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Characterization of a Putative Clone for the 67-Kilodalton Elastin/Laminin Receptor Suggests That It Encodes a Cytoplasmic Protein Rather than a Cell Surface Receptor†

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ABSTRACT: The 67-kDa elastin binding protein shares many immunological and structural properties with the high-affinity 67-kDa tumor cell laminin receptor. Taking advantage of these similarities, we have screened a bovine cDNA library with a partial cDNA probe for the laminin receptor and have isolated and characterized a cDNA clone of 1038 bp that hybridizes to a single-size mRNA of 1.3 kb. The clone encodes a protein with a predicted molecular weight of 33K that lacks an N-terminal leader sequence, shows no posttranslational processing when translated in vitro in the presence of microsomes, and does not bind to elastin affinity columns. Although the bovine clone is nearly identical with clones encoding human and mouse proteins proported to be 67-kDa laminin receptor, physical and functional characteristics of the encoded protein suggest that it is a cytoplasmic protein that does not bind elastin. This finding calls into question the earlier conclusion that the clone encodes the 67-kDa receptor.

It is becoming increasingly evident that most extracellular matrix (ECM) macromolecules interact with specific binding proteins on the surface of cells. The best characterized receptors are members of the integrin family (Hynes, 1987), although other matrix binding proteins have been identified. One component of the extracellular matrix that lacks Arg-Gly-Asp (RGD) sequences and does not interact with integrin receptors is elastin, an abundant ECM component of elastic tissues (Mecham et al., 1989a). Instead, elastin associates with one or more cell surface proteins that exhibit properties of typical receptors as assessed by high-affinity binding that is both saturable and reversible (Blood et al., 1988; Hornebeck et al., 1986; Mecham et al., 1989a). One of these elastin receptors is a 67-kDa protein that is structurally and functionally similar to, if not identical with a high-affinity 67-kDa laminin receptor (Graf et al., 1987; Lesot et al., 1983; Rao et al., 1983; Terranova et al., 1983).

cDNA clones thought to encode the 67-kDa protein have been identified and characterized. Using a monoclonal antibody generated against the 67-kDa laminin binding protein, Wewer et al. (1986) isolated a partial clone (clone pLR4-4) from a human umbilical vein endothelial expression cDNA library. This partial sequence was then used to select full-length clones from a transformed murine fibroblast cDNA library [clone pMLR21 (Rao et al., 1989)]. Yow et al. (1988) characterized a human cDNA clone, J9, selected because of its increased expression in colon carcinomas. J9 was found to have extensive homology with the mouse cDNA, suggesting that receptor sequences are highly conserved between species.

Because the 67-kDa elastin binding protein exhibits immunological and functional similarity to the laminin receptor, we used a partial cDNA clone to the laminin receptor to screen a cDNA library made from mRNA isolated from bovine ligamentum nuchae. We felt that there was a high likelihood that selected hybridizable clones would be the elastin receptor because the predominant cell type in the ligament is a fibroblast that produces elastin but not laminin. In this report, we describe the isolation, characterization, and DNA sequence of a bovine clone that hybridizes with the putative laminin receptor probe. One clone is highly homologous to the laminin receptor clones, but functional and physical characteristics suggest it encodes a cytoplasmic protein that lacks a transmembrane domain. This protein neither binds elastin nor undergoes posttranslational processing that might account for the difference in size between the encoded protein and the isolated elastin/laminin receptor.

Materials and Methods

Materials. T_4 DNA ligase, T_4 DNA polymerase, exonuclease III, SP6 RNA polymerase, pGEM-4Z, rabbit reticulocyte lysate, canine pancreatic microsomes, and mRNAs encoding the *Escherichia coli* β-lactamase and *Saccharomyces cerevisiae* α-factor were obtained from Promega (Madison, WI). *Eco*RI, *Bam*HI, Klenow DNA polymerase, and DNA polymerase 1 were from New England Biolabs (Beverly, MA).

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dNTP's and ddNTP's were from Pharmacia (Piscataway, NJ). Affigel-10 was purchased from Bio-Rad (Richmond, CA). [α-^{35}S]dATP (1250 Ci/mmol), [^{35}S]methionine (800 Ci/mmol), and Gene Screen$^+$ were purchased form DuPont (Wilmington, DE), and [α-^{32}P]dCTP (3000 Ci/mmol) was from ICN (Leslie, IL).

Library Screening and Subcloning. A λgt-11 library constructed from RNA isolated from neonatal calf ligamentum nuchae RNA (Grosso et al., 1991) was plated on *E. coli*, strain Y1088, at approximately 50 000 plaques per plate (150 cm) and screened by plaque hybridization using as a probe the 650 bp insert of the proposed human laminin receptor clone pLR4-4, which had been labeled with ^{32}P by nick translation. DNA was isolated from plaque-purified bacteriophage by plate lysis and DE-52 column chromatography (Helms et al., 1985). Restriction enzyme digestion, agarose gel electrophoresis, Southern and Northern blotting, and DNA subcloning were performed by standard protocols (Maniatis et al., 1982). The plasmid vector used for subcloning was pGEM-4Z.

Probe Isolation, Radioactive Labeling, and DNA Sequencing. Inserts of the partial cDNA clone of the human laminin receptor, pLR4-4 (Wewer et al., 1986), and clone C10 selected from our ligament library with pLR4-4, were excised by restriction enzyme digestion from plasmid vectors and gel-purified. Nick-translation of DNA in the presence of [α-^{32}P]dCTP to a specific activity of $>10^8$ cpm/μg of DNA was by the method of Rigby et al. (1977). Nitrocellulose membranes were hybridized at 65 °C in 5× SSC, 0.1% SDS, 100 μg/mL DNA, 0.02% BSA, 0.02% Ficoll, and 0.02% poly(vinylpyrrolidone) and washed in 2× SSC/0.1% SDS at 55 °C. GeneScreen$^+$ membranes were hybridized and washed as recommended by the manufacturer.

Dideoxy plasmid sequencing was performed using [α-^{35}S]dATP and synthetic primers corresponding to the T7 and SP6 RNA polymerase binding sites of pGEM-4Z. Internal sequences of the cDNAs were obtained by subcloning of appropriate DNA restriction fragments, *Exo*III/S1 nuclease digestion (Henikoff, 1984), and the use of internal DNA primers. RNA was synthesized in vitro with bacteriophage SP6 RNA polymerase under the manufacturer's recommended conditions.

In Vitro Transcription/Translation and Affinity Chromatography. A rabbit reticulocyte lysate translation system with [^{35}S]methionine was used for cell-free translation of the synthetic RNA (Grosso & Mecham, 1988). To assess post-translational modifications, canine pancreatic microsomes were added to selected translation reactions (Grosso & Mecham 1988). Alkaline extraction, immunoprecipitation, and SDS–PAGE analysis of the translation products were as previously described (Grosso & Mecham, 1988). To determine the ability of the canine pancreatic microsomes to incorporate and process translation products, two in vitro produced RNAs were translated in parallel reactions. Translation of *E. coli* β-lactamase and *S. cerevisiae* α-factor was used to assess the ability of the microsomal preparation to cleave signal sequences and glycosylate proteins, respectively. Changes in the electrophoretic mobility of the proteins when translation was carried out in the presence of microsomes were considered evidence of microsomal incorporation and posttranslational processing.

Elastin affinity chromatography was performed by incubating the products of cell-free translation with either elastin peptide–Affigel-10 or ethanolamine-blocked Affigel-10 for 1 h at room temperature with constant agitation (Mecham et al., 1989a). After being extensively washed with buffer, the retained proteins were identified by boiling the column matrix in gel sample buffer followed by SDS–PAGE as described (Mecham et al., 1989a).

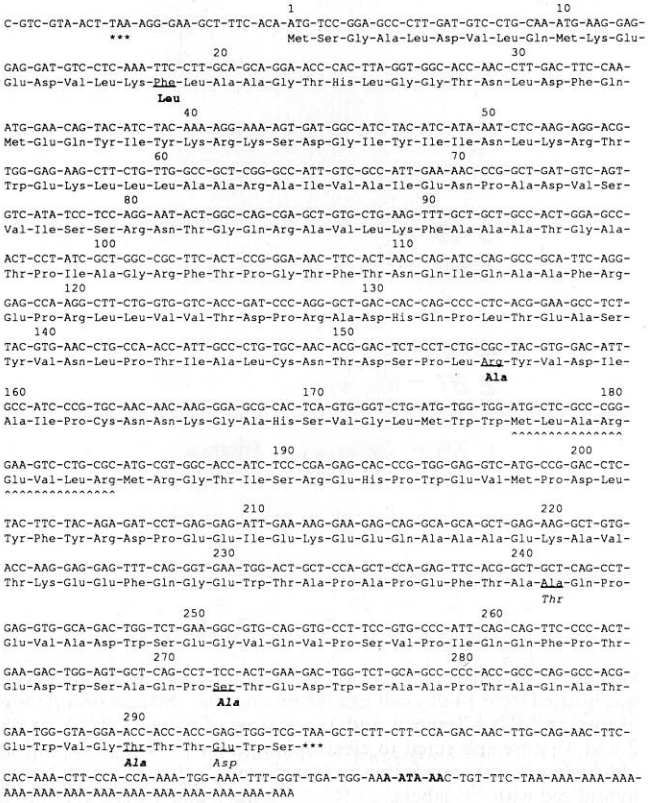

FIGURE 1: Structure of the C10 clone. DNA sequence with the predicted amino acid sequence of the long open reading frame. In-frame stop codons are indicated by three asterisks. The polyadenylation signal is in boldface. Amino acids different from those of the mouse or human cDNAs are underlined; beneath them in boldface are the mouse amino acids, and italicized are the human amino acids (Rao et al., 1989; Yow et al., 1988). The amino acid sequence obtained from CNBr-cleaved purified human placental laminin receptor is indicated by circumflexes (Wewer et al., 1986).

Results

Characterization of the Nuchal Ligament Library and Isolation of C10 Clone. Screening 3.5×10^5 plaques (approximately 10% of the neonatal calf ligamentum nuchae library) with pLR4-4 laminin receptor clone resulted in 200 positive plaques. Forty-three were purified to homogeneity by repeated screenings, and DNA from each bacteriophage was analyzed by agarose gel electrophoresis after *Eco*RI digestion. cDNA inserts in five of the larger clones were subcloned into pGEM-4Z at the *Eco*RI site. On the basis of sequences at the termini and the relative insert sizes, clone C10, having an electrophoretic mobility of 1.1 kbp, was selected for further study.

The complete sequence of C10 is shown in Figure 1. The cDNA is 1038 bp and contains a single long open reading frame of 885 bases. This open reading frame begins with a methionine and is preceded by an in-frame stop codon. A consensus polyadenylation signal sequence and a small poly(A) region are found at the 3′ end of the insert. These findings establish the orientation of the clone with respect to the mRNA and indicate that the entire coding sequence is contained within C10. The long open reading frame encodes a protein of 295 amino acids with a predicted molecular weight of 33K. Interestingly, neither an N-terminal hydrophobic signal peptide structure nor N-linked glycosylation sites (Asp-X-Ser/Thr) are present. C10 demonstrated at least 88% identity at the nucleotide level with previously reported mouse and human

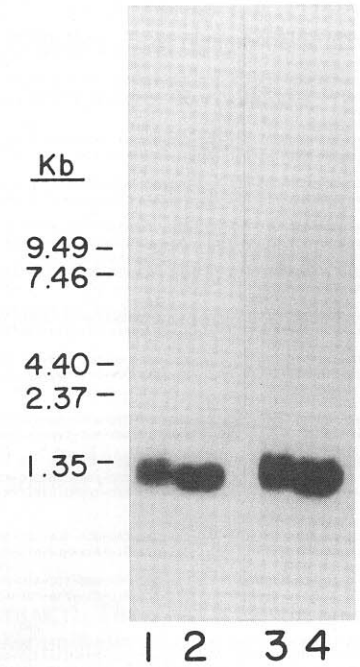

FIGURE 2: Northern analysis of ligamentum nuchae RNA. RNA was isolated from 14-day calf ligamentum nuchae. Sixteen micrograms of total cell RNA (lanes 1 and 3) and 3 μg of poly(A) RNA (lanes 2 and 4) were subjected to electrophoresis in a 1% agarose–formaldehyde gel and transferred to GeneScreen$^+$. Lanes 1 and 2 were hybridized with ^{32}P-labeled pLR4-4. Lanes 3 and 4 were hybridized with ^{32}P-labeled C10. RNA size standards are 9.49, 7.46, 4.40, 2.37, and 1.35 kb (from top to bottom).

laminin receptor clones (Rao et al., 1989; Yow et al., 1988).

C10 and pLR4-4, in Northern blot analysis of total cell and poly(A)-containing RNA isolated from bovine ligamentum nuchae, detect a single, abundant RNA species (Figure 2) that migrates on formaldehyde–agarose gels with a mobility of 1.3 kb. Since most mammalian mRNAs have poly(A) tails between 200 and 300 bases, the size of C10 is consistent with it being essentially a full-length cDNA clone.

In Vitro Translation of C10 RNA. Because the predicted size of the C10 protein is significantly smaller than the 67-kDa receptor, we utilized in vitro translation in the presence of dog pancreas microsomes to determine whether the molecular weight difference was due to posttranslational processing. This in vitro system also allowed us to investigate whether the C10 protein associates with the microsome as a transmembrane protein as has been postulated by Rao et al. (1989). C10 RNA was synthesized from the pGEM-4Z construct with SP6 RNA polymerase and, on formaldehyde–agarose gel electrophoresis, migrated as a single band with an electrophoretic mobility of 1 kb (data not shown). While translation reactions lacking mRNA produced multiple faint protein bands, translation of C10 RNA in the reticulocyte lysate system produced a [^{35}S]methionine-labeled protein with mobility on high-resolution SDS–PAGE of 39 kDa (Figure 3). Consistent with the absence of both a "classic" signal peptide and known glycosylation sites, no change in electrophoretic mobility was detected when pancreatic microsomes were present in the translation reaction (Figure 3). Furthermore, less than 3% of the total C10-incorporated [^{35}S]methionine sedimented with the microsomal fraction after centrifugation (Figure 3), suggesting that C10 protein does not associate with microsomes as a nonintegral protein. The inability of C10 protein to associate with microsomes was confirmed by showing that no C10-associated ^{35}S counts sedimented with the microsomal pellet following extraction with 0.1 M $NaCO_3$ (pH 11.5),

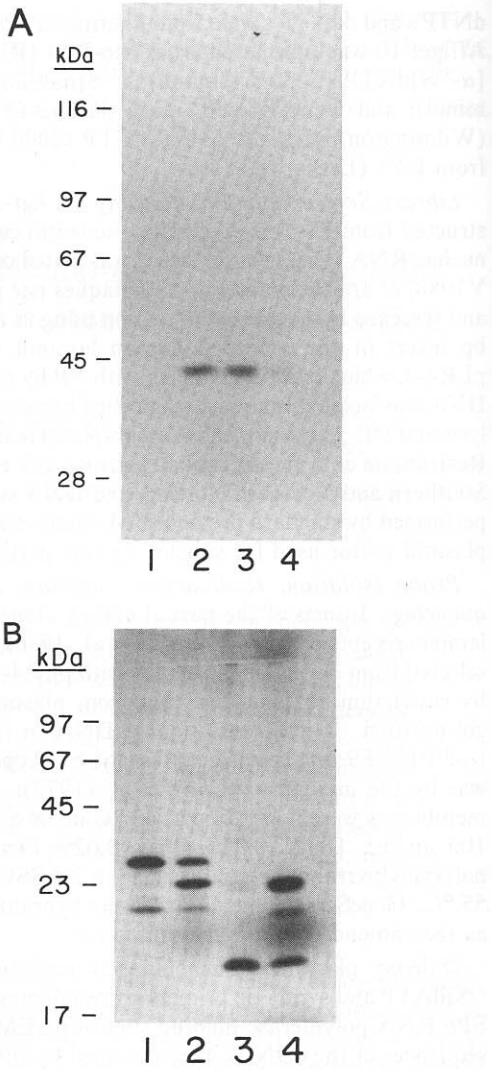

FIGURE 3: SDS–PAGE analysis of C10 RNA cell-free translation products. RNA was translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. Autoradiogram of 10% acrylamide gel. (A) RNA coding for C10 protein. Identical volumes of the translation reactions were used in each lane. Lane 2 is the reaction products in the absence of canine microsomes. Lane 3 is the total translation reaction products in the presence of microsomes. Lane 4 is the products associated with the isolated microsomes. Lane 1 is the products of alkaline-extracted isolated microsomes. (B) RNA coding for β-lactamase, a protein with a cleavable signal significance (lanes 1 and 2), and RNA coding for α-factor, a protein with N-linked glycosylation sites (lanes 3 and 4). Canine microsomes were added to reactions for lanes 2 and 4. Total reaction products were subjected to electrophoresis.

conditions which remove nonintegral but not integral membrane proteins (Mostov et al., 1981). Confirmation that the microsome preparation was capable of processing appropriate proteins was obtained by demonstrating cleavage of the signal peptide from β-lactamase (Figure 3B, lanes 1 and 2) and glycosylation of α-factor (Figure 3B, lanes 3 and 4).

Affinity Chromatography with C10 Protein. To determine whether the C10 protein was capable of binding elastin, C10 protein (translated either in the presence or in the absence of pancreatic microsomes) was incubated with immobilized elastin peptides. Under conditions shown to produce optimal binding of native receptor, no preferential binding of C10 protein was identified (Figure 4). A small degree of nonspecific binding to the Affigel support was evident, but this constituted less than 5% of the total C10 protein applied to the column. Total specific binding was not enhanced by increasing the NaCl concentration to 100 mM or by the addition

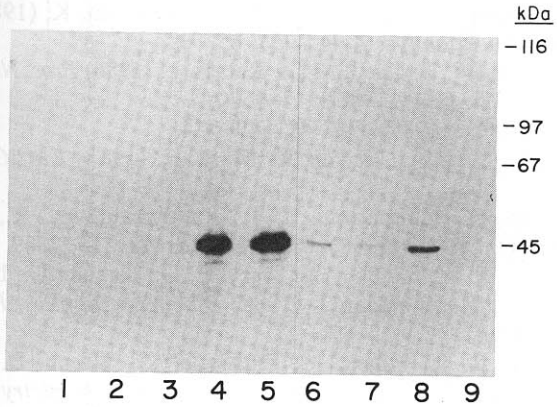

FIGURE 4: Affinity chromatography of in vitro translation products. Autoradiogram of a gradient PAGE gel showing proteins retained on elastin affinity columns. Affigel-10 blocked with ethanolamine served as a control resin for assessing nonspecific binding. Lanes 1–4 are from reactions containing microsomes; lanes 5–9 are translation reactions without microsomes. Lanes 1 and 6 are translation products that bound to elastin–peptide–Affigel. Lanes 2 and 7 are translation products incubated with elastin–peptide–Affigel in the presence of 10 μg/mL elastin–peptides. Lanes 3 and 8 are translation products incubated with ethanolamine–Affigel. Lane 9 is translation products incubated with elastin–peptide–Affigel in the presence of 0.1% Triton X-100. Lanes 4 and 5 show the total in vitro translation product used in each binding reaction.

of divalent cations (either 10 mM $MgCl_2$ or 10 mM $CaCl_2$), BSA (20 μg/mL), or Triton X-100 (0.1%) (data not shown).

Immunoreactivity of C10 Protein with Receptor Antibodies. The immunological relationship of C10 protein to the 67-kDa elastin receptor was investigated by using two antibodies that react with the 67-kDa protein: a mouse monoclonal antibody (BCZ_{67}) to the 67-kDa elastin receptor (Mecham et al., 1988), and a polyclonal antibody (A 14.5) raised to a small molecular weight (14K) galactoside binding lectin that was found to cross-react with several galactoside lectins including the elastin receptor (Cerra et al., 1984; Hinek et al., 1988). As shown in Figure 5, attempts to immunoprecipitate C10 protein with either antibody following cell-free translation were unsuccessful.

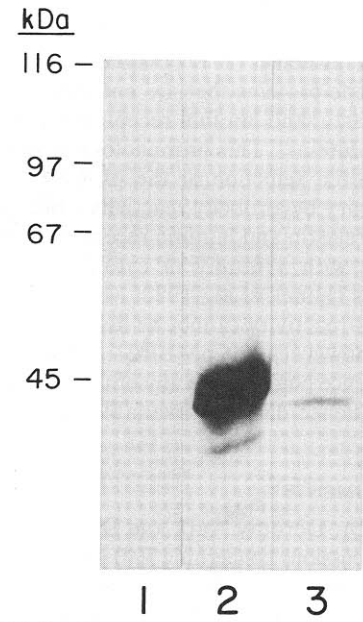

FIGURE 5: SDS–PAGE analysis of immunoprecipitated in vitro translation products. RNA coding for C10 protein was translated in the presence of [^{35}S]methionine. Autoradiogram of a 10% acrylamide gel. The reaction products were immunoprecipitated with A14.5, a polyclonal anti-galactoside lectin antibody (lane 1), or BCZ_{67} (lane3). Lane 2 is the amount of translation products used for immunoprecipitation.

Discussion

The elastin receptor mediates cell recognition of tropoelastin and proteolytic fragments of elastin (Mecham et al., 1989a; Senior et al., 1984) and, in cells that produce elastin, may function as a matrix assembly protein by directing the incorporation of newly synthesized tropoelastin into extracellular elastic fibers (Hinek et al., 1988). The 67-kDa receptor is a bifunctional protein with a binding site for elastin and a lectin site that regulates the affinity of the receptor for its protein ligands (Hinek et al., 1988; Mecham et al., 1989a). The 67-kDa protein also interacts with laminin and shares immunological determinants with the 67-kDa human laminin receptor (Mecham et al., 1989b).

To more fully characterize the 67-kDa elastin receptor, we have undertaken the molecular cloning of this protein. Our experimental approach was to take advantage of similarities between the bovine elastin binding protein and human laminin receptor and use a putative laminin receptor probe to screen a cDNA library made from bovine ligamentum nuchae mRNA. The clone with the longest open reading frame (C10) demonstrated at least 88% identity at the nucleotide level with previously reported mouse and human laminin receptor clones (Rao et al., 1989; Yow et al., 1988). When one considers the amino acid sequence, the identity increases to greater than 98%, with the majority of amino acid changes conservative in nature. The Arg-Ala substitution at amino acid 155 is an exception. Only a single RNA band with an electrophoretic mobility of 1.3 kb was detected by Northern analysis, which is identical with the data reported for mouse laminin receptor (Rao et al., 1989). In contrast, some human tumors have been found to have multiple RNAs which hybridize to J9 (Yow et al., 1988).

Despite the close homology between C10 and clones J9, pMLR21, and pLR4-4, several characteristics of C10 are inconsistent with it encoding the 67-kDa elastin/laminin receptor, the most obvious differences being its small size (39 vs 67 kDa for the purified receptor), the failure of antibodies that react with the elastin receptor to recognize the C10 protein, and the fact that C10 does not bind to elastin affinity columns. In addition, the absence of a leader sequence or a traditional transmembrane domain[1] suggests that C10 and pMLR21, the full-length clone of pLR4-4 (Rao et al., 1989), are not processed as secretory or integral membrane proteins and calls into question whether either can function as a cell surface receptor. Additionally, these proteins lack the conserved S-type lectin domain (Drickamer, 1988).

It is interesting to note that the sequences of C10 and the putative laminin receptor clones pMLR21 and J9 are essentially identical with a previously identified cytoplasmic protein P40 (Makrides et al., 1988). Although a precise function for the P40 protein has not been demonstrated, it has been localized immunologically as a cytoplasmic protein that is not associated with the plasma membrane.[2] Furthermore, in many cell types, the mRNA for P40 occurs to a considerable extent associated with mRNP particles that do not interact with the translation apparatus of the cell (Makrides et al., 1988; Yenofsky et al., 1983). These characteristics, together with the findings described in this report, strongly suggest that

[1] Rao et al. (1989) have predicted a short transmembrane domain at amino acid residues 86–101.

[2] D. Auth and G. Brawerman, personal communication.

proteins encoded by C10 and pMLR21 reside in the cytoplasm of the cell and do not, by themselves, function as transmembrane receptor proteins. It is possible that C10 and hence, pMLR21 encode proteins that are antigenetically related to the 67-kDa elastin and laminin receptors but are themselves not receptor proteins. This immunological similarity would explain the initial selection of the pLR4-4 clone from the expression library by antibody reactivity. Because antibodies to the 67-kDa elastin and laminin receptors have been shown to cross-react with a family of cytoplasmic β-galactoside binding lectins (Hinek et al., 1988; Mecham, et al., 1989b), antibody cross-reactivity cannot be discounted. It should be emphasized, however, that pMLR21 and C10 proteins lack extensive sequence homology to any of the β-gal binding lectins for which sequence data are available (Hirabayashi et al., 1989).

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