weeks. The images were photographed and digitized. The hybridization signal of the radiolabeled probe appears as white grains. All specimens were observed under dark field illumination after nuclear counterstain with hematoxylin. Immunostaining for factor VIII–related antigen (*4*) confirmed that injury was limited to endothelium.

23. Binding was carried out in buffer containing 50 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 2.5 mM Hepes (pH 7.9), 1 μg of polydeoxyinosinic-deoxycytidylic acid, and 20 μg of BSA for 30 min at 22°C. Polyclonal antipeptide antibodies to Sp1 and Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with nuclear extracts 15 min before the addition of the probe.
24. d77mEgr-CAT was constructed with the use of an oligonucleotide bearing the Oligo Bm (*12*) sequence as the 5′ primer for the polymerase chain reaction. Transfections in BAECs were performed with 10 μg of reporter plasmid and the calcium phosphate protocol (*10*). The cells were incubated with PMA (100 ng/ml), cotransfected with CMV–Egr-1, or injured with a sterile comb (*8*), and then incubated for 36 hours at 37°C. CAT activity was assessed by the two-phase fluor diffusion technique (*13*) and was normalized to the amounts of protein in the cell lysate.
25. Recombinant Egr-1 was incubated with ^{32}P–Oligo B (*12*) for 30 min at 22°C and applied to a running nondenaturing 5% polyacrylamide gel at the times indicated. Alternatively, a 1000-fold molar excess of the unlabeled cognate was added after the 30-min incubation and applied to the gel (*21*).
26. Increasing amounts of recombinant Egr-1 were applied to a solution in which Sp1 was preincubated with ^{32}P–Oligo B (*12*) for 30 min at 22°C (Fig. 3B, left side). Alternatively, ^{32}P–Oligo B was incubated with a fixed concentration of Sp1 and decreasing amounts of Egr-1 (Fig. 3B, right side) (*21*).
27. Protein immunoblots were analyzed with polyclonal antibodies to Egr-1 and Sp1 (1:2500; Santa Cruz Biotechnology) and to PDGF-B (1:200; Genzyme). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham) with horseradish peroxidase–linked donkey secondary antiserum to rabbit immunoglobulin at 1:10,000 dilution.
28. J. T. Kadonaga, K. A. Jones, R. Tjian, *Trends Biochem. Sci.* **11**, 755 (1986); K. A. Jones, J. T. Kadonaga, P. A. Luciw, R. Tjian, *Science* **232**, 755 (1986).
29. B. Christy and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8737 (1989).
30. We thank F. J. Rauscher III for recombinant Egr-1, V. P. Sukhatme for CMV–Egr-1, M. A. Frosch for critical review of the manuscript, and M. A. Gimbrone Jr. for enthusiastic support. Supported in part by grants from NIH (T.C.) and the American Heart Association (V.L.). L.M.K. is a C. J. Martin Postdoctoral Research Fellow (National Health and Medical Research Council of Australia) and a recipient of a J. William Fulbright Postdoctoral Research Award. T.C. is an Established Investigator of the American Heart Association.

6 November 1995; accepted 7 February 1996

TECHNICAL COMMENTS

Sperm-Egg Binding Protein or Proto-Oncogene?

Recently, D. J. Burks *et al.* (*1*) identified and characterized a human sperm receptor protein tyrosine kinase (RPTK), called Hu9, as a receptor for ZP3, a glycoprotein of the egg-surrounding matrix, zona pelucida. The finding is a step forward in our understanding of the initiation of the acrosome reaction. However, the sequence presented bears some features that are problematic:

1) The RPTK Hu9 is virtually identical over large parts with the human putative proto-oncogene c-*mer* (*2*); in these regions, Hu9 is more similar to human c-*mer* than is the mouse orthologue of c-*mer* (*3*) (Fig. 1). c-*mer* is a member of the growing *axl* subfamily of RPTK genes that is characterized by high similarity and conservation of special features among the tyrosine kinase domains and by the presence of two immunoglobulin (Ig) and two fibronectin type III (FNIII) domains in its extracellular parts (*3*) (features that are missing in Hu9). This family includes c-*eyk* (4), which has [as reported by Burks *et al.* (*1*)] 55% identity to Hu9 in the catalytic domain.

2) Although Burks *et al.* (*1*) did not find any similarity to other proteins in the extracellular part of Hu9, the first 70 amino acids are almost identical with an extracellular region of human c-*mer* and still 48% identical to the corresponding region in c-*eyk* (Fig. 1A).

3) The proposed signal peptide of Hu9 is unusual as it is almost entirely composed of hydrophilic rather than hydrophobic residues. This putative signal peptide is identical with a region within the second FNIII domain in the much larger extracellular part of c-*mer* and similar to other *axl*-like RPTKs. FNIII modules are globular domains common to many extracellular proteins (*5*); it is extremely unlikely that a part of a globular domain with a known β-sandwich fold is able to function as a membrane-spanning helical segment. The presence of a signal sequence upstream from the stated (*1*) NH_2-terminus is also supported by 73 bases of the *hu*9 complementary DNA (cDNA) (GenBank database accession no. L08961) upstream of the predicted protein that are identical to human c-*mer*.

4) Two regions of Hu9 seem to be frameshifted as compared with c-*mer* and the other *axl*-like RPTKs; one is located proximal to the transmembrane region and the other within the kinase domain (Fig. 1A). In both cases, the cDNA is 100% identical to human c-*mer*, but the reported (*1*) amino acid sequence of Hu9 is totally different.

5) Hu9 contains two inserts compared to the *axl*-like RPTKs. The insert in the extracellular part contains a segment of 22 residues that is identical (except for two small gaps) to a part of an Ig-like domain in rat PDGF receptor β (Fig. 1B). The two frameshifted regions are located at the end of these inserts. In the case of alternative splicing this could be explained by difficulties in finding the exact termination of the introns.

How can one make sense out of these observations? I offer two extreme interpretations:

1) Hu9 could contain an extremely high sequencing error rate and could be identical to with human c-*mer*: (i) the cDNA is far from being complete; it can be extended in both directions, by about 400 amino acids upstream from the NH_2-terminus as well as by 30 amino acids or so downstream from the COOH-terminus. (ii) As the Hu9 RNA was apparently extracted from a testis library that includes cell nucleii (*1*), the *hu*9 cDNA could correspond with a nuclear pre-mRNA transcript or a defectively spliced transcript, implying that the two inserts would be spliced out of the mature RNA. (iii) The putative (double) frameshifted regions could be corrected by insertion or deletion of four bases in the appropriate positions (Fig. 1) leading to 100% c-*mer* identity. (iv) Numerous small errors such as multiple omission of bases could be corrected. Finally, a protein with an estimated mass of about 95 kD could result, very similar in size to a ZP3 receptor characterised earlier in mouse and human by the same group (*6*). The interaction of the Ig domains with ZP3 would make sense, as ZP3 also contains a module (ZP) that is common to other zona pelucida proteins (*7*). Thus, a fine-tuned network of Ig-ZP interactions could initiate the signaling processes required for the complex acrosome reaction.

2) Although unlikely, one could argue that the sequence presented shows very recent evolution at work. (i) Hu9 would then be one of the first traceable cases where evolution incorporates frameshifts to create variety in proteins. (ii) The inserts in Hu9 compared to c-Mer and other *axl*-like RPTKs could be natural and would be explained by alternative splicing or exon shuffling. The high similarity to a part of a globular Ig-like domain in PDGF receptor β supports the latter; reports of an alternative splicing site in human c-*mer* proximal of the membrane (*2*) point to the former. (iii) Alternative splicing might also lead to a truncated c-*mer*–like receptor that misses a large fraction of the NH_2-terminus and does not contain a signal sequence (that is, a nearly full-length cDNA has been presented). (iv) As Hu9 is closer to human c-*mer* in the regions of similarity than the putative mouse c-*mer* ortholog, one conclusion would be that divergence of Hu9 occurred after the descent of humans and rodents. This would, however, exclude a mouse or-

A

```
c-mer diff.                              #               #   #                #    #   #   #    # # #  #           #    #    #
chicken c-eyk      363  WITASTTEGAPTTQPLNVTVSLNESSSFLEIRWVKPPLERTHGELQGYHIWHTWQDSKGLQNISLEAQPNATVAILPVVATNATCSVRVAAVTKGGVGPFSSPV
mouse   c-mer      368  WILASTTEGAPSVAPLNITVFLNESNNILDIRWTKPPIKRQDGELVGYRISHVWESAGTYKELSEEVSQNGSWAQIPVQIHNATCTVRIAAITKGGIGPFSEPV
human   c-mer      373  WILASTTEGAPSVAPLNVTVFLNESSDNVDIRWMKPPTKQQDGELVGYRISHVWQSAGISKELLEEVGQNGSRARISVQVHNATCTVRIAAVTRGGVGPFSDPV
human hu9            1  ---------------------------------MKPITKQQ-GELVGSRISHVWQSAGISKELLEEVGQNGSRARISVQVHNATCTVRIAAVTKGGVGPFSDPV
alternative        -24  WILASTTEGAPSVAPLNVTVFLNE                                                                     *
differences                                     ********* * *    *     *
domains              ...-Fn3(1)--><--------------------------------------------Fn3(2)-------------------------------------------

c-mer diff.                 #    #              # ###  #             #    # #        #  #    #     #              #
chicken c-eyk      ....EVFVPAS   GLITSSPSSTPASGNTDSFIVALGFVCGTVAVGLILCLSVVIQKRCMETKYGNAFSRNDSELVVNYTAKKSYCRRAVELTLGSLGVSSELQQKLQ
mouse   c-mer      ....NIIIPEH   SKVDYAPSSTPAPGNTDSMFIILGCFCGFILIGLILCISLALRRRVQETKFGGAFSEEDSQLVVNYRAKKSFCRRAIELTLQSLGVSEELQNKLE
human   c-mer      ....KIFIPAH   GWVDYAPSSTPAPGNADPVLIIFGCFCGFILIGLILYISLAIRKRVQETKFGNAFTEEDSELVVNYIAKKSFCRRAIELTLHSLGVSEELQNKLE
human hu9          (63)gnvtpct   rlgrlcplfnsgawqrrscahhlwlllwIILIGLVLYISLAIRKRVQETKFGNAFTEEDSELVVNYIAKKSFCRRAIELT-HSLGVSEELQNKLE
alternative            GTSHPAHD*AGSVRYL---TQAPGNADPVLIIFGCFCG
differences            ****   *** * * **** *                !      *                                             *
domains                ---->                       <-----transmembrane----->

c-mer diff.                  #   #
chicken c-eyk       DVVIDRNALSLGKVLGEGEFGSVMEG--RLSQPEGTPQKVAVKTMKLDNFSHREIEEFLSEAACMKDFDHPNVIKLLGVCIELSSQQIPKPMVVLPFMKYGDLHSFLL
mouse   c-mer       DVVIDRNLLVLGKVLGEGEFGSVMEG--NLKQEDGTSQKVAVKTMKLDNFSQREIEEFLSEAACMKDFNHPNVIRLLGVCIELSSQGIPKPMVILPFMKYGDLHTFLL
human   c-mer       DVVIDRNLLILGKILGEGEFGSVMEG--NLKQEDGTSLKVAVKTMKLDNSSHREIEEFLSEAACMKDFSHPNVIRLLGVCIEMSSQGIPKPMVILPFMKYGDLHTYLL
human hu9           DVVIDRNLLILGKILGEGEKGTVYEGLWNIPEGKEVKIPVAIKTLKLDTMANKEI---LDEASVMKGFGNPHVVRLLGICMTSTIYVITE-YCLLVYRRNKDKAEQHR
differences                            * * *  ** **********  *  *   *****  *** *  **  * ** * *    * ******* ****** ****** ******
domains             <-----------------------------------------------------------------------------------------------------------

c-mer diff.                    #        #   #
chicken c-eyk       RSRLEMAPQFVPLQMLLKFMVDIALGMEYLSSRQFLHRDLAARNCMLRDDMTVCVADFGLSKKIYSGDYYRQGRIAKMPVKWIAIESLADRVYTTKSDVWAFGVTMWE
mouse   c-mer       YSRLNTGPKYIHLQTLLKFMMDIAQGMEYLSNRNFLHRDLAARNCMLRDDMTVCVADFGLSKKIYSGDYYRQGRIAKMPVKWIAIESLADRVYTSKSDVWAFGVTMWE
human   c-mer       YSRLETGPKHIPLQTLLKFMVDIALGMEYLSNRNFLHRDLAARNCMLRDDMTVCVADFGLSKKIYSGDYYRQGRIAKMPVKWIAIESLADRVYTSKSDVWAFGVTMWE
human hu9           SNCAELNP---PLQTLLKFMVDIALGMEYLSNRNFLHRDLAARNCMLRDDMTVCVADFGLSKKIYSGDYYRQGRIAKMPVKWIAIESLADRVYT-KSDVWAFGVTMWE
differences         **** ** ***                                                                                   *
domains             -----------------------------------------tyrosine kinase domain---------------------------------------------

c-mer diff.                                                     #       #                  #     #######  #           #  #
chicken c-eyk       IAT--RGMTPYPGVQNHEIYEYLFHGQRLKKPENC....LDELYDIMSSCWRAEPADRPTFSQLKVHLEKLLESLPAPRGSKDVIYVNTSLPEESPDSTQDLG...
mouse   c-mer       ITT--RGMTPYPGVQNHEMYDYLLHGHRLKQPEDC....LDELYDIMYSCWSADPLDRPTFSVLRLQLEKLSESLPDAQDKESIIYINTQLLE----SCEGIA...
human   c-mer       IRT--RGMTPYPGVQNHEMYDYLLHGHRLKQPEDC....LDELYEIMYSCWRTDPLDRPTFSVLRLQLEKLLESLPDVRNQADVIYVNTQLLE----SSEGLA...
human hu9           IATTLRGMTPYPGVQNHEMYDYLLHGHRLKQPRTA(39)wmncmkkctlagepiPKTGPTFSVLRLQLEKLLESLPDVRNQADVIYVNTQLLE-----SEGLA...
alternative                                                LDELYEKMYTSRGTD
differences            **                           !**          *  **** ! *                                         *
domains             --------------------------------------------------------------------->
```

B

```
human hu9 SFKLNNTLHIPCRGR--PQPNVT---CRDLKRCN
rPDGFRb   SHPANGEQILRCRGRGMPQPNVTWSTCRDLKRCP
```

Fig. 1. (**A**) Multiple alignment of Hu9 with three members of the *axl*-RPTK family. Position of the displayed regions within the respective sequences is given. # above the sequences (c-*mer* diff.) indicates positions at which human and mouse c-*mer* differ, but human c-*mer* and human Hu9 are identical. Below Hu9, "alternative" translations are displayed. They can be made by inserting or deleting a single base. These were revealed by two independent programs designed to detect frameshifts, CheckSeq and PairWise (*8*). Positions at which frameshifts are predicted using PairWise (*9*) are indicated by an exclamation mark ("!"). Amino acids identical to c-*mer* are boldface, and the respective region in Hu9 is lower case. "Difference" line highlights positions that are different between human c-*mer* and Hu9; they are clustered in groups along the alignment. Length and positions of the two inserts in Hu9 relative to the *axl*-family are indicated by numbers in parentheses. Apart from the two predicted double frameshifts, a segment proximal to the proposed initial methionine of Hu9 also has 100% amino acid identity to c-*mer* when the frame is shifted. Carboxyl termini of the sequences are omitted as the alignment becomes unreliable; nevertheless, Hu9 is considerably shorter than the other *axl*-like RPTKs. (**B**) Alignment of a part of the NH_2-terminal insert with rat PDGF receptor β. Identical amino acids are shown in bold.

tholog of Hu9, the presence of which has been reported by Burks *et al.* (*1*).

The answer might be in between these two views. We will probably have the definitive evidence in less than 5 years with the almost complete human genomic sequence. Meanwhile, one should be cautious with the published Hu9 sequence, as most of the observations presented here point to a considerable number of cloning artifacts, or sequencing errors, or both.

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REFERENCES AND NOTES

1. D. J. Burks, R. Carballada, H. D. M. Moore, P. M. Saling, *Science* **269**, 83 (1995).
2. D. K. Graham, T. L. Dawson, D. L. Mullaney, H. R. Snodgrass, H. S. Earp, *Cell Growth Diff.* **5**, 647 (1994).
3. D. K. Graham *et al.*, *Oncogene* **10**, 2349 (1995).
4. R. Jia and H. Hanafusa, *J. Biol. Chem.* **269**, 1839 (1994).
5. P. Bork and R. F. Doolittle, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8990 (1992).
6. L. Leyton and P. Saling, *Cell* **57**, 1123 (1989).
7. P. Bork and C. Sander, *FEBS Lett.* **300**, 237 (1992).
8. CheckSeq (J. Boyle, N. P. Brown, P. Bork, in preparation) and PairWise (J. Birney, J. Thompson, T. Gibson, in preparation) are available from the authors on request: <borg@embl-heidelberg.de> and <gibson@embl-heidelberg.de>, respectively.

9. I am indebted to T. Gibson for help in the PairWise error analysis and to T. Gibson, C. Sander, and R. Wade for valuable comments.

28 July 1995; accepted 18 January 1996

Burks *et al.* (*1*) point out that the Hu9-encoded product shows homology to the phosphotyrosine kinase domain of c-*eyk*. On the basis of our interest in evolutionary aspects of fertilization, we decided to compare the Hu9 sequence (GenBank accession no. L08961) with the human homolog of c-*eyk*—called c-*mer* (GenBank accession no. U08023)—which is composed of 999 amino acids and has a predicted molecular weight of 110,321 daltons (*2*). Unexpectedly, Hu9 and human c-*mer* showed regions of extremely high similarity in both DNA and amino acid sequences. Four stretches of the Hu9 polypeptide sequence (Fig. 1), which account for over half of the entire sequence, together show a 97% identity with corresponding regions of human c-*mer*.

The supposition that Hu9 encodes p95 is

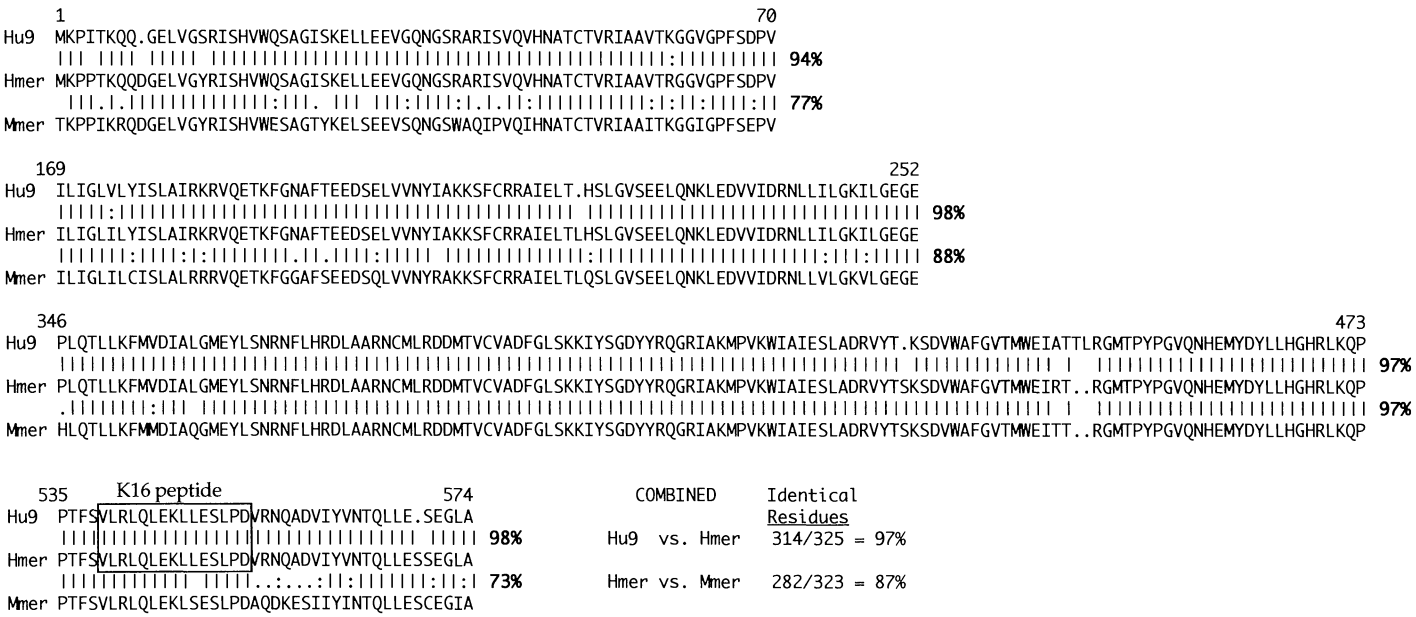

Fig. 1. Comparison of polypeptide sequences in the regions of homology between human Hu9, human c-*mer* (Hmer), and mouse c-*mer* (Mmer). Numbers above each similar region indicate positions within the published Hu9 polypeptide sequence. Pairwise percentage identity is indicated to the right of the similar region. Region defined by the K16 peptide is boxed.

based entirely on the results of a single experiment in which an anti-peptide antibody (called K16) directed against residues 539–553 from the postulated Hu9 product was used to achieve a positive signal on a protein immunoblot in the 95-kD range [lane 2 in figure 4A in the report by Burks *et al.* (*1*)]. However, the identical 15-residue peptide is also presented in the c-*mer* polypeptide (Fig. 1). This identity invalidates the only experimental evidence for distinguishing between Hu9 and c-*mer* as the coding sequence for the 95-kD polypeptide. The use of the K16 antibody and other antibodies based on identical or nearly identical peptides from Hu9 and c-*mer* in functional assays for the role of Hu9 in sperm-egg binding is also invalidated [figures 5A and lane 2 in figure 5B in (*1*)].

The statement by Burks and colleagues (*1*) that Hu9 is expressed uniquely in spermatogenic cells is also called into question by the extreme similarity between Hu9 and c-*mer* at the level of nucleotide sequence. Expression of a 4.4-kb c-*mer* transcript has been demonstrated in blood cells, the spleen, prostate, testes, ovary, lung, liver, and kidney (*2*). In contrast, Burks *et al.* see only a 2.2-kb transcript in testes and nothing in RNA from other tissues on a Northern blot probed with Hu9. However, the Hu9 sequence shows 99% identity to c-*mer* in two regions (bases 1–74 and 514–826 in Hu9) that together total to 387 nucleotides, and 97% identity in a third region (bases 1108–1486 from Hu9) with 379 nucleotides. Based on the Northern blot washing conditions reported by Burks *et al.* (*1*) (2× standard saline citrate; 0.1% SDS at 55°C), the Hu9 probe must cross-hybridize to the c-*mer* transcript. Thus, the Northern blot results obtained with Hu9 are incompatible with the results reported previously for c-*mer* (*2*). What can explain this discrepancy? One possibility is that the RNA samples used for the Hu9 Northern blot are partially degraded to a degree that eliminates most material in the 4.4-kb range, but not in the 2.2-kb range; in the absence of control hybridization with an independent probe that detects transcripts in the higher range, this cannot be ruled out. An alternative possibility is that the testes transcript observed by Burks *et al.* was incorrectly sized, and RNA from other tissues was degraded. The certainty that cross-hybridization will occur between Hu9 and c-*mer* also calls into question the interpretation of the in situ hybridization results presented in figure 3B of the report (*1*).

With the degree of similarity that exists between Hu9 and human c-*mer*, it is critical to evaluate the genetic relationship between the two sequences. According to the available data, the Hu9 clone seems to represent a truncated version of c-*mer* with several small insertions and deletions. This relationship between cDNA sequences could be explained by two different scenarios. First, one could postulate the existence of a single gene that encodes both c-Mer and Hu9. In this case, large differences in the cDNA sequences would be ascribed to alternative splicing events, and small differences would be ascribed to naturally occurring polymorphisms within the human population. In the second scenario, c-Mer and Hu9 would be derived from two different genetic loci that resulted from a recent duplication event.

Two pieces of data argue in favor of the single gene hypothesis. Although Burks *et al.* state that the NH_2-terminal portion of the putative Hu9 polypeptide represents a cleavable signal peptide, it has none of the features normally ascribed to such a peptide. Furthermore, the region of the c-*mer* polypeptide that is homologous to this putative Hu9 signal peptide lies within an external FNIII-like domain. This observation suggests that the coding sequences determined for Hu9 by Burks *et al.* is incomplete at the NH_2-terminal. In support of this postulate is the observation of high identity (99%) between the Hu9 and c-*mer* nucleotide sequences in the region that is supposed to represent the 5′ untranslated region of Hu9, but is contained within the coding sequence of c-*mer*.

Nevertheless, these observations do not rule out the two gene hypothesis. With the assumption that there are two genes, we decided to evaluate the likely time of emergence of the Hu9 gene in relation to the speciation event that separated humans and mice. To accomplish this task, we compared the human c-*mer* sequence to both the mouse c-*mer* sequence [GenBank accession no. U21301 (*3*)] and the human Hu9 sequence in the four regions of similarity observed among these three genes (see Fig. 1). If Hu9 was present as a separate gene prior to the evolutionary divergence of humans and mice, we would expect it to show less similarity to either human c-*mer* or mouse

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*).

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REFERENCES

1. D. J. Burks, R. Carballada, H. D. M. Moore, P. M. Saling, *Science* **269**, 83 (1995).
2. D. K. Graham, T. L. Dawson, D. L. Mullaney, H. R. Snodgrass, H. S. Earp, *Cell Growth Diff.* **5**, 647 (1994).
3. D. K. Graham *et al.*, *Oncogene* **15**, 2349 (1995).
4. L. Leyton and P. Saling, *Cell* **57**, 1123 (1989).
5. P. Kalab, P. Visconti, R. Leclerc, G. Kopf, *J. Biol. Chem.* **269**, 3810 (1994).

26 September 1995; accepted 19 January 1996

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 *hu9* 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 *hu9* 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 testis, 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 *hu9* 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 *axl* (*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 *hu9* 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-I 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 *hu9* 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 *hu*9 is shared by c-*mer* and *axl*, other members of this new family encoding PTKs (*2*, *3*). Intracellular and extracellular structural differences exist between c-*mer* and *hu*9. For example, the extracellular region of both c-Mer and Axl possess two Ig domains and two fibronectin type II (FNII) domains; *hu*9 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. Retrospectively, these were useful tissues to assess, as the c-*mer* transcript has been shown to be expressed in all of them.

7) Evidence that *hu*9 encodes a receptor on sperm: We provided the following evidence that *hu*9 may encode a sperm receptor molecule: (i) *hu*9 is expressed in spermatogenic cells. (ii) K16 recognizes a 95-kD protein in human sperm which is also detected by anti-PY 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 *hu*9 and c-*mer*. However, the general rules for the evolutionary relationship of proteins involved in sex are different from those for non–sex-related proteins (*7*–*9*). In species as far ranging as *Chlamydamonas*, 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 *hu*9, 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 *hu*9-encoded protein is a member of a conserved protein family is not unanticipated. Among other examples which parallel our results with *hu*9 are fertilin as an ADAM protein (*5*) and PH-20 as a hyaluronidase (*6*).

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REFERENCES

1. D. J. Burks, R. Carballada, H. D. M. Moore, P. M. Saling, *Science* **269**, 83 (1995).
2. D. K. Graham, T. L. Dawson, D. L. Mullaney, H. R. Snodgrass, H. S. Garp, *Cell Growth Diff.* **5**, 647 (1994).
3. J. P. O'Bryan *et al.*, *Mol. Cell Biol.* **11**, 5016 (1991).
4. A. Ullrich, *EMBO J.* **5**, 2503 (1986).
5. P. Bork and C. Sander, *FEBS Lett.* **300**, 327 (1992).
6. R. Yanagimachi, in *The Physiology of Reproduction*, E. Knobil and J. D. Neill, Eds. (Raven, New York, 1994), p. 189.
7. W. J. Swanson and V. D. Vacquier, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4957 (1995).
8. P. K. Tucker and B. L. Lundrigan, *Nature* **364**, 715 (1993).
9. L. S. Whitfield, R. Lovell-Badge, P. N. Goodfellow, *Nature* **364**, 713 (1993).
10. T. G. Wolfsberg *et al.*, *J. Cell Biol.* **131**, 275 (1995).
11. M. Gmachl *et al.*, *FEBS Lett.* **336**, 545 (1993).

19 December 1995; accepted 14 February 1996