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In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment

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ABSTRACT **The particle bombardment method of gene transfer provides an alternative approach for analysis of *in vivo* promoter activity and transgene expression. Transient expression of the firefly luciferase gene from five viral and five cellular promoters was assessed after *in vivo* gene transfer using this method. The relative strengths of these promoters were quantitatively determined in five different rat tissues: skin epidermis, dermis, muscle, liver, and pancreas. Cytomegalovirus immediate early enhancer/promoter activity was consistently the highest in each tissue, whereas other promoters displayed tissue-specific preferences. In liver, the mouse phospho*enol*pyruvate carboxykinase and metallothionein promoters were stimulated *in vivo* by inducing agents at 1 and 5 days posttransfection. In dermis, sustained luciferase activity was observed for over 1.5 years after gene delivery. *In vivo* transgene expression was also detected in bombarded mouse, rabbit, and rhesus monkey tissues. These results suggest that particle bombardment provides an effective system for studies of *in vivo* gene transfer and gene therapy.**

Currently, the expression patterns of most mammalian transgene constructs are analyzed by using cell culture systems (1, 2), which may not reflect the behavior of specific promoters or transgenes under *in vivo* conditions (3). With the recent progress in gene therapy research (4, 5), there is a need for an efficient gene delivery and assay system to characterize promoter–therapeutic gene constructs *in vivo*, allowing examination of transgene expression levels and physiological effects. It is also highly desirable that this system be applicable to the many different somatic tissues and mammalian species pertinent to gene therapy experiments.

One of the few systems currently being used to analyze promoter and transgene activity *in vivo* is the transgenic mouse (6, 7). Although this system has proven to be a valuable and powerful approach for studying transgene expression *in vivo*, it is, however, limited in its ability to provide a convenient and quantitative analysis of transgene activity. When compared with the same tissue type, extremely variable levels of transgene activity were observed among different lines of transgenic mice created using the same promoter–gene construct (8). This is because transgene expression levels have been shown to be readily influenced by position effects at the site of integration on host chromosomes (9). Techniques for generating transgenic mice are not yet routinely applicable to other mammalian systems and are also costly, laborious, and time-consuming. These various limitations make it impractical as a routine assay system for *in vivo* transgene expression in targeted somatic tissues of test animals.

We have previously demonstrated that the particle bombardment method of gene transfer is effective in various somatic tissues *in vivo* and *ex vivo* (10). High levels of transgene activity have been readily detected both in tissue extracts and at the cellular level from various reporter genes bombarded into the skin and liver tissues of rats and mice (10, 11). The Accell bombardment method, which utilizes an electric discharge device to accelerate DNA-coated microscopic gold particles into target tissues, requires minimal manipulation of the target organs of test animals, and results can be readily obtained after gene transfer. Since particle bombardment utilizes a physical force for gene delivery, it is apparently not dependent on cell membrane properties of the various target cell types (10–12).

In this study, we evaluate its use for *in vivo* promoter characterization in various rat somatic tissues, for long-term transgene expression, and for application to other mammalian systems. Future applications to gene transfer and gene therapy are discussed.

MATERIALS AND METHODS

Plasmids. A series of vectors expressing the firefly luciferase (luc) gene under the regulatory control of various viral and cellular gene promoters was used. pNASSluc (no promoter) and pADluc (containing the adenovirus 2 major late promoter) were made by replacing the *lacZ* gene from pNASSβ and pADβ (Clontech) with the luciferase gene as a *Not* I fragment from pCMVluc. Plasmids pCMVluc, pRSVluc, pSVluc, and pMLVluc, which contain the cytomegalovirus (CMV) immediate early gene enhancer/promoter, Rous sarcoma virus (RSV) long terminal repeat, Simian virus 40 (SV40) early enhancer/promoter, and the U3 region of the murine leukemia virus (MLV) long terminal repeat, respectively, have been described (13). Plasmids pmMTluc, pPEPluc, pBLGluc, and pPLluc, which contain the mouse metallothionein (mMT) gene promoter (14), mouse phospho*enol*pyruvate carboxykinase (PEP) gene promoter (15), bovine β-lactoglobulin (BLG) gene promoter (16), and bovine prolactin (PL) gene promoter (17), respectively, were constructed by inserting the isolated promoters into the *Xho* I site of pNASSluc. pCMVβ-gal consists of the CMV promoter driving expression of β-galactosidase (β-gal) (10). The pPGKluc plasmid expressing the luciferase gene from the mouse phosphoglycerate kinase (PGK) gene promoter, a gift from J. A. Wolff (University of Wisconsin, Madison), was previously referred to as pKJ-1-Lux (18).

***In Vivo* Bombardment of Various Organs.** Particle bombardment was performed as described using the Accell gene delivery system (Agracetus, Middleton, WI) (10). Briefly, adult male Holtzman rats were anesthetized by i.m. injection of a mixture of ketamine (70 mg/kg of body weight) and xylazine (6 mg/kg of body weight). Rabbits and mice were anesthetized by i.m. injection of a mixture of ketamine (50 mg/kg of body weight) and xylazine (10 mg/kg of body weight). Rhesus monkeys were anesthetized with ketamine

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Abbreviations: CMV, cytomegalovirus; β-gal, β-galactosidase; mMT, mouse metallothionein; PEP, phospho*enol*pyruvate carboxykinase; PGK, phosphoglycerate kinase; RLU, relative light unit.

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(10–15 mg/kg, i.m.). After induction of anesthesia, abdominal hair was removed by treatment with Nair (Carter–Wallace, New York), and the exposed skin was sterilized with povidone-iodine and rinsed with 70% ethanol. Abdominal muscle and dermis targets were exposed s.c. by making a 3-cm-long incision in the abdomen. Abdominal dermis tissue was separated from the underlying abdominal muscle tissue with blunt-ended forceps. The thin fascia layer of dermis was carefully removed using fine tweezers. The liver and pancreas were bombarded by making a 2-cm incision into the abdominal cavity and exposing the organ. Each targeted rat tissue site was then bombarded *in situ* at 21 kV with 0.8 μg of plasmid DNA coated onto 0.32 mg of gold particles (1–3 μm in diameter). Details of Accell particle bombardment techniques for DNA loading capacity and effects of discharge voltage have been described (10, 12). After bombardment, the muscle tissue layer was sutured, and the skin was closed with stainless steel clips.

***In Vivo* Induction of Luciferase Transgene Expression.** Rat livers were bombarded *in situ* with pPEPluc or pmMTluc DNA. After 1 or 5 days, pPEPluc-bombarded animals were given two i.p. injections of N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (dibutyryl-cAMP) and theophylline (each at 30 mg/kg of body weight) over a 2-hr interval. The pmMTluc-bombarded rats were injected with $ZnSO_4$ (20 mg/kg) following the same injection regime for induction. Control (uninduced) rats were injected with a 0.9% saline solution. Tissue samples corresponding to the bombarded sites were collected 4 hr after the first injection and assayed for luciferase activity.

Tissue Extraction and Enzyme Assays. Animals were sacrificed 16 hr postbombardment, and target sites were excised and frozen in liquid N_2. Liver and muscle tissue samples were sliced horizontally to obtain a 1.5-mm-thick section of the bombarded tissue of a uniform size (3.24 cm^2). Skin and pancreas tissue samples were not sectioned. All tissue samples analyzed weighed ≈0.5 g, corresponding to 29.8, 11.7, 11.7, 19.4, and 15.5 mg of total extractable protein in liver, skin epidermis, skin dermis, pancreas, and muscle, respectively, quantified using a Coomassie blue-based protein assay (Pierce). Extracts were prepared by addition of 500 μl of luciferase extraction buffer containing 0.5 M potassium phosphate (pH 7.5), protease-free bovine serum albumin (0.1 mg/ml), 2.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol to tissue samples, followed by scissor mincing, pestle grinding, sonication, and then centrifugation of cell debris. Crude tissue extract was then analyzed for luciferase activity as described (10) with a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego). Histochemical staining for β-gal activity was performed as described (10). Tissue sections were made from excised dermis tissue 9 days postbombardment and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Molecular Probes).

RESULTS AND DISCUSSION

In vivo promoter activity was investigated in a variety of rat tissues by assaying the transient activity levels of the luciferase reporter gene. Five different rat tissues were transfected with a variety of viral and cellular promoter constructs containing a common plasmid-vector component. The optimal voltage applied for *in vivo* gene transfer to various target tissues using the Accell device was found experimentally to be 21 kV, selected from a range evaluated between 12 and 24 kV. Three lots of gold particles, including beads 0.95 μm, 1–3 μm, and 5–7 μm in diameter, were tested, and the highest luciferase activities in test samples were obtained from the 1- to 3-μm beads. Optimal DNA and gold bead loading rates were determined to be 2.5 mg of DNA per mg of beads and 0.1 mg of beads per cm^2 per uniform tissue site, respectively.

The abdominal skin epidermis and dermis, muscle, liver, and pancreas tissues of anesthetized animals were exposed and bombarded over a uniform target area (3.24 cm^2). Transient luciferase activity was found to peak around 16 hr after bombardment for all tested tissues; thus, uniform size tissue samples from the bombarded sites (3.24 cm^2 × 1.5 mm thick; ≈0.5 g) were obtained from sacrificed test animals at this time point. Tissue extracts from these samples were assayed for luciferase activity, with the specific activity for luciferase gene expression defined as relative light units (RLUs) per uniform tissue sample.

Fig. 1 shows that wide variations in specific luciferase activity can be observed between different promoters in test tissues. The CMV immediate early enhancer/promoter consistently displayed high specific activities relative to other tested promoters in all tissues. The specific activities of the other promoters, relative to each other, varied with tissue type. Two mouse cellular promoters, from the PGK gene and the PEP gene, were as active as the CMV promoter in dermis tissue (Fig. 1*A*). The mMT promoter was very active in epidermis (Fig. 1*B*); it expressed a level similar to that of the CMV promoter. The Rous sarcoma virus long terminal repeat and simian virus 40 early region enhancer/promoter, two commonly used viral promoters, expressed luciferase in muscle, liver, and pancreas at higher levels than any of the cellular promoters tested (Fig. 1 *C–E*). The adenovirus major late promoter was as active as the other viral promoters in muscle tissue (Fig. 1*C*), but was relatively ineffective in other tested tissues. The commonly used murine leukemia virus promoter consistently displayed the lowest activity among the promoters in all tested tissues. These results show that *in vivo* expression can vary drastically among various transgenic promoters in different somatic tissues, and these differences may be readily identified using particle-mediated gene transfer.

Within the same tissue type, promoter strengths can be directly and effectively compared by using the specific activity for luciferase gene expression. Among different tissue types, however, transgenic promoter activity can only be compared in relative terms, since the efficiency of gene delivery into cells of different tissues using particle bombardment may vary depending on a number of cellular and tissue characteristics. The heterogeneity of cell types between different tissues, the rigidity of the tissue, the cell's ability to process foreign DNA, and the intrinsic transcriptional capacity of different cell types may all be factors that affect transgene expression levels. It is possible that these factors contribute to the up to 1000-fold difference in pCMVluc expression observed between epidermis and muscle tissues (Fig. 1 *B* and *C*).

Taking this consideration into account, we evaluated the relative strength of expression of specific promoters in different tissues by using pCMVluc activity as an internal standard (i.e., percentage of CMV activity). A quantitative analysis of the relative activities of the PGK, mMT, and PEP promoters in liver, epidermis, and dermis tissues demonstrated the differences in promoter preference among tissues (Fig. 1*F*). The relative activity of the nominally constitutive PGK promoter was found to vary drastically in different tissues; pPGKluc relative activity in dermis was expressed at levels over 10-fold higher than that in liver. A large difference in relative activity was also observed with pmMTluc between liver (16% of CMV) and epidermis (94% of CMV). The inducible PEP promoter was expected to be more effectively expressed in liver than in other tissues (19, 20), but under normal physiological conditions *in vivo*, much higher relative activity of the PEP promoter was observed in the dermis (67%) than in the liver (1%). This difference can also be observed at the specific activity level (Fig. 1 *A*, *B*, and *D*). This result suggests that the PEP promoter can be effectively

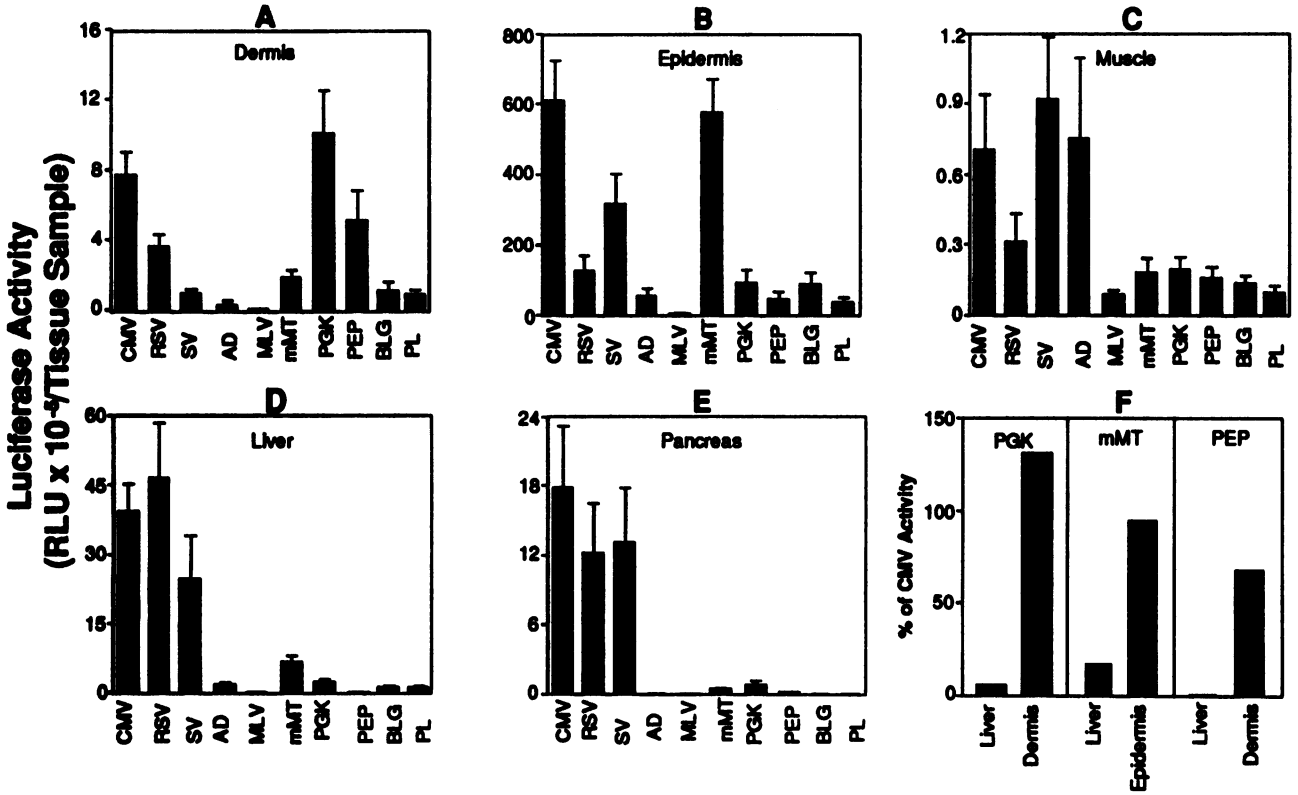

FIG. 1. Comparison of *in vivo* transgenic luciferase activities driven by various viral and cellular promoters in different rat tissues. Luciferase activity was obtained from tissue extracts of abdominal dermis (*A*), abdominal epidermis (*B*), abdominal muscle (*C*), liver (*D*), and pancreas (*E*) bombarded with pCMVluc (CMV), pRSVluc (RSV), pSVluc (SV), pADluc (AD), pMLVluc (MLV), pmMTluc (mMT), pPGKluc (PGK), pPEPluc (PEP), pBLGluc (BLG), or pPLluc (PL) at 21 kV. (*F*) Relative activity of pPGKluc, pmMTluc, and pPEPluc in various tissues compared to pCMVluc activities, which were employed as an internal standard (100%) for each test tissue. Graphed values represent an average of the luciferase activity ± SEM per uniform target tissue. Six to 12 separate tissue samples were collected from three to six experimental rats, with no more than two samples from each animal. Control tissue samples bombarded with gold particles alone (with no coated DNA) showed no detectable luciferase activity.

employed for *in vivo* expression of transgenes in rat epidermis and dermis tissues, as well as liver. The observed tissue type preferences in promoter usage (Fig. 1) may have useful applications to various mammalian gene transfer and gene therapy experiments.

The transgene activities from the PEP and mMT promoters presented in Fig. 1*D* were obtained using otherwise untreated test animals. Within 4 hr after i.p. injection of the inducing agents dibutyryl-cAMP and theophylline (each at 30 mg/kg of body weight), pPEPluc activity in the livers of treated animals increased by 20- to 30-fold over the levels detected in the livers of control (0.9% saline-injected) animals (Fig. 2*A*). *In vivo* expression from the PEP promoter was similarly induced at 1 and 5 days following *in situ* bombardment of the liver with pPEPluc (Fig. 2*A*). Parallel experiments using pPGKluc-transfected rats treated with dibutyryl-cAMP and theophylline showed little or no induction (data not shown).

The mMT promoter was also inducible in liver (Fig. 2*B*). Two injections of $ZnSO_4$ (20 mg/kg of body weight) were made into the peritoneal cavity during a 4-hr period. This resulted in a 2- to 4-fold increase of luciferase activity in liver tissue from rats whose livers had been bombarded on the previous day with pmMTluc. Injections made 5 days post-transfection resulted in a 42-fold increase in pmMTluc activity over nonstimulated animal samples. These results suggest that the particle-mediated gene transfer technique can provide an effective and convenient approach for evaluating regulated and inducible gene expression *in vivo*.

The *in vivo* time course of pCMVluc expression in various rat tissues was investigated to evaluate the useful time frame for transgene analysis using particle bombardment. Expression levels in the epidermis, liver, and pancreas tissues peaked within 3 days after bombardment and then declined to 1–5% of peak levels after 1 week. Rat dermis, however, showed a very different pattern of pCMVluc expression. Fig. 3 shows that transgenic luciferase activity was continuously detected in dermis tissue at significant and stable levels during the entire experimental period of 1.5 years. Since skin tissue is a readily accessible major organ, the finding of

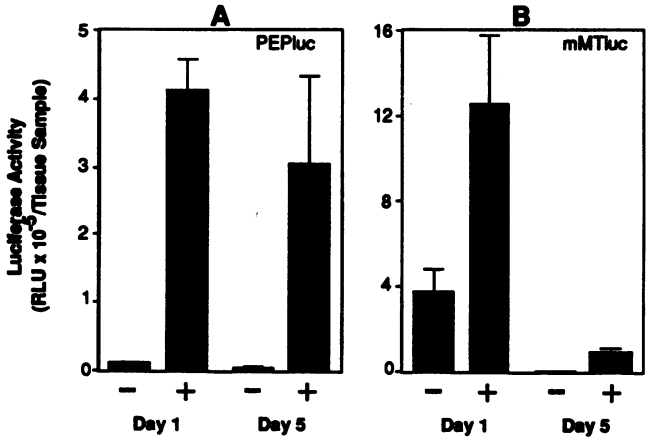

FIG. 2. *In vivo* induction of PEP (*A*) and mMT (*B*) promoter activities in rat liver 1 and 5 days postbombardment. Tissue samples corresponding to the bombarded sites were collected 4 hr after the first injection of inducer and assayed for luciferase activity. Graphed values represent the average luciferase activity (RLUs) per sample ± SEM obtained from 6 to 12 liver samples.

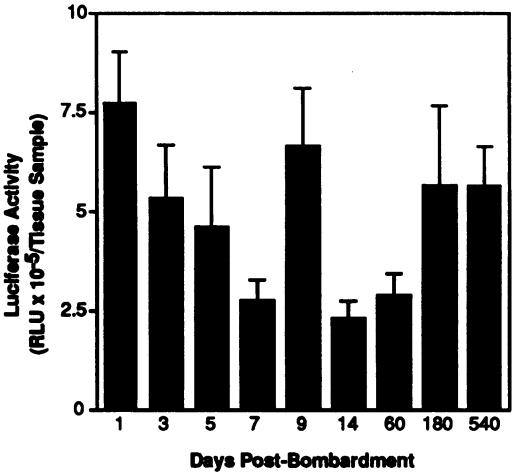

FIG. 3. Time course of luciferase activity detected in pCMVluc-bombarded rat dermis tissue. Luciferase activity was determined at indicated time points after bombardment. Data presented are the average luciferase activity per sample ± SEM obtained from four to eight independent dermis samples, with no more than two samples from a single experimental animal.

long-term transgene expression in dermis tissue suggests that the skin's dermis layer may be employed as a useful target tissue for gene therapy experiments.

Histochemical studies revealed that the majority of β-gal-expressing cells in the dermis were located in the panniculus carnosus layer, which consists of a specific type of muscle cell (Fig. 4). Wolff *et al.* (21) demonstrated that plasmid DNA injected into the skeletal muscle tissue of mice provided long-term transgene expression *in vivo* and that the injected plasmid DNA was maintained in a free, circular form, without integration into the cell's nuclear genome. We could not determine the molecular state of the luciferase DNA in the dermis tissue since Hirt extractions (22) were not successfully performed on these samples in our experiments. We have also observed sustained gene expression in abdominal muscle after bombardment with pCMVluc. Expression was maintained at the initial activity levels for 2 weeks (no further time point was tested, data not shown). However, the differences observed in promoter preference for luciferase expression between the dermis and muscle tissues (Fig. 1 *A* and *C*) suggest that the muscle cells in the panniculus carnosus of the dermis behave differently in their regulation of gene expression as compared to abdominal muscle cells.

The applicability of particle bombardment to various mammalian species was quantitatively evaluated by *in situ* bombardment of the abdominal epidermis tissue of mice, rats, rabbits, and rhesus monkeys. Transient luciferase activity was readily detected in target site tissue extracts from all test animals 16 hr after gene transfer. The average level of luciferase activity expressed per unit of epidermis sample (from 4 to 10 independent samples) was 1.8×10^7 RLUs for mice, 6.1×10^7 RLUs for rats, 1.0×10^7 RLUs for rabbits, and 8.2×10^7 RLUs for rhesus monkeys. Transgene expression was also readily detected in bombarded dermis, liver, and muscle tissues in these four mammals (data not shown). Hence, particle bombardment can be effectively applied to a variety of mammalian species and may thus provide an attractive alternative for experimental studies of *in vivo* transgene expression.

In conclusion, we have demonstrated that particle bombardment can be effectively employed for the *in situ* delivery of transgenic promoter constructions into various somatic tissues of experimental rats and three other mammalian species. Almost lifetime expression of a marker gene was demonstrated in rat dermis tissue. Tissue type preferences for *in vivo* expression of various cellular and viral promoters were also observed and evaluated. These results suggest that particle bombardment is a convenient method for characterization of the *in vivo* expression of specific promoters and functional transgenes in various somatic tissues of animal systems. This technology may have special application for evaluating and verifying the *in vivo* expression level and physiological effects of candidate therapeutic genes in gene therapy experiments. Particle bombardment may, therefore, have multiple applications as an effective *in vivo* gene transfer approach to basic research in molecular genetics and clinical research in gene therapy.

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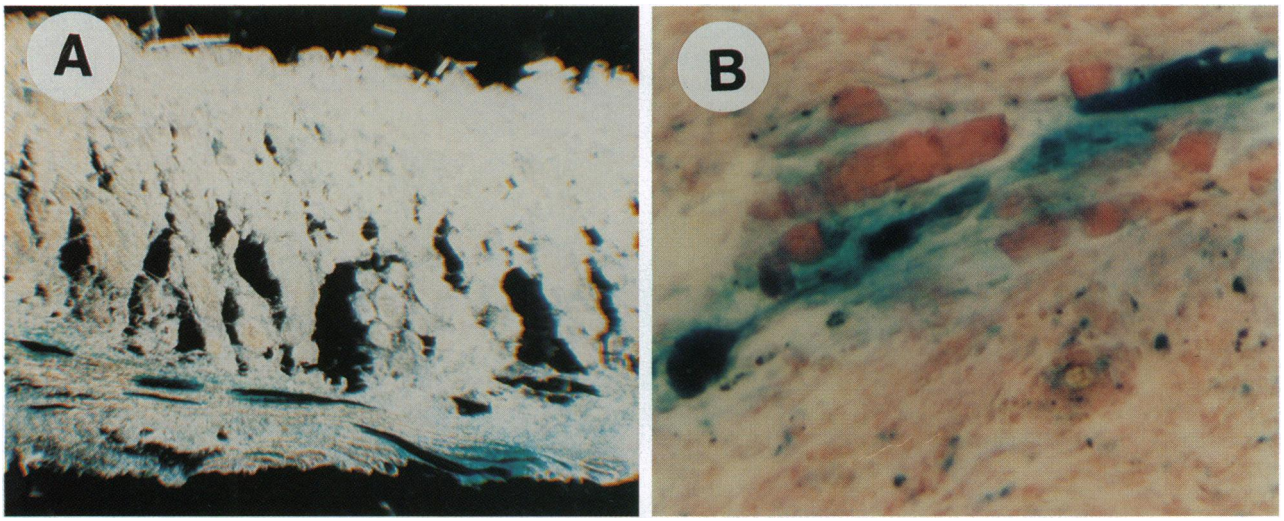

FIG. 4. Expression of β-gal activity in rat dermis at the cellular level. Photomicrographs show the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside staining of dermis tissue sections at low magnification (×16) in dark field (*A*) and at high magnification (×100) in bright field (*B*). The figures are oriented with the dermis layer on the bottom of each photomicrograph. Rat skin dermis tissues were bombarded *in situ* with pCMVβ-gal DNA. Nine days after gene transfer, transfected dermis samples were excised from test animals, fixed, sectioned, and stained for β-gal activity. These results show that β-gal expression in the dermis is localized in the muscle type cells of the panniculus carnosus layer.

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