phate dehydrogenase gene (Xq28) retained the V2 receptor gene. These results suggest that the V2 AVP receptor gene is located at Xq27-q28, although a region just proximal to Xq26 cannot be rigorously excluded. Definite linkage between the V2 AVP receptor and nephrogenic diabetes insipidus will require the characterization of a mutant V2 receptor gene from an affected individual. □

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Changing role of *even-skipped* during the evolution of insect pattern formation

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THE development of *Drosophila* is typical of the so-called long germband mode of insect development, in which the pattern of segments is established by the end of the blastoderm stage[1,2]. Short germband insects, such as the grasshopper *Schistocerca americana*, by contrast, generate all or most of their metameric pattern after the blastoderm stage by the sequential addition of segments during caudal elongation[3]. This difference is discernible at the molecular level in the pattern of initiation of the segment polarity gene *engrailed*[4], and the homeotic gene *abdominal-A* (ref. 5). For example, in both types of insects, *engrailed* is expressed by the highly conserved germband stage[4,6] in a pattern of regularly spaced stripes, one stripe per segment[7–9]. In *Drosophila*, the complete pattern is visible by the end of the blastoderm stage, although *engrailed* appears initially in alternate segments in a pair-rule pattern[9,10] that reflects its known control by pair-rule genes such as *even-skipped*[11–15]. In contrast, in the grasshopper, the *engrailed* stripes appear one at a time after the blastoderm stage as the embryo elongates[4]. To address the molecular basis for this difference, we have cloned the grasshopper homologue of the *Drosophila* pair-rule gene *even-skipped* and show that it does not serve a pair-rule function in early development, although it does have a similar function in both insects during neurogenesis later in development.

Figure 1 shows a comparison of the proteins encoded by the *Drosophila even-skipped* gene and the putative grasshopper homologue. A high degree of amino-acid identity exists within the homeodomain (56 out of 60 amino acids), in the adjacent C-terminal region (region A in Fig. 1; 17 out of 24 amino acids), and in a short stretch at the C-terminal end of the protein (region B in Fig. 1; 7 out of 10 amino acids). Comparisons of the grasshopper sequence with other homeodomain-containing proteins of *Drosophila* reveal that no other homeodomain has more than 60% amino-acid identity and none has significant sequence similarity outside of the homeodomain. Thus the sequence comparisons indicate that the grasshopper gene described here is a homologue of the *Drosophila even-skipped* gene.

We used antibodies raised against grasshopper *even-skipped* to investigate the protein distribution during development. Midway through *Drosophila* embryogenesis, *even-skipped* is expressed in a segmentally repeated subset of identified neurons, an ectodermal ring at the anal pad, and in segmentally reiterated mesodermal cells at the dorsal edge of the embryo[13,14,16] (Fig. 2*a*). At an equivalent stage of development, grasshopper *even-skipped* is expressed in an almost identical pattern (Fig. 2*b*). The neural expression of *even-skipped* is known to play an important part in the differentiation of particular identified neurons in *Drosophila*[16], homologues of which are readily identifiable in grasshopper[17]. A careful examination suggests that *even-skipped* is expressed in homologous neurons in the nervous systems of both insects; for example, on the dorsal surface of the central nervous system (CNS), *even-skipped* is expressed by only the aCC, pCC and RP2 neurons (Fig. 2*c* and *d*). The similarities in expression pattern, particularly in the nervous system, are further evidence that we have indeed isolated the grasshopper homologue of *Drosophila even-skipped*.

To analyse the potential pair-rule function of grasshopper *even-skipped*, we examined embryos at a much earlier stage of development. During the blastoderm stage, *Drosophila even-skipped* expression is resolved into a series of seven stripes[13,14]. Shortly thereafter, *engrailed* expression is initiated in a pattern of fourteen stripes, with the anterior margin of each *even-skipped* stripe coinciding with an odd-numbered *engrailed* stripe[12]. At the onset of gastrulation, *even-skipped* stripes have sharply defined anterior borders and *engrailed* stripes show a pair-rule pattern of intensity, reflecting their order of initiation (Fig. 3*a* and *d*). Detailed genetic analysis has indicated that the pair-rule pattern of *Drosophila even-skipped* expression is required to establish the proper pattern of *engrailed* expression[11,13,15].

To determine the potential for *even-skipped* regulation of *engrailed* in grasshopper, we examined the expression of *even-skipped* at stages before (12% to 16% of development) and during (17% to 30%) the appearance of *engrailed* stripes[4]. Figure 3*b* and *c* shows an embryo in which the first two *engrailed* stripes (T1 and T2) have just started to appear. At this stage, grasshopper *even-skipped* (Fig. 3*e* and *f*) does not show any pair-rule pattern of expression and does not overlap with *engrailed* expression. At no time in development do we observe an expression pattern that would suggest that grasshopper *even-skipped* is involved in the initiation of *engrailed* striped expression. In fact, with the exception of a few neurons, we have not observed grasshopper even-skipped and engrailed localization within the same nucleus.

If pair-rule patterning were involved in short germband development, one might expect to find grasshopper homologues of at least two of the three *Drosophila* primary pair-rule genes (*hairy*, *runt* and *even-skipped*) expressed in a pair-rule fashion before the appearance of *engrailed* stripes. Grasshopper

even-skipped, however, does not display a pair-rule pattern of expression and therefore does not appear to play a part in setting up the pattern of *engrailed* expression during grasshopper development. These observations strengthen the argument that pair-rule patterning is not involved in the segmentation of short germband embryos, such as grasshopper, which are representative of the primitive mode of insect development. One caveat to this analysis would be the existence of a second *even-skipped* gene in grasshopper that does play a part in pair-rule patterning, but we have not found any indication of such a second gene from our polymerase chain reaction experiments, nor from the analysis of genomic Southern blots probed with the existing grasshopper *even-skipped* gene.

Despite the lack of pair-rule expression, grasshopper *even-skipped* is expressed in the early embryo. At the onset of gastrulation, expression is observed in the posterior region of the embryo and is predominantly mesodermal, with low levels in the overlying ectoderm. At no point does this expression pattern resolve into a pattern of stripes. As the embryo begins to elongate, expression is maintained in the most posterior region of mesoderm (Fig. 3*e*), with the highest levels of expression in mesodermal cells in medial regions of the gastral groove. At about 25% of development, this mesodermal staining disappears. Thus, early expression in grasshopper seems to be limited to posterior mesoderm during the time that the embryo is gastrulating—an expression pattern unlike anything reported for *Drosophila even-skipped*[13,14].

This expression in posterior mesoderm, however, is reminiscent of the early expression patterns of *Xhox*-3 and *Evx*-1, *Xenopus* and mouse *even-skipped* homologues, respectively[18–20]. For these vertebrate genes, expression is predominantly in the posterior mesoderm; a variety of manipulations in *Xenopus* suggest that they may be involved in early axial patterning[21,22]. Later in development, these vertebrate *even-skipped* homologues are expressed within the central nervous system[18,19]. Interestingly, the vertebrate *even-skipped* homologues appear to be associated with Hox gene clusters. For example, the human *even-skipped* homologues, *EVX*-1 and *EVX*-2, are located at the extreme 5′ ends of the clusters on chromosomes 7 and 2, respectively[23,24]. In *Drosophila*, however, *even-skipped* is located on a different chromosome from the one containing the Antennapedia and Bithorax complexes. We do not yet know if the

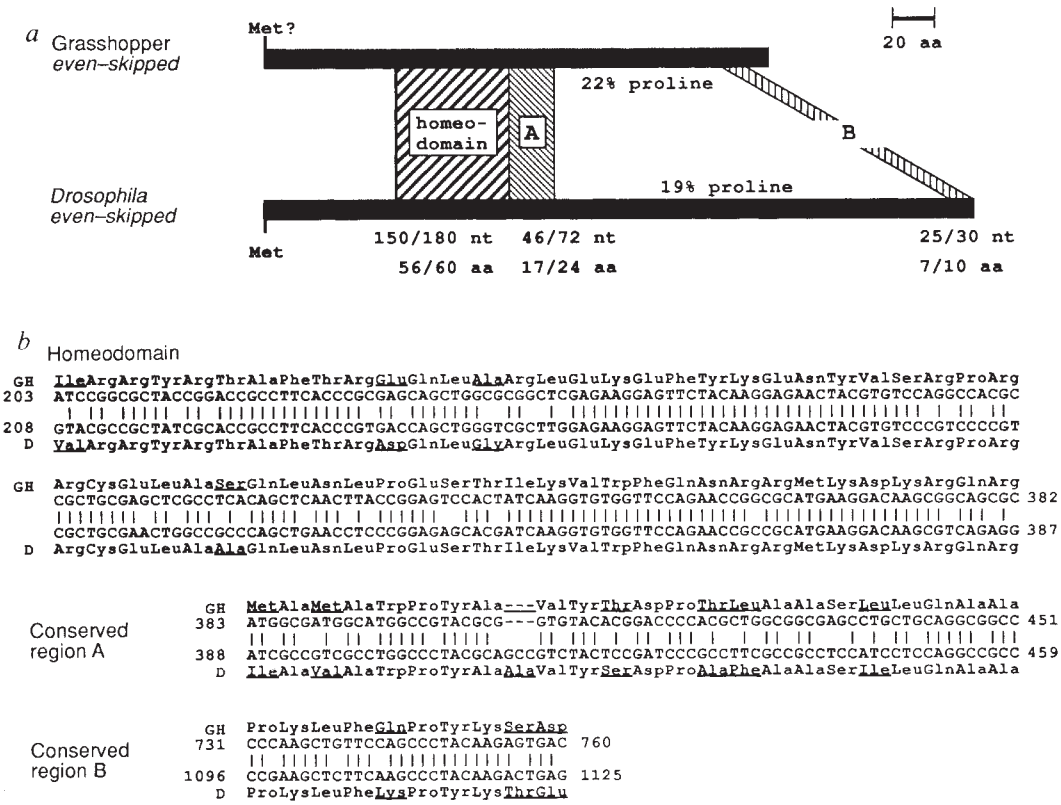
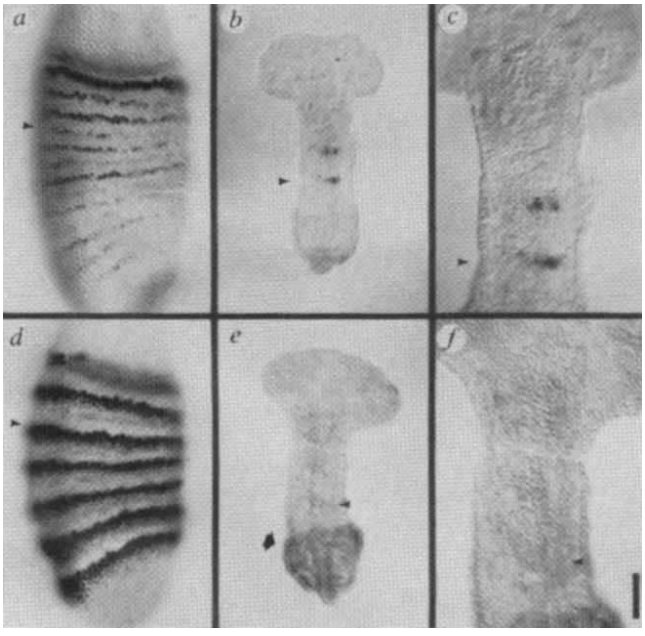


FIG. 1 Alignment of *Drosophila* and grasshopper *even-skipped* sequences. *a*, Schematic representations of the grasshopper and *Drosophila even-skipped* proteins[15,16] indicating the three regions of amino-acid sequence similarity: the homeodomain, region A (extended homeodomain) and region B. Below each region, the ratios of nucleotide (nt) and amino-acid (aa) identities are given. Between regions A and B, the predicted sequence of both proteins indicates a high proportion of proline residues. *b*, Nucleotide and amino-acid alignments of the three regions of similarity. Amino-acid mismatches are underlined and a single amino-acid gap has been inserted in grasshopper region A (dashed lines) for the purposes of sequence alignment. For *Drosophila even-skipped*, nucleotide numbering (shown at the beginning and end of each domain) begins with the adenine of the start methionine codon of the protein[13,14]. For the grasshopper sequence, numbering begins with the first nucleotide of the cDNA sequence after the *Eco*RI cloning site. There is an in-frame methionine starting at the fifth base pair, but the surrounding nucleotides match poorly with the consensus seen around start methionine codons[25]; thus we believe that the actual start methionine may not be included in our cDNA clone.

METHODS. mRNA was isolated from grasshopper embryos ranging from 28% to 35% of embryonic development. First strand cDNA, synthesized using oligo(dT) and M-MLV reverse transcriptase, was used as a template for PCR reactions[26]. Based on the comparison of the homeobox sequences of *Drosophila eve* and mouse *Evx-1* with other homeoboxes, four different primers were made to amplify *even-skipped* class homeoboxes specifically by polymerase chain reaction (PCR): (1) 5′-GGCAAGCTTGGARAARGART-TYTAYA-3′; (2) 5′-CGCCTCGAGAARGARTTYTAYARGGARAAYT-3′; (3) 5′-CATCCGCGGRTTYTGRAACCANACYTTXAT-3′; and (4) 5′-TTTGGATCCY-TGNCGYTTRTCYTTCAT-3′ (where N is A,C,G or T; R is A or G; Y is C or T; and X is A,G or T). Primers were used at a 1 μM final concentration in a total volume of 25 μl. Primers 1 and 4 were used for 35 cycles (95 °C for 1 min, 55 °C for 1 min, 72 °C for 45 s), and 2.5 μl of this product was then used as the template for another 35 cycles of amplification using primers 2 and 3. The final product was purified after electrophoresis in a 1.9% agarose gel, kinased, subcloned into M13, and sequenced. Twelve M13 clones were sequenced, and all contained the identical amplification product (corresponding to nucleotides 276–335 of the grasshopper *even-skipped* cDNA sequence shown above). This product was also used to screen a total of 5×10^6 plaques from grasshopper λgt11 and λgt10 cDNA libraries[27]. No positives were found from three different pools of a λgt11 library, but a screen of 1×10^6 plaques from the λgt10 library yielded three identical cDNA clones. The grasshopper *even-skipped* cDNA was sonicated, and fragments were then subcloned into M13 and sequenced. The total length of the cDNA is 2,140 base pairs, of which 1,341 base pairs are 3′ untranslated sequence. The complete grasshopper *even-skipped* cDNA sequence is available through the EMBL, GenBank and DDBJ Databases (accession number Z11845).

FIG. 2 Comparison of *even-skipped* expression in *Drosophila* and grasshopper at mid-embryogenesis. *a* and *c*, Expression of *even-skipped*, as visualized with monoclonal antibody 3C10, in a *Drosophila* embryo at 12 h of development; *b* and *d*, *even-skipped* expression in a 45% grasshopper embryo as detected with an affinity-purified rat antiserum. In both *Drosophila* (*a*) and grasshopper (*b*) embryos, *even-skipped* protein accumulates in a subset of neurons (large arrowhead), in dorsal mesoderm (open arrow) and in an ectodermal ring at the anal pad (small arrowhead in *a*; not shown in *b*). The *Drosophila* embryo appears to have more neural staining, but this is simply because the greater thickness of the grasshopper embryo prevents visualizing all stained neurons in a single focal plane. Homologous neurons express *even-skipped* in both insects. For example, *c* and *d* show the dorsal-most neurons that express *even-skipped* in *Drosophila* (*c*) and grasshopper (d). In both, *even-skipped* protein is detected in RP2 (arrowhead), aCC (wide arrow), and pCC (thin arrow), but not in any of the other well-characterized dorsal neurons. Scale bar, 70 μm (*a* and *b*), 8 μm (*c*), 10 μm (*d*). Anterior is up in all panels.

METHODS. To produce antibodies against grasshopper *even-skipped*, the cDNA was subcloned into the *Eco*RI site of Bluescript KS$^+$, and then a *Sac*I to *Xba*I fragment (*Sac*I site at nucleotide position 304 of grasshopper *even-skipped*, *Xba*I site provided by the Bluescript polylinker) was subcloned into both pRIT31 (ref. 28) and pATH11 (ref. 29) cut with *Sac*I and *Xba*I. These were used to generate protein A and *trpE* fusion proteins containing the C-terminal two-thirds of grasshopper *even-skipped*. The entire *Eco*RI fragment was also subcloned into the *Eco*RI site of pGEX-3X (Pharmacia) to generate a glutathione *S*-transferase fusion protein containing the entire protein encoded by the cDNA. The protein A fusion (50 μg) was injected into rats at two-week intervals. After four injections, the resulting antiserum was affinity-purified on a column of the *trpE* fusion protein coupled to Affigel 10/15 (Bio-Rad) and eluted with glycine–HCl, pH 2.3. Fractions were tested on western blot strips of the glutathione *S*-transferase fusion. For the production of a monoclonal antibody against *Drosophila even-skipped*, a plasmid producing *Drosophila even-skipped* under the control of the T7 promoter was provided by T. Hoey and M. Levine. The bacterially produced *Drosophila even-skipped* protein was used to immunize mice and generate monoclonal antibody 3C10. All immunohistochemistry followed published methods[30]. For both 3C10 and the rat antiserum, staining was improved by limiting the fixation time to 10 min.



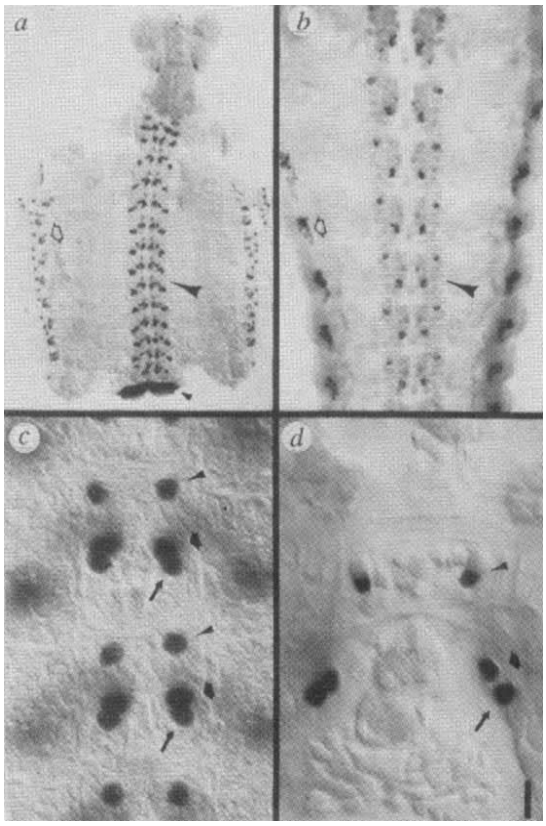


FIG. 3 Expression of *even-skipped* during *Drosophila* and grasshopper segmentation. *Drosophila engrailed* (*a*) and *even-skipped* (*d*) protein accumulation at the end of gastrulation shows that *even-skipped* stripes (*d*) are sharply defined at the time that *engrailed* stripes (*a*) are forming. Arrowhead marks the position of the posterior portion of the second thoracic (T2) segment (*engrailed* stripe 5, anterior part of *even-skipped* stripe 3). The anterior margin of each *even-skipped* stripe corresponds to each of the odd-numbered *engrailed* stripes[1,2]. Note the pair-rule pattern of stripe intensity in which the odd-numbered *engrailed* stripes are weaker than the even-numbered ones at this stage. At 17% of grasshopper development, *engrailed* stripes (*b*, and at higher magnification in *c*) have just appeared in T1 and T2 (arrowhead marks T2). The next stripes to appear will be further anterior in S1 and S3 (the mandibular and labial segments). The stripes of S1 and S3 represent the only pair of grasshopper *engrailed* stripes to appear in any pattern reminiscent of the pair-rule pattern of initiation seen for *Drosophila engrailed*[4]. In grasshopper embryos of the same stage stained for *even-skipped* expression (*e* and *f*), no pair-rule stripe pattern is visible. The position of T2, which can be determined even in unstained preparations by bulges in the mesoderm which are visible shortly after *engrailed* stripes appear, is marked by an arrowhead. The higher magnification view in *f* shows that no stripe of *even-skipped* expression is evident in T2, where *engrailed* expression has started, or more anteriorly in S1 and S3, where *engrailed* stripes will next appear. There is, however, a domain of grasshopper *even-skipped* expression in the more posterior mesoderm (arrow in *e*). At no earlier or later stage do we observe even-skipped expression in a pattern of ectodermal stripes that would be consistent with a role in the regulation of *engrailed* expression. Scale bar represents 200 μm (*b* and *e*), 100 μm (*c* and *f*), and 75 μm (*a* and *d*). Anterior is up in all panels.

grasshopper *even-skipped* gene is located near any of the homeotic gene homologues.

Our results provide some initial answers to long-standing questions concerning the evolution of insect segmentation. We suggest that *even-skipped* had a role in neurogenesis and/or axial patterning in the common ancestor to vertebrates and arthropods. The extremely similar expression patterns in identified neurons, dorsal mesoderm and the ring of tissue at the anal pad, suggest a highly conserved role in the germband stage of all insects. During the course of insect evolution, insects using a long germband mode of development arose and *even-skipped* acquired an additional function, that of pair-rule patterning. □

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Location of MHC-encoded transporters in the endoplasmic reticulum and *cis*-Golgi

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IMMUNE recognition of intracellular proteins is mediated by major histocompatibility complex (MHC) class I molecules that present short peptides to cytotoxic T cells[1–4]. Evidence suggests that peptides arise by cleavage of proteins in the cytoplasm and are transported by a signal-independent mechanism into a pre-Golgi region of the cell, where they take part in the assembly of class I heavy chains with β_2-microglobulin (reviewed in refs 5–7). Analysis of cells that have defects in class I molecule assembly and antigen presentation[8–14] has shown that this phenotype can result from mutations in either of the two ABC transporter genes located in the class II region of the MHC[15–22]. This suggested that the protein complex encoded by these two genes[20,22] transports peptides from the cytosol into the endoplasmic reticulum. Here we report additional evidence by showing that the transporter complex is located in the endoplasmic reticulum membrane and is probably oriented with its ATP-binding domains in the cytosol.

To locate the MHC-encoded transporter proteins, an antiserum (AK1.7) was raised against the carboxy-terminal peptide of the TAP1(RING4) sequence[20]. This reagent precipitated the TAP1 protein and coprecipitated the associated TAP2 product[20]. Preliminary cell staining experiments were done to compare the mutant lymphoblastoid cell line LCL721.174 (termed .174), that has deleted both TAP genes[9,17], with the wild-type control LCL721 (termed 721). The unpurified serum gave high background staining on .174 cells (data not shown; ref 20), but after affinity purification on the TAP1 peptide used for immunization, it stained 721 cells specifically (Fig. 1*a*). The improved specificity of staining after affinity purification was accompanied by the loss of two prominent proteins of M_r ~33–34K in immunoprecipitates from both .174 and 721 cells (Fig. 1*b*), which may have arisen through cross-reactions with the carrier (KLH) used for immunization. Additional proteins of ~28K and ~52K were precipitated from the wild-type 721 cells but not from .174, and were retained after affinity purification of the AK1.7 serum. We are investigating the nature of these bands. They may have co-precipitated with the TAP1 protein, because western blot analysis with the affinity purified AK1.7 antiserum detected a single ~71K band in total cell lysates from 721 cells, which was absent in similar extracts from .174 (Fig. 1*c*).

TABLE 1 Numbers of anti-TAP1 immunogold particles on cryosections of LCL721 and .174 cells

	LCL721 cells	.174 cells	Specificity factor
Rough endoplasmic reticulum	671	96	7.0
Golgi-complex	59	22	2.7
Irrelevant*	696	845	0.8
Total gold	1,426	963	

Cells were randomly selected in the electron microscope viewer and were photographed at a magnification of ×3000 until a section surface of 1,815 μm^2 had been included for each cell type. In these cell profiles gold particles were counted and assigned to the structures listed. A gold particle was considered to label a membrane when lying within 20 nm of it. Totals of 19 LCL 721 cells and 24 mutant .174 cells were quantified. Of each cell type ~180 μm^2 endoplasmic reticulum and 40 μm^2 Golgi complex were evaluated. The specificity factor of TAP1 labelling is the ratio of LCL721 over .174 labelling for a particular compartment.

* 'Irrelevant', for this study included nuclei, mitochondria, cytosol and unidentifiable structures.

The distribution of staining revealed by light microscopy on acetone-fixed cytospin preparations was predominantly perinuclear, consistent with location in the endoplasmic reticulum (Fig. 1*a*). To determine the precise location of the TAP1 protein we performed high resolution immuno-electron microscopy on ultrathin cryosections of 721 and .174 cells. Immunogold particles were counted on sections of the two cell lines and were found specifically on the membranes of the endoplasmic reticulum and Golgi complex (Figs 2, 3*a*, *b* and Table 1). Because the antibody was raised to a peptide from the C terminus of the TAP1 ATP-binding domain and labelling occurred predominantly on the cytosolic side of the membrane (Fig. 2) we suggest that the TAP1 protein is oriented in the endoplasmic reticulum and Golgi membranes with its ATP-binding domain in the cytosol.

The distribution of TAP1 in the Golgi complex was defined by comparison to class I MHC molecules (which can be detected throughout the stack)[23], and p53 (which is localized to the *cis*-Golgi)[24,25]. Although MHC class I molecules were found throughout the stacks of Golgi cisternae, TAP1 was confined to only one side of the stacks (Fig. 3*a*). Comparison with p53 staining showed that TAP1 was localized to the *cis*-Golgi (Fig. 3*b*). We estimate that the density of TAP staining of the *cis*-Golgi is close to that of the endoplasmic reticulum, because the *cis*-Golgi cisternae comprises only ~1/4 to ~1/3 of the total Golgi membranes. The specificity factor for *cis*-Golgi, as opposed to total Golgi (in Table 1), would therefore be ~9. Expression of the TAP complex in the *cis*-Golgi as well as the endoplasmic reticulum may provide a hint as to the sites of peptide loading. In fact, it has been suggested that class I molecules cycle between the endoplasmic reticulum and *cis*-Golgi[41].

The TAP proteins are the only members of the ABC superfamily of transporters that localize to the endoplasmic reticulum and *cis*-Golgi. The mechanism of localization of the TAP1 protein is not apparent, because it does not contain any previously characterized endoplasmic reticulum retention signals[26,27]. To see if retention of TAP1 required its association with TAP2, we stained mutant T2 cells that expressed the TAP1