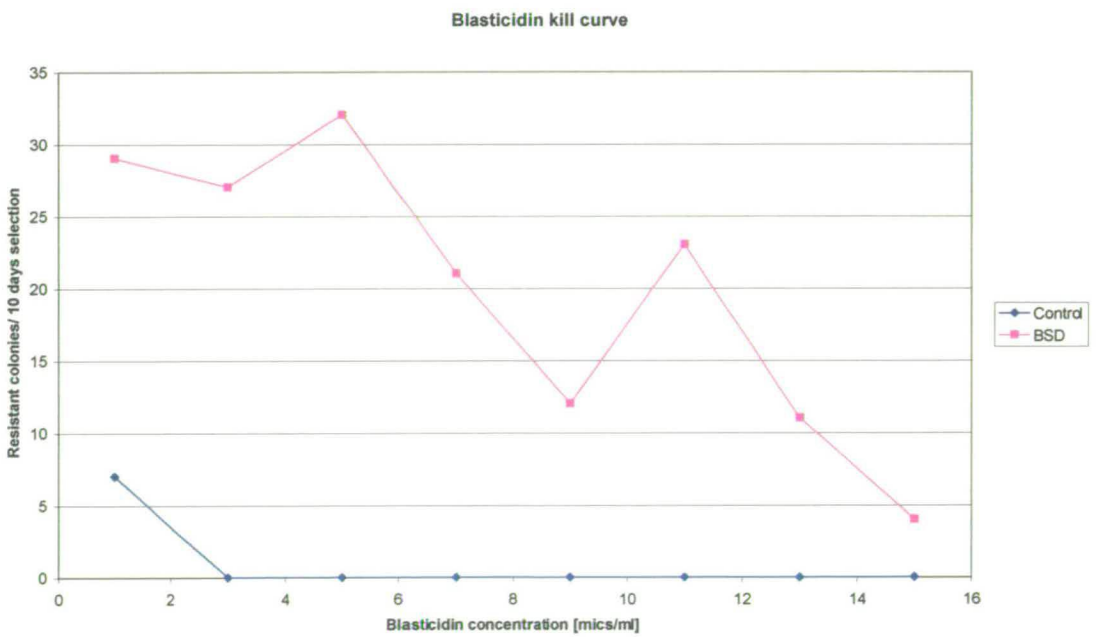

Figure 18. Blasticidin kill curve.

iii.targeting vectors

a.p129-ΔHPRT and pCBA-ΔHPRT

The targeting replacement vectors p129-ΔHPRT and pCBA-ΔHPRT (see page 80) were originally designed to inactivate the X-linked HPRT gene in ES cells obtained from the mouse strains 129 and CBA, respectively. Successful targeting produces a deletion between exons 3 and 6 (figure 19). As a first step to developing a reliable system for comparing E.T.F.'s under different experimental conditions, it was decided to assess the ability of the above constructs to knockout the *HPRT* locus in 129/5.4 and CBA ES cell lines. Once tested in isogenic conditions, both CBA- and 129-derived non- isogenic vectors would be respectively assayed in 129 and CBA cell

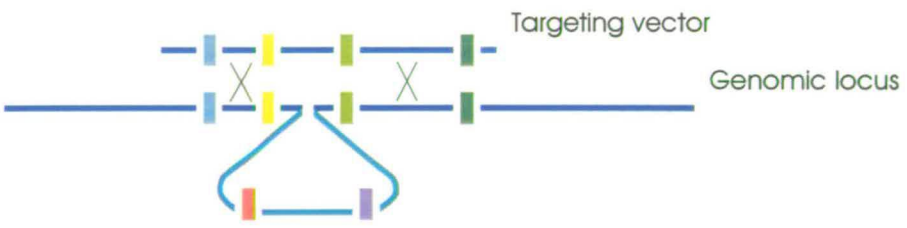

Figure 19. Schematic representation of the targeting event induced by p129–ΔHPRT and pCBA–ΔHPRT vectors. Coloured boxes in the aligned regions, from left to right: *HPRT* exon 2 (blue), 3 (yellow), 6 (light green) and 7 (dark green). Homologous recombination yields a deletion of the region between exons 3 and 6, comprising exons 4 (red) and 5 (violet). Distances are not to scale.

lines, in order to examine the effect of mismatches in gene targeting frequency.

We were unable to recover 6-TG resistant clones in pilot experiments using these vectors. It was initially thought that the reason for this negative result could be a poor optimisation of the conditions for the recovery of targeted clones. First, it is necessary to maintain the plates for up to 6 days in non-selective conditions, to allow degradation of previously synthesised HPRT protein in targeted cells. This causes overconfluence and extensive differentiation in 129/5.4 and, above all, CBA cell lines. Second, the proximity of non-targeted cells to targeted ones favours the exchange of different metabolites, amongst them PRPP/6-TG. Only a few imported molecules of this compound may kill targeted cells. This is one of the reasons why most of the studies on *HPRT* targeting have been done to correct a defective locus rather than to inactivate it (Thompson *et al.*, 1989).

b. p129- and pCBA- zeocin variants

In order to eliminate metabolic cooperation, a CMV-zeocin cassette was added to p129-ΔHPRT and pCBA-ΔHPRT between the two *HPRT* regions of homology in both possible orientations, as described in page 81. A primary selection during the first six days after transfection would ensure that targeted and non-targeted colonies are separated and that translocation of PRPP/6-TG does not take place. Two versions of both targeting constructs (in which the zeocin resistance gene is driven by the Oct3/4 promoter) were also made. The gene Oct3/4 is preferentially expressed in non-differentiated cells, and its promoter has been successfully used to drive neomycin expression in CBA cell lines (McWhir *et al.*, 1996). Homologous recombination of any of these vectors with the genomic *HPRT* locus must yield HPRT-defective/ zeocin resistant cells, given a proper level of expression of the zeocin cassette. Targeted clones are selectable in medium supplemented with 6-TG and zeocin, while clones arising from illegitimate recombination events can be selected in the presence of zeocin, but are not 6-TG-resistant.

Pilot experiments with these vectors (p129-zeo and p129-oct/zeo) are summarised in table 5. These results confirmed the previous observation that this antibiotic is unsuited for the development of a testing system. First, zeocin sensitivity seems to be strongly affected by plating density. The higher the cell concentration, the less efficient the selection. It was observed that particularly high cell densities (i.e., 3 x 10^4 cells/cm^2) remain virtually unaffected by zeocin, even at high concentration (100μg/ml). Also, when cells are not evenly distributed across the plate, local agglutination of arising colonies generate false resistants. This observation may account for the experiment in which a dozen colonies were recovered after 14 days of zeocin selection at 50 μg/ml after transfection with p129/oct-zeo. However, Southern blots with two different zeocin probes did not reveal any band in any of the genomic extracts (data not shown).

Cell line	Number of cells/ plating density	Plasmid DNA/ μg	Number of colonies recovered/ Zeocin cocentration (μg/ml)										
			0	5	7	10	12	20	25	40	50	70	100
E14	10^7/3 x 10^4 cells/ cm^2	p129-zeo/100	*	*	*	*	*	*	*	*	*	*	*
E14	10^7/3 x 10^4 cells/ cm^2	NO DNA	*	*	*	*	*	*	*	*	*	*	*
E14	10^7/1.5 x 10^4 cells/ cm^2	p129/oct-zeo/300									12	0	0
HG-287	10^7/3 x 10^4 cells/ cm^2	p129-zeo/100	*			*			*		*		0
R72-1	10^7/3 x 10^4 cells/ cm^2	p129-zeo/100	*			*			*		*		0

Table 5. Zeocin kill curve using zeo-targeting vectors. (*) No colony formation.

Second, zeocin selection may be affected by growth rate. Both HG-287 and R72-1 cell lines carry mutations in the *p53* gene, and they have been observed to have abnormally faster cell division times. This may lead to undesirable local increases in cell density during the 24 hour period following electroporation, in which cells are not yet under selection.

Although zeocin selection has been used by other groups in ES cells, the sensitivity of a system intended to accurately measure gene targeting frequency would be hindered by the high incidence of false positives and narrow window of selection that we have observed. Due to these disadvantages it was decided not to proceed with zeocin-based vectors in subsequent targeting experiments.

c.p129-Hyg

A hygromycin positively selectable marker was subcloned into p129-ΔHPRT as an alternative to zeocin (page 83). A kill curve (table 2 and figure 16) shows that a concentration of 55 μg/ml is enough to select colonies from a population of 129/5.4 ES cells after transfection with a *hyg* cassette. No colonies are recovered at such concentration when using untransfected ES cells as a control, although it was necessary to maintain selection for up to 16 days. Since the differences between control and transfected plates were not evident until the second week of selection the vector was not further utilised.

d.p129-Neo

A *neo* cassette was engineered into p129-ΔHPRT in both orientations (page 82). As ES cells transfected with *neo* can be easily selected when the medium is supplemented with the drug G418 (normally at 300-500 μg/ml), the use of these vectors would unequivocally determine whether the defect of p129-ΔHPRT lay in the selection system or rather in the vector itself. Four experiments were conducted using p129-neo ($\leftarrow/\rightarrow$) to electroporate a variety of wild-type ES cell lines.

Cell line / passage n.	Construct	[DNA]/ linearisation	N. cells electrop.	G418^R colonies	G418^R + 6TG^R colonies	E.T.F. (%)
129/5.4/13	p129-neo (←)	300 μg/ *Kpn*I	10^7	70	0	0
RI/25	p129-neo (→)	200 μg/ *Kpn*I	10^7	65	0	0
RI/25	p129-neo (←)	200 μg/ *Kpn*I	10^7	89	0	0
E14-27/33	p129-neo (→)	250 μg/ *Kpn*I	10^7	815*	0	0

Table 6. Targeting experiments with p129-neo. The arrow in brackets indicates the orientation of the *neo* cassette. Cells were plated in ten 90 mm dishes in experiments 1-3. (*) Two 15 cm plates were used in experiment 4. A.T.F., absolute targeting frequency; E.T.F., effective targeting frequency.

The results summarised in tables 6 and 7 indicate that: (a) Plating efficiency seems to correlate to plating density (table 7). (b) The vector p129 is intrinsically ineffective to target *HPRT*, irrespective of the selection system used. Further experiments carried out in our laboratory suggest that the construct may work, although at an absolute targeting frequency (A.T.F.) $<10^{-7}$. This order of magnitude is very close to the estimated rate of spontaneous mutation of *HPRT*, and clearly insufficient for our purposes.

Number of cells plated	Dishes	Plating density	Total number of colonies in G418
10^7 (experiments 1-3)	10 of 90 mm	1,5 x 10^4 cells/ cm^2	390 (mean)
10^7 (experiment 4)	2 of 150 mm	2.8 x 10^4 cells/ cm^2	815

Table 7 . Effect of plating density on G418 selection

e.p2016

Despite the time and effort employed in the p129-derived constructs, it was decided to use a targeting vector of well-proven efficacy, such as the pM44-derived p2016 (page 79), in subsequent experiments. This replacement vector has been designed to disrupt the *HPRT* locus by inserting a *neo* cassette into exon 6 (figure 20).

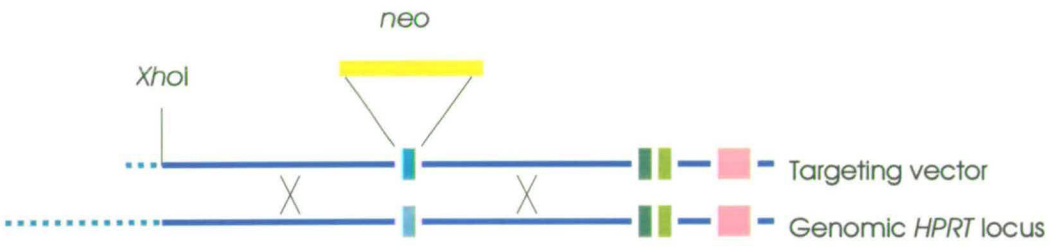

Figure 20. p2016 gene targeting event (see text for details). Coloured boxes, from left to right: exon 6 (light blue), 7 (dark green), 8 (light green) and 9 (pink). *Xho*I, linearisation site. Distances are approximate, and exon sizes are not to scale.

In a series of pilot experiments, 10^7 cells were electroporated (800 V, 3 μF) with variable amounts of *Xho*I-linearised DNA and plated in two 150 mm dishes at a density of 5 x 10^6 cells/plate (2,8 x 10^4 cells/ cm^2). G418 selection (300 μg/ml) was applied 24 h after electroporation. One of the two plates was further supplemented with 6-TG after 6 days of G418 selection. These experiments are outlined in table 8.

From these initial results it can be concluded that: (a) p2016 is proficient at targeting the *HPRT* locus in wild-type ES cells. Effective targeting frequency oscillates between 4 and 8 %. (b) There is a linear correlation between E.T.F. and the amount of DNA used/ electroporation up to 300 μg. This observation has been verified using a p2016 derivative, p2017 (page 110).

Cell line / passage	Construct	[DNA] / linearisation	N. cells electrop.	G418^R col. (Plate 1)	G418^R + 6TG^R col.	E.T.F. (%)
E14-27/33	p2016	75 μg/ *Xho*I	10^7	467	19	4.06
E14-27/33	p2016	150 μg/ *Xho*I	10^7	632	38	6.01
E14-27/33	p2016	300 μg/ *Xho*I	10^7	729	59	8.09
E14-27/33	p2016	300 μg/ *Xho*I	10^7	685	54	7.88
E14-27/33	p2016	300 μg/ *Xho*I	10^7	648	48	7.40
E14-27/33	p2016	300 μg/ *Xho*I	10^7	509	39	7.66
E14-27/33	p2016	300 μg/ *Xho*I	10^7	518	42	8.10
E14-27/33	p2016	300 μg/ *Xho*I	10^7	607	47	7.74
E14-27/33	p2016	300 μg/ *Xho*I	10^7	603	42	6.96

Table 8. Pilot targeting experiments with p2016.

f.p2017

Another version of the previous construct, p2017 (provided by H. Wallace, page 79), features a puromycin positively selectable cassette in place of *neo*. Due to the speed of puromycin selection even at very low concentrations (page 99) p2017 was used in most subsequent experiments, regardless of the presence of *neo* in the target cells.

g.p2000

The targeting vector p2000 (page 83) is another derivative of pM44, with a blasticidin selectable cassette in exon 6. It was used for the determination of gene targeting frequency in PARP -/- ES cells, which already have *neo* and *pac* cassettes.

h.experimental design

Unless otherwise stated, p2017 was used in all subsequent targeting experiments, with the following standard conditions: Upon transfection (800 V, 3 μF, 300 μg of targeting vector), cells were split in two 15 cm dishes (5 x 10^6 cells / plate, 2.8 x 10^4 cells / cm^2). Puromycin selection was applied 24 hours later, and maintained for 5 days. The sixth day after transfection, one plate (A)

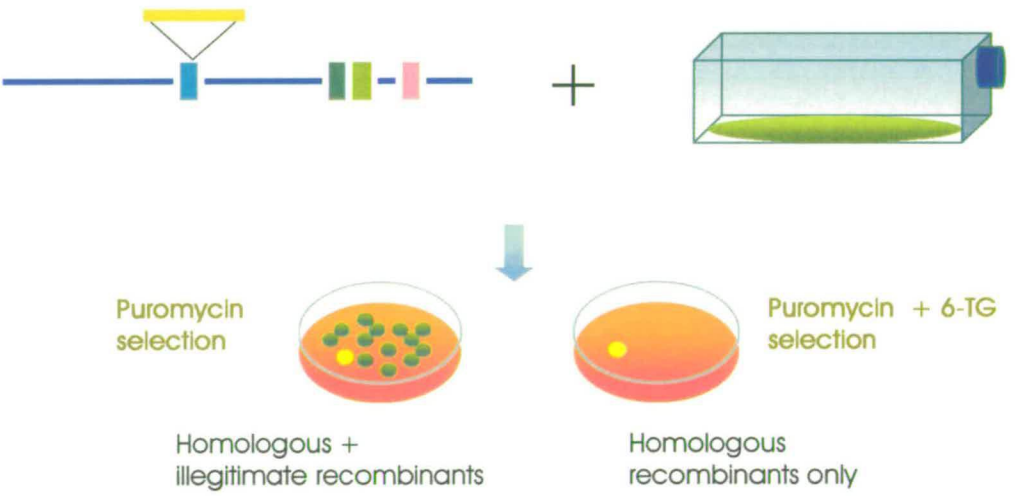

Figure 21. Experimental strategy (see text for details). Effective Targeting Frequency (E.T.F.) was calculated as the ratio of homologous (puromycin + 6-TG resistant colonies) to total integrants (puromycin resistant colonies)

was fixed and stained for colony counting (homologous and non-homologous recombinants) and the other (B) further supplemented with 6-TG. 4-5 days after 6-TG

selection, plate B was also fixed and stained. Effective targeting frequency (E.T.F.) was calculated as the ratio of homologous recombinants (number of colonies in B) to total integrants (number of colonies in A) (figure 21). Southern analysis of colonies randomly picked from plate B before staining shows that 100 % of clones in this sample were successfully targeted (figure 22).

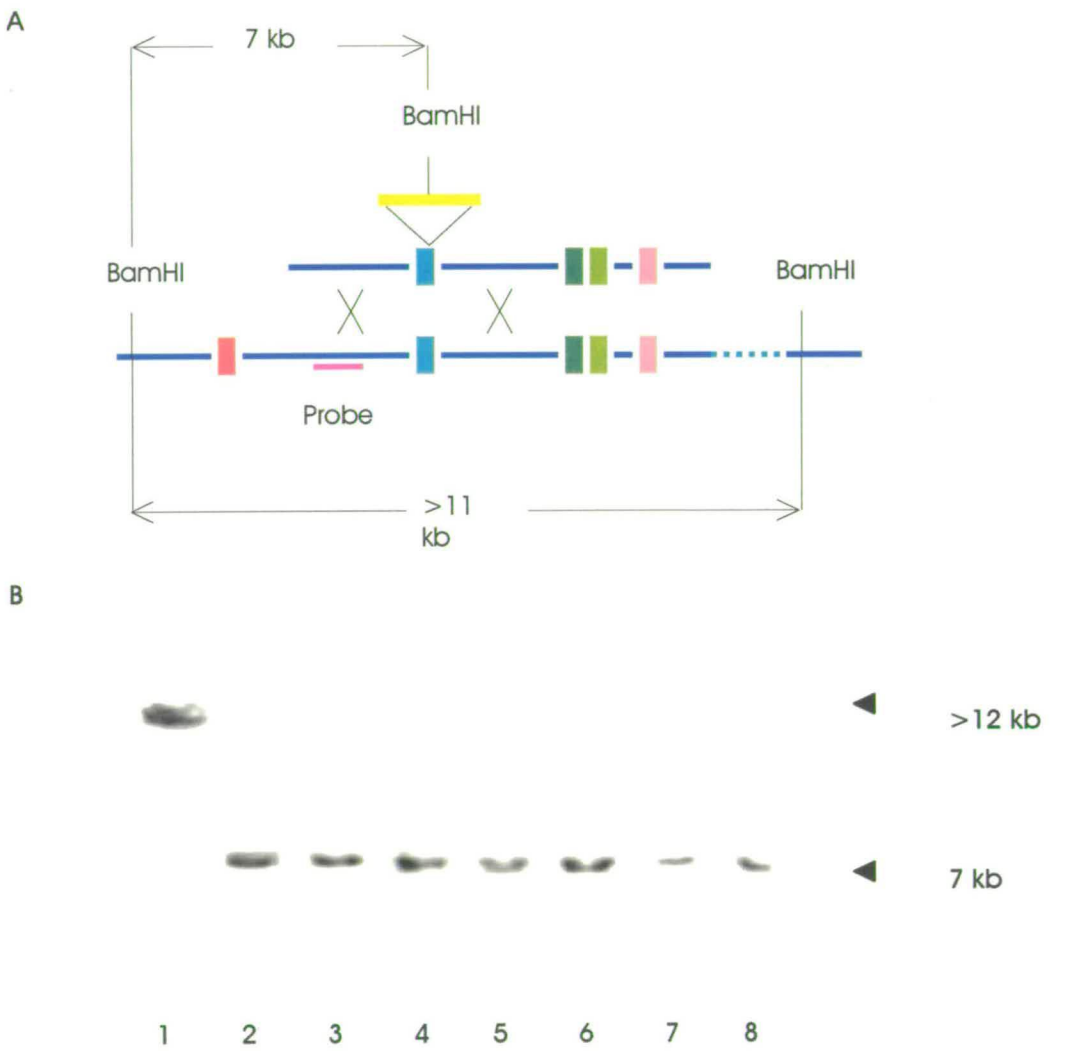

Figure 22. targeting event induced by p2017 (A) and Southern blot of randomly picked 6-TG-resistant colonies (B). Genomic DNA was digested with *Bam*HI and probed with a 500 bp *Sph*I/*Bgl*II fragment located within intron 5 of the targeting vector. Successfully targeted clones display a 7 kb band, and wild-type cells show a band >12 kb (heavier than the larger band of the DNA size marker used). Lane 1: untransfected control. Lanes 2-8: 6-TG resistant clones.

i.DNA concentration

In the course of previous experiments it was observed that the amount of targeting vector/electroporation seemed to influence the number of colonies recovered, both after positive (G418) and negative (6-TG) selection (page 106). In order to study this effect in depth, five groups of E14 ES cells (passage 35) were transfected with increasingly higher concentrations of p2017. The results of these experiments are summarised in table 9 and figure 23.

DNA amount/ electroporation	Puromycin^R colonies	6-TG^R colonies	E.T.F (%)
50 µg	500	18	3.6
100 µg	694	30	4.33
200 µg	776	57	7.35
300 µg	1176	113	9.6
500 µg	1350	161	11.93

Table 9. Effect of DNA concentration on gene targeting frequency.

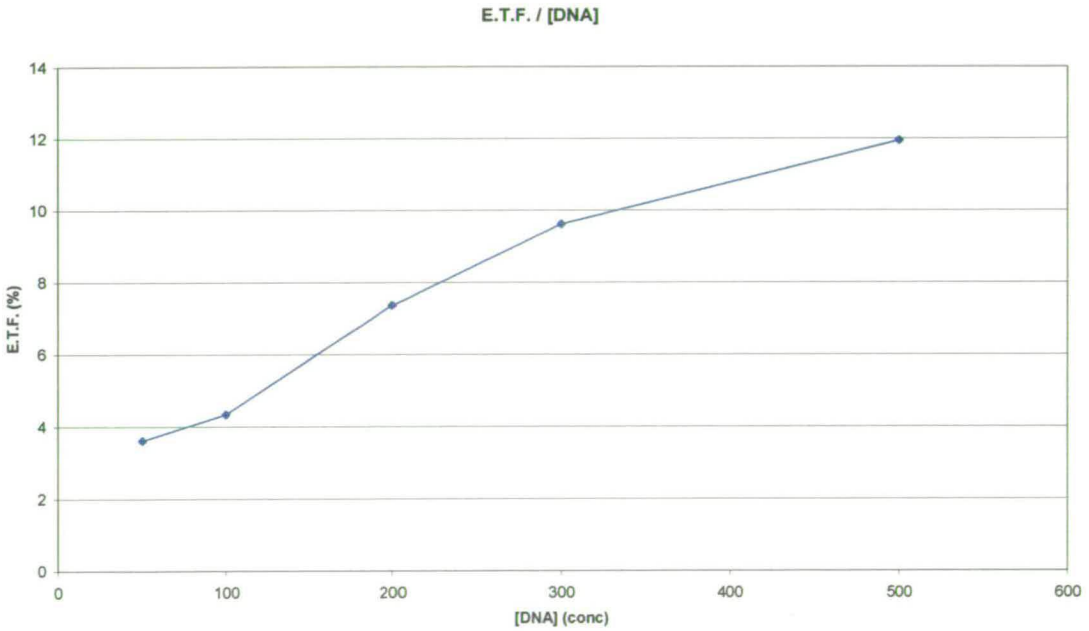

Figure 23. Effect of DNA concentration on E.T.F.

It was found that there is a linear correlation between DNA concentration and the number of colonies recovered after both positive and negative selections. However, the arithmetic progression of 6-TG resistant colonies/ [DNA] is much steeper than that of puromycin resistant clones/ [DNA]. This means that higher concentrations of DNA/electroporation result in correspondingly enhanced effective targeting frequencies (E.T.F.), at least within the interval examined in this work.

It was also observed that experimental variation of E.T.F. was slightly higher when using <200 μg DNA/experiment. Since the use of 300 μg DNA gave more consistent and repeatable E.T.F. values under the same electroporation conditions, it was decided to use this amount of targeting vector in subsequent experiments (unless otherwise indicated).

Chapter IV. modifications of the vector

i.introduction
ii.incubation with NLS
iii.dsRNA vectors

i.introduction

Conventional targeting vectors are based on double-stranded DNA fragments homologous to the target locus. The introduction of the desired modification occurs upon homologous recombination, through the general mechanism described in pages 6-16. In recent years, vectors have been modified in a number of ways in order to increase targeting efficiency. These include, among others, the protection of the ends to avoid intracellular degradation (Chang and Wilson, 1987) and coating with nuclear localisation signals (Collas and Alleström, 1996). Alternative vectors, such as PNAs, TFOs and RDOs, have also been developed with the same purpose in mind (pages 43-46). In these cases, gene targeting occurs through mechanisms less studied than homologous recombination, but frequently more efficient.

This chapter will describe two strategies aimed at increasing gene targeting frequency based on the modification of the targeting vector. The first approach takes advantage of the properties of the SV40 nuclear localisation signal (page 42). The second is based on the use of double-stranded RNA vectors, which in *C. elegans* and other species is responsible for the effect known as RNA interference (RNAi). Although there is now compelling evidence that RNAi is effected at the mRNA level, the inclusion of this strategy in the present work is justified by the fact that the target of dsRNA molecules was unknown at the time we started working on it. Based on the original observation that RNAi effects were evident even in F1 (Fire *et al.*, 1998), the possibility of a DNA target could not be ruled out (Wagner and Sun, 1998).

ii.nuclear localisation signals

a.introduction
b.objective
c.results
d.discussion

a.introduction

Since the efficiency of homologous recombination depends on the length of homology between the two recombinant partners (Deng and Capecchi, 1987), targeting vectors tend to be large (>10 kb) to ensure reasonably high targeting frequencies. However, size is likely a critical factor affecting nuclear entry of DNA. Whereas small oligonucleotides can diffuse freely into the nucleus, larger DNA molecules are transported less efficiently (Ludtke *et al.*, 1999). The use of nuclear localisation signals (NLSs) as a means to improve import of DNA molecules into the nucleus has already been discussed in page 42. NLSs can be chemically or electrostatically attached to DNA molecules in order to facilitate active transport through the nuclear pore complex (NPC) (rev. by Collas and Alëstrom, 1996). Enhancement of nuclear transport has also been shown to result in higher frequency of transgene expression (measured as transfection efficiency) in cultured cells (Zanta *et al.*, 1999; Ludtke *et al.*, 1999).

Based on this evidence, it was reasoned that transfection of targeting vectors pre-incubated with a NLS might increase the concentration of recombinogenic DNA molecules within the nucleus. This enhanced accumulation may have an effect on targeting efficiency, by increasing homologous recombination, non-homologous end-joining, or both. This section examines the influence of incubating targeting vectors with the NLS of the SV40 T antigen on E.T.F.

b.objective

To determine the effect of pre-treatment of targeting vectors with the SV40 T antigen NLS on effective targeting frequency.

c.results

In order to assess whether NLS binding would improve nuclear transport in ES cells, 2 μg of a fluorescein-12 dUTP-labelled, PCR-amplified 3 kb fragment from the targeting vector p129-ΔHPRT, were incubated for 10 minutes with 0.72 μg of the nuclear localisation oligopeptide of the SV40 T antigen (PKKKRKV) (kindly given by P. Collas), in a total volume of 6 μl of distilled water (0.3 μg DNA/μl). This ratio DNA:NLS (1: 0.33) has been found to result in efficient electrostatic NLS binding to DNA. Treated samples display a band shift compared to untreated DNA when run in a 0.6 % agarose gel. Although NLS dissociate in EDTA-containing buffers (P. Collas, personal communication), band shift is not altered upon formation of DNA-liposome complex. This complex was lipofected (Lipofectamine) into exponentially growing ES cells, and nuclear uptake examined by fluorescence microscopy. The observations were not conclusive, since the volumetric ratio nucleus/cytoplasm is very high in ES cells and it was difficult to distinguish fluorescent nuclei from the cellular fluorescent background (data not shown). Differences between cells transfected with NLS-treated and non-treated DNA were not evident. Since one proposed measurement of the efficiency of NLS-mediated DNA nuclear transport is transfection efficiency (Arohnson and Hughes, 1998; Zanta *et al.*, 1999), it was decided to proceed directly with targeting experiments. 2 μg of *Xho*I-linearised targeting vector p2017 (page 79) were incubated as above with the SV40 NLS. Treated and non-treated plasmids were lipofected (Effectene, Qiagen) into ES cells and colonies were counted after puromycin (for total integrants) and puromycin + 6-TG (for homologous recombinants) selection. The results are shown in table 10 and figure 24.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
NLS-treated	1	812	17	2.09	2.79 +/- 0.376
	2	915	31	3.38	
	3	896	26	2.9	
Control	1	798	17	2.13	1.69 +/- 0.451
	2	876	19	2.16	
	3	751	6	0.79	

Table 10. Effect of treatment of DNA with SV40 NLS on E.T.F. Control: untreated DNA. Cells were lipofected with 2 μg of p2017. S.E.: standard error for each group. Every entry represents an independent experiment.

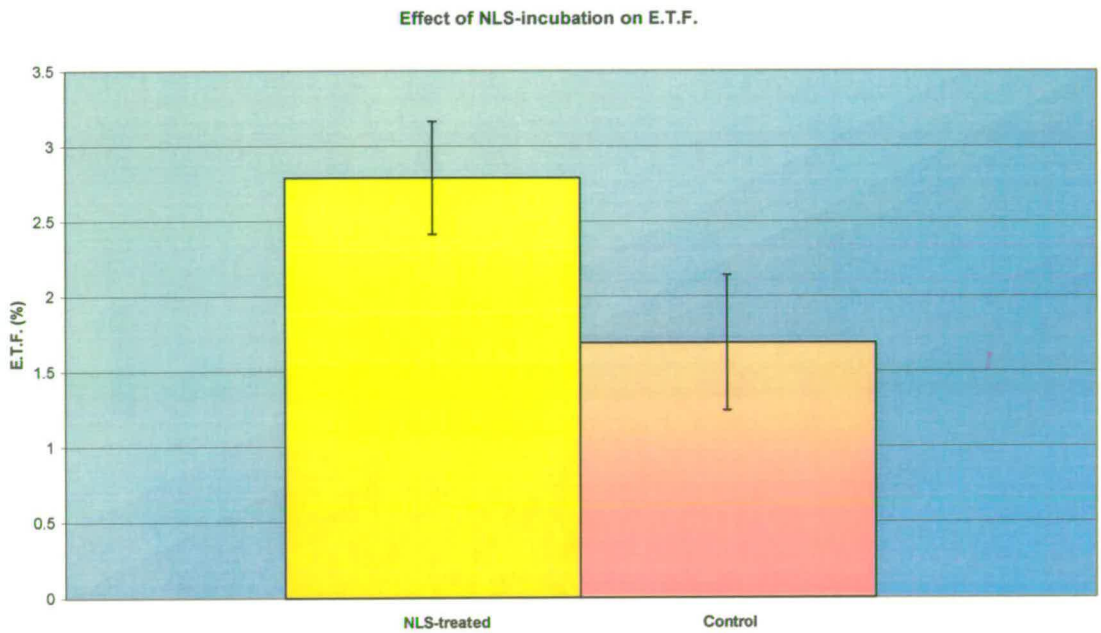

Figure 24. Effect of NLS-incubation on E.T.F.

A one-way ANOVA analysis of these data ($F_{1,4}$ = 3.47; F_{crit} = 7.70; P = 0.135 > 0.05), shows that the null hypothesis (group E.T.F. means are equal) cannot be rejected. A similar conclusion can be drawn from the ANOVA analysis of transfection efficiency, measured as number of puromycin resistant colonies ($F_{1,4}$ = 1.86; F_{crit} = 7.70; P = 0.243 > 0.05). Therefore we conclude that differences between E.T.F. means and between transfection efficiency means are not statistically significant.

d.discussion

The above data demonstrate that neither transfection efficiency nor gene targeting frequency are significantly improved upon pre-incubation of our targeting vectors with the nuclear localisation signal of the SV40 T antigen. Based on experiments described in page 110, it was hypothesised that an increased amount of targeting vector molecules in the nucleus may lead to higher targeting frequencies. Two explanations can be proposed to account for this observation. First, although there is convincing evidence that this NLS actively facilitates nuclear transport of DNA in a variety of cells, both by microinjection (Collas *et al.*, 1996; Collas and Aleström, 1997) and lipofection (Aronsohn and Hughes, 1998; Zanta *et al.*, 1999), the use of this strategy in ES cells has not been reported thus far. We have not been able to unequivocally show enhanced nuclear uptake of DNA following transfection with fluorescein-dUTP-labelled fragments of DNA pre-incubated with NLS. Thus, the possibility that ES cells are not as proficient as other cell types for NLS-mediated nuclear transport of DNA cannot be ruled out. Second, to our knowledge this approach has not been used for DNA molecules larger than ~3.6 kb (Collas *et al.*, 1996). Indeed, it has been reported that the overall level of NLS-mediated nuclear DNA uptake was considerably reduced for fragments > 1.5 kb (Ludtke *et al.*, 1999). The size of the targeting vector used in this work (12.5 kb) may represent an obstacle for efficient translocation. Many NLS distributed along the DNA (as it occurs when NLS and DNA are electrostatically coupled) may inhibit nuclear transport if the molecule is longer than the distance between adjacent pores (figure 25).

If vector size proved to be the reason behind the apparent lack of effect of NLS-preincubation on targeting efficiency, it would be difficult to optimise this strategy using conventional targeting vectors. Improvements in nuclear transport using smaller vectors would possibly be compensated by a decrease in gene targeting frequency associated with reductions in the length of homology. This problem could be overcome by chemically linking one single NLS to the end of a gene (Zanta *et al.*, 1999), which would facilitate entry of the DNA molecule through a single pore. However, this approach has the disadvantage that cytoplasmic DNA degradation may result in the loss

of the signal before nuclear translocation takes place (Ludtke *et al.*, 1999). Alternatively, the use of NLS-based strategies to improve nuclear uptake could be used in conjunction with TFO (Zanta *et al.*, 1999) or PNA (Cutrona *et al.*, 2000) targeting vectors, whose small size makes them specially suited for this purpose.

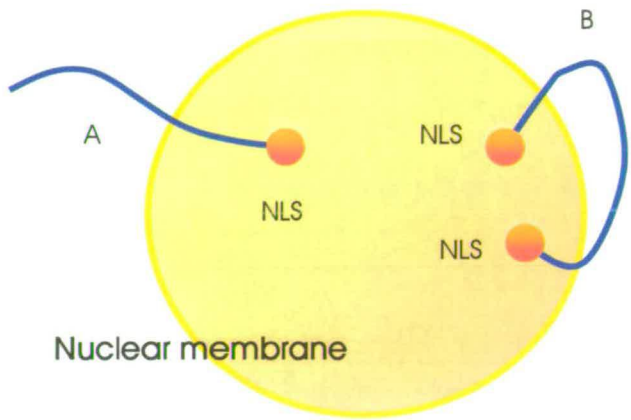

Figure 25. Hypothetical effect of NLS-binding on DNA entry into the nucleus. A single signal favours translocation through the nuclear pore complex (A), but the presence of several NLS in long DNA molecules (B) may actually prevent efficient transport (adapted from Zanta *et al.*, 1999).

iii.dsRNA vectors

a.introduction
b.objective
c.results
d.discussion

a. introduction

There are a number of features of RNA interference (RNAi) technology that led us to seek its application in mammalian cell culture: (1) Long-term down-regulation of the target gene (up to 100x more potent than that of either sense or antisense RNA) is achieved without permanent modification at the genetic level. (2) Work in *C. elegans* shows that there is an active, non-induced transport of dsRNA molecules across cellular boundaries (Fire *et al.*, 1998). Feeding worms with dsRNA-expressing bacteria, or soaking them in dsRNA solutions, is sufficient to reproduce the levels of RNAi observed by microinjection (Timmons and Fire, 1998; Tabara *et al.*, 1999). This active transport could greatly facilitate transfection in cell culture systems. (3) The possibility of using dsRNA to target several genes at once with a very high efficiency (Kennerdell and Carthew, 1998) makes RNAi one of the most powerful tools available for the analysis of gene function in developmental studies *in vivo*. *In vitro*, it could also be used to strongly and simultaneously down-regulate more than one gene, particularly when the nature of the biological culture (e.g., limited lifespan *in vitro*) prevents the completion of several rounds of gene targeting before senescence occurs.

However, the use of RNAi in mammalian cells faces a well-documented dsRNA-triggered 'panic' response. One of the better characterised components of this response is the dsRNA-activated protein kinase (PKR). This protein has been implicated in the regulation of cellular growth and differentiation, as well as interferon-mediated antiviral defense (rev. by Proud, 1995). Activation of PKR by exogenous dsRNA leads

to phosphorylation of the translation initiation factor eIF-2, which blocks its activity. As a result, translation is globally suppressed and the cell initiates apoptosis (Lee and Esteban, 1994).

Since the minimum dsRNA length required for PKR activation is 33 bp (Proud, 1995), we sought to overcome the apoptotic response by designing shorter dsRNA oligonucleotides for *in vitro* transfection of ES cells. *HPRT* was chosen as target gene, for direct selection of *HPRT*-deficient clones in 6-TG after dsRNA-mediated down-regulation.

b.objective

To prove *HPRT* down-regulation by RNAi in ES cells *in vitro*.

c. results

A 30 bp-long dsRNA vector designed against exon 1 of the *HPRT* gene was made by annealing the following sense and antisense oligoribonucleotides (MGW):

5' GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC3'
5' GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC GTAC3'

Formation of dsRNA was confirmed by migration on 3 % agarose and 15 % polyacrylamide gels (figure 26).

A first set of experiments was aimed at determining whether simple incubation of ES cells with medium containing dsRNA in solution is sufficient for effective transfection. In order to assess the stability of dsRNA in ES cell medium (where serum may contain traces of ribonucleases), 1 μg of dsRNA was preincubated for two hours at

37 °C in 500 µl of (a) GMEM ES cell culture medium; and (b) Sterile PBS. As shown in figure 26 c, no apparent degradation was observed in either case.

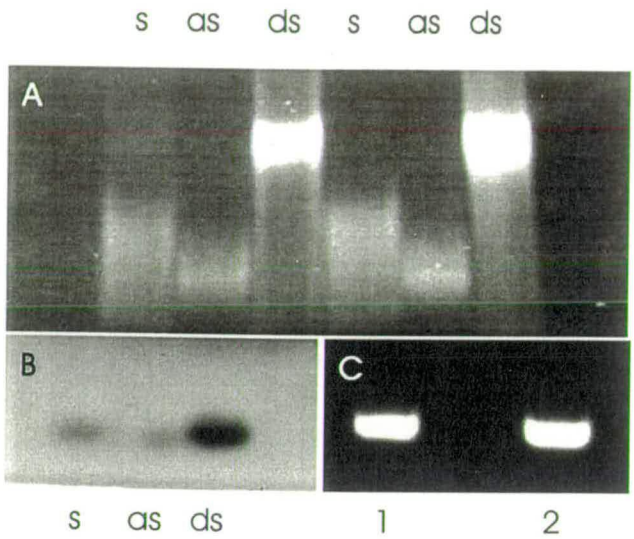

Figure 1. (a) Migration of sense (s), antisense (as) and double stranded RNA (ds) in a 15 % polyacrilamyde gel. Lanes 1, 2, 3, after annealing in 1mM Tris HCl, 1 mM EDTA buffer; lanes 4, 5, 6, annealing in Tris HCl 10 mM, NaCl 20 mM pH 7.6 buffer. **(b)**Migration pattern of s, as and dsRNA in a 2.5 % agarose gel. **(c)** dsRNA after incubation in PBS (1) and GMEM (2), 2 hours at 37 °C.

6-well plate/70 % confluent ES cell cultures (E14 p39) were incubated for 24 hours with 2 ml GMEM containing 0.25; 0.5; 1; 2; 3; and 5 µg of anti-*HPRT* dsRNA. The following day, the cells were trypsinised and separately plated at low density (10^6 cells/ 90 mm dish). 6-TG selection was applied 6 days after incubation, but no colonies were recovered.

In a second series of experiments, 1, 1.5 and 2 µg of dsRNA were lipofected (Effectene, Qiagen) into 6-well plate/50 % confluent ES cell cultures (E14 p39). Cells were trypsinised 24 hours after incubation and selected as above, but no 6-TG resistant colonies could be obtained after 7 days of selection.

d.discussion

Several assumptions were made when designing these experiments: (1) Cultured cells can be directly transfected by dsRNA vectors dissolved in the medium; (2) PKR needs to be down-regulated, or dsRNA vectors have to be designed to avoid PKR activation, in order to produce gene-specific RNAi; and (3) Short dsRNA oligonucleotides are able to interfere with the expression of target genes as efficiently as the bigger (~0.5-1 kb) vectors described thus far in the literature.

With the only possible exception of the first, the remaining suppositions proved to be wrong, according to data published by other groups in the course of these experiments. First, a recent report on the induction of RNAi in mouse embryos (Wianny and Zernicka-Goetz, 2000) demonstrated that concerns about PKR activation in response to dsRNA treatment had been raised prematurely (unless PKR is not expressed yet in early mouse embryonic development, in which case there may still be difficulties in using dsRNA at later stages). Second, studies in *drosophila* S2 cells (Hammond *et al.*, 2000) and syncytial blastoderm cell-free extracts (Tuschl *et al.*, 1999) indicated that RNAi requires a minimum length of dsRNA estimated in ~100 bp, well above the 30 bp of our dsRNA vector. In addition, the selection system utilised (loss of HPRT function) would require an almost complete downregulation of the target gene, since leakiness may lead to residual HPRT activity which would kill the cell in the presence of 6-TG. Thus, although the results of this work do not rule out the possibility that dsRNA fragments can produce RNAi in mammalian cell cultures, longer dsRNA vectors and a more appropriate selectable system (such as GFP downregulation in GFP-expressing cell lines) would be necessary to establish proof of principle.

Chapter V. manipulating the expression of homologous recombination-related genes

i.down-regulation of *p53*
ii.up-regulation of *Rad51*
iii.down-regulation of NHEJ enzymes

i.down-regulation of *p53*

a.introduction

The role of p53 in regulating homologous recombination-based DNA repair has been examined by many groups over the last few years (page 54). A mechanistic explanation of the phenomenon by which cells devoid of p53 regulation exhibit hyperrecombination phenotypes was suggested by the discovery of the direct physical interaction of p53 with Rad51 and recA *in vitro* (Stürzbecher *et al.*, 1996; Buchhop *et al.*, 1997). According to this model, p53 activity would be critical to repress Rad51 post-translationally, thus minimising the risks associated with homologous recombination in the absence of DNA damage. The present work aims at establishing proof of principle that gene targeting frequency is also enhanced in *p53*-defective ES cells. While permanent inactivation of *p53* cannot be considered as a practical approach to improve targeting efficiency, strategies could be envisioned for the transient down-regulation of p53, either transcriptionally or by inhibiting the protein activity. This would create a time window during which homologous recombination processes –and therefore gene targeting- would be highly facilitated.

b.objective

To test the hypothesis that *p53*-defective ES cells show an enhanced frequency of gene targeting compared to wild-type ES cell lines.

c.results

E.T.F. was calculated for three murine ES cell lines following electroporation with p2017. R72.1 (p53 $^{+}/_{-}$) was generated from E14.27 (p53 $^{+}/_{+}$) by targeting a *neo* cassette into one copy of the *p53* gene. HG-287 (p53 $^{-}/_{-}$) was derived from R72.1 by culture in high G418 concentration. Inactivation of *p53* in HG-287 was confirmed both by Southern blot (work carried out by Dr A. Clarke at the Department of Pathology, University of Edinburgh) and loss of detectable protein in a Western blot after γ-irradiation (figure 27).

Five independent experiments were performed for each genotype. All cell lines were karyotypically normal (N. Sphyris, Department of Pathology, University of Edinburgh, personal communication). 10^7 cells and 300 μg of *Xho*I-linearised DNA were used in each electroporation (800 V, 3 μF) and cells were plated in two 150 mm dishes (5 x 10^6 cells/ dish). Puromycin selection (1 μg/ml) was applied 24 h after electroporation. One of the two plates was further supplemented with 6-TG after 6 days of Puromycin selection. A sample of Puro^R/6-TG^R colonies was picked on each experiment for amplification and Southern blot confirmation of the targeting event (figure 28). Both Puro and Puro + 6-TG plates were stained (Giemsa) and resistant colonies were counted (table 11 and figure 29).

Figure 27. p53 protein expression was determined by Western Blot after γ-ray induction (5 Gy). Primary (NCL-p53-CM5) and secondary antibodies were obtained from Novo Castra. Lane 1: wild type; lane 2: p53 +/-; lane 3: p53 -/-; lane 4: human positive control (Oncogene). Loading (20 μg/lane) was controlled by Coomasie blue staining of the polyacrylamide gels prior to blotting (data not shown).

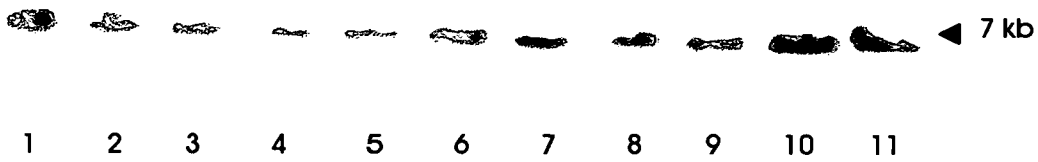

Figure 28. Southern blot of randomly picked colonies from each genotype. Genomic DNA was digested with *BamHI* and probed with a 500 bp *SphI/BglII* fragment located within intron 5 of the targeting vector (figure 22). Successfully targeted clones display a 7 kb band. Lanes 1-4: wild type; lanes 5-7: p53 +/-; lanes 8-11: p53 -/-.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
p53 +/+	1	1589	158	9.943	8.72 +/- 0.349
	2	1707	136	7.967	
	3	1679	140	8.338	
	4	1617	135	8.348	
	5	1318	119	9.028	
p53 +/-	1	1713	168	8.782	9.02 +/- 0.136
	2	1590	149	9.371	
	3	1789	155	8.664	
	4	1638	152	9.279	
	5	1668	151	9.052	
p53 -/-	1	1606	136	8.468	8.81 +/- 0.358
	2	1754	152	8.665	
	3	1553	128	8.242	
	4	1626	167	10.27	
	5	1210	106	8.76	

Table 11. Effect of p53 genotype on gene targeting frequency. Every entry represents and independent experiment. E.T.F., Effective Targeting Frequency. S.E.: standard error for each group.

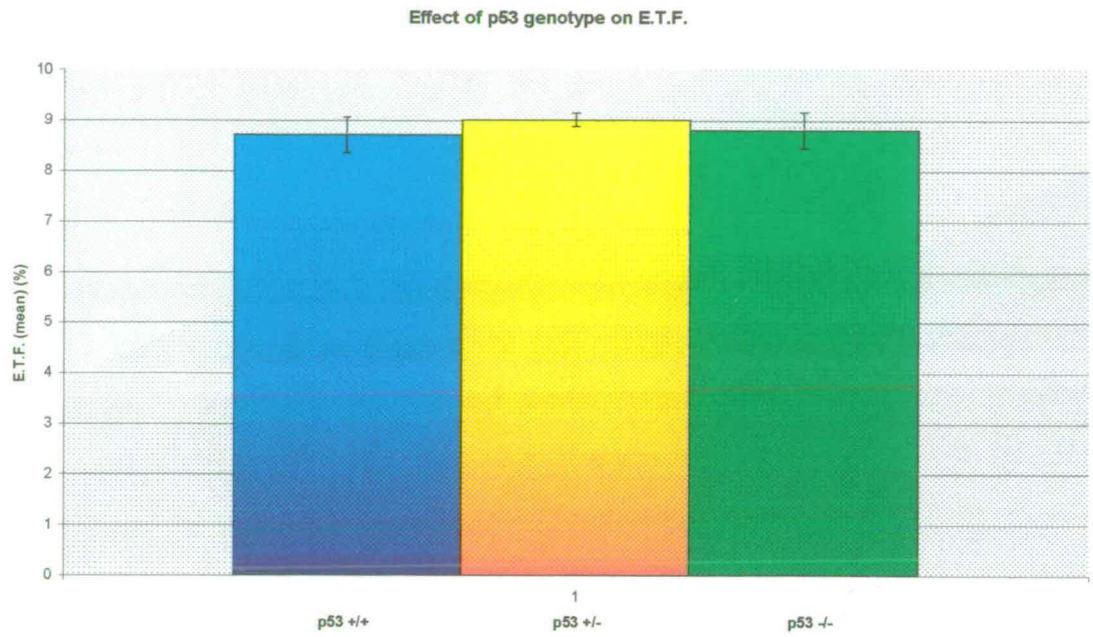

Figure 29. Effect of p53 genotype on E.T.F. using 300 μg of DNA/electroporation. Y bars: standard error for each group.

A one-way ANOVA analysis (Excel) shows that neither E.T.F. ($F_{2,12} = 0.25$; $F_{crit} = 3.88$; $P = 0.78$) nor A.T.F. ($F_{2,12} = 1.88$; $F_{crit} = 3.88$; $P = 0.19 > 0.05$) means differ significantly.

In order to discard the possibility that saturating DNA concentrations might mask any possible effect of *p53* inactivation on gene targeting frequency, these experiments were repeated for wild-type and double knockout genotypes using 150 μg of plasmid DNA/ electroporation. The results are shown in table 12 and figure 30.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
E14 p53 +/+	1	919	53	5.76	**5.34 +/- 0.328**
	2	879	42	4.77	
	3	782	49	6.26	
HG287 p53 -/-	1	763	48	6.29	**5.59 +/- 0.437**
	2	699	37	5.29	
	3	718	45	6.26	

Table 12. Effective targeting frequency in HG-287 and E14 (control) cells after electroporation with 150 μg of p2017. S.E.: standard error for each group.

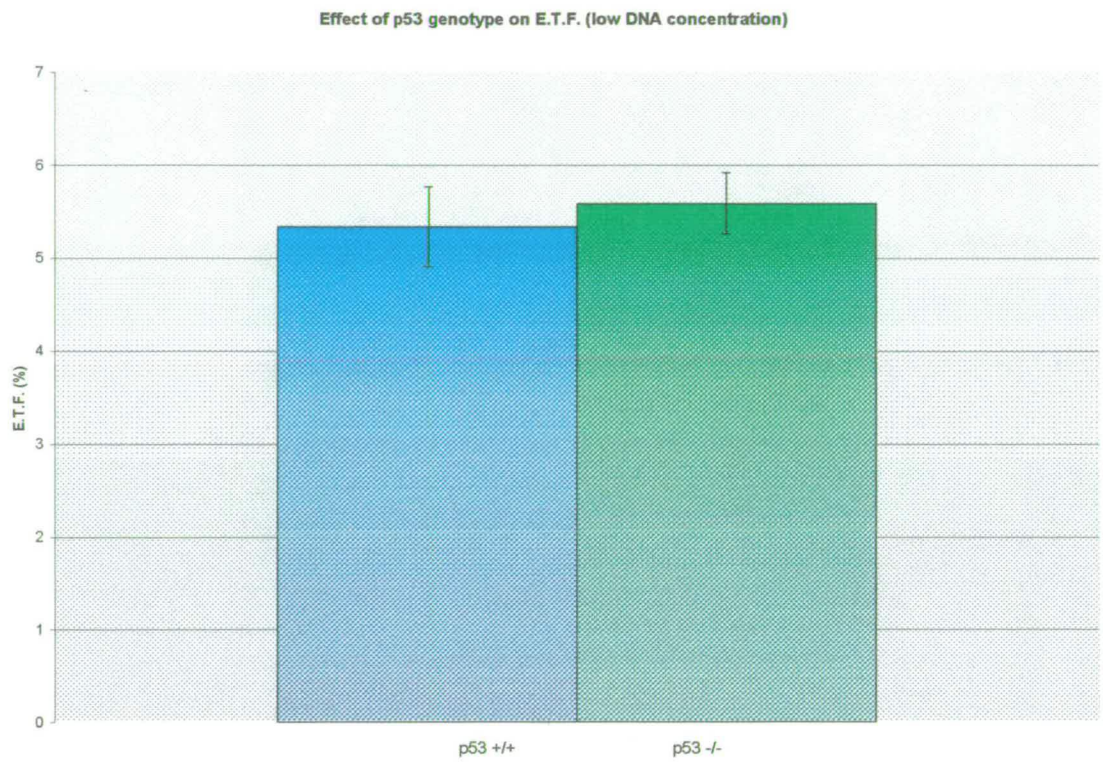

Figure 30. Effect of p53 genotype on E.T.F. using 150 μg of DNA/electroporation. Y bars: standard error for each group.

Again, there is no significant effect of the p53-defective genotype on E.T.F. when using lower concentrations of targeting vector (ANOVA $F_{1,4} = 0.408$; $F_{crit} = 7.708$; $P = 0.557 > 0.05$).

To determine whether or not the absence of p53 function may affect gene targeting frequency in other cell types, E.T.F. was calculated in p53-deficient, spontaneously immortalised mouse embryonic fibroblasts (MEFs) (kindly given by DW Melton). Although it was not possible to obtain a valid immortalised control for this experiment, E.T.F. was also calculated in a MEF line immortalised with the SV40 large T antigen (given by G. Li), which also inactivates p53. 3 μg/ml of puromycin were used to select total integrants after electroporation with 150 μg of p2017. 6-TG selection was applied 6 days after electroporation. As shown in table 13, E.T.F. could not be

calculated in either case due to the absence of 6-TG resistant colonies after 10 days of selection.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
MEF p53 $^{+/+}$	1	870	0	n/a	n/a
	2	985	0	n/a	
MEF p53 $^{-/-}$	1	154	0	n/a	n/a
	2	125	0	n/a	

Table 13. Effect of MEF's p53 genotype on E.T.F. Every entry represents an independent experiment. S.E.: standard error for each group.

d.discussion

The fact that p53 down-regulation is associated with higher levels of homologous intrachromosomal recombination in different biological systems has been well established (page 54). It was therefore predicted that targeting efficiency would be enhanced in p53-defective cells. However, this study demonstrates that the down-regulation of p53 in ES cells has no effect on E.T.F. One possible explanation to account for these results is that the cellular mechanisms behind gene targeting may be different from those mediating intrachromosomal recombination (ICR). There is evidence that ICR and extrachromosomal recombination (ECR) differ in many properties, such as the requirement of homology (Puchta *et al.*, 1992). Whereas mismatches reduce the efficiency of ICR drastically, they have only a minor effect on ECR (Waldman and Liskay, 1987). Interestingly, it has been reported that p53 has a role in controlling the rate of homologous recombination by specific mismatch recognition (Dudenhöffer *et al.*, 1998). The small effect of mismatches on ECR could be explained if p53 is not directly involved in this pathway. The data presented in this study support the hypothesis that p53 controls homologous recombination only at the chromosomal level, but not when one (gene targeting) or both (ECR) recombinant substrates are extrachromosomal.

An alternative explanation is that p53 may be naturally inactive in ES cells. It has been observed that some ES and EC cells show absence of p53-dependent cell cycle regulation, which has led to the hypothesis that inactivation of p53 is a necessary event in the immortalisation of embryonic stem cells (Prost *et al.*, 1998; Schmidt-Kastner *et al.*, 1998). However, we have observed that p53-null ES cells grow faster than their wild-type counterparts (page 103). In addition, they contain numerous genomic abnormalities (DW Melton, personal communication), which is inconsistent with the hypothesis that p53 is naturally inactive in ES cells. In any case, there is increasing evidence that G1/S cell cycle checkpoint control and regulation of homologous recombination are separate and independent functions of p53 (Saintigny *et al.*, 1999; Willers *et al.*, 2000b). Thus, even if p53 is not involved in the control of the cell cycle, it could still repress homologous recombination in wild-type ES cells. The observations described in the present chapter suggest that p53-dependent control of homologous recombination is disabled in ES cells. This would not be the case in somatic cells, where the involvement of p53 in the control of homologous recombination is well documented. In order to test this hypothesis, we aimed at determining whether E.T.F. is increased in p53-defective somatic cells. Targeting experiments were conducted using p53-nullizygous MEFs, which were immortalised as a result of spontaneous downstream effects of p53 inactivation. E.T.F. was also calculated in wild type cells immortalised with the SV40 large T antigen, which also inactivates p53. Since no immortalised cells with a wild-type p53 phenotype were available, this experiment did not have a proper control. The two cell lines used in this study have dissimilar morphologies (data not shown) and exhibit significantly different transfection efficiencies (table 13). A very low targeting efficiency associated with the different genetic backgrounds of the target mice and the 129 strain used in the construction of the targeting vector can be proposed to explain the absence of 6-TG-resistant colonies in either case. Ideally, this experiment should be repeated using primary somatic cells isolated from wild-type and p53-null embryos. This would have required material not available at the time of this study, as well as a thorough optimisation of gene targeting in primary fibroblasts.

ii.up-regulation of *Rad51*

a.introduction
b.objective
c.results
d.discussion

a. introduction

The essential role of Rad51 in promoting the early stages of homologous recombination-mediated DNA repair has already been discussed in page 14 of this work. The present section will focus on the development of strategies to increase gene targeting frequency based on the recombinogenic properties of this protein.

It is thought that, under normal conditions, Rad51 is transcriptionally downregulated to avoid uncontrolled recombination between the widespread DNA repeats across the genome (pages 17-22). Since homologous pairing requires stoichiometric amounts of Rad51 relative to ssDNA, subsaturating concentrations of the protein are unable to catalyse strand exchange (Baumann and West, 1998). Only under exceptional circumstances, where the benefits of 'unleashing' the protein exceed the risks of hyperrecombination, Rad51 is de-repressed. This occurs mainly during meiosis (Shinohara *et al.*, 1992) and following extensive DNA damage (Shinohara *et al.*, 1992; Abbousekhra *et al.*, 1992; Basile *et al.*, 1992). While little is known about the molecular mechanisms that keep Rad51 under control, a few groups have been able to overcome them by overexpressing the enzyme. Thus, a 2-3 fold overexpression of Rad51 in CHO cells was found to stimulate homologous recombination between integrated genes (20-fold) and increase resistance to ionising radiation (Vispé *et al.*, 1998). Enhanced homologous recombination was also observed when mammalian (Shcherbakova *et al.*, 2000) and plant (Reiss *et al.*, 1996) cells were transfected with the Rad51 bacterial counterpart gene, recA, fused to a nuclear localisation signal.

Given that gene targeting is a form of homologous recombination, it was reasoned that enhancement of the rate of this process by overexpressing its key enzyme would also result in a higher targeting efficiency. This work demonstrates that murine ES cells stably transfected with a *Rad51* transgene under the control of a constitutive promoter exhibit increased gene targeting frequencies. We next sought to reproduce these results by transiently inducing high Rad51 concentrations at the time of gene targeting, by (a) co-transfecting the targeting vector with a Rad51 expression cassette; (b) incorporating a Rad51 cassette in the targeting vector; (c) co-culturing ES cells with feeder layers stably transfected with the fusion protein VP22-Rad51; and (d) transiently pre-transfecting cells with a VP22/Rad51 expression vector prior to gene targeting. Like other proteins such as HIV-1 Tat (Ensoli *et al.*, 1993), interleukin 1β (Rubartelli *et al.*, 1990) and fibroblast growth factors (FGFs) (Jackson *et al.*, 1992), the herpes simplex virus tegument protein VP22 is secreted by a Golgi-independent mechanism from the cells where it is synthesised (Elliot and O'Hare, 1997). What makes VP22 unique is its unusual ability to 'transfect' adjacent cells via a yet unknown non-endocytotic pathway. In addition, despite the lack of any recognisable nuclear localisation signal, VP22 is directly transported to the nucleus, where it binds to chromatin and seggregates upon cell division. Remarkably, chimaeric proteins made by fusing VP22 to other peptides retain the ability to spread between cells (Elliot and O'Hare, 1997), which has been exploited in recent years to develop non-transgenic protocols for the delivery of markers (Elliot and O'Hare, 1999), prodrugs (Dilber *et al.*, 1999) and therapeutic proteins (Phelan *et al.*, 1998).

b.objective

To examine the effect of constitutive and transient overexpression of Rad51 on E.T.F.

c.results

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c.1.*Rad51*-transgenic ES cell lines

The construct pSV-Zeo/Rad51 (given by M. Defais) was used to stably transfect E14 ES cells (100 μg of *Kpn*I-linearised vector, 10^7 cells). This plasmid contains the hamster *Rad51* (*hsRad51*) cDNA cloned downstream from the SV40 constitutive promoter, as well as a *zeo* selectable marker for selection in zeocin (10 μg/ml). Ten zeocin-resistant clonies (R1-10) were picked and grown for hsRad51 expression analysis. Integration of the transgene was confirmed by Southern blotting, which revealed random insertion of at least 1-2 copies/genome in all cases (figure 31).

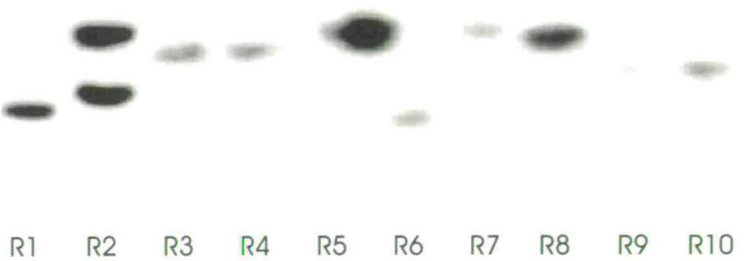


Figure 31. Southern blot analysis of clones R1-R10. A pSV-Zeo/Rad51 589 bp *Rsr*II/*Sac*I fragment corresponding to the zeocin cassette was used as a probe to confirm successful integration of the vector. Genomic DNA was digested with *Eco*RV, which does not cut within the sequence of the transgene. Therefore, every band represents an independent integration event. Sizes vary between 5 and 12 kb.

Transgene transcription was demonstrated by RT-PCR, using primers that non-selectively amplify both endogenous and transgenic Rad51. As shown in figure 32, digestion of the retrotranscribed cDNAs with *Nsi*I (whose recognition sequence is present in the endogenous gene, but not in the transgene) reveals the existence of two different Rad51 mRNA populations in the cell extracts. The possibility that the uncut 1 kb fragment represented only incomplete digestion of the endogenous transcript was eliminated by digestion with *Xho*I (which gave two extra 0.5 kb bands corresponding to the transgenic transcript) and Northern blotting, which specifically showed expression of HsRad51. Under stringent conditions, a HsRad51-derived probe specifically hybridises with the transgenic transcript, as confirmed by the absence of signal in non-transgenic controls (figure 33).

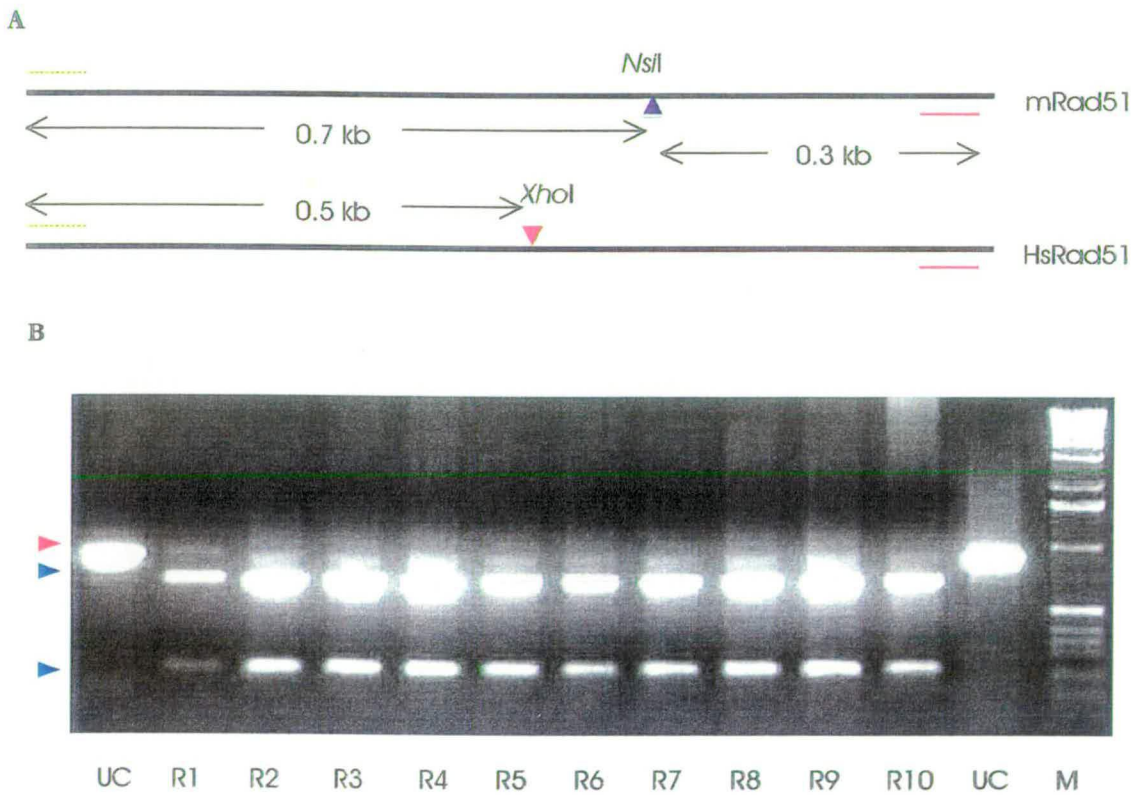


Figure 32. (A) Schematic depiction of the two mRNA populations, mouse (m-) and hamster (Hs-)Rad51, amplified by RT-PCR using the primers 5'T G C A G A T A C T T C A G T G G A A G3' (forward, green) and 5'A G A C A G G G A G A G T C A T A G A3' (reverse, red). Relative position of the *Nsi*I and *Xho*I recognition sequences in mRad51 and HsRad51 are indicated. **(B)** *Nsi*I overdigestion (5 hours) of the ~1kb Rad51 band RT-PCR-amplified from transgenic RNA extracts (UC) yields three bands in all clones examined (R1-R10). The two lighter bands (~700 and 300 bp, in blue) correspond to endogenous Rad51 transcripts. The heaviest band (~1 kb, in red) may represent transgenic hsRad51 transcripts, which lack the *Nsi*I recognition sequence.

Using human Rad51 antibodies (no commercial anti-HsRad51 is available), Rad51 signals could not be detected by Western blot (figure 34). Thus, clonal variability in transgene expression was assessed by Northern analysis.

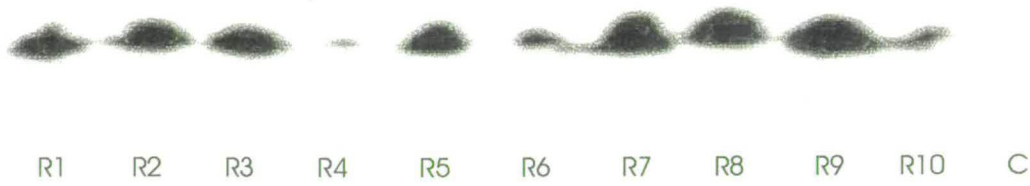


Figure 33. Northern analysis of clones R1-10. Membranes were probed with a 0.38 kb *Xho*I-*Sph*I HsRad51-derived cDNA probe. Using stringent hybridisation conditions, this probe does not hybridise to endogenous mRad51 transcripts (C, control). Loading (15 μg/lane) was similar in all lanes, as determined by ethidium-bromide staining of the gel prior to blotting. An actin control would have been necessary to unequivocally demonstrate Rad51 overexpression.

Figure 34. Western analysis of clones R1-10. Mouse monoclonal anti- human Rad51 antibodies (GeneTex) were used in conjunction with anti-actin (Sigma) antibodies as loading control. The two primary antibodies were added in different combinations, both sequentially and simultaneously. The observed bands represent the ubiquitous 42 kD actin protein. Rad51 bands (38 kD) could not be detected (loading: 20 μg/lane).

In order to study the effect of Rad51 overexpression on gene targeting, *HPRT* effective targeting frequency (E.T.F.) was calculated for two clones with strong transgene expression (R7 and R9). Following the experimental design described in page 108, E.T.F. was found to be up to 4-fold that observed in wild-type parental E14 ES cells (table 14 and figure 35). This increase is statistically significant within experiments ($F_{1,8}$ = 1220.17; F_{crit} = 5.317; P = 4.93 x 10^{-10} < 0.05) and reflects a net increase in the absolute number of *HPRT*-targeted (6-TG resistant) clones rather than a decrease in the rate of illegitimate recombination.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
Clone R7	1	1732	567	32.73	**32.61 +/- 0.552**
	2	1584	518	32.7	
	3	1607	496	30.86	
	4	1677	576	34.34	
	5	1554	504	32.43	
Clone R8	1	1119	200	17.87	**15.26 +/- 0.834**
	2	1256	204	16.24	
	3	1472	223	15.15	
	4	1312	175	13.33	
	5	1524	209	13.71	
E14 (control)	1	1628	136	8.35	**8.42 +/- 0.417**
	2	1500	107	7.13	
	3	1227	99	8.06	
	4	1198	115	9.59	
	5	1325	119	8.98	

Table 14. E.T.F. in Rad51-transgenic clones R7 and R8. 300 μg of p2017 were used per experiment. Every entry represents an independent experiment. S.E., standard error.

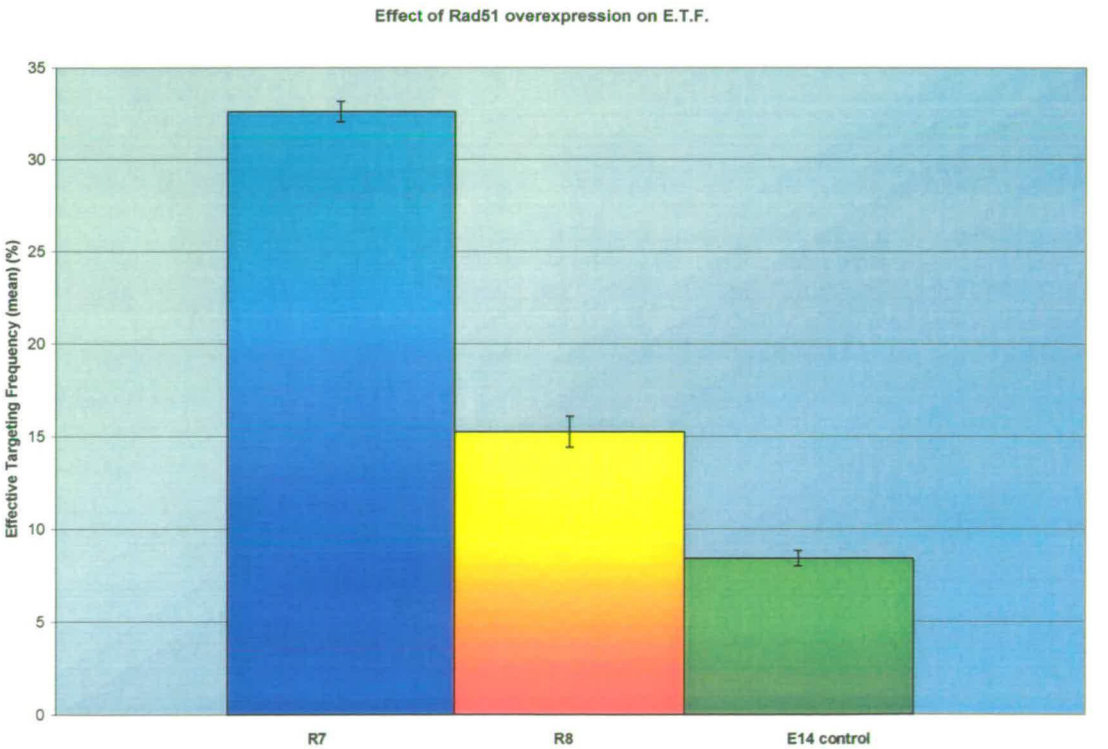


Figure 35. Effect of constitutive Rad51 overexpression on E.T.F. Y bars: Standard error for each group.

c.2.E.T.F. in *Rad51*-transgenic/*p53* null cells

Although it has already been proven that p53 inactivation *per se* has no effect on gene targeting frequency in ES cells (pages 124-130), it was thought that overexpression of Rad51 protein in cells devoid of this mechanism of control may result in even higher rates of homologous recombination. In order to test this hypothesis, *p53*-nullizygous/Rad51-overexpressing ES cell lines were generated by stably transfecting HG-287 cells (given by A. Clarke) with pSV-Zeo/Rad51. Ten zeocin-resistant clones were selected and analysed for Rad51 expression as previously described (figure 36).

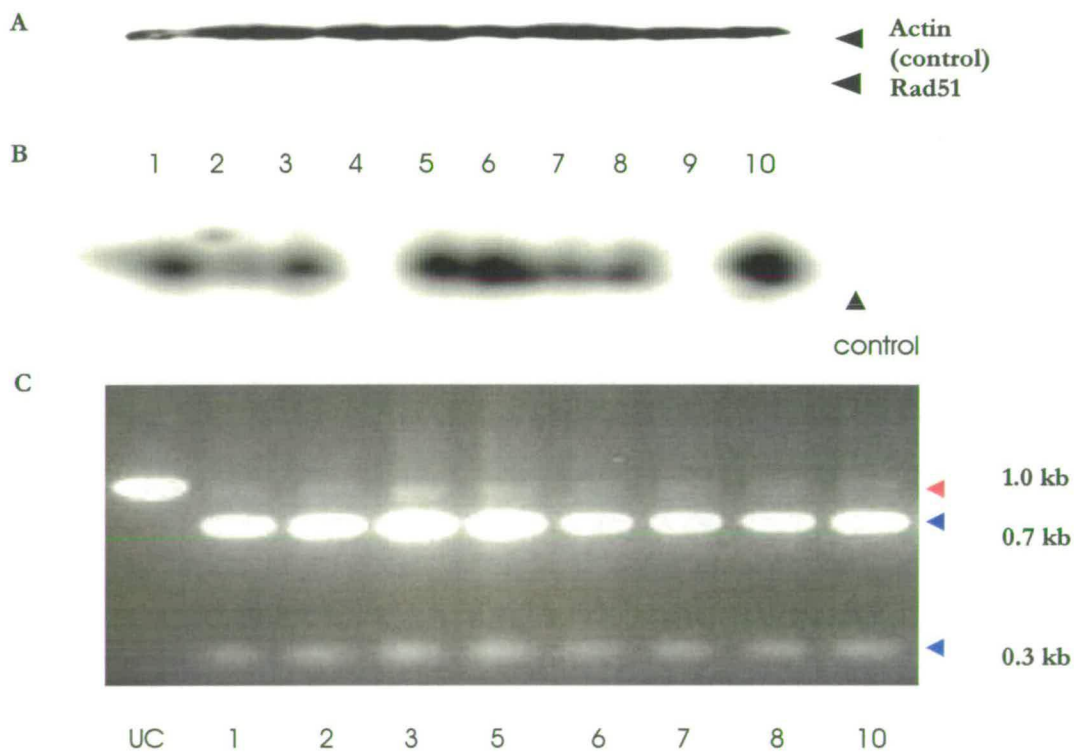


Figure 36.(A) Western analysis of Rad51 expression in p53-/- cells transfected with pSVZeo/Rad51. As previously explained, the lack of Rad51 signal might be due to the low affinity of the human antibody utilised (loading, 20 μg/lane). **(B)** Northern analysis shows HsRad51 expression in transgenic p53 -/- clones (loading, 15 μg/lane) **(C)** RT-PCR analysis of clones 1-3, 5-8 and 10 shows the presence of HsRad51 and endogenous mRad51 in the amplified transcript (see figure 4 for more details).

E.T.F. was calculated as above for clones 53/6 and 53/10, which showed strong levels of Rad51 expression. HG-287 ES cells were used as controls. As summarised in table 15 and figure 37, E.T.F. is consistently higher in both transgenic clones than in HG-287 ($F_{2,6} = 50.764$; $F_{crit} = 5.143$; $P = 0.00017 < 0.05$). A two-fold increase in E.T.F., comparable to that observed in R8, was observed in 53/10 cells.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
Clone 53/6	1	1815	232	12.78	**12.74 +/- 0.765**
	2	1718	196	11.40	
	3	1800	253	14.05	
Clone 53/10	1	1379	222	16.09	**17.52 +/-0.724**
	2	1652	298	18.03	
	3	1702	314	18.44	
HG 287 control	1	1567	142	9.06	**8.39 +/- 0.347**
	2	1698	134	7.89	
	3	1761	145	8.23	

Table 15. Effect of constitutive Rad51 overexpression on E.T.F. Every entry represents an independent experiment. S.E.: standard error for each group.

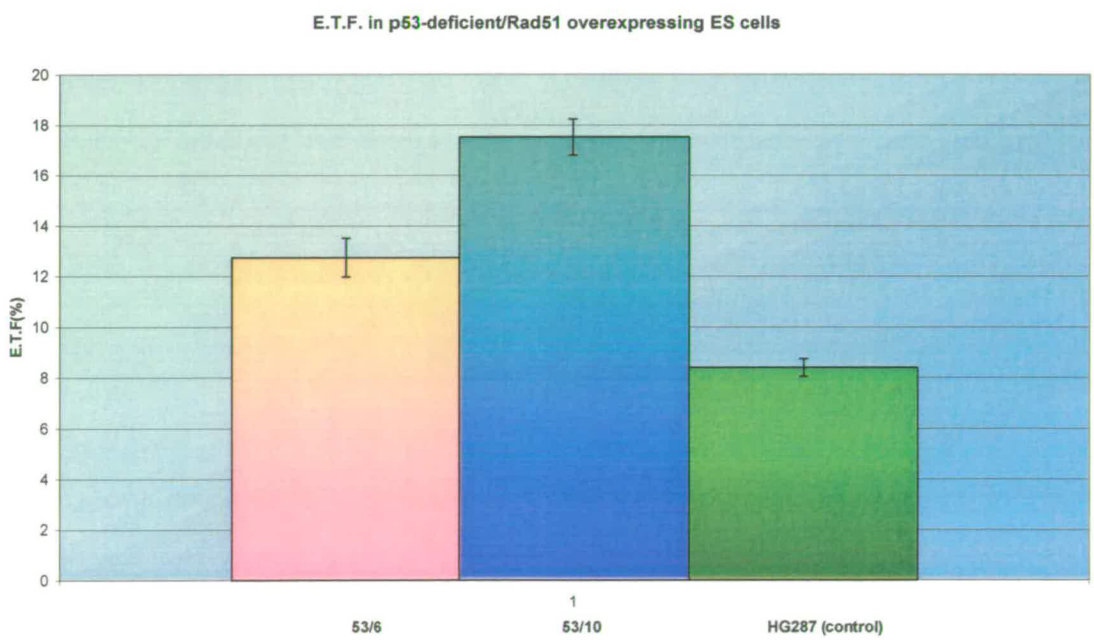


Figure 37. Effect of constitutive Rad51 overexpression in p53-deficient cells on E.T.F. Y bars: Standard error for each group.

c.3 co-electroporation approach

Having demonstrated that constitutive overexpression of Rad51 results in higher targeting frequencies, we next decided to investigate whether this could also be achieved by transient up-regulation of Rad51. One possible strategy would be the simultaneous transfection of the targeting vector and a Rad51 supercoiled expression plasmid. This idea is based on the well-known fact that circular DNAs tend to remain episomal and maintain their expression for a few days before getting degraded. A molar excess of the *Rad51* construct over the targeting vector would ensure that most of the cells transfected with the latter also incorporate the former. Finally, critical for the success of this strategy was the assumption that there is enough time for Rad51 to accumulate upon transfection before targeted integration takes place.

To investigate this possibility, E14 ES cells were electroporated with two different DNA mixtures. A first group of cells (test group) received circular pSVZeo/Rad51 (230 μg) and *Xho*I-linearised p2017 (200 μg). This represents a 3:1 molar ratio of the Rad51 expression plasmid over the targeting vector. As a control, a second group of cells was co-electroporated with p2017 and pTracer (Invitrogen), an empty expression vector with a structure similar to that of pSVZeo/Rad51 and roughly the same size. While keeping the amount of p2017 unchanged (200 μg), 238 μg of pTracer were added to maintain the same proportion between the two DNA species. *HPRT* effective targeting frequency was calculated for the two groups of cells. As shown in table 16 and figure 38, there is an statistically significant 4.5-fold stimulation of gene targeting in cells co-transfected with pSVZeo/Rad51 compared to the control group (ANOVA $F_{1,,8} = 233.55$; $F_{crit} = 5.317$; $P = 3.37 \times 10^{-7} < 0.05$). Compared with a second control (cells electroporated with targeting vector only), Rad51-cotransfection results in a 1.7-fold E.T.F. increase (ANOVA $F_{1,,8} = 58.47$; $F_{crit} = 5.317$; $P = 6.03 \times 10^{-5} < 0.05$).

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
T.V. + Rad51	1	1264	209	16.53	**15.502 +/- 0.77**
	2	1243	196	15.76	
	3	1150	184	16	
	4	1345	225	16.72	
	5	1400	176	12.5	
T.V. alone	1	1156	105	9.08	**9.104 +/- 0.326**
	2	1201	97	8.07	
	3	1261	116	9.19	
	4	1056	107	10.13	
	5	1358	123	9.05	
T.V. + pTracer	1	3148	119	3.78	**3.548 +/- 0.102**
	2	3408	117	3.43	
	3	3075	105	3.41	
	4	2980	118	3.96	
	5	3014	100	3.31	

Table 16. Effect of co-electroporation of the targeting vector (p2017, T.V.) with pSVZeo/Rad51 (Rad51) or pTracer (Invitrogen). A 3:1 molar ratio circular plasmid (230 μg): T.V. (200 μg) was used in both cases. Targeting vector alone (T.V., 200 μg) was used as control.

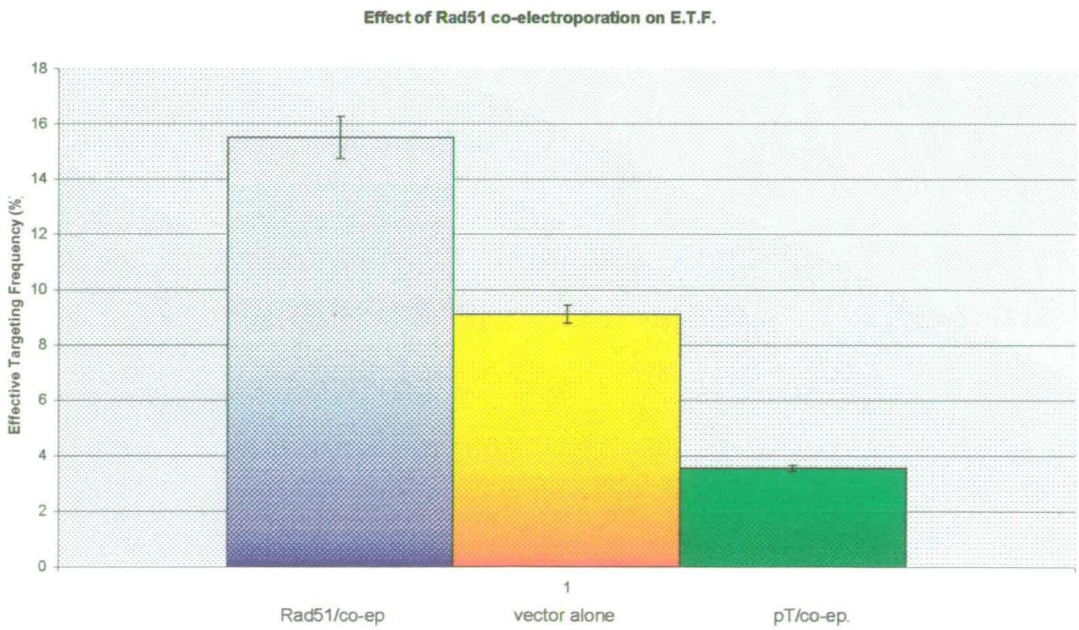


Figure 38. Effect of Rad51 co-electroporation on E.T.F. Y bars: Standard error for each group.

c.4.p2017/51

The observation that co-electroporation with pTracer results in a marked decrease in E.T.F. suggests that there may be side effects associated with the entry of a high number of DNA molecules into the cell (see Discussion). It was thought that this problem could be overcome by engineering the *Rad51* expression cassette into the targeting vector. This would ensure the simultaneous presence of both elements in all transfected cells without increasing the molar amount of DNA. To this purpose, a 1.9 kb *Eag*I fragment of pSVZeo/Rad51 encompassing the *HsRad51* ORF, the SV40 immediate-early promoter and the SV40 poly-adenylation tail, was subcloned outside the region of *HPRT* homology in p2017 (see Materials and Methods). In order to determine whether or not Rad51 is expressed from this vector (p2017/51), RNA was recovered from transiently transfected cells and RT-PCR- amplified using Rad51 primers. As previously discussed in page 134, these primers amplify both endogenous and transgenic transcripts. After five cycles of PCR, DNA was overdigested with *Xho*I, which selectively cuts mouse Rad51 cDNA but leaves intact the transgenic HsRad51 cDNA (see Materials and Methods). PCR was allowed to proceed for 30 cycles more and samples were run in a 1 % agarose gel. A 1 kb band corresponding to the endogenous transcript is observed in *Xho*I digests. Samples digested with *Nsi*I show a weaker signal that may correspond to the transgenic transcript, but not those digested with both *Nsi*I and *Xho*I (which cuts the transgene) (figure 39).

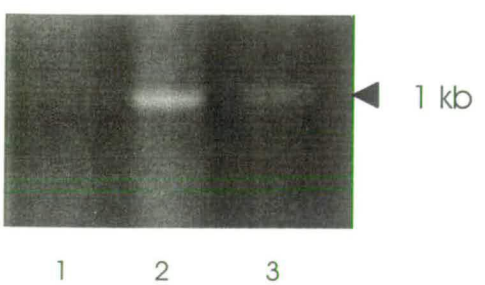


Figure 39. RT-PCR-amplified Rad51 transcripts (mRad51 and HsRad51) from p2017/51-transiently transfected cells were selectively digested (5 hours) with *Xho*I (which cuts HsRad51) and/or *Nsi*I (which cuts mRad51) after 5 PCR cycles. Following digestion, PCR was allowed to proceed for 30 cycles. Lane 1: double *Xho*I/*Nsi*I digestion (no band); Lane 2: *Xho*I digestion; Lane 3: *Nsi*I digestion. These experiments were not controlled for contaminating genomic DNA amplification (discussion in page 150).

p2017/51 was subsequently linearised (*Not*I) and used to conduct targeting experiments in E14 wild-type cells (200 μg/electroporation). 150 μg of p2017 (molar ratio 1:1) were used to transfect control cells.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
p2017/51	1	442	31	7.013	**7.622 +/- 0.755**
	2	526	48	9.125	
	3	431	29	6.728	
p2017	1	631	49	7.765	**7.438 +/- 0.217**
	2	598	45	7.525	
	3	612	43	7.026	

Table 17. Effect of p2017/51 on E.T.F. Every entry represents an independent experiment. S.E.: standard error for each group.

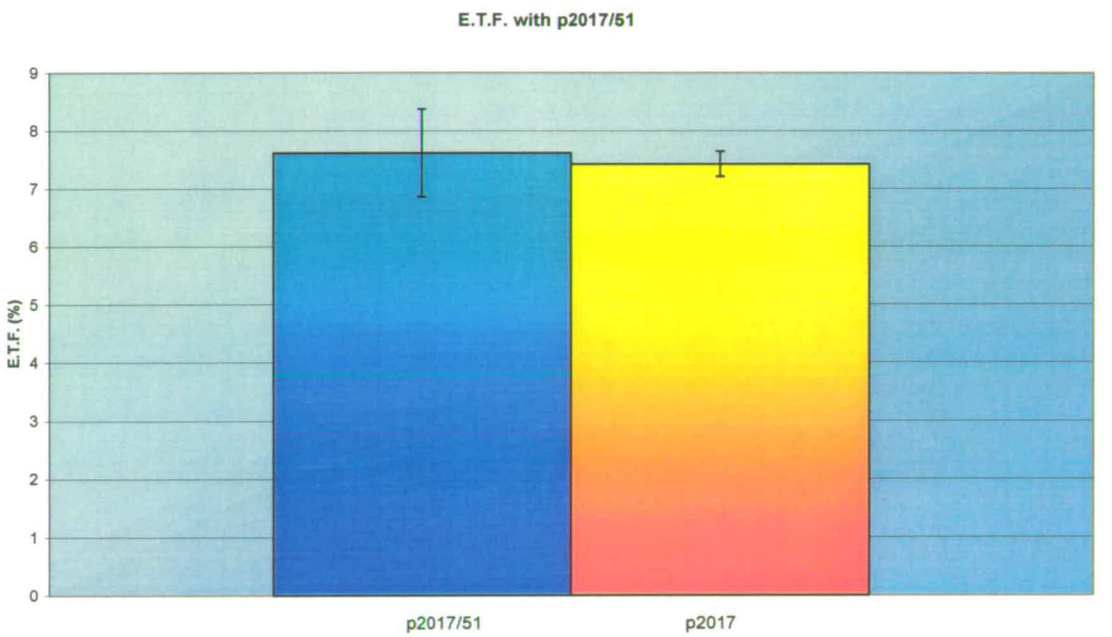


Figure 40. Effect of p2017/51 on E.T.F. Y bars: standard error for each group.

A one-way ANOVA analysis of the data shown in table 17 and figure 40 indicates that the differences between the E.T.F. means obtained with p2017 and p2017/Rad51 are not statistically significant ($F_{1,4}$ = 0.054; F_{crit} = 7.708; P = 0.827 > 0.05).

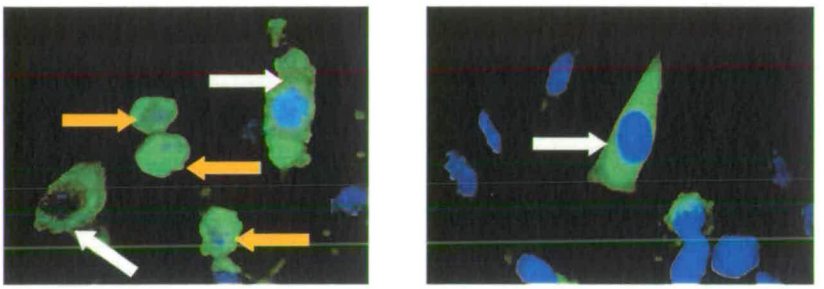
c.5.VP22-Rad51

It has already been proven that an excess of Rad51 results in enhanced rates of homologous recombination. Ideally, we would like to transiently provide this extra amount of enzyme at the time of transfection via a non-transgenic approach. The properties of the viral protein VP22 (discussed in Introduction) seemed to be specially suited for this purpose. Using a VP22-Rad51 fusion transgene (page 84), two approaches were designed to transport the chimaeric protein into target cells: (1) Co-culturing ES cells in the presence of VP22-Rad51-stably transfected STO feeder fibroblasts; and (2) Sequential electroporation with the VP22/Rad51 transgene (day 1) and the targeting vector (day 2).

VP22-Rad51-transgenic feeder cells were created by electroporating STO's with 100 μg of linearised pVP22/Rad51. Seven G418 resistant clones were amplified and screened by Southern blot for the presence of the transgene (data not shown). VP22-Rad51 expression was determined by Western blot in five positive clones, as shown in figure 41.

Figure 41. Western blot of VP22-Rad51-transgenic STO feeder cell lines. Primary antibody: rabbit anti-*myc* tag (NBL). Secondary antibody: mouse anti IgG-HRP (Sigma). Lanes 1-5, stable VP22-Rad51 transfectants (as determined by Southern blot). Lane 6: Control (untransfected cells). Coomasie-blue staining of a replica polyacrylamide gel was used as loading control. The size of the protein is in the region corresponding to the 75 kD rainbow marker (Amersham). This is approximately the predicted size of the fusion protein (38kD Rad51 + 32 kD VP22 + 6 kD 6xHis and *myc* tags). Loading: 15 μg/lane.

In order to establish the translocation properties of VP22-Rad51, clone 4 (which showed strong expression of the protein in the Western blot) was co-cultured with wild-type STO fibroblasts in a 1:50 ratio. Slides were fixed after 48 hours and then immunostained with an antibody raised against the 6xHis tag of the fusion protein (figure 42)



A B

Figure 42. Staining resembling the pattern expected of VP22-Rad51 trasnslocation was observed by fluorescein-immunostaining of VP22-Rad51 STO cells co-cultured with normal STOs (1:50 ratio) for 48 hours **(A)**. Primary antibody: mouse anti-6xHis (Invitrogen); secondary antibody: goat anti-IgG-fluorescein conjugate (Calbiochem). White arrows show cytoplasmic staining (fluorescein, green) of VP22-Rad51-expressing cells. Orange arrows indicate VP22-Rad51-stained nuclei. Blue is nuclear counter-staining (DAPI). However, the frequency of this observation was low. VP22-Rad51-expressing cells were normally surrounded by non-stained cells, as shown in **(B)**. Therefore, the possibility of a staining artifact cannot be ruled out.

Although it was not possible to unequivocally prove widespread occurrence of this phenomenon, staining resembling that expected of VP22 translocation (cytoplasmic staining in VP22-Rad51 transgenic cells and nuclear staining of neighbouring cells) was occasionally observed. Despite its low frequency, it was decided to proceed with targeting experiments.

In a first set of experiments, wild-type ES cells were cultured for 48 hours in the presence of (a) VP22/Rad51 STO clone 4 feeder layers, or (b) normal STO feeder layers. E.T.F. was calculated following transfection with 150 μg of p2017. A one-way ANOVA analysis of the results presented in table 18 and figure 43 indicates that the hypothesis that the means of the two groups analysed are the same cannot be rejected ($F_{1,4} = 7.033$; $F_{crit} = 7.708$; $P = 0.056 > 0.05$).

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
VP22-Rad51 STOs	1	536	44	8.208	7.444 +/- 0.420
	2	638	47	7.366	
	3	577	39	6.759	
Normal STOs	1	576	36	6.25	6.229 +/- 0.182
	2	525	31	5.904	
	3	612	40	6.535	

Table 18. Effect of incubation with VP22/Rad51 STO's on E.T.F. Every entry represents an independent experiment. S.E.: standard error for each group.

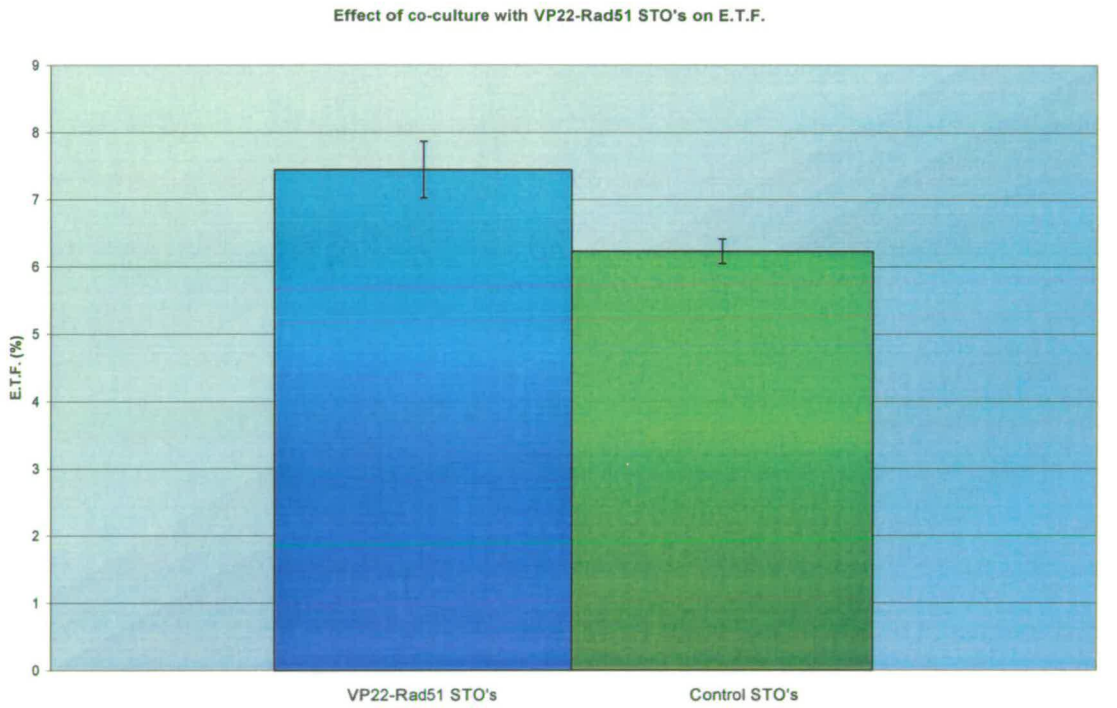


Figure 43. Effect of incubation with VP22/Rad51 STO's on E.T.F. Y bars: standard error for each group.

In the second experimental approach, 10^5 ES cells were lipofected (Effectene, Qiagen) with 1 μg of circular pVP22/Rad51. Three controls were done by transfecting similar ES cell populations with equimolar amounts of pVP22 (Invitrogen), pTracer

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
pVP22-Rad51	1	108	5	4.62	**4.256 +/- 0.197**
	2	95	4	4.21	
	3	76	3	3.94	
pVP22	1	84	3	3.57	**3.163 +/- 0.267**
	2	92	3	3.26	
	3	75	2	2.66	
pTracer	1	64	2	3.12	**2.480 +/- 0.430**
	2	75	2	2.66	
	3	60	1	1.66	
pSVZeo/Rad51	1	91	2	2.19	**2.976 +/-0.425**
	2	82	3	3.65	
	3	97	3	3.09	

Table 19. Effect of transient pre-lipofection (1 μg) with pVP22/Rad51, pVP22, pTracer or pSVZeo/Rad51 (day 1) on E.T.F. (calculated after transfection with 1 μg of p2017 on day 2). Every entry represents an independent experiment. S.E.: standard error for each group.

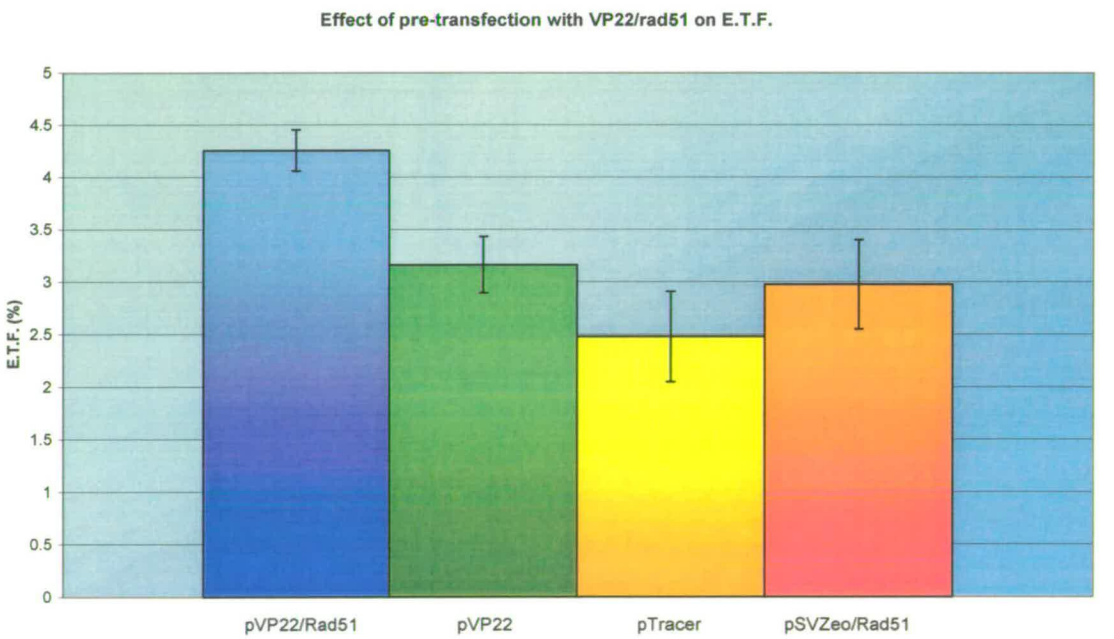


Figure 44. Effect of transient pre-lipofection (1 μg) with pVP22/Rad51, pVP22, pTracer or pSVZeo/Rad51 (day 1) on E.T.F. (calculated after transfection with 1 μg of p2017 on day 2). Y bars: standard error for each group.

(Invitrogen) and pSVZeo/Rad51. 24 hours later medium was removed and all four plates lipofected again with 1 μg of p2017. Puromycin selection and 6-TG selections were applied as usual. The results are summarised in table 19 and figure 44. In this case, an ANOVA analysis of the four groups shows that the difference between E.T.F. means are statistically significant ($F_{3,8} = 4.709$; $F_{crit} = 4.066$; $P = 0.035 < 0.05$).

d. discussion

The Northern blots described in pages 135 and 137 (using a probe that specifically hybridises with the transgenic transcript) suggest that hamster Rad51 may be overexpressed in transgenic ES cell clones. Untransfected controls show no signal. Although a proper loading control (i.e. actin) would have been desirable, RNA was carefully quantitated and absence of RNA degradation in all lanes was confirmed by ethidium bromide staining prior to blotting. The level of Rad51 overexpression could not be calculated by Western blot, because the human Rad51 antibodies used in this work do not hybridise with the hamster protein. RT-PCR experiments show a band that may correspond to the transgenic transcript. The weakness of this band compared to that corresponding to the endogenous transcript might reflect the preferential amplification of the latter over the transgenic mRNA, rather than their relative expression levels. Sequencing of the DNA regions used to design the primers would be necessary to confirm that there are no point mutations in the transgene that could impair RT-PCR amplification.

This study demonstrates for the first time that targeting efficiency can be enhanced in ES cells following stable transfection with a Rad51 cassette. Similar results have been independently reported in human cells by Yáñez and Porter (1999), which proves the validity of this approach in other testing systems. These findings are in agreement with the idea that Rad51 levels are rate-limiting in homologous recombination (Baumann and West, 1998), and raise interesting possibilities for the design of novel strategies to enhance targeting frequency.

The physical interaction of p53 with Rad51 *in vivo* (Stürzbecher *et al.*, 1996) has been interpreted as a manifestation of the critical role of p53 in controlling genomic stability. In this context, it has been proposed that hyperrecombination phenotypes in p53-deficient cells are the direct consequence of the loss of p53 control over the process of homologous recombination (Buchhop *et al.*, 1997). Direct evidence to support this model comes from the observation that specific biochemical activities of the Rad51 bacterial homologue recA, such as DNA strand exchange, are inhibited by p53 *in vitro* (Stürzbecher *et al.*, 1996). We show here that overexpression of Rad51 in p53-deficient ES cells results in increased (up to 2-fold) targeting frequencies compared to non-transgenic controls, but not higher than those observed in p53 +/+/Rad51-overexpressing clones. This result confirms that p53 is not directly involved in the regulation of homologous recombination in ES cells (pages 124-130)

Although these experiments unequivocally establish the principle that constitutive expression of Rad51 enhances the rate of homologous recombination, generation of stable *Rad51* transfectants is impractical as a general scheme to improve gene targeting frequency. Homologous recombination-based gene therapy protocols, for instance, cannot rely on the previous introduction of a transgene in the target cells. Besides, it can be reasoned that permanent up-regulation of the recombination machinery may destabilise the genome and ultimately lead to cell death.

In contrast, transient overexpression of Rad51 could be the basis of experimental approaches aimed at creating a temporary window of time during which the process of homologous recombination would be highly favoured. This would minimise the risk of uncontrolled genomic rearrangements in the long term. To this purpose, three novel strategies have been examined, namely: (1) co-transfection of the targeting vector with a Rad51-expression cassette; (2) engineering of a Rad51 expression cassette within the targeting vector; and (3) non-transgenic delivery of a VP22-Rad51 fusion protein.

We report here a ~4.5-fold enhancement in E.T.F. when the targeting vector (p2017) is co-electroporated with a Rad51-expression cassette (pSVZeo-Rad51), compared to controls where the co-transfected plasmid does not contain any Rad51

sequence (pTracer). However, unlike previous observations in experiments conducted in *Rad51*-transgenic lines, this result is mainly a consequence of an average ~2.5-fold enhancement of illegitimate recombination in cells co-transfected with p2017 and pTracer compared to those receiving p2017 and pSVZeo-Rad51 (puromycin resistant colonies mean = 3125 and 1280, respectively) (table 16).

In contrast, there are no significant differences between the groups transfected with p2017 + pSVZeo-Rad51 and p2017 alone in the average number of colonies recovered after puromycin selection (puromycin resistant colonies mean = 1280 and 1206, respectively). The average number of 6-TG resistant colonies observed in the p2017 + pSVZeoRad51 group (198) is ~1.8-fold higher than in the other two groups (112 and 109 for p2017 + pTracer and p2017 alone, respectively).

Since none of the circular plasmids (pSVZeo-Rad51 and pTracer) is selectable in puromycin, these results suggest that there may be a specific hyperactivation of the illegitimate recombination pathway associated with the entrance of a relatively high number of non-homologous DNA molecules into the cell. This would affect the integration pattern of both the targeting and the expression vectors, although the selection used (puromycin) only allows measurement of the integration level of the former. Up-regulation of the illegitimate integration pathway would therefore result in a higher number of puromycin resistant colonies and a proportional decrease in E.T.F. (as observed in the p2017 + pTracer group).

However, when the molecule in excess contains a Rad51 expression cassette (pSVZeo/Rad51), the frequency of non-homologous integration of p2017 is not enhanced. This could be explained if transient Rad51 overexpression down-regulates illegitimate integration in favour of the homologous integration pathway. The notion that high Rad51 levels may result not only in enhanced homologous recombination levels (as observed in cells co-transfected with p2017 and pSVZeo-Rad51), but also in down-regulation of the NHEJ pathway, is introduced to account for the observation that the total number of integrants in this group is approximately the same as in cells electroporated with the targeting vector alone. The fact that NHEJ is not significantly decreased in Rad51 stable transfectants (table 14) suggests that this hypothetical Rad51-

mediated down-regulation of NHEJ may be an specific response to the overactivation of this pathway resulting from the cellular uptake of a high number of DNA molecules. A speculative model is proposed in figure 45 to explain these observations.

In order to circumvent the side effects associated with the co-electroporation approach, it was decided to engineer a Rad51 expression cassette directly into the targeting vector. By placing this cassette outside the region of *HPRT* homology, homologous recombination events result in its loss. Based on the results of the co-transfection experiments, it was thought that there may be a time window sufficient for the expression and accumulation of Rad51 prior to integration. However, targeting experiments using p2017/51 and p2017 as a control show no difference in E.T.F. This can be explained by several reasons. First, RT-PCR data about Rad51 expression from p2017/51 (page 141) are not conclusive, since *Nsi*I-uncut mouse Rad51 cDNA may theoretically account for the band observed in lane 3 of figure 39. Expression of Rad51 from a linear targeting vector may be less efficient than from the supercoiled Rad51 plasmid. In addition, the RT-PCR results cannot rule out the possibility of point mutations in the Rad51 ORF, which would result in the synthesis of a defective protein. Finally, even if the protein is functional and expressed normally, the overall size of the vector (over 16 kb) may decrease the efficiency of nuclear uptake.

As an alternative to strategies based on the overexpression of Rad51 from a transgene, it was thought to transiently provide the target cells with a supplement of the protein itself. Since large peptides do not spontaneously penetrate the cellular membrane, Rad51 was fused in frame with the VP22 carrier protein, which translocates between adjacent cells and accumulates in the nucleus of recipient cells. The moderate E.T.F. increase observed after co-culturing target ES cells with VP22-Rad51-transgenic STO feeder layers is not statistically significant. There is a significant enhancement of E.T.F. when cells are pre-electroporated with pVP22/Rad51, but the low number of 6-TG resistant colonies recovered per experiment (1-5) makes it difficult to unequivocally determine whether this enhancement is due to VP22-Rad51 import. Optimisation of this experimental design would serve to draw more consistent conclusions. It is clear from the immunostaining analysis that, even if VP22-Rad51 translocation takes place, its extent may be lower than expected. This can be one of the reasons for the lack of a

more dramatic enhancement of E.T.F. As previously discussed, the presence of undetected point mutations in key regions of VP22 and/or Rad51 may also severely impair the properties of the fusion protein. Lastly, the possibility that VP22 may alter the recombinogenic properties of Rad51 cannot be discarded. Further studies about the biochemical properties of VP22/Rad51 *in vitro* would be necessary to clarify this point.

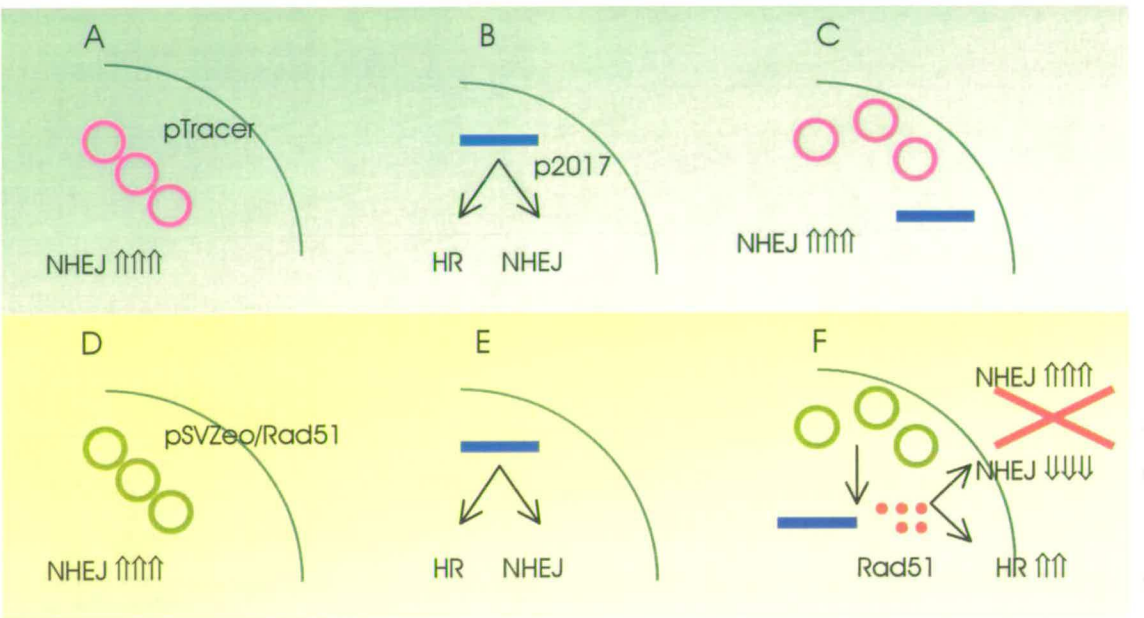


Figure 45. Hypothetical model to explain the effect of the electroporation of pSVZeo/Rad51 or pTracer with p2017 on E.T.F. Top (A, B, C), pTracer co-electroporation; bottom (D, E, F), pSVZeo/Rad51 co-electroporation. When any two plasmid species are co-transfected, three basic situations can be considered: in (A) and (D) only the circular, non-homologous plasmid enters the cell. We propose that this event may up-regulate NHEJ, but is undetectable due to the selection used (puromycin). In (B) and (E), only p2017 enters the cell. This may result in either homologous (HR) or illegitimate (NHEJ) recombination. In (C), both p2017 and pTracer enter the cell. This improves the frequency of non-homologous integration of p2017, which results in a very low E.T.F. However, when pSVZeo/Rad51 is co-electroporated (F), the plasmid number-mediated up-regulation of NHEJ is compensated by the repression of this process by Rad51 (red dots), and the overall rate of illegitimate recombination remains unaffected. The effects of plasmid number and Rad51 cancel each other out, as indicated by a red cross. Rad51 also has a positive effect on homologous recombination and therefore targeting frequency is enhanced.

iii.down-regulation of PARP and NHEJ enzymes

a.introduction

Illegitimate recombination or non-homologous end-joining (NHEJ) is a major pathway for the repair of chromosomal DSBs in the DNA of somatic cells, as well as the specific process of V(D)J recombination in the immune system. In contrast with yeast, homologous recombination seems to play only a complementary role in mammalian cells. The only exception of this general rule is meiosis. Over the past few years, inactivation of genes involved in these two pathways has been used to gain a clearer understanding of their interaction and regulation. The fact that knockouts of some key NHEJ enzymes (such as Ku70, Ku80 or DNA-PK_{cs}) are viable suggests that other mechanisms may take over the general process of DNA repair when the NHEJ mechanism is impaired. This is consistent with the observation that Ku proteins are strongly downregulated during meiosis, which has led to the hypothesis that Ku levels may determine the choice between homologous and non-homologous recombination pathways (Goedecke *et al.*, 1999).

In this context, the aim of this chapter is to assess whether down-regulation of NHEJ results in up-regulation of homologous recombination, measurable in terms of gene targeting frequency. To this purpose, we have examined effective targeting frequency in DNA-PK_{cs} and Ku80 nullizygous cells, as well as following PARP down-regulation. Although PARP is not directly involved in NHEJ, it has been proposed that its activation may act as a switch between the two main DNA-repair processes.

According to the model described in page 22, DSB-induced poly(ADP-ribosyl)ation of this enzyme facilitates NHEJ by preventing the access of the HR machinery to the DNA break. Numerous studies show that inactivation of PARP results in lower NHEJ and higher homologous recombination rates (Farzaneh *et al.*, 1988; Waldman and Waldman, 1990; Semionov *et al.*, 1999; Waldman and Waldman, 1991). Thus, it was hypothesised that gene targeting frequency would be increased in PARP knockouts and/or following PARP chemical inhibition.

b.objective

To determine the effect of Ku80, DNA-PK$_{cs}$ and PARP down-regulation on E.T.F.

c.results

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c.1.DNA-PK$_{cs}$ -/- ES cells

E.T.F was calculated in #2-35-2 (DNA-PK$_{cs}$ -/-), #2 (+/-) and parental TC-1 (+/+) ES cell lines (kindly given by Y. Gao), following electroporation with the targeting vector p2017 (250 μg). Puromycin and 6-TG selections were applied as previously described (page 108).

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
DNA-PK$_{cs}$ +/+	1	1120	130	11.6	10.76 +/- 0.702
	2	1259	118	9.37	
	3	1314	149	11.33	
DNA-PK$_{cs}$ +/-	1	698	48	6.87	7.34 +/- 0.417
	2	456	36	7.89	
	3	607	54	8.89	
DNA-PK$_{cs}$ -/-	1	120	7	5.83	6.80 +/- 0.490
	2	97	7	7.21	
	3	149	11	7.38	

Table 20. Effect of DNA-PK$_{cs}$ genotype on E.T.F. 250 μg of p2017 were used per experiment. S.E. = Standard error for each group.

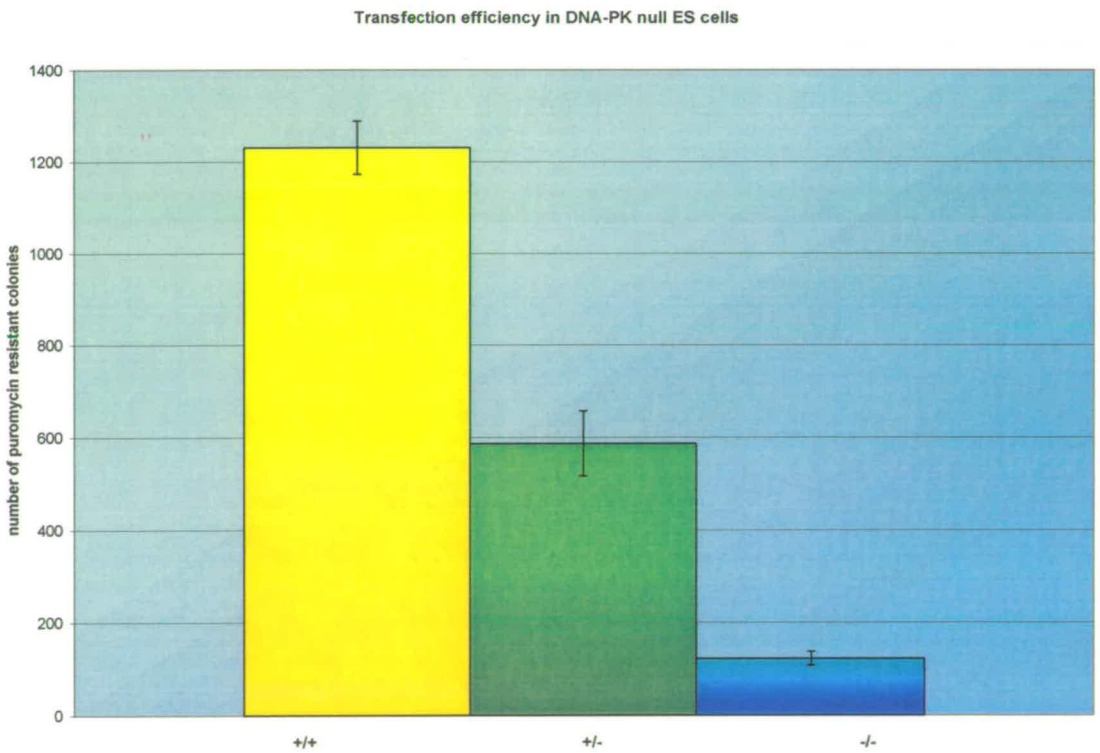

Figure 46. Effect of DNA-PK$_{cs}$ genotype on transfection efficiency (measured as the number of puromycin resistant colonies following transfection with p2017). Error bars: Standard error for each group.

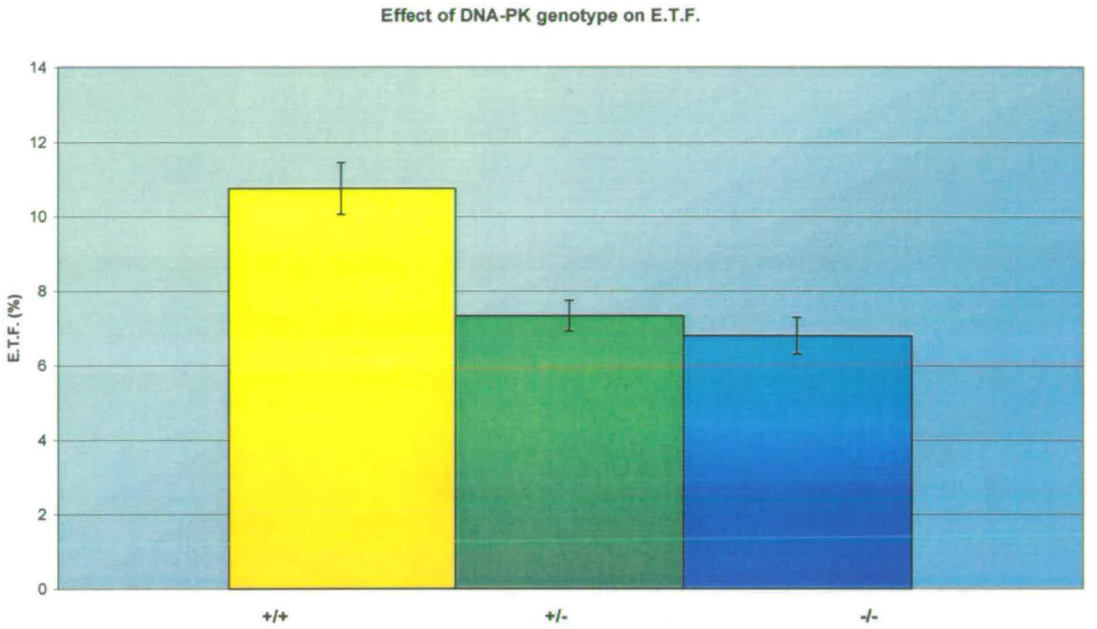

Figure 47. Effect of DNA-PK$_{cs}$ genotype (+/+, +/-, -/-) on E.T.F . Y bars: Standard error.

A one-way ANOVA analysis of the data presented in table 20 and figures 46 and 47 shows that the differences observed both between transfection efficiency means ($F_{2,6}$ = 108.96; F_{crit} = 5.143, P = 1.92 x 10^{-5} < 0.05) and between E.T.F. 's ($F_{2,6}$ = 11.70; F_{crit} = 5.143, P = 0.008 < 0.05) of the three groups studied, are statistically significant.

c.2.Ku80 -/- mouse embryonic fibroblasts

Using the same targeting vector (250 μg), E.T.F. was subsequently determined in Ku80 -/- mouse embryonic fibroblasts (kindly given by G. Li), immortalised with the large T antigen of SV40 (Nussenzweig *et al.*, 1996) A concentration of 2.5 μg of puromycin/ml was found to kill non-transfected cells and yield resistant colonies in p2017-transfected cells after 10 days of antibiotic selection.

		Puromycin^{R} colonies	6-TG^{R} colonies	E.T.F (%)	Group E.T.F. mean $^{+}$/- S.E.
Ku80 $^{+/+}$	1	207	0	n/a	**n/a**
	2	326	0	n/a	
	3	239	0	n/a	
Ku80$^{-/-}$	1	62	0	n/a	**n/a**
	2	51	0	n/a	
	3	32	0	n/a	

Table 21. Effect of Ku80 genotype on E.T.F. 250 μg of p2017 were used per experiment. S.E. = Standard error for each group. N/A, non applicable.

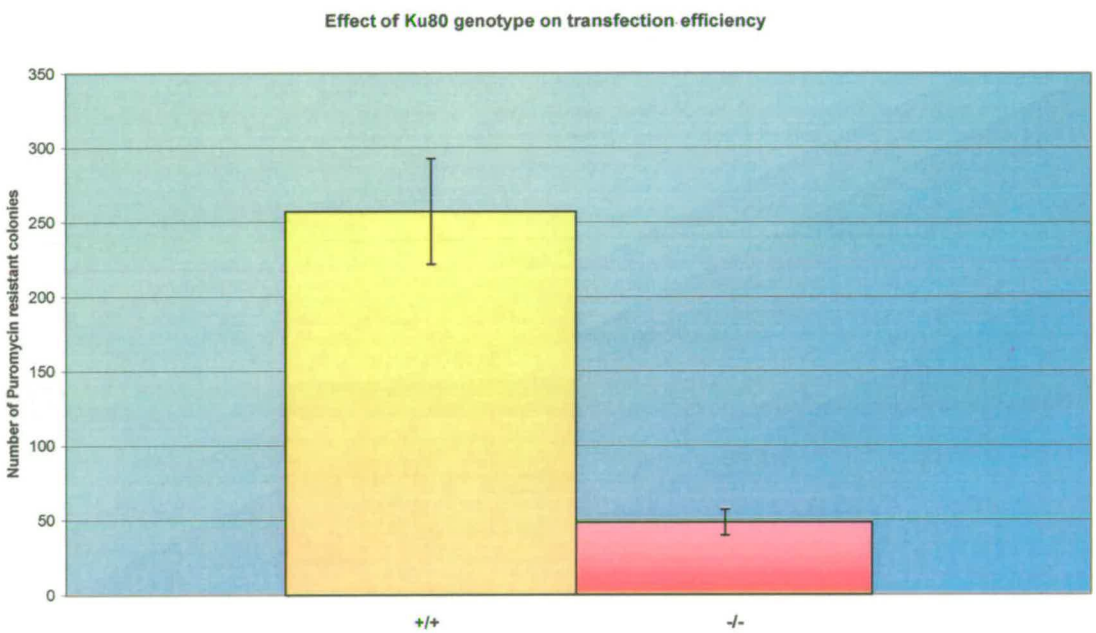

Figure 48. Effect of Ku80 genotype on transfection efficiency (measured as the number of puromycin resistant colonies following p2017 transfection). Y bars, standard error for each group.

As shown in table 21 and figure 48 , there is an statistically significant decrease in transfection efficiency associated with the loss of Ku80 function ($F_{1,4}$ = 32.57; F_{crit} = 7.70; P = 0.0046 < 0.05). E.T.F. cannot be calculated because no colonies were recovered after 6-TG selection.

c.3.PARP -/- ES cells

E.T.F. was calculated for the ES cell lines 210 (PARP -/-), 226 (+/-) and parental wild type J1 (kindly given by M. Matsutani). 300 μg of the targeting vector p2000 were used per electroporation. This vector (which contains a blasticidin selectable marker) was utilised instead of p2016 and p2017, due to the presence of neomycin and puromycin cassettes in the double knockouts. An antibiotic concentration of 5 μg/ml was found to effectively select blasticidin-resistant clones in six days (page 100). The results of the targeting experiments are shown in table 22 and figure 49.

A one-way ANOVA analysis of these data shows that the 3.3-fold increase in E.T.F. observed in PARP -/- cells compared with wild-type controls is statistically significant ($F_{1,4}$ = 279.68; F_{crit} = 7.708; P = 7.49 x 10^{-5} < 0.05). In contrast, the null hypothesis that PARP +/- and PARP +/+ means are the same cannot be rejected ($F_{1,4}$ = 0.916; F_{crit} = 7.708; P = 0.39 > 0.05).

		Blasticidin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
PARP +/+	1	745	101	13.55	**12.89 +/- 0.755**
	2	693	79	11.39	
	3	618	85	13.75	
PARP +/-	1	599	92	15.35	**13.95 +/- 0.799**
	2	625	87	13.92	
	3	588	89	12.58	
PARP -/-	1	600	278	46.3	**43.04 +/- 1.636**
	2	683	285	41.7	
	3	615	253	41.13	

Table 22 . Effect of PARP genotype on gene targeting frequency. E.T.F., Effective Targeting Frequency. S.E., Standard Error .

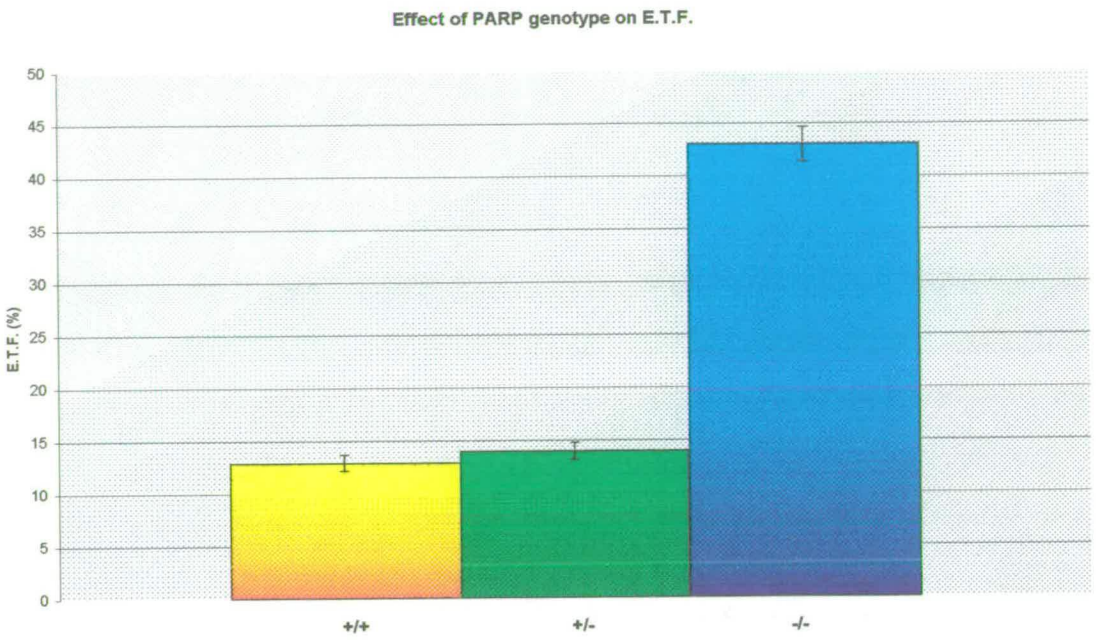

Figure 49. Effect of PARP genotype on E.T.F. Y bars: standard error for each group.

c.4.chemical inhibition of PARP

As shown in the previous section, effective targeting frequency is enhanced in PARP-deficient ES cells. The aim of the following experiments was to reproduce this effect by transiently down-regulating PARP activity in wild-type cells. Among the many inhibitors of poly(ADP-ribosyl)ation, 3-aminobenzamide (3-AB) is commercially available and has proved to be effective in a variety of cell types (Ueda and Hayaishi, 1985; Lindahl *et al.*, 1995; Guo *et al.*, 1998; Latour *et al.*, 2000).

J1 ES cells were incubated in different concentrations of 3-AB (Sigma) for 24 hours before electroporation with p2017 (250 μg). Puromycin and 6-TG selections were applied as described (page 108). The results are shown in table 23 and figure 50.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
1 mM 3-AB	1	1672	142	8.49	8.38 +/- 0.274
	2	1422	125	8.79	
	3	1780	140	7.86	
5 mM 3-AB	1	1578	179	11.34	11.15 +/- 0.120
	2	1705	191	11.2	
	3	1372	150	10.93	
10 mM 3-AB	1	516	21	4.06	3.71 +/- 0.173
	2	509	18	3.53	
	3	619	22	3.55	
Control	1	1455	130	8.93	8.81 +/- 0.188
	2	1444	122	8.44	
	3	1567	142	9.06	

Table 23. Effect of 3-AB-mediated downregulation of PARP activity on gene targeting frequency. E.T.F., Effective Targeting Frequency. S.E., Standard Error .

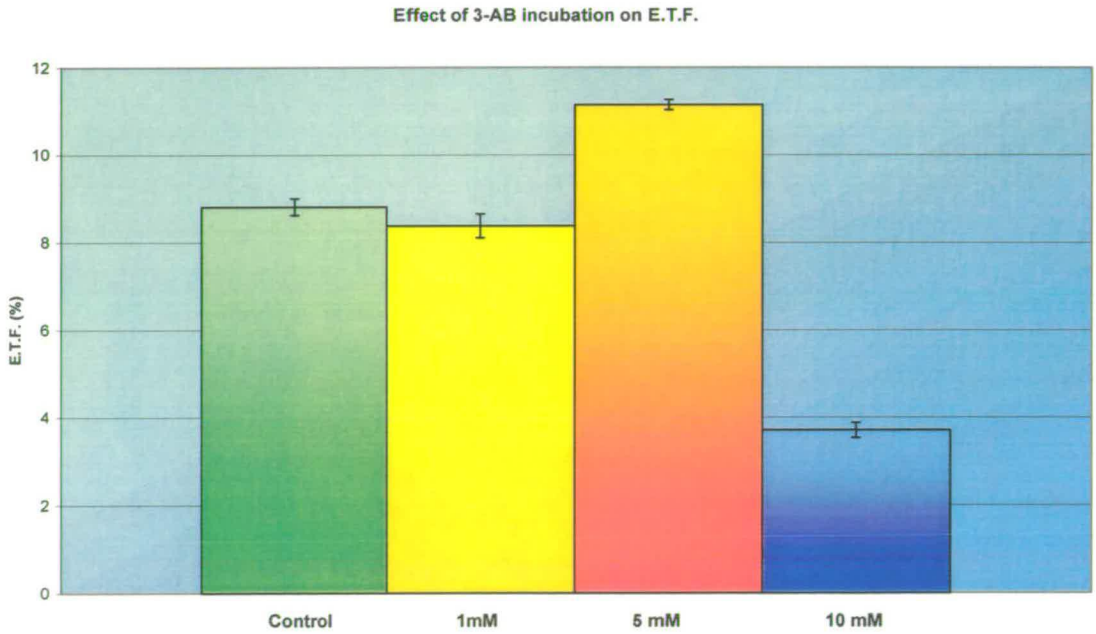

Figure 50. Effect of PARP down-regulation by 3-AB on E.T.F. Y bars: Standard error for each group.

A one-way ANOVA analysis of the above data demonstrates that E.T.F. is significantly enhanced (~30 %) in ES cells treated with 5 mM of 3-AB ($F_{1,4}$ = 109.89; F_{crit} = 7.708; P = 0.000468 < 0.05) and decreased (2.4 - fold) after treatment with 10 mM of 3-MB ($F_{1,4}$ = 395.30; F_{crit} = 7.708; P = 3.78 x 10^{-5} < 0.05) when compared with untreated control cells. Treatment with 1 mM of 3-AB has no effect on E.T.F. ($F_{1,4}$ = 1.669 ; F_{crit} = 7.708; P = 0.265 > 0.05).

d.discussion

Inactivation of Ku80 has been shown to result in severe impairment of the NHEJ DNA-repair pathway in mammalian cells (Nussenzweig *et al.*, 1996). Both Ku70 and Ku80 knockout mice are viable but immunocompromised due to defects in V(D)J recombination (Featherstone and Jackson, 1999b). These animals are usually much smaller than their heterozygous littermates, and somatic cells isolated from them often fail to proliferate and show premature senescence *in vitro* (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). It has been suggested that this phenotype is a consequence of a slower rate of cell division, given the inability of Ku-deficient cells to efficiently repair DNA damage after checkpoint arrest (Featherstone and Jackson, 1999a,b). Disruption of Ku70 and Ku80 results in very similar phenotypes, and it has been observed that the loss of one subunit destabilises the other (Smider *et al.*, 1994; Taccioli *et al.*, 1994; Errami *et al.*, 1996). Unlike Ku80, however, a small level of lymphocyte development has been reported in Ku70 null mice (Gu *et al.*, 1997). Homodimerisation of Ku80 in the absence of Ku70 has been proposed to account for this residual NHEJ activity (Featherstone and Jackson, 1999a). For this reason Ku80 was chosen over Ku70 as the subject of this study. Based on observations by Goedecke *et al.* (1999) and Parsons *et al.* (2000), our working hypothesis was that down-regulation of the non-homologous recombination pathway may result in homologous recombination taking over the process of genomic integration of exogenous DNA, and therefore in enhanced targeting efficiency.

Ku80 -/- mouse embryonic fibroblasts (MEF's) were used instead of null ES cells, which were not available at the time this work was started. Immortalisation of these cells with the large T antigen of SV40 (G. Li, personal communication) was essential to maintain them in culture. Targeting experiments using wild-type immortalised MEF's as a control show that transfection efficiency is decreased ~5-fold in Ku80-deficient cells. This result is consistent with the prevailing idea that integration of exogenous DNA occurs preferentially through illegitimate recombination. No 6-TG resistant colonies could be recovered, which would be the case if the frequency of homologous recombination is too low to be detected in this system. This can be explained either by (a) the different genetic backgrounds of the nullizygous mice and the 129 strain from which the targeting vector was developed or (b) the observation that Ku80-deficient cells exhibit excess degradation of extrachromosomal DNA (Liang and Jasin, 1996). In any case, the fact that none of the puromycin resistant colonies obtained from null MEF's were homologous recombinants (page 156) shows that illegitimate integration takes place in the absence of functional Ku80 protein. This result also rules out the hypothesis that the choice of NHEJ or homologous recombination to integrate exogenous vectors depends on the level of Ku protein. As previously proposed to explain V(D)J NHEJ residual activity in Ku70 -/- mice, homodimerisation of the remaining Ku70 protein can be responsible for a small number of non-homologous integrants. Alternatively, other enzymes of the NHEJ pathway may account for the random integration of the targeting vector in the absence of Ku. One of such enzymes is DNA-PK_{cs}. In most systems studied, this protein is recruited by Ku70/80 to DSBs, where it has been proposed to play structural and signal transduction roles in NHEJ-mediated DNA repair (Cary *el al.*, 1997). DNA-PK_{cs} knockout mice have a phenotype very similar to that observed in Ku70-null animals, including impaired V(D)J recombination and severe immunodeficiency (Bogue *et al.*, 1998). Unlike Ku knockouts, however, DNA-PK_{cs}-deficient mice have a normal size (Gao *et al.*, 1998) and cultured cells do not show slow proliferation or early onset of senescence (Featherstone and Jackson, 1999a). This, together with the identification of DNA-PK_{cs}-independent functions for Ku in V(D)J recombination (Gao *et al.*, 1998) has led to the hypothesis that although both enzymes function in the same DNA repair pathway, DNA-PK_{cs} is only necessary to repair a subset of lesions.

To determine whether DNA-PK_{cs} is responsible for the phenotypic leakiness observed in Ku80-deficient cells, it would be necessary to conduct the same targeting experiments in Ku80-null, DNA-PK_{cs}-null cells. Such a double knockout has not been reported thus far. However, since DNA-PK_{cs}-defective cells were available, it was decided to use them to confirm that down-regulation of the NHEJ pathway does not result in a higher incidence of homologous recombination. The involvement of DNA-PK_{cs} in illegitimate integration was confirmed by the observation that transfection efficiency was decreased 10-fold in the double knockouts and 2-fold in the hemizygous cells. Although the efficiency of DNA uptake was not controlled in these experiments, it is very unlikely that this factor may be affected by the knockout of a gene known to function at the nuclear level. As in the Ku80-null experiments, this decrease in the overall frequency of integrants corroborates that homologous recombination does not compensate for the loss of NHEJ capacity. More surprising is the fact that homologous recombination also decreases almost proportionally to the number of puromycin-resistant colonies in the three genotypes analysed. If homologous and illegitimate recombination were completely separate pathways, the number of 6-TG-resistant colonies would have remained basically unaffected in the hemizygous and nullizygous cell lines. Although this would not have changed absolute targeting frequency, E.T.F. would have been significantly increased.

Although neither DNA uptake nor plating efficiency were controlled, these results raise the possibility that there is a more intimate biochemical link between NHEJ and homologous recombination than expected. Thus far, the fact that both mechanisms can be independently manipulated *in vitro* (Waldman and Waldman, 1990; Sonoda *et al.*, 1999; Semionov *et al.*, 1999; and page 17) has been interpreted as a reflection of their functional separation. However, several enzymes such as Mre11, Rad50 and Xrs2 have been associated with both routes (Jeggo, 1998; Haber, 1998; Goedecke *et al.*, 1999). As an initial hypothesis for further studies, this work suggests that DNA-PK_{cs} may be involved in homologous recombination as well as in NHEJ. This enzyme may stabilise DSBs or act as a signal transductor for the up-regulation of both NHEJ and homologous recombination pathways. If this is the case, then the 'switch' between both mechanisms would be downstream DNA-PK_{cs} activation. As previously discussed in pages 18-22, one candidate for such a switch is PARP. Although many aspects of PARP

biology, function and regulation remain to be elucidated, the most commonly accepted models indicate that the binding of this protein to DSBs serves as a catalyst for efficient NHEJ, while down-regulating homologous recombination (Lindahl *et al.*, 1995). This hypothesis has been tested by Park *et al.* (1983), Farzaneh *et al.* (1988), Waldman and Waldman (1991), Morrison *et al.* (1997) and Semionov *et al.* (1999), who proved that selective down-regulation of PARP not only results in a marked decrease of NHEJ, but also in enhanced sister chromatid exchange, intra- and extra-chromosomal recombination. In this work we aimed at determining whether PARP down-regulation also leads to higher targeting frequencies. In order to establish proof of principle, targeting frequencies were calculated in PARP -/-, +/- and -/- cells. As shown in pages 157-158, E.T.F. is enhanced up to 3.3-fold in PARP-deficient ES cells (with a maximum E.T.F. value of up to 46 %). It was next sought to reproduce this effect by transiently down-regulating PARP by incubating wild-type cells in the presence of chemical inhibitors of poly(ADP-rybosyl)ation. Moderate increases in E.T.F. of up to 30% were observed when treating cells with 5 mM of 3-AB, a common PARP inhibitor. Lower concentrations of the inhibitor proved to be ineffective, whereas a higher concentration (15 mM) resulted in increased cell death and decreased targeting efficiency. The latter observation can be explained by the unspecific side effects of 3-AB, which include nicotinamide N-methyltransferase inhibition and impaired *de novo* synthesis of DNA (Ueda and Hayaishi, 1985). Increases of up to 4.6-fold in the frequency of extrachromosomal recombination have been reported following PARP down-regulation in mouse Ltk-fibroblasts by a much more specific inhibitor, 1,5-isoquinolinediol (Semionov *et al.*, 1999). Although comparisons cannot be drawn between gene targeting and extrachromosomal recombination frequencies in two different biological systems, it is likely that 1,5 isoquinolinediol (which was not commercially available at the time of this work) would also result in higher E.T.F.'s in ES cells, without the deleterious effects of 3-AB.

In summary, this study demonstrates that (1) targeting efficiency is not enhanced by partially inactivating the NHEJ-mediated DNA repair route in mammalian cells; (2) a residual level of exogenous DNA integration is observed in DNA-PK_{cs} and Ku80 knockouts, which is not associated with homologous recombination; (3) DNA-PK_{cs} may be involved in both homologous and non-homologous recombination pathways; (4)

E.T.F. is significantly enhanced in PARP-deficient cells; and (5) strategies can be developed to increase targeting efficiency by transiently down-regulating PARP using specific inhibitors. In this respect, it would be interesting to determine whether these approaches are more successful in somatic than in ES cells.

Chapter VI. modifications of the target

i.alteration of gene expression
ii.alteration of cell conditions

i.alteration of gene expression

a.introduction
b.methylation
c.chromatin remodelling

a.introduction

The control of gene expression cannot be fully explained by simplistic models based on stage- or tissue- specific activity of transcription factors: most of them are ubiquitously expressed, which suggests that there may be other equally important mechanisms for regulating transcription. Two of such mechanisms are DNA methylation and histone acetylation.

It is generally acknowledged that DNA methylation is involved in the regulation of developmental gene expression (rev. by Bird, 1992; Jones, 1999). Mammalian DNA is heavily methylated at cytosine residues within the dinucleotide sequence CpG. It has been hypothesised that increases in methylation at CpG islands or critical CpG sites in promoter/enhancer regions lead to transcriptional downregulation and gene silencing (Boyes and Bird, 1991; Eden and Cedar, 1994). Several studies have established that methylation of the 5'-end of many genes is incompatible with their expression, both *in vitro* and *in vivo* (Razin and Riggs, 1980; Yisraeli *et al.*, 1988; Boyes and Bird, 1991; Qu and Ehrlich, 1999; Siegfried *et al.*, 1999). At a global scale, changes in the pattern of methylation are associated with specific developmental stages (Razin and Shemer, 1995), imprinting (rev. by Reik and Walter, 1998), X chromosome inactivation (Latham, 1996) and carcinogenesis (Counts and Goodman, 1995).

Two basic models have been proposed to explain the silencing effects of methylation on transcription. A 'passive' model (cit. by Boyes and Bird, 1991) postulates that essential transcription factors cannot attach to their DNA binding sequences when these are methylated. The 'active' model, in contrast, suggests that methylated sites are recognised by positive or negative trans-acting proteins, which modulate gene

expression accordingly (Zhang *et al.*, 1989, 1993; Nan *et al.*, 1998). Many of these factors work by sequestering genes in inactive, highly condensed chromatin structures (Meehan *et al.*, 1989). The repeated observation that actively transcribed genes are typically in an open configuration confirms that regulation of chromatin structure plays a major role in the utilisation of genomic information (Gross *et al.*, 1988).

Chromatin structure is the result of a delicate balance between the activities of histone acetyltransferases (HATs) and deacetylases (HDACs). HATs contribute to relaxing chromatin by weakening the association of the core histones with DNA (Norton *et al.*, 1989), which facilitates the access of transcription factors *in vitro* (Lee *et al.*, 1993). Similarly, inhibition of HDACs normally results in upregulation of gene expression (Dion *et al.*, 1997). Recent studies indicate that histone deacetylation and DNA methylation may operate along a common biochemical pathway to repress transcription (Nan *et al.*, 1998; Jones *et al.*, 1998; Zhang *et al.*, 1999; Fuk *et al.*, 2000). Consistent with this hypothesis is the discovery that the enzyme Dnmt1, which is responsible for 'maintenance' CpG methylation in mammals, has a transcriptional repression domain that binds histone deacetylase I (HDAC I) *in vivo* (Fuks *et al.*, 2000).

The observation that homologous recombination and gene expression rates are directly correlated is well documented, as discussed in page 50 of this work. Although the possibility that these two processes are biochemically linked cannot be ruled out (Kassavettis and Geidushek, 1993), the widespread consensus is that this relationship simply reflects the increased accessibility of the chromosome to recombinases and homologous DNA substrates during transcription. In this context, enhanced levels of homologous recombination would be only a downstream and indirect effect of transcriptional up-regulation.

The aim of this study was to determine whether controlled modulation of gene expression can be used to increase gene targeting frequency *in vitro*. Two experimental approaches have been explored to this purpose: (1) Changing the overall methylation pattern of recombinant substrates and (2) Relaxing chromatin structure.

b.methylation

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b.1.introduction

It has been proposed that methylation alters chromatin structure and regulates the ability of recombination enzymes to catalyse strand exchange (Thomas and Rothstein, 1989). In plants with large genomes, such as maize, recombination seems to be restricted to hypomethylated regions (Whitkus *et al.*, 1992). Also, experiments in Ascobolus show that DNA methylation suppresses crossing-over (Maloisel and Rossignol, 1998). The influence of methylation in mammalian recombination is less clear. Although CpG methylation has no effect on extrachromosomal recombination (Liang and Jasin, 1995), different recombination rates at identical chromosomal regions between male and female cells indicate that the pattern of methylation may have a key role in this process (Paldi et al., 1995).

The DNA methyltransferase Dnmt1 is responsible for cytosine methylation in mammalian cells, and its involvement in gene silencing has been unequivocally established (Tate and Bird, 1993). One mechanism by which Dnmt1-mediated repression is exerted might be the indirect recruitment of histone deacetylase activity, as discussed in the previous section. It has been recently reported that murine ES *Dnmt1* nullizygous cells display global DNA hypomethylation, which results in elevated mutation rates and chromosomal abnormalities normally associated with hyperrecombination (Chen *et al.*, 1998). The first priority in this study is to examine whether the *Dnmt1* -/- genotype also has an effect on the frequency of gene targeting. A

complementary line of research focuses on the properties of the recently identified DNA demethylase (Bhattacharya *et al.*, 1999), an enzyme that specifically removes methyl groups from cytosines in CpG islands.

b.2.objective

To assess the influence of methylation on gene targeting frequency.

b.3.results

b.3.i.targeting frequency in Dnmt1-/- cells

Effective *HPRT* targeting frequency (E.T.F.) was calculated for *Dnmt1*-/- and parental +/+ ES cells (kindly given by A. Bird) following the transfection protocol described in page 108. As shown in figure 51 and table 24, E.T.F. is ~50 % higher in *Dnmt1*-/- than in wild-type controls (ANOVA $F_{1,8} = 111.52$; $F_{crit} = 5.317$; $P = 5.64 \times 10^{-6} < 0.05$).

In order to determine whether the methylation status of the vector also affects the efficiency of homologous recombination due to steric hindrance between the recombinant substrates, a parallel set of experiments was conducted using plasmid DNA isolated from the DM-1 *E.coli* strain, which is defective in the two major prokaryotic DNA methyltransferases (*dam* and *dcm*). Hypomethylation of the targeting vector was confirmed by restriction analysis using methylation-sensitive enzymes. As summarised in table 25 and figures 52 and 53, differences in E.T.F. between cells transfected with 'normal' and hypomethylated plasmid are not statistically significant, regardless of their genotype (ANOVA $F_{1,8} = 2.555$; $F_{crit} = 5.317$; $P = 0.148 > 0.05$ for wild-type cells; $F_{1,4} = 0.535$; $F_{crit} = 7.708$; $P = 0.504 > 0.05$ for *Dnmt*1 -/- cells).

		Puro^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
J1(*Dnmt1* +/+)	1	1372	90	6.55	8.29 +/- 0.505
	2	1304	105	8.05	
	3	1200	109	9.08	
	4	1150	109	9.47	
	5	1166	97	8.31	
B (*Dnmt1* -/-)	1	1107	193	17.43	15.62 +/- 0.476
	2	1238	185	14.94	
	3	1129	167	14.79	
	4	1367	209	15.28	
	5	1324	208	15.7	

Table 24. Effect of *Dnmt1* genotype on E.T.F. B (*Dnmt1*-/-) and J1 (wild-type) cells on E.T.F. Cells were transfected with 300 μg of p2017. Every entry corresponds to an independent experiment. S.E.: standard error.

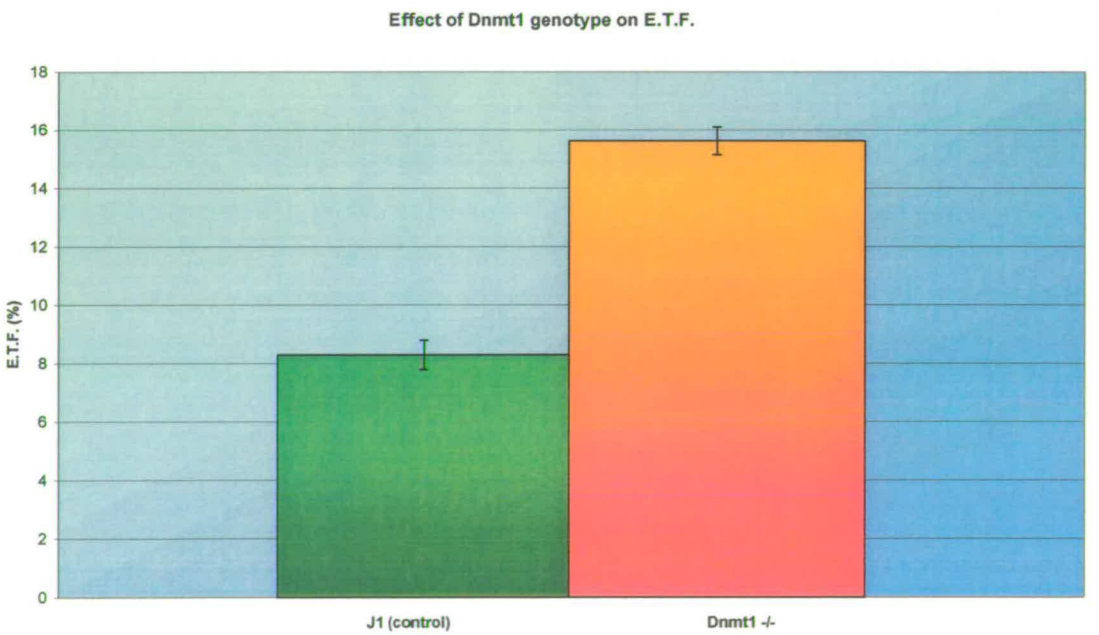

Figure 51. Effect of *Dnmt1* genotype on E.T.F. Y bars: standard error for each group.

		Neo^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
J1 (+/+) p2016	1	632	42	6.64	**7.46 +/- 0.433**
	2	748	45	6.01	
	3	783	56	7.15	
	4	712	47	6.61	
	5	624	40	6.41	
J1 (+/+) p2016*	1	615	51	8.29	**6.56 +/- 0.184**
	2	697	45	6.45	
	3	728	43	5.91	
	4	656	54	8.23	
	5	603	51	8.45	
		Puro^R colonies			
B (-/-) p2017	1	1234	181	14.6	**14.26 +/- 0.712**
	2	1193	183	15.3	
	3	1384	179	12.9	
B (-/-) p2017*	1	1134	165	14.55	**15.13 +/- 0.940**
	2	1052	146	13.87	
	3	960	163	16.97	

Table 25. Effect of vector methylation on E.T.F. Host cell lines: J1 (wild type, figure 52) and B (*Dnmt1*$^{-/-}$, figure 53). p2016 and p2017: normally methylated targeting vectors (see Materials and Methods); p2016* and p2017*: hypomethylated vectors. p2017 was used in B cells because they already have an integrated *neo* cassette. Average E.T.F.'s obtained with different targeting vectors cannot be compared, because p2016 targets the *HPRT* locus less efficiently than p2017. 300 μg of DNA were used per electroporation. S.E., standard error.

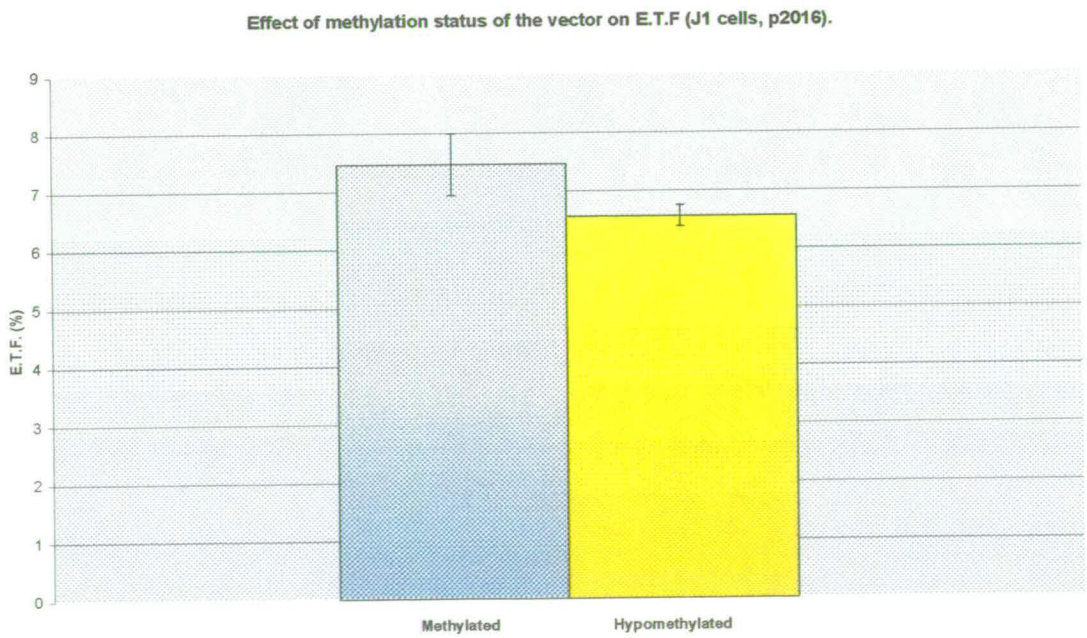

Figure 52. Effect of p2016 methylation on E.T.F. in wild-type J1 cells. Y bars: standard error for each group.

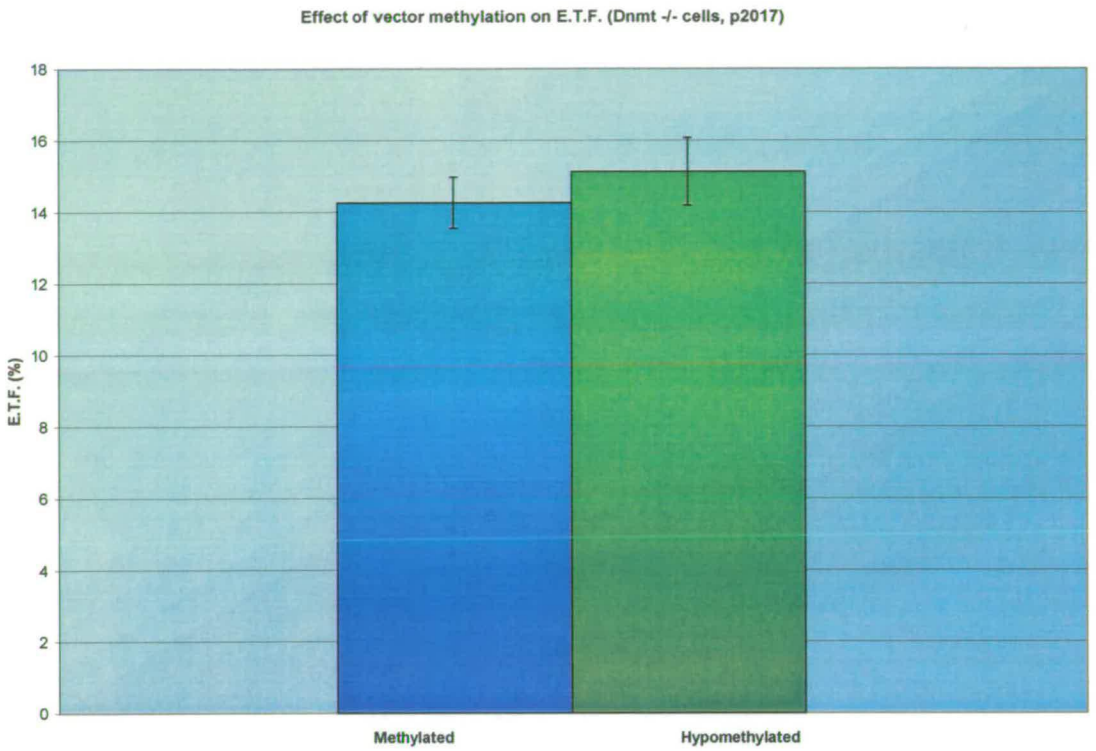

Figure 53. Effect of p2017 methylation on E.T.F. in B (*Dnmt*1 -/-) cells. Y bars: standard error for each group.

b.3.ii.DMTase-transgenic cells

Although *Dnmt1* mutations lead to general hypomethylation, the activity of other methyltransferases may account for a basal level of methylation in *Dnmt1*-/- ES cells. An active induction of demethylation may still be necessary to overcome this activity and achieve more dramatic increases in gene targeting frequency. The recent characterisation of a putative mammalian DNA demethylase (DMTase) (Bhattacharya *et al.*, 1999) was therefore seen as an opportunity to assess this hypothesis. A vector containing DMTase cDNA under the control of the CMV constitutive promoter and a *neo* selectable marker (kindly provided by M. Szyf) was used to generate stable ES cell transgenic clones overexpressing DMTase. One clone with high transgene expression (D3) was chosen for subsequent targeting experiments (figure 54). As shown in table 26 and figure 55, DMTase-transfected

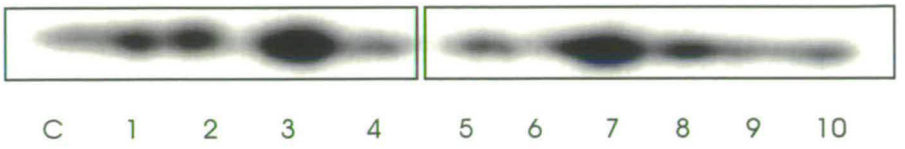

Figure 54. Northern blot of DMTase-overexpressing clones. The entire DMTase ORF (~1 kb) cDNA was used as a probe. Lanes 1-10: DMTase-transfected clones; C: untransfected control. 15 μg of RNA were loaded/ lane. The ethidium bromide-stained gel was used as a loading control.

		Puro^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
J1(*Dnmt1* +/+)	1	1418	99	6.981	7.363 +/- 0.285
	2	1401	111	7.922	
	3	1266	91	7.187	
J1-DMTase	1	1524	116	7.611	7.802 +/- 0.926
	2	1222	77	6.301	
	3	1464	139	9.494	

Table 26. Effect of DMTase constitutive expression on E.T.F. J1 (wild-type) cells and D3 (DMtase-transgenic) on E.T.F. Cells were transfected with 300 μg of p2017. Every entry corresponds to an independent experiment. S.E.: standard error.

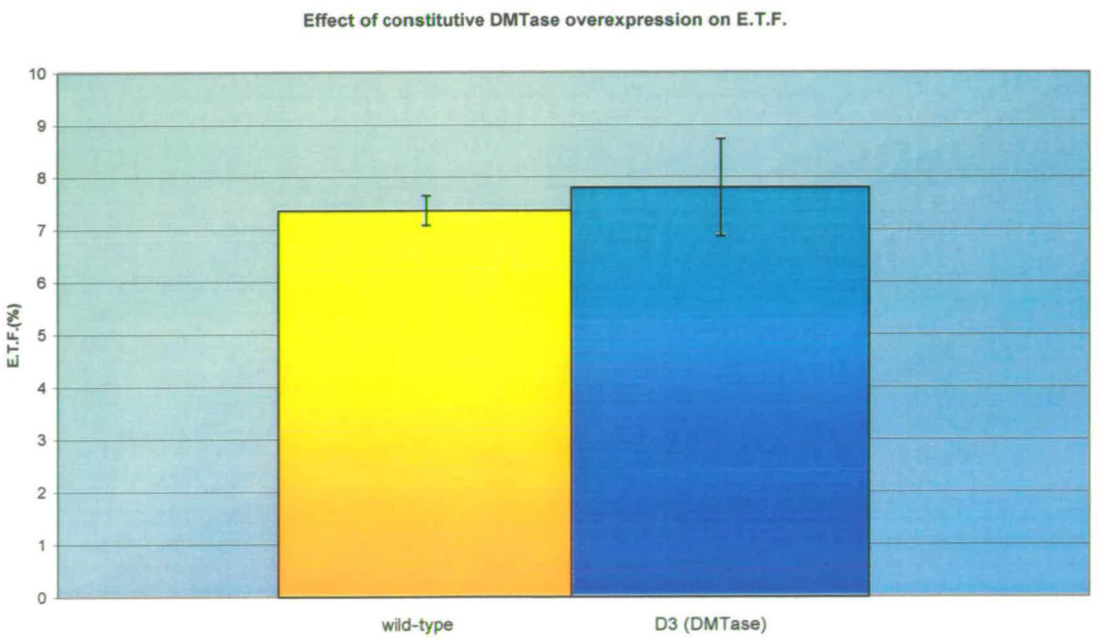

Figure 55. Effect of DMTase constitutive overexpression on E.T.F. Y bars: standard error for each group.

ES cells do not exhibit any significant enhancement in effective targeting frequency compared to untransfected controls. We next attempted the generation of *Dnmt1* null/DMTase-overexpressing clones. If constitutive expression of DMTase resulted in even lower methylation levels of the cell, then the additive effects of both genetic modifications may result in higher targeting frequencies. Since a *neo* gene is already present in *Dnmt1*-/- cell lines (Chen *et al.*, 1998), DMTase-transfected clones were selected in blasticidin (5 μg/ml) or puromycin (1 μg/ml) upon co-lipofection of the transgene with a *bsd* or *pac* resistance cassette (1:4 molar ratio), respectively. No colonies were recovered in either case.

b.4.discussion

This work demonstrates that decreasing the level of methylation can be used as a strategy to enhance gene targeting frequency. The molecular mechanism behind this effect, however, remains unclear. One possible explanation for the influence of methylation on homologous recombination is that methyl groups introduce steric hindrance preventing the correct alignment of the recombinant substrates during heteroduplex formation. Alternatively, it has been proposed that methylation may restrain homologous recombination by impairing the stability of recombination intermediates, rather than by preventing their formation (Maloisel and Rossignol, 1998). We report here that hypomethylated vectors are not more proficient at targeting the *HPRT* locus than their normally methylated counterparts, both in wild-type and *Dnmt1* nullizygous cells. Although this observation seems to argue against the above possibilities, an exhaustive analysis of the methylation status of the recombinant partners would be necessary to rule them out.

A more likely explanation, however, involves the proven ability of methylation to influence chromatin structure (Kass *et al.*, 1997; Nan *et al.*, 1998; Jones *et al.*, 1998; Zhang *et al.*, 1999; Fuks *et al.*, 2000). According to this model, increased targeting frequencies observed in *Dnmt1*-/- cells may be a consequence of a more open

chromatin configuration at the target locus, which would facilitate the access of the targeting vector and recombination enzymes. The inability of hypomethylated vectors to promote more efficient recombination seems to confirm that methylation determines genomic accessibility rather than heteroduplex formation.

In this context, it was hypothesised that transient induction of hypomethylation levels could lead to improved targeting frequencies. We aimed at establishing proof of principle by constitutively expressing a recently identified mammalian demethylase (Bhattacharya *et al.*, 1999) in ES cells. However, as shown in the previous section, E.T.F. is not significantly enhanced in DMTase-transgenic cells. One possible explanation is that demethylase activity may not be restricted to one single enzyme. The demethylase activity of the enzyme itself has proved controversial. For instance, Ng *et al.* (1999) have been unable to reproduce in HeLa cells the demethylase activity of the protein identified by Bhattacharya *et al.* (1999), suggesting that this protein is actually a transcriptional repressor.

Alternatively, it can be reasoned that active genomic demethylation may simultaneously need *Dnmt*1 repression and DMTase upregulation. Thus, we attempted to create *Dnmt*1-defective/DMTase-overexpressing lines. The fact that no transgenic colonies could be recovered was interpreted as a consequence of inducing hypomethylation levels incompatible with cell viability. This hypothesis could be tested by transfecting *Dnmt*1-defective cells with an inducible DMTase cassette and examining overall methylation levels and other phenotypic consequences following gradual activation of the demethylase.

c.chromatin remodelling

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c.1.introduction

The fact that DNA condensation in chromatin functions not only to constrain the genome within the nucleus, but also to silence gene expression in a general manner, has been well established over the past decade (rev. by Lu *et al.*, 1994; Farkas *et al.*, 2000). Chromatin structure has consistently been associated with transcriptional regulation, and more indirectly, with the rate of homologous recombination. It has been proposed that relaxation of chromatin conformation is essential for facilitating the access of not only transcriptional, but also recombinogenic enzymes (Thomas and Rothstein, 1989; Adams and Workman, 1993; Kornberg and Lorch, 1995; Krude and Elgin, 1996; Farkas *et al.*, 2000). The involvement of methylation in chromatin remodelling and the prospects for improving gene targeting frequency by manipulating the overall methylation level of the cell have already been discussed in the previous section. Here we focus on histone acetylation, the other major mechanism known to be involved in chromatin conformation. It has been shown that acetylation reduces the net positive charge of histones and therefore results in weaker histone-DNA interactions (Norton *et al.*, 1989). Hyperacetylation of histones is observed in open genomic regions, and is commonly associated with highly expressed genes. In contrast, hypoacetylation normally correlates with heterochromatin and transcriptionally silent genes (rev. by Loidl, 1994). Acetylation levels are regulated by the relative activities of histone acetyltransferases (HATs) and

(HATs) and deacetylases (HDACs), in a balance known to be affected by the methyltransferase Dnmt1 (Fuks *et al.*, 2000).

Specific inhibitors of histone deacetylation have become common tools for the study of chromatin configuration and the regulation of gene expression. Amongst these, n-butyrate and trichostatin A (TSA) have proved to be effective at reducing histone deacetylase activity, increasing the overall level of nucleosomal acetylation and changing the pattern of expression of several genes (Yoshida *et al.*, 1990; Takahashi *et al.*, 1996; Dion *et al.*, 1997; Xu *et al.*, 1997; García Villalba *et al.*, 1997; Ruh *et al.*, 1999; Niki *et al.*, 1999; Garrison *et al.*, 2000). It was hypothesised that TSA-mediated deacetylase inhibition in ES cells could lead to a global relaxation of chromatin structure, which may have a positive effect on targeting efficiency. The effect of phytohemagglutinin (PHA) on E.T.F. is also examined in this context. This compound has been shown to increase up to 10-fold the level of expression of the *HPRT* gene in lymphocytes (Steen *et al.*, 1990; 1991). Higher levels of transcription may be a consequence of a more relaxed chromatin configuration at the *HPRT* locus. Since transcription and homologous recombination rates are directly related in a variety of test systems (page 50), it was thought that a similar enhancement of *HPRT* transcription in ES cells may result in higher E.T.F.'s.

c.2.objective

To study the effect of TSA- and PHA-pretreatment of ES cells on E.T.F.

c.3.results

c.3.i.TSA

J1 ES cells were incubated for 48 hours prior to electroporation with GMEM complete medium supplemented with TSA (Sigma) at

concentrations ranging from 10 to 300 nmol/l. However, growth arrest followed by extensive cell death was observed very quickly after TSA addition to the medium, even at the lowest concentration examined. In contrast, control cells supplemented with equivalent volumes of the TSA solvent (PMSO) showed no changes in growth or death rate (table 27)

TSA concentration	Observations
0 nmol/l	Normal growth
10 nmol/l	Growth arrest. Changes in cell morphology and cell death observed 24 hours after TSA addition.
50 nmol/l	Growth arrest. Changes in cell morphology and cell death observed 24 hours after TSA addition.
100 nmol/l	Growth arrest. Changes in cell morphology and cell death observed 8 hours after TSA addition.
200 nmol/l	Growth arrest. Changes in cell morphology and cell death observed 8 hours after TSA addition.
300 nmol/l	Growth arrest. Changes in cell morphology and cell death observed 8 hours after TSA addition.

Table 27. Effect of TSA treatment on ES cells.

Given the toxic effect of long exposures to TSA, it was subsequently decided to conduct targeting experiments in ES cells incubated with a relatively high TSA concentration (100 nmol/l) for a short period (2 hours) immediately before electroporation (p2017, 300 μg). Control cells were similarly treated with DMSO only. The results of these experiments are summarised in table 28 and figure 56.

		Puro^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
J1 control	1	1777	168	9.454	**8.908 +/- 0.287**
	2	1571	138	8.790	
	3	1686	143	8.481	
J1 TSA-treated	1	1598	119	7.446	**8.331 +/- 0.533**
	2	1429	118	8.257	
	3	1367	127	9.290	

Table 28. Effect of TSA preincubation (2 hours) of ES cells on E.T.F. Cells were transfected with 300 μg of p2017. Every entry corresponds to an independent experiment. S.E.: standard error.

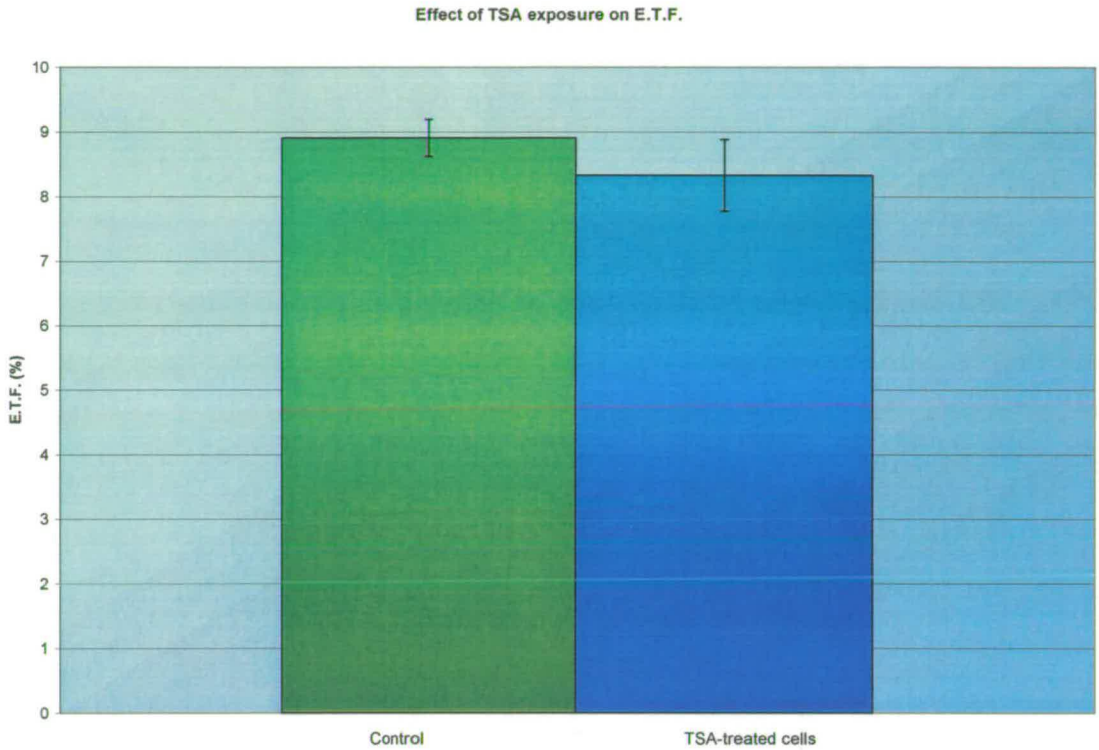

Figure 56. Effect ofTSA-preincubation on E.T.F. Y bars: standard error for each group.

A one-way ANOVA analysis of the data presented above demonstrates that E.T.F. is not significantly affected by a 2 hour TSA exposure (100 mmol/l) prior to electroporation ($F_{1,4} = 0.907$; $F_{crit} = 7.708$; $P = 0.394 > 0.05$).

c.3.ii.PHA

E.T.F. was calculated in wild-type ES cells following preincubation (24 hours) with different concentrations of PHA. As shown in table 29 and figure 57, differences in E.T.F. means observed after PHA treatment (any concentration) are not statistically significant (ANOVA $F_{3,8} = 0.419$; $F_{crit} = 4.066$; $P = 0.743 > 0.05$).

		Puro^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
No PHA	1	1200	98	8.166	8.074 +/- 0.420
	2	1451	127	8.752	
	3	1369	100	7.304	
PHA 10 µg/ml	1	1325	107	8.075	8.406 +/- 0.166
	2	1616	138	8.539	
	3	1348	116	8.605	
PHA 30 µg/ml	1	1109	79	7.123	7.629 +/- 0.555
	2	1224	86	7.026	
	3	1316	115	8.738	
PHA 60 µg/ml	1	1274	119	9.34	8.019 +/- 0.674
	2	1207	86	7.125	
	3	1304	99	7.592	

Table 29. Effect of PHA preincubation (48 hours) of ES cells on E.T.F. Cells were transfected with 300 µg of p2017. Every entry corresponds to an independent experiment. S.E.: standard error.

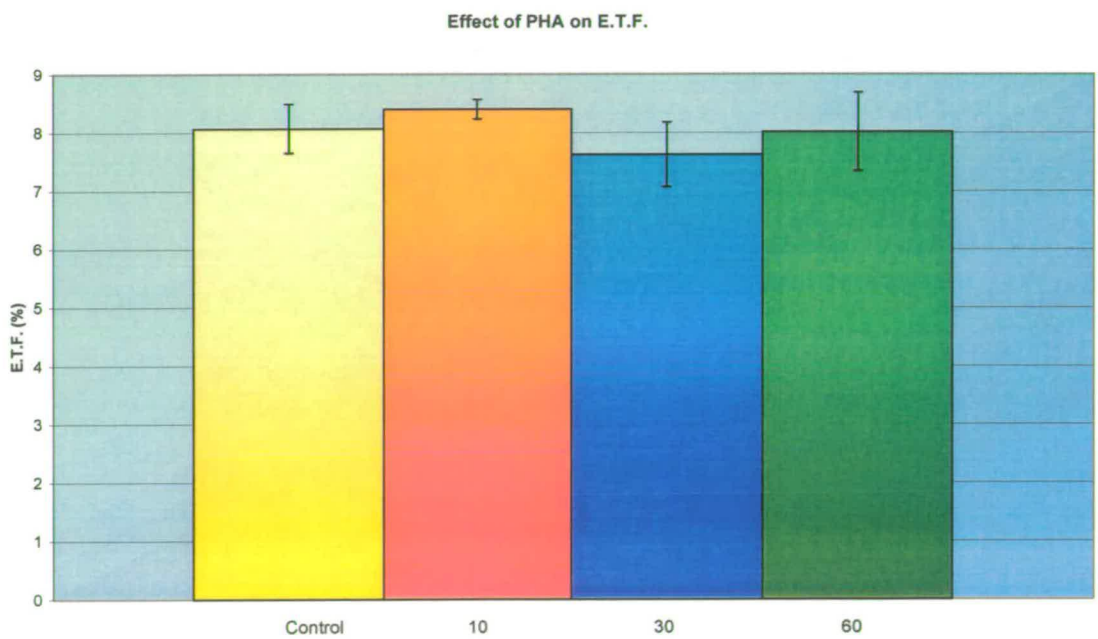

Figure 57. Effect of PHA on E.T.F. Y bars: standard error for each group.

c.4.discussion

The use of TSA to down-regulate histone deacetylation has been extensively documented in a variety of somatic cell types (pages 176-177). We have found that ES cells die when this chemical is present in the medium, even at concentrations 10 times lower than those usually reported for other cell types (Takahashi *et al.*, 1996; Dion *et al.*, 1997; Xu *et al.*, 1997; García Villalba *et al.*, 1997; Ruh *et al.*, 1999; Niki *et al.*, 1999; Garrison *et al.*, 2000). Unlike n-butyrate, which has been shown to interfere with other cellular processes (Tang and Taylor, 1990; Charollais *et al.*, 1990; Nudelman *et al.*, 1992), TSA has not been related to actions other than HDAC inhibition. This suggests that TSA-induced changes in gene expression in ES cells may be sufficient to result in growth arrest and cell death. Our observation that cells are viable after a shorter exposure to TSA indicates either that (1) this hypothetical change in the pattern of gene expression has to be maintained in order to kill the cell; or (2) TSA has no measurable effect on histone acetylation during the first two hours of incubation. The latter hypothesis is consistent with results reported by Dion *et al.* (1997) who showed a time-dependent response of HeLa cells to TSA. This would also explain why E.T.F. was not affected following a short pre-incubation with TSA. As for what possible changes in gene expression induced by inhibition of histone deacetylation may be responsible for cell death in ES cells, any conclusion drawn from the available data would be a matter of speculation. However, since the aim of this work is to explore the possibility of using HDAC inhibition as a strategy to increase targeting efficiency, it would be interesting to repeat these experiments in somatic cells known to be sensitive to TSA.

As is the case with many other 'housekeeping' genes, HPRT is expressed at a very low level in most cells examined, both primary and immortalised (Steen *et al.*, 1990). It has been shown that PHA administration increases *HPRT* expression up to 10-fold in lymphocytes (Steen *et al.*, 1990; 1991), which led us to think that ES cells may also upregulate expression of this gene. Since higher levels of gene expression are usually associated with enhanced homologous recombination rates (page 50), ES cell incubation with PHA-containing medium may therefore result in improved targeting efficiencies. However, we report here that E.T.F. is not affected by PHA at concentrations known to

have mitogenic effects on lymphocytes. This result can be explained if the transcriptional up-regulation observed in lymphocytes is simply a reflection of their activation. Lymphocytes are usually non-proliferative and need PHA stimulation. Since ES cells are immortal, they are already, in a sense, 'activated' and insensitive to PHA. Further studies along this line would require the precise determination of HPRT levels of expression before and after addition of PHA.

ii. modification of cell conditions

a.introduction
b.passage number
c.oxygen partial pressure

a.introduction

The fact that cell culture conditions may affect targeting efficiency has attracted little attention thus far. However, the variability between targeting experiments is probably a reflection of changes in factors such as passage number, cell density, medium composition or growth rate. Another source of variability could be the relative proportion of cells in any given phase of the cell cycle. The relevance of this factor is highlighted by the observation that homologous recombination occurs preferentially in early to mid S-phase (Wong and Capecchi, 1987), whereas illegitimate recombination peaks at G_2/M phase. Work by Udy *et al.* (1997) suggests that some of these factors (particularly cell cycle rate) may have an effect on targeting frequency even more dramatic than those derived from the use of isogenic/ non isogenic vectors. This section focuses on two possible sources of variability associated with the optimisation of culture conditions, namely oxygen partial pressure and cell passage number. The influence on E.T.F. of the controlled modification of these factors is examined.

b.passage number

The study of the influence of age (measured as passage number) on E.T.F. would be of particular interest in somatic cells, since they show an age-related loss of proliferative activity in culture (Start *et al.*, 1991; Chang-Liu *et al.*, 1997; Rubin, 1997; Ruiz-Torres *et al.*, 1999). In contrast, the growth rate of ES cells (as well as other immortalised cell lines) does not appear to be affected by age. However, it has been observed that prolonged culture of ES and ES-like cells results in a decrease of their capacity to contribute to chimaeras, and particularly to colonise the germ line (Mitalipov *et al.*, 1994). To explain this observation, it has been suggested that the prolonged *in vitro* culture of rapidly dividing ES cells may lead to accumulated changes and chromosomal abnormalities (Brown *et al.*, 1992). The possibility that age-related physiological alterations in ES cells may also have consequences in the rate of homologous recombination (and therefore gene targeting) cannot be ruled out.

In this section, we aimed at determining whether E.T.F. is affected by passage number in ES cells. To this purpose, targeting experiments were conducted in the cell line E14 at passages 19, 45 and 73. The results are outlined in table 30 and figure 58.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
E14 –p19	1	1566	142	9.067	**8.699 +/- 0.524**
	2	1161	89	7.665	
	3	1324	124	9.365	
E14 – p45	1	1600	134	8.375	**7.936 +/- 0.366**
	2	1228	101	8.224	
	3	1387	100	7.209	
E14 – p73	1	1225	96	7.836	**8.287 +/- 0.237**
	2	1111	96	8.640	
	3	1395	117	8.387	

Table 30. Effect of passage number on E.T.F. Five independent experiments were conducted in each group (300 μg p2017). Passage number (19, 45 and 73) is indicated. S.E.: standard error.

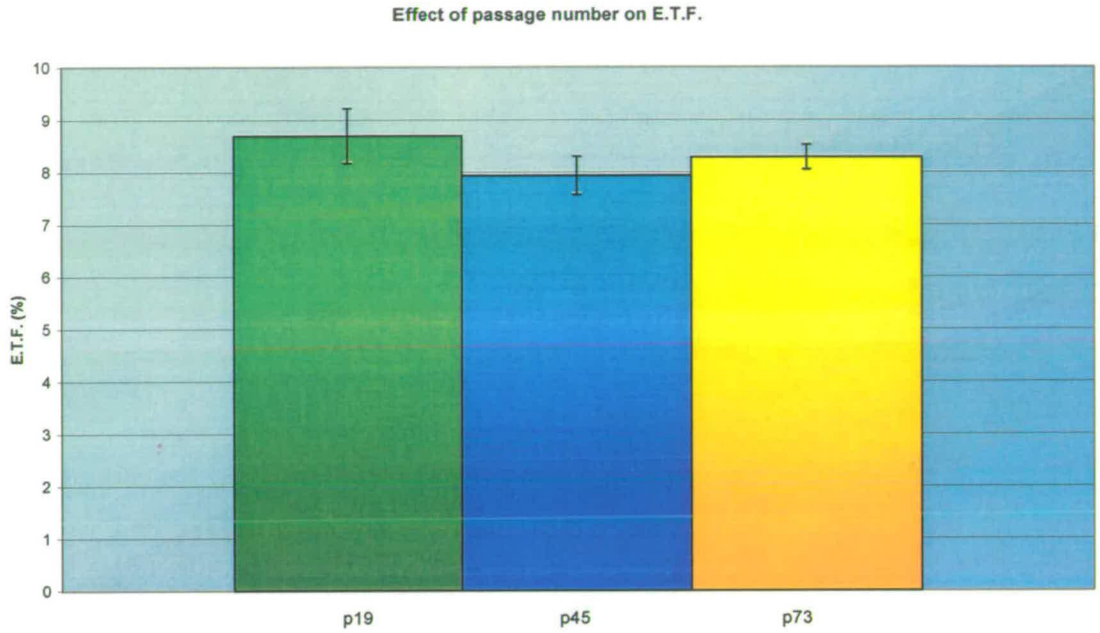

Figure 58. Effect of passage number on E.T.F. Y bars: standard error for each group.

A one-way ANOVA analysis of the data presented above ($F_{2,6} = 0.940$; $F_{crit} = 5.143$; $P = 0.44 > 0.05$) indicates that the null hypothesis that all E.T.F. means are the same cannot be rejected.

Although it can be reasoned that changes in targeting frequency may be noticeable at even higher passage numbers, the examined interval greatly exceeds the usual age we may expect of ES cells in routine targeting experiments. Therefore we conclude that passage number has no practical influence on E.T.F., at least with this cell line up to passage 73.

c. oxygen partial pressure

Optimising the conditions for colony survival after electroporation is essential when designing protocols for enhanced targeting frequency. In this context, oxygen partial pressure has been suggested as a limiting factor for clone viability. The environmental oxygen concentration of 20 % normally used in cell cultures is certainly higher than that of cells *in vivo*, which may lead to oxidative stress and changes in growth rate and lifespan. Several studies have demonstrated that lowering ambient oxygen concentrations stimulates proliferation of human cells (Balin *et al.*, 1984; Falanga and Kirsner, 1993) and extends their lifespan *in vitro* (Packer and Fuehr, 1977).

The effect of high oxygen concentrations on gene targeting frequency has remained unexplored thus far. On one hand, it can be reasoned that hyperoxia may be inhibitory for growth and survival of clones after the stress of electroporation. On the other, homologous recombination might be enhanced due to the higher incidence of DNA damage in oxidative conditions. To gain a better understanding of the influence of oxygen partial pressure on targeting efficiency, a number of targeting experiments were conducted after culturing E14 ES cells either on high (20 %) or low (2%) oxygen concentrations for one week.

		Puromycin^R colonies	6-TG^R colonies	E.T.F (%)	Group E.T.F. mean +/- S.E.
E14 - 20 % O_2	1	1376	120	8.72	**9.81 +/- 0.47**
	2	1385	146	10.54	
	3	1324	143	10.8	
	4	1280	110	8.59	
	5	1335	139	10.4	
E14 – 2 % O_2	1	1229	118	9.6	**9.48 +/- 0.41**
	2	1103	121	10.97	
	3	1200	109	9.08	
	4	1218	103	8.45	
	5	1197	112	9.3	

Table 31. Effect of oxygen partial pressure on E.T.F. Five independent experiments were conducted in each group (300 μg p2017). S.E., standard error.

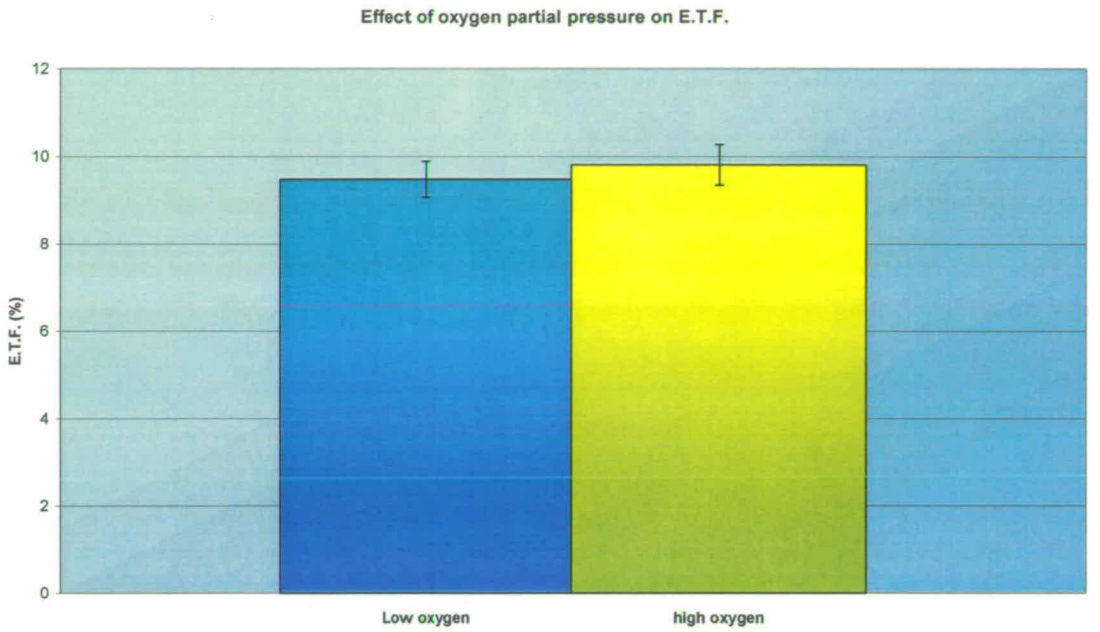

Figure 59. Effect of oxygen partial pressure on E.T.F. in ES cells. Y bars: standard error for each group.

A one-way ANOVA statistical analysis of the data presented on table 31 and figure 59 demonstrates that there is no significant difference between the mean E.T.F.'s of the two groups examined. Thus, it can be concluded that lowering oxygen partial pressure is irrelevant for gene targeting purposes, at least in ES cells and in the conditions described above. This precision is important, because unlike other cell types, ES cells are already immortal and might be less sensitive to the effects of variable oxygen concentrations in the environment. Therefore, these results cannot rule out the possibility that this factor has a measurable influence on clone survival and targeting frequency in somatic cells.

Chapter VII. Concluding remarks

Despite the great success achieved over the past fifteen years in the generation of targeted animals, the use of gene targeting for therapeutic purposes still remains a distant possibility. The major limitation has been the very low efficiency of this technology, as the absolute incidence of homologous integration in mammalian cells is consistently lower than that of illegitimate recombination. However, it is likely that this observation does not accurately reflect the relative contribution of each process in maintaining genomic stability. In fact, whereas mutations of several key enzymes of the NHEJ pathway (such as Ku70, Ku80 or DNA-PK_{cs}) do not compromise the viability of knockout animals, Rad51 mutations are invariably lethal. The extraordinary sensitivity to DNA damage observed in mutants of other genes involved in homologous recombination confirms the essential physiological role of this process, specially during the S phase of the cell cycle (Thompson and Schild, 1999).

These findings have somehow challenged the notion that targeted gene therapy is impractical in mammalian cells. In recent years, several groups have directed their efforts towards enhancing targeting efficiency by 'tricking' the cell into making full use of the homologous recombination pathway. In this context, our primary goal has been to identify those lines of research with most promising perspectives, rather than focusing exclusively in any given strategy. Thus, starting from a comprehensive review of the latest trends in molecular biology aimed at improving effective targeting frequency, this work explores novel avenues that could be the basis of more effective targeting protocols.

Amongst the examined approaches, overexpression of the mammalian recombinase Rad51 and down-regulation of poly(ADP-rybosyl) polymerase (PARP) were the most successful at enhancing gene targeting frequency (4- and 3.3-fold, respectively). These results confirm that Rad51 levels are rate-limiting in homologous recombination (Baumann and West, 1998) and suggest that PARP might act as a switch between the default process of NHEJ and homologous recombination in mammalian cells (pages 19-22). However, constitutive up- or down-regulation of these genes prior to gene targeting would be impractical as a general strategy to stimulate the rate of homologous recombination. For this reason, we have also devised approaches for the transient reproduction of these effects at the time of transfection with the targeting

vector. Thus, we have been able to obtain a 2-fold increase in E.T.F. by co-electroporating the targeting vector with a Rad51 expression cassette. Although there are side effects associated with the entry of a high number of circular DNA molecules into the cell, this result proves that Rad51 protein transiently expressed from a plasmid can be sufficiently accumulated as to have an effect on the mode of integration of a simultaneoulsy introduced targeting vector. This encouraging observation may be the basis for novel strategies aimed at temporarily increasing the concentration of Rad51 (and perhaps other recombinases, such as Rad52 and Rad54) at the time of gene targeting. Two of such schemes (engineering a Rad51 expression cassette into the targeting vector and creating a VP22-Rad51 fusion protein) have been outlined in chapter V. As discussed in pages 149-151, further research along these lines would require confirmation of the absence of point mutations both in p2017/51 and pVP22/Rad51, as well as a precise determination of the biochemical properties of the fusion protein *in vitro*.

We have also demonstrated that E.T.F. can be moderately enhanced by inhibiting PARP activity with 3-AB. Other inhibitors of poly(ADP-rybosyl)ation (such as 1,5 isoquinolinediol) remain to be tested for their ability to improve the rate of homologous recombination without negatively affecting cell viability.

Significant increases in E.T.F. were also observed in *Dnmt*1-defective cells when compared to wild-type controls. Future work would require a more detailed knowledge of the molecular mechanisms linking homologous recombination to the processes of DNA methylation and histone acetylation.

Although other strategies examined in this work have proven ineffective at enhancing gene targeting frequency, they have served to gain a better understanding of homologous recombination and its regulation. For instance, the observation that the absolute frequency of homologous recombinants in DNA-PK_{cs} is considerably lower than in wild-type cells (page 153) suggests that this enzyme may be involved in the early stages of both illegitimate and homologous recombination. Experiments with Ku80-deficient cells also show that down-regulation of the NHEJ pathway does not result in homologous recombination taking over the process of genomic integration of targeting

vectors, as proposed by Goedecke *et al.* (1999) in yeast. Finally, our results suggest that the mechanism of control of homologous recombination by p53 (which is well documented in a variety of somatic cells, as discussed in pages 129-130) may be inactive in ES cells. In this respect, due to the unique biological properties of these cells, it would be most informative to repeat many of the experiments described in this work in other biological systems.

Other negative results, such as the pre-incubation of the targeting vector with SV40 NLS or the use of dsRNA vectors, do not rule out the possibility that these strategies may work in different experimental conditions. For instance, the use of NLS in shorter targeting vectors (pages 117-118) may still result in a higher concentration of recombinogenic molecules in the nucleus. Similarly, longer dsRNA molecules remain to be tested for RNAi in cell cultures (page 122).

In summary, this work presents evidence, obtained from a variety of experimental strategies, that the frequency of gene targeting can be manipulated *in vitro*. These results also contribute to a better understanding of the molecular mechanisms underlying homologous recombination in mammalian cells. Although there is still a long way to achieve a 100 % targeting efficiency (which would be the ultimate goal in order to design *in vivo* gene targeting-based therapeutic protocols), the basic principle that gene targeting frequency can be enhanced has been established. More interestingly, we demonstrate that this objective can be successfully approached from diverse perspectives. This raises the possibility that the combination of some of the research lines described here (for instance the simultaneous transient up-regulation of Rad51 and chemical down-regulation of PARP) may result in even higher targeting frequencies.

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