c-mer than the two c-mer homologs would show to each other along their entire lengths on the basis of the molecular clock hypothesis. On the other hand, if Hu9 was derived by a recent duplication event along the human evolutionary lineage, we would expect to see greater similarity between its sequence and that of human c-mer in regions of homology, relative to the similarity between human and mouse c-mer in these same regions. (Regions of nonhomology between Hu9 and human c-mer could be accounted for in this scenario by exon shuffling or similar mechanisms occurring during Hu9 evolution.)

In three of the four similar regions, Hu9 and human c-mer are much more similar to each other than either is to mouse c-mer; in the fourth region, there is a three-way tie (Fig. 1). Overall, Hu9 and human c-mer polypeptide sequences show 97% identity, while the corresponding human and mouse c-mer polypeptide regions show only 87% identity. To evaluate the significance of this difference in identity levels, we performed a one-way analysis of variance on the raw data (setting all amino acid identities equal to 1, and all amino acid differences equal to 0). The value of P obtained is 0.000012, indicating high statistical significance.

Our conclusion from this evolutionary analysis is that if Hu9 is a separate gene, it cannot be present in all mammals. Furthermore, as it would have emerged after the divergence of humans and mice, there cannot be a mouse homolog of this putative gene. Thus, even if there is a human Hu9 product involved in human sperm-egg binding, a homologous product cannot exist in the mouse, and the 95-kD mouse protein previously described by Leyton and Saling with egg binding properties (4) cannot be homologous to the putative Hu9 product. Other investigators have presented evidence that a prominent mouse 95-kD phosphotyrosine-containing sperm protein is a unique form of hexokinase (5). However, if another mouse 95-kD phosphotyrosine-containing sperm protein exists with homology to the human 95-kD sperm protein, our analysis would suggest that these proteins are likely to be products of the c-mer gene homologs in each species; such homologs are expressed broadly among somatic tissues in addition to the testes and ovary. Thus, even if the c-mer products are involved in sperm-egg binding, they do not provide suitable targets for contraceptive development as suggested by Burks et al. (1).

**REFERENCES**


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Response: We thank Bork and Tsai and Silver for their thorough analysis of our work.

1) Relationship between Hu9 and c-mer: At the time of submission of our manuscript, the database entry most similar to hu9 was the mouse sequence c-eyk, as presented in figure 2 of our report (1). However, the putative human cellular homologue of v-ryk has been cloned recently (1) and designated c-mer. The overall similarity between c-Mer and Hu9 protein sequences is 61%; the majority of this similarity is found in the intracellular domain, as the extracellular domains present 46% identity. Overall, c-Mer and c-Eyk are 74% similar. On the basis of degree of similarity to both c-mer and c-eyk, hu9 may represent a new member of the axl-like kinase family, as suggested in our report (1).

2) Hu9 as a full-length clone: Bork does not account for salient features of the ha9 cDNA sequence and manually frameshift the sequence of hu9. With the published hu9 sequence, there are a variety of reasons to suspect that it is a full-length cDNA and is not the result of a cloning artifact. First, as stated in our report (1), the cDNA sequence contains three stop codons upstream of the putative initiating methionine. On the basis of the presence of these upstream elements and a reasonable Kozak consensus sequence, the Met at nucleotide position 70 of the hu9 cDNA was assigned as start codon. Moreover, when the hu9 nucleotide sequence is translated, the open reading frame which begins with a Met and encodes amino acid sequences similar to c-mer or other PTKs is the one we reported putatively initiated by the ATG described at nucleotide position 70. Second, the presence of a stop codon and polyadenylation signal at the 3' end of the ha9 cDNA suggests that this transcript is complete. These features of the hu9 cDNA strongly argue that the clone we have identified is complete. Third, the entire hu9 cDNA was sequenced in both directions, a strategy that is likely to exclude the many sequencing errors implied by the alignments created by Bork. Finally, Northern analysis of hu9 expression, presented in figure 3 of our report, detected a 2.2-kb transcript in human tissues, which suggests that Hu9 is a full-length clone.

3) Northern analysis: Tsai and Silver suggest that our Northern analysis is flawed, possibly due to degraded RNA. Ethidium bromide staining of RNA samples used in our Northern analysis verified intact ribosomal RNA and equal loading of samples. As Tsai and Silver indicate, regions of high similarity exist between Hu9 and c-mer. However, these regions constitute considerably less than half of the full Hu9 sequence, which may explain our failure to detect the c-Mer transcript using full-length hu9 as the probe. The exposure reproduced in our report (1) shows the 2.2-kb transcription (in the testis lane) and a larger band at about 4.5 kb that is considerably fainter. Close examination of a much longer exposure reveals that two additional bands are also detected in the testis lane, at about 3.0 and 4.5 kb (data not shown). Whether these are actual bands or background due to overexposure is under investigation, but it is possible that ha9 hybridizes weakly with a c-mer transcript in the testis sample.

Bork discusses possible generation of hu9 via alternative splicing; alternatively spliced products have been described for both c-mer (2) and c-Eyk (3). One isoform of C-Mer reportedly contains an insertion with an in-frame stop codon, possibly yielding a truncated, secreted form of c-mer.

4) Molecular weight of hu9-encoded product: The primary structure of ha9 predicts a product of about 70 kD, whereas a product of about 110 kD is predicted for c-mer. The c-mer sequence predicts the structure of a transmembrane tyrosine kinase with a large extracellular domain, which is likely to be modified by glycosylation. The latter is also true for Hu9, which contains four sites for potential N-linked glycosylation and numerous sites for potential O-linked glycosylation. For both proteins, modification of this type can be expected to increase their apparent molecular weight. In the case of several known proteins, the size increase due to secondary modification (glycosylation or phosphorylation) is substantial, as in the case of the insulin and IGF-1 receptors. Both of these receptors are generated from precursors. Analogous to the insulin receptor, the IGF-1 precursor generates α (80,423) and β (70,866) subunits, which correspond to the 135,000 (α) and 90,000 (β) fully glycosylated receptor subunits (4). Thus, both the Hu9 and the C-Mer proteins would be expected to migrate on SDS gels slower than predicted by their primary structure. The protein that we reported as encoded by ha9 migrates on SDS gels as a 95-kD protein is fully consistent with this pattern, and detectably smaller than the minimum size predicted for C-Mer.

5) Structural considerations: Although not a typical feature of signal peptides, the
unusually hydrophilic putative signal peptide encoded by hu9 is shared by c-mer and ax, other members of this new family encoding PTKs (2, 3). Intracellular and extracellular structural differences exist between c-mer and hu9. For example, the extracellular region of both c-Mer and Axl possess two Ig domains and two fibronectin type II (FNII) domains; hu9 does not fully encode either of these motifs. Bork suggests that if Ig domains were present in Hu9, these could be responsible for interacting with the conserved "ZP domain" found in many zona pellucida proteins, which he has described previously (5). This predicted Ig-ZP interaction is difficult to reconcile with the long-standing observations that ZP2 and ZP3 play different roles in gamete interaction (6) yet both contain "ZP" motifs. A prediction that fits the accumulated data more closely is that a sperm protein involved in primary zona binding or signal transduction, or both, would interact with regions unique to ZP3, and not be represented in ZP2, as ZP3 is the egg matrix component responsible for primary binding and sperm activation leading to acrosomal exocytosis (6). Furthermore, as we demonstrate through binding inhibition experiments in figure 5 of our report (1), peptides contained in the extracellular region of Hu9 are apparently important in sperm-egg recognition, suggesting a unique function for the extracellular domain of this molecule. Although peptide 1 (residues 57 to 71) is also present in c-Mer, peptide 3 (residues 94 to 105), which was the most effective peptide and inhibited sperm-egg binding by 80%, appears to be unique to Hu9 and is not found in any recognized motif.

6) Specificity of the K16 antibody. Although we did not know of the existence of c-Mer at the time we prepared the K16 antibody, as a matter of interest and standard procedure we characterized the antibody's reactivity toward other tissues. Tissue extracts were prepared from human liver, spleen, and kidney, and analyzed by immunoblot alongside human sperm samples. We found that K16 reacted with a 95-kD protein in the sperm samples alone, and no protein in the 70- to 150-kD range was recognized in these somatic tissues. Respectively, these were useful tissues to assess, as the c-mer transcript has been shown to be expressed in all of them.

7) Evidence that hu9 encodes a receptor on sperm: We provided the following evidence that hu9 may encode a sperm receptor molecule: (i) hu9 is expressed in spermatogenic cells. (ii) K16 recognizes a 95-kD protein in human sperm which is also detected by antip8 and monoclonal antibody 97.25. (iii) Two peptides present in the predicted Hu9 extracellular domain inhibit sperm-zona binding. One of these peptides (residues 57 to 71) is common to c-Mer while the other, peptide 3 (amino acids 94 to 105), is unique to Hu9. This unique Hu9 peptide inhibits sperm-zp binding by 80%, suggesting that this portion of Hu9, or ZRK, may be involved in sperm-egg recognition. (iv) K16 immunoprecipitates contain kinase activity that is stimulated by recombinant human ZP3.

8) Evolutionary analysis: We have performed neither detailed Southern (DNA) blot analysis nor chromosomal mapping to probe the evolutionary relationship of hu9 and c-mer. However, the general rules for the evolutionary relationship of proteins involved in sex are different for those for non-sex-related proteins (7–9). In species as far ranging as Chamrydamonas, abalone and mice, key proteins involved in sex determination or gamete interaction are encoded by genes that display increased mutational activity, little-to-no codon bias, remarkably divergent genomic organization, and related somatic cell genes. Only a few representative proteins have been identified and analyzed so far, but the evidence to date suggests that different rules apply for the evolution of genes important in speciation and sex. We do not yet know whether any of these altered gene characteristics apply to hu9, but its encoded protein fits well within the criteria for a protein involved in gamete interaction. Thus, before fundamental information concerning the genes that encode Hu9 and c-Mer is determined, it appears premature to attempt to categorize their relationship.

Our finding that the hu9-encoded protein is a member of a conserved protein family is not unanticipated. Among other examples which parallel our results with hu9 are fertilin as an ADAM protein (5) and PH-20 as a hyaluronidase (6).

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