INVESTIGATION INTO THE EFFECTS OF THE TARGETED DISRUPTION OF THE MURINE GENE *CIAP2*

by

David Smookler

A thesis submitted in comformity with the requirements for the degree of Master's of Science, Graduate Department of Medical Biophysics, University of Toronto

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Master's of Science 2000, David Stephen Smookler, Department of Medical Biophysics, University of Toronto.

ABSTRACT

In 1995 two members of the inhibitors of apoptosis (IAPs) family, cIAP1 and cIAP2, were discovered to participate in the tumour necrosis factor receptor 2 (TNFR2) signalling complex. Subsequently they have been shown to be involved in suppression of apoptosis, or programmed cell death, triggered by TNFR1 activation. At least one mechanism by which the IAPs can suppress apoptosis is suggested by *in vitro* studies which show IAPs can prevent activation of certain caspases. The caspases are proteolytic enzymes that play a central role in the execution of apoptosis. The physiological importance of these IAPs has yet to be established. To investigate the role of cIAP2 in vivo, gene targeting was used to create a strain of mice deficient in one allele of *cIAP2*. Breeding of the heterozygous mice produced no homozygous *CIAP2* mutant pups, and subsequent typing of embryos suggest that loss of cIAP2 leads to pre-implantation lethality.

This thesis is dedicated to the memory of Sheldon Glasser, who died earlier in 1999.

Shelly was the first scientist I ever met; he was a terrific storyteller and an inspiring man.

I am sorry his story is over.

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ABBREVIATIONS

BIR ---------- baculovirus inhibitory repeat
CARD ---------- caspase recruitment domain
C. elegans ---------- Caenorhabditis elegans
CARD ---------- CAspase Recruitment Domain
CHO ---------- Chinese hamster ovary
DD ---------- death domain
DED ---------- death effector domain;
DISC ---------- death-inducing signalling complex
ES cell ---------- embryonic stem cell
FGF ---------- fibroblast growth factor
FLICE ---------- FADD-like ICE
hygro ---------- hygromycin resistance gene
IAP ---------- inhibitor of apoptosis
ICE ---------- IL-1β converting enzyme
ICM ---------- inner cell mass
LA ---------- long arm
LIF ---------- leukemia inhibitory factor
MALT lymphoma -- mucosa-associated lymphoid tissue lymphoma
mdn ---------- *morula decompaction*
NAIP ---------- neuron apoptosis inhibitory protein
neo ---------- neomycin resistance gene
PGK ---------- 3-phosphoglycerate kinase
RAIDD ---------- RIP-associated, ICH-1/CED-3-homologous protein with a death domain o n a ham and rye
RIP ---------- receptor interacting protein
SA ---------- short arm
TNF ---------- tumour necrosis factor
TNFR ---------- tumour necrosis factor receptor
TRAF ---------- TNF associated factor
VDAC ---------- voltage-dependant anion channel

INTRODUCTION

APOPTOSIS - PROGRAMMED CELL DEATH

Much of what is known of programmed cell death can be understood according to the paradigm developed from the pioneering genetic studies in *Caenorhabditis elegans* (C. elegans). Of fourteen genes identified in the worm that affect apoptosis, three are essential to promote or protect from cell death: ced-3, ced-4 and ced-9 (Hengartner and Horvitz, 1994). Ced-3 and ced-9 are now considered archetypes of two important families of vertebrate genes found in apoptosis; respectively the caspase and Bcl-2 family. More recently ced-4 has also been shown to have vertebral homologues: Apaf-1, which acts at a critical point in the signalling pathway of apoptosis (Zou, et al., 1997) and FLASH, the role of which is currently less well defined (Imai, et al., 1999).

Bcl-2 family members are pro- and anti-apoptotic factors

The Bcl-2 family members are either pro- or anti-apoptotic. In C. elegans CED-9 can block apoptosis by binding to CED-4, holding it in an inactive conformation (Hengartner, 1998). In the mammalian system the CED-9 homologue Bcl-xL can bind to the CED-4 homologue Apaf-1. Apaf-1, unlike CED-4, requires the co-factor cytochrome c to be activated. A major mechanism by which the mammalian Bcl-2 family members control

the activation of apoptosis may be via the control of the release of this co-factor into the cytosol from mitochondria (Yang, et al., 1997).

Recent evidence suggests cytochrome c is released through the a pore in the mitochondria known as the voltage-dependant anion channel (VDAC) (Heiden, et al., 1999), and that anti-apoptotic Bcl-2 family members bind to this pore to keep it closed while pro-apoptotic members bind to the pore to accelerate its opening (Shimizu, et al., 1999). Bcl-2 family members can heterodimerize, so the pro-apoptotic members may act by directly interfering with the function of the anti-apoptotic proteins.

Apaf-1 is involved in activating caspase-9

Apaf-1 has been shown to bind to the mitochondrial membrane protein Bcl-x_L. Apaf-1 can also bind to itself via its Ced-4 homology domain. Binding of released cytochrome c and dATP to Apaf-1 exposes the CAspase Recruitment Domain (CARD domain) of Apaf-1 to caspase-9, allowing the caspase to bind to Apaf-1 (Srinivasula, et al., 1998). Taken together, pro-apoptotic Bcl-2 family members promote the release of cytochrome c from the mitochondria. The cytochrome c causes a conformational change in Apaf-1 which allows caspase-9 molecules to form oligomers, or to be in close proximity to each other, as they bind oligomers of Apaf-1. This may allow self-cleavage of procaspase 9.

The caspases amplify apoptotic signals

Caspases are proteases that contain a cysteine residue in their catalytic site. Caspases are very specific in the cleavage of their substrates. They target certain pentapeptides, cutting after the most C-terminal amino acid, which is always an aspartate residue. Caspases are expressed as pro-enzymes, containing a highly variable N-terminal regulatory region followed by two catalytic regions known as the large and small subunits. The three regions are separated by aspartate residues that are targets for proteolysis by other members of the caspase family, other proteases, such as granzyme B, or by other caspases of the same type as the substrate itself (Figure 1). It follows that the proteolytic effect of a few active caspases can be quickly amplified since their activity can activate more caspase molecules of the same and different type. Several caspases, such as caspase-4, -8 and -9 contain a CARD domain in the N-terminal region, which can mediate binding with non-caspase proteins that also contain CARD domains (Li, et al., 1997).

To date over 40 substrates have been identified for the caspases. Cleavage by caspases can either activate or inactivate proteins. They can inactivate proteins that maintain the integrity of the cell, such as nuclear lamins, which keep the nuclear membrane intact (Buendia, et al., 1999), proteins involved in DNA replication, such as the large subunit of the DNA replication complex C (Ubeda and Habener, 1997), and can even attack anti-

Figure 1. Caspase structure

Based on Thornberry & Lazebnik Science v. 281 Aug. 28 1998

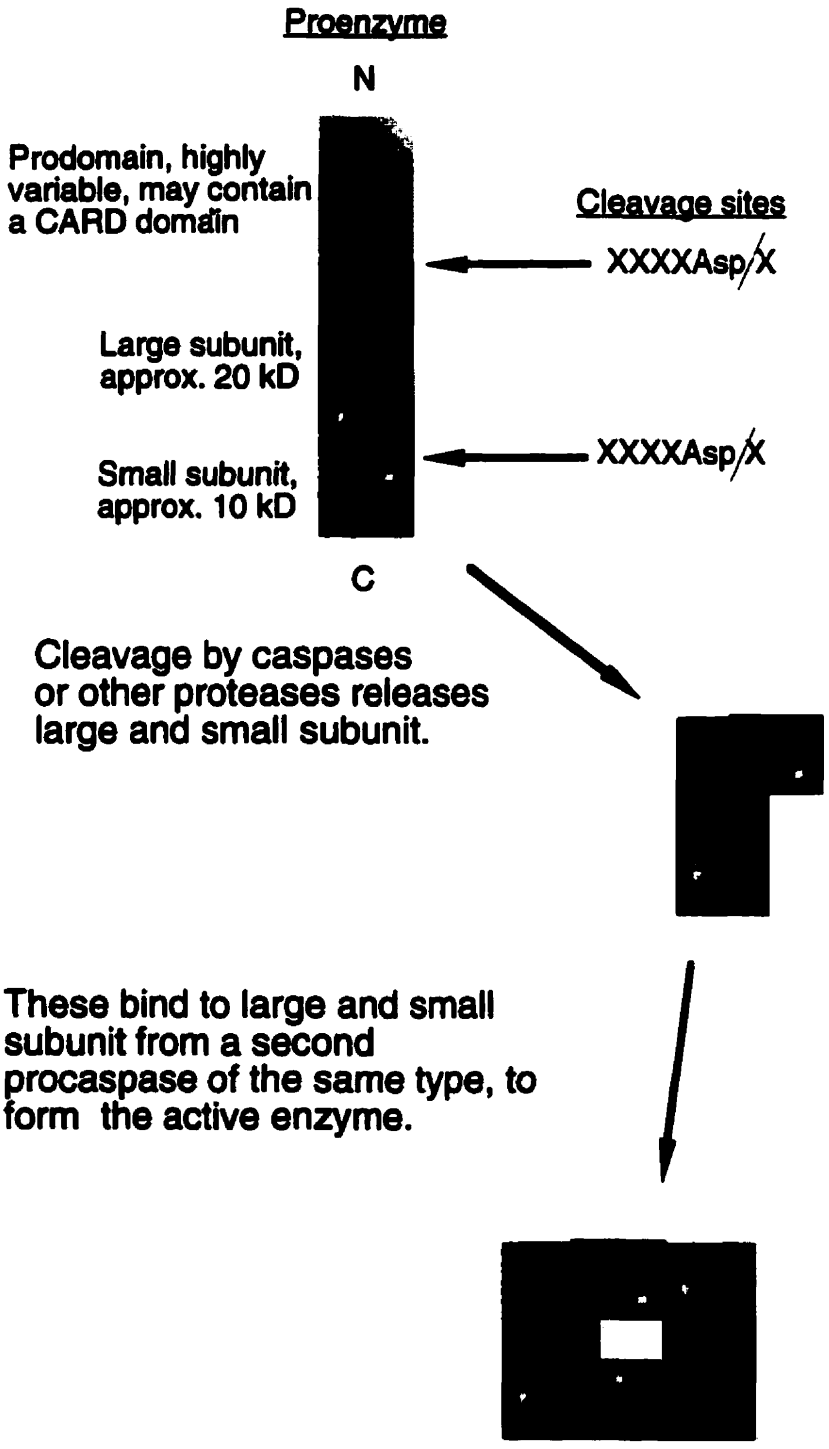


Fig. 1 Most caspases are activated via cleavage by other caspases. Effector caspases may also be cleaved by the activity of their procaspase form after oligomerization or by one of the few other proteases that target after aspartate, such as granzyme B (Shi, L *et al*, PNAS 1996).

apoptotic proteins, for example the cleavage of Bcl-2 by caspase-3 (Cheng, et al., 1997).

Proteolysis by caspases can also activate proteins that promote cell death or the ordered dismantling of the cell. These include BID, a Bcl-2 antagonist (Li, et al., 1998); DNA fragmentation factor (Liu, et al., 1997); and gelsolin, which can dissociate the cell's cytoskeleton (Geng, et al., 1998). Thus the caspases trigger many of the effects seen in the process of apoptosis, which leads to the death of cells in as quickly as 30 to 60 minutes after the initial death signal (reviewed by Thornberry and Lazebnik, 1998).

At least two of the caspases, caspase-8 and -9, function as initiators, rather than effectors, of this organized cell destruction. They represent two linked but separable pathways by which apoptosis can be triggered.

Caspase-9 is active in the pro-enzyme state

Caspase-9 has some proteolytic activity in its unprocessed, proenzyme state, about 1-2% of the activity of the processed enzyme. On binding of cytochrome c to Apaf-1, oligomerization of caspase-9 occurs and the enzymatic activity in the proenzyme initiates cleavage of neighbouring proenzymes, yielding active caspase-9 molecules. Once cleaved, the enzyme can activate downstream caspases, leading to the orderly destruction of the cell. Caspase-9, therefore, links the release of

cytochrome c from the mitochondria to apoptosis. Many factors, such as nitric oxide can damage mitochondria and lead to cytochrome c release (Ushmorov, et al., 1999). In addition the pro- or anti-apoptotic effects of the various Bcl-2 family members can in part be explained by their ability to block or promote the activation of caspase-9 by cytochrome c.

Caspase-8 is activated by signals from the cell surface

Caspase-8, also known as FLICE, appears in the cluster of proteins that form on the cytosolic side of certain death inducing TNF receptors when the receptors are activated by ligand-induced trimerization. These clusters are called DISCs for death-inducing signalling complexes. Like caspase-9, the caspase-8 proenzyme has some catalytic ability, and it has been suggested that it is activated by autocatalysis when recruited to the DISC (Muzio, et al., 1998). The signalling of cell death by two TNFR family members, Fas and TNFR1, is mediated by the activation of caspase-8 (Figure 2). The prodomain of caspase-8 contains a consensus sequence called a death effector domain (DED) which binds to the DED sequence of the adaptor protein FADD. With both TNFR1 and Fas, recruitment of caspase-8 to the DISC is via FADD. Activation of caspase-8 at the surface of the cell quickly leads to the cleavage of effector caspases including caspase-2, -7 and -3. In addition, active caspase-8 can activate the Bcl-2 family member BID. 'Active' BID translocates to the mitochondrial membrane inducing the release of cytochrome c, leading to an

Figure 2. Signalling pathway of fas and TNFR1

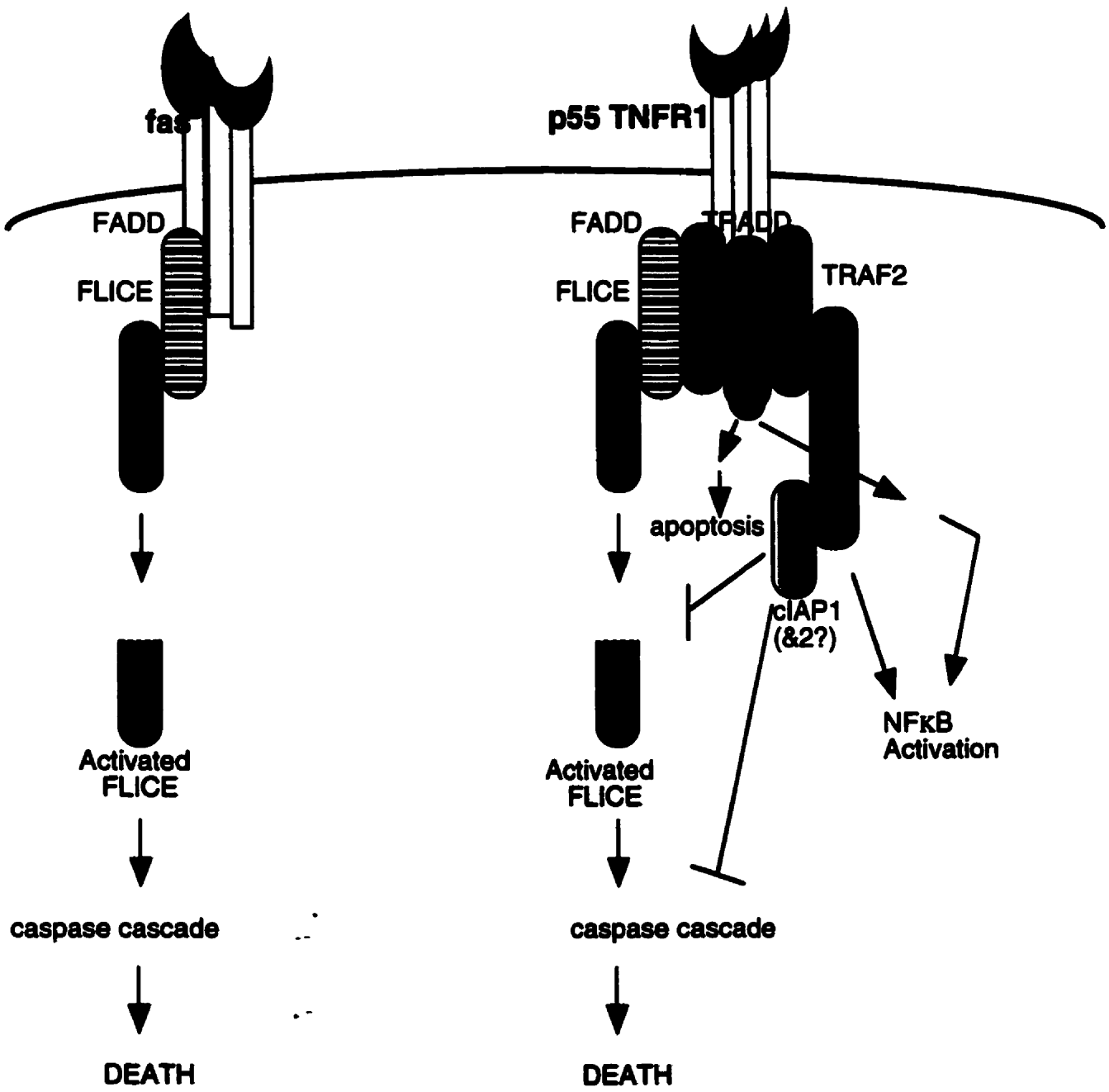


Fig. 2 Data from Yang et. al. Cell Vol. 89 June27 97 and Nagata Cell Vol. 88 Feb 7 97 showing some of the signalling pathways downstream of the TNFR family members fas and TNFR1.

amplification of the death signal by the activation of caspase-9 (Luo, et al., 1998). XIAP, cIAP1 and cIAP2 (see below) have been shown to directly bind to and prevent the cleavage of procaspase-9, thus blocking a powerful mechanism for amplification of the cell death pathway mediated by caspase-8. In addition, these IAPs have been shown to directly bind to and inhibit the cleavage of some of the effector caspases, caspase-3 and -7, that are downstream of both caspase-8 and caspase-9 (Deveraux, et al., 1998).

THE TNFα SIGNALLING PATHWAY

Tumour necrosis factor and the TNFα signalling pathways are involved in a variety of cellular activities

The physiological effects of TNFα activation include cytotoxicity, antiviral activity and fibroblast proliferation. In addition, TNFα causes endothelial cells to express adhesion molecules, recruiting a variety of leukocytes including neutrophils, monocytes and lymphocytes to the site of inflammation. TNFα binding can also activate neutrophils and stimulate monocytes to release cytokines such as IL-1 and TNFα itself. TNFR-1 and-2 are the only known receptors of TNFα; they are both widely expressed throughout the body.

TNFα receptors constitute a large family of proteins

Both cIAP1 and 2 have been shown to be associated with the TNFR2 and TNFR1 signalling pathway. TNFR1 and TNFR2 are two of a large family of receptors distinguished by repeated cysteine-rich domains found in their extracellular regions. Typically these receptors are activated by ligand induced homo-trimerization. In addition, several of the family members, including TNFR1, contain a cytosolic region related to the CARD domain known as the death domain (DD) through which they are able to transduce a signal leading to the death of the cell via a caspase cascade. In the case of TNFR1 activation the DD binds to the adaptor molecule TRADD which can transduce either a death signal via a caspase cascade, or alternatively, can signal to protect the cell against death through a family of adaptor molecules known as the TRAFs (Boldin, et al., 1995; Gray, et al., 1990; Hsu, et al., 1996b; Hsu, et al., 1995).

TRADD is at the apex of signalling pathways leading to apoptosis or NF-κB activation

TRADD contains a C-terminal death domain that binds to the DD of TNFR1 and the DD of FADD, linking the two proteins. This provides the transduction pathway required for TNFR1 activation to lead to cell death. In addition, TRADD contains an N-terminal TRAF-binding domain which links TRAFs to the activated TNFR1 complex. TRADD can also bind

another DD containing protein known as Receptor Interacting Protein or RIP, which can transduce signals leading to either cell death or to the activation of NF-κB (Hsu, et al., 1996a).

TRAFs are involved in many TNFR signalling pathways

The TRAF family of cytosolic proteins is distinguished by a C-terminal TRAF domain, which can be subdivided into N-terminal and C-terminal subdomains. They are adaptor proteins with no known enzymatic activity. cIAP1 and 2 have been shown to bind to the N-terminal coiled-coil region of the N-terminal subdomain of TRAFs 1 and 2 (Figure 3). Five of the six known TRAFs also share an N-terminal RING finger, followed by five tandem zinc finger structures. The TRAFs have been shown to bind many of the TNF receptor family members. Both TRAF1 and TRAF2 are involved in several signal transduction pathways; TRAF2 binds directly to the receptors CD40, CD30 and TNFR2 and indirectly binds to TNFR1 via TRADD. TRAF1 is known to bind to TNFR2 and CD30. Thus cIAP1 and cIAP2 could potentially be downstream of several receptors other than TNFR1 and TNFR2.

NF-κB promotes cell survival and cytokine production

NF-κB is a transcription factor sequestered in the cytosol by an inhibitory protein known as IκB, of which there are several forms. Phosphorylation

Figure 3. cIAP1 and 2 binding to TRAF 2

Rothe et. al. Cell, Dec. 29 1995

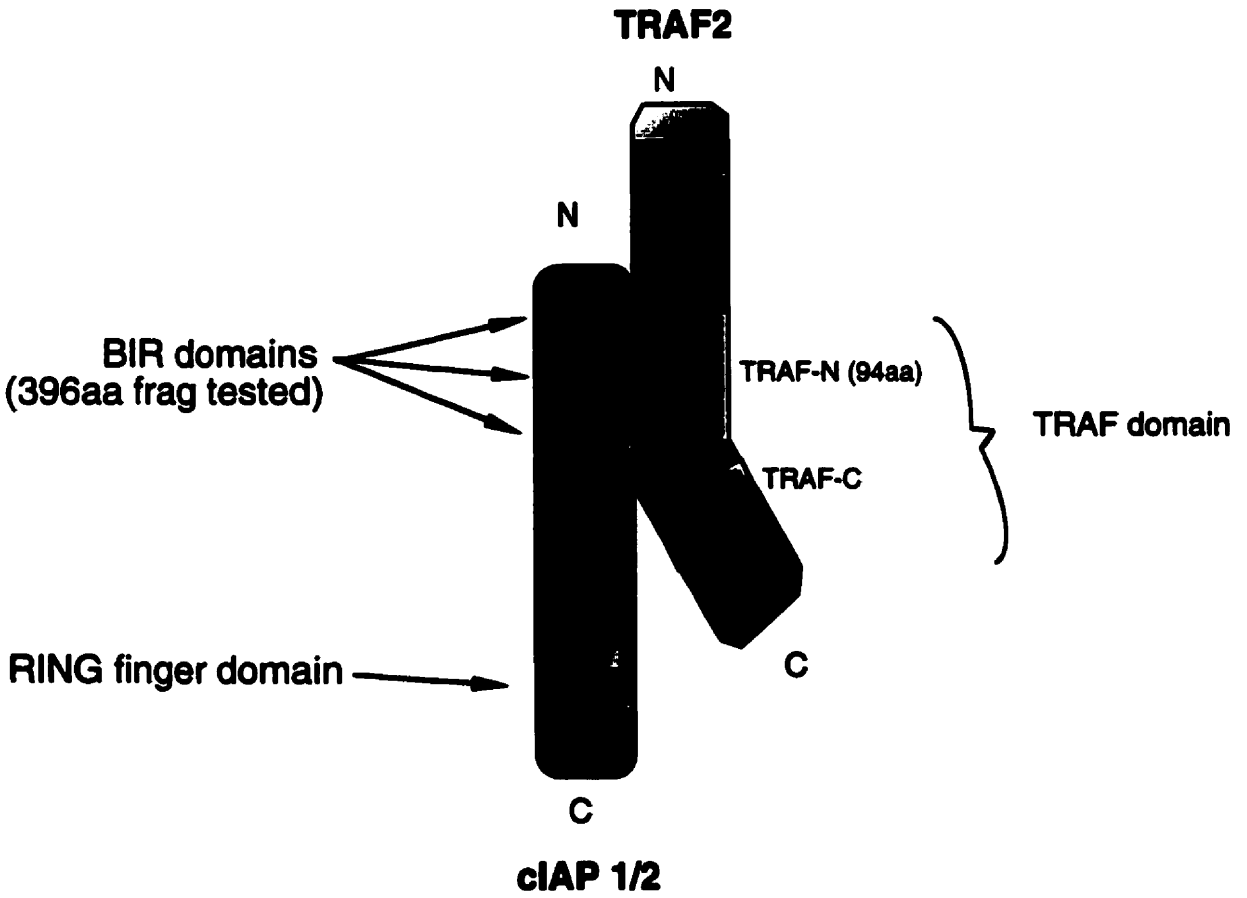


Fig. 3 Yeast 2-hybrid and co-immunoprecipitation studies with truncated portions of TRAF1 and 2 and cIAP1 and 2 demonstrate the TRAF-N domain and the BIR region of these IAPs are required for binding (Rothe et. al., Cell 1995).

of IκB by the kinases IKKα or IKKβ leads to its degradation uncovering the nuclear localization signal of NF-κB, allowing the transcription factor to be translocated to the nucleus. NF-κB is responsible for the transcription of a variety of cytokines, cell adhesion molecules and growth factors (reviewed by Baeuerle and Baltimore, 1996). As well, its activation can block cell death by TNF. Evidence suggests that at least one way NFκB prevents cell death is by promoting the transcription of TRAF1 and cIAP2 (Wang, et al., 1998)

INHIBITOR OF APOPTOSIS PROTEINS (IAPs)

The Inhibitor of Apoptosis Proteins are a novel family of cytosolic proteins involved in the suppression of apoptosis. The archetypes of the family were originally discovered in baculoviruses as proteins that complemented the loss of p35, an unrelated viral protein that also inhibits apoptosis (Crook, et al., 1993). Subsequently IAP members have been found in a variety of organisms including flies, chickens and mammals (Table 1).

The potential importance of this family is exemplified by studies of the IAP survivin, which is normally only expressed in fetal tissue. Survivin is found to be expressed in over 40% of certain cancers (reviewed by (LaCasse, et al., 1998). In addition cIAP2 itself has recently been implicated in mucosa-associated lymphoid tissue (MALT) lymphomas (Dierlamm, et al., 1999).

Table 1. List of IAPs

last update June 4 1999

Mouse
cIAP 1/mIAP2/MIHB
cIAP2/mIAP1/MIHC/API2
xIAP/miap-3
TIAP
BRUCE (?)
Human
cIAP1/hIAP2/ MIHB
cIAP2/hIAP1/ MIHC
xIAP/hILP
NAIP
Survivin
Chicken
IAP1/ch-IAP1/ita
Drosophilia
DIAP1
DIAP2/dILP
Viral
Op-IAP
Cp-IAP
C elegans
BIR-1
BIR-2

Table 1. All IAPs listed have been demonstrated to have anti-apoptotic effects when overexpressed, except BRUCE (no test published) and BIR-1 and -2.

Figure 4. Structure of mammalian IAPs

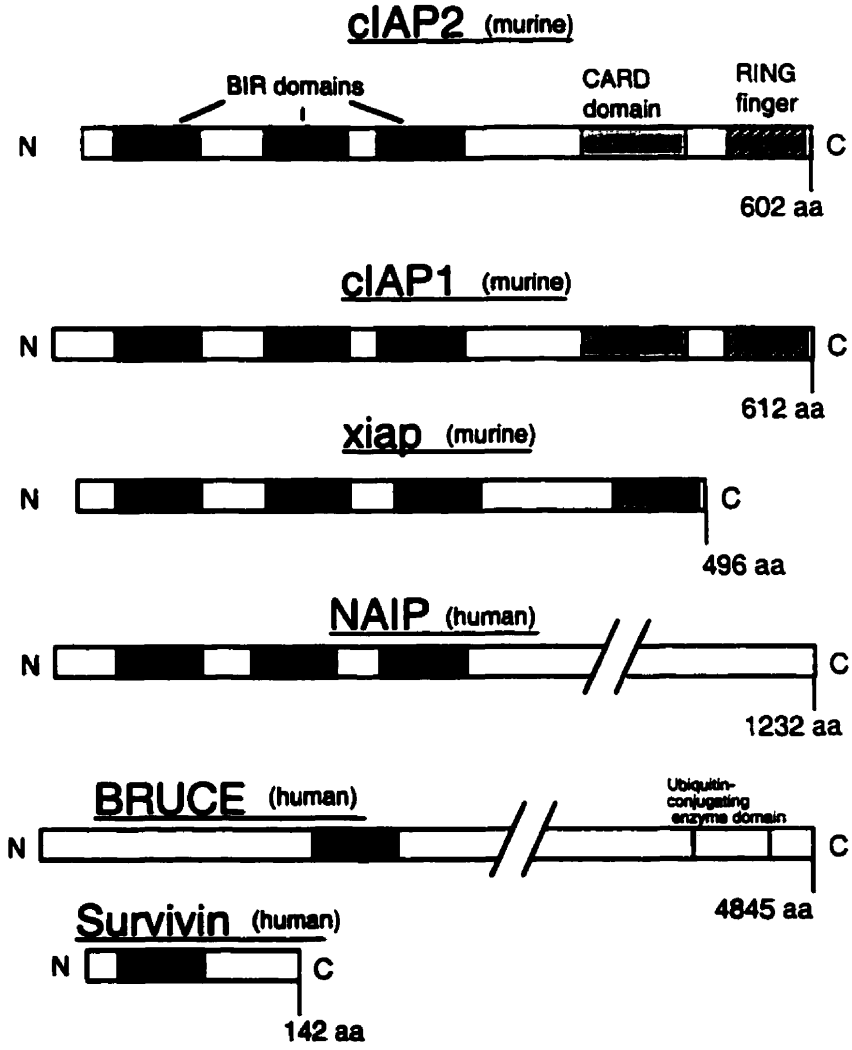


Fig. 4 Mammalian IAPs all contain a least one BIR domain. All except BRUCE have shown antiapoptotic effects in cells when overexpressed.

Structure

The common feature of the IAP family of proteins is the presence of at least one baculovirus inhibitory repeat (BIR) motif. BIR domains are approximately 70 amino acids long and are generally found at the N termini of the proteins as one to 3 repeats (Figure 4). Sequence analysis of the BIR domains suggests they contain a zinc binding motif, recently confirmed by NMR analysis (Hinds, et al., 1999).

The BIR domains in both cIAP1 and cIAP2 have been shown to bind to a putative coiled-coil structure in TRAF1 and 2 known as the TRAF-N domain (Rothe, et al., 1995). (Figure 3). In almost all IAPs, including cIAP2, there is a second type of zinc binding domain called a RING finger. RING fingers are thought to be involved in protein-protein interactions (Freemont, 1993) (Figure 4).

In addition, cIAP1 and 2 specifically contain a CARD domain (Figure 5) that falls between the BIRs and the RING finger (Hofmann, et al., 1997). This domain is not shared with other IAPs such as NAIP or XIAP. CARD domains are found in many caspases and in several caspase-binding proteins such as Apaf1, Bcl-10 (a human homologue of herpesvirsus protein E10, mutated in B-cell lymphomas), and the RIP-associated, ICH-1/CED-3-homologous protein with a death domain (RAIDD).

Figure 5. cIAP1 and 2 both contain a CARD domain

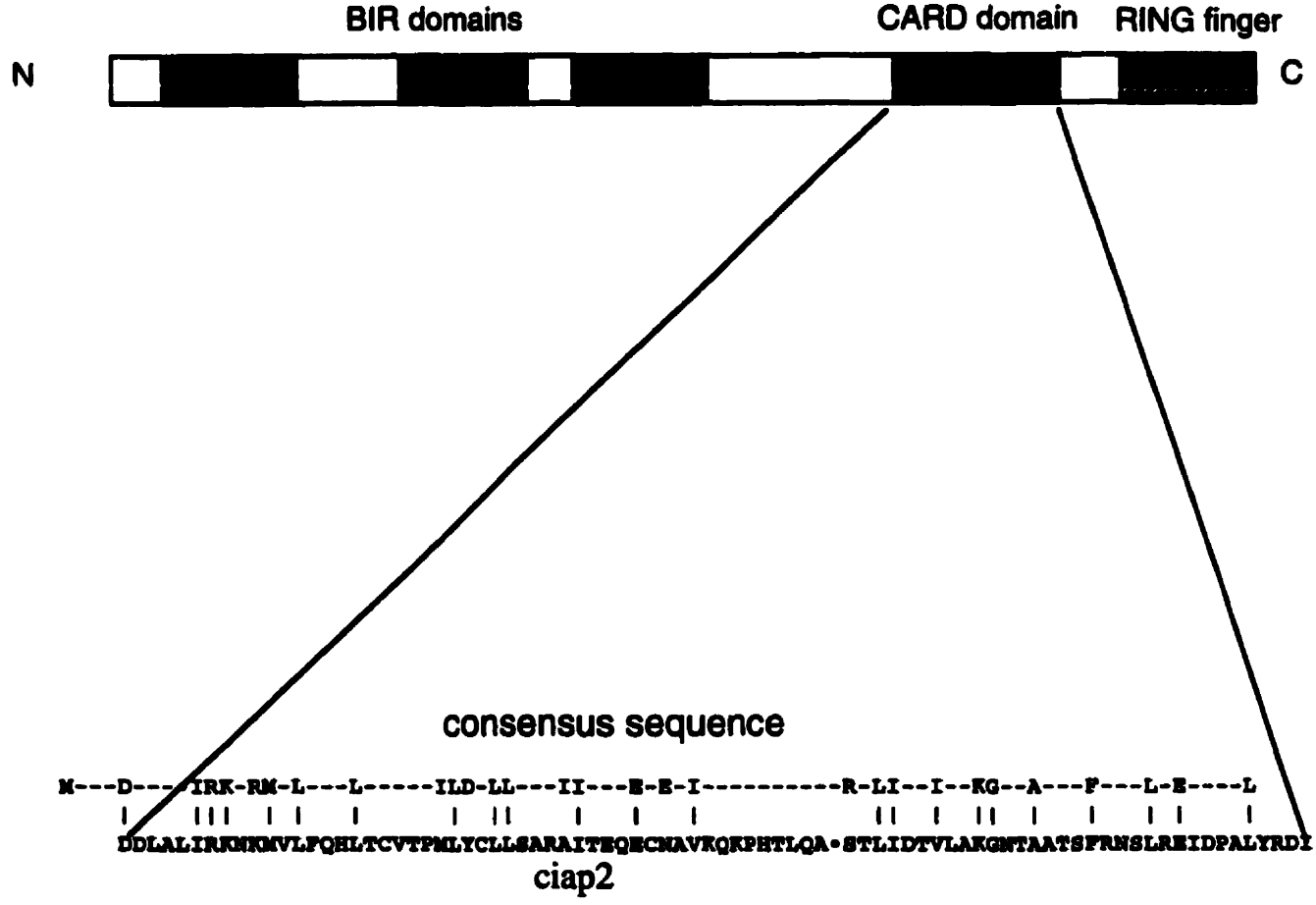


Fig. 5 Top row shows CARD domain consensus sequence (Hoffman and Bucher, TIBS, 1997). Bottom row shows corresponding sequence in the mouse cIAP2 protein, amino acids 439 to 522.

Murine cIAP2 and cIAP1 are both approximately 600 amino acids in length. The proteins have 69% sequence identity and 81% sequence similarity. The *cIAP2* and *cIAP1* genes are 12 kbp apart on chromosome 9A2 (Liston, et al., 1997). The sequence similarity of the genes along with their close proximity suggest the two were the product of a gene duplication event (Figure 7).

cIAPs are widely expressed

Analysis of cIAP1 mRNA expression in human tissue shows it expressed ubiquitously in adult and fetal tissue, with particularly high expression in the adult thymus and testes (Rothe, et al., 1995). The mRNA for cIAP2 is more selectively expressed, primarily found in the thymus, spleen, peripheral blood lymphocytes, and in the fetal lung and kidney (Rothe, et al., 1995, Liston, et al., 1996).

Known functions of cIAP1 and cIAP2

Liston and colleagues (Liston, et al., 1996) have shown that over-expression of cIAP1 and cIAP2 can prevent death in Chinese Hamster Ovary (CHO) cell caused by serum withdrawal or treatment with menadione, a source of superoxides which damage mitochondria (Saxena, et al., 1995). More recently it has been shown that cIAP2 is involved in NFκB protection from cell death triggered by TNF. Specifically, Wang et. al. published in 1998 that in

the absence of functioning NFκB, over-expression of cIAP1, cIAP2, TRAF1 and TRAF2 can prevent cell death due to TNF, in mouse embryonic fibroblasts and in a fibrosarcoma cell line. Furthermore, stimulation with TNF in cells that contain functional NFκB induces the transcription of TRAF1 and cIAP2, suggesting that it is increased expression of these proteins specifically that mediates the NFκB controlled cell protection(Wang, et al., 1998).

cIAPs are potentially important in controlling response to TNF

Biochemical and cell culture analysis of the IAPs suggest that they could be powerful modulators of caspase activation under physiological conditions. They potentially play a critical role in a cell's readiness to die in response to caspase activation. cIAP1 and cIAP2 are of particular interest since they are associated with the TNF signalling pathway which is an important mediator of the inflammatory response.

Genetic analysis is required to confirm or disprove the importance of cIAP1 and cIAP2 in the physiological setting. The generation and analysis of *cIAP1 -/-* mice has already been carried out in our lab. Surprisingly, targeted gene disruption of *cIAP1* resulted in almost no detectable phenotype. Gross anatomical and histological analysis was normal in these mice. Exposure of mice to lysteria, a pathogen known to require a

functional TNF signalling pathway to be cleared, also revealed no difference in the *cIAP1 -/-* mice. The majority of tissue culture analyses also revealed little difference in these mice. Lymphocytes responded normally to a variety of apoptotic stimuli. In response to TNF, programmed cell death of embryonic fibroblasts derived from these mice was the same as in normal cells. The only difference found at all was a moderate increase in apoptosis of the mutant embryonic fibroblasts in response to TNF if protein synthesis was also blocked, by the addition of cyclohexamide (Albert, 1998).

Generation of a strain of *cIAP2-/-* mice was begun concurrently with the creation of the *cIAP1-/-* mice to determine the role cIAP2 plays in the TNF receptor signalling pathway. In addition, an attempt was made to generate *cIAP1-/- cIAP2-/-* mice on the assumption that the two proteins play a similar and redundant role.

WORK DONE BY AUTHOR

This work was a collaborative effort. David Ferrick, a former post-doctoral fellow in the lab, performed the genomic screen, the subcloning, and the designing of the neo targeting construct for cIAP2. Petra Arck, a visiting scientist, transformed and isolated ES cells containing the targeting vector. I designed a flanking probe for the Southern Blot of the cIAP2 locus, designed new primers for PCR detection of homologous

recombination and for loss of the wild-type gene. I made the cIAP2-hygro targeting vector, as well as the necessary primers for detecting integration into the targeted locus. I grew and transform *cIAP1+/-* ES cells with the *cIAP2-hygro* vector, and isolated hygromycin-resistant clones. I carried out the Southern Blot and PCR analysis of the *cIAP2-hygro* ES clones. I designed and carried out the RT-PCR and Northern Blot analysis of *cIAP2* levels in embryonic tissue. Injection of the mutant ES cell lines into blastocysts and the subsequent implantation into foster mothers was done by technicians. I was also responsible for the isolation, culture and typing of blastocysts, and the isolation and typing of embryos at all stages of development.

METHODS

Two partial cIAP2 genomic clones were cloned from a 129J mouse genomic library cloned into the λ Dash II vector (Figure 6). The library was probed using full length mouse cIAP1 cDNA, rather than cIAP2 cDNA; this was possible due to the high homology between the two genes, 69% (Liston, et al., 1997). One of the genomic cIAP2 clones was digested with Eco RI and subcloned as an 8kb fragment into the plasmid pBS.

Generation of cIAP2 heterozygous ES clones and mice

Genes can be modified in ES cells by introducing linear DNA that matches the locus of interest on either end but includes a novel region in the middle. The regions flanking the novel DNA are called the long arm (LA) and short arm (SA). The novel region includes stop codons which will prevent the complete translation of the target gene and will introduce a gene that can be used for cell selection, such as the neomycin resistance gene (*neo*). The SA is short enough to allow a PCR reaction to occur using primers that will flank the SA region if the integration is at the homologous site in the genome. The LA is longer to increase the probability of homologous recombination of the construct into the target locus (Figure 7).

Figure 6. Lambda DASH II library

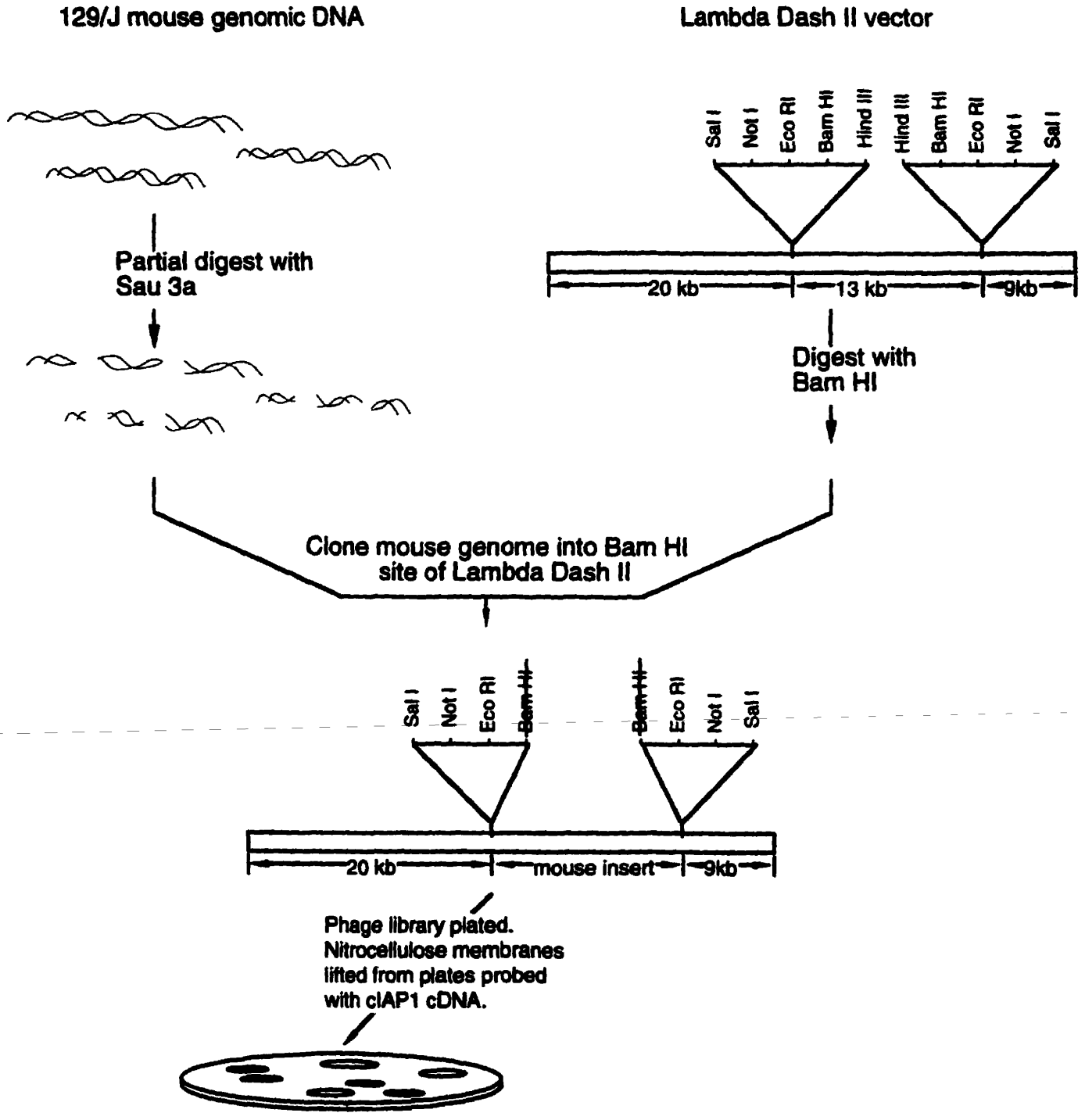


Fig. 6 Steps involved in obtaining and probing a genomic library.

Figure 7. Genomic Structure of murine cIAP1, 2

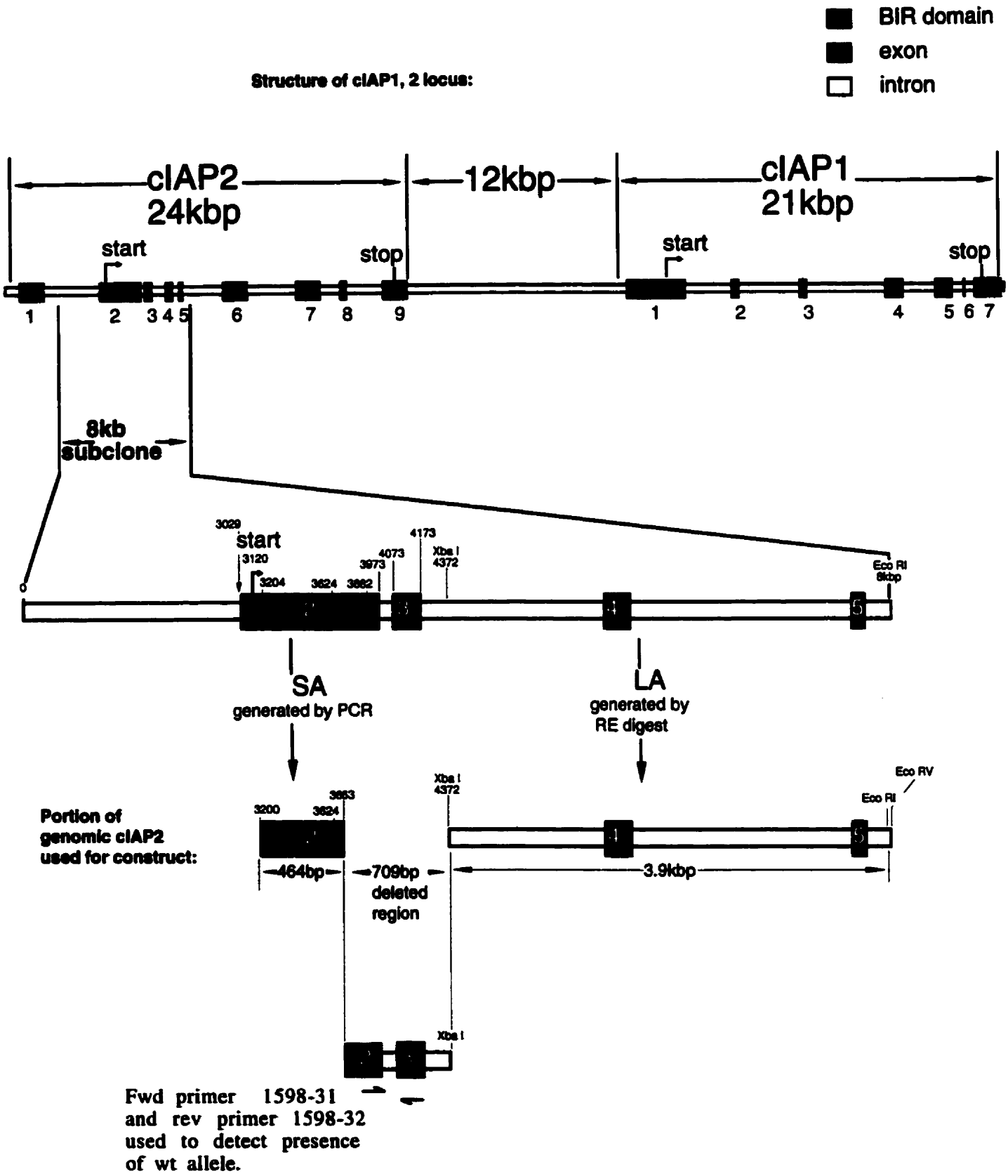


fig. 7 Genomic structure of cIAP1, cIAP2 locus from Liston et. al. Genomics 1997

A gene targeting construct for deleting cIAP2 was made by PCR synthesis of a 464 nucleotide short arm within exon 2, and an EcoRV-XbaI digest of a 3.9kb long arm within introns 3 and 5 (Figure 8). SA primers were fwd(1391-31) 5′-CCC GTC GAC TGT GAG CTG TAC CGA TTG-3′ and rev(1391-32) 5′-CCC AAG CTT GAC AAT GGC CAT GTT TCA-3′; 62° annealing, 30 cycles. The short and long arm were cloned into a plasmid containing the *neo* gene driven by the mammalian promoter 3-phosphoglycerate kinase (PGK) (Figure 9).

Correct assembly of the construct was confirmed by restriction mapping and sequencing reactions that spanned the four splice sites where the short and long arm were inserted into the plasmid containing *neo*.

The construct was linearized and transfected into mouse 129J E14 embryonic stem (ES) cells. Cells were selected in 0.3mg/ml G418 for 10 days. Antibiotic selection identifies clones that express the neomycin resistance protein. Most of those clones contain the gene as a random integration into their genome. To identify clones containing a homologous integration, the surviving colonies were screened by PCR using a forward primer (1605-97) 5′-GAG TGC TGA CAC CTT TGA GTT GAA AT-3′ specific for a portion of exon 2 of *cIAP2* just upstream of the short arm -- i.e. not in the targeting vector -- and a reverse primer (1605-98) 5′-AGA TCA GCA GCC TCT GTT CCA CAT AC-3′ specific for a region in

Figure 8. Detail of Long and Short Arm creation from 8kb genomic subclone

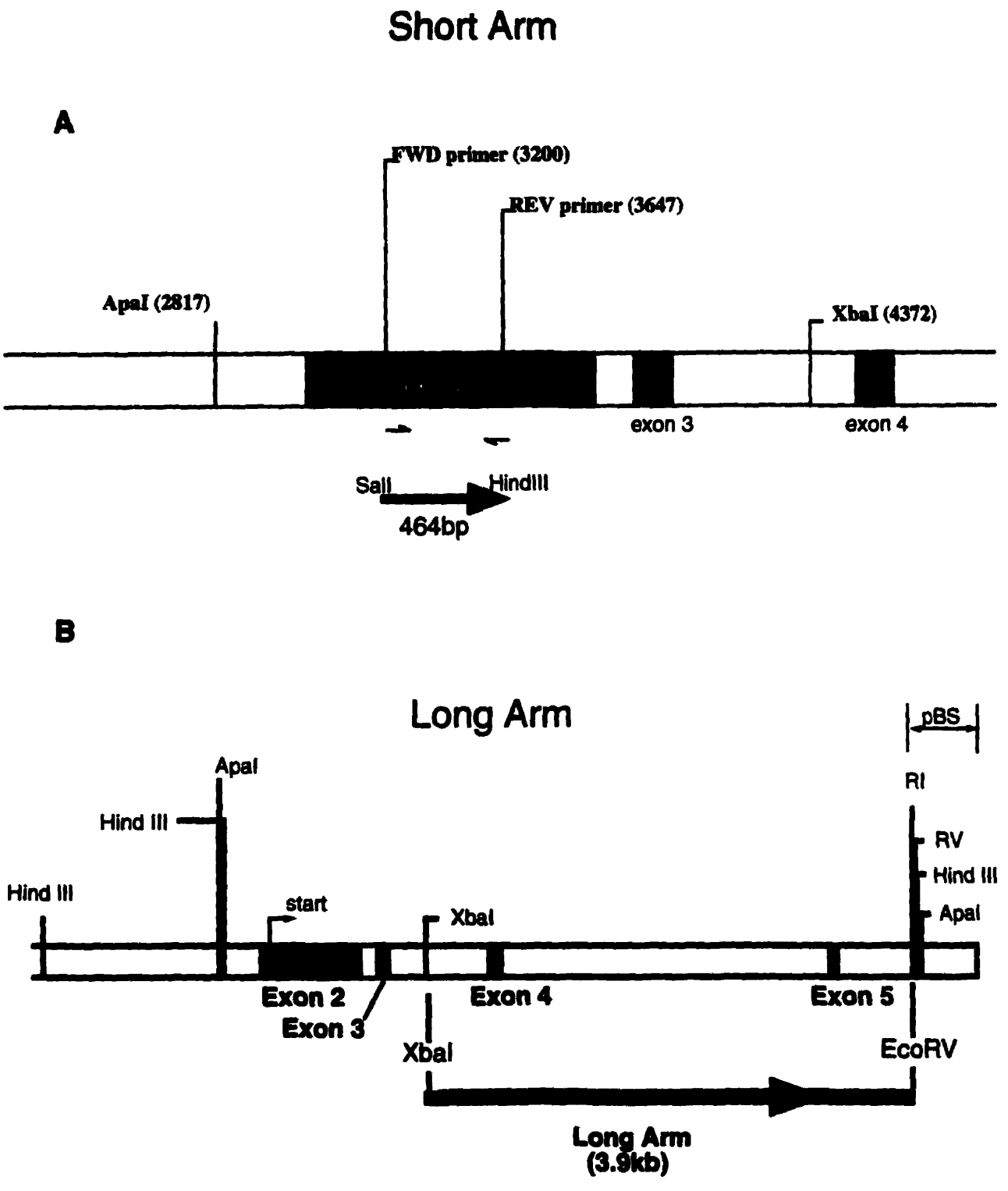


Fig. 8 A: cIAP2 Short Arm created by PCR using genomic subclone as template. B: Long Arm created by restriction digest of genomic subclone using an endogenous Xba I site and an Eco RV site from the plasmid multiple cloning site.

Figure 9. clAP2-neo construct

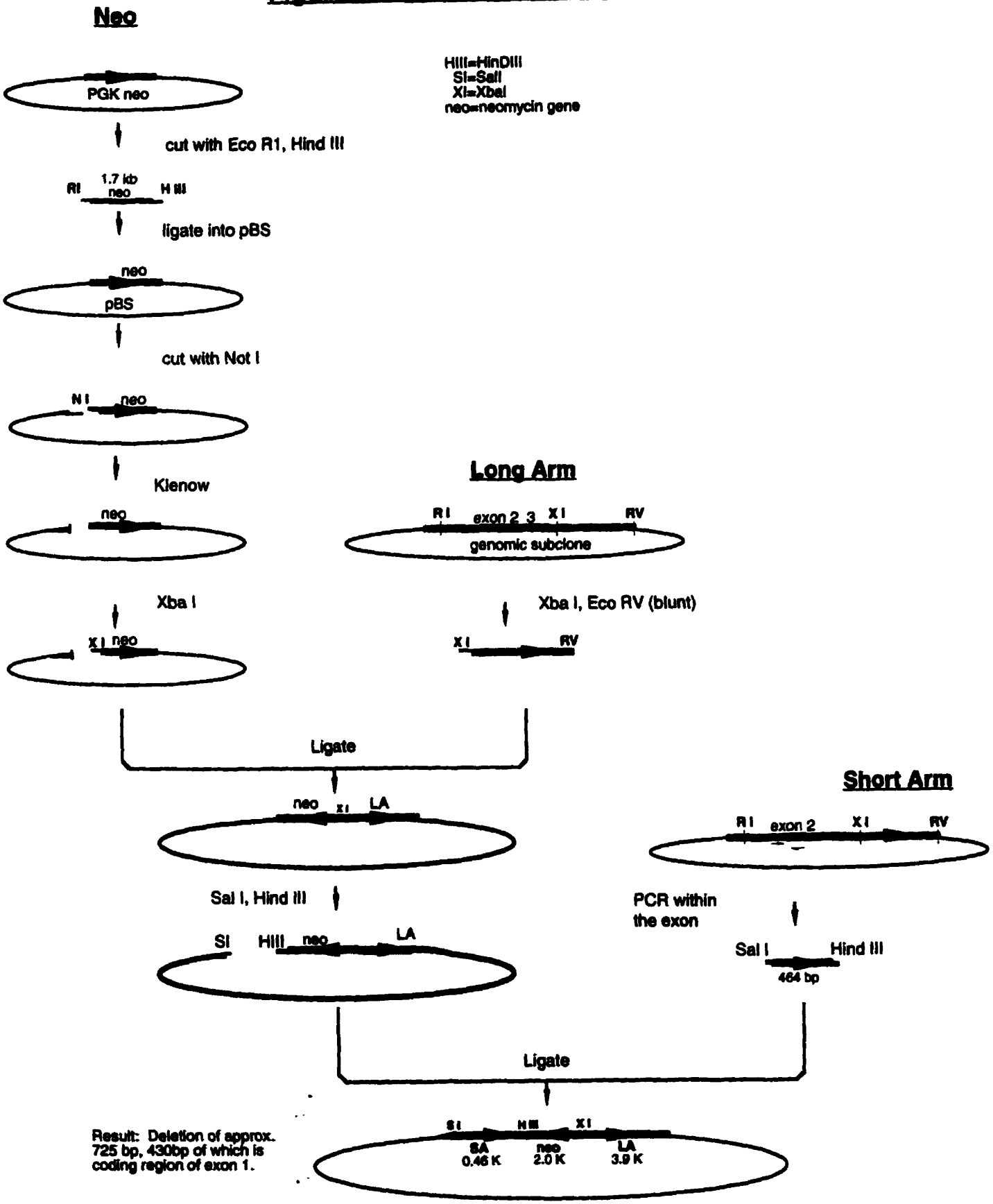


Fig. 9 Steps involved in assembling PGK-neo, the Long Arm and the Short Arm.

the *neo* cassette. The annealing temperature was 59°, and the reaction was usually run for 35 cycles. The PCR reaction results in a 576 bp product for the homologous recombinant clones.

Homologous recombinants were confirmed by Southern blot analysis. Typically 15 μg of DNA was digested with EcoRI and eletrophoresed overnight in a .7% agarose gel. The DNA was then transferred overnight to a Genescreen Plus charged nylon membrane under alkali conditions, cross-linked to the membrane with ultra-violet light and exposed to a radioactive probe. A 0.9kbp probe generated by PCR from the genomic subclone was used to identify the EcoRI fragment targeted by the construct; primers: fwd(1438-54) 5′-CCT GTG CCT CAG TGC TCA CCT CAT GC, rev(1438-57) 5′-GGG GTG TGG GTG TCT CTG GGG TTG GG-3′; 62° annealing, 30 cycles. The probe hybridized to a region upstream of the SA of the construct. The probe was labelled by random priming (Multiprime DNA Labelling Kit, Amersham) with ^{32}P αdCTP. Probing of wild-type DNA identified a band of approximately 9.4 kbp.

Homologous recombination of the targeting vector was predicted to shorten the EcoRI band by 2.6 kb from that of the wild-type (Figure 10). Homologous recombinants were confirmed to contain a single integration of the construct by probing with a fragment of *neo*.

Figure 10. clAP2 wild-type and mutant loci

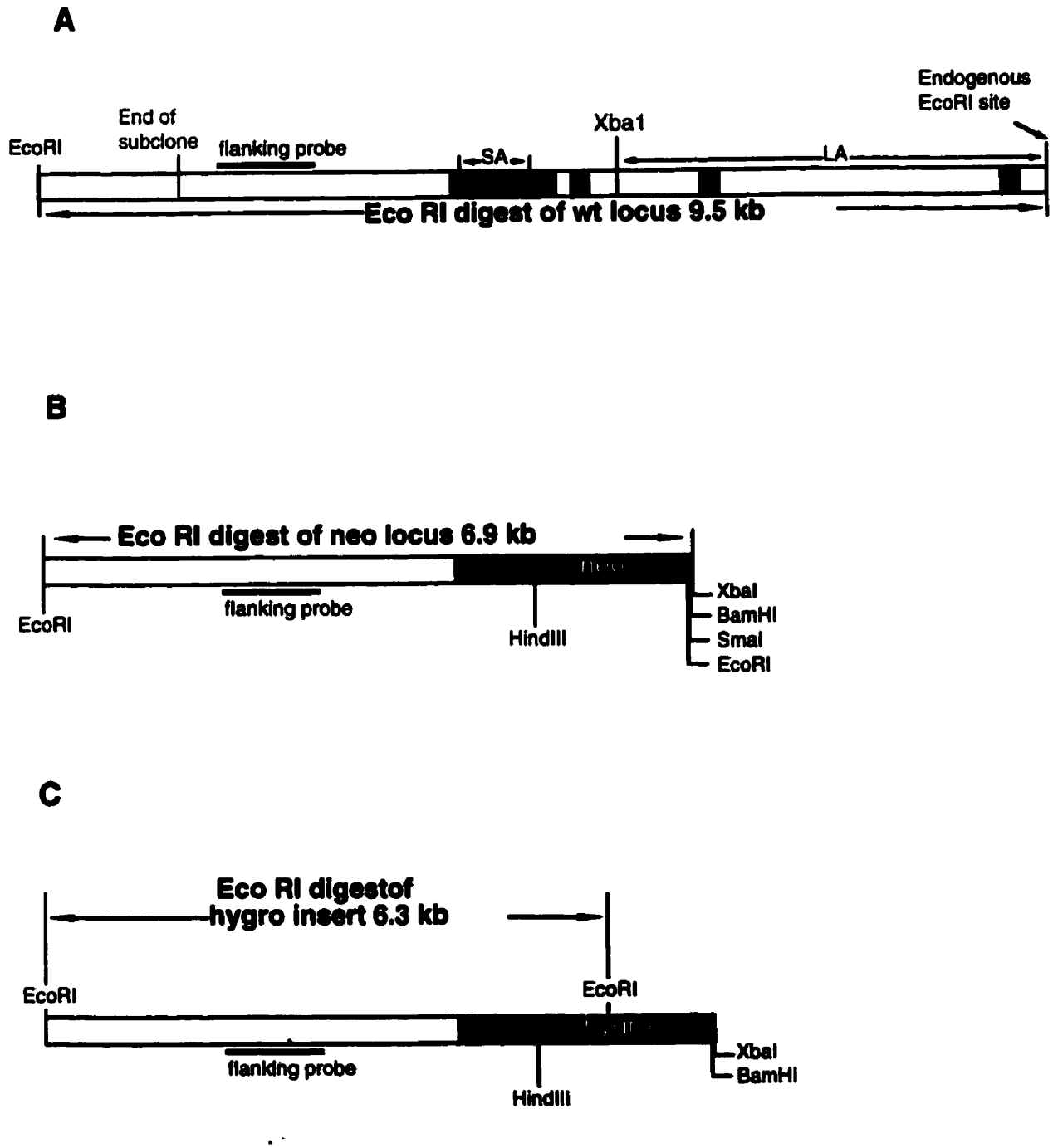


Fig. 10 A: Wild-ype locus contains a 9.5 kbp Eco RI fragment. identified by a labelled flanking probe. B: The neo targeting vector introduces an Eco RI restriction site which reducse the size of the fragment identified by the flanking probe from9.5 kb in the wild-type locus to to 6.9 kb in the mutant locus. C: The hygro targeting vector introduces an Eco RI site which reduces the fragment identified by the flanking probe to 6.3kbp.

Recombinant ES clones were injected into day 3.5 blastocysts harvested from C57BL/6 females and implanted into CD1 pseudopregnant mice. The chimeras produced were bred with C57BL/6 mates. The chimeric mice contain tissue that is from the original C57BL/6 blastocysts as well as from the injected 129J ES cells. Germ cells from the chimeras, however, must come from either one or the other source. The source is detectable once the offspring of the chimera grow hair.

Mice of the 129J strain from which the ES cells came are agouti in coat colour. This is a dominant trait, so coat colour is used to identify the offspring of chimeras which are descendants of the 129J ES cells from the chimeras. Agouti coloured offspring were typed by Southern blot as described above or by PCR with the same conditions used to screen the ES colonies.

Mice that were heterozygous for the cIAP2 gene were bred together in an attempt to obtain cIAP2 homozygous mutants (*cIAP2-/-*). The offspring or embryos from these breedings were typed either by Southern blot as above or by PCR. No pups were born that genotyped as *cIAP2-/-*, therefore it was required to genotype embryos. Detection of the loss of the wild type allele by Southern blot could not be done with embryos younger than d9.5 due to the small quantity of tissue.

For these early embryos the only test possible to confirm a homozygous mutant genotype was PCR testing to show the presence of the mutant allele (using the primers described above) in conjunction with PCR tests which failed to show the presence of the wild-type alleles. The following primers were used to test for the presence of the wild-type allele: fwd (1598-31) 5′-AGG CAT TTC CCC AGC TGT CCG TTC TTA A-3′ and rev(1598-32) 5′-CCT CAG CCC ACC ATC ACA GCA AAA ACA C-3′; 59° annealing, 35 cycles. These primers identify a portion of exons 2 and 3 that are absent in the mutant allele (Figure 7). The PCR reaction produces a 322 bp product in wild-type and heterozygous mice. There is no homology between the fwd primer 1598-31 and cIAP1, ensuring that the cIAP1 gene can not act as a template for the reaction to produce a false positive result.

Analysis of RNA expression of cIAP2 in early embryo

Northern blot:

Whole RNA from ES cells, E8.0, E9.0 and adult spleen was the gift of Jose de la Pompa. RNA was electrophoresed in a gel contained 1% agarose, 1xMOPS and 5.6% Formaldehyde. For each sample 20μg RNA was denatured at 65° and loaded into each well. Electrophoresis and transfer

to Hybond N^+ filter was done according to standard procedure. The SA was used to probe the blot according to standard procedure.

RT-PCR:

RT-PCR primers were selected from exons 2 and 3 of *CIAP2*. Fwd (1689-69): 5′-CAC GCA GCC CGT ATT AGA ACA TTC TC-3′; rev (1697-04): 5′-CAT CTC CAG ATT CCC AGC ACC TCA G-3′. PCR product from a cDNA template was predicted to be 157bp; from a genomic template 257bp. cDNA was reverse transcribed using the Ready to Go kit with total RNA isolated from 129J ES cells using TRIZOL (GibcoBRL). ES cells are typically grown on a layer of embryonic fibroblast (EF) cells. In this case the ES cells were grown and passaged approximately 10 times on gelatin plates before RNA isolation, to ensure that no EF cells contributed RNA.

Growth of blastocysts

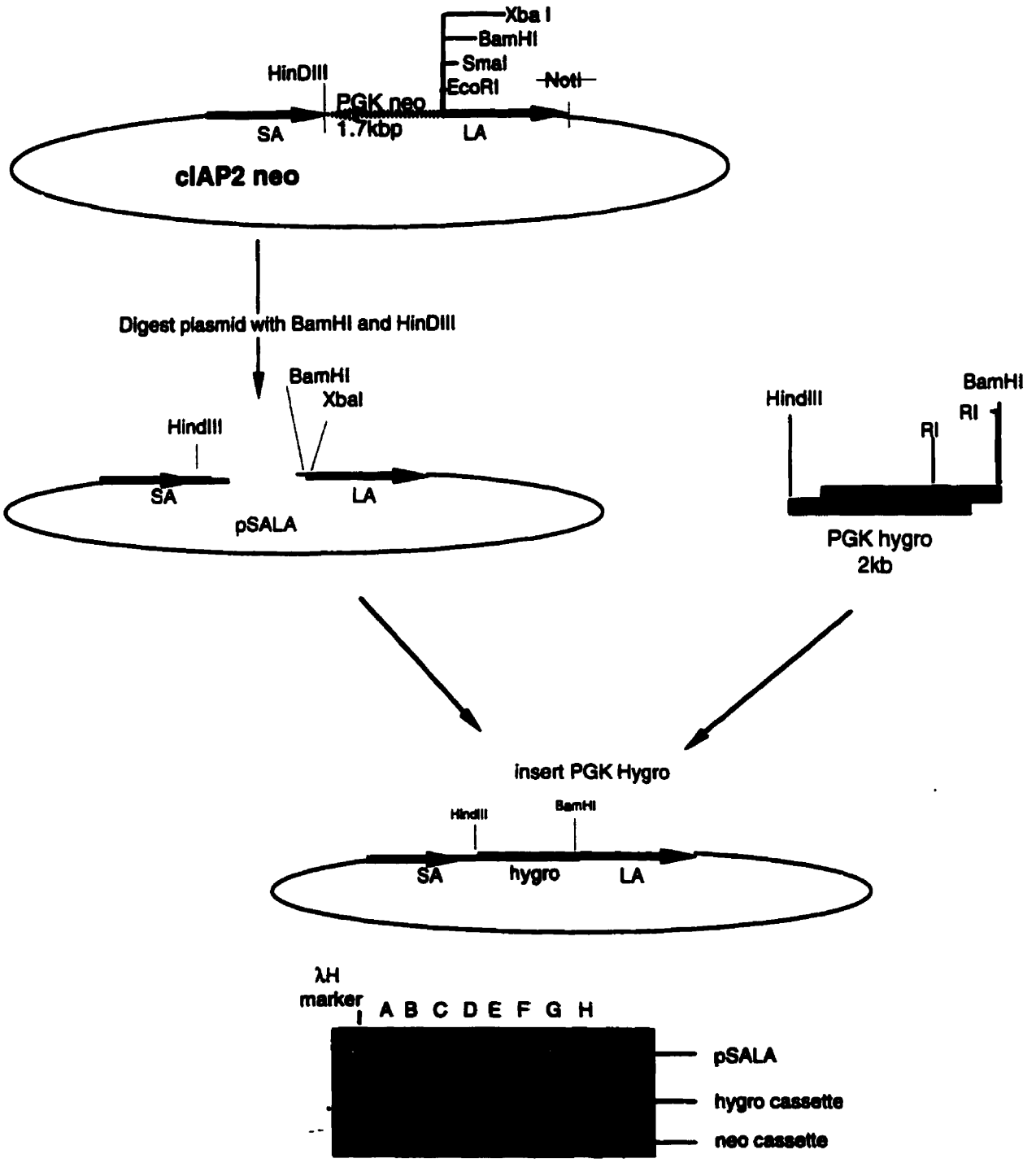
Blastocysts and morula were obtained by flushing the uteri of pregnant heterozygous females approximately 3.5 days following matings with heterozygous males. Embryos were grown in ES media on 24 well plates covered in 1% gelatin, in the absence of leukemia inhibitory factor (LIF). Embryos which adhered to the plates and continued to grow after 9 to 13 days were typed by PCR.

Generation of a targeting vector containing *hygro*

It was expected that the loss of cIAP2 might be compensated for by the presence of cIAP1, due to the similarity of structure and expression patterns of the two genes. Therefore a second project was begun to generate a mouse missing both cIAP1 and cIAP2 by making an ES cell line containing mutations in both genes. It was necessary to mutate both genes within a single ES cell clone rather than breed the two strains of heterozygous mice together because the two genes are located on the same chromosome and are only 24kbp apart (Figure 7).

David Ferrick, a former post doctoral fellow in our lab, had already generated an ES cell line containing a targeted disruption of the cIAP1 gene. This cell line had been employed successfully to produce germ-line transmission of the *cIAP1* targeted mutation. Because the *cIAP1* ES clone was originally transfected with *neo* for selection in the antibiotic G418, a second system of selection was necessary for this new construct. PGK-*neo* was removed from the *cIAP2* targeting construct described above using a double digest of BamHI and HindIII. The hygromycin resistance gene was then ligated into the plasmid, confirmed by restriction digest (Figure 11). The mutant *cIAP1* ES cells were then transfected with the new construct and selected over 10 days in ES media containing 150µg/ml of hygromycin, instead of G418.

Figure 11. cIAP2 hygro construct made using the cIAP2 neo construct



A. Neo released from cIAP2 targeting construct using BamHI HindIII
B. Hygro gene released from pBS hygro plasmid using BamHI HindIII
C-H. Putative cIAP2 hygro plasmids digested with BamHI and HindIII

Fig. 11 Replacement of neomycin gene with hygromycin gene in cIAP2 targeting vector. Photo insert lane A shows Hind/Bam digest releases neo from original vector. Lanes C-H show Hind/Bam digest of new construct, releasing the hygro cassette.

Selection of *cIAP2-hygro* homologous recombinants

ES clones with the homologous integration of the *cIAP2-hygro* targeting construct were detected using the same procedure as that used to identify *cIAP2-neo* clones, with the following changes: for PCR detection of homologous recombination of the *cIAP2-hygro* targeting construct into the *cIAP2* locus the following primers were used: fwd (1605-97) GAG TGC TGA CAC CTT TGA GTT GAA AT; rev (1613-07) GTG GGA TTA GAT AAA TGC CTG CTC TT. The forward primer is the same flanking primer described above for the original cIAP2 selection. The reverse primer identifies a portion of the hygromycin resistance gene that has no homology to the neomycin resistance gene. The PCR product of the recombinant locus was predicted to be 819 base pairs long.

Detection of homologous recombination by Southern blot assay was performed as before with the *cIAP2-neo* construct: DNA was digested with EcoRI, run on a 0.7% agarose gel overnight, denatured and transferred to a Genescreen Plus charged nylon membrane. The same flanking probe described above was used to identify the target band. Homologous integration of the *cIAP2-hygro* construct was predicted to introduce an EcoR I restriction site into the cIAP2 locus that would make a recombinant fragment 3.2 kb shorter than the wild-type fragment.

Confirmation that the targeted disruption of cIAP2 was on the same chromosome would have to wait until the generation of F1 mice.

RESULTS

cIAP1 and *cIAP2* expression in early embryogenesis

Northern blot analysis confirmed low but detectable levels of *cIAP2* expression in ES cells, and higher levels in day 8 and day 9 embryos. RT-PCR of whole RNA also confirmed the expression of *cIAP2* in ES cells, as well as expression of cIAP1 (Figure 12).

Confirmation of homologous recombination

Three ES cell clones were shown to contain a single integration of *neo* at the *cIAP2* locus after transfection with the *cIAP2 neo* construct (Figure 13). Of these three, it was assumed clones 1F5 and 1F6 were originally from a single clone: it was clear by their clone numbers that they had been picked consecutively, and so they were originally adjacent or overlapping on the tissue culture plate. Therefore only 1F5 and 5D7 were injected into blastocysts.

Clone 1F5 produced chimeras but they generated only wild-type agouti pups. DNA from 20 agouti pups was tested repeatedly with a number of PCR primers and Southern conditions, but failed to show the presence of the mutant locus. The other clone, 5D7, produced 2 chimeras which

Figure 12. wild-type expression of cIAP2 RNA in early embryonic tissue

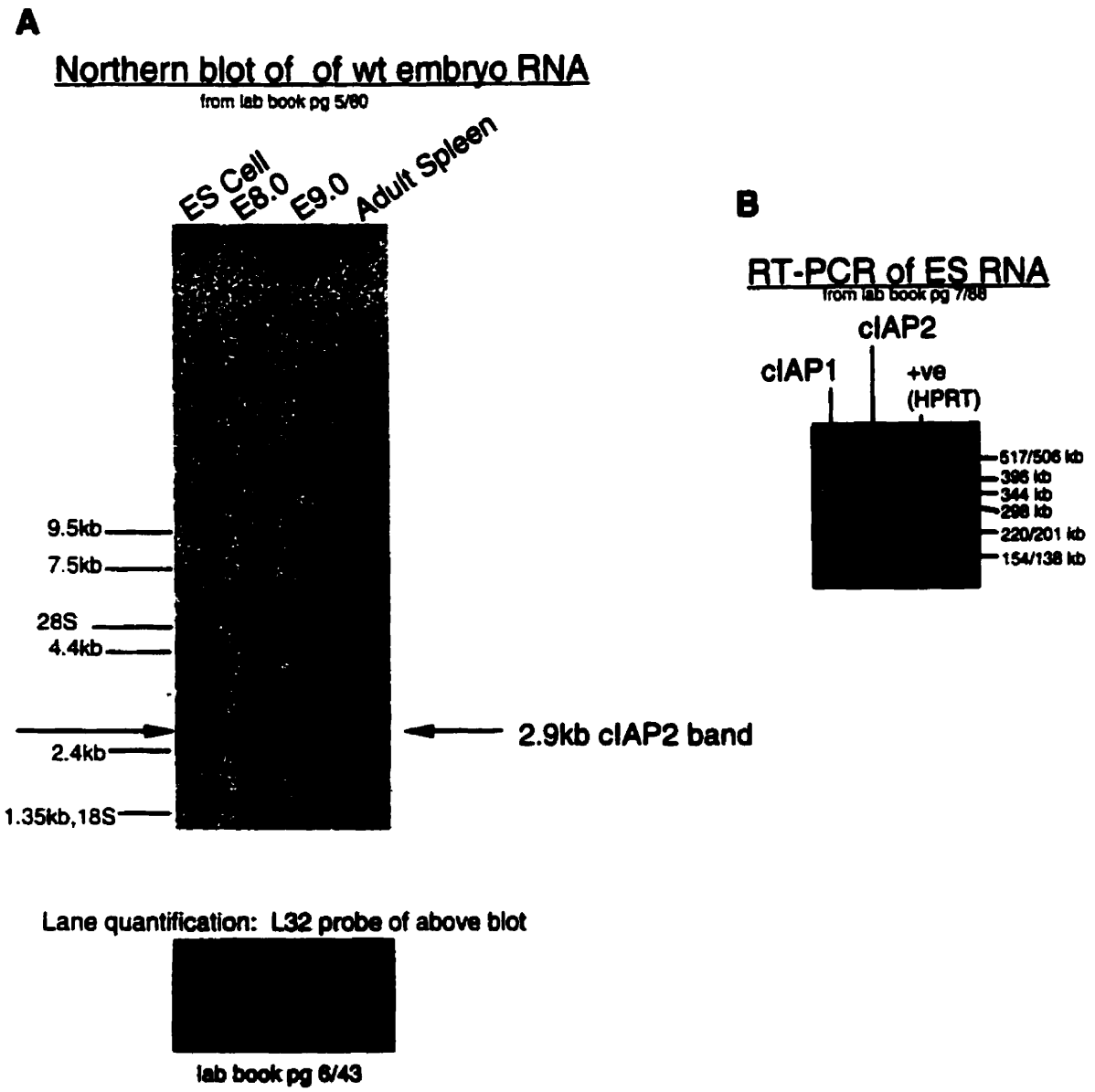
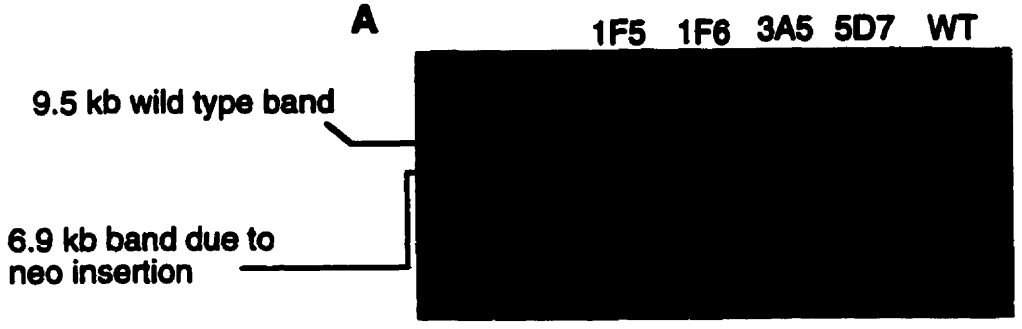


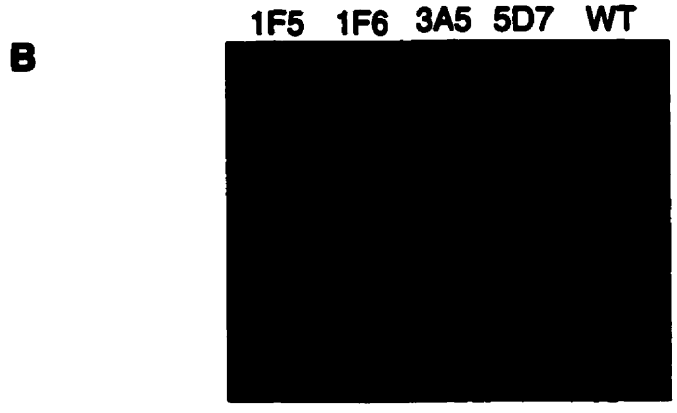
Fig. 12 A: Northen blot analysis of embryonic tissue shows cIAP2 mRNA is present to some degree in all stages of development tested. B: RT-PCR also demonstrates presence of cIAP2 mRNA, as well as cIAP1 mRNA in ES cells.

Figure 13. Southern blot confirms homologous recombination

from lab book pgs 5/48, 5/56



4 clones probed with clAP2 genomic flanking probe



Same blot as above probed with neo gene

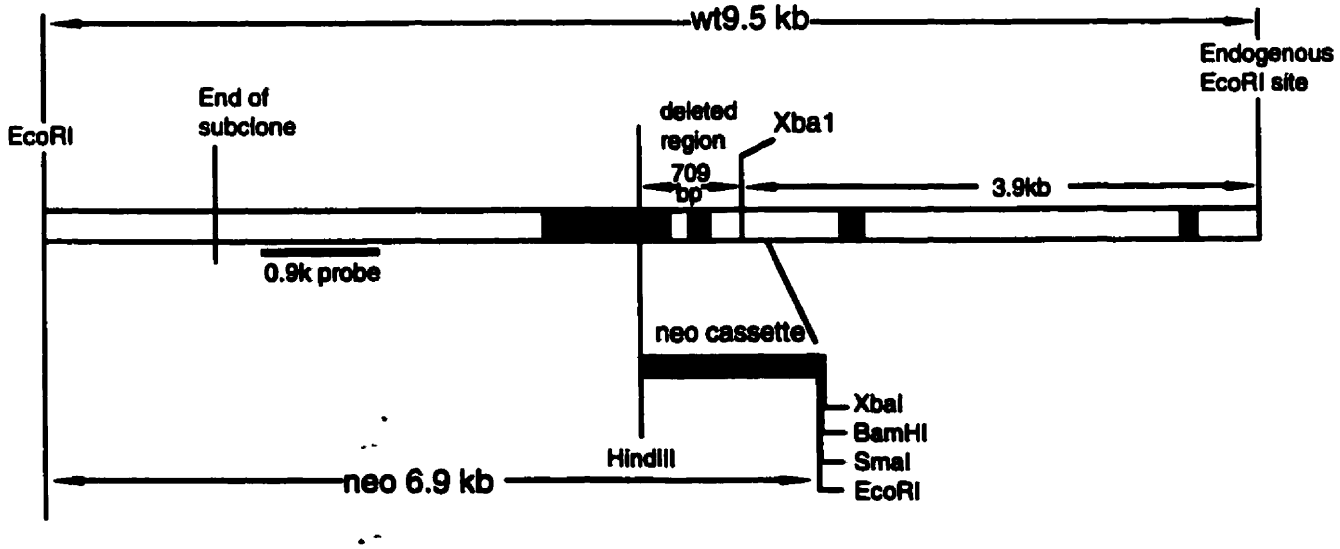


Fig. 13 A: clAP2 flanking probe of EcoRI digested DNA from wild-type and mutant ES cells show three mutant clones, 1F5, 1F6 and 5D7 contain the predicted change of restriction fragment size. B: Probing with the neo gene confirms all three of these clones contain only one integration of the targeting vector.

generated heterozygous agouti pups, clearly shown by Southern blot (Figure 14). It should be noted that the EcoR1 digests of the tail DNA always produced a large non-specific band, in addition to the expected wild-type band. However, since this large band was found to appear in wild-type animals as well as heterozygous mutants it was not seen as an indicative of any mis-integration of the construct. This band was not seen in DNA from wild-type or mutant ES cell lines and rarely seen in DNA from wild-type or heterozygous embryos, and therefore was assumed to be an artifact of incomplete digestion of tail DNA.

Selection for cIAP2 homozygous mutant ES cells was unsuccessful

An attempt to make 5D7 cIAP2 homozygous mutant ES cells by exposing cells to different amounts of G418 failed to select for any survivors. 4.0 mg/ml of active G418 killed all cells, and lower concentrations failed to select for homozygous mutant cells, according to PCR analysis.

ANALYSIS OF THE MICE

Viability of *cIAP2* +/- mice

The first of the cIAP2 heterozygous pups has survived over 1 year. Over 100 heterozygous mice have been born, and no sign of disease or

Figure 14. Southern blot of DNA from F1 agouti mice born of clAP2 chimeras

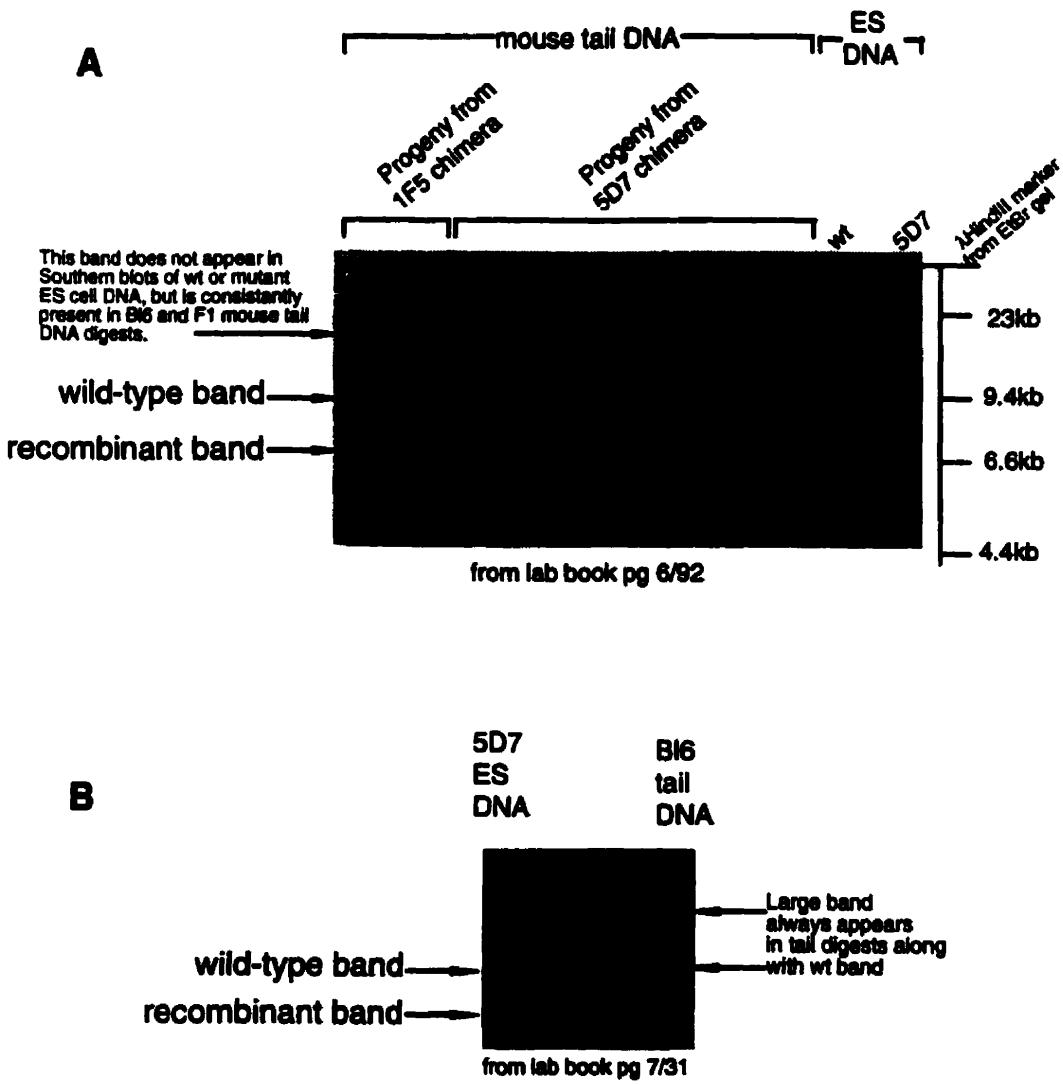


Fig. 14 A: Southern blot of agoutis offspring from 1F5 chimera and 5D7 chimera. Note there is no recombinant band for the two 1F5 mice. B: Southern blot of Bl6 mouse DNA. Note large band appears in DNA from wild-type animals and therefore is not an artifact of the transformation.

phenotype is evident with any of them, suggesting that a single copy of the intact cIAP2 gene is sufficient for normal function.

Requirement for cIAP2 in early embryogenesis

Breeding of heterozygous mice produced no homozygous mutant offspring. Typing of embyros from day 13.5, 9.5, 8.5 and 6.5 also revealed no homozygous mutant offspring (Table 2). Backcrossing the mice several generations also led to no homozygous offspring from heterozygous parents (Table 3).

Over 300 offspring have been typed. The ratio of heterozygous pups and embryos to homozygous pups and embryos is approximately 2:1 (211:95). This suggests the mutant allele can come from either sperm or egg. If the loss of cIAP2 in oocytes prevented oogenesis then the ratio of heterozygous pups to wild-type pups would be expected to be 1:1.

The lack of *cIAP2* homozygous mutants indicates that the gene is required for embryogenesis. Preliminary studies with E13.5 litters and E9.5 litters suggest the lethality occurs earlier, since no homozygous mutant embryos were found at these time-points. For embryos younger than E9.5 there is not enough DNA to do a Southern blot, so genotyping must rely on PCR analysis. PCR typing of tail DNA using the primers previously described

Table 2. Typing of cIAP2 hetero-hetero progeny (5D7 clone)

	total typed	+/+	+/-	-/-	resorbtions
live pups (15 litters)	111	43 39%	68 61%	0 0%	N/A
e13.5	14	4 29%	10 71%	0 0%	8
e9.5	18	6 33%	12 66%	0 0%	2
e8.5	41 (PCR)	11 27%	30 73%	0 0%	10
e7.5	40 (PCR)	14 35%	26 65%	0 0%	0
e6.5	82 (PCR)	17 21%	65 79%	0 0%	5
Total	306	95 31%	211 69%	0 0%	
cultured blastocysts	62 (PCR)	15 24%	47 76%	0 0%	N/A

Table 3. Genomic background of progeny of cIAP2 5D7 hetero-hetero breeding

Live pups

Litter#	background	#of -/-
19	BL6 F1	0/11
23	BL6 F1	0/4
24	BL6 F1	0/8
25	BL6 F1	0/10
33	BL6 F1	0/9
34	BL6 F1	0/8
35	BL6 F1	0/8
36	BL6 F1	0/10
38	BL6 F1	0/9
43	BL6 F1	0/17
44	BL6 F1	0/5
45	BL6 F1	0/4
55	BL6 F1	0/1
56	BL6 F1	0/5
57	BL6 F1	0/2
		Total: 0/111

embryos

name	Background	# of -/-
1a-10a	BL6 F1	0/10
1-9	BL6 F1	0/9
10-20	BL6 F1	0/11
20-22	BL6 F1	0/2
23-34	ICR F1	0/12
35-42	BL6 F1	0/8
43-51	BL6 F1	0/8
52-59	BL6 F1	0/7
61-68	ICR F1	0/7
69-79	BL6 F2	0/11
80-90	ICR F1	0/11
90a-96	BL6 F1	0/7
97-100	BL6 F1	0/4
101-109	BL6 F1	0/9
110-120	ICR F1	0/10
121-126	BL6 F2	0/5
127-134	BL6 F1	0/8
135	BL6 F2	0/1
136-144	ICR F1	0/9
146-155	BL6 F1	0/10
156-163	BL6 F2	0/8
164-173	BL6 F4	0/10
174-185	BL6 F3	0/12
	Total:	0/195

Table 3: The original germcells came from 129J. Backcrosses indicate the number of generations the mice have been bred into other strains, either C57BL/6 or ICR

reliably correlates with typing by Southern blot, and regularly produces clear and unambiguous results (Figure 15).

Eighty-two E6.5 embryos were analyzed. There were some variations in their stages of development, but otherwise all embryos observed looked healthy and normal. Notably, only 5 empty decidua were found in the uteri of the 11 females killed at day 6.5 of pregnancy. If the -/- embryos had successfully implanted into the uterus and then died before day 6.5, 25% of the decidua would be expected to be empty. Instead only 5/87, or 6%, were empty, suggesting the -/- embryos failed to implant, an event which normally occurs at between day 4.5 and 5.0 of embryogenesis.

Analysis of blastocyst cultures from heterozygous matings produced ambiguous results

Sixty-two blastocysts were successfully cultured, meaning they adhered to the plates, developed trophoblast giant cells around their base, and grew up into the media to form embryoid bodies within 9 or 10 days. An additional 17 blastocysts which did not grow into embryoid bodies could not be typed. Some failed to adhere or grow at all, whereas others adhered but remained extremely small (Table 4, figure 16).

Figure 15. Example of PCR test of pups from clAP2 hetero-hetero crosses

data from Typing Book pg 24

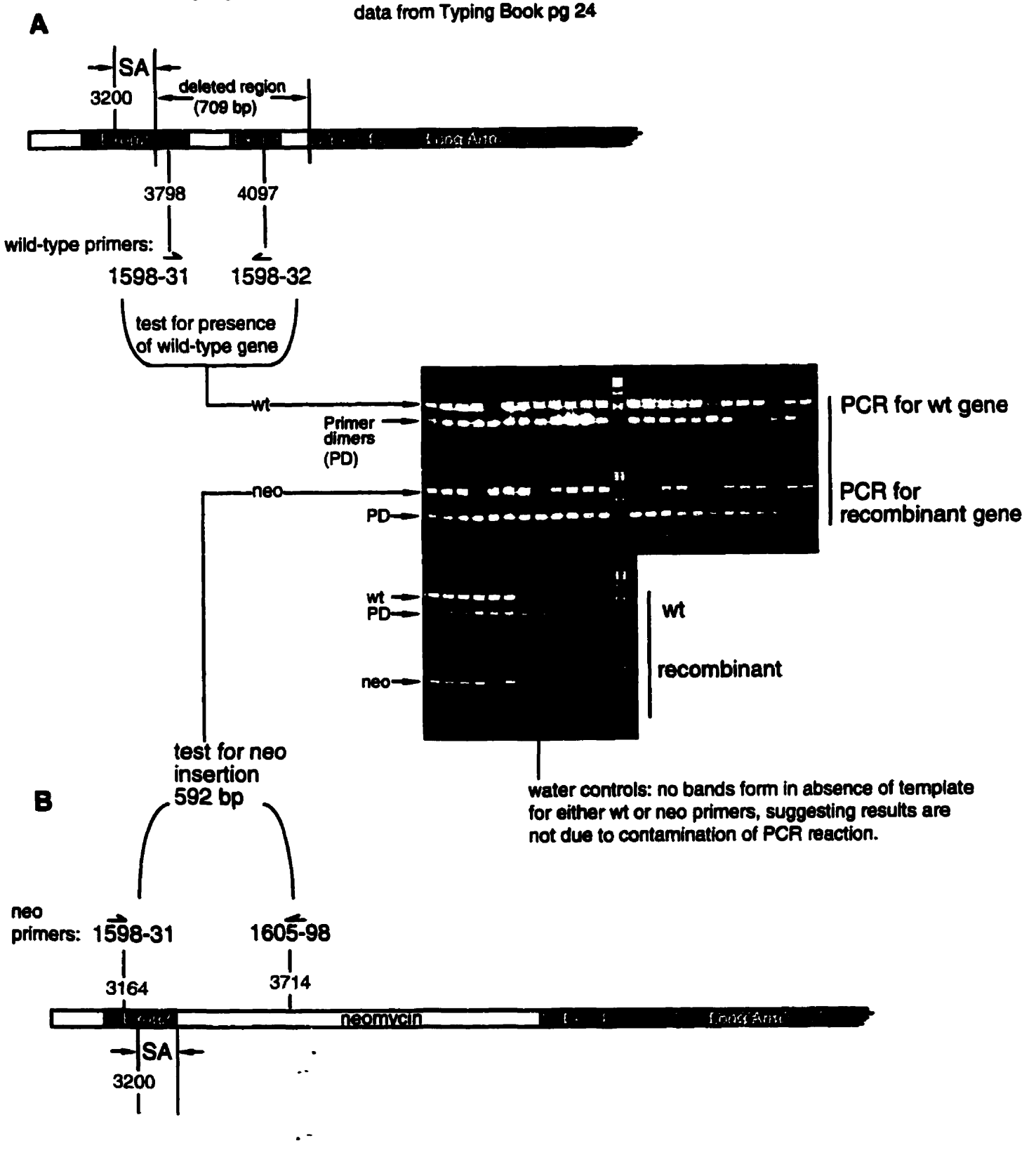


Fig. 15 A: Primers targeting the delete portion of the clAP2 mutant locus are used to identify mice containing a wild-type copy of clAP2. B: Primers that target a portion of the neo gene identify offspring that contain the mutant locus.

Table 4: Results of blastocyst platings

Page	plate	normal size	abnormally small	failed to adhear or progress beyond blastocyst stage (not typed)
7/64	1	6	1?*	0
7/64	2	6	1?	0
7/65	3	6	0	0
7/66	4	8	1?	2
7/68	4a	4	2??	1
7/68	4a	1	5???	1
7/69	4a	8	0	0
7/70	4a	4	1?	2
7/70	5	6	0	1
7/73	5	5	2??	2
7/73	6	4	2?	0
		61	15	9

*?=could not be typed

Total # of blastocysts grown in culture:	85
Number that failed to hatch:	9
Number that hatched but were extremely small after 9 or 10 days:	15
Number of small embryoid bodies that could not be typed	12

Table 4. Phenotypic analysis of embryos removed at the blastocyst stage from cIAP2 hetrozygous matings. Wild-type embryos typically hatch out of the zona pellucida surrounding the blastocyst around day 4.0 or 4.5.

Figure 16. Healthy blastocyst culture vs. abnormally small culture

typical size of +/+ or +/- after 9 days

abnormally small after 9 days

Fig. 16 Typical examples of healty blastocyst cultures after 9 or 10 days of growth vs. those cultures which did not grow and could not be typed.

TRANSFECTION OF cIAP1 ES CELLS WITH *cIAP2-neo*

Several chimeras were produced

Transfection of the *cIAP1* mutant ES cells with the *cIAP2-hygro* construct resulted in 5 clones that contained a single integration targeted to the *cIAP2* locus, as determined by Southern blot (Figure 17). Probing with the hygromycin resistance gene confirmed for all five clones that the construct had integrated into the genome only once. All five clones were injected leading to the production of chimeric mice from each. None of the chimeras has produced agouti pups (Table 5).

Figure 17. Clones made from transfection of cIAP1 ES cells with cIAP2-hygro

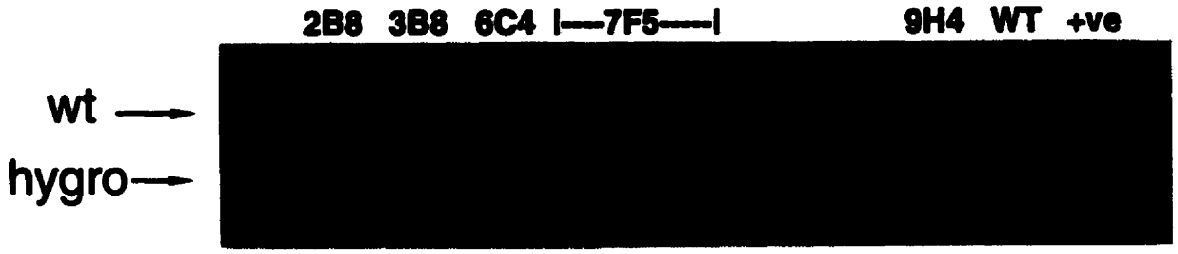


Table 5. Results of injections of cIAP2-hygro ES clones

Injected clones:	Litters:
2B8: 1 chimera	5 all black
3B8: 1 chimera	12 all black
6C4: 11 chimeras	17 all black
9H4: 3 chimeras	7 all black
7F5: 3 chimeras	4 all black

Fig. 17 DNA from five clones show homologous recombination of hygro targeting construct when probed with flanking probe. Probe with hygro demonstrated each contained a single integration of the hygro gene (not shown).

Table 5 All five clones injected produced chimeras, however none of these chimeras produced agouti pups: the mutant ES cells failed to produce viable germline cells.

DISCUSSION

Lack of *cIAP2-/-* embryos

One hypothesis for the absence of any *cIAP2* homozygous mutant embryos is that in normal embryogenesis the activity of the caspases is critically inhibited by cIAP2. Loss of cIAP2 may reduce the threshold for stimuli that triggers a caspase cascade, leading to cell death. Northern blot and RT-PCR analysis of the ES cell and embryo RNA indicates that *cIAP1* and *cIAP2* are expressed very early in embryogenesis, allowing for this possibility. *In vitro* data have shown that cIAP2 is a direct inhibitor of caspase-3 and caspase-9 (Deveraux, et al., 1998). As well, caspase-3 and caspase-9 have been shown to be expressed in early embryogenesis (Hakem, et al., 1998, Woo, et al., 1998). All of this is consistent with the possibility that cIAP2 is a necessary inhibitor of caspases in early embryogenesis.

An alternative hypothesis is that cIAP2 has an unknown function that is essential for embryogenesis. In either case the early lethal phenotype in mice due to cIAP2 loss is in strong contrast to the phenotype of other murine IAP gene mutants. The loss of cIAP1 produced a very weak effect (Albert, 1998), and the ablation of XIAP in mice (generated in the lab of Craig B. Thompson but not published) produced no detectable phenotype at all. The crossing of these two strains to create a double mutation of *cIAP1-/-* and *XIAP -/-* has also produced no obvious phenotype in the mice (Albert,

1998). On the other hand the loss of the drosophila gene, *diap1*, like cIAP2 ablation in mice, produces an early embryonic lethal phenotype. All of the cells in the early embryo undergo apoptosis in the *diap1-/-* fly (unpublished data from the lab of Hermann Steller (cited by Raff, 1998)).

A third possibility that must be considered is that the embryonic lethal phenotype is an artifact. It is possible that a random genetic mutation in the 5D7 mouse ES clone from which all of the data are derived is responsible for the lack of -/- mice or embryos. Attempts to generate mice from a second independent *cIAP2* +/- ES cell line were unsuccessful. A second cell line would be necessary to confirm that the lethal phenotype is in fact due to the loss of *cIAP2*. If there is a lethal mutation of an unknown gene then the gene must be on the same chromosome as *cIAP2*, since the phenotype is linked to the *cIAP2* locus and appears to track through at least four generations of backcrosses.

The purpose of the *cIAP2-hygro* gene targeting of a *cIAP1* disrupted ES cell line was twofold. In the case that *cIAP2* ablation produced no phenotype it would have explored the possibility that the two genes were redundant. The second purpose, however, was to generate a second ES cell line containing just the *cIAP2* disruption, to confirm whatever phenotype was associated with *cIAP-/-*.

In the absence of a strain derived from a second cell line, another test, less conclusive, is to continue to breed the *cIAP2* mutation back into a healthy strain of mice, to separate the *cIAP2* mutation from the putative unknown mutation. The problem with this approach, however, is that the two genes might be close together on chromosome 9A. Close proximity reduces the chances that the two genes would be separated during chromatid exchange in cells undergoing meiosis.

The mice have been back-crossed into the BL6 background for four generations. None of the of these F4's or the F3's produced any -/- embryos. Of course this does not prove that there is no second mutation, since a second mutation could be so close to the cIAP2 locus that several more back-crosses to separate the two genes are required.

Lack of *cIAP2*-/- ES clones after high G418 selection

cIAP2+/- ES cells were exposed to increased G418 concentrations with the aim of deriving -/- ES cells by gene conversion. The failure to produce *cIAP2*-/- ES cells may have been due to an inability of the ES cells to survive in the stressful environment of high antibiotic levels once they had lost the second *cIAP2* allele. Potentially the loss of an inhibitor of the caspases renders cell hypersensitive to the stress of the G418 or the stress of growth in culture.

In support of this possibility is the finding that attempts to generate -/- ES cell lines from heterozygous FLIP ES cells by G418 gene conversion have failed (Ite, 1999). FLIP is an inactive homologue of caspase 8 that can act as a decoy, reducing the availability of sites for caspase 8 to bind to at the DISC of activated receptors (Scaffidi, et al., 1999). In the case of both cIAP2 and FLIP the inability to generate -/- ES cell lines could be due to increased caspase activity with the loss of the gene.

Contrast with other pre-implantation embryonic lethal mutations

Several mouse strains have been developed that result in pre-implantation embryonic lethal phenotypes (Copp, 1995). These include the βS12 transgenic line (Cheng and Costantini, 1993), the *E-cadherin* -/- mouse (Larue, et al., 1994), the *fibroblast growth factor receptor (FGFR) 2*-/- mouse (Arman, et al., 1998), All three of these result in a pre- or peri-implantation embryonic death that does not involve the normal house-keeping function of the cells of the embryo.

The βS12 transgenic mouse line contains an insertion of multiple copies of the human β-globin gene. The insertion results in a deletion of approximately 2 centimorgans of mouse DNA from chromosome 1. The loss of a gene or genes from this region results in the *morula*

decompaction (*mdn*) phenotype. Embryos undergo normal development to the 8-cell stage, they compact and form a normal morula after 48 hours, but then decompact, failing to form a blastocyst over the next 24 hours (Cheng and Costantini, 1993). In normal embryos compaction would be followed by differentiation into the two cell types of the blastocyst: those of the trophoectoderm and those of the inner cell mass (ICM).

E-cadherin is a cell adhesion protein expressed in the early embryo. The *E-cadherin* -/- mouse embryo compacts normally at the 8 and 16 cell stage. Following this, however, the -/- embryo decompacts, fails to develop to the blastocyst stage and cannot hatch out of the zona pellucida, the membrane surrounding the early embryo. However, cells are still viable: individual cells removed from the zona pellucida will adhere to a feeder layer and develop into trophoectoderm giant cells (Larue, et al., 1994).

The fibroblast growth factor receptor (FGFR) 2 is involved in cellular proliferation and differentiation in embryogenesis and in the adult animal. *FGFR2-/-* embryos appear normal as blastocysts. *In vivo* the embryos implant into the uterus but fail to trigger deciduization. *In vitro* the embryos hatch from the zona pellucida and can adhere to culture plates. Trophoectoderm giant cells grow but the ICM cells fail to develop and eventually disintegrate (Arman, et al., 1998).

-/- embryos may not have developed beyond blastocyst stage

In each of these cases of a pre- or peri-implantation lethal phenotype the embryos fail to develop normally *in vitro*. It would be expected that the *cIAP2-/-* blastocysts would also fail to develop normally *in vitro*, since they failed to trigger deciduization *in vivo*. Seventy-eight blastocysts were plated, but seventeen of these cultured blastocysts were too small to be typed. It could be that most or all of these blastocyst that either failed to hatch, or failed to grow once they did hatch, were the *cIAP2-/-* embryos, and that like the *E-cadherin-/-* embryos they could not progress beyond the blastocyst stage.

Future work is required to confirm or disprove phenotype

It would be possible to further analyze early embryos from the existing 5D7 strain. Careful PCR typing of the blastocysts from the hetero-hetero matings may confirm that -/- embryos remain normal to that stage of development. The strain should also be further backcrossed to confirm that the phenotype is not due to a hidden gene linked to *cIAP2*. As well the existing strain could be crossed with strains containing disruptions in the apoptotic machinery, such as caspase-3, caspase-9, or Apaf-1 +/- mice. The loss of one or more of these genes may delay death of the *cIAP2-/-* embryo to a later stage of development. This could allow for the establishment of a double mutant cell line, which could lead to a wealth of

experiments. Most importantly, however, it is essential that a second strain of mice be established from an independent *cIAP2+/-* ES clone, to confirm this surprisingly powerful phenotype of very early embryonic lethality in the *cIAP2* -/- embryo.

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