Characterization of a protein tyrosine phosphatase (RIP) expressed at a very early stage of differentiation in both mouse erythroleukemia and embryonal carcinoma cells

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Abstract From our previous studies, several protein tyrosine phosphatases (PTPase) are implicated in the early events leading to in vitro differentiation of both mouse erythroleukemia (MEL) and embryonal carcinoma (F9) cells. Among the PTPases, recent experiments suggest that a new PTPase (RIP) plays a critical role in differentiation processes, particularly at their early stages. We isolated cDNA clones for RIP from a RNA preparation isolated from differentiating MEL cells, and determined the total 7932 bp base sequence for RIP cDNA. The cDNA codes for a putative 269.8 kDa (2450 amino acids) protein with a PTPase catalytic domain. We have demonstrated that the transcripts exist in multiple forms, and among mouse tissues they were found predominantly in kidney and, to a lesser extent, in lung, heart, brain and testis. The RIP gene was mapped between *D5Mit90* and *D5Mit25* on mouse chromosome 5.

Key words: Protein tyrosine phosphatase; Erythroleukemia cell; Chromosomal mapping; Differentiation

1. Introduction

Previously, we presented evidence that the dephosphorylation of phosphorylated tyrosine moieties of specific cellular proteins plays an important role in triggering differentiation of mouse erythroleukemia (MEL) as well as embryonal carcinoma (F9) cells [1–5]. Besides extensive dephosphorylation of tyrosine-phophorylated cellular proteins at a very early stage of differentiation [3], other experimental evidence for involvement of tyrosine dephosphorylation in cellular differentiation includes; induction of MEL and F9 cell differentiation by protein tyrosine kinase inhibitors [1], inhibition of differentiation by a specific inhibitor (Na_3VO_4) of PTPases [3], and lack of tyrosine dephosphorylation in differentiation-defective mutant cells [3]. Furthermore, we found that the level of transcripts for several specific PTPases sharply increases at an early stage of differentiation of MEL and F9 cells [4,5]. Interestingly, among four PTPase transcripts which displayed an increase, three of them were commonly induced during both MEL and F9 cell differentiation, despite the fact that these cells exhibit entirely different patterns of differentiation.

Among these PTPases, the induction of RIP, an apparently new PTPase, was most striking. RIP transcripts, which are usually present at a very low level in the cells, are induced at an early stage after addition of inducers. Furthermore, the induction was not observed in MEL and F9 mutant cells defective in differentiation [4,5]. In this paper, we report cloning of cDNA for RIP, its total base sequence, characterization and tissue distribution of the transcripts, and chromosomal location of the gene.

2. Materials and methods

2.1. Cells and cell culture

MEL (Friend) cells (745A) and differentiation-resistant MEL cells (Dif-1 and Dif-2) [4] were cultured at 37°C in a CO_2 (5%) incubator in minimal essential medium supplemented with 12% (v/v) fetal calf serum.

2.2. Northern blot analysis

Northern blot analysis was carried out as described previously [4]. A mouse multiple tissue Northern blot membrane (MTN, Clontech) was used for the analysis of the tissue distribution of the transcripts.

2.3. Cloning of RIP cDNA clones

RNA was isolated from MEL cells which had been incubated with Me_2SO for 24 h and poly(A)$^+$ RNA was then obtained by using Oligotex beads (Roche, Japan) as described before [4]. A cDNA library was constructed from the poly(A)$^+$ RNA using a ZAP cDNA synthesis kit (Stratagene). In essence, the poly(A)$^+$ RNA (7 μg) was annealed to an oligo dT primer (5′-GAGAGAGAGAGAGAGAGAGAACTCGAG-TTTTTTTTTTTTTTTT-3′) and the first and second cDNA strands were synthesized by MMLV reverse transcriptase (Superscript II; BRL), and by *E. coli* RNase H and DNA polymerase I. The double-stranded cDNA was blunted by T4 DNA polymerase, ligated to *Eco*RI adapters and phosphorylated. After *Xho*I digestion, the cDNA was purified by a Sephacryl S-400 column, cloned into Uni-Zap XR II vector and packaged using a Gigapack II packaging extract (Stratagene).

Phage clones were transferred onto nylon membranes (Hybond-N$^+$; Amersham) and hybridized with a 367 bp RIP (MTC) probe [6] which had been labeled with [^{32}P]dCTP (111TBq/mmol; ICN) using the randomly primed DNA labeling kit (Boeringer Mannheim) as described previously [4]. Inserts of several dozens of clones which gave positive signals by the probe were subjected to structural analysis and a clone (45-29) with the largest insert (~6850 bp) was selected. After partial sequencing of the insert, 5′-RACE was performed to obtain sequence information for the full-length RIP cDNA.

5′ RACE was performed by using the 5′-Amplifinder RACE kit (Clontech). Briefly, poly(A)$^+$ RNA (2 μg) was annealed to primer P3 (5′-CACTGCTGGATTCAAACCTC-3′) (nt. 1315–1296) and the first cDNA strand was synthesized by AMV reverse transcriptase. After alkaline RNA hydrolysis, cDNA was purified by Genobind suspension (Clontech) and ligated with an anchor (5′-GGAGACTTCCAAGGT-CTTAGCTATCACTTAAGCAC-3′) by T4 RNA ligase. The cDNA was then subjected to the first PCR. The primer sets used were an anchor primer (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAG-

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AATCGATAG-3′) and primer P2 (5′-CTCGCTCGCCCACTCGAGGAAATAACAGATGGGCTTTCACTG-3′) (nt. 1250–1227). The PCR conditions were: denaturation at 94°C (2 min), followed by 30 cycles at 94°C (1 min), 55C° (2 min) and 72C° (4 min), and the final incubation at 72°C for 7 min. Amplified DNA was electrophoresed on an agarose (1.5%) gel, extracted from the gel, purified and ligated to pGEM T vector (Promega). A clone with DNA extended to the furthest 5′ position (nt. 60) was selected and the DNA sequenced. A second PCR was then performed on the same first cDNA strand as a template using the anchor primer and primer P6 (5′-ATCCGTAAAGGACACACTGC-3′) (nt. 204–185) as described above to clone 5′ DNA sequences proximal to the first PCR products. Significant portions of the amplified DNA consisted of ~250 bp (or slightly less) sequences and they were cloned into pGEM T vector as for the first PCR products and their sequences were determined. We performed another round of 5′-RACE to find 5′ sequences further extended from the second PCR products, but no extended products were detected (data not shown).

2.4. DNA sequencing

The DNA sequences were determined for both strands by the dideoxy chain-termination method [7] with denatured double-stranded DNA as a template after constructing nested deletions. Sequencing was performed by using Sequenase sequencing kit (United States Biochemicals) and *Taq* Dye primer cycle sequencing kit (Applied Biosystems).

2.5. RT-PCR

RT-PCR was performed by using SuperScript pre-amplification system (Gibco BRL). cDNA was synthesized from total RNA (2 mg) using random hexamers, treated with RNase H and subjected to PCR. Primers used were P1 (5′-GTAGCAGTGAACAAAAACCTGACCGGAG-3′) (nt. 560–587), P2 (see above), P7 (5′-ACCCAAACTGCATAACACCT-3′) (nt. 2217–2238) and P8 (5′-CCTAAGCCGTGTCTTGGATC-3′) (nt. 3284–3265). PCR conditions were: denaturation at 94°C (3 min), followed by 35 cycles at 94°C (0.5 min), 55°C (1 min), 72°C (1.5 min) and 72°C (4 min).

2.6. Interspecific backcross mapping for the chromosomal positioning of the RIP gene

The 6.5-kb *Spe*I–*Kpn*I fragment was hybridized to *Taq*I-digested DNA from European Collaborative Interspecific Backcross (EUCIB). F1 females from the cross of two parental mice, C57BL/6 and *Mus spretuss*, were backcrossed to C57BL/6 or *Mus spretus* males. DNA from each backcross progeny mouse was scored for 3–4 microsatellite markers per chromosome, completing an anchor map of 70 loci across the mouse genome. A total of 36 mice were used to map the RIP gene.

Microsatellite marker primer sets (Map Pairs) were purchased from Research Genetics (Huntsville, AL) and used as described [8]. Both C57BL/6 and *Mus spretus* specific RFLP and sequence variations were followed in backcross mice. The informative RFLP was a 8.6-kb fragment of *Mus spretus* and 7.1-and 6.4-kb fragments of C57BL/6. Data were analyzed in the EUCIB database, Mbx.

Fig. 2 (A). For caption, see p. 236. →

3. Results and discussion

We have reported recently that several specific PTPase transcripts are induced at an early stage of in vitro differentiation of MEL as well as F9 cells [4,5]. The PTPase transcripts include those for RIP, PTPβ2, PTPε, and PTPμ. Among the PTPases, we concentrated our efforts on characterization of RIP, an apparently novel PTPase, because it exhibited the most drastic increase in transcript levels at an early stage of MEL as well as F9 cell differentiation while the transcripts were not detected (or barely detected) in mutant MEL and F9 cells defective in differentiation. In Fig. 1 we show the pattern of induction of RIP transcripts during erythroid differentiation of mouse MEL (745A) cells and their two mutant (Dif-1 and Dif-2) cells.

Employing the previously isolated RIP (originally termed MTC) cDNA fragment [6] as a probe, we isolated cDNA clones for a full-length RIP gene product from a poly(A)$^+$ RNA preparation isolated from differentiating MEL cells. We subsequently determined the total base sequence of RIP cDNA (to be submitted to GenBank/EMBL Data Bank). The cDNA consists of 7932 bp, which is consistent with the molecular size of the RIP transcripts (~8 kb) detected by Northern hybridization [4]. In the sequence, the initiation codon is present at the 41th nucleotide from the 5′ terminus, which is followed by a single

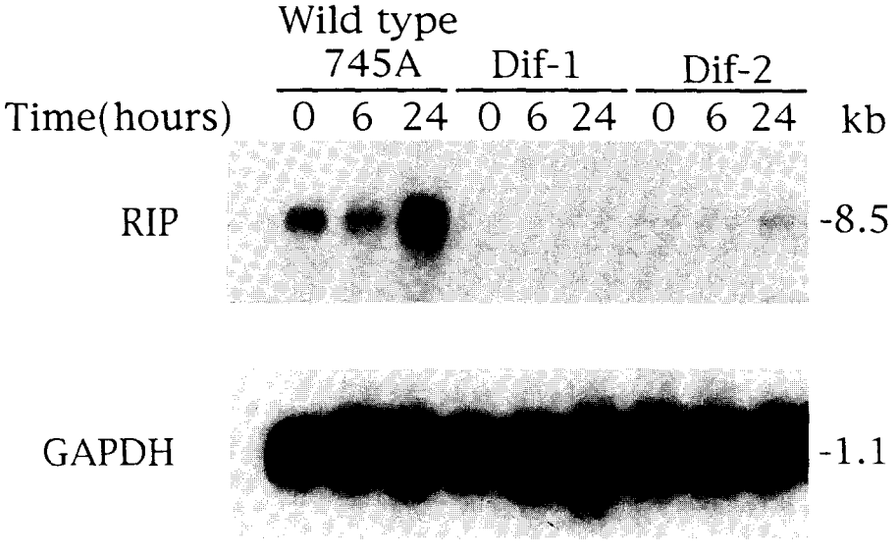

Fig. 1. Induction of RIP transcripts during MEL cell differentiation. Exponentially grown MEL cells were exposed to Me_2SO when the cell density reached 2–3 × 10^5 cells/ml. Aliquots (200 ml) were withdrawn at the time (hours) indicated and, after washing with cold PBS (2 times), the cells (~5 × 10^7 cells) were lysed and total RNA was prepared as described previously [4]. Poly(A)$^+$ RNA (2 μg) was electrophoresed on a formaldehyde-agarose (1%) gel, blotted onto a Hybond-N$^+$ membrane and hybridized with a 367 bp RIP (MTC) cDNA [6] and GAPDH (glyceraldehyde phosphodehydogenase) cDNA probes which had been labeled with [^{32}P]dCTP using a random priming kit as described previously [4].


A

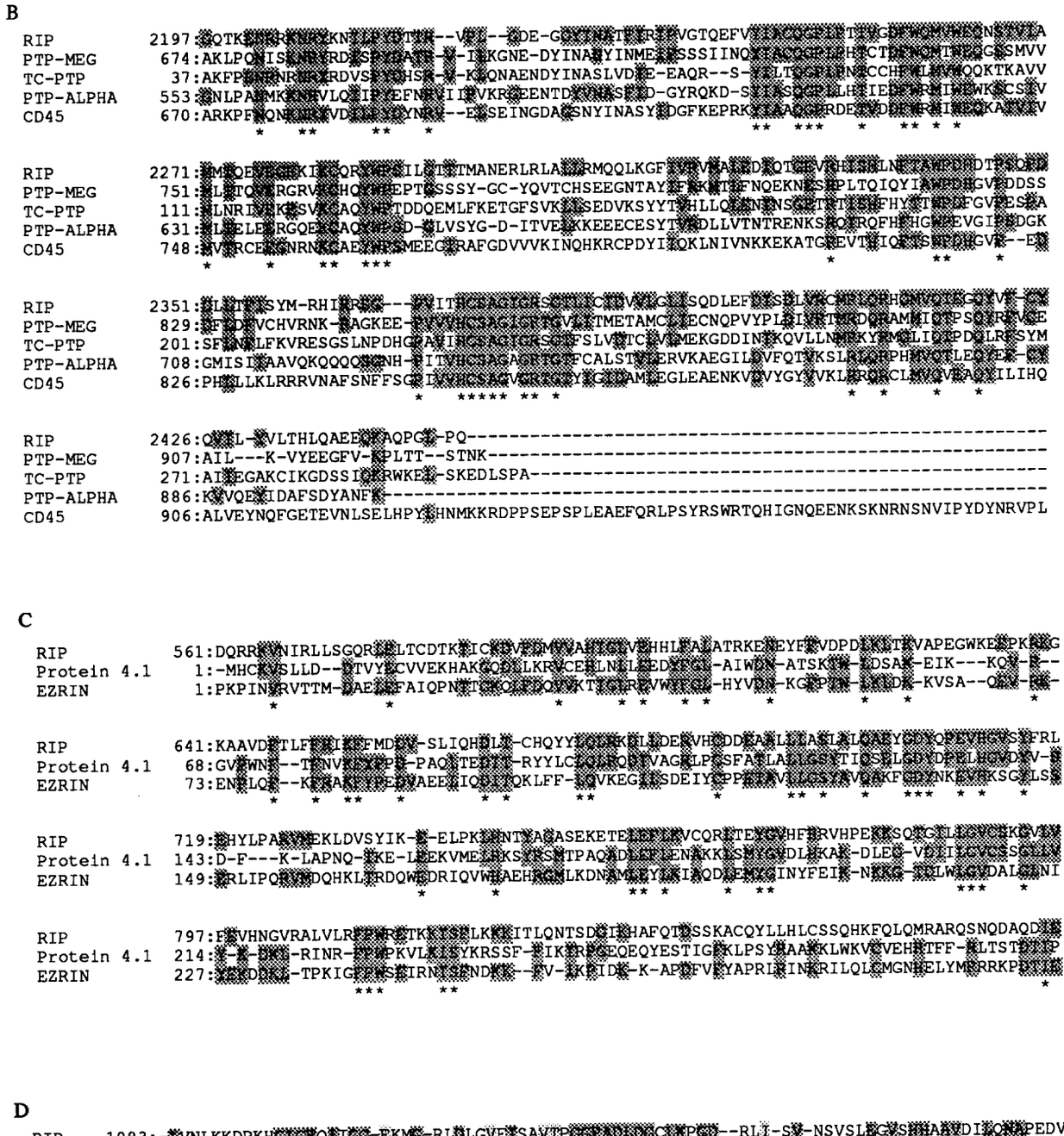

Fig. 2. The total base sequence of RIP cDNA and deduced amino acid sequence, and alignment of RIP with conserved domains in related proteins. (A) The total base sequence and predicted amino acid sequence in one letter code of RIP. The open box is the segment homologous to the N-terminal of protein 4.1 super family. The shaded box is the segment homologous to the catalytic domain of PTPases, in which the active site for PTPase activity is underlined by a bold line. GLGF repeats are underlined. (B) Proposed alignment of the PTPase catalytic domain of RIP with that of PTP-MEG (11), TC-PTP [12], PTP-ALPHA [13] and CD45 [14]. Conserved residues in these proteins are shaded. (C) Proposed alignment of the putative membrane binding domain of RIP and that of protein 4.1 [15] and Ezrin [16]. Conserved residues in these proteins are shaded. (D) Proposed alignment of GLGF repeats of RIP and that of psd-95 [17], dlg [18] and NOS [19]. Conserved residues in these proteins are shaded. Throughout A–D, the residue numbers of the initial amino acid of each PTPase are indicated at the left hand.

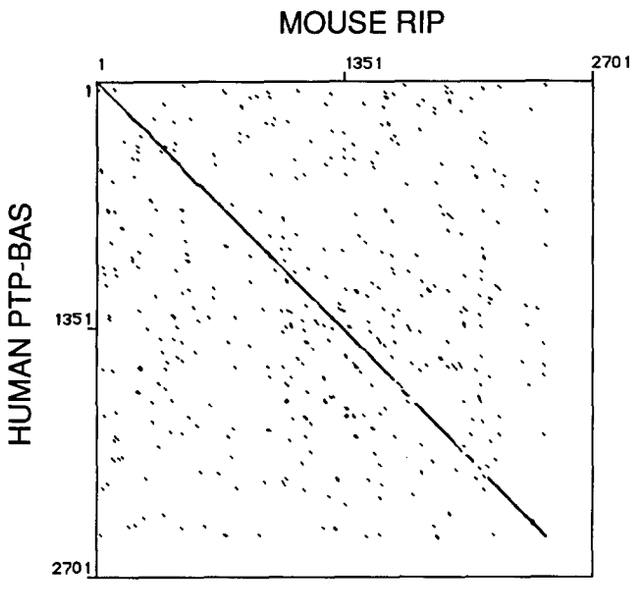

Fig. 3. Comparison of amino acid sequences of RIP and human PTP-BAS. Diagonal plots were made using the HarrPlot Program [10]. The axes are labeled with amino acid numbers where the amino acid at the N-terminus (methionine) is 1.

open reading frame (7350 bp). In Fig. 2A, we show the total base sequence of RIP cDNA and the deduced amino acid sequence with 2450 amino acid residues (MW of 269.8 kDa). An homology search (Swiss Plot Data Base) of the amino acid sequence revealed a typical PTPase catalytic domain at the position close to the C-terminus (amino acid 2198–2450), which is conserved among almost all PTPases [9,10] (Fig. 2B). No transmembrane domains were detected in the sequence, suggesting that RIP is a cytoplasmic PTPase. However, the search also revealed the presence of a domain at amino acid sequence 561–576 which is believed to be essential for the association of protein 4.1 and its super family (ERM family) members with transmembrane proteins [20] (Fig. 2C). This may indicate that RIP also associates with transmembrane protein(s) through this domain. The possible biological significance of the domain in RIP in the cell differentiation cascade will be discussed later. Another characteristic of RIP is the presence of a GLGF repeat, the function of which is still unknown (Fig. 2D).

We found that the base sequence of RIP has homology with the recently reported human PTPase (PTP-BAS, hPTP1E, PTPL1) cDNA [22–24] the biological function of which is yet to be understood. The homology of the amino acid sequence between RIP and human PTP-BAS (2485 amino acids) is 79.0%, and the comparison of the sequence is shown in Fig. 3.

In addition to the full-length cDNA clone we isolated several RIP cDNA clones the sequences of which were deleted at two specific places (sites A and B), as shown in Fig. 4A. To confirm the presence of mRNA species to these cDNA in the cells, we performed RT-PCR with the total RNA isolated from MEL cells. As shown in Fig. 4B, RT-PCR at site A gave two DNA bands of approximately 700 and 150 bp, respectively, which are explained by the presence of a RIP mRNA species spliced (544 bp) at site A. Similarly, at site B we also detected two RT-PCR products which were expected from the sequence analysis (1028 bp and 463 bp, respectively) (Fig. 4C). RT-PCR covering both of the A and B sites gave at least three bands of approximate molecular sizes of 2700, 2200 and 1600 bp, which should correspond to the expected molecular sizes of 2725 bp, 2182 plus

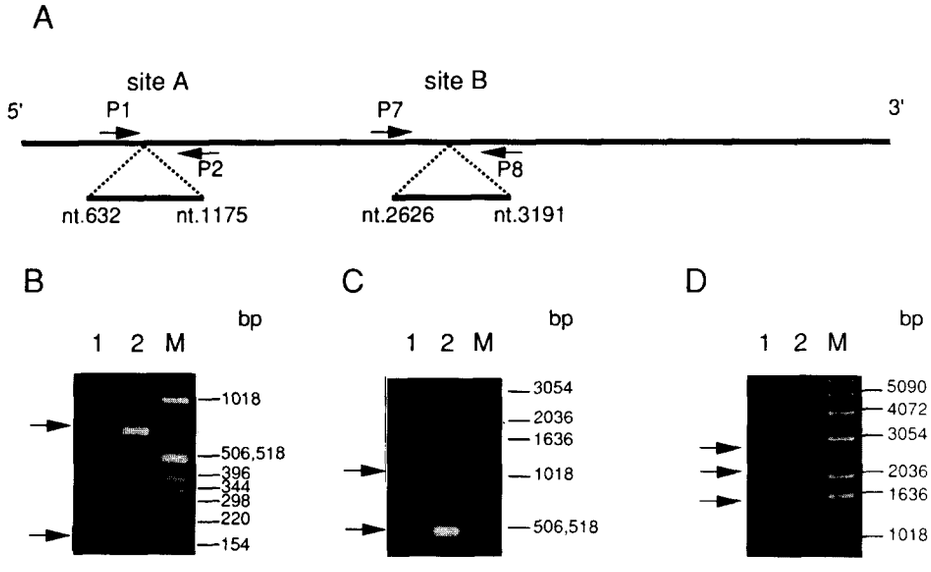

Fig. 4. Structures of RIP cDNA variants and analysis of RT-PCR products. (A) A diagrammatic representation of structures of RIP cDNA variants and the sites for PCR primers used for RT-PCR. The positions of site A and B on the full-length RIP cDNA (bold line) are shown along with the sites and directions of PCR primers used for RT-PCR (arrows). The nucleotide (nt) numbers are expressed as the number of nucleotides from the first base of the initiation codon (nt 1). (B–D) RT-PCR analysis of RIP transcripts in MEL cells. Total RNA (2 μg) from Me_2SO-treated (24 h) cells were subjected to RT-PCR as described in section 2. The amplified DNA was electrophoresed on an agarose gel (A, 2%; B, 1%; and C, 0.8%) and visualized by ethidium bromide staining. (B) PCR with primers P1 and P2. (C) PCR with primers P7 and P8. (D) PCR with primers P1 and P8. Because of the proximity of the molecular size, the bands corresponding to 2182 and 2159 bp (see text) can not be distinguished from each other and seen as a single band (the middle band) in the picture. Throughout B–D: lane 1, control (PCR without reverse transcription); lane 2, RT-PCR products; and lane M, DNA size markers (1 kb DNA ladder, BRL). Arrows indicate amplified DNA bands.

2159 bp and 1616 bp PCR products (Fig. 4D). These RT-PCR experiments indicate that mouse RIP transcripts exist in multiple forms. Interestingly, preliminary experiments indicated that the relative amount of each transcript was altered during MEL cell differentiation when the total RIP transcript level was sharply increased, as shown in Fig. 1 (data not shown).

We conducted Northern hybridization experiments to examine the distribution of the RIP transcripts among various mouse tissues. As shown in Fig. 5, the RIP transcripts were predominantly found in kidney and, to a lesser extent, in lung, heart, brain and testis. The transcripts were also present in RNA in the fetuses at day 9, 10, 11 and 12 postcortum (data not shown).

We attempted to determine the chromosomal location of the mouse RIP gene by analyzing 36 interspecific backcross DNA between two mouse species C57BL/6 and *Mus spertus*. Our initial analysis indicated that the RIP gene maps between *D5Nds2* and *D5Nds6* on chromosome 5. To locate the RIP gene more precisely on the chromosome, we selected 10 recombinants between *D5Nds2* and *D5Nds6* , which were further typed for *D5Mit30*, *D5Mit25*, *D5Mit240*, *D5Mit175* and *D5Mit90* loci. The genetic order of the loci and distances (± one standard error), determined by the haplotype analysis, are as follows: Cen------*D5Nds2*-8.3 ± 4.6*cM-D5Mit90*-2.8 ± 2.7*cM-RIP/D5Mit-175/D5Mit240*-5.7 ± 3.9*cM-D5Mi t25*-5.7 ± 3.9*cM-D5Mit30*-2.9 ± 2.8*cM-D5Nds6* . Thus, the RIP gene is located on mouse chromosome 5 between *D5Mit90* and *D5Mit25*, as diagrammatically presented in Fig. 6. Finer mapping of the RIP gene is now in progress to obtain information regarding a possible linkage between RIP and any human genetic disorders.

In this letter, we have reported the total base sequence of RIP cDNA and basic characteristics of the gene product. The putative RIP protein is quite large in size (269.8 kDa) with a PTPase catalytic domain close to the C-terminus. The protein does not have transmembrane domain(s), suggesting that RIP is involved in cellular differentiation through protein tyrosine dephosphorylation in the cytoplasm. As described above, RIP has another domain which is likely to be essential for the association of cytoplasmic proteins with transmembrane proteins. Although the role played by the domain in RIP in the cellular differentiation cascade must await further investigation, it is attractive to speculate that transmembrane signals generated by differentiation inducers such as Me_2SO (MEL cells) and retinoic acid (F9 cells) are transmitted to RIP through this domain. As a natural extention of the results presented here and previously, we are currently investigating whether differentiation of MEL and F9 cells can be triggered (without differentiation inducers) when transfected RIP cDNA with an appropriate promoter is inducibly expressed. It might be interesting to note that the previously reported proteinaceous differentiation inducing factor (DIF-II), which induces MEL cell differentiation upon introduction into the cells, is of cytoplasmic origin and has characteristics of a PTPase with MW of slightly over 250 kDa. [2,25].

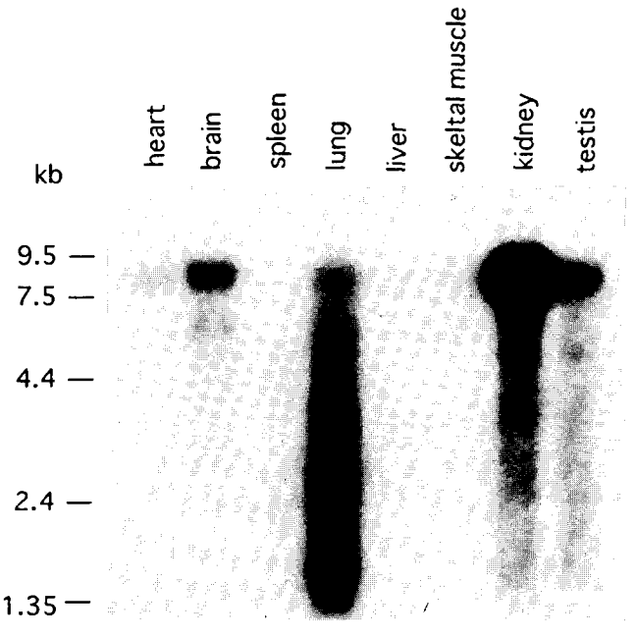

Fig. 5. Tissue distribution of RIP transcripts. A membrane immobilized with 2 μg of poly(A)$^+$ RNA from various mouse tissues (MTN; Clonetech) was hybridized with a 367 bp ^{32}P-labeled RIP (MTC) probe [6] and autoradiographed as described previously [4]. Positions of RNA size markers (kb) are indicated.

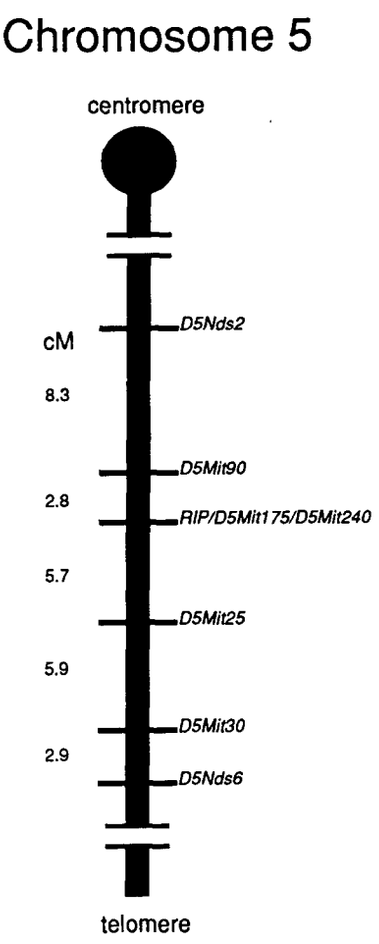

Fig. 6. Chromosomal location of RIP gene. Genetic distances are given in centimorgans (cM) and are shown for each pair of loci on the left of the chromosome map.

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