Transcriptional Targeting of Recombinant Adenoviruses to Human and Murine Melanoma Cells

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ABSTRACT

One potential avenue for future cancer therapy involves the specific targeting of effector genes to cancer cells throughout the body, including distant metastatic sites. As a first step toward this goal, we tested the ability of the transcriptional regulatory elements of the human and mouse tyrosinase genes to promote high levels of pigment cell-specific transcription. A construct consisting of 209 bp of the human tyrosinase promoter linked to two enhancer elements was demonstrated to drive high-level, melanoma-specific expression of a β-galactosidase (β-gal) reporter gene in transient transfection assays. In studies of the murine tyrosinase promoter region, constructs containing up to 2500 bp of the 5' regulatory region were found to have very low transcriptional activity in murine melanoma cells. However, as with the human system, addition of two tandem repeats of an upstream enhancer element resulted in high levels of lineage-specific transcriptional activation. The murine tyrosinase promoter-enhancer expression cassette was introduced into the E1 region of a recombinant adenovirus to generate the virus AdmTyr-βgal. This virus grows to high titer and maintains transcriptional specificity for pigment cell lineages. Strikingly, AdmTyr-βgal is extremely active in human melanoma cells, in some cases exceeding the transcriptional activity of a cytomegalovirus promoter-driven recombinant β-gal virus. Tissue specificity of gene expression is maintained, with very low levels observed in tumors and primary human cells derived from other lineages. These data provide evidence that it is possible to target human melanoma cells with high efficiency and specificity using high-titer recombinant adenovirus vectors.

INTRODUCTION

Recent advances in delineating the genetic and molecular bases of cancer have aroused a great deal of interest in the possibility of correcting the inherent genetic defects responsible for cancer pathophysiology, including growth dysregulation, angiogenesis, metastasis, and avoidance of homeostatic apoptosis pathways. An obvious approach to correct genetic lesions is to devise techniques for efficient introduction of therapeutic genes into cancer cells, thereby restoring normal physiological regulatory functions or rendering them more susceptible to standard therapies. The concept of gene therapy as a cancer treatment modality is in its infancy, and several obstacles to successful implementation remain (1). These include the systemic delivery of therapeutic genes to metastatic cancer cells scattered throughout the body and the targeting of these genes to the desired cell population while avoiding potential toxicity to normal cells.

Novel approaches have been described with the goal of targeting gene expression specifically to tumor cells. Progress has been made in targeting infectious recombinant viral vectors to cells expressing specific cell surface receptors, including specific subsets of differentiated cells. Retroviruses engineered to express a chimeric envelope protein containing the polypeptide hormone erythropoietin were more infectious for murine cells bearing the erythropoietin receptor and became infectious for human cells expressing the erythropoietin receptor (2). Retroviruses have also been engineered to express env fused to the Fv region of a monoclonal antibody that binds the low-density lipoprotein receptor (3). A major limitation in the use of similar techniques for cancer therapy has been the difficulty in identifying cell surface receptors and their ligands that are specific for cancer cells. However, growth factor receptors are overexpressed in some common malignancies (4), and other highly expressed or aberrant cell surface proteins could provide targets for future cancer cell-targeting endeavors (5, 6).

An alternative approach is to target cancer cells at the transcriptional level using lineage-specific promoters that restrict expression of effector genes to tumor cells and related normal cells derived from the same developmental lineage (7). Examples of tumor types that have been targeted in this manner include colon (8, 9), lung (8, 10), breast (11), and hepatocellular carcinomas (12–14) and melanoma (15). A critical appraisal of claims to have developed useful transcriptional targeting vectors requires knowledge of the efficiency of gene expression in the targeted cell populations as well as the level of undesirable expression in other cell types that would be exposed to the vector in the in vivo setting. Because a variety of systems and methods of data analysis have been used by different laboratories, a comparison of these data is not always possible. Nonetheless, there are sufficient data available to suggest the feasibility of targeting tumor cells using lineage-specific characteristics of the tumor cell population.

Metastatic melanoma is a highly aggressive, lethal tumor for which no standard form of medical therapy currently exists; therefore, novel therapeutic approaches are needed. Immunotherapy trials during the past decade have demonstrated that tumor regressions, including durable complete remissions, can be achieved using interleukin 2-based regimens (16). Tumor-infiltrating lymphocytes with specificity for lineage-specific antigens such as MART-1 and gp100 can mediate tumor regression, suggesting that in the case of melanoma, it is possible to target lineage-specific molecules without incurring undue toxicity to normal host cells (16, 17). In fact, the appearance of vitiligo (patchy loss of skin pigmentation due to death of melanocytes) has correlated with immunotherapeutic efficacy in some human melanoma trials (18). Tyrosinase, the product of the albinoc locus (19, 20), is a pigment cell-restricted enzyme that catalyzes the rate-limiting step in melanin synthesis. The tyrosinase promoter-enhancer elements have been described in both the human and murine systems (21–27).

In this article, we describe the construction and testing of plasmids containing tyrosinase promoter-enhancer cassettes derived from the human or mouse tyrosinase genes and compare lineage-specific expression with previously described constructs. Our data demonstrate that promoter regions alone are insufficient for high levels of lineage-specific expression and that tandem enhancer elements are also required. Incorporation of the murine promoter-enhancer cassette into an adenovirus vector has led to the generation of a high-titer virus that expresses effector genes with high efficiency and specificity in murine and human melanoma cell lines. These data provide a basis for in vivo experiments designed to test the efficacy of transcriptional targeting in tumor models of murine melanoma and human xenografts.
MATERIALS AND METHODS

Cell Lines and Culture Conditions. Cell lines were maintained at 37°C in a 5% CO₂ incubator and grown in DMEM or RPMI 1640 (Life Technologies, Inc.) supplemented with 100 units/ml penicillin/streptomycin and 5% fetal bovine serum. The murine cell lines B16F10 (melanoma), REN (renal cell carcinoma), NIH3T3 (fibroblast), DA3 (breast carcinoma) and C26M1 (colon carcinoma) were kindly provided by Dr. Robert Wiltrout (National Cancer Institute). The murine melanoma cell line S91MEL-3 was obtained from the American Type Culture Collection. The human cell lines SK-MEL-28 (melanoma), DM13 (melanoma), T98G (glioblastoma), Panc-1 (pancreatic cancer), and OVCAR-3 (ovarian carcinoma), SK-N-SH (neuroblastoma), H464 (lung cancer), and 786-0 (renal cell carcinoma) were obtained from the American Type Culture Collection or have been passaged at the National Cancer Institute for many years (DM13 and OVCAR-3). HUVEC were grown as recommended by the supplier (Clonetics Corp.) and used at passage 4. The fibroblast-like primary cell lines HEL (human fetal lung tissue) and MRHF (normal human foreskin) were obtained from BioWhittaker, Inc. Adenoviruses were propagated on 293 cells provided by Dr. Toren Finkel (NIH).

Construction of Expression Plasmids. The human promoter/enhancer units were generated by PCR amplification of the individual promoter-enhancer units followed by ligation into a single transcriptional cassette. Human genomic DNA, isolated from DM13 cells using a commercial DNA extraction kit (Stratagene), was used as the template. An antisense primer including sequences complementary to positions +51 to +32 (underlined) of the human tyrosinase promoter and encoding an XhoI site (CCCGCTCGAGTAGTCACAGACCTTCTCA) was used to prime the 3' end of the promoter. The 3' regulatory region of the human tyrosinase promoter was amplified using a primer including a sequence complementary to −2689 to −2670 (underlined) and an EcoRI site (CCGGAATTCCTCACTGGAAGAAGGACCTCCT). PCR was performed using a Gene Amp PCR reagent kit (Perkin Elmer Corp.). Reaction conditions were denaturation (94°C, 1 min), annealing (55°C, 1 min), and elongation (72°C, 2 min) for a total of 25 cycles. The PCR product was digested with the EcoRI and XhoI (Life Technologies), purified using the Qiaex II gel extraction kit (Qiagen), and cloned into pNASSβ (Clontech Laboratories, Inc.). Plasmids were propagated in the Escherichia coli strain DH5α (Life Technologies) and analyzed by restriction mapping and DNA sequencing (Sequenase version 2.0 DNA-sequencing kit; United States Biochemical Corp.). An EcoRI site at −461 to −456 of the human tyrosinase promoter was used to generate constructs containing a "medium" promoter element (−461 to +51). For amplification of the minimal promoter, a sense primer complementary to nucleotides −209 to −190 (underlined) incorporated a 5'-EcoRI site was used (CCGGAATTCATCTTCTACATAGATAAATTTAA) in conjunction with the 3'-XhoI primer described above. Following EcoRI and XhoI digestion, this fragment was cloned into the promoterless pNASSβ expression vector. The region containing the human tyrosinase enhancer element was amplified by PCR using a sense primer complementary to −1811 to −1830 (underlined) also incorporating an EcoRI site (CCGGAATTCATGTTGCAAGAGAATTTAA) and an antisense primer complementary to −1811 to −1830 (underlined) also incorporating an EcoRI site (CCGGAATTCATGTTGCAAGAGAATTTAA). The DNA containing the enhancer element was inserted into each of the tyrosinase promoter-pNASSβ constructs at the EcoRI site.

PCR amplification of the murine tyrosinase 5' regulatory region from −815 to −46 was performed using the sense primer containing a 5'-EcoRI site (CCGGAATTCCTCACTGGAAGAAGGACCTCCT) and an antisense primer (CCGCGCCTGAATCTCACTGGAACCCCTTTTTTTTTTTT) containing a 5'-XhoI site. Genomic DNA isolated from B16F10 melanoma was used as the template for these reactions. The PCR product was digested with EcoRI and XhoI and ligated into pNASSβ. The mouse tyrosinase enhancer region was amplified using a sense primer (CAAGCITTCACTAGTCCCTGCCA) and an antisense primer (TATTGTTGTTGGCCAGAGCCTGCAATCT) corresponding to sequences located 12.1–12.3 kb upstream of the murine tyrosinase mRNA start site. The PCR product was cloned into PCRII using the original TA cloning kit (Invitrogen).

RESULTS

Cell Type Specificity of the Tyrosinase Enhancer-Promoter Construct. Tyrosinase catalyzes the rate-limiting step in the synthesis of melanin, and its expression is limited to pigmented cells, including melanocytes, pigmented retinal epithelium, and melanoma. DNA sequences contained within a 2.1-kb region upstream of the human tyrosinase mRNA cap site have been demonstrated to direct melanoma-specific expression (22–24, 27, 30). Key elements include a melanoma-specific enhancer region located at position −2014 to −2004 and a promoter region extending 209 bp upstream of the initiation site and including melanocytes, pigmented retinal epithelium, and melanoma. DNA sequences contained within a 2.1-kb region upstream of the human tyrosinase mRNA start site have been demonstrated to direct melanoma-specific expression (22–24, 27, 30). Key elements include a melanoma-specific enhancer region located at position −2014 to −2004 and a promoter region extending 209 bp upstream of the initiation site and including melanocytes, pigmented retinal epithelium, and melanoma.

To generate the mouse tyrosinase promoter-enhancer constructs, the enhancer fragment was excised from PCRII with EcoRI and ligated into the EcoRI site of pNASSβ immediately upstream of the tyrosinase promoter.

Transfection and Analysis of β-gal Expression. Plasmids used for transfection experiments were purified using Qiaex 500 columns. Cells were plated on Falcon 60-mm tissue culture plates (Becton Dickinson) at a density of 2–4 × 10⁵ cells/dish (depending on the cell type) in the appropriate complete medium and transfected with 4 μg of DNA using Lipofectamine as described by the manufacturer (Life Technologies). β-gal assays were performed 48 h after transfection using the Galacto-Plus chromogenic- cent detection system (Tropix) as described by the manufacturer. To normalize the level of β-gal expression between cell lines of different transfection efficiencies, the luciferase expression vector pGL3 (Promega) was included as an internal control. Luciferase activity in the lysates of transfected cell lines was determined (Luciferase Assay System; Promega), and β-gal activity was normalized to the level of luciferase expression. Protein concentrations were determined using the Bio-Rad protein assay. Light unit values obtained on a Lumat luminometer (B Eldh orb) were normalized to the protein concentrations for each sample (to light units/μg). Results are expressed as numerical values that indicate the fold increase above promoterless control plasmid.

Construction of AdTyr-β-gal. The recombinant adenovirus was constructed and purified using the procedures described by Hint et al. (28). Briefly, DNA sequences containing the human tyrosinase enhancer-promoter cassette, the β-gal gene, and the SV40 splice site and polyadenyllic acid site were excised from the vector with NarI and SalI. The resulting 5.2-kb fragment was subcloned into the CflI and SalI sites of the adenoviral shuttle plasmid pΔE1-Δp1A (Microbiex Biosystems, Inc.). The resulting shuttle vector was cotransfected into 293 cells with plasmid pBGH10, which contains the adenoviral genome with a deletion of E1 and E3 early region genes (29). Primary plaques were isolated, amplified, and screened by PCR to verify the presence of the tyrosinase enhancer-promoter cassette. Recombinant viruses were plaque purified twice, and a single plaque was chosen for large-scale amplification. Amplification was performed using 293 cells grown in T150 tissue culture flasks. Following growth on 293 cells, large-scale preparations of virus were purified by CsCl gradient centrifugation, dialyzed to remove excess CsCl, and frozen at −70°C. Viral titer was determined by plaque assay on 293 cells. AdCMV-β-gal was kindly provided by Dr. Toren Finkel.

Infection of Cell Lines with Recombinant Adenoviruses. The day prior to infection, cells were seeded in six-well tissue culture plates at 5 × 10⁵ cells/well. For infection, media were aspirated from each well, and the amount of virus required for the indicated MOI was diluted in infection media (Iscove’s modified eagle medium + 2% FCS) and added to the cells. After 60 min of incubation, 2 ml of normal growth media were added to each well. Cells were incubated for an additional 48 h to allow for expression of the β-gal reporter gene. Cells were either fixed in situ for β-gal staining or harvested and extracts prepared for quantitative β-gal assays.
construct was analyzed by transient transfection into the human melanoma cell line SK-MEL-28. As shown in Fig. 1A, 5' regulatory sequences, including those extending from −209, −461, or −2689 to +51, were all weak transcriptional activators, although the latter construct contains a single enhancer element in its normal position (−2014 to −1810). Addition of a single enhancer to the minimal or medium promoter fragments increased activity to levels similar to that observed with the −2689 construct. In contrast, addition of a second enhancer element to the −209 to +51 minimal promoter fragment dramatically increased β-gal expression compared with constructs containing no or only a single enhancer element. Interestingly, addition of a second enhancer element to the −461 fragment did not increase β-gal expression, suggesting the presence of a negative regulatory element between −461 and −209 that prevents the enhanced level of transcriptional activity mediated by enhancer dimers.

Analysis of transcriptional regulatory elements derived from the murine tyrosinase gene required for high-level, tissue-specific expression yielded results similar to those we observed for the human gene. Previous studies indicated that the 5' regulatory region extending 2500 or 815 bp upstream of the murine tyrosinase mRNA cap site were effective transcriptional activators in B16F10 melanoma cells (15). We tested two independently isolated clones of the −815 to −46 promoter fragment and observed only minimal transcriptional activity (Fig. 1B). A construct extending to −2500 had similarly low levels of activity (data not shown). The low level of transcriptional activity by the promoter region alone may be explained by the recent description (26) of a 200-bp melanoma-specific enhancer region that maps 12 kb upstream of the promoter region (and, hence, is not included in either of the promoter-proximal constructs). Therefore, we amplified this enhancer region and generated constructs containing one or two tandem enhancer elements upstream of the −815 to −46 promoter fragment. Significant increases in transcriptional activity were observed by the addition of a single enhancer element to the −815 to −46 promoter-proximal fragment. Addition of a second enhancer element, in this case generating a tail-to-tail tandem dimer, dramatically increased the activity in a manner very similar to that observed with the human tyrosinase constructs. Attempts to generate constructs with more than two enhancer elements were unsuccessful, perhaps due to instability of multiple repetitive sequences. The dimer constructs, however, were very stable through multiple rounds of plasmid purification. A similar pattern of high-level transcriptional activation was observed when these constructs were transfected into a second murine melanoma cell line, TK1735 (data not shown). In addition, constructs containing enhancer dimers in the 5'-to-3' orientation yielded similar results, consistent with the orientation-independent function of enhancer elements (data not shown). Thus, in both the human and murine tyrosinase systems, high-level expression in melanoma cells was dependent on tandem enhancer elements associated with minimal promoter elements.

Cell Type Specificity of the Tyrosinase Promoter-Enhancer Dimer Cassette. To determine whether transcriptional activation from the human and murine promoter-enhancer cassettes was lineage specific, a variety of human and murine cell lines were transiently transfected with the enhancer dimer constructs determined to be most active in the previous experiments (Fig. 1). The human promoter-enhancer dimer construct drove high levels of expression in melanoma cell lines (SK-MEL-28 and DM13) but only background levels in tumor cell lines derived from a variety of other tissue types (Fig. 2A). Similar data were obtained in the murine system (Fig. 2B). Expression was very efficient in B16F10 cells, but only background levels were observed in NIH3T3 cells as well as in tumor cells derived from distinct differentiated cell types. These data confirm that expression from both the human and murine promoter-enhancer cassettes is restricted to cells derived from the pigment cell lineage.

Generation of a Recombinant Adenovirus Containing the Murine Tyrosinase Promoter-Enhancer Cassette. Practical use of the melanoma-specific nature of these promoter-enhancer elements required that they be incorporated into an efficient gene transduction system capable of infecting both proliferating and noncycling cells and maintaining the tissue specificity of expression demonstrated above. For this purpose, we recombinated the murine tyrosinase expression cassette into an adenovirus vector. DNA sequences encompassing 5200 bp including the tyrosinase promoter-enhancer, splice site, β-gal gene, and polyadenylic acid addition site were cloned into the shuttle vector pΔE1sp1A. This plasmid was cotransfected into 293 cells along with the E1- and E3-deleted adenovirus type 5 vector plasmid pBH110. One recombinant clone, AdmTyr-βgal, was plaque purified and used for the subsequent analyses.

Tissue-specific Expression by AdmTyr-βgal in Murine Cell Lines. To test the transcriptional efficiency and tissue specificity of AdmTyr-βgal, a panel of murine cell lines were infected with this virus, and data were compared with parallel infections with AdCMV-βgal. A relatively high MOI is used in these experiments, because the tropism of adenovirus is for human cells, and infection of murine cells
is less efficient. Infection of two murine melanoma cell lines (B16F10 and S91Mel-3) resulted in very high levels of β-gal expression that are 30 and 21%, respectively, of the activity of the CMV promoter at a MOI of 250 (Table 1). In contrast, very low levels of β-gal expression were observed in cells of other lineages, including kidney epithelium (Renca), colon (C26M1), and breast (DA3). Although some expression was observed in NIH3T3 fibroblasts, only low levels were observed in primary mouse embryo fibroblasts. Fig. 3 shows in situ β-gal activity after infection of B16F10, C26M1, and NIH3T3 cells with the AdmTyr-βgal or AdCMV-βgal virus. Taken together, the enzymatic assays and in situ data demonstrate that AdmTyr-βgal retains the transcription pattern observed with the plasmid construct, both in terms of the high levels of transcriptional activity in melanoma cells and restricted tissue specificity.

AdmTyr-βgal Infection of Human Cell Lines. Our decision to use the murine promoter-enhancer system to generate the initial recombinant adenovirus was based on two factors: (a) experiments to demonstrate the potential uses of these viruses would require studies in murine melanoma models; and (b) the murine enhancer dimer construct also directed significant levels of β-gal expression in human melanoma cells in transient transfection assays. Therefore, we tested transcriptional activity of the AdmTyr-βgal virus in a panel of human tumor and primary cell lines. Infection of the human melanoma cell lines DM13 and SK-MEL-28 with AdmTyr-βgal resulted in very high levels of β-gal expression, even at low MOIs. The data are presented as percentages of AdCMV-βgal activity at MOIs of 1, 10, 50, 100, and 250 (Table 2) and plotted as actual β-gal units at each MOI (Fig. 4). It is striking that infection of DM13 cells with AdmTyr-βgal at most MOIs resulted in levels of expression that were greater than that observed with AdCMV-βgal. In fact, at MOIs of 10 and 50, the AdmTyr-βgal levels were more than twice as great. Extremely high levels of expression were also noted in SK-MEL-28 cells. As shown by the graphs of β-gal units in Fig. 4, these percentage levels correspond to very high absolute levels of enzyme activity.

In contrast to the melanoma cell lines, infection of other cell lines with AdmTyr-βgal resulted in very low absolute and percentage levels of β-gal expression (Table 2 and Fig. 4). Of critical importance are the low levels observed in the primary cell lines HUVEC (endothelial), HEL (embryonic lung, fibroblast-like), and MRHF (foreskin fibroblast). Infection of HUVECs resulted in <0.1% of the expression level observed with the AdCMV-βgal. Adenoviral infection of HUVECs is not inefficient, because infection with the AdCMV-βgal virus led to very high levels of expression (Fig. 4). The peak β-gal activity in HUVECs infected with AdmTyr-βgal occurred at a MOI of 250 and equaled approximately 74,000 β-gal units. At similar MOIs, β-gal activity in SK-MEL-28 and DM13 cells was greater than 12 and 17 million units, respectively. Thus, absolute levels of β-gal activity observed in HUVECs were 0.6 and 0.4% of that observed in SK-MEL-28 and DM13 cells, respectively. Similar analysis holds for the HEL and MRHF cell lines. Infection of tumor cells derived from colon (HT-29), ovarian cancer (OVCAR-3), and neuroblastoma (SK-N-SH) further attest to the tissue specificity of AdmTyr-βgal compared with the broad range of expression of the CMV-promoted virus. These data are confirmed by the in situ analysis of β-gal expression following infection of these cell lines with AdmTyr-βgal or AdCMV-βgal. As shown in Fig. 5, infection of SK-MEL-28 cells with either virus resulted in high levels of staining in the majority of cells. In contrast, whereas infection of HUVECs or HEL cells with AdCMV-βgal resulted in very efficient expression, AdmTyr-βgal infection incurred no obvious staining, even after infection at a MOI of 250. Taken together, these data demonstrate the very high efficiency and specificity of AdmTyr-βgal for human melanoma.

Table 1 Percentage of expression of AdmTyr-βgal in murine cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AdCMV-βgal (%)</th>
<th>MOI</th>
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<tbody>
<tr>
<td></td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>Melanoma</td>
<td></td>
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</tr>
<tr>
<td>B16F10</td>
<td>30 ± 5</td>
<td>47 ± 14</td>
</tr>
<tr>
<td>S91Mel-3</td>
<td>21 ± 3</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Nonmelanoma</td>
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<tr>
<td>C26M1</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>DA3</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
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</tr>
<tr>
<td>MEF</td>
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<td>1.3 ± 0.9</td>
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<tr>
<td>Renca</td>
<td>0.7 ± 0.5</td>
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MELANOMA-SPECIFIC ADENOVIRUS

DISCUSSION

The human and murine tyrosinase expression cassettes described in this manuscript were designed by incorporation of cis-acting regulatory elements that have been demonstrated to be essential for high-level, tissue-specific transcription in melanoma cell lines (21–27). Sequence elements encoded by the E-box (consensus sequence CANNTG) play a key role in tissue-specific transcriptional activation of the tyrosinase gene, as well as TRP-1, another pigment cell-specific gene (22, 24, 25, 30). In some contexts, the CATGTG motif observed in pigment cell-specific promoters forms the core of an 11-bp conserved motif referred to as the M-box (24, 25, 30). bHLH-Zip transcription factors form a large family of proteins that bind to E-box motifs and activate the transcription of genes that are tissue specific and developmentally regulated [as in the case of the myogenic transcription factor Myo D (32)], or genes with more ubiquitous expression (i.e., as with upstream stimulatory factor; Refs. 31 and 33). The identity of a melanocyte-specific member of the bHLH-Zip family resulted from studies of the mi gene defects in mice (34–36), the

Fig. 3. In situ staining for ß-gal activity following infection of murine cell lines with AdCMV-ßgal or AdmTyr-ßgal. Cell lines were infected at a MOI of 250 with the indicated virus, and ß-gal activity was determined after 48 h of culture. A, B16F10 cells infected with AdCMV-ßgal. B, B16F10 cells infected with AdmTyr-ßgal. C, C26M1 colon cancer cells infected with AdCMV-ßgal. D, C26M1 cells infected with AdmTyr-ßgal. E, NIH3T3 fibroblasts infected with AdCMV-ßgal. F, NIH3T3 cells infected with AdmTyr-ßgal. ×100.
Table 2 Percentage of expression of AdmTyr-βgal in human cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MOI</th>
<th>1</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>250</th>
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<td>DM13</td>
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<td>56</td>
<td>66</td>
<td>86</td>
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<tr>
<td>Nonmelanoma</td>
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<td>0.80</td>
<td>1.3</td>
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<tr>
<td>MRH</td>
<td>0.17±0.24</td>
<td>&lt;0.1</td>
<td>0.12</td>
<td>0.15</td>
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<tr>
<td>OVCAR-3</td>
<td>0.26±0.10</td>
<td>3.1±0.1</td>
<td>1.0±0.1</td>
<td>0.70</td>
<td>0.81</td>
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<tr>
<td>SK-N-SH</td>
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We have used homologous recombination between a shuttle vector containing the murine promoter-enhancer-β-gal transcription unit and an E1- and E3-deleted adenoviral vector to generate the recombinant virus AdmTyr-βgal. Two important features of the expression pattern of AdmTyr-βgal should be stressed. First, infection of human melanoma cells results in very high levels of expression that are similar to those observed with AdCMV-βgal, the standard of reference for potent transcriptional activation in a variety of cell types (40, 41). This is evident both from the in situ staining of infected cells and by comparative assays of β-gal enzymatic activity over a range of MOIs. High levels of expression are also noted in murine melanoma cells, although higher MOIs were required. The high transcriptional activity of AdmTyr-βgal was surprising, especially in human melanoma cells. As discussed above, transfection of enhancer dimer plasmid constructs into melanoma cell lines resulted in significant increases in efficacy compared with promoterless constructs; however, in multiple experiments, the percentage of activity compared with that of pCMVβ was never greater than 25% (data not shown). One possible reason for the increased activity of the recombinant virus was a contribution of adenovirus DNA sequences that were retained in the shuttle vector and, hence, the recombinant virus. Deletion analysis has identified a region extending between -141 and -305 upstream of the E1A cap site (retained in the recombinant virus) that appears to be important for E1A enhancer activity (42, 43). It was possible that transcription from the tyrosinase promoter-enhancer was potentiated by viral enhancer elements through cooperative interactions between Mi or MITF and transcription factors and coactivators associated with the E1A enhancer elements. We have performed experiments in which human melanoma cells were transfected with either the murine enhancer dimer construct (as described in Fig. 1B) or the same transcription unit after ligation into the pDEIspIαA shuttle vector (which contains E1A enhancer elements). No transcriptional enhancement was observed (data not shown). It is still possible that within the context of the viral genome these or other viral regulatory elements potentiate the activity of the tyrosinase transcription elements.

A second feature of AdmTyr-βgal expression is the very tight tissue-specific transcriptional regulation, as shown by the lack of significant activity after infection of HUVECs and two primary fibroblast-like cell lines (HEL and MRH). Endothelial cells and connective tissue fibroblasts are likely sites of viral infection following any route of viral administration, and the lack of expression of effector genes at these sites will eliminate one source of potential toxicity. The lack of significant β-gal expression in tumor cells derived from other tissue lineages, including the neural crest-derived neuroblastoma cell line SK-N-SH, further attests to the specific nature of the tyrosinase promoter-enhancer elements. Although our survey of the pattern of AdmTyr-βgal transcriptional activation in various cell types is not exhaustive, the data indicate a potent transcriptional activation in melanoma cells with minimal cross-expression in cells of other lineages.

Other groups have described recombinant adenoviruses with properties of tissue-specific transcriptional targeting. The promoter region from the CEA gene has been incorporated into a recombinant adenovirus upstream of the HSV-TK gene (44). The 420-bp promoter region confers selectivity of gancyclovir toxicity to those cells that normally express CEA; however, the 420-bp region was acknowledged to be a week transcriptional activator. Recently, two upstream enhancers of the CEA gene were described, which, when added to tandem repeats of a promoter element, increased transcriptional activity of the promoter-only constructs by 4–5-fold (9). This appears to be similar to our experience with the tyrosinase promoter region and suggests that, by analogy, adenoviruses incorporating the upstream enhancer elements of the CEA gene will have improved transcriptional activation efficiency but will maintain tissue specificity. Upstream regulatory sequences of the αFP gene have been incorporated into an adenovirus

phenotypes of which include complete absence of melanocytes and small, nonpigmented eyes. Defective expression of the human homologue of Mi, MITF, occurs in the human genetic disease Waardenburg syndrome type 2 (37, 38), characterized by hearing loss and pigmented disturbances. Cotransfection of Mi or MITF expression plasmids and reporter constructs containing tyrosinase transcription elements including the CATGTG motif resulted in high-level activation of transcription in melanoma cells and lower but significant levels of activity in cells that normally do not express tyrosinase (i.e., fibroblasts or HeLa cells; Refs. 24, 26, and 30). Mutational analysis has demonstrated the absolute requirement of E-box sequences for this trans-activation, suggesting that the activity of Mi at the E-box is an essential step in activation of pigment cell-specific genes (26, 30).

Our data demonstrate that high level expression from the murine 769-bp promoter region requires the addition of a tandem repeat of the enhancer fragment, the normal position of which is 12 kb upstream of the transcriptional start site of the endogenous murine tyrosinase gene (26). This enhancer contains three CANNTG E-box elements, one of which forms part of an 11-bp palindrome that is required for enhancer function (26). The upstream enhancer region of the human tyrosinase gene also contains an essential CATGTG motif that is the target of MITF-induced transcription of the tyrosinase promoter (22, 30). Thus, the addition of multiple enhancer elements in close proximity to the tyrosinase promoter may promote cooperative interactions between E-box-binding transcription factors, specifically Mi or MITF, and the basal transcription machinery. This may be a common feature of bHLH-Zip family members. Constructs containing multiple repeats of the E-box-containing μE3 binding site for the Mi-related factor TFE3 also results in synergy of transcriptional activation (39).

We have used homologous recombination between a shuttle vector containing the murine promoter-enhancer-β-gal transcription unit and an E1- and E3-deleted adenoviral vector to generate the recombinant virus AdmTyr-βgal. Two important features of the expression pattern of AdmTyr-βgal should be stressed. First, infection of human melanoma cells results in very high levels of expression that are similar to those observed with AdCMV-βgal, the standard of reference for potent transcriptional activation in a variety of mammalian cell types (40, 41). This is evident both from the in situ staining of infected cells and by comparative assays of β-gal enzymatic activity over a range of MOIs. High levels of expression are also noted in murine melanoma cells, although higher MOIs were required. The high transcriptional activity of AdmTyr-βgal was surprising, especially in human melanoma cells. As discussed above, transfection of enhancer dimer plasmid constructs into melanoma cell lines resulted in significant increases in efficacy compared with promoterless constructs; however, in multiple experiments, the percentage of activity compared with that of pCMVβ was never greater than 25% (data not shown).
Fig. 4. Enzymatic assays of β-gal activity in human cell lines after infection with AdCMV-βgal or AdmTyr-βgal. Human melanoma cell lines (DM13 and SK-MEL-28), nonmelanoma primary cell lines (HUVEC, HEL, and MRHF), and nonmelanoma tumor cell lines (SK-N-SH, OVCAR-3, and HT-29) were infected with either AdCMV-βgal or AdmTyr-βgal at MOIs of 10, 50, 100, and 250. Forty-eight h after infection, cell lysates were prepared, and β-gal enzymatic activity was determined. Y axis, average absolute β-gal units from triplicate infections at each MOI; data are recorded as the exponentials of the determined values. Note that the scale on the Y axis differs for some control cell lines; this reflects differences in activity of the CMV promoter in cells of different lineages and/or a difference in their inherent infectibility with adenovirus. ○, infection with AdCMV-βgal; ■, infection with AdmTyr-βgal; bars, SD.
vector driving HSV-TK as the effector gene (14). Levels of HSV-TK produced by infected cells correlated with the level of endogenous αFP produced by each cell line. Comparison with the level of expression with an adenovirus vector producing HSV-TK under control of the Rous sarcoma virus promoter showed that the αFP-regulated expression was <1% of the Rous sarcoma virus level. Thus, it appears that promoter elements can confer tissue specificity; however, high-level expression may require enhancer elements not present in sequences immediately adjacent to the gene. Other tissue-specific promoter-enhancer elements will soon be tested in the adenoviral system.

These include those derived from the DF3/MUC1 gene (11) and regulatory sequences from the prostate-specific antigen gene (45).

We describe regulatory sequences derived from the mouse and human tyrosinase genes that are sufficient for high-level, tissue-specific transcription in pigment lineage cells. The virus AdmTyr-βgal incorporates the murine promoter-enhancer elements and was shown to retain the desired transcriptional properties. By comparison with a CMV-promoted virus, AdmTyr-βgal was shown to be equally efficacious in human melanoma cells but highly regulated when tested in a panel of cells form other cell lineages. Experiments are under way.
to combine the promoter-enhancer elements described here with a variety of effector genes for in vivo studies of therapeutic efficacy in both human xenograft models and murine melanoma models. Effector genes of interest include HSV-TK, interleukin-12, and combinations of effector genes that might synergize for antitumor activity with establishment of immunological memory (46, 47). A number of obstacles remain before transcriptional targeting vectors of the type described in this article can be used in a clinical setting. Foremost is the inability to distribute genetic material throughout the body to all sites of potential metastatic deposits. Given that cancer patients with metastatic disease have $10^6$–$10^{12}$ tumor cells, targeting each tumor cell does not appear feasible. Using current technology, transcriptional targeting systems could only be used in conjunction with effector mechanisms that do not require expression in every cancer cell. The continued evolution of new vector systems, including novel viruses as well as nonviral formulations, should serve to improve our ability to transfect tumor cells in vivo. Transcriptional targeting cassettes could be incorporated as a component of novel vector systems and, as such, could be used as part of targeted, systemic gene therapy systems.

REFERENCES


Transcriptional Targeting of Recombinant Adenoviruses to Human and Murine Melanoma Cells

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