Molecular Cloning of cDNA for Rat Liver Gap Junction Protein

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Abstract. An affinity-purified antibody directed against the 27-kD protein associated with isolated rat liver gap junctions was produced. Light and electron microscopic immunocytochemistry showed that this antigen was localized specifically to the cytoplasmic surfaces of gap junctions. The antibody was used to select cDNA from a rat liver library in the expression vector lambda gt11. The largest cDNA selected contained 1,494 bp and coded for a protein with a calculated molecular mass of 32,007 daltons. Northern blot analysis indicated that brain, kidney, and stomach express an mRNA with similar size and homology to that expressed in liver, but that heart and lens express differently sized, less homologous mRNA.

Gap junctions are present between cells in many tissues and in a wide range of species (Bennett and Goodenough, 1978). The gap junctions in these tissues all share a basic structural similarity, but exhibit a variation both in their detailed morphologies as revealed by electron microscopy (Friend and Gilula, 1972) and in their sensitivities to physiological manipulations (Rose and Loewenstein, 1975; Turin and Warner, 1977; Spray et al., 1981). Evidence is available in several systems which suggests that individual cells within a given tissue are capable of forming physiologically and structurally distinct junctions with their neighbors (Weir and Lo, 1982; Miller and Goodenough, 1986). Thus the question arises: are the junctions in these different tissues composed of the same, similar, or completely different protein components?

One approach to this question has been to develop procedures by which gap junctions can be isolated from different tissues. The polypeptides associated with isolated junctions have been compared directly. Antibodies raised against junctional polypeptides have been used to identify related proteins in tissues in which junctions cannot be isolated. It has been possible to directly isolate enriched preparations of gap junctions from a few tissues and species: mouse and rat liver (Goodenough and Stoeckenius, 1972; Henderson et al., 1979; Hertzberg and Gilula, 1979; Finbow et al., 1985), mouse, rat, and rabbit heart (Kensler and Goodenough, 1980; Gros et al., 1983; Manjunath et al., 1982), and bovine lens fibers (Alcala et al., 1975; Goodenough, 1979).

Detailed biochemical comparisons have been performed on polypeptides enriched by these procedures. Most hepatocyte gap junction preparations contain a low number of protein components which are dominated by a single protein with a range of reported molecular masses of 26–29 kD. However, Warner et al. (1984) and Paul (1985) report the presence of a 54-kD protein in a number of tissues that cross-reacts with antibody to rat liver 27-kD protein. In addition, Finbow et al. (1985) have isolated gap junction structures from mouse liver which are comprised of an 18-kD protein. This 18-kD protein exhibits no homology with rat liver 27-kD by two-dimensional peptide mapping. Antibodies against rat liver 27-kD do not cross-react with the 18-kD species. Thus, while there seems to be a general consensus that the liver gap junction is composed primarily of a 27-kD species, there are still data which need clarification.

Lens fiber membranes display a 26-kD protein, designated MP26, as a major protein component. MP26 has been suggested as a structural component of the lens fiber junction although this is a matter of controversy (see Bok et al., 1982; Paul and Goodenough, 1983*a*, *b*). Biochemical comparisons reveal no structural similarities between the liver 27-kD and the lens fiber MP26. Peptide mapping of liver 27-kD and lens MP26 reveals no obvious homology (Hertzberg et al., 1982; Gros et al., 1983) and this finding is supported by partial amino acid sequencing (Nicholson et al., 1983). In addition, antisera and monoclonal antibodies generated against MP26 show no affinity for rat liver 27-kD protein (Paul and Goodenough, 1983*a*; Fitzgerald et al., 1983; Hertzberg et al., 1982 but see Traub and Willecke, 1982).

Myocardial gap junctions have been isolated by several investigators and a number of different major proteins have been reported. These include proteins of 30–34 kD (Kensler and Goodenough, 1980), 27 kD (Hertzberg and Skibbens, 1984), 45 and 29 kD (Page and Manjunath, 1985), and 29 kD (Gros et al., 1983). The heart 29-kD protein has been peptide mapped and some NH_2-terminal sequence has been determined which has revealed a partial homology with the rat liver 27-kD protein (Nicholson et al., 1985). Peptide maps of liver, heart, and uterine junction polypeptides performed by Zervos et al. (1985) suggest a very close homology between all three. They also demonstrate that an antibody to a synthetic oligopeptide based on the liver NH_2-terminal sequence cross-reacts with heart and uterine junction proteins.

Antisera produced against complete liver 27-kD protein have been reported both to cross-react with heart (Hertzberg and Skibbens, 1984) and not to cross-react (Paul, 1985; Dermietzel et al., 1984), suggesting that conserved and non-conserved regions may exist between the proteins of these different tissues.

The results of these studies suggest that a variety of gap junction proteins may exist. In each tissue, a number of potential candidates for major structural protein have been proposed. The relationship of the proteins in different tissues remains unclear.

To confirm the identity of the 27-kD protein as a structural protein(s) of rat liver gap junction, affinity-purified anti-27-kD antibodies were generated and used to determine the location of 27-kD protein by electron microscopic (EM)[1] immunocytochemistry. To provide another method with which to identify and compare possible gap junction components, the antibodies were used, in conjunction with an expression vector, to isolate cDNA clones from a rat liver library.

A 1.1-kb cDNA was cloned using the expression screening technique. A longer 1.5-kb cDNA was cloned using a hybridization screen using the 1.1-kb cDNA as a probe. The DNA sequence of the 1.5-kb cDNA predicts a 32-kD protein. Northern blot analysis revealed a major 1.6-kb band in liver and in a variety of other tissues excepting heart and lens. In heart and lens, several bands were detected. These bands were less homologous to, and differed in size from, the major liver band. These results suggest that there may be distinct, tissue-specific gap junction mRNAs encoding significantly different proteins.

Methods and Materials

Preparation of Anti-27-kD Protein Antibodies

Gap junctions from calf liver were prepared according to Fallon and Goodenough (1981) with the modification of the addition of 5×10^{-4} M diisopropylfluorophosphate (Sigma Chemical Co., St. Louis, MO) to the sarkosine solutions. Purified 27-kD protein was obtained from the isolated junctions by preparative SDS PAGE as described in Paul and Goodenough (1983*a*). Antiserum was raised in rabbits by injecting whole isolated junctions emulsified in Freund's adjuvant. Primary immunization consisted of multiple subdermal and subcutaneous injections (~50–100 μl/site) of 1 mg of isolated whole gap junctions at a concentration of 0.5 mg/ml in complete Freund's adjuvant. Animals were hyperimmunized at monthly intervals in a similar manner except that incomplete Freund's adjuvant was used. Animals were bled via ear vein 5, 7, and 11 d after hyperimmunization. Useful titers were observed after the first boost. The affinity-purified anti-27-kD antibodies used in these experiments were derived from animals that had been hyperimmunized twice. The anti-fusion protein antiserum was produced in a similar manner except that 0.5 mg of SDS PAGE purified fusion protein was injected each time. The anti-fusion antiserum used for these experiments was not affinity purified and was derived from an animal that had been boosted only one time.

Anti-27-kD antibodies were produced by affinity purification of the serum using isolated 27-kD protein coupled to Sepharose described in Paul and Goodenough (1983*a*). Affinity-purified anti-27-kD antibodies and anti-fusion protein antiserum were characterized by immunoblot (Towbin et al., 1979) as described in Paul and Goodenough (1983*a*). The amounts of protein loaded in the companion gel lanes that were Coomassie Blue-stained are the same as those loaded in gel lanes that were transferred to nitrocellulose. Electrophoretic transfer was performed in a Hoefer Transblot apparatus (Hoefer Scientific Instruments, San Francisco, CA) using Laemmli gel sample buffer (Laemmli, 1970) containing 10% methanol with continuous tap water cooling (~10°C) at 25 V for 1 h.

Immunofluorescence was performed on 5-10-μm cryostat sections (Bright Instruments, U.K.) of adult mouse liver perfused with 10 mM sodium *m*-periodate and 0.3% freshly dissolved paraformaldehyde in 0.075 M lysine-HCl and 0.037 M phosphate, pH 7.4, (PLP fixative) according to Brown and Farquhar (1984). Sections were incubated with anti-27-kD antibody at 10 μg/ml in phosphate-buffered saline (PBS), and with unfractionated anti-fusion protein antiserum at 1:200 dilution in PBS, for 45 min at room temperature. Slides were washed three times for 5 min each in Coplin jars in PBS. Rhodaminated goat anti-rabbit secondary antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 1:200 dilution in PBS was applied to the sections and incubated an additional 45 min at room temperature. After two 3-min rinses in PBS, the slides were incubated in 5% goat serum overnight at 4°C, rinsed again in PBS, and mounted in 50% glycerol/PBS with 0.4% *n*-propyl gallate. Immunofluorescence microscopy was performed as described previously (Goodenough et al., 1980).

EM localization of 27-kD protein was performed on fragments of rat liver plasma membrane. Membranes were isolated according to Hubbard et al. (1983). Antibody binding was visualized with colloidal gold-conjugated goat anti-rabbit IgG (Janssen Pharmaceuticals, Westchester, PA). 50 μl of 0.5 mg/ml isolated membranes were incubated for 2 h at room temperature with both anti-27-kD antibody and anti-fusion protein antiserum at the same concentrations used for immunofluorescence. The membranes were washed three times in PBS by centrifugation (8,000 rpm for 5 min) in a BiofugeA (Heraust-Christ, West Germany) in Eppendorf centrifuge tubes. The final membrane pellets were resuspended in colloidal gold-conjugated goat anti-rabbit IgG which had been previously preadsorbed with three 50-μl aliquots of the same membrane suspension used for the localizations. Incubations were carried out for 2 h at room temperature, and the membranes washed by centrifugation in PBS as above. Membranes were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate pH 7.4 for 30 min at room temperature. Specimens were subsequently stained with osmium and uranium and embedded in Epon as described previously (Paul and Goodenough, 1983*a*).

Construction and Screening of cDNA Library

cDNA clones were isolated from a rat liver library in lambda gt11. This library was provided by M. Mueckler of the Whitehead Institute and its preparation has been described (Mueckler and Pitot, 1985). The library was screened according to Young and Davis (1983) except that horseradish peroxidase-conjugated goat anti-rabbit IgG (CooperBiomedical, Inc., Malvern, PA) was used to detect antibody binding. Anti-27-kD antibody was incubated with nitrocellulose lifts at 100 ng/ml in PBS containing 5% normal goat serum and 0.1% Tween 20 (Sigma Chemical Co.) for 1–4 h at room temperature with constant shaking. Filters were never stacked. Filters were rinsed three times for 15 min each with PBS/Tween then incubated with secondary antibody that had been extensively absorbed with lysates of plating bacteria (*Escherichia coli* Y1090) to reduce background. After washing, bound horseradish peroxidase was detected by incubation with 0.5 mg/ml diaminobenzidine and 0.01% hydrogen peroxide in PBS/Tween.

The cDNA selected by antibody was used to rescreen the same library by plaque hybridization (Maniatis et al., 1982). After EcoRI digestion of the parent lambda DNA, the cDNA inserts were isolated after electrophoresis in low-melt agarose (Bethesda Research Laboratories, Gaithersburg, MD). Labeling was performed in low-melt agarose using random hexanucleotide primers as described by Feinberg and Vogelstein (1983).

DNA Sequence Analysis

Lambda clones were harvested from plate lysates on DEAE cellulose columns according to Helms et al. (1985). The cDNAs were recloned into M13mp18 (Messing and Vieira, 1982). A series of overlapping deletions in M13 were constructed according to Dale et al. (1985). Sets of overlapping deletions from both strands were sequenced by a modification (Biggin et al., 1983) of the method of Sanger et al. (1977). Some regions were sequenced by a modification of the chemical degradation procedure described by Bencini et al. (1984) supplied by Richard Tissert (Columbia University) (manuscript in preparation). Sequence data were analysed using Intelligenetics computer programs (Intelligenetics, Palo Alto, CA).

Production of Fusion Protein

The cDNA insert from the lambda clone picked up by antibody screening was recloned into the high-level plasmid expression vector pMAM-17 (Muesing et al., 1984). This vector contains the ColE1 rop gene under the control of a thermally inducible lambda P^1 promoter. A unique PvuII site in the rop gene provides for insertion of foreign sequences. To achieve ex-

1. *Abbreviation used in this paper*: EM, electron microscopic.

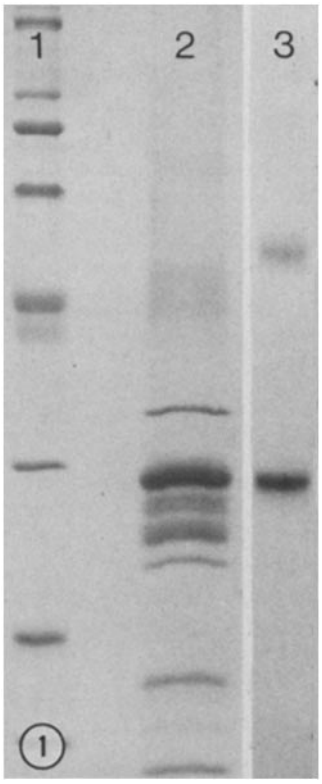


Figure 1. Coomassie Blue-stained SDS polyacrylamide gel of samples used to produce and purify antisera. (Lane *1*) Molecular mass standards: myosin (200 kD), phosphorylase A (95 kD), BSA (68 kD), gamma globulin (50 kD), actin (43 kD), aldolase (40 kD), carbonic anhydrase (29 kD), RNase (13 kD), cytochrome c (11 kD). (Lane *2*) Isolated rat hepatic gap junctions. (Lane *3*) Gel-purified 27 kD protein used for affinity purification. Higher molecular mass bands are aggregates caused by dissolving in SDS.

pression of the cDNA released from lambda by EcoRI, an appropriately sized synthetic EcoRI linker (New England Biolabs, Beverly, MA) was introduced at the PvuII site of pMAM-17. The selected cDNA was introduced in the new EcoRI site and the appropriate host bacteria (K-12 H1 trp) were transformed. Fusion protein was produced by shifting log phase bacteria growing in 2XTY broth at 30–42°C for 3 h. Bacteria were then cooled to 4°C, centrifuged (5 kg for 5 min) and sonicated in Laemmli gel sample dissolving buffer. Fusion protein was isolated by bulk preparative SDS PAGE on 15% Laemmli gels. Antiserum was prepared in rabbits as described above.

Northern Blot Analysis

Total RNAs from rat liver and heart and from bovine lens were isolated by homogenization in guanidine isothiocyanate followed by CsCl gradient centrifugation as described by Chirgwin et al. (1979). Poly(A) RNAs were isolated by chromatography on oligo-dT cellulose (Collaborative Research, Inc., Waltham, MA) according to Aviv and Leder (1972). RNAs were displayed on 1% agarose/formaldehyde gels as described by Maniatis et al. (1983) except that formaldehyde was added to 0.22 M. Gels were loaded with 5 μg of total RNA or 1.5 μg of poly(A) RNA. Gels were capillary blotted in 10× SSC onto nitrocellulose (Schleicher & Schuell, Keene, NH) and baked. Blots were prehybridized in 5× SSC, 1% sarcosine (IBI, New Haven, CT) for 1 h at 65°C, then hybridized in the same buffer at 65°C overnight with probes prepared and labeled as described above. The blot containing total RNA was washed twice for 30 min each time in 2.5× SSC, 0.5% sarcosine at room temperature, then twice for 30 min each time in 0.1× SSC, 0.5% sarcosine at 65°C before exposure to Kodak XAR-5 film for autoradiography at −80°C with an intensifying screen. The blot of poly(A) RNA was washed twice for 30 min each time in 2.5× SSC, 0.5% sarcosine at room temperature then twice for 30 min each time in 2.5× SSC, 0.5% sarcosine at 50°C before exposure to film. The blot was rewashed in 2.5× SSC, 0.5% sarcosine at 65°C and exposed again. Another wash in 0.5× SSC, 0.5% sarcosine at 65°C was performed followed by final exposure (1× SSC = 150 mM NaCl, 15 mM Na Citrate, pH 7.0).

Results

Preparation of Anti-27-kD Antibody

The protein profile of isolated gap junctions, which were used for immunizations, is shown in Fig. 1, lane *2*. The gel-purified 27-kD protein, used to affinity purify the anti-gap junction antiserum, is shown in Fig. 1, lane *3*. The higher molecular mass band at ~48 kD in lane *3* is an aggregate of the 27-kD band which appears upon heating or concentrating the protein in SDS solution (Henderson et al., 1979).

A Western blot characterization of the affinity-purified anti-27-kD antibody is presented in combination with a characterization of an antibody against a fusion protein derived from cloned cDNA (Fig. 2) and will be discussed later.

Immunolocalization of 27-kD Antigen in Liver

Anti-27-kD antibodies were used for immunohistochemistry on frozen sections of PLP-fixed rat liver (Fig. 3 *A*). The staining consisted of numerous macular and threadlike regions of intense fluorescence localized at the lateral but absent from the sinusoidal surfaces of the hepatocytes. This staining was consistent with the size, shape, and distribution of gap junctions in this tissue as revealed by numerous EM studies. EM localization of antibody binding was used to demonstrate that gap junctions were responsible for the staining pattern observed at the light microscopic level. The EM study was performed on crude plasma membranes isolated from rat liver. Anti-27-kD antibody binding was de-

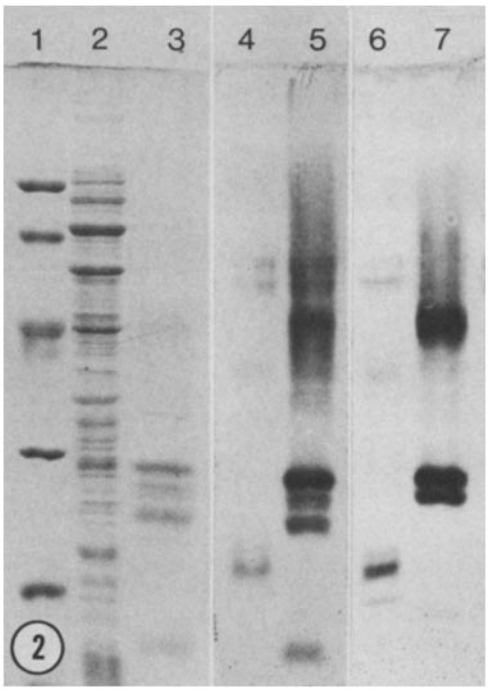


Figure 2. Western blot comparison of anti-27-kD antibody and anti-fusion protein antiserum. Lanes *1–3* display a Coomassie Blue-stained companion of the gels transferred to nitrocellulose. (Lane *1*) Molecular mass standards. (Lane *2*) Lysates of induced bacteria expressing fusion protein encoded by the cloned 1.1-kb cDNA (see text). (Lane *3*) Isolated gap junctions. Lanes *4* and *5* show staining with anti-27-kD antibody; lanes *6* and *7* show staining with anti-fusion protein antiserum. Both antibodies label fusion protein (lanes *2*, *4*, and *6*), and 27-kD protein and 48-kD aggregates (lanes *3*, *5*, and *7*). Not all of the presumed breakdown products of the 27-kD protein which are recognized by the anti-27-kD antibody are labeled by the anti-fusion protein antiserum. Neither preimmune sera label any bands under these conditions (data not shown).

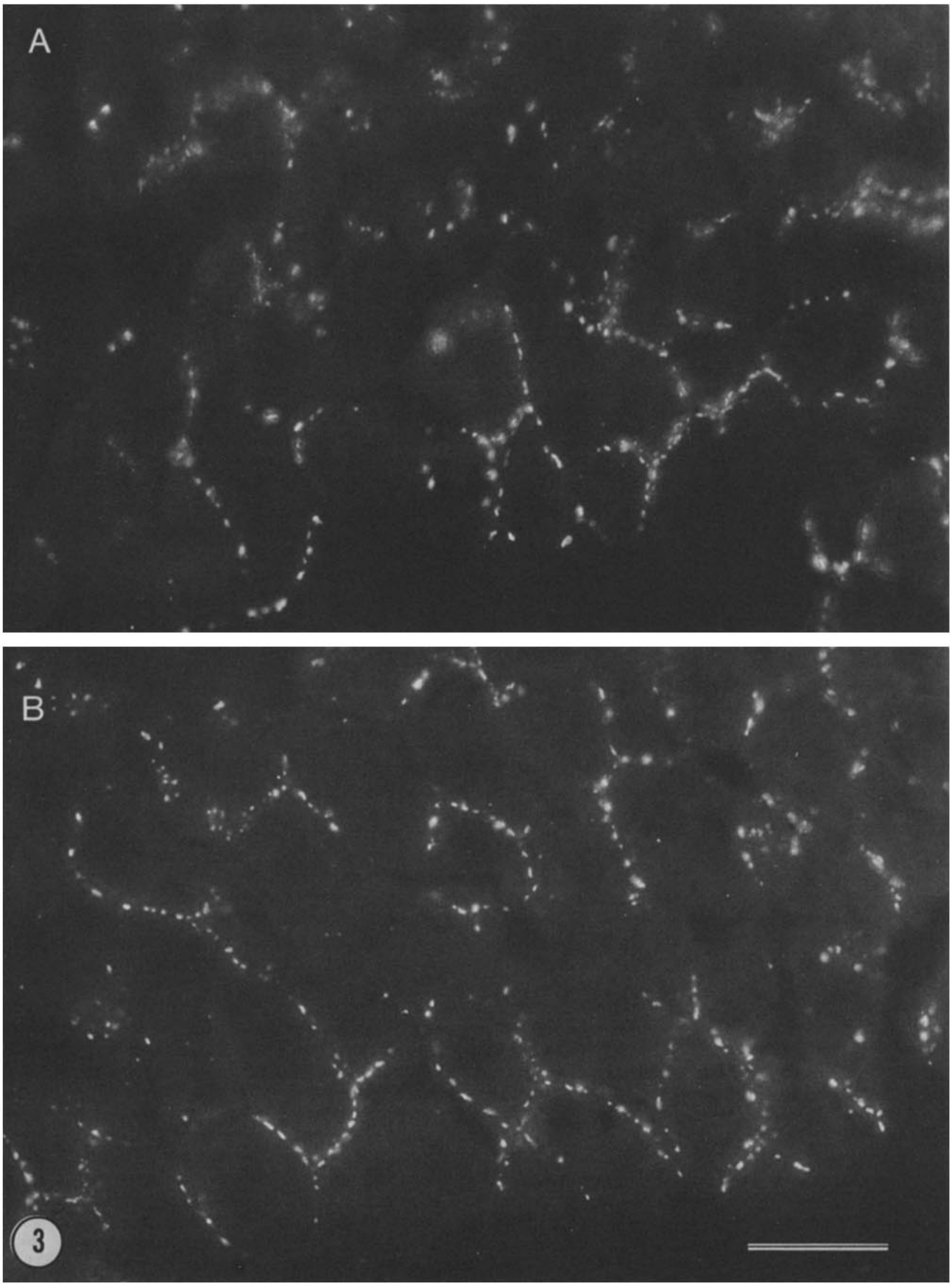
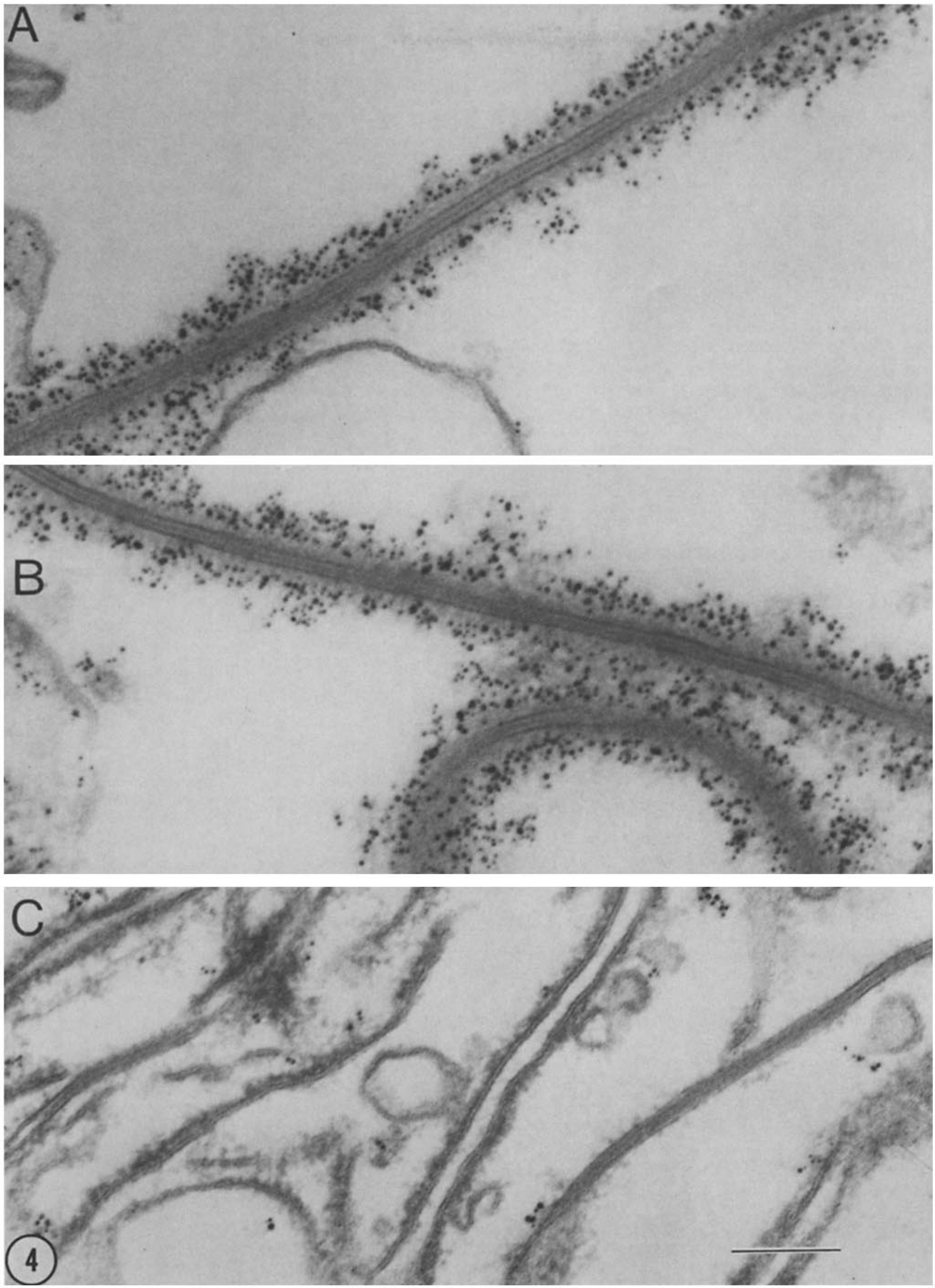


Figure 3. Immunofluorescent localization of gap junction protein (*A*) and cDNA-encoded fusion protein (*B*) in PLP-fixed frozen sections of rat liver. Specific labeling consisted of macular or thread-like areas along the lateral aspects of the cell borders. The distribution of these antigens is extremely similar. Bar, 20 μm.


A
B
C
4

tected using colloidal gold coupled to goat anti-rabbit IgG (Fig. 4, *A* and *B*). Gold particles were observed only on the cytoplasmic aspects of the gap junctional regions. No specific staining was detectable on either cytoplasmic or extracellular surfaces of non-junctional membranes. Preimmune Ig showed a low, nonspecific background binding of gold (Fig. 4 *C*).

cDNA Cloning with Antibody

Affinity-purified anti-27-kD antibodies were used to screen a rat liver cDNA library in lambda gt11. 250,000 plaques were plated for the initial screen in which one unambiguous positive was detected. This positive plaque was picked and replated to purity. EcoRI digestion of the lambda DNA released a single fragment that was 1.1 kb in size. This selected cDNA will be referred to as the 1.1-kb clone.

Two strategies were used to verify that the selected cDNA coded for a gap junction protein. First, an antibody was raised against the fusion protein. This antibody was expected to exhibit characteristics similar if not identical to that of the original anti-27-kD antibody. Second, the library was rescreened by hybridization to select longer cDNAs, hopefully containing the complete protein-coding region. The NH_2-terminal sequence predicted by these clones was expected to match that of the published amino acid sequence.

Production of an Anti-fusion Protein Antibody

Two problems were experienced in producing an antibody against the lambda gt11 fusion protein. First, the absolute levels of expression for the 1.1-kb cDNA were extremely poor. Second, the antigenic response to the beta-galactosidase portion of the fusion protein, which represents over 110 kD of the total protein, was much more prominent than the response to the protein encoded by the cDNA. Therefore, the 1.1-kb cDNA was subcloned into the high level expression vector pMAM-17 (Muesing et al., 1984). This vector produces a fusion protein consisting of 6 kD of a bacterial protein, rop, plus whatever is coded by the inserted cDNA. Production of fusion protein is thermally induced by growing the bacteria carrying the construct at 42°C. Fig. 5 shows an SDS polyacrylamide gel of lysates of such bacteria grown at 30°C, during which time expression was inhibited (lane *2*), and at 42°C, during which time expression was induced (lane *3*). Induced bacteria displayed a major new band at 21 kD. This fusion protein was collected by preparative SDS PAGE (lane *5*) and injected into rabbits.

Comparison of Anti-fusion Protein Antiserum and Anti-27-kD Antibody

The unfractionated anti-fusion protein antiserum was characterized by Western blot (Fig. 2). The affinity-purified anti-27-kD antibody (Fig. 2, lanes *4* and *5*) and anti-fusion protein antiserum (Fig. 2, lanes *6* and *7*) were used to label blots consisting of lysates of induced bacteria (Fig. 2, lanes *2*, *4*, and *6*) and whole isolated gap junctions (Fig. 2, lanes *3*, *5*, and *7*). With both antisera, labeling of the fusion protein, the 27-kD gap junction protein, and the 48-kD aggregate were observed. The anti-fusion protein antiserum did not label all of the lower molecular mass bands which were labeled by the anti-27-kD antibody. These fragments may consist of NH_2-terminal sequences which are not present in the fusion protein. Both the anti-fusion protein antiserum and the anti-27-kD antibody labeled a number of higher molecular mass proteins whose identities are unclear. No bands were labeled by preimmune serum from either rabbit (data not shown). The anti-fusion protein antiserum was also characterized by light microscopic immunohistochemistry (Fig. 3 *B*), revealing a pattern of labeling indistinguishable from that of the original anti-27-kD antibody (Fig. 3 *A*). EM immunocytochemistry using the anti-fusion protein antiserum was indistinguishable from Fig. 4 (data not shown).

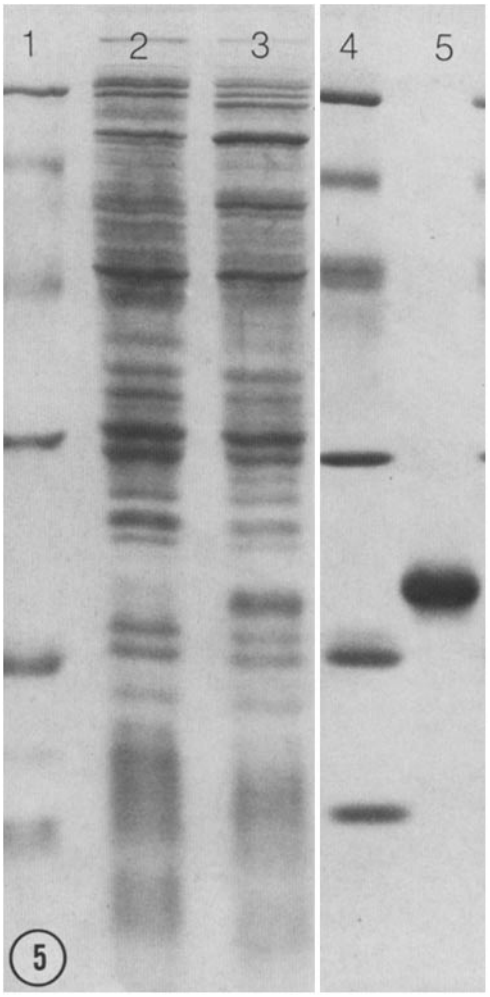


Figure 5. Coomassie Blue-stained SDS polyacrylamide gel of fusion protein produced in pMAM-17. (Lanes *1* and *4*) Molecular mass standards. (Lane *2*) Lysate of bacteria grown at 30°C during which expression is repressed. (Lane *3*) Lysate of bacteria grown at 42°C during which expression is induced. (Lane *5*) Sample of SDS PAGE-purified fusion protein used for production of antisera.

Figure 4. EM localization of 27-kD protein in rat liver plasma membranes. (*A* and *B*) Membranes stained with anti-27-kD followed by colloidal gold-conjugated goat anti-rabbit IgG. Staining is observed only on the cytoplasmic surfaces of junctional membranes. No specific stain is detected on non-junctional membranes. (*C*) Preimmune control. Bar, 100 nm.

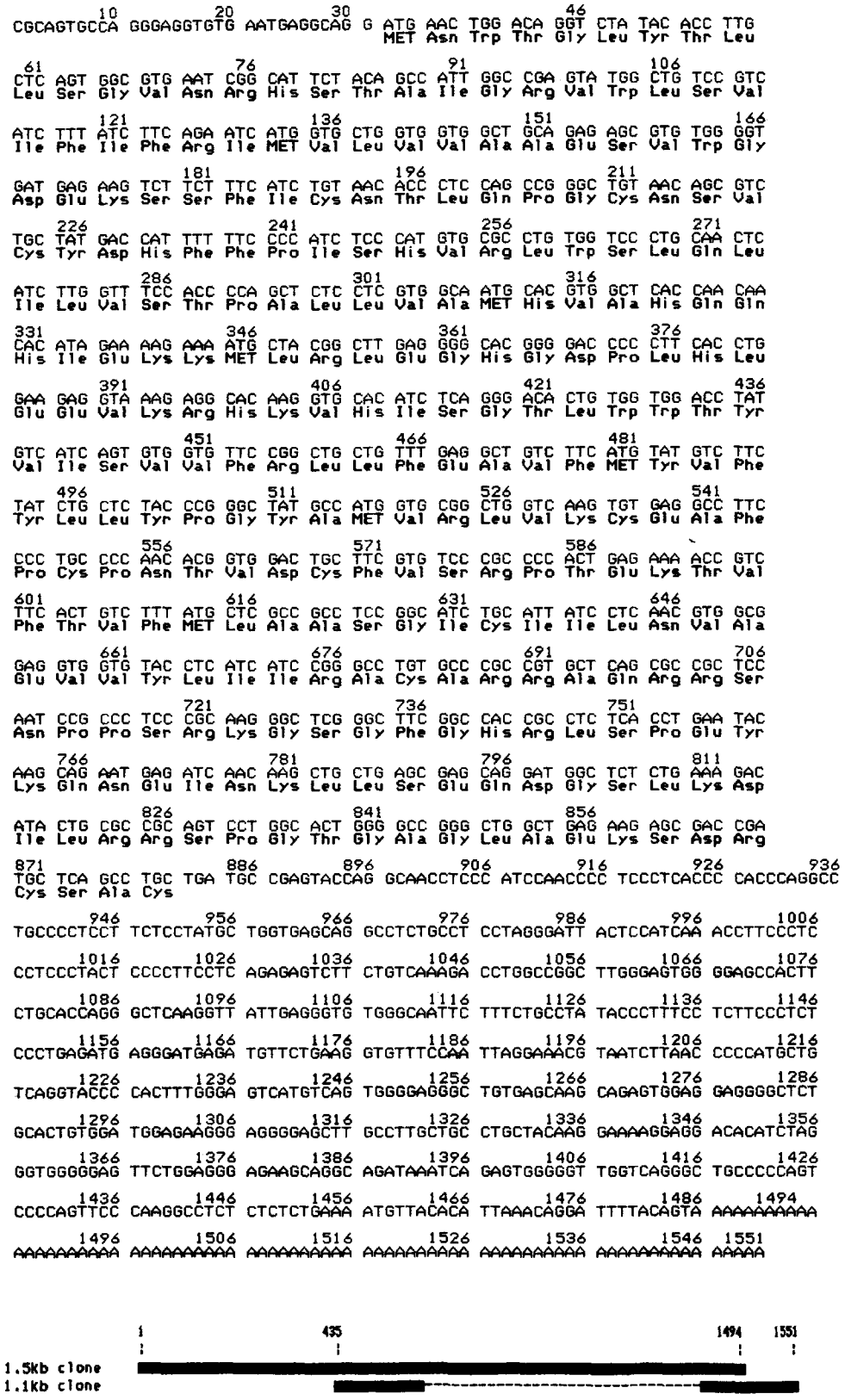


Figure 6. Nucleotide sequence of rat liver gap junction cDNA and derived amino acid sequence. This sequence is constructed by overlapping sequences from the 1.1-kb cDNA and the 1.5-kb cDNA. The nature of the overlap between these cDNAs is shown in the diagram at the bottom of the figure. Solid bar indicates regions of the cDNA that were completely sequenced. Dotted line indicates region where sequence data was not obtained. The largest open reading frame codes for a 32-kD protein. The first 56 amino acids have been sequenced by Edman degradation (Nicholson et al., 1983) and match the derived sequence precisely.

cDNA Cloning by Hybridization

To isolate full length cDNA clones, the original library was rescreened by plaque hybridization with the 1.1-kb cDNA. Initially, 150,000 plaques were plated; 22 positives were picked and replated to purity. A 1.5-kb cDNA clone was selected for complete sequencing. The original 1.1 kb clone was partially sequenced. The nucleotide sequences of these two clones overlapped and provided a major portion of the sequence of rat liver gap junction mRNA. The derived nucleotide and protein sequence is shown in Fig. 6. The nature of the overlap in the sequenced regions of the two

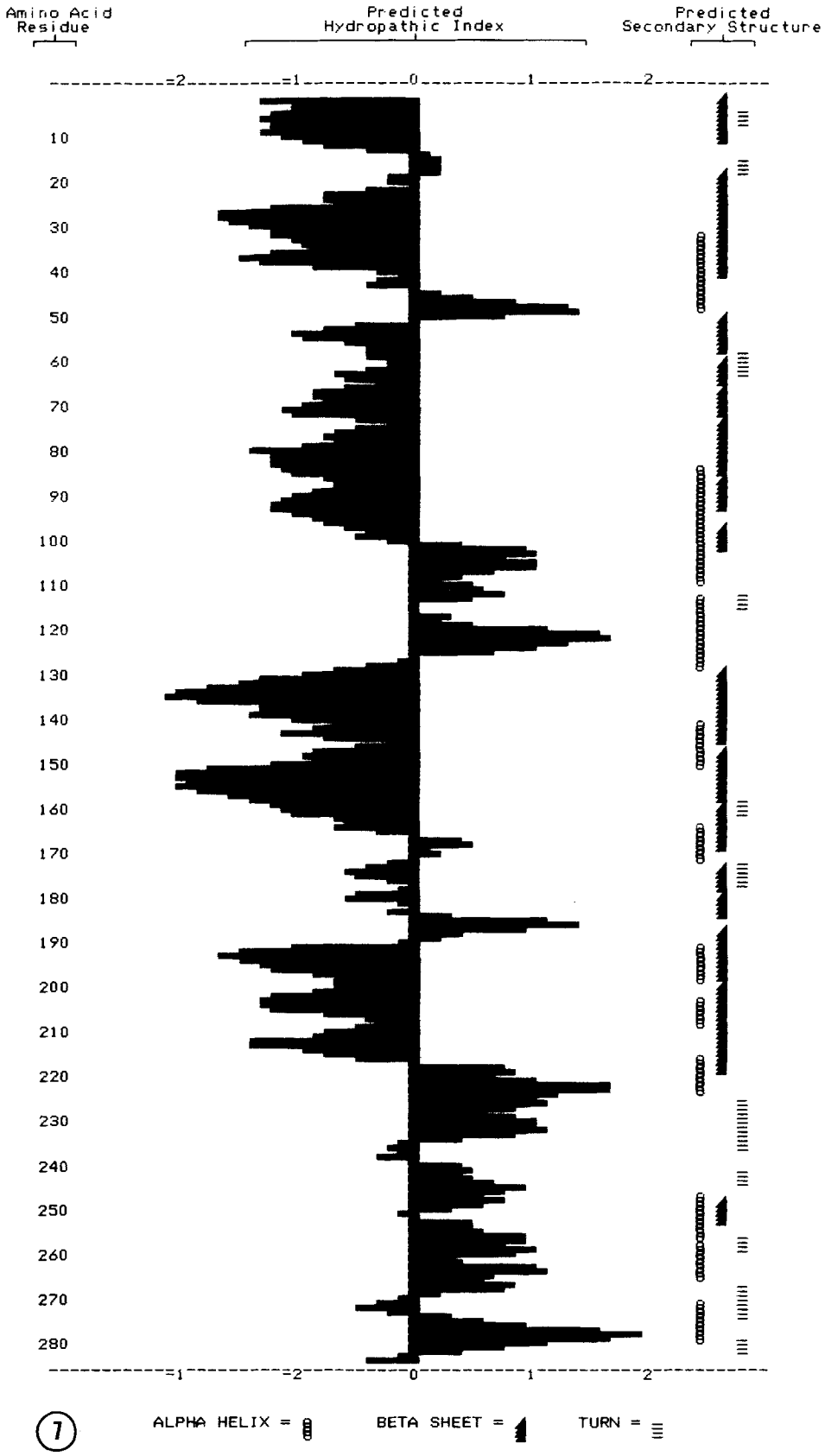


Figure 7. Plot of hydropathicity and secondary structure of rat liver gap junction protein. Hydropathicity values have been plotted with respect to position along the derived amino acid sequence of gap junction protein. Column at left displays amino acid residue number. Negative peaks (left of center zero) indicate hydrophobic areas, positive peaks (right of center zero) are hydrophilic. Secondary structure calculated by the paradigm of Chou and Fasman (1974) is indicated at the right. The positions of the major hydrophobic regions correspond closely to the prediction of beta-sheet secondary structure.

cDNAs is shown in the diagram at the bottom of Fig. 6. The nucleotide sequence of the 1.1-kb cDNA was determined for 220 bp starting at the 5′ (coding region) end. The 1.1-kb cDNA sequence overlaps the 1.5-kb cDNA beginning at base 435 of the longer clone. The sequence of the 1.1-kb cDNA was also determined 176 bp from the 3′ (noncoding) end. The 1.1-kb cDNA contains a 71-base poly(A) region at its 3′ end not found in the 1.5-kb cDNA. The predicted mRNA se-

quence exhibits a 3′ untranslated region of 668 nucleotides and does not display a consensus poly-adenylation signal (Proudfoot and Brownlee, 1976). The sequence ATTAAA near the 3′ end of the cDNA at base 1,466 may serve as such a signal. The reading frame in the cDNA sequence is closed near the 5′ side of the presumed initiation codon.

The cDNA sequence predicts a protein containing 283 amino acids with a calculated molecular mass of 32,007 daltons. The predicted NH_2-terminal amino acid sequence matches exactly the published NH_2-terminal amino acid sequence determined by Nicholson et al. (1983). This agreement, coupled with the behavior of the anti-fusion protein antibody, confirms that the cloned cDNA can code for a hepatocyte gap junction protein. It also suggests that there is no cleaved leader or signal sequence at the NH_2 terminal.

The predicted protein contains one potential site for N-linked glycosylation (Fig. 6, starting at base 35) although no appreciable carbohydrate has been detected in the 27-kD protein in isolated gap junctions. An interesting feature of this predicted sequence is the pronounced imbalance of charged residues. There are 42 positively charged and 21 negatively charged residues at neutral pH. The amino acid sequence was analyzed by the procedure of Kyte and Doolittle (1982) which predicts the hydropathic character of local regions in the sequence (Fig. 7, center). There are four major hydrophobic, potentially membrane-embedded areas: residues 1–40, 55–100, 130–165, and 190–215. The COOH-terminal 60 residues exhibit a pronounced hydrophilic character.

A prediction of secondary structure by the procedure of Chou and Fasman (1974) is also displayed in Fig. 7 (right). The hydrophobic regions of this protein exhibit a striking tendency toward beta sheet conformation. Although this is an unusual characteristic for a membrane protein, it has been experimentally observed by x-ray diffraction of isolated rat liver gap junctions by Makowski et al. (1982).

Comparison to Lens MP26 and Other Proteins

The lens membrane protein MP26 has been suggested as a structural component of the unusual gap junctions between lens fibers. Bovine lens MP26 cDNA has been cloned and the derived amino acid sequence is available (Gorin et al., 1984). The predicted amino acid sequences of rat liver gap junction protein and bovine lens MP26 were are compared by the matrix homology search method of Pustell and Kafatos (1982) (data not shown). This procedure revealed no obvious homology at any point between these proteins. The National Biomedical Research Foundation (Dayhoff) protein sequence data bank was also searched for homologous proteins. No obvious matches were detected.

Northern Blot Analysis

Total RNAs from rat liver, brain, stomach and kidney (Fig. 8, lanes *1–4*, respectively) were examined for homologous sequences by Northern blot analysis using the 1.5-kd cDNA as probe. For all tissues, hybridization was observed to a single band, or possibly a heterogeneous smear of similarly sized bands. In each case, a band of 1.6 kb, migrating slightly faster than the 18s ribosomal small subunit (lower arrowhead), was observed. This result suggests that each of these tissues expresses a single gap junction mRNA which is very homologous to that expressed in rat liver. Under these conditions of washing (0.1× SSC at 68°C), no signal was detected in total RNA from rat heart or bovine lens. To examine these tissues more closely, poly(A) RNA was prepared and blotted. Fig. 9 displays a panel of exposures of the same blot containing poly(A) RNA from rat liver (lanes *1*), rat heart (lanes *2*), and bovine lens (lanes *3*), washed under conditions of increasing stringency. Fig. 9 *A* shows the results of a very low stringency wash (2.5× SSC at 50°C); bands are detected in liver at 1.6 kb and at ~9 kb (lane *1*). A single band is detected in heart at 1.3 kb (lane *2*). Two bands are detected in lens at ~3.0 kb and 8 kb (lane *3*). As stringency of the wash is increased (Fig. 9 *B* and *C*) first the hybridization to the band in heart is lost (Fig. 9 *B*, lane *2*), then hybridization to the two bands in lens (Fig. 9 *C*, lane *3*). The blot in *C* was subjected to prolonged exposure to film; no trace of hybridization to heart and lens remained (data not shown). This result suggests that heart and lens express gap junction proteins which share some but not complete homology to the protein expressed by liver, brain, kidney, and stomach.

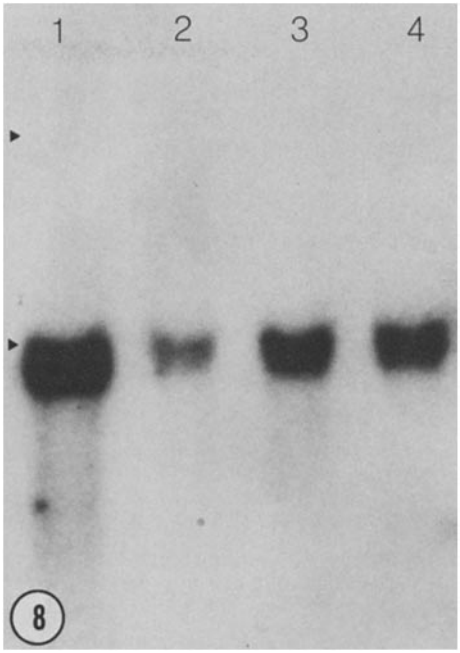


Figure 8. Hybridization of rat liver gap junction cDNA to total RNAs from liver, brain, stomach, and kidney of adult rats (lanes *1–4*, respectively). Each lane contains 5 μg RNA. Blot is probed with full length cDNA. Hybridization is observed in each case to a single band at ~1.6 kb. Arrowheads indicate the positions of 28s and 18s ribosomal subunits.

Discussion

It has been demonstrated by immunocytochemistry that the 27-kD protein associated with isolated rat liver gap junctions is specifically localized to morphologically recognizable junctions. This strongly supports the notion that the 27-kD protein is a structural component of gap junctions in that tissue. cDNAs have been cloned from a rat liver library which encodes a protein with immunocytochemical localization and an NH_2-terminal amino acid sequence identical to the 27-kD protein. The calculated molecular mass of the protein predicted by the cDNA is 32 kD. Northern blot analysis indicates that liver-type mRNA is present in a variety of tissues but is specifically absent from heart and lens. This supports

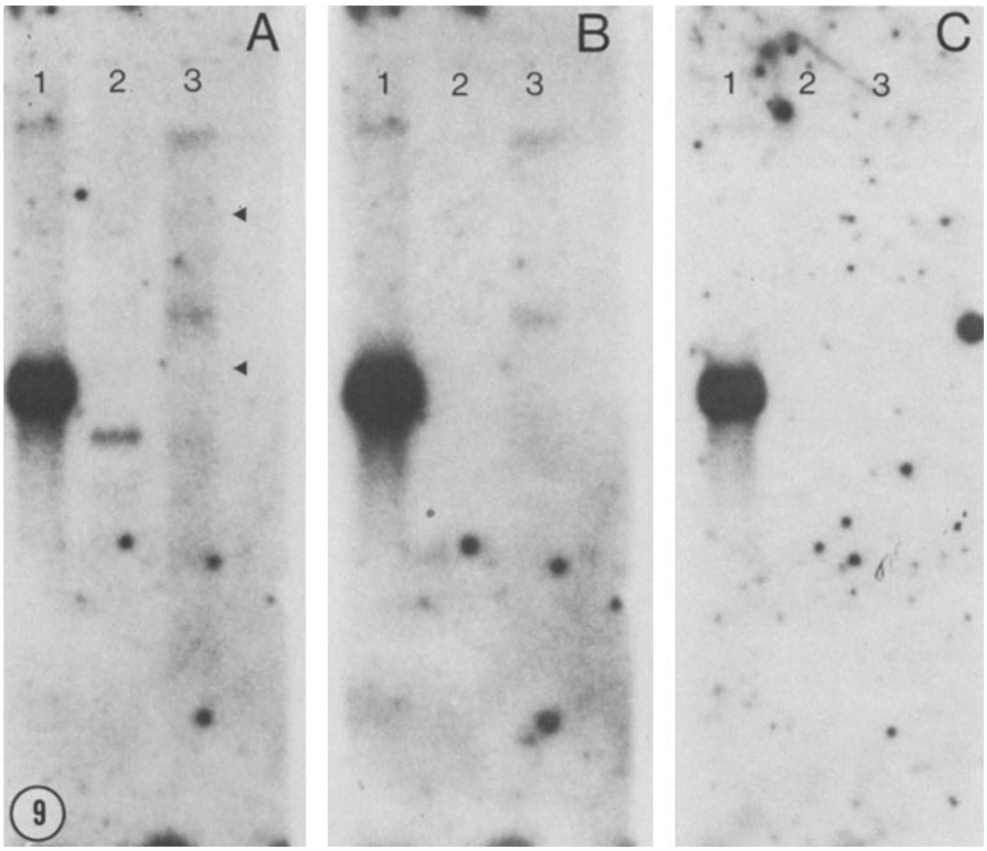


Figure 9. Hybridization of rat liver gap junction cDNA to liver, heart, and lens poly(A) RNA under conditions of varying stringency. Blot consists of lanes of 1.5 μg poly(A) RNA from rat liver (lanes *1*), rat heart (lanes *2*), and bovine lens (lanes *3*). Blot was washed in 2.5× SSC, 50°C (*A*), 2.5× SSC, 65°C (*B*), and 0.5× SSC, 65°C (*C*). At lowest stringency, hybridization is observed to two bands in liver (1.6 kb and 9.0 kb), to one band in heart (1.3 kb) and two bands in lens (3.0 kb and 8.0 kb). At higher stringencies, hybridization in heart is abolished first, followed by hybridization in lens. Hybridization in liver is retained under all conditions tested. Arrowheads indicate the positions of 28s and 18s ribosomal subunits.

the suggestion (Nicholson et al., 1983; Paul, 1985) of the existence of at least three distinct, tissue-specific gap junctions proteins.

There are three alternative explanations for the disparity between the observed molecular mass upon SDS PAGE and the molecular mass predicted from the cDNA. First, the 27-kD protein observed in isolated gap junctions could be the result of proteolytic cleavage of a 32-kD precursor. This cleavage could be physiologically relevant or may be artifactual due to the isolation procedure. Second, the difference in calculated and observed molecular masses may be an artifact of the SDS PAGE system. Anomalous mobility of proteins in SDS PAGE is occasionally observed (Weber and Osborn, 1975). Finally, a nucleotide sequencing error could result in the prediction of incorrect protein sequence. In fact, several regions of the 1.5-kb cDNA were difficult to sequence due to gel compressions and other artifacts of the dideoxy sequencing procedure. To overcome this problem, sequence was obtained on both strands of the 1.5-kb cDNA by the dideoxy method and for the first 960 bases on both strands by the Maxam-Gilbert chemical degradation method.

A 54-kD protein that cross-reacts with antibody specific for the rat liver 27-kD protein has been reported by Warner et al. (1984) and Paul (1985). This protein is not detected in isolated gap junctions but is seen in crude homogenates of a variety of tissues. The results of the Northern analysis do not directly support the notion of a homologous but higher molecular mass protein, but they do not rule it out. The 54-kD protein may share too little homology with the 27-kD protein to be observed under the conditions used. Alternatively, the 54-kD may be coded by an mRNA close enough in size to that which codes for the 27-kD protein so that they are not resolved by the gel. In either case, it is clear that no precursor/product relationship obtains for any 54-kD protein and the protein predicted by the cDNA we have cloned.

A possible explanation of our failure to observe an open reading frame long enough to code for a 54-kD protein is that the cDNA we obtained resulted from a "recombination" artifact occurring during the preparation of the library. To address this issue, the 1.5-kb cDNA clone was separated into two fragments by digestion with Sau3A, which cuts at base 771. Both fragments were used independently to probe Northern blots of liver RNA. No difference in quality or quantity of hybridization was observed (data not shown).

The prediction of a large amount of beta conformation in the secondary structure of the 27-kD protein is extremely unusual. In fact, only one other integral membrane protein, *E. coli* porin (Engle et al., 1985) has been demonstrated to exhibit significant beta structure. x-ray diffraction measurements reveal a transmembrane domain of the gap junction channel which is characterized by a high proportion of beta-sheet running parallel to the membrane surfaces (Makowski et al., 1982). Thus the membrane intercalated regions of this protein may not span the membrane in short, perpendicular traverses of the type observed in bacteriorhodopsin (Henderson and Unwin, 1975).

The amino acid sequence predicted by the cloned cDNA exhibits one potential site for N-linked glycosylation (asn-X-ser/thr) at residue 2. The 27-kD protein associated with isolated junctions is apparently not glycosylated. This could mean that the potential sites are not exposed in the lumen of the rough endoplasmic reticulum where such glycosylation

normally occurs. If so, it predicts an either membrane embedded or cytoplasmic disposition for the NH_2 terminus.

Northern blot analysis reveals the presence of very high molecular mass RNAs in poly(A) liver and lens. The nature of these RNAs is unknown. Possibly, they represent partially processed primary transcripts. If so, an interesting possibility exists that the different lower molecular mass RNAs observed in liver, heart, and lens are derived by differential processing of the same primary transcript. Another important consideration in the analysis of these Northern blots is that most of the tissues examined contain more than one type of cell. For example, lens exhibits two major cell types and at least two types of gap junctions that can be distinguished physiologically (Miller and Goodenough, 1986). It is not known which of these types, if any, are responsible for the bands observed in Northern blots of lens RNAs.

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