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Molecular cloning of two novel *rab* genes from human melanocytes

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Abstract

We isolated the genes of two small GTP-binding proteins of the rab family from a human melanocyte cDNA library and from melanoma cells. One gene, *rab30* codes for a novel rab protein of 203 amino acids with minimal homology to previously documented GTPases. The other, *rab22b*, appears to be an isoform of the human homologue of canine *rab22*. Both *rab* mRNAs displayed a nearly ubiquitous pattern of expression in the various tissues examined. *Rab22b* and *rab30* were mapped to chromosomes 18 and 11, respectively.

Keywords: GTPase; Melanosome; Dense body; Lysosome; Vesicles; Membranes

1. Introduction

Rab proteins represent a family of nontransforming monomeric GTP-binding proteins in the ras superfamily. Similar to ras, these proteins have four highly conserved regions (designated I to IV) involved in GTP binding and hydrolysis. They also contain a consensus carboxy-terminal amino acid sequence containing two cysteine residues. Geranyl-geranylation of a carboxy-terminal cysteine allows for anchoring of the rab to the plasma membrane or to a vesicular membrane. Subsequently, in a fashion not fully elucidated, rabs regulate the critical intracellular processes of membrane trafficking and vesicular fusion and targeting. These functions predict that a number of different rab proteins should be expressed in eukaryotic cells, each rab associated with a distinct intracellular membrane sorting process. Indeed, since the first rab cDNAs were isolated in 1987 from a rat brain library, approximately 30 different rabs have been identified in a variety of mammalian species (Pfeffer, 1994; Simons and Zerial, 1993; Novick and Brennwald, 1993). Nineteen of these rabs (including isoforms) have been isolated from human cells, including one, *rab*28, which involves alternative mRNA splicing (Brauers et al., 1996).

Although most *rab* genes are expressed in a wide variety of cell types, a few exhibit tissue or cell type specificity. For example, *rab17* is expressed primarily in epithelial cells (Lutcke et al., 1993) and *S10*, a rab not designated by standard nomenclature, is expressed exclusively in lymphoid cell lines (Koda and Kakinuma, 1993). Tissue specificity can also be achieved by the expression of different rab isoforms. The *rab3* subfamily, for instance, has three isoforms, with *rab3a* expressed predominantly in neuronal tissues, *rab3b* preferentially in epithelial cells and *rab3d* largely in adipocytes (Weber et al., 1994; Baldini et al., 1992). The expression of certain *rab* genes may prove critical to the differentiated function of cells requiring unique vesicle targeting or fusion pathways.

Melanocytes typify such differentiated cells. Their unique vesicles, melanosomes, achieve the cells' primary function, i.e., producing melanin pigment. Although melanocyte-specific rab proteins have not been described, certain rab proteins may function specifically in melanosome genesis and fusion. On the other hand,

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Abbreviations: bp, base pair(s); C, cysteine; cDNA, DNA complementary to RNA; GCG, Genetics Computer Group (Madison, WI); GTPase, guanosine triphosphatase; kb, kilobase pair(s); kDa, kilodalton; ORF, open reading frame; PCR, polymerase chain reaction; pI, isoelectric point; RACE, rapid amplification of cDNA ends.

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melanosomes share membrane proteins with lysosomes, e.g., LAMPs (Zhou et al., 1993), and with platelets, e.g., granulophysin or ME 491 (Nishibori et al., 1993), and melanocytes have general cellular functions besides those related to pigment formation. Therefore, melanocytes should contain rabs found in other cells as well.

We explored these possibilities by documenting a complex array of 17 rabs and other small GTP-binding proteins in pigmented human melanoma cells using a PCR-based strategy. In this report, we present the complete cDNA sequences of two novel *rab* genes, their expression in a variety of tissues and cell lines and their chromosome assignment.

A

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1      CGAGGATGCTGCTGAGCCCCGGCACTGCCTGGCTGCGAGCACATGATGGCGATACGGGAG
                                                  M  A  I  R  E        5

61     CTCAAAGTGTGCCTTCTCGGGGACACTGGGGTTGGGAAATCAAGCATCGTGTGTCGATTT
        L  K  V  C  L  L  G  D  T  G  V  G  K  S  S  I  V  C  R  F     25

121    GTCCAGGATCACTTTGACCACAACATCAGCCCTACTATTGGGGCATCTTTTATGACCAAA
        V  Q  D  H  F  D  H  N  I  S  P  T  I  G  A  S  F  M  T  K     45

181    ACTGTGCCTTGTGGAAATGAACTTCACAAGTTCCTCATCTGGGACACTGCTGGTCAGGAA
        T  V  P  C  G  N  E  L  H  K  F  L  I  W  D  T  A  G  Q  E     65

241    CGGTTTCATTCATTGGCTCCCATGTACTATCGAGGCTCAGCTGCAGCTGTTATCGTGTAT
        R  F  H  S  L  A  P  M  Y  Y  R  G  S  A  A  A  V  I  V  Y     85

301    GATATTACCAAGCAGGATTCATTTTATACCTTGAAGAAATGGGTCAAGGAGCTGAAAGAA
        D  I  T  K  Q  D  S  F  Y  T  L  K  K  W  V  K  E  L  K  E     105

361    CATGGTCCAGAAAACATTGTAATGGCCATCGCTGGAAACAAGTGCGACCTCTCAGATATT
        H  G  P  E  N  I  V  M  A  I  A  G  N  K  C  D  L  S  D  I     125

421    AGGGAGGTTCCCCTGAAGGATGCTAAGGAATACGCTGAATCCATAGGTGCCATCGTGGTT
        R  E  V  P  L  K  D  A  K  E  Y  A  E  S  I  G  A  I  V  V     145

481    GAGACAAGTGCAAAAAATGCTATTAATATCGAAGAGCTCTTTCAAGGAATCAGCCGCCAG
        E  T  S  A  K  N  A  I  N  I  E  E  L  F  Q  G  I  S  R  Q     165

541    ATCCCACCCTTGGACCCCCATGAAAATGGAAACAATGGAACAATCAAAGTTGAGAAGCCA
        I  P  P  L  D  P  H  E  N  G  N  N  G  T  I  K  V  E  K  P     185

601    ACCATGCAATCCAGCCGCCGGTGCTGTTGACCCAAGGGCCGTGGTCCACGTACTTGAAGA
        T  M  Q  S  S  R  R  C  C                                      194

661    AGCCAGAGCCCACATCCTGTGCACTGCTGAAGGACCCTACGCTCGGTGGCCTGGCACCTC
721    ACTTTGAGAAGAGTGAGCACACTGGCTTTGCATCCTGGAAGACCTGCAGGGGCGGGCAGG
781    AAATGTACCTGAAAAGGATTTTAGAAAACCCTGGAAAACCCACCACACCACCACCACAAA
841    ATGGCCTTTAGTGTATGAAATGCACATGGAGGGGATGTAGTTGCATTTTTGCTAAAAAA
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B

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  1 MAIRELKVCLLGDTGVGKSSIVCRFVQDHFDHNISPTIGASFMTKTVPCGNELHKFLIWD  60
    ||:||||||||||||||||||| |||:| || || |||||||||||| : ||||||||||
  1 MALRELKVCLLGDTGVGKSSIVWRFVEDSFDPNINPTIGASFMTKTVQYQNELHKFLIWD  60

 61 TAGQERFHSLAPMYYRGSAAAVIVYDITKQDSFYTLKKWVKELKEHGPENIVMAIAGNKC 120
    ||||| |: ||||||||||||:|||||||:: | ||| |||||::||| |||:|||||||
 61 TAGQEAFRALAPMYYRGSAAAIIVYDITKEETFSTLKNWVKELRQHGPPNIVVAIAGNKC 120

121 DLSDIREVPLKDAKEYAESIGAIVVETSAKNAINIEELFQGISRQIPPLDPHENGNNGTI 180
    || |:|||  :|||:||:|| || |||||||||||:||| :||| ||  |::  :    :
121 DLIDVREVMERDAKDYADSIHAIFVETSAKNAININELFIEISRRIPSADANPPSGGKGF 180

181 KVEKPTMQSSRRCC  194
    |: :   :  | ||
181 KLRRQPSEPQRSCC  194
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Fig. 1. **(A)** Nucleotide and deduced amino acid sequence of human *rab22b* (GenBank accession No. U57091). The conserved GTP-binding domains I–IV are underlined. **Methods:** The 3′ portion of the cDNA was first obtained by RACE. Two primers, 22S1 (5′-CGGTTTCATTCATTGGCTCCCATG-3′) and 22S2 (5′-GCGTCTAGATCGAGGCTCAGCTGCAGCTG-3′) were used as external and nested primers, respectively, with a common 3′-RACE primer dTn 5′-CCTCTGAAGGTTCCAGAATCGATAGGAATTC$(T)_{18}$VN-3′ (V is A, C or G; N is A, C, G or T). The PCR was carried out at 94°C, 30 s; 65°C, 30 s; and 72°C, 90 s for 30 cycles on a Perkin Elmer Cetus DNA Thermal Cycler 480. The nested PCR product of 750 bp was cut with *Bam*HI and *Xba*I, cloned into pGEM11 (Promega), and sequenced. The human melanocyte cDNA library, constructed from melanocyte poly(A)$^+$RNA (1 μg) in lpCEV27 vector, was screened essentially as described (Miki and Aaronson, 1993). The probe was the 3′-RACE fragment (see above) excised from the vector by *Eco*RI digestion. Two clones were sequenced. **(B)** The alignment of human *rab22b* and canine *rab22*. Identical amino acids are indicated by vertical bars, similar ones by dots.

2. Experimental and discussion

2.1. *Nomenclature:* rab22b *and* rab30

Using degenerate PCR primers derived from the conserved GTP-binding domains II and III of *rab* genes, we identified in human melanoma cells 17 different partial cDNA clones of approximately 200 bp which encode small GTP-binding proteins of the rab family (D. Chen and W.A. Gahl, unpublished). While most of the sequences corresponded exactly to known *rab* sequences, two showed only partial sequence homology. One of these (Fig. 1A) had significant homology to canine *rab22* (Fig. 1B); hence, it was named *rab22b* in accordance with the currently accepted nomenclature system (Kahn et al., 1992). The other (Fig. 2) was predicted to encode a peptide containing all the conserved amino acids between rab GTP-binding domains II and III; it was tentatively named *rab30*.

2.2. *Determination of the 3′ ends of* rab22b *and* rab30

Since sequences between GTP-binding domains II and III are well conserved among all *rabs*, use of these sequences for probing a cDNA library or northern blot might result in cross-hybridization. Therefore, we performed 3′ rapid amplification of cDNA ends (3′-RACE) on the *rab22b* and *rab30* partial clones to isolate sequences sharing little homology among rab genes, i.e., sequences encoding the carboxy termini and 3′ untranslated regions. Using two gene-specific primers (nested PCR) and a common poly(T) primer for each gene, specific fragments of 750 bp and 1100 bp were obtained for *rab22b* and *rab30*, respectively. The sequences of both fragments were typical for *rab* genes, containing a GTP-binding domain IV (coding for a conserved ETSA sequence) and exhibiting the highly conserved Lys and Phe residues at predicted positions (Figs. 1A and 2). The *rab22b* sequence had a stop codon

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1     AAAATTGAAGCTGTGTAAATGAGTATGGAAGATTATGATTTCCTGTTCAAAATTGTTTTA
                              M  S  M  E  D  Y  D  F  L  F  K  I  V  L     14

61    ATTGGCAACGCTGGTGTGGGGAAGACGTGCCTCGTCCGAAGATTCACTCAGGGTCTTTTC
       I  G  N  A  G  V  G  K  T  C  L  V  R  R  F  T  Q  G  L  F     34

121   CCCCCAGGTCAAGGAGCCACAATTGGAGTTGGTTTTATGATTAAGACAGTGGAGATTAAT
       P  P  G  Q  G  A  T  I  G  V  G  F  M  I  K  T  V  E  I  N     54

181   GGTGAAAAAGTAAAGCTACAGATCTGGGACACAGCAGGTCAAGAGAGATTTCGGTCCATT
       G  E  K  V  K  L  Q  I  W  D  T  A  G  Q  E  R  F  R  S  I     74

241   ACCCAGAGTTACTACCGAAGCGCCAATGCCTTGATCCTCACCTATGACATTACCTGTGAG
       T  Q  S  Y  Y  R  S  A  N  A  L  I  L  T  Y  D  I  T  C  E     94

301   GAATCCTTCCGTTGCCTTCCTGAGTGGCTGCGGGAGATAGAACAATATGCCAGCAACAAG
       E  S  F  R  C  L  P  E  W  L  R  E  I  E  Q  Y  A  S  N  K     114

361   GTCATCACTGTGTTAGTGGGCAACAAGATTGACCTGGCTGAAAGGAGAGAGGTTTCCCAG
       V  I  T  V  L  V  G  N  K  I  D  L  A  E  R  R  E  V  S  Q     134

421   CAGCGAGCTGAAGAATTCTCAGAAGCTCAGGACATGTATTATCTGGAGACCTCAGCCAAG
       Q  R  A  E  E  F  S  E  A  Q  D  M  Y  Y  L  E  T  S  A  K     154

481   GAATCTGATAATGTGGAGAAACTCTTCCTTGACTTAGCATGCCGACTCATCAGTGAAGCC
       E  S  D  N  V  E  K  L  F  L  D  L  A  C  R  L  I  S  E  A     174

541   AGACAGAACACACTTGTGAACAATGTATCCTCACCCTTACCTGGAGAAGGAAAAGCATC
       R  Q  N  T  L  V  N  N  V  S  S  P  L  P  G  E  G  K  S  I     194

601   AGCTATTTGACTTGTTGTAATTTCAACTAAAGGCTGAGGCACGGAGAAGAAAAGGAATCA
       S  Y  L  T  C  C  N  F  N                                       203

661   GCAACTGCCCTGATGCGGCAATGAGATGCTGGGGAGATCTGGCGATGACTGTGGCTCCCG
721   CTCTCTGTCCTTCTGACTCCTGTGGGCTCCTGAGCTTACAAAGCATGGCAGGCCAAGGGC
781   TCGACCACAGGCCAGCATTAGCAGAACATATAATGGTTTCACCCTTTT
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Fig. 2. Nucleotide and deduced amino acid sequence of human *rab30* (GenBank accession No. U57092). The conserved GTP-binding domains I–IV are underlined. **Methods**: 3′-RACE was carried out as described in Fig. 1. The gene-specific primers were 30S1 5′-CCTTCCGTTGCCTTCCTGAGTG-3′ (external) and 30S2 5′-GCGTCTAGAGTGGCTGCGGGAGATAGAAC-3′ (nested). 5′-RACE was performed using the Marathon cDNA amplification kit (Clontech). Two rab30 specific primers used for nested PCR were 30A1 5′-GCTGGGAAACCTCTCTCCTTTCAGCCAGG-3′ (external) and 30A2 5′-CAGGTAATGTCATAGGTGAG-3′ (nested). The upstream primers, adaptor primer 1, and nested adaptor primer were provided by the kit. The PCR conditions were 94°C, 30 s; 55°C, 30 s; and 72°C, 15 s for 35 cycles. The PCR product (550 bp) was cloned into pCRIII (Invitrogen) and four independent clones were sequenced to rule out the possible misincorporation of nucleotide during PCR amplification.

35 amino acids from the invariable Phe and ended with a CC motif (Fig. 1A). The *rab30* sequence had a stop codon 40 amino acids from the Phe and ended with a CCAAX motif (Fig. 2). Both deduced sequences were in good agreement with the expected sizes and carboxy termini of known rab proteins.

2.3. *Isolation of the complete* rab22b *cDNA from a melanocyte library*

Using the 3′-RACE product of *rab22b*, a human melanocyte cDNA library was screened under stringent conditions. Of approximately 2 x 10^5 plaques, 7 positive clones having a 1.0-kb cDNA insertion were isolated and purified. The nucleotide sequences were determined for two clones, which showed an identical 582 bp ORF. The 3′ sequence corresponded to that determined by 3′-RACE.

The full length cDNA of *rab22b* and its deduced amino acid sequence is shown in Fig. 1A. The carboxy terminus has two cysteines, as for human rab1, rab2, rab9, rab10, and rab12. Of the two methionine codons at the beginning of the ORF, the second probably represents the true amino terminus of rab22b, since its surrounding sequence has a better match with the Kozak consensus sequence for a translation initiation site, i.e., an A at position −3 (Kozak, 1989). In addition, the canine rab22 only has one methionine at its amino terminus and shows close sequence homology to rab22b in the region of the second methionine (Fig. 1B). Hence, human *rab22b* has 194 amino acids with a calculated molecular weight of 21.6 kDa and a pI of 6.7. By this calculation, *rab22b* is the smallest human rab family member identified to date.

A homology search using the Blast program showed that *rab22b* shared 71% identity and 82% similarity with canine *rab22*, suggesting that the two sequences are indeed closely related. However, this identity is lower than that for many rab orthologues, which are often more than 90% identical. Consequently, we believe that our *rab22b* is probably not the human homologue of canine *rab22*, but an isoform of the human homologue of canine *rab22*. Among known human *rabs*, *rab22b* has the highest identity, 46%, with isoforms of the *rab5* subfamily (Table 1).

2.4. *Isolation of* rab30 *cDNA using 5′-RACE*

Attempts to isolate *rab30* cDNA from the melanocyte cDNA library, as well as from a human melanoma library (Clontech), yielded no positive clones. Therefore, 5′-RACE was performed using cDNA transcribed from total melanoma RNA as template. A fragment of 550 bp was obtained after nested PCR; sequencing showed it contained the 5′ portion of *rab30* cDNA.

The cDNA sequence and deduced amino acid sequence of *rab30* (Fig. 2) revealed a 612-bp ORF predicting a 203 amino acid protein with a molecular weight of 23 kDa and pI of 4.8. The amino acid sequence exhibited the characteristic features of rab proteins, including four conserved domains involved in GTP binding and hydrolysis, and a CCAAX isoprenylation motif at its carboxy terminus, similar to those of human *rab11* (Drivas et al., 1991) and *H-YTP3*, an isoform of *rab11* (Zhu et al., 1994). Using the Blast program, comparison of the rab30 amino acid sequence between residues 5 and 179 showed 52% identity and 73% homology with the first rab protein identified, yeast small GTP-binding protein YTP1. However, if the amino and carboxy termini were included, the overall identity dropped to 45%. Among known human *rabs*, *rab30* shares the highest homology (48%) with *S10* (Table 1), a distinct rab family member with preferential expression in cells derived from hematopoietic stem cells (Koda and Kakinuma, 1993). It also shows a similar level of identity with *rab1*, the human homologue of yeast *YTP1* (Zahraoui et al., 1989), and with *H-ray*, another human *rab* without a standard nomenclature which was initially isolated from skeletal muscle, but showed a ubiquitous expression pattern (Zhu et al., 1994) (Table 1). In order for a *rab* gene to be considered a new member of the family, it generally must exhibit less than 50% identity to other *rab* sequences, with the bulk of the homology clustered in the four GTP-binding regions. Using this criterion, *rab30* represents a new member of the family.

Table 1
Amino acid sequence comparisons of *rab22b* and *rab30* with known human rab proteins

	% Identity compared with	
	rab22b	*rab30*
Rab1	39	46
Rab2	37	39
Rab3a	31	39
Rab3b	32	38
Rab4	35	41
Rab5a	46	39
Rab5b	45	38
Rab5c	46	37
Rab6	35	39
Rab7	42	36
Rab8	37	44
Rab11	40	40
HYTP3	40	40
Rab13	34	42
Hray	36	47
Rab22b	100	37
Rab27a	35	35
Rab27b	35	39
Rab28	30	26
Rab30	37	100
S10	37	48

The sequences were compared using the GCG program.

2.5. Tissue expression

Human multiple tissue blots were probed using 3′-RACE fragments of *rab22b* and *rab30* to examine their tissue distribution. Both *rab22b* and *rab30* showed multiple transcripts, which have also been observed for some other *rab* messages (Zahraoui et al., 1989). *Rab22b* displayed a major transcript of 4.0 kb in all tissues and cell lines examined (Fig. 3A), including melanoma cells and fibroblasts. The ubiquitous expression was similar to that seen for canine *rab22* using a mouse RNA blot (Olkkonen et al., 1993). A minor transcript of 1.0 kb was also observed in tissues with relatively high expression levels, such as testis, ovary, lung and leukocytes (Fig. 3A). This is probably the form isolated by 3′-RACE. In leukocytes, however, the smaller message is abundant, exceeding the 4.0-kb transcript in density (Fig. 3A). The 4.0-kb transcript likely reflects a large 3′ untranslated region due to multiple polyadenylation signals, although we cannot eliminate the possibility that there exists an additional small coding region 5′ or 3′ to the 1.0-kb fragment.

Northern blot analysis of *rab30* using the 3′-RACE fragments showed several transcripts with roughly equal abundance (Fig. 3B). The sizes ranged from 1.7 kb to 11 kb. This multi-transcript expression pattern was detected in most of the lanes on the blots, although several tissues such as liver, lung, kidney and leukocyte showed much lower expression levels. The 1.7-kb message, which was weak on the multiple tissue blots but relatively abundant on a blot containing human fibroblast and melanoma RNA (Fig. 3B), was likely the form isolated by 3′-RACE. The origin of two large transcripts of 11 and 5.8 kb, which showed weakly on a blot

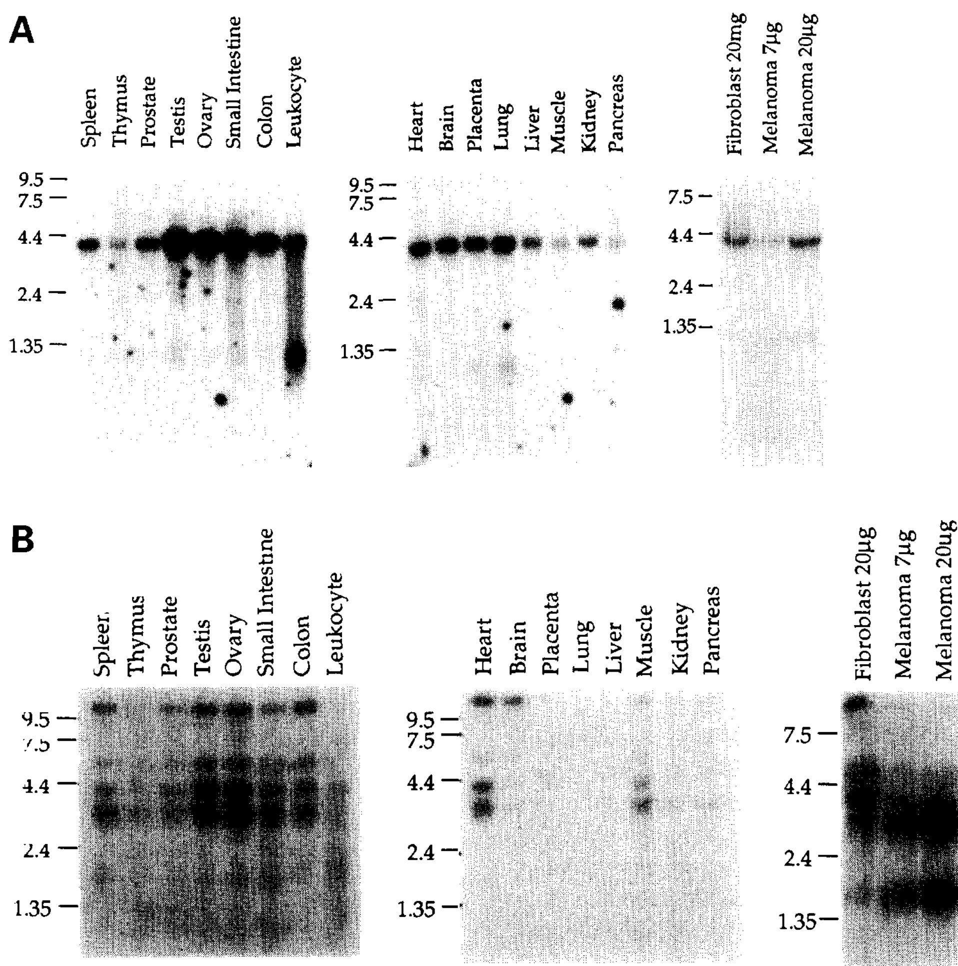

Fig. 3. Northern blot analysis of human *rab22b* (**A**) and *rab30* (**B**). The tissues and cells from which RNA was prepared are indicated above each lane. Nearly equivalent amounts of RNA were loaded, as demonstrated by similar band densities when probed with human elongation factor 2 partial cDNA (not shown). **Methods:** Total RNA was extracted from normal human fibroblast and pigmented melanoma cells (American Type Culture Collection) using RNAzol (Tel Test). The indicated amounts of RNA were fractionated on a 1% denaturing gel and transferred to Nytran membrane (Schleicher and Schuell). Human multiple tissue northern blots (Clontech) containing 2 µg of polyA$^+$ RNA in each lane were probed with the same fragments used for library screening at 68°C in ExpressHyb Solution (Clontech). The blots were washed with 2 × SSC, 0.1% SDS at 37°C for 30 min followed by 0.1 × SSC, 0.1% SDS at 60°C for 30 min. The blots were exposed to X-ray film for different lengths of time to obtain a proper image.

containing melanoma RNA, is unknown. The overall abundance of *rab30* was lower than that of *rab22b*, since a much weaker signal was obtained when the same blots were hybridized to equivalently labeled probes (data not shown).

PCR amplification was also performed, using two *rab30* specific primers 30S4 (5′-AGGAATCCTTCCGTTGCC-3′) and 30A3 (5′-CTGCCATGCTTTGTAAGCTC-3′), with cDNA transcribed from human melanocytes as template. (RNA prepared from human melanocytes was kindly provided by Dr. Raymond Boissy, University of Cincinnati College of Medicine). This PCR showed that *rab30* was also expressed in melanocytes (data not shown).

2.6. Chromosome mapping

The chromosomes on which the *rab22b* and *rab30* genes reside were identified using a single human chromosome/rodent somatic cell hybrid panel (National Institute of General Medical Sciences, Camden, NJ). Genomic DNA from all 24 hybrid cell lines (22 autosomes and the X and Y chromosomes) was amplified by PCR using primers selected from the 3′ untranslated regions of both cDNAs. The primers for *rab22b* were 22bS3 (5′-CCCATGAAAATGGAAACAATG-3′) and 22bA3 (5′-CATTTCATACACTAAAGGCC-3′). The primers for *rab30* were 30S3 (5′-CAACTAAAGGCTGAGGCAC-3′) and 30A3 (see above). A single band of expected size (300 bp for *rab22b* and 150 bp for *rab30)* was detected with both pairs of primers. *Rab22b* was mapped to chromosome 18 and *rab30* to chromosome 11. Chromosome band 19p13 may contain a cluster of *rab* genes, since both *rab3a* and *rab8* have been mapped to this region (Rousseau-Merck et al., 1989). Other *rab* genes are scattered on different chromosomes, but none on 11 or 18 (Rousseau-Merck et al., 1991).

3. Conclusions

(1) The complete cDNA sequences of two novel small GTPases of the rab superfamily were isolated from human melanocyte and melanoma cells. One of them, *rab22b*, is a distinct isoform of the previously documented canine *rab22*. The other rab cDNA encodes a novel member of the family, *rab30*.

(2) Neither rab is limited to melanocytes and melanoma cells. Most of the tissues examined expressed both *rab22b* and *rab30* mRNA with multiple transcripts and various expression levels.

(3) We speculate that these rab proteins are involved in vesicular membrane trafficking and fusion processes that are present in a variety of cell types.

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