

# Identification of Radiation-Responsive Genes *In Vitro* Using a Gene Trap Strategy Predicts for Modulation of Expression by Radiation *In Vivo*

Katherine A. Vallis,<sup>a,b,d,1</sup> Zhuo Chen,<sup>d</sup> William L. Stanford,<sup>e</sup> Mei Yu,<sup>d</sup> Richard P. Hill<sup>a,b,d</sup> and Alan Bernstein<sup>c,e</sup>

<sup>a</sup> Departments of Radiation Oncology, <sup>b</sup> Medical Biophysics and <sup>c</sup> Molecular and Medical Genetics, University of Toronto; <sup>d</sup> Division of Experimental Therapeutics, Ontario Cancer Institute; and <sup>e</sup> Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada

---

Vallis, K. A., Chen, Z., Stanford, W. L., Yu, M., Hill, R. P. and Bernstein, A. Identification of Radiation-Responsive Genes *In Vitro* Using a Gene Trap Strategy Predicts for Modulation of Expression by Radiation *In Vivo*. *Radiat. Res.* 157, 8–18 (2002).

A large number of genes are known to be responsive to ionizing radiation, and there is strong evidence for the existence of inducible radiation resistance in mammalian cells. We have developed a gene trap insertional mutagenesis strategy to identify novel genes involved in responses to radiation. Using this approach, we have isolated four gene-trap integrations in embryonic stem cells. In three cases (9A, 3E and 9H) the trapped genes are radiation-inducible, and in one (7D) the gene is down-regulated. Sequence analysis of fusion transcripts from three of the integrations indicate one novel gene (3E), the mouse homologue (9A) of a known but uncharacterized human gene that encodes a protein with significant homology to several GTPase-activating proteins and a murine locus, *Mym* (9H). The embryonic stem cell clone with the 9A insertion was introduced into the mouse germline, and the *in vivo* expression pattern of 9A was studied in detail. A unique, spatially restricted pattern of expression in embryos and adult animals was observed. There is tissue-specific *in vivo* induction of the 9A gene in adult mice by radiation. This study demonstrates the potential of the gene trap approach for the identification and functional analysis of novel radiation-regulated genes. Similar strategies may facilitate the discovery and characterization of genes involved in other cellular stress responses. © 2002 by Radiation Research Society

---

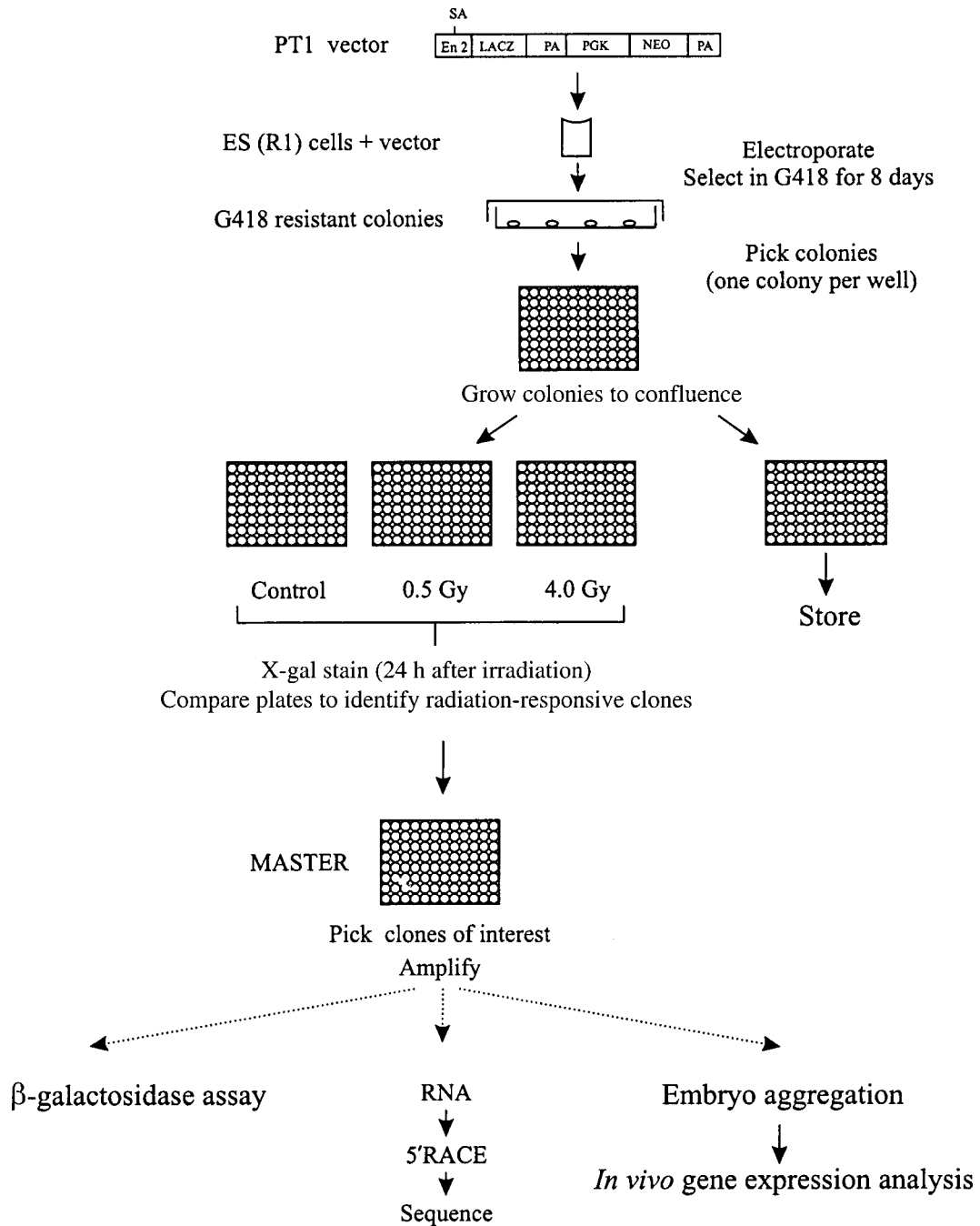
## INTRODUCTION

Ionizing radiation is one of the main treatment modalities used in the management of cancer. Thus there is considerable interest in understanding the cellular response to DNA-damaging agents, particularly because the ability to deliver

a curative dose of radiation is frequently limited by the adverse reaction of normal tissues within the radiation treatment field. One approach to this problem is to understand the molecular mechanisms underlying the radiation responses of normal tissue so that critical molecular pathways can be manipulated to improve the therapeutic ratio and hence the chance of cure. It is well established that cells adapt to environmental challenges such as radiation by activating co-regulated groups of genes that affect a variety of cellular functions (1). DNA damage results in alteration of the expression of mammalian genes involved in regulating progression through the cell cycle, apoptosis, DNA repair and maintenance of cellular architecture [reviewed in ref. (2)]. For example, the tumor suppressor gene, *TP53*, encodes a sequence-specific DNA-binding protein that regulates expression of a number of genes including the cell cycle inhibitor *CDKN1A* (also known as p21<sup>waf1/cip1</sup>), which regulates the cyclin-CDK complexes required for progression of cells from G<sub>1</sub> to S phase.

Genes involved in determining the biological outcome after radiation exposure often act in a tissue- and cell-specific manner (3–5). This heterogeneity of genotoxic stress responses has recently been highlighted in a report by Amundson *et al.*, who studied the expression of 12 radiation-regulated genes in a panel of 12 human cell lines (6). Nine of the genes were identified as radiation-responsive using quantitative fluorescence cDNA microarray hybridization and three were well-characterized radiation-inducible genes (*CDKN1A*, *BAX* and *MDM2*). Responses varied widely in cell lines that have different tissues of origin and different genetic background. It is becoming apparent that cell–cell and cell–matrix interactions also influence the effect of radiation *in vivo* (7). The biological significance of newly identified radiation-responsive genes can therefore be truly appreciated only when placed in the context of the intact animal. To address this complexity, we have developed a new approach to the investigation of radiation stress responses that has the potential to place novel radiation-regulated genes within their cellular context in the intact

<sup>1</sup> Author to whom correspondence should be addressed at Division of Experimental Therapeutics, Ontario Cancer Institute, 610 University Avenue, Toronto, Ontario M5G 2M9, Canada. e-mail: katherine.vallis@rmp.uhn.on.ca.



**FIG. 1.** *In vitro* screening strategy. The PT1 vector, which contains a promoterless *lacZ* gene immediately downstream of a splice acceptor (SA) site and the *neo<sup>R</sup>* gene driven by the PGK-1 promoter, was introduced into R1 cells by electroporation. After selection in G418 for 8 days, resistant colonies were picked and seeded into 96-well plates. These cultures were then expanded and replica-plated. One plate was used as the control and two were irradiated (0.5 Gy and 4 Gy). One plate was frozen and stored at  $-70^{\circ}\text{C}$  and one was used as a master. Cells in control and irradiated plates were stained for  $\beta$ -gal activity 24 h after irradiation and compared to identify radiation-responsive clones.

animal. This strategy, gene trapping, allows the simultaneous identification, sequencing, *in vivo* expression analysis, and phenotyping of genes of interest. Gene trapping is an insertional mutagenesis technique in which an exogenous DNA vector integrates randomly into the genome. The vectors used in this approach typically include a splice acceptor site immediately upstream of a promoterless reporter gene (*lacZ*) and the selectable *neo* gene driven by an au-

tonomous promoter. Reporter gene activity accurately reflects the activity of the endogenous gene into which the vector has integrated (8, 9). The endogenous locus is usually (but not always) inactivated by vector integration, leading to a loss of function. When applied to totipotent embryonic stem cells, the gene-trap method may be used to generate mutant animals by introducing these cells into the mouse germline. Thus gene trapping in embryonic stem

**TABLE 1A**  
**Relative  $\beta$ -Gal Activity<sup>a</sup> in R1 and Gene-Trap Cell Lines after Exposure to 4 Gy  $\gamma$  Radiation**

Cell line	Hours after irradiation				
	0	4	6	24	48
R1	1.00 $\pm$ 0.16	1.00 $\pm$ 0.03	1.00 $\pm$ 0.24	0.88 $\pm$ 0.07	0.92 $\pm$ 0.02
9A	1.00 $\pm$ 0.02	1.89 $\pm$ 0.21*	2.11 $\pm$ 0.02*	2.33 $\pm$ 0.25*	1.05 $\pm$ 0.07
3E	1.00 $\pm$ 0.15	1.25 $\pm$ 0.16	1.96 $\pm$ 0.45*	2.33 $\pm$ 0.18*	3.52 $\pm$ 0.19*
9H	1.00 $\pm$ 0.13	1.79 $\pm$ 0.03*	2.21 $\pm$ 0.04*	1.09 $\pm$ 0.02	1.53 $\pm$ 0.08
7D	1.00 $\pm$ 0.08	0.55 $\pm$ 0.08	0.73 $\pm$ 0.15	0.41 $\pm$ 0.02*	1.18 $\pm$ 0.06

cells provides an approach to generate novel insertional mutations in the mouse germline (10). The large size of the mammalian genome has necessitated the development of additional strategies to select from amongst these insertional events for those that may be of particular interest. For example, we have previously described strategies that pre-select *in vitro* for insertions into genes whose expression is modulated by retinoic acid (11) or is limited to specific cell types (12). In this study, we have further extended this approach to identify insertion events into genes whose expression is altered after irradiation.

## METHODS

### Gene-Trap Vector

The gene-trap vector PT1-ATG (henceforth referred to as PT1) contains the *En-2* splice acceptor site fused to the promoterless *lacZ* reporter gene with an ATG translational start site (13). Integration of PT1 into the intron of a gene will result in a fusion transcript between *lacZ* and 5' exons of the trapped gene. PT1 also contains the bacterial neomycin-resistance gene, *neo*, which is driven by the PGK-1 promoter, allowing selection with G418 for cells in which the vector has integrated successfully.

### Embryonic Stem Cell Culture and Selection of Radiation-Responsive Clones

R1 embryonic stem cells were maintained on primary embryonic fibroblasts as described previously (14). After electroporation and selection in G418 for 8–10 days, resistant colonies were picked and transferred to 96-well plates (Fig. 1). These cultures were expanded and replica-plated on five 96-well plates. One plate was used as control and two were irradiated with <sup>137</sup>Cs  $\gamma$  rays (dose rate, 1.14 Gy/min). The first 2,400 gene

trap clones were screened with radiation doses of 2 Gy and 6 Gy. Subsequently, 0.5 Gy and 4 Gy were used. Cells in control and irradiated plates were stained for  $\beta$ -galactosidase ( $\beta$ -gal) activity 24 h after irradiation. Stronger staining in irradiated compared to control cells indicated radiation inducibility. Weaker staining in irradiated compared to control cells indicated that the trapped gene was down-regulated by radiation. A lack of alteration in staining indicated either integration into a non-radiation-responsive or housekeeping gene or that the integration site was intergenic. Even trapped genes induced rapidly after irradiation would be expected to lead to increased  $\beta$ -gal activity at 24 h because  $\beta$ -gal is a stable protein. One 96-well plate was frozen and stored at  $-70^{\circ}\text{C}$  and one was used as a master plate. Clones that appeared to be radiation-responsive were trypsinized, transferred from the master plate to culture dishes, and expanded for further study.

### Reporter Gene Expression In Vitro and In Vivo

To detect  $\beta$ -gal activity, cell cultures were rinsed in 100 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5) then fixed in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl<sub>2</sub> and 100 mM Na<sub>2</sub>HPO<sub>4</sub> for 5 min. Cells were washed three times for 5 min each in 2 mM MgCl<sub>2</sub>, 0.02% NP-40 and 100 mM Na<sub>2</sub>HPO<sub>4</sub> and stained with X-gal (5-bromo-4-chloro 3-indoyl  $\beta$ -galactoside) overnight at 37°C.  $\beta$ -Gal activity was detected in 9A embryos and adult tissues as described above, except that the fixative included 1.5% formaldehyde and the embryos were fixed for 30 min and washed three times for 15 min each. X-Gal-stained embryos and adult tissues were embedded in paraffin, sectioned and counterstained with Nuclear Red. To investigate tissue-specific *in vivo* modulation of reporter gene expression in response to radiation, 6-week-old male mice were killed humanely 24 h after whole-animal irradiation (4 Gy). Unirradiated animals were used as controls. X-Gal staining of several organs (cerebellum, brain, liver, kidney, heart, spleen, lung, skin, skeletal muscle, intestine, urinary bladder and testis) was performed, and  $\beta$ -gal activity in control and irradiated tissues was compared.

**TABLE 1B**  
**Relative  $\beta$ -Gal Activity<sup>a</sup> in R1 and Gene-Trap Cell Lines 24 Hours after Exposure to  $\gamma$  Radiation**

Cell line	Dose (Gy)					
	0	0.5	2	4	10	15
R1	1.00 $\pm$ 0.17	1.06 $\pm$ 0.10	1.08 $\pm$ 0.12	1.00 $\pm$ 0.19	0.75 $\pm$ 0.05	0.75 $\pm$ 0.02
9A	1.00 $\pm$ 0.03	1.83 $\pm$ 0.08*	3.02 $\pm$ 0.13*	3.20 $\pm$ 0.16*	4.05 $\pm$ 0.22*	2.83 $\pm$ 0.20*
3E	1.00 $\pm$ 0.49	1.75 $\pm$ 0.53	2.93 $\pm$ 0.14	3.09 $\pm$ 0.47*	2.97 $\pm$ 0.39*	3.96 $\pm$ 0.14*
9H	1.00 $\pm$ 0.07	1.17 $\pm$ 0.11	1.34 $\pm$ 0.20	2.00 $\pm$ 0.08*	2.50 $\pm$ 0.55*	2.00 $\pm$ 0.19*
7D	1.00 $\pm$ 0.10	0.35 $\pm$ 0.00*	0.43 $\pm$ 0.10*	0.70 $\pm$ 0.08	0.30 $\pm$ 0.09*	0.20 $\pm$ 0.08*

*Notes.* The numbers shown are the relative (fold) induction compared to untreated control samples. All values are presented as means  $\pm$  standard errors for triplicate samples from one representative experiment. Statistical significance was determined using ANOVA and the Dunnett adjustment for multiple comparisons.

<sup>a</sup>  $\mu\text{g}$   $\beta$ -Gal/mg total protein.

\* Statistically significant ( $P < 0.05$ ).

**TABLE 2**  
**Summary of Characterization of Radiation-Responsive Gene-Trap Clones**

Cell line	Response to radiation	Size of endogenous gene sequence obtained by 5'RACE (in bp)	<i>lacZ</i> expression <i>in vivo</i> (adult tissues)	Identity
9A	Induced	116	Brain Cerebellum Spleen Kidney	Human gene (homology to GTPase activating proteins) Spleen EST Embryo EST
3E	Induced	186	Unknown	Mouse mammary gland ESTs Human fetal heart EST
9H	Induced	114	Unknown	<i>Mym</i>
7D	Down-regulated	20	Unknown	Unknown

#### Quantitative $\beta$ -Galactosidase Assay

Time course and dose dependence of  $\beta$ -gal activity in radiation-responsive gene trap cell lines was measured using a chemiluminescence assay (15). The Glacto-Star™ luminescent  $\beta$ -galactosidase reporter system II (Tropix) was used according to the manufacturer's instructions. The R1 cell line was used as a control. For each cell line,  $2 \times 10^6$  cells were seeded onto 24-well plates. For time-course experiments, cells were exposed to 4 Gy  $\gamma$  radiation at 48, 24, 6 and 4 h before being harvested for the  $\beta$ -gal assay. For dose-dependence experiments, cells were irradiated with doses of 0.5, 2, 4, 10 and 15 Gy and harvested 24 h later.  $\beta$ -Galactosidase activity was measured in triplicate samples for each time or dose. Statistically significant differences between values for irradiated samples and unirradiated controls were determined using ANOVA and the Dunnett adjustment for multiple comparisons. An aliquot of cell lysate was retained and used to measure protein content using the Bradford method (Bio-Rad).

#### 5'RACE-PCR

Total RNA was prepared from gene-trap cell lines. 5'RACE was performed using a 5'RACE kit (Gibco/BRL) according to the manufacturer's instructions. Superscript II and GT-*lacZ*-1 (5'-GCAAGCGCATTAAGTTGGGT-3') primer were used for reverse transcription. DNA amplification of the RACE products was performed in two rounds in 50- $\mu$ l volumes containing PCR buffer II (Perkin Elmer), 1.25 mM MgCl<sub>2</sub>, 200 mM deoxynucleoside triphosphates, 200 nM of each primer, and 2.0 U of AmpliTaq DNA Polymerase (Perkin Elmer) in a DNA thermal cycler (one cycle at 94°C for 5 min; one cycle at 80°C for 8 min; 35 cycles at 94°C for 1 min, at 60°C for 2 min and at 72°C for 3 min; one cycle at 72°C for 5 min). The second round of amplification used the same conditions, nested primers, and 5  $\mu$ l of the reaction product from the first round. The primers used for DNA amplification were as follows: For the first round, GT-*lacZ*-2A (5'-CCGTCGACTCTGGCGCCGCTGCTCTGT-CAG-3') and Anchor (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') (Life Technologies). For the second round, Nested 2AU (5'-CAUCAUCAUATTGTGCGACCTGTTGGTCTGAACTCAGCCT-3') and Universal Amplification primer (5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3') (Life Technologies). The RACE-PCR products were digested with *Eco*R1/*Sac*I and cloned into pAMP1 plasmid by using the CloneAmp system (Life Technologies). The cDNA inserts were sequenced on both strands using T7 and SP6 primers. Sequences were analyzed by comparison to the nonredundant GenBank and EST bank of NCBI using the BLAST program.

#### Generation of Chimeras

The gene-trap cell lines were aggregated with CD-1 blastomeres and transferred to foster mothers to generate male chimeras that were mated with CD-1 female mice. Chimeras transmitted the mutation through the germline in the case of 9A. DNA from tail biopsies of F<sub>1</sub> and F<sub>2</sub> offspring was analyzed by Southern blot analysis (16) and hybridized to an *En-2/lacZ* probe.

#### Northern Blot Analysis

To study the radiation dose and time dependence of 9A gene expression, total RNA was prepared from R1 cells after irradiation with doses of 0.2 to 10 Gy and at 0.5 to 24 h after 4 Gy irradiation, according to published methods (17). After RNA transfer, membranes were hybridized using a 9A-specific cDNA probe (EST, AA183903 obtained from Research Genetics). Membranes were rehybridized using a *GAPDH* cDNA probe as a loading control. To study *in vivo* 9A gene expression, wild-type (+/+) mice and mice heterozygous for the 9A insertion (9A/+) were irradiated with a dose of 4 Gy and killed 6 h later. Unirradiated +/+ and 9A/+ mice were used as controls. Tissues (brain, cerebellum, liver, kidney, heart, spleen, lung, skin, skeletal muscle, intestine and testis) were removed and used to prepare total RNA for northern blot analysis. To further study 9A gene expression in the spleen, +/+ mice were exposed to 0, 2, 4 or 6 Gy  $\gamma$  radiation and killed at 6 h after irradiation. Splens were removed and used to prepare total RNA. Northern blot analysis was performed using a 9A-specific probe.

## RESULTS

#### Identification of Radiation-Responsive Clones

Using the screening strategy shown in Fig. 1, we screened 6,669 G418-resistant embryonic stem cell colonies for integrations of the PT1 vector into radiation-responsive genes. In brief, undifferentiated gene trap clones were replica-plated into 96-well plates. Two plates were exposed to ionizing radiation and one unirradiated plate was used as a control. Twenty-four hours after irradiation, the plates were assayed for  $\beta$ -gal activity and compared to identify radiation-responsive gene-trap events. *LacZ* expression was observed in 791 "blue" colonies (11.3%), and 30 clones that appeared radiation-responsive on initial

## A

9A (KIAA0580)		AETLSDYEVAEKIWFNESNRSCADCKAPDPDASINLCVVICKKRCAGQHRSLGPK
KIAA0782		AEALSTSEVAERIWAAPNRFACDCGAPQPDWASINLCVVICKRCAGEHRGLGAG
centaurin $\beta$ 2	393	EKLLKGESALQRVQCI PGNASCDCGLADPRWASINLGITLCIECSGIHRSLGVH
gcs1	5	KVDPDTRRRLLQLQKIGANKKCMDCGAPNPQWATPKFGAFICLECAGIHRGLGVH
arfl gap	1	MASPRTRKVLKEVRAQDENNVCFECGAFNPQWVSVTYGIWICLECGRHRGLGVH

9A (KIAA0580)		DSKVRSLKMDASIWSNELIELFIVIGNKRANDFWAGN----LQKDEELHMDS--PV
KIAA0782		VSKVRSLKMDRQVWVETLIELFLQLGNGACNRFWAAN----VPPSEALQPS--SP
centaurin $\beta$ 2	446	FSKVRSLTLDT--WEPPELLKLMCELGNDVINRVYEAN----VEKMGIKKPPGQR
gcs1	58	ISFVRSITMDQ--FKPELLRMEKGGNEPLTEWFKSHN-IDLSLPQVKVYDNPVA
arfl gap	54	LSFVRSVTMDK--WKDIELEKMKAGGNAKRFEELEAQDDYEPSWSLQDKYSSRAA

9A (KIAA0580)		EKRKNFLTQKYKE
KIAA0782		STRCHLEAKYRE
centaurin $\beta$ 2	493	QEKEYIRAKYVE
gcs1	108	EDYKEKLTCLCED
arfl gap	105	ALFRDKVATLAEG

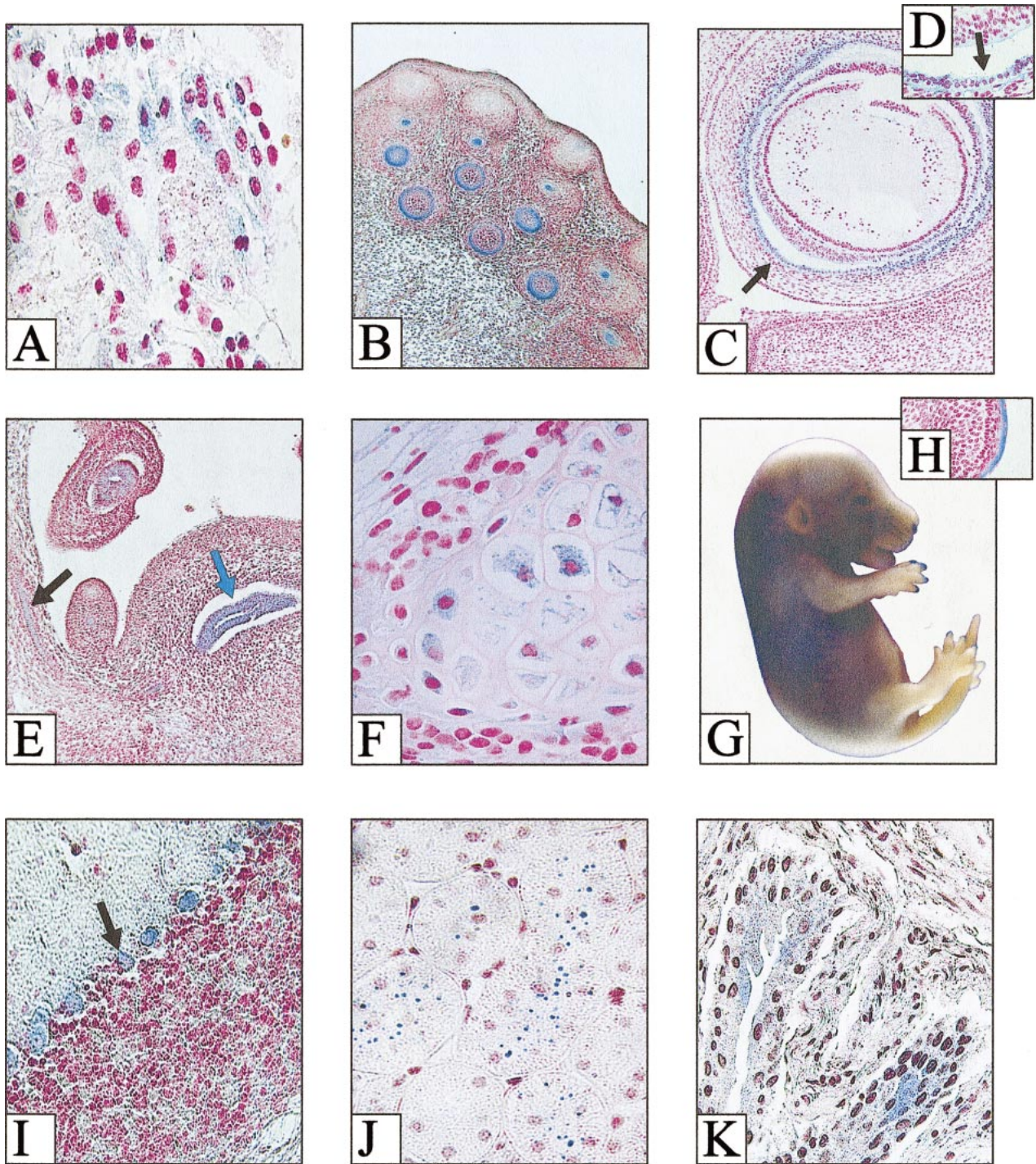
## B

9A (KIAA0580)		KNDVPIIVNSCIAFVTQYGLGCKYIYQKNGDPLHISELLESFKKD-ARSFKLRAG
KIAA0782		DSDIPVIVYRCVDYITQCGLTSEGIYRCKGQTSKTQRLLLESRLQD-ARSVHLKEG
bcr	1064	RSKVPYIVRQCVVEEIERRGMEEVGIYRVSGVATDIQALKAAFVNV-NKDVSVMMMS
abr	657	RSKVPYIVRQCVVEEVEKRGIEEVGIYRISGVATDIQALKAVFDAN-NKDILMLLS
n-chimerin	153	TTKRPMVDMCIREIESRGLNSEGLYRVSGFSDLI EDVKMAFDRDGEKADISVNM
beta-chimerin	287	NTQRPMVVDICIREIEARGLKSEGLYRVSGFTEHIEDVKMAFDRDGEKADISANV
myr 7	2140	DRAVPLVVEKLYNYIEMHGLYTEGIYRKSSTNKIKELRQGLDITD---AESVNL

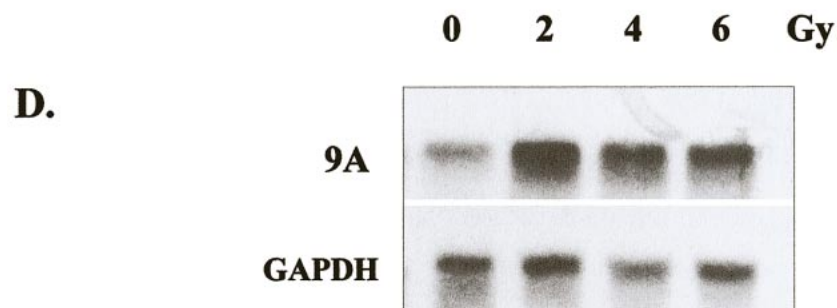
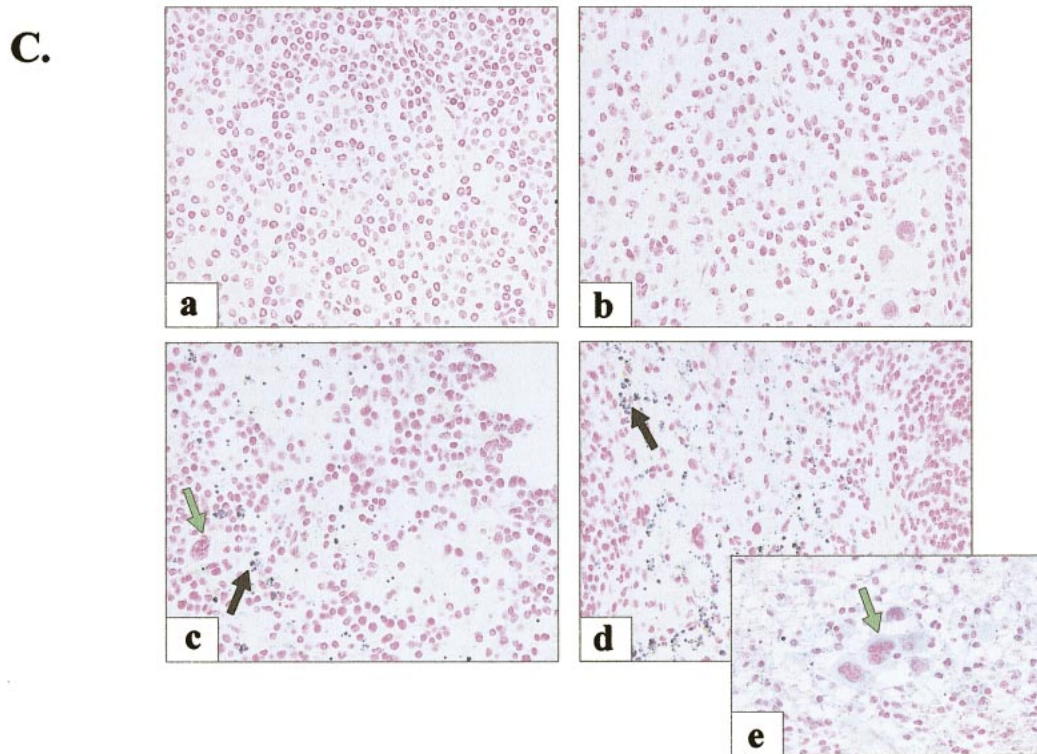
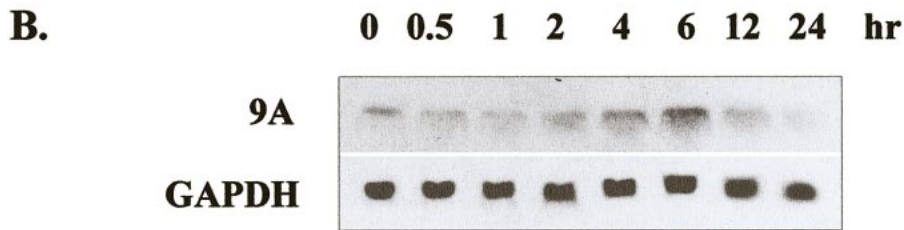
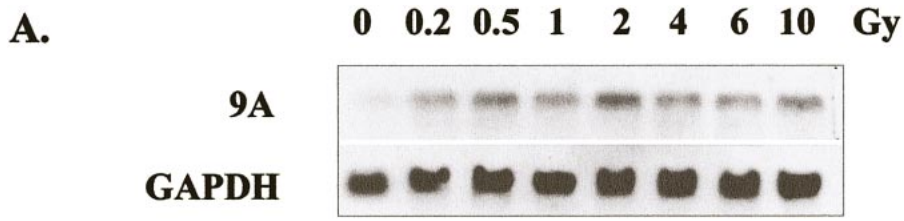
9A (KIAA0580)		KHQLEDVTAVLKSFLSDIDDALLTKELYPYWISALDTQDDKERIKKYGAFIRSLP
KIAA0782		EQHVDDVSSALKRFLRDLPLGLFTRAQRLTWLEASEIEDEEEKVSRVRELLVRLP
bcr	1128	EMDVNAIAGTLKLYFRELPEPLFTDEFYPNFAEGIALSDPAKESCMMLLLSLP
abr	711	DMDINAIAGTLKLYFRELPEPLTDRLYPAFMEGIALSDPAKENCMMHLLRSLP
n-chimerin	208	YEDINIIITGALKLYFRDLPIPLITYDAYPKFIESAKIMDPDEQLETLHEALKLLP
beta-chimerin	342	YPDINIIITGALKLYFRDLPIPVITYDTYSKFIDAAKISNADERLEAVHEVLMLLP
myr 7	2192	DYNIHVIASVFKQWLRDLNPLMTFELYEEFLRAMGLQERKETIRGVYSVIDQLS

9A (KIAA0580)		GVMNRATLAAIIEHLYRVQKCEINHMNAHNLALVFS SCLFQTKGQTS EEVNV
KIAA0782		PVNRATVKALISHLYCVQCFSDTNQMNHNLAIVFGPTLFQTDGQDYKAGRV
bcr	1173	EANLLTFLFLLDHLKRVAEKEAVNKMSLHNLATVFGPTLLRPSEKESKLPAN
abr	766	DPNLITFLFLLEHLKRVAEKEPINKMSLHNLATVFGPTLLRPSEVESKAHLT
n-chimerin	263	PAHCETLRYLMAHLKRVTLHEKENLMAENLGI VFGPTLMRSP ELDAMAALN
beta-chimerin	397	PAHYETLRYLMIHLKRVTMNEKDNFMNAENLGI VFGPTLMRPPEDSTLTTLH
myr 7	2247	RTHLSTLERLIFHLVRIALQEDTNRMSANALAI VFA PCILRC PDTTDPLQSV

**FIG. 2.** Sequence comparison of 9A (KIAA0580). Sequences were aligned using the Clustalw program; identical residues are shaded. Panel A: Alignment of the Arf GAP domain. The accession numbers of the proteins shown in the alignment are shown in parentheses. 9A/KIAA0580 (BAA25506) is compared with KIAA0782 (BAA34502), an uncharacterized human protein encoded by a gene isolated from a brain cDNA library; human centaurin  $\beta$  2 (CAB41450), residues 393–509; Gcs1, a *Saccharomyces cerevisiae* Arf GAP (NP\_010055), residues 5–124; and rat Arf1 GAP (AAC52337), residues 1–121. The cDNAs for KIAA0580 and KIAA0782 were incomplete, and therefore residue numbers were not assigned. The positions of conserved cysteines of the zinc finger motif are marked with dots. Panel B: Alignment of the Rho GAP domain. The accession numbers of the proteins shown in the alignment are shown in parentheses. 9A/KIAA0580 is compared with KIAA0782; human breakpoint cluster region protein (BCR) (P11274), residues 1064–1224; human active breakpoint cluster region protein (ABR) (Q12979), residues 657–817; human n-chimerin (P15882), residues 153–314; human beta-chimerin (P5257), residues 287–448; and rat myosin-rhoGAP protein (Myr7) (CAA04946), residues 2140–2298. The invariable arginine that is critical for GTPase rate enhancement (28), but which is absent in KIAA0580, is indicated by a dot.



**FIG. 3.** *In vivo lacZ* expression in the 9A mouse strain. The 9A cell line was introduced into embryos by aggregation and germline transmission of the mutated gene was established. In heterozygote embryos (9A/+) reporter gene expression is restricted to (panel A) the myocardium at 13.5 days postcoitus; (panel B) primordium follicles of vibrissae (12.5 days postcoitus) (also seen in panel G); (panel C) the cornea (arrow) at 15.5 days postcoitus (also shown in inset, panel D, at higher magnification); (panel E) the urogenital tract including ureters (black arrow) and bladder epithelium (blue arrow) at 14.5 days postcoitus; (panel F) chondrocytes, shown here in the head of a rib in a 15 days postcoitus embryo; (panel G) the epidermis (shown in 15.5 days postcoitus embryo) with inset; (panel H) showing histological section of the tip of a digit. In tissues of adult heterozygote (9A/+) mice, there is strong expression of *lacZ* in the (panel I) Purkinje cells (arrow) of the cerebellum and low-level expression in (panel J) renal tubules and (panel K) bladder.



screening were expanded for further investigation. Of these, four clones showed reproducible alteration in  $\beta$ -gal expression in response to radiation on repeated testing. In three clones, *lacZ* expression was induced (9A, 3E and 9H), and in one it was down-regulated (7D). All four of the trapped genes were expressed in undifferentiated embryonic stem cells.

#### *Time and Dose Dependence of $\beta$ -Galactosidase Activity in Response to Radiation*

The response of the gene-trap cell lines to radiation was confirmed using a chemiluminescence quantitative  $\beta$ -gal assay. For time-course experiments, cell cultures were exposed to 4 Gy  $\gamma$  radiation and harvested at several times up to 48 h after irradiation. For dose-response experiments, cell cultures were exposed to a range of doses (0.5 to 15 Gy) and harvested 24 h later. The results of these experiments are shown in Table 1. Maximum induction of clones 9A, 3E and 9H and maximal down-regulation of clone 7D occurred at 24, 48, 6 and 24 h after irradiation, respectively. Clones 9A, 3E and 9H were induced and 7D was down-regulated by a maximum of 4.1-, 4.0-, 2.5- and 0.2-fold, respectively. Alteration in  $\beta$ -gal activity after irradiation was observed at a low dose (0.5 Gy), but was greatest at 10 or 15 Gy for each clone.

#### *Identification of Trapped Genes*

To determine the sequence of the radiation-responsive trapped genes, we carried out 5'RACE-PCR on these clones. The PCR products were cloned and sequenced, and the sequences obtained were used to search public databases for genes with identity or homology to the trapped genes. The identification of the trapped genes is summarized in Table 2. Clone 9A exhibited 86% homology to an uncharacterized human gene (Accession number AB011152) over 70 bases. This sequence was isolated from a human brain cDNA library (18). The predicted human protein (KIAA0580) contains two GTPase-activating (GAP) domains; one for the ADP-ribosylation factor (Arf) family and the other for the Rho family of GTPases (Fig. 2). The Arf family of small GTP-binding proteins are involved in membrane traffic and hydrolysis of GTP on Arf requires GTPase-

activating proteins (19). The protein encoded by the 9A gene contains a sequence of approximately 90 amino acids that is similar to sequences found in Arf1 GAP and GCS-1 (a yeast Arf GAP) (Fig. 2A). Within this conserved region, there is a zinc finger motif that is known to be essential for GAP activity in Arf1 GAP and GCS-1. GAPs for the Rho family of GTP-binding proteins belong to a family whose members share significant sequence homology in a conserved GAP domain. Rho GAPs act as downstream effectors in signal transduction events and control the activities of Rho proteins (20). 5' RACE revealed splicing of the engrailed-2 splice acceptor of the gene trap construct to a base equivalent to base 2207 of the published human cDNA. Clone 9A was also 100% homologous to two murine ESTs over all 116 bp of the endogenous gene sequence (obtained by 5' RACE). The ESTs were isolated from spleen and embryonic cDNA libraries (Accession numbers AA183903 and AA545344, respectively). One of these (AA545344) was obtained from Research Genetics. This EST, which is 1.6 kb in length, was sequenced and shows 80% homology to the putative human homologue of the 9A gene.

Clone 3E shows 95% identity to two murine mammary gland ESTs (Accession numbers AA620183 and AI607237) over 140 bp and 149 bp, respectively. Clone 3E also showed 90% identity to a human EST derived from a fetal heart cDNA library (Accession number AA348215). Clone 9H corresponds to a murine locus, *Mym* (Accession number AF019615). Only 20 bp of the trapped gene sequence in clone 7D has been obtained to date. One possible explanation for this is that the vector integrated at the 5' end of a gene and that no further sequence can be obtained using 5' RACE. Another method that may be used to clone the endogenous gene associated with the gene-trap insertion in this situation is inverse PCR (21).

#### *In Vivo Expression Pattern of 9A Gene*

The 9A gene-trap cell line was introduced into blastomeres by aggregation and germline transmission of the mutated gene was established. 9A F<sub>1</sub> males and F<sub>1</sub> females were bred to obtain F<sub>2</sub> progeny. The availability of live homozygous animals was confirmed by test breeding for

←

**FIG. 4.** Induction of the 9A gene by radiation *in vitro* and *in vivo*. Panel A: To determine the dose dependence of 9A gene expression in response to irradiation, total RNA was prepared from R1 cells 6 h after various doses (0 to 10 Gy) of radiation. The dose is indicated above each lane. After RNA transfer, membranes were hybridized using a 9A-specific cDNA probe (EST, AA183903 obtained from Research Genetics). Membranes were rehybridized using a *GAPDH* cDNA probe as a loading control. Panel B: To determine the time dependence of 9A gene expression in response to irradiation, total RNA was prepared from R1 cells at various times (0.5 to 24 h) after 4 Gy. Time after irradiation is indicated above each lane. RNA from unirradiated cells was used as a control and loaded in lane 1. After RNA transfer, membranes were hybridized using 9A-specific and *GAPDH* cDNA probes as for panel A. Panel C: Histological sections of the spleens of (a) +/+, unirradiated; (b) +/+, irradiated; (c) (9A/+), unirradiated; and (d) (9A/+) irradiated animals. In (e) (inset), megakaryocytes are shown at greater magnification; *lacZ* staining is observed (black arrow) in both control and irradiated 9A spleens. However, induced expression is noted in cells in the red pulp and particularly in megakaryocytes (green arrow). Panel D: +/+ mice received 0, 2, 4 or 6 Gy and were killed at 6 h. Spleens were removed and total RNA was prepared. Northern blot analysis, using a 9A gene-specific probe, was performed. RNA transcripts complementary to the *GAPDH* gene are shown as loading controls. There was a 3.5-fold increase in RNA transcripts corresponding to the 9A gene after 2 to 6 Gy of radiation.

100% transmissibility of the 9A gene trap. Ten F<sub>2</sub> 9A male mice were crossed with CD-1 females. Genomic DNA from 11.5 days postcoitus embryos was analyzed by Southern blot analysis and hybridized to an *En-2/lacZ* probe. The offspring of one of the 10 males tested were all +/-, indicating homozygosity for the insertion in this animal, which appeared phenotypically normal.

In embryos heterozygous for the 9A insertion (9A/+), reporter gene expression was observed in the epidermis of 12.5 days postcoitus embryos. Staining in the skin was particularly obvious at the tips of the digits and was most intense at 15.5 days postcoitus (Fig. 3G and H). Marked expression was also seen in the primordium follicles of vibrissae (from 12.5 days postcoitus onward) (Fig. 3B and G), in the cornea (Fig. 3C and D), in the urogenital tract including ureters throughout development (Fig. 3E), in the chondrocytes (Fig. 3F) and in the myocardium (Fig. 3A). Expression was observed in the heart at 11.5 days postcoitus but was not apparent from 14.5 days postcoitus onward. In tissues of adult 9A/+ mice, there was strong *lacZ* expression in the Purkinje cells of the cerebellum (Fig. 3I) and low-level expression in renal tubules (Fig. 3J), cardiac atria, bladder (Fig. 3K) and spleen (Fig. 4C). When tissues of irradiated and control mice were compared, induction of  $\beta$ -gal activity was observed in the spleen but not in other organs.

#### *Induction of the 9A Gene by Radiation In Vitro and In Vivo*

Northern analysis was used to confirm that the endogenous gene in the 9A gene-trap clone was radiation-responsive. The wild-type embryonic stem cell line, R1, was irradiated and the RNA was harvested. A three- to fourfold induction of 9A at the mRNA level was observed over a dose range of 0.2 to 10 Gy of  $\gamma$  radiation (Fig. 4A). Maximal induction occurred at 6 h after irradiation (Fig. 4B). There was a rapid decrease of mRNA levels after irradiation, with a return to steady-state levels at 24 h after irradiation. There was low-level expression of the 9A mRNA transcript in control, unirradiated conditions on both of the Northern blots shown in Fig. 4A and B. The difference in the intensity of this band on the two Northern blots was due to minor differences in experimental conditions and to differences in the length of exposure of the membrane to the radiograph.

Northern blot analysis of RNA prepared from adult tissues (brain, cerebellum, liver, kidney, heart, spleen, lung, skin, skeletal muscle, intestine and testis) taken from control and irradiated +/+ mice demonstrated an 8.0-kb transcript after hybridization to a 9A gene-specific cDNA probe. Six hours after whole-body irradiation (4 Gy), induction of 9A gene expression was observed in the spleen (Fig. 4C) but not in other tissues (data not shown). Reporter gene expression was investigated in spleen taken from +/+ and 9A/+ control and irradiated animals (Fig. 4C, a-d).

Low-level  $\beta$ -gal activity was observed in spleen taken from unirradiated 9A/+ mice. The pattern of staining was punctate and was located mainly in the red pulp and particularly in macrophages. Increased levels of staining were observed after irradiation, and this was observed particularly in megakaryocytes (Fig. 4C, e). Induction of 9A gene expression at the mRNA level in the spleen was also investigated using northern blot analysis. 9A gene expression was elevated 3.5-fold in the spleen after doses of 2, 4 and 6 Gy (Fig. 4D).

## DISCUSSION

Many mammalian genes have been implicated in radiation responses (2). These genes are involved in a wide spectrum of cellular processes including DNA repair, apoptosis, oxidative stress responses, and growth control. The discovery of radiation-inducible genes has been serendipitous in some cases, but several investigators have undertaken systematic searches for mammalian homologues of bacterial and yeast genes involved in DNA repair and have used various subtraction techniques to identify genes whose expression is selectively altered after irradiation (22, 23). These differential hybridization methods are efficient for genes that are highly expressed in stimulated cells, but mRNAs of low abundance are difficult to isolate using these approaches. Subtraction hybridization improves the chances of cloning rare cDNAs; using this strategy and UV radiation as a selecting agent, Fornace *et al.* identified the *GADD* genes (24). Serial analysis of gene expression (SAGE) compares expression between two mRNA populations and allows quantitative and simultaneous analysis of a large number of transcripts (25). It has been used to identify genes whose expression is increased in the presence of functional *TP53* (26). Recently gene array technology has been used as a new approach to isolate radiation-responsive genes (6). Although all these methods facilitate the identification of differentially expressed genes, they do not simultaneously introduce mutations into the genes of interest or allow for introduction of tagged genes into the mouse germline to study expression and phenotype *in vivo*.

The strategy of gene trapping is a powerful technique that simultaneously integrates gene identification and sequence analysis with expression and function into one process. Thus gene trapping allows the study of gene regulation and gene function within the context of a live animal. We are aware of one other report of the use of gene trapping to identify genes regulated by genotoxic stress (27). Fibroblasts containing gene-trap integrations were stained with the fluorescent substrate of  $\beta$ -gal, fluorescein-di $\beta$ -D-galactopyranoside, and analyzed by fluorescence-activated cell sorting (FACS). *LacZ*-positive clones were discarded because the trapped genes were regarded as constitutively expressed. *LacZ*-negative cells were replica-plated and exposed to UV or ionizing radiation. Three clones that ap-

peared to show induced  $\beta$ -gal activity after UV irradiation were analyzed further, but in two,  $\beta$ -gal activity seemed to be only slightly increased. In the third, a threefold induction by UV radiation was observed, and the endogenous gene sequence obtained using 5'RACE was novel. The FACS approach allowed a high-throughput screen of over 30,000 gene-trap clones, and one that was selected for further analysis showed reproducible and significant induction after genotoxic stress. However, because fibroblasts were used, this method does not allow introduction of tagged genes into the mouse germline to study expression and phenotype *in vivo* and during embryological development. In our study, we have applied the functional genomics approach of gene trapping in embryonic stem cells to isolate genes whose expression is altered after irradiation. Using this approach, we identified 4 embryonic stem cell clones out of nearly 7,000 analyzed that contained insertions of the gene-trap vector into genes whose expression is altered, at least *in vitro*, in response to irradiation. In one case, regulation by radiation has been demonstrated *in vivo*.

One of the radiation-responsive genes that we have identified is novel (3E), one corresponds to an uncharacterized human gene (9A), and for one (7D) we have insufficient sequence data so far for a valid comparison with known sequences. The gene-trap clone, 9H, has been identified as *Mym*, which was originally isolated in a previous gene-trap screen (11).

In the case of gene-trap cell line 9A, germline transmission has been achieved, and a specific restricted pattern of reporter gene activity has been demonstrated in embryos and adult mice. The level of 9A mRNA rises rapidly after exposure to radiation, with a peak at 6 h, and declines to control levels by 24 h after treatment. The kinetics of induction of 9A follows the pattern of the rapid response typical of many stress-induced immediate-early genes. By analogy, and given that the human homologue of 9A shows significant similarity to GTPase-activating proteins (GAP), we speculate that 9A may participate in a radiation-induced signaling pathway. However, the putative protein encoded by the human homologue of the 9A gene does not contain an invariant arginine residue in the Rho GAP domain that is a critical element for GTPase rate enhancement (28). Whether the protein encoded by 9A is a true Rho GAP therefore remains to be determined. Induction of 9A expression was observed in the spleen after irradiation *in vivo*. This is the first demonstration of *in vivo* modulation of gene expression by an agent used to select the gene in an *in vitro* gene-trap screen. Within the spleen, expression of 9A was clearly evident in macrophages and megakaryocytes. Expression of 9A was also observed in a variety of other organs but appeared constitutive, with no apparent alteration in steady-state levels after irradiation. These findings emphasize the tissue-specific and cell lineage-specific nature of radiation-inducible genetic responses. We have demonstrated the feasibility of using the gene trap approach to

identify, analyze *in vitro* and *in vivo*, and mutate genes that are regulated by radiation.

Note added in proof: 9A is now referred to as *PARX* (*PH/ArfGAP/RhoGAP/X-ray inducible protein*; Accession no. AF439781).

## ACKNOWLEDGMENTS

This work was supported by the Agnico-Eagle Research Fund and the National Cancer Institute of Canada.

Received: February 14, 2001; accepted: August 23, 2001

## REFERENCES

1. W. Welch, How cells respond to stress. *Sci. Am.* **268**, 56–64 (1993).
2. G. Woloschak, Radiation-induced responses in mammalian cells. *In Stress-Inducible Processes in Higher Eukaryotic Cells* (T. Koval, Ed.), pp. 185–219. Plenum Press, New York, 1997.
3. N. Crompton, Programmed cellular response to ionizing radiation damage. *Acta Oncol.* **37**, 129–142 (1998).
4. J. Wang, Cellular responses to DNA damage. *Curr. Opin. Cell Biol.* **10**, 240–247 (1998).
5. C. Midgley, B. Owens, C. Briscoe, D. Thomas, D. Lane and P. Hall, Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type *in vivo*. *J. Cell Sci.* **108**, 1843–1848 (1995).
6. S. Amundson, M. Bittner, Y. Chen, J. Trent, P. Meltzer and A. Fornace, Fluorescent cDNA microarray hybridization reveals complexity and heterogeneity of cellular genotoxic stress responses. *Oncogene* **18**, 3666–3672 (1999).
7. M. Barcellos-Hoff, How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. *Radiat. Res.* **150** (Suppl.), S109–S120 (1998).
8. W. C. Skarnes, J. E. Moss, S. M. Hurlley and R. S. Beddington, Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl. Acad. Sci. USA* **92**, 6592–6596 (1995).
9. W. C. Skarnes, B. A. Auerbach and A. L. Joyner, A gene trap approach in mouse embryonic stem cells: the *lacZ* reporter is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice. *Genes Dev.* **6**, 903–918 (1992).
10. F. Cecconi and B. Meyer, Gene trap: A way to identify novel genes and unravel their biological function. *FEBS Lett.* **480**, 63–71 (2000).
11. L. M. Forrester, A. Nagy, M. Sam, A. Watt, L. Stevenson, A. Bernstein, A. L. Joyner and W. Wurst, An induction gene trap screen in embryonic stem cells: Identification of genes that respond to retinoic acid *in vitro*. *Proc. Natl. Acad. Sci. USA* **93**, 1677–1682 (1996).
12. W. L. Stanford, G. Carauna, K. A. Vallis, M. Inamdar, M. Hidaka, V. L. Bautch and A. Bernstein, Expression trapping: Identification of novel genes expressed in hematopoietic and endothelial lineages by gene trapping in ES cells. *Blood* **92**, 4622–4631 (1998).
13. D. P. Hill and W. Wurst, Screening for novel pattern formation genes using gene trap approaches. *Methods Enzymol.* **225**, 664–681 (1993).
14. A. Nagy, J. Rossant, R. Nagy, W. Abramow-Newerly and J. Roder, Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424–8428 (1993).
15. V. Jain and I. Magrath, A chemiluminescent assay for quantitation of beta-galactosidase in the femtogram range: Application to quantitation of beta-galactosidase in lacZ-transfected cells. *Anal. Biochem.* **199**, 119–124 (1991).
16. J. Sambrook, E. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

17. P. Chomczynski and N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).
18. T. Nagase, K-I. Ishikawa, N. Miyajima, A. Tanaka, H. Kotani, N. Nomura and O. Ohara, Prediction of the coding sequences of unidentified human genes. IX. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. *DNA Res.* **5**, 31–39 (1998).
19. J. Moss and M. Vaughan, Molecules in the ARF orbit. *J. Biol. Chem.* **273**, 21431–21434 (1998).
20. A. Hall and C. Nobes, Rho GTPases: Molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philos. Trans. R. Soc. Lond. B* **355**, 965–970 (2000).
21. J-I. Jonsson, Q. Wu, K. Nilsson and R. A. Phillips, Use of a promoter-trap retrovirus to identify and isolate genes involved in differentiation of a myeloid progenitor cell line *in vitro*. *Blood* **87**, 1771–1779 (1996).
22. D. A. Boothman, M. Meyers, N. Fukunaga and S. Lee, Isolation of x-ray-inducible transcripts from radioresistant human melanoma cells. *Proc. Natl. Acad. Sci. USA* **90**, 7200–7204 (1993).
23. S. Sadekova, S. Lehnert and T. Chow, Induction of PBP74/mortalin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: A possible role in induced radioresistance. *Int. J. Radiat. Biol.* **72**, 653–660 (1997).
24. A. J. Fornace, I. Alamo and M. C. Hollander, DNA damage-inducible transcripts in mammalian cells. *Proc. Natl. Acad. Sci. USA* **85**, 8800–8804 (1988).
25. V. Velculescu, L. Zhang, B. Vogelstein and K. Kinzler, Serial analysis of gene expression. *Science* **270**, 484–487 (1995).
26. S. Madden, E. Galella, J. Zhu, A. Bertelsen and G. Beaudry, SAGE transcript profiles for p53-dependent growth regulation. *Oncogene* **15**, 1079–1085 (1997).
27. P. Menichini, S. Viaggi, E. Gallerani, G. Fronza, L. Ottaggio, A. Comes, J. W. Ellwart and A. Abbondandolo, A gene trap approach to isolate mammalian genes involved in the cellular response to genotoxic stress. *Nucleic Acids Res.* **25**, 4803–4807 (1997).
28. T. Barrett, B. Xiao, E. Dodson, G. Dodson, S. Ludbrook, K. Nurmahomed, S. Gamblin, A. Musacchio, S. Smerdon and J. Eccleston, The structure of the GTPase-activating domain from p50rhoGAP. *Nature* **385**, 458–461 (1997).