

Identification of a heat-shock pseudogene from *Caenorhabditis elegans*

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While characterizing the hsp70 gene family from *Caenorhabditis elegans* we encountered an unusual member of this family. Sequence data reveal that the *hsp-2ps* gene is a pseudogene of the constitutively expressed, heat-inducible *hsp-1* gene. Two stop codons generated near the 5' end of the sequence as well as several frameshift mutations and a large internal deletion confirm the identification of *hsp-2ps* as a pseudogene. The nucleotide substitution rate of the third codon position was twice that of the first and second codon positions, suggesting that the *hsp-2ps* gene was nonfunctional since the time of the duplication event. The *hsp-2ps* gene duplicates a region of the *hsp-1* gene that lies exclusively within the transcribed region and retains the introns. We feel that the *hsp-2ps* gene was produced by a transpositional duplication event, which occurred approximately 8.5 million years ago.

Key words: heat shock, pseudogene, *Caenorhabditis elegans*, hsp70.

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Au cours de la caractérisation de la famille de gènes hsp70 chez le *Caenorhabditis elegans*, un membre inhabituel de cette famille a été décelé. Les données séquentielles révèlent que le gène *hsp-2ps* est un pseudogène du gène *hsp-1* qui est inducible par la chaleur et qui s'exprime de façon constitutive. Deux codons d'arrêt générés près de l'extrémité 5', plusieurs mutations transpositionnelles ainsi qu'une importante suppression interne, ont confirmé l'identification du gène *hsp-2ps* en tant que pseudogène. Le taux de substitution des nucléotides dans la localisation du troisième codon a été de deux fois supérieur à ceux des localisations du premier et du deuxième codons, ce qui suggère que le gène *hsp-2ps* n'a pas été fonctionnel depuis l'époque où sa duplication est survenue. Le gène *hsp-2ps* duplique une région du gène *hsp-1* qui n'est située qu'à l'intérieur de la région transcrite et qui retient les introns. Nous avançons donc que le gène *hsp-2ps* résulte d'un processus de duplication transpositionnelle qui serait survenu il y a environ 8,5 millions d'années.

Mots clés : choc de chaleur, pseudogène, *Caenorhabditis elegans*, hsp70.

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Introduction

All organisms often respond to a sudden elevation of temperature or the introduction of physiological stress by synthesizing a specific set of proteins called heat-shock proteins (hsps). During heat shock, the synthesis of most proteins normal to development is inhibited. The stress-induced proteins are highly conserved throughout evolution in both size and sequence. There are several closely related gene sequences encoding the 70 000 dalton (Da) or hsp70 gene family within each eukaryotic genome examined to date (reviewed in Craig 1985; Lindquist 1986). In addition to heat-inducible genes, the hsp70 gene family encodes a number of isoforms of the hsp70 proteins termed heat-shock cognates (hsc70). The hsc70s are normal constituents of the unstressed cell (Ingolia and Craig 1982; Craig *et al.* 1983). Until recently, little has been known about the function of the hsp70 proteins, but recent advances have given us clues to some properties of the hsp70 proteins (Pelham 1986).

Caenorhabditis elegans is a relatively simple eukaryote ideal for the study of both the genetics and the biochemistry of genes and proteins. With this in mind, the members of our laboratory are trying to identify and characterize the hsp70 genes to complement the genetic studies done on the unicellular eukaryote *Saccharomyces cerevisiae* (Craig and Jacobsen 1984, 1985; Craig *et al.* 1987; Werner-Washburne *et al.*

1987). The heat-shock response has been characterized in *C. elegans* (Snutch and Baillie 1983) and several members of the hsp70 gene family have been isolated (Snutch 1984; Snutch *et al.* 1988). This family consists of at least nine genes. Two of the genes, *hsp-1* and *hsp-2ps* (heat shock protein; formerly hsp 70A and hsp70B, respectively), cross hybridize under high stringency conditions, suggesting that these two genes are closely related (Snutch *et al.* 1988). The *hsp-1* gene has been mapped to linkage group (LG) IV (right) (Snutch *et al.* 1988), while *hsp-2ps* has been mapped to the left arm of LG X, using *in situ* hybridizations (D. Albertson, personal communication). At the level of transcription, the *hsp-1* gene is constitutively expressed and heat inducible, while no transcripts have been detected for the *hsp-2ps* gene (Snutch *et al.* 1988). Only a homolog of the *hsp-1* gene and not the *hsp-2ps* gene has been found in the sister species *C. briggsae* (Snutch 1984). Based on these features, it was suggested that the *hsp-2ps* gene was a pseudogene of *hsp-1*. Several pseudogenes from three different *C. elegans* multigene families have been identified to date: the *hsp-70* family (Snutch *et al.* 1988), the vitellogenin (*vit*) family (Spieth *et al.* 1985a) and the sperm-specific (*msp*) family (Ward *et al.* 1988).

Here we describe the *hsp-2ps* gene and its DNA sequence. The *hsp-2ps* gene is unusual in that it duplicates only a part of the *hsp-1* transcription unit including the introns but does not duplicate any of the flanking region. We present our arguments suggesting that the *hsp-2ps* gene was nonfunctional from the time of the duplication event.

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Materials and methods

Construction of plasmids for sequencing

The phage containing the *hsp-2ps* gene HSL 143 (Snutch *et al.* 1988) was digested with *EcoRI* and subcloned in the plasmid vector pUC19 (Norlander *et al.* 1983). The plasmid containing the *hsp-2ps* gene was identified from the other fragments by its restriction pattern.

Overlapping plasmid deletions were made using either exonuclease III (Henikoff 1987) or restriction enzymes.

Plasmid DNA preparation

Plasmid DNA for deletion and sequencing reactions was prepared using the mini alkali lysis method (Maniatis *et al.* 1982) except that (i) two phenol-chloroform extractions were done and (ii) after the first ethanol precipitation, the air-dried pellet was resuspended in 0.25 M sodium acetate and reprecipitated with ethanol. After digestion with RNase, the plasmid DNA samples were precipitated with polyethylene glycol (Hattori and Sakaki 1986).

DNA sequencing and sequence analysis

Dideoxy sequencing was performed on collapsed plasmids (Sanger *et al.* 1980; Chen and Seeburg 1985).

DNA sequences were analyzed with the computer program Microgenie (Beckman). Visual inspection and preparation of the DNA sequences for publication were done with the aid of the computer program ESSE (E. Cabot, personal communication).

Gel electrophoresis

Genomic DNAs (2 µg) were digested with 20 units of *EcoRI* for 4 h (Davis *et al.* 1980). The reactions were stopped by the addition of 1/10 volume 50% sucrose, 25 mM EDTA, 2% bromophenol blue, and 1× TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA pH 8.3). The DNA was electrophoresed through a 0.7% agarose gel containing 10 µg ethidium bromide/100 mL and 1× TBE. Marker DNA was λ (cl857 Sam7) *HindIII*-digested DNA. The DNA was visualized and photographed under a 300 nm wavelength trans-illuminator.

DNA transfer, nick translation, and hybridizations

The DNA was transferred to nitrocellulose, using the bidirectional method of Smith and Summers (1980). The nick translations were done according to Davis *et al.* (1980). The reactions were stopped by the addition of sodium dodecyl sulphate and EDTA to a final concentration of 0.3% and 10 mM, respectively. The specific activity of the resulting probes was approximately 1×10^8 cpm/µg DNA.

Hybridization of the filters to the ³²P-labelled probe was done essentially according to the procedure outlined in Davis *et al.* (1980) except that the carrier DNA and formamide were omitted and 2.5× Denhardt's (1× = 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) was used. All the hybridization and posthybridization steps were carried out at 68°C. Posthybridization washes were done using 1× SSPE (0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA pH 7.0) instead of 2× SSPE. The filters were air dried and exposed to Kodak X-Omat K film at -70°C for 3 days.

Results and discussion

Characterization of the *hsp-2ps* gene sequence

The *hsp-2ps* gene was sequenced and homology with the *hsp-1* gene was determined to be 88%. A comparison of the *hsp-2ps* gene with the *hsp-1* gene revealed that the *hsp-2ps* sequence homology was truncated and missing the last one-third of the sequence corresponding to the 3' end of the *hsp-1* gene (Fig. 1). Sequence homology between *hsp-2ps* and *hsp-1* begins just upstream of the start of translation and ends within the transcription unit. The homology extends through the introns to a point two-thirds of the way down the *hsp-1* sequence. At this point, the sequence shows no identity (i.e., approximately 25% identity) (Fig. 2). Sequences upstream of the start of transcription, including the *hsp-1* gene TATA box,

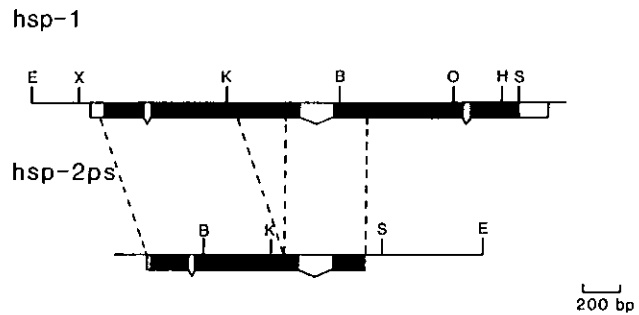


FIG. 1. Restriction maps of the *hsp-1* and *hsp-2ps* genes. Limits of homology are defined by the dotted lines (see Fig. 2). Coding regions are solid. Transcribed, nontranslated regions are open. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; O, *Xho*I; S, *Sal*I; X, *Xba*I.

were not conserved. Sequences corresponding to a 5' trans spliced leader sequence (Krause and Hirsh 1987) found in the *hsp-1* cDNA (Heschl 1988) and mRNA (Bektesh *et al.* 1988) were not seen at the 5' end of the *hsp-2ps* gene sequence. No 3' flanking sequences from the *hsp-1* gene were found flanking the *hsp-2ps* gene (Snutch 1984; Snutch *et al.* 1988). There is a large internal deletion of 243 base pairs (bp), from +723 to +966, of the corresponding *hsp-1* sequence (Fig. 1). There was one single-nucleotide insertion and a number of single-nucleotide deletions and substitutions. These changes are shown in Fig. 2. Two of the substitutions generated stop codons occurring near the 5' end of the sequence. These features have led us to confirm the identification of *hsp-2ps* as a pseudogene.

Estimation of time of divergence and inactivation of *hsp-2ps*

The rate of nucleotide substitution between the corresponding coding regions was examined. The substitutions were not randomly located with respect to the codon positions. Instead, a substitution rate of 7.5% (23/306), 5.6% (17/306), and 13.3% (41/306) at the first, second, and third positions of the codons, respectively, was observed. Determination of the nucleotide substitution rate between a pseudogene and the functional gene must take into account the fact that both sequences have been accumulating nucleotide substitutions at different rates. In a functional gene, there is very little selection pressure occurring at the third codon position, while there is high selection pressure at the first and second codon positions. In a pseudogene, there is no selection pressure at any of the three codon positions. Nucleotide substitutions will accumulate randomly (with respect to the ancestral gene before the duplication event) at the third codon positions in both genes, whereas nucleotide substitutions will accumulate randomly (with respect to the ancestral gene before the duplication event) at the first and second codon positions in the pseudogene only. Therefore, if inactivation accompanied duplication, we would expect the percent divergence at the third codon position to be approximately double that of the first or second codon positions. We observed a twofold difference in the substitution rate at the third codon position with respect to the first and second codon positions.

The nucleotide substitution rate of the introns was also examined. Since most of the intron sequences are not under selective pressure, we would predict that the nucleotide substitution rate of the introns should be similar to that of the third codon position. Taking into account the six nucleotides at the


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      A A I L S G D K S E A V Q D L L L L D V A P L S L G I E T A G G V
1374 AGCCGCTATCCTCTCCGGAGACAAGTCTGAGGCTGTCCAGGATCTTCTTCTTGACGTTGCCCACTTCCCTTGGTATTGAGACCGCTGGAGGAGTC
1116 .....AC.C.A...G....AGC.GGAC..T.CT..A.TT..CA.T.CTCCG.C.GA..ACAA.TTTC.TAG
      D K E A G H F S T F H F S V G T Q V S #

      M T A L I K R N T T I P T K T A Q T F T T Y S D N Q P G V L I Q V
1474 ATGACTGCTCTCATCAAGAGAACACCACCATCCCAACCAAGACCCTCAGACCTTCAACCTATTCTGATAACCAACCAGGAGTGTGATCCAGGTTT
1216 TCA..CT..AATT...GT..TGTGT.G.CAAGCC.A.CT.....GTC.TTGCCGC.TC.A..CAAG.CCTC.TTGTGCCCTA.T..CTC..CCC.GG
      SalI

      Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V
1574 ACGAAGGAGAAGCTGCCATGACCAAGGACAACAACCTGTCTCGGAAAGTTCGAGCTCTCCGGAATCCACCAGCACCACCGGAGTCCCACAAATCGAAGT
1316 T.ATCTC.TC.ATC.TGTCA.TGCCACTA..ATCG..TAGT.AC.TT..T.TAA.TGAT....A.TT.TGGTG..T.GAGCT.TAGAGCTT.G.T..TTG

      T F D I D A N G I L N V S A T D K S T G K A K Q I T I T N D K D R
1674 CACTTTCGATATTGACGCCAACGGAATCTTGAACGCTCTGCCACTGACAAGTCCACCGGAAAGGCAAAACAGATCACCATCACCAACGACAAGGATCGC
1416 TGAAAGTAG.T.CATT.AAGC.CACCGTGG.TT.A..A.C.A...GAG..CCGACT.TACTCC.AC..TA.CGAG.TG.CA..TGT.A.ACTATGG.AT
                                                                                               XhoI

      F S K D D I E R M V N E A E K Y K A D D E A Q K D R I G A K N G L
1774 TTTTCCAAGGATGACATTGAACGCATGGTCAACGAAGCTGAGAATACAAGGCTGACGATGAGGCCAAAAGGACCCTATTGGAGCCAAGAACGGACTCG
1516 CG..T.CG.CT.ATAT.GCCGTTGTC.CAA...ATTT.CT.TGCCACTGTACTGATTTTCGATATGTAT.T.T.TA.C.C.CCCCAGT.TTC.TCA.T.AT

      E S Y A F N L K Q T I E D E K
1874 AGTCATACGCCTTCAACCTTAAAGCAGACCATTGAGGACGAGAAGGtttagttaattatattttgtcaacaagtttttaatttccattttttcagC
1616 GA.AGCGATT.GC.CCTT.CGTC..CTTT...A.T.TGATTTCTT.C.CC.CT.CT..A.TA...ACTCTTTTCC..G...TCAGTTCGA...G.A.AT

      L K D K I S P E D K K K I E D K C D E I L K W L D S N Q T A E K E E
1974 TCAAGGATAAGATCAGCCCAGAAGACAAGAAGATCGAGGACAAGTGCAGCAGATCTTGAAGTGGCTCGACAGCAACCCAGCCGAGAGAAGGAGGA
1716 .TGGAAATGCCAT.C.TTCCTCATGTT.C..A..ATCG.GCCT.TTT.TTTTA.AT...GCTCT..C.ATTT..C.GTTTG...ATT..ATTC
                                                                                               EcoRI

                                                                                               HindIII

      F E S Q Q K D L E G L A K P D L S K L Y Q S A G G A P P G A A P G
2074 GTTCGAGTCACAACAGAAGGATTGGAAAGATTGGCCAAGCCCGATCTTCCAAGCTTACCAGAGTGCCGGAGGAGCCACCAGGTGCTGCTCCAGGA

      SalI

      G A A G G A G G P T I E E V D -
2174 GGAGCCCGGAGGAGCTGGAGGACCAACGATCGAGGAGGTGCGACTAATTATTATCTTCTTTTTGATCTCGGTTTTATCTTTATTCTTTTTCCCCC

                                                                                               poly A

2274 AAATCCCCTATATAATCGATCTTCTGCCCTTCCCTGTCAACCACCAACCGTTATTACGCCTAGAATGGTGAACAATAAAAAAATTAATAATCCA
2374 AGTGTGTTGCTTTCGTGTTCTTCCAGCAGAATCGGTTTTAAGGATGCGAGCTTTTTTTCACAATCGGCATATTGTGTGACGCTTGTGCTCTAATC
2474 GTCTTGACAAGCAAGAAATGGATAAAATCAACTCTACAG

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Fig. 2 (concluded).

5' and 3' ends of the intron which are highly conserved (Karn *et al.* 1983; Spieth *et al.* 1985b) the nucleotide substitution rate for the two introns was found to be 12.2% (26/212). The nucleotide substitutions occurred randomly regardless of "codon position." This percentage divergence is similar to the percent divergence of 13.3% calculated for the third codon position. Based on these arguments and the structure of the *hsp-2ps* gene, we conclude that the *hsp-2ps* gene has been non-functional since the duplication event.

To estimate the time since duplication, the number of codons that could allow all four nucleotide substitutions without an amino acid change was determined. There are 157 of these codons shared between the *hsp-1* and *hsp-2ps* genes, 24 of which are substituted at the third position. A percent divergence of these codons was calculated to be 15.3%. This proportion was corrected for multiple hits by assuming a Poisson distribution of mutations ($-3/4 \ln(1 - 4/3f)$, where $f = 15.3\%$) (Ochman and Wilson 1987). The corrected percent divergence is 17.1%. A rate of 1% divergence per million years at synonymous sites has been estimated for the *hsp82* genes in *Drosophila* (Blackman and Meselson 1986) as well as frogs, sea urchins, plants, mammals, and bacteria (Ochman and Wilson 1987). If this is used, then we estimate that the

hsp-1 and *hsp-2ps* genes have been diverging for approximately 17 million years. This suggests that the duplication event probably occurred approximately 8.5 million years ago. G. Poinar has estimated that the time of separation of *C. elegans* and *C. briggsae*, based on taxonomy, the fossil record, and plate tectonics, occurred 20–40 million years ago (personal communication). Our estimate of the time of duplication falls well after the estimated point of species divergence and is consistent with the results of DNA hybridizations involving the *hsp-1* and *hsp-2ps* genes to *C. briggsae* (Snutch 1984).

Hybridization of the *hsp-2ps* gene to several *C. elegans* strains collected from different geographical locations indicates that the *hsp-2ps* gene is present in all laboratory strains tested (Fig. 3). Thus, it appears these strains have originated since the duplication event occurred.

Generation of the *hsp-2ps* gene

The *hsp-2ps* gene is unlike the vitellogenin pseudogene, *vit-1* (Spieth *et al.* 1985a) and the *msp* pseudogenes (Klass *et al.* 1988; Ward *et al.* 1988). These pseudogenes are found within their respective multigene family clusters. The 5' flanking DNA corresponding to the regulatory region is also

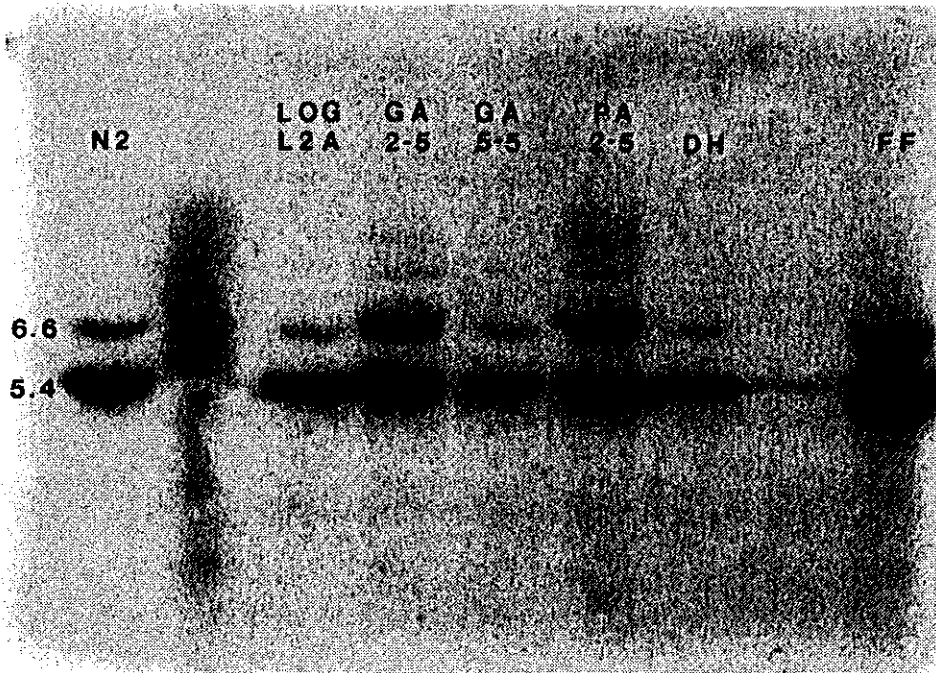


FIG. 3. Hybridization of *hsp-2ps* to different *C. elegans* strains. The *hsp-2ps* plasmid, pCes440, was hybridized to *EcoRI*-digested genomic DNAs of various *C. elegans* laboratory strains. The strain designations are indicated on the autoradiograph. The 5.4 kbp *hsp-2ps* band and the cross hybridizing 6.6 kbp *hsp-1* band are indicated. Hybridizations were done at 68°C in 5× SSPE, 0.3% SDS, and 2.5× Denhardt's (Davis *et al.* 1980).

conserved between these pseudogenes and their functional counterparts. We propose two possible models for the origin of the *hsp-2ps* gene. First, the *hsp-2ps* gene could have arisen through an RNA intermediate, since it has only transcribed DNA sequences from the ancestral gene. It is known that in *Drosophila*, if the heat stress is great enough, introns of all transcripts are not excised (Yost and Lindquist 1986). Based on this, it is possible that the ancestral genome underwent a heat stress great enough to induce heat-shock transcription of the *hsp-1* gene without intron excision. The unprocessed pre-mRNA would then be available to be reverse transcribed. Integration of the cDNA into the DNA of the X chromosome would result in the duplication of the *hsp-1* gene. Second, the *hsp-2ps* gene could have arisen by a DNA duplication event involving a transposition of a copy of the *hsp-1* (LG IV) DNA to LG X. A high amount of restriction fragment length differences has been observed around the *hsp-1* gene between the Bristol and Bergerac strains (Snutch and Baillie 1984). This suggests that the *hsp-1* gene region is prone to nucleotide changes and DNA damage. As such, a stretch of DNA corresponding only to the *hsp-1* gene may have been excised and then transposed to the X chromosome. This may also account for the large internal deletion seen in the *hsp-2ps* gene. Either model could explain the duplication of the *hsp-1* gene. However, as a result of the lack of a proven reverse transcriptase activity in *C. elegans*, it is more likely that the *hsp-2ps* gene arose as an unusual DNA-mediated transposition—duplication event of the *hsp-1* gene.

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