Molecular Biology of the Cell
Vol. 11, 2459–2470, July 2000

Two *Drosophila* Innexins Are Expressed in Overlapping Domains and Cooperate to Form Gap-Junction Channels

Lucy A. Stebbings,* Martin G. Todman, Pauline Phelan,† Jonathan P. Bacon, and Jane A. Davies

Sussex Centre for Neuroscience, School of Biological Sciences, University of Sussex, Brighton, BN1 9QG, United Kingdom

Submitted December 17, 1999; Revised April 3, 2000; Accepted May 4, 2000
Monitoring Editor: Guido Guidotti

Members of the innexin protein family are structural components of invertebrate gap junctions and are analogous to vertebrate connexins. Here we investigate two *Drosophila* innexin genes, *Dm-inx2* and *Dm-inx3* and show that they are expressed in overlapping domains throughout embryogenesis, most notably in epidermal cells bordering each segment. We also explore the gap-junction–forming capabilities of the encoded proteins. In paired *Xenopus* oocytes, the injection of *Dm-inx2* mRNA results in the formation of voltage-sensitive channels in only ~ 40% of cell pairs. In contrast, Dm-Inx3 never forms channels. Crucially, when both mRNAs are coexpressed, functional channels are formed reliably, and the electrophysiological properties of these channels distinguish them from those formed by Dm-Inx2 alone. We relate these in vitro data to in vivo studies. Ectopic expression of *Dm-inx2* in vivo has limited effects on the viability of *Drosophila,* and animals ectopically expressing *Dm-inx3* are unaffected. However, ectopic expression of both transcripts together severely reduces viability, presumably because of the formation of inappropriate gap junctions. We conclude that Dm-Inx2 and Dm-Inx3, which are expressed in overlapping domains during embryogenesis, can form oligomeric gap-junction channels.

INTRODUCTION

Gap-junction channels allow small molecules and ions to pass between cells, thus mediating processes such as electrical coupling, maintenance of homeostasis, and cell–cell signaling (reviewed in Bruzzone *et al.*, 1996). In vertebrates, these channels are composed of proteins called connexins. Six connexins associate to form a hexameric ring structure (connexon) in the plasma membrane that intercellularly docks with a corresponding connexon in an adjacent cell to form a continuous channel linking the cytoplasms (Yeager and Nicholson, 1996; Unger *et al.*, 1999). Despite the fact that gap junctions are also found throughout invertebrate tissues, no connexins have been identified in the *Caenorhabditis elegans* or *Drosophila* genomes, for which near complete sequence data are available (Wilson, 1999; Flybase website: http://fly.ebi.ac.uk:7081/).

It has recently been shown that invertebrate gap-junction channels are composed of proteins now named innexins (Phelan *et al.*, 1998a,b; Landesman *et al.*, 1999; reviewed in Phelan, 2000). These bear no sequence homology to the connexins but possess an identical predicted topology of four transmembrane domains and intracellular N- and C-termini (Crompton *et al.*, 1995; Starich *et al.*, 1996).

Innexin genes have been identified in several invertebrates. *C. elegans* has at least 24 innexins (Barnes and Hekimi, 1997), but few of these genes have been investigated in detail. Mutations in the *unc-7* and *unc-9* genes result in uncoordinated phenotypes (Starich *et al.*, 1993, 1996; Barnes and Hekimi, 1997), and in *eat-5* mutants electrical and dye coupling are abolished between some pharyngeal muscle cells, leading to feeding defects (Avery, 1993; Starich *et al.*, 1996). In *Drosophila*, five innexin gene loci have been described, and products of two of these loci, *shaking-B(lethal)*, *shaking-B(neural)* (previously known as *passover* [Krishnan *et al.*, 1993]), *shaking-B(N2)-(N4)* (Zhang *et al.*, 1999), and *ogre*, have been characterized. *shaking-B(lethal)* mutations result in the animal's death after an extended first larval instar (Crompton *et al.*, 1995). Mutations such as *shak-B*^{2} that disrupt the other products of the locus result in the loss of electrical synapses (essentially gap junctions, Bennett, 1997) in the giant fiber (Phelan *et al.*, 1996; Blagburn *et al.*, 1999) and haltere neural systems (Trimarchi and Murphey, 1997) and the abolition of dye coupling between some muscles

* Corresponding author. E-mail address: l.a.stebbings@sussex.ac.uk.
† Present address: Department of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ UK.

during embryogenesis (Todman *et al.*, 1999). A mutation in *ogre* leads to a reduced number of neurons in the optic lobes and an abnormal electroretinogram (Lipshitz and Kankel, 1985; Watanabe and Kankel, 1990). Three additional *Drosophila* innexin genes have been identified recently (Curtin *et al.*, 1999) but mutations are not yet available.

These mutant phenotypes are consistent with the involvement of innexin genes in gap-junction function. However, the most compelling evidence that innexins are structural gap-junction proteins, and not merely accessory factors, is that both Shaking-B(lethal) (Phelan *et al.*, 1998a) and one of the *C. elegans* innexins, Ce-Inx-3 (Landesman *et al.*, 1999) can form functional channels in paired *Xenopus* oocytes, a heterologous system commonly used to model connexin function.

Shaking-B(neural) (Phelan *et al.*, 1998a), which is partially identical to Shaking-B(lethal), and Eat-5 (Landesman *et al.*, 1999) fail to form homotypic channels (composed of just one innexin type) in the *Xenopus* oocyte system. This raises the possibility that innexins, like connexins, form mixed junctions with a different type of hemi-channel in each membrane (heterotypic channels) (Swenson *et al.*, 1989; Werner *et al.*, 1989; Barrio *et al.*, 1991; reviewed in Bruzzone *et al.*, 1996) or with more than one type of innexin in each hemi-channel (heteromeric channels) (Stauffer, 1995; Jiang and Goodenough, 1996; Lee and Rhee, 1998; Ebihara *et al.*, 1999; He *et al.*, 1999). In view of the large number of innexins identified and the overlapping expression patterns of those that have been investigated so far (Crompton *et al.*, 1995; Curtin *et al.*, 1999), the occurrence of mixed channels seems likely. Here we show that the innexin, *Dm-inx2* (*prp33*, Curtin *et al.*, 1999), and the newly identified family member, *Dm-inx3*, exhibit overlapping expression domains throughout *Drosophila* embryogenesis. We provide electrophysiological evidence that the encoded proteins interact to form functional channels in paired *Xenopus* oocytes and support this with in vivo data from ectopic expression studies in *Drosophila*.

MATERIALS AND METHODS

cDNA and Genomic Clone Characterization

I.M.A.G.E. Consortium (Lawrence Livermore National Laboratory, Livermore, CA) cDNA clones, LD11362 and LD17559 (Lennon *et al.*, 1996), were identified in the Berkeley *Drosophila* Genome Project (BDGP)/Howard Hughes Medical Institute EST project databases and obtained from Genome Systems (St. Louis, MO). We have named the corresponding genes *Dm-inx2* and *Dm-inx3,* respectively (hereafter referred to as *inx2* and *inx3*). To obtain genomic sequence, gridded genomic P1 clone filters (Genome Systems) and gridded genomic cosmid clone filters (Human Genome Mapping Project Resources Centre, Hinxton, Cambridge, UK) were screened using standard techniques. A cosmid clone, Dros17F19 (HGMP Resource Centre), containing the *inx2* gene and two P1 clones, DS03216 (Hartl *et al.*, 1994) and DS04968 (BDGP), containing *inx3* were identified. Sequencing was performed directly on these genomic clones and on fragments subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA) using primers designed to the corresponding cDNAs and to pBluescript. DNA was prepared using QIAprep spin columns (Qiagen, Crawley, West Sussex, UK) and sequencing was either performed on site using an Applied Biosystems 370A DNA sequencer or off site by MWG-Biotech UK (Milton Keynes, UK) using the LI-COR 4200 system. Sequence analysis was performed using LaserGene software (DNAstar, Madison, WI), and the multiple sequence alignment was assembled using CLUSTAL X (Thompson *et al.*, 1997) and decorated using SeqVu 1.1 (Garvan Institute of Medical Research, Sydney, Australia).

Chromosome In Situ Hybridization

In situ hybridization to salivary gland polytene chromosomes was performed according to the method of Laverty *et al.* (BDGP, detailed at: http://www.fruitfly.org/methods/cytogenetics.html) using digoxigenin-labeled DNA probes and a horseradish peroxidase–conjugated antidigoxigenin antibody for probe detection (Roche Diagnostics, Lewes, East Sussex, UK).

mRNA In Situ Hybridization to Embryos

In situ hybridization, using digoxigenin-labeled RNA probes (Roche Diagnostics), was carried out as described by Lehmann and Tautz (1994) except that Proteinase K treatment was with 25 μg/ml Proteinase K for 3 min. In the case of LD11362 (*inx2*), downstream AT-rich regions were removed before probe synthesis to reduce nonspecific background staining. A 1.5-kb *Eco*RI fragment of LD11362, containing the coding sequence for Inx2 and part of the noncoding upstream and downstream regions, was subcloned into the *Eco*RI site of pBluescript II KS+ (Stratagene). LD17559 (a pBluescript II SK+ clone), which encodes Inx3, was used for probe synthesis without modification.

Transcription of mRNAs

inx2 and *inx3* coding regions were cloned into the SPJC2L vector (gifted by H. Woodland, Warwick, UK) between upstream and downstream *Xenopus* β *globin* gene sequences to give *inx2-SPJC2L* and *inx3-SPJC2L*. These plasmids were linearized using *Xho*I and *Not*I, respectively, and transcribed in the presence of $m^7G(5')ppp(5')G$ (Roche Diagnostics) from the SP6 promoter. The resulting capped mRNAs were stored in aliquots at −20°C and thawed only once before use.

Translation of inx2 *and* inx3 *in* Xenopus *Oocytes*

Xenopus oocytes were injected with *inx2* and/or *inx3* mRNAs (18.4 nl of 0.5 ng/nl) and L-methionine [^{35}S] (0.23 μCi, ICN) using a Drummond Nanoject (Laser Laboratory Systems, Southampton, UK). The cells were incubated for 24 h at 20°C, and membrane extracts were prepared using the "sucrose cushion" method (Colman, 1984). Protein samples were solubilized in SDS gel-loading buffer (2.5× stock: 312.5 mM Trizma base pH 6.8, 5% SDS, 25% glycerol, 12.5% β-mercaptoethanol, 2.5 mM dithiothreitol, 0.1% bromophenol blue) at room temperature for 1 h and then at 80°C for 10 min before separating on a 12.5% SDS-polyacrylamide gel alongside a prestained, broad range protein marker (New England Biolabs, Beverly, MA). After washing in 30% methanol/3% glycerol for 30 min, the gels were heat-dried under vacuum and exposed to Super RX medical x-ray film (Fuji, Tokyo, Japan) for up to a week. Densitometry readings were obtained from scanned autoradiographs imported as TIFF files into ImageMaster 1.10 (Pharmacia, Uppsala, Sweden).

Expression in the Paired Xenopus *Oocyte System*

Methods for oocyte isolation, injection and pairing were essentially as previously described (Swenson *et al.*, 1989; Phelan *et al.*, 1998a). Cells were preinjected with 20 ng Cx38 DNA antisense oligonucleotides (5′-CTGACTGCTCGTCTGTCCACACAG-3′), 24 h before the injection of 2–20 ng innexin mRNA in 18.4 nl H_2O or H_2O only (Barrio *et al.*, 1991). After pairing the oocytes and incubating in Barth's saline at 20°C for 24–48 h, each oocyte of a pair was impaled with two 1–5 mΩ borosilicate glass microelectrodes (filled with 3 M KCl, 10 mM HEPES, 10 mM EGTA; pH 7.5) and recorded using a double voltage-clamp procedure (Spray *et al.*, 1981). Junctional conductance (g_j) and channel sensitivity to transjunctional voltage (V_j)

and inside-outside voltage (V_{i-o}) were determined using methods described previously (Verselis *et al.*, 1991; Phelan *et al.*, 1998a). Data were analyzed and exponentials fitted using Axograph 4 software.

Drosophila Transformation

Flies were raised on standard *Drosophila* medium at 25°C. To prepare constructs for transformation, a 1.5-kb *Eco*RI fragment of LD11362 (*inx2*) and the complete LD17559 cDNA (*inx3*) were cloned into pUAST (Brand and Perrimon, 1993) giving *UAS-inx2* and *UAS-inx3* constructs, respectively. Each was purified twice over a CsCl gradient before coinjection with pπ25.7wc (a transposase source; Karess and Rubin, 1984) into *yellow white* (*y w*) embryos at concentrations of 400 and 100 μg/ml, respectively. Standard methods for P-element–mediated transformation were used (Spradling and Rubin, 1982). Multiple lines were obtained in a *y w* background, and the chromosomal positions of the insertions were mapped by standard genetic methods.

Ectopic Expression Studies

The UAS/GAL4 system (Brand and Perrimon, 1993) was used to ectopically express innexins in *Drosophila,* and the hatch and eclosion rates of these animals were determined. *24B-GAL4* was obtained from A. Brand (Cambridge, UK) and *armadillo-GAL4* from the Bloomington Stock Center (stock number 1561; donated to the Center by J.-P. Vincent, National Institute for Medical Research, London, UK). Stocks homozygous for both *UAS-inx2* and *UAS-inx3* were constructed using standard genetic techniques. Males from these stocks (*UAS-inx2;UAS-inx3*) and the original *UAS-inx2* and *UAS-inx3* lines were each crossed to *24B-GAL4* or *arm-GAL4* virgin females, and the embryos were collected in batches of 50, gridded onto media, and transferred to vials. These embryos were kept in humidified conditions at 25°C. Hatch rates were assessed after 2 days, and the numbers of adults that eclosed also were counted.

Innexin Nomenclature

cDNA clones LD11362 and LD17559 were identified in the BDGP EST database on the basis of their homology to other innexins. The corresponding genes were named *Dm-inx2* and *Dm-inx3,* respectively, (shortened to *inx2* and *inx3* for convenience in this article). The two-letter prefix identifies the organism, and *inx* denotes innexin.

Ganfornina *et al.* (1999) have recently presented a *Schistocerca americana* protein, Sa-Inx(1), which is an orthologue of *Drosophila* Ogre. In light of this work, Dm-Inx1 must now be considered as an alternative nomenclature for Ogre. Additionally, Ganfornina *et al.* (1999) have identified another grasshopper innexin, Sa-Inx(2). Subsequently, Curtin *et al.* (1999) isolated a gene encoding its *Drosophila* orthologue (*prp33*). However, a partial sequence corresponding to this *Drosophila* gene had already been deposited in the BDGP EST database, and it was from here that we obtained the cDNA clone for *inx2* described in this article. *inx3*, which was also identified in the BDGP database, has not been reported previously and, as yet, has no known orthologues in other organisms.

RESULTS

Molecular Characterization of inx2 *and* inx3

Clones LD11362 (*inx2*) and LD17559 (*inx3*) were sequenced, and the chromosomal positions of the corresponding genes were mapped to 6E4–5 and 98E4–6, respectively, by in situ hybridization to polytene chromosomes. P1 clones containing *inx3*, DS03216 and DS04968, have also been mapped to 98E3–6 and 98E4–6, respectively, by the BDGP. We have confirmed that the predicted polypeptide encoded by *inx2* is 367 amino acids in length (Prp33, Curtin *et al.*, 1999) and

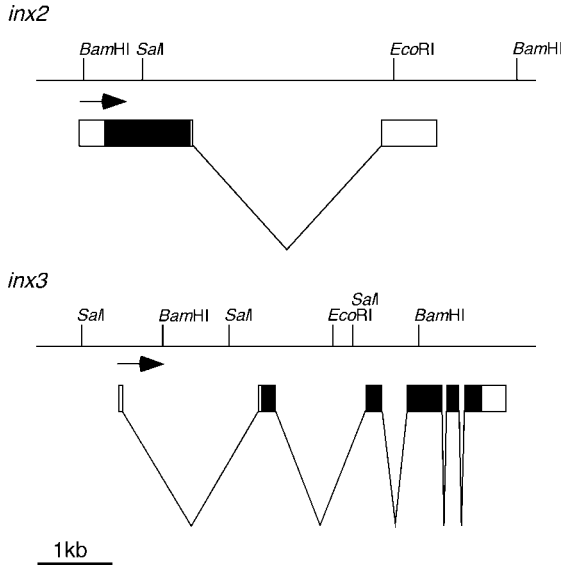

Figure 1. The genomic organizations of the *inx2* and *inx3* genes. *inx2* and *inx3* map to chromosome positions 6E4–5 on the X chromosome and 98E4–6 on chromosome 3, respectively. *Eco*RI, *Bam*HI, and *Sal*I restriction sites are shown, and the direction of transcription is given by the arrows. Genomic DNA is indicated by horizontal lines, coding regions by filled boxes, noncoding sequences by open boxes, and intronic regions by a "v."

42.49 kDa in mass with an isoelectric point (pI) of 6.096. *inx3*, a newly identified innexin, is predicted to encode a polypeptide of 395 amino acids with a mass of 45.36 kDa and a pI of 8.4.

To obtain data on both the sequence and genomic organization of the *inx2* and *inx3* genes, gridded genomic library filters were screened using the cDNAs as probes. A cosmid clone covering *inx2* and two P1 clones covering the *inx3* region were identified and partially mapped (Figure 1). *inx2* includes one intron that lies downstream of the coding region. It is likely that there are no other splice forms resulting in additional proteins from the *inx2* locus because the intron is outside the coding region. However, the cDNA isolated by Curtin *et al.* (1999) that corresponds to *inx2* is unspliced, and the sequence of this transcript continues into the intron for 240 bases before terminating. The polypeptides encoded by both cDNAs are identical, so this differential splicing could only have an effect on the regulation or localization of the transcripts. *inx3* possesses five introns, four of which interrupt the open reading frame. No other splice forms of *inx3* were detected in 12- to 24-h embryonic cDNA libraries (N. Brown, Wellcome/CRC Institute, Cambridge, UK) using PCR and primers to either end of the existing cDNA clone (Todman, unpublished data).

The polypeptide sequences for Inx2 and Inx3 are 42% identical and are homologous to other innexin sequences in *Drosophila melanogaster* (Shaking-B, Ogre), *Caenorhabditis elegans* (Eat 5, Unc 7, Unc 9), *Schistocerca americana* (Sa-Inx(1), Sa-Inx(2)), and *Bombyx mori* (Bm-Inx2). Strongest homology is seen in the transmembrane domains and around the conserved cysteine residues in the extracellular loops (Figure 2). Inx2 is 76% identical to Sa-Inx(2) (Ganfornina *et al.*, 1999). A

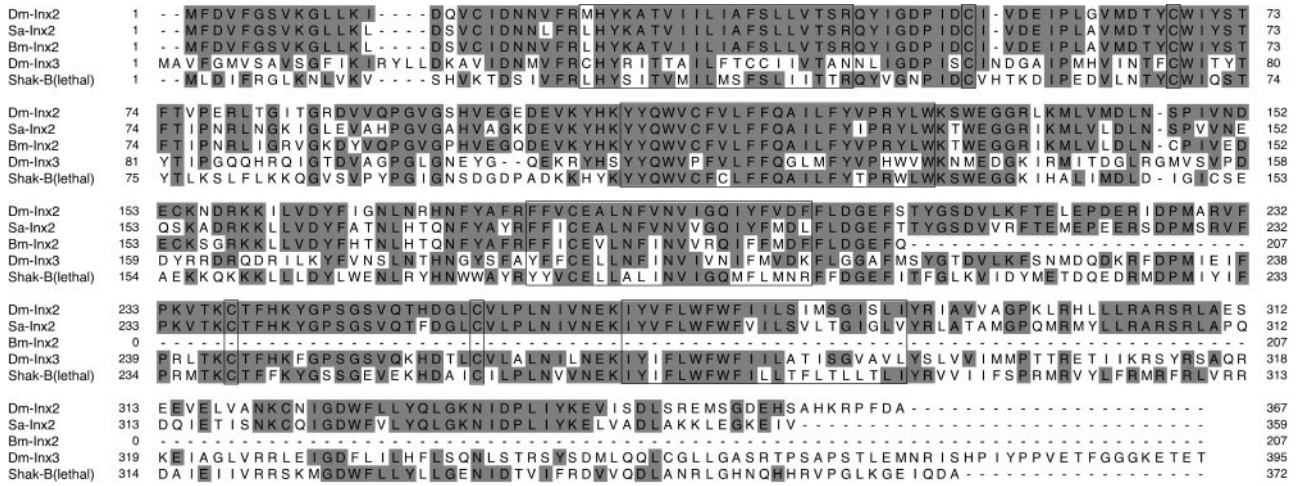

Figure 2. Multiple polypeptide sequence alignment of some innexins. From top to bottom; *Drosophila* Innexin2 (Dm-Inx2), *Schistocerca americana* Innexin2 (Sa-Inx(2)), *Bombyx mori* Innexin2 (Bm-Inx2, only a partial sequence is available), *Drosophila* Innexin3 (Dm-Inx3) and Shaking-B(lethal). Predicted transmembrane regions and conserved cysteines are boxed. Amino acids that are identical to those in Dm-Inx2 are shaded. These sequence data are available from GenBank under the following accession numbers: Dm-Inx2, AF172257; Sa-Inx(2), AF115854; Bm-Inx2, AU003649; Dm-Inx3, AF172258; Shaking-B(lethal), S78495.

third orthologue is found in the silk moth, *Bombyx mori* (Bm-Inx2), for which only a partial N-terminal sequence is available in the GenBank nucleotide sequence database. Inx2 is 84% identical to Bm-Inx2 and 81% identical to Sa-Inx(2) over this N-terminal region, and Sa-Inx(2) and Bm-Inx2 show 86% identity over the same region.

Embryonic Expression of inx2 *and* inx3

In general, *inx2* and *inx3* exhibit very similar expression patterns in the embryo, although there are some differences. In the blastoderm, *inx3* mRNA is asymmetrically localized to anterior and ventral regions, whereas *inx2* transcripts are evenly distributed (our unpublished results). Expression of both transcripts was detected throughout the germ band during early gastrulation until stage 10, when some modulation in the pattern began to be detectable. A segmentally reiterated pattern of expression emerges during germ band extension (stage 11, Figure 3, A and B). The pattern is refined further as the germ band retracts (stage 12, Figure 3, C and D) until only one or two rows of cells at each side of the segment borders express both transcripts strongly (stage 13, Figure 3, E, F, I, and J). Likewise, *inx2* and *inx3* epidermal expression in the head and terminal regions becomes restricted to the segment borders from stage 12 onward as the germ band retracts.

Both transcripts were also detected in the hindgut and possibly the foregut with little or no expression in the midgut. This expression is strong from stage 11 onward and is particularly apparent in the hindgut of stage 14 embryos (Figure 3G, arrowhead). Expression was also detected in a few segmentally repeated cells around the spiracular openings to the immature tracheal system (stage 14, Figure 3H, arrowhead).

The most noticeable difference between *inx2* and *inx3* expression was that only *inx2* was detected in the dorsal trunk (the main anterior-posterior tracheal branch) and in the precursor cells of this structure from stage 11 onward (Figure 3, C, E, and G, arrows). *inx2*, but not *inx3*, is expressed strongly in the segmentally repeated tracheal placodes once they become internalized (stage 11). Tracheal expression was most clearly seen as cells of the placodes in adjacent segments migrate to meet at stage 13 and form a continuous tube (stage 14, Figure 3G, arrow).

Translation of Inx2 and Inx3 in Xenopus *Oocytes*

Membrane extracts prepared from *Xenopus* oocytes injected with *inx2* and/or *inx3* mRNA(s) and a radiolabeled methionine source were separated on SDS-polyacrylamide gels. Protein bands with apparent sizes of ~ 41 and 39 kDa can be seen in the Inx2 and Inx3 lanes, respectively (Figure 4, Inx2 and Inx3 lanes), both protein bands are present in the Inx2+Inx3 membrane extract, (Figure 4, Inx2+Inx3), and neither are detected in the membrane preparations from oocytes injected with water (Figure 4, H_2O). The apparent protein sizes are smaller than expected, considering the predicted sizes of the Inx2 (42.49 kDa) and Inx3 (45.36 kDa) polypeptides (deduced from sequence data). Additionally, Inx3, which was predicted to be larger than Inx2, gives an apparent band size that is slightly below that of Inx2. The difference, however, is unlikely to be significant because other innexins run anomalously on SDS-polyacrylamide gels (Phelan *et al.*, 1998a).

When densitometry measurements were taken and standardized to take loading into account, Inx2 bands had optical densities ~ 1.5 times greater than those for Inx3 bands, both when the proteins were expressed singly (Figure 4, Inx2 and Inx3 lanes) and when expressed together (Figure 4, Inx2+Inx3). Because there are half the number of methionines in Inx2 than in Inx3 (8 and 16, respectively), the translation and/or membrane insertion of Inx3 is less efficient by a factor of three.

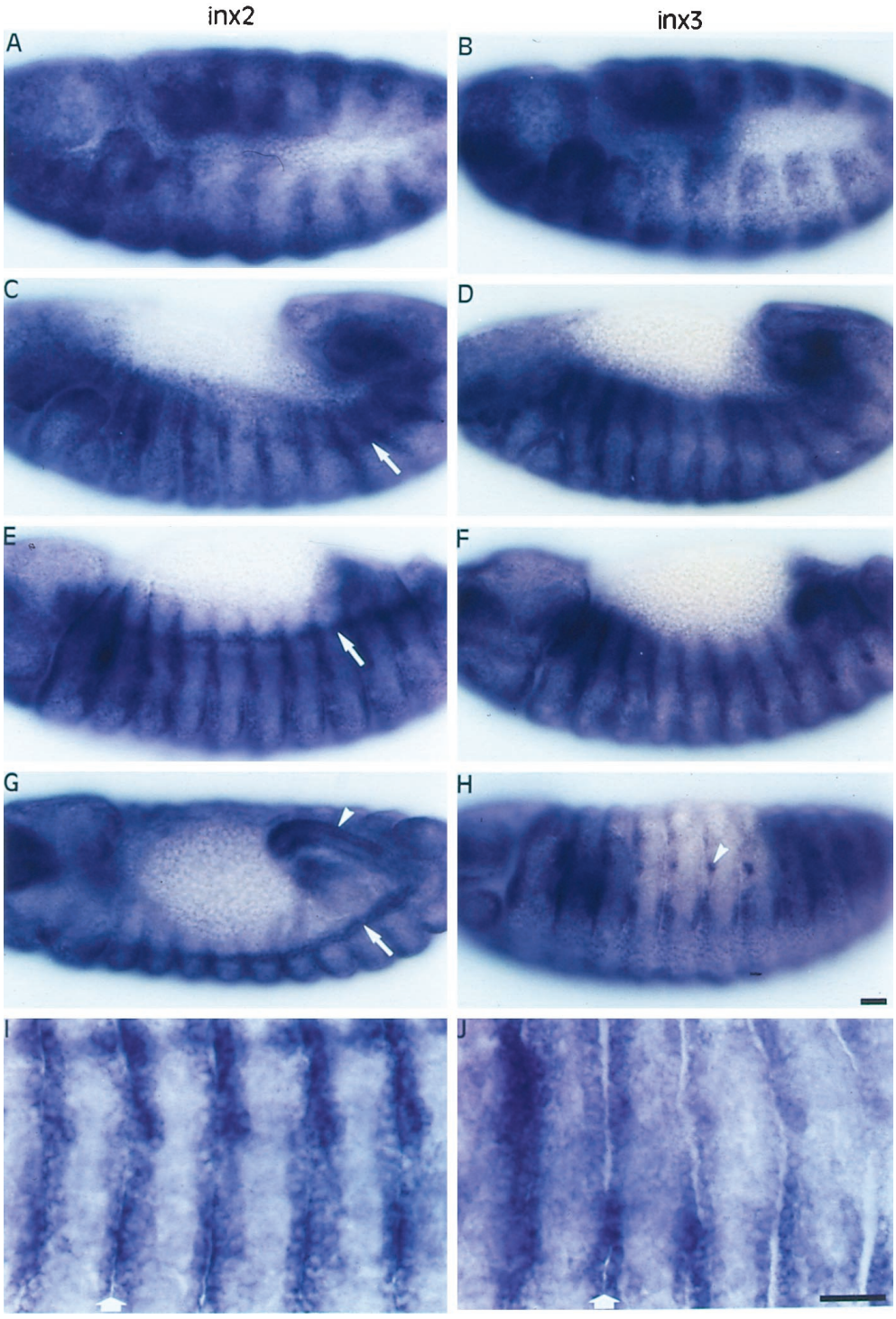

Figure 3. Distribution of *inx2* (A, C, E, G, I) and *inx3* (B, D, F, H, J) mRNAs during embryogenesis. Anterior is to the left and dorsal is up unless otherwise stated. In germ band extended embryos (stage 11), *inx2* (A) and *inx3* (B) are expressed in broad segmentally repeated bands. As the germ band retracts (stage 12), expression becomes localized to the segment borders (*inx2*, C; *inx3*, D), where it is maintained through stage 13 (*inx2*, E; *inx3*, F). In stage 14 embryos, both transcripts were detected in the foregut (out of focus) and hindgut (arrowhead, only shown for *inx2*, dorsal view, G) and in lateral cell clusters around the spiracular openings (arrowhead, only shown for *inx3*, H). Unlike *inx3*, *inx2* is expressed in the tracheal system dorsal trunk and the cell placodes that give rise to this structure (arrows, C, E, G). (I) and (J) show regions of epidermis, comprising 5 segments, at higher magnification in stage 13 embryos. The expression of *inx2* (I) and *inx3* (J) is clearly highest around the segment borders (arrows). Bars, 20 μm.

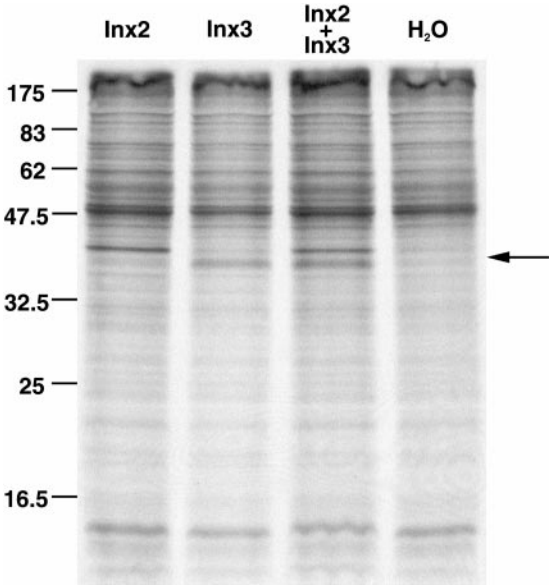

Figure 4. Translation of Inx2 and Inx3 in *Xenopus* oocytes. Ten nanograms *inx2* mRNA, 10 ng *inx3* mRNA, or 5 ng each of *inx2* and *inx3* mRNAs were translated in *Xenopus* oocytes. Membrane preparations (one oocyte equivalent loaded) were separated on SDS-polyacrylamide gels. Lanes were loaded as follows from left to right: Inx2; Inx3; Inx2+Inx3; H_2O (no mRNA added). The positions of the protein size marker bands are given on the left, and protein bands unique to the Inx2, Inx3 and Inx2+Inx3 lanes are indicated by the arrow on the right.

Table 1. Junctional conductances in oocyte pairs injected with *inx2* or *inx2+inx3* mRNAs

RNA injected	Number coupled/total[a]	Conductance (μS)[b]
H_2O (—)	0/19 (0)	0.00 ± 0.05 (19)
inx2 (10 ng)	17/39 (44)	4.62 ± 1.05 (17)
inx2+inx3 (2+2 ng) and (5+5 ng)	76/78 (97)	17.99 ± 1.58 (76)

mRNAs were injected at the amounts indicated (ng); cells were paired and recorded electrophysiologically to determine intercellular coupling. In the *inx2+inx3* experiments, both mRNAs were injected into each cell. Junctional conductance (g_j) was calculated from double voltage-clamp recordings as described in Figure 5. Values are the maximum g_j, recorded at transjunctional voltage steps of 10–20 mV. Note that the mean g_j value for *inx2* and *inx2+inx3* experiments does not include noncoupled cell pairs.

[a] Values in parentheses are percentages.

[b] Values are means ± SEM for n cell pairs, in parentheses.

Expression in Paired Xenopus *Oocytes*

We expressed the proteins encoded by *inx2* and *inx3* mRNAs in paired *Xenopus* oocytes to determine whether they are sufficient to form intercellular channels. Although in this series of experiments we never detected coupling in water-injected oocyte pairs, as a precaution, the oocytes were routinely pretreated with Cx38 antisense oligonucleotides to deplete any endogenous channels (Barrio *et al.*, 1991; Phelan *et al.*, 1998a).

inx3 mRNA, at amounts up to 20 ng, never induced channel formation in oocytes; the average junctional conductance (g_j) in *inx3*-injected cell pairs (0.02 ± 0.02 μS, for 24 cell pairs), was not significantly different from g_j in H_2O-injected control pairs (0.00 ± 0.05 μS, for 19 cell pairs, H_2O; Table 1). By contrast, *inx2* mRNA induced measurable conductances in some cell pairs. However, even at amounts of 10 ng mRNA, only 44% were coupled.

Because the expression domains of *inx2* and *inx3* were found to be partially overlapping in the *Drosophila* embryo (Figure 3), we were interested to determine whether the encoded proteins might interact to form functional channels in the oocyte system. To investigate the possibility that they form heterotypic channels, we paired cells expressing *inx2* with cells expressing *inx3*; such pairs did not develop conductances (*inx2/inx3*, mean g_j = 0.01 ± 0.02 μS, for 16 cell pairs). In contrast, coexpression of *inx2* and *inx3* in both oocytes of pairs at amounts that failed to induce (*inx3*), or unreliably induced (*inx2*), conductances when expressed alone, resulted in the formation of intercellular channels (*inx2+inx3*, Figure 5, A, B, and D and Table 1). When 2 ng of each mRNA was injected, channels were formed in essentially all cell pairs (97.4% of cell pairs electrically coupled) and the magnitude of g_j (17.57 ± 2.12 μS) increased significantly when compared with cells expressing *inx2* alone (mean g_j = 4.62 ± 1.05 μS; Table 1). Increasing the amounts of each mRNA from 2 to 5 ng did not significantly increase the magnitude of g_j, possibly because the translational machinery was saturated (*inx2+inx3*, 5 ng of each, mean g_j = 18.54 ± 2.40 μS). These data suggest that Inx3 in some way either promotes channel formation by Inx2 or directly interacts with Inx2 in the hemi-channel to assemble heteromeric junctions. In an attempt to distinguish between these possibilities we compared the electrical properties of the homotypic (Inx2) and presumptive heteromeric (Inx2+Inx3) channels.

The sensitivity of the intercellular conductance to transjunctional voltage (V_j, the voltage difference between the two cells) and transmembrane voltage ($V_{i\text{-}o}$, the voltage difference between the cytoplasm and the extracellular space) was examined in cell pairs injected with *inx2* only or with *inx2+inx3* mRNAs. We measured V_j by depolarizing one cell of the pair from a holding potential of −80 mV, and therefore the observed V_j sensitivity may have included a component of $V_{i\text{-}o}$ sensitivity. Both *inx2* and *inx2+inx3*–injected cell pairs showed similar sensitivity to V_j when depolarizing voltage steps were applied (Figure 5, A and B, shows typical recordings from *inx2* and *inx2+inx3* cell pairs). In both cases, the near steady-state g_j (measured at the end of the step change) decreased as V_j increased from 10 to 80 mV (Figure 5, A and B). When larger V_j steps were imposed, the curves of declining I_j with V_j (Figure 5, A and B) did not follow single exponentials, suggesting that the transition is not simply from one state to another but involves an intermediate state(s). At V_j steps larger that 30 mV, time constants for the fitted bi- and tri-exponentials at each V_j were very similar for both channels. However, at 10- and 20-mV voltage steps, Inx2+Inx3 channels tended to be slightly more voltage sensitive than Inx2 channels, and the fitted curves differed. When g_j (normalized to maximum

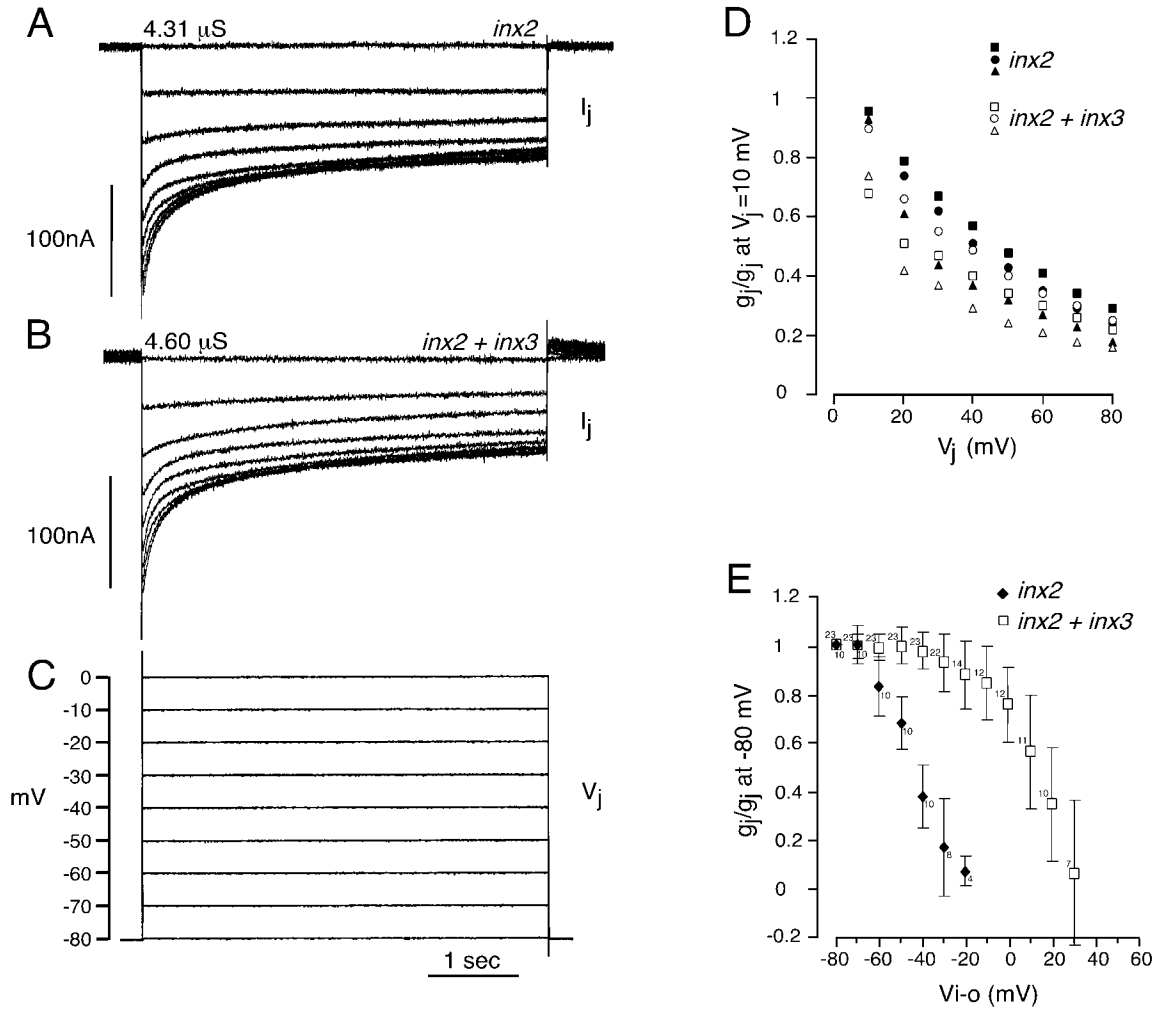

Figure 5. Electrical properties of Inx2 and Inx2+Inx3 channels. The intercellular channels formed when *inx2* was expressed alone, and when *inx2* was coexpressed with *inx3* were differentially sensitive to applied voltage. Cells were individually microinjected with the mRNAs alone or in combination, paired, and recorded 1 day later using the double voltage-clamp technique. (A–C) Recordings from cell pairs injected with *inx2* mRNA only (10 ng, A) and *inx2*+*inx3* mRNAs (2 ng each, B). Both oocytes of a pair were initially clamped to a holding potential of −80 mV. Transjunctional voltages (V_j) were generated by depolarizing one cell of the pair in 10-mV steps (C) and the current (I_j) required to maintain its paired neighbor at the holding potential was simultaneously recorded (A, B). For the pairs shown, the maximum junctional conductances (I_j/V_j, measured at the beginning of the 10-mV step) were 4.31 μS (Inx2) and 4.60 μS (Inx2+Inx3). For the *inx2*+*inx3*-injected cell pair, this was slightly greater than the steady-state g_j, because the channels were strongly voltage-dependent and began to close at V_j steps of 10 mV. V_j-dependent closure was very obvious for the second (20 mV) and subsequent, larger voltage steps for both *inx2*- and *inx2*+*inx3*-injected cell pairs (A, B). (D) g_j/V_j relation for the *inx2* and *inx2*+*inx3* pairs shown in (A) and (B) (*inx2*+*inx3*, □; *inx2*, ■) and additional cell pairs with conductances up to 6.04 μS. Data were normalized to the maximum instantaneous g_j at a V_j of 10 mV. Cell pairs injected with *inx2* and *inx3* tended to show slightly more voltage sensitivity than pairs injected with *inx2* only, particularly for smaller V_j steps. (E) $g_j/V_{i\text{-}o}$ relation. Both oocytes of a pair were stepped equally and simultaneously over a range of negative and positive membrane potentials ($V_{i\text{-}o}$). At each potential, a 10-mV depolarizing pulse was delivered to one cell to measure g_j; values (±SD for numbers of pairs indicated) were normalized to g_j at −80 mV. Data shown are from pairs with conductances in the range 0.5–20 μS. Restricting the analysis to pairs with g_js of 5 μS or below did not change these curves significantly. Intercellular conductances in oocyte pairs expressing *inx2* only (filled symbols) were significantly more sensitive to $V_{i\text{-}o}$ than g_js in pairs expressing both innexins (open symbols).

instantaneous g_j at 10 mV) was plotted against V_j (Figure 5D), only slight differences were apparent in the overall sensitivity of these channels to voltage. *inx2*+*inx3* cell pairs tended to be marginally more voltage dependent than *inx2* cell pairs at smaller V_j steps (Figure 5, A–D). This difference was unlikely to be due to access resistance effects because channels with conductances >6.04 μS were excluded from these data (Wilders and Jongsma, 1992). We were unable to examine the symmetry of the g_j/V_j relationship around 0 mV because we could not maintain the holding potential of

the passive cell when the stepped cell of the pair was hyperpolarized. Asymmetry of the g_j/V_j plot would have indicated channel sensitivity to V_{i-o}.

However, we were able to measure V_{i-o} directly by stepping cell pairs over a range of negative and positive membrane potentials. The junctional conductances in cell pairs injected with *inx2* mRNA only (Figure 5E, filled symbols) were significantly more sensitive to V_{i-o} than conductances in cell pairs in which *inx3* was also injected (Figure 5E, open symbols). The majority of the channels in *inx2* cell pairs were closed at V_{i-o} values of −20 to −10 mV. In contrast, there was little significant reduction in the g_j of *inx2+inx3* cell pairs at negative V_{i-o}, and half maximal g_j was evident only at approximately +10 mV (Figure 5E). Because the imposition of V_j steps necessarily alters V_{i-o}, one might have expected this clear difference in V_{i-o} sensitivity to be more obvious in the g_j/V_j relation (Figure 5, A–D). One possibility is that Inx2+Inx3 channels are more sensitive to transjunctional voltage than Inx2 channels, partially compensating for the greater V_{i-o} sensitivity of Inx2 channels and resulting in the very similar traces seen in Figure 5, A and B. An alternative explanation could be that V_{i-o} sensitivity develops relatively slowly and was not resolved during the time course (4.5 s) of the V_j steps used in our recordings. In some insect preparations, full resolution of V_{i-o} requires a longer time course (Bukauskas *et al.*, 1992; Churchill and Caveney, 1993).

These physiological data show that coexpression of *inx2* and *inx3* causes channels to form between oocyte pairs that are distinct, in terms of probability of formation, conductance, and voltage sensitivity, from those formed when *inx2* is expressed alone. The simplest interpretation of these results is that Inx2 and Inx3 proteins do collaborate to form heteromeric channels.

Ectopic Expression in Drosophila

Inx2 and Inx3 clearly cooperate to form gap-junction channels between *Xenopus* oocytes. To determine whether these proteins might also cooperate to form gap-junction channels in vivo, we ectopically expressed *inx2* and *inx3* in *Drosophila* embryos and assessed the survival rates of these animals (Figure 6). Using the UAS/GAL4 system (Brand and Perrimon, 1993), expression was driven in embryonic muscles (*24B-GAL4,* A. Brand) and, more ubiquitously, using *armadillo-GAL4* (FlyBase website: http://fly.ebi.ac.uk:7081/). *UAS-shaking-B(lethal)* was crossed to each of these GAL4 lines as a positive control; this line was used because we had previously noted that in vivo ectopic expression of this innexin (which forms fully functional gap junctions in the *Xenopus* oocyte system) results in a lethal phenotype. As a negative control, each of these driver lines was crossed to the *yellow white* (*y w*) injection stock.

Progeny of the *UAS-inx3* × *24B-GAL4* cross exhibited hatch and eclosion rates very similar to those of *y w* × *24B-GAL4* progeny. Ectopic expression of *inx2* using the *24B-GAL4* driver line resulted in a hatch rate similar to that of *24B-GAL4* × *y w* progeny, but only 15% of the total number of embryos survived to adulthood. When both *inx2* and *inx3* were coexpressed, viability was significantly lower than that seen in *UAS-inx2* × *24B-GAL4* experiments; only 27% of embryos hatched and only 5.5% reached adulthood. Survival rates of flies resulting from the *UAS-inx2* × *arm-GAL4* and the *UAS-inx2/UAS-inx3* × *arm-GAL4* crosses were also significantly reduced compared with the *y w* × *arm-GAL4* progeny (Figure 6). Experiments using independently isolated lines of *UAS-inx2* and *UAS-inx3* confirmed that the observed results were not due to position effects. Additionally, mRNA in situ hybridization using *inx2* and *inx3* as probes was performed on all the progeny, and we found no obvious difference between the expression levels of *inx2* or *inx3* when ectopically expressed singly or in combination (our unpublished results). We conclude that the simultaneous ectopic expression of Inx2 and Inx3 proteins significantly reduces the viability of *Drosophila*.

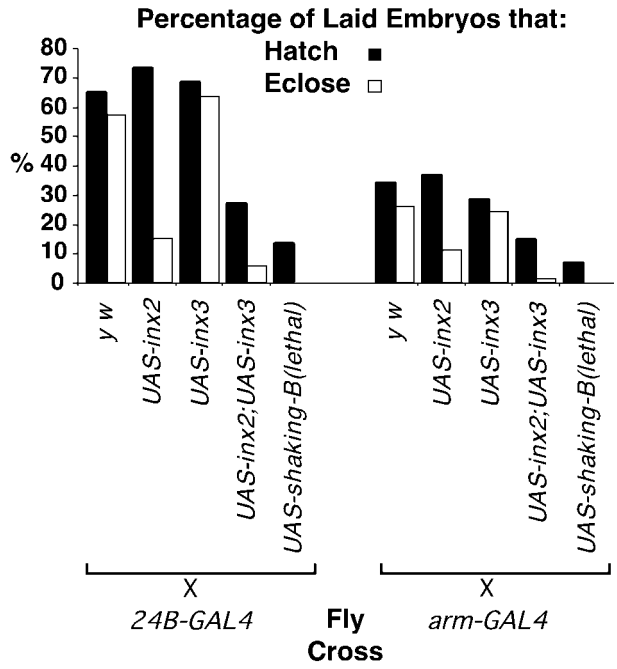

Figure 6. Fly crosses were performed as shown using two different GAL4 driver lines (all UAS lines are in a *y w* background). Hatch (black bars) and eclosion (white bars) rates are expressed as a percentage of the total number of embryos examined. For *24B-GAL4* crosses $n = 400$ and for *arm-GAL4* crosses, $n = 600$. Note that none of the embryos in which *shaking-B(lethal)* was ectopically expressed reached adulthood.

DISCUSSION

We have characterized two new members of the *Drosophila* innexin gene family, *inx2* and *inx3*. Both are expressed at high levels throughout embryogenesis; the transcripts become localized to epidermal cells bordering each embryonic segment at stage 12, and *inx2* exhibits additional expression in parts of the tracheal system. In *Xenopus* oocytes Inx2 alone, but not Inx3, formed channels in ~ 40% of oocyte pairs. As might be expected of a channel forming protein, Inx2 reduced viability when ectopically expressed in *Drosophila* embryos. In contrast, when both innexin proteins were coexpressed in the same cells, channels that were clearly distinct from Inx2 channels were reliably formed in oocyte pairs, and there was a much more profound effect on

Drosophila viability. We conclude that Inx2 and Inx3 are likely to interact to form heteromeric gap-junction channels.

Additional Members of the Innexin Family

cDNAs corresponding to *inx2* and *inx3* were identified in the BDGP EST databases on the basis of their homology to known innexins. *inx3* is a newly identified gene; *inx2* has been independently isolated by PCR methods (*prp33*; Curtin *et al.*, 1999). This increases the number of published *Drosophila* innexin proteins to eight.

Innexins have also been found in other insects. Three orthologues of *inx2* have now been sequenced, from *Drosophila*, *Bombyx mori* (partial sequence, Mita, Morimyo, Shimada, Okano, and Maeda, unpublished results) and *Schistocerca americana* (Ganfornina *et al.*, 1999). Sa-Inx(2) and Bm-Inx2 are more similar to each other (86% identity) than to Dm-Inx2 (81 and 84% identity, respectively) over the N-terminal region available for Bm-Inx2. Their degree of identity to each other is much higher than identity between innexin family members within *Drosophila,* which ranges from 29 to 47% (Curtin *et al.*, 1999) (excluding the high identity between Shaking-B{N + 16} [Zhang *et al.*, 1999], Shaking-B(neural) and Shaking-B(lethal)). Sequence comparison between orthologues in different species allows some examination of functional conservation. For example, the C-terminal regions of Sa-Inx(2) and Dm-Inx2 are divergent, whereas the N-terminal cytoplasmic tails of all three orthologues are highly conserved, suggesting that this region may be crucial to the functioning of this specific innexin. Shaking-B(neural) and Shaking-B(lethal) differ only in their N-terminal regions and they behave very differently both in vivo and in the *Xenopus* oocyte system. Additionally, the N-terminal regions of some connexins have been implicated in specifying which connexins can associate to form hetero-oligomeric hemi-channels (Falk *et al.*, 1997).

inx2 *and* **inx3** *Are Expressed in Embryonic Tissues that are Known to Possess Gap Junctions*

inx2 and *inx3* expression was examined in the embryonic epidermis, hindgut, foregut, and tracheal system, all of which are ectodermal in origin and possess gap junctions during embryogenesis (Tepass and Hartenstein, 1994). In the insect epidermis, all cells, regardless of their position with respect to the segment border, are electrically coupled. However, although larger ions such as Lucifer Yellow transfer freely from cell to cell within a segment, movement across the segmental border is restricted in both larval and adult epidermis (Warner and Lawrence, 1982; Blennerhassett and Caveney, 1984; Ruangvoravat and Lo, 1992). This suggests that gap junctions at segmental borders may have permeability properties different from those within a segment. Because *inx2* and *inx3* are expressed most strongly around the borders of each segment, they may contribute to these differences. Both *inx2* and *inx3* transcripts were detected in the hindgut and foregut, which are ectodermally derived, but not in the midgut, which is mainly endodermal in origin. The type and number of cell junctions in the midgut is known to differ from those in the hindgut and foregut (Tepass and Hartenstein, 1994). Although gap junctions are present throughout the gut, our data suggest that the constituents of the gap-junction channels in these endodermally and ectodermally derived regions differ. We have also demonstrated that *inx2,* but not *inx3,* is expressed in the dorsal trunk, a multicellular primary branch of the tracheal system, and in precursors of this structure. During germ band retraction, segmentally repeated clusters of cells, the tracheal placodes, reorganize to form the initial outgrowths of the tracheal branches (Manning and Krasnow, 1993; Samakovlis *et al.*, 1996). Some of these cells then migrate to link adjacent segments and form the dorsal trunk. Gap junctions are known to be present between the cells of this structure (Tepass and Hartenstein, 1994), and they may be involved in coordinating the migration and reorganization of dorsal trunk cells from adjacent placodes. Given that *inx2* mRNA is expressed in these cells, Inx2 protein is likely to be a constituent of at least some of these tracheal gap-junction channels.

Inx2 Forms Homomeric Channels and Cooperates with Inx3 to Form Heteromeric Channels in Paired **Xenopus** *Oocytes*

Paired *Xenopus* oocytes have been used extensively for functional expression of proteins of the connexin family and, with the exception of Cx31.1, Cx32.7 and Cx33, all connexins so far characterized form homotypic channels in this system (Hennemann *et al.*, 1992; Bruzzone *et al.*, 1995; Chang *et al.*, 1996; reviewed in Bruzzone *et al.*, 1996). In a previous study, we expressed the two partially identical innexins, Shaking-B(neural) and Shaking-B(lethal), in oocyte pairs and found that only the latter forms homotypic channels (Phelan *et al.*, 1998a). In the present study, we found that a second innexin, Inx2, also forms voltage-sensitive channels in the oocyte system. However, although Inx2 was clearly competent to form homotypic channels, it did so much less readily than Shaking-B(lethal). Injecting only 0.5 ng of *shaking-B(lethal)* mRNA gives rise to junctional conductances (mean g_j = 15.87 ± 1.45 μS, Phelan *et al.*, 1998a) in essentially all cell pairs whereas at 10 ng *inx2* mRNA, only 44% of cell pairs developed measurable conductances (mean g_j = 4.62 ± 1.05 μS). This could be accounted for if essential assembly/regulatory molecules are missing from the oocyte or if, ordinarily, Inx2 is a component of hetero-oligomeric channels. Although we cannot rule out a requirement for additional cofactors or channel subunits, we have presented evidence in this article that Inx3, the distribution of which overlaps that of Inx2 in some *Drosophila* tissues, may partner Inx2 in heteromeric channels.

Inx3, like Shaking-B(neural) (Phelan *et al.*, 1998a), did not form homotypic channels in paired oocytes. However, when Inx3 was present in cells expressing Inx2, channels were formed that had voltage properties distinct from the Inx2 homotypic channels (which clearly also might have assembled in these cell pairs). Notably, the two channel subtypes were differentially sensitive to transmembrane voltage. The conductance of Inx2 channels dropped dramatically upon depolarization, to negligible levels at membrane potentials of −10 mV; the channels in *inx2*+*inx3* cell pairs, although also influenced by $V_{i\text{-}o}$, showed no significant reduction in g_j at negative potentials. Considering the results of ectopic expression in selected *Drosophila* tissues (see below), we interpret our oocyte expression data to imply that Inx2 and Inx3 form heteromeric channels. Similarly, electrical proper-

ties (Ebihara *et al.*, 1999; He *et al.*, 1999) and also pH sensitivity (Bevans and Harris, 1999) have been shown to distinguish connexin heteromeric channels from homotypic channels formed by the same proteins. Alternative approaches such as coimmunoprecipitation (Stauffer, 1995; Jiang and Goodenough, 1996) would be required to directly demonstrate protein–protein interactions within a channel but as yet suitable probes are not available for Inx2 and Inx3.

In terms of their sensitivity to $V_{i\text{-}o}$, the innexin channels described here differ from both Shaking-B(lethal) and Ce-Inx-3 channels, which are $V_{i\text{-}o}$ insensitive (Phelan *et al.*, 1998a; Landesman *et al.*, 1999). However, the Inx2+Inx3 channels, in particular, are reminiscent of intercellular channels (of unknown molecular composition) characterized in many invertebrate tissues. Gap junctions in *Drosophila* embryonic muscle cells (Gho, 1994), salivary gland cells from *Drosophila* and *Chironomus* (Obaid *et al.*, 1983; Verselis *et al.*, 1991), and several insect cell lines (Bukauskas *et al.*, 1992, 1997) are gated by $V_{i\text{-}o}$, such that the conductances decline as the membrane potential becomes more positive.

The Activity of Inx2 and Inx2+Inx3 Channels In Vivo Reflects Their Activity in Paired Xenopus *Oocytes*

Because *inx2* and *inx3* expression domains partially overlap and the encoded proteins appeared to interact in the paired oocyte system, we were prompted to investigate whether they might also cooperate in vivo. The rationale behind these experiments was based on unpublished observations (M.G.T.) that ectopic expression of Shaking-B(neural) has no effect on viability (our unpublished results) and that this protein fails to form functional junctions in the oocyte system (Phelan *et al.*, 1998a). In contrast, ectopic expression of Shaking-B(lethal) in vivo results in death (Figure 6), and this protein consistently forms intercellular channels in oocyte pairs (Phelan *et al.*, 1998a).

Similarly, when *inx2* and *inx3* were ectopically expressed together, only very few animals survived to adulthood, mirroring the efficacy of presumptive Inx2+Inx3 oligomeric channels in *Xenopus* oocyte pairs. Ectopic expression of *inx2* alone was less harmful to the organism, in keeping with its reduced ability to form functional gap junctions in oocytes. These data could, if taken in isolation, be accounted for by a variety of interactions between *inx2*, *inx3* and/or their products, or by non–gap-junction–related effects resulting from the misexpression of membrane proteins. However, because we have presented evidence that heteromeric channels are formed in *Xenopus* oocytes when both proteins are present, the most parsimonious explanation is that heteromeric channels are also formed in vivo. The most direct way to confirm this would be to look for differences in intercellular dye-coupling between epidermal cells in wild-type, *inx2*-, *inx3*-, and *inx2/inx3*-deficient embryos. These studies await the generation of appropriate mutant stocks.

Conclusions

Of the four *Drosophila* innexins so far expressed in paired oocytes, two are unable to form functional homotypic channels. Similarly, some *C. elegans* innexins also appear not to form homotypic channels (Landesman *et al.*, 1999; reported as a personal communication in Curtin *et al.*, 1999). So what additional factors are required for these innexins to form channels? This article has provided strong evidence that two innexins form heteromeric channels, raising the possibility that hetero-oligomerization is a common feature of invertebrate gap-junction channels. Some data from mutational studies in *C. elegans* support this; mutations in two innexin genes, *unc-7* and *unc-9*, exhibit almost identical phenotypes, possibly because their encoded proteins are components of the same gap-junction channel (Barnes and Hekimi, 1997). As more family members are functionally expressed, some general rules of innexin compatibility should emerge.

ACKNOWLEDGMENTS

We thank Elizabeth Brint for maintaining and crossing fly stocks, Richard Baines and Kirsten Jacobs for advice and discussion, and Chris Ford for help with the initial stages of this project. This work was supported by the Biotechnology and Biological Sciences Research Council, UK, to J.P.B. and J.A.D., and in part by a Wellcome Trust grant to P.P.

REFERENCES

Avery, L. (1993). The genetics of feeding in *Caenorhabditis elegans*. Genetics *133*, 897–917.

Barnes, T.M., and Hekimi, S. (1997). The *Caenorhabditis elegans* avermectin resistance and anesthetic response gene *unc-9* encodes a member of a protein family implicated in electrical coupling of excitable cells. J. Neurochem. *69*, 2251–2260.

Barrio, L.C., Suchyna, T., Bargiello, T., Xu, L.X., Roginski, R.S., Bennett, M.V.L., and Nicholson, B.J. (1991). Gap junctions formed by Connexin 26 and 32 alone and in combination are differently affected by applied voltage. Proc. Natl. Acad. Sci. USA *88*, 8410–8414.

Bennett, M.V.L. (1997). Gap junctions as electrical synapses. J. Neurocytol. *26*, 349–366.

Bevans, C.G., and Harris, A.L. (1999). Regulation of connexin channels by pH—Direct action of the protonated form of taurine and other aminosulfonates. J. Biol. Chem. *274*, 3711–3719.

Blagburn, J.M., Alexopoulos, H., Davies, J.A., and Bacon, J.P. (1999). Null mutation in *shaking-B* eliminates electrical, but not chemical, synapses in the *Drosophila* giant fiber system: a structural study. J. Comp. Neurol. *404*, 449–458.

Blennerhassett, M.G., and Caveney, S. (1984). Separation of developmental compartments by a cell type with reduced junctional permeability. Nature *309*, 361–364.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Bruzzone, R., White, T.W., Yoshizaki, G., Patino, R., and Paul, D.L. (1995). Intercellular channels in teleosts: functional characterization of two connexins from Atlantic croaker. FEBS Lett. *358*, 301–304.

Bruzzone, R., White, T.W., and Paul, D.L. (1996). Connections with connexins: the molecular basis of direct intercellular signaling. Eur. J. Biochem. *238*, 1–27.

Bukauskas, F.F., Kempf, C., and Weingart, R. (1992). Electrical coupling between cells of the insect *Ades albopictus*. J. Physiol. *448*, 321–337.

Bukauskas, F.F., Vogel, R., and Weingart, R. (1997). Biophysical properties of heterotypic gap junctions newly formed between two types of insect cells. J. Physiol. *499*, 701–713.

Chang, M., Werner, R., and Dahl, G. (1996). A role for an inhibitory connexin in testis? Dev. Biol. *175*, 50–56.

Churchill, D., and Caveney, S. (1993). Double whole-cell patch-clamp characterization of gap junctional channels in isolated insect epidermal cell pairs. J. Membrane Biol. *135*, 165–180.

Colman, A. (1984). In: Transcription and Translation-A Practical Approach, ed. B.D. Hames and S.J. Higgins, Oxford, UK: IRL, 271–302.

Crompton, D., Todman, M., Wilkin, M., Ji, S., and Davies, J. (1995). Essential and neural transcripts from the *Drosophila shaking-B* locus are differentially expressed in the embryonic mesoderm and pupal nervous system. Dev. Biol. *170*, 142–158.

Curtin, K.D., Zhang, Z., and Wyman, R.J. (1999). *Drosophila* has several genes for gap junction proteins. Gene *232*, 191–201.

Ebihara, L., Xu, X., Oberti, C., Beyer, E.C., and Berthoud, V.M. (1999). Co-expression of lens fiber connexins modifies hemi-gap-junctional channel behavior. Biophys. J. *76*, 198–206.

Falk, M.M., Buehler, L.K., Kumar, N.M., and Gilula, N.B. (1997). Cell-free synthesis and assembly of connexins into functional gap junction membrane channels. EMBO J. *16*, 2703–2716.

Ganfornina, M.D., Sanchez, D., Herrera, M., and Bastiani, M.J. (1999). Developmental expression and molecular characterization of two gap junction channel proteins expressed during embryogenesis in the grasshopper *Schistocerca americana*. Dev. Genet. *24*, 137–150.

Gho, M. (1994). Voltage-clamp analysis of gap junctions between embryonic muscles in *Drosophila*. J. Physiol. *481*, 371–383.

Hartl, D.L., Nurminsky, D.I., Jones, R.W., and Lozovskaya, E.R. (1994). Genome structure and evolution in *Drosophila*: applications of the framework P1 map. Proc. Natl. Acad. Sci. USA *91*, 6824–6829.

He, D.S., Jiang, J.X., Taffet, S.M., and Burt, J.M. (1999). Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells. Proc. Natl. Acad. Sci. USA *96*, 6495–6500.

Hennemann, H., Dahl, E., White, J.B., Schwarz, H.J., Lalley, P.A., Chang, S., Nicholson, B.J., and Willecke, K. (1992). Two gap junction genes, connexin 31.1 and 30.3, are closely linked on mouse chromosome 4 and preferentially expressed in skin. J. Biol. Chem. *267*, 17225–17233.

Jiang, J.X., and Goodenough, D.A. (1996). Heteromeric connexons in lens gap junction channels. Proc. Natl. Acad. Sci. USA *93*, 1287–1291.

Karess, R.E., and Rubin, G.M. (1984). Analysis of P transposable element functions in *Drosophila*. Cell *38*, 135–146.

Krishnan, S.N., Frei, E., Swain, G.P., and Wyman, R.J. (1993). *Passover*: a gene required for synaptic connectivity in the giant fiber system of *Drosophila.* Cell *73*, 967–977.

Landesman, Y., White, T.W., Starich, T.A., Shaw, J.E., Goodenough, D.A., and Paul, D.L. (1999). Innexin-3 forms connexin-like intercellular channel. J. Cell. Sci. *112*, 2391–2396.

Lee, M.-J., and Rhee, S.K. (1998). Heteromeric gap junction channels in rat hepatocytes in which the expression of connexin26 is induced. Molecules and Cells *8*, 295–300.

Lehmann, R., and Tautz, D. (1994). In situ hybridization to RNA. Methods Cell. Biol. *44*, 575–598.

Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M.B. (1996). The I.M.A.G.E. consortium: an integrated molecular analysis of genomes and their expression. Genomics *33*, 151–152.

Lipshitz, H.D., and Kankel, D.R. (1985). Specificity of gene action during central nervous system development in *Drosophila melanogaster*: analysis of the *lethal (1) optic ganglion reduced* locus. Dev. Biol. *108*, 56–77.

Manning, G., and Krasnow, M.G. (1993). Development of the *Drosophila* tracheal system. In: The Development of *Drosophila melanogaster*, vol. I, ed. M. Bate and A. Martinez-Arias, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 609–685.

Obaid, A.L., Socolar, S.J., and Rose, B. (1983). Cell-to-cell channels with two independently regulated gates in series: analysis of junctional conductance modulation by membrane-potential, calcium, and pH. J. Membrane Biol. *73*, 69–89.

Phelan, P., Nakagawa, M., Wilkin, M.B., Moffat, K.G., O'Kane, C.J., Davies, J.A., and Bacon, J.P. (1996). Mutations in *shaking-B* prevent electrical synapse formation in the *Drosophila* giant fiber system. J. Neurosci. *16*, 1101–1113.

Phelan, P., Stebbings, L.A., Baines, R.A., Bacon, J.P., Davies, J.A., and Ford, C. (1998a). *Drosophila* Shaking-B protein forms gap junctions in paired *Xenopus* oocytes. Nature *391*, 181–184.

Phelan, P., *et al.* (1998b). Innexins: a family of invertebrate gap-junction proteins. Trends. Genet. *14*, 348–349.

Phelan, P. (2000). Gap junction communication in invertebrates: the innexin gene family. Curr. Top. Membranes *49*, 389–422.

Ruangvoravat, C.P., and Lo, C.W. (1992). Restrictions in gap junctional communication in the *Drosophila* larval epidermis. Dev. Dynamics *193*, 70–82.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D.C., Guillemin, K., and Krasnow, M.A. (1996). Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. Development *122*, 1395–1407.

Spradling, A.C., and Rubin, G.M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science *218*, 341–347.

Spray, D.C., Harris, A.L., and Bennett, M.V.L. (1981). Equilibrium properties of a voltage-dependent junctional conductance. J. Gen. Physiol. *77*, 77–93.

Starich, T.A., Herman, R.K., and Shaw, J.E. (1993). Molecular and genetic analysis of *unc-7*, a *Caenorhabditis elegans* gene required for coordinated locomotion. Genetics *133*, 527–541.

Starich, T.A., Lee, R.Y.N., Panzarella, C., Avery, L., and Shaw, J.E. (1996). *eat-5* and *unc-7* represent a multigene family in *Caenorhabditis elegans* involved in cell-cell coupling. J. Cell Biol. *134*, 537–548.

Stauffer, K.A. (1995). The gap junction proteins beta1 connexin (connexin 32) and beta2 connexin (connexin 26) can form heteromeric hemi-channels. J. Biol. Chem. *270*, 6768–6772.

Swenson, K.I., Jordan, J.R., Beyer, E.C., and Paul, D.L. (1989). Formation of gap junctions by expression of connexins in *Xenopus* oocyte pairs. Cell *57*, 145–155.

Tepass, U., and Hartenstein, V. (1994). The development of cellular junctions in the *Drosophila* embryo. Dev. Biol. *161*, 563–596.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. *25*, 4876–4882.

Todman, M.G., Baines, R.A., Stebbings, L.A., Davies, J.A., and Bacon, J.P. (1999). Gap-junctional communication between developing *Drosophila* muscles is essential for their normal development. Dev. Genet. *24*, 57–68.

Trimarchi, J.R., and Murphey, R.K. (1997). The shaking-B^2 mutation disrupts electrical synapses in a flight circuit in adult *Drosophila.* J. Neurosci. *17*, 4700–4710.

Unger, V.M., Kumar, N.M., Gilula, N.B., and Yeager, M. (1999). Three-dimensional structure of a recombinant gap junction membrane channel. Science *283*, 1176–1180.

Verselis, V.K., Bennett, M.V.L., and Bargiello, T.A. (1991). A voltage dependent gap junction in *Drosophila melanogaster*. Biophys. J. *59*, 114–126.

Warner, A.E., and Lawrence, P.A. (1982). Permeability of gap junctions at the segmental border in insect epidermis. Cell *28*, 243–252.

Watanabe, T., and Kankel, D.R. (1990). Molecular cloning and analysis of *l(1)ogre*, a locus of *Drosophila melanogaster* with prominent effects on the postembryonic development of the central nervous system. Genetics *126*, 1033–1044.

Werner, R., Levine, E., Rabadan-Diehl, C., and Dahl, G. (1989). Formation of hybrid cell-cell channels. Proc. Natl. Acad. Sci. USA *86*, 5380–5384.

Wilders, R., and Jongsma, H.J. (1992). Limitations of the dual voltage clamp method in assaying conductance and kinetics of gap junction channels. Biophys. J. *63*, 942–953.

Wilson, R.K. (1999). How the worm was won—the *C. elegans* genome sequencing project. Trends Genet. *15*, 51–58.

Yeager, M., and Nicholson, B.J. (1996). Structure of gap junction intercellular channels. Curr. Opin. Structural Biol. *6*, 183–192.

Zhang, Z., Curtin, K.D., Sun, Y.-A., and Wyman, R.J. (1999). Nested transcripts of gap junction gene have distinct expression patterns. J. Neurobiol. *40*, 288–301.