Presence of the *Caenorhabditis elegans* spliced leader on different mRNAs and in different genera of nematodes

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Several different mRNAs from *Caenorhabditis elegans* contain the same 22-nucleotide leader sequence at their 5' ends that is acquired in a *trans*-splicing reaction. About 10 to 15% of the major proteins are translated from mRNAs that contain the spliced leader, among them two ribosomal proteins, ubiquitin, GAPDH, a heat shock protein (hsp70a), and three actins. The same spliced leader sequence is present in mRNAs isolated from nematodes from several different genera; but it is not present in mRNAs from other organisms. The spliced leader is encoded as a spliced leader (SL) RNA about 100 nucleotides long. The gene for the SL RNA is located in the 5S rDNA repeat in *C. elegans*; however, this association with the 5S repeat is not preserved in other genera. The 22-nucleotide spliced leader sequence is conserved in three genera of nematodes.

[Key Words: *Caenorhabditis elegans*; spliced leader; *trans*-splicing]

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Krause and Hirsh (1987) reported finding a common RNA leader sequence on the 5' ends of three different actin mRNAs in the nematode *Caenorhabditis elegans*. The 22-nucleotide leader sequence is spliced onto the actin mRNAs from a separate 100-nucleotide precursor RNA, and this 22-nucleotide sequence became known as the spliced leader (SL), and its precursor, the spliced leader RNA (SL RNA). The mechanism by which the SL is transferred onto the mRNAs appeared to be through *trans*-splicing because (1) the SL and actin genes are widely separated in the genome, (2) a consensus splice donor sequence is present in the SL RNA, and (3) the actin genes contain a consensus splice acceptor where the SL joins the actin mRNA. Detection of a Y-branched intermediate in the SL addition reaction is further support for a *trans*-splicing mechanism (Bektesh and Hirsh 1988).

Trans-splicing in *C. elegans* provoked special interest for several reasons: (1) It was the first discovery of *trans*-splicing of nuclear transcripts in a multicellular eukaryote. (2) *C. elegans* mRNAs contain introns whereas trypanosome mRNAs do not. Therefore, *C. elegans* must carry out conventional *cis*-splicing of internal introns in its actin mRNAs as well as *trans*-splicing of the SL onto the 5' ends of actin mRNAs. (3) Although *C. elegans* contains four actin genes, transcripts from only three receive the SL; transcripts from the fourth gene do not undergo *trans*-splicing. Therefore, only some members of a closely related gene family undergo *trans*-splicing. (4) Examination of the vitellogenin and *unc-54*

myosin genes and their mRNAs indicate that they do not acquire the SL (Spieth et al. 1985; Krause and Hirsh 1987). Therefore, not all of the mRNAs in *C. elegans* undergo *trans*-splicing.

These observations raise questions regarding the generality of the SL. Do other mRNAs in *C. elegans* contain the SL or is it a peculiarity of the three actin genes in which it was originally described? Further, is the SL present in other organisms? As shown here, other mRNAs in *C. elegans* acquire the SL, as do mRNAs from other nematodes. Although we could not find the *C. elegans* 22-nucleotide SL sequence in RNA from other more distantly related organisms, *trans*-splicing may occur, but with different SL sequences.

Results

mRNAs in addition to actin contain SL

The possibility that mRNAs other than actins-1, -2, and -3 contain SL derives from an earlier observation. Some RNAs of higher molecular weights hybridize to a sequence complementary to SL in a Northern blot (Krause and Hirsh 1987). However, at that time the specificity of the hybridization was not established. We show here by additional Northern blot analyses of *C. elegans* RNAs that other mRNAs contain SL. We used a synthetic oligodeoxynucleotide complementary to the SL sequence as the hybridization probe (Fig. 1). This oligodeoxynucleotide hybridizes to many discrete RNAs which partition preferentially to the poly(A)⁺ RNA fraction. The

Figure 1. Analysis of *C. elegans* RNA for SL sequences. *C. elegans* RNA [Poly(A)⁺ and poly(A)⁻, as indicated] was fractionated on a 1.5% agarose-formaldehyde gel and transferred to Zetaprobe. One-half of the blot was hybridized with an oligodeoxynucleotide complementary to the SL sequence. The other half was hybridized with an oligodeoxynucleotide complementary to a portion of the actin coding sequence which is identical in all four actin genes.

most prevalent mRNA species averages about 700 nucleotides. However, larger mRNAs are present, even ones larger than the actin mRNAs which are 1450, 1550, and 1650 nucleotides. The larger RNAs are not actin precursors and the smaller RNAs are not degraded actin mRNAs. This is shown by the control experiment in which an oligodeoxynucleotide complementary to actin coding sequences is used as the hybridization probe (Fig. 1). Only the expected 1450-, 1550-, and 1650-nucleotide actin mRNAs are visible.

The question arises as to how many different mRNAs contain SL. We used hybrid arrest of in vitro translation to estimate the fraction of the proteins in *C. elegans* encoded by mRNAs with an SL. Approximately 200 spots are distinguishable on the two-dimensional gel after the in vitro translation of *C. elegans* RNA in a rabbit reticulocyte lysate (Fig. 2A). However, 32 of these spots are either undetectable or much reduced in intensity if the RNA is first incubated with a synthetic oligodeoxynucleotide complementary to the SL (Fig. 2B).

To verify that synthetic oligodeoxynucleotides specifically inhibit translation of complementary mRNAs, RNA was transcribed in vitro from an individual SL cDNA clone, designated SL52. This cDNA clone, made from an mRNA that contains SL, was constructed as described in the next section. In vitro translation of SL52

mRNA produces a 6-kD protein. Addition of an oligodeoxynucleotide complementary to sequences 5' to the ATG initiation codon of SL52 inhibits SL52 mRNA translation as does addition of an oligodeoxynucleotide complementary to the SL (data not shown). The presence of a nonhybridizing oligodeoxynucleotide has no effect on SL52 translation.

Characterizing SL cDNA clones

To identify the individual mRNAs in addition to the actins that contain SL, we constructed a cDNA library biased for mRNAs containing SL sequences (SL mRNAs). The cDNA library was made by priming

Figure 2. Hybrid arrest of in vitro translation of *C. elegans* RNA. Poly(A)⁺ RNA from *C. elegans* was translated in vitro in rabbit reticulocyte lysates containing [³⁵S]methionine. Labeled products were separated by two-dimensional gel electrophoresis. The products were visualized by fluorography. (A) Translation products from poly(A)⁺ RNA. The arrowheads indicate proteins that are absent or greatly reduced when translation is arrested using an oligodeoxynucleotide complementary to the SL. (B) Translation products from poly(A)⁺ RNA that was first annealed with an oligodeoxynucleotide complementary to the SL before in vitro translation.

second-strand synthesis with a synthetic oligodeoxynucleotide with the same sequence as the SL. To characterize the SL cDNAs, 25 different SL cDNA clones were partially sequenced from both ends of the inserts, and three clones were sequenced completely (Table 1). All 25 clones have the SL at their 5' ends, and the sequence is identical to the SL sequence on the 5' ends of actin-1, -2, and -3 mRNAs (Krause and Hirsh 1987). Eight of the clones (SL118, 24, 10, 77, 57, 52, 109, 5) have a poly(A) region indicating that they are full-length cDNAs. The sequences of the SL clones show that the SL is located from 0 to 38 nucleotides upstream of the ATG; therefore, a fixed distance is not required for either the splicing or function of SLs.

We compared the sequence information from the 25 clones to the NBRF (protein) Database and determined that two clones encode known proteins. Clone SL118 is 87% homologous at the amino acid level to the yeast ribosomal protein L29; clone SL5 is 54% homologous to the rat ribosomal protein L37. Therefore, we believe that SL118 and SL5 encode *C. elegans* ribosomal proteins. Searches of the GenBank nucleic acid database did not identify any other *C. elegans* clone.

We wanted to be certain that SL is present on the 5' ends of *C. elegans* ribosomal protein mRNAs, and that we were not observing nonspecific hybridization or artifacts resulting from cDNA cloning. Therefore, we carried out primer-extension sequencing of *C. elegans* mRNA using oligodeoxynucleotide primers comple-

mentary to sequences found in our cDNA clones. The results confirmed that the SL is present on the 5' ends of the mRNAs that correspond to SL5 and SL118 (Fig. 3).

We also wanted to know if the SL cDNA clones are members of gene families and/or belong to a specific size class of mRNAs. Seventeen clones were used as hybridization probes to Northern blots and to genomic Southern blots. Although most cDNAs hybridized to RNA of 600–700 nucleotides, some hybridized to mRNAs larger than 2 kb (Table 1). The abundances of the mRNAs carrying SL vary depending on the particular mRNA: Clone SL13 appears to represent the most abundant SL mRNA yet observed. Most SL cDNAs hybridized to single-copy genes on genomic Southern blots; however, SL10 is a member of a large, multigene family, and SL42 is a member of a small gene family, possibly containing as few as two members.

Because only two of the 25 sequenced clones could be identified by database searches, we took another approach to identify genes whose mRNAs contain SL. If conventional *cis*-splicing occurs upstream of an ATG, we would expect to find splice donor sequences a short distance upstream of the acceptor sites because most *C. elegans* introns are small (~54 nucleotides). By contrast, if an RNA obtains SL through *trans*-splicing, we would expect to find an acceptor sequence upstream of the

Table 1. Characterization of SL cDNA clones

Clone number	SL-ATG distance	mRNA size ^a	Known gene
118	3	600	L29 yeast
44	17	700	
10	1	1.2 kb/5 kb	
24	8	700	
31	6	800	
17	19	500	
15	13		
77	7	600	
13	6	600	
57	14	500	
37	21	600	
18	3		
47	2	700	
52	38	350	
42	5	400	
109	2	600/900	
72	1	550	
100	17		L37 rat
64	15		
5	0		
33	10		
70	8		
27	0		
101	18		
46	1		

^aIn nucleotides unless designated.

Figure 3. Primer extension sequencing of mRNAs with SL. Synthetic oligodeoxynucleotides were used to sequence the 5' ends of SL5 and SL118 mRNAs. For SL5 the oligodeoxynucleotide primer is complementary to nucleotides 9–26 of the protein coding region (the A of the ATG is position 1). For SL118 the primer is complementary to nucleotides 6–25 of the protein coding region. (Lane E) Extended product in the absence of ddNTPs.

ATG, but no donor site nearby. We searched the available *C. elegans* genomic sequences for genes with this motif. Genes that meet these criteria include glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1a), ubiquitin, and the heat shock gene hsp70a (Yarborough et al. 1987; Graham et al. 1988; Snutch et al. 1988). Therefore, we believed they would have an SL on their mRNAs. Accordingly, we synthesized oligodeoxynucleotides complementary to sequences near, but downstream of, the splice acceptor sequence for each of these genes. These oligodeoxynucleotides were used in primer extension sequencing reactions with *C. elegans* RNA. These reactions revealed that all three of these genes have SL at the 5' ends of their mRNAs (data not shown).

Genomic clones corresponding to SL cDNA clones

The three actin genes whose mRNAs carry an SL are clustered in a 12-kb region of chromosome V; the *act-4* gene, whose mRNA does not have an SL, is on the X chromosome (Files et al. 1983; Albertson 1985). The gene encoding the SL RNA is also on chromosome V, separated by several map units from the actin cluster. We wanted to know the chromosomal locations of other genes whose mRNAs carried SL and, in particular, whether they were also located on chromosome V. Accordingly, we isolated genomic clones from an EMBL-4 library of *C. elegans* DNA that correspond to five of the SL cDNA clones. The clones were mapped to contigs in order to assign them to linkage groups (Coulson et al. 1986). Clone SL52g belongs to a contig that had been mapped to chromosome IV; the other four clones belong to contigs that have not yet been assigned to linkage groups. In addition, the ubiquitin gene is on chromosome III, GAPDH 1a on chromosome II, and hsp70a on chromosome IV (R. Hecht, pers. comm.; Graham et al. 1988; Snutch et al. 1988). Therefore, genes encoding mRNAs that acquire SL are on at least four of the six *C. elegans* linkage groups.

Presence of the *C. elegans* SL in other organisms

We used the *C. elegans* SL hybridization probe to seek a similar SL in other nematode RNAs. RNAs were isolated from: (1) *C. elegans* var. Bergerac (which interbreeds with *C. elegans* var. Bristol, the strain in which the SL was originally found); (2) *C. briggsae* (another species of *Caenorhabditis*); (3) *Panagrellus redivivus* (another free-living nematode); (4) *Ascaris suum* (a parasitic nematode from pigs); (5) *Haemonchus contortus* (a sheep parasitic nematode); and (6) *Anisakis* spp. (a parasitic nematode from fish). The *C. elegans* SL probe hybridizes to a broad spectrum of RNAs of different molecular weights in all of these nematodes (Fig. 4). Based on these hybridization results the SL seems well conserved in the nematodes. However, this SL probe gave no detectable signal with RNA from the trematode *Schistosoma mansoni* (data not shown). We also examined RNAs from distantly related organisms such as yeast, trypanosomes, *Dictyostelium*, *Drosophila*, *Xenopus*,

Figure 4. Analysis of the RNAs from various nematodes using Northern blots. RNAs from different nematodes was separated on a 1.5% agarose-formaldehyde gel and transferred to Zeta-probe. The blot was hybridized with an oligodeoxynucleotide complementary to the SL. (Lane 1) *C. elegans* var. Bristol; (lane 2) *C. elegans* var. Bergerac; (lane 3) *C. briggsae*; (lane 4) *Panagrellus redivivus*; (lane 5) *Ascaris suum*; (lane 6) *Haemonchus contortus*; (lane 7) *Anisakis* spp.

and humans. No hybridization to the SL probe occurred with these RNAs, even at reduced stringency of hybridization (data not shown).

We compared the organization of SL RNA genes in *P. redivivus* and *H. contortus* with that of *C. elegans* by screening genomic libraries. In *C. elegans* most of the SL RNA genes are on a 1-kb tandem repeat (Krause and Hirsh 1987). This repeat also contains the 5S RNA gene (Nelson and Honda 1985). Of the 10,000 phage screened from a *C. elegans* genomic library, 47 hybridized to both SL and 5S probes (Table 2). No phage were isolated that contained only 5S or SL genes.

In *P. redivivus*, most of the clones hybridized either to the SL probe or to 5S probe but not to both (Table 2). However, seven phage were identified that hybridized to both probes. In *H. contortus* no phage were found to contain both the SL and 5S genes, although many were found that contain either SL or 5S. Therefore, the SL is

Table 2. Occurrence of SL and 5S clones in genomic libraries

Species	SL	5S	SL and 5S
<i>C. elegans</i>	0	0	47
<i>P. redivivus</i>	120	29	7
<i>H. contortus</i>	25	20	0

not obligatorily linked to the 5S RNA gene in all nematodes because the average size of genomic DNA in these phage is >12 kb.

We sequenced clones containing SL from *P. redivivus* and *H. contortus*. The 22-nucleotide SL sequence in these nematodes is identical to *C. elegans* (data not shown). The rest of the SL sequence has diverged and we are presently investigating SL RNAs in these other nematodes.

Discussion

The original discovery of *trans*-splicing in *C. elegans* was based on finding the same 22-nucleotide leader sequence on mRNAs transcribed from three different actin genes. The SL probe which is complementary to the central 20 nucleotides of the 22-nucleotide sequence hybridized to other RNAs but the targets of the probe were not known (Krause and Hirsh 1987).

We have now shown that many mRNAs other than actin have an SL sequence at their 5' ends. From hybrid arrest of translation experiments we estimate that 10 to 15% of the proteins are translated from SL mRNAs. This contrasts with results in trypanosomes where complete arrest of protein synthesis was observed in a similar experiment (Walder et al. 1986). In our experiment we used a rabbit reticulocyte in vitro system that reveals the products of approximately 200 of the most highly translated mRNAs. If SL serves either to enhance or to repress translation in vitro then this estimate may be either too high (enhancement) or too low (repression).

The mechanism by which hybridization to the complementary SL sequence blocks translation is not known. It has been shown that oligodeoxynucleotides complementary to the initiation codon region or to sequences 5' of the initiator ATG inhibit translation in a rabbit reticulocyte in vitro system (Liebhaber et al. 1984; Blake et al. 1985). However, oligodeoxynucleotides complementary to coding sequences have no effect on translational elongation (Liebhaber et al. 1984). The heteroduplexes between the oligodeoxynucleotide and the mRNA prevent translational initiation but not elongation. Therefore, our results indicate that 10 to 15% of the mRNAs have SL at their 5' ends and their translation is most likely inhibited by duplex formation.

The fact that mRNAs containing SL are a minority class in *C. elegans* differs strikingly from the situation in trypanosomes. Apparently all mRNAs in these protozoa receive an SL sequence (Walder et al. 1986). That only a minority class of mRNAs in *C. elegans* contains SL raises the question of what special feature they might share. This led us to ask whether the proteins encoded by SL mRNAs share an obvious common physiological function. This required identifying proteins in addition to actins whose mRNAs contain SL.

We used two different approaches to identify proteins in *C. elegans* encoded by SL mRNAs. One approach was to examine known genomic sequences upstream of the open reading frame for a splice acceptor sequence that does not have a splice donor nearby and then do primer

extension sequencing of the corresponding mRNA. This strategy exploits the fact that most *C. elegans* introns are small, so that a donor site for a conventional intron should not be far upstream. Three genes with SL mRNAs (ubiquitin, hsp70a, and GAPDH Ia) were identified in this way. This strategy might also prove useful for finding genes in other organisms that acquire a spliced leader despite the presence of larger introns.

Our other approach was to make a library enriched for SL cDNAs and compare their sequences to the sequences of known genes. In this way we were able to identify 2 of 25 clones. As the sequence databases expand, particularly for *C. elegans* genes, we will be able to identify more of these SL clones. From the genes identified so far, actin, ubiquitin, two ribosomal proteins, hsp70a, and GAPDH Ia, no clear common physiological function emerges. One can envision roles for SL other than a common physiological function for the proteins. For example, a likely role for SL may be to regulate translation either by affecting ribosome binding or mRNA stability.

Actin genes 1, 2, and 3 reside on chromosome V, which is also the location of the 5S rDNA repeat encoding the SL RNA, although the 5S repeat and the actins are far from each other on chromosome V. Nevertheless, this did raise the possibility that linkage was a prerequisite for *trans*-splicing. It is clear now that SL mRNAs are encoded by genes on chromosomes II, III, IV, and V. Although there are some dispersed copies of the SL RNA, no SL RNA gene has been found associated with the 12-kb actin gene cluster (Krause and Hirsh 1987). Nor was a copy of the SL RNA gene detected on the DNAs of the contigs encoding two SL mRNAs, SL5 and SL13 (S. Bektesh and B. Rosenzweig, unpubl.). This is further support for discontinuous synthesis of SL mRNAs.

Although the SL sequence is evolutionarily highly conserved, its location relative to the 5S rDNA repeat is not. In *C. elegans* the 5S rRNA and SL RNA genes are transcribed divergently and separated by only 176 nucleotides (Krause and Hirsh 1987). This finding raised the possibility that their transcription is coupled and possibly regulated by *cis* elements. A second possibility is that the location of the SL RNA in the 5S repeat is a means to insure the abundance of the SL RNA genes. However, in *P. redivivus* and *H. contortus*, there are many copies of SL RNA genes, and they are not associated with the 5S rDNA repeats. Therefore, in these organisms other factors are operating to maintain the high copy number of SL RNA genes. In addition, if transcription of 5S rRNA and SL RNA is coupled, it is probably through *trans* rather than *cis* factors.

Although there does not appear to be a strongly cross-hybridizing SL sequence in other taxa examined, it is possible that other organisms have an SL of different sequence. The trypanosome SL sequence is 35 nucleotides and the *C. elegans* SL only 22 nucleotides, but there are two regions of homology that extend for a total of 11 of 22 nucleotides of the *C. elegans* SL. However, even with reduced stringency, the *C. elegans* SL does not hybridize

with the trypanosome SL. It is not known whether the homology is fortuitous or actually represents conservation of functionally important regions. Only further studies on the mechanisms of *trans*-splicing and the functions of SL will answer these questions.

Materials and methods

RNA isolation and analysis

Total and poly(A)⁺ RNAs were isolated as described previously (Cox et al. 1985). RNA from *Anisakis* spp. and *Schistosoma mansoni* was a gift from Judy Sakanari. RNA from *Ascaris suum* was a gift from Karen Bennett. RNA from *Haemonchus contortus* was a gift from George Cox. For Northern analyses, RNAs were separated on 1.5% agarose/2.2 M formaldehyde/50 mM HEPES (pH 7.8) gels and transferred to Zetaprobe (Bio-Rad) charged nylon membranes in 2× SSC. Oligodeoxynucleotide probes were treated with T4 polynucleotide kinase (Boehringer) by standard procedures and hybridized to filters in 6× SSC, 2× Denhardt's solution, 1% SDS, and 100 µg/ml of tRNA. Hybridizations were done overnight at 2–4°C below the theoretical *T_m* of the oligodeoxynucleotide.

Probes

The SL complementary oligodeoxynucleotide has the sequence TCAAACTTGGGTAATTAAAC. The actin oligodeoxynucleotide probe is complementary to all four actin mRNAs from nucleotides 35 to 52 of the coding region. The sequence is ACATTCCGGATCCATTGT. To prepare a 5S rRNA probe, a cloned 1-kb *Bam*HI 5S rDNA fragment was digested with *Pvu*II which separates 5S and SL RNA sequences (Krause and Hirsh 1987). The 695-bp *Bam*HI–*Pvu*II fragment was gel-purified and nick-translated with [α -³²P]CTP.

Construction of an SL-enriched cDNA library

First-strand cDNA was synthesized using reverse transcription and priming with oligo(dT) on poly(A)⁺ RNA. The RNA was hydrolyzed with 0.25 N NaOH for 30 min at 45°C, neutralized with 0.25 N HCl and 0.25 M Tris-HCl (pH 7.6), phenol-extracted, and ethanol-precipitated. Synthesis of second-strand cDNA was primed with a synthetic oligodeoxynucleotide identical in sequence to the spliced leader. T4 DNA polymerase was used to blunt end the double-stranded cDNA. The cDNA was methylated with *Eco*RI methylase, *Eco*RI linkers were added, and the cDNA library was cloned into the *Eco*RI site of pUC13.

Genomic libraries

The *C. elegans* and *P. redivivus* genomic libraries were a gift from Chris Link (Link et al. 1987). The *H. contortus* EMBL 3 library was a gift from George Cox.

Primer extension sequencing of RNA

Five nanograms of specific α -³²P-labeled oligodeoxynucleotide primer was annealed to 10–60 µg of total RNA in a 12-µl final volume containing 250 mM KCl, 10 mM Tris-HCl (pH 8.3). The mixture was heated to 80°C for 3 min and allowed to anneal for 45 min at a temperature that was 5–7°C below the *T_m* of the oligodeoxynucleotide. Following annealing, 2 µl of template/primer was aliquoted to microfuge tubes containing 1 µl of either ddATP, ddGTP, ddCTP, ddTTP (0.2–1 mM) or no ddNTPS. Then 3.3 µl of reverse transcription buffer containing 24 mM

Tris-HCl (pH 8.3), 16 mM MgCl₂, 8 mM dithiothreitol, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dTTP, 0.8 mM dGTP, 100 µg/ml actinomycin D, and 3–6 units of avian myeloblastosis virus reverse transcriptase were added to each sample and incubated at 50°C for 45 min. Reactions were stopped by addition of 2 µl of 100% formamide containing 0.3% bromphenol blue and 0.3% xylene cyanol. Samples were boiled for 3 min and loaded on either 8% or 10% acrylamide gels containing 8 M urea and 0.5× TBE. Electrophoresis was at 30 watts constant power until bromphenol blue reached the bottom of the gel. Gels were dried and the products visualized by autoradiography.

Hybrid arrested translation

One microgram of poly(A)⁺ RNA from *C. elegans* was translated in rabbit reticulocyte lysates (Promega; 30 µl reaction) containing 50% lysate, 3 µl of [³⁵S]methionine (1400 Ci/mmol), and 33 µM amino acids except methionine. Incubation was at 30°C for 30 min. In reactions containing oligodeoxynucleotides, the RNA was annealed to the oligodeoxynucleotide prior to translation or added to the translation directly. Oligodeoxynucleotides were used at 10 µM in the translation reactions. Reactions were stopped by quick-freezing in dry ice/ethanol.

Two-dimensional gel electrophoresis

Five microliters of in vitro translation products were separated essentially as described by O'Farrell (1975). Electrophoresis in the first dimension was in isoelectrofocusing tube gels and the second dimension was in 10% SDS–polyacrylamide gels.

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