Involvement of ITF2 in the Transcriptional Regulation of Melanogenic Genes*

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Minao Furumura‡, S. Brian Potter‡, Kazutomo Toyofuku‡, Jun Matsunaga‡, Jacqueline Muller§, and Vincent J. Hearing‡‡§

From the Pigment Cell Biology Section, Laboratory of Cell Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the Division of Virology, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

In response to agouti signal protein, melanocytes switch from producing eumelanin to pheomelanin concomitant with the down-regulation of melanogenic gene transcription. We previously reported that a ubiquitous basic helix-loop-helix transcription factor, known as ITF2, is up-regulated during this switch, and we now report that treatment of melanocytes with melanocyte-stimulating hormone down-regulates expression of ITF2. To more fully characterize the involvement of ITF2 in regulating melanogenic gene transcription, ITF2 sense or antisense constructs were introduced into melan-a melanocytes. Gene and protein expression analyses and luciferase reporter assays using promoters from melanogenic genes showed that up-regulation of ITF2 suppressed melanogenic gene expression as well as the expression of Mitf, a melanocyte-specific transcription factor. In addition, stable ITF2 sense transfectants had significant reductions in pigmentation and a less dendritic phenotype compared with mock transfectants. In contrast, ITF2 antisense-transfected melanocytes were more pigmented and more dendritic. These results demonstrate that up-regulation of ITF2 during the pheomelanin switch is functionally significant and reveal that differential expression of a ubiquitous basic helix-loop-helix transcription factor can modulate expression of melanogenic genes and the differentiation of melanocytes.

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† To whom correspondence should be addressed: Laboratory of Cell Biology, Bldg. 37, Rm. 1B25, NCI, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-1566; Fax: 301-420-8787; E-mail: hearingy@nih.gov.
‡ The abbreviations used are: ASP, agouti signal protein; bHLH, basic helix-loop-helix; MC1R, melanocortin 1 receptor; MSH, melanocyte-stimulating hormone; PMSF, phenylmethylsulfonyl fluoride; db-cAMP, N²,²-O-dibutyryl adenosine 3':5'-cyclic monophosphate; (R)⁻-cAMP-S, adenosine 3':5'-cyclic monophosphorothioate; (R)⁻-isomer, 8-bromo-cAMP, adenosine 3':5'-cyclic monophosphate, 8-bromo-sodium salt; PBS, fetal bovine serum; Pipes, 1,4-piperazinediethanesulfonic acid; kb, kilobase.

In animals, melanocytes can produce two types of melanin, black/brown eumelanin and/or red/yellow pheomelanin; skin and hair color is determined by the relative proportions of these two melanin in those tissues (1, 2). The eumelanin/pheomelanin ratio is regulated in mice by at least three genetic loci termed agouti, POMC, and extension. Those loci encode agouti signal protein (ASP), ¹ proopiomelanocortin (a precursor of melanocyte-stimulating hormone (MSH)), and the MSH receptor, called the melanocortin 1 receptor (MC1R), respectively (for review, see Refs. 3 and 4). ASP, which is produced in hair follicles, acts on follicular melanocytes to switch them from producing eumelanin to pheomelanin, whereas MSH has the reverse effect (3, 5). In mice wild-type at the agouti locus, eumelanin is usually produced by follicular melanocytes during hair growth (6). However, ASP is transiently expressed from days 4 to 7 of the hair growth cycle and signals follicular melanocytes to produce pheomelanin rather than eumelanin (5). Eumelanin and pheomelanin differ not only in their gross appearance but also in their chemical composition and in the structure of melanosomes in which they are synthesized and deposited (2, 7, 8).

Several studies have shown that ASP acts as a competitive antagonist of the MC1R and that the switch between eu- and pheomelanogenesis involves the opposing effects of ASP and MSH as ligands for that receptor (4, 9, 10). It has recently been reported that the interactions of MSH and ASP through the MC1R are modulated by attractin, the product of the mahogany locus (11, 12). The MC1R is a G protein-coupled receptor whose intracellular signaling is mediated via the cAMP pathway. Stimulation of MC1R up-regulates the expression of melanogenic genes including tyrosinase, Tyr1 (also known as TRP1 or gp75), and Dct (also known as TRP2) (13–15). The activation of Mitf (see below), a transcription factor that regulates melanogenic gene expression, is known to be a critical event during this signaling (16–18). Although ASP opposes the action of MSH by blocking MSH activation of the MC1R and thus suppresses the cAMP pathway, its mechanism of action is still controversial. Several studies (14, 16, 19) have shown that ASP not only inhibits the effects of MSH but also inhibits cAMP stimulation induced by forskolin. This suggests that ASP could affect the downstream signaling of the MC1R leading to the transcriptional down-regulation of melanogenic genes.

We have recently shown (20–22) that down-regulation of melanogenic genes in cultured melanocytes treated with recombinant ASP mimics the physiological switch to pheomelanogenesis seen during the agouti hair cycle. All genes currently known to be important to eumelanin production (including tyrosinase, Tyr1, Dct, Pmel17, and pink-eyed-dilution) are transcriptionally down-regulated during pheomelanogenesis (20, 23). However, the dramatic differences elicited during pheomelanogenesis, both with respect to the structural reorganization of melanosomes and the chemistry of melanins produced, suggest that there must be genes that are up-regulated during this switch. In a recent study (24), we used high-resolution two-dimensional gel electrophoresis and differential display to begin identifying such up-regulated genes. Several genes were identified with one of the most interesting being a transcription factor termed ITF2 (also known as SEF2).
Itf2 is a member of the ubiquitous Class A basic helix-loop-helix (bHLH) transcription factor family termed E proteins. Members of that family, which include the isoforms Itf2a and Itf2b, bind a consensus DNA sequence CACXXTG also known as an E-box (25). E-box-binding proteins share a common bHLH motif that mediates their dimerization and a basic region located upstream of the HLH domain serves for DNA binding (26). Itf2 has the capacity to regulate the expression of various tissue-specific genes that results from its ability to form homo- or heterodimers with other bHLH proteins. In melanocytes, a melanocyte-specific Class C bHLH-zip (or bHLH leucine zipper) transcription factor, termed microphthalmia (Mitf in mice and Mitf in humans), is critically important for melanoblast development and also for regulation of melanogenic gene expression in mature melanocytes (16, 27–29). Mitf (30) is down-regulated during pheomelanogenesis induced by ASP and is up-regulated following treatment with MSH (17, 30–32).

Based on those findings and our own results, our hypothesis is that Itf2 might form homodimers or heterodimers with other bHLH proteins, and because Itf2 is up-regulated during pheomelanogenesis, this might result in the transcriptional down-regulation of melanogenic genes. Because Mitf is the principal regulatory transcription factor that regulates the expression of melanogenic genes, opposing effects of Itf2 and Mitf might be important in modulating melanocyte differentiation. The functional significance of ubiquitously expressed bHLH proteins has recently become well recognized in terms of the regulation of tissue-specific gene expression in various types of cells such as myocytes, pancreatic cells, and B-lymphocytes (26, 33). Furthermore, Itf2 has been shown to inhibit the activities of various tissue-specific gene promoters (34–37).

In this study, we present evidence that Itf2 can modulate melanogenic gene expression in concert with its effects on Mitf expression and function. Our findings suggest that these ubiquitous bHLH proteins function in melanocytes as regulators of melanogenesis that control melanocyte differentiation and modulate the expression of melanogenic enzymes in response to extrinsic physiological factors.

**MATERIALS AND METHODS**

**Reagents**— Forskolin, 3-isobutyl-1-methylxanthine, db-cAMP, cholera toxin, [Nle4,D-Phe7]-MSH, phorbol 12-myristate 13-acetate, Nonidet P-40, sodium deoxycholate, PMSF, aprotinin, and leupeptin were from Sigma Chemical Co. (R), -cAMP-S triethylammonium salt and 8-hydroxy-cAMP were from Calbiochem-Novabiochem Corp. RPMI 1640 medium and trypsin-EDTA were from Life Technologies, Inc. SuperFect transfection reagent was from Qiagen, Inc. (Valencia, CA). FBS was from Hyclone Laboratories (Logan, UT). The C5 monoclonal antibody generated against a histidine fusion protein expressed from the NH2-terminus of human MITF cDNA was from LabVision Corp. (Fremont, CA) and recognizes mouse Mitf and human MITF (these proteins are 94% identical). The ePEP7, ePEP1, ePEP8, and ePEP16 polyclonal antibodies were raised against the carboxyl termini of murine tyrosinase, TyrP1, Dct, and ASP proteins, respectively (38–41). Peroxidase-conjugated anti-mouse and -rabbit immunoglobulin were from Sigma (St. Louis, MO). Anti-MITF antisera was kindly provided by Dr. Dorothy C. Bennett (St. George’s Hospital Medical School, London, United Kingdom). Melanocytes were cultured at 37 °C with 10% CO2 in RPMI 1640 medium containing penicillin, streptomycin, sodium pyruvate, nonessential amino acids, 1% FBS, 70 µM phorbol 12-myristate 13-acetate, and 100 µM 2-mercaptoethanol at pH 7.2 until reaching semiconfluence as reported previously (21). Typically 2 × 106 cells were seeded in 150-mm-diameter dishes and allowed to attach for 24 h; treatment with 100 nM MSH or 10 nM ASP was then initiated for 4 days. Controls were cultured in the absence of MSH and ASP but with similar volumes of ASP storage buffer (20 µmol NaCl).

**Preparation of Skin Samples and Cultured Melanocytes**—Anesthetized mice were sacrificed by cervical dislocation, and their dorsal skins were dissected. Immediately after removing fat and subcutaneous tissue, hair follicles were scraped off with a razor blade and were snap-frozen in liquid nitrogen. Frozen samples were pulverized and homogenized in 5 volumes (v/v) of lysis buffer (50 mM sodium borate, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1 mg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin) in a Microson Ultrasonic Cell Disruptor XL (Misonix Inc., Farmingdale, NY) using three 30-s pulses.

Melan-a melanocytes were treated with 100 nM MSH or 10 nM ASP for a total of 4 days as described above. For immunoblot analyses of nuclear proteins, nuclear extracts were prepared as described previously (35). Whole cell extracts were prepared for Western immunoblot analyses by sonicating harvested cells in Nonidet P-40/SDS cell lysis buffer (1% Nonidet P-40, 0.01% SDS, 0.1 mM Tris-HCl, pH 7.2, 1 µg/ml aprotinin, and 100 µM PMSF), as described previously (21).

**Western Blot Assays**—Equal amounts of protein (10 µg/lane) were separated on 8% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis. Following blotting onto Immobilon-P membranes (Millipore Corp., Bedford, MA) for 1.5 h at 25 V, membranes were blocked in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Antibodies to detect tyrosinase, TyrP1, or Dct were used at a 1:1500 dilution (normal rabbit serum was used as a control), whereas antibodies to Mitf were used at a 1:1000 dilution. Membranes used to detect Itf2 were treated overnight with phosphate-buffered saline containing 3% nonfat milk and 0.25% Nonidet P-40 as previously described (35), and this blocking solution was also used for antibody incubation and washing steps instead of TBS-T. Antibodies to Itf2 were used at a 1:1000 dilution. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit or -mouse immunoglobulins at 1:20000 dilution. Immunoreactive bands were detected by enhanced chemiluminescence using the ECL kit from Amersham-Pharmacia Biotech.

**RNA Protection Assays**—To produce the desired Itf2 antisense RNA for the RNase protection assay, we obtained a 0.8-kb fragment of ITF2A cDNA from a melan-a melanocyte cDNA library (34). That clone contains 798 base pairs of the ITF2A cDNA 5'-end that overlaps 1073–1711 of ITF2B cDNA. Total RNAs from skin tissues and melan-a melanocytes were extracted with a TRIZOL Total RNA Isolation Reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. For RNase protection analyses, template plasmid DNA was linearized with the appropriate restriction endonuclease, and antisense RNA was synthesized in vitro using Riboprobe System-T7 (Promega Corp., Madison, WI) in the presence of [α-32P]CTP according to the manufacturer’s protocol. One µg of total RNA and 107 cpm of riboprobe were co-precipitated and allowed to anneal prior to RNase digestion using a HybSpeed RPA kit (Ambion, Inc., Austin, TX) according to the manufacturer’s protocol. Protected RNA fragments were separated on 6% polyacrylamide, 40% urea gels and visualized with a Storm Phosphorimage (Molecular Dynamics, Sunnyvale, CA).

**Northern Blot Assays**—Total RNA was extracted from cells using an RNeasy total RNA isolation kit (Qiagen, Inc.) following the manufacturer’s instructions. Total RNA (5–10 µg) was denatured, electrophoresed through 1.25% agarose gels, and then blotted to Nytran membranes (Schleicher & Schuell, Keene, NH) using a TurboBlotter System (Schleicher & Schuell) and UV light cross-linking (UV Stratalinker 1800, Stratagene, La Jolla, CA). Radioactive probes were generated with a PrimeIt Random Prime Labeling kit (Stratagene) with 32P-dCTP. Hybridizations were performed in 10× Denhardt’s solution with 0.1% SDS and 1× formamide at 50 °C for 30 min. The filters were then washed in 1% SSF, 3% sodium dodecyl sulfate, 0.1 mM 2-mercaptoethanol at pH 7.2 until reaching semiconfluence as reported previously (21). Typically 2 × 106 cells were seeded in 150-mm-diameter dishes and allowed to attach for 24 h; treatment with 100 nM MSH or 10 nM ASP was then initiated for 4 days. Controls were cultured in the absence of MSH and ASP but with similar volumes of ASP storage buffer (20 µmol NaCl).

**Coexpression of Expression and Reporter Plasmids**—Expression vectors PGK-ITF2-2A and PGK-ITF2-2B mouse cDNAs containing the NotI-SalI and SalI-AscI fragments of the full-length cDNAs including the coding regions of ITF2A and ITF2B, respectively, were kindly provided.
Transient Transfection and Luciferase Reporter Assays—Melan-a melanocytes were plated 2 days before transfection, and COS1 cells were plated 16 h before transfection in six-well plates. Using SuperFect reagent, melanocytes (80% confluent) or COS1 cells (60% confluent) were co-transfected with 2.5 μg of DNA consisting of 2 μg reporter plasmid, 0.3 μg of expression plasmid, and 0.05 μg of Renilla luciferase expression vector pRL CMV (Promega Corp.). The total amount of DNA was adjusted to 2.5 μg with sheared salmon sperm DNA (Life Technologies). Plasmid DNA was prepared using the Qiagen plasmid Maxi kit (Qiagen, Inc.). Transfected cells were incubated for 48 h, then washed with phosphate-buffered saline, pH 7.4, lysed, and assayed for luciferase activity using the Dual-luciferase Reporter Assay System (Promega Corp.) and a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Luciferase activities were normalized for Renilla luciferase activity using the Dual-luciferase Reporter Assay system as described in the manufacturer’s protocol.

Stable Transfection—Melan-a cells (5 × 10^6 cells/dish) were seeded into 150-mm culture dishes in RPMI 1640 medium, pH 7.4, supplemented with 5% FBS and 10 mM sodium pyruvate. Ten days later, surviving blasticidin-resistant clones were picked by cloning loop and incubated for 48 h before transfection. Cells were then incubated for 48 h in RPMI 1640 medium containing 5% FBS, then trypsinized, and seeded at a 1/5 dilution in 150-mm culture dishes in RPMI 1640 medium with 5% FBS. The cells were switched the next day to a selective RPMI 1640 medium containing 5 μg/ml blasticidin S (Invitrogen). After 7 days of culture in the selective medium, 12 representative blasticidin S-resistant clones from each of the sense or antisense transfectants were expanded in 12-well dishes. Ten days later, surviving blasticidin-resistant clones were trypsinized, mixed together, and used for additional experiments as stable ITF2 sense or ITF2 antisense transfectants.

Light and Electron Microscopy—For light microscopy, cells growing in six-well plates (treated as noted in the legend to Fig. 6) were photographed using an inverted Olympus microscope under phase contrast and bright field microscopy. For electron microscopy, cells were harvested, centrifuged for 5 min at 14,000 × g at 4 °C, and fixed for 2 h at 23 °C in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3; the fixative was then removed and the samples were washed with Tris-buffered saline containing 0.5% sucrose at 4 °C. Samples were subsequently processed with graded alcohols and embedded in epoxy resin for electron microscopy in the usual manner. Thin sections were stained with uranyl acetate and lead citrate, viewed, and photographed with a LEO EM912 Omega electron microscope as detailed previously (21).

Immunoprecipitation Assays—For immunoprecipitation assays, 10^7 melan-a melanocytes were harvested and resuspended in 1 ml of cell lysis buffer containing 50 mM sodium borate, pH 8.0, 150 mM NaCl, 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1% Nonidet P-40, and 0.5% sodium deoxycholate. For immunoprecipitation of hair follicle proteins, 200 μg of protein from hair samples were diluted to 1 ml with immunoprecipitation buffer (IPB) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1 mg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1% Nonidet P-40, and 0.5% sodium deoxycholate. These extractions were subsequently sonicated at 4°C in a Microson Ultrasonic Cell Disruptor XL for three 30-s pulses at setting 8 followed by incubation for 30 min at 4 °C. Supernatants were recovered by centrifugation at 14,000 × g for 10 min at 4 °C. To biotinylate proteins in cell extracts, 0.25 mg/ml biotin-16-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS, Roche Molecular Biochemicals) was added and incubated for 15 min at 4 °C. The labeling reaction was stopped by the addition of 50 μl of 1 M NH4Cl. The biotin-labeled cell lysates were mixed with 50 μl of a 50% slurry of protein G-Sepharose beads (Gam- maBind G Sepharose, Amersham Pharmacia Biotech) and incubated for 1 h at 4 °C with agitation. After centrifugation to sediment the beads, supernatants were recovered, and 10 μl of antibody were added and incubated for 1 h at 4 °C. Fifty μl of a 50% slurry of protein G-Sepharose beads in dilution buffer were added, and the incubations were continued overnight at 4 °C with agitation. Beads were then washed three times with 1 ml of IPB, resuspended in 30 μl of SDS-polyacrylamide gel electrophoresis buffer, boiled for 10 min, and separated on 4–20% gradient SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes, and biotinylated proteins were detected with a streptavidin biotin labeling kit (Roche Molecular Biochemicals). The specificities of bands identified were checked using nonbiotinylated protein samples for immunoprecipitation, and transferred proteins were detected by specific primary antibodies and horse-radish peroxidase-labeled secondary antibodies using enhanced chemiluminescence as noted above.

RESULTS

Expression of Melanogenic Proteins and Transcription Factors in Response to Extrinsic Stimuli—In our previous study (24), Northern blot analysis showed that ITF2 mRNA levels are increased during pheomelanogenesis such as occurs in ASP-treated melanocytes and in mouse hair follicles overexpressing ASP due to the lethal yellow mutation (Ay/a). In this study, we...
thus further examined the expression patterns of ITF2 in ASP- or MSH-treated melanocytes and in agouti hair follicles (Fig. 1). Analysis of mRNA levels (Fig. 1, panel A) and protein levels (Fig. 1, panel B) confirmed the decreases in tyrosinase and Tyrp1 mRNAs and proteins that are elicited by ASP treatment and the increases that follow treatment with MSH as previously reported (20, 21, 24). Levels of Mitf mRNA and protein were similarly regulated by MSH and ASP as recently reported by other groups (16, 17). In contrast, ITF2 expression at the mRNA and protein levels showed contrasting patterns and was strikingly up-regulated in ASP-treated cells and down-regulated following treatment with MSH. Four days is the standard treatment time used to elicit maximal hormonal effects on melanocytes, but further analysis (as shown below) confirms that such changes occur relatively quickly (within hours) particularly with respect to Mitf and ITF2 expression.

To examine whether ITF2 levels are modulated in vivo in response to transient ASP expression, hair follicles from 0- to 11-day-old newborn agouti and non-agouti black mice were examined by Western blot and by immunoprecipitation analyses (Fig. 2). Hair follicles obtained at 4–7 days after birth (i.e. during the pheomelanogenic phase) had dramatically higher levels of ITF2 protein that coincided with increased ASP expression. In contrast, levels of Mitf and of the melanogenic enzymes tyrosinase and Dct remained stable or were even down-regulated during the pheomelanogenic phase of hair growth. At day 9, immediately following the decreases in ASP and ITF2 expression, levels of Mitf, tyrosinase, and Dct increased dramatically. As a control, proteins extracted from hair follicles of sibling non-agouti black mice were similarly examined, but no apparent modulation of ITF2 expression was seen during any phase of the hair growth cycle nor was ASP detectable (not shown).

Expression of ITF2 Splice Variants in Melanocytes in Vitro and in Vivo—ITF2 transcripts can be alternatively spliced in their 5'-flanking regions to generate isoforms known as ITF2A and ITF2B (34). An RNase protection assay was used to analyze the relative abundance of ITF2 splice variants produced in melanocytes particularly during pheomelanogenesis (Fig. 3). The riboprobe used for RNase protection contains the region

![Figure 3: Expression of ITF2 isoforms by melanocytes in vitro and in vivo.](http://www.jbc.org/)

![Figure 4: Effects of transient ITF2 transfection on melanogenic gene expression.](http://www.jbc.org/)

![Figure 5: Effects of stable transfection of ITF2 on melanogenic gene expression.](http://www.jbc.org/)
that differs between ITF2A and ITF2B (and also their common region), so that after electrophoresis and autoradiography two protected fragments that represent ITF2A and ITF2B could be seen. In cultured melanocytes and in mouse skin, ITF2A and ITF2B could be detected as protected fragments with the expected lengths, and their expression was up-regulated during pheomelanogenesis, i.e. upon ASP treatment of melanocytes in culture or in yellow skin compared with black skin. ITF2B was by far the more abundant splicing isoform.

**Suppression of Melanogenic Gene Expression by ITF2**—The ability of ITF2A and/or ITF2B to affect transcription of melanogenic genes was then assessed using transient transfection of melanocytes with luciferase reporter constructs attached to promoter sequences of tyrosinase, Tyrp1, or Dct (Fig. 4). Basal levels of tyrosinase, Tyrp1, and Dct expression were significant because of endogenous expression of Mitf and perhaps other transcription factors even in mock-transfected melanocytes, i.e. upon ASP treatment of melanocytes in culture or in yellow skin compared with black skin. ITF2B was by far the more abundant splicing isoform.

**Effects of Stable Transfection of ITF2 Sense or Antisense on Melanogenic Gene Expression**—To further elucidate the regulatory function of ITF2, melan-a melanocytes were transfected with a stable expression vector, pcDNA3.1-ITF, that contains the full-length cDNA of ITF2B. ITF2B was used for these experiments because it was the dominantly expressed variant and was more potent in down-regulating melanogenic gene expression in melanocytes than was ITF2A as shown above. Stable ITF2 (sense and antisense) transfectants were selected using blasticidin S, and representative clones were isolated; cells transfected with the pcDNA3.1 vector alone (mock transfection) were used as controls (Fig. 5). Stable transfectants were analyzed for expression of Mitf mRNA (Fig. 5, panel A) and protein (Fig. 5, panel B) and for the melanogenic proteins tyrosinase, Tyrp1, and Dct. Overexpression of ITF2 sense suppressed levels of all three melanogenic enzymes as well as of Mitf mRNA and protein. ITF2 sense transfectants showed a less pigmented phenotype that was also characterized by a less dendritic and polygonal cell shape and a faster growth rate compared with mock transfectants (Fig. 6). Ultrastructural analysis revealed that melanosomes visible in mock-transfected controls were virtually completely absent from the ITF2 sense-transfected cells. Thus, these ITF2 sense transfectants were phenotypically identical to ASP-treated melanocytes.

Because the endogenous expression levels of ITF2 in melan-a melanocytes are significant during pheomelanin and eumelanin synthesis as shown above, an antisense strategy was used to examine the effects of blocking ITF2 function on melanogenic
gene expression. ITF2 antisense transfectedants (ITF2-RV) were more pigmented and grew more slowly compared with mock-transfected controls (Fig. 6). Ultrastructural analysis showed that the number of densely pigmented melanosomes was greater in ITF2 antisense transfectedants than in controls. ITF2 antisense transfectedants showed significant decreases in expression of endogenous ITF2 (Fig. 5), and all three melanogenic enzymes (tyrosinase, Tyrp1, and Dct) were dramatically up-regulated at the mRNA (not shown) and protein levels (Fig. 5).

Northern and Western blot analyses demonstrate clearly that Mitf is also significantly up-regulated in ITF2 antisense transfectedants. Thus, ITF2 antisense transfectedants are phenotypically similar to melanocytes treated with MSH. Furthermore, this pattern of regulation suggests that ITF2 might modulate melanocyte differentiation and pigmentation by directly regulating Mitf expression and/or function.

That possibility was assessed with an Mitf promoter-luciferase construct co-transfected into melanocytes with ITF2 (sense or antisense). The results (Fig. 7, panel A) show clearly that ITF2 sense down-regulates Mitf expression in melanocytes compared with mock-transfected controls, whereas ITF2 antisense up-regulates Mitf mRNA levels. Treatment of melanocytes with MSH stimulates expression of Mitf mRNA as recently reported (16), but significantly decreases Mitf expression also as recently determined (16, 49). A cAMP-responsive element in the promoter region of Mitf (TG motif) and, to a greater extent, its binding to XXE-box (a CAAT box motif) has been implicated in various forms of Waardenburg syndrome (15, 50–53).

DISCUSSION

The importance of the cAMP pathway during the induction of melanogenesis is well established, and regulation of Mitf transcription seems to be a critical control point (28, 29, 31, 32, 46, 49). A cAMP-responsive element in the promoter region of Mitf appears to account for most of the cis-acting regulation induced by cAMP-stimulating agents (see Fig. 9). PAX3 and SOX10 transcription factors also regulate Mitf expression, and mutations in PAX3, SOX10, or Mitf have been implicated in various forms of Waardenburg syndrome (15, 50–53).

Other more recent studies on the transcriptional regulation of melanosinogenic genes have revealed that Mitf plays a pivotal role in mediating MSH and ASP signaling (16, 17). Up-regulation of melanosinogenic gene expression induced by MSH via the MC1R is primarily through activation of the cAMP-dependent protein kinase/cAMP pathway. In turn, increased cAMP levels regulate transcription of melanosinogenic genes by stimulating production of Mitf, which homodimerizes to form the active transcription factor. In addition to the transcriptional up-regulation of Mitf expression through the cAMP-responsive element, cAMP also increases the binding affinity of Mitf to the E-box (a CAAT box motif) present in the promoters of genes encoding melanosinogenic enzymes (46). Our results suggest that ITF2 may be involved in this regulation physiologically because its expression is up-regulated at exactly the same time as ASP is expressed in hair bulbs in situ. The expression of Mitf, tyrosinase, and Dct at those same times is stable or decreased, but immediately following the decrease of ASP and ITF2 expression at day 8, their expression is dramatically increased.
ITF2 may actually function in two distinct manners (1) by inhibiting the transcription of melanogenic gene promoters and perhaps more importantly (2) by inhibiting the transcription of Mitf itself. The suppressive effect of ASP may be mediated, at least in part, via the inhibition of MSH binding to the MC1R even when MSH is present. However, even in the absence of MSH, ASP can directly suppress cAMP signaling below the constitutive level (21, 22). Such suppression could be explained theoretically by the putative function of ASP as an inverse agonist of the MC1R (12, 43).

Whatever mechanism(s) might be involved in the action of ASP on melanocytes, one important down-stream effect is its up-regulation of ITF2, a ubiquitous bHLH transcription factor. We have shown in this study that ITF2 is up-regulated during the switch to pheomelanogenesis and that ITF2 serves as a negative transcriptional regulator of Mitf (by 70%), giving them unique functions depending on the cell type in which they are expressed. In conclusion, our data demonstrate that a cAMP-dependent pathway ultimately regulates the transcriptional regulation of melanogenesis.

The biological effects of ASP can be attributed to (1) antagonizing the binding of MSH to the MC1R and (2) suppression of spontaneous MC1R signaling as an inverse agonist resulting in the abrogation of basal cAMP levels. Modulation of ITF2 expression seems to be another important subcellular pathway that may function as a suppressive modulator of melanogenesis. CRE, CAMP-responsive element; AC, adenylate cyclase; CAGNTG, CAXXTG; Tyr, tyrosinase.

**FIG. 8. Effects of cAMP modulators on ITF2 and Mitf mRNA levels.** Melanocytes were treated for 4 h with reagents as noted and then Northern blotting was used to examine levels of ITF2 or Mitf mRNAs (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control). Concentrations of reagents used were as follows: 10 nM MSH, 10 nM ASP; 10 μM (R)-cAMP-S, 20 μM 8-bromo-cAMP, 10 μM forskolin, 20 μM 3-isobutyl-1-methylxanthine (IBMX), 20 μM db-cAMP, 100 nM cholra toxin, or buffer only (control).

**FIG. 9. Schematic of melanogenic gene regulation.** The biological effects of ASP can be attributed to (1) antagonizing the binding of MSH to the MC1R and (2) suppression of spontaneous MC1R signaling as an inverse agonist resulting in the abrogation of basal cAMP levels. Modulation of ITF2 expression seems to be another important subcellular pathway that may function as a suppressive modulator of melanogenesis. CRE, CAMP-responsive element; AC, adenylate cyclase; CANNTG, CAXXTG; Tyr, tyrosinase.
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cantly more complex than has been previously recognized. The complete functional significance of these bHLH proteins in melanogenesis remains to be elucidated.

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