Transgenic Techniques
(Molecular Biology)

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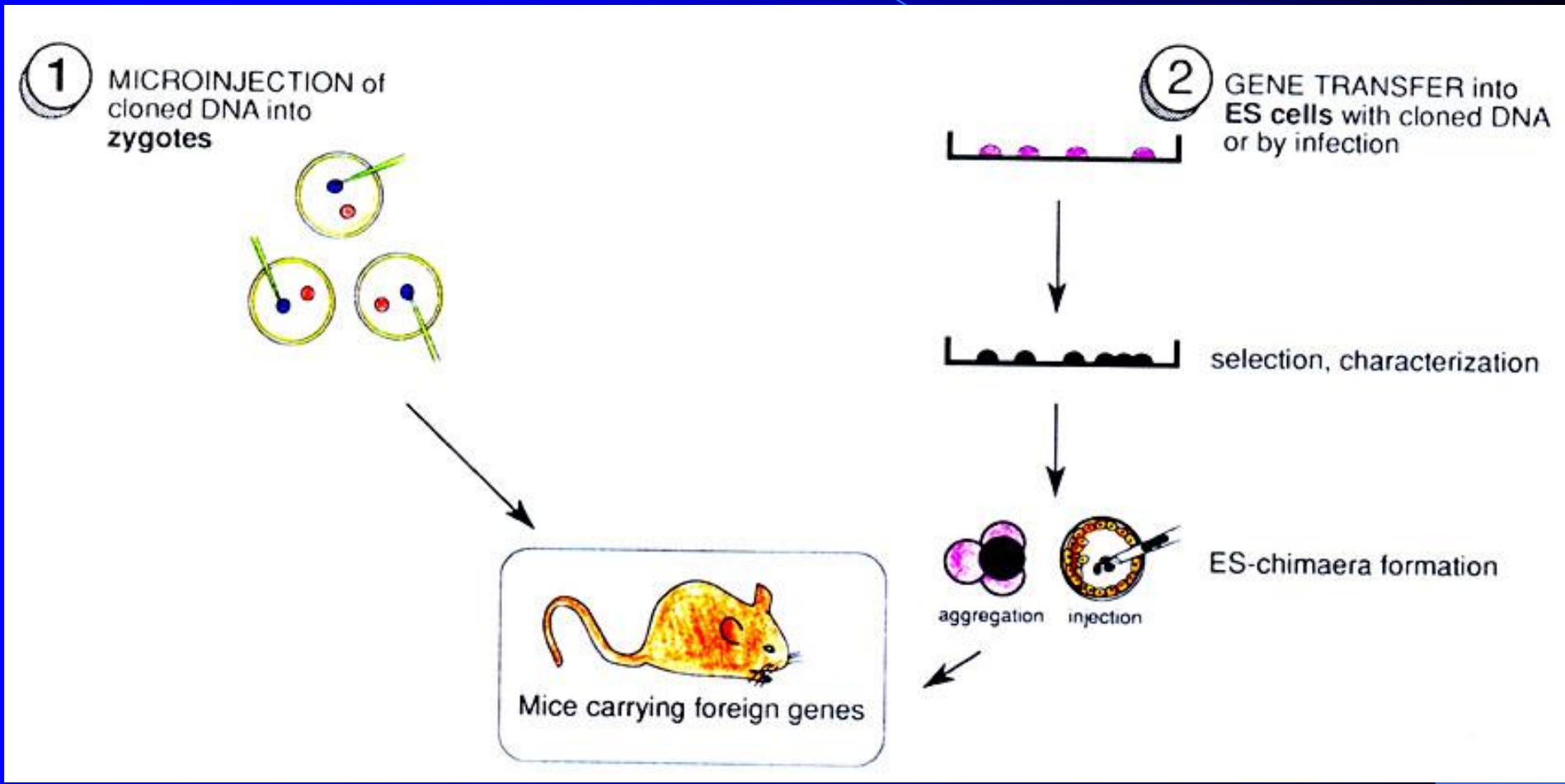
解剖學暨細胞生物學科
錢宗良

Methods for introducing genes into mouse embryos



I. 傳統基因轉殖： 將欲探討的基因直接打入動物的受精卵

DNA injection into fertilized eggs (over-expression, multiple copies of transgene)

- To study gene control
- To change the physiology of mice
- To study oncogene function

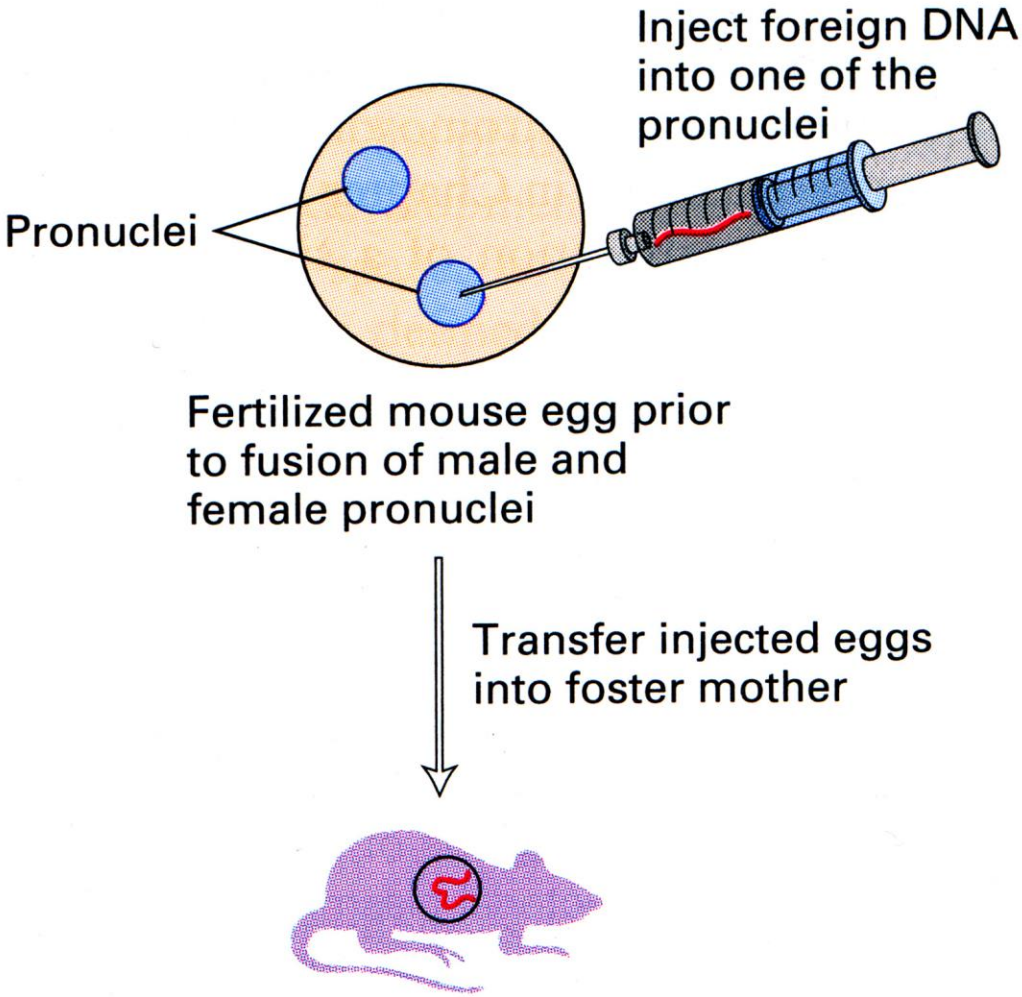
II. 基因敲毀轉殖(knock out): 將欲探討的基因在胚胎種細胞 (embryonic stem cells) 內先行破壞，在利用複雜的胚胎轉殖技術，獲得基因敲毀轉殖動物。

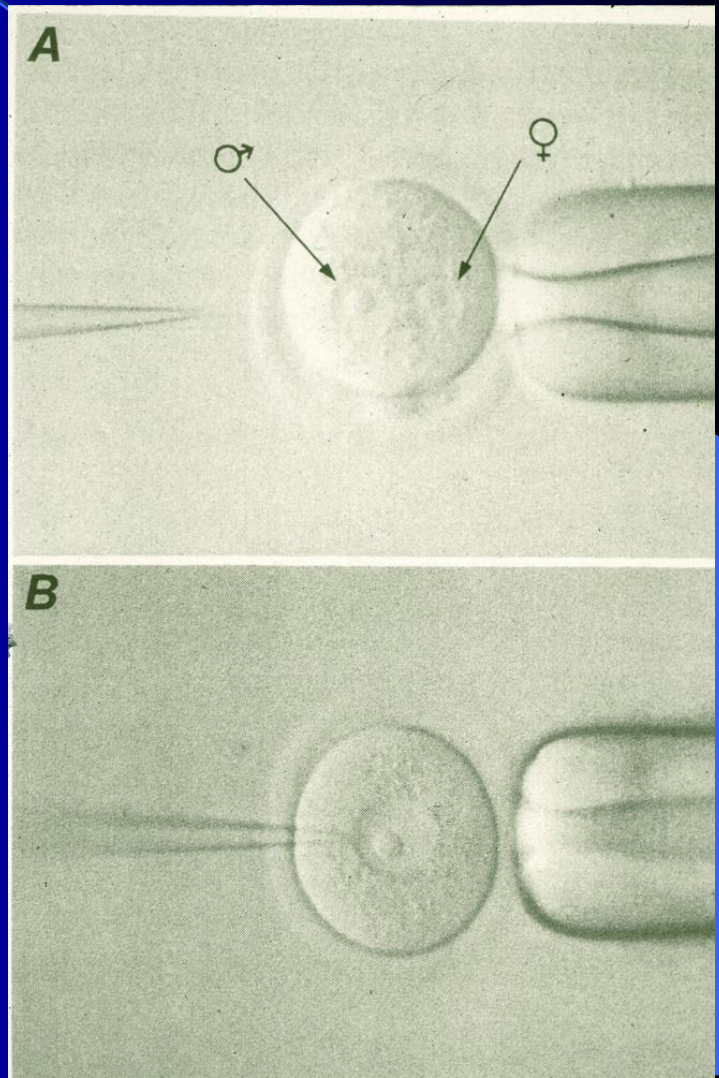
Gene transfer using embryonic stem (ES) cells (gene knock-out, null mutation)

- To study gene function in vivo
- To change the phenotype of mice
- To examine the gene redundancy (gene knock-in)

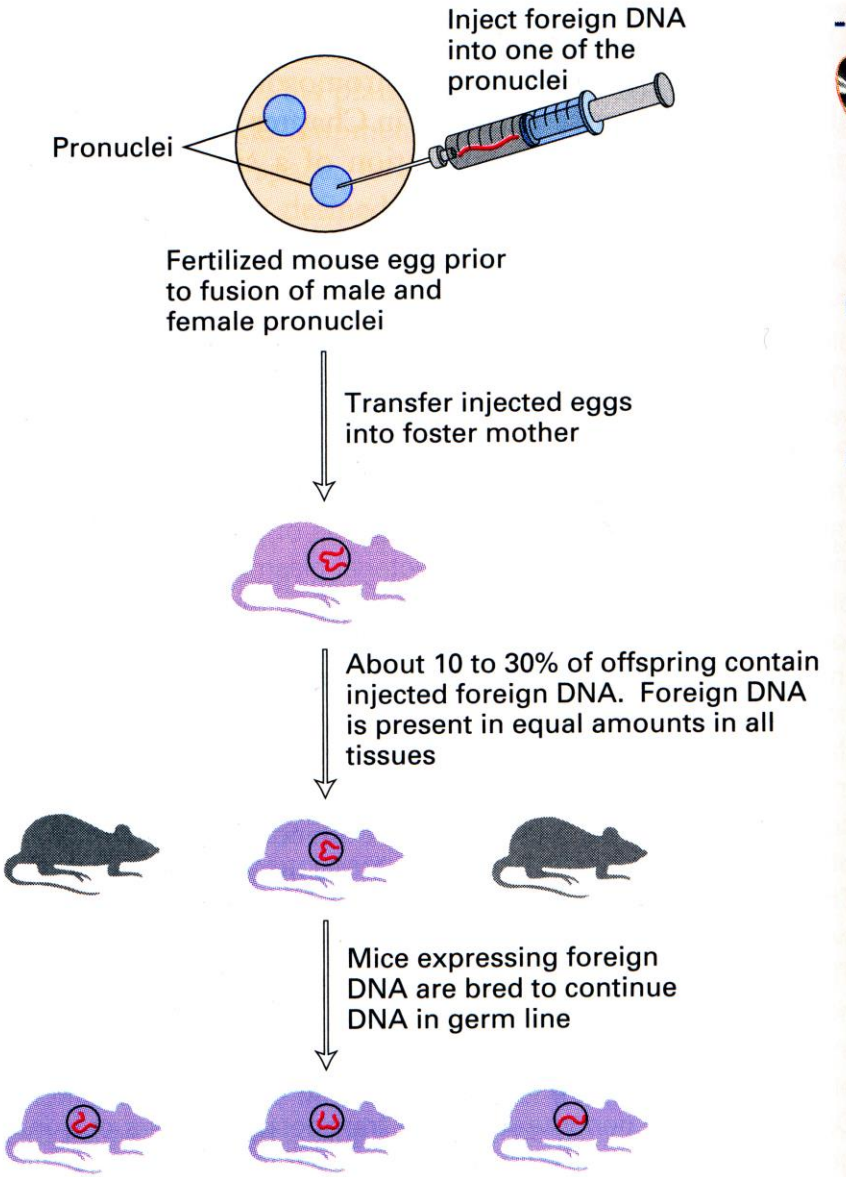
傳統基因轉殖：

將欲探討的基因直接打入動物的受精卵。





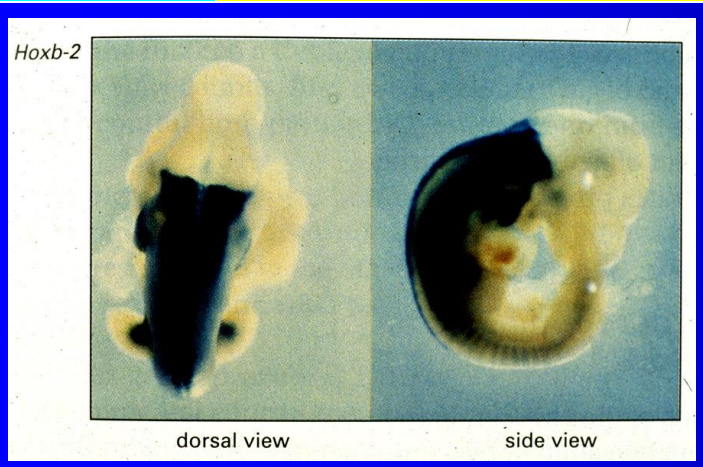
Creating a Transgenic Mouse (with a movie)



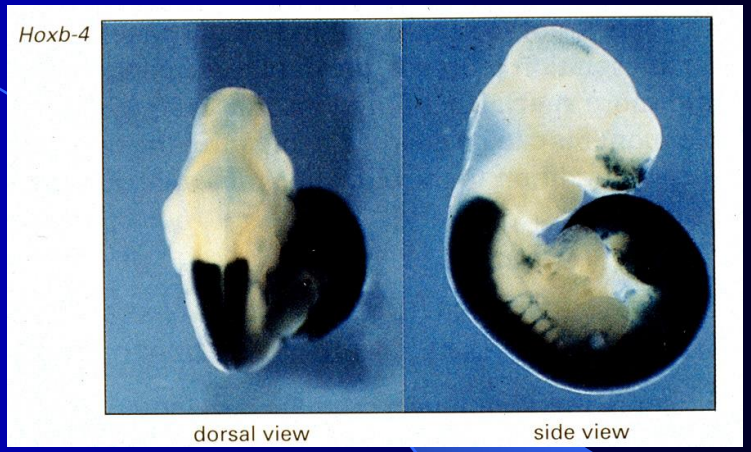
▲ **FIGURE 8-36 General procedure for producing transgenic mice.** [See R. L. Brinster et al., 1981, *Cell* **27**:223.]

MEDIA CONNECTIONS
Creating a Transgenic Mouse

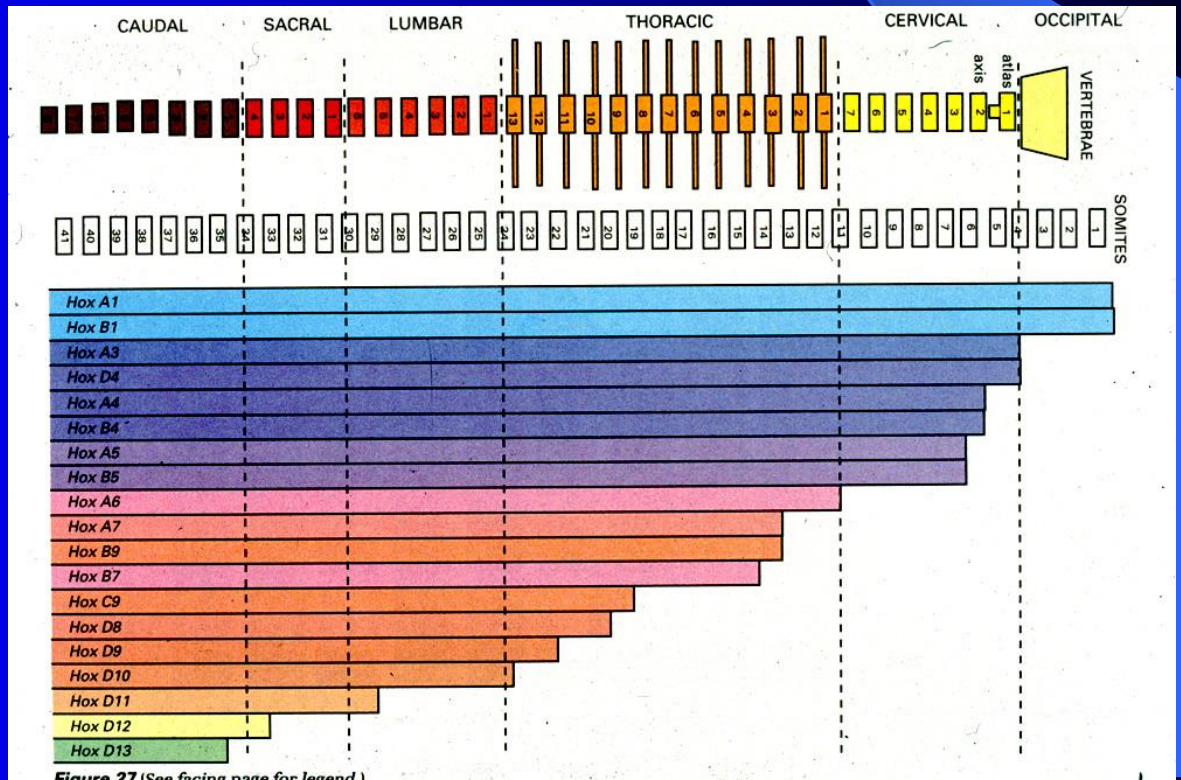
Hoxb-2 β-*galatosidase*



Hoxb-4 β-*galatosidase*



Transgenic mice with Hox gene promoter and a reporter β-*gal*



阿茲海默氏病 (Alzheimer's disease)
基因轉殖動物模式

類澱粉前驅蛋白 (Amyloid Precursor Protein, APP)基因轉殖動物 (Nature 373:523-527, 1995; Nature 395:755-756, 1998)探討神經退化機制

LETTERS TO NATURE

Alzheimer-type neuropathology in transgenic mice overexpressing V717F β-amyloid precursor protein

Dora Games*, David Adams†‡, Ree Alessandrini†, Robin Barbour*, Patricia Berthelette†‡, Catherine Blackwell†‡, Tony Carr*, James Clemens§, Thomas Donaldson†‡, Frances Gillespie†‡, Terry Guido*, Stephanie Hagopian†‡, Kelly Johnson-Wood*, Karen Khan*, Mike Lee*, Paul Leibowitz†‡, Ivan Lieberburg*¶, Sheila Little§, Eliezer Masliah‖, Lisa McConlogue*, Martin Montoya-Zavala†‡, Lennart Mucke☆, Lisa Paganini*, Elizabeth Penniman†, Mike Power*, Dale Schenk*, Peter Seubert*, Ben Snyder†, Ferdie Soriano*, Hua Tan*, James Vitale†‡, Sam Wadsworth†‡, Ben Wolozin** & Jun Zhao*

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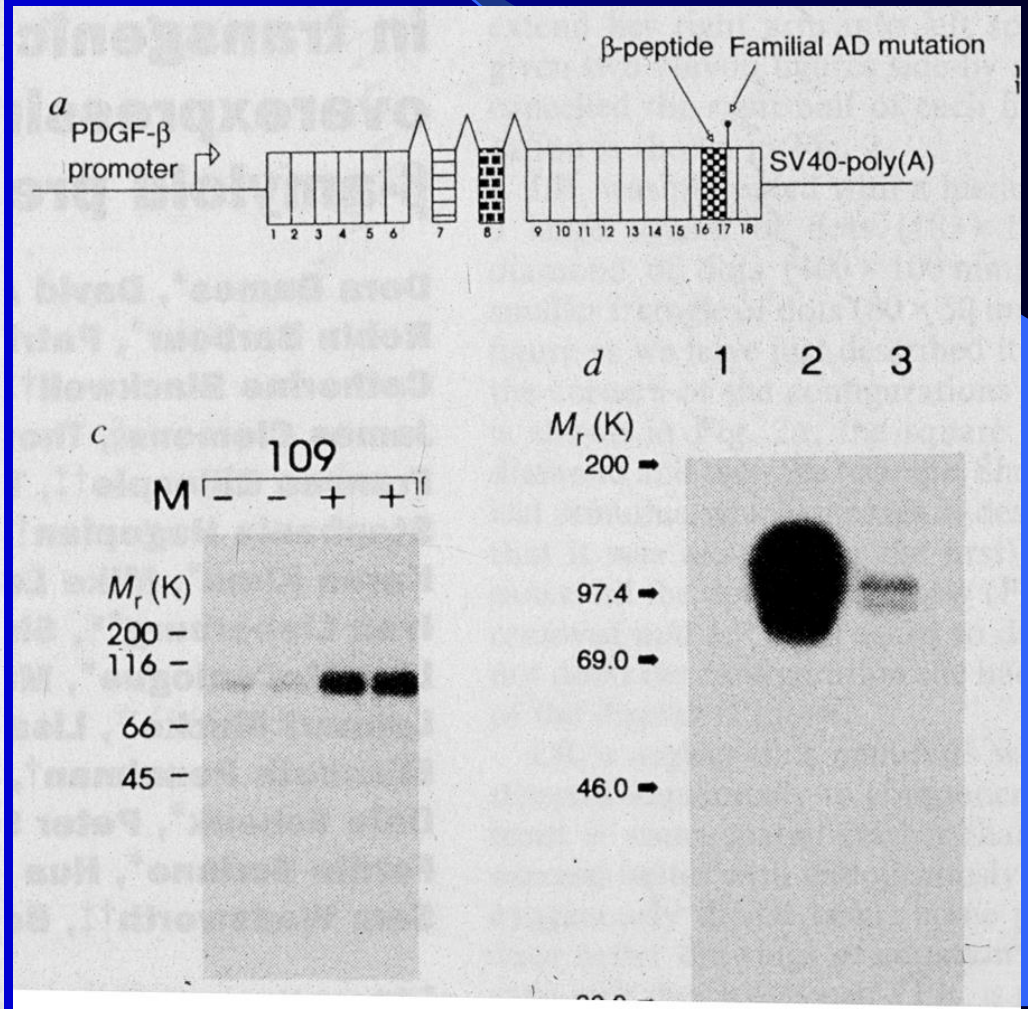
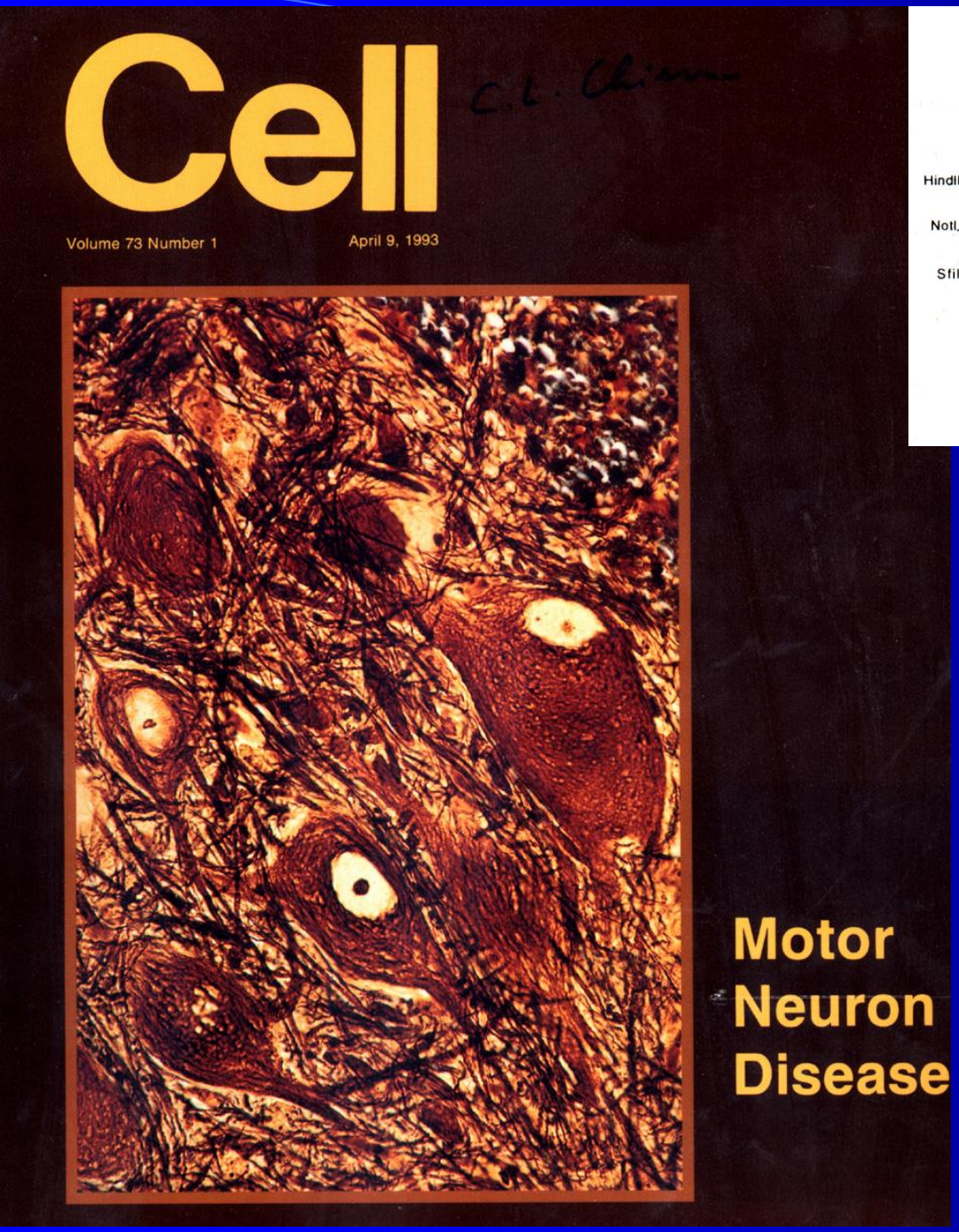
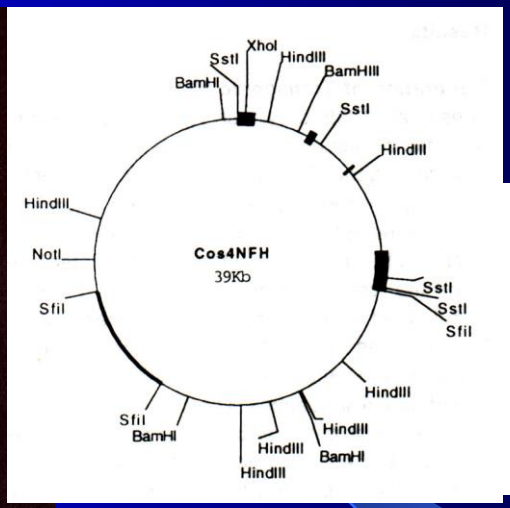
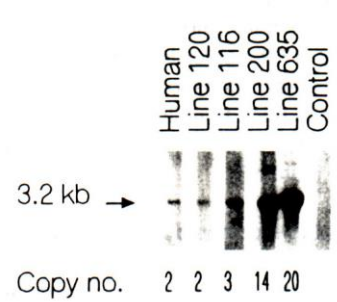
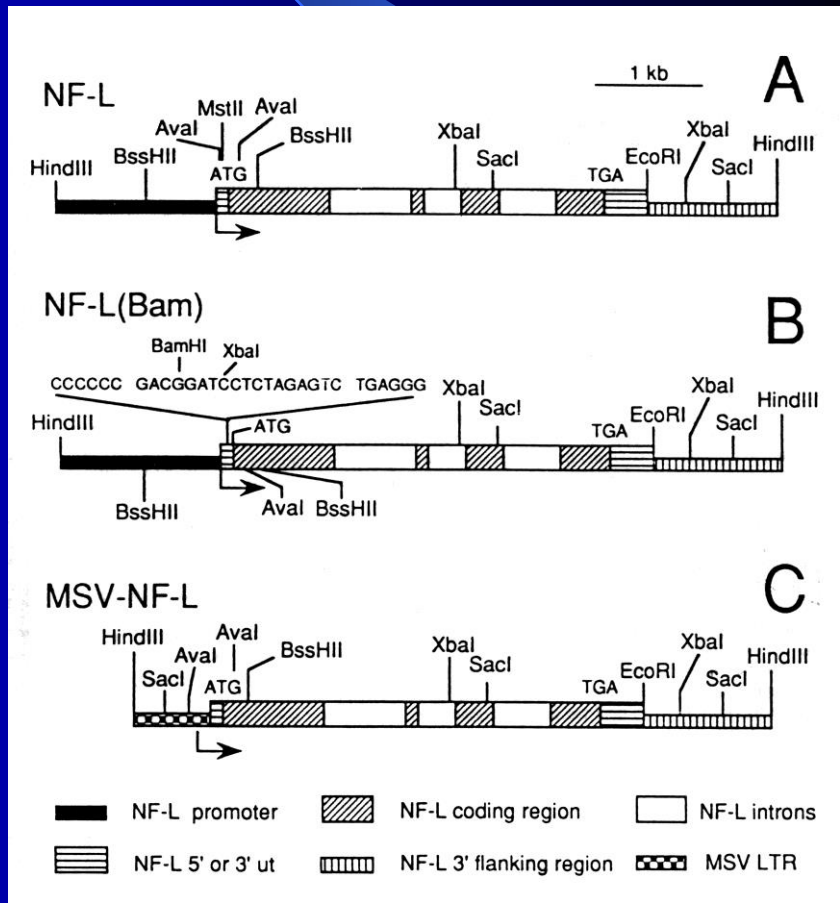
§ Lilly Research Laboratories, Indianapolis, Indiana 46285, USA
☆ The Scripps Research Institute, Department of Neuropharmacology, 10666 North Torrey Pines Road, La Jolla, California 92037, USA
‖ Department of Neurosciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, USA
** Laboratory of Clinical Science, National Institute of Mental Health,



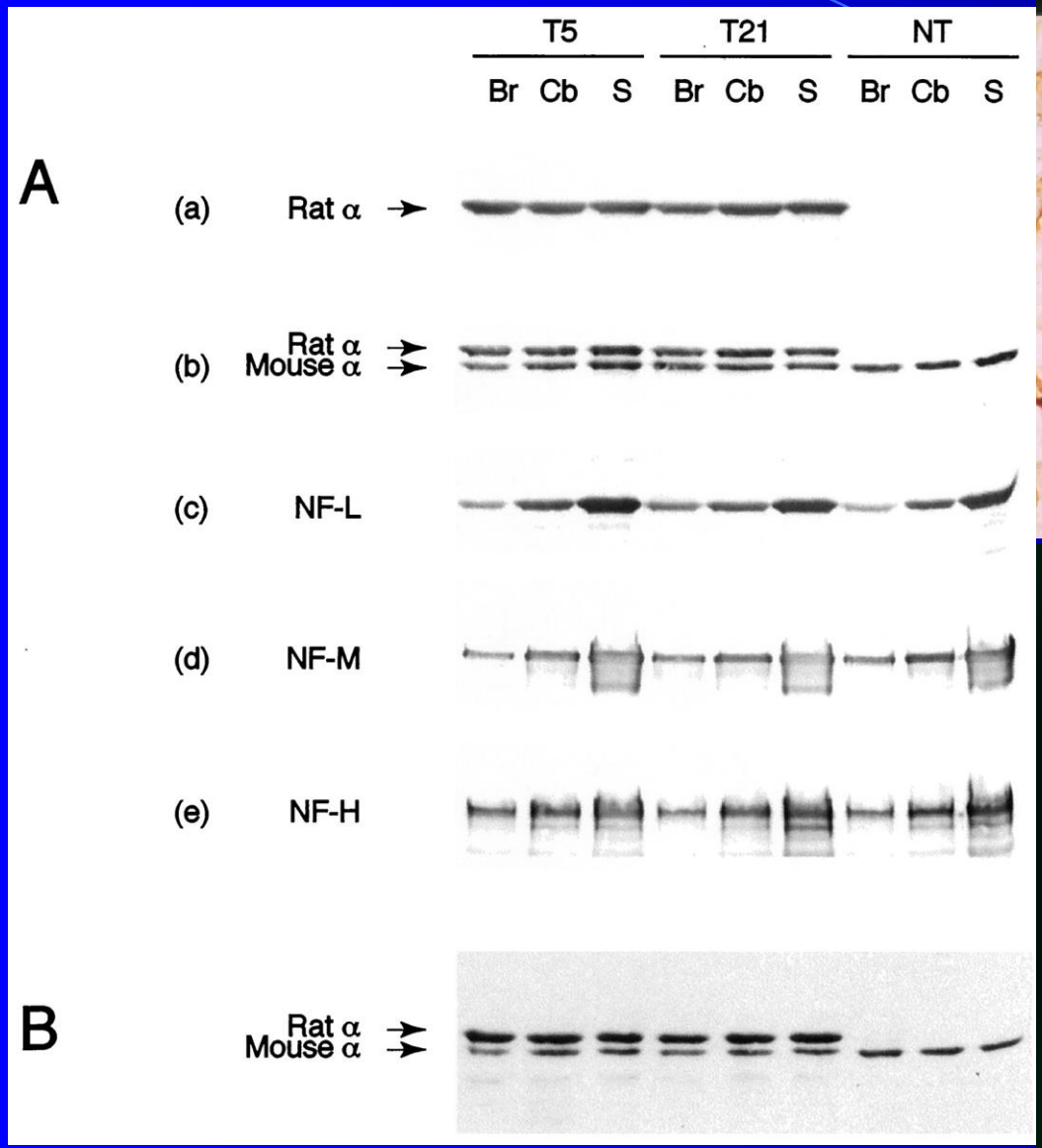

Cell
Volume 73 Number 1
April 9, 1993
Motor
Neuron
Disease

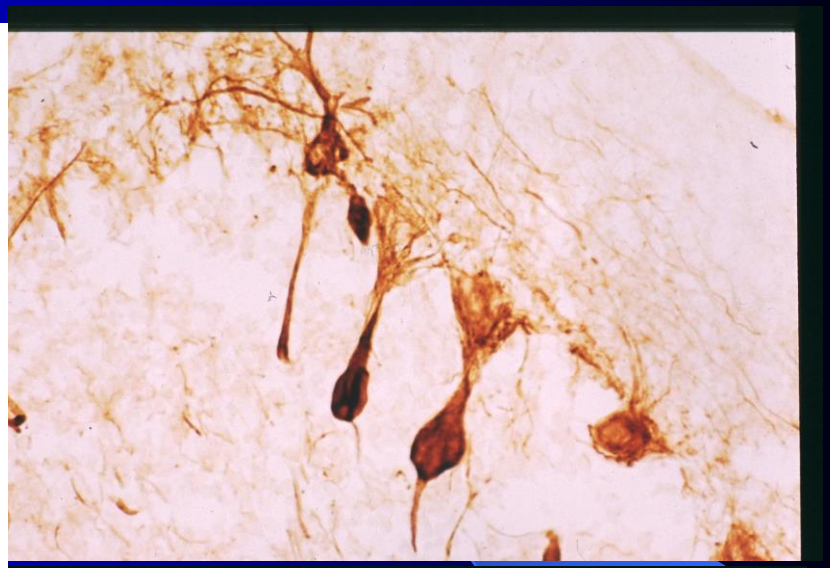

Cos4NFH
39Kb
XhoI
HindIII
BamHI
SstI
NotI
SfiI

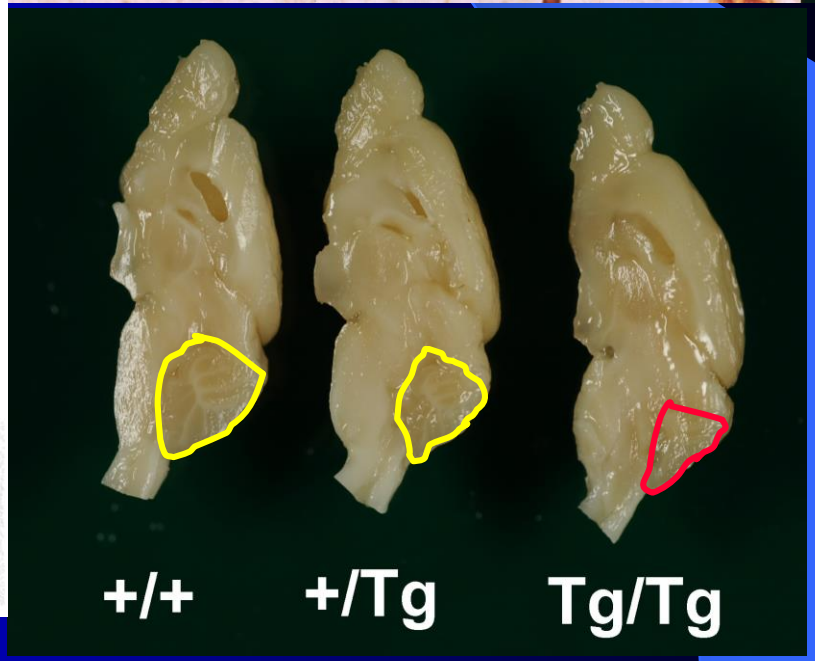

Human
Line 120
Line 116
Line 200
Line 635
Control
3.2 kb
Copy no. 2 2 3 14 20


NF-L
1 kb
A
MstII
AvaI
BssHII
XbaI
SacI
EcoRI
HindIII
ATG
TGA
NF-L(Bam)
B
BamHI
CCCCCC GACGGATCCTCTAGAGTC TGAGGG
MSV-NF-L
C
NF-L promoter
NF-L coding region
NF-L introns
NF-L 5' or 3' ut
NF-L 3' flanking region
MSV LTR

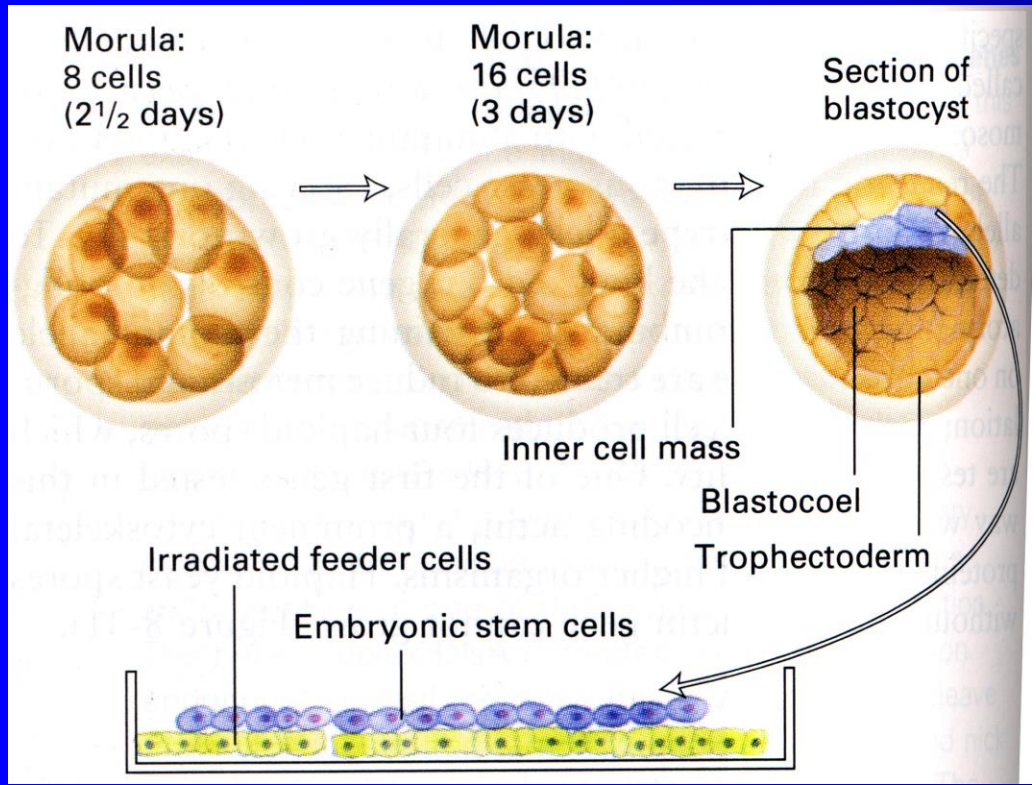
Animal model for cerebellar atrophy (J. Neurosci. 19:2974-2986, 1999)

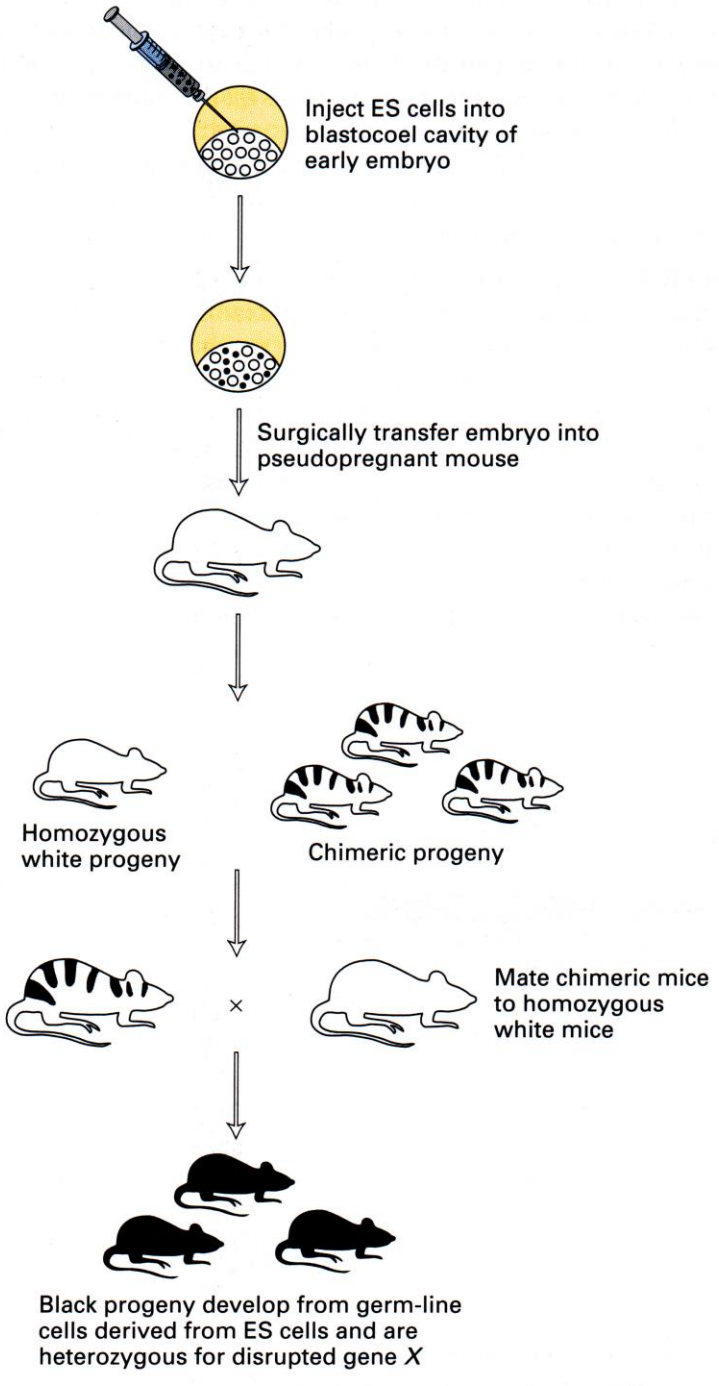


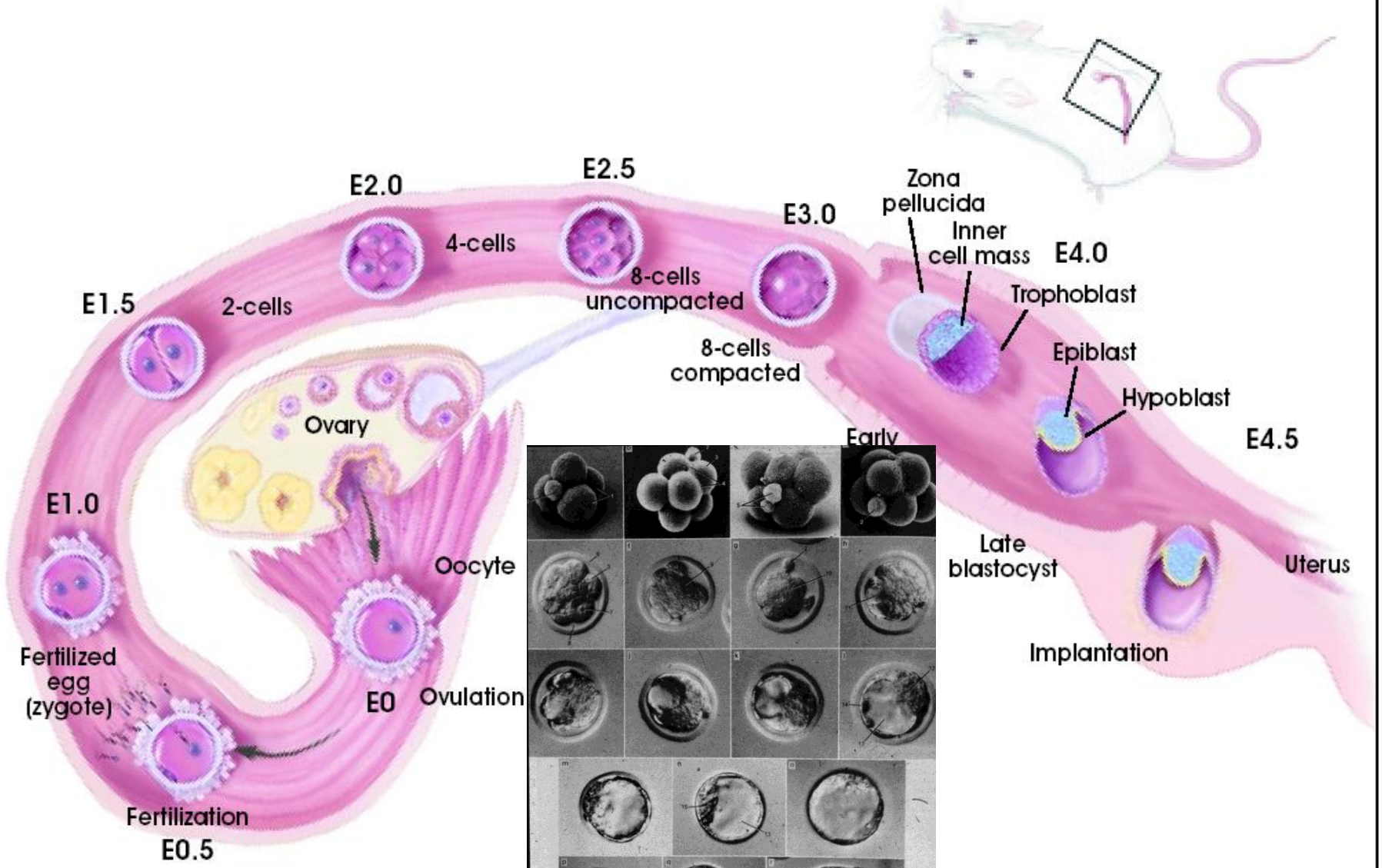




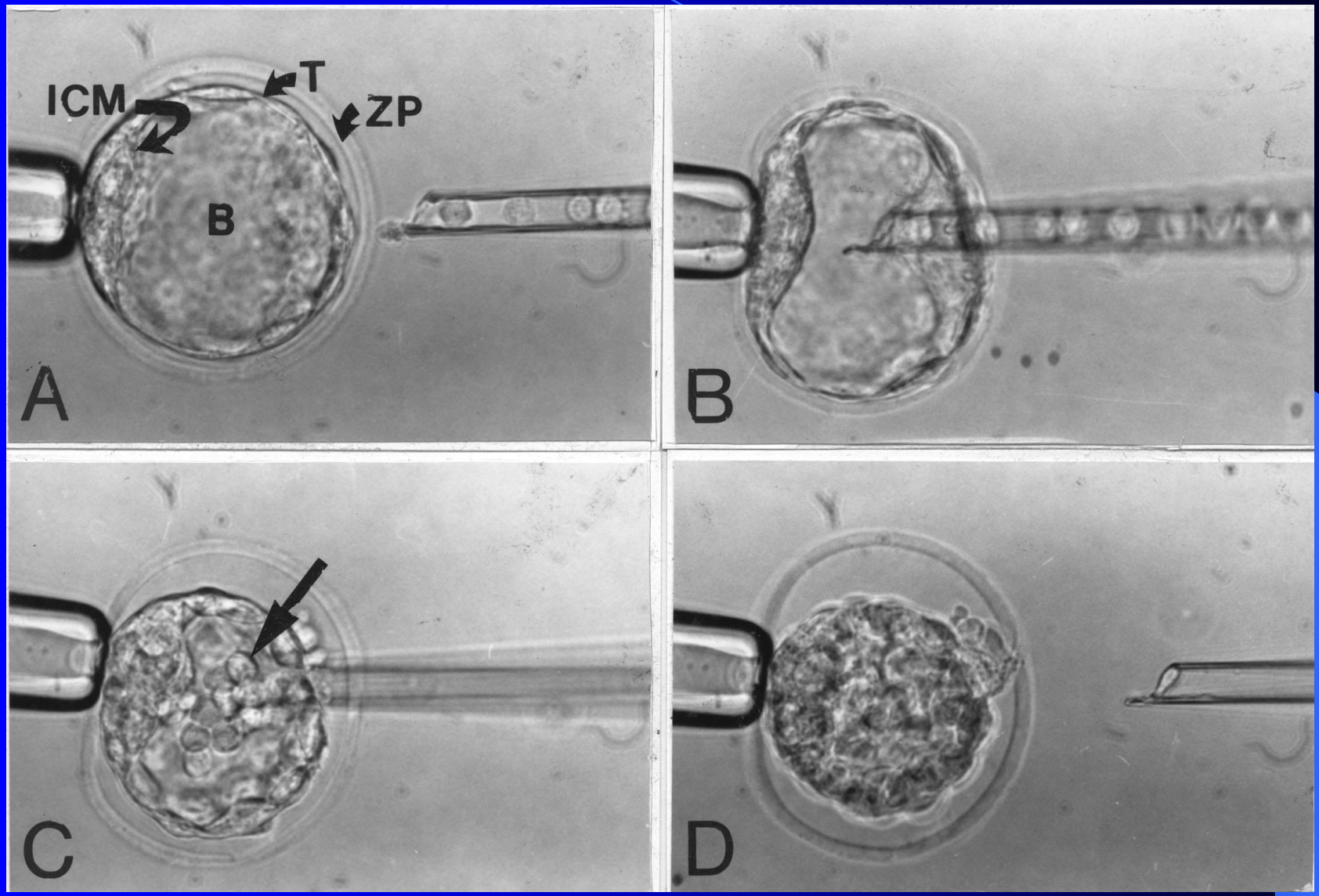
Gene transfer using embryonic stem (ES) cells (gene knock-out, null mutation)



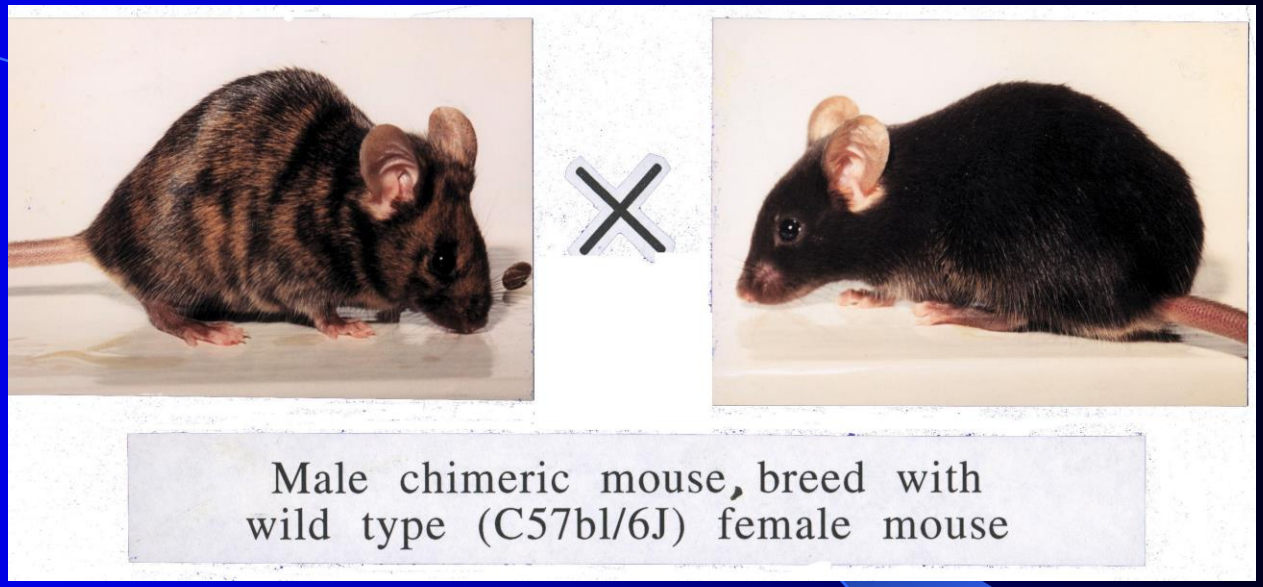




ES cells microinjected into blastocyst



Germ-line transmission analysis



Aa x Aa:

- ¼ AA (wild type)
- ½ Aa (heterozygote)
- ¼ aa (homozygote)

Cell, Vol. 62, 1073–1085, September 21, 1990.

14

The *Wnt*-1 (*int*-1) Proto-Oncogene Is Required for Development of a Large Region of the Mouse Brain

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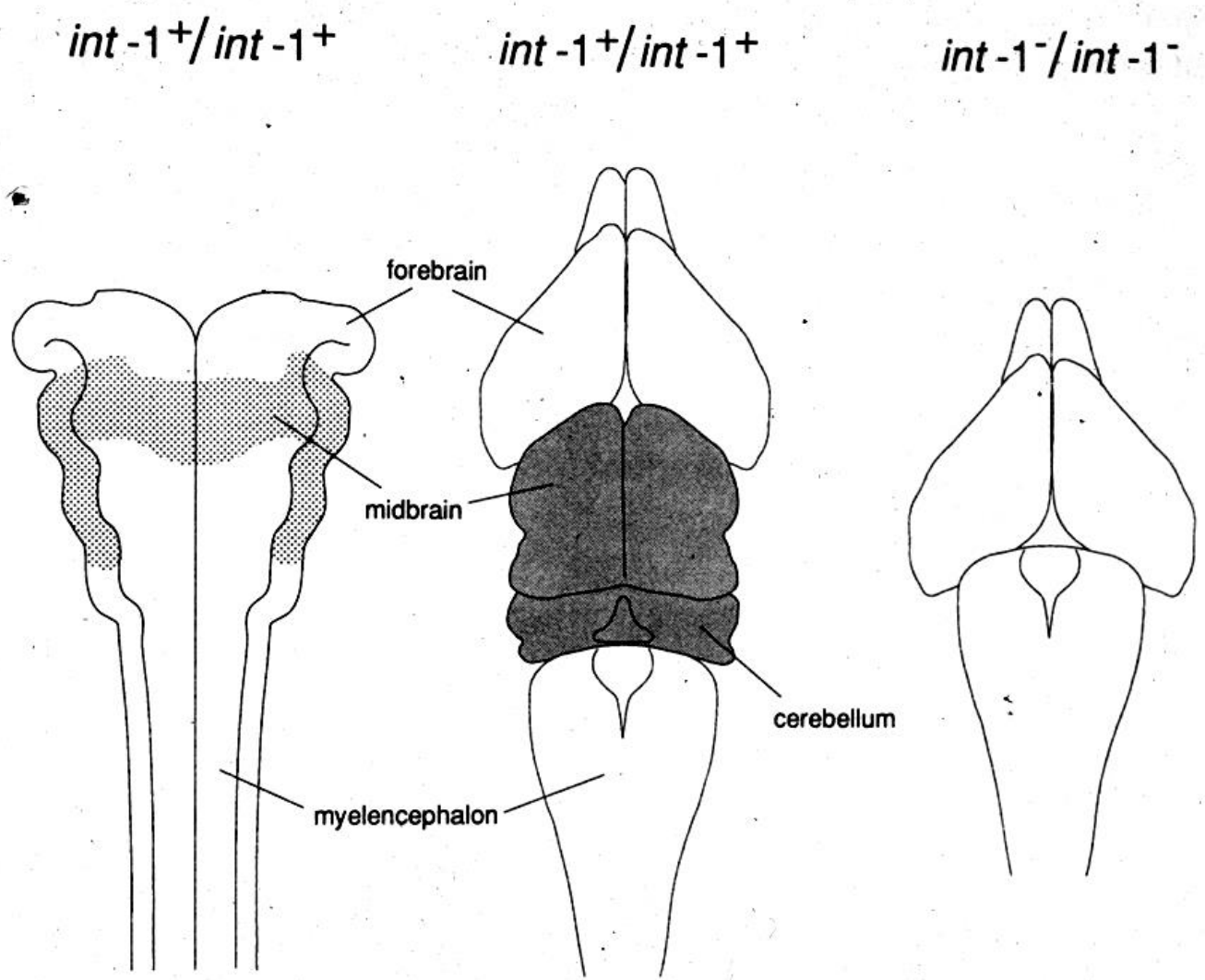
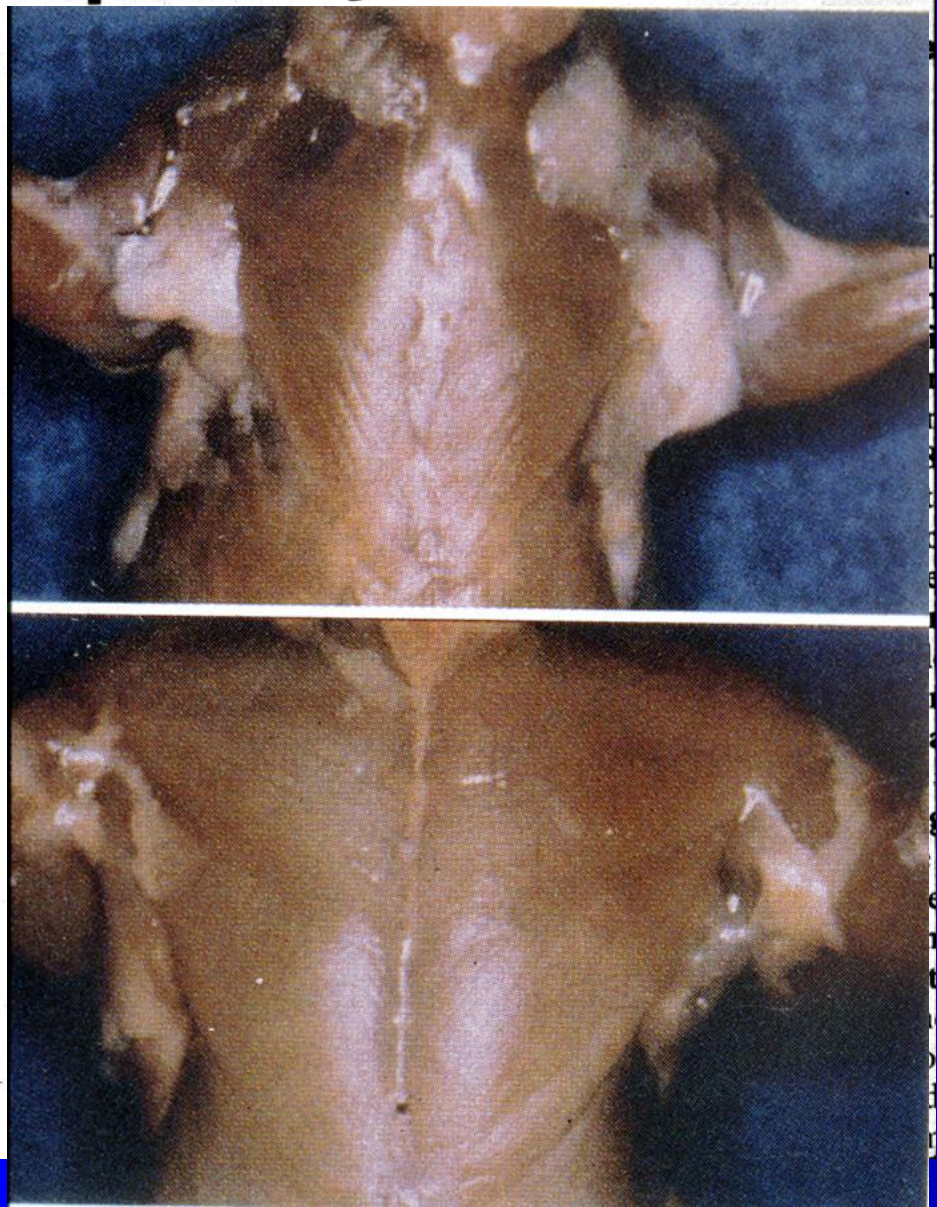
Roche Institute of Molecular Bi
Roche Research Center
Nutley, New Jersey 07110
† Institute for Molecular Genetic
Baylor College of Medicine
Houston, Texas 77030

Summary

**The *Wnt*-1 (*int*-1) proto-onco
putative signaling molecule, i
in the developing central ne
testes. To examine the role of
independent embryonic stem
tion of a *neo*^{R} gene by homolo
activated a *Wnt*-1 allele. G**

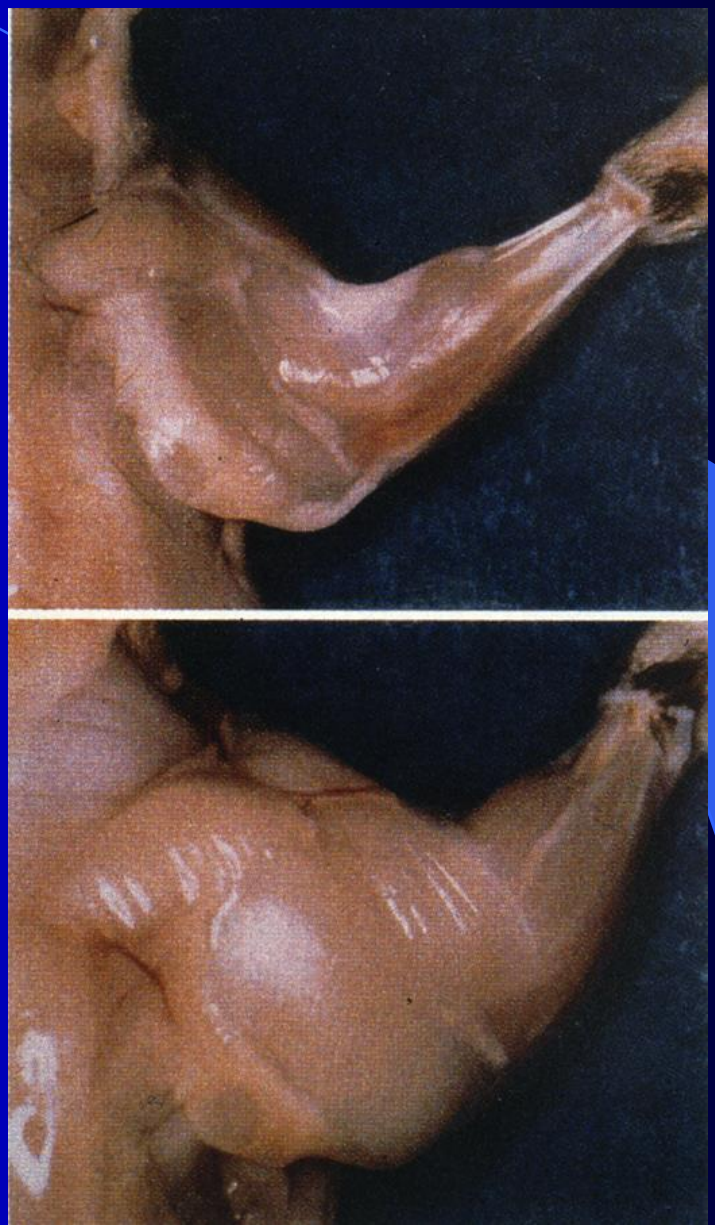


Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member



Myostatin gene Knock-out

(Nature 387:83-90, 1997)



Nature KO mutants: in the Belgian Blue

Belgian Blue Mutation at the myostatin gene

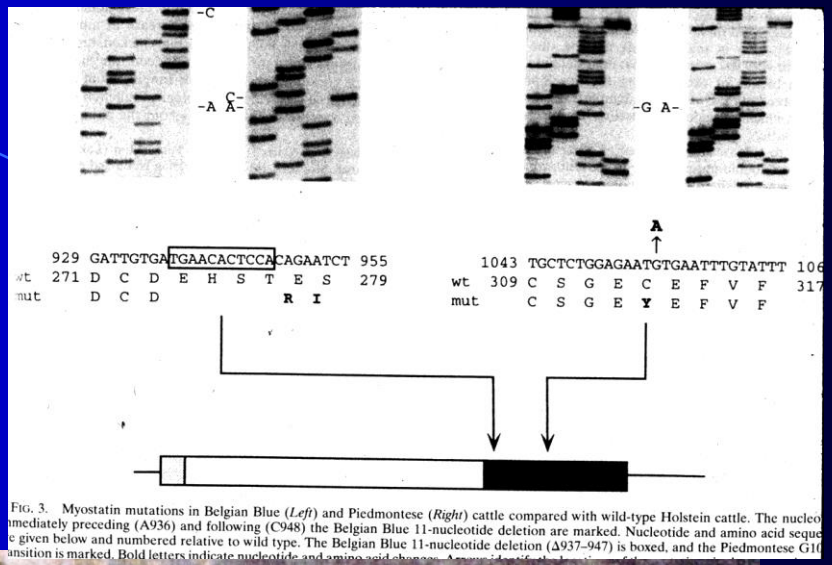
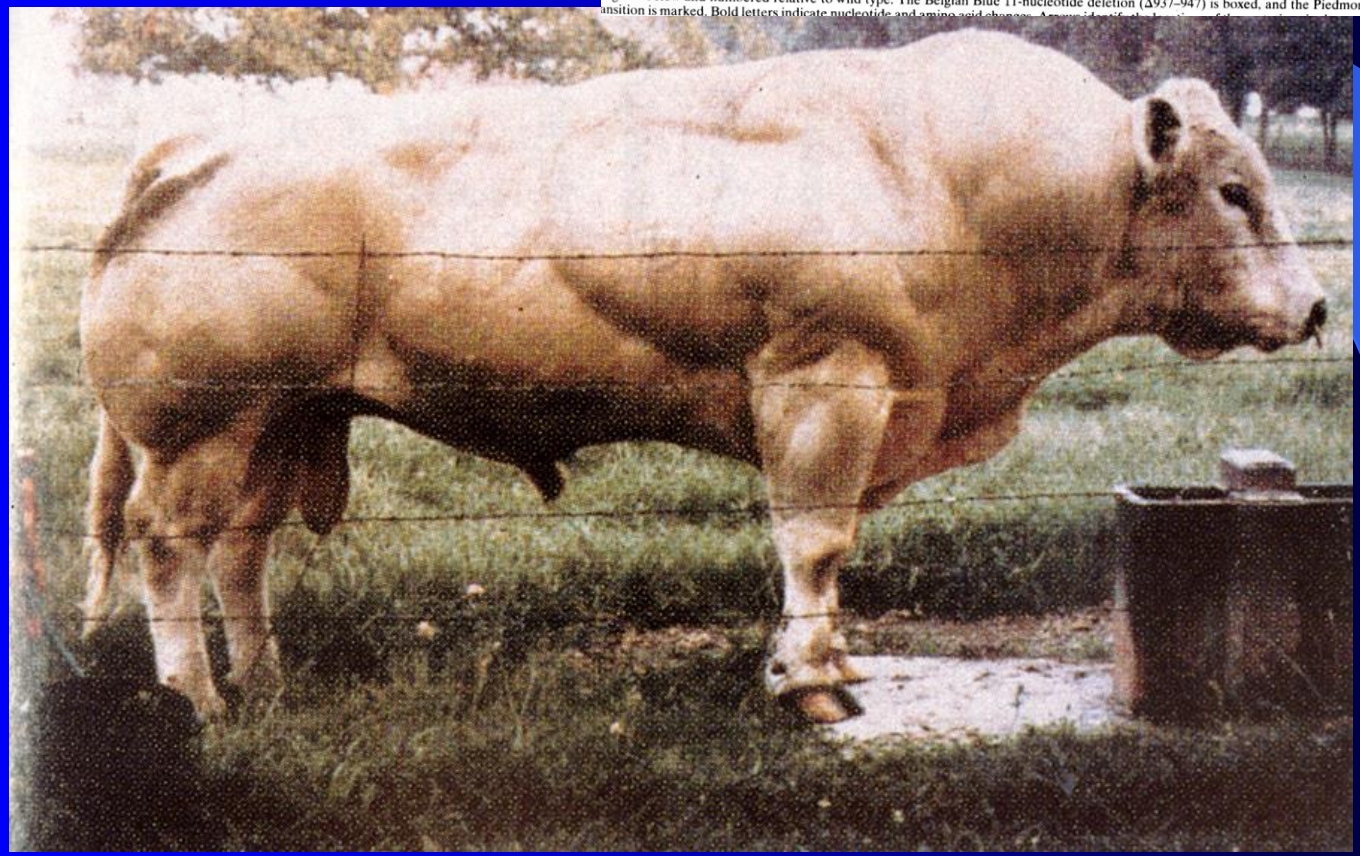
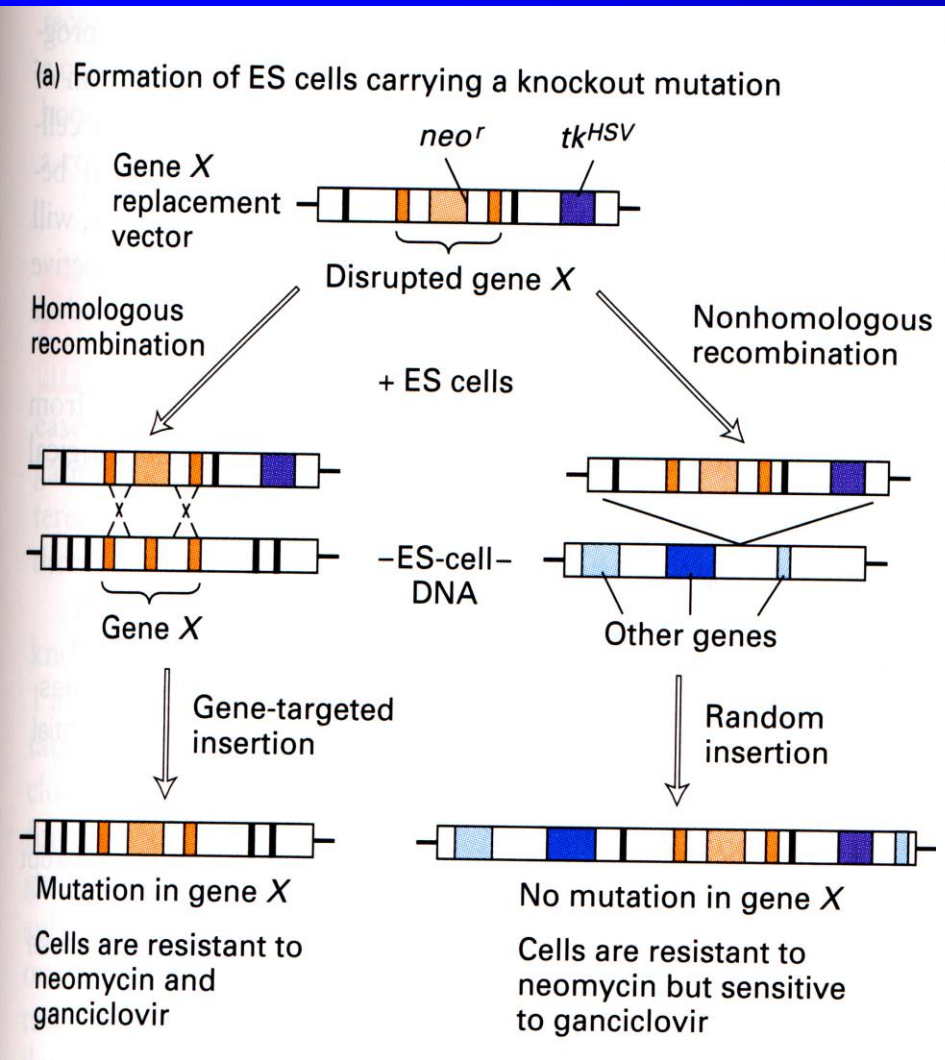
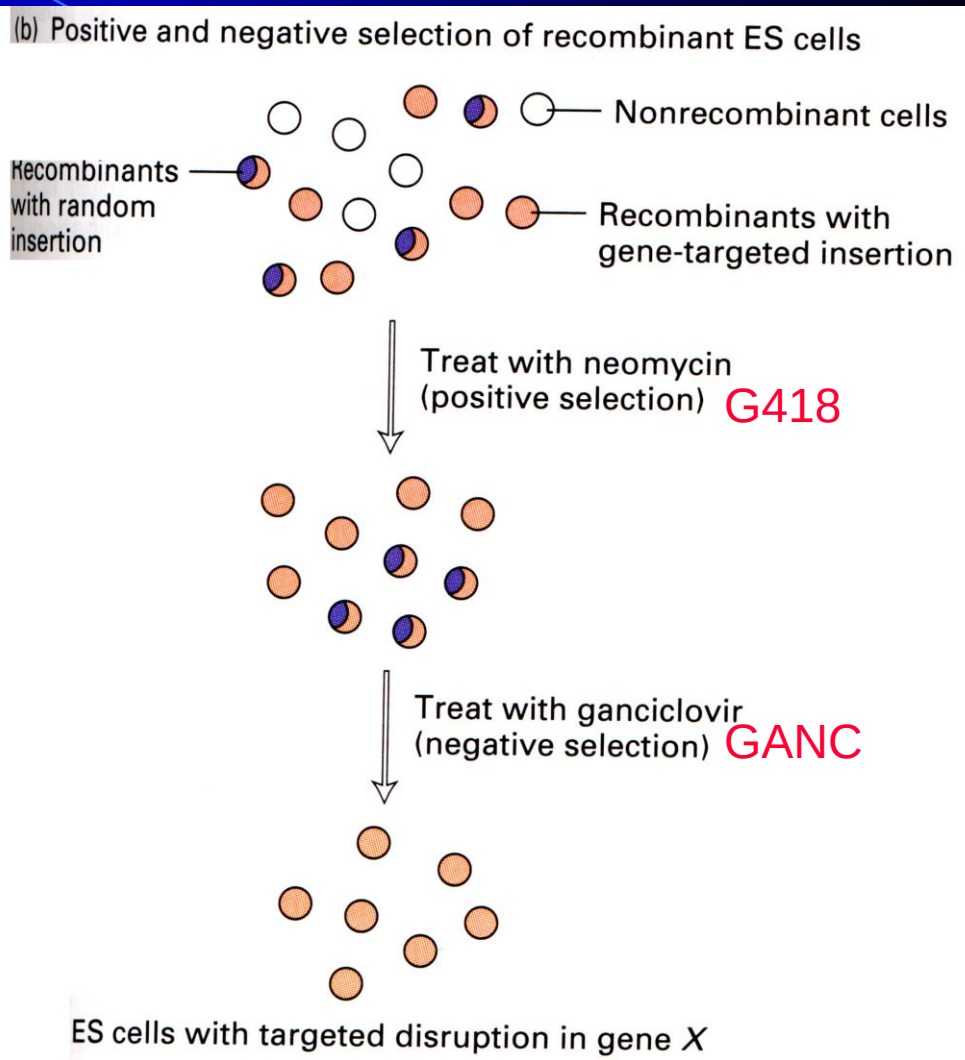


FIG. 3. Myostatin mutations in Belgian Blue (*Left*) and Piedmontese (*Right*) cattle compared with wild-type Holstein cattle. The nucleo
mediately preceding (A936) and following (C948) the Belgian Blue 11-nucleotide deletion are marked. Nucleotide and amino acid seque
e given below and numbered relative to wild type. The Belgian Blue 11-nucleotide deletion (Δ937–947) is boxed, and the Piedmontese G1
ansition is marked. Bold letters indicate nucleotide and amino acid changes.

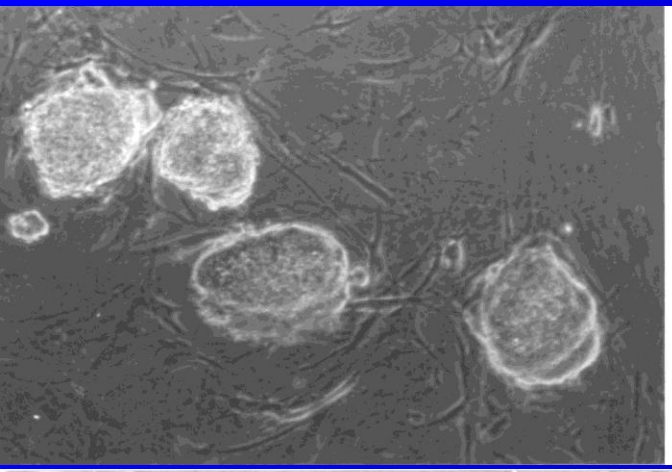


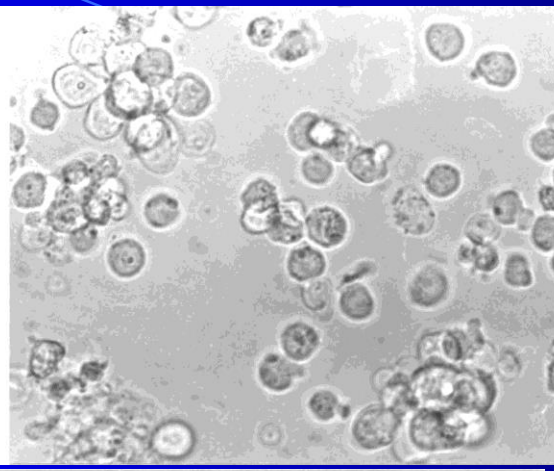
Positive and Negative Selection of ES cells

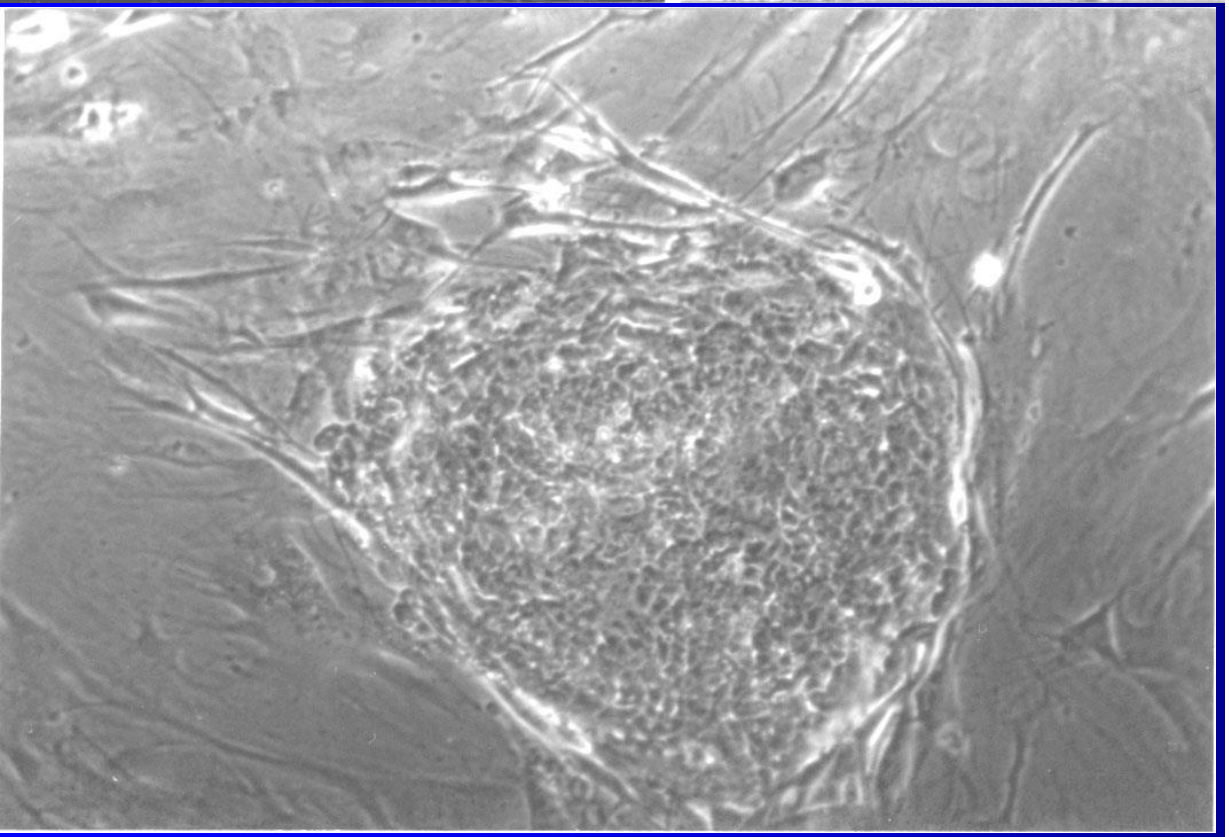


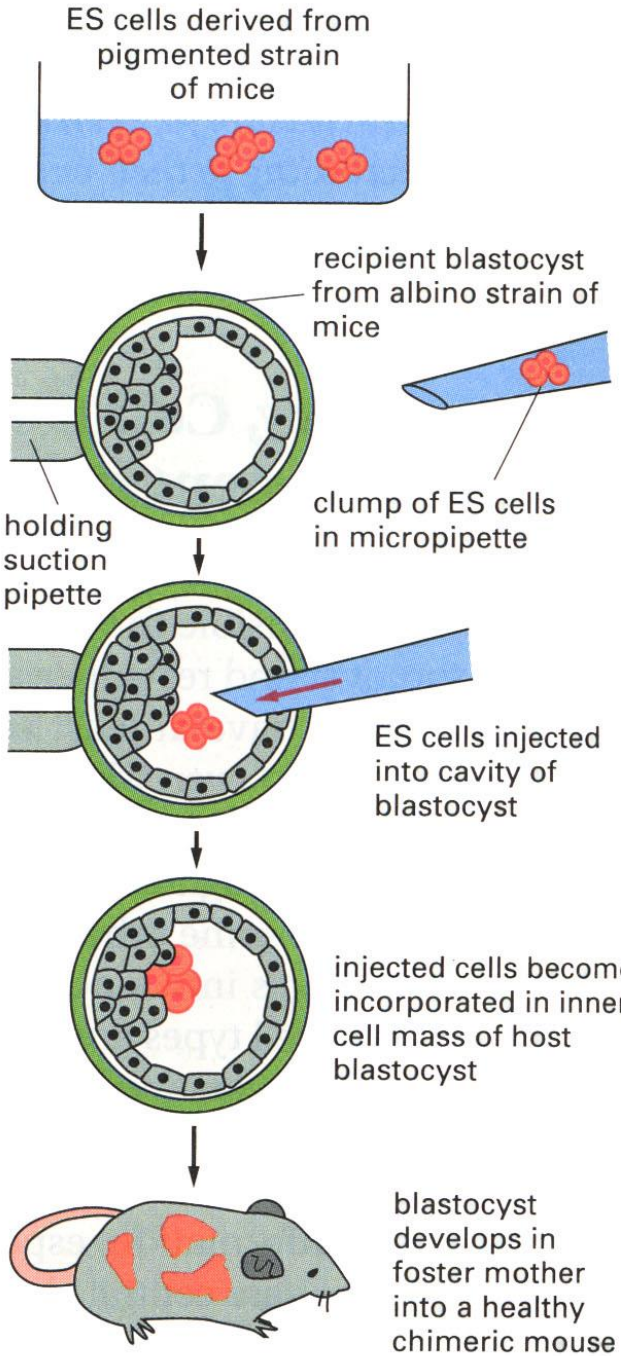


Selection of Targeted ES Clones









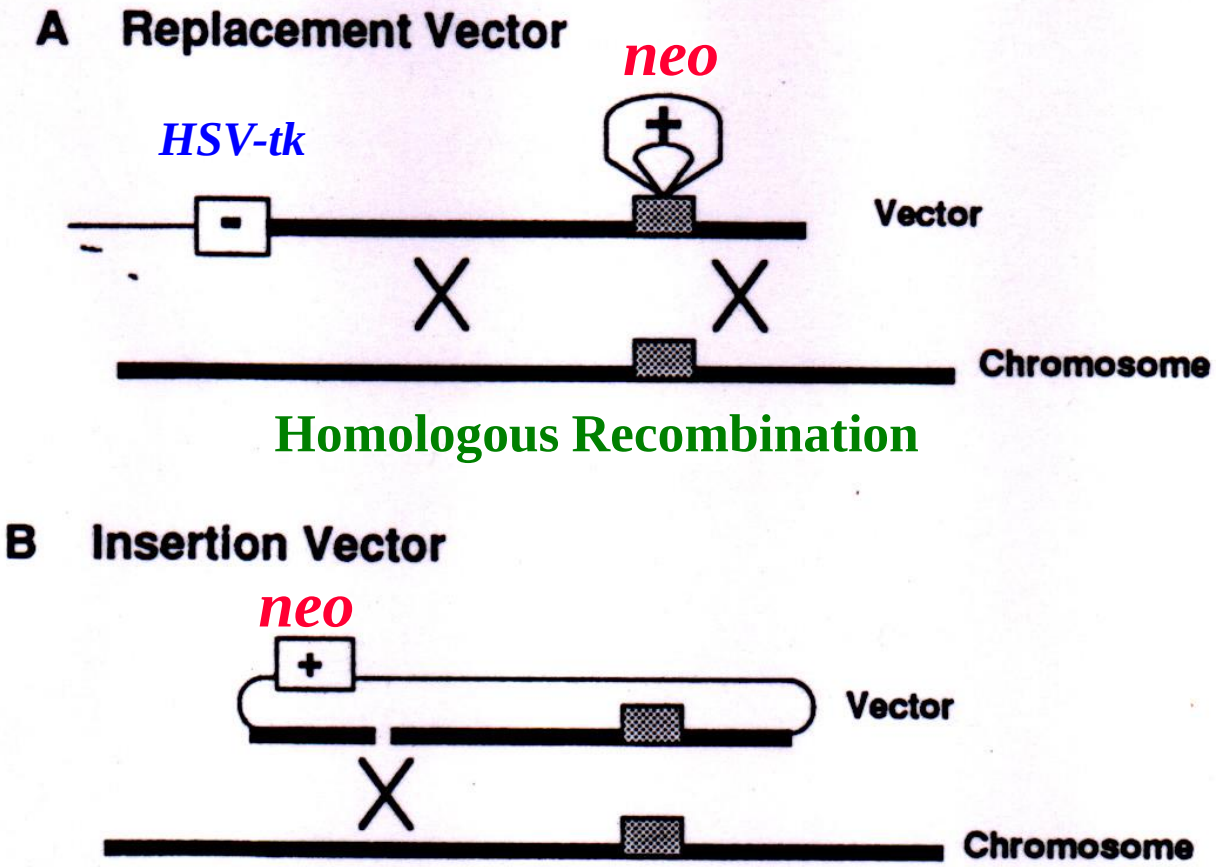
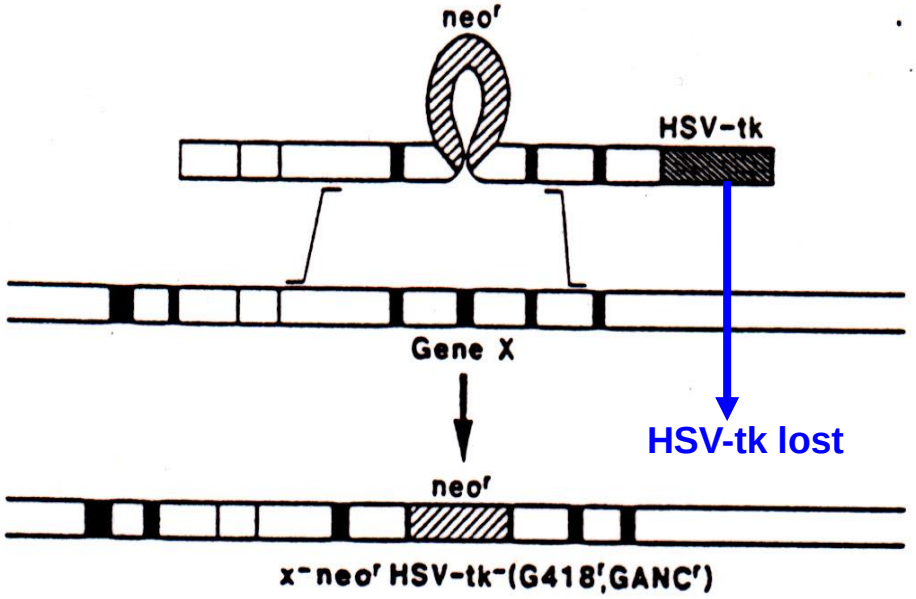
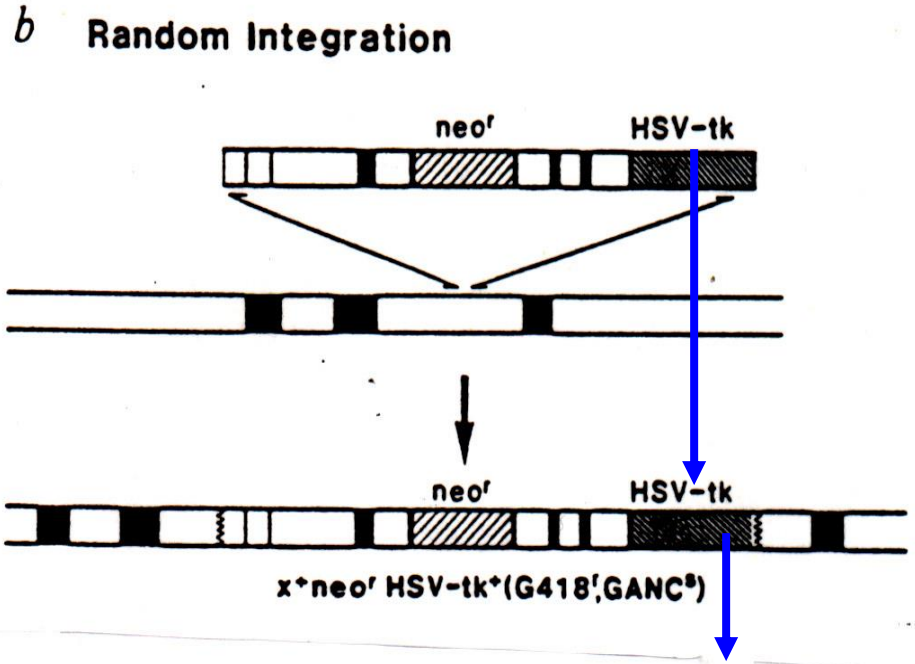


Figure 5. Diagram of a replacement and insertion vector. The thick line represents the vector homology to the target locus; the thin line represents bacterial plasmid. The stippled rectangle represents an exon. The positive selection marker is shown as a box that contains a +. (A) The replacement vector. The positive selection marker interrupts the target homology. This is required for a replacement vector. The negative selection marker is shown as a rectangle that contains a −. The replacement vector is linearized outside the target homology prior to transfection. (B) An insertion vector. A positive selectable marker may be cloned into the homologous sequences or the vector backbone. A double strand break is generated in the target homology prior to transfection.





Targeted ES cell

G418 and GNAC resistant

Fig. 4 The PNS procedure used to enrich for ES cells containing a targeted disruption of gene *X*. *a*, A gene *X*-replacement vector, that contains an insertion of the *neo*^{r} gene in an exon of gene *X* and a linked HSV-*tk* gene, is shown pairing with a chromosomal copy of gene *X*. Homologous recombination between the targeting vector and genomic *X* DNA results in the disruption of one copy of gene *X* and the loss of HSV-*tk* sequences. Such cells will be X^-, *neo*^{r} and HSV-*tk*$^-$ and will be resistant to both G418 and GANC. *b*, Because non-homologous insertion of exogenous DNA into the genome occurs through the ends of the linearized DNA[9-11], the HSV-*tk* gene remains linked to the *neo*^{r} gene. Such cells will be X^+, *neo*^{r} and HSV-*tk*$^+$ and therefore resistant to G418 but sensitive to GANC. Open boxes denote introns or flanking DNA sequences, closed boxes denote exons and cross-hatch boxes denote the *neo*^{r} or HSV-*tk* genes.

Active Thymidine kinase

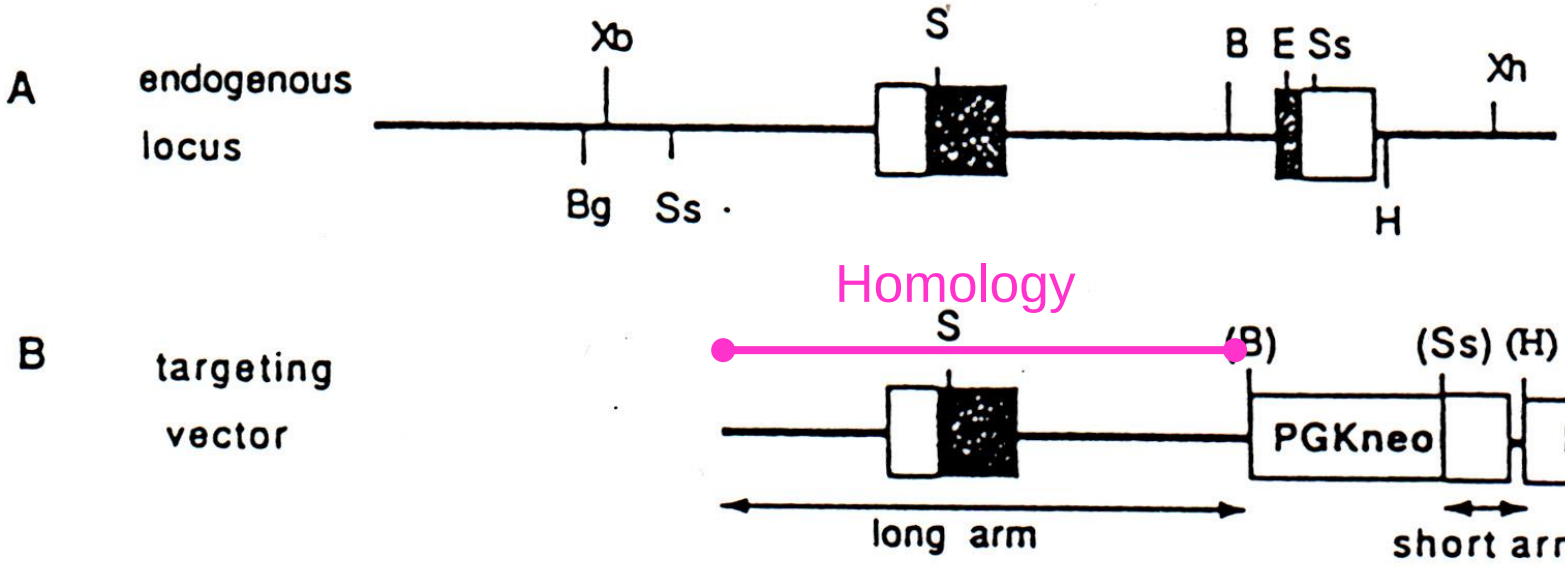
GNAC → GNAC-p

DNA polymerase

Inhibition of DNA polymerase activity

Cell can't proliferate

Cell death



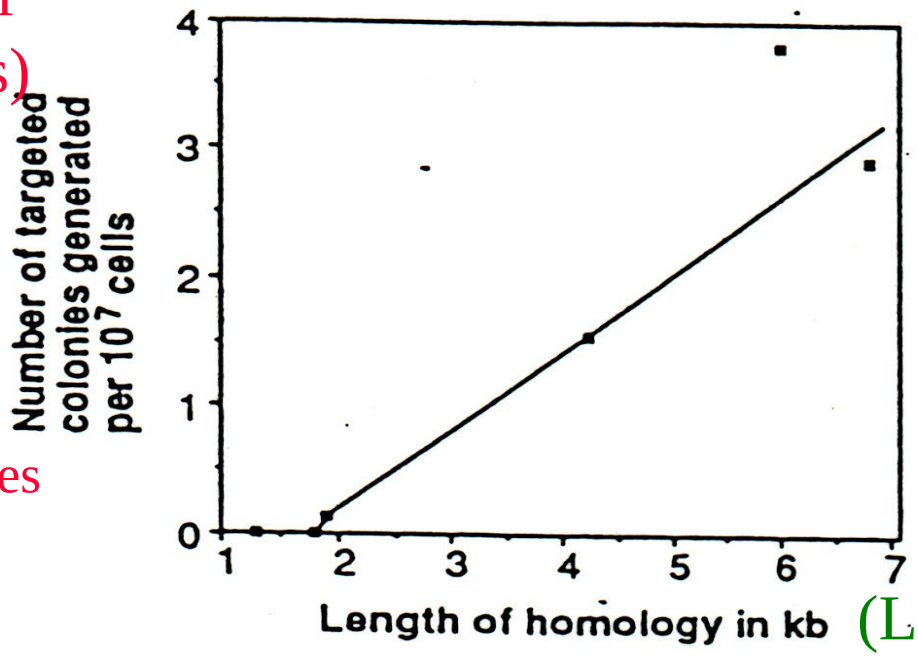
e.g. Cell number
$1x10^6$ (10 plates)

↓ **PNS**

1,000 ES colonies

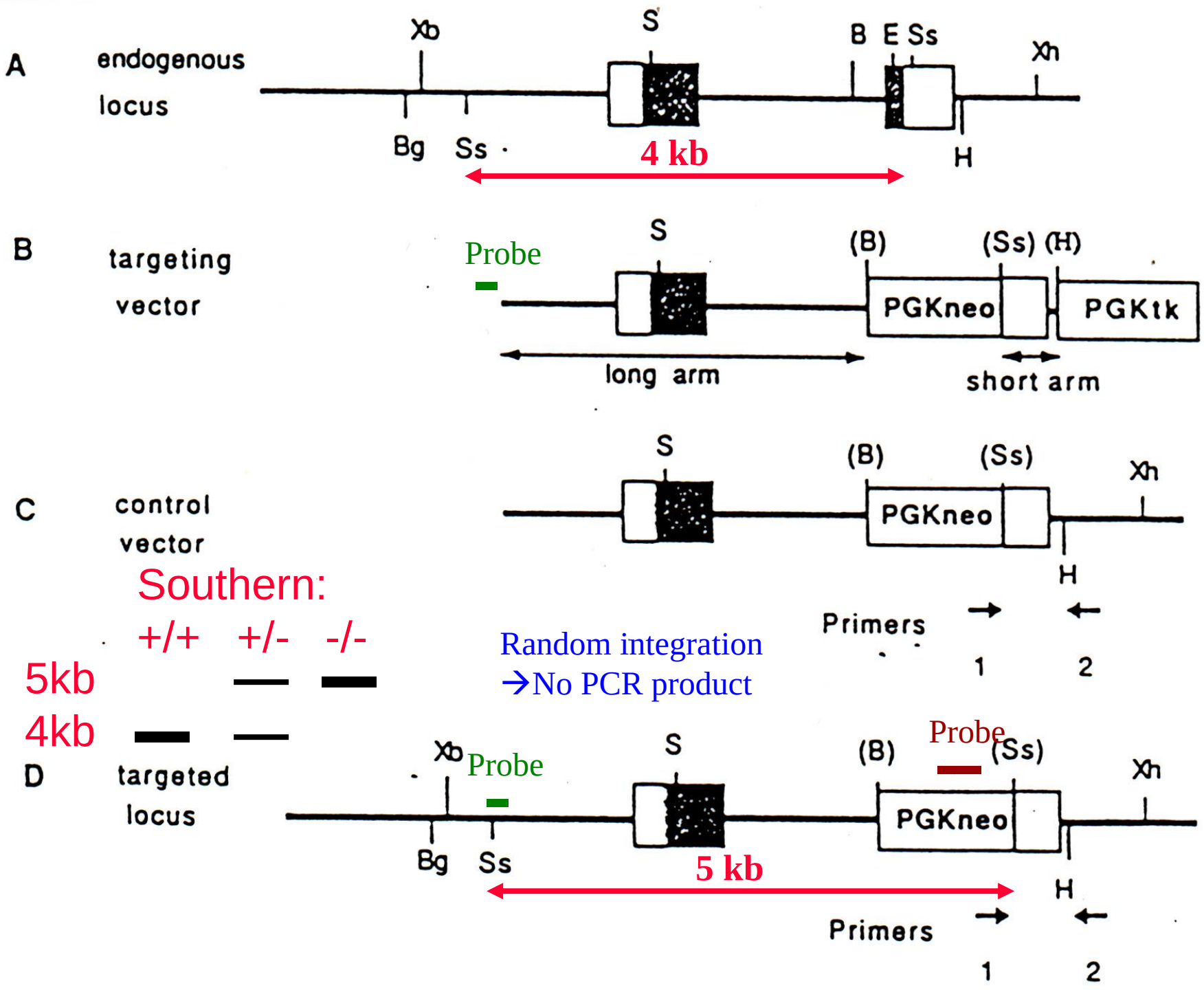
↓ **PCR or Southern**

1- 10 targeted clones



(Longer is better)

Figure 5. The relationship between the targeting frequency and the length of homology in a replacement vector (12).


A
endogenous
locus
Xb
S
B E Ss
Xh
Bg
Ss
4 kb
H
B
targeting
vector
Probe
S
(B)
(Ss) (H)
PGKneo
PGKtk
long arm
short arm
C
control
vector
S
(B)
(Ss)
Xh
PGKneo
H
Primers
1
2
Southern:
+/+
+/-
-/-
5kb
4kb
Random integration
→No PCR product
D
targeted
locus
Xb
Probe
S
Probe
(B)
(Ss)
Xh
PGKneo
Bg
Ss
5 kb
H
Primers
1
2

Positive selectable *neo* cassette

Promoter Trap (YFG highly expressed in ES cells)

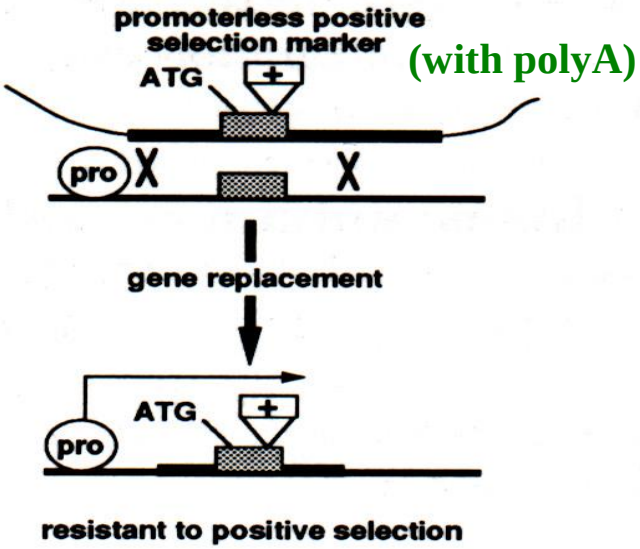
(Replace the 1st Exon)

(Insertion in the intron)

PolyA Trap

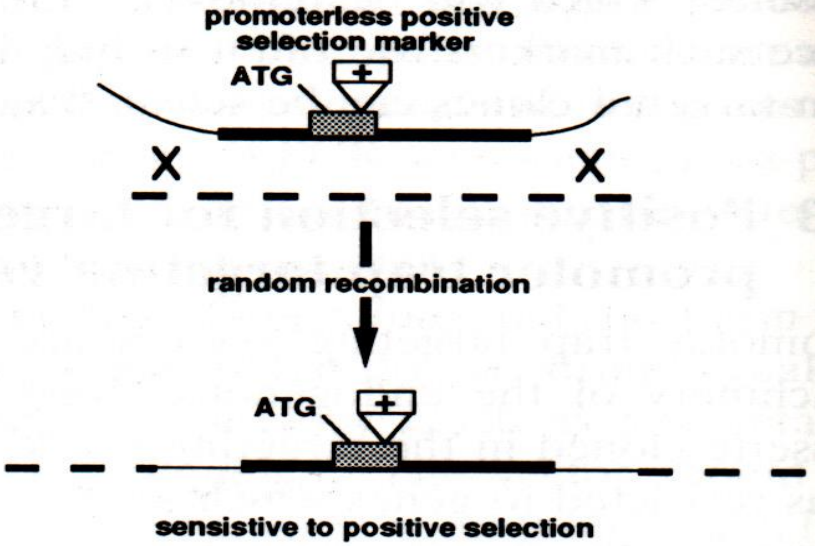
(Insertion in the intron)

A Promoter Trap Positive Selection



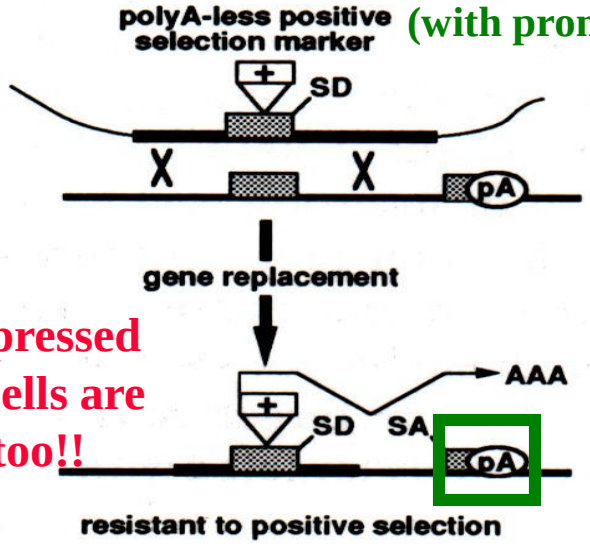
(with polyA)

Neo expressed
→ ES cells are happy!



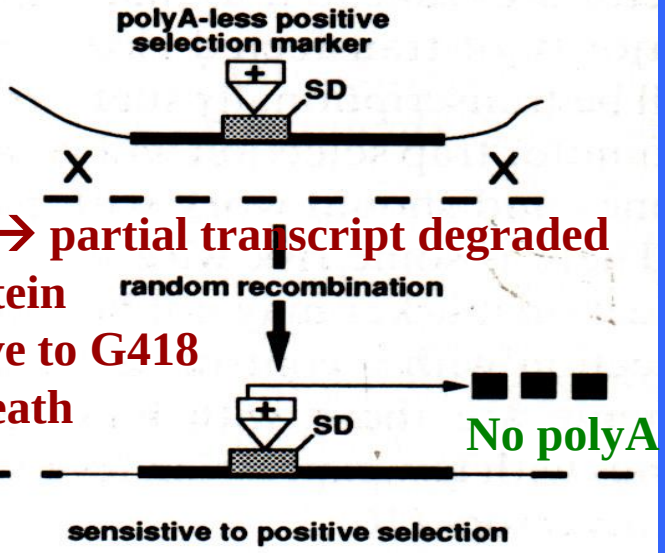
No promoter →No *Neo*
→ ES cells sensitive to G418
→ Cell Death

B Polyadenylation Trap Positive Selection



(with promoter)

Neo expressed
→ ES cells are happy too!!



No polyA→ partial transcript degraded
→No Protein
→Sensitive to G418
→ Cell Death

No polyA

Useful Targeting Vectors

pMC1neo and pMC1neo Poly A Vectors

- Facilitates gene targeting and lineage marking in mammalian stem cells
- G418-resistance cartridge is engineered for high-level expression of the Tn5 neomycin-resistance coding sequence in single-copy integrants of eukaryotic cells

APPLICATIONS

- pMC1neo vector used when cloned fragment provides poly(A) adenylation signal for neomycin gene
- pMC1neo Poly A vector used when polyadenylation signal is not present

CLONING SITES Unique *Sal* I and *Xho* I sites for cloning sequences of interest

SELECTION *E. coli*: ampicillin resistant, kanamycin sensitive; Eukaryotic cells: G418 resistance

Homologous Recombination

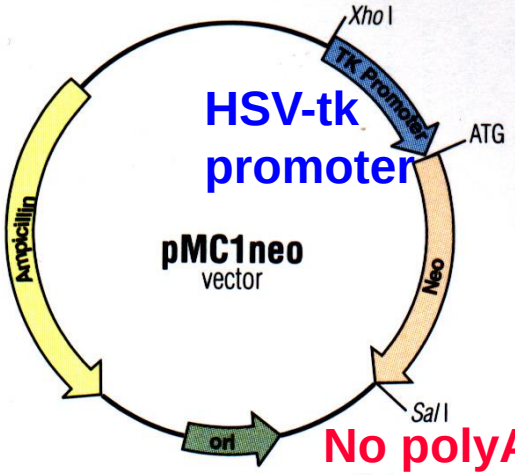
The G418-resistance cartridge is introduced by homologous recombination between the cloned sequence of interest and its genomic copy following transfection of cells. Cells that have undergone homologous recombination are G418 resistant. In a model system, 1/1000 cells that were G418-resistant had also undergone a gene targeting event.[1]

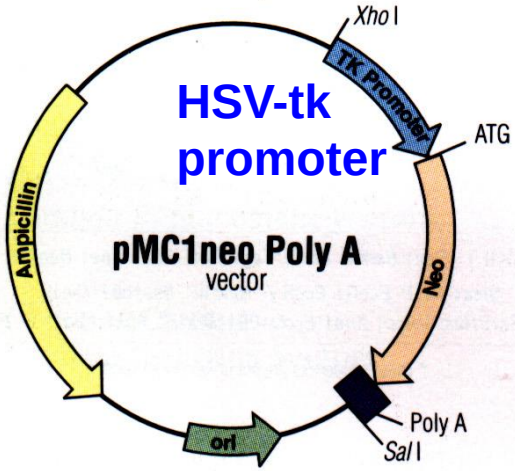
REFERENCE

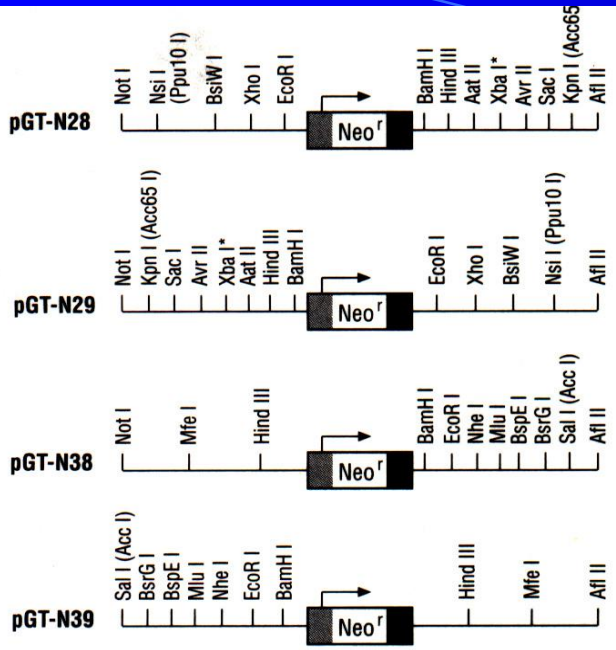
1. Thomas, K.R. and Capecchi, M.R. (1987) *Cell* 51: 503-512.

pMC1neo and pMC1neo Poly A Vector Kit

	CONTENTS	
	25 µg pMC1neo vector 25 µg pMC1neo Poly A vector Host strain: AG1	#213201







pGT Vector Polylinkers: Only unique sites are indicated. Note: Xba I sites can only be considered unique when using a dam+ strain.

PGK promoter *PGK polyadenylation site*

Description: New England Biolabs has developed a series of backbone plasmids that make the design and construction of gene targeting vectors easier and faster. These plasmids, derived from LITMUS vectors (1), combine a large number of unique restriction sites in two multiple cloning sites (MCS) with the neomycin resistance cassette for positive selection. The Neo gene is driven by the phosphoglycerate kinase (PGK) gene promoter. *Not* I and/or *Sal* I sites are included to facilitate plasmid linearization prior to transfection into ES cells. In addition, as all plasmids have an M13 origin of replication, single-stranded DNA can be easily prepared for site-directed mutagenesis and DNA sequencing (2). Using these vectors it is possible to introduce a point mutation within a coding sequence,

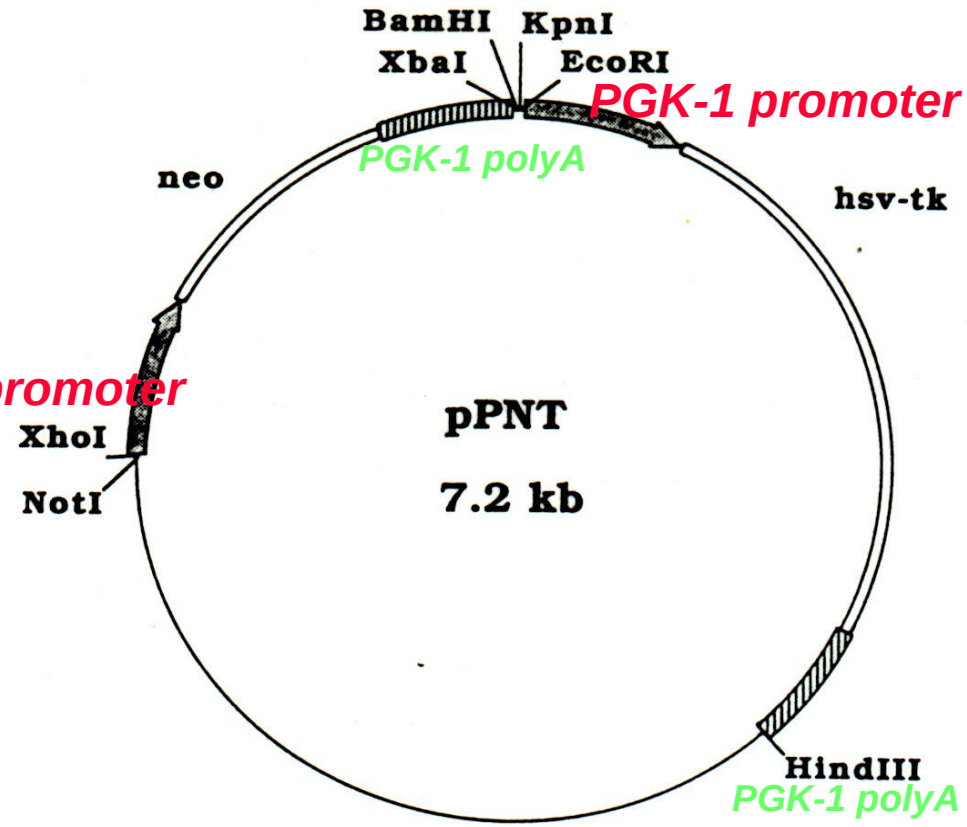


Figure 1. Structure of pPNT

The shaded arrows represent the PGK-1 promoter, the hatched boxes represent the PGK-1 poly(A) addition sequences, the open boxes are the *neo* and HSV-*tk* genes as labeled, and the line represents the plasmid backbone. Unique restriction sites are indicated. The precise nature of each of the fragments is described in Experimental Procedures.

Reference:

1. Tybulewicz et al. (1991) *Cell* 65: 1153-1163.

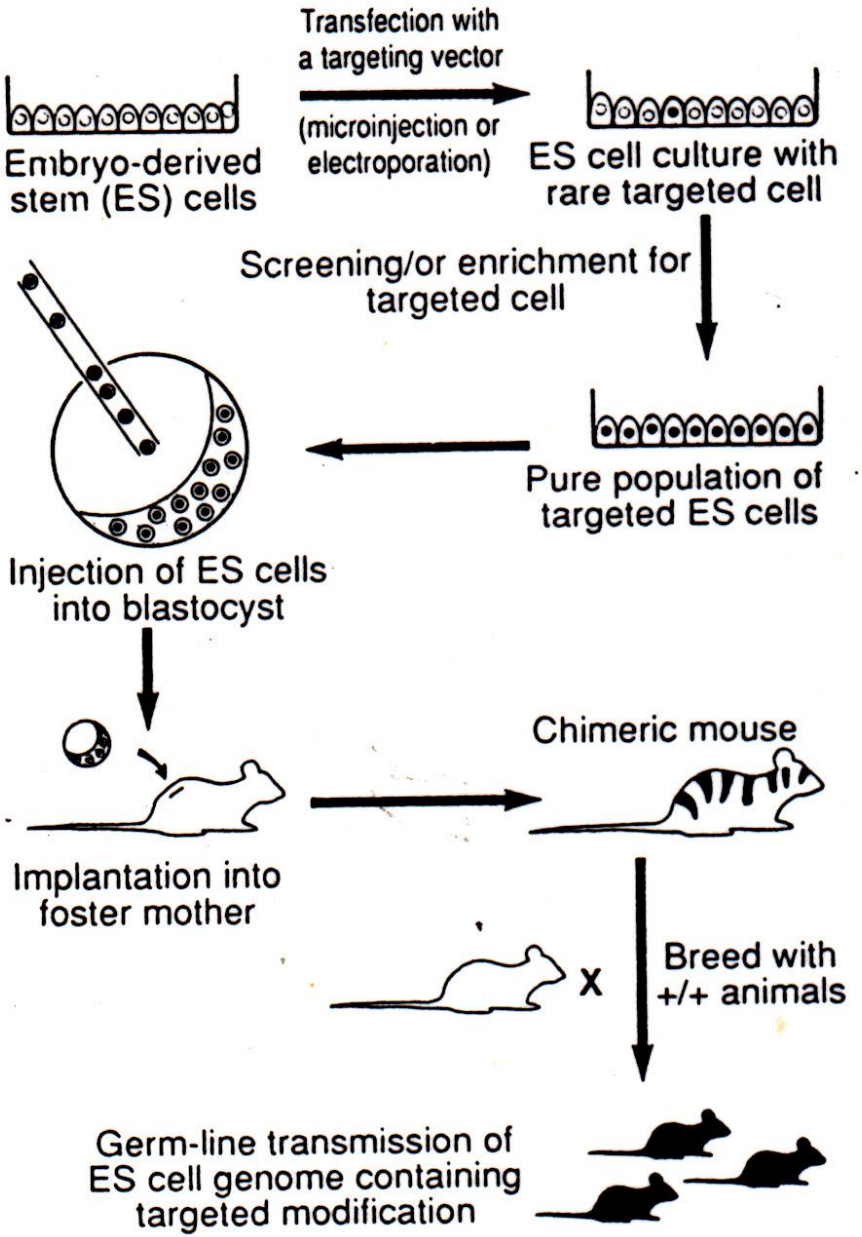
Table 2. Transfection efficiency of *neo* expression cassettes in ES cells (6).

	G418 ^{R}colonies 10^{7} cells	Relative efficiency
pMC1neopA	13	1
RV4.0	1632	125
MC1neobpA	464	36
TKneobpA	124	10
Pol2sneobpA	324	25
Pol2neobpA	788	61
PGKneobpA	940	72

PGK promoter is better than **HSV-tk promoter**!!

From ES Cells to Germ Line Chimera

Fig. 9. Generation of mouse germ line chimeras from embryo-derived stem (ES) cells containing a targeted gene disruption.



Aa x Aa:

¼ AA (wild type)

½ Aa (heterozygote)

¼ aa (homozygote)

Conditional Gene Targeting (KO)

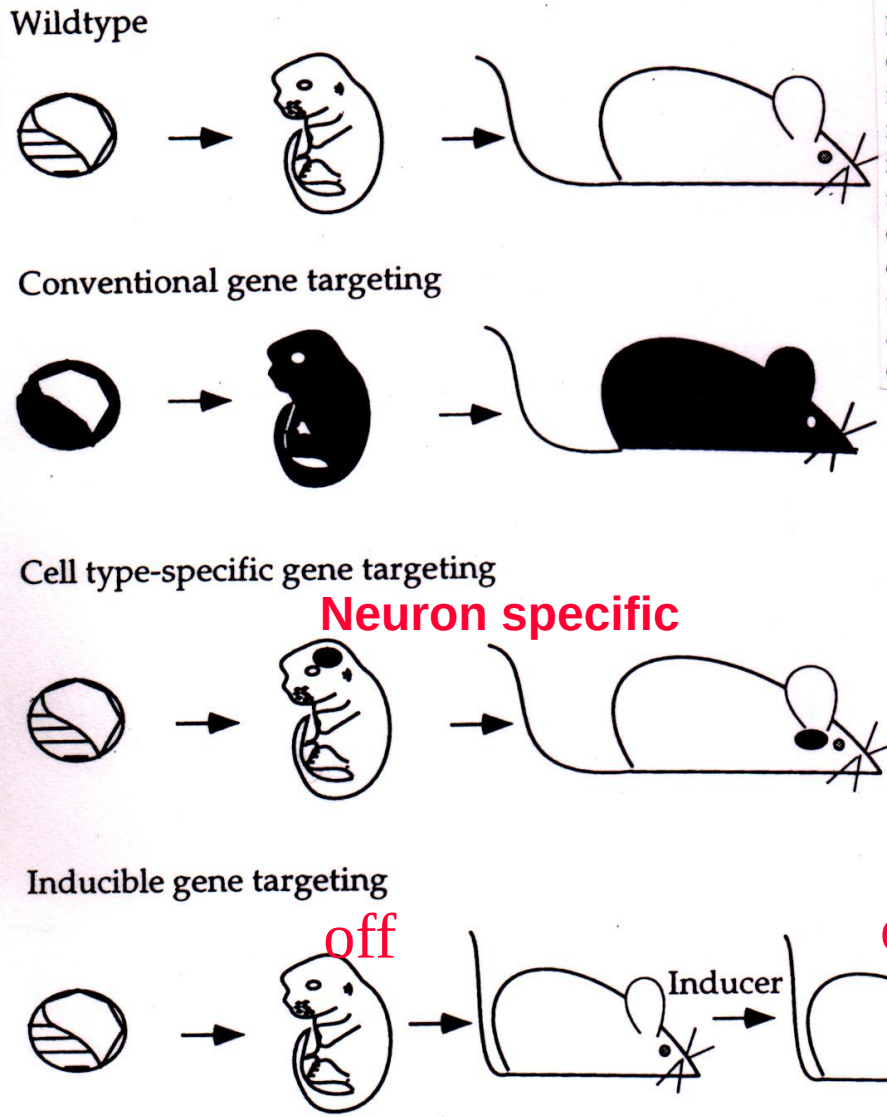
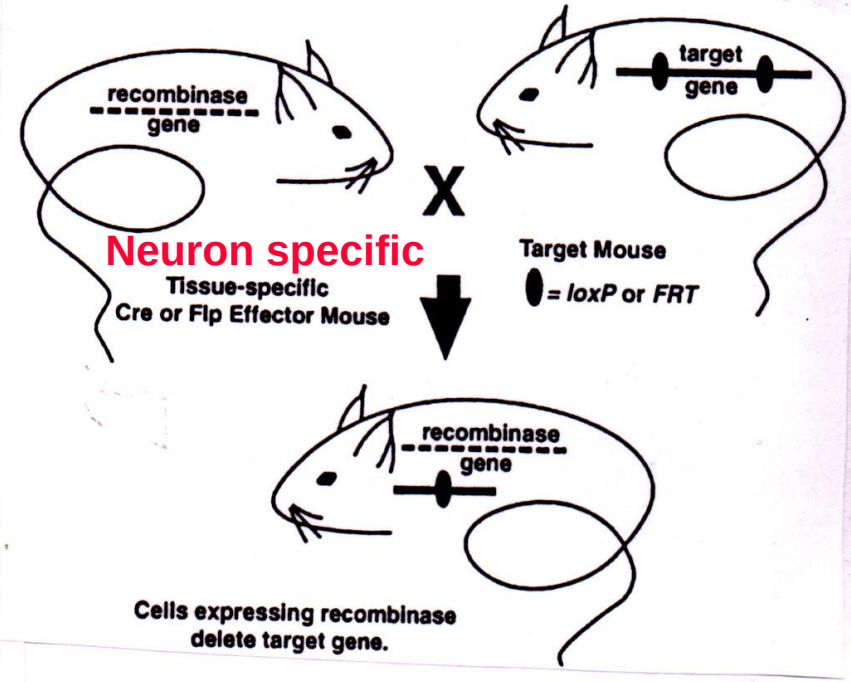
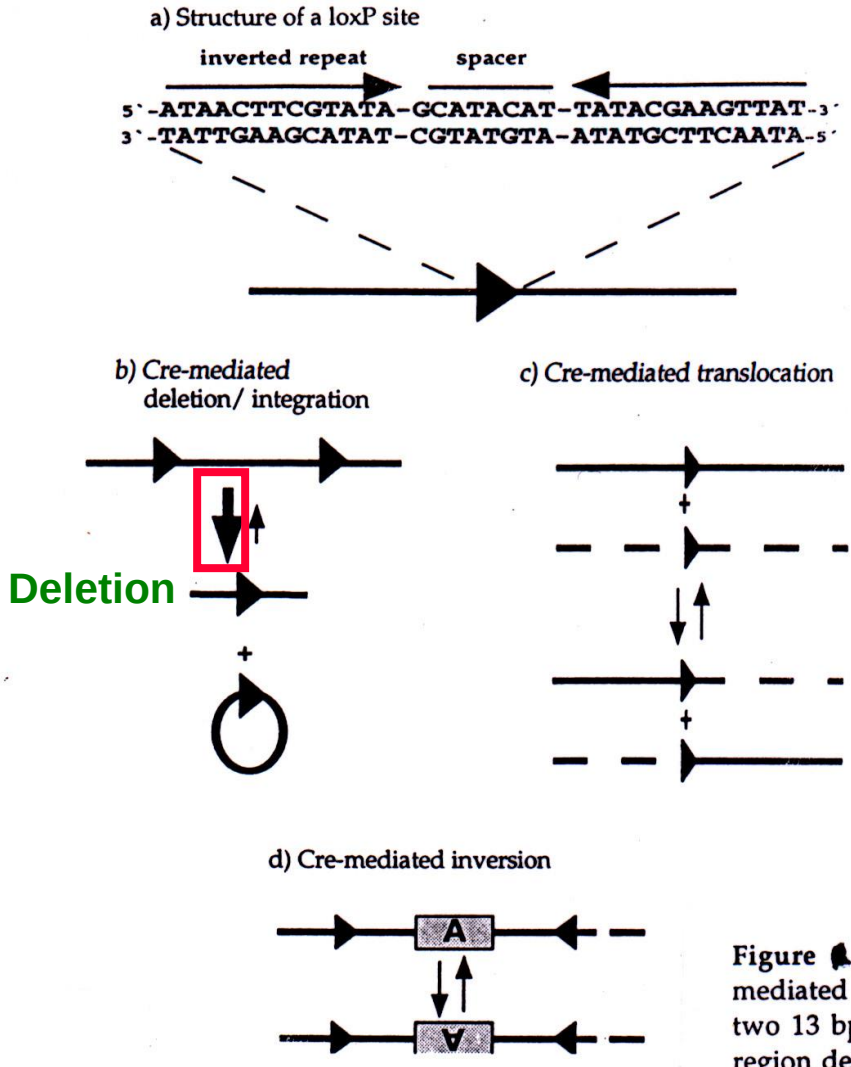


Figure 1. Comparison of conventional and conditional gene targeting strategies at different ontogenetic stages (blastocyst; embryo; adult). Filled, black regions indicate tissues/organs expressing the modification of a target gene whereas white regions symbolize its wildtype function. **(a)** Wildtype mouse. **(b)** Conventional mutant expressing a mutation in all tissues throughout life. **(c)** Constitutive, cell type-specific gene targeting. In this example a promoter region is used for Cre expression which becomes active in a certain cell type and region of the brain (black oval) during embryonic development. **(d)** Inducible, cell type-specific gene targeting. The activity of Cre recombinase is switched on in certain cell types upon administration of an inducer; in the example shown Cre should be inducible in a certain cell type and region of the brain.





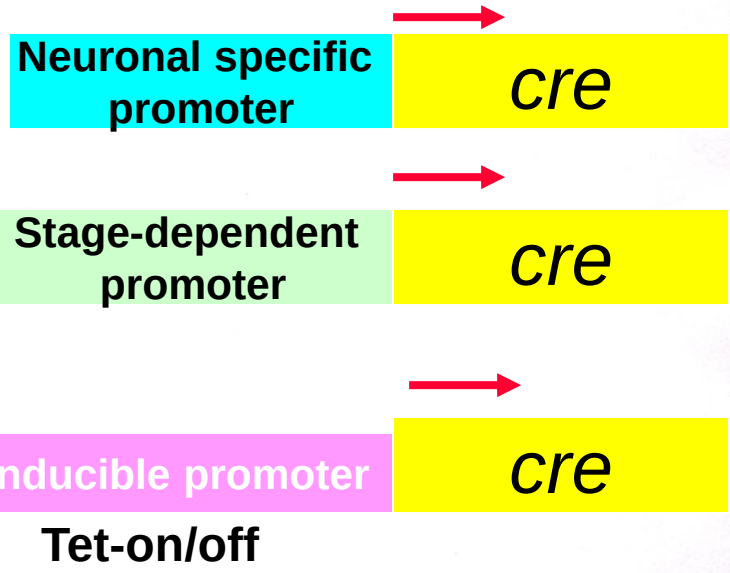
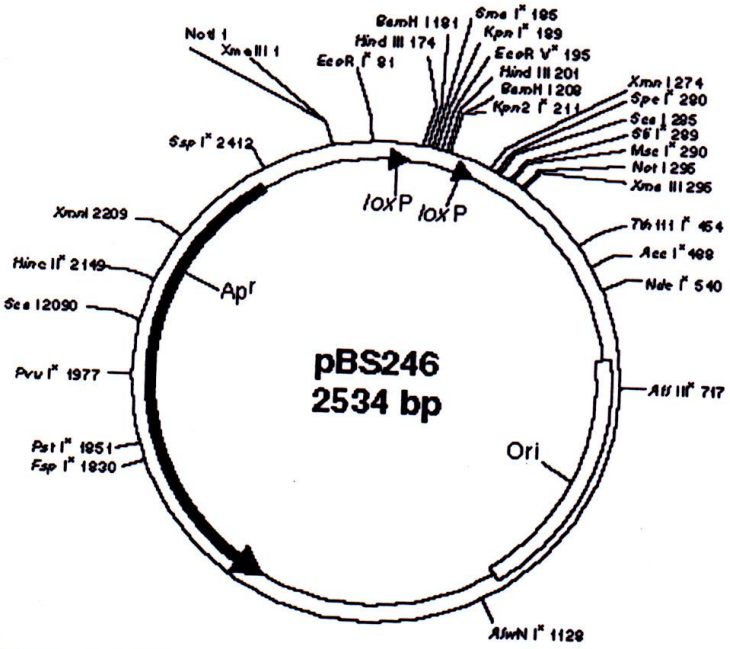
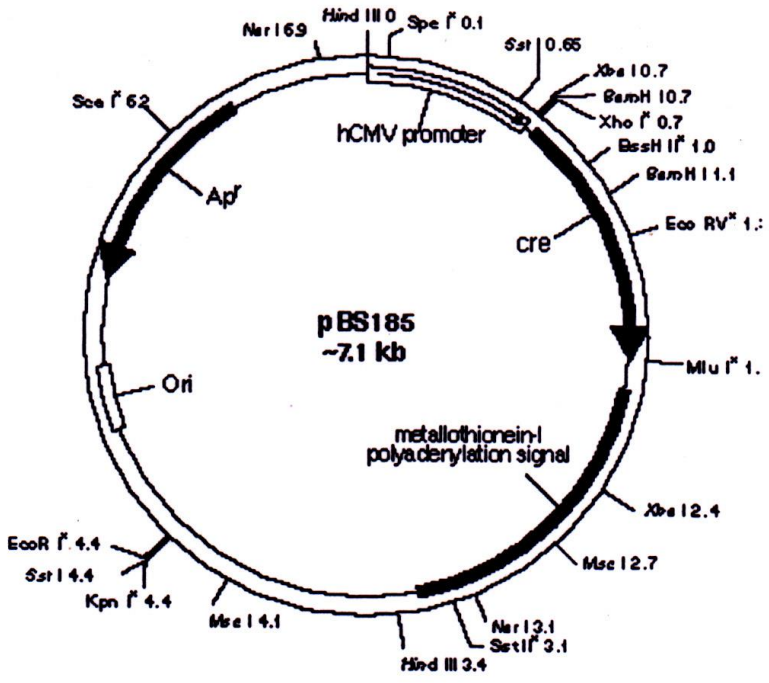
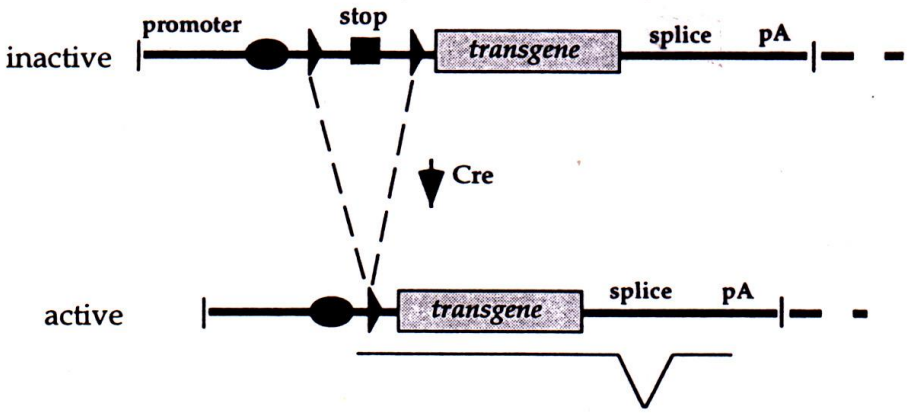


Figure LoxP site structure and products of intra- and intermolecular Cre-mediated recombination between two loxP sites. **(a)** A 34 bp loxP site consisting of two 13 bp inverted repeats and an asymmetric 8 bp spacer region. The spacer region defines the orientation of the loxP site represented as a filled triangle. **(b)** Cre-mediated recombination between two directly repeated loxP sites on a linear DNA molecule leads to the excision and circularization of the loxP flanked DNA segment. One loxP site remains on each of the reaction products. In the reverse reaction a loxP containing circle is integrated into a linear DNA molecule. **(c)** Cre-mediated intermolecular recombination of two linear DNA molecules, each containing one loxP site. The regions flanking the loxP sites are reciprocally exchanged between the reaction partners as a result of recombination. **(d)** Cre-mediated inversion of a DNA segment flanked by two loxP sites in opposite orientation.

Cre- loxP system







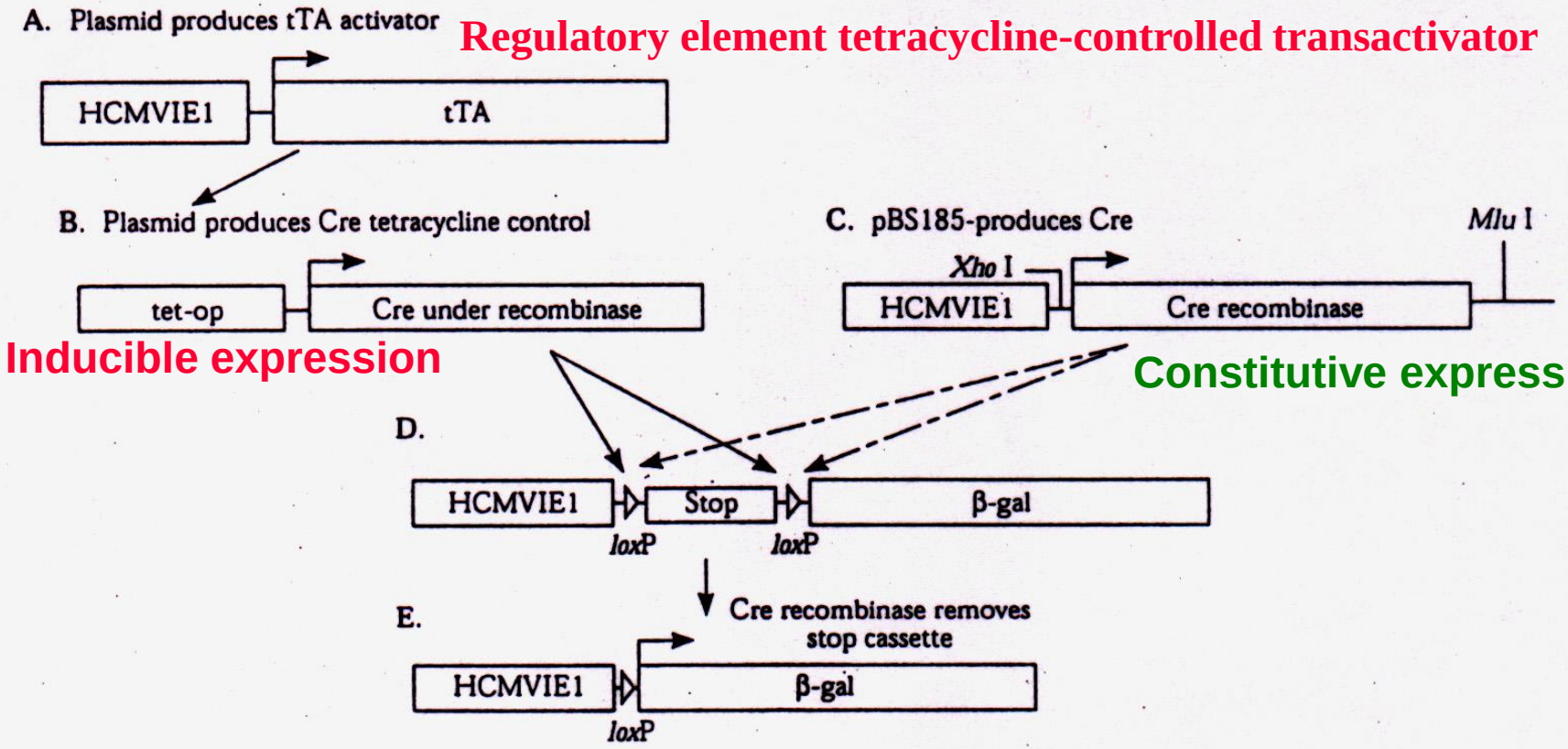
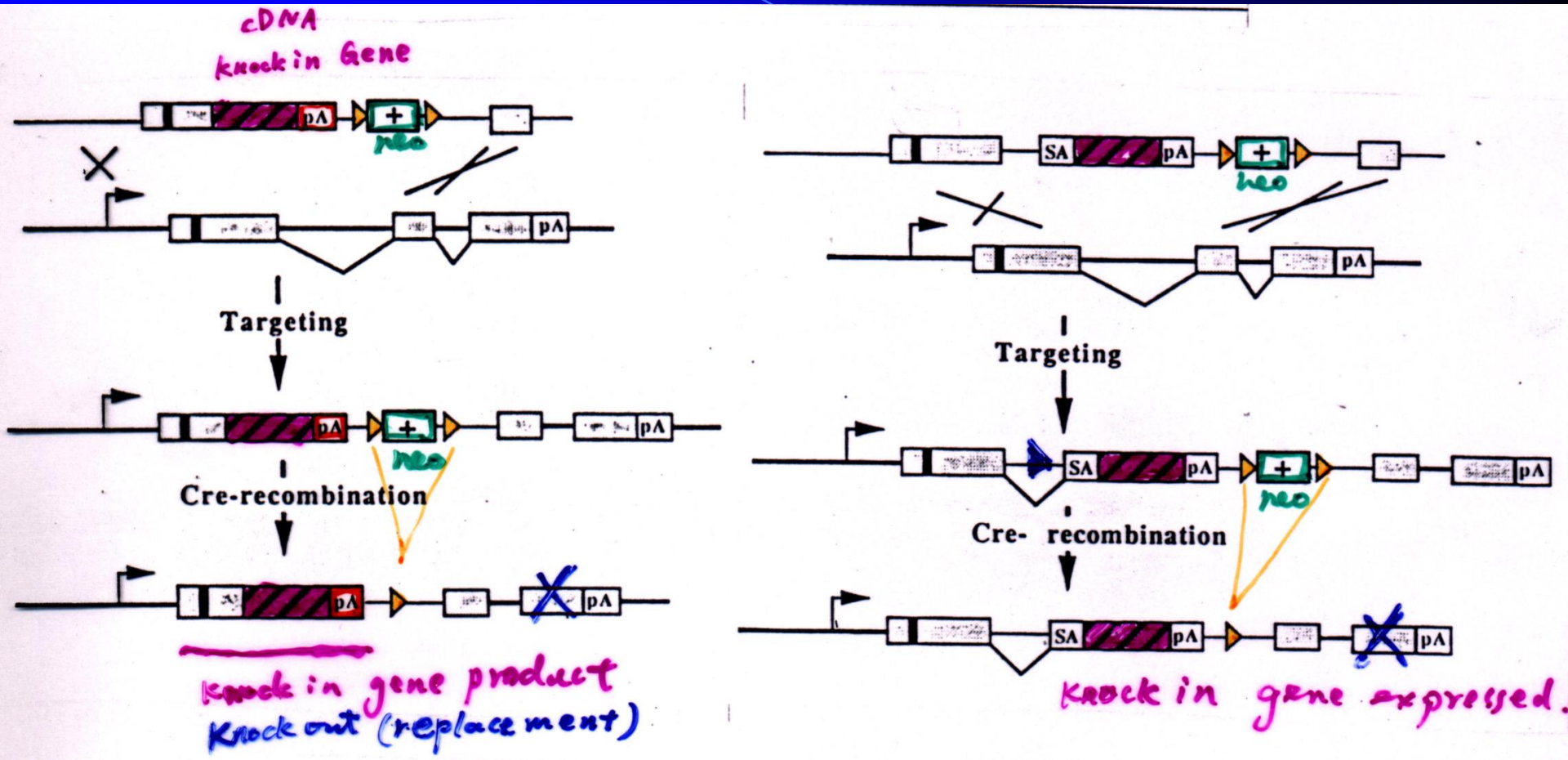


FIGURE 1. Schematic of vectors and experiment. tTA expressed from the HCMVIE1-tTA expression vector (A) activates the tet-op Cre (B). The Cre recombinase excises the stop sequence between the *lox*P sites in the HCMVIE1-Stop-β-gal (D). An expression vector able to transcribe β-gal is generated (E). Similarly, Cre expressed from pBS185 (C) can generate an active HCMVIE1-β-gal (E).

Table 6. Generation of recombinase mice

Advantages	Disadvantages
Knock-in	
• Proper control of recombinase expression.	• More laborious to generate mice.
• Minimizes mosaicism in recombinase expression.	• Could necessitate maintaining mice as heterozygotes.
• Does not require prior isolation of defined promoter/enhancer sequences.	• Many genes are expressed in numerous tissues.
Transgenics	
• Straightforward to generate mice by zygote injection.	• Often mosaic expression of recombinase.
• Frequently can maintain as homozygotes	• Requires prior isolation of promoter/enhancer sequences.
	• Requires screening many lines to obtain correct expression pattern and level.

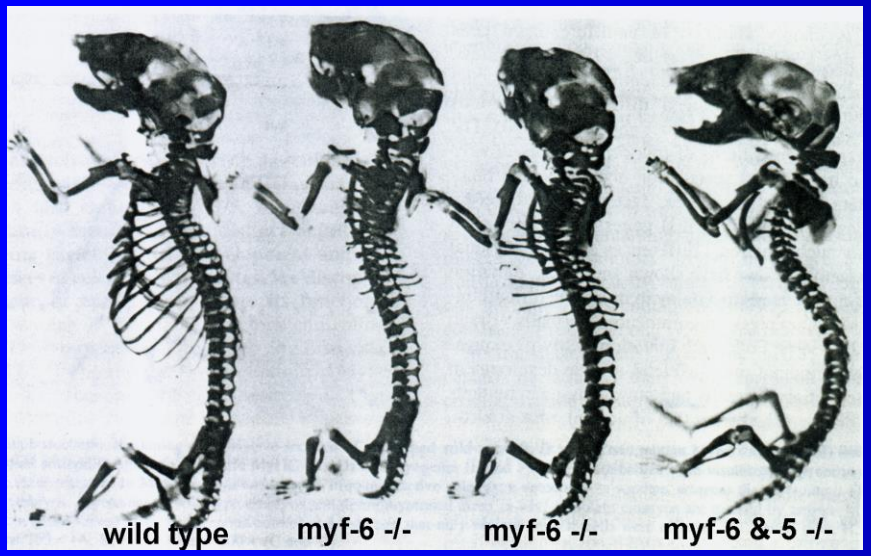
Knock in approach

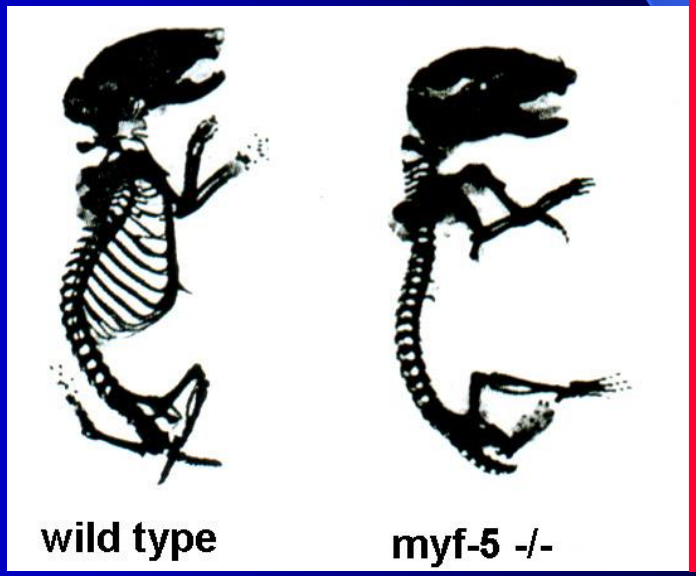


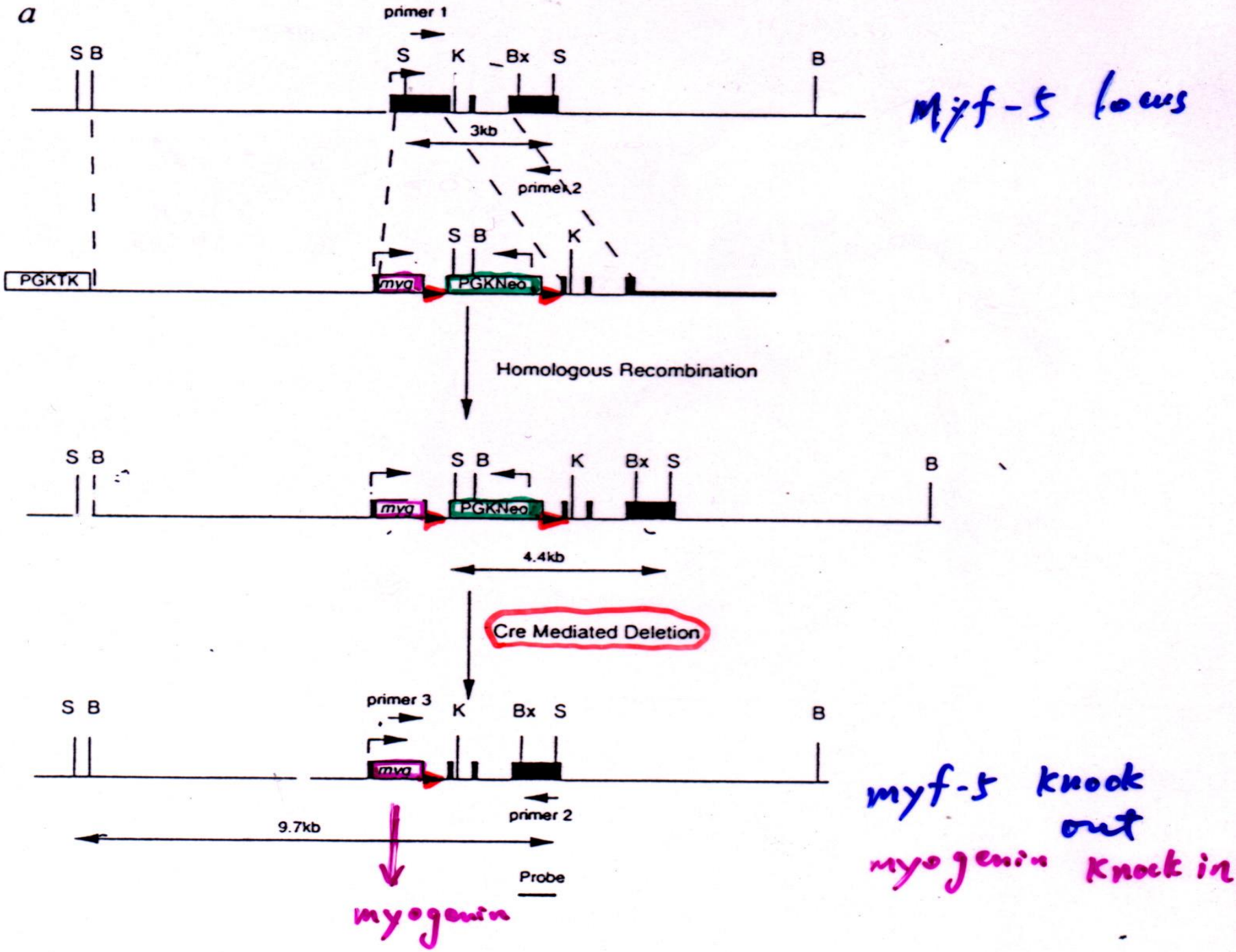
Myogenin KO: Muscle deficiency and neonatal death (Nature 364:501-506, 1993)

Myf-5 and Myf-6 double KO: alterations in skeletal muscle development (EMBO J. 14: 1176-1186, 1995)

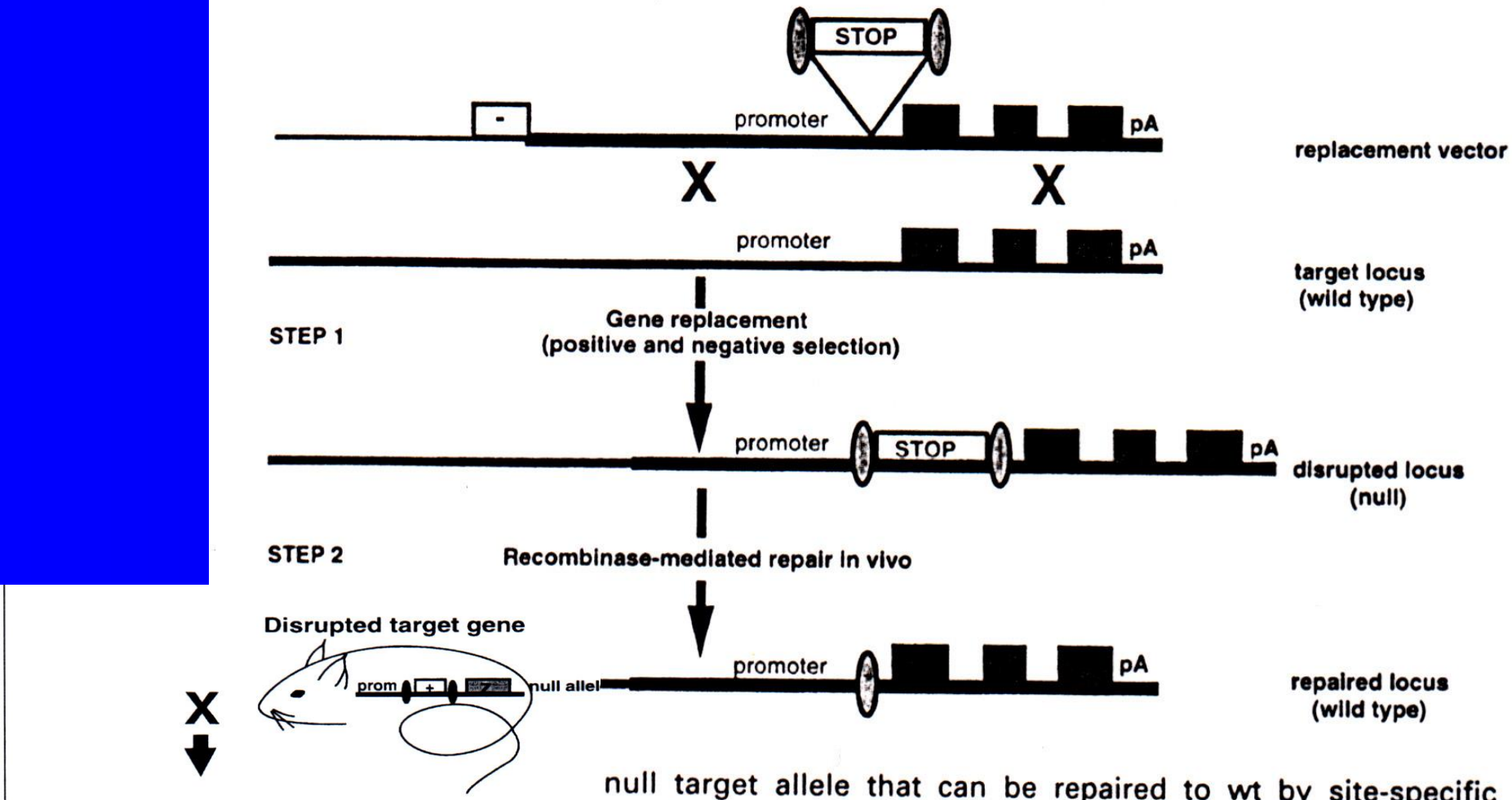
Myogenin knock-in in myf-5 KO mice: Functional reduundancy of the muscle-specific transcription factors Myf5 and myogenin (Nature 379: 823-825, 1996)





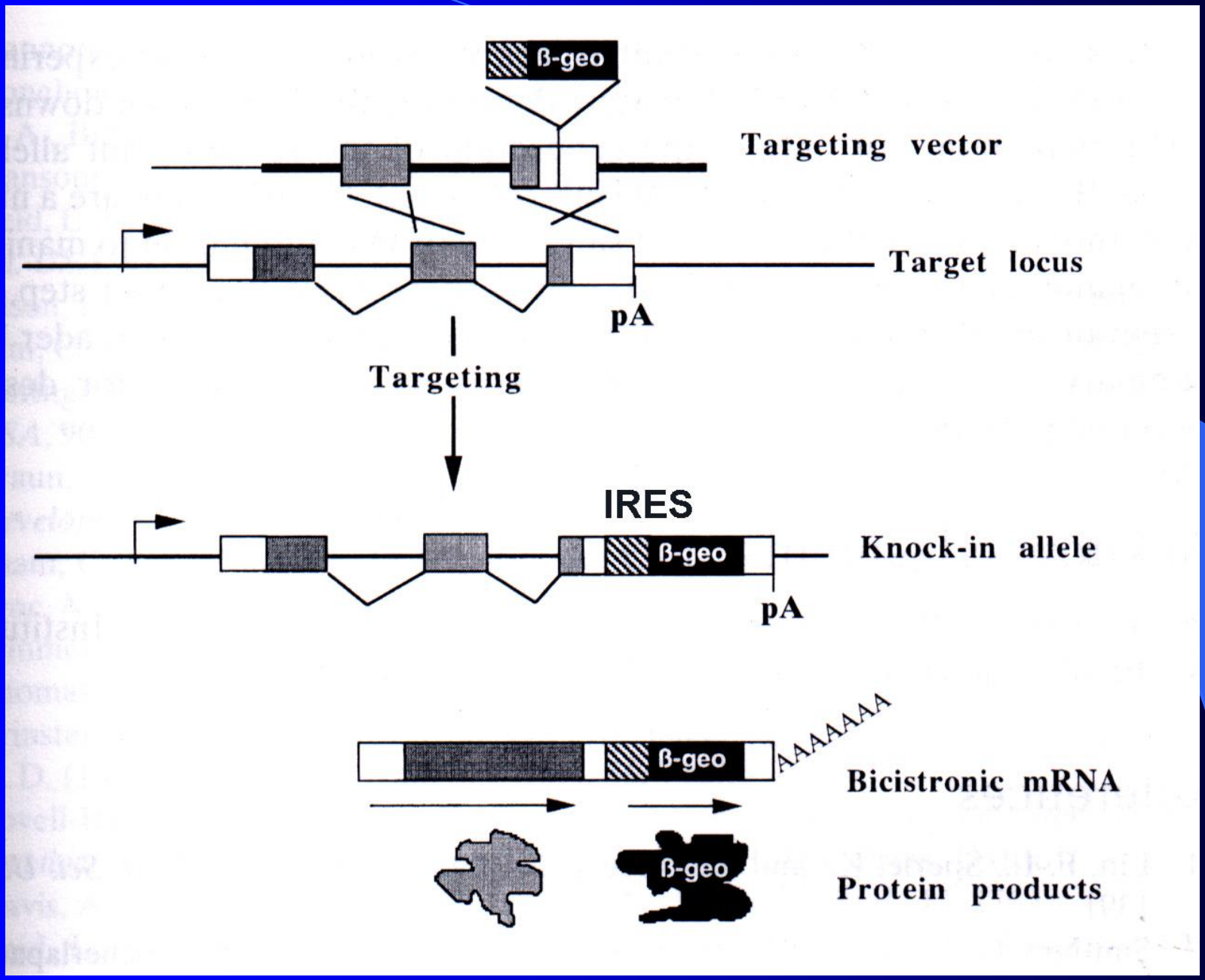

a
primer 1
S B
S K Bx S
B
3kb
primer 2
S B K
PGKTK
myo
PGKNeo
Homologous Recombination
S B
S B K Bx S
B
myo
PGKNeo
4.4kb
Cre Mediated Deletion
S B
primer 3
K Bx S
B
myo
primer 2
9.7kb
Probe
myogenin
Myf-5 locus
myf-5 knock out
myogenin Knock in

Gene Replacement Followed by Recombinase-Mediated Repair



null target allele that can be repaired to wt by site-specific
s and boxes are as indicated in *Figure 4*; grey stippled ovals
RT repeats. A floxed or flrted STOP cassette is inserted between
ation start sequence of the target locus so as to completely
ssion. Care must also be taken to ensure that the residual *loxP*
s neutral with respect to target gene function.

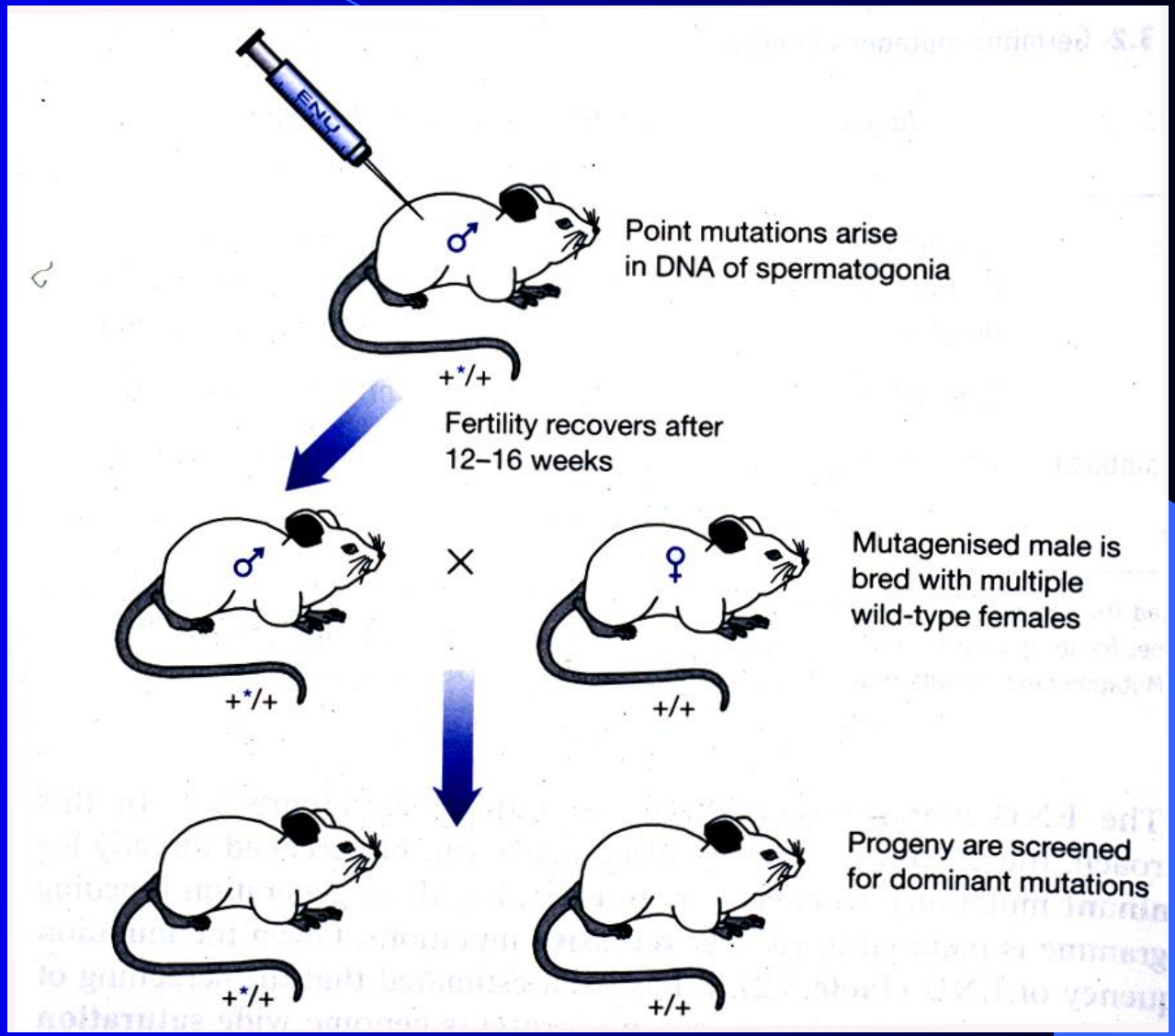
Knock in reporter gene (*β-geo*) with IRES (Internal Ribosomal Entry Site)

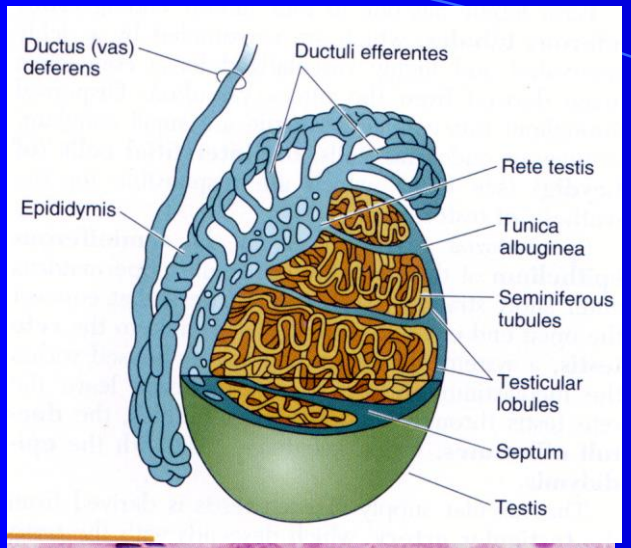


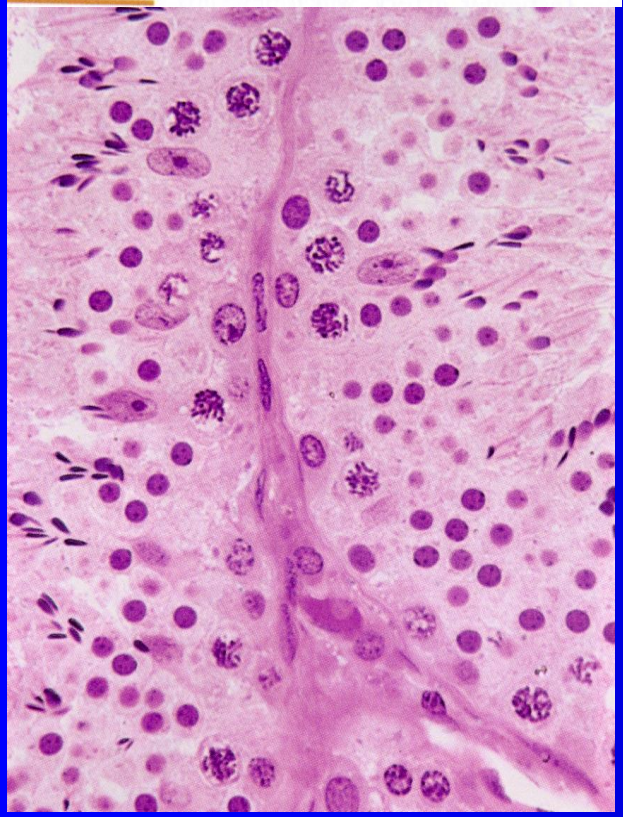
隨機突變法 (Random mutagenesis)

將致使生殖細胞基因突變藥物 **N-ethyl-N-nitrosourea (ENU)** 打入睪丸使精母細胞產生基因突變後，再針對其衍生具有基因突變動物做廣泛形態與行為分析。

Nature Genetics
24: 314-317, 2000 (ES)
24: 318-321, 2000 (ES)
25: 440-443, 2000
25: 444-447, 2000




Ductus (vas) deferens
Ductuli efferentes
Rete testis
Epididymis
Tunica albuginea
Seminiferous tubules
Testicular lobules
Septum
Testis



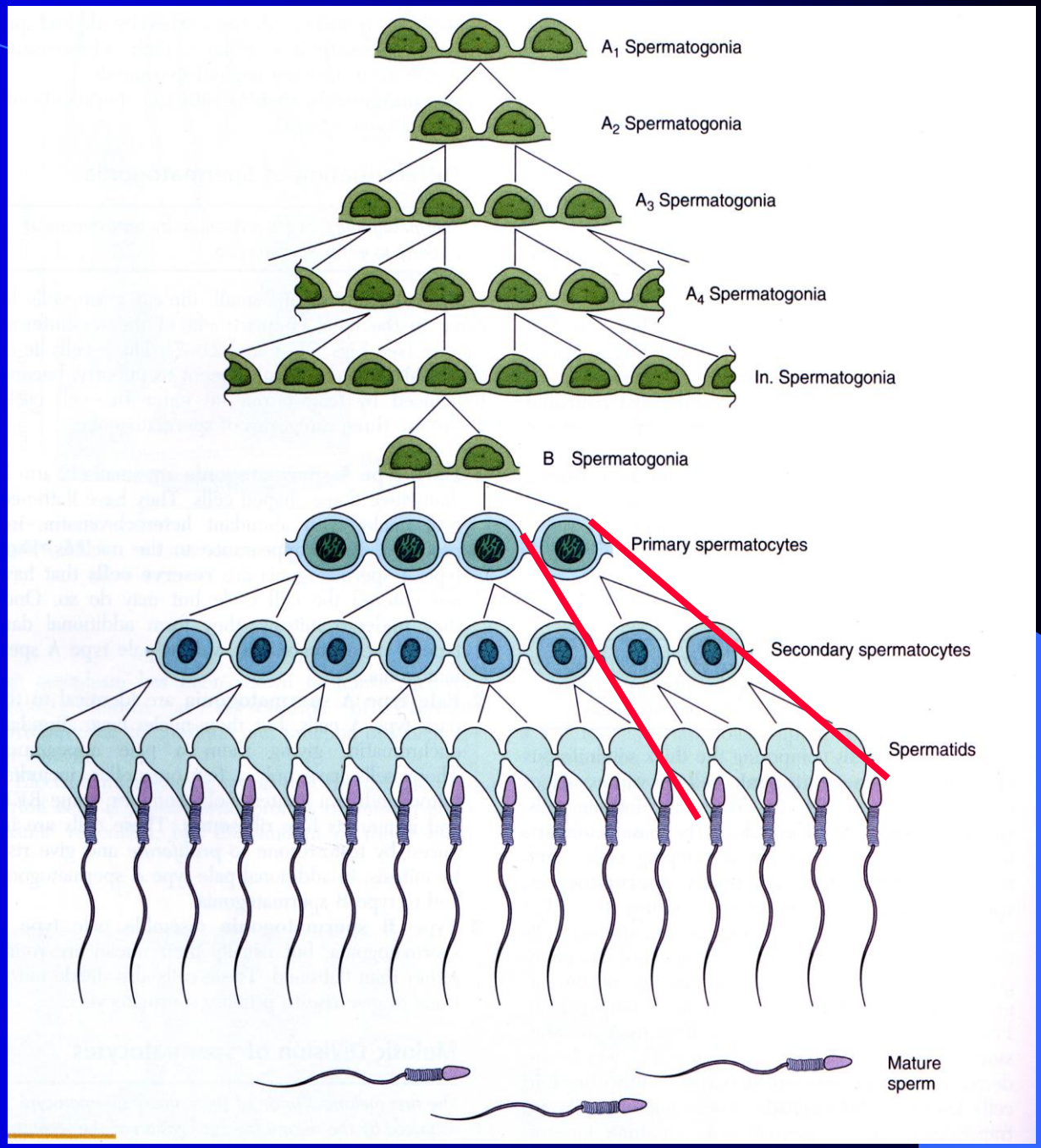

A1 Spermatogonia
A2 Spermatogonia
A3 Spermatogonia
A4 Spermatogonia
In. Spermatogonia
B Spermatogonia
Primary spermatocytes
Secondary spermatocytes
Spermatids
Mature sperm

Table 1 • Screening protocols

Screening for visible defects	Birth	Pre-weaning	Weaning	5 weeks	6 weeks	8–12 weeks
Category				SHIRPA	Behavioural testing	Clinical chemistry
				semi-quantitative	LMA:	
size	large/small	large/small	large/small	battery of tests*	activity recorded for	sodium
sense organs	eye size/colour		eye size/colour	deficits recorded in:	35 min. in cages equipped	potassium
	low set ears		ear size/position	lower motor	with beam-splitting devices	chloride
skin and hair	anaemia	stripes	coat colour/texture	neuron/muscle function		creatinine
	skin colour/texture	skin colour	loose/tight skin	spinocerebellar function	Acoustic startle response	urea
		blotchy coat	greasy/rough coat	sensory function	and PPI of the	total calcium
	curly whiskers		curly coat/whiskers	neuropsychiatric function	acoustic startle response	inorganic phosphat
			thin/balding coat	autonomic function	40 min test in soundproofed	glucose
			dark footpads		startle chambers	bicarbonate
behaviour	activity	activity	activity		incorporating both startle	alkaline phosphatas
		tremors/fits	tremors/fits		and PPI sessions	alanine
		circling	circling			aminotransferase
		head weaving	head weaving			asparate
		ataxia/gait	ataxia/gait			aminotransferase
skeleton	micrognathia		micrognathia			total protein
	agnathia		short/wide/thin head			albumin
	short head					total cholesterol
	scoliosis		scoliosis			HDL cholesterol
	hare lip					triglycerides
tail/extremities	short/bent tail		short/bent tail			
	poly/syndactyly		poly/syndactyly			
	fused toes		fused toes			
	limbs					
	bent/short limbs		bent/short limbs			
	puffy limbs/tail		puffy limbs/tail			
colour/spotting		belly spot	belly spot			
		head blaze	head blaze			
		coat colour	coat colour			
various	blebs/bruising					
	oedema	hydrocephaly	hydrocephaly			
	hydrocephaly					
	chylous ascites					
	spina bifida					

*See http://www.mgu.har.mrc.ac.uk/mutabase.