mutations in *S. typhimurium* that show decreased levels of invasion in epithelial cells and macrophages (*9*). We examined these mutant strains to determine whether any of the regulated proteins were affected by these mutations. We found that bacteria belonging to one class (class 6) of the Tn*phoA* mutations of *S. choleraesuis* no longer made the regulated proteins in the presence of fixed monolayers (Fig. 2C, lane 18), and only small numbers of bacteria adhered. The other five classes, including rough mutations in core and O–side chain lipopolysaccharides, all synthesized the induced proteins (*5*). The two *S. typhimurium* Tn*10* mutants did not synthesize the induced proteins in the presence of epithelial cells (Fig. 2D, lanes 25 and 26), nor did they adhere well. These two mutants had a median lethal dose (LD_{50}) five orders of magnitude greater than that of the parent strain when injected intraperitoneally into mice (*9*), an indication that these proteins are required for virulence of *S. typhimurium*. The results obtained with these mutants confirm that *Salmonella* must synthesize these proteins to stably adhere to and invade epithelial cells.

We used various agents to alter the surface of epithelial cells to determine the nature of the compounds of the eukaryotic cell that were required for induction of these proteins. Treatment of polarized MDCK monolayers with periodic acid, trypsin, and neuraminidase before fixation all caused lowered levels of stable bacterial adherence (Table 1). Treatment of viable monolayers with periodic acid and neuraminidase also significantly decreased invasion levels (Table 1). If *S. choleraesuis* or *S. typhimurium* was added to fixed monolayers that had been treated with any of these three agents, the induction of these proteins was no longer observed (Fig. 2C, lanes 14 to 16). Thus if the epithelial cell surface is modified by proteolytic or neuraminidase treatment, these bacteria bind poorly and do not synthesize proteins essential for stable adherence and invasion, indicating that glycoprotein-like structures are necessary for these processes. These experiments do not distinguish whether the receptor responsible for initial adherence or that required for stable adherence is disrupted, because altering either receptor would produce the observed results.

Bacteria contain many operons that are regulated by different environmental signals (*10*). These systems usually allow the bacterium to synthesize the proteins required to cope with the new environments and are often regulated by conserved two-component systems, one of which interacts with the stimulus while the other regulates the necessary genes (*10*). The interaction of *Salmonella* species with epithelial cell surfaces, and the resultant changes in bacterial proteins, may be similar to these other regulated systems. We believe these bacteria adapt to intracellular life by synthesizing proteins required for entry into and possibly intracellular growth within this niche. Although the concept of pathogenic organisms adapting to new environments inside hosts is an attractive one, there are few reported examples (*11*). We think that the system that we have reported represents one such system, and, as the tools become available, more systems analogous to this one will be identified in other intracellular pathogens.

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A Family of Putative Potassium Channel Genes in *Drosophila*

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Mutant flies in which the gene coding for the *Shaker* potassium channel is deleted still have potassium currents similar to those coded by the *Shaker* gene. This suggests the presence of a family of *Shaker*-like genes in *Drosophila*. By using a *Shaker* complementary DNA probe and low-stringency hybridization, three additional family members have now been isolated, *Shab*, *Shaw*, and *Shal*. The *Shaker* family genes are not clustered in the genome. The deduced proteins of *Shab*, *Shaw*, and *Shal* have high homology to the *Shaker* protein; the sequence identity of the integral membrane portions is greater than 50 percent. These genes are organized similarly to *Shaker* in that only a single homology domain containing six presumed membrane-spanning segments common to all voltage-gated ion channels is coded by each messenger RNA. Thus, potassium channel diversity could result from an extended gene family, as well as from alternate splicing of the *Shaker* primary transcript.

THE *SHAKER* GENE OF *DROSOPHILA melanogaster* codes for a potassium channel of the transient type (*1*) that is present in both nerves and muscles (*2*). In addition to the primary sequence of the peptide, the cloning of the K^+ channel coded by the *Shaker* gene revealed a possible mechanism for generating the diverse K^+ currents in different cells and tissues: the *Shaker* gene primary transcript is alternately spliced so as to produce a host of transcripts that encode proteins differing at their amino and carboxyl termini (*3*). Expression of these transcripts in *Xenopus* oocytes has shown that the alternative peptides produced by this gene form channels with different biophysical properties (*4*).

In addition, *Shaker* may be but one member of a K^+ channel gene family. Other ion currents in *Drosophila* with similar biophysical properties to the current coded by the *Shaker* gene are coded by at least one additional gene (*5, 6*). Solc *et al.* (*6*) reported that at least one *Shaker*-like current was present in larval neurons of some *Shaker* mutants. We have extended these studies and have found that a variety of *Shaker*-like currents are present in pupal neurons after the *Shaker* gene is removed genetically (*7*).

We isolated several *Shaker*-like cDNAs by screening a cDNA library at low stringency; a *Shaker* cDNA probe containing all pre-

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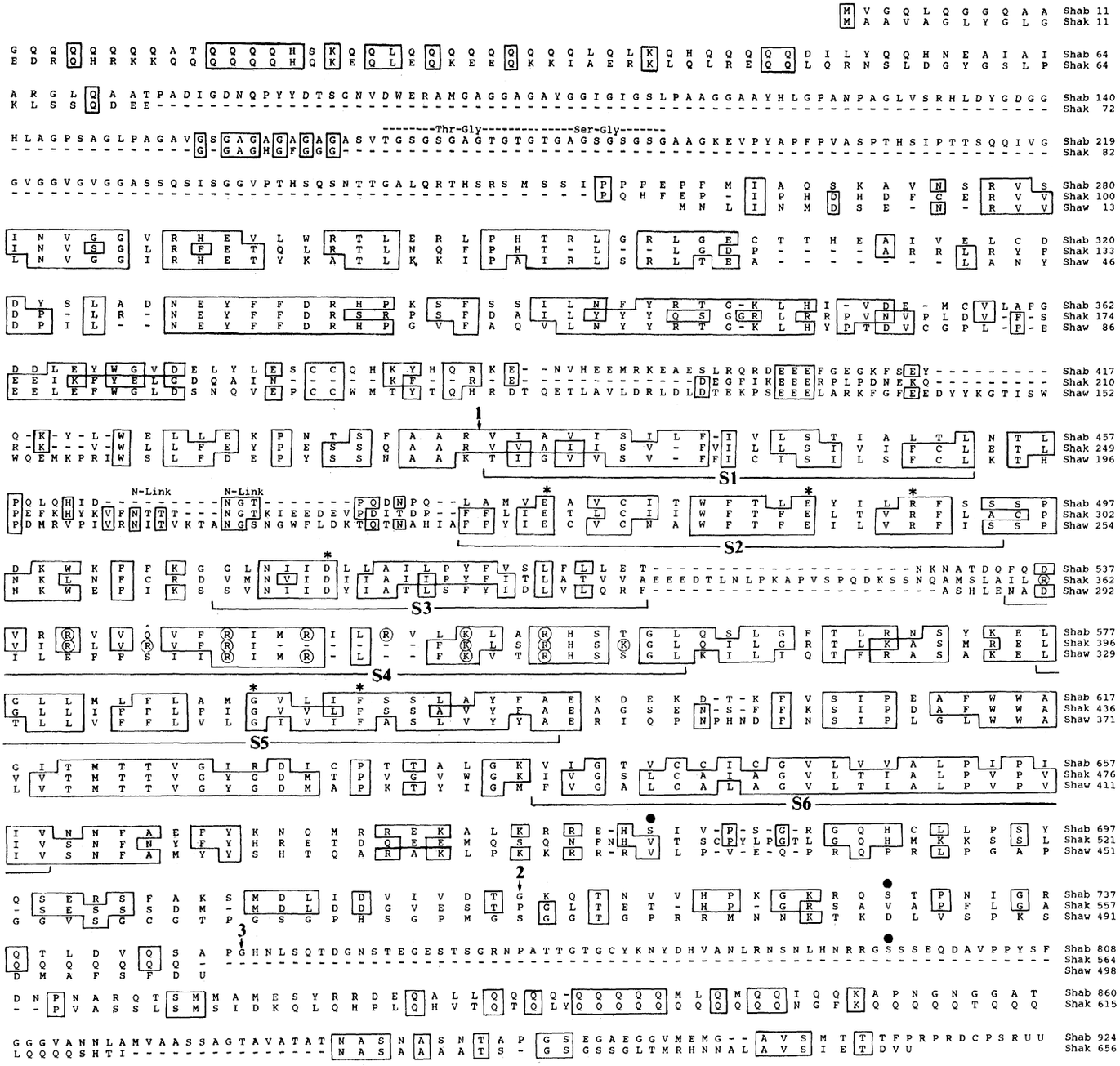

Fig. 1. The deduced proteins from the cDNAs Shab11 (top sequence) and Shaw2 (lower sequence) and their homology to the protein predicted by the cDNA ShakerB1 (middle sequence). Identical residues are shown in enclosed boxes. The deduced sequence of the *Shaker* protein is from ShakerB1 of Schwarz *et al.* (*3*) and was chosen for comparison to the *Shab* sequence because it showed higher homology to *Shab* than other *Shaker* sequences at the amino and carboxyl termini because of the opa sequences. The presumed membrane spanning segments are underlined and designated S1 through S6. N-Link, possible glycosylation sites (Asn-X-Ser/Thr) (*10*). Arrows 1 and 2, sites of introns in inappropriately spliced *Shab* cDNAs. The *Shab* sequence is from the Shab11 cDNA, which has an additional exon (between arrows 2 and 3) that is not present in two other cDNAs, Shab8 and Shab9. Excision of the small exon regenerates the Gly residue and leaves the downstream sequence in the same reading frame. ...Thr-Gly...Ser-Gly..., the area homologous to proteoglycans. Filled circles, potential cAMP-dependent phosphorylation sites (Arg/Lys-Arg/Lys-X-Ser/Thr or Arg/Lys-Arg/Lys-X-X-Ser/Thr) (*12*). Circled residues, positively charged amino acids. The Gln residue in the *Shab* sequence that is at a position where a positively charged residue might be expected (Shab543) is designated with a ^ mark. Asterisks, see text. The *Shaker* cDNA used as a probe was isolated from an adult stage *Drosophila* cDNA bank by using a synthetic oligonucleotide DNA probe (to region S5) that was end-labeled with ^{32}P. The cDNA was incomplete but included regions S1 to S6 and was identical to *ShakerB1* reported by Schwarz *et al.* (*3*). The following conditions were used for low-stringency screening to isolate the *Shaker* family cDNAs: Hybridizations were in 6× SSPE (1.1*M* NaCl, 60 m*M* NaH_2PO_4, 6 m*M* Na_2EDTA, *p*H 7.7) with 30% formamide at 42°C; wash in 1× SSC (standard sodium citrate) with 0.1% SDS at 50°C. For high-stringency hybridization the following conditions were used: 6× SSPE without formamide at 65°C; wash in 0.1× SSC with 0.1% SDS at 50°C. Bidirectional sequencing was achieved with m13 single-strand cloning vectors mp18 and 19. Overlapping deletions in single-stranded DNA were constructed with the single-strand exonuclease activity of T4 polymerase (IBI Cyclone Kit). Alternately, synthetic primers were used. The single letter amino acid code is A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; U, Stop.

sumed membrane spanning domains (S1–S6) was used to isolate the cDNA from the Shaker-like gene *Shab*, and a *Shab* cDNA containing the analogous region was used, in turn, to isolate the *Shaw* cDNA. A mixed probe from *Shaker*, *Shab*, and *Shaw* was used to isolate *Shal*. The cytological location of each gene was determined by hybridizing a cDNA or corresponding genomic DNA clone to denatured polytene chromosomes with established techniques (*8*) modified for biotin labeling. *Shab* and *Shal* are both on chromosome 3L, *Shab* at 63A, and *Shal* at 76B. *Shaw* is on 2L at 24 B-C. The *Shaker* gene is on chromosome 1 at 16F (*2*).

The deduced amino acid sequence from a *Shab* and *Shaw* cDNA is shown in Fig. 1 and their primary structures are compared to a *Shaker* protein. Six *Shab* cDNAs were isolated and partial or complete sequence was obtained for all. The cDNAs fell into three categories and all three diverged at the same splice junction 3′ to membrane spanning region S6 (Fig. 1, arrow 2, Shab residue 717). Two cDNAs were incompletely spliced and contained an intron; two were alternatively spliced and contained an additional exon of 90 nucleotides. The remaining two lacked the exon containing the 30 amino acids shown between arrows 2 and 3. The longest correctly spliced cDNA (Shab11) was 3373 nucleotides and contained an open reading frame of 2772 nucleotides coding for 924 amino acids. The *Shaw* cDNA of 1660 nucleotides contained an open reading frame of 1494 nucleotides. The deduced protein of 498 amino acids contains two potential initiator methionines; a second methionine is present six residues downstream from the first. Both *Shab* and *Shaw* cDNAs were blocked up- and downstream of the open reading frames by nonsense codons.

In Fig. 1 the proposed transmembrane segments are labeled S1 through S6 in accordance with the original designations of Noda *et al.* (*9*) and followed in the *Shaker* literature by Pongs *et al.* (*3*). Tempel *et al.* (*3*) have proposed an additional intramembrane segment between S5 and S6.

The homology domains of the *Shab*, *Shaw*, *Shal*, and *Shaker* proteins show substantial amino acid identity with each other and in addition are conserved with respect to the corresponding regions of both the Na^+ and Ca^{2+} channels (*9*). About 109 amino acids (depending on alignment) are identical in *Shab*, *Shaw*, and *Shaker*, more than are shared between any member of the *Shaker* gene family and either the Na^+ and Ca^{2+} channel proteins. This suggests a specialization for K^+ channel function among the *Shaker* family members that is separate from the common functions of voltage-gated ion channels. The membrane-spanning regions contain six conserved residues (Fig. 1, asterisks) that are also present in equivalent positions in Na^+ and Ca^{2+} channels (*9*). One of these residues, the arginine in S2, has undergone a conservative shift to lysine in the Na^+ and Ca^{2+} channels.

The similarity of all of the transmembrane segments in *Shab*, *Shaw*, and *Shaker* is evident (Fig. 1); in contrast, two of the presumed extracellular linker regions (*3*, *9*), those connecting transmembrane segments S1 to S2 and S3 to S4, are dissimilar in both length and composition. However, possible sites of N-linked glycosylation (*10*) are present in all three proteins in the S1 to S2 linker (Fig. 1, N-Link). The highest identity (86%) is between *Shaw* and *Shaker* in S6. Downstream from S6, outside of the presumed intramembrane portion of the protein, the homology among the proteins is less except for the polyglutamine (opa) repeat (*11*) in *Shab* and *Shaker* but not in *Shaw*. In both *Shab* and *Shaker* there are possible adenosine 3′,5′-monophosphate (cAMP)–dependent phosphorylation sites (*12*) in this region; the deduced protein from the cDNA ShakerB1 has one such site at residue 520 (*3*). Shab11 has three possible sites (Fig. 1, filled circles). One of these sites is present in the small exon between arrows 2 and 3 (Fig. 1), which is not included in two other cDNAs, Shab8 and Shab9.

The conservation of the central parts of these three *Shaker* family proteins is reflected in the similarity of the presumed membrane spanning regions S1 to S6 in the hydrophilicity plots (Fig. 2). The similarity also extends into the very hydrophilic portions of the proteins on either side of the membrane spanning portions. Further upstream from S1 outside of the presumed intramembrane region there is another area where high amino acid identity is again evident (Fig. 1, *Shab* 278 to 351). This area is unique in having a more hydrophilic profile than the other highly conserved areas.

Distinctive features of *Shab* not found in *Shaw* or *Shaker* include the large size of translated sequence 5′ to the first membrane spanning region of the channel protein. In this amino terminal area is an amino acid sequence rich in glycine, serine, threonine, and alanine. Curiously, the Ser-Gly and Thr-Gly repeats in this region occur in the extracellular matrix proteins proteoglycans (*13*) where they indicate sites of O-linked glycosylation. Thus they may be sites of channel glycosylation. However, current

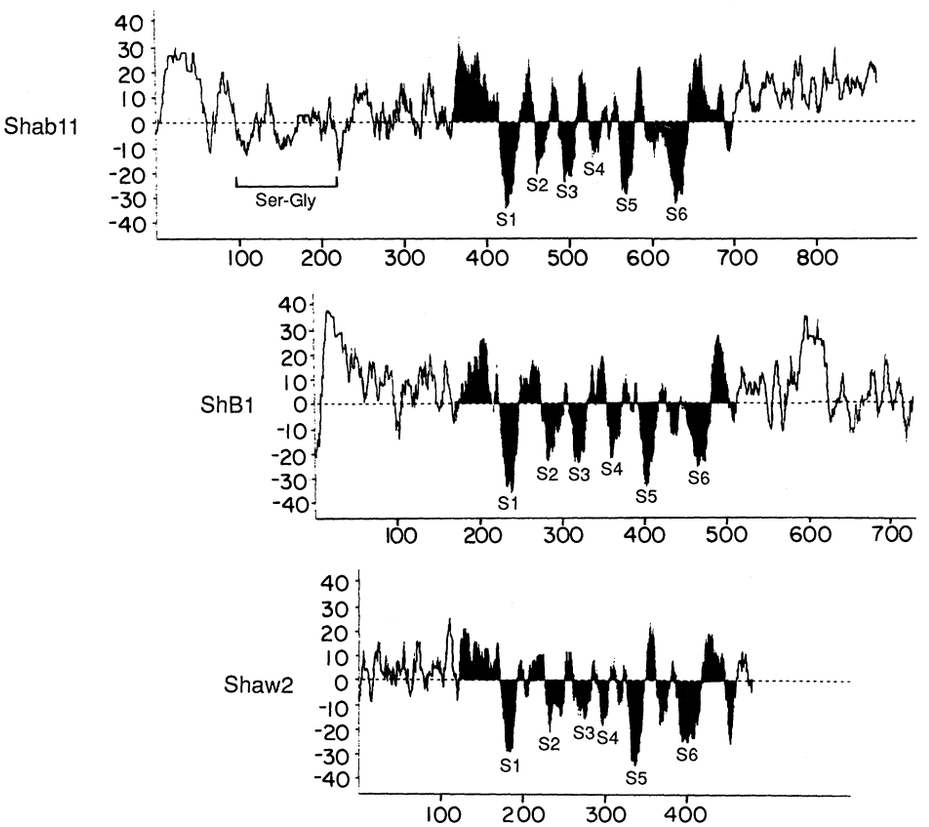

Fig. 2. Hydrophilicity profile of the *Shaker* family genes predicted by the cDNAs Shab11, ShakerB1 (ShB1), and Shaw2. The profiles were computed according to Kyte and Doolittle (*18*) with a window size of nine amino acids. Negative (downward) index values indicate hydrophobic groups, and positive values indicate hydrophilic groups. ShakerB1 is a *Shaker* cDNA reported by Schwarz *et al.* (*3*).

models of the channel protein suggest that the amino terminal is cytoplasmic and that the S1 region spans the membrane from the inside out (*3, 9*). Nevertheless, an examination of the hydrophilicity plot for this region (Fig. 2, Shab11, Ser-Gly), shows sufficient hydrophobic stretches to allow for the protein to exit and then reenter the cell. This area also shows homology to the product of the *Drosophila per* locus, which is also rich in these residues (*14*). Outside of the core region, the *Shab*-deduced protein is similar to some *Shaker* products in having opa [poly(Gln)] repeated sequences near both the amino and carboxyl termini. These repeated sequences are present in other developmentally regulated genes expressed in the *Drosophila* nervous system (*11*). The significance of the opa repeat is unknown.

The degree of homology among the *Shab, Shaw*, and *Shaker* protein is less than that for *Shaker* among species; the mammalian homolog of the *Shaker* protein (*15*) has long stretches of identity with the *Drosophila*

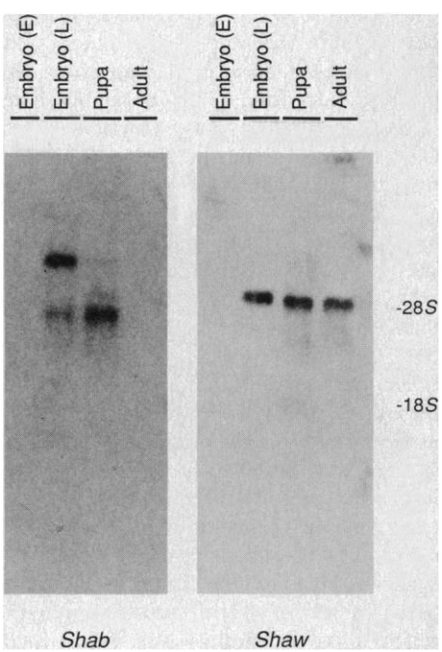

Fig. 3. Developmental expression of the *Shab* and *Shaw* genes shown by RNA Northern blot analysis. Five micrograms of polyadenylated mRNA were loaded in each lane. The mRNA was selected from flies of different developmental stages as indicated: Embryo (E), 0- to 9-hour embryos; Embryo (L), 9- to 18-hour embryos; Pupa, 30- to 96-hour post-pupariation pupae; Adult, 1- to 2-week-old adults. The RNA blot was probed under the high-stringency conditions in Fig. 1. The *Shab* and *Shaw* cDNA probes included the regions corresponding to the S1 to S6 membrane spanning portions of the protein. As a control for mRNA loading, the RNA blots were washed and reprobed with a cDNA for a polyadenylated mRNA coding for a small ribosomal protein that is present in equal amounts at all developmental stages (*19*). No appreciable differences in mRNA loading were noted.

Shaker protein; the homology is thus higher than that among *Shab, Shaw*, and *Shaker*. This is also true of *Shaw*; the *Drosophila* protein has higher identity to a mammalian *Shaw* homolog (*16*) than to *Shaker* or *Shab*. Thus the individual members of this gene family evolved their distinctive structural features prior to the separation of vertebrate and invertebrate species.

All homology domains of all voltage-gated ion channel proteins deduced thus far have a string of positive charges in the S4 region, arginines or lysines in a string at every third position, and two intervening uncharged residues separating each pair (Fig. 1, circles). This structure may form the voltage sensor of the channel (*9*). The *Shaker* protein has a string of seven positive charges, whereas *Shab* has a total of six and *Shaw* a total of four. The arrangement of the gating charge region in both *Shab* and *Shaw*, however, is unique. In *Shab* the expected position of the second positively charged residue is occupied by the polar residue glutamine. In both *Shab* and *Shaw* the position corresponding to the first gating charge in *Shaker* is occupied by negatively charged aspartates. In *Shaw*, the position corresponding to the second *Shaker* gating charge is also occupied by a negative residue, glutamate.

The four S4 regions of the Na^+ and Ca^{2+} channels are not identical; the number of positive gating charges differs among regions. For the Na^+ channel the numbers are four, five, six, and eight. The fact that this configuration is virtually identical for both *Drosophila* and vertebrates (*9*) suggests that this asymmetry reflects an important optimization of channel structure. The Ca^{2+} channel has one S4 region with four charges, two with five, and one with six. In contrast, all products of the *Shaker* locus have the same S4 gating region, which has seven positive charges. The differences in *Shab, Shaw*, and *Shaker* when compared to the Na^+ and Ca^{2+} channels may reflect an optimization for a heteromultimeric K^+ channel structure. If, on the other hand, *Shab* and *Shaw* form homomultimeric structures, the channels may be less responsive to voltage because of the lower number of gating charges.

To examine the expression of the *Shab* and *Shaw* genes at different developmental stages, we performed RNA Northern blots with polyadenylated RNA from different developmental stages and probed them with a *Shab* and *Shaw* cDNA (Fig. 3). Two discrete mRNAs are detectable from *Shab*, one of approximately 4.3 kb and a larger one of approximately 6.8 kb. Only a single mRNA of approximately 4.9 kb is detectable from *Shaw*. The *Shab* gene also shows a change during development in the relative abundance of the two expressed forms; the larger mRNA is more abundant in the embryo, while the smaller form is more abundant in pupa.

Alternate splicing of *Shab* mRNAs involves the coding portion of the gene; the Shab11 cDNA contained an additional exon not found in two other cDNAs (Fig. 1, arrows 2 and 3). This additional exon was associated only with the larger mRNA; an oligonucleotide probe specific to this exon labeled only the larger message on a duplicate Northern blot. Although the extent of alternative splicing of these genes is not yet known, our preliminary results suggest that it is not as extensive as in the *Shaker* gene (*3*).

Hille (*17*) has suggested that all voltage-gated ion channels have evolved from a common progenitor, an ancient nonspecific cation channel. The Na^+ and Ca^{2+} channels each evolved by intragenic duplication so that both modern forms are composed of four homologous domains in a single polypeptide.

Our data indicate that several K^+ channels may have followed a separate evolutionary path; intragenic duplication has not occurred and the modern genes code for only a single homology domain, much like the genes for the acetylcholine receptor, which each code for only a single subunit. Some or all K^+ channels may have originally been homomultimeric and may remain as homomultimeric structures. This possibility is supported by experiments that show that mRNA transcribed from a single *Shaker* cDNA can produce a transient voltage-dependent K^+ current when injected into *Xenopus* oocytes (*4*).

Some K^+ channels, however, may be heteromultimers. Both the Na^+ and Ca^{2+} channels have a pseudosymmetrical rather than a true symmetrical structure. Some K^+ channels may be similarly constructed but from the products of different genes. Notably, the alternately spliced products of the *Shaker* locus are all identical in the S1–S6 region except for two conserved residues (*3*).

Our findings suggest that there are several sources of K^+ channel diversity in addition to alternative splicing of primary RNA transcripts observed at the *Shaker* locus; the products of several similar genes may form homo- or heteromultimeric channel proteins.

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Antisense RNA–Induced Reduction in Murine TIMP Levels Confers Oncogenicity on Swiss 3T3 Cells

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Mouse 3T3 cell lines capable of constitutively synthesizing an RNA complementary to the messenger RNA encoding TIMP, tissue inhibitor of metalloproteinases, were constructed by transfection with appropriate plasmid constructs. Many of the lines were down-modulated for TIMP messenger RNA levels and secreted less TIMP into the culture medium. In comparison to noninvasive, nontumorigenic controls, these cells not only were invasive in a human amnion invasion assay, but also were tumorigenic and metastatic in athymic mice. These results indicate that TIMP suppresses oncogenicity, at least in immortal murine 3T3 cells.

A MURINE CDNA CLONE CALLED 16C8 was identified initially by differential screening of a cDNA library with radiolabeled cDNA probes reflecting the mRNA content of quiescent versus serum-stimulated Swiss 3T3 cells (*1*). Synthesis and cytoplasmic abundance of the cognate 0.9-kb mRNA are enhanced by serum, platelet-derived growth factor, double-stranded RNA, and 12-*O*-tetradecanoylphorbol-13-acetate; the product of the mRNA is a secreted glycoprotein (M_r ~29,000) called TIMP, tissue inhibitor of metalloproteinases (*2*).

An inverse correlation between TIMP levels and the invasive potential of murine and human cells has been reported (*3*), implying an important role for TIMP in the control of tumor invasion in vivo. We report here that Swiss 3T3 cells modified genetically to synthesize an RNA (antisense RNA) complementary to the mRNA encoding TIMP are down-modulated for TIMP expression. This down-modulation caused the previously noninvasive cells to become invasive as assessed by an amnion invasion assay. In contrast to control cells, cells producing less TIMP gave rise to tumors and to metastatic lesions 2 to 3 months after subcutaneous or intravenous injection into nude mice.

A full-length cDNA clone encoding mouse TIMP was inserted in both orientations into the Bam HI cloning site of the mammalian expression vector pNMH, and constructs capable of producing sense and antisense transcripts were identified by restriction analysis (*4*). Transcription of the insert is ostensibly driven by the mouse metallothionein-1 promoter; the human growth hormone 3′ untranslated region provides a splice site and termination signals for RNA processing. Plasmid pNMH also has a neomycin resistance gene that confers resistance to G418 sulfate. pNMH-aT, the plasmid designed to produce antisense TIMP RNA, was introduced into Swiss 3T3 cells by coprecipitation with calcium phosphate (*5*), and stable G418-resistant transformants were isolated.

The structure of the integrated plasmid DNA was determined in individual clones by DNA blot analysis (Fig. 1). Four out of ten clones screened had a 2.3-kb Eco RI fragment that is diagnostic of the unrearranged expression cassette; the larger Eco RI fragments of 8.0 kb and 3.1 kb carry portions of the endogenous TIMP gene. LA1 showed a signal at least 100-fold more intense than the signals from the LA6, LA7, and LA8 clones, which appeared to have single-copy inserts. The higher copy number of LA1 could be the result either of gene amplification or of insertion of the plasmid as a concatemer at the site of integration. The differences in the sizes of the Hind III and Bam HI fragments among the various clones indicate that the plasmid had recombined into different sites in the genome and that the cell lines were unique. The G418-resistant MC2 line derived from pNMH-transfected 3T3 cells served as a control.

The extent of stable antisense RNA expression and its effect on the endogenous TIMP mRNA levels was investigated by Northern blot analysis (*1, 6*). Cytoplasmic RNA was extracted from exponentially growing cells, separated by electrophoresis, and blotted onto nitrocellulose. The resulting blot was probed with strand-specific probes to identify sense (Fig. 2B) or antisense (Fig. 2C) TIMP RNA (*7*). One of several controls performed to verify that RNA loading and transfer were uniform is shown in Fig. 2A. An amount of antisense TIMP RNA comparable to mRNAs of moderate abundance was present in the cytoplasm of LA1, but not LA6, LA7, or LA8 cells. Analysis of total cellular RNA by

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