Incidence of sperm dysfunction in normal men and those with mild factor infertility

Male factor infertility, primarily diagnosed by abnormal semen parameters, is the single most common defined cause of infertility. Although the underlying biochemical, molecular and genetic causes are poorly understood (e.g. Matzuk and Lamb, 2002), treatment using intracytoplasmic sperm injection (ICSI) is very effective and routinely used for men with mild and severe forms of infertility. With the success and widespread availability of ICSI, conventional IVF is now reserved for men with normal or nearly normal semen parameters (mild male factor infertility). Such selection is aimed at eliminating men with sperm dysfunction and avoiding fertilization failure. It is therefore surprising that total fertilization failure is still a relatively common occurrence in IVF (for review, see Ola et al., 2001; Tournaye et al., 2002). For example, a randomized controlled trial comparing ICSI and IVF in mild male factor infertility reported that fertilization failure occurred in 25% of IVF cycles (Tournaye et al., 2002). Interestingly, a meta-analysis of nine studies comparing the success of IVF versus ICSI in men with normal semen parameters or mild male factor infertility showed that fertilization failure occurred in 37% of IVF cycles with a relative risk of failure of 10.8 (confidence limits: 3.1–38.1) compared with ICSI. Flexible clinical and laboratory protocols can reduce this, for example, insemination of higher number of spermatozoa. However, even in such circumstances, the fertilization failure rate is still high (~5%) (Tournaye et al., 2002). This emphasizes the fact that sperm dysfunction is a significant cause of infertility. Until recently, very little was known about the detailed clinical nature of sperm dysfunction in so-called ‘hidden’ male factor infertility.

Abnormalities in sperm–zona pellucida (ZP) interaction are associated with fertilization failure and poor fertilization in IVF. Large-scale clinical studies highlight the importance of this interaction and underscore the need to understand its cellular and biochemical basis. For example, Liu and Baker (2000) examined sperm zona binding and penetration in 563 couples...
with poor (<25% of oocytes fertilized) or no fertilization during IVF. Remarkably, of the 310 couples with a zero fertilization rate, 52% had a normal semen analysis. Seventy-five percent of these men had low sperm binding and 47% had no spermatozoa penetrating the ZP. In a second study of 186 men with normal semen parameters and normal sperm binding to the ZP, 25% had a disordered ZP-induced acrosome reaction (DZPIAR) (Liu et al., 2001; Liu and Baker, 2003). Interestingly, in these men, high levels of fertilization can be achieved using ICSI; consequently, IVF is an inappropriate therapy and ICSI should be used as a first-line treatment option. Such studies, in combination with data from other groups (Mackenna et al., 1993; Esterhuizen et al., 2001; Oehninger 2001; for review, see Barratt and Publicover, 2001) suggest that defects in capacitation, sperm binding and/or DZPIAR are significant causes of sperm dysfunction in men with apparently normal semen or mild male factor infertility.

The question is what are the causes of sperm dysfunction in prefertilization events? Very little is known of the nature of such defects, but clinically based experiments, for example using inducers of the acrosome reaction, show that failure of progesterone-induced calcium influx (and subsequent acrosome reaction) can predict fertilization success at IVF in over 95% of unselected cases (male and non-male factor) (Krausz et al., 1996). In fact, there is a subgroup of men in whom defective acrosome binding to the plasma membrane is associated with infertility (Tesarik and Mendoza, 1992). These findings strongly suggest both that failure of Ca<sup>2+</sup> mobilization is a significant factor in male infertility and also that the Ca<sup>2+</sup> signalling pathway activated by progesterone binding is required for the acrosome reaction. Currently it is not known if men with, for example, DZPIAR, are non-responsive to progesterone, but it is now possible to ascertain the clinical nature of these lesions to determine if single or multiple defects do occur. Defects in sperm capacitation, apart from observations of reduced levels of hyperactivation (Mackenna et al., 1993), are rarely reported. As virtually nothing is known about the molecular nature of sperm–ZP interaction, at least in the human, little progress has been made in this area.

Consequently, the current research programme has two primary objectives: firstly to understand, in cellular and molecular terms how a mature human spermatozoon is prepared for fertilization and secondly, to document what factors are involved in the initial signalling interactions (calcium influx and mobilization) between the egg and the human spermatozoon. The underlying premise is that once a rudimentary understanding of these processes has been established, an attempt can be made to determine how such interactions go wrong in subfertile and infertile men and hence appropriate drug based therapy can eventually be developed.

Technical and scientific approaches to understanding prefertilization events

Approach A: The cell physiology of spermatozoa: calcium signalling – dealing with heterogeneity and size

During the last 25 years, a series of technical advances in physiological and molecular techniques have permitted significant progress in understanding of cell signalling – the processes used by cells to recognize each other, to ‘talk’ to each other and to regulate cellular activities. These signals, between and within cells, regulate both on-going activity on a milliseconds to minutes timescale and longer-term function, by control of gene expression. Researchers are beginning to understand the immense complexity of these processes and the fact that many diseases reflect lesions of cell signalling activities. However, the progress made in understanding regulation of spermatozoa by cell-signalling processes, let alone how defects in signalling cause subfertility or infertility, has been comparatively poor. The major reason for the failure to progress rapidly is, almost certainly, the very small size of spermatozoa and the limitations that this puts on the application of technologies that are effective when applied to larger cells. Furthermore, since it is relatively straightforward to obtain near-pure preparations of spermatozoa, it is tempting to investigate the characteristics of spermatozoa in fertile and subfertile individuals by using cell populations rather than investigating the characteristics and behaviour of individual cells. Many studies have therefore used biochemical and fluorometric techniques to investigate second messenger signalling associated with capacitation and interaction with egg-derived factors. These approaches have been, and continue to be, of great value. However, by their nature, such studies provide data on the activity of the cell population and are therefore most useful for the study of processes that occur in a synchronized manner, with acceptably similar kinetics and amplitude, in the majority of cells. Though some aspects of sperm function are appropriate for such analysis, it is becoming increasingly apparent that this approach should be supplemented by examination of the activities of individual cells.

Single-cell imaging

It has been known for many years that human spermatozoa are a functionally heterogeneous population. Although single-cell characterization of physiology and function is still relatively unsophisticated, a recurring observation is that populations of spermatozoa from humans and other mammals are heterogeneous in their activity and responses to stimuli (e.g. Florman et al., 1989; Kirkman-Brown et al., 2000, Sutton and Roman, 2003). A technique that has been used extensively to investigate signalling events in individual somatic cells is imaging of cells loaded with fluorescent probes, which provides real-time information on changes in ion concentration and membrane potential. Though considerably more difficult to apply to spermatozoa than somatic cells, with suitable techniques for cell immobilization and an imaging system of sufficient resolution and sensitivity, useful single-cell data are obtained. In human spermatozoa, the best-characterized signalling response is the biphasic [Ca<sup>2+</sup>], response evoked by micromolar concentrations of progesterone (P4), which will be encountered by spermatozoa as they approach the cells of the cumulus surrounding the oocyte. The response to P4 is well characterized in suspended populations (Figure 1A; for review, see Baldi et al., 1998) and initial imaging studies showed that a P4-induced [Ca<sup>2+</sup>], signal occurs in the vast majority of cells and that a biphase response, similar to that seen in populations, can occur in individual cells (Kirkman-Brown et al., 2000). However, analysis of large numbers of single cell responses shows not only that there is variation between cells, but also that responses fall into a number of classes, reflecting which Ca<sup>2+</sup> influx pathways are present and/or available for activation
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(Figure 1B; Harper et al., 2003; Kirkman-Brown et al., 2003). Furthermore, many cells, after an initial response to P4, generate repeated, slow [Ca^{2+}]_i oscillations (a form of complex Ca^{2+}-signalling previously seen only in somatic cells and undetectable in population studies) (Fukami et al., 2003; Kirkman-Brown and Harper, unpublished observations), which continue for 10s of minutes and therefore may well be occurring as spermatozoa penetrate the ZP and enter the perivitelline space. In sea urchin spermatozoa, faster oscillations, generated in the sperm tail and probably related to control of motility, have recently been described (Wood et al., 2003). Again, these were not detectable in population measurements. The significance of these observations is yet to be determined, but what they clearly show is that population measurements, though valuable, give a simplified version of events. Spermatozoa are capable of more complex signalling than previously believed and populations of spermatozoa include cells that show a variety of responses to the same stimulus, reflecting heterogeneity of the cells themselves or possibly variation in capacitation-regulated processes.

Electrophysiological recording

Another technique that has been used extensively in studying signalling in somatic cells is electrophysiological recording (usually patch clamping), which can provide information on cell membrane potential and on activity and regulation of ion channels in the cell membrane. Since this technique is dependent upon direct placement of recording electrodes, it is particularly difficult to apply to spermatozoa, since they are both minute and motile. An alternative that has proved very useful has been the application of the patch clamp technique to immature male germ cells (spermatogenic cells) that are sufficiently large to be ‘patched’. Since spermatozoa are transcriptionally inactive, these cells may express the same channels as mature spermatozoa. These studies have provided valuable data on the expression and function of T-type voltage-operated Ca^{2+} channels (VOCC) and K^+ channels in mouse germ cells and have significantly advanced understanding of mouse fertilization (Arnoult et al., 1996; Lievano et al., 1996; Florman et al., 1998; Darszon et al., 1999). However, the available evidence suggests that many of the channels that are important in the functioning of mature spermatozoa are not detected by this method. For instance, functional expression of the recently discovered Catsper channel, which is reportedly involved in regulating mouse sperm motility, is seen only in mature spermatozoa (Ren et al., 2001). Other channels that are believed to function in mature mouse spermatozoa, such as high voltage activated VOCC types (Wennemuth et al., 2000), are not detected in patch clamped spermatogenic cells. Patch clamping of human spermatogenic cells was particularly disappointing.

Not only are fresh male germ cells extremely difficult to obtain, but expression of any channel activity was very rare (Jagannathan et al., 2002). These observations show that critical changes in channel expression occur as human (and probably mouse) germ cells develop into mature ejaculated spermatozoa. The alternative is to apply patch clamp to mature spermatozoa. This has been achieved on a number of occasions and recently, the application of sophisticated micropositioning techniques has greatly increased success rates. Using patch clamping, characterization of ion channel expression and regulation of membrane potential and channel activity during cell activity will be achieved in the next few years.

Approach B: use of proteomics to study spermatozoa and the zona pellucida

Proteomics, the study of proteins in a genome, is a key area of emerging research in the post-genomic era (Brewis, 1999; Tyers and Mann, 2003). Since proteins and protein–protein interactions are responsible for cellular function, it is critical that a comprehensive and systematic identification and quantification of proteins expressed in cells and tissues is undertaken to gain new insights into these processes. With the completion of the Human Genome Project, it is estimated that there are between 30,000 and 40,000 genes (Lander et al., 1998; Darszon et al., 1999). However, the available evidence suggests that many of the channels that are important in the functioning of mature spermatozoa are not detected by this method. For instance, functional expression of the recently discovered Catsper channel, which is reportedly involved in regulating mouse sperm motility, is seen only in mature spermatozoa (Ren et al., 2001). Other channels that are believed to function in mature mouse spermatozoa, such as high voltage activated VOCC types (Wennemuth et al., 2000), are not detected in patch clamped spermatogenic cells. Patch clamping of human spermatogenic cells was particularly disappointing.

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**Figure 1.** (A) typical fluorometric record of the population [Ca^{2+}]_i signal induced by progesterone (3 µM) in human spermatozoa labelled with fura 2. Progesterone was added at time 0 s. The response is biphasic, comprising an initial [Ca^{2+}]_i plateau followed by a sustained phase. (B) Four single cell records from a field of cells labelled with Oregon Green 488 BAPTA-1 and exposed to progesterone (3 µmol/l; arrow). Images are taken every 10 s (indicated by data points). Responses are slower than in (A) due to carrying out the experiment at a lower temperature. Though the biphasic nature of the population response is clearly present, transient responses vary between cells in kinetics and latency. Analysis shows that the transient incorporates two discrete components (indicated on the traces by markers a and b). Contributions of the two components to the [Ca^{2+}]_i transient vary greatly between cells (Kirkman-Brown et al., 2003).
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2001; Venter et al., 2001) and that there are around 10 times more proteins than genes, meaning that there are around 300,000 proteins in the human proteome (Harrison et al., 2002). Proteomics, therefore, represents a considerable challenge at present, as there are no methods for automated analysis to the level that was possible with the genome.

The main approach presently in proteomics is to resolve proteins using high-resolution two-dimensional (2D) electrophoresis. Following separation, proteins are detected (for example by silver staining or immunoblotting) and gel spots corresponding to distinct proteins are cut out from the gel and digested with trypsin. The resulting peptides are subjected to mass spectrometry and the results are compared with current databases to enable definitive protein identification (Brewis, 1999; Aebersold and Mann, 2003). There are a number of types of mass spectrometry approaches available. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) is used to produce peptide ‘fingerprints’, while quadrupole-time of flight (Q-TOF) associated with tandem mass spectrometry (MS/MS) allows for amino acid microsequencing of peptides to be obtained from trypsin digestion (Celis et al., 1998; Aebersold and Mann, 2003). As well as facilitating protein identification by mass spectrometry, 2D electrophoresis provides basic information such as the molecular size and isoelectric point of a protein as well as relative abundance. In addition, comparison of two or more samples prepared from different physiological conditions (or pathological versus normal) can provide profile signatures.

One of the aims of applying proteomics to study reproductive biology has been to discover possible target proteins for the development of contraceptive vaccines. These approaches have concentrated on isolating proteins that are abundant, expressed at the cell surface, immunogenic, testis- or ovary-specific and conserved between species (Naaby-Hansen et al., 1997; Shetty et al., 2001; Coonrod et al., 2002; Shibahara et al., 2002). This technology has also been used to probe signal transduction pathways (Sato et al., 2002) such as target proteins that are tyrosine phosphorylated during human sperm capacitation (Ficarro et al., 2003). Moreover, proteomics has also been used for mapping and/or characterising proteins putatively involved in post-testicular sperm maturation (Syntin et al., 1996; Fouchécourt et al., 2000; Starita-Geribaldi et al., 2001).

**Proteomic profiling of human spermatozoa**

Based on the current literature it is impossible to predict how many proteins constitute the sperm proteome. Studies by Naaby-Hansen et al. (1997) have shown there to be at least 1400 distinct protein moieties of which approximately 100 are certainly localized to the cell surface (plasma membrane). However, both of these figures are likely to be underestimates. Proteins on the sperm surface are critical for cellular function and are responsible for cell regulation during sperm capacitation in the female reproductive tract, binding to the ZP and induction of the signalling cascades that trigger the sperm acrosome reaction (Evans and Florman, 2002). Although many surface proteins have been proposed to be involved in these events, there is little consensus.

A long-term goal in relation to sperm function should therefore be to map all of the proteins present in the sperm plasma membrane proteome. Such a resource will represent an invaluable tool to researchers in the future, and facilitate studies on already known and novel sperm surface proteins to

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**Figure 2.** Fluorescent labelling pattern of human spermatozoa. Intact (A, B) or nitrogen cavitated (C, D) human spermatozoa were incubated with wheat germ agglutinin conjugated with fluorescein as a marker for the plasma membrane. Fluorescence (A and C) and phase-contrast (B and D) images of a ×1000 magnification.
further understand these key processes. One of the current approaches is to use proteomic strategies similar to those previously described by Flesch et al. (1998), using nitrogen cavitation and ultracentrifugation in order to isolate a sperm plasma membrane-enriched fraction. This technique induces the loss of the plasma membrane in approximately 70% of the cells as assessed using lectins, which target different sperm membranes (Figure 2). This may allow specific investigation of potential changes occurring within the plasma membrane during capacitation, as well as identification of key proteins involved in sperm-egg binding/fusion.

A number of clinical studies have also been carried out. The objective is to use proteomics to determine if there are any significant differences in protein expression (for example increased/decreased and/or absence) between controls (normozoospermic fertile men) and selected men with defined infertility (fertilization failure at IVF or globozoospermia). Evidence from a series of studies on knockout mice show that a absence of a key protein leads to defects in pre-fertilization events. For example–calmegin (Ikawa et al., 1997), phospholipase Cδ4 (Fukami et al., 2001) and casein kinase II α (Xu et al., 1999) all showed impaired fertility. However, there are a plethora of proteins that can affect sperm function. In addition, there is no reason to believe that men with similar pathology have the same defect(s). Therefore, in order to determine which proteins are differentially expressed in men with dysfunctional spermatozoa, a non-bias objective approach is required. Analysis of the sperm proteome enables this approach, where more than 1000 proteins maybe analysed at the same time, some of which may be novel.

Critical to the detection of real differences in protein expression is reliability of the technique and establishing the range of normal variation. To date, sample preparation (sperm preparation from semen and protein solubilization) and the reproducibility of 2D electrophoresis have been optimized so that a high level of accuracy can now be achieved within duplicate samples from the same ejaculate. The human sperm proteome is now being categorized in normozoospermic (fertile) donors in detail, and in particular, intra- and inter-donor variability is being addressed.

In initial experiments, several differences were found between the sperm proteome of one patient with failed fertilization at IVF compared with controls. In contrast, the sperm proteome of men with globozoospermia shows approximately 60 2D gel spots missing compared with controls (Figure 3). Of the proteins that have been characterized, many are associated with the acrosome such as proacrosin-binding protein. This is not surprising, as these patients have complete globozoospermia with no acrosome visible at the electron microscopic level in any of the spermatozoa. The absence of an acrosomal vesicle suggests a defect during spermiogenesis, and hence there is interest in gaining further insights into this process. In addition, it should be noted that men with globozoospermia require ICSI to achieve conception, as the spermatozoa are incapable of binding and penetrating the ZP (Aitken et al., 1990). Hence, such samples may be informative about proteins involved in sperm–zona binding/penetration.

**Proteomic profiling of human zona pellucida**

Studies are also being directed towards the composition and molecular characteristics of the human ZP, which is an extracellular matrix composed of a small number of related proteins that surrounds all mammalian oocytes and is responsible for species specificity, prevention of polyspermia and induction of the acrosome reaction. From studies in the mouse, it is proposed that the ZP consists of three glycoproteins, ZP1, ZP2 and ZP3 (also known as ZPB, ZPA and ZPC, respectively) conserved in several mammals (Carino et al., 2002). Using a bioinformatic approach, a fourth human ZP gene (ZP1) has been demonstrated, showing that the ZP1 and ZPB genes are not orthologs but paralogs (Hughes and Barratt, 1999). Consequently, the revised gene nomenclature for the human is now ZP1, ZP2, ZP3 and ZPB (also known as ZP4). The separate identification of the ZP1 and ZPB genes in the human is consistent with the finding of distinct ZP1 and ZPB genes in both the chicken and rat (see Table 1), and with recent molecular phylogenetic analysis of vertebrate ZP genes (Spargo and Hope, 2003; Conner and Hughes, 2003). Thus at the genomic level, there is evidence that the composition of the ZP is more complex than previously realized, and the implications of this for the ZP function need to be fully investigated. Current experiments are aimed at determining if

![Figure 3. Silver stained 2D PAGE of spermatozoa from control donor (A) and globozoospermic patient (B). Highlights represent proteins present within the normal sperm proteome that are missing from the globozoospermic sample.](image)
the human ZP contains all four proteins and to examine their respective function(s). Analysis of the control region of ZP1 showed it possesses all the machinery necessary for expression. Thus there is no reason to believe that the human ZP does not contain four proteins and if correct, this will challenge both the widely held three protein model for the structure of the ZP, and the nature of the interaction of spermatozoa with the ZP, both of which are based on work in the mouse. Furthermore, the currently accepted model that ZP3 is the primary sperm receptor comes from experiments employing mouse ZP-derived protein fractions that had been separated electrophoretically (Bleil and Wassarman, 1980). This is now known to be insufficient to separate ZPB and ZP3 in the pig (Yurewicz et al., 1987). Consistent with the possibility that sperm-binding activity in ‘ZP fractions’ may reflect, at least in part, contamination with ZPB, there is evidence from a variety of species that ZPB, as well as ZP3, has sperm-binding activity (Topper et al., 1997; Yurewicz et al., 1998; Govind et al., 2000). Therefore, the possibility that ZPB or a ZP3-ZPB complex is responsible for sperm–zona binding in the human should be considered. The current focus on ZP3 as the sole zona-derived sperm ligand may underlie the difficulties than have been encountered in identifying and characterizing such proteins to date.

Since the availability of human ZP is limited for research purposes, it is crucial to perform preliminary experiments using other species. Isolated porcine ZP has been used to identify the different glycoproteins present. Each ZP protein exhibits considerable heterogeneity and consist of several differently glycosylated isomers, i.e. a structure characteristic of glycoproteins, distinctly visible after 2D electrophoresis, which produces protein trains as observed in Figure 4 (see also Hedrick and Wardrip, 1987). Such heterogeneity in a ZP population has been described in other species: mouse (Bleil and Wassarman, 1980), cow (Florman and First, 1988) and human (Bercegeay et al., 1995). At present, 2D electrophoresis coupled with MS/MS is an indispensable method to visualize and identify proteins. However, there are limitations to this approach, such as suboptimal protein solubilization, particularly for membrane proteins, and for highly acidic or alkaline proteins, as well as the need for a large amount of starting material, since as in some cases less than 1% of proteins are recovered from gel spots. Experience suggests that identification by MS/MS of porcine ZP proteins needs approximately 10,000 ZP (Figure 4). This is not a feasible approach for use in humans.

An alternative technique for protein identification is direct MS/MS analysis, a non-2D electrophoresis approach. This allows direct identification of individual proteins from complex mixtures. This technological advance in performing proteomic analysis is currently gaining favour over gel-based approaches (Peng and Gygi, 2001). The advantages of the direct MS/MS is that it is more sensitive, since it needs less starting material and it eliminates sample solubilization issues associated with electrophoretic methods as well as loss of material from the gel spots during trypsin digestion procedures. Figure 5 highlights the peptides obtained using only 10 isolated porcine ZP which was enough to identify all three known pig ZP proteins (ZP2, ZPB and ZP3). Direct MS/MS is now being used to determine if four proteins are present in the human ZP. A proteomic approach to study the human ZP represents a definitive analysis relying on protein sequencing compared with previous analysis using antibodies. Once the function of the four proteins can be determined, the molecular nature of sperm–zona interaction(s) can be investigated in detail.

Table 1. The presence of the four ZP genes in different vertebrates. Table based on sequence information available as of April 2003.

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<th>ZP2</th>
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<td>Human</td>
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Figure 4. Silver stained 2D PAGE of approximately 10,000 isolated porcine ZP. MS/MS analysis revealed that the protein trains 1 and 2 corresponded to ZP2, while the protein train 3 consisted of ZP3 and ZPB (a) and ZPB only (b).
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Figure 5. Pig ZP2 (P42099), ZPB (S35712) and ZP3 (S70433) amino acid sequences. The bold underlined sequences represent the tryptic peptides obtained from the direct MS/MS analysis.

ZP2
1 MACRHRGDSGRPLSWLSASWSRLLLFFELTVTQSNIGVQQLVNTAFPGIV
51 TCHNPMVVEFPRILGTMQYRSTSVVDPLGLEMNMTYTVLELPNLTVKAPY
101 EACTKVRGHEQMTPLILDQNAALRQALMYHICSPVMGEGPDHGSGT
151 ICMDKEMTSMFTMNFPCCMADEFVKREDKQGRGWSSLWVGQLMERARTLTQFP
201 AMTOSQNYFLINQQMNQVSQHATVGRSYQGNSHYLVMVNLKHKVHSGQ
251 SLILASQLICAVDPVTCNATHTTVLAIPEFGKGLKVNLGSQGNAVSLKHK
301 HGIEMEFTNGLLLPHTNQLTKTNVSEKCLPQHLYLSSLKTLTFHSLERAVS
351 MVIVFECLEELSVSLVSEELCTQDGMPDVKVHSQHTKPAKLNLTLRVNGDS
401 SCQPTFKAPAQLVQFRIPNLNCGCRTHKFNDKVIYNEIHALWADFPSA
451 VSRRBEFNNTRRCSYSNMLNINTNVESLPSPEASVKGPGPILTLTQTYPD
501 NAYLQPYGDKYFVVKYLRQPIYLEVRILMNTDPMNCLVLVDDCWATESTRD
551 PASLPQWNVMDGCEYNLDNHTTFHGPVSSTYTPNHQDVRKTFAFVS
601 GAQVSQQLVYFHCSVIFCQNLSPFTSLCSVFTCHGFSRRRATGTTEEKEM
651 IVSLFGFILLSDLSSLRDAVSNKSRTNGVYAFKTMVMVASAGIVATL
701 GLISYLHKKRLNMLNH

ZPB
1 MWLRSIPWLCFLPLCALPQSGPKAADDLQGLYCGPSSFHFSSBLSQDT
51 ATTFPAVLWDRRRILKQIDSGCGTWYKQGFGSSMGVEA SYRGGYWTED
101 DSHYLMPIGELQEDADGGHRVTETKLFKCPVDLALDVPTIGLICDAPFPW
151 DRLPCEAPPIATTGECQGQGCYNEEVPSCPYYGNVTNVRCTQDGHFISAV
201 SRNVTSPPLLWDWHLAFRNSESECKFVMETHFVLFROFSSCGTAKVRT
251 GNQAVYENELVAARVBTWRSHGISTRDSIFRLRVSCIYVSSSLAPVNIQ
301 VFTLPPPLEEFTHGQLTLLEQAIAKEDYGSYSNASDPVFVLKLEPMIVYE
351 VSIRHRDFTLGLLHLLCQWATPGMSPLQQWFMPLVNGCYPHTGYNQTC
401 IPVQKASNNLPPSHYQRFSVSTFSFVDSVQKQALKGVPYHCMTAVCPCA
451 GAPIVCVTCCAAARRRRSDHIHFQNGTGISKGMILQAQTRDSSERLHK
501 YSRPVPADSHALVWAGLQLSILIGALLVSYLFVRFKWR

ZP3
1 MAPSWRFVFCFLLWGGTLECSPQPVWQDEGQRLRPSKPTQVMVECOAQQL
51 VVIVSVDLFLGTQKLRPAPQSGPKACFLVQSDAQAVFFEVGLHCQG
101 LOVTVDVALYTFTELHRDPRPGNLSSLRTNRAEVPIECHQPRQVNVSWMA
151 IILPTTVFWERTGTGSEEEKLVFLRRLMEENWASEMPTFQGDRAHLAOQY
201 HTGSHVPLRLRVDHCVATTLPDWNTSPSHTIDVFSGLCLVDGLTEASSPAF
251 AGRPPGETLPQFTVNDFHSANDNSRTVICYTOLHLKTVTADFVHQDNKNCASF
301 SXSNNRWSVPEGPAVICCRCQHKGCGTPSLSRKLMPKQEAPRSHRHTV
351 DEAODTVGGFPFLGKTSRHVQEGVSTSSPTSVMVGLALTAVTVTLATLVL
401 GVARRRRRAALHLVCQFSAQ
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