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‘VIT1’, A Novel Gene Associated with Vitiligo

I. CAROLINE LE POOLE¹, RANGAPRASAD SARANGARAJAN², YANG ZHAO², LAWRENCE S. STENNETT¹, THOMAS L. BROWN³, PRANAV SHETH², TORU MIKI⁴, and RAYMOND E. BOISSY²

¹ Department of Pathology, Loyola University Chicago, Illinois

² Department of Dermatology, University of Cincinnati, Ohio

³ Department of Microbiology and Immunology, Wright State University, Dayton, Ohio

⁴ Molecular Tumor Biology Section, NIH/NCI, Bethesda, Maryland

Abstract

To define genes associated with the pigmentary disorder vitiligo, gene expression was compared in non-lesional melanocytes cultured from three vitiligo patients and from three control melanocyte cultures by differential display. A basic local alignment search tool search did not reveal homology of six differentially expressed cDNA fragments to previously identified expressed sequence tags; thus, one was used to screen a melanocyte cDNA library. The underlying VIT1 gene maps to chromosome 2p16. The 3' portion of the VIT1 message is complementary to the 3' end of hMSH6 mRNA, enabling the formation of RNA–RNA hybrids, which may interfere with G/T mismatch repair function. Moreover, the aligned cDNA sequence revealed an open reading frame identical to a hypothetical protein expressed in brain, with a similarity to *Drosophila* calmodulin, and containing a zinc-finger motif partially identical to *N*-recogin. Expression of ORF mRNA was confirmed for multiple skin cell types, suggesting its importance for skin physiology.

Keywords

depigmentation; gene expression; differential display; calmodulin; hMSH6

Abbreviations

bFGF, basic fibroblast growth factor; BLAST, basic local alignment search tool; BSA, bovine serum albumin; dNTP, 2'-deoxynucleoside 5'-triphosphate; DTT, dithiothreitol; ORF, open reading frame; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; SSPE, saline-sodium phosphate-EDTA; TAE, tris-acetate-EDTA; TPA, phorbol 12-myristate 13-acetate

INTRODUCTION

Vitiligo is a skin disorder presenting with progressive depigmentation of the skin. It affects approximately 0.5% of the world population, without preference for a specific skin tone or gender (1). The disease frequently initiates before the age of 20; however, late-onset vitiligo is not uncommon (1). Depigmented lesions are cosmetically disfiguring, and consequently patients experience social isolation. In countries where leprosy is prevalent, this problem is accentuated by the fact that tuberculoid leprosy can present with similar depigmented skin patches (2). Melanocytes, the pigment-producing cells of the skin, are lost from vitiligo lesions

*Address reprint requests to I. Caroline Le Poole, Ph.D., Loyola University Medical Center, Department of Pathology, Cardinal Bernardin Cancer Center, Room 303, 2160 South 1st Avenue, Maywood, Illinois 60513. E-mail: ilepool@lumc.edu.

(3). As pigment cells cannot be found within lesions either by morphologic criteria or through detection by antibodies commonly recognizing melanocytes, the consensus of opinion now is that the etiology of vitiligo involves a process that explains the loss of the pigment cell rather than inhibition of melanogenesis. Spontaneous repigmentation is rare and total therapeutic repigmentation is infrequent (1).

The disease clusters in families (4). The pattern of inheritance, skipping generations but clearly clustering within families, suggests that vitiligo is a multigenic disorder (5). Moreover, there is a clear association with certain communities in which consanguineal marriages are common (6). Thus far, the genes underlying sensitivity to melanocyte loss remain an enigma. To find such genes, it has been attempted to link the disorder with genes affected in animal models for vitiligo (7,8). In this respect, it is known that in the *vit*-mouse, the *microphthalmia* locus is affected (9). However, it has recently been shown that this does not hold true for the human variant of the disease (10).

In the present article, the search for genes associated with vitiligo pathogenesis has been approached by finding genes that are differentially expressed in melanocytes cultured from generalized vitiligo patients as compared with controls. It has been shown that melanocytes from these vitiligo patients display morphologic and physiologic abnormalities. Specifically, melanocytes from vitiligo patients cultured under limiting conditions expressed a lag time in growth and could not be subcultured (11). Moreover, melanocytes from normally pigmented vitiligo skin displayed melanosome compartmentalization and dilated endoplasmic reticulum profiles in vitro as well as in situ, in humans as well as in animal models of the skin disorder (12–15). The presence of dilated RER profiles was recently confirmed in the stable vitiligo melanocyte cell line PIG3V as well (16). The fact that such abnormalities are intrinsic to the melanocyte prompted us to use cultured melanocytes for analysis of differential gene expression at the RNA level. As melanocytes represent a minor subpopulation of cells within the epidermis; this method is preferred over analysis of gene expression in total skin biopsies or in complete epidermis. A similar approach for analysis of genes affected in melanocytes exposed to phenolic agents, representing the pathogenesis of occupational/contact vitiligo, has led to the association of the A2b receptor for adenosine with this clinical subform of the disease (17). This receptor has also been described as being differentially expressed during activation of T cells (18). The ligand for this receptor, adenosine, is generated in increased quantities during cell stress (19). Adenosine can induce apoptosis in several cell types (20,21).

Differential display can thus be used to identify genes associated with vitiligo etiopathogenesis without prior knowledge of the pathogenic pathway. In the present article, this approach was applied to melanocytes cultured from generalized vitiligo patients. Their mRNA profiles were compared with those observed in control melanocyte cultures.

MATERIALS AND METHODS

Cell Culture

Melanocytes were isolated from non-lesional skin tissue of patients diagnosed with generalized vitiligo as well as from skin of control individuals. This study was approved by the appropriate institutional review boards and where skin tissue was obtained for research purposes, informed consent was obtained from all subjects involved. Melanocytes were isolated from full-thickness skin biopsies or from blister suction material. From full-thickness skin biopsies, melanocytes were isolated by treatment with 0.25% trypsin in phosphate-buffered saline (PBS) at 37°C for 2 hr. If only epidermal tissue was obtained, blister roof-tops were incubated in 0.1% trypsin in PBS for 30 min. Cells were propagated essentially as previously described (13,22). Briefly, the culture medium consisted of MCDB-153 (Sigma, St Louis, MO, USA) containing 4% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA), 0.2% bovine pituitary

extract (Clonetics, San Diego, CA, USA), 32 nM phorbol 12-myristate 13-acetate (TPA), 10^{-8} M α -melanocyte stimulating hormone, 1 μ g/ml transferrin, 1 μ g/ml vitamin E, 5 μ g/ml insulin, 1.8 ng/ml basic fibroblast growth factor (Sigma) and penicillin 100 IU/ml plus streptomycin 100 μ g/ml (Gibco). The elevated concentrations of TPA and bFGF, used for all melanocytes included in the study, were chosen to accommodate slow-growing vitiligo melanocytes. Contaminating fibroblasts were removed by treatment with 0.1 mg/ml geneticin (Gibco) for 3 consecutive days, making use of the relative insensitivity of slow-growing melanocytes to this antibiotic compound (23). Cells were routinely split 1:2 at confluency. Melanocyte cultures used for differential display are further described in Table 1, whereas other cell cultures used are designated by culture codes and are described in the figure legends.

Keratinocytes were isolated from normal skin as described above for melanocytes, and were plated in serum-free medium M154 (Cascade Biologicals, Portland, OR, USA) with keratinocyte growth supplements (Cascade) and penicillin 100 IU/ml plus streptomycin 100 μ g/ml.

Fibroblasts were propagated from normal skin by placing the dermal component left after removal of the epidermis by trypsin treatment of skin biopsies in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum and penicillin 100 IU/ml plus streptomycin 100 μ g/ml.

RNA Isolation

At 80% confluency, RNA was isolated from cells propagated in 75-cm² flasks. To this end, adherent cells were washed in Hanks' balanced salt solution (HBSS) and scraped in Tri-Reagent (MRC Inc., Cincinnati, OH, USA). RNA was isolated essentially according to Chomczynski (24). RNA was subsequently treated with DNase I according to specifications accompanying a MessageClean Kit (GenHunter Corp., Nashville, TN, USA) to avoid amplification of contaminating DNA in subsequent polymerase chain reactions (PCRs).

Differential Display

A differential display kit (GenHunter Corp.) was used for reverse transcription of mRNA and for subsequent PCR amplification of the resulting cDNA (25). Based on one-anchor priming, cDNA for each of the six RNA samples used was generated in reverse transcription reactions using either HindIII-linked T₁₁A, T₁₁C, or T₁₁G as poly-A complementary primers (26). PCR amplification was performed using the same one-anchor primer in combination with each of 16 HindIII-linked arbitrary 7-mers according to the manufacturer's specifications. Amplification involved 40 rounds of denaturation at 94°C for 15 s, annealing at 40°C for 2 min, and polymerization at 72°C for 5 min, followed by a single round of elongation at 72°C for 5 min. The 48 resulting PCRs generated approximately 150 separate reaction products each, specific for the primer combinations involved.

PCR amplification was performed in presence of ³³P-dATP (NEN, Boston, MA, USA) and amplification products were displayed on a 6% polyacrylamide buffer gradient gel. The dried gel was exposed to Kodak X-Omat XAR-5 film for 16 hr. Only bands representing mRNA fragments consistently differentially expressed in all vitiligo versus all control samples in two separate experiments were considered of interest for further analysis. In such cases, the RNA was subjected to another round of RT-PCR using the same primer combination to verify differential expression.

Fragment Cloning

Consistently differentially expressed cDNA fragments were cut from dried acrylamide gels, reamplified using the original primer combination, checked for correct size on a low-melt

agarose gel, isolated from the gel and cloned into a T-vector derived from pBluescript SK + (27). Plasmids were transformed into XL1-Blue and positive clones were recognized as white colonies on X-gal/IPTG-coated agar plates. Plasmids were reisolated from bacteria grown in L-broth with tetracyclin to select for XL-1 Blue, and with ampicillin to select for presence of the plasmid. The amplified plasmid was reisolated and the fragment of interest was restricted by HindIII-digestion. Vector and fragment were separated by agarose gel electrophoresis, and the fragment was isolated using a Qiaex II gel extraction kit (Quiagen, Valencia, CA, USA).

Reverse Dot Blotting

As emphasized in our recent description of the differential display technique, a substantial number of fragments isolated from differential display gels are never confirmed as differentially expressed (28). Reverse dot blotting offers a more sensitive method than Northern blotting to confirm differential expression of a cloned fragment, and is used to separate accidentally differentially amplified fragments from truly differentially expressed transcripts and to distinguish differentially expressed fragments from transcripts of equal length (17). Briefly, the fragment of interest was heat-denatured and applied to a nitrocellulose membrane in four spots of 10 μ l in concentrations of 5 ng, 500 pg, 50 pg, and 5 pg per spot. Different amounts of the fragment are spotted because the most suitable amount to visualize differences in expression cannot easily be predicted from the differential display results. Simultaneously, the PCR was repeated using reverse transcribed mRNA from two samples, representing vitiligo melanocytes in one sample and control melanocytes in the other. 32 P-dATP was used to label the fragments generated in this PCR. Labeled samples were cleared of unincorporated nucleotides by separation on a BioSpin30 chromatography column (BioRad). The Southern dot blots were exposed to pre-hybridization buffers for 3 hr prior to hybridization. The samples were subsequently heat-denatured and added in fresh hybridization buffer. After incubation for 16 hr at 42°C, the blots were washed in 2 $\times$ saline-sodium phosphate-EDTA (SSPE) with 0.1% sodium dodecyl sulfate (SDS) at room temperature and at 42°C for 30 min each. Washed blots were exposed to Kodak-XR film for 76 hr. A decreased amount of the fragment of interest present within the mix of PCR products was detected as less hybridization to dots generated with the same concentration of plasmid DNA. An aliquot of the labeled sample was also loaded on a 6% SDS-polyacrylamide gel to verify successful PCR amplification.

Sequence Analysis and Identification

Cloned cDNA fragments of interest were sequenced at the DNA core facility of the University of Cincinnati by automated sequencing using fluorescent dye terminator. Priming was to M13 forward and reverse primer sites flanking the cloned fragment of interest in pBlueScript. For longer fragments, further sequencing was performed by primer walking, generating new primers based on the sequencing products. The sequences generated were compared by BLAST search of databases containing GenBank sequences of previously identified genes and expressed sequence tags (EST).

cDNA Library Screening

Library screening was performed to find a longer cDNA sequence for a fragment not identified by the BLAST search, further confirmed by reverse dot blotting for differential expression and down-regulated in vitiligo melanocytes. The cDNA library used was generated from normal human melanocyte-derived RNA and was first described when used for the identification and cloning of human MITF (29). In the first round of screening, LE392 bacteria and 10^7 pfu were plated in NZY top agar in the presence of 10 mM MgSO₄. Plaque DNA was blotted onto nylon filters and cross-linked. The probe was radioactively labeled by a random hexamer labeling kit (Gibco), followed by removal of unincorporated nucleotides with a BioSpin 30 chromatography column. Following pre-hybridization, filters were exposed to the heat-

denatured probe in hybridization solution for 48 hr. Filters were washed and exposed to Kodak X-Omat AR film. Plaques containing hybridizing DNA were used for a second and third round of screening. Phage DNA of interest was subsequently isolated in bulk from a liquid culture. Ten micrograms of the resulting DNA solution was restricted with XhoI and the resulting DNA fragments were religated to generate plasmid pCEV27 containing the cloned cDNA sequence of interest. The ligation product was used to transfect XL1Blue bacteria. The plasmid was reisolated from a liquid culture and 2 µg was restricted with SalI to release the fragment of interest. Sequencing and identification of the inserts was performed as described above, initially using SP6 and T7 primer sites flanking the cloned cDNA sequences. Sequencing was completed by primer walking. The cloned DNA was compared with GenBank and EST databases for further identification.

RT-PCR

Expression of an open reading frame (ORF) as defined by detection of a 287-bp fragment following RT-PCR amplification in presence of primers 5'-TCACGCAACTGCAACACT-3' and 5'-TGAGGGGGAAAGTAATTGGG-3' was considered indicative of its expression in cells of interest. RT was carried out at 42°C for 50 min with 1–5 µg of RNA in presence of 3.5 mM MgCl₂, 3 mM nucleotides, 200 U SuperScript reverse transcriptase (Gibco), 2.5 µM oligo-dT primer (Perkin Elmer) and 1 × PCR buffer (Perkin Elmer). PCR was performed in presence of 50 ng/ml forward and reverse primers, 20 µM nucleotides, 2 U Ampli-Taq polymerase (Perkin Elmer) and 1 × PCR buffer. PCR amplification was carried out as 40 cycles at 94°C for 30 s, at 40°C for 2 min', and at 72°C for 30 s, followed by 5 min at 72°C. The resulting PCR products were size fractionated on a 1% agarose gel in tris-acetate-EDTA (TAE) for 45 min at 80 V, together with a 100 nucleotide ladder (Gibco).

For semi-quantitative RT-PCR, RNA was reverse transcribed to investigate relative expression of VIT1 versus hMSH-6. Samples were obtained from cultured cells as well as from frozen sections of control and progressive marginal vitiligo skin. To this end, 5 g of RNA was reverse transcribed at 42°C for 50 min in presence of 3.5 mM MgCl₂, 40 U (1 µl) Rnase inhibitor (Rnase out; Gibco Life Technologies), 1 mM nucleotides, 200 U SuperScript reverse transcriptase (Gibco), 0.5 µg oligo-dT primer (Gibco), 1 × RT buffer (Gibco) and 10 mM DTT. RNA samples included in this analysis were from non-lesional vitiligo melanocytes, control melanocytes, keratinocytes, and fibroblasts. Primers used for PCR amplification were VIT1-ORF primers 5'-TCACGCAACTGCAACACT-3' and 5'-CCCAATTACTTTCCCCCTCA-3', and conversely hMSH-6 primers 5'-CCCTCAGCCACCAAAGAAGCA-3' AND 5'-CTGCCACCACTTCCTCATCCC-3' (30). Primers were chosen to exclude regions of homology among resulting PCR products. Reverse transcription was performed at 42°C for 1 hr. The enzyme was subsequently inactivated for 10 min at 94°C. PCR amplification of 25% of the RT reaction was carried out in presence of 1 × PCR buffer, 1 µg/µl BSA, 0.4 mM dNTPs, 2.5 mM MgCl₂, and 7.5 U of Taq polymerase. Amplification was carried out as 35 cycles of 15 s at 95°C, 2 min at 59°C, and 30 s at 72°C, ending with a 10-min elongation step at 72°C. Products were electrophoresed in a 1% agarose gel in TAE buffer in the presence of ethidium bromide.

RESULTS

Differential display

A total of 22 differentially expressed candidate fragments were detected in the first round of screening using 48 primer combinations. The numbering of cell cultures described in Table 1 corresponds to lanes represented from left to right in Fig. 1. Of these 22 fragments, 6 were eventually confirmed in a second round of RT-PCR, successfully reamplified, cloned, and sequenced and termed fragments 9, 12, 18, 21, 28, and 33, as shown in Table 2. Using NIH

Image 1.62 software, the intensity of individual bands was confirmed to be increased (or, in the case of fragment 12, reduced) in every control sample compared with every vitiligo sample. Ratios of intensity in vitiligo versus control lanes are included in Table 2; for the control band, the ratio was measured as 1.00. None of these fragments revealed their identity by a BLAST search, yet a chromosomal location was identified for fragments 9 (2p16), 21 (19q13.2), and 33 (chromosome 22). Two carried significant stretches of repetitive DNA (fragments 18 and 28), making interpretation of homology searches difficult. Following further confirmation of differential expression by reverse dot blotting, fragment 9 was chosen for further analysis. Differential expression of the cloned fragments is depicted in Fig. 1.

Reverse Dot Blotting

In Fig. 2, results of reverse dot blotting are shown for fragment 9. Down-regulated expression of fragment 9 in vitiligo melanocytes was confirmed; the intensity of the spots resulting from hybridization to 500 pg of the spotted fragment show a ratio of 2.26 for control versus vitiligo melanocyte RNA as measured by NIH Image 1.62 software. Based on these results, fragment 9 was chosen to screen a cDNA library.

Library Screening

Probing a melanocyte cDNA library with radiolabeled fragment 9, three individual clones were isolated and melanocyte cDNA contained within these clones was sequenced by primer walking. Sequence analysis revealed that clones 1 and 2 contained the complete differentially expressed fragment, whereas hybridization of plaque number 3 was based on partial homology. Clones 1 and 2 contained identical cDNA sequences except for two introns retained in clone 2, which is most likely derived from a nuclear and thus incompletely spliced mRNA sequence. The introns of 330 and 726 nucleotides, respectively, are flanked by consensus GT and AG sequences on the 5' and 3' ends, supporting their identity as introns. Alternatively, the two clones represent splice variants. After eliminating the putative introns from clone 2, the cDNA sequence contained within clones 1, 2, and 3 spanned 4.094 kb, as shown in Fig. 3. The sequence encoded by clones 1 and 2 has been submitted to Genbank (Accession no. AF264714). Interestingly, clone 3 contained cDNA encoded in the opposite direction from clones 1 and 2 and from originally differentially expressed fragment 9. In this respect it is important to note that the coding strand for the differentially expressed message is not arbitrary and is determined by the one-anchor primer complementary to mRNA, which is used in the RT reaction. The 3'-terminus of the aligned sequence submitted to Genbank was complementary to the hMSH6 gene, which is located on chromosome 2p16. This signifies that the sequence of interest may be located at the same site in the genome. The hMSH6 gene encodes a G/T mismatch repair enzyme. The stretch of homology to this gene is depicted in Fig. 4.

Interestingly, clones 1 and 2 contained an ORF potentially encoding an approximately 14-kDa protein. Alternatively, if clone 2 represents a splice variant rather than an incompletely spliced message, the resulting transcript encodes a protein truncated at 34 amino acids. The ORF consisted of 141 triplets starting from methionine. The ORF and structural features of the encoded protein are indicated in Fig. 5, and a putative zinc-finger motif 45% identical to the one in *N*-recognin is underlined. The encoded protein appears to be identical to a hypothetical protein described following analysis of brain mRNA, except for 94 *N*-terminal amino acids not included in the VIT1 encoded protein and a stretch of 26 amino acids not reported for the hypothetical brain protein. Both sequences have been aligned in Fig. 6.

Vitiligo Gene Expression in Other Skin Cells

A fragment of the correct size generated from DNase free RNA of keratinocytes, fibroblasts, and melanocytes by RT-PCR is shown in Fig. 7, indicating that the gene of interest is ubiquitously expressed.

Semi-quantitative RT-PCR

Fig. 8 shows that vitiligo melanocytes expressed diminished levels of VIT1 mRNA as well as elevated levels of hMSH-6 as compared with control samples. These preliminary results further confirm that VIT1 is indeed down-regulated in vitiligo melanocytes, and suggest that hMSH-6 is simultaneously induced in vitiligo.

DISCUSSION

Genetic factors have long been suspected as contributors to vitiligo etiopathogenesis. It was noted that the disease clusters in families, and that certain communities with high rates of consanguinal marriages display an increased incidence of the disease. Hereditary factors have been overlooked as generations tend to be skipped, supporting the notion that multiple genes act in concert to precipitate the disease. The search for genes underlying depigmentation has been further complicated by the fact that depigmentation can in fact result from separate etiological pathways, perhaps giving rise to different clinical entities known as occupational/contact vitiligo, inflammatory vitiligo, generalized vitiligo, and segmental vitiligo (16). In fact, differential penetration of the disease can potentially be attributed to the involvement of environmental factors including exposure to UV, emotionally destabilizing conditions, mechanical injury, and contact with phenolic compounds (summarized as 'stress') in progressive depigmentation. In this respect, it is of interest that on comparison of messages associated with generalized vitiligo as described here, and messages associated with 4-tertiary-butyl-phenol (4-TBP) treatment of melanocytes to represent conditions encountered in occupational vitiligo as described earlier (17), one message was down-modulated in either condition [28, marked XXVIII in the earlier publication]. The other fragments described as differentially expressed were unique to the condition under study.

In the present article, the search for vitiligo-associated genes has been approached by identification of messages differentially expressed in generalized vitiligo versus control melanocytes. This is justified by the fact that melanocytes from vitiligo patients were previously shown to exhibit characteristic abnormalities, which were retained in culture over multiple passages. Such abnormalities should be reflected in their gene expression pattern, which can be analyzed by differential display (13).

Analysis was performed primarily on RNA isolated from three individual vitiligo melanocyte cultures versus three individual control cultures and was limited to this number by practical constraints only (namely the number of available melanocyte cultures from patients with generalized vitiligo). This group size may not fully represent the comparison among diseased versus normal melanocytes, for which the availability of a large number of cultures would be ideal; in particular in the stage where differential expression is verified. It should be noted that vitiligo was the main discriminator separating both groups, avoiding the selection of amplified gene fragments associated with age, gender, site of biopsy, passage number, or racial background. Genes associated with a condition as revealed by differential display are not necessarily causally related to that condition. Nevertheless, with the limited materials available, differential display has offered a first glance at messages associated with the disease condition in generalized vitiligo.

Among the six fragments representing differentially expressed messages, a fragment for which differential expression was further confirmed by reverse dot blotting and which could not be identified by a BLAST search was used to screen a melanocyte cDNA library to identify a larger segment of the underlying gene. The ORF contained within cDNA of the hybridizing plaques revealed a message that was expressed in melanocytes, keratinocytes, and fibroblasts, as shown by RT-PCR. As all cell types assessed express this mRNA sequence, it is of potential importance to skin cell physiology. However, vitiligo is a disease that selectively eliminates melanocytes from the epidermis, and it is perceivable that differential expression, as noted here, primarily targets melanocytes. Apparently, the gene is expressed at very low levels because the message was undetectable by Northern blotting (results not shown). This confirms a recurring difficulty in confirming differential expression by Northern blotting, as frequently a message of interest may be expressed at low levels (28).

The 3'-end of the VIT cDNA sequence was complementary to the 3'-end of hMSH6, a G/T mismatch repair gene [GenBank Accession no. U73737; (31)]. Preliminary results indicate that decreased levels of VIT1 are associated with increased levels of hMSH6. Complementary stretches of mRNA can hybridize within the cell, and as double-stranded RNA can mediate post-transcriptional gene silencing, the VIT1 transcript potentially modifies the level of G/T mismatch repair protein in the cell (32). In fact, high levels of hMSH6 may reflect increased DNA damage in vitiligo melanocytes. Homology between VIT1 and hMSH6, as well as a BLAST search of the human genome looking for identity, the VIT1 contig confirms the location of the gene at 2p16, with the complete contig demonstrating homology to a span of 5 kb at 2p16, including three potential introns. Coincidentally, 2p16 is also a site to which the Carney complex was previously mapped (33,34). This syndrome presents with multiple neoplasms and spotty skin pigmentation. Mutations in the *PRKAR1A* gene encoded by chromosome 17q22–24 appear to be responsible for the disease in a subset of patients, yet mutations in this gene do not correlate with the pigmentation phenotype and the gene encoded by 2p16 has not been identified to date (34). It is of interest that the Carney complex shares a genomic site with a gene underlying loss of melanocytes in vitiligo. It was in fact shown that the hMSH6 gene itself was not the gene responsible for Carney syndrome (33). One might speculate that the VIT1 vitiligo gene is down-regulated in vitiligo yet up-regulated in other diseases such as Carney complex, with opposing consequences.

It would be of interest to learn the expression status of the VIT1 gene reported here in other forms of vitiligo as well in other tissues, and to ascribe a function to the gene. The size of the ORF is small. The protein encoded by this ORF was noted to be identical to a hypothetical protein derived from a brain mRNA library sequence (GenBank Accession no. AL117620). Importantly, the similarity to *Drosophila* calmodulin is suggestive of a function in calcium homeostasis. As numerous cell functions are regulated by intracellular calcium, including cell adhesion, further analysis of a potentially related function for the VIT1 gene could reveal its role in vitiligo pathogenesis (35). In this respect, it is important to note that defective calcium transport in vitiliginous melanocytes has previously been reported (36). Moreover, treatment aimed at modulating epidermal calcium homeostasis can be beneficial for vitiligo patients (37,38).

A 45% identity of amino acids 37–67 to a putative zinc-finger motif found in *N*-recognin is of interest because the latter is involved in binding *N*-terminal degradation signals in cellular proteins, thereby defining the efficiency of protein degradation (39). With the zinc-finger-like motif found in VIT1, the protein may similarly support protein degradation, and reduced VIT1 expression can therefore help to explain protein accumulation leading to dilated ER profiles, a hallmark of vitiligo melanocytes (12–15). Future studies will hopefully lead to a better understanding of the role of the present and other gene products to melanocyte physiology in general, and to vitiligo in particular.

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Fig. 1. Differentially expressed mRNA fragments. Fragments of differential display autoradiograms showing differentially expressed cDNA fragments in three vitiligo samples (first three lanes) versus three control samples (three right-hand lanes). Samples shown from left to right are numbered accordingly in Table 1. In some lanes additional bands are visible representing cDNA fragments for which expression levels are not associated with vitiligo. All but # 12 of the fragments were down-regulated in vitiligo samples compared with controls.

Fig. 2.

Reverse dot blot of fragment 9. Four circles on each filter indicate where fragment 9 was spotted in concentrations of 5 ng (top left), 500 pg (bottom left), 50 pg (top right) and 5 pg (bottom right). The experiment was repeated twice with the same result. The radiolabeled and PCR amplified cDNA derived from a control sample hybridized more readily to fragment 9 than the vitiligo-derived sample, indicating that the message is indeed down-regulated in vitiligo melanocyte RNA.

Fig. 3.

Sequence of aligned library cDNA's hybridizing to fragment 9. The sequence shown represents aligned cDNA contained in three library clones hybridizing to fragment 9 and sequenced by primer walking. Individual sequencing reactions are represented by numbered arrows mentioning the stretch of nucleotides contained within. A loop of approximately 1 kb representing introns and flanked by consensus splice sites, most likely derived from incompletely spliced nuclear RNA, was eliminated from the sequence as shown to reveal the 423-nucleotide ORF. Complementarity to hMSH-6 cDNA over a stretch of 0.45 kb was also indicated.

VIT1	3474	gtaactcagttttgtgaaaggtaaaaaactactatgttttaagtacactttagaaagtct
hMSH6	2819	gtaactcagttttgggaaaggtaaaaaactactatgttttaagtacactttagaaagtct
VIT1		cttcaaaaacaaacagtcctg-tatttgaactctgttcatagttttttttgaac-agttt
hMSH6		cttcaaaaacaaacagtcctggtatctgnactctgttcatagttttttttgaaccagttt
VIT1		agacaaaactgttgaaattaaaccaatttcctgcattaagctgagacaagtataataac
hMSH6		agacaaaactgttgaaattaaaccaatttcctgcattaagctgagacaagtataataac
VIT1		agccctatacagtttcatagtcttttctccccatttatgtgtattggtgacagtgaggta
hMSH6		agccctatacagtttcatagtcttttctccccatttatgtgtattggtgacagtgaggta
VIT1		taaaacagcctgaaagtgtatgcatgtaccataaacatttagacttaatatattgtggagt
hMSH6		taaaacagcctgaaagtgtatgcatgtaccataaacatttagacttaatatattgtggagt
VIT1		attcaataaccattatgtagaaggtagataagaattaaaagggtttaatttcctagaaag
hMSH6		attcaataaccattatgtagaaggtagataagaattaaaagggtttaatttcctagaaag
VIT1		aaaatggaaaaatgggtcatttttaaaaaataaagtttatttagatcataatgttgtctgaa
hMSH6		aaaatggaaaaatgggtcatttttaaaaaataaagtttatttagatcataatgttgtctgaa
VIT1		tttaccacctttgtcagaagtcactcaaagcttccaatgtagtcagtcctataattcctt
hMSH6		tttaccacctttgtcagaagtcactcaaagcttccaatgtagtcagtcctataattcctt
VIT1		aatcaaagtcagcaatttatggacagcttcagcatttacagttgacctttcactagccag
hMSH6		aatcaaagtcagcaatttatggacagcttcagcatttacagttgacctttcactagccag
VIT1		gcaaaactcccttaaaatt 4030
hMSH6		gcaaaactcccttaaaatt 2261

Fig. 4.

Homology between VIT1 and hMSH-6. Complementary nucleotide sequences among VIT1 and hMSH-6 cDNA were revealed by a BLAST search. As both messages are encoded in opposite directions, the mRNA species can potentially form RNA-RNA hybrids of 0.5 kb, covering part of the coding region for the G/T mismatch repair gene.

Amino acid sequence *VIT1**(secondary structure: H = helix, E = strand, - = no prediction)****Underlined= region 44.4% identical to N-recognin putative zinc finger motif):***

```

MKDNKIMNQDAIEKAVSRGQCLYKISSYTSYPMHDFYRCHTCNTTDRNAICVNCIKKCH
-----HHHHHHHHH-----EEEE-----EE--H-----
QGHDVEFIRHDRVVAHLLDILPNYFPFHFSNIWVSFCFRFFCDGAGTLSNPCTLAGEPT
-----EEHHHHHHHHHH-----EEEEEE-EEE-----
HDTDTLYDSAPPIESNTLQHN
-----

```

Fig. 5.

Amino acid sequence encoded by the ORF shown in Fig. 4. Starting with methionine, a 423-bp fragment encodes a 141-amino acid protein before a stop codon is encountered. If translation starts from valine, the protein encoded is 226 amino acids in length. No signal sequence was detected, suggesting that the differentially expressed message may translate into a cytoplasmic protein. Homology was noted for the underlined stretch of amino acids to a putative zinc-finger motif found in *N-recognin*.

```

Score = 221 bits (563), Expect = 5e-57
Identities = 114/141 (80%), Positives = 114/141 (80%), Gaps = 27/141 (19%)

VIT1 : 1 MKDNKIMNNQDAIEKAVSRGQCLYKISSYTSYPMHDFYRCHTCNTTDRNAICVNCIKKCH 60
      MKDNKIMNNQDAIEKAVSRGQCLYKISSYTSYPMHDFYRCHTCNTTDRNAICVNCIKKCH
Sbjct: 95 MKDNKIMNNQDAIEKAVSRGQCLYKISSYTSYPMHDFYRCHTCNTTDRNAICVNCIKKCH 154

VIT1 : 61 QGHDVEFIRHDRVVAHLLDILPNYFPPHFSNIWVSFCFRFFCDGAGTLSNPCTLAGEPT 120
      QGHDVEFIRHD RFFCDGAGTLSNPCTLAGEPT
Sbjct: 155 QGHDVEFIRHD-----RFFCDGAGTLSNPCTLAGEPT 187

VIT1 : 121 HDTDTLYDSAPPIESNTLQHN 141
      HDTDTLYDSAPPIESNTLQHN
Sbjct: 188 HDTDTLYDSAPPIESNTLQHN 208

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Fig. 6.

Homology between the putative VIT1 protein and a hypothetical protein encoded by brain mRNA. Homology is shown among the putative VIT1 protein and a hypothetical protein encoded by fetal human brain mRNA (Genbank Accession no. AL117620).

Fig. 7. Expression of the VIT1-ORF and hMSH 6 in melanocytes, fibroblasts and keratinocytes. Expression of the VIT1 message in skin cells (KC = keratinocytes) was revealed by RT-PCR. The length of amplified fragments observed upon agarose gel electrophoresis corresponds to the expected 287-bp determined by the primer combination used, covering the putative ORF.

Fig. 8.

Relative expression of VIT1 and hMSH6 revealed by semi-quantitative RT-PCR. A 864-bp fragment was amplified by VIT1 primers and a 288-bp fragment was generated using primers for hMSH6 from the same RT samples in a limited number of PCR cycles, such that the amount of product will reflect the amount of substrate in the original RNA samples. Results are represented by the inversed image of bands observed in an EtBr-stained gel. The samples represented in this image are (1) vitiligo melanocytes culture Mk9904 in 3rd passage, (2) keratinocytes (passage 2), skin fibroblasts (passage 3) and (4) control melanocytes culture Mf9925 in second passage. An inverse relationship was apparent between the intensity of bands amplified by both primer combinations. Moreover, this experiment further supported down-regulated expression of VIT1 in vitiligo melanocytes and demonstrated ubiquitous expression of VIT1 mRNA among skin cells.

Table 1
Description of melanocyte cultures analyzed for differential gene expression

Culture No.	Condition	Pigmentary background	Gender	Age (years)	Donor site	Passage No.
1	Vitiligo	Caucasian	Male	42	Arm	15
2	Vitiligo	Caucasian	Male	28	Thigh	10
3	Vitiligo	Caucasian	Female	34	Arm	6
4	Control	Caucasian	Male	0	Foreskin	24
5	Control	Caucasian	Male	0	Foreskin	17
6	Control	Caucasian	Male	43	Arm	6

Table 2

Cloned differentially expressed fragments

Fragment	Primers used	Length (nucleotides)	Intensity ratio control:vitiligo bands
9 (down-regulated)	HT11C/HAP1	261	3.15
12 (up-regulated)	HT11C/HAP2	136	0.69
18 (down-regulated)	HT11G/HAP4	232	3.07
21 (down-regulated)	HT11G/HAP9	166	1.93
28 (down-regulated)	HT11G/HAP16	194	3.35
33 (down-regulated)	HT11C/HAP16	378	3.72