The sequence of the mouse 14 kDa β -galactoside-binding lectin and evidence for its synthesis on free cytoplasmic ribosomes

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The partial amino acid sequence of the mouse 14 kDa β -galactoside-binding lectin has been deduced from cDNA clones corresponding to 86 % of the coding sequence and extending to the polyadenylation signal. The deduced amino acid sequence for the murine lectin shows 94 % identity with the rat, 89 % with human, 86 % with bovine and 46 % with the chicken 14 kDa lectins. A cDNA probe has been used to analyse genomic DNA and identify a single mRNA of approx. 570 bp in 3T3 fibroblasts, murine erythroleukaemia cells and the murine basement-membrane-secreting Engelbreth–Holm–Swarm tumour. Analysis of free and bound polyribosomes has shown that the lectin message is translated on free cytoplasmic ribosomes.

INTRODUCTION

A family of soluble β -galactoside-specific lectins are widely distributed in many species, from electric eel to man. They are present in a number of cell types and their tissue concentration varies during differentiation and tissue organization (Barondes, 1984). It has recently been shown that the mouse 35 kDa lectin is a component of the heterogeneous nuclear ribonucleoprotein complex (hnRNP) (Jia & Wang, 1988), but the location of the small subunit lectins (13–14 kDa) in cells and their extracellular matrix is uncertain, and their function remains undetermined (Chaudhuri *et al.*, 1988; Lotan & Raz, 1988).

A number of cDNA clones from different species, encoding the whole or part of the 13–14 kDa lectin, have been sequenced (Ohya *et al.*, 1986; Clerch *et al.*, 1988; Raz *et al.*, 1988; Abbott *et al.*, 1989) and the deduced amino acid sequences, together with sequences derived from peptide fragments (Ohya *et al.*, 1986; Clerch *et al.*, 1988; Hirabayashi & Kasai, 1988), show a high degree of sequence identity between species. We report here 86 % of the sequence of the mouse lectin deduced from nucleotide sequencing of two overlapping clones isolated from a 3T3 cell cDNA library.

A preliminary report of this work has been published previously (Wilson *et al.*, 1989).

EXPERIMENTAL

Materials

The Clontech 3T3 cell cDNA library (Cambridge Bioscience, Cambridge, U.K.) was screened with sheep anti-(mouse lectin) antiserum and with a cDNA, clone 2, kindly supplied by Professor S. H. Barondes (University of California, San Francisco, CA, U.S.A.). The antiserum was raised as described previously (Fitzgerald *et al.*, 1984), against the 14 kDa mouse lectin purified from

thymus by affinity chromatography on lactose-Sepharose and stabilized by alkylation with iodoacetamide (Whitney & Powell, 1986). Clone 2 was isolated by immunoscreening a human hepatoma cDNA library in bacteriophage λ gt11 with antiserum raised against the rat 14.5 kDa lectin, and sequence analysis suggested that clone 2 encoded the C-terminal half of a human 14 kDa lectin (Gitt & Barondes, 1986).

3T3 fibroblasts and murine erythroleukaemia cells (MEL cells) were grown in Hepes-buffered (5 mM) Dulbecco's Minimum Essential Medium (DMEM; Gibco BRL) with 10 % (v/v) foetal-bovine serum. Differentiation was induced in MEL cells by addition of 4 mM-*NN'*-hexamethylenebisacetamide (HMBA; Sigma). After 4 days, 98 % of cells contained haemoglobin, assessed by benzidine staining. Basement-membrane-producing-tumour tissue, from Engelbreth–Holm–Swarm (EHS) tumours passaged in Balb/c mice, was excised and rapidly frozen in liquid N₂ before storage at –70 °C. Neonatal lung tissue was taken from 9-day-old mice (Balb/c) and 9-day-old rats (Wistar) bred at Babraham.

Methods

Sequencing. The 3T3 cDNA library in λ gt11 was grown up in *Escherichia coli* according to the manufacturer's instructions and screened for lectin expression (Huynh *et al.*, 1985) with anti-(mouse lectin) antiserum, or for lectin cDNA (Mason & Williams, 1985) with clone 2 cDNA labelled with [α -³²P]dCTP (3000 Ci/mmol) by the random-priming method of Feinberg & Vogelstein (1984). Phage from positive plaques were subcloned into the plasmid pUC 18 as described by Maniatis *et al.* (1982) and transformants produced in DH1 *E. coli* (Hanahan, 1983). Mini preps of plasmid, prepared by the boiling method of Maniatis *et al.* (1982), were analysed after restriction-endonuclease-*Eco*R1 digestion, by Southern blotting, probing being done with [α -³²P]-

Abbreviations used: MEL, murine erythroleukaemia; DMEM, Dulbecco's Minimal Essential Medium; HMBA, *NN'*-hexamethylenebisacetamide; EHS, Engelbreth–Holm–Swarm; GuITC, guanidinium isothiocyanate/0.2 M-sodium acetate, pH 5.6; DEPC, diethyl pyrocarbonate; 1 $\times$ SSC, 0.15 M-NaCl/0.015 M-sodium citrate; hnRNP, heterogeneous nuclear ribonucleoprotein complex.

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dCTP-labelled clone 2. Plasmids from clones of interest were purified on CsCl gradients and dialysed extensively against 10 mM-Tris/1 mM-EDTA, pH 8.0, as described by Maniatis *et al.* (1982). Double-stranded sequencing was carried out using Sequenase (Cambridge Bioscience) and [α - 32 S]dATP (1200 Ci/mmol) as directed by the manufacturer, after separation of plasmid strands on spin columns (Murphy & Kavanagh, 1988).

Preparation and analysis of DNA and RNA. Genomic DNA was prepared from mouse liver and EHS-tumour tissue and electrophoresed as described by Davis *et al.* (1986), then transferred to Gene Screen Plus (Du Pont) by capillary blotting according to the manufacturer's instructions.

Total cellular RNA was extracted in 6 M-guanidinium isothiocyanate/0.2 M-sodium acetate, pH 5.6 (GuITC) by a modification of the method of Chirgwin *et al.* (1979). Fibroblasts were scraped off Petri dishes in GuITC using a plastic transfer pipette and MEL cells were pelleted and lysed in GuITC (1 ml/ 0.5×10^7 cells). Tissue (200 mg) was homogenized in 5 ml of GuITC for 20 s in an Ultra-Turrax disperser, followed by ten strokes at 250 rev./min in a glass/Teflon Potter-Elvehjem homogenizer. Ethanol (0.5 vol.) was added with rapid vortex mixing, and RNA was precipitated overnight at -20°C . After centrifugation at 16000 *g* for 15 min at 4°C , RNA was dissolved in 200 μl of urea solution [7 M-urea/350 mM-NaCl/50 mM-Tris (pH 7.5)/1 mM-EDTA/0.2% SDS]/ml of GuITC, extracted (10 min) with an equal volume of phenol/chloroform (1:1, v/v), spun at 3500 *g* for 10 min at room temperature and then re-precipitated overnight at -20°C from the upper aqueous layer by addition of 2 vol. of ethanol. After washing with 70% ethanol, precipitated RNA was dried, dissolved in DEPC (diethyl pyrocarbonate; Sigma)-treated water (Milli Q) (Maniatis *et al.*, 1982) and stored at -70°C . Total cellular RNA was size-fractionated on a 0.6 M-formaldehyde/1%-agarose denaturing gel as described by Davis *et al.* (1986), except that a 50 mM-Hepes (pH 7.8)/1 mM-EDTA/0.6 M-formaldehyde buffer was used, then transferred to Gene Screen Plus by capillary blotting according to manufacturer's instructions and baked at 80°C for 3 h.

Northern and Southern blots were probed in a Hybaid (Twickenham, Middlesex) apparatus with clone C (corresponding to amino acid residues 20–104 of the mouse 14 kDa lectin; see the Results and discussion section) prepared by electrophoresis of *Eco*RI-digested plasmid, stored in low-melting-point agarose and labelled with [α - 32 P]dCTP by the random-priming method of Feinberg & Vogelstein (1984). Unincorporated label was removed by gel filtration (Nick Columns; Pharmacia) and the probe denatured by boiling (10 min) before addition to filters prehybridized with 100 μg of denatured salmon sperm DNA/ml. Filters were prehybridized for at least 15 min and hybridized (overnight) at 60°C (RNA) or 65°C (DNA) in 1 M-NaCl/1% (w/v) SDS/10% (w/v) dextran sulphate. After probing, blots were washed with $2 \times \text{SSC}$ at room temperature, $2 \times \text{SSC}/1\%$ SDS at hybridization temperature (30 min), and finally with 0.1% SSC at room temperature, wrapped in Saran Wrap ('clingfilm'; Dow Chemical Co.) and autoradiographed at -70°C by using Konica Medical X-ray film with two intensifying screens.

Isolation and analysis of polyribosomes. Free and bound polysomes were isolated from neonatal-mouse and -rat lung by the method of Ramsey & Steele (1976) with the addition to all solutions of cycloheximide (100 $\mu\text{g}/\text{ml}$) and RNA Guard (Pharmacia-LKB) at 50 units/ml instead of liver-cell sap. Final polysome pellets were resuspended in 500 μl of DEPC-treated water and stored at -70°C . Equivalent amounts of free and bound polysomes in 50–100 μl [15 μg of RNA as estimated by absorption at 260 nm (A_{260}/A_{280} values were consistently between 1.7 and 1.9)], were denatured by 10 min incubation at 70°C in 1 ml of formaldehyde/formamide [15 mM-Hepes (pH 7.8)/0.3 mM-EDTA/2% (v/v) formaldehyde/51% (v/v) formamide (deionized)] and cooled rapidly on ice. Serial 1.5-fold dilutions (200 μl) were prepared in DEPC-treated water and loaded on to Hybond N (Amersham International) in dot-blot apparatus. After 30 min incubation, the supernatant was gently sucked through the filter and the dots washed with 200 μl of DEPC-treated water. The filter was then removed and air-dried before fixation of the RNA by 2 min exposure to u.v. light on a transilluminator. Triplicate blots were probed for (i) actin mRNA with a 1255 bp fragment generated by *Pst*I digestion of clone pAM 91-2 encoding mouse cardiac muscle actin cDNA (Minty *et al.*, 1982), for (ii) the transplantation antigen H2-II with 246 pb fragment generated by *Pst*I digestion of a pH2-II cDNA (Steinmetz *et al.*, 1981) and for (iii) lectin mRNA with clone C. All probes were prepared and labelled as for Northern and Southern blotting. Hybridization procedures were as described for Northern blots.

RESULTS AND DISCUSSION

The nucleotide sequence corresponding to 86% of the coding region and extending to the polyadenylation signal was determined by sequencing two clones. Both extend from amino acid residue 20, but whereas the shorter clone, clone C, runs 256 residues to the *Eco*RI site (see Fig. 1), the larger, clone A, presumably generated by incomplete digestion when subcloning into pUC 18, extends through this site into the 3' non-coding region. Secondary structure of the template, perhaps due to the poly(A) tail, has so far precluded accurate sequencing beyond the polyadenylation signal. Both clones had identical nucleotide sequences, as did one other clone, isolated and subcloned independently from a positive screening of the λ gt11 library. This suggests, therefore, a single coding sequence for the 14 kDa lectin in 3T3 cells.

Part of this sequence (residues 190–428 in Fig. 1) proved to be virtually identical with that of clone 2, used to probe the library. The published sequence of clone 2 (Gitt & Baronides, 1986) contains an extra adenine nucleotide at the position marked with an asterisk (*) on Fig. 1. Such equivalence between mouse and human nucleotide sequences, even in the 3' non-coding region, is rather unexpected. However, clone 2 was isolated from a cDNA library generated from a human hepatoma excised from a mouse host (deWet *et al.*, 1984). The tumour would therefore have been infiltrated by host connective and vascular tissues which could have resulted in the inadvertent cloning of a cDNA derived from mouse lectin mRNA rather than from the human lectin.

The mouse nucleotide sequence has considerable sequence identity with that of the rat lung 14 kDa lectin

RLL	61	CGG GGA GAG <u>CTG</u> GCC <u>CCG</u> GAC GCC AAG AGC TTT GTG <u>TTG</u> AAC CTG GGG <u>AAA</u> GAC AGC
M3T3L		CGG GGA GAG <u>GTG</u> GCC TCG GAC GCC AAG AGC TTT GTG CTG AAC CTG <u>GGA</u> <u>AAA</u> GAC AGC
MIP3L		GGG CAG AGG TGG <u>ACC</u> TCG GAC <u>GAC</u> <u>AGG</u> AGC TTT GTG CTG ACC CTG <u>AAG</u> <u>AAA</u> GAC AGC
		↑
RLL	118	AAC AAC CTG TGC CTA CAC TTC AAC CCC CGC TTC AAC GCC <u>CAC</u> <u>GGA</u> <u>GAT</u> <u>GCC</u> AAC ACC
M3T3L		AAC AAC CTG TGC CTA CAC TTC AAT CCT CGC TTC AAT GCC CAT GGA GAC GCC AAC ACC
MIP3L		AAC AAC <u>CGT</u> TGC CTA CAC TTC AAT CCT CGC TTC AAT GCC CAT GGA GAC GCC AAC ACC
		↑
RLL	165	ATT GTG TGT AAC <u>AGC</u> AAG <u>GAC</u> <u>GAT</u> GGG ACC TGG GGA <u>ACA</u> <u>GAA</u> <u>CAA</u> <u>CGG</u> <u>GAG</u> <u>ACT</u> GCC
M3T3L		ATT GTG TGT AAC ACC AAG GAA GAT GGG ACC TGG GGA ACC GAA CAC CGG GAA CCT GCC
MIP3L		ATT CTG TGT AAC ACC AAG GAA GAT GGG ACC TGG GGA ACC GAA CAC CGG GAA CCT GCC
		↑
RLL	232	TTC <u>CCT</u> TTC CAG <u>CCT</u> GGG AGC ATC <u>ACG</u> <u>GAG</u> GTG TGC ATC ACC TTT GAC CAG GCT GAC
M3T3L		TTC CCC TTC CAG CCC GGG AGC ATC ACA GAG GTG TGC ATC ACC TTT GAC CAG GCT GAC
MIP3L		TTC CCC TTC CAG CCC GGG AGC ATC ACA GAG <u>TGT</u> GCA <u>TGC</u> <u>ACC</u> TTT GAC CAG GCT GAC
		EcoRI ↓
RLL	265	CTG ACC ATC AAG CTG CCA GAC GGG CAT GAA TTC <u>AAA</u> <u>TTC</u> CCC AAC CGC CTC AAC ATG
M3T3L		CTG ACC ATC AAG CTG CCA GAC GGA CAT GAA TTC AAG TTC CCC AAC CGC CTC AAC ATG
MIP3L		CTG ACC <u>ATG</u> CAA GCT GCC AGA CGG ACA T
		↑
RLL	346	GAG GCC ATC AAC TAC ATG GCG GCG GAT <u>GCT</u> GAC TTC AAG ATT AAG <u>TGT</u> <u>GTG</u> GCC TTT
M3T3L		GAG GCC ATC AAC TAC ATG GCG GCG GAT GGA GAC TTC AAG ATT AAG TGC GTG GCC TTT
		*
RLL	403	GAG TGA AGCC AGCC AGCC CCC <u>AGCC</u> <u>GTT</u> AGCC CCC AATAAA GGCAGCTGCT
M3T3L		GAG TGA AGCC AGCC AGCC TGT <u>AGCC</u> <u>.T</u> C AATAAA GGCAGCTGCT
		▲

Fig. 1. Nucleotide sequences of rat and mouse 14 kDa lectin cDNAs

Abbreviations: RLL, rat lung lectin (Clerch *et al.*, 1988); M3T3L, murine 3TE cell lectin; MIP3L, murine fibrosarcoma lectin (Raz *et al.*, 1988). The numbering system is taken from the full-length RLL cDNA clone. 'Boxes' enclose regions of sequence identity; ▲, termination codon;, spaces introduced to maximize alignment; $\rightarrow$ EcoRI, EcoRI cleavage site; —, poly(A) signal; ↑, positions where an extra guanine residue is inserted in the MIP3L sequence; *, position of an additional adenine residue in clone 2 (Gitt & Barondes, 1986).

(Clerch *et al.*, 1988) (Fig. 1). In the coding region there are 22 single base differences (94% identity), only six of which result in amino acid changes (95% identity). The 3' untranslated sequences before the poly(A) signal also show extensive similarity, both containing AGCC repeating units (five in rat and four in mouse).

A partial sequence of a mouse 14 kDa lectin has previously been reported (Raz *et al.*, 1988), determined from a single cDNA clone generated from the fibrosarcoma cell line UV-2237-IP3 (MIP3L in Fig. 1). The central part of this sequence also shows extensive identity with that from the 3T3 cell cDNA library. The major discrepancies between the mouse sequences appear to arise from the insertion of an extra guanine residue in the MIP3L sequence at each of the positions indicated by an arrow (↑) in Fig. 1.

Deduced amino acid sequence

The deduced amino acid sequence of the 3T3 cell lectin is aligned in Fig. 2 with the previously reported sequences of the 14 kDa lectin from other species, determined both by cDNA and amino acid sequencing. Totally conserved residues are enclosed in boxes, illustrating the very high degree of sequence conservation in the 14 kDa lectin between all species investigated (46%). Among mammalian species, sequence identity is considerably higher (78%), and most amino acid substitutions are conservative. The mouse and rat 35 kDa β -galactoside-binding lectins also show considerable structural similarity to the 14 kDa lectin. The rat 35 kDa lectin was

originally identified as an IgE-binding protein by affinity chromatography on IgE-Sepharose (Liu *et al.*, 1985). The sequence alignment of the 14 kDa and 35 kDa lectins (Fig. 2) suggests that the identical residues 44–49 (HFNPRF) and several blocks of sequence between residues 58 and 82 (IVCNS-K----WG-E-R---FPF--G), probably constitute the β -galactoside-binding site which these lectins share. This domain is encoded in a single exon in the chicken (Ohyama & Kasai, 1988) (see Fig. 2).

The requirement of reducing agents to maintain the haemagglutinating activity of all these lectins suggests that a cysteine residue is crucial in the maintenance of an active conformation. Cysteine is not thought to be at the active site, since derivatization of the lectin with iodoacetamide stabilizes lectin activity rather than blocking saccharide binding (Whitney & Powell, 1986). Only one cysteine residue is conserved in all β -galactoside-binding lectins, (▲) in the sequence IVCNS/TK. This reacts readily with iodoacetic acid (Chaudhuri *et al.*, 1988), indicating a location at the surface of the molecule. The cysteine at position 2 may also be conserved among 14 kDa lectins. In the rat lung lectin this residue and Cys-130 react most rapidly with iodoacetamide, again indicating a surface location and implying a possible role in the maintenance of an active conformation.

Southern-blot analysis

Hybridization of clone C (corresponding to amino acid residues 20–104) gave identical labelling patterns

Fig. 2. Alignment of amino acid sequences of soluble β -galactoside-specific lectins

Abbreviations: M3T3L, mouse 3T3 cell lectin; RLL, rat lung lectin (Clerch *et al.*, 1988); HPL, human placental lectin (Hirabayashi & Kasai, 1988); BHL, bovine heart lectin (Abbott *et al.*, 1989); CSL, chicken skin lectin (Ohyama *et al.*, 1986); CBP35, 35 kDa carbohydrate-binding protein (Jia & Wang, 1988); IGE BP, IgE-binding protein (Albrandt *et al.*, 1987). Amino acids are numbered from the N-terminal residue where this has been determined. 'Boxes' enclose regions of sequence identity; . . . , undetermined residues; -, spaces introduced to maximize alignment; *, termination signal; $\blacktriangle$, cysteine residue conserved in all lectins; $\downarrow$, positions of introns in the chicken gene (Ohyama & Kasai, 1988).

with genomic DNA from two strains of mice, DBA and Balb/c (Fig. 3). Since it has been suggested that these lectins are associated with extracellular glycoconjugates (Barondes, 1984), genomic DNA from a basement-membrane-secreting mouse tumour, EHS, was also analysed. However, this generated an identical pattern on Southern blotting, suggesting there is no rearrangement or amplification of lectin genes in this tumour.

Northern-blot analysis

Cytoplasmic lectin mRNA was identified after electrophoresis in denaturing gels by hybridization to clone C and sized by comparison with internal ribosomal RNA bands and an RNA ladder (Gibco BRL). A single message of relatively low molecular mass (approx. 570 bp)

was detected in neonatal mouse lung, in fibroblast and MEL cells and in EHS-tumour tissue (results not shown). (No change in mRNA size was detected in MEL cells after induction of differentiation with HMBA.) A total of 408 residues are required to code the amino acid sequence, and at least 40 residues precede the polyadenylation tail, indicating the presence of a relatively short 5' leader sequence and polyadenylation tail in the cytoplasmic mRNA.

Subcellular site of lectin synthesis

Much controversy exists concerning the cellular site of action of these lectins. Their carbohydrate-binding activity initially suggested an extracellular function (Barondes, 1984), but there have been repeated failures

Fig. 3. Southern-blot analysis

Genomic DNA from a DBA and Balb/c mouse and an EHS tumour were cleaved with (1) *Eco*R1, (2) *Hind*III and (3) *Bam*HI and probed with clone C lectin cDNA after electrophoresis and blotting. The positions of λ DNA fragments (23.1–0.5 kb) generated by *Hind*III digestion (Gibco BRL) are shown.

Fig. 4. Dot-blot analysis of mRNA on free and bound polyribosomes

Dilutions (1.5-fold) of isolated polyribosomes (15 μ g of RNA) from mouse lung (1 and 2), free and bound respectively, and rat lung (3 and 4), free and bound respectively, were probed for actin mRNA (A), H-2II mRNA (P) and lectin mRNA (C) as described in the text.

to detect extracellular lectin in various tissues and tissue-culture systems (Chaudhuri *et al.*, 1988; Clerch *et al.*, 1988; Powell, 1988). The sensitivity of these lectins to oxidation has also suggested an intracellular function should be considered. To determine the intracellular site of synthesis of these lectins, free and bound polyribosomes were isolated from neonatal-mouse and -rat lung and the relative distribution of the lectin mRNA compared with that of actin mRNA, translated on free ribosomes and the MHC antigen, H-2II, translated on bound ribosomes. Analysis performed by dot-blot hybridization showed that the distribution of the lectin message paralleled that of actin mRNA, indicating cytoplasmic synthesis of the lectin (Fig. 4). This, together with the failure to detect an *N*-terminal signal sequence for secretion in any cell type, indicates that, if indeed these lectins function extracellularly, their secretion must involve either post-translational translocation into the endoplasmic reticulum or an entirely novel mechanism.

The data presented here emphasize the high degree of sequence conservation among the 13–14 kDa β -galactoside-binding lectins in different species, but sequence data has not so far helped to elucidate their function. On the other hand, the 35 kDa lectins are clearly bifunctional proteins. The proline- and glycine-rich *N*-terminal sequence shows some structural similarity to other hnRNP proteins (Jia & Wang, 1988), whereas the *C*-terminal sequence contains a saccharide-binding site closely related to that of the 14 kDa lectins, as described in the text. This saccharide-binding structure may constitute a peptide domain found in several different proteins, for example, immunological cross-reactivity suggests the carbohydrate-binding properties of the 67 kDa component of the elastin/laminin receptor may be due to a structurally related domain (Hinek *et al.*, 1988). It is becoming clear that the term 'galactin', which was derived from 'gal', their saccharide specificity, and 'hapto', meaning 'to touch', and proposed to describe these related proteins (Harrison & Chesterton, 1980), will only serve to classify them until their other, probably diverse, properties are identified.

Note added in proof (received 25 May 1989)

Recent investigations strongly support the conclusion that the IgE-binding protein (IGE-BP, Fig. 2) is the rat homologue of the mouse 35 kDa β -galactoside lectin, CBP35 [Laing J. G., Robertson, M. W., Gritzmacher, C. A., Wang, J. L. & Liu, F-T. (1989) *J. Biol. Chem.* **264**, 1907–1910].

We are grateful to Ms J. Douglas, Department of Clinical Oncology, Addenbrookes Hospital, Cambridge, U.K. for the RNA-extraction procedure.

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Received 10 January 1989/22 February 1989; accepted 3 March 1989