

# メラニン生合成の調節

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Tyrosinase is a rate-limiting enzyme in melanin biosynthesis and tyrosinase-related protein (TRP) is responsible for the formation of black melanin rather than brown. To identify the cis-acting element(s) required for pigment cell-specific gene transcription, we analyzed the promoter function of two pigment cell-specific genes encoding human tyrosinase and TRP by transient expression analysis. The fusion genes were constructed by inserting the 5'-flanking region of the human tyrosinase gene or TRP gene upstream from the firefly luciferase gene and were introduced into human melanoma cells and cervical cancer cells (HeLa cells). We thus identified the enhancer sequence of 39 base pairs (bp), located about 1.8 kb upstream from the transcription initiation site of the human tyrosinase gene, that is responsible for its pigment cell-specific expression. Furthermore, we found the presence of enhancer-like activity in the first intron of the human TRP gene that enhances the transient expression of the reporter (luciferase) gene. However, this enhancer-like activity is detected not only in melanoma (pigment) cells but also in HeLa cells.

## 1. 緒言

色素細胞はメラニン合成という特異な形質を持つ細胞であり、高等動物ではメラノサイトとして全身の皮膚に分布する神経堤 (neural crest) 由来のものと、色素上皮細胞として脳由来の網膜に存在するものの二種類がある。日常経験する“日焼け”という現象からも明らかのように、メラニンの主な機能は紫外線に対する防護作用である。事実、メラニンを欠損する先天性白皮症 (アルビニズム) 患者では、日光に曝露される部位の皮膚がんの発生頻度が高い。すなわち、日焼けは一種の生体防御反応でもある。日焼け以外にも、様々な皮膚の炎症後に色素沈着の起こることが知られているが、このような色素沈着 (メラニン量の増加) が起こる機構はよくわかっていない。

本研究では、メラニン合成の律速酵素であるチロシナーゼとその関連蛋白である tyrosinase-

related protein (TRP) という二つの色素細胞特異的蛋白に着目し、メラニン合成の調節機構の一端を明らかにする。チロシナーゼはメラニン合成の最初の二つの反応 (チロシンからドーパ、ドーパからドーパキノン) を触媒する銅酵素であり、ドーパキノン以降は非酵素的にも反応が進みメラニンが生成される。TRP はチロシナーゼとアミノ酸レベルで約40%の相同性を持ち、黒いメラニンの生成に必要とされる。

本研究では、メラニン生合成に関与するチロシナーゼと TRP という二つの色素細胞特異遺伝子の転写に必須な領域を決定する。

## 2. 実験

2.1 無細胞転写系におけるヒトチロシナーゼ遺伝子と TRP 遺伝子のプロモーター機能解析  
マウスメラノーマ細胞より無細胞転写系を確立

し、上記色素細胞特異遺伝子のプロモーター活性を調べる。5'上流領域に種々の欠損を持つ鋳型DNAを用いて、転写に必要な領域を決定する。

## 2.2 一時的発現による色素細胞特異遺伝子のプロモーター機能解析

当該遺伝子の5'上流領域をプロモーターとして持ち、ホタルルシフェラーゼ(luc)をレポーターとして持つような融合遺伝子を構築し、ヒトメラノーマ細胞とHeLa細胞に導入しlucの一時的発現を調べることにより、色素細胞に特異的なプロモーター活性とその発現に必要な領域を調べた。この際、内部コントロールとしてSV40プロモーターの下流に $\beta$ -ガラクトシダーゼ遺伝子を持つプラスミドを上記融合遺伝子と同時に細胞に導入・発現させた。

## 2.3 色素細胞特異遺伝子の転写因子の同定

ヒトメラノーマ細胞より核抽出液を調製し、ゲルシフト法やフットプリント法を駆使して、色素細胞特異遺伝子の5'上流領域に結合する蛋白質を解析する。

# 3. 結果

## 3.1 無細胞転写系におけるチロシナーゼ遺伝子とTRP遺伝子のプロモーター活性

チロシナーゼ遺伝子とTRP遺伝子はメラノーマ由来の無細胞転写系において転写されるが、ヒトHeLa細胞由来の無細胞転写系では転写されなかった。よって、メラノーマ細胞にはこれら二つの遺伝子の色素細胞特異的転写に必須な因子が存在すると思われる。

## 3.2 チロシナーゼ遺伝子の色素細胞特異的発現の分子機構

### 3.2.1 マウスとヒトチロシナーゼ遺伝子のプロモーター機能は同等ではないトランスジェニックマウスを利用したマウスチ

ロシナーゼ遺伝子のプロモーター機能の解析により、転写開始点の上流270塩基対(bp)内に色素細胞特異的発現に必要なシスエレメントが存在することが報告されている。しかし、ヒトチロシナーゼ遺伝子のプロモーター機能を一時的発現により解析すると、転写開始点の上流600bp内には色素細胞特異的発現に必要なシスエレメントの存在を確認できなかった。すなわち、同遺伝子のプロモーター領域の下流にレポーター遺伝子(luc)を持つ融合遺伝子をメラノーマ細胞とHeLa細胞に導入し、その一時的発現を調べると、両者で有意な違いは検出できなかった。一方、このような系でも、マウスチロシナーゼ遺伝子の転写開始点の上流270bpが存在すれば、レポーター遺伝子はメラノーマ細胞(色素細胞)特異的に発現した。

### 3.2.2 ヒトチロシナーゼ遺伝子の色素細胞特異的エンハンサーの同定

ヒトチロシナーゼ遺伝子のさらに上流領域を同様に解析したところ、転写開始点の上流約1.8キロbpの部位に色素細胞特異的発現に必要なシスエレメント(約200bp)が存在することが判明した。しかも、この部位をSV40遺伝子プロモーターの上流につなげても、レポーター遺伝子の一時的発現は色素細胞特異的であった。また、そのエレメントを逆向きに組み込んでも、その機能に変化はなかった。よって、このシスエレメントはヒトチロシナーゼ遺伝子の色素細胞特異的発現に必須なエンハンサーである。

### 3.2.3 色素細胞特異的エンハンサーの決定

ヒトチロシナーゼ遺伝子の転写開始点の上流約1.8キロbpの部位に存在する上記シスエレメント(約200bp)の機能をさらに詳細に解析し、39bpの領域があれば、レポーター遺伝子は色素細胞特異的に発現された。

## 3.3 ヒトTRP遺伝子のプロモーター機能解析

ヒトTRP遺伝子のプロモーター機能を3.2と同様に解析した。ヒトチロシナーゼ遺伝子と異なり、ヒトTRP遺伝子の上流約4キロbpの領域内には、

色素細胞特異的エンハンサーの存在を示唆する結果は得られなかった。しかし、ヒトTRP遺伝子の第一イントロン内に細胞非特異的なエンハンサーの存在が示唆された。すなわち、このイントロンが存在すると、レポーター遺伝子の発現がメラノーマ細胞とHeLa細胞の両方で著明に増加した。

#### 4. 考 察

ヒトチロシナーゼ遺伝子の色素細胞特異的発現に必須なエンハンサーの領域(39bp)を決定した。同エンハンサーは転写開始点の上流約1.8キロbpの部位に存在する。この39bpの中にはCATGTGモチーフが存在しており、このモチーフはヘリックス-ループ-ヘリックス構造を持つDNA結合蛋白質の認識部位であることが知られている。今後、このエンハンサーに作用する蛋白質因子を同定し、精製する。

一方、ヒトTRP遺伝子では、色素細胞特異的発現に必須なエンハンサーを見いだせなかった。その代わり、第一イントロン内に細胞非特異的なエンハンサー様活性を検出した。今後このイント

ロン内のエンハンサー様活性の実体を解明すると共に、ヒトTRP遺伝子の色素細胞特異的発現に必須なエンハンサーの検索を行う。

#### 5. 研究発表

1. Shibata, K., Takeda, K., Tomita, Y., Tagami, H. and Shibahara, S. :Down-stream region of the human tyrosinase-related protein gene enhances its promoter activity. *Biochem. Biophys. Res. Commun.* 184:568-575, 1992.
2. Shibata, K., Muraosa, Y., Tomita., Y., Tagami, H. and Shibahara, S. :Identification of a cis-acting element that enhances the pigment cell-specific expression of the human tyrosinase gene. *J. Biol. Chem.* 267, 20584-20588, 1992.
3. Shibahara, S. :Functional analysis of the tyrosinase gene and brown-locus protein gene promoters. *J. Invest. Dermatol.* 100, 146S-149S, 1993.

別 添 資 料  
—成果発表論文抜粋—

発 表 1

1 - 1 掲載誌

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS  
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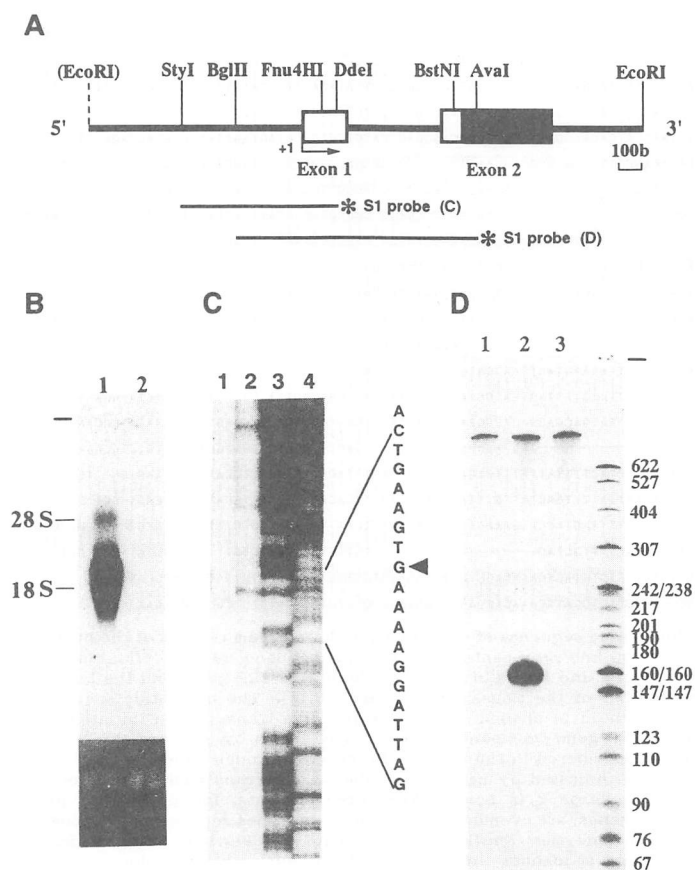
1 - 2 表 題

DOWNSTREAM REGION OF THE HUMAN TYROSINASE-RELATED PROTEIN GENE  
ENHANCES ITS PROMOTER ACTIVITY

1 - 3 要 約

We have cloned and sequenced the human genomic DNA segments encoding the 5'-flanking region and the first two exons of the tyrosinase-related protein (TRP) gene, a pigment cell-specific gene. Functional analysis of its promoter suggests that the downstream region of the TRP gene, including the first intron, enhances the transient expression of the luciferase gene under control of the TRP gene promoter about 16-to 20-fold. This enhancer-like activity is detected not only in melanoma cells but also in HeLa cells whose TRP gene expression is assumed to be repressed. We suggest a possibility that the downstream region is not sufficient to confer pigment cell-specific expression, but is required for efficient transcription of the TRP gene in pigment cells. ©1992 Academic press, Inc.

1 - 4 図表等 (次頁)



**Fig. 1.** Structural organization of the 5'-region of the human TRP gene.

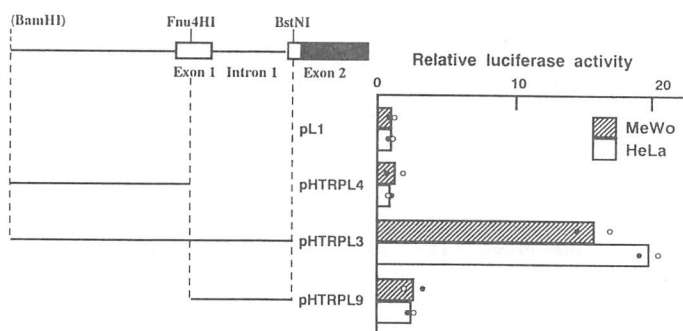
A. Schematic representation of the human TRP gene. The direction of transcription is from left to right. Shown are the sites for only relevant restriction enzymes. The *EcoRI* site located at the 5'-end of the gene represents the linker sequence. Solid lines represent flanking and intronic DNA segments. The 5'-untranslated and the protein-coding regions are indicated by open boxes and a closed box, respectively. The S1 probes used in C and D were also shown and asterisks indicate the sites of end-labeling. B. Northern blot analysis. Each lane contained 10  $\mu$ g of total RNA prepared from MeWo melanoma cells (lane 1) and HeLa cells (lane 2). A small horizontal bar indicates the origin of lanes. A bottom panel shows the stained rRNA (18S and 28S) representing variability in the amounts of RNA loaded. The hybridization probe used was the *RsaI/RsaI* fragment (806/1554) excised from human TRP cDNA (11). C. Identification of the transcription initiation site. Shown is an autoradiogram of a sequencing gel. Lane 1, protected products with HeLa cell RNA; lane 2, protected products with human melanoma RNA. Lanes 3 and 4, chemically cleaved products of the S1 probe, representing purines and pyrimidines, respectively. Capital letters, shown along the sequencing ladder, denote the sequence of the sense strand (see Fig.2). An arrow indicates the putative transcription initiation site. D. S1 mapping analysis for the presence of correctly spliced transcripts. Lane 1, protected products with HeLa cell RNA; lane 2, protected fragments with human melanoma RNA; and lane 3, protected products with yeast tRNA. The size markers shown are pBR322 plasmid DNA digested with *MspI* and given in bases.

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-200          GGTAAATGTTGAATAACAGTTGAATA-ACAGAAATAAAA-AA
-203'          TCATCTTGTTTCTTCCTTTGGTATACAGATAAGAAAAATAA
-159  AATCTCTGAAAGTAAAC-AAATCTCACTAGTTTATCTGACTTGTATTCCAAATTAGTGCTTCTGGCCCTTTCTTAAAAAC
-160' AATCACTACAACGAAAGCAAAAATCTCTTCAGCGTCTCTAATACATCTTCCAAATCAGTGTGCTGACCTTTCTTAAAGAC
-80   TTTAAGCATCACAAAGAAATCAGTTGGAAGGGAATCATGTGCTGATCAAGTCTTAAAGGGCAGAAATATTCACCTGAAGT
-80'  TTTAACCATCACAAAGAAACAGTGGGGAGGGAGTCATGTGCTG-CCTAGTAGTTAAAGGGCAGGAGAAATTCACCTGGTGT
1    *GA-AAAGGATTAGTAAAGGTTGAAAAAAGACCAGCCGCCCCCTAGTTGGGTGAGCAGATTTGGGATTAATATCAG
-1'   GAGAAAGGATTAGTAAAGGTTGAAAAAAGACCAGCCGCCCCCTAGTTGGGTGAGCAGATTTGGGATTAATATCAG
80   GCAGCAATCCACAT---GCACCTAACAGTCCGAGGTGAGAGGACAAAGAAACACAAGCAAAATATAAAACATCAATTTCTA
78'  GCAGAAAATCTCTTCGGGCAATTAACAG---CTGGCATCAGGGGAAAAG-CAGAC-ATCCAA---CAACACI-AGCCTCTG
157  AGAGAAGTTCATCAGAGACATCCTTCCAGGATTTGTCAGTACTGGAAAGAAGTCCATGGGGAGTGGGTGGACACGTGCCA
149' AAGGAGATCAGCAGAGAAAACCTTCCAGGATTTGTCAGTACTGGTGGAGCAG--CTCTGTG--GTGGGT---AC---CCT
237  AAATCCCATTAGTGTAAAGGACCTTAAATCACAGAAAATTAACCTTGGTGGAAATCTGTCCCAATTCCTCTCAGCTCCTA
217' TGTGACCA-AAGCTCTAGGAAC----ATGAAGGAGATTGCTTGTCTATAAACCTGTTTCCTATCTCCTTTCAATTTCCA
317  AGGTTAAATTAATGTAATTAATGATGGTACCTGCTAATTCATGCTTTGATAACTGATATCTA--GTATGATATATA
291' TGG-----TTAACTATTACT-ATGGTAGTCACCAACTAGTGGATGCTTTGGTAAATGACATCTATGGAAGCTCTTTTGG
395  TATAAACAATAAGCAGGAGCAGGGAATTAATATTTGGGTATCACACATCGAGGTGTTATATATGCCAAAATTTTAAAG
365' GATCA          240 bp          TGAGT-TCTGTCATCTAGCTATTATGCTTCAGATATTTAAAG
475  GTAATAACTACTCTTTTATTTATTTGTGAAATGTCATTTACAT-ATGGGTCCATTTTGAAGTGGTTTGGGAAGGGGG
652' GTGAATAATATTTTACTATTGTTTGAATAATGTTTTCACATGAAAGATTCATTTCCGGAGTGGGTTGAAAAGTATG
554  CATACCAATTTAAGTACCAAGAAAACCTTGCATAATCTCATTTT-ACTTTCTCTTTTTCAGCTGGATTTTCTCTCAGCTG
732' CAAAAGAACCTTTTGAAC-----TCTGTTTTGCTTTCTGTTTTTTCAGCTGGATTTTTCATCTGAGCA
633  CTTCACTCTCTCTCATCACAAAGAGGCTGCAAAACAGGCTTTGTTTTGCACTCTATTTCAAGCAGATAG
795' CCCCTGTCTCTCCATGCAAAAGAGCAGCATAGGAGACCTGTGTTCTGAACTCTTGCCTTCGAGAAGATAG

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**Fig. 2.** Nucleotide sequence of upstream and downstream regions of the human TRP gene. The top line represents the nucleotide sequence of the 5'-flanking region, exon 1, intron 1 and a part of exon 2 of the human TRP gene, and the bottom line represents that of the mouse counterpart (13,14). The nucleotide sequences of exons are printed in shaded areas. The putative transcription initiation site of the human TRP gene (nucleotide residue 1) is shown by an asterisk. Nucleotide residues are numbered in the 5' to 3' direction, and nucleotides on the 5' side of residue 1 are indicated by negative numbers. The translational initiation codon located in the exon 2 is boxed. The three elements, found in other pigment cell-specific genes, are overlined (13). Double overlines represent the recognition sites for three enzymes: *Fnu4HI*, *DdeI* and *BstNI* (see Fig.1A). Small vertical lines between both sequences indicate identical nucleotide residues and small horizontal bars represent gaps. Two other 5'-splicing sites of the mouse intron 1 are indicated by closed triangles (14). The middle portion (240 bp) of the mouse intron 1 is not shown, because no sequence similarities are found.



**Fig. 3.** Functional analysis of the human TRP gene promoter. MeWo human melanoma cells and HeLa cells were cotransfected with  $\beta$ -galactosidase expression vector, pCH110 and the fusion genes containing the human TRP gene promoter upstream of the luciferase gene. Both upstream and downstream regions of the TRP gene used for construction of fusion genes are shown at the left panel. The *BamHI* site located at the 5'-end of the upstream region represents the linker sequence used for construction. Cellular extracts were prepared from transfected cells and assayed for luciferase and  $\beta$ -galactosidase activity as an internal control. Luciferase activity was divided by  $\beta$ -galactosidase activity and shown as a ratio to the value obtained with pL1, a promoterless luciferase expression vector. The data shown are averages of two independent experiments as indicated by closed or open circles.

## 発表 2

### 2-1 掲載誌

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### 2-2 表題

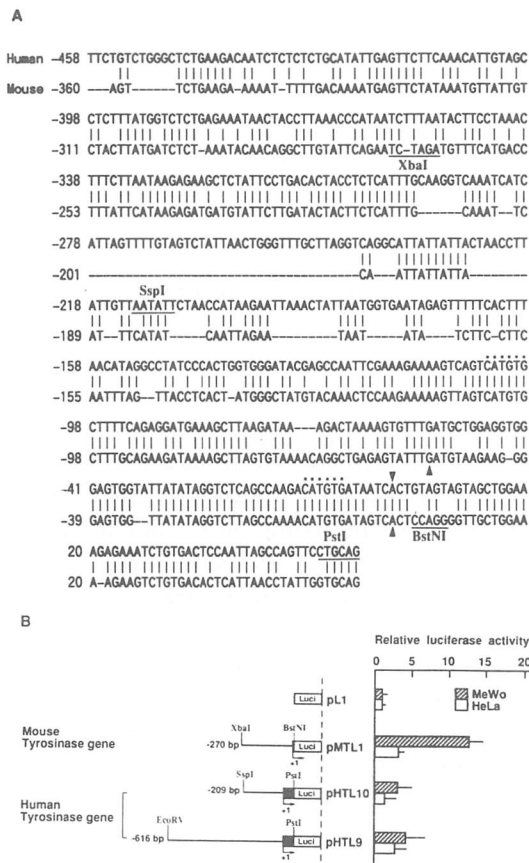
Identification of a *cis*-Acting Element That Enhances the Pigment Cell-specific Expression of the Human Tyrosinase Gene

(Received for publication, June 8, 1992)

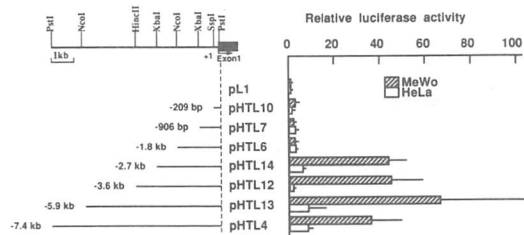
### 2-3 要約

To identify the *cis*-acting element that is responsible for the pigment cell-specific expression of the human tyrosinase gene, we analyzed the promoter activity of its 5'-flanking region by transient expression assays. The fusion genes were constructed by inserting the 5'-flanking region of the human tyrosinase gene upstream from the firefly luciferase gene and were introduced into human melanoma cells and HeLa cells. We thus found the element, located between 2.7 and 1.8 kilobase pairs upstream from the transcription initiation site, that enhances the transient expression of the luciferase reporter gene in melanoma cells, but not in HeLa cells, the tyrosinase gene expression of which is not detectable. Using the fusion genes containing putative enhancer elements under the control of the heterologous simian virus 40 promoter, we identified the pigment cell-specific enhancer of ~200 base pairs (bp) between -2.0 and -1.8 kilobase pairs and localized the core sequence to a 39-bp region. This 39-bp core element was then confirmed to direct the melanoma cell-specific expression of the reporter gene under the tyrosinase gene promoter. We thus propose that this core element is responsible for the pigment cell-specific expression of the human tyrosinase gene.

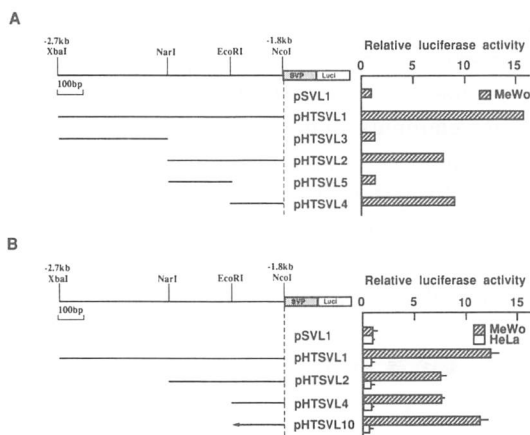
### 2-4 図表等 (次頁)



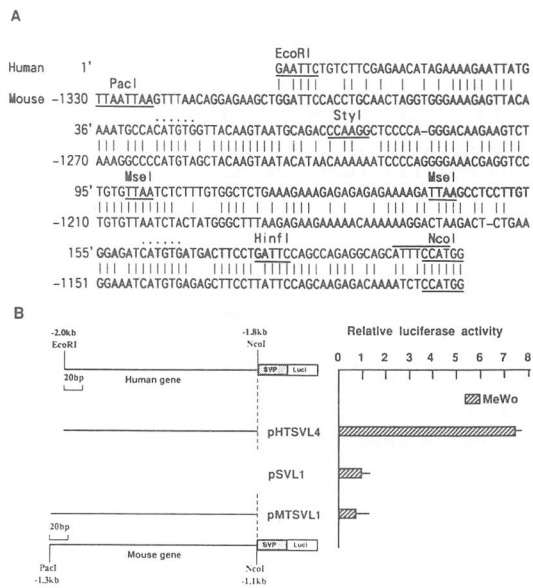
**FIG. 1. Comparison of nucleotide sequences and function of human and mouse tyrosinase gene promoters.** *A*, comparison of the nucleotide sequences. The *top line* represents the nucleotide sequence of the human tyrosinase gene promoter (8, 9), and the *bottom line* represents that of the mouse gene (12, 14). *Closed triangles* indicate the major transcription initiation sites (residue 1) and the second initiation site of the mouse gene (12). The nucleotides on the 5'-side of residue 1 are indicated by *negative numbers*. Relevant restriction sites and the CATGTG motif are also indicated. *B*, transient expression analysis. MeWo human melanoma cells and HeLa cells were cotransfected with  $\beta$ -galactosidase expression vector pCH110 and the fusion genes containing the tyrosinase gene promoters upstream from the luciferase gene. The promoter regions used for construction of fusion genes are schematically shown to the *left*. The direction of transcription is from left to right as indicated by *arrows*, and the transcription initiation site is numbered +1. The exons coding for the 5'-untranslated regions are indicated by *closed boxes*. The number shown at the 5'-end of each upstream region represents the position from the transcription initiation site. Luciferase activity was divided by  $\beta$ -galactosidase activity (an internal control) and is shown as a ratio to the value obtained with pL1 (relative luciferase activity). The data shown are the mean  $\pm$  S.D. of three independent experiments.



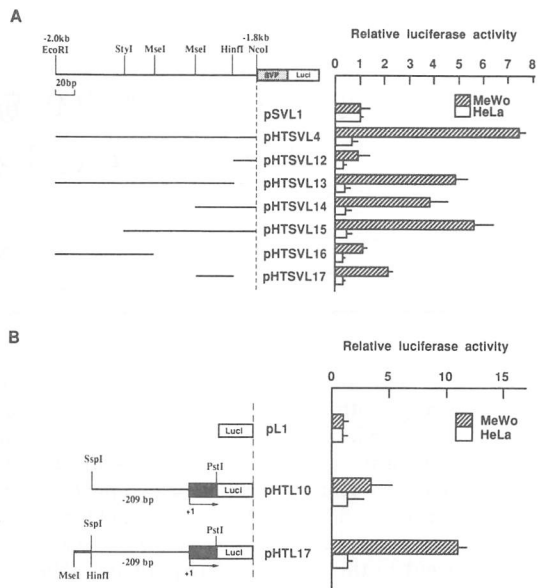
**FIG. 2. Functional analysis of human tyrosinase gene promoter.** The upstream regions of the human tyrosinase gene used for construction of fusion genes are shown to the *left*. The relative luciferase activity is shown to the *right*. Other conditions were the same as described for Fig. 1.



**FIG. 3. Functional analysis of enhancer element using heterologous promoter.** *A*, presence of the enhancer element. The *XbaI/NcoI* fragment, identified in Fig. 2, is enlarged, and the upstream regions of the human tyrosinase gene used for enhancer analysis are shown to the *left*. The upstream regions were inserted upstream from the SV40 promoter. The data shown were obtained from one experiment. *B*, presence of the enhancer element conferring pigment cell-specific gene expression. pHTSVL10 carries the enhancer element in the opposite orientation, as indicated by the *arrow*.



**FIG. 4. Comparison of pigment cell-specific enhancer of human tyrosinase gene with homologous sequence found in mouse counterpart.** A, nucleotide sequence of the pigment cell-specific enhancer of the human tyrosinase gene. Shown is the nucleotide sequence of the *EcoRI/NcoI* fragment (-2.0/-1.8 kb), containing the enhancer element of the human tyrosinase gene (top line). The bottom line represents the nucleotide sequence of the putative mouse homolog (14). Nucleotide residues are tentatively numbered in the 5'- to 3'-direction for the human element (see "Experimental Procedures"), and the nucleotide residues of the mouse homolog were renumbered according to our assignment of the transcription initiation site (12). Indicated are the recognition sites for the enzymes used for construction of the fusion genes. Vertical lines between both sequences indicate identical nucleotide residues, and dashes represent gaps. The core element is from 142' to 180', and the CATGTG motif is dotted. The overline indicates the sequence similar to the consensus sequence of octamer-binding proteins. B, functional analysis of the mouse sequence homologous to the human enhancer element. The *PacI/NcoI* fragment (-1325/-1105) of the mouse tyrosinase gene was located upstream from the SV40 promoter/luciferase fusion gene.



**FIG. 5. Identification of core element in pigment cell-specific enhancer of human tyrosinase gene.** A, functional analysis using the SV40 promoter. The *EcoRI/NcoI* fragment, identified in Fig. 3, was dissected, and its enhancer activity was analyzed. B, functional analysis using the tyrosinase gene promoter. The *MseI/HinfI* fragment, containing the core sequence of the enhancer, was analyzed for enhancer activity.