V. RESULTS

Chapter I

Induction of cellular immunity by direct pDNA transfer of a mutated p53 allele into Balb/c mice

In recent years tumor immunotherapy has received a great deal of attention. Two developments have contributed to this. The first is the identification of target antigens expressed by tumors. Tumor specific antigens are generated through mutation or by chromosomal translocation. In many tumors, these antigens are overexpressed or altered by translational modification and can be identified by cytotoxic T cells and antibodies. So far over- or aberrantly expressed normal antigens have proved the most promising therapeutic targets, since in contrast to point mutations, they usually provide a relatively large number of potential T cell epitopes.
The second reason for the resurgence of interest in the immunotherapy of tumors is the increased understanding of the factors regulating the immune response. Over the last decade there have been great strides in elucidating the nature of thymic selection and the heterogeneity of peripheral lymphocytes including the recognition of effector cells with distinct Th1 and Th2 cytokine profiles. Of particular importance to tumor immunology have been advances in the understanding of antigen presentation. The realization that T cells can recognize antigens originating within cells means that intracellular tumor proteins are now potential targets for immunotherapy. The immune repertoire of individuals with tumors contains both T and B cells capable of responding to antigens present within malignant cells (236, 237, 238). Immunotherapeutic approaches to cancer therapy are thus aimed at improving tumor antigen presentation, at enhancing effector cell response, or both. This study aims to develop a strategy for the immunotherapy of cancer utilizing plasmid DNA encoding antigenic tumor specific proteins. T cells patrol in the body as policemen in constant search for infected or degenerated cells. On the other hand malignant cells form in the process of natural selection protective mechanisms to evade detection and elimination by the immune system. Upon direct injection of tumor antigen encoding plasmid DNA T cells obtain a description of the "bad boys", they are preactivated and more efficient to look for cells that show distinctive features of a malignant phenotype.

5.1 Reporter gene constructs

The plasmid pZeoSV (Invitrogen, NV Leek, The Netherlands) is a constitutive mammalian expression vector. High level transient and stable expression of inserted genes is controlled by the Simian Virus 40 (SV40) early enhancer-promoter sequences. To construct pZeoSVLuc, a Hind III - BamH I fragment containing the firefly luciferase gene was cloned into the pZeoSV vector. pZeoSVLacZ, encoding the bacterial beta-galactosidase reporter gene was obtained from Invitrogen, NV Leek, The Netherlands. To construct pCMXLuc, the Hind III - BamH I luciferase fragment was cloned into the mammalian expression vector pCMX (gift from Roland Schüle, Tumor Biology Center, Freiburg, Germany). In this vector eukaryotic expression of the luciferase gene is under control of the cytomegalovirus (CMV) immediate early promoter.

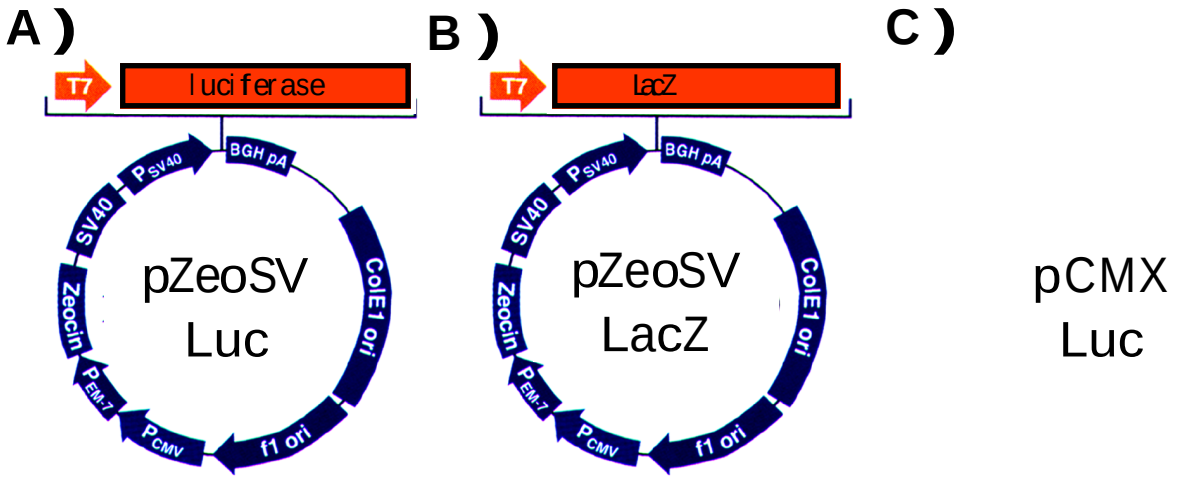

Fig. 5.1 Reporter gene constructs. The pZeoSV expression vectors are designed for high level constitutive expression in mammalian cells. Gene products are expressed via the SV40 enhancer-promoter. The zeocin resistance gene can be used for selection. In the pCMX vector the luciferase expression is regulated by the immediate-early cytomegalovirus promoter.

5.2 *In vivo* application of pDNA

It has been shown that intramuscular or intradermal injection of naked plasmid DNA (pDNA) encoding for viral or other protein antigens results in the uptake of the pDNA by the muscle or skin cells and subsequent synthesis of the protein by these cells. In skin dermal keratinocytes, fibroblasts, and cells with the morphology of Langerhans cells and macrophages showed prolonged expression of the intracellular antigen (239). The transfected cells process the antigen, present immunogenic peptides on major histocompatibility complexes and induce both long-lived humoral and cellular immune responses. pDNA based immunization offers a new, promising tool, especially for inducing cellular immune responses to viral infection and tumors. Before immunization with tumor antigen encoding plasmids, we analyzed the *in vivo* expression of reporter genes and optimized the transfer conditions for pDNA.

5.2.1 Expression of the luciferase-reporter gene construct in mice

Six to eight weeks old female Balb/c mice were injected with 40 µg of reporter pDNA. To obtain best transfection efficacy different transfer conditions were tested. pDNA was diluted in H_2O, PEI, PBS, HBS, or a cationic lipofection reagent and injected intradermally into mice. Alternatively pDNA was coated to gold particles and used for biolistic transfer (Fig. 5.2). Biopsies of the injection site were homogenized in 400 µl ice-cold lysis buffer by a Ultratorax 100™ cell homogenizer (1 min., 15000 rpm), centrifuged and 200 µg total protein were analyzed in a 200 µl volume in the luminometer AutoLumat LB953, Berthold, Germany. Transfection results are shown in Fig. 5.2. Luciferase expression after 48 hrs. was highest by high velocity particle bombardment or when pDNA was diluted in PBS or mixed with a liposomal transfection reagent (Fig. 5.2).
Cationic liposomal gene transfer is thought to be based on the compensation of the negative charges of the phosphate DNA backbone and favors fusion of pDNA-liposomal complexes with negatively charged cell membranes. Additionally pDNA-liposomal complexes are less susceptible for proteolytic degradation in cellular lysosomes. To determine the optimum liposome-pDNA ratio, liposome-pDNA complexes were formed by mixing 30 µl of the cationic transfection reagent DOTAP with variable amounts of the pZeoSVLuc reporter gene. Mixtures were injected intradermally into mouse ears and luciferase reporter gene expression was analyzed 48 hrs. post pDNA injection. The ratio of the positively charged cationic lipofection reagent and the negatively charged phosphates of the DNA backbone was

shown to be of fundamental importance for transgene transfer and expression of the protein antigen (Fig. 5.3).

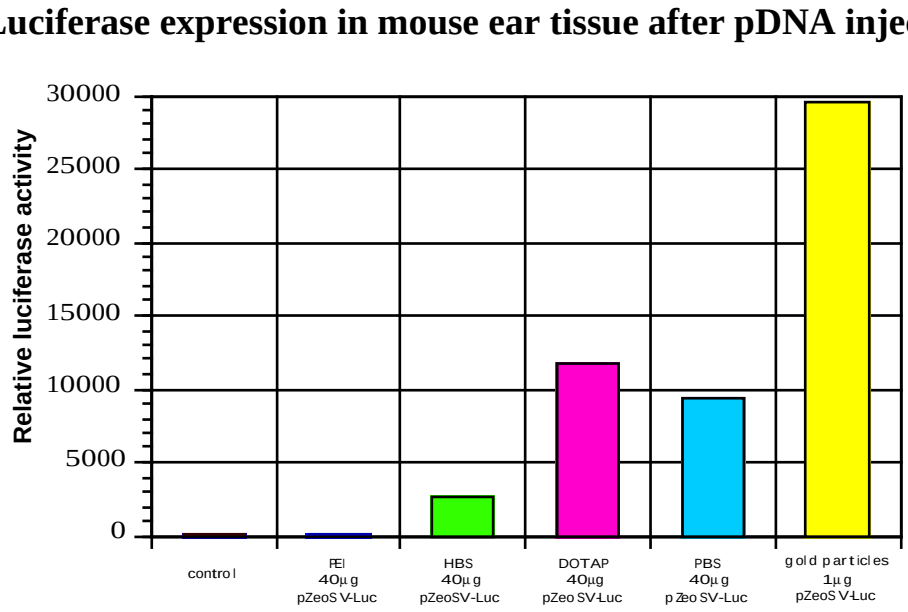

Fig. 5.2 Determination of optimal transfection conditions for naked DNA in *in vivo* applications. pZeoSVLuc-pDNA was injected subcutaneously in different solubilization buffers (40 µg pDNA) or coated to gold particles (1 µg pDNA) for high velocity particle bombardment into ears of Balb/c mice. After 48 hrs. transfected cell lysates were normalized for cytoplasmatic protein content and analyzed for activity of the luciferase reporter gene.

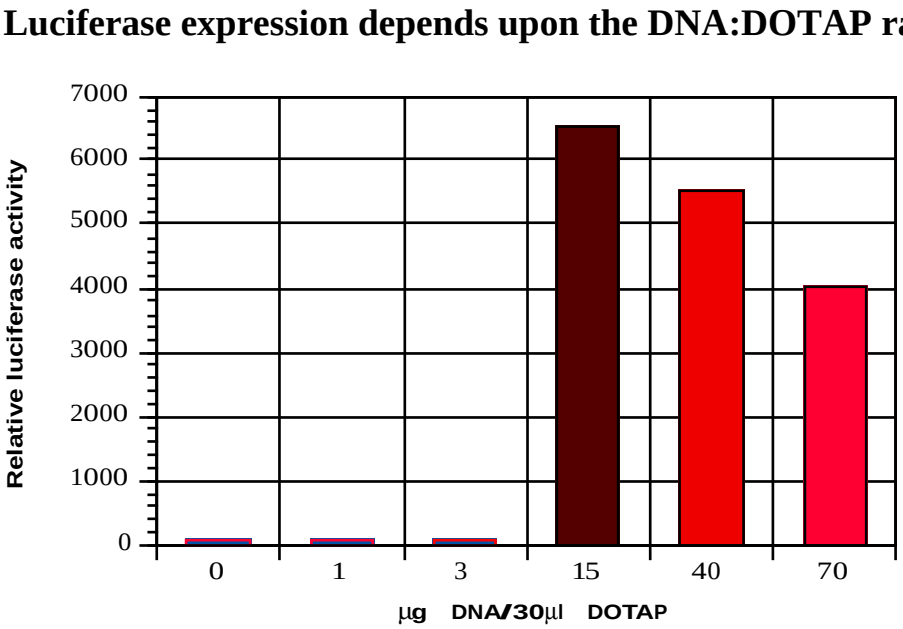

Fig. 5.3 Luciferase reporter gene expression is a function of the liposomal transfection reagent/DNA phosphate ratio. A pZeoSVLuc-liposomal transfection mixture including various pDNA amounts and 30 µl of the cationic lipofection reagent DOTAP was injected subcutaneously into mouse ears. Transfected cell lysates were normalized for cytoplasmatic protein content and luciferase expression was measured 48 hrs. post immunization.

5.2.2 Expression of the lacZ-reporter gene construct in mice

For the detection of β-galactosidase expression, biopsies of injected tissue were snap frozen in liquid nitrogen/isopentane, cut to 5-10 µm sections, mounted on glass slides, fixed in 4% formaldehyde for 10 min., rinsed twice with PBS containing 1 mM $MgCl_2$, and stained for 20 hrs. at 37°C in PBS, 2 mM $MgCl_2$, 16 mM $K_3Fe(CN)_6$, 16 mM $K_4Fe(CN)_6$, and 5 mg/ml X-Gal substrate. Expression of the reporter genes was localized in the epidermal and dermal region (Fig. 5.4), 100-300 µm below the epithelial cell layer. This area is known to be enriched by keratinocytes, Langerhans cells and dendritic cells. These cells perform critical immune regulatory and antigen presenting function *in vivo* (240, 241). In general high velocity particle bombardment by the gene gun is transfecting upper cell layers within the epidermal region. Acceleration of gold particles was achieved at 15 to 45 bars. Depth of transgene expression was not substantially influenced by the pressure that was applied to accelerate gold particles to the tissue (data not shown). Best results and highest expression were observed with a pressure adjusted to 35 bar (Fig. 5.4) These conditions were also used for gene gun immunization with mutant p53 vector constructs. However, injection of naked DNA effectively transfected cells also in lower dermal layers at multiple sites (Fig. 5.4). The level of transgene transfer and expression was comparable to the gene gun method. However, as little as 1 µg of pDNA coated to gold particles resulted in similar expression as injection of 40 µg of the reporter gene construct.

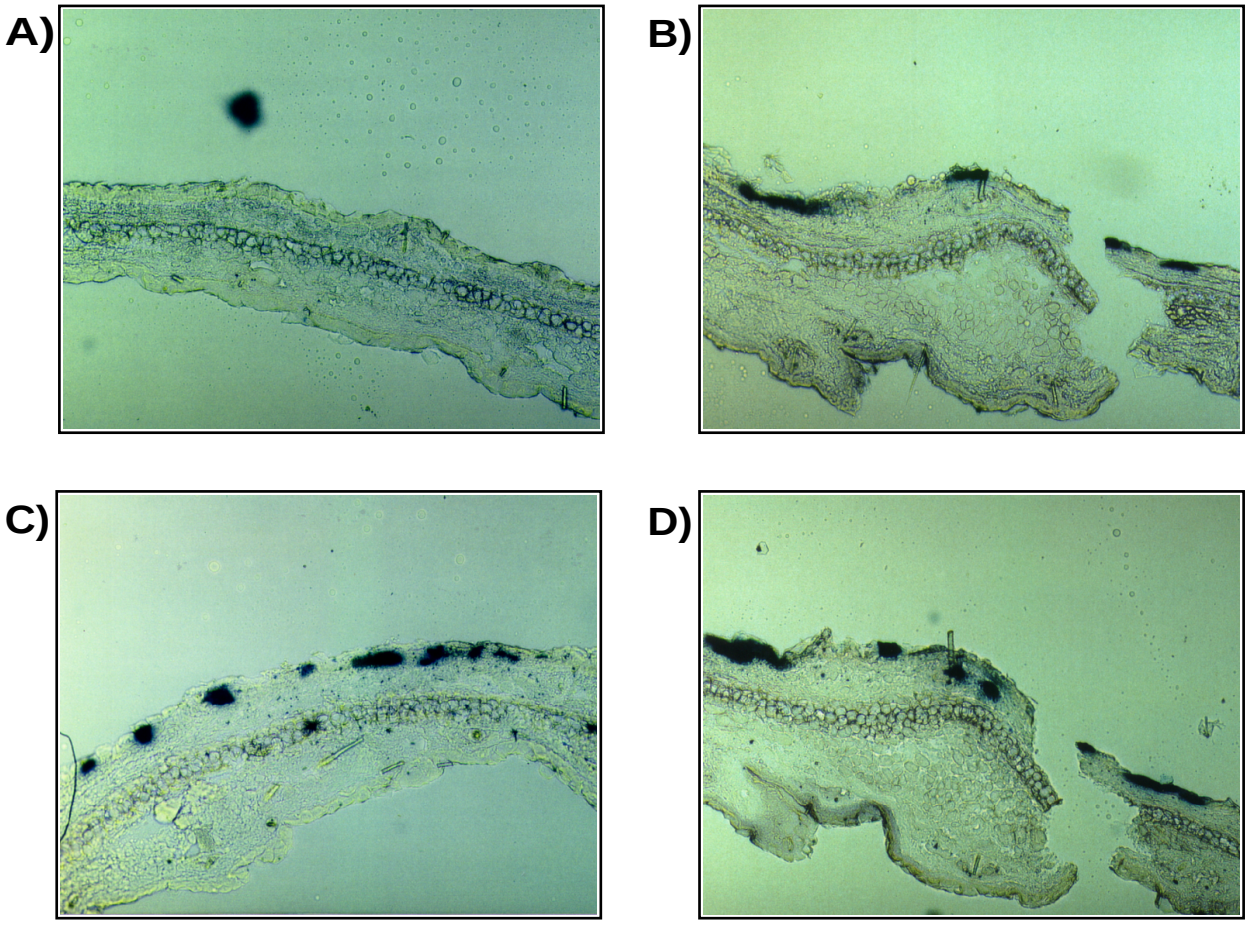

Fig. 5.4 Delivery of naked LacZ-DNA to the ears of Balb/c mice by direct intradermal injection. Stain indicates gene activity in the dermal tissue cells after staining with X-gal substrate. In the control (a), gene activity was not detectable. Gene gun mediated transfer (b) showed strong expression of the LacZ-gene with as little as 1 µg pDNA coated to gold particles. LacZ-gene expression was also detectable after intradermal injection of 40 µg pDNA solubilized in PBS (c) or as liposomal complexes when mixed with the cationic liposomal transfection reagent DOTAP (d). Skin tissues were processed for cryohistology by standard histological procedure and photographed under a light microscope.

5.2.3 Detection of reporter gene expression in lymphoid tissue

Cutaneous immunity is initiated via skin-associated lymphoid tissues that are located in the epidermis and dermis, and are separated by the dermal-epidermal junction and the basement membrane. Langerhans cells and dermal dendritic cells reside in the epidermis and dermis to capture antigens. Once activated they can enter blood capillaries, post-capillary venules and lymphatic channels present in the dermis. Afferent lymphatic ducts carry lymph contents to the draining lymph nodes containing lymphoreticular cells and post-capillary venules. Here, immune effector cells are mobilized and upon activation they migrate to sites of antigen exposition to eliminate cells that are presenting the antigen. We were interested if the injected transgene was transported to lymphoid tissue, probably due to dermal APCs. Lymph nodes are the central region for activation of resting lymphocytes by presentation of the antigen protein by professional APCs. In our experiment 6 to 8 weeks old female Balb/c mice were injected with the transgene and the lymph nodes were excised and analyzed for transgene expression. We could demonstrate short termed luciferase reporter gene expression in cervical lymph nodes after intradermal injection of pCMX Luc-pDNA into mouse ears (Fig. 5.5). Transport of injected fluids to lymph organs was visualized by injection of 100 µl Isovit-300 (Schering, Berlin, Germany) and autoradiography (data not shown).

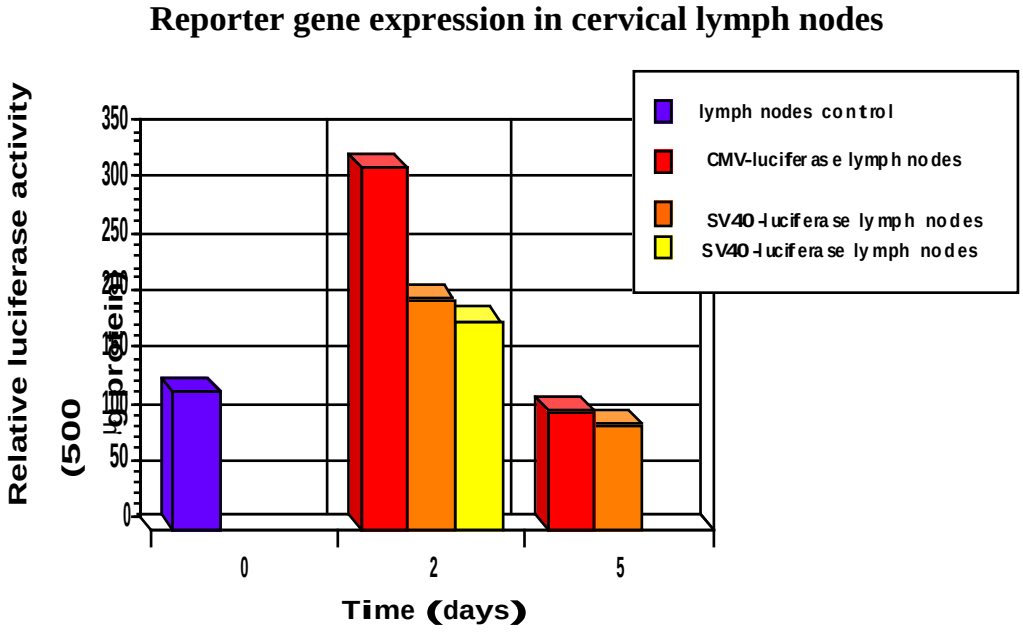

Fig. 5.5 Analysis of draining lymph nodes for luciferase gene expression. Ears of six to eight weeks old female Balb/c mice were injected with 70 µg pCMXLuc-pDNA/DOTAP (red bar) or 70 µg pZeoSVLuc-pDNA/DOTAP (yellow/orange bar). Draining lymph nodes were isolated and analyzed for reporter gene expression by standard procedure at day 2 or day 5 after DNA inoculation. At day 2 two to three-fold expression of the luciferase reporter gene could be detected in the lymph nodes in comparison to control tissue. At day 5 transgene expression was not measurable anymore.

5.2.4 Time kinetics of reporter gene expression

Long term expression of the transgene has been reported by different groups in animal tissue (239). Ideally, the expression of the transgene leads to a permanent induction of the immune system and a persistent activation of immune effector cells. Analysis of the pattern of the transgene expression allows drawing conclusions for additional booster immunizations to maintain a strong antigen stimulus. In an animal experiment pZeoSVLuc-pDNA/DOTAP (Fig. 5.6) or pCMXLuc-pDNA/DOTAP (Fig. 5.7) were injected subcutaneously into ears of mice and transgene expression was measured at different time points post injection. A rapid

decrease of reporter gene activity was observed in both cases. The CMV driven reporter gene showed 10 to 15 times higher expression in comparison to SV40 driven constructs. However, expression of the reporter gene constructs could be observed for months in both cases. Due to a rapid decrease of reporter gene expression, we suppose delivery of pDNA-vaccination with the tumor antigen vaccine in 10 days intervals to maintain high expression of the transgene and to boost the immune response.

Time kinetics of luciferase expression post pDNA injection (SV40 promoter)

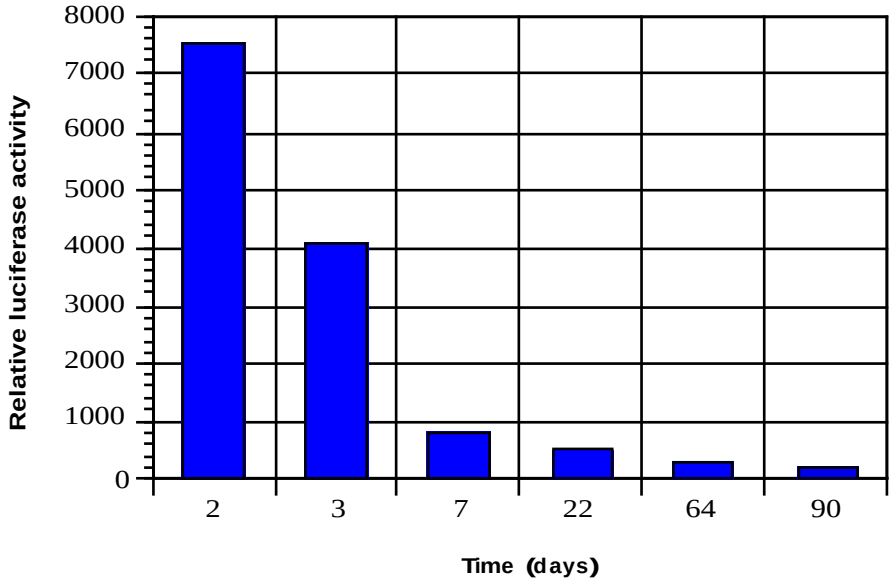

Fig. 5.6 Injection of pZeoSVLuc into ears of Balb/c mice. Luciferase reporter gene activity was analyzed by standard procedure at day 2, 3, 7, 22, 64, and 90 post injection. The amount of protein was standardized to 200 µg. A rapid decline of luciferase activity could be observed. Maximum expression was measured at day two. To maintain high expression of the transgene, delivery of pDNA in 10 days intervals appears appropriate.

Time kinetics of luciferase expression post pDNA injection (CMV promoter)

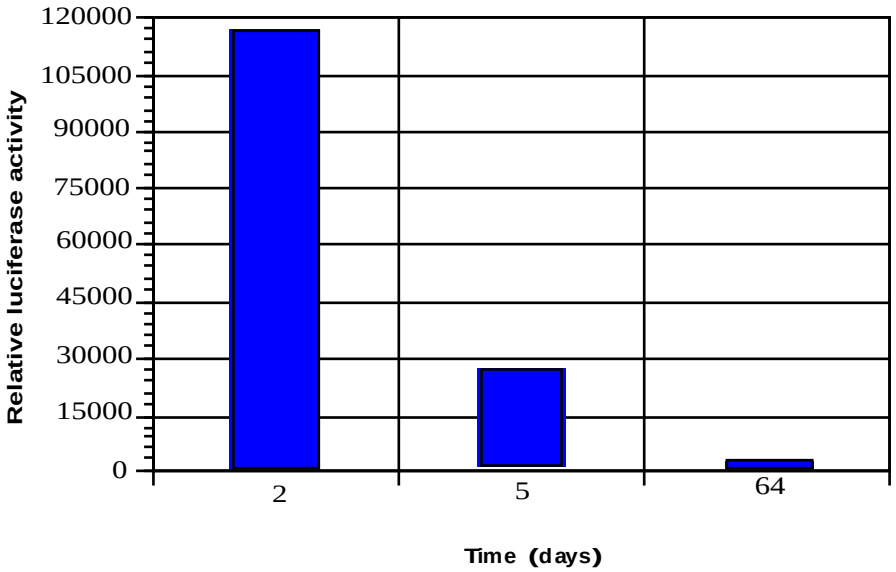

Fig. 5.7 Injection of pCMXLuc-pDNA/DOTAP into ears of mice. Reporter gene activity was measured at day 2, 5, and 64 post injection. Highest expression of the luciferase gene was measured at day two. At day 5 expression was reduced by a factor of 5 to 6. Two months post injection expression of the reporter gene was still detectable, 3 to 4 fold higher than in untreated control mice.

5.3 Immunization of mice with mutant p53 tumor antigen

CTLs play a crucial role in the host's immune response to viral infections and cancer. These effector cells recognize short peptide antigens in association with class I MHC molecules. Virtually all class I MHC-associated peptides sequenced today consist of 8 to 10 amino acid residues. They conform to allele specific sequence motifs, in which the amino acids found in the second and ninth positions are relatively invariant. CTL defined tumor peptides have been identified and used for the development of peptide-based cancer immunotherapy (82, 83, 84). One obvious candidate for such T cell recognition is the product of p53, a gene frequently mutated in tumors of experimental animals and humans. The Meth A sarcoma is one of the best studied of all murine tumors and is extremely lethal unless protective immunity is induced. Meth A tumors express high levels of p53 that is known to have three missense point mutations in codons 132, 168, and 234 (Fig. 5.9)(242, 243). Spleen cells, pulsed with peptides containing the 234 mutation induced specific long-term reactive CTLs**.** 234 peptide immunized mice also showed heightened resistance to Meth A tumor challenge and a proliferative $CD4^+$ T cell response (244-246). No consistent response was seen with peptides spanning codon 132 or 168.

5.3.1 Generation of mutant p53 expressing pDNA

To explore p53 as a DNA-based tumor vaccine, two mutant p53 alleles were isolated from Meth A tumor cells by RT-PCR (Fig. 5.8) and inserted into mammalian expression vectors. Features of the expression vectors are described in Fig. 5.9. The two mutant p53 alleles were distinguished by restriction analysis utilizing the Afl III restriction endonuclease site (Fig. 5.8). The sequence of mutant p53 was verified by sequence analysis utilizing the Cycle Sequencing Kit, Perkin Elmer (data not shown).

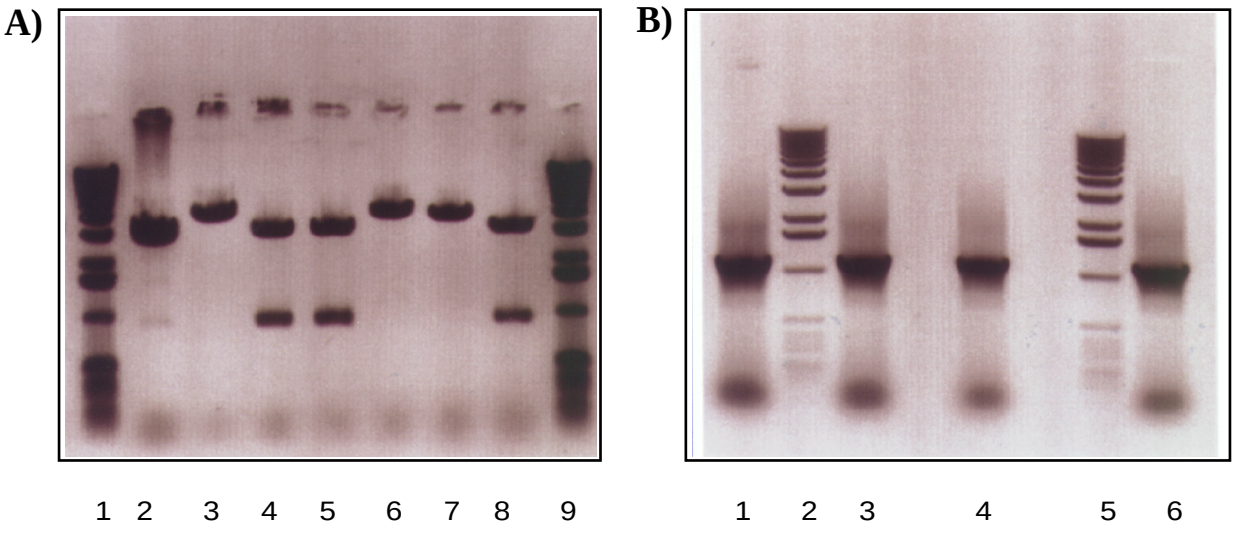

Fig. 5.8 Two mutant p53 variants were isolated from the mouse tumor cell line Meth A by RT-PCR. A 1.2 kb EcoR I, Hind III DNA fragment (lane 1, 3, 4, 6) was isolated from the agarose gel and inserted into mammalian expression vectors (B). Different alleles were identified by restriction analysis (A). A point mutation in the DNA binding region of one p53 allele eliminates an Afl III restriction endonuclease site resulting in a 4.7 kb fragment as visualized on the gel after Afl III endonuclease treatment (allele 1, lane 3, 6, 7). On lane 2 the pZeoSV vector molecule was loaded.

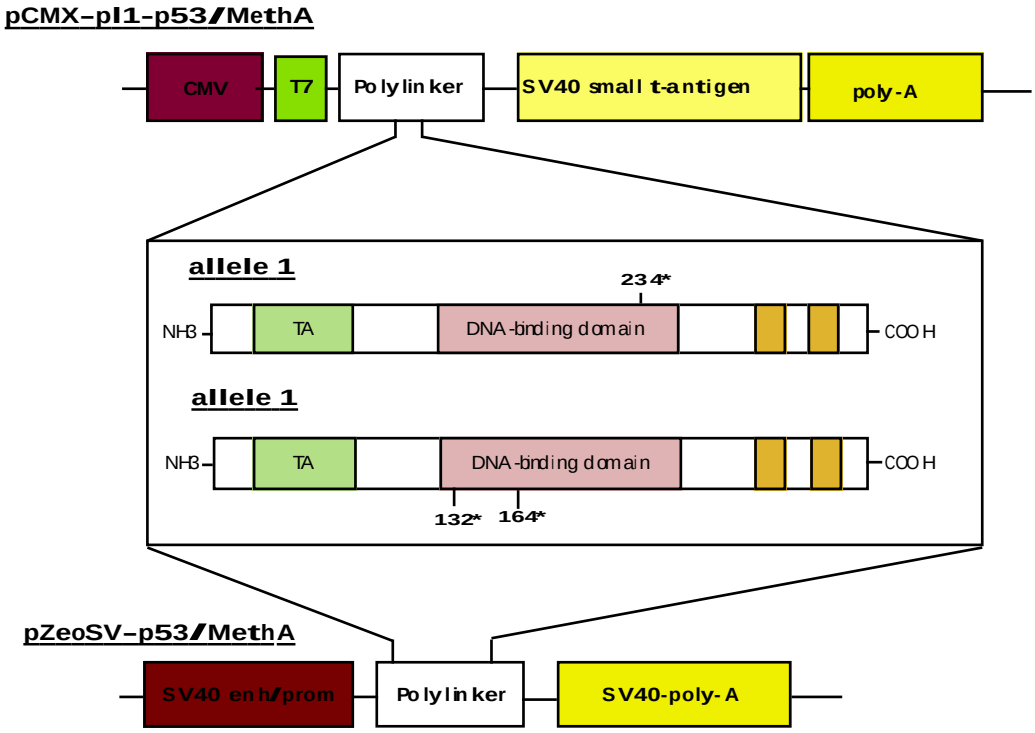

Fig. 5.9 Constructs for pDNA-vaccination. The mutant p53 genes were cloned into mammalian expression vectors. Expression of the tumor antigens is controlled by a viral CMV or SV40 promoter. Mutations are localized in the DNA binding domain, whereas the transactivation domain (TA) or the carboxyterminal oligomerization domain corresponds to wild type sequences. Vector constructs were used as a pDNA-vaccine.

5.3.2 pDNA vaccination with the mutant p53 genes

Mutant p53 encoding pDNA constructs were used for immunization of mice. 70 µg of pDNA were injected intradermally into the ear or food pad of six to eight weeks old Balb/c mice (Fig. 10a). In epidermal specimen of these regions high surface densities of epidermal Langerhans cells have been identified (247). These cells perform critical antigen-presenting function *in vivo* and activate cellular immune responses (248-250). After pDNA immunization mice were inoculated with tumor cells and tumor growth was analyzed. All non-treated control mice developed large tumors and died within 4 weeks. Mice injected with the DNA vaccine containing the mutated p53 gene showed long termed resistance to Meth A tumor growth for at least 12 months whereas growth of Q6 tumor cells was not influenced by pDNA injection. The p53 gene in Q6 tumor cells is mutated at different regions and tumor cells harbor a phenotype with low p53 expression as determined by FACS analysis (data not shown). Protection from tumor growth by the DNA vaccine is most probably dependent on the presence of specific mutations within the p53 tumor antigen. Mice injected with pDNA encoding a bacterial β-galactosidase or a firefly luciferase gene were not protected against Meth A tumor cells. DNAimmunization was also ineffective when the pDNA-vaccine was administered to nude mice. These athymic mice lack the T lymphocyte dependent immune responses that seem to be necessary for rejection of Meth A tumor cells.