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The Transcription Factors SOX9 and SOX10 Are Vitiligo Autoantigens in Autoimmune Polyendocrine Syndrome Type I*

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Vitiligo is common in the hereditary disorder autoimmune polyendocrine syndrome type I (APS I). Patients with APS I are known to have high titer autoantibodies directed against various tissue-specific antigens. Using sera from APS I patients for immunoscreening of a cDNA library from human scalp, we identified the transcription factors SOX9 and SOX10 as novel autoantigens related to this syndrome. Immunoreactivity against SOX9 was found in 14 (15%) and against SOX10 in 20 (22%) of the 91 APS I sera studied. All patients reacting with SOX9 displayed reactivity against SOX10, suggesting shared epitopes. Among the 19 patients with vitiligo, 12 (63%) were positive for SOX10 ($p < 0.0001$). Furthermore, three of 93 sera from patients with vitiligo unrelated to APS I showed strong reactivity against SOX10, which may indicate a more general role of SOX10 as an autoantigen in vitiligo.

The autosomal recessively inherited disease autoimmune polyendocrine syndrome type I (APS I),[1] also called autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), is characterized by organ-specific autoimmunity, mucocutaneous candidiasis, and ectodermal manifestations (1). The gene responsible for the syndrome has been identified recently and named autoimmune regulator (*AIRE*), coding for a protein with the characteristics of a transcription factor (2, 3).

Two of the classical triad of mucocutaneous candidiasis, hypoparathyroidism and adrenocortical insufficiency, are required for the clinical diagnosis of APS I(4). Other severe manifestations such as autoimmune chronic active hepatitis, gonadal failure, malabsorption, and insulin-dependent diabetes mellitus may also affect the patients. Furthermore, ectodermal manifestations such as vitiligo, alopecia, and nail and enamel dystrophy are seen frequently (1, 5). High titer autoantibodies against affected tissues is a hallmark of APS I. The antigenic structures identified, tissue-specific key enzymes (6–13), have been shown to be important, some of which seem to act as autoantigens also in other more common autoimmune diseases (14).

Vitiligo is a depigmenting skin disorder with a prevalence in the general population of about 1% (15). The etiology is largely unknown, but autoimmune mechanisms are thought to be involved. It is associated with various autoimmune disorders (16) such as insulin-dependent diabetes mellitus (17), thyroid disease (18, 19), and pernicious anemia (20, 21) and is often a component in autoimmune polyendocrinopathies (4) including APS I (1, 5). Autoantibodies against melanocytes have been reported in patients with vitiligo unrelated to APS I (22, 23), but the major autoantigens in vitiligo are yet to be identified. The possible roles of tyrosinase, tyrosinase-related protein 1 (TPR-1), and tyrosinase-related protein 2 (TPR-2), key enzymes in the synthesis of melanin as well as the melanosomal matrix glycoprotein, pmel17 (24), as melanocyte autoantigens remain largely unresolved. Some studies report frequent immunoreactivity against these proteins in patients with vitiligo (25), whereas other studies show only a low frequency of immunoreactivity (26). In the majority of patients studied with vitiligo and APS I, complement-fixing antibodies directed against melanin-producing cells were identified (27, 28). This immunoreactivity was not demonstrated in patients with isolated vitiligo or vitiligo associated with other autoimmune disorders (28) and can be found in patients with APS I many years before the development of vitiligo (29).

Sera from patients with APS I are known to contain high titer autoantibodies, which makes APS I a suitable disease model for the identification of autoantigens. We recently demonstrated the presence of immunoreactivity against melanocyte nuclei in both hair follicles and epidermis in APS I patients (30). Because vitiligo is common in APS I, affecting 8–15% of the patients (1, 5), this study aims at further characterization of the antigenic structures related to this manifestation.

EXPERIMENTAL PROCEDURES

Sera—Samples from 91 APS I patients and 93 patients with vitiligo unrelated to APS I were tested. In addition, sera from 35 healthy Swedish and 30 Finnish blood donors were used as controls. Clinical characteristics of the 10 Swedish (11, 30, 31), 7 Norwegian (12), 12 Italian (5) and 62 Finnish patients (1) with APS I have been described previously. The male/female ratio was 45/46. Vitiligo was present in 3 of 10 (30%) Swedish, 2 of 7 (29%) Norwegian, 3 of 12 (25%) Italian, and 11 of 62 (18%) Finnish patients, thus altogether in 19 of the 91 (21%) patients. There were 35 males and 58 females among the 93 patients with vitiligo not associated to APS I. Thirty-seven of these patients had accompanying autoimmune diseases, of which autoimmune thyroid dis-

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[1] The abbreviations used are: APS I, autoimmune polyendocrine syndrome type I; PCR, polymerase chain reaction; ITT, *in vitro* transcription and translation.

ease was the most prevalent and found in 21 of the patients. None of the 93 patients had a clinical picture of APS I.

Specific Antibodies—A mouse anti-human monoclonal antibody directed against a yet uncharacterized but melanocyte-specific cell-surface protein was purchased from ANAWA (Zürich, Switzerland). A fluorescein isothiocyanate-conjugated donkey anti-mouse immunoglobulin and a Cy3-conjugated donkey anti-human immunoglobulin was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). A polyclonal rabbit anti-rat Sox10 serum was a kind gift from Dr. M. Wegner (Zentrum für Molekulare Neurobiologie, Hamburg, Germany) (32).

Construction of cDNA Library—Preparation of mRNA from human scalp containing intact hair follicles was performed with Dynabeads® mRNA DIRECT kit (Dynal AS, Oslo, Norway) followed by synthesis of the library (λZAP-EXPRESS cDNA synthesis kit, Stratagene) according to the protocols supplied by the manufacturers. Fractions containing cDNA fragments larger than 500 base pairs were pooled and ligated into the vector arms. The library, containing 3.0×10^6 unique cDNA clones, was then amplified once. Scalp tissues were taken in connection with neurosurgical interventions and with the approval of the local ethics committee.

Isolation of the SOX9 Clone—Serum from an APS I patient with vitiligo was diluted 1:3000 and used for immunoscreening of the library as described previously (9). After *in vitro* excision of pBK-CMV phagemid vectors according to the Stratagene protocol, DNA sequence analysis was performed by primer walking with AmpliTaq DNA Polymerase FS, dye terminator, and dye primer kits (PerkinElmer Life Sciences) on a 373A DNA Sequencer (Applied Biosystems, Foster City, CA). The DNA sequence was then compared with international data bases using BLAST (NCBI).

Subcloning of Human SOX10—A DNA fragment containing the open reading frame of human *SOX10* was PCR-amplified from the vector huSOX10 (kindly donated by Dr. M. Wegner) (32). The primers used (Cybergene, Huddinge, Sweden) were 5′-GGCGGCGGCCGGGGGCGACAT-3′, annealing 20 bases upstream of the first ATG, and 5′-TCAGGGCAGGAGCCAGACAGAAA-3′, annealing 158 bases downstream of the stop codon. The PCR product was subsequently subcloned using the pGEM-T vector systems kit (Promega, Madison, WI) according to manufacurer protocol.

In Vitro Transcription and Translation (ITT) and Immunoprecipitation—Plasmids were purified with the Qiagen miniprep kit (Qiagen GmbH, Hilden, Germany). ITT of the purified plasmids was performed using the TNT T3- and T7-coupled reticulocyte lysate system (Promega). The incorporation rate of total [^{35}S]methionine was ~1% for both SOX9 and SOX10. The correct size of the radioactively labeled proteins was analyzed on an SDS-polyacrylamide gel electrophoresis minigel (Bio-Rad) according to standard protocols. The ^{35}S-radiolabeled SOX9 and SOX10 proteins were used for immunoprecipitation with the sera from patients in a 96-well plate assay as described elsewhere (33). The results were expressed as SOX9 and SOX10 indices ((cpm sample − cpm-negative control)/(cpm-positive control − cpm-negative control) × 100), respectively. Each sample was analyzed in triplicate. One APS I patient with a high titer of antibodies against SOX9 and SOX10 was used as a positive control, and one of the blood donors was used as a negative control in each microwell plate. The upper normal limits of SOX9 and SOX10 antibody indices were set at 4 and 24, respectively. These values divided the cohort of APS I patients into those with clearly elevated indices and those with normal or slightly elevated indices.

Preparation of Melanocytes—Human epidermal melanocytes were prepared as described elsewhere (34). Cells were cultured for 3 weeks in serum-free M2 melanocyte medium (PromoCell, Heidelberg, Germany) and were then resuspended in 8% Me_2SO in fetal calf serum for storage at −150 °C until needed (35).

Western Blot—Ten million cells prepared from Caucasian human skin taken during mammary plastic surgery were washed two times in PBS, lysed in loading buffer with 10 mM EDTA, and loaded on an SDS-polyacrylamide gel electrophoresis gel (36). Lysates from 5×10^5 cells were loaded in each lane. Western blot was performed as described elsewhere (37) with sera from eight Swedish APS I patients and three healthy blood donors diluted 1:1000. The normal rabbit serum (DAKO A/S, Glostrup, Denmark) and the polyclonal rabbit anti-rat SOX10 antiserum were diluted 1:100.

Preabsorption of Serum with in Vitro Expressed SOX Proteins and Indirect Immunofluorescence—A biopsy was taken from the dorsal surface of an upper limb of a healthy Caucasian female and used for subsequent cryosection. Serum from the APS I patient used for the initial immunoscreening of the scalp cDNA library was preincubated with SOX9 and SOX10 proteins expressed *in vitro* as described above. The serum was diluted 1:300 in a total volume of 100 μl of incubation buffer (50 μl of the ITT mixture and 50 μl of PBS) and was preincubated overnight at 4 °C. A similar ITT mixture without cDNA template served as a negative control. The mouse anti-human melanocyte antibody was added to each preabsorption mixture in a final dilution of 1:200. Fifty microliters of all three overnight-incubation mixtures were then used for indirect immunofluorescence staining of the cryosections. A secondary donkey anti-human (Cy3) antibody and a donkey anti-mouse (fluorescein isothiocyanate) antibody were diluted 1:200 and 1:50, respectively. Tissue preparation and immunohistochemical incubation procedures have been described elsewhere (30). Photographs were taken with a Leica DMRB microscope (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland).

Reverse Transcriptase-PCR—Melanocytes were obtained from two sources: 1) mammary skin from one healthy Caucasian female undergoing plastic surgery and 2) normal gluteal skin from a Caucasian female with vitiligo. Cells (8×10^6) from each person were lysed in 300 μl of lysis buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 1% lithium dodecyl sulfate, 500 mM LiCl, and 5 mM dithiothreitol), and mRNA was extracted with Dynabeads® mRNA DIRECT kit (Dynal A.S). After cDNA synthesis with Superscript-Reverse Transcriptase (Life Technologies, Inc.), PCR was performed with specific primers designed to anneal to separate exons to avoid amplification of genomic sequences. The primers were: *SOX9* sense (exon 2) 5′-GCCACGGAGCAGACGCAC-3′ and antisense (exon 3) 5′-GCGCCTGCTGCTTGGACA-3′); *SOX10* sense (exon 4) 5′-GACCGCCGCCATCCAGGC-3′ and antisense (exon 5) 5′-AGTAGCTGCTCACATGGCCT-3′; and β-actin, which was used as a positive control, sense (exon 2) 5′-CAACTGGGACGACATGGAGA-3′, and antisense (exon 3) 5′-TGTCAGGCAGCTCGTAGCTG-3′. PCRs were done with Expand™ High Fidelity PCR system (Roche Molecular Biochemicals) according to manufacturer protocol at an annealing temperature of 62 °C for 35 cycles.

Statistical Methods—Fisher's exact test was used to compare the frequencies of reactivity against SOX9 and SOX10 with various symptoms.

RESULTS

Identification of SOX9 as an Autoantigen—Four positive clones were identified when the λZAP-cDNA library was screened with serum from one of the Swedish APS I patients with vitiligo. After *in vitro* excision of pBK-CMV vectors corresponding to the positive clones, *in vitro* transcription and translation with T3 polymerase was performed followed by immunoprecipitation with a panel of 10 APS I sera. One of the four clones was recognized solely by the serum used for the screening, whereas the other three were identified by four of the 10 sera. DNA sequence analysis revealed that these latter clones all coded for the transcription factor SOX9. Two of the clones were identical with the reported full-length cDNA (38), and one clone represented a truncated *SOX9* cDNA.

Immunoprecipitation of the Recombinant SOX9 and SOX10 with Patient Sera—Fourteen (15%) of the 91 APS I patients had antibodies against SOX9 with an index above 4. Of the 19 patients with vitiligo, 9 (47%) had autoantibodies, in contrast to 5 (7%) of 72 patients without vitiligo ($p < 0.0001$).

SOX10 is expressed in embryonic melanoblasts (39) and is structurally related to SOX9 with an overall homology of 59%. This prompted us to assess SOX10 as a possible autoantigen in vitiligo. All APS I patients with immunoreactivity against SOX9 also had reactivity against SOX10. In addition, six patients only reacting with SOX10 were identified, three of these having vitiligo. Altogether, 20 (22%) of the 91 APS I sera displayed SOX10 antibodies (Fig. 1). Twelve (63%) sera from 19 patients with vitiligo, in contrast to 8 (11%) of 72 without vitiligo, had an index above 24 ($p < 0.0001$). Of the 19 APS I patients with vitiligo, 12 (63%) also had alopecia areata, and 9 (75%) of them were SOX10 antibody-positive. Among 93 patients with vitiligo that is unassociated with APS I, three (3%) showed strong reactivity against SOX10. Two of these had symmetrical active vitiligo, one had focal static vitiligo, and one also showed weak SOX9 reactivity.

Analysis of the *in vitro* transcribed and translated products

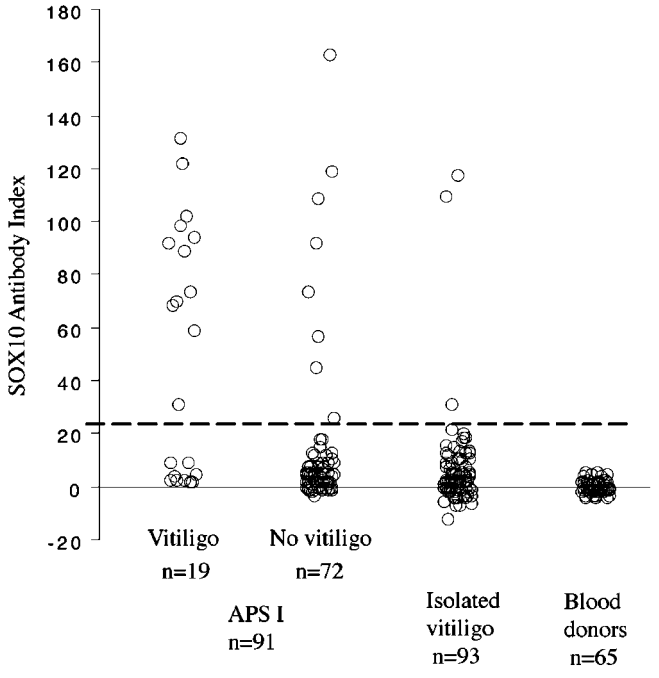

FIG. 1. **Serum immunoreactivity against SOX10.** Reactivity against *in vitro* expressed SOX10 in the ITT assay with sera from 19 APS I patients with vitiligo, 72 APS I patients without vitiligo, 93 patients with isolated vitiligo, and 65 healthy blood donors. The *dotted line* indicates the upper normal limit dividing the cohort of APS I patients into those with clearly elevated indices and those with normal or slightly elevated indices.

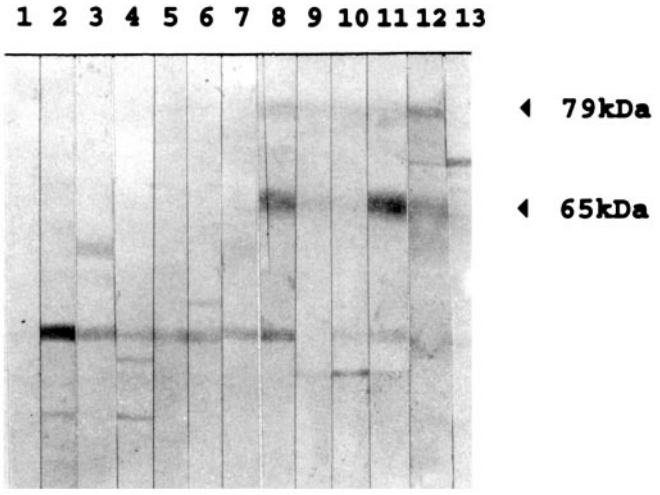

FIG. 2. **Western blot analysis.** Whole cultured human melanocytes were analyzed with human and rabbit sera. *Lanes 1–3*, three healthy blood donors; *lanes 4–11*, eight Swedish APS I patients; *lane 12*, rabbit anti-rat SOX10 serum; *lane 13*, normal rabbit serum. All human sera were diluted 1:1000, and rabbit sera were diluted 1:100.

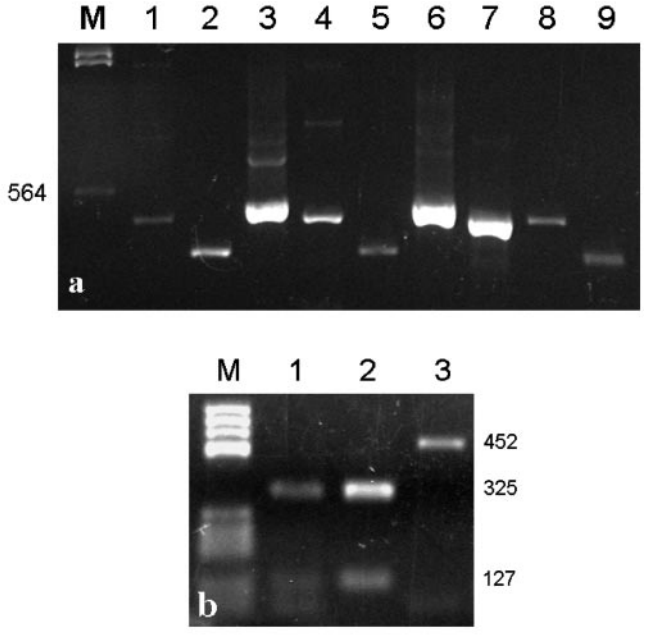

FIG. 3. **Detection of mRNA expression.** *a*, reverse transcriptase-PCR with *SOX9*- (*lanes 1* and *4*), *SOX10*- (*lanes 2* and *5*), and β-*actin*- (*lanes 3* and *6*) specific primers against cultured human melanocyte mRNA from a healthy person (*lanes 1–3*) and a vitiligo patient (*lanes 4–6*). PCR was carried out with the *SOX9*-specific primers against a pBK-CMV-SOX9 vector (*lane 7*) and 4 μl of the scalp cDNA library (*lane 8*) and with the *SOX10*-specific primers against 4 μl of the scalp cDNA library (*lane 9*) as templates. *b*, subsequent restriction enzyme *Apa*I digestion of the *SOX9* PCR fragments generated from the vitiligo patient cDNA (*lane 1*) and the pBK-CMV-SOX9 template (*lane 2*). *Lane 3*, nondigested *SOX9* PCR fragment from the pBK-CMV-SOX9 template. *M*, molecular size markers.

on SDS-polyacrylamide gel electrophoresis showed bands with the expected sizes of ~60 kDa for SOX9 and 55 kDa for SOX10 (data not shown).

Western Blot—When lysates from melanocytes were used for immunoblotting, four of the eight APS I patients showed reactivity against a broad band (Fig. 2, *lanes 8–11*) migrating with a molecular mass of ~65 kDa (Fig. 2), and a less intensively stained band of ~79 kDa, co-migrating with bands detected by the specific rabbit antiserum against SOX10. In addition, a third protein of ~45 kDa was also weakly recognized by the same four patient sera (Fig. 2), but this was not detected by the specific rabbit antiserum (Fig. 2, *lane 12*). No reactivity against the 79-, 65-, or the 45-kDa bands was seen with sera from healthy blood donors or with the normal rabbit serum.

Reverse Transcriptase-PCR—SOX9 and SOX10 bands with the expected sizes were obtained when mRNA from isolated melanocytes from both a healthy female (Fig. 3, *lanes 1–3*) and a female patient with vitiligo (*lanes 4–6*) was used for reverse transcriptase-PCR. Restriction enzyme digestion with *Apa*I, only present in the *SOX9* sequence, generated fragments of the expected size (Fig. 3*b*), confirming the amplification of *SOX9* mRNA.

Preabsorption Studies—A nuclear staining was seen in melanocytes close to or just above the basal membrane when serum from one APS I patient was used for immunofluorescence (Fig. 4). After preincubation with the *in vitro* expressed proteins, only the SOX10 protein eliminated the nuclear melanocyte staining (Fig. 4).

DISCUSSION

We report on the identification of the transcription factors SOX9 and SOX10 as autoantigens associated with vitiligo in APS I. Autoantibodies against SOX10 were also found in three patients with vitiligo unassociated with APS I, implying that SOX10 is an autoantigen in a subgroup of patients with idiopathic vitiligo.

SOX9 and SOX10 belong to a family of more than 20 transcription factors sharing a motif homologous to the high mobility group box in the sex-determining region Y (SRY) factor (35, 36). SOX and other high mobility group box proteins bind to DNA in a highly sequence-specific manner (40) and are thought to play important roles in the embryonic development of various organs (41, 42). SOX9 and SOX10 are structurally

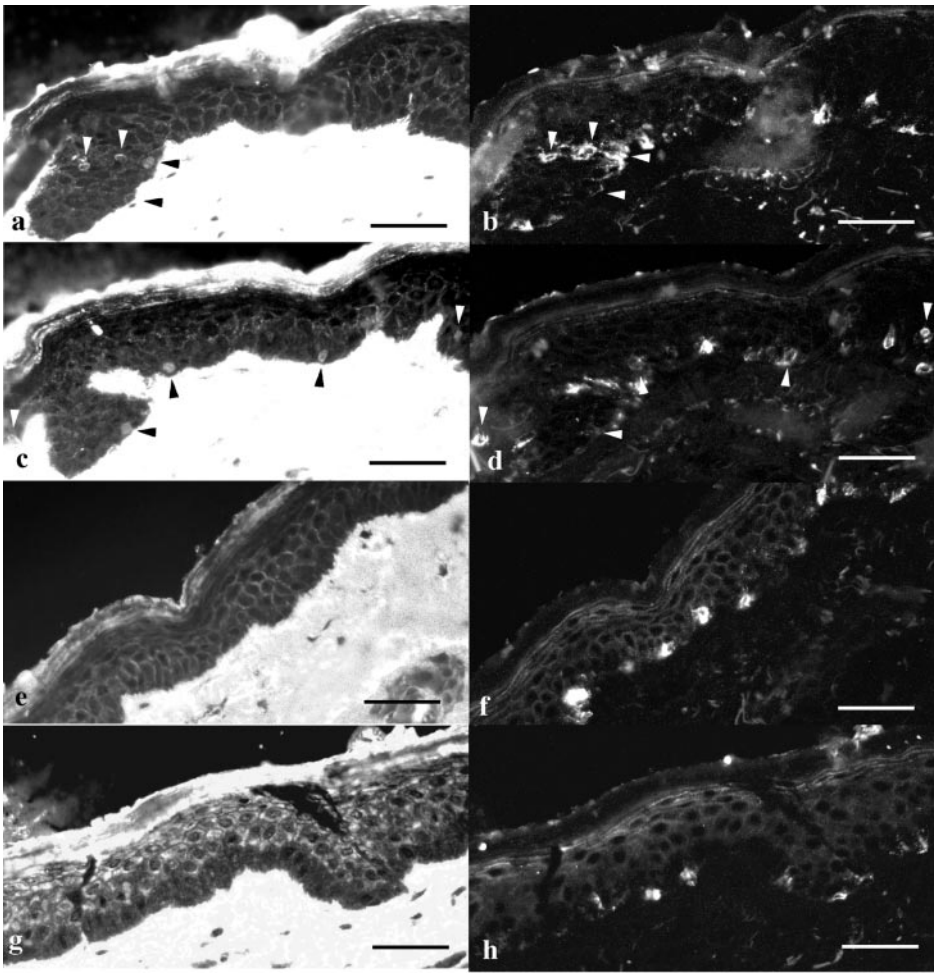

FIG. 4. **Preabsorption assay.** Indirect immunofluorescence of normal human epidermis with sera from one APS I patient (*a*, *c*, and *e*) known to be SOX9/10-immunoreactive in the ITT assay and one serum from a healthy blood donor (*g*) diluted 1:300. The APS I serum was preabsorbed with ITT reactions without ITT product (*a*), with the SOX9 product (*c*), and with the SOX10 product (*e*). All sections were double-stained with a mouse anti-human melanocyte antibody (*b*, *d*, *f*, and *h*). The *arrowheads* indicate nuclear staining (*a* and *c*) of corresponding melanocytes (*b* and *d*). *Scale bars*, 50 μm.

closely related with almost 100% homology within a stretch of 110 amino acids and an overall amino acid identity of 59% (32). SOX10 is involved in the differentiation of tissues derived from the neural crest, and its expression has been detected in the central nervous system, peripheral nervous system, sympathetic and parasympathetic ganglia, and enteric ganglia throughout the gastrointestinal tract during human embryogenesis. Expression has also been shown in the submandibular glands, the pancreas, the heart, and the lung. In adults, expression of SOX10 has been detected in the brain, prostate, small intestine, colon, and heart (43).

Melanocytes are derived from the neural crest, and SOX10 expression has been reported in migrating melanoblasts in developing mouse dermis (39). SOX10 has been shown to bind to the promoter of microphtalmia-associated transcription factor (44), a gene important for the development of melanocytes and other neural crest-derived cells (45). Interestingly, mutations in the *SOX10* gene in patients with Waardenburg-Hirschprung disease give rise to pigmentary abnormalities as well as deafness and agangliotic megacolon (46).

We found reactivity against SOX10 in 63% of the APS I patients with vitiligo but only in 11% without vitiligo ($p < 0.0001$), indicating a strong association between these autoantibodies and vitiligo. Reactivity against SOX9 was found in a subgroup of the patients with reactivity against SOX10. This may imply cross-reactivity against epitopes shared by SOX9 and SOX10 and that SOX10 is the major autoantigen. This notion is supported by the finding that only *in vitro* expressed SOX10, but not SOX9, abolished the immunohistochemical staining of melanocytes by APS I sera in preabsorption experiments (Fig. 4). In addition, in the three patients with vitiligo unrelated to APS I, a strong reactivity was only detected against SOX10.

Unlike SOX10, expression of SOX9 has not been reported in melanocytes. Mutations in the *SOX9* gene, causing skeletal malformation syndrome, campomelic dysplasia, and autosomal sex reversal (female or ambiguous genitalia in individuals with an XY genotype) (38, 47), have not been associated with pigment abnormalities. Using reverse transcriptase-polymerase chain reaction we were able, however, to detect both *SOX9* and *SOX10* transcripts in isolated human melanocytes. Also, using Western blot on lysate from human melanocytes, we identified three bands recognized by sera from four of eight patients with APS I (Fig. 2). Two of these, the 79- and 65-kDa bands, corresponded to proteins also detected by the rabbit anti-rat SOX10 antiserum. Their electrophoretic mobility was lower than that calculated for the molecular mass of SOX10. We have no explanation for this difference, but similar discrepancies have been described (32). The third 45-kDa band was not seen with the specific SOX10 antibody and may represent another melanocyte antigen that has yet to be identified. Thus, it is possible that the autoimmune attack against melanocytes in vitiligo may involve both SOX9 and SOX10 as autoantigens despite the findings mentioned above that would support SOX10 as the major autoantigen.

Of the vitiligo patients with APS I, 63% also had alopecia areata ($p < 0.002$), and we found that both SOX9 and SOX10 reactivity correlate with this combination of symptoms,

whereas no correlation was found with alopecia areata alone. Other studies have found correlations between vitiligo and alopecia areata (18, 48), and antibodies reactive against hair follicle melanocytes (49) and melanoma cell lines (50) in sera from patients with alopecia areata have been demonstrated using Western blot technique. In our recent study APS I sera also stained melanocyte nuclei in hair follicles (30). In the present study these sera were shown to be reactive against both SOX9 and SOX10. This indicates that SOX10, and possibly also SOX9, are present in melanocytes in hair follicles and points to a possible role of melanocytes in the etiology of alopecia areata.

Nuclear autoantigens have been described in systemic autoimmune diseases such as primary Sjögren's syndrome and systemic lupus erythematosus (51). These antigens are, in contrast to SOX9/10, present in most cells throughout the body. Immunoreactivity against other members of the high mobility group family of transcription factors has been demonstrated previously in sera from patients with primary pulmonary hypertension (52), systemic lupus erythematosus (53), juvenile rheumatoid arthritis (54), and drug-induced autoimmunity (55).

Recently, increased frequencies of autoantibodies against SOX13 have been found in sera from patients with type I diabetes mellitus and primary biliary cirrhosis (56, 57), and high titer autoantibodies against SOX group B transcription factors (SOX1, -2, -3, and -21) were found in sera from 30–40% of patients with small cell lung cancer (58). This may further indicate important immunogenic properties of this family of transcription factors.

Antimelanocyte autoantibodies have been identified in sera from patients with vitiligo, but different frequencies have been reported. Autoantibodies precipitating melanocyte surface proteins were found in 78% of sera from patients with vitiligo, compared with only 14% of the control sera, although only a fraction of the proteins were shown to be melanocyte-specific (22). The frequency of autoantibodies against the melanocyte-specific enzyme tyrosinase, however, ranged from 10 to 60% of sera from patients affected by vitiligo, and even lower frequencies of autoantibodies against TRP-1, TRP-2, and Pmel17 were found (24–26, 59).

In this study we have shown that SOX9 and SOX10 are expressed in human adult skin melanocytes and identified them as autoantigens in APS I. Immunoreactivity against these proteins is associated with vitiligo in APS I. Strong reactivity against SOX10 is also detected in a subgroup of patients with idiopathic vitiligo. The relevance of the serum immunoreactivity against SOX9 and SOX10 for the development of vitiligo in these patients must be studied further. Epitopes recognized by autoantibodies may not be the same as epitopes recognized by T-cell receptors on autoreactive T-lymphocytes, and the development of autoantibodies may also be the consequence of cell lysis rather than the course of it. We still believe that these autoantigens may provide a new tool in the diagnosis and understanding of vitiligo, and furthermore the identification of SOX9 and SOX10 as autoantigens may also help in the development of new immunotherapeutic approaches for the treatment of melanoma.

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