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Table 2 Inhibition of cell growth by compound IV renders bacteria susceptible to serum killing

Pretreatment	% Survival following treatment with 50% human serum for 40 min at 37 °C
None	113 ± 16.2
Chloramphenicol (50 μg ml^{-1})	98 ± 2.9
Erythromycin (750 μg ml^{-1})	86 ± 2.3
Tetracycline (5 μg ml^{-1})	94 ± 9.8
Compound IV (100 μg ml^{-1})	1.3 ± 0.2*

Cells were grown in MOPS medium containing 0.2% glucose (w/v) and pretreated as indicated. Drug concentrations in the pretreatment were chosen so that growth ceased in 2–3 h. An equal volume of phosphate-buffered saline (PBS) or normal human serum was added, and samples incubated at 37 °C for 40 min. No loss of viability occurred in PBS. Survival values in serum are given with the standard deviation as determined by viable count in triplicate.

* Most (>95%) of the bacteria were killed in 20 min and addition of fresh serum at 40 min resulted in continued killing, that is, serum complement is exhausted when large numbers of bacteria are used.

pmol per min per mg protein at 100 μM substrate). Second, release of inhibitor from IV, V, VI and VII was inhibited by the aminopeptidase inhibitor bestatin; and third, there was a correlation between inhibitor release in cell extracts and antibacterial potency (Table 1).

Inhibition of LPS synthesis, accumulation of lipid A precursor, and bacteriostasis, however, are not the final consequences of treatment with compound IV. Translocation of drug-induced lipid A precursor to the outer membrane (S. Kadam *et al.*, in preparation) causes development of sensitivity to host defences and increased sensitivity to antibiotics known to have difficulty crossing the outer membrane permeability barrier. When compared to other bacteriostatic agents, induction by compound IV of susceptibility to complement killing is striking (Table 2). No killing is observed following bacteriostasis induced by tetracycline, chloramphenicol, or erythromycin; however, extensive killing results from pretreatment with IV. In addition, cells become 10 times more sensitive to erythromycin following pretreatment with IV, such that 50% inhibition of protein synthesis rate is brought about by 10 μg ml^{-1} rather than 100 μg ml^{-1} erythromycin.

In conclusion, peptide prodrugs of compound III represent antibacterial agents which specifically inhibit LPS synthesis at the site of KDO metabolism. Previously, diazaborine was considered to act in this manner[15], but further studies revealed that this was not the case and suggested that its antibacterial action was due to inhibition of fatty acid biosynthesis (K. Fuchs and G. Hogenauer, personal communication). We have confirmed these results, finding that synthesis of all classes of fatty acid are inhibited by diazaborine, probably by inhibition of acetyl CoA carboxylase (unpublished data).

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A liver-stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning

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The liver phase of development of malaria parasites has been studied only recently and remains poorly understood compared to the other stages such as sporozoites, merozoites and gametes[1,2]. Access to liver forms of *Plasmodium falciparum* has been improved by the development of *in vivo*[3] and *in vitro*[4] propagation methods, but the yield of mature schizonts remains limited and does not allow a detailed antigenic analysis. To date, only immunofluorescence assays (IFA) have permitted a description of a species and liver-stage-specific antigen(s) (LSA; ref. 3). Monospecific antibodies to these antigens have not been obtained due either to difficulty in immunizing mice (against LSA), or to poor stability of human monoclonal antibodies. Therefore, as a means of characterizing the LSA, we used an alternative immunological approach to identify clones of the corresponding LSA genes. We describe here the isolation of a DNA sequence coding for a *P. falciparum* liver-stage-specific antigen composed of repeats of 17 amino-acids, which is immunogenic in man.

We looked for human sera with restricted specificity to the pre-erythrocytic stages of development of *P. falciparum* by screening individuals living in a malaria endemic area and undergoing continuous drug prophylaxis. One such serum taken from a subject ingesting 100 mg of chloroquine daily, whilst being under continuous exposure to malaria for 26 years, had high antibody titres to sporozoites (1/3,200 by IFA) and liver stages (1/6,400) yet was essentially negative with blood-stage parasites (<1/100). The serum was used to screen a genomic expression library of *P. falciparum* Tak9.96 DNA[5] cloned in λgt11 (ref. 6). The recombinant phages were first screened with a pool of hyper-immune sera and 2,000 antigen-producing clones were detected. Of these only 15% were positive with the sporozoite and liver-stage-restricted serum. This indicates that the antibodies to sporozoite- and liver-stage antigens are a restricted subset of the total antibody responses to malaria parasites. Of these clones the 22 most positive were selected for further study. Human antibodies from the original serum were affinity-purified on β-galactosidase fusion proteins from these clones. The stage specificity was assayed by IFA using *P. falciparum* sporozoites, liver- and blood-stage parasites. Antibodies selected on three clones (DG145, DG199 and DG307) reacted specifically with liver schizonts: location of the fluorescence was very similar to that considered characteristic of LSA (ref. 3; Fig. 1). The reaction was *P. falciparum*-specific as the affinity-purified antibodies were negative with *P. vivax* liver schizonts prepared in *Saimiri* monkeys and with *P. yoelii* liver stages grown in BALB/c mice. Moreover, the three recombinant antigens were negative with sera to *P. vivax*, *P. ovale*, *P. cynomolgi* and *P. yoelii* (data not shown).

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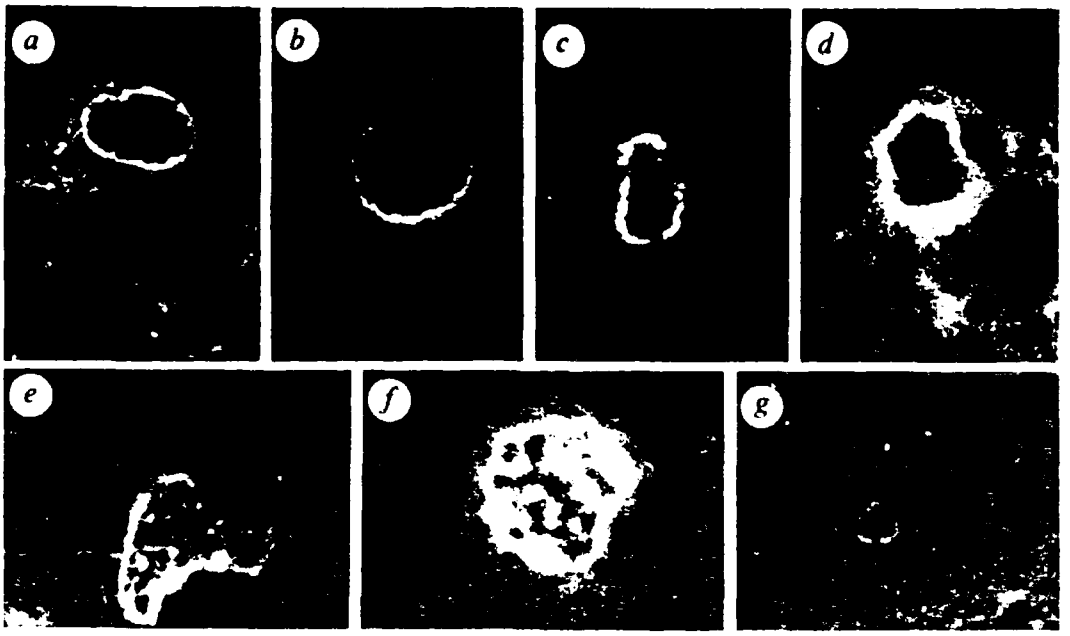

Fig. 1 Reaction and localization of antibodies with liver-stage schizonts. *a*, Typical immunofluorescence using adult African serum diluted 1/2,000 and reacted with 5-μm sections of Carnoy fixed liver fragments taken from *Cebus apella* monkeys infected with *P. falciparum* (ref. 3). Same antigen reacted with antibody eluted from protein expressed by: *b*, clone DG307; *c*, clone DG145; *d*, clone DG199; *e*, *f*, the same as *a* and *b*, using more mature schizonts to show the internal distribution of the antigen. *g*, Liver schizonts reacted with 1/250 dilution of a rabbit serum raised to clone DG307 fusion protein (one i.m. injection with FCA of the recombinant fusion protein isolated by preparative gel electrophoresis followed by four additional i.v. injections at 15-day intervals).

The recombinant fusion proteins of DG145, DG199 and DG307 were positive with the original human serum (Fig. 2*a*) and with ten other African sera, but negative with sera from transfusion malaria patients and anti-sporozoite sera (data not shown). The three fusion proteins were found to be heat-stable as the LSA epitopes remained antigenic after boiling at 100 °C for 15 min. This is shown for DG307 in Fig. 2*a* track 2. Full immunological cross-reactivity between the three recombinant antigens was demonstrated by immunoblot analysis of the fusion proteins. Antibodies affinity-purified on the DG307 protein reacted to the same extent with the recombinant antigens of clones DG307, DG145 and DG199, but they did not react with the recombinant antigens of unrelated clones. The same cross-reactivity was also observed using affinity-purified antibodies corresponding to clones DG145 and DG199 (data not shown).

The liver-stage-specificity of LSA was further established by raising a polyclonal monospecific antiserum to the DG307 fusion protein. The anti-DG307 rabbit serum gave the characteristic LSA immunofluorescence image (Fig 1*g*). Like the affinity-purified human antibodies selected using the three clones, this serum was negative by IFA and immunoblot with sporozoites and blood-stage antigens (Fig. 2*b*).

To analyse the relationship between clones and to characterize the LSA gene(s), DNA from the three LSA phages DG145, DG199 and DG307 was prepared and the individual *P. falciparum* DNA inserts recloned into the plasmid pUC9 and bacteriophage M13 (ref. 8). DG145 contained two *P. falciparum* DNA fragments of 400 and 700 base pairs (bp) (Fig. 3*a*). The 700-bp fragment cross-hybridized with the 196-bp insert in phage DG307, but did not cross-hybridize with the 600-bp fragment of DG199. Genomic DNA was cut with four different restriction enzymes (both single and double digests) and was used in a series of Southern transfer analyses (one example is shown in Fig. 3*b*). The analysis indicated that the DG307, DG199 and DG145 700-bp fragments were derived from the same region of the genome as they all hybridized to a *Rsa*1 fragment of 2.2 kilobase (kb), a *Dra*1 fragment of 4.5 kb and an *Alu*1 fragment

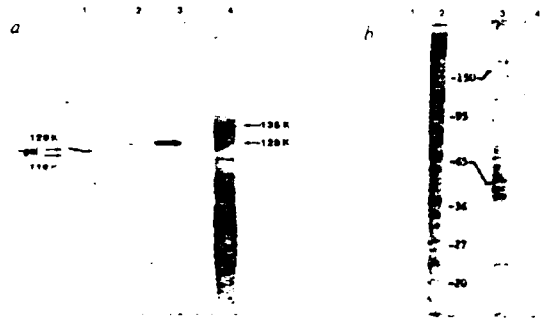

Fig. 2 *a*, Identification of the fusion proteins of the three clones expressing LSA antigen by human antibodies. SDS-PAGE electrophoresis of proteins from cultures of DG199 (track 1), DG307 (tracks 2 and 3) and DG145 (track 4). *b*, Stage specificity of DG307. Rabbit antibodies to DG307 (1/20 dilution) did not react on immunoblots with antigens extracted from blood stages (track 1) and sporozoites (track 4). Shown as a control is an immune African serum (1/100 dilution) which is positive with both erythrocyte (track 2) and sporozoite (track 3) stage antigens. Note that the recombinant protein of DG307 remains antigenically reactive after boiling and also that the fusion protein of DG145 appears somewhat degraded.

Methods. The three LSA clones were isolated from a genomic expression library constructed in the following way. Random fragments of T9.96 DNA were generated by the action of DNaseI, methylated to protect the endogenous *Eco*RI sites and cloned by the addition of linkers into the *Eco*RI site of λgt11. The fusion protein of DG307 was boiled in PBS at 100 °C for 15 minutes before gel electrophoresis (track 2). Proteins were transferred to nitrocellulose filters according to Biorad recommendations and antigenically reactive proteins detected by incubation with 1/100 dilution of the selective immune human serum, or 1/20 dilution of rabbit serum depleted of anti β-galactosidase and anti *Eschenchia coli* antibodies. Immune complexes were revealed using anti-human and rabbit IgG, A, M peroxidase labelled antibody (Biosys, diluted 1/500) and diamino-benzidine substrate.

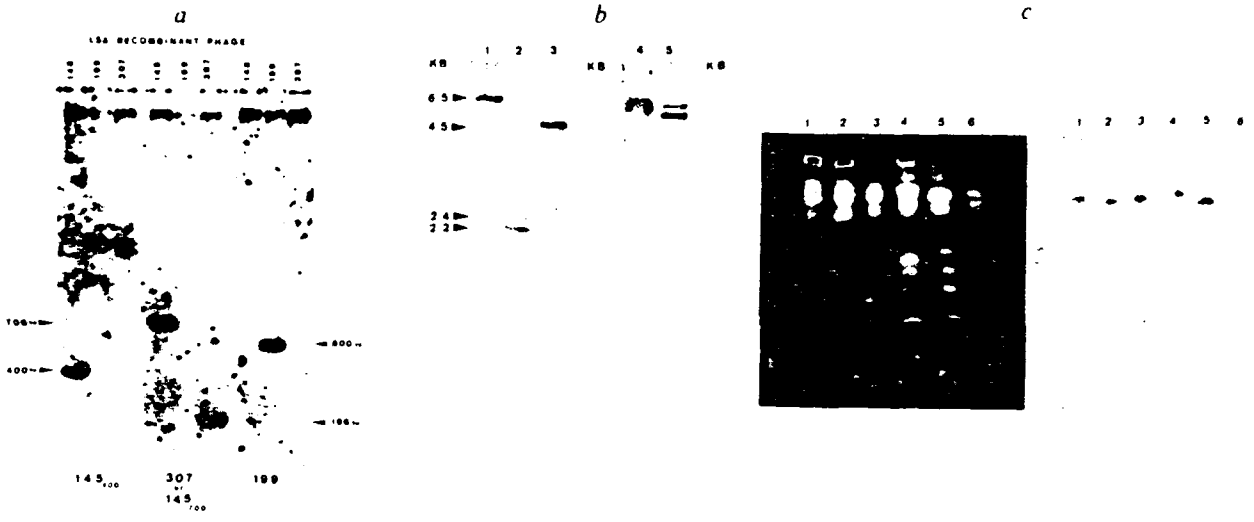

Fig. 3 Analysis of the LSA gene. *a*, Analysis of *P. falciparum* DNA inserts in the recombinant LSA phages. DNA was prepared from DG145, DG199 and DG307, restricted with *Eco*R1 and size fractionated on a 1% agarose gel. The DNA was transferred[9] to Hybond-N according to Amersham protocol and hybridized at very low stringency (2 × SSC, 37 °C) with the radiolabelled pUC9 plasmid recombinants containing separately each of the *Eco*R1 fragments derived from the LSA recombinant phages. The 600-bp sequence of DG199, like the 400-bp insert of DG145, hybridized only to itself whereas the 700-bp DG145 insert cross-hydridized to the 196-bp fragment contained in DG307 (and vice versa), indicating that they contain homologous sequences. *b*, Parasite DNA restriction fragments hybridized with DG307. Track 1, *Eco*RI; 2, *Rsa*I; 3, *Dra*I acting on Tak9.96 DNA. Tracks 4, *Eco*RI; 5, *Rsa*I acting on Palo Alto DNA (FUP Uganda). The restricted DNA was transferred[9] to Hybond-N (Amersham) and hybridized under stringent conditions (0.5 × SSC, 65 °C). Note that two *Rsa*I fragments of different size are identified in Palo Alto DNA. *c*, Chromosomes separated by pulse field gradient electrophoresis[10,11]. The karyotypes presented are for strains (1) Palo Alto (2) D3, (3) 7G8, (4) B9, (5) C11 (6) Tak9.96. B9 and C11 are clonal derivatives of a single Thai isolate (P.D., unpublished) and D3 is a clonal derivative of FCR3 (a kind of gift of W. Trager). The gel (1% agarose) was run at 250 V using a 75-s pulse for 20 h. The variation in size is due to chromosome polymorphism[12,13]. The chromosomal gel was transferred[9] to HyBond-N and probed with the inserts of DG145, DG199 and DG307 cloned in pUC9 and radiolabelled. The same large, poorly separated chromosome (number 6 counting from bottom) was identified in all strains by clones DG307, DG145 and DG199; the result obtained with plasmid recombinant DG307 is shown.

of 1.3 kb. The observation that antibodies to the DG199 fusion protein cross-reacted with the fusion proteins of DG307 and DG145, whereas its nucleotide sequence differs from the 51-bp repeat, suggests that DG199 encodes a different type of repetitive cross-reacting peptide. This situation has already been observed for several other *P. falciparum* blood-stage antigens such as FIRA and RESA[15,16].

The LSA locus shows restriction polymorphism for the enzymes *Rsa*1 and *Alu*1 as DNA fragments of different size were found when other strains were examined. Figure 3*b* shows the result obtained with *Rsa*1 and the *P. falciparum* Ugandan isolate Palo Alto (compare tracks 2 and 5). Karyotype analysis demonstrated that the three LSA clones are located on one of the large chromosomes and were found to be conserved in all the *P. falciparum* strains examined (Fig. 3*c*). Consistent with this observation is that the clones were derived from a Thai parasite and were selected by human sera from Africa. Finally, no sequences homologous to the *P. falciparum* LSA clones were found in heterologous species by probing the genomes of *P. vivax* and *P. chabaudi* (data not shown).

In order to allow further immunological studies and to complement the rabbit LSA-specific serum raised to the DG307 β-galactosidase fusion protein, we generated the corresponding synthetic peptide. The DNA sequence of DG307 was determined and a synthetic peptide prepared. Figure 4 shows that clone DG307 contains a DNA fragment of 196 bp composed entirely of a 51-bp repeat. Only one reading frame is in frame with the β-galactosidase *lacZ* gene and the clone produces a fusion protein that carries the epitopes recognized by the human sera (Fig. 2). The inferred amino-acid sequence corresponding to this frame is also presented. It is composed of a 17-residue repeat rich in glutamine, glutamic acid and leucine. Unlike the DNA sequence, the amino-acid sequence of the repeats is highly conserved. The one change in the third repeat results from an A to G substitution at the second position in the eighth codon.

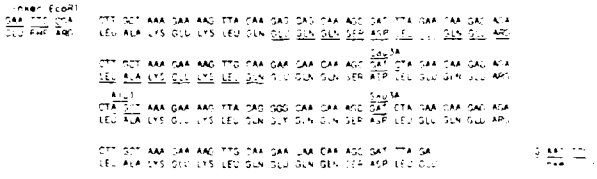

Fig. 4 The DNA and amino-acid sequence of DG307. DNA sequence of the 196-bp *P. falciparum* genomic DNA fragment expressed in clone DG307. The *Eco*RI linkers are underlined, as are some restriction sites. Note that the repeats are not perfectly conserved. Substitution of an A for a T creates an *Alu*I site in the third repeat. A change from a T to a C gives *Sau*3A sites in the second and third repeats. None of these changes result in an amino-acid substitution. The sequence overall is 61% A + T. Shown below the DNA sequence is the amino-acid sequence expressed by clone DG307. The amino acids corresponding to the *Eco*RI linker are also given as they denote the reading frame. The first arginine and last glycine are therefore artificial as they are encoded in part by the linker and in part by *P. falciparum* DNA. Shown underlined are the amino acids of the synthetic peptide used to confirm the sequence. The 196 bp of *P. falciparum* DNA of DG307 were excised by digestion with *Eco*RI, cloned in both orientations into the *Eco*RI site of the single-stranded phage m13mp8 and sequenced by the chain-termination method[14].

Otherwise all other substitutions are silent. No homology with known DNA and protein sequences was detected (at the 66% level) when the Los Alamos and NBRF data banks were screened. The peptide (EQQSDLEQERLAKEKLQ) corresponding to the amino-acids underlined (see Fig. 4) was synthesized and used in ELISA assays. Its reactivity with the rabbit anti-DG307 serum confirmed the deduced DNA sequence. The synthetic peptide reacted equally with the human antibodies affinity-purified on the fusion proteins of clones DG307, 145 and 199. This emphasizes the antigenic similarity of the three

recombinant antigens. Moreover, the reaction of the synthetic peptide with the serum used in the initial screening, together with its reaction with ten other African sera indicate that a single 17 amino-acid repeat carries at least one epitope corresponding to an antibody specificity in human sera.

The 17-amino-acid repeat described here is the first such reported for a malaria antigen. As repeated epitopes have been described for several *P. falciparum* antigens[17] it is not surprising that LSA, which is immunogenic in humans, also possesses repeated structures. Computer analysis predicts that, in contrast to the CS protein, the LSA repeat with seven or eight charged amino acids may assume a helical structure. The observation that the amino-acid sequence is more highly conserved than the DNA sequence argues for a functional or structural role for these repeats. Finally the above results, obtained using a novel approach to a poorly accessible stage, provide the first data on the structure of a protein specific for the liver stage of development of *P. falciparum*. Availability of recombinant antigens, synthetic peptides and the corresponding antibodies now provide a way to evaluate the role and biological function of this stage-specific protein.

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Involvement of the *pumilio* gene in the transport of an abdominal signal in the *Drosophila* embryo

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A small set of maternal genes controls the basic longitudinal pattern of the *Drosophila* embryo. For the posterior pattern, five genes are necessary both for abdominal segmentation and for pole cell formation[1-3]. Cytoplasmic transplantations involving *oskar* (*osk*) mutant embryos have suggested that the pole plasm serves as a source of a signal required in the more anterior abdominal region[3]. Here we present evidence that the maternal gene *pumilio* (*pum*) is involved in the transport of this signal to the abdominal region. In *pum* embryos (that is, embryos from females of genotype *pum/pum*) only the abdomen is affected, whereas the pole plasm seems normal. Transplantation experiments reveal that *pum* pole plasm contains the abdominal signal but that it cannot reach the target site, the abdomen. Abdominal segmentation is restored when the physical separation between pole plasm and abdominal region is overcome either by transplantation of *pum* pole plasm into the abdominal region of *pum* embryos or by genetic means in double mutants with *torsolike* (in mutant *torsolike* embryos the abdominal region is juxtaposed to the pole plasm).

Embryos derived from females homozygous for a strong *pum* allele form no more than two of the normal eight abdominal segments whereas head, thorax and the posterior end (the telson) are normal (Fig. 1*b*). A similar phenotype has been described for the zygotic mutant *knirps* (ref. 4) and for five maternal effect loci: *tudor*, *vasa*, *staufen*, *valois* and *oskar* (refs 1-3). In contrast to these maternal mutants, *pum* embryos do form pole cells, the germ-line precursors. These pole cells are functional when transplanted into otherwise sterile host embryos, such as the progeny of osk^{301}/osk^{301} females[5]. Morphologically, we cannot detect a difference between *pum* and wild-type pole plasm. Polar granules[6] are present. Thus, the embryonic function of the *pum* gene product is restricted to the development of the abdomen.

We tested whether the abdominal phenotype of *pum* embryos could be rescued by transplantation of wild-type cytoplasm. Using our standard injection procedure[3] the abdominal phenotype of *pum* embryos can be partially rescued by transplantation of cytoplasm from wild-type donors into the abdominal region of *pum* recipients (Fig. 2*a*). However, unlike in mutant *osk* embryos, completely normal abdominal segmentation is not restored. In mutant *pum* embryos the rescuing activity does not seem to spread but acts directly at the site of injection. For example, injections into the dorsal side of the prospective abdomen lead to the development of dorsal structures and leave the ventral side strongly mutant (Fig. 1*c*). Injections anterior and posterior to the abdominal region (20-50% egg length, where 0% is the posterior pole) rarely lead to a rescue (Fig. 2*a*). Despite this specificity of *pum* for abdominal development, rescuing activity is recovered only from the posterior pole of donor embryos: homotopic transplantation of abdominal cytoplasm into the abdomen of *pum* embryos does not lead to any change in phenotype (Table 1). Thus, although the mutant phenotype of *pum* suggests independence of abdominal development and pole cell formation, the transplantation experiments show identical localization of rescuing activity for *pum* and *osk* to the posterior pole. This may indicate that the abdominal defects in *pum* and *osk* embryos are caused by the lack of the same signal.

To test whether the activity that rescues *pum* embryos is present in *osk* embryos, we transplanted cytoplasm from *osk* embryos into *pum* embryos. No rescue occurred (Table 1), indicating that the repair of the *pum* mutant phenotype is dependent upon osk^+ activity. In the reciprocal experiment (transplantation of *pum* pole plasm into the abdominal region of *osk* embryos) the *osk* phenotype is rescued to the same extent as after transplantation of wild-type cytoplasm (Fig. 2*b*). One explanation for this finding is that *pum* embryos possess a functional signal, but that the signal is restricted to the pole plasm region and is therefore unable to reach the abdominal region. In which case *pum* pole plasm should rescue the *pum* phenotype.

Injection of posterior pole plasm from *pum* embryos into the abdominal region of *pum* embryos does indeed restore abdominal segmentation (Table 1). As in the wild type, rescuing activity is recovered only from the posterior pole region (0-20% egg length). Thus, *pum* and *osk* may lack the same signal in the abdominal region: the signal cannot be detected in *osk* embryos; it is present in *pum* embryos, but is trapped at the posterior pole. If this interpretation is correct, the distribution of the signal might be altered in *pum* embryos. We compared the spatial distribution of rescuing activity in wild-type and *pum* donors. In earlier experiments, when about 5% egg volume was

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