

Mice lacking ADPRT and poly(ADP-ribosylation) develop normally but are susceptible to skin disease

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Poly(ADP-ribosylation) is catalyzed by NAD⁺:protein(ADP-ribosyl)transferase (ADPRT), a chromatin-associated enzyme which, in the presence of DNA breaks, transfers ADP-ribose from NAD⁺ to nuclear proteins. This post-translational modification has been implicated in many fundamental processes, like DNA repair, chromatin stability, cell proliferation, and cell death. To elucidate the biological function of ADPRT and poly(ADP-ribosylation) in vivo the gene was inactivated in the mouse germ line. Mice homozygous for the ADPRT mutation are healthy and fertile. Analysis of mutant tissues and fibroblasts isolated from mutant fetuses revealed the absence of ADPRT enzymatic activity and poly(ADP-ribose), implying that no poly(ADP-ribosyl)ated proteins are present. Mutant embryonic fibroblasts were able to efficiently repair DNA damaged by UV and alkylating agents. However, proliferation of mutant primary fibroblasts as well as thymocytes following γ -radiation in vivo was impaired. Moreover, mutant mice are susceptible to the spontaneous development of skin disease as ~30% of older mice develop epidermal hyperplasia. The generation of viable ADPRT^{-/-} mice negates an essential role for this enzyme in normal chromatin function, but the impaired proliferation and the onset of skin lesions in older mice suggest a function for ADPRT in response to environmental stress.

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Post-translational modification of proteins is believed to be an important mechanism that regulates many cellular processes, such as gene transcription, cell cycle progression, and cell differentiation. In contrast to phosphorylation and acetylation, poly(ADP-ribosylation) is a unique post-translational modification for nuclear proteins, which is strongly stimulated in cells following environmental insults (for review, see Althaus and Richter 1987). Poly(ADP-ribosylation) is catalyzed by NAD⁺:protein(ADP-ribosyl)transferase (polymerizing) (ADPRT or PARP, EC 2.4.2.30), which has been characterized extensively at biochemical as well as molecular levels (for review, see de Murcia et al. 1991). ADPRT is a chromatin-associated protein that catalyzes the covalent attachment of ADP-ribose units from its substrate, NAD⁺, to numerous nuclear proteins, including ADPRT itself (Althaus and Richter 1987). The protein contains three functional domains: (1) an amino-terminally located DNA-

binding region that comprises two zinc fingers involved in the recognition of DNA strand breaks; (2) a central domain for automodification by poly(ADP-ribosylation); and (3) a carboxy-terminal fragment containing the catalytic domain (see Fig. 1A, below). The gene encoding ADPRT is highly conserved and present in almost all eukaryotic cells except yeast (for review, see de Murcia and Ménissier-de Murcia 1994), and the genomic organization of the murine and human gene is very similar (Auer et al. 1989). Only a single genomic locus harboring a functional ADPRT gene has been mapped in the human (Baumgartner et al. 1992) as well as in the murine genome (Berghammer et al. 1992). Expression of ADPRT has been detected in almost all eukaryotic cells from plants to vertebrates as well as in many mouse organs (Ogura et al. 1990).

Many studies on the functional role of ADPRT have been made, and most nuclear functions have been associated with poly(ADP-ribosylation) (for review, see Althaus and Richter 1987). In particular, a critical role for ADPRT has been predicted in DNA repair because the activity of this enzyme is increased 100-fold following

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DNA damage (Benjamin and Gill 1980). Further support for this hypothesis comes from studies showing that cells treated with specific chemical inhibitors of the enzyme are hypersensitive to DNA-damaging agents (for review, see Shall 1984; Althaus and Richter 1987). In addition, expression of antisense ADPRT mRNA in HeLa cells resulting in the loss of 80% of enzyme activity led to inhibition of strand break rejoining of damaged DNA (Ding et al. 1992). Finally, DNA repair was shown to be impaired in mutant cell lines that have reduced expression levels of ADPRT (Chatterjee et al. 1990, 1991; Berger et al. 1992).

Poly(ADP-ribosyl)ation seems to be also indirectly involved in other cellular processes because a number of DNA metabolic enzymes, for example, endonucleases, topoisomerases I and II, DNA polymerases α and β , as well as DNA ligase II, have been identified as acceptors of poly(ADP-ribosyl)ation (for review, see Althaus and Richter 1987). In addition, nuclear scaffold proteins, such as lamins and histones, are acceptors of poly(ADP-ribosyl)ation, and a large proportion of poly(ADP-ribosyl)ated proteins is observed in chromatin following treatment of cells with DNA-damaging agents (for review, see Althaus and Richter 1987). A role for ADPRT in maintaining chromatin stability is supported by the observation that repression of ADPRT synthesis causes alterations of chromatin structure (Ding et al. 1992). Although several models have been proposed for the possible involvement of poly(ADP-ribosyl)ation in chromatin function, there is no evidence that this enzyme or poly(ADP-ribosyl)ation is directly involved in the DNA repair process.

ADPRT has also been proposed to play a role in cell cycle progression, cell proliferation, and neoplastic transformation. Poly(ADP-ribosyl)ation activity was elevated specifically in the G₂ phase of the cell cycle (Tanuma et al. 1978; Kidwell et al. 1982); however, other studies indicated that levels of poly(ADP-ribosyl)ation are high throughout the entire cell cycle (Adolph 1985). The involvement of ADPRT in cell proliferation is supported by the observation that expression of ADPRT is induced when cells are stimulated to proliferate (Menegazzi et al. 1988; McNerney et al. 1989; Cesarone et al. 1990). With respect to neoplastic transformation, inhibitors of ADPRT can prevent oncogenic transformation of cultured cells (Tseng et al. 1987; Nakagawa et al. 1988); however, ADPRT activity has also been inversely correlated with transformation (Strain 1985; Yamagami et al. 1985; Miller et al. 1989). ADPRT is also implicated in cytotoxicity, because activation of ADPRT causes intracellular NAD⁺ depletion and thereby energy depletion, which leads to cell death (Berger 1985; Gaal et al. 1987). Recent studies demonstrated that free radical-induced cell toxicity in neuronal cells as well as in pancreatic β -islet cells is mediated by ADPRT-dependent NAD⁺ depletion, and that inhibitors of the enzyme can prevent cell death (Radons et al. 1994; Zhang et al. 1994).

Many insights into possible functions of ADPRT in different biological processes were obtained from in vitro experiments employing NAD⁺ analogs as inhibitors of

poly(ADP-ribosyl)ation (e.g., nicotinamide, benzamide, and their derivatives). However, whereas these substances do inhibit ADPRT activity, they may also interfere with normal cellular metabolism (Willmore and Durkacz 1993). Thus, it is possible that effects unrelated to ADPRT inhibition also influence the results obtained from those studies. Much effort has been made to eliminate the side effects of inhibitors either by selecting mutant cell lines in which expression of ADPRT is reduced (Chatterjee et al. 1990), or by introducing a dominant-negative mutation in cells (Küpper et al. 1990; Molinete et al. 1993). However, the results are often contradictory most likely because of different experimental systems and potential side effects of the inhibitors used. To circumvent these problems and to better define the biological function of ADPRT and poly(ADP-ribosyl)ation both in vitro and in vivo, we have taken a genetic approach by generating mice with a disrupted ADPRT gene using homologous recombination in embryonic stem (ES) cells. Mice lacking ADPRT exhibit no poly(ADP-ribosyl)ation but are viable and fertile. They are, however, susceptible to skin lesions and frequently develop epidermal hyperplasia with increasing age. Whereas normal DNA repair was observed in fibroblasts derived from mutant embryos, the absence of ADPRT affected the proliferation of these cells in vitro and delayed the recovery of thymocytes in vivo after exposure to γ -irradiation.

Results

Generation of mice with a disrupted ADPRT gene

Inactivation of the ADPRT gene in ES cells was performed by homologous recombination using a promoterless targeting construct. This strategy was chosen because we found that ADPRT was expressed at high levels in D3 ES cells (data not shown). A *Hind*III fragment derived from the 5' murine genomic ADPRT gene was used to construct a targeting vector in which the neomycin resistance gene (*neo*) replaced a *Kpn*I fragment containing part of the second exon and second intron (Fig. 1A). The *neo* cassette, which lacked promoter and ATG elements but contained a TGA stop codon and a synthetic polyadenylation site, was fused in-frame to the ADPRT-coding sequence. A 2.3-kb *Hind*III fragment of the targeting vector was isolated, purified, and introduced by electroporation into D3 ES cells. After G418 selection, 264 clones were analyzed first by the polymerase chain reaction (PCR) for a gene targeting-specific junction fragment and 3 correctly targeted clones were identified by Southern blot analysis containing the expected wild-type and mutant alleles (Fig. 1A,B). Using 5' and 3' external probes as well as an internal probe, we determined the absence of additional integration events (data not shown). Injection of these cells into mouse blastocysts gave rise to a number of chimeras that transmitted the targeted allele of the ADPRT gene to their offspring (Fig. 1C). Heterozygous mice appeared phenotypically normal and were intercrossed to produce mice homozygous for

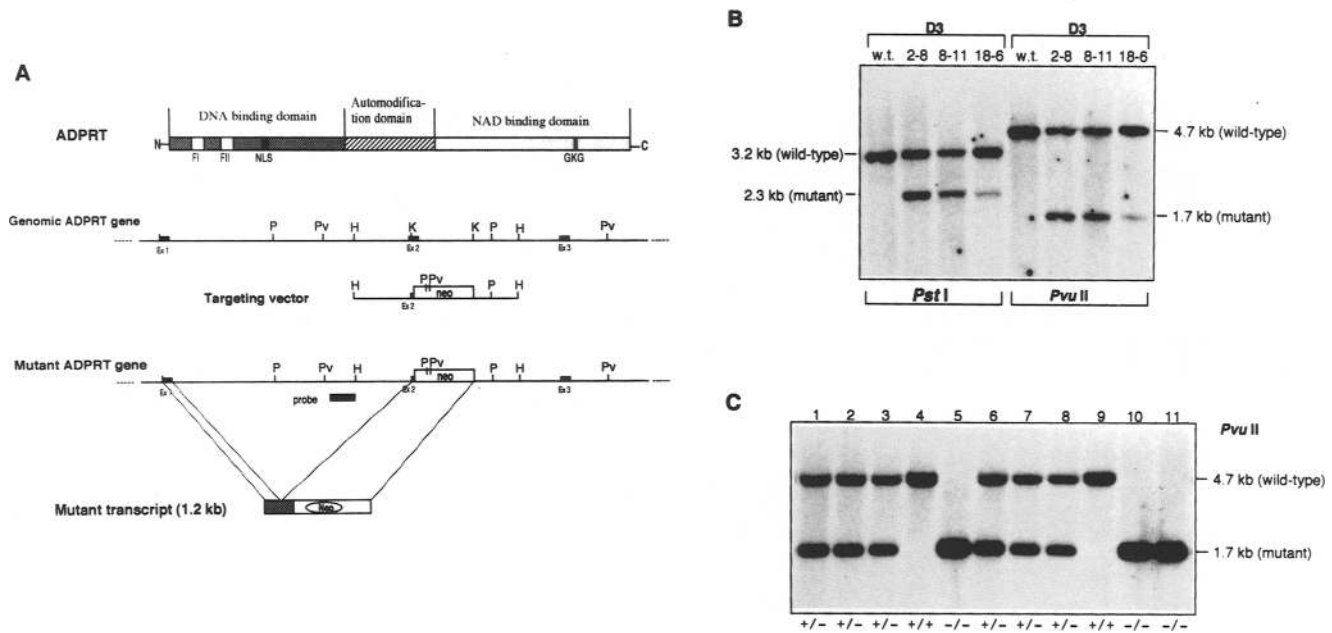


Figure 1. (A) Structure of the targeting vector and partial restriction map of the ADPRT locus before and after targeted integration. The ADPRT protein structure is shown at the *top*. The targeting vector contains a *Hind*III fragment of the ADPRT gene in which a *Kpn*I fragment was replaced by the *neo* gene that does not contain the promoter and the start codon ATG. The *Pst*I and *Pvu*II sites introduced by *neo* facilitate the identification of the correct targeting event. Because the *neo* gene containing a TGA stop codon and a poly(A) site was fused in-frame to the second exon of the ADPRT gene, the expected fusion transcript is indicated at the *bottom*. (FI and FII) Zinc finger I and II, (NLS) nuclear localization signal; (GKG) NAD binding motif. (K) *Kpn*I; (P) *Pst*I; (Pv) *Pvu*II; (H) *Hind*III. (B) Southern blot analysis of ES cell clones containing a single disrupted allele of the ADPRT gene. Genomic DNA from wild-type D3 ES cells, single targeted cell lines D-2-8, D-8-11, and D-18-6 was digested with either *Pst*I or *Pvu*II and analyzed by Southern blot hybridization using a 5' external probe as shown in A (see also Materials and methods). The fragment sizes of wild-type and mutant ADPRT alleles are indicated. (C) Southern blot analysis of progeny derived from two heterozygote intercrosses. Tail DNA from 7-day-old mice was digested with *Pvu*II and hybridized with the 5' external probe. The genotype at the ADPRT locus is indicated below each lane, and the sizes of the DNA fragments are shown.

the mutation (Fig. 1C). Homozygotes were viable, fertile, and exhibited no macroscopic abnormalities at birth.

Absence of ADPRT mRNA and enzyme activity in mutant mice

To examine whether the inactivation of the ADPRT gene was a null mutation in homozygous mice, we analyzed the expression of ADPRT mRNA in wild-type and homozygous mutant mice. Northern blot analysis showed the expected 3.7-kb ADPRT mRNA present only in tissues of the wild-type mouse at low levels in brain, lung, kidney, and muscle, and at relatively higher levels in liver and testes (Fig. 2A). In contrast, full-length ADPRT mRNA was not detected in any tissues from the homozygous ADPRT mutant mouse (Fig. 2A), but a 1.2-kb mRNA, corresponding to the truncated ADPRT-*neo* fusion transcript terminating at the poly(A) site of the *neo* gene was present in most tissues tested (Fig. 2A).

To verify that ADPRT activity was abolished in the homozygous mutant mice, we performed enzymatic assays on tissue extracts isolated from wild-type and homozygous mutant mice (ADPRT^{-/-}). Tissue extracts were separated on polyacrylamide gels (SDS-PAGE) con-

taining fragmented DNA, which were then incubated with ³²P-labeled NAD⁺. Incorporation of isotope into the ADPRT protein band was revealed by autoradiography. Whereas normal levels of ADPRT activity were present in all tissues tested from wild-type mice, ADPRT activity was completely abolished in ADPRT^{-/-} mice (Fig. 2B). These data indicate that a null mutation was introduced into the ADPRT locus of homozygous mice.

To investigate whether poly(ADP-ribosyl)ation can occur in the absence of ADPRT activity, we performed immunofluorescence studies using an antibody against poly(ADP-ribose). Primary embryonic fibroblast cells were isolated from either ADPRT wild-type (+/+), heterozygous (+/-), or homozygous (-/-) embryos at day 13.5 of gestation (E13.5). These cells were treated with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) to induce DNA damage and poly(ADP-ribose) formation, and then incubated with an antibody against the (ADP-ribose) polymers. A bright, uniform staining was present in almost all nuclei of +/+ cells (and +/- cells; data not shown); however, the ADPRT^{-/-} cells were devoid of any positive signal (Fig. 2C). Moreover, no ADPRT activity was detectable

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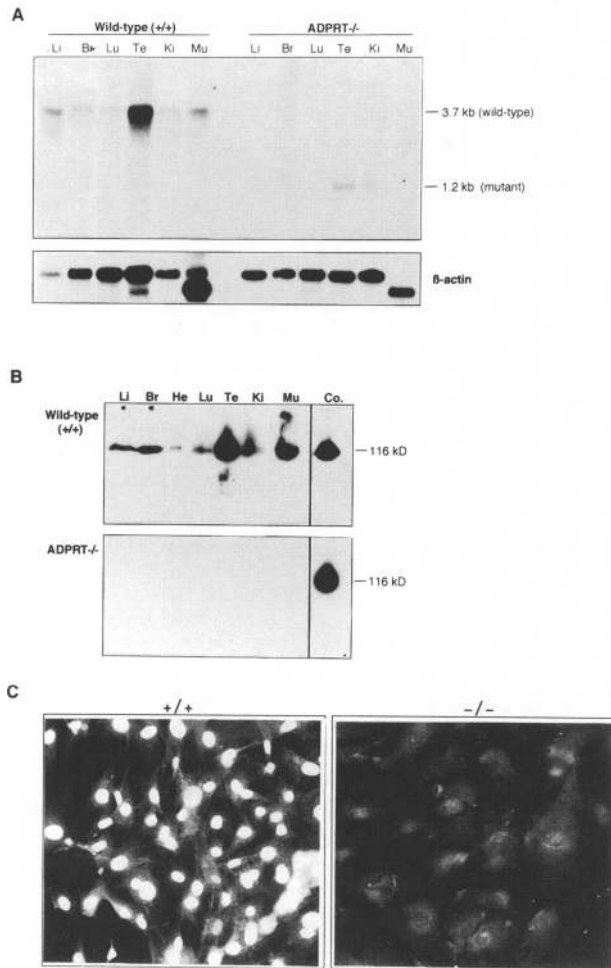


Figure 2. (A) Northern blot analysis of ADPRT mRNA in different tissues from a wild-type and a homozygous ADPRT $-/-$ mouse. Four micrograms of poly(A)⁺ mRNA was analyzed by hybridization with a human cDNA probe. The signals of wild-type ADPRT mRNA (3.7 kb) were detectable in liver, brain, lung, testis, kidney, and muscle tissues from the wild-type mouse. Only ADPRT-*neo* fusion transcripts (~1.2 kb) were expressed at low levels in tissues from the mutant mouse. The film was exposed for 5 days at -80°C . The β -actin signal served as an internal control for RNA loading and the film was exposed for 24 hr. (B) Enzymatic analysis of ADPRT activity in wild-type and homozygous mutant mice. The protein extracts were prepared from various tissues and electrophoresed in a polyacrylamide gel followed by incubating with ^{32}P -labeled NAD⁺. A human cell extract served as a positive control (Co.). ADPRT activity, revealed by incorporation of ^{32}P -labeled NAD⁺ into the gel in situ, was detected in all samples from wild-type mice after 12 hr of exposure. No signal was detected in extracts from ADPRT $-/-$ mice even after a longer exposure of the gel (48 hr). The molecular mass of ADPRT is indicated in kD. (Li) Liver; (Br) brain; (He) heart; (Lu) lung; (Te) testis; (Ki) kidney; (Mu) muscle. (C) Immunofluorescence analysis of poly(ADP-ribose) formation. Wild-type (+/+) and mutant (-/-) cells were treated with MNNG and then stained with a monoclonal antibody (10H) against the polymers. No signal was detected on -/- cells (right), whereas +/+ cells (left) showed a strong nuclear staining.

by the enzymatic activity assay in these fibroblasts (data not shown). These data imply that ADPRT is a unique enzyme for the formation of (ADP-ribose) polymers following DNA damage and that there is no alternative pathway by which nuclear proteins can be poly(ADP-ribosyl)ated.

Spontaneous development of skin lesions in mutant mice

Mice homozygous for the ADPRT mutation appeared healthy after birth; however, starting from 5–6 months of age ~30% of mutant mice originating from a mixed genetic background (129/Sv \times C57BL/6) developed a severe skin disease with increasing age (Fig. 3A,B). Skin lesions were only seen in homozygous mutant mice, although all mice (wild-type, heterozygous, and homozygous genotypes) were housed under the same conditions, that is, in the same room and in some cases, in the same

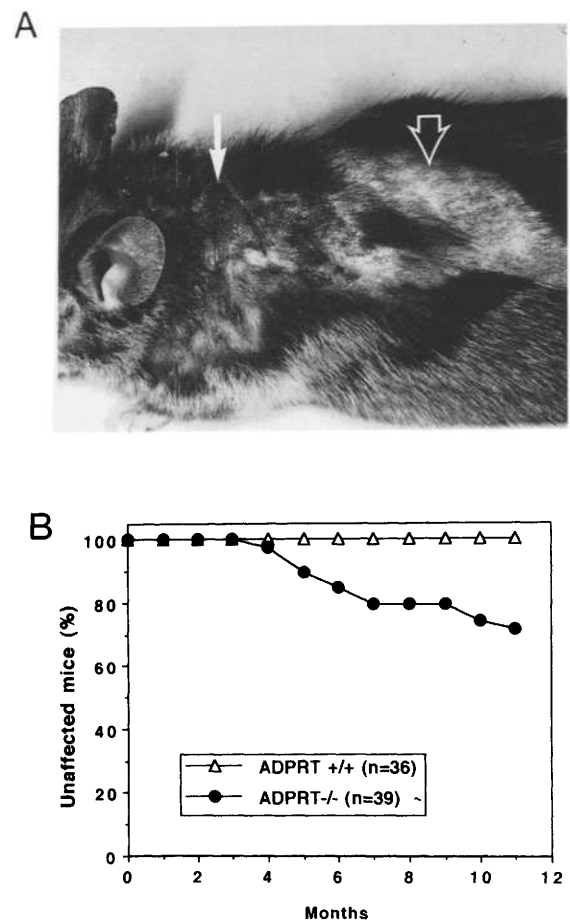


Figure 3. (A) Manifestation of the skin disease in a 10-month-old ADPRT $-/-$ mouse. Note the large patches of alopecia (open arrow) some of which show central ulceration (solid arrow). (B) The skin lesions develop at a high incidence specifically in ADPRT mutant mice on a mixed genetic background (129/Sv \times C57BL/6). A group of wild-type and mutant (-/-) mice was maintained for 11 months, and the appearance of the inflammatory skin diseases was scored. (x-axis) Age (months).

age. The cutaneous changes began characteristically with extensive hair loss on the trunk. This was followed by the appearance of rapidly enlarging and coalescing erythematous patches with occasional central ulcer formation (Fig. 3A). In rare instances wild-type littermates also exhibited patches of hair loss but did not develop inflammatory lesions. Several of the diseased mice were sacrificed for autopsy and for histopathological analysis. A detailed examination of the affected skin revealed striking pathological changes. The epidermis showed massive acanthosis as evidenced by a thickening of all epidermal layers (basal layer, spinous layer, granular layer, and stratum corneum) in all lesions examined compared with the wild-type skin (Fig. 4A,B). The stratum corneum exhibited focal signs of parakeratosis (presence of nucleated corneocytes) (Fig. 4C), indicative of a high turnover rate of newly formed keratinocytes. Close examination revealed the presence of many mitotic keratinocytes in the basal layer of epidermis (Fig. 4B), which is a sign of elevated keratinocyte proliferation. This massive epidermal hyperplasia was also observed in the epithelium of the hair follicles (Fig. 4C). Patchy spongiosis (intercellular edema of the epidermis), another characteristic feature of these lesions, was particularly prominent beneath the parakeratotic foci (Fig. 4C). The upper dermis contained a significant inflammatory infiltrate composed of granulocytes and, to a less extent, mononuclear cells (Fig. 4C). In the deeper layer of the dermis, numerous fibroblasts and capillaries were seen, indicative of granulation tissue formation (Figure 4C). This process was particularly prominent in ulcerated areas (data not shown). In addition, multinucleated keratinocytes were seen in the epidermis and inflammatory cells, predominantly neutrophils, occasionally obscured

the dermal–epidermal junction (Fig. 4D). Histological analysis of the nonlesional area of diseased mice and the skin of unaffected homozygous mutant mice revealed no differences from that of wild-type mice (data not shown).

Proliferation deficiencies in cells lacking ADPRT

Because several *in vitro* studies have indicated a potential role of ADPRT in cell proliferation, next we measured the growth parameters of primary ADPRT mutant cells. Primary embryonic fibroblasts were isolated from E13.5 sibling fetuses from heterozygous intercrosses and growth rates as well as accumulated cell numbers were determined. The growth rates of homozygous mutant (ADPRT^{-/-}) fibroblasts were indistinguishable from that of wild-type (+/+) cells for the first 2 culture days. Thereafter, ADPRT^{-/-} cells grew more slowly during the remaining 12-day observation period (Fig. 5A). Mutant cells never reached the same cell densities as wild-type cells by measuring the accumulated cell number (data not shown). No differences were seen between wild-type and heterozygous (+/-) primary fibroblasts with regard to the growth parameters analyzed and the cell morphology from all three genotypes (data not shown).

To investigate whether the apparent growth deficiency of ADPRT^{-/-} fibroblasts could be extended to other cell types, we tested thymocyte proliferation following exposure of mice to γ -radiation. It is well known that following irradiation, immature thymocytes (CD4⁺/CD8⁺) are depleted in the thymus attributable to apoptosis and that progenitors for these cells proliferate very rapidly and restore the population in the thymus after several days (Ashwell 1994). A group of wild-type mice

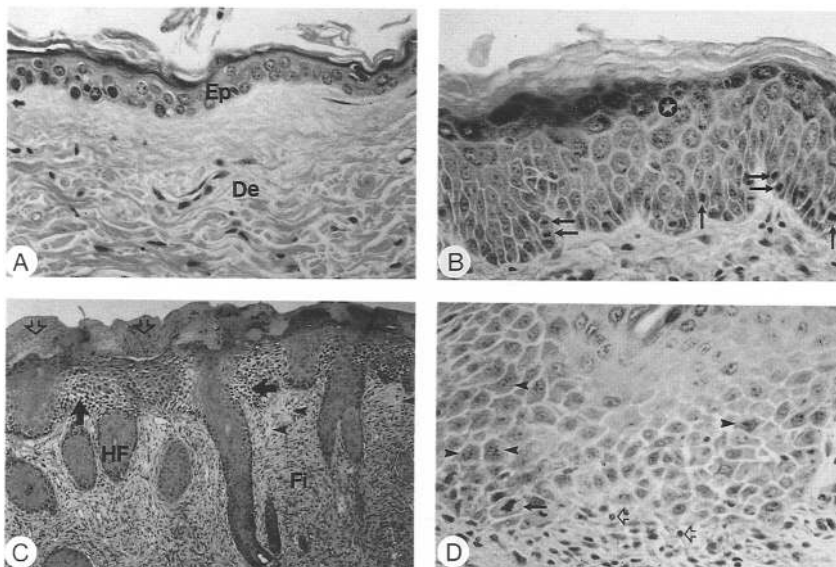


Figure 4. Histological analysis of the skin lesions of ADPRT^{-/-} mice. (A) Section through dorsal skin of a wild-type mouse showing normal morphology: A thin, orthokeratotic stratum corneum; epidermis composed of two to three keratinocyte layers and dermis with few cellular components and abundant fibers. (B) Section through a region showing a pronounced acanthosis in a mutant mouse. Layers of keratinocytes in the epidermis are increased with many mitotic figures (arrows) in the basal layer. Note the pronounced granular layer (star). (C) Lesional skin of mutant mice shows a typically hyperplasia in epidermis with parakeratosis, as evidenced by packed layer of nucleated keratinocytes in stratum corneum (open arrows); acanthosis (also visible in the hair follicles) and patchy spongiosis (solid arrows) in the epidermis as well as leukocytic infiltration, papillary edema (arrowheads), and fibrosis (Fi) in the dermis.

(D) Higher magnification reveals many multinucleated keratinocytes (arrowheads), several mitotic basal cells (arrow), and abundant inflammatory cells (open arrows) invading and partially destroying epidermal layer. (Ep) Epidermis; (De) dermis; (HF) hair follicle. Magnification, 200 \times (A, B, D); 50 \times (C).

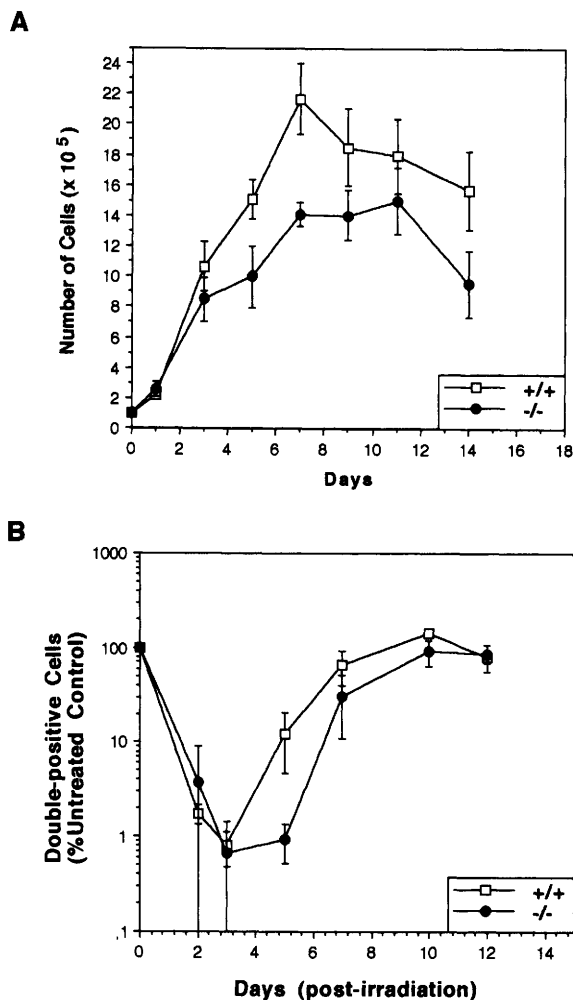


Figure 5. (A) Growth curves of primary fibroblasts from wild-type (+/+) and mutant (-/-) embryos. Cells were plated at a density of 10^5 cells/well in a 6-well plate (~ 10 cm²) (day 0), and cell numbers were counted at the indicated times. Data represent the means of triplicate cultures with standard deviation (\pm S.D.) of two cell lines of each genotype. Data represent one of three independent experiments. (B) Recovery of CD4⁺/CD8⁺ thymocytes following γ -irradiation in vivo. Wild-type (+/+) and mutant (-/-) mice received a sublethal dose (4.5 Gy) of radiation at day 0. Mice from each genotype were sacrificed at the indicated times, and CD4⁺/CD8⁺ thymocytes were analyzed by flow cytometry following specific antibody staining. Results are plotted as percentage of CD4⁺/CD8⁺ thymocytes in γ -irradiated mice compared with that of the untreated group. Data are mean values \pm S.D. of at least four mice from two independent experiments.

and their homozygous mutant littermates were sublethally irradiated (4.5 Gy) at the age of 4–6 weeks. At various times mice were sacrificed together with the untreated controls and the number of immature CD4⁺/CD8⁺ thymocytes was measured by flow cytometry. As expected, the number of CD4⁺/CD8⁺ thymocytes in all irradiated mice was drastically reduced in the first 2 days compared with that of nonirradiated controls, reaching

the lowest levels at 3 days postirradiation (Fig. 5B). However, whereas the content of thymocytes in wild-type thymus recovered by day 5 to $\sim 15\%$ of untreated control mice, mutant mice exhibited a significantly delayed recovery by ~ 2 days (Fig. 5B). This deficiency was not permanent as after an additional 9 days the number of mutant CD4⁺/CD8⁺ cells reached wild-type control levels (Fig. 5B). These studies suggest that either ADPRT or poly(ADP-ribosylation) have biological importance in fibroblast and thymocyte proliferation following experimental treatment.

Normal DNA repair in embryonic fibroblasts devoid of ADPRT

To study the role of ADPRT in DNA repair processes, we analyzed the efficiency of DNA repair in embryonic fibroblasts lacking ADPRT by (1) measuring the recovery of transcriptional activity of an in vitro-damaged reporter plasmid (Klocker et al. 1985), and (2) examining unscheduled DNA synthesis (UDS) by measuring incorporation of ³H-labeled thymidine into repaired DNA of cells treated with UV or MNNG (Schiffmann et al. 1984). First, a chloroamphenicol acetyltransferase (CAT) expression plasmid (SV40-CAT) was exposed in vitro to either UV radiation or MNNG and subsequently transfected into primary wild-type and ADPRT -/- fibroblasts. The DNA repair capability was measured by quantifying the restoration of CAT expression levels. To control the transfection efficiency, an untreated luciferase expression plasmid was cotransfected with the UV- or MNNG-damaged CAT plasmid and the amount of cell extract for the CAT assay was determined based on the luciferase values. Twelve hours after transfection a reduction in CAT activity from both UV- and MNNG-treated plasmids was observed in both ADPRT wild-type (+/+) and mutant (-/-) cells, indicating that the CAT plasmids were damaged, when compared with that seen in corresponding cells transfected with untreated CAT plasmid (Fig. 6A). After an additional 28 hr of culture, expression of CAT activity, regardless of treatment, reached levels comparable to the corresponding control values, demonstrating that UV- and MNNG-damaged plasmid DNA had been repaired with similar efficiency in both wild-type and ADPRT -/- cells (Fig. 6B).

The UDS assay was then employed to examine DNA repair synthesis further in mutant immortalized 3T3-like fibroblasts following DNA damage. Endogenous DNA replication was blocked by arresting these fibroblasts with 1% fetal calf serum and hydroxyurea, and the incorporation of [³H]thymidine into DNA following DNA damage reflects the efficiency of DNA repair synthesis (Schiffmann et al. 1984). Increased levels of thymidine incorporation were seen in both wild-type and ADPRT -/- cells, with increasing UV radiation dosage and concentration of MNNG, indicating that DNA damage and repair synthesis had occurred (Fig. 6C,D). However, there were no significant differences in the incorporation of [³H]thymidine into ADPRT -/- cells compared with the levels in wild-type cells, regardless of the

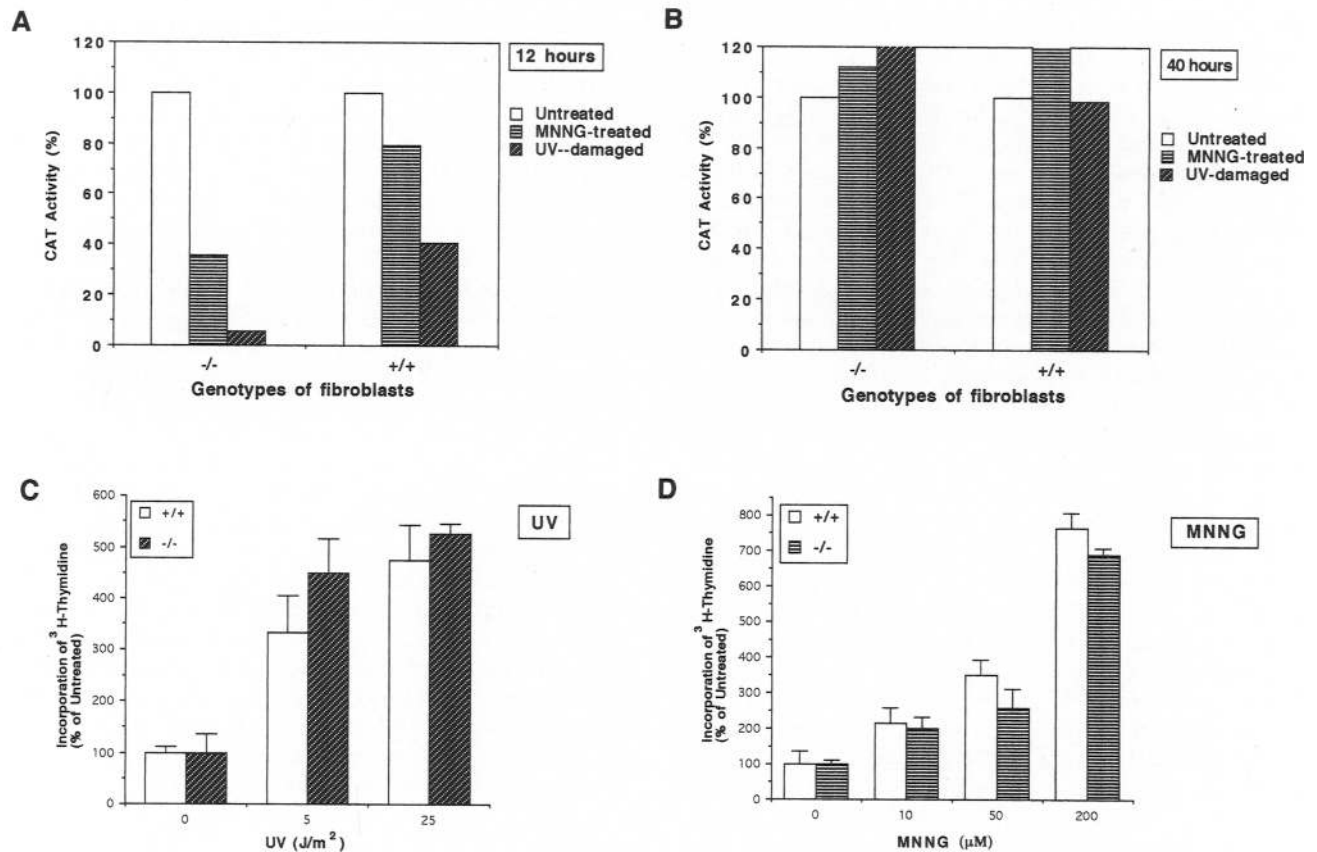


Figure 6. DNA repair assays in wild-type (+/+) and ADPRT mutant (-/-) fibroblasts. (A,B) An SV40-CAT plasmid was treated either with UV or MNNG and transfected into primary fibroblasts together with a luciferase plasmid that served as an internal control for transfection. The CAT activity of the damaged plasmid was compared with the CAT activity of the untreated plasmid (100%). At the 12-hr point, 100% activity of wild-type and ADPRT -/- cells equals 13.3% and 36.4% conversion of acetylated chloramphenicol respectively; at the 40-hr point, 100% activity of wild-type and ADPRT -/- cells equals 55.5% and 50.5%. Data represent one of four independent experiments. At 12 hr post-transfection the reduced CAT activity reflects the damage of the plasmid (A), and restoration of CAT activity at the 40-hr time point indicates DNA repair efficiency (B). (C) UDS assay in immortalized wild-type (+/+) and ADPRT -/- fibroblasts that were treated with different doses of UV. (D) UDS assay in +/+ and -/- cells that were treated with different concentrations of MNNG. The amount of incorporated [³H]thymidine was normalized to the untreated cells and is presented as percentage of the control. 100% equals to ~20,000 cpm.

DNA-damaging agents used (Fig. 6C,D). To further substantiate the kinetics of the DNA repair process, UDS assays were performed at different time points (from 15 min to 7 hr) after UV and MNNG treatment. Regardless of the genotypes, the efficiency of DNA repair was similar in both wild-type and ADPRT -/- 3T3-like fibroblasts (data not shown), indicating further that the DNA repair syntheses are comparable in both cell types.

Discussion

Homozygous ADPRT mutant mice generated from ES cells following gene disruption by homologous recombination were employed to investigate the biological function of poly(ADP-ribosyl)ation. The absence of an overtly strong phenotype in ADPRT-less mice is not attributable to incomplete gene inactivation because the introduced mutation appears to be a null allele as determined by the absence of any wild-type ADPRT mRNA and enzyme

activity in mutant tissues. Furthermore, immunofluorescence analysis showed that mutant fibroblasts are devoid of poly(ADP-ribose) following DNA damage. These findings are surprising in view of the widely held notion that poly(ADP-ribosyl)ation represents a fundamental post-translational modification of proteins that plays an important role in many chromatin functions, including chromatin stability and DNA repair. The use of genetic means to abolish the function of ADPRT in vitro and in vivo has allowed a direct evaluation of the biological function of poly(ADP-ribosyl)ation in murine development, cell proliferation, and DNA repair.

Poly(ADP-ribosyl)ation is not essential for mouse development and normal cellular processes

ADPRT -/- mice displayed normal fetal and postnatal development. A systematic histological examination of young ADPRT -/- mice revealed no gross abnormali-

ties in all tissues tested despite the fact that ADPRT is expressed in wild-type mouse tissues (Ogura et al. 1990; Grassi Zucconi et al. 1992). These results indicate that ADPRT/poly(ADP-ribosyl)ation is not required for cell proliferation and differentiation during development *in vivo*, and are striking in view of the importance assigned to this protein modification. One possible explanation for these results is that the defect is so subtle that a selective disadvantage would only emerge in older animals. An alternative possibility is that the function of ADPRT is substituted by another, yet unknown, enzyme. However, this latter possibility is unlikely because we have no evidence for the existence of any isoenzymes of ADPRT. No ADPRT enzymatic activity was detectable in protein extracts from ADPRT^{-/-} mouse tissues or embryonic fibroblasts. Moreover, the NAD⁺ depletion caused by activation of ADPRT following treatment of cells with free radicals was largely abolished in ADPRT^{-/-} lymphocytes or pancreatic islet cells (B. Heller, Z.-Q. Wang, E.F. Wagner, J. Radons, A. Bürkle, K. Febsel, V. Burkhart, and H. Kolb, in prep.), thereby demonstrating that ADPRT is responsible for the consumption of NAD⁺ as a substrate. Finally, the formation of poly(ADP-ribose) following DNA damage by MNNG could not be detected by staining with monoclonal antibodies against (ADP-ribose) polymers in embryonic fibroblasts derived from ADPRT mutant mice. However, this antibody may be unable to detect oligomers of ADP-ribose smaller than tetramers (Kawamitsu et al. 1984); therefore, we cannot rule out the formation of oligomers in mutant cells. It is possible that the function of ADPRT on DNA damage may be substituted by another mechanism that is presently unknown. A candidate for an alternative pathway could be the DNA-dependent protein kinase (for review, see Gottlieb and Jackson 1994).

Cells devoid of ADP-ribosylation exhibit proliferation defects

Although mice lacking ADPRT showed no apparent developmental defects *in vivo*, poly(ADP-ribosyl)ation may be important for cells particularly in response to environmental stress. Cultivation of primary embryonic fibroblasts revealed that ADPRT-deficient cells have a reduced proliferation rate. A similar proliferation deficiency was also observed in thymocyte progenitors, which showed a delayed recovery following whole-body γ -radiation. The mechanism of the impaired proliferation in ADPRT^{-/-} cells is not clear and a preliminary analysis showed that there are no apparent differences in cell cycle progression or in the ability of quiescent ADPRT^{-/-} fibroblasts to re-enter the cell cycle when compared with wild-type controls (L. Stingl, unpubl.). One explanation might be that the lack of poly(ADP-ribosyl)ation may affect chromatin structure in a manner that influences DNA replication and/or chromosome segregation, because chromatin contains a large proportion of poly(ADP-ribosyl)ated proteins and poly(ADP-ribosyl)ation facilitates chromatin relaxation. However,

DNA fragmentation following treatment of thymocytes *in vitro* with apoptotic agents (e.g., γ -radiation and glucocorticoid hormones) was not affected by the ADPRT mutation (data not shown), suggesting that poly(ADP-ribosyl)ation does not appear to be involved in chromatin fragmentation. It is also possible that DNA metabolic enzymes might require poly(ADP-ribosyl)ation to function efficiently, because several enzymes involved in DNA replication, such as topoisomerases and DNA polymerases, have been shown to be acceptors of poly(ADP-ribosyl)ation. An association between the DNA metabolic enzymes and poly(ADP-ribosyl)ation was found for topoisomerase β expression, which correlates with the expression of ADPRT *in vivo* (Ogura et al. 1990). The fact that proliferation deficiencies in ADPRT^{-/-} cells emerged only under stress conditions may suggest that target molecules, such as DNA metabolic enzymes and perhaps cell cycle regulatory molecules, like p53 and cyclins, may need to be poly(ADP-ribosyl)ated when the cells are challenged by external stimuli. Alternatively, it is possible that the impaired proliferation of mutant cells might be attributable to a higher frequency of misrepaired DNA following imposed stress. Thus, it is conceivable that whereas poly(ADP-ribosyl)ation is dispensable for normal cellular activity *in vivo*, it may play an important role in the cellular response to environmental disturbances.

Epidermal hyperplasia as a consequence of lacking a protective response?

Despite the fact that young ADPRT-deficient mice exhibited no apparent phenotypic changes, ~30% of mice older than 6 months developed a characteristic skin disease on a mixed genetic background. The disease was histologically identical in all affected mice and was characterized by acanthosis, parakeratosis, and spongiosis. These lesions are apparently itchy, as the mice often scratch themselves. However, the hyperplasia in the epidermis was not caused by inflammatory infiltrate because there were no signs of inflammation in mild lesions of the skin (data not shown). In addition, transplantation of haematopoietic stem cells from affected mice into lethally irradiated wild-type mice did not transfer the disease (data not shown), implying that the keratinocyte hyperplasia is not a secondary response to abnormal function of lymphoid and myeloid lineages. Rather, our results suggest that the increased keratinocyte proliferation may be attributable to metabolic changes in these cells. One possibility is that ADPRT or poly(ADP-ribosyl)ation is a negative regulator of cell proliferation. This is very unlikely because mice lacking the gene are of normal size and are not tumor prone, and primary fibroblasts grow even slower *in vitro*. In addition, immortalized ADPRT^{-/-} fibroblasts exhibit no significant proliferation differences compared with wild-type cells (data not shown). Another possibility is that cells devoid of ADPRT lack a protective mechanism to eliminate cells prone to escape growth control. In support of this hypothesis is the fact that free radicals induced the

same extent of DNA damage in wild-type and ADPRT^{-/-} pancreatic β -islet cells; however, mutant cells showed a significantly higher resistance to cell lysis (B. Heller, Z.-Q. Wang, E.F. Wagner, J. Radons, A. Bürkle, K. Fehsel, V. Burkhardt, and H. Kolb, in prep.). It is likely that whereas normal cells have a mechanism to exclude DNA-damaged cells by a cell death pathway through lowering NAD⁺ levels (Berger 1985; Gaal et al. 1987), resistance of the ADPRT^{-/-} cells to death may increase the risk of error-prone DNA repair and thus mutagenesis in these cells. Because the skin is an organ constantly exposed to environmental stress, it is conceivable that the lack of ADPRT activity in keratinocytes may prevent elimination of cells that contained damaged DNA and may therefore be susceptible to additional events leading to hyperproliferation. This increased susceptibility may also explain, at least in part, why only a fraction of mutant mice develop the skin disease.

DNA repair processes are not affected by the lack of poly(ADP-ribosyl)ation

Two independent DNA repair assays were used to demonstrate that damaged DNA can be repaired efficiently in cells devoid of ADPRT and poly(ADP-ribosyl)ation. Our analysis showed that ADPRT^{-/-} cells can repair both MNNG-induced methylation damage (base excision repair) and UV radiation-induced photoproducts (nucleotide excision repair). This suggests that ADPRT/poly(ADP-ribosyl)ation is most likely not directly involved in both repair pathways. These data are striking, as cells treated by chemical inhibitors or cells with reduced ADPRT expression displayed increased sensitivity to DNA-damaging agents (Durkacz et al. 1980; Chatterjee et al. 1991; Berger et al. 1992). Furthermore, recent studies using dominant-negative mutants of ADPRT in cells showed that inhibition of ADPRT activity blocked unscheduled DNA synthesis induced by MNNG but not by UV radiation, thereby underscoring the importance of ADPRT in the repair of DNA damage (Molinete et al. 1993). The discrepancies in the possible role of ADPRT in DNA repair may be attributable to the fact that previous studies used either ADPRT inhibitors, which may have additional nonspecific effects, or spontaneous ADPRT mutant cell lines, which contain reduced levels of ADPRT. In agreement with our data showing that ADPRT is not a critical regulatory component in the DNA repair process are studies demonstrating that DNA repair was apparently independent of ADPRT (Satoh and Lindahl 1992; Satoh et al. 1993; Smulson et al. 1994). These results suggest that ADPRT acts as a "nick-protection" molecule to prevent accidental homologous recombination events and is needed for the maintenance of chromatin structure (Althaus et al. 1990; Satoh et al. 1994).

In conclusion, the data presented here strongly suggest a more sophisticated function for ADPRT than appreciated previously. ADPRT/poly(ADP-ribosyl)ation appears to play a protective role in mice in damaging environ-

ments, which is in agreement with the hypothesis that poly(ADP-ribosyl)ation might be a cellular emergency reaction in response to environmental challenges (Berger 1985; Wintersberger and Wintersberger 1985; Gaal et al. 1987). The spontaneous skin disease observed in older mice may be related to the lack of a protective mechanism by poly(ADP-ribosyl)ation, which ensures that the damaged cells can be eliminated. However, the proliferation deficiency seen in mutant fibroblasts and thymocytes following γ -irradiation may be explained by another mechanism involving poly(ADP-ribosyl)ation of DNA metabolic enzymes as well as nuclear matrix proteins. Although the molecular mechanisms remain to be defined, ADPRT^{-/-} mice and cells isolated from them will undoubtedly provide a useful experimental tool for dissecting the exact role of this enzyme in normal physiological conditions and following environmental insults.

Materials and methods

Construction of the targeting vector

The ADPRT gene contains 23 exons; we targeted to the second exon using a promoterless targeting vector. A 5.2-kb *Sall* fragment isolated from a 129/Sv genomic library contains the entire second exon of the genomic ADPRT gene with 5'- and 3'-flanking sequences (Berghammer et al. 1992). This fragment was digested with *Asp718*, releasing part of the second exon and intron, and was fused in-frame with a *neo* cassette, derived from pHA14 (te Riele et al. 1990), which lacked ATG but contained a TGA stop codon and a polyadenylation site. The *PstI* and *PvuII* restriction sites that were introduced by the *neo* fragment facilitated the Southern blot analysis of the recombination events in targeted clones. The *HindIII* fragment (2.3 kb) was subcloned further into a pUC12 plasmid to generate the final target vector pMAII2.3H.

Gene targeting in ES cells and generation of mutant mice

A 2.3-kb *HindIII* fragment was isolated from the clone pMAII2.3H and electroporated into D3 ES cell lines. The cells were then plated onto four 100-mm dishes containing mitomycin C-treated G418^r mouse embryo fibroblast feeder cells. The ES cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplied with 15% fetal calf serum (FCS), 1% penicillin and streptomycin, 1×10^{-4} M β -mercaptoethanol, 1000 U/ml leukemia inhibitory factor (LIF) and an additional 0.3 mg/ml of G418 for *neo* selection. The selection started 24–48 hr after plating and was maintained for up to 10 days. Three *neo*-resistant colonies were found to contain homologous recombination events when analyzed by PCR and verified by Southern blot. Primers for the PCR reaction were outside of the sequence of the targeting vector (5'-GTTGTGAACGACCTTCTGGG-3') and inside the *neo* gene (5'-GTTGTGCCAGTCATAG-3'). The probe for Southern blot was a 600-bp *XhoI*-*HindIII* fragment of the gene that is not present in the targeting vector (pMAII2.3H). Screening of ES cells with PCR and Southern blot, and the generation of germ-line chimeric mice were described previously (Wang et al. 1992). For testing germ-line transmission, chimeric males originating from clone D3-18-6 were bred to C57BL/6 females and germ-line offspring were determined by the presence of an agouti coat color in F₁ progeny and by Southern blot analysis to detect the targeted allele.

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RNA isolation and Northern blot analysis

Isolation of poly(A)⁺ RNA from mouse tissues and Northern blot analysis were performed as described previously (Wang et al. 1991). The probe was a 2-kb fragment of human cDNA that covers the amino-terminal region of the mRNA and recognizes the ADPRT-*neo* fusion transcript (Schneider et al. 1987; Auer et al. 1989).

Enzymatic analysis

Approximately 50 mg of mouse tissue was ground in liquid nitrogen. The resulting fine powder was added to 100 μ l of sample-buffer (40 mM Tris at pH 6.8, 5% glycerol, 1% SDS, 0.001% bromophenol blue, 100 mM β -mercaptoethanol), and the suspension was mixed vigorously and centrifuged. Ten microliters of the supernatant was applied to SDS-PAGE [10% acrylamide gel containing 100 μ g/ml of activated DNA, described by Loeb (1969)]. After electrophoresis, the gel was treated as described by Scovassie et al. (1984). The ADPRT reaction was carried out in 5 ml of reaction buffer [100 mM Tris at pH 8.0, 10 mM MgCl₂, 1 mM DTT, 15 mM NAD⁺, 10 μ Ci/ml of ³²P-labeled NAD⁺ at 800 Ci/mmol] at 25°C for 12 hr. The gel was exposed to Kodak XAR films at -80°C for autoradiography.

Flow cytometry of thymocytes and histological examination of the skin

Eighteen wild-type and mutant mice at 4–6 weeks of age were sublethally irradiated (4.5 Gy) from a dual Cesium 137 source (Gammacell 40; Nordion, Kanata, Canada) at a dose of 1.2 rad/min. Analyses of thymocytes by flow cytometry and histological analysis of skin were performed as described by Wang et al. (1992).

Isolation and immortalization of primary embryonic fibroblasts

Embryonic fibroblasts were isolated and immortalized essentially as described by Brüsselbach et al. (1995). The A11, A12 (ADPRT -/-), and A16, A19 (+/+) cell lines were used for the proliferation and DNA repair experiments. To examine the growth property of the primary embryonic fibroblasts, 1 \times 10⁵ cells were plated into six-well plates in DMEM containing 10% FCS. The medium was changed every 2 days, and cells were counted using a hemocytometer.

Immunofluorescence assay on fibroblasts

Wild-type and ADPRT -/- fibroblasts were cultured as described above. For induction of DNA damage and antibody staining, the cells were cultured on coverslips at subconfluence. The cells were incubated with 100 μ M of alkylating agent MNNG (made from a fresh stock) at 37°C for 30 min. The cells were then washed immediately and fixed in cold 10% TCA/PBS and dehydrated through a graded serial ethanol washing and subsequently incubated with the monoclonal antibody 10H directed against poly(ADP-ribose) (Kawamitsu et al. 1984). The specific signal of poly(ADP-ribose) was visualized by the staining of the cells with FITC-conjugated goat-anti-mouse IgG.

DNA repair assay (CAT assay and UDS)

An SV40-CAT plasmid (Promega, cat. no. E-1031) was damaged by UV radiation (450 J/m²; emission peak 254 nm, Stratagene) or by incubation with 0.4 mM MNNG. Six micrograms of dam-

aged or untreated (control) plasmid, together with 0.6 μ g of luciferase reporter plasmid, was cotransfected into 1 \times 10⁵ fibroblasts in 6 cm plates. Transfection and CAT assay were described previously by Hilberg et al. (1987). Luciferase activity was determined according to the protocol of de Wet et al. (1987). Unscheduled DNA synthesis was performed according to the protocol described by Schiffmann et al. (1984) with minor alterations. To reduce background of replicative DNA synthesis the cells were cultured for 48 hr in DMEM medium containing 1% fetal calf serum and 10 mM hydroxyurea was added to the cultures 30 min before UV or MNNG treatment. Thereafter, the cells were incubated with [³H]thymidine (10 μ Ci/3 \times 10⁵ cells) for 5 hr.

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Note added in proof

We recently observed that a large fraction of ADPRT -/- female mice older than 15 months become obese, suggesting an important role for ADPRT/poly(ADP-ribosylation) in general metabolism.

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Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease.

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