CHAPTER 3. EVALUATING SIZES OF HOMOZYGOUS DELETIONS

3.1 Introduction

Ionizing radiation has been used to generate random deletions in mouse ES cells and mice. Combining gene targeting technique and negative selection strategy, mutant cells with deletions at specific loci can be isolated (Kushi *et al.* 1998; Schimenti *et al.* 2000; Thomas *et al.* 1998; You *et al.* 1997). Kushi has measured the sizes of deletions generated by X-rays around the *Hprt* (Hypoxanthine-guanine phosphoribosyltransferase) locus on the X chromosome of the mouse E14 ES cell line (Kushi *et al.* 1998). He found that these deletions ranged from 200 to 700 kb (kilobase pairs). Schimenti used F1 hybrid mouse ES cells and made the deletions selectable by targeting a negative selection cassette Herpes Simplex Virus thymidine kinase (*HSV-tk*) to 3 loci on chromosome 5. The targeted ES cell clones were irradiated with 4 Gray of gamma rays and clones with deletions were isolated by negative selection for loss of the *HSV-tk* gene in FIAU (1-2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl-5-iodouracil) (Schimenti *et al.* 2000). Using this approach, heterozygous deletions up to approximate 70 Mb of proximal mouse chromosome 5 were generated in cells. Sizes of the deletions were measured by examing the presence of the microsatellite markers. Forty per cent (11/26) of these clones injected were able to produce germ line chimeras.

However, these approaches can only measure the sizes of heterozygous deletions. As I planned to generate homozygous deletions in *Blm*-deficient cells, the sizes of this type of deletions need to be determined for at least three main reasons: First to determine if deletions can be detected using a 200 kb resolution array CGH; second these data are needed to estimate the size of the library; and the response of the *Blm* cells to irradiation was unknown. If homozygous deletions cover any viability associated genes, cells cannot survive then these deletions will not be observed. Therefore, a gene rare region is more appropriate to conduct experiments to measure sizes of homozygous deletions.

3.1.1 Gene deserts

A gene desert is defined as a genomic region larger than 500 kb in length without a gene. About 20–25% (~605 Mb) of the human genome is gene desert regions. This number falls to 208 Mb if a gene desert is defined as a region larger than 1 Mb without a gene (Nobrega

et al. 2003; Venter *et al.* 2001). Although gene deserts are devoid of genes, in many cases conserved elements can be identified across species. These elements have been shown to have enhancer activity in humans by constructing transgenic mice carrying a beta-galactosidase reporter system and these 300–800 bp conserved sequences upstream of a mouse heat shock protein 68 minimal promoter (Nobrega *et al.* 2003). Despite this, another study from the same group demonstrated that large homozygous deletion of gene desert regions does not have any impact on mouse development (Nobrega *et al.* 2004). Gene desert deletions (1.5 Mb, 0.8 Mb on chromosome 3, 19, respectively) were generated by Cre mediated recombination between targeted *loxP* sites. Mice homozygous for either deletion were viable and fertile without any distinguishable physiological features compared to wild type mice. A gene desert spanning 2.7 Mb (9.2–11.9 Mb position) on mouse chromosome 6 was chosen for this study (Figure 3-1). This region is highly conserved with the human genome (Figure 3-2, Figure 3-3). Like the mouse region, these regions in the human genome are gene poor, too.

In this chapter, a strategy was developed which could determine sizes of homozygous deletions generated by irradiation without complications arising from deleting essential genes. In order to do this, a design similar to that employed by Kushi *et al*. 1998 and Schimenti *et al*. 2000 was used. Briefly, a positive-negative selection marker (*PuroΔtk*) was targeted into a gene desert. A targeted single clone was expanded then irradiated. After irradiation, cells were selected in puromycin for the presence of the *PuroΔtk* marker. Then cells were cultured for multiple generations to allow LOH to occur. As a result of LOH, homozygous deletions at the targeted locus led to the loss of the *PuroΔtk* marker gene, therefore these cells can be selected in FIAU. Homozygous deletions can be analysed by comparative genomic hybridization (CGH) array.

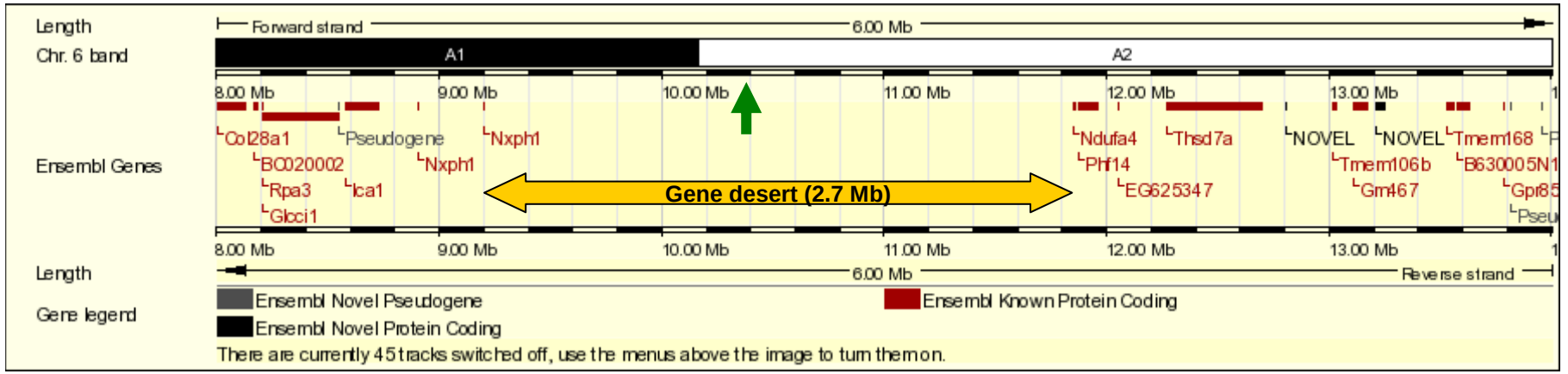

Figure 3-1 A gene desert on chromosome 6

The figure shows the 8–14 Mb region of mouse chromosome 6. From Ensembl gene *Nxph1* to *Ndufa4*, there is a 2.7 Mb gene desert region. The green arrow indicates the locus selected for targeting. From NCBI m36, Ensembl release 46.

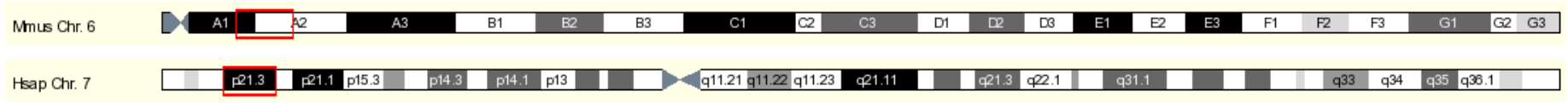

Figure 3-2 Conserved linkage between mouse chromosome 6 gene desert region and human genome

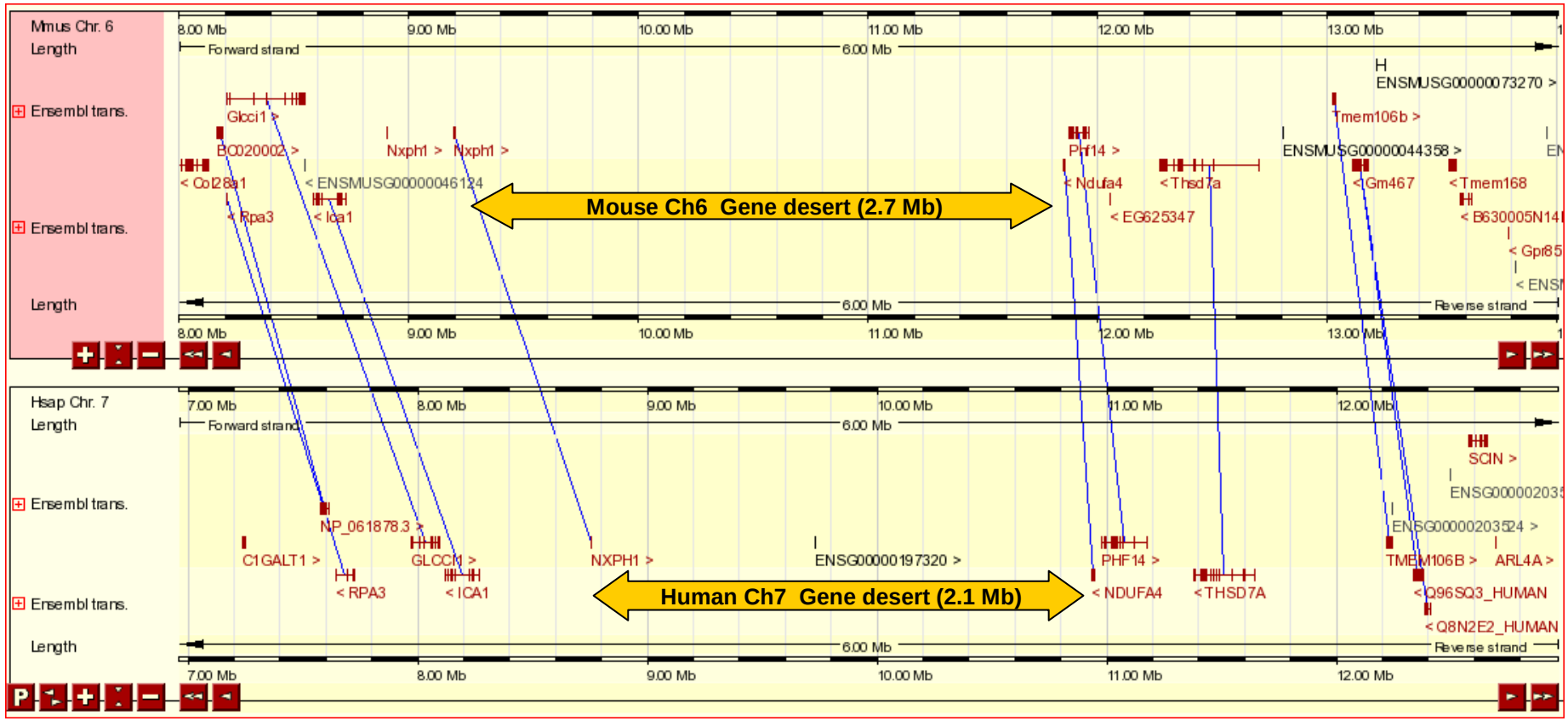

Figure 3-3 Syntenic genes around the chromosome 6 gene desert

The upper panel and the lower panel show 8 to 14 Mb of mouse chromosome 6 and 7 to13 Mb of human chromosome 7, respectively. Blue lines indicate conserved genes in these genomes. Sequence alignments from Ensembl also indicate the gene deserts in the above regions (yellow arrows) are conserved. From NCBI m36 (mouse) and NCBI 36 (human), Ensembl release 46.

3.1.2 A strategy to isolate mutants with homozygous deletions

To determine the relationship between radiation dose and deletion length in a neutral part of the genome, a positive-negative selection (PNS) marker (*PuroΔtk*) (Chen, Y. T. *et al.* 2000) was targeted into 10,440,045bp–10,440,812bp (replacing 766 bp sequence, NCBI m36, Ensembl release 46) in the gene desert region on chromosome 6 in the NGG5-3 *Blm*$^{-/-}$ cells.

The *PuroΔtk* cassette is selectable in both a positive and negative direction. Targeting events can be selected in puromycin media. Heterozygous deletions with loss of the marker can be directly selected in FIAU. Similarly, deletions of the targeted allele can be selected in FIAU after loss of heterozygosity (LOH).

Homozygous deletions of a gene desert region can be selected through the strategy illustrated in Figure 3-4. When the *PuroΔtk* cassette is targeted on one allele of the gene desert, targeted colonies can contain wild type cells. The colonies were expanded in a high concentration of puromycin media so that the contaminating wild type cells are killed because they do not contain the *PuroΔtk* cassette. After expansion the cells are irradiated to generate mutations, including deletions. A second round of puromycin selection is then initiated. Two populations of cells will survive and they are the cells with *PuroΔtk* at one allele and a deletion in the other allele on the homologous chromosome of the gene desert region, and the cells with the *PuroΔtk* cassette and mutations at other loci. By culturing the population for 15 days, sister chromatid exchange and LOH events occur. Homozygous deletions at the targeted locus will result in loss of the *PuroΔtk* cassette. A rough estimation of the frequency of sister chromatid exchange on chromosome 6 resulting in homozygous deletions at the targeted locus is about 7% (10Mb/150Mb, length of the proximal region to the gene desert/the whole length of chromosome 6). These cells are viable in media containing FIAU (Figure 3-4). The lengths of deletions can be identified using array CGH. However, other types of cells without the *PuroΔtk* cassette could be selected by FIAU, too. For example, cells with either heterozygous or homozygous deletions on the same chromosome as the gene desert will be FIAU-resistant; and some cells with deletions in other chromosomes can also be resistant to FIAU.

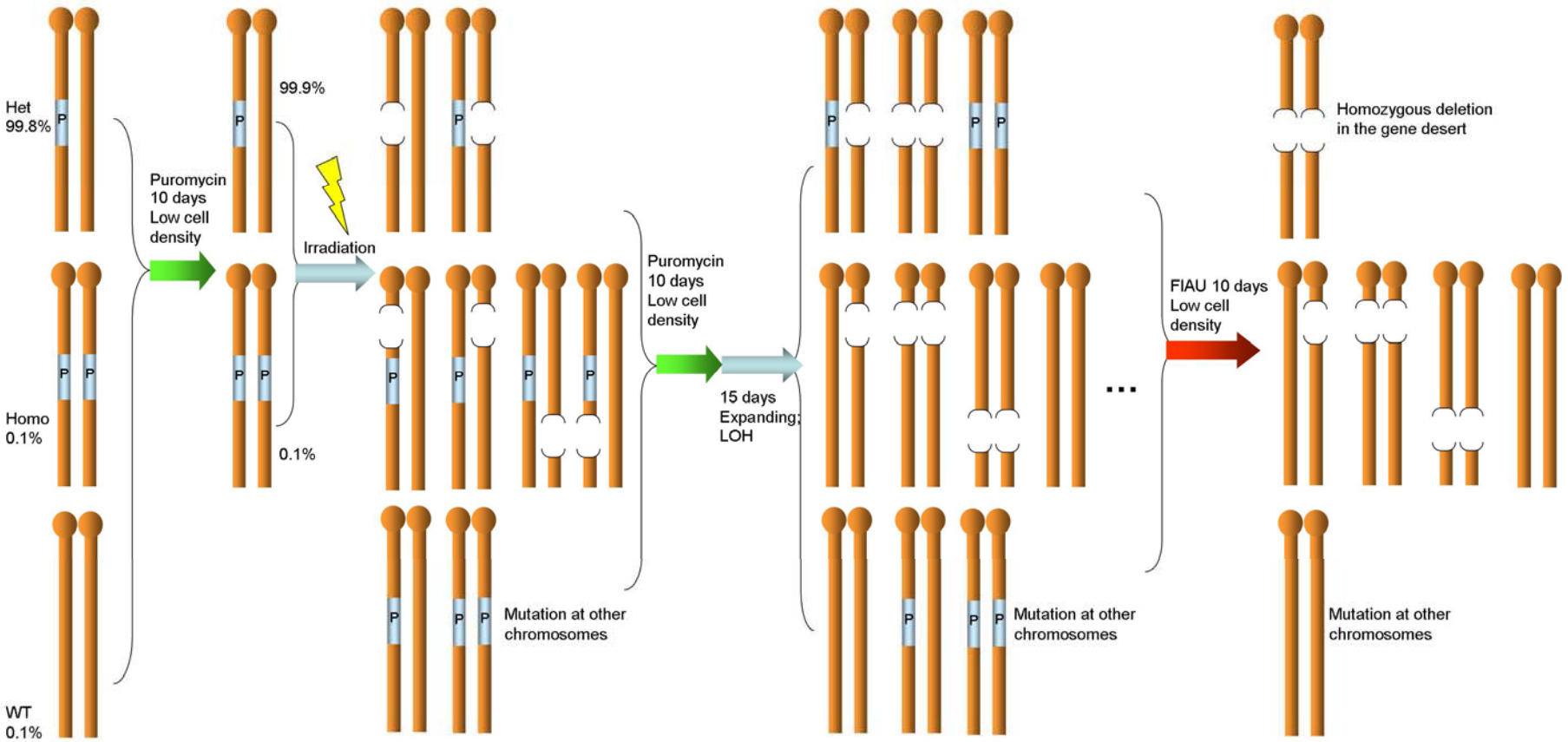

Figure 3-4 Design to generate homozygous deletions in gene desert region

The starting population is a targeted clone, which has the *PuroΔtk* cassette at the gene desert region. After 10 days puromycin selection on low density plated cells, wild type cells are removed. Irradiation generates deletions. Another 10 days of puromycin selects only those cells with *PuroΔtk* at one allele and a deletion in the other allele of the gene desert region or those cells with a deletion elsewhere. Sister chromatid exchange occurs and LOH in 15-day culture then FIAU selects homozygous deletions in the gene desert in spite of the presence of cells with both heterozygous and homozygous deletions at other loci.

3.2 Results

3.2.1 Chromosome 6 gene desert knock-in cell ZK2.1

The chromosome 6 gene desert targeting vector pZK-GD31 was constructed by recombineering and used for targeting in the *Blm*-deficient ES cell line NGG5-3 (Guo *et al.* 2004). pZK-GD31 was linearized with *Not* I, checked on an agarose gel to confirm the digestion, precipitated with ethanol and redissolved in water prior to electroporation.

Approximately ten million *Blm*-deficient ES cells (NGG5-3) and 20 µg linearized pZK1 were used in the electroporation. After electroporation, cells were plated onto 90mm feeder plates and puromycin selection was initiated 24 hours later. After 10 days selection, puromycin resistant colonies were visible. There were about 300 colonies per 10^7 cells plated. A total of 96 colonies were clonally isolated and screened for targeted clones by Southern blot analysis.

Using a 5' external Southern blot probe, targeted and wild type alleles can be distinguished. This 718 bp probe was amplified from genomic DNA (10434089 to 10434806 bp, NCBI m36, Ensembl release 46, primers 142.1 and 142.2, sequences at section 2.3.3). Genomic DNA was digested with *Hind* III, fractionated on a 0.8% agarose gel and then transferred to Hybond XL membrane and hybridized using a standard protocol. From 96 clones, one targeted clone (ZK2.1 G4) was identified with both a 15.3 kb wild type band and an 11.7 kb targeted band (Figure 3-5).

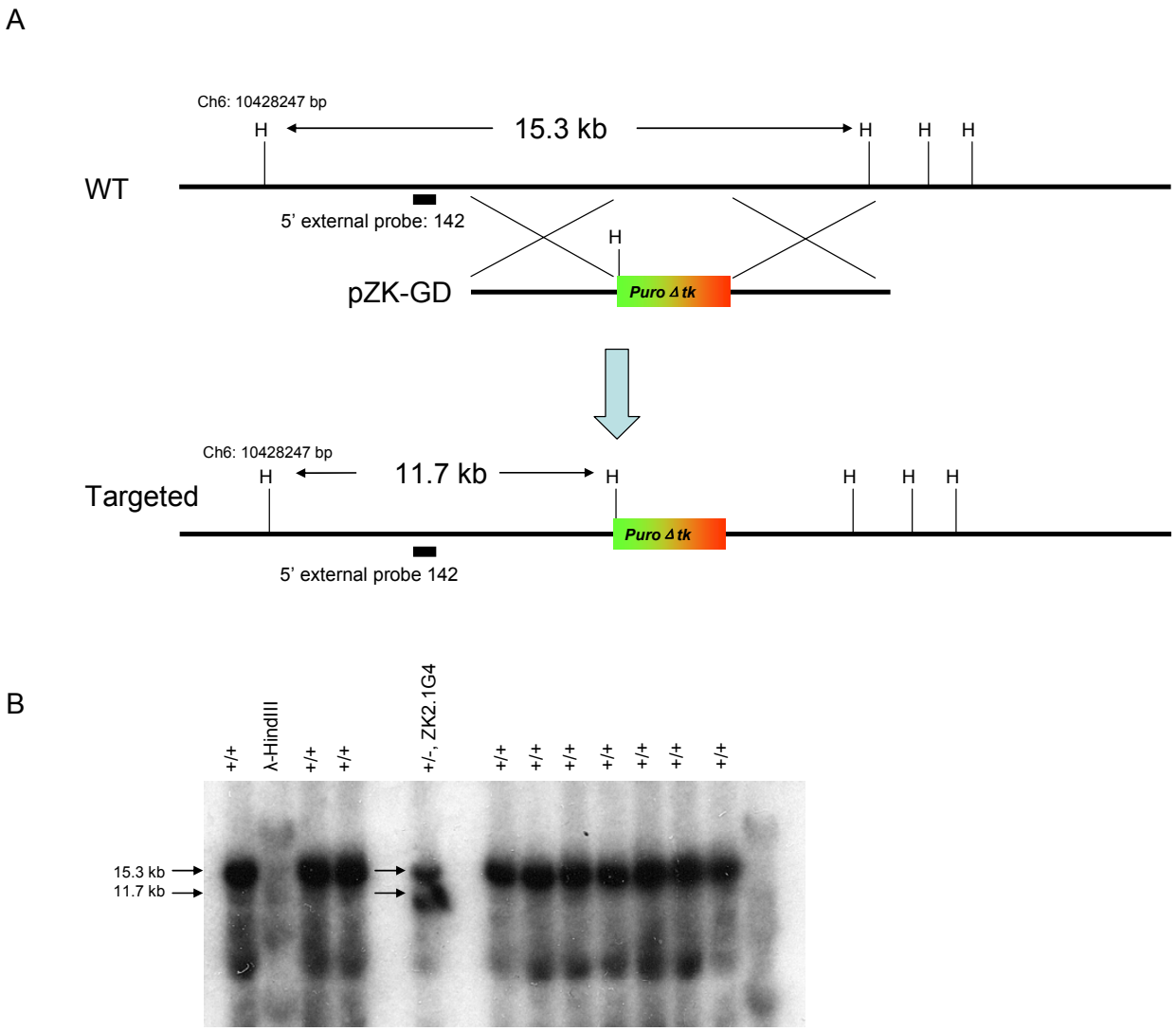

Figure 3-5 Chromosome 6 gene desert targeting

A. Schematic figure of Southern blot strategy for detecting gene targeting. DNA was digested with *Hind* III (H). Using a 5' external probe (probe 142), the wild type fragment is 15.3 kb while the targeted fragment is 11.7 kb. **B**. Southern blot screen targeted clones. Clone ZK2.1 G4 has both a wild type allele and a targeted allele.

3.2.2 Testing background FIAU resistance levels prior to selection for homozygous deletions

The targeted ES cell clone ZK2.1G4 was used to determine sizes of homozygous deletions. Clone G4 was cultured and expanded in puromycin media at low cell density for 10 days to eliminate trace numbers of wild type cells then the cells were pooled together. Before the purified ZK2.1G4 population was irradiated, one million cells were plated and cultured 90mm plates with FIAU media as a control. This control showed that FIAU-resistant background was high. A potential reason for the high background was the purity of the clone plated in FIAU. Therefore the cell line was cloned by dilution and single cell clones were isolated. Twelve clones were expanded and the targeted allele was confirmed by Southern blot. The FIAU resistance background was examined by a double selection in the puromycin and FIAU media. $1–2\times10^6$ cells were plated at the beginning of the double selection. The degree of the double resistance background in different clones varied as shown in Figure 3-6. Among clones 4, 6, 7, 8, 11 and 12, puromycin and FIAU-resistant background is between 10^{-4} and 10^{-2} per plated cell.

Because these 12 clones were subcloned, the chance of impurity was very low. In addition, none of them showed full resistance to either FIAU or puromycin+FIAU. One explanation was that mutations, which occurred during cell culture lead to the FIAU or puromycin+FIAU resistance. To test this assumption, another electroporation of the gene desert targeting vector was performed in both wild type and the *Blm*-deficient cells (NGG5-3) to generate random insertions. Transfected cells were selected in puromycin and two sets of 96-well plates were clonally isolated. ZK2.9, ZK2.10 (*Blm*$^{-/-}$ background) and ZK11, ZK12 (wild type background), were tested for resistance to puromycin and FIAU (Figure 3-7). As expected all (100%) of the transfected puromycin^R clones in the control AB1 cell line (wild type) were sensitive to FIAU. However, half of the puromycin resistant clones isolated in the *Blm*-deficient cells were resistant to FIAU though levels varied from 10^{-5} to 10^{-2}. This indicates that the *PGK-PuroΔtk*-pA cassette was intact and functional in wild type cells. While *PGK-puro* was still functional in the *Blm*$^{-/-}$ cells, the *Δtk* component seemed to have lost function in some of the cells in half of the clones. However, not all the cells in each clone were FIAU-resistant since individual colonies could be seen in individual wells instead of a lawn of resistant cells, suggesting that the *PGK-PuroΔtk*-pA cassette was not lost at the stage when it was integrated into cell genome, rather this occurred during the expansion of the clone. Because of this, FIAU selection could not be used to isolate homozygous mutations in the *Blm*-deficient ES cells.

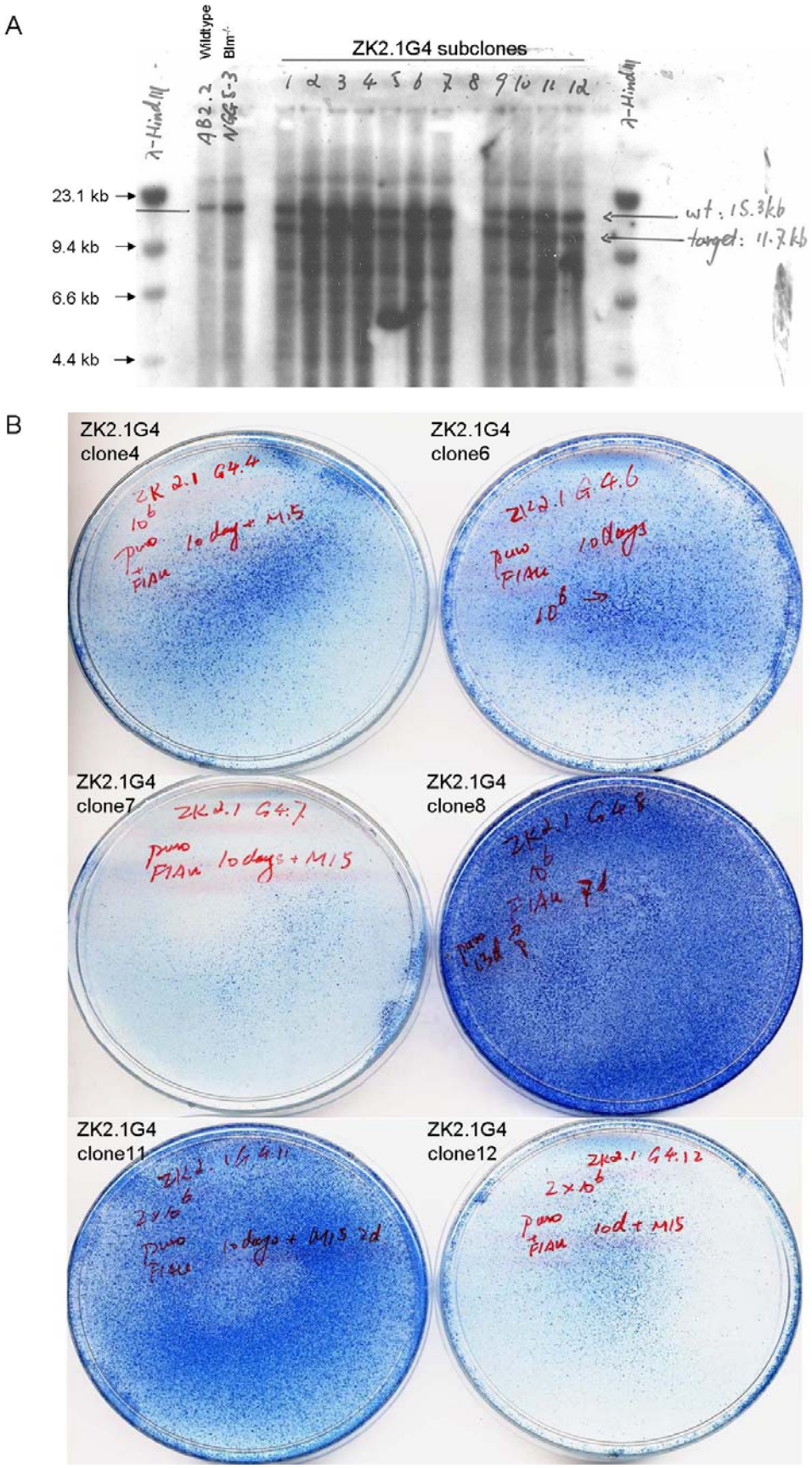

Figure 3-6 **Gene desert targeted clone with resistance to puromycin and FIAU**

Figure 3-6 desert targeted clone with resistance to puromycin and FIAU

A. Southern blot analysis confirmed subclones 1–12 from ZK2.1G4 gene desert targeted cell. Wild type cell (AB2.2) DNA and *Blm*-deficient cell (NGG5-3) DNA were used as controls. Both controls have the wild type allele at 15.3 kb. Subclones 1–12 all have the wild type allele and the targeted allele (11.7 kb). **B**. Puromycin and FIAU double resistance background of two subclones of gene desert targeted cells. 2 million cells of both clone 11 and 12 and one million cells of clone 4, 6, 7 and 8 were plated in 90mm plate. These subclones were cultured in puromycin+FIAU media for 10 days then cells were grown in M15 normal media for 2 days before they were stained by methylene blue. The clones have different levels of background.

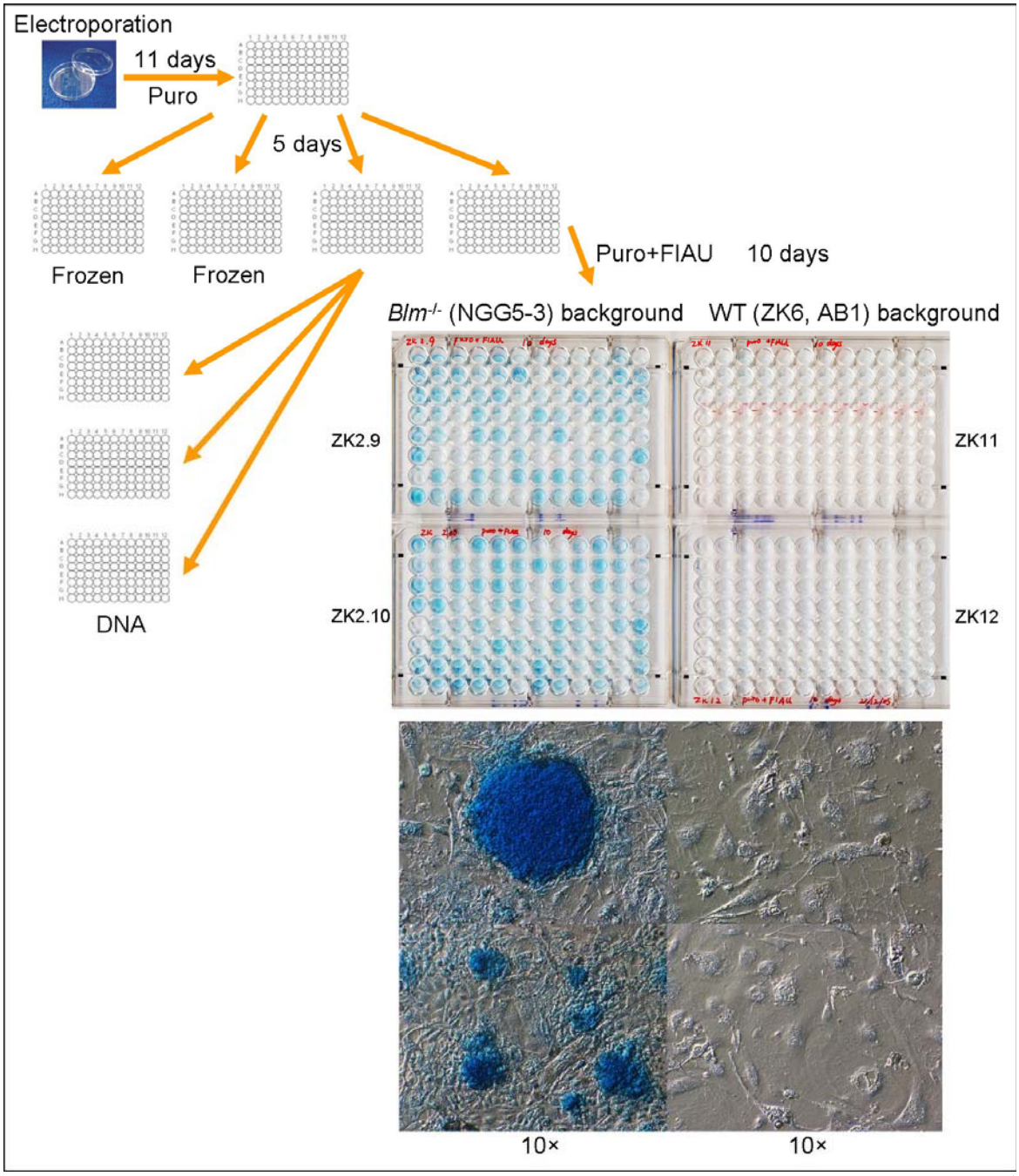

Figure 3-7 Puromycin and FIAU double resistant background in NGG5-3 cells

The gene desert targeting vector pZK-GD.31 was electroporated into wild type (ZK6) and *Blm*-deficient (NGG5-3) cells, which were selected in puromycin media for 11 days. Then colonies were picked up, passaged and duplicates were plated for selection in (puromycin+FIAU) media. Cells were stained with methylene blue. Resistant clones could be seen in half of the wells in the *Blm*-deficient cell plates, but not in wild type cell plates.

3.2.3 Puromycin and FIAU double resistant clones contain the *PuroΔtk* cassette

It is known that the *Blm*-deficient ES cells have an elevated rate of mitotic recombination between non-sister chromatids (Luo *et al.* 2000). Thus, the occurrence of puromycin and FIAU double resistant clones in gene desert targeted *Blm*-deficient cells but not wild type cells can be explained by loss-of-function mutations of the *Δtk* part of the *PuroΔtk* cassette, such as deletion or point mutations, which must leave the *PGK-puro* cassette functionally active and result in lost of the function of *Δtk*. To test this hypothesis, *Eco* RI digested DNA samples from 22 double resistant gene desert targeted clones and the *Blm*-deficient cells were hybridized with the *PuroΔtk* probe and the *Δtk* probe separately (Figure 3-8). All DNA samples of these mutants, except the *Blm*-deficient cell DNA, showed the expected band after Southern blotting, suggesting the *PuroΔtk* fusion gene did not experience any large fragment loss. Another possible explanation of the puro^R, FIAU^R phenotype is the *Δtk* cassette contains small frame-shift mutations, which did not affect the function of puromycin resistance cassette. Therefore these clones remain puromycin and FIAU resistant. However, the frequency of FIAU resistance in puro^R cells is about 10^{-5} to 10^{-2} (Figure 3-7), which is much higher than the spontaneous mutation rate of about 5×10^{-6} per locus per generation in normal cells and the LOH rate of ~4.2×10^{-4} events per locus per cell per generation in the *Blm*-deficient ES cells. Thus, small frame-shift mutations may not be the reason of the fluctuating frequency of puromycin and FIAU double resistant cells.

One other hypothesis is raised after considering that the fluctuating frequency of the double resistant cells indicates the loss-of-function of the *Δtk* part was generated in the process of cell culture and the original mutants regenerated various numbers of offspring. Such a phenomenon is similar to the LOH events of a pre-existing heterozygous mutation. Therefore, it is assumed that a gene required for FIAU metabolism was heterozygously mutated in the *Blm*-deficient cells. Homozygous mutations of this gene led by LOH can result in FIAU-resistance although the *Δtk* part of the *PuroΔtk* fusion gene may remain functional. And LOH can occur anytime during cell culture so that the number of FIAU^R cells varies. The NGG5-3 *Blm*-deficient ES cell line was derived from wild type ES cells after multiple gene targeting experiments and subclonings, thus this cell line is very likely to have acquired mutations prior to my experiments.

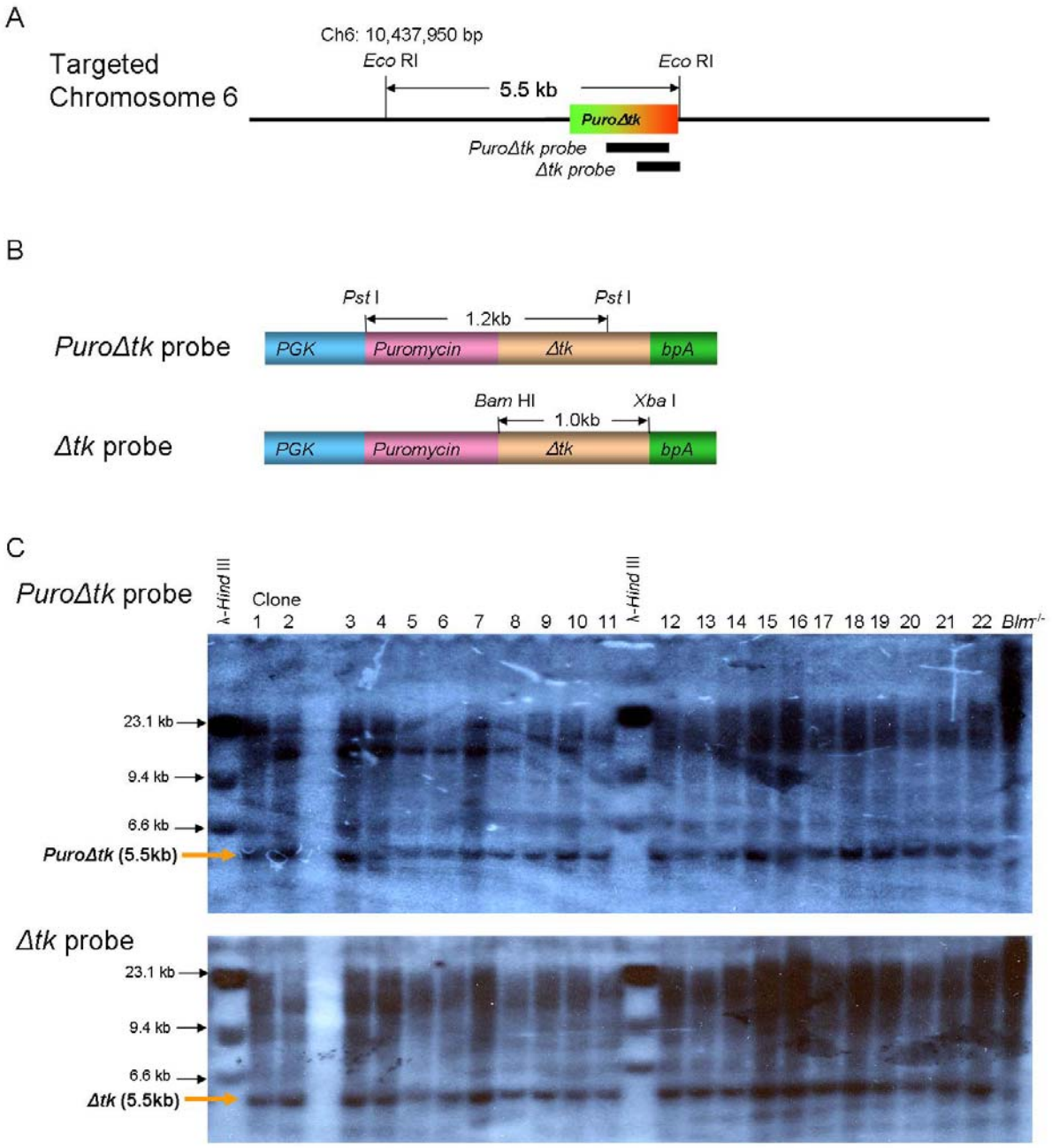

Figure 3-8 **Southern blot analysis confirms the presence of *PuroΔtk* cassette**

Figure 3-8 Southern blot analysis confirms the presence of *PuroΔtk* cassette

A. Southern blot strategy to identify if the *PuroΔtk* cassette is intact. The *PuroΔtk* targeted allele is shown. After *Eco* RI digestion, hybridization with either *PuroΔtk* probe or the *Δtk* probe will generate a 5.5 kb band. **B**. Generation of the *PuroΔtk* probe or the *Δtk* probe. The *PuroΔtk* probe is a 1.2 kb fragment cut by *Pst* I from vector YTC37 (Chen, Y. T. *et al.* 2000). The *Δtk* probe is a 1.0 kb fragment cut by *Bam* HI and *Xba* I from vector YTC37. **C**. Southern blots show that 22 puromycin and FIAU double resistant gene desert targeted clones contain the whole *PuroΔtk* cassette. DNA from these clone and the *Blm*-deficient cells was hybridized with the *PuroΔtk* probe or the *Δtk* probe. The 5.5 kb band can be seen in all double resistant clones but not in *Blm*-deficient cells. There is a weak background band between 9 to 24 kb in all these cells. And its existence was also observed in other experiments when hybridizing the *PuroΔtk* probe with DNA of cells derived from the NGG5-3 *Blm*-deficient cell line (Figure 7-11–14).

3.3 Discussion

The positive-negative marker *PuroΔtk* was successfully targeted into chromosome 6 of *Blm*-deficient mouse ES cells. One targeting event was found in 96 puromycin resistant colonies. This targeting efficiency is much lower than what was typically achieved in the laboratory (10–50%). The low targeting efficiency was probably caused by the accessibility of the chromatin in the gene desert region. This locus is near the Centromere (proximal 10Mb out of 150 Mb) of mouse chromosome 6 and is repetitive. This might also play a role in the low targeting efficiency.

It was expected that targeting of *Δtk* would facilitate the selection of deletions in FIAU. The selection of such deletions relies on a low background of loss of *Δtk*. However, a high background was observed in the gene desert targeted cells. One explanation of this high background was the impurity of the isolated targeted clone. Therefore this targeted cell line was subcloned by dilution. Twelve subclones with the confirmed gene desert targeted allele also showed similar resistance to FIAU. Because these clones were not fully resistant to FIAU, loss of *Δtk* function did not occur at an early time point. The frequency of the puromycin and FIAU double resistant background in these subclones is between 10^{-4} and 10^{-2}. It was assumed loss of *Δtk* function occur during expansion of the clones. To test this assumption, the gene desert targeting vector was electroporated into wild type cells and *Blm*-deficient cells. Transfected cells were selected in puromycin and 192 colonies from each genetic background were clonally isolated. All of the transfected puromycin^R clones in the control AB1 cell line (wild type) were sensitive to FIAU, which was expected. Some cells in half of the puromycin resistant clones isolated from the *Blm*-deficient cells were resistant to FIAU. This indicates that the *PGK-PuroΔtk*-pA cassette was intact and functional in wild type cells. However, the *PGK-puro* component was functional in the *Blm*$^{-/-}$ cells but the *Δtk* component had lost its function in some cells of the half of the clones. The loss-of-function of the *Δtk* component occurred during expansion instead of prior to homologous recombination or random integration because individual FIAU-resistant colonies appeared in FIAU selection instead of a lawn of resistant cells. Therefore, the puromycin and FIAU double resistance must have a genetic basis, which occurs in the process of cell growth. In addition, these events are independent of genomic loci because they were found in both targeted cells and random integrated cells. Moreover, it is associated with the *Blm* deficiency.

To examine if this genetic events occurred at the *PuroΔtk* locus, a Southern blot analysis

was conducted. DNA of twenty-two double resistant clones from the gene desert targeted clone ZK2.1G4 was hybridized with both the *PuroΔtk* probe and the *Δtk* probe. The analysis confirmed the presence of the whole fusion *PuroΔtk* gene in those mutants. However, this does not rule out the possibility of small frame-shift of the *Δtk* cassette, though this is unlikely to occur with such a frequency.

Another hypothesis to explain the presence and the fluctuating frequency of the double resistant cells is that these phenomena are due to the LOH events of a pre-existing heterozygous mutation within FIAU metabolism pathway. The NGG5-3 *Blm*-deficient ES cell line had experienced four homologous recombination experiments and a number of subcloning procedures, thus this cell line is very likely to have acquired mutations prior to my experiments. LOH events of this mutation can cause a homozygous mutation, which lead to the resistance to FIAU in cells. When a cell with such a homozygous mutation expand, more and more cells are resistant to FIAU although the number of these cells are determined by the time point of the LOH event and the doublings of the cells after the LOH event. However, this process does not necessarily involve the inactivation of the *Δtk*.