Chromosomal localization and expression pattern of the RNase L inhibitor gene

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Abstract 2-5A-Dependent RNase (RNase L), an important component of the 2-5A pathway, is directly implicated in the molecular mechanism of interferon action. We have cloned and sequenced following immunoscreening, a full-length cDNA that encodes the RNase L inhibitor (RLI). Northern blot analysis from a variety of human tissues revealed that two transcript forms (3.8 kb and 2.4 kb) are ubiquitously expressed but differences in levels of expression suggest a tissue-specific regulation. The RLI gene was localized to locus 4q31 by in situ hybridization indicating that this gene and other enzymes of the 2-5A pathway are not organized in cluster in the human genome.

Key words: RNase L inhibitor; mRNA expression; cDNA; Chromosomal localization

1. Introduction

Interferons (IFNs) constitute a family of polypeptides with pleiotropic biological effects. IFNs affect a wide variety of cellular functions by altering the expression of specific genes [1]. They are implicated in the regulation of cell proliferation and differentiation, and of the immune response [2,3]. Several genes, including those encoding for oligo 2-5A synthetases, are regulated by IFNs and involved in the antiviral response. These synthetases are activated by double-stranded RNAs, frequently found following viral infection, and convert ATP into unusual 5′-phosphorylated 2′,5′-linked oligoadenylates known as 2-5A [4]. This component activates a unique IFN-induced endoribonuclease, the 2-5A-dependent RNase (RNase L), capable of degrading both viral and cellular single-stranded RNAs [5,6]. The levels of RNase L and/or 2-5A synthetase vary with growth conditions, hormone status, liver regeneration and cell differentiation [7–10], suggesting a broader role for the 2-5A system in the general control of RNA stability.

Human and murine RNase L cDNAs have been cloned by means of ligand screening [11]. The predicted amino acid sequence encoded by this cDNA reveals a repeated phosphate loop motif (P-loop), a zinc finger motif homologue and similarities with protein kinases, zinc fingers and *Escherichia coli* RNase E. Interestingly, the duplicated P-loop has been shown to be involved in the binding of 2-5A by deletion analysis and site-directed mutagenesis [11]. It has been proposed that the 2-5A-dependent RNase is a heterodimer of a 2-5A binding and catalytic subunits [12]. However, on the one hand, study of expression of the human 2-5A-dependent RNase in insect cells using baculovirus as vector [13] has shown that both the 2-5A binding and catalytic domains are encoded by a single cDNA expressed as one polypeptide. On the other hand, a recent study has shown that the 2-5A-dependent RNase molecules dimerize during activation by 2-5A [14]. These findings suggest a highly complex molecular mechanism for the 2-5A system and the discovery of a new protein acting as a negative regulator of the RNase L (referred to RLI for RNase L inhibitor) has further increased the complexity of this pathway [15]. RLI inhibits binding of 2-5A to endogenous or in vitro expressed RNase L in a ratio-dependent and reverse manner. This inhibition results neither from competition for 2-5A nor from degradation of these components. In addition, RLI antagonizes the 2-5A-dependent nucleolytic activity of RNase L and overexpression of RLI in stably transfected HeLa cells inhibits both the binding of 2-5A by RNase L and the antiviral activity of IFN.

We report here the molecular cloning and the chromosomal localization of a human cDNA encoding the RNase L inhibitor. The study of RLI mRNAs expression has shown that two mRNA species of 3.8 and 2.4 kilobases (kb) are expressed in various human tissues with a tissue-specific regulation.

2. Materials and methods

2.1. Isolation and sequencing of cDNA

An MCF7 cell line λgt11 cDNA library was subjected to immunoscreening with the monoclonal antibody B1N (IgM), according to the method of Young et al. [16]. The sequence of the positive recombinant clone 3λgt11 isolated was performed using the Sanger dideoxy method (T7 sequencing kit, Pharmacia) and allowed us to identify a 235 bp fragment that was used to screen a λZAP II phage library prepared from HeLa cells (cDNA λZAPII and λgt11 libraries were a gift from Prof. Chambon, IGBMC, Strasbourg). From this second screening, six positive clones were isolated. The nucleotide sequence of each DNA insert was determined by primer walking on both strands.

2.2. Northern blot analysis

Human I and II multiple tissues Northern blots (MTN) were purchased from Clontech. Each lane contains a minimum of 2 μg of pure poly(A)$^+$ RNA from human tissues. Radiolabeled probes A or B were obtained by PCR from clone 8 and correspond either to the 3′ end or to the medium part of this clone. Blot hybridizations were performed according to the manufacturer's instructions. To normalize the mRNA levels, MTN blots were stripped and reprobed with rat GAPDH cDNA [17]. The amount of mRNA was quantitated by PhosphorImager System (Molecular Dynamics) [18] and were normalized with data from GAPDH hybridization.

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Abbreviations: IFN, interferon; RLI, RNase L inhibitor; bp, base pair(s); PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate-deshydrogenase; kb, kilobase(s); aa, amino acid(s); MTN, multiple tissues Northern blot.

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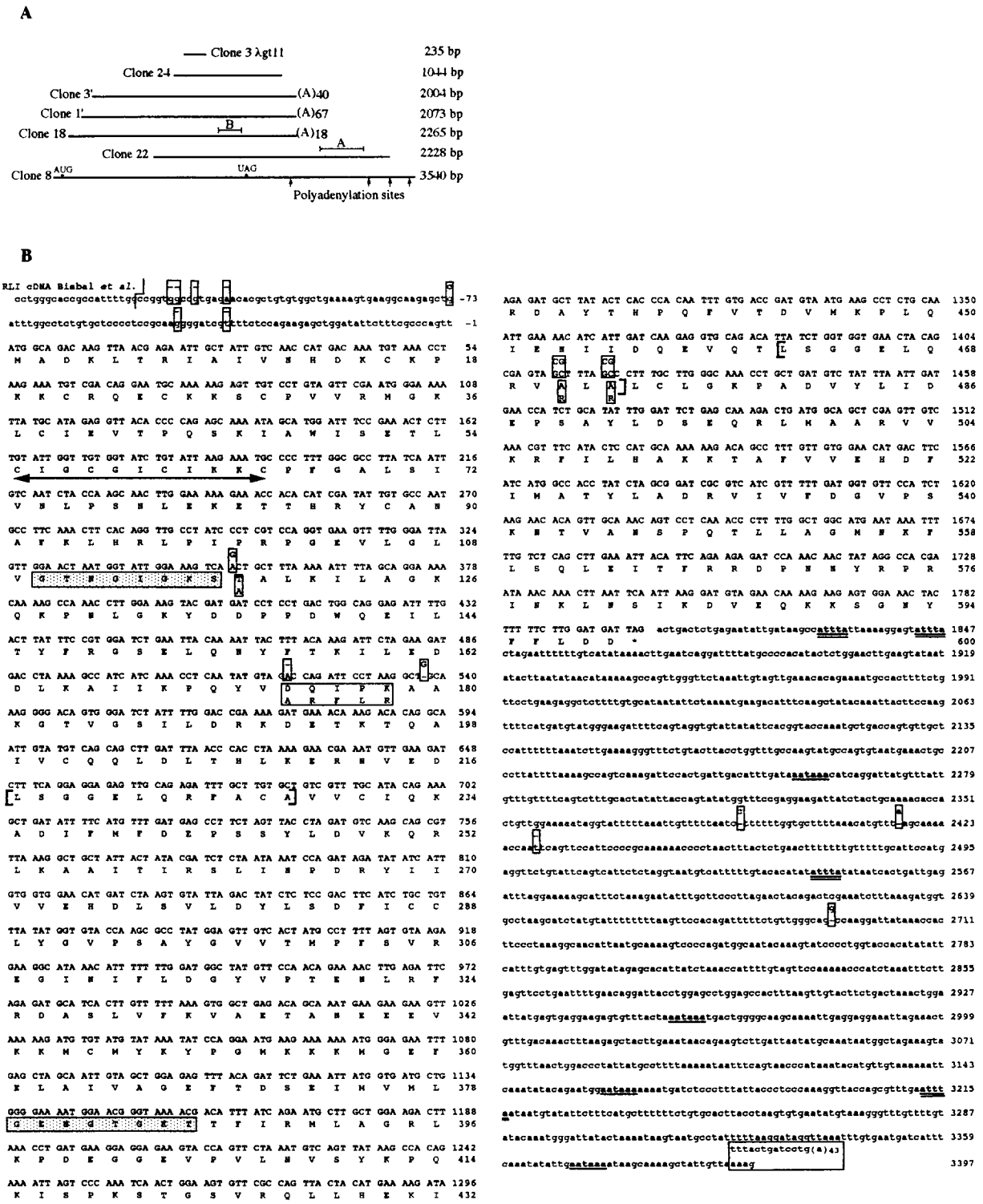

Fig. 1. Cloning of full-length RLI cDNA. (A) A first clone was isolated by immunoscreening of a λgt11 library and was designated as clone 3λgt11. Using the 235 bp radiolabeled cDNA corresponding to the clone 3λgt11 insert, six positives clones were isolated from a λZAPII library. Clones 1′, 3′ and 18 have polyadenylation tails of different length. Clone 8 is the largest and corresponds to the complete coding sequence of RLI cDNA. It contains the first in-frame ATG codon, a stop codon UAG (marked with asterisk) and four polyadenylation sites (indicated by arrows). Probes A and B, obtained by PCR from clone 8, are used to detect specific transcripts. (B) Clone 8 cDNA and the deduced amino acid sequence are aligned with RLI sequences. Nucleotides and amino acids indicated by open boxes correspond to differences with the sequence described by Bisbal et al. [15]. The position of nucleotides and amino acids is indicated by numbers on the right. Stuctural motifs include consensus sequence for iron–sulfur binding (delimited by arrow), the two P-loop motifs (indicated by shadowed boxes) and the duplicated consensus pattern of the ATP-binding proteins family (within brackets). Polyadenylation signal sequences are underlined and the ATTTA putative instability sequences are double underlined.

2.3. Southern blot analysis

Human genomic DNA was prepared from blood and digested with *Eco*RI or *Hin*dIII. Southern blots were hybridized with a α-^{32}P-labelled probe corresponding to clone 8. All these procedures have been performed as described by Sambrook et al. [19].

2.4. Chromosomal in situ hybridization

In situ hybridization was carried out on metaphase chromosome preparations according to our method previously described [20]. The clone 8 pBluescript was tritium-labeled and used as probe.

3. Results and discussion

3.1. Isolation and characterization of seven distinct overlapping cDNA clones

In order to identify a new proliferation-associated nuclear antigen, we screened a λgt11 expression library of the MCF7 cell line using a previously described monoclonal murine B1N [21,22]. Three different clones have been identified in the course of immunoscreening. One of these (clone 3 λgt11) was used to screen a λZAP library of the Hela cell line by plaque hybridization to obtain full-length cDNA. This second screening allowed us to isolate six additional independent positive clones.

Sequence analysis of the seven cDNA fragments has shown that they are overlapping (Fig. 1A). Three clones (3′, 1′, 18) have the same 3′ end displaying a stretch of respectively 40, 67 and 18 adenine residues, preceded by the putative polyadenylation signal AATAAA. The largest one, clone 8, comprises 3540 pb in length and possesses three other putative polyadenylation signals but no polyadenylation tail. This sequence contains a single open reading frame encoding a 599 amino acid (aa) protein with a calculated pI of 8.3 and a molecular weight of 67.3 kDa (Fig. 1B).

Quite recently, a similar cDNA sequence, differing from our cDNA sequence at some nucleotide positions in both the coding and non-coding sequences, has been reported by Bisbal et al. [15]. This cDNA encodes a potent inhibitor designated as RLI (for RNAse L inhibitor). Our cDNA is 20 nucleotides longer at the 5′-unstranslated end (Fig. 1B). The first in-frame ATG is preceded by an in-frame translational stop codon and is in a favorable Kozak environment [23], indicating that it corresponds to the translation initiation codon. This sequence has been confirmed in three other small independent clones isolated during a third screening (data not shown).

Sequence comparison of clone 8 and H2ABP cDNAs described by Bisbal et al. [15] reveals several nucleotide differences in the coding region at positions 352, 1411, 1412, 1417, 1418 in Fig. 1B, inducing amino acid substitutions at positions 118, 471 and 473. Futhermore, an additional A at position 521 and the absence of G at position 538 in clone 8 ORF explain the divergence of five amino acids residues (aa 174–178). Other differences are observed at the 5′ and 3′ untranslated ends. In particular, the last five nucleotides of clone 8 differ from those of clone H2ABP (Fig. 1B). Since we carefully checked our original sequence data on several overlapping clones, errors should be excluded in our sequence. The persistent discrepancies might reflect either natural polymorphism or a cloning artefact.

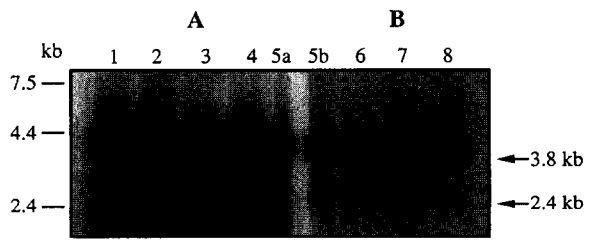

Fig. 2. Northern blot analysis of RLI transcripts. MTN I was hybridized with the α-^{32}P-labeled probe A (part A) or probe B (part B). The position of the RNA size markers is shown on the left side. Lanes: 1 = spleen; 2 = thymus; 3 = prostate; 4 = testis; 5a and 5b = ovary; 6 = small intestine; 7 = colon; 8 = peripheral blood leukocyte.

The predicted amino acid sequence contains a ferredoxins iron–sulfur binding region signature CX(2)-CX(2)-CX(3)-C-(PEG) [24,25] (aa 55–65 in Fig. 1B) and a P-loop consensus motif (G/AXXXXGKS/T) [26] at two positions (aa 110–117; 379–386), already mentioned by Bisbal et al. [15]. In addition, we found an ATP-binding site (aa 217–228) with the consensus pattern: (LIVMFY)-S-(SAG)-GX(3)-(RKA)-(LIVMYA)-X-(LIVMF)-(SAG). A duplicated form of this conserved region is also found at positions 462–473. A P-loop motif is often associated with this ATP-binding region in the ATP-binding cassette (ABC) of the transporter protein superfamily [27]. These proteins are involved in a variety of distinct biological processes, but most of them are involved in active transport of small hydrophilic molecules across the cytoplasmic membrane. However, they share a conserved domain of about 200 amino acids including the ATP-binding site. Despite the absence of this domain in RLI, we are faced with the question of the significance of the simultaneous presence of these duplicated sequences and of the P-loop motif. We hypothesize that this protein has an ATP-dependent activity. This idea could be compatible with the observation that ATP stimulates at least the 2-5A-dependent RNase activity, probably by stabilizing the active form of the enzyme [13].

3.2. Distribution of RLI mRNAs in various adult human tissues by Northern blot analysis

Poly(A)$^+$ mRNAs from various human tissues were analysed by using probes A and B (Fig. 1A). As shown in Fig. 2, probe B, the sequence of which is shared by clones 1′, 3′ and 18, detects two mRNA species of 3.8 kb and 2.4 kb, while probe A, specific for the 3′ end (clones 22 and 8), hybridizes only to the larger mRNA (see ovary lane in Fig. 2). Thus on the one hand, the cDNA determined from the 5′ end of clone 8 up to the 3′ end of clones 3′, 1′ and 18 probably corresponds to the small mRNA (2419 pb without the poly(A) tail), confirming that the first polyadenylation site (position 2255) is biologically active. On the other hand, clone 8 (3540 bp) most probably corresponds to the large 3.8 kb mRNA. We can conclude that the polyadenylation signal located 25 bp upstream of the 3′ end of clone 8 (position 3371) is also active in vivo, although no polyadenylation tail has been found on this clone. This location is in agreement with the position already described in eucaryotes cDNA [28].

To evaluate the expression level of the two specific mRNAs, multiple tissue Northern blots were successively analysed with PCR probe B and with a glyceraldehyde-3-phosphate deshydrogenase (GAPDH) probe as control. This enzyme is constitutively expressed in most tissues, except in fast twitch muscle where it can be induced to high levels [29]. This is the reason why values from heart and skeletal muscles have not been taken into account for comparative analyses.

As shown in Fig. 3A and B, both mRNAs (3.8 kb and

2.4 kb) are present in various tissues, but at different levels suggesting a transcriptional and/or post-transcriptional regulation. The 2.4 kb transcript is generally less abundant than the 3.8 kb, except in skeletal muscle and testis, where they have equivalent levels (Fig. 1A). Peripheral blood leukocytes, for example, show a lower 3.8 kb mRNA level and a dramatic decrease in 2.4 kb mRNA. Sequence data have shown that the two mRNAs differ in the length of their 3′-unstranslated end. There is evidence that the 3′-untranslated regions are involved in mRNA stability through secondary structure and protein–RNA interactions, both elements being also able to control translation initiation at the 5′ end [30–32]. Interestingly, 4 putative instability ATTTA sequences are detected in the 3′ untranslated end (Fig. 1B). Two of them are absent from the small transcript, suggesting differences in the decay rate of the two mRNAs. However, both transcripts encode the same RLI protein, the ratio difference between the two mRNAs and their biological and functional signification needs to be elucidated. It can be noted that the 3.8 kb mRNA is expressed to a lesser extent in non-proliferating tissues, such as brain, peripheral blood leukocyte and kidney, than in ovary and testis, known to have a high proliferative index. The pattern of 2.4 kb mRNA distribution is similar, except in testis, where 2.4 kb mRNA is overexpressed. In this tissue, high levels of both mRNAs are available for translation.

3.3. Genomic blot analysis and chromosome mapping

Southern blot analysis of human leukocyte genomic DNA was performed using α-^{32}P-labelled clone 8 cDNA in pBluescript vector as probe. Genomic DNA was digested with *Eco*RI or *Hin*dIII restriction endonuclease. Hybridization revealed 6 *Hin*dIII fragments and 10 *Eco*RI fragments of equal intensities indicating more likely a single copy gene per haploid genome (data not shown). As the whole cDNA probe has no such restriction site, RLI gene should be splat in at least ten exons. However, the existence of a second gene or a pseudogen cannot be fully excluded.

Chromosomal localization of the RLI gene was determined by in situ hybridization with clone 8 cDNA probe on metaphase chromosome spreads from phytohemagglutinin-stimulated human peripheral blood lymphocytes. In the 100 metaphases examined, 34 of 155 silver grains (21.9%) were located

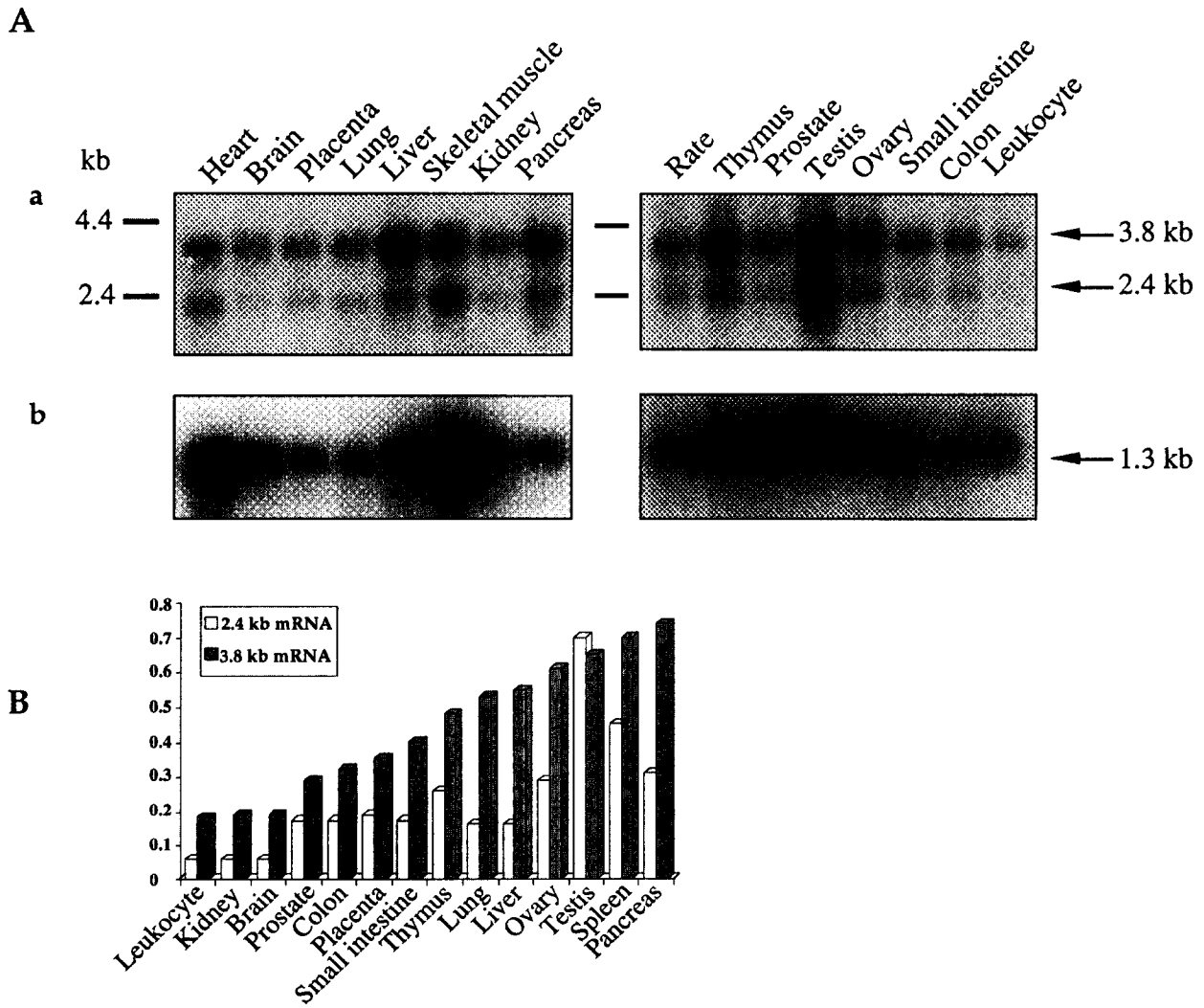

Fig. 3. Relative distribution of 3.8 kb and 2.4 kb mRNA in various human tissues by Northern blot analysis. (A) MTN I and II were hybridized with the α-^{32}P-labeled probe B (a) or with a GAPDH probe as normalizing control (b). (B) The resulting histogram represents relative abundance of RLI transcripts in growing expression order in various human tissues. These relative values were estimated from the ratio of data obtained with specific mRNA (3.8 kb or 2.4 kb) to GAPDH mRNA.

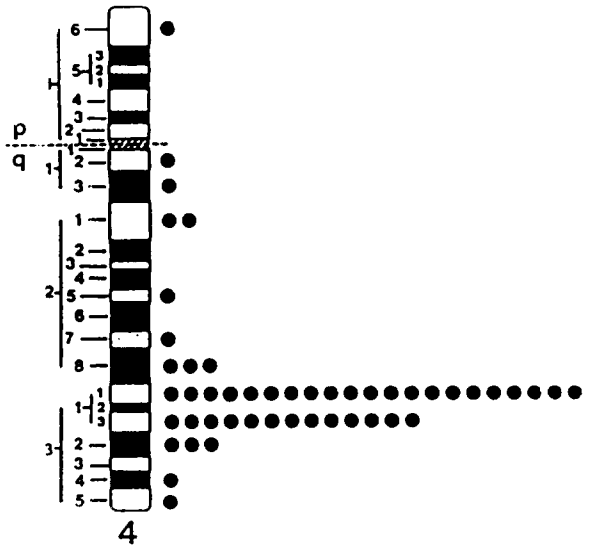

Fig. 4. Chromosomal localization of the RLI gene by in situ hybridization. Ideogram of the human G-banded chromosome 4 illustrating the distribution of labeled sites for the RLI gene probe with the maximum concentration of silver grains at locus 4q31.

on the long arm of chromosome 4 in the q31 region (Fig. 4). These results allow us to map the RLI gene to 4q31 band of the human genome.

Region q31 has been shown to be involved in translocation associated with a form of oculocutaneous albinism [33]. The RLI probe might constitute a physical mapping tool for chromosome 4 region q31 analysis. Because of the localization of the small forms of 2-5A synthetase in human chromosomes 11 [34] and 12 [35], and of 2-5A-dependent RNase gene RNS4 in human chromosome 1 [36], the genes for these different enzymes in the 2-5A system and RNase L inhibitor do not appear to form a gene cluster system in the human genome.

Immunoscreening with the previously described monoclonal antibody B1N allowed us to isolate and sequence several cDNAs. Surprisingly, these cDNAs correspond to three sets of mRNAs coding for three different proteins: HSP90 [37], an unknown protein bearing some similarities to the helicase family (manuscript in preparation) and RLI. To substantiate which one of the cDNAs encodes the authentic B1N protein, we are currently preparing polyclonal antibodies against a bacterially expressed part of each polypeptide deduced from isolated cDNAs. These antibodies are expected to show in immunoblot and immunohistochemical assays staining patterns identical to those obtained with monoclonal antibody B1N [21,22]. If the B1N antigen does indeed correspond to the RLI protein, polyclonal and especially monoclonal antibodies will constitute a usefull tool to study the role of RLI in the 2-5A pathway.

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