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Expression of Functional Cell-Cell Channels from Cloned Rat Liver Gap Junction Complementary DNA

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An oocyte expression system was used to test the relation between a complementary DNA (cDNA) clone encoding the liver gap junction protein and cell-cell channels. Total liver polyadenylated messenger RNA injected into oocytes induced cell-cell channels between paired oocytes. This induction was blocked by simultaneous injection of antisense RNA transcribed from the gap junction cDNA. Messenger RNA selected by hybridization to the cDNA clone and translated in oocyte pairs yielded a higher junctional conductance than unselected liver messenger RNA. Cell-cell channels between oocytes were also formed when the cloned cDNA was expressed under the control of a heat-shock promoter. A concentration-dependent induction of channels was observed in response to injection with in vitro transcribed gap junction messenger RNA. Thus, the liver gap junction cDNA encodes a protein that is essential for the formation of functional cell-cell channels.

IT IS GENERALLY ASSUMED THAT GAP junctions represent assemblies of cell-cell channels. This is based on several lines of circumstantial evidence such as the correlation between the presence of gap junctions and intercellular communication (*1, 2*). Over the past 15 years, procedures for isolating gap junctions have been developed that allowed an analysis of their protein components. Molecular sizes for the major gap junction protein ranging from 18,000 to 54,000 daltons have been reported (*3–8*). In part, this wide range of observed molecular sizes may be due to the fact that the gap junctions were obtained from a variety of tissues. However, there is a consensus that the major component of the liver gap junction is a 28,000-dalton protein [but see (*8*)].

It is not known whether the 28,000-dalton protein from liver gap junctions is an essential component of the cell-cell channel, and, if so, whether it can be used to form a functional channel. Two recent developments make it possible to address these questions directly. First, a complementary DNA (cDNA) clone specific for the rat liver gap junction protein has been isolated (*9*). Second, a functional assay for testing channel-specific messenger RNA (mRNA) is now available (*10, 11*). We therefore used the *Xenopus* oocyte cell-cell channel assay in combination with the cDNA clone to examine (i) hybrid arrest of channel formation induced by liver mRNA with antisense RNA, (ii) enrichment of channel-inducing mRNA from rat liver by hybrid selection with gap junction cDNA, (iii) expression of the cloned cDNA under the control of a heat-shock promoter, and (iv) in vitro synthesis of gap junction mRNA from the cloned cDNA and its expression in oocytes.

To test whether the cloned cDNA encodes a critical component of functional cell-cell channels, we used the hybrid-arrest technique (*12–14*). In these experiments antisense RNA specific for the gap junction protein was added to a preparation of total polyadenylated mRNA that is capable of inducing cell-cell channels when injected into oocytes. The induction of such channels in oocytes should be abolished if the cDNA clone, from which the antisense RNA was transcribed, corresponds to the channel gene. To avoid potential problems that could arise from insufficient homology between transcripts in different tissues, we used total polyadenylated mRNA from liver instead of from uterus (*11*) for this experiment. Messenger RNA isolated from rat liver induced a junctional conductance in paired oocytes (Fig. 1 and Table 1). The induction can be seen above a low background (undetectable in several experiments) of endogenous oocyte channels, and the degree of induction is comparable to that observed with uterus or heart mRNA (*15*). Channel induction by liver mRNA was blocked by simultaneous injection of antisense RNA synthesized in vitro from the gap junction cDNA clone. The level of endogenous cell-cell channels, however, was not significantly affected by injection of antisense RNA (Table 1). The observed inhibition of channel formation indicates that an mRNA species that is homologous to, if not identical to, the cloned cDNA is essential for the observed channel induction.

To corroborate these results we used the cloned cDNA to enrich homologous liver mRNA by hybrid selection (*16*). Such hybrid-selected liver mRNA induced channels with higher efficiency than did total mRNA (Table 1), even though the concentration of the selected mRNA was lower than that of polyadenylated total mRNA. In addition, partial degradation probably had occurred. Thus, the cDNA clone can be used to enrich a channel-specific mRNA species from total liver polyadenylated mRNA.

Both hybrid selection and hybrid arrest can be accomplished with incomplete cDNA sequences. Therefore, if the cDNA clone unexpectedly, should not contain the entire sequence, it should still be usable for these experiments. In the next series of experiments, we tested whether the cDNA clone itself can be functionally expressed in oocytes. First, the cDNA sequence, shortened by 500 bases in the 3′-nontranslated region was linked to the promoter of a human heat-shock gene (Fig. 2). Transcription of this construct in heat-treated oocytes was dem-

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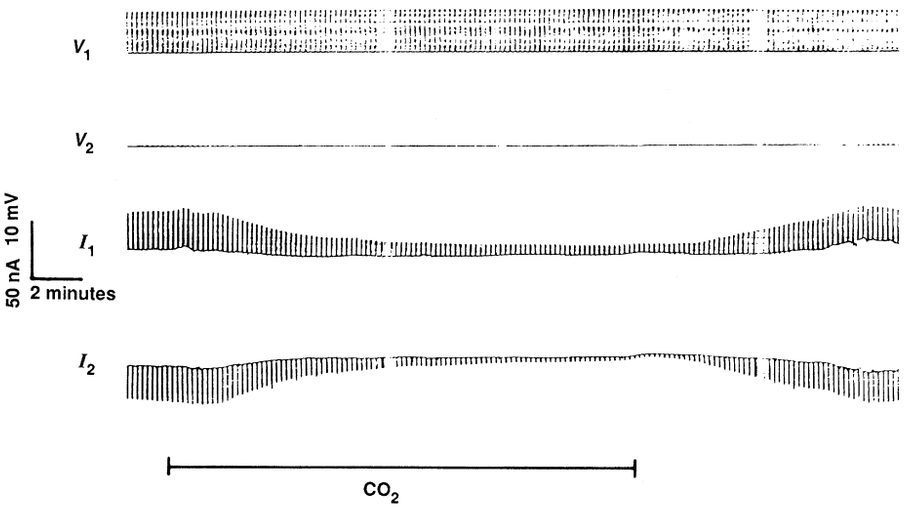

Fig. 1. Recording of junctional conductance between paired oocytes injected with in vitro transcribed mRNA. Oocytes were prepared as described (*11*). Junctional conductance was measured by the dual–voltage clamp technique (*29, 30*). Each of the two oocytes within the pair is independently voltage-clamped at its resting membrane potential. A voltage step is applied to oocyte 1 (V_1), which generates the clamp current I_1. Oocyte 2 is held at a constant potential (V_2). Current flowing from oocyte 1 to oocyte 2 in response to the voltage step generates a current in the clamp circuit of oocyte 2, holding its membrane potential constant. This current (I_2) is equal in magnitude, but of opposite sign, to the transjunctional current (I_j). Junctional conductance is given by I_2/V_j, where V_j is the transjunctional voltage. The voltage steps are between 5 to 10 mV lasting approximately 1 second. Perfusion of the recording chamber with medium saturated with 100% CO_2 results in a reversible reduction of junctional conductance.

onstrated by Northern blot analysis (Fig. 3) (*17*). The polyadenylated mRNA synthesized by the oocytes was of the expected size. A different construct, in which a *Drosophila* heat-shock promoter was linked to the complete cDNA sequence, produced a longer mRNA species as predicted from the construct. No hybridization of the liver cDNA with any endogenous oocyte message was observed (Fig. 3, lane 4). Because the oocytes used in this experiment had an unusually high number of endogenous channels (mean conductance: 1 μS), this result suggests that the mRNA encoding the endogenous channels does not exhibit a high degree of homology to liver gap junction mRNA. This is consistent with the observation that the formation of endogenous channels is not affected by antisense RNA.

When assayed for junctional conductance, the gap junction gene under the control of the heat-shock promoter yielded an induction higher than that observed with total liver mRNA (Table 1) (*18*). This result suggests that the gap junction cDNA codes for a protein essential for cell-cell channel formation. The fact that the induction was only about twice as high as that observed with liver mRNA can have a variety of causes. One of the possibilities is that the junctional protein encoded by the cloned cDNA may not be sufficient for channel formation. It is conceivable, for example, that participation of additional proteins provided by the oocytes is required, and that these proteins may determine the rate of channel formation.

In a second approach to test the capability of the liver gap junction protein to produce cell-cell channels, we used the cloned cDNA, linked to an SP6 promoter, to synthesize in vitro capped channel-specific mRNA. This mRNA, when injected into oocytes, produced the highest increase in junctional conductance observed (Table 1). Such high conductance between oocytes would also be seen if the two oocytes had undergone fusion (*11*). To test this possibility, coupled oocyte pairs were subjected to acidification with carbon dioxide, a treatment that reversibly uncouples hepatocytes

Table 1. Conductances between paired oocytes. The detection limit for junctional conductance is less than 0.01 μS. The number of oocyte pairs analyzed is given in parentheses. In the hybrid-arrest, hybrid-select, and in vitro transcribed mRNA experiments, mRNA was injected into oocytes immediately after pairing. About 20 to 30 ng of liver mRNA or antisense RNA was injected per oocyte. Junctional conductance was measured 16 to 18 hours after injection. Antisense mRNA was synthesized in vitro with SP6 RNA polymerase from the gap junction cDNA clone inserted into the Eco RI site of pGEM-3. For the hybrid-arrest experiment, this RNA was mixed with an equal amount of liver mRNA before injection into *Xenopus* oocytes. For the hybrid selection of gap junction mRNA, total liver mRNA was hybridized to gap junction cDNA immobilized on nitrocellulose filters. After repeated washings under stringent conditions the selected mRNA was eluted with boiling water and precipitated with ethanol in the presence of yeast transfer RNA. Messenger RNA selected from 30 μg of total liver polyadenylated mRNA was dissolved in a final volume of 10 μl. This solution was injected (20 to 30 nl per oocyte). Heat-shock experiments were as described in Fig. 3 except that oocytes were paired 60 minutes after termination of the heat treatment, and junctional conductance was measured 16 to 18 hours later. Messenger RNA was synthesized in vitro from gap junction cDNA inserted into the Eco RI site of pGEM-4 (Promega Biotech). pGEM-3 and pGEM-4 differ only by the orientation of the SP6 promoter in relation to the polylinker site. Thus, SP6 RNA polymerase could be used for the synthesis of both mRNA and antisense RNA. The plasmid was linearized with Pvu II, and transcription was carried out in the presence of an excess of GpppG over guanosine 5′-triphosphate (GTP) to allow formation of a 5′-cap structure (*27*). After degradation of the template DNA with deoxyribonuclease I, the transcripts were extracted with phenol-chloroform, precipitated with ethanol, and redissolved at a concentration of 3 μg/μl (based on OD_{260}, actual amount of full-length capped transcripts is probably lower). Quantities of this solution (20 to 30 nl), as well as of 10-fold and 100-fold dilutions, were microinjected into oocytes. (Note the variation in the background of endogenous channels. Experimental data can only be compared between oocytes with similar levels of background endogenous channel expression). Experimental and control data points were always obtained with oocytes from the same ovary. In the hybrid-arrest experiment, conductances in controls and liver mRNA–injected oocytes were significantly different ($P < 0.01$), whereas no significant difference can be detected for data between controls and liver mRNA plus antisense RNA or antisense RNA (Wilcoxon test) (*28*).

Experimental system	Conductance (μS) (mean ± SEM)
Hybrid-arrest experiment	
Control	0.06 ± 0.03 (19)
Liver mRNA	0.26 ± 0.17 (18)
Liver mRNA + antisense RNA	0.03 ± 0.02 (20)
Antisense RNA	0.07 ± 0.02 (14)
Hybrid-select experiment	
Control	0.18 ± 0.03 (16)
Liver mRNA	0.57 ± 0.17 (17)
Selected mRNA	1.30 ± 0.29 (18)
Heat-shock experiment	
Control	0.05 ± 0.01 (40)
D3G13 DNA	0.43 ± 0.14 (36)
In vitro transcribed mRNA	
Control	0.23 ± 0.07 (28)
mRNA	7.42 ± 2.90 (18)
Control	0 (13)
mRNA	1.49 ± 0.52 (9)
mRNA (1:10 dilution)	0.65 ± 0.38 (9)
mRNA (1:100 dilution)	0.06 ± 0.03 (8)

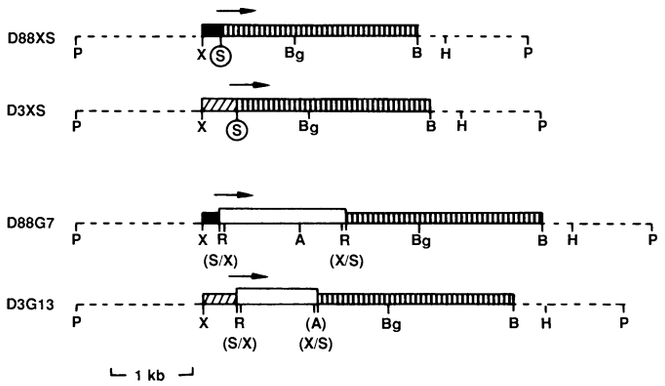

Fig. 2. Heat-shock expression vectors D88XS and D3XS and heat-shock–gap junction hybrid genes D88G7 and D3G13. The expression vectors were derived from previously described heat-shock hybrid genes (*31*, *32*). A unique Sal I (S) site is located between heat-shock gene 5′-nontranscribed and nontranslated sequences and 3′-nontranslated sequences. To construct D88G7, a 1.5-kb rat liver gap junction cDNA plasmid (*9*) was digested with Eco RI, ends were filled in, and Xho I linkers (CCTCGAGG) were added (*17*). After linker activation, the 1.5-kb cDNA fragment was isolated electrophoretically and ligated to the Sal I site of D88XS. The mixture was digested with Sal I and then used for transformation of *Escherichia coli*. Correct recombinants were identified by restriction analysis. Clone D3G13 was prepared in an analogous fashion except that a shorter gap junction cDNA fragment was used, which still included the entire gap junction protein-coding region [from Eco RI to Avr II at nucleotide (nt) 981], and insertion was into the Sal I site of D3XS. *Drosophila melanogaster hsp*70 gene sequences from nt −88 to +84 from clone 132E3 (*33*) (filled portion); 2.4 kb of 3′-nontranslated sequences from a *D. melanogaster hsp*70 gene clone 56H8, segment begins at nt 588 of the sequence in figure 2b of Torok and Karch (*34*) (portion with vertical lines); human *hsp*70 gene sequences from nt −270 to +113 (*32*) (hatched portion). pSVOd vector sequences (*35*) including an SV40 origin-of-replication segment are indicated by dashed lines. Gap junction cDNA segments are shown by open boxes. A, Avr II; B, Bam HI; Bg, Bgl II; H, Hind III; P, Pst I; R, Eco RI (only sites in the gap junction DNA are shown); X, Xho I. Sites that have been destroyed in the constructions are in parentheses.

(*19*). Junctional conductance in oocyte pairs injected with SP6-transcribed mRNA was reversibly reduced by acidification (Fig. 1) in a manner similar to that observed with hepatocytes.

The induction of cell-cell conductance was dependent on the concentration of the in vitro transcribed mRNA over a range of two orders of magnitude. At low concentrations, conductance increased proportionally with mRNA concentration. The highest mRNA concentration used was in the range where others have found saturation of the oocyte's translational capacity for exogenous proteins (*20*). This result indicates that the supply of the junctional protein is itself rate-limiting in the observed increase in junctional conductance.

The induction of cell-cell channels in oocytes expressing the liver gap junction protein is not only observed as a quantitative increase of junctional conductance over background provided by endogenous channels, the cDNA-directed channels also are qualitatively different from the endogenous channels. The oocyte channels exhibit gating properties that are very similar to those described for early *Xenopus* embryos (*21*, *22*): (i) their junctional conductance decreases with transjunctional voltage, (ii) the channels are insensitive to an increase in the concentration of cytoplasmic Ca^{2+}, and (iii) they close readily in response to cytoplasmic acidification. By contrast, the cDNA-directed channels (i) are voltage-insensitive, (ii) close in response to cytoplasmic injections of Ca^{2+}, and (iii) require pronounced cytoplasmic acidification (*23*). Thus, the cDNA-directed channels exhibit gating properties similar to those in hepatocytes (the parent tissue) and several other mammalian differentiated tissues (*1*, *19*, *22*, *24*, *25*). Endogenous and cDNA-directed channels, therefore, can easily be distinguished (*26*).

Because of their size and durability, *Xenopus* oocytes are amenable to experimental procedures difficult for many other cells. This durability and their ability to express cell-cell channels encoded by injected nucleic acids make them an excellent experimental tool for studying the mechanisms involved in the channel formation process, which so far have remained elusive. Furthermore, the fact that the cDNA-directed channels exhibit gating properties like those in the parent tissue indicates that the oocyte channel assay will be useful in defining the different domains of the channel-forming protein that are involved in the gating processes. Site-directed mutagenesis of the cDNA clone site-specific antibodies derived from synthetic peptides can be used in such studies.

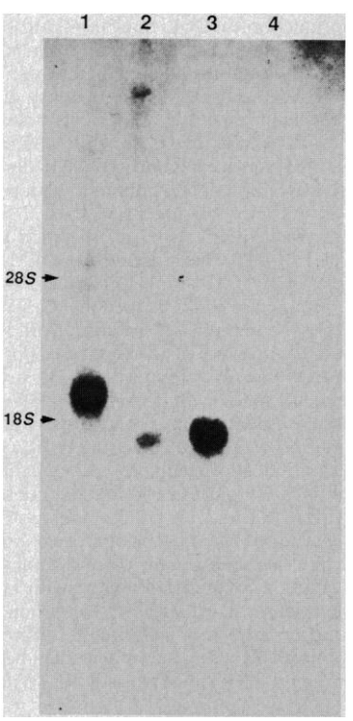

Fig. 3. Transcription from heat-shock–gap junction hybrid genes. Polyadenylated mRNA was extracted from groups of 30 oocytes (*36*) and fractionated by agarose gel electrophoresis. After blotting onto nitrocellulose (*17*), gap junction–specific mRNA was detected by hybridization with ^{32}P-labeled gap junction cDNA under conditions of high stringency [hybridization in 50% formamide, 42°C, washing in 0.1× standard saline citrate (SSC) at 25°C]. Lanes 1 and 2 are from heat-treated oocytes injected with construct D88G7 and D3G13, respectively. Lane 3 contains 5 μg of rat liver mRNA. Arrows indicate the positions of 18*S* and 28*S* ribosomal RNA. Lane 4 represents mRNA isolated from noninjected heat-treated oocytes. DNA (5 to 7 μg per nucleus) was injected into the nuclei of oocytes (lanes 1 and 2). After 30 minutes, the oocytes (lanes 1, 2, and 4) were heat-treated for 90 minutes at 36°C. Messenger RNA was extracted 60 minutes after termination of the heat treatment.

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Selective Inactivation of Influenza C Esterase: A Probe for Detecting 9-*O*-Acetylated Sialic Acids

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The influenza C virus (INF-C) hemagglutinin recognizes 9-*O*-acetyl-*N*-acetylneuraminic acid. The same protein contains the receptor-destroying enzyme (RDE), which is a 9-*O*-acetyl-esterase. The RDE was inactivated by the serine esterase inhibitor diisopropyl fluorophosphate (DFP). [^{3}H]DFP-labeling localized the active site to the heavy chain of the glycoprotein. DFP did not alter the hemagglutination or fusion properties of the protein, but markedly decreased infectivity of the virus, demonstrating that the RDE is important for primary infection. Finally, DFP-treated INF-C bound specifically and irreversibly to cells expressing 9-*O*-acetylated sialic acids. This provides a probe for a molecule that was hitherto very difficult to study.

SIALIC ACIDS (NEURAMINIC ACIDS) can be *O*-acetylated at positions 4, 7, 8, or 9. We and others have demonstrated that these modifications are developmentally regulated (*1–3*), and that the addition of a single *O*-acetyl group can markedly affect the biological properties of the parent molecule (*4–6*). The biochemical study of *O*-acetylation of sialic acids in biological systems is fraught with many problems, especially loss and migration of *O*-acetyl groups during analysis (*7*). Since 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac_2) is the preferred receptor for the hemagglutinin of influenza C (INF-C) (*8*) we reasoned that the virus could be used as a direct and specific probe for 9-*O*-acetylation. However, when INF-C is incubated with cells expressing Neu5,9Ac_2, an esterase [called the receptor-destroying enzyme (RDE)] causes a rapid and marked decrease in amount of the *O*-acetylation (*9*). We therefore sought ways to selectively block the esterase activity. As shown in Fig. 1, 1 m*M* diisopropyl fluorophosphate (DFP) completely inactivated the esterase. Phenylmethylsulfonyl fluoride (PMSF), another serine-active site inhibitor, was less effective, giving 25 to 50% inactivation at a 1 m*M* concentration.

After DFP treatment, the direct and specific binding of INF-C to cells could be demonstrated. Mouse erythrocytes, which are known to contain high levels of 9-*O*-acetylated sialic acids (*10*), were incubated with DFP-treated, biotinylated INF-C and then with fluorescent avidin. The INF-C–treated cells showed greater fluorescence (6.6-fold) than control cells (Fig. 2). This binding was completely blocked by bovine submaxillary mucin (which is rich in Neu5,9Ac_2) (Fig. 2) but not by an equivalent amount of bovine serum albumin. Thus, DFP-treated biotinylated INF-C is a specific probe for the presence of 9-*O*-acetylated sialic acids on cell surfaces.

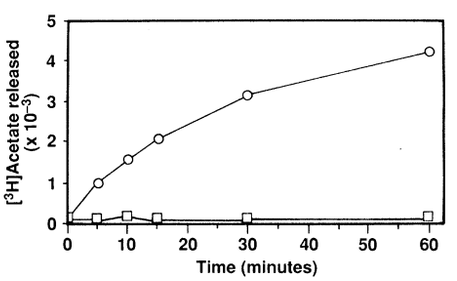

Fig. 1. Inactivation of INF-C esterase by DFP. INF-C (ATCC VR 104, C/Taylor/1233/47,5/80) was incubated with 1% isopropyl alcohol (IPA) or 1 m*M* DFP in 1% IPA for 30 minutes at 4°C. Treated virus was incubated with [^{3}H-acetyl] Neu5,9Ac_2 at 37°C. Reactions were stopped with an equal volume of a solution of 1*M* chloroacetic acid, 2*M* NaCl, and 0.5*M* NaOH, and contents of vials were added to 10 ml of scintillation cocktail which contained 20% isoamyl alcohol, 80% [0.5% 2,5-diphenyloxazole (PPO), 0.3% [1,4-bis-2(5-phenyloxazoyl)-benzene (POPOP) in toluene]. The principle of this assay and the details of the preparation of the substrate are as described (*20*). □, DFP-treated; ○, control.

These results also suggest that, although the hemagglutination and RDE activities of INF-C are both contained in the same glycoprotein and recognize the same structure (Neu5,9Ac_2), they do not share the same binding site. To confirm this, we studied the hemagglutination properties of the treated virus. Hemagglutination activity was not blocked by DFP-treatment of INF-C (Fig. 3). When the cells were warmed to 22°C, esterase activity caused the predicted loss of hemagglutination in the controls and the PMSF-treated sample. However, hemagglutination was preserved in the DFP-treated sample, and persisted for several days at 4°C. Thus, the binding of DFP to the serine active site of the esterase did not block the hemagglutination function; indeed, it made it stable rather than rapidly reversible.

To confirm the binding of DFP to the glycoprotein, we labeled INF-C virions with [^{3}H]DFP and then examined the virus proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Previous studies have indicated that the activated influenza C glycoprotein (gpII)

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