

## BRIEF COMMUNICATIONS

## Zebrafish Yolk-Specific *not really started* (*nrs*) Gene Is a Vertebrate Homolog of the *Drosophila spinster* Gene and Is Essential for Embryogenesis

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**ABSTRACT** By using retroviral insertional mutagenesis in zebrafish, we have identified a recessive lethal mutation in the *not really started* (*nrs*) gene. The *nrs* mutation disrupts a gene located in linkage group 3 that is highly homologous to the *spinster* gene identified in *Drosophila* and to *spinster* orthologs identified in mammals. In flies, *spinster* encodes a membrane protein involved in lysosomal metabolism and programmed cell death in the central nervous system and in the ovary. In *nrs* mutant fish embryos, we detect an opaque substance in the posterior yolk cell extension at approximately 1 day after fertilization. This material progressively accumulates and by 48 hr after fertilization fills the entire yolk. By day 3 of embryogenesis, mutant embryos are severely reduced in size compared with their wild-type siblings and they die a few hours later. By *in situ* hybridization, we show that the *nrs* mRNA is expressed in the yolk cell at the time the mutant phenotype becomes apparent. In wild-type embryos, *nrs* message is present maternally and zygotically throughout embryogenesis and is also detected in adult animals. In *nrs* homozygous mutant embryos, *nrs* transcripts are undetectable at the time the phenotype becomes apparent, indicating that the retroviral insertion has most likely abolished expression of the *nrs* gene. Finally, the *nrs* phenotype can be partially rescued by microinjection of *nrs* encoding DNA. These results suggest that the *nrs* mutation affects an essential gene encoding a putative membrane-bound protein expressed specifically in the yolk cell during zebrafish embryogenesis. © 2002 Wiley-Liss, Inc.

**Key words:** *Danio rerio*; lipofuscin; ceroid; insertional mutagenesis

### INTRODUCTION

In recent years, the search for genes that are essential for the early development of vertebrate embryos has been aided by the introduction of forward genetic screens in the zebrafish. Hundreds of mutations induced with ethyl nitrosourea (ENU) have been identified, which affect many aspects of embryogenesis, organ and tissue formation, and behaviors (Haffter et al., 1996; Driever et al., 1996). However, molecular identification of the mutant loci relies on positional cloning methods, which in the zebrafish are still laborious and slow. To date, only a small number of mutations have been associated with a specific gene, most of these by using the candidate gene approach. An alternative strategy, in which mutations are induced by insertion of foreign DNA, was introduced recently (Gaiano et al., 1996; Allende et al., 1996; Amsterdam et al., 1999). Although less efficient than ENU, insertional mutagenesis has the advantage of allowing the extremely rapid identification of the disrupted genes (for a review see Amsterdam et al., 1997). This strategy uses pseudotyped mammalian retroviral vectors that are concentrated to high titers and used to infect early fish embryos. Insertion-carrying fish are identified and each integration is bred to homozygosity to assay for

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the presence of an induced mutation. To date, over three hundred insertional mutants have been identified, and more than one hundred of the disrupted genes have been cloned (Gaiano et al., 1996; Allende et al., 1996; Becker et al., 1998; Amsterdam et al., 1999; Golling et al., 2001). Here, we report the identification of the *not really started* (*nrs*) insertional mutant and describe the disrupted gene. The *nrs* locus encodes a *trans*-membrane domain-containing protein highly similar to a protein that is conserved in metazoans from *Drosophila* to humans, called Spinster. The *spinster* (*spn*) gene was originally identified in a behavioral mutant screen in *Drosophila*: *spn* mutant females display strong rejection to courting males (Suzuki et al., 1997). More recent studies have shown that *spinster* mutant flies present abnormal programmed cell death in neurons and glia in the central nervous system and in nurse cells in the ovary (Nakano et al., 2001). Mutant flies also accumulate lipofuscin-like pigments, suggesting a role for the Spinster protein in lysosomal turnover (Nakano et al., 2001). We show here that zebrafish *nrs* mRNA is maternally supplied and that, during embryogenesis, it is expressed in the yolk cell of the developing zebrafish embryo. The insertional mutation we have isolated interrupts the 5' region of the *nrs* gene such that its mRNA cannot be detected in homozygous mutants. Mutant embryos show a visible phenotype in the yolk cell beginning at 24 hr after fertilization and they die at day 3 of embryogenesis. Finally, microinjection of DNA encoding the *nrs* gene delays the onset of the phenotypic defect and prolongs the life of mutant embryos. This evidence indicates that we have molecularly identified a novel gene essential for zebrafish embryogenesis.

## RESULTS AND DISCUSSION

Retroviral insertions are bred to homozygosity and potential mutant embryos are screened on days 1, 2, and 5 for the presence of visible, lethal, or both, phenotypes. In intercrosses of fish harboring insertion 891, 25% of the offspring show a phenotype. The defect in mutant animals first becomes apparent at 24 hr after fertilization. In mutant embryos, an opaque material accumulates within the caudal end of the yolk extension which lies under the posterior trunk (compare Fig. 1a and b). During the following hours, this opacity extends anteriorly eventually filling the entire yolk cavity at 48 hr. At this time, there is no visible yolk material remaining in the yolk extension of mutant embryos (Fig. 1d). Until 48 hr after fertilization, no apparent defects are observed in any other embryonic structures and mutants exhibit normal blood circulation and touch responses. However, by day 3, mutant embryos lag behind their siblings and decline rapidly thereafter; they do not survive past the fourth day. We have named this mutant *not really started* or *nrs* (allele designation *nrs*<sup>hi891</sup>).

Phenotypically identified mutant and wild-type embryos were genotyped by testing for the presence of

proviral sequences. All phenotypically mutant embryos ( $n = 119$ ) were positive for the presence of a proviral-specific polymerase chain reaction (PCR) product, indicating linkage between the mutation and the provirus. To strengthen the linkage data, we cloned a DNA fragment adjacent to the proviral insertion by inverse PCR (see below). A subfragment of this sequence was shown to behave as a single copy hybridization probe which allowed us to distinguish the mutant and wild type chromosomes in Southern blot experiments. Although phenotypically wild-type embryos ( $n = 39$ ) were genotypically either homozygous wild-type or heterozygous, mutant embryos ( $n = 45$ ) were always homozygous carriers of the proviral insertion (data not shown).

We sought to identify the *nrs* gene by searching the genomic region surrounding proviral insertion 891. A 2.9-kb DNA fragment flanking the provirus on the 5' side was cloned by inverse PCR but database searches by using this DNA failed to reveal any significant homology to sequences in Genbank. Therefore, we used a 0.5-kb subfragment of this sequence to screen a zebrafish genomic BAC library. A BAC clone containing approximately 100 kb of genomic sequence was identified by hybridization to this probe. By using this DNA, we conducted a search for functional genes by exon trapping (Buckler et al., 1991). Several exons, possibly encoded by three different genes, were identified by exon trapping within the BAC sequence. One of the exons corresponded to a transcript shown to be absent in *nrs* mutant embryos (see below) and was considered to be the *nrs* gene. Partial mapping of the *nrs* genomic region showed that the exon containing the ATG codon of the *nrs* gene is located 4.3 kb from the 5' end of the proviral insertion and is transcribed away from the virus (Fig. 2A). Because we have not determined whether there are additional exons upstream of the ATG-containing exon, we do not know whether the provirus integrated within an intron or 5' to the *nrs* transcription initiation site.

To determine whether the *nrs* gene is expressed in mutant animals, we performed reverse transcriptase (RT)-PCR experiments by using RNA isolated from 24-hr-old phenotypically mutant embryos or from their wild-type siblings (Fig. 2B). The expected 612-bp product is amplified from RNA isolated from phenotypically wild-type embryos. A large intron (5.5 kb) spans the region between the *nrs* primer binding sites, which precludes the amplification of contaminating genomic DNA in the RNA preparation under the PCR conditions used (see Fig. 2A). When RNA from phenotypically mutant embryos is used, we cannot detect this amplification product indicating that expression at the RNA level is abolished, or diminished beyond detection, by the proviral insertion.

We determined the temporal pattern of expression of the *nrs* gene in wild-type embryos by RT-PCR. *nrs* message was detected in all embryonic stages examined (Fig. 2C), including fertilized zygotes, indicating that *nrs* mRNA is supplied maternally, and in RNA

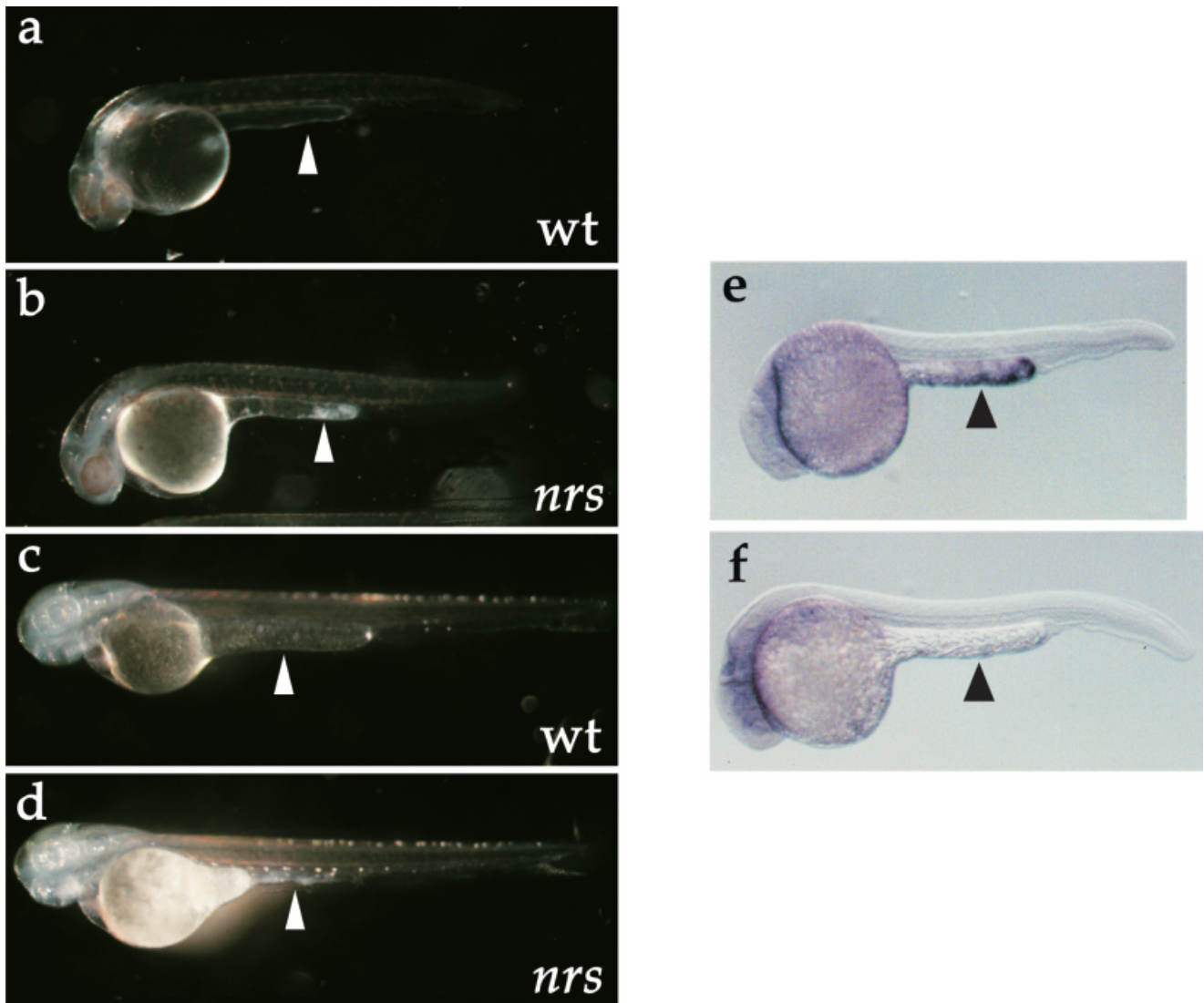


Fig. 1. *nrs* mutant phenotype. **a**: At 24 hr after fertilization, an extension of yolk is present under the trunk and part of the tail in wild-type (wt) zebrafish embryos. **b**: In *nrs* homozygous mutants, an opaque substance begins to accumulate in the posterior part of the yolk extension (arrowhead) and in the periphery of the yolk sac. **c,d**: At 48 hr after fertilization, the yolk extension in *nrs* mutants has emptied (compare arrowheads in wt [c] and *nrs* [d]) and the dark material now fills the entire yolk sac. Note that mutant embryos are otherwise normal. **e,f**: Expression of the *nrs* RNA. Expression was monitored by whole-mount in situ hybridization by using antisense (e) and sense (f) RNA probes. Note expression in the yolk extension (arrowhead) and yolk sac periphery only seen with the antisense probe (f). In all photos anterior is to the left and dorsal is up.

prepared from whole adult animals (not shown). The *nrs* cDNA was used to synthesize sense and antisense RNA probes for in situ hybridization experiments (Fig. 1e–f). We were able to detect specific hybridization stain in 24-hr-old embryos with antisense strand probe (Fig. 1e) but not with the control sense strand probe (Fig. 1f). The hybridization stain is localized to the yolk extension at 24 hr, which coincides spatially and temporally where the phenotype first becomes visible in *nrs*<sup>-/-</sup> embryos. Mutant embryos 24-hr-old do not stain with the *nrs* RNA probe (not shown), which is consistent with the RT-PCR result described above. This finding indicates that the maternal stores of *nrs* mRNA

are consumed by the end of the first day of embryogenesis. *nrs* RNA was detected at later stages (2–4 days after fertilization) localized to the interface between the dorsal yolk and the overlying embryonic tissues (not shown). We have not determined the localization of transcripts in subsequent stages of development nor in adults.

A 2145-bp cDNA encoding the entire putative translation product of the *nrs* gene was isolated by screening a cDNA library (Genbank accession no. AF465772). The *nrs* cDNA was tested against the Genbank database by using the BLAST algorithm (Altschul et al., 1990). Identical sequences (99% ho-

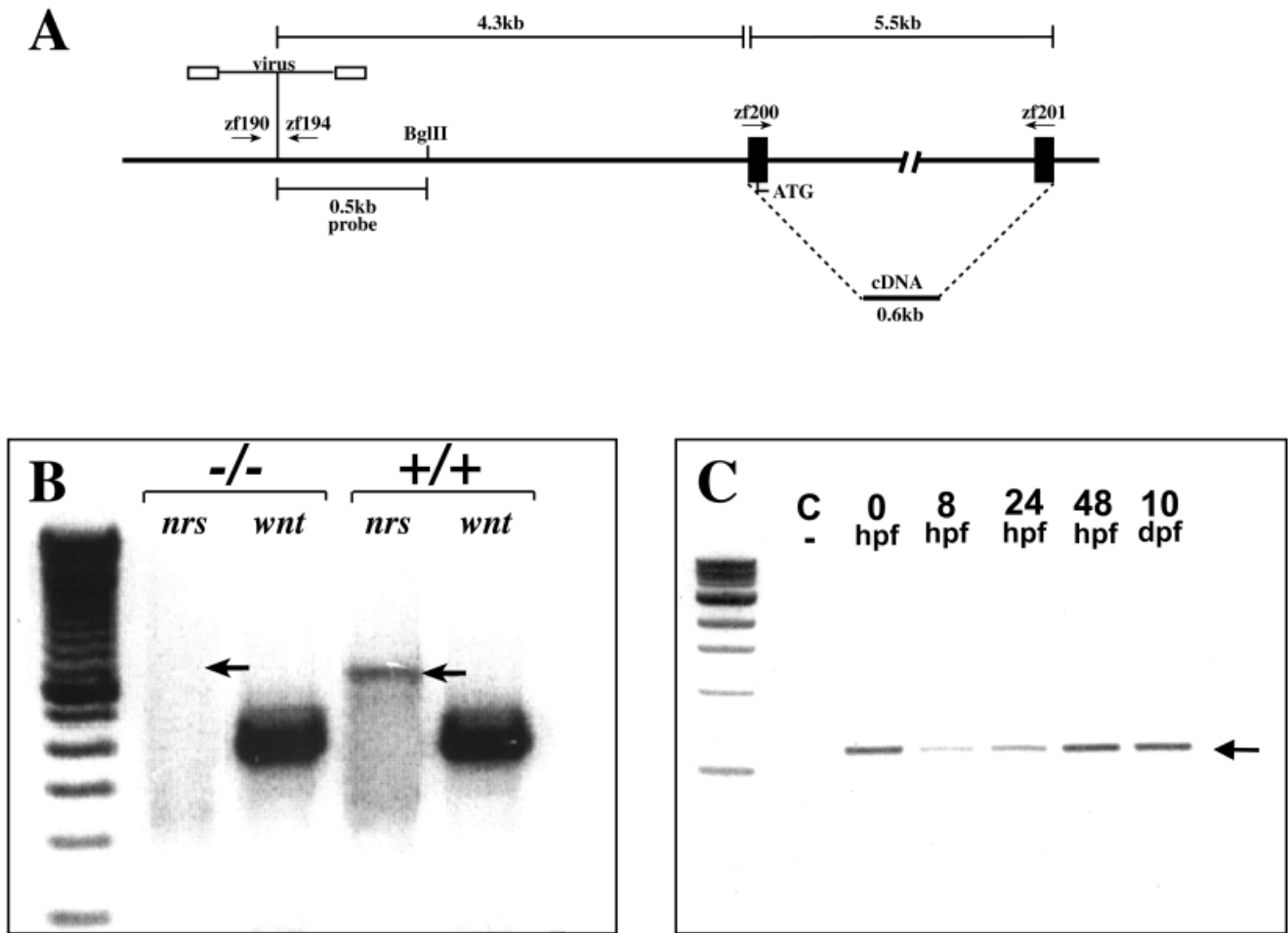


Fig. 2. **A:** Partial genomic structure of the *nrs* locus. Proviral insertion 891 lies 4.3 kb from the *nrs* exon, which contains the ATG codon. The filled boxes are the exons identified in the exon trapping experiment. Analysis of the *nrs* cDNA indicates that 0.6 kb of sequence corresponding to one or more exons lies between the trapped exons and is not shown in this diagram; the trapped exons lie 5.5 kb away from each other in the genome. Also shown is the position of the 0.5-kb genomic DNA fragment isolated by inverse PCR (between the end of the provirus and a genomic *Bgl*III site). This fragment was used as a probe to screen the zebrafish BAC library and to distinguish between mutant and wild-type chromosomes in the linkage experiments. Genotyping of mutant and wild-type embryos in the rescue experiments (Table 1) was done with PCR primers zf190 and zf194. RT-PCR experiments were performed by using the zf200 and zf201 primers. **B:** *nrs* RNA is not expressed in *nrs*  $-/-$  mutants. RT-PCR was performed by using RNA prepared from 10 phenotypically mutant ( $-/-$ ) or wild-type ( $+/+$ ) 2-day-old embryos. The primers used in each case are specific for *nrs* (zf200 and zf201) or for the *wnt5a* gene (*wnt*) (Lin et al., 1994). Arrows indicate the absence of *nrs*-specific product in the mutant RNA sample (lane 2) and the presence of the expected band in the wild-type RNA sample (lane 4). **C:** Temporal time course of *nrs* RNA expression. RT-PCR was performed from RNA prepared from 50 embryos for the time points indicated. The negative control corresponds to a parallel experiment by using 24 hpf RNA in which no RT was included. Hpf, hours postfertilization; dpf, days postfertilization.

mology) were found which correspond to zebrafish ESTs sequenced in the Washington University Zebrafish EST Project (e.g., Genbank accession no. AI585178). The gene has been sequenced as an EST at least four times independently. The *nrs* gene was mapped by using the Research Genetics Zebrafish Radiation Hybrid Panel. This analysis places the *nrs* locus 36-37 cM from the top of Linkage Group 3, between markers z15457 and z21679 (logarithm of odds score of 17.66).

When comparing the *nrs* cDNA to sequences from other organisms, closely related genes were found in human, mouse, and *Drosophila* corresponding to the

*spinster* genes. Alignment of the predicted Nrs protein (458 amino acids) and the mouse, human, and fly Spinster proteins is shown in Figure 3. The degree of identity is highest between Nrs and the identified mammalian proteins (70%), whereas identity with the *Drosophila* Spinster protein is 51%. As was shown for the Spinster proteins (Nakano et al., 2001), a hydrophobicity plot of the Nrs protein indicates eight transmembrane domains (not shown). Besides showing homology to the *spinster* genes, comparison of the Nrs protein sequence against the Swissprot database indicates a much lower similarity to a family of prokaryotic transporters involved in hexose metabolism. Nonethe-



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nrs      1  -----MSQADA
Hspin1  1  -----MAGS
Mspin1  1  -----MAGS
spinster 1  MSLKHQKQSYQPLPTAAAMDNPMIQSSGSSGSSSSSEEGGSREDVANLSPLGLPTIYSQ

nrs      7  DITPFFADDNEGSGP-----VENGVG-----SPLPEDEEEESPSGVDRRAIMTVIV
Hspin1  5  DTAPFLSQADDPDGGPVPGTPLPGSTGNPKSEEPVDPQEGLQRIITGLSPGRSALIVAV
Mspin1  5  DTAPFLSQADDPDGGPAPGHPGLPGPMGNPKSGELEVPDCEGLQRIITGLSRGISTLIVIV
spinster 61  QLMPSDTDSMEEERHRLRPHHHHHHPILG-ERHHHPGIPPSAVVPSRLSSVGRSQWFTVIV

nrs      54  LCYINLLNYMDRFTVAGVLPDIEHFFGIGDGSGLQOTVFI CSYMF LAPVFGYLGDRYNR
Hspin1  65  LCYINLLNYMDRFTVAGVLPDIEQFFNIGDSSSGLQOTVFISSYMLAPVFGYLGDRYNR
Mspin1  65  LCYINLLNYMDRFTVAGVLPDIEQFFNIGDGSGLQOTVFISSYMLAPVFGYLGDRYNR
spinster 120  LCFVNLINNYMDRFTVAGVLPDVRNDFDIGNDSAGLQOTVFI SYMVCAPVFGYLGDRYSR

nrs      114  KLIMCVGIFFWSVVTLASSFIKDHFWALLLTRGLVGVGEASYSTIAPT IADLFVKEKR
Hspin1  125  KYIMCGGIAFWSLVTLGSSFIPGEHFWLLLLTRGLVGVGEASYSTIAPT IADLFVADQR
Mspin1  125  KYFMCGGIAFWSLVTLGSSFIPREHFWLLFLTRGMVGVGEASYSTIAPT IADLFVADQR
spinster 180  PWIMAVGVGLWSTTTLLGSFMK--QFGWFI AFRALVGI GEASYSTIAPT IISDLFVHDMR

nrs      174  INMLSIFYFAIPVGSGLGYIVGSKVDTVAKDHWALRVTPGLGVA VAVLLMLLVQEPKRG
Hspin1  185  SRMLSIFYFAIPVGSGLGYIAGSKVKDAGDHWALRVTPGLGVVAVLLFLVVRPPRG
Mspin1  185  SRMLSIFYFAIPVGSGLGYIAGSKVKDVAGDHWALRVTPGLGVA VAVLLFLVQEPKRG
spinster 238  SKMLAIFYFAIPVGSGLGYIVGSKTAHIANWRWALRVTPILGVA VAVLLMLLVQEPKRG

nrs      234  AIEAHP-EHTLHRTSWLADMKALCRNPSFILSTFGFTAFAVFTGSLALWAPAFLEFRACVF
Hspin1  245  AVERHSDLPPLNPTSWWADLKALARNPSFVLSLGFSTAFAVFTGSLALWAPAFLLRSRVV
Mspin1  245  AVERHSGSPPLSPTSWWADLKALARNPSFVLSLGFSTAFAVFTGSLALWAPAFLLRSRVV
spinster 298  SEGSHN----LEATTYKQDIKALVRNRSFMLSAGFTCVAFVAGALAWWQPSFVYLGKQV

nrs      293  TGVKQPCFKAP-CDDSDSLIFGAIIVVTGLGVASGVQASKLLRTRTPRADPLVCAAGLL
Hspin1  305  LGETPPCLPGDSCSSSDSLIFGLITCLTGVLGVGLGVEISRRLRHSNPRADPLVCATGLL
Mspin1  305  LGETPPCLPGDSCSSSDSLIFGLITCLTGVLGVGLGVEISRRLRHSNPRADPLVCAAGLL
spinster 354  QPGNENIVQDD-----VAFNFGVITMLAGILGVPLGSFLSQYLVRKRYPTADPVICAFGLL

nrs      352  LAAPFLVLSIIFAQSTIVATYVFI FGETFLSMNWAIVADILLYVVIPTRRSTAEAFQIV
Hspin1  365  GSAPFLVLSLACARGSIVATYIFIFIGETLLSMNWAIVADILLYVVIPTRRSTAEAFQIV
Mspin1  365  GSAPFLVLSLACARGSIVATYIFIFIGETLLSMNWAIVADILLYVVIPTRRSTAEAFQIV
spinster 409  VSAPLLTGACLLVNSNSVCTYALIFFGQALALNINWAIVADILLYVVIPTRRSTAEAFQIV

nrs      412  LSHLLGDATSPYLIGVSDSIKESN-----
Hspin1  425  LSHLLGDAGSPYLIGLISDRIRRNWPP-----
Mspin1  425  LSHLLGDAGSPYLIGLISDRIRRSWPP-----
spinster 469  ISHALGDAGSPYLVGATSEAIMKHLHKNPSDSGLTTELRSMSQVAGSAISNATQVIAEAT

nrs      437  -----SYMWEFRSLQMSLLCSFVA VAGGAFFLTA V FIEKDRDLA NYV
Hspin1  452  -----SFLSEFRALQFSLMLCAFV GAGLGGAAFLGTA V FIEADRRRAQLHV
Mspin1  452  -----SFLSEFRALQFSLMLCAFV GAGLGGAAFLGTA V FIEADRRRAQLHV
spinster 529  TSLMETARSSASQEYS DVEQFEQLQYALFSTS FVEVVLGGIFF FTACFIKDYNA TRG

nrs      482  P-----SDDAPIVVPRSGRSTKVS VSSVLI-----
Hspin1  497  QGLLHEAGSTDDRIVVPQRGRSTRVPVAVSVLI-----
Mspin1  497  QGLLHEAGSPSDDRIVVPQRGRSTRVPVAVSVLI-----
spinster 589  QGD-QGAQAVRSSVALASGQKDVESFNSDCLV LCTDIALRERT

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Fig. 3. Alignment of the predicted Nrs protein sequence with predicted Spinster protein sequences from human (Hspin1), mouse (Mspin1), and *Drosophila* (*spinster*). Homology searches were performed with the BLAST program (Altschul et al., 1990), and alignments were compiled by using the ClustalW algorithm (Thompson et al., 1994). Dark shading indicates identity, and light shading indicates similarity.

TABLE 1. Rescue of the *nrs* Mutants<sup>a</sup>

	No. of embryos	Phenotypically mutant at day 1 pf	Surviving to day 4 pf	Surviving to day 6 pf	Genotypically mutant at day 6 pf
Control	136	27	98	90	0
Injected	150	3	141	124	22

<sup>a</sup>Embryos obtained from a cross of *nrs* heterozygous parents were raised as controls or injected with 100 ng/μl plasmid DNA encoding the *nrs* gene. Survival and the appearance of the *nrs* phenotype were assayed at days 1 and 4 postfertilization. At day 6 postfertilization, all surviving embryos were genotyped by polymerase chain reaction (see Experimental Procedures section). pf, postfertilization.

less, the precise biochemical function of the *nrs* and *spinster* products in metazoans remains to be elucidated.

To obtain unequivocal proof that we had cloned the gene responsible for the observed phenotype, we placed the *nrs* cDNA into a eukaryotic expression vector and injected this construct into a pool of embryos derived from a cross of *nrs* heterozygous parents. In uninjected controls, *nrs* homozygous mutants are easily visible by the first day after fertilization (Fig. 1) and they do not live past the third day after fertilization. We confirmed this finding by genotyping the surviving embryos at day 6 after fertilization. No homozygous mutant *nrs* embryos were found out of 98 total animals analyzed (Table 1). On the other hand, we failed to see any manifestation of the mutant phenotype until day 3 in the injected batch of embryos and mutant embryos began to die only after day 5 or 6 after fertilization. When we genotyped the surviving injected embryos at day 6 after fertilization by PCR, 22 of 124 total embryos analyzed were *nrs*<sup>-/-</sup> mutants, indicating that supplying exogenous *nrs* is able to extend the life of homozygous mutants for at least 3 days (Table 1). However, complete rescue was not possible because we could not find any surviving *nrs* homozygotes when genotyping beyond day 6 after fertilization (not shown).

Thus, we have identified a gene essential for zebrafish embryogenesis which is expressed in the yolk cell. In zebrafish, the yolk becomes a syncytium containing zygotic nuclei during the cleavage stages (Kimmel and Law, 1985). These nuclei migrate vegetally, leading the overlying blastoderm during the morphogenetic process of epiboly. Although the yolk cell has been shown to be the source of localized inductive signals (Ober and Schulte-Merker, 1999), it is unclear what role the syncytial nuclei play in this process. The present work suggests that essential zygotic genes are being expressed from these nuclei as late as 24 hr after fertilization.

The phenotype of the *nrs* mutant is novel. To our knowledge, this type of embryonic mutation has not been seen in the large-scale screens carried out in zebrafish. It is possible that the phenotype is unusual and that most mutations in genes expressed in the yolk cause "general" retardation or death without the "dark yolk" phenotype visible in the *nrs* mutant embryos. The proviral insertion responsible for the mutation inte-

grated in the 5' region of the *nrs* gene and apparently abolished transcription from this locus. We do not know yet whether the proviral insertion interrupts regulatory sequences present upstream of the transcription start site or whether it is within the gene, in an intron. Of the previous insertional mutations analyzed at the genomic level, most correspond to proviral insertions that integrated into either the first exon or the first intron of the affected genes.

*nrs* mRNA is present from the one cell stage—indicating a maternal contribution of this gene product—and remains present throughout the life of the animal. That the *nrs* phenotype becomes apparent starting at 24 hr after fertilization does not preclude the possibility that the gene is essential from the earliest stages of development. In this case, the maternal stores of *nrs* mRNA could maintain the viability of zygotically mutant embryos during the first day of embryogenesis. Similarly, we were only partially successful in our rescue experiments, as we could delay the onset of the lethal phenotype for 2 to 3 days at most. Therefore, the *nrs* gene product seems to be required throughout the embryonic and larval stages of zebrafish. Because the yolk is consumed by the fifth day after fertilization, the *nrs* gene product must be expressed thereafter in other tissues.

The deduced sequence for the Nrs protein indicates that it has eight putative membrane-spanning domains and that it is closely related to the *spinster* family of genes identified in *Drosophila*, *C. elegans*, human, and mouse (Nakano et al., 2001). The *spinster* gene was first discovered in flies as the gene responsible for a striking behavioral phenotype in which mutant females reject male courtship (Suzuki et al., 1997). *Drosophila spinster* has been proposed to have a function in lysosomal metabolite turnover as accumulation of a lipofuscin-like substance in the brain and in the ovary—accompanied by abnormal cell death in these tissues—is observed in mutant flies (Nakano et al., 2001). The neuronal ceroid lipofuscinoses in humans are neurodegenerative disorders characterized by the accumulation of autofluorescent lipopigment in various tissues. We speculate that, if the zebrafish Nrs protein is indeed a transporter or a lysosomal membrane protein involved in yolk metabolism, the dark material could be due to accumulation or oxidation of a metabolite or by-product. However, because we were unable

to detect the presence of autofluorescent material accumulating in *nrs* mutant embryos, it is unclear whether the function of the Nrs and Spinster proteins is conserved between flies, humans, and fish. Experiments are under way in our laboratory to determine whether the Nrs protein becomes localized to the plasma membrane or to other subcellular destinations such as lysosomes. In mice, the *mspin1* gene is differentially spliced and is expressed widely throughout development and in most tissues examined (Y.N., unpublished results).

The location of the proviral insertion with respect to the cloned gene, the absence of transcription from this locus in mutant embryos, the spatial and temporal coincidence of the expression of *nrs* mRNA with the mutant phenotype, and the rescue of the phenotype by injection of the cloned gene constitute strong evidence that we have molecularly identified a yolk-specific gene essential for embryonic survival in the zebrafish.

### EXPERIMENTAL PROCEDURES

Fish were maintained and raised in our facility essentially as described by Westerfield (1993). The generation of proviral insertions in fish as well as the mutant screen have been described previously (Lin et al., 1994; Gaiano et al., 1996; Allende et al., 1996; Amsterdam et al., 1999).

Standard molecular cloning methods were performed as in Ausubel et al., (1997). PCR to detect viral sequences for linkage and Inverse PCR for cloning genomic DNA fragments adjacent to a proviral insertion have been described (Allende et al., 1996). Exon trapping was carried out as described by using the pSPL- vector system (Buckler et al., 1991). Briefly, DNA from a BAC clone containing the *nrs* locus was digested with *Bam*HI, *Bgl*II, and partially with *Sau*III. After filling in with A and G, the digested fragments were ligated with the vector pSPL3 that was digested with *Xho*I and filled in with C and T. The ligation was transformed into HB101 *E. coli* competent cells, and ampicillin-resistant colonies were mixed and used to prepare DNA for transfection into COS7 cells. Twenty-four hours after transfection, total RNA was prepared to perform RT-PCR and subsequently a second PCR reaction was carried out to amplify the trapped exons. An exon encoding a fragment of the *nrs* cDNA was used to screen a zebrafish cDNA library to identify full-length cDNAs. To detect the *nrs* mRNA in RT-PCR experiments, we used primers zf200 (5'-GATGTCACAAGCAGATGCAGA-3') and zf201 (5'-TCTT-TAGCCACAGTATCCACC-3') and, as a PCR control, we used primers wnt5aF and wnt 5aR (Lin et al., 1994). PCR conditions were 30 cycles of 92°C 30 sec, 55°C 60 sec, 68°C 60 sec. To genotype wild-type and mutant animals, we designed PCR primers flanking the proviral insertion site which amplify a 400-bp DNA fragment if a wild-type chromosome is present and no product if both chromosomes are interrupted by the provirus. The primers used are zf190 (5'-ATCGGT-

TAACACCCAACAGTCCTC-3') and zf194 (5'-TAAGTCGGTCCGGCTGCACGGTT-3') and PCR conditions are as above, except that the annealing temperature is 60°C. To map the *nrs* locus, we used the Zebrafish Radiation Hybrid Panel (Research Genetics). PCR primers for mapping were C-F (5'-GGAG-GAGTCTTCCAGCGGAGTCACCCATAG-3') and D-R (5'-ATCACAAATGCCGAAGAAATGCTCAATGTC-3') with an annealing temperature of 60°C for 35 cycles. Reactions were done in triplicate. In situ hybridization was as described in Allende et al. (1996). For the rescue experiments, the *nrs* coding sequence was cloned in the pCS2 vector placing it under the control of the CMV promoter. Plasmid was linearized and injected into one-cell stage embryos at a concentration of 100 µg/ml in 0.1 M KCl and 0.025% phenol red.

Sequences were analyzed by the BLAST algorithm (Altschul et al., 1994) and protein alignments were compiled by using the ClustalW program (Thompson et al., 1994). Protein structure analysis was done according to Kyte and Doolittle (1982).

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