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Short and highly efficient synthetic promoters for melanoma-specific gene expression

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Abstract **Here, we report the construction and functional analysis of synthetic promoters designed for gene therapy applications requiring strong and specific gene expression in melanoma cell lines. We have analysed the transcriptional activity of different combinations of two transcriptional regulatory modules, a melanocyte-specific element from the human tyrosinase promoter and a cell-cycle-specific element from the human α-fetoprotein promoter. Transient expression assays in different cell lines show that several of these composite synthetic promoters can drive a strong and selective expression of a reporter gene in melanoma cell, providing us with a new powerful tool for gene therapy of melanomas.**
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Keywords: Melanoma; Tyrosinase promoter; Gene therapy; Expression vector

1. Introduction

Melanoma is a highly aggressive tumour and effective therapeutic agents are not currently available, therefore gene therapy represents a promising approach for the treatment of this disease. Several strategies have been developed for gene therapy of cancer and many efforts have been directed to the construction of transcriptionally targeted vectors to obtain the expression of cytotoxic genes in a tumour-specific manner, avoiding non-specific toxicity [1–4].

Tumour-cell-specific expression can be achieved by the use of a tumour-specific promoter. The majority of melanoma cells synthesise melanin, and the rate-limiting step of this metabolic pathway is the hydroxylation of tyrosine catalysed by the enzyme tyrosinase [5]. Tyrosinase is the key enzyme in melanogenesis and it is expressed in a tissue-specific manner in pigmented cells, including melanoma [6].

Transcriptional regulatory elements that are crucial for the expression and the tissue-specificity of the tyrosinase gene have been identified and characterised [7,8]. The human tyrosinase proximal promoter is regulated by one negative and four positive elements. There are no data on the identity of the factors involved in the negative regulation. The positive elements contain a binding site for the SP1 transcription factor, a highly conserved M-box, a CR2 element containing the initiator E box and an octamer element. The M-box is associated in the activation of tyrosinase gene expression through the product of microphthalmia gene (Mi), which is known to be involved in melanocyte differentiation [9]. In addition to the proximal promoter region, a strong and cell-specific expression of the tyrosinase gene requires an enhancer element located 2 kb upstream of the transcriptional start site, containing a tyrosinase distal element (TDE) which enhances tissue-specificity through the binding of Mi [10]. This is in line with the general organization of eukaryotic promoters, in which the tissue-specificity is achieved through the cooperation of multiple elements usually located at considerable distance from one another.

Several attempts have been made to use tyrosinase promoter and enhancer elements in vectors for melanoma gene therapy [11–16]. However, viral vectors employing long promoter sequences, although able to drive-specific gene expression, often display a reduction in viral titre that correlates with the size of the inserted promoter [12]. Furthermore, many vectors have a size-constrain that does not allow the insertion of a full promoter. On the other hand, shorter promoter sequences are often too weak or lose specificity. Therefore, the availability of compact and highly specific promoters will multiply the possible gene therapy approaches to melanoma.

In this context, we developed a strategy for the construction of compact synthetic promoters to be utilised in melanoma gene therapy. We have previously found that the polymerisation of a 100 nucleotides region of the human α-fetoprotein proximal promoter, a combination of an AP1/GRE (cell-cycle specific) and a HNF1 (liver specific) regulatory elements, is very effective for the production of synthetic promoters with a high hepatoma-specificity (our unpublished results). Here, we describe the construction and analysis of synthetic promoters containing different combinations of the human tyrosinase promoter M-box and of the previously described AP1/GRE transcriptional elements. Transient expression assays in different cell lines show that these synthetic promoters can drive a strong expression of a CAT reporter gene selectively in melanoma cell, providing us with a new powerful tool for gene therapy of melanomas.

2. Materials and methods

2.1. Cell culture and cell lines

The human cell lines MeWo and A2058 (melanoma), Hela and the murine melanoma cell line B16, were obtained from American Type Culture Collection (Manassas VA, USA). All cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a 5% CO_2, 37 °C.

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2.2. Transfection and analysis of CAT expression

Cells were transfected with 20 μg of plasmid DNA using N',N-bis(2-hydroxyethylpiperazine)-2-aminoethanesulfonic acid method [17] with some modifications. Transfections were carried out overnight at 3% CO_2 and 35 °C and followed by a 3 min 10% glycerol shock. After a three times rinse with PBS, cells were reefed and incubated at 5% CO_2, 37 °C. CAT assays were performed 48 h after transfections using the CAT ELISA colorimetric immunoassay (ROCHE) as described by the manufacturer. To normalise the transfection efficiency among the samples of the same experiment, the test plasmids were cotransfected with a cytomegalovirus (CMV)-luciferase reporter construct. All transfections were repeated at least three times and with different plasmid preparations. In order to compare the results obtained in different cell lines, the data reported in Fig. 2 have been further normalised by dividing them for the positive control values (pSV2-CAT) obtained in that specific cell line.

2.3. Construction of promoter-reporter vectors

The CAT constructs for the analysis of synthetic promoters are based on the expression vectors pTKshortCAT and EI2XCAT [18]. Two complementary oligonucleotides containing the M-box and the functional binding sites for AP1 and GRE were phosphorylated at 5′ termini using T4 polynucleotide kinase, annealed and ligated to form concatenamers. The ends of ligated products were filled-in by using the Kleenow fragment of *Escherichia coli* DNA polymerase I and cloned into pTKshortCAT. The oligonucleotides containing the TDE elements were phosphorylated, annealed and ligated as described before. Plasmids were propagated in *E. coli* strain DH5α and analysed by restriction mapping and DNA sequencing.

3. Results

We designed two complementary synthetic oligonucleotides containing the tissue-specific M-box of the human tyrosinase gene, associated to the AP1 and GRE cell-cycle-specific elements (Section 2). The oligonucleotides were multimerised, cloned randomly in the expression vector pTKshortCAT [18] and recombinant clones containing different copies and combinations of these regulatory elements were selected. The schematic structure of the different promoters is illustrated in Fig. 1.

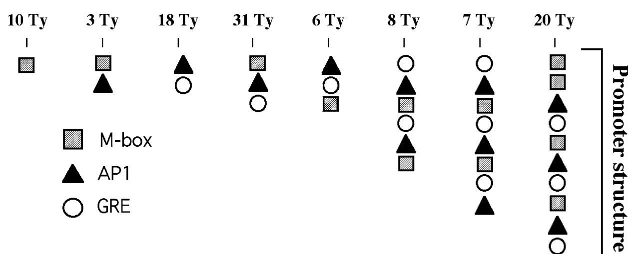

Fig. 1. Architecture of our synthetic Ty promoters. The topside is toward the reporter gene.

The transcriptional efficiency and specificity of these different combinations were analysed by transient expression in three different melanoma cell lines (B16, MeWo and A2058) and in Hela cells as control (Fig. 2). Quantisation of CAT activity demonstrated that single copies of the M-box and AP1 elements, either alone or together, make very poor promoters. However, the addition to the M-box and AP1 of a single GRE element (compare 3Ty with 31Ty) results in a significant increase of transcriptional activity in mouse melanoma cells (B16) and in human melanoma cells (MeWo), while it is completely ineffective in the highly metastatic human melanoma cell line A2058. This combination of elements cannot be considered cell-specific, since the transcriptional activity observed in Hela cells is comparable to that observed in B16 melanoma cell lines (see also construct 31-Ty in Fig. 3).

The 7-Ty promoter, containing 3 GRE, 3 AP1 and 2 M-box, appears to be the most effective and specific combination in melanoma cells. The comparison of CAT activity of 7-Ty and pSV2CAT clearly shows that in melanoma cells the 7-Ty promoter is stronger than pSV2CAT. A further increase of the number of regulatory elements may result in a loss of specificity, as evident for recombinant 20-Ty, whose expression in HeLa cells increases as compared to 7-Ty.

The use of a synthetic enhancer element containing three copies of the tyrosinase distal enhancer (TDE) has been reported to generate transcriptionally targeted vectors for gene therapy of melanoma [13]. Therefore, we decided to compare the efficiency and cell-specificity of this synthetic enhancer to those of our promoters. A synthetic oligonucleotide containing three copies of TDE element was subcloned in both orientations in pTKshortCAT. We also generated a recombinant clone containing six copies of TDE element. Fig. 3 shows that, in melanoma cells, our synthetic promoters are more effi-

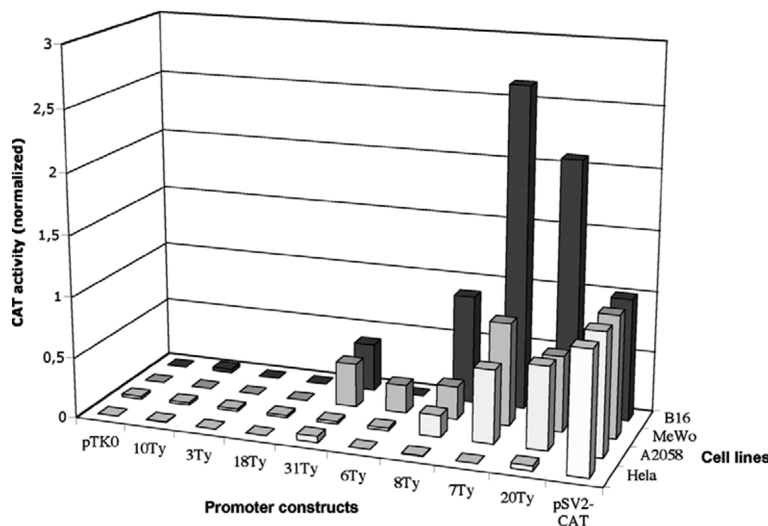

Fig. 2. Transient expression assays of Ty constructs in human (A2058, MeWo) and murine (B16) melanoma cell lines. CAT activity is expressed as the ratio between the absolute value and the positive control value (pSV2-CAT).

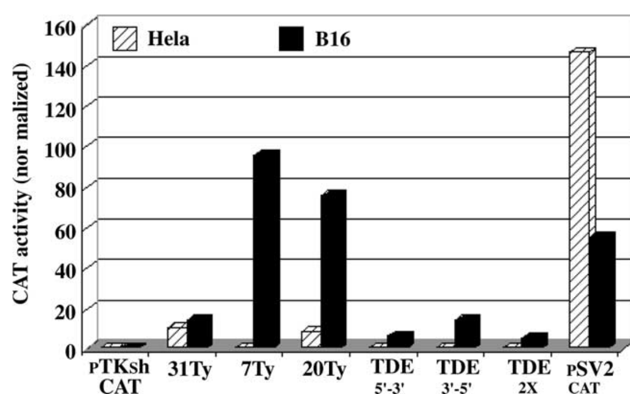

Fig. 3. Comparison of Ty and TDE constructs transient expression in B16 melanoma and in HeLa cell lines. TDE, trimer of the Tyrosinase Distal Enhancer; TDE 2X, further dimerisation of TDE.

cient as compared to those containing the TDE elements, while their expression is lower in non-melanoma cells. These results suggest that our promoters may be better suitable to drive melanoma-specific expression of a therapeutic gene.

4. Discussion

A main problem of gene therapy approaches is how to target a specific tissue or a group of cells within the whole organism, leaving the other tissues unaffected. Virus-derived vectors may take advantage of natural viral tropism or, alternatively, the viral molecules involved in the cell entry pathway can be modified to redirect a virus toward a different host. However, the availability of naturally host-restricted vectors is limited to a minority of cell types, e.g., lymphocytes or neural cells. Moreover, despite the huge efforts of several research groups, manipulation of the viral entry mechanisms almost invariably results in a marked reduction of infection efficiency and/or virus stability.

A relatively easier way to obtain selectivity is to use tissue or cell-specific transcription regulatory signals to drive the expression of the therapeutic gene. In this case, even if the vector can infect different cell types, the infection will result in the expression of the therapeutic gene only in the desired cell type. This result can be achieved by incorporating in gene therapy vectors natural tissue-specific promoters, and there are several reports in which this approach has been successfully employed [12–16].

Nevertheless, Pol II promoters are often very large and complex, and tissue-specific transcription results from the combinatory co-operation of several control elements, each of them contributing only partially to specificity, spread over very large DNA fragments. On the other hand, in the most widely used vectors for gene therapy space constrains prevent the incorporation of very large promoter region and impose to use only part of them, thus reducing transcriptional efficiency and specificity.

The majority of gene therapy approaches to melanomas are currently based on selective expression in melanoma cells of a variety of therapeutic genes: "suicide" genes, tumour suppressor genes, inhibitors of aberrant oncogene expression, or genes encoding immunologically relevant molecules. All of these approaches have shown to be effective in vivo, albeit at different degrees and often in combination with other therapeutic protocols [19]. Nevertheless, in all of them the main issues are the strength and the tightness of the transcriptional control.

Here, we describe a group of synthetic promoters engineered to obtain high levels of melanoma-specific expression, the larger of them not exceeding 300 nucleotides. These promoters are strong enough to achieve in melanoma cells transcriptional levels higher than those obtained with the very strong and compact SV40 enhancer/promoter region, still retaining their melanoma-specificity. Experiments to assess the transcriptional activity of these promoter in vivo are in progress.

Our promoters have been specifically designed to operate in the context of SV40-derived vectors for a suicide gene approach. Nevertheless, we believe that these promoters may be very useful tools for all gene therapy approaches based on selective expression in melanoma cells, especially when planning the use of space-limited vectors.

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References

[1] Vile, R.G., Russel, S.J. and Lemoine, N.R. (2000) Cancer gene therapy: hard lessons and new courses. Gene Therapy 7, 2–8.
[2] Moolten, F.L. (1994) Cancer Gene Ther. 1, 279–287.
[3] Pardoll, D.M. (1995) Paracrine cytokine adjuvants in cancer immunotherapy. Annu. Rev. Immunol. 13, 399–415.
[4] Miller, N. and Whelan, J. (1997) Progress in transcriptionally targeted and regulatable vectors for genetic therapy. Hum. Gene Ther. 8, 803–815.
[5] Korner, A. and Pawelek, J. (1982) Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. Science 217, 1163–1165.
[6] Hearing, V. and Tsukamoto, K. (1991) Enzymatic control of pigmentation in mammals. FASEB J. 5, 2902–2909.
[7] Lowings, P., Yavuzer, U. and Goding, C.R. (1992) Positive and negative elements regulate a melanocyte-specific promoter. Mol. Cell. Biol. 12, 3653–3662.
[8] Bentley, N., Eisen, T. and Goding, C.R. (1994) Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol. Cell. Biol. 14, 7996–8006.
[9] Yasumoto, K.I., Yokoyama, K., Shibata, K., Tomita, Y. and Shibahara, S. (1994) Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. Mol. Cell. Biol. 14, 8058–8070.
[10] Shibata, K., Muraosa, Y., Tomita, Y., Tagami, H. and Shibahara, S. (1992) Identification of a *cis*-acting element that enhances the pigment cell-specific expression of the human tyrosinase gene. J. Biol. Chem. 267, 20584–20588.
[11] Vile, R.G., Diaz, R.M., Miller, N., Mitchell, S., Tuszynski, A. and Russel, S.J. (1995) Tissue-specific gene expression from Mo-MLV retroviral vectors with hybrid LTRs containing the murine tyrosinase enhancer/promoter. Virology 214, 307–313.
[12] Diaz, R.M., Eisen, T., Hart, I.R. and Vile, R.G. (1998) Exchange of viral promoter/enhancer elements with heterologous regulatory sequences generates targeted hybrid long terminal repeat vectors for gene therapy of melanoma. J. Virol. 72, 789–795.
[13] Siders, W.M., Halloran, P.J. and Fenton, R.G. (1998) Melanoma-specific cytotoxicity induced by a tyrosinase promoter-enhancer/herpes simplex virus thymidine kinase adenovirus. Cancer Gene Ther. 5, 281–291.
[14] Cao, G., Zhang, X., He, X., Chen, Q. and Qi, Z. (1999) A safe, effective in vivo gene therapy for melanoma using tyrosinase promoter-driven cytosine deaminase gene. In Vivo 13, 181–187.

[15] Nettelbeck, D.M., Rivera, A.A., Balague, C., Alemany, R. and Curiel, D.T. (2002) Novel oncolytic adenoviruses targeted to melanoma: specific viral replication and cytolysis by expression of E1A mutants from the tyrosinase enhancer/promoter. Cancer Res. 62, 4663–4670.
[16] Banerjee, N.S., Rivera, A.A., Wang, M., Chow, L.T., Broker, T.R., Curiel, D.T. and Nettelbeck, D.M. (2004) Analyses of melanoma-targeted oncolytic adenoviruses with tyrosinase enhancer/promoter-driven E1A, E4, or both in submerged cells and organotypic cultures. Mol. Cancer Ther. 3, 437–449.
[17] Demartis, A., Vignali, R., Maffei, M., Barsacchi, G. and De Simone, V. (1994) Cloning and developmental expression of LFB3/HNF1 beta transcription factor in *Xenopus laevis*. Mech. Dev. 47, 19–28.
[18] Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7, 2745–2752.
[19] Sotomayor, M.G., Yu, H., Antonia, S., Sotomayor, E.M. and Pardoll, D.M. (2002) Advances in gene therapy for malignant melanoma. Cancer Contr. 9, 39–48.