Identification of a rat liver cDNA and mRNA coding for the 28 kDa gap junction protein

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By screening of a rat liver cDNA library with complex and deoxyinosine containing oligonucleotide probes a cDNA clone was isolated and shown by sequencing to code for the amino-terminal half of the rat liver 28 kDa gap junction protein. The insert hybridized to a 1.9 kb species from rat and mouse liver poly(A)$^+$ RNA in Northern blot analysis. In embryonic mouse hepatocytes the amount of the 1.9 kb mRNA increased 3-fold between 24 and 96 h in culture. This correlates with the previously described increase of the 28 kDa gap junction protein under these conditions.

Colony screening *Deoxyinosine oligonucleotide* *Gap junction* *cDNA* *Northern blot* *Hepatocyte (Mouse)*

1. INTRODUCTION

Gap junctions consist of intercellular channels in organs or between cultured cells. Ions and metabolites can diffuse through these channels which are likely to be formed from protein subunits of molecular masses between 26 and 28 kDa [1,2]. The significance of other proteins, also isolated from rodent liver gap junctions, for example the 21 kDa (cf.[1]) or the 16 kDa protein [3], still remains to be clarified. The cDNA sequence of the bovine lens major intrinsic protein (MIP) has been reported [4] which is presumed to be a gap junction protein but which does not hybridize to any poly(A)$^+$ RNA species from liver [4].

In an attempt to isolate a cDNA for the 28 kDA rat liver protein we used the published sequence of its N-terminal amino acid residues [5] in order to deduce oligonucleotide probes. The incorporation of deoxyinosine [6] instead of 3 or 4 different nucleotides during the synthesis led to an oligonucleotide mixture of little complexity. Besides the obvious advantages for synthesis and hybridization this decreased complexity allowed us to deduce the deoxyinosine containing probe from a region more distant from the N-terminus of the corresponding amino acid sequence (fig.1).

Recently it was preliminarily reported, that a cDNA clone corresponding to the 28 kDa rat liver gap junction protein had been isolated by immunoscreening of a λgt11 expression library [7].

2. MATERIALS AND METHODS

2.1. *Preparation of oligodeoxyribonucleotide probes*

Oligonucleotide synthesis and the removal of protecting groups were done according to the manual of Applied Biosystems. Full length oligonucleotides were separated from shorter by-products and protecting groups by polyacrylamide gel electrophoresis [8]. Urea and salts were removed on Sep-Pak C_{18} cartridges (Waters Associates) [8]. After ^{32}P-labelling with T_4 polynucleotide kinase (BRL) [9] the oligonucleotides were separated from unreacted [γ-^{32}P]ATP by chromatography on Whatman DE52 cellulose [10].

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2.2. *Replica preparation*

The rat liver library was constructed by Dr G. Ostroff (University of Massachusetts, Medical Center, Worcester, MA, USA) and was obtained in 1983 from Dr R.A. Weinberg (Whitehead Institute, Cambridge, MA, USA). The S_1-nuclease treated cDNA was inserted into the *Pst*I-site of pUC8 by dG:dC-tailing. Before amplification the library contained 217 000 independent transformants of *E. coli* JM83. 10 000–20 000 bacteria per NC-filter (132 mm diameter; Schleicher and Schüll) were plated as described [11] without any pretreatment of the filters. Two replicas were made from each master filter as described [11] with some simplifications [12]. Plasmids were amplified as described [10]. Colonies were lysed and their DNA was denatured and immobilized on the filters by washing twice with slight agitation for 5 min in each of the following solutions: 0.5 M NaOH, 1.5 M NaCl, 1 M Tris-HCl, pH 7.5, 1.5 M NaCl, 2 × SSC [10]. The filters were dried on paper towels and baked in vacuo at 80°C for 2 h.

2.3. *Hybridization with oligonucleotides*

Filters from colony screening, Southern blots, or gels were hybridized [10] in batches without carrier DNA and prehybridization overnight in a sealed glass beaker at temperatures calculated according to [13]. Hybridization solutions were used up to 80 days in several cycles of hybridization and 5000 cpm of the radioactive oligonucleotides/ml per sequence were found to be sufficient for high-density colony screening [12]. Gels and filters were washed 4 times for 5 min (nitrocellulose) or 20 min (other materials) in 6 × SSC [10] while the temperature was elevated from room temperature to 1–2°C below hybridization temperature. Then the filters and gels were drained, sealed in plastic bags, and exposed to Kodak XAR-5 films at −80°C.

2.4. *Rescreening*

Several colonies were picked from each area of the master plate in which a hybridizing colony had been localized. The colonies were cultivated in distinct wells of microtiter plates. Replicas were obtained on Whatman 541 filters and the cells were lyzed as described [14].

2.5. *cDNA and Northern blot analysis*

DNA restrictions and ligations, agarose gel electrophoresis and Southern blotting were carried out as described [9]. DNA sequencing was carried out by the M13-derived dideoxyribonucleotide chain terminating method [15]. Total RNA from primary cultures of mouse embryonic hepatocytes [16] and from livers of adult BD IX rats or Balb/c mice was isolated as described [17]. Poly(A)$^+$ RNA was isolated by affinity chromatography on poly dU paper [18]. Nick translation of the cDNA insert, Northern blot preparation as well as hybridization, and stringent washing were carried out according to [9].

3. RESULTS AND DISCUSSION

Each of the oligonucleotide mixtures 3 and 4 (fig.1) identified 1 of 333 000 rat liver cDNA colonies on 22 pairs of replicas. Both clones were isolated and retested with the less complex probe I. The insert that hybridized with probe 3 also reacted with mixture I and was therefore sequenced. An open reading frame (342 bp) was found to span the whole cDNA and to code for the amino-terminal half of the rat liver gap junction

–	Gly	Val	Asn	Arg	His	Ser	Thr	Ala	Ile	Gly	Arg	Val	Trp	Leu	–
3′	CCG	CAC	TTA	GCC	GT	5′ 3′	TGI	CGI	TAI	CCI	GCI	CAI	ACC	GA	5′
	A	A	G	A							T			A	
(3)	C	G		G				(I)							
	T	T		T											

3′	CCG	CAC	TTA	TCC	GT	5′
	A	A	G	T		
(4)	C	G				
	T	T				

Fig.1. Deduction of the probes 3, 4 and I from amino acids 12–25 of the 28 kDa gap junction protein published as partial sequence [5].

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                                G TAC ACC TTG CTC AGT GGC GTG AAT CGG
Met Asn Trp Thr Gly Leu Tyr Thr Leu Leu Ser Gly Val Asn Arg
                                                  10

CAT TCT ACA GCC ATT GGC CGA GTA TGG CTG TCC GTC ATC TTT ATC
His Ser Thr Ala Ile Gly Arg Val Trp Leu Ser Val Ile Phe Ile
                  20                                      30
                                        PstI
TTC AGA ATC ATG GTG CTG GTG GTG GCT GCA GAG AGC GTG TGG GGT
Phe Arg Ile Met Val Leu Val Val Ala Ala Glu Ser Val Trp Gly
                                                  40

GAT GAG AAG TCT TCT TTC ATC TGT AAC ACC CTC CAG CCG GGC TGT
Asp Glu Lys Ser Ser Phe Ile Cys Asn Thr Leu Gln Pro Gly Cys
                  50                                      60

AAC AGC GTC TGC TAT GAC CAT TTT TTC CCC ATC TCC CAT GTG CGC
Asn Ser Val Cys Tyr Asp His Phe Phe Pro Ile Ser His Val Arg
                                                  70

CTG TGG TCC CTG CAA CTC ATC TTG GTT TCC ACC CCA GCT CTC CTC
Leu Trp Ser Leu Gln Leu Ile Leu Val Ser Thr Pro Ala Leu Leu
                  80                                      90

GTG GCA ATG CAC GTG GCT CAC CAA CAA CAC ATA GAA AAG AAA ATG
Val Ala Met His Val Ala His Gln Gln His Ile Glu Lys Lys Met
                                                 100

CTA CGG CTT GAG GGG CAC GGG GAC CCC CTT CAC CTG GAA GAG GT
Leu Arg Leu Glu Gly His Gly Asp Pro Leu His Leu Glu Glu Val
                  110                                     120
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Fig.2. cDNA sequence and deduced amino acid sequence of the 28 kDa rat liver gap junction protein. The positions of the underlined amino acids have already been reported [5].

protein that had been partially sequenced before [5]. The cDNA and the deduced amino acid sequence showed no significant homology to any other sequence in the DNA data base (1985) of the University of Wisconsin Genetics Computer Group and the protein data base (1985) of the National Biomedical Research Foundation. As expected for a channel forming protein the hydropathy plot [19] of the first N-terminal 120 amino acids (fig.3) shows two highly hydrophobic regions which are likely to span the plasma membrane and a shorter, possibly membrane-associated region near the N-terminus.

The cDNA recognized a species of 1.9 kb in poly(A)$^+$ RNA from rat and mouse liver (fig.4). The less intensive reaction with mouse liver mRNA suggests sequence differences in the liver 28 kDa gap junction proteins between the two species. Furthermore the amount of the 1.9 kb mRNA in mouse embryonic hepatocytes after 1 and after 4 days in culture was compared by densitometric

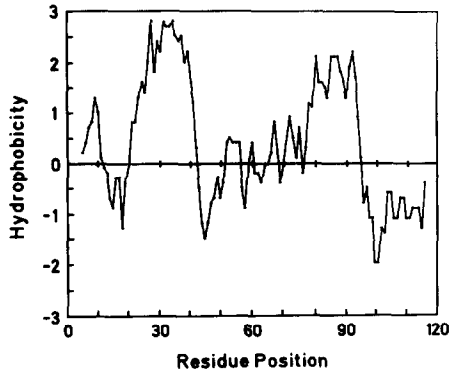

Fig.3. Hydropathy plot of the deduced N-terminal amino acid sequence of the 28 kDa rat liver gap junction protein. Hydropathy values [19] for windows of 9 amino acid residues were averaged, assigned to the middle residue of the span, and plotted with respect to position along the amino acid sequence.

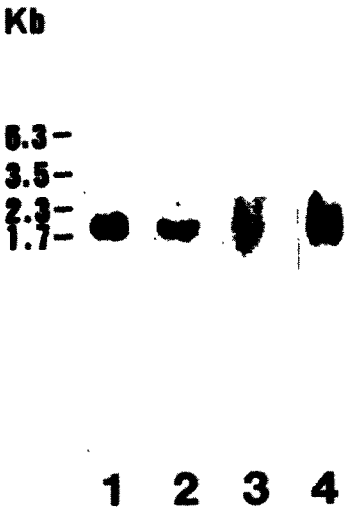

Fig.4. Northern blot analysis. Poly(A)$^+$ RNA was electrophoresed in 1.2% agarose gel containing formaldehyde, transferred to 'Gene screen' membranes (NEN) and hybridized with the nick-translated pUC8 plasmid containing the 342 bp cDNA. On lanes 1 and 2, 10 μg each of poly(A)$^+$ RNA from rat and mouse liver were applied. Lanes 3 and 4 contained 12 μg each of poly(A)$^+$ RNA from primary embryonic mouse hepatocytes harvested after 24 and 96 h in culture, respectively. Markers of molecular mass are 28 S and 18 S ribosomal RNA from mouse liver as well as 23 S and 16 S ribosomal RNA from *E. coli*.

evaluation of the corresponding autoradiograph. Fig.4 illustrates a 3-fold increase of the 1.9 kb mRNA during this time interval which corresponds to the previously described increase of the mouse liver gap junction protein under these conditions [16,20]. Possibly the transcription of the gene coding for the 28 kDa gap junction protein is similarly dependent on cell-cell contact or on intact tissue organization as has recently been shown for other liver specific mRNAs [21]. Alternatively transcription of the gap junction gene or the stability of the primary transcript and mRNA may decrease in proliferating hepatocytes. The corresponding protein has been shown to be decreased after partial hepatectomy [22] or in cultured hepatocytes when they are in the S phase of the cell cycle [23].

Besides its use for studying the regulation of gap junction biosynthesis the 342 bp cDNA can be employed to investigate to what extent gap junction proteins in different tissues contain heterologous sequences [1,2,16]. Furthermore the availability of gap junction cDNA opens new possibilities to study the function of the gap junction channel, for example by site-directed mutagenesis or with anti-sense RNA.

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