

Genomic Organization and Biosynthesis of Secreted and Cytoplasmic Forms of Gelsolin

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Abstract. Gelsolin is an actin regulatory protein which is unique among vertebrates in that it is found as both an intrinsic cytoplasmic protein and as a secreted plasma protein. We demonstrate that plasma and cytoplasmic gelsolins are derived by alternative transcriptional initiation sites and message processing from a single gene 70 kb long, containing at least 14 exons. Their message and amino acid sequences are identical except at the 5' end/NH₂ termini. The cytoplasmic-specific 5' sequence is derived from two exons

that encode untranslated sequence, while the plasma message-specific 5' sequence is derived from a single exon that encodes untranslated sequence, the signal peptide, and the first 21 residues of the plasma protein. The two transcriptional initiation sites are separated by ≥ 32 kb. Biosynthetic and RNase protection studies indicate that a number of cell types make both plasma and cytoplasmic gelsolin in widely varying amounts and ratios.

GELSOLIN is a multifunctional actin-binding protein which was first identified in rabbit macrophages and subsequently found in a wide variety of vertebrate cells (Yin and Stossel, 1979; Yin et al., 1981; Stossel et al., 1985; Carron et al., 1986; Nodes et al., 1987). Gelsolin binds actin monomers, nucleates actin filament growth, and caps the fast-growing end of actin filaments, thus preventing actin monomer exchange (Bryan and Kurth, 1984; Janmey et al., 1985; Byran and Coluccio, 1985; Stossel et al., 1985). In addition, it severs actin filaments by nonproteolytically breaking the bond between actin monomers in a filament. Gelsolin is activated by μM Ca²⁺, and its effects on actin could cause profound changes in the cytoskeleton of a cell. Recent studies have indicated that, besides Ca²⁺, other regulatory mechanisms may also affect gelsolin-actin interactions in cells (Chaponnier et al., 1987). In vitro studies show that polyphosphoinositides inhibit actin severing by gelsolin and dissociate actin-gelsolin complexes (Janmey and Stossel, 1987; Janmey et al., 1987). These observations suggest that gelsolin may be regulated by a two-signal mechanism involving changes in Ca²⁺ and polyphosphoinositide concentration, and may have an important role in the reorganization of the cytoskeleton during receptor-mediated signaling.

A closely related, slightly larger form of gelsolin has been found in the plasma of every vertebrate species examined (Chapponier et al., 1979; Norberg et al., 1979; Harris et al., 1980). As it binds actin with high affinity and severs actin filaments, plasma gelsolin may be essential for the clearance of actin filaments released into the bloodstream or extracellular space during tissue injury and cell senescence. Consistent with this hypothesis, gelsolin-actin complexes have been

demonstrated in the serum of rats treated intravenously with oleic acid, which causes an acute lung injury syndrome, and in the serum of patients with the adult respiratory distress syndrome (Smith et al., 1988).

Plasma gelsolin is present at ~ 220 $\mu\text{g}/\text{ml}$ in human plasma (Smith et al., 1987). The source of such a substantial amount of extracellular gelsolin has not yet been defined. Biosynthetic labeling studies have shown that the human hepatoma cell line, HepG2, secretes large amounts of plasma gelsolin and synthesizes only a small amount of cytoplasmic gelsolin (Yin et al., 1984). We have recently isolated full-length plasma gelsolin cDNA clones from a HepG2 library and showed that plasma gelsolin mRNA encodes a 27 amino acid NH₂-terminal peptide with characteristic features of a signal peptide, which is not found in the mature protein (Kwiatkowski et al., 1986). At the protein level, plasma gelsolin is very similar to cytoplasmic gelsolin, except that it contains a 25 amino acid residue extension (plasma extension) at its NH₂ terminus, which appears to account for the 3-kD size difference between the two proteins on SDS-polyacrylamide gels (Yin et al., 1984). The plasma gelsolin amino acid sequence, as inferred from cDNA cloning, is identical to all available amino acid sequence (from three regions of the protein, encompassing 89 amino acids) of rabbit macrophage gelsolin, confirming that the two proteins have similar primary structures. Furthermore, Southern blotting studies show that 5' and 3' fragments of the plasma gelsolin cDNA bind to a single band in restriction enzyme-digested human DNA, suggesting that a single gene encodes both human plasma and cytoplasmic gelsolin (Kwiatkowski et al., 1986).

The existence of gelsolin as an intrinsic cytoplasmic and

closely related secreted protein is unique among vertebrates. Its dual localization is distinct from that described for fibronectin or IgM, which are either secreted or inserted into the membrane. However, an example of secreted and cytoplasmic variants of the same protein has been described in yeast. The yeast sucrose-hydrolyzing enzyme invertase is both an intracellular and secreted protein and was demonstrated to be derived from a single gene by alternative processing of message RNA, with selective addition of a signal peptide on the secreted form (Perlman and Halvorson, 1981; Carlson et al., 1983).

To determine how the two forms of gelsolin are derived from the same gene and assess the extent of their structural homology, we have isolated cytoplasmic gelsolin cDNA and gelsolin genomic clones. We report that plasma and cytoplasmic cDNA clones are identical at every base apart from their 5' region, where they diverge entirely. Analysis of genomic clones establishes that a single gene encodes plasma and cytoplasmic gelsolin, with organization of this 5' region [(cytoplasmic) exon 1] intron [(cytoplasmic) exon 2] intron [(plasma) exon 3] intron [(common) exon 4] over a region of 35 kb. RNase-mapping analysis, S1 nuclease analysis, and primer extension studies indicate that initiation of transcription of plasma gelsolin message occurs at the 5' end of exon 3, establishing that the two gelsolins have distinct transcription initiation sites. The common 3' remainder of the gene comprises another 32 kb and ≥ 10 exons. We also show that human fibroblasts, uterus, macrophages, and all cell lines examined contain message for both plasma and cytoplasmic gelsolin, with amounts correlating with biosynthetic rates for the two protein forms.

Materials and Methods

Cell Culture and Biosynthetic Labeling

Human monocytes were isolated from the blood of healthy donors after informed consent and maintained in culture for 3–7 d as described (Cole et al., 1982). During this time, they become adherent to the surface, extend processes, and phagocytose particles, thus acquiring properties of mature macrophages and herein are referred to as macrophages. Human uterus was obtained from surgical specimens of patients undergoing hysterectomy. Primary cultures of human neonatal foreskin fibroblasts were established and maintained in culture as described (Goldring et al., 1979), and used between passages 4 and 15. HepG2 cells were maintained as described previously (Yin et al., 1984). The human epidermoid carcinoma line A431 and the human colonic adenocarcinoma cell line HT29 were both obtained from American Type Culture Collection, Rockville, MD, and maintained as recommended.

Confluent macrophage and fibroblast cultures were labeled for 3 h with L-[35 S]methionine (1.0 Ci/mM, 100 μ Ci/ml; New England Nuclear, Boston, MA) in DME lacking methionine. Supernatant and cellular lysate fractions were collected and equivalent amounts were immunoprecipitated with an anti-gelsolin monoclonal antibody coupled to sepharose beads (Chaponnier et al., 1987). Immunoprecipitated proteins were separated by electrophoresis on linear gradient 5–10% polyacrylamide-SDS gels (Laemmli, 1970).

RNA Isolation and General DNA Methods

RNA was isolated as described (Chirgwin et al., 1979). Briefly, fresh tissue was minced on ice into 0.5-g pieces and homogenized in an eightfold excess volume of guanidinium thiocyanate solution at top speed for 1 m using a polytron homogenizer (Brinkman Instruments Co., Westbury, NY). 1 g CsCl was added per 3.5 ml homogenate and after clarification, was layered over a cushion of 5.7 M CsCl and centrifuged in a rotor (model AH650; Sorvall Instruments Div., Du Pont de Nemours & Co., Inc., Newton, CT) at 35,000 rpm for 8 h. For tissue culture cells, a fivefold excess volume of guanidinium thiocyanate solution was added to the drained dish and addition of CsCl was

omitted. The RNA precipitate was rinsed with 70% ethanol, resuspended in TE, and ethanol precipitated. RNA was quantitated by UV spectroscopy (OD 260) and inspection of ethidium bromide stained formaldehyde gels. Poly A⁺ RNA was selected by chromatography over an oligo (dT) cellulose column (Aviv and Leder, 1972). Phage DNA preparation, plasmid DNA preparation, restriction and other enzyme use was as described (Maniatis et al., 1982). Labeled probes were prepared from gel-purified subcloned cDNA and genomic inserts by the random hexanucleotide method (Feinberg and Vogelstein, 1983). DNA and RNA (formaldehyde) agarose gels were transferred to Genescreen Plus, dried, prehybridized, probed, and washed as recommended by the manufacturer (New England Nuclear, du Pont Co., Wilmington, DE).

Construction and Screening of Libraries

A λ GT11 cDNA library was prepared as described (Ginsburg et al., 1985) from poly A⁺ RNA isolated from day-7 macrophages in culture. This library was the generous gift of Alan Ezekowitz, Children's Hospital Medical Center, Boston, MA. An EMBL3 human genomic library was prepared from a size-fractionated partial Sau3A digest of genomic DNA and was the generous gift of David Bonthron, Children's Hospital Medical Center, Boston, MA.

DNA Sequencing

cDNA and genomic clones were subcloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain termination method (Sanger et al., 1977). All sequencing data presented is the result of determinations on both strands by forced cloning of appropriate small restriction fragments and/or deletional subcloning (Dale et al., 1985). Primers used include the universal primer (No. 1211; New England Biolabs, Beverly, MA), a series of 17 base oligonucleotides complementary to the plasma gelsolin cDNA sequence selected at 300-bp intervals along the cDNA and a 25-base oligonucleotide (complementary to bases 191–215 of the plasma gelsolin cDNA). The oligonucleotides were prepared on a synthesizer (model 470A; Applied Biosystems, Inc., Foster City, CA) by the phosphotriester method. In some cases, ambiguous sequences in GC-rich regions were sequenced by substituting 7-deaza-dGTP (Boehringer-Mannheim Diagnostics, Inc., Houston, TX) for dGTP in the reaction mixtures.

Genomic Structure Determination

Genomic structure was determined by a combination of methods. Eco RI, Bgl II, Hind III, and combined digests of isolated genomic clones were transferred to Genescreen Plus and probed with fragments of the plasma and macrophage gelsolin cDNAs to identify fragments containing coding regions and to delineate a partial ordered map of restriction sites at the genomic level. To complete the map, restriction fragments of genomic clones were used to confirm overlapping regions of the clones and to localize fragments not containing exons.

Primer Extension

2 pmol of the 25-mer oligonucleotide (orientation antisense to plasma gelsolin cDNA, bases 191–215) was labeled with 100 μ Ci γ - 32 P ATP using T4 polynucleotide kinase, phenol/chloroform extracted and ethanol precipitated. 0.01 pmol of the labeled oligonucleotide was annealed to 0.5 μ g polyA⁺ RNA in 10 λ 50 mM Tris-HCl, 0.3 M NaCl, pH 8.0, by heating to 85°C for 5 min, then incubating at 60°C for 1 h. The annealed mixture was then ethanol precipitated and resuspended in 20 λ 140 mM KCl, 100 mM Tris-HCl pH 8.3, 0.5 mM dNTPs, 10 mM MgCl₂, 4 mM dithiothreitol DTT, 8.5 U AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL) and 20 U RNasin (Promega Biotech, Madison, WI) were added and the mixture incubated at 42°C for 1 h. The reaction mix was then treated with 1 λ 0.5 M EDTA and 3 λ 1 M NaOH at 65°C for 30 min to degrade the RNA, ethanol precipitated, and analyzed by gel electrophoresis on a 6% polyacrylamide/7 M urea gel. Molecular weight standards were prepared by T4 polynucleotide kinase/ γ - 32 P ATP labeling of Hinf I and Msp I digests of pBR322.

RNase Protection

Uniformly 32 P-labeled RNA probes were synthesized as described using the pGEM3 and pGEM4 plasmids and SP6 RNA polymerase (Melton et al., 1984). The plasmid constructs were (a) the 5' Eco RI-Bgl II 224-bp fragment of the plasma cDNA (bases 1–224, Fig. 2, b and c) was subcloned in the Eco RI-Bam HI site of pGEM3; (b) the 5' Eco RI-Bgl II 211-bp frag-

ment of the macrophage cDNA (bases 1–211, Fig. 2, *a* and *c*) was subcloned in the Eco RI–Bam HI site of pGEM3. Probes were phenol/chloroform extracted and ethanol precipitated. 1% of the labeled probe was then hybridized to 10 µg RNA or poly A⁺ equivalent in 10λ 80% formamide, 40 mM Pipes, pH 6.7, 0.4 M NaCl, 1 mM EDTA for 6 h at 45°C. 0.1 ml of digestion buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.3 M NaCl, 4 µg/ml RNase A [Sigma Chemical Co., St. Louis, MO]) was then added and incubated at 37°C for 30 min. 7λ 10% SDS and 2λ proteinase K (5 mg/ml) were added and incubated at 37°C for 15 min, and the digestion product was extracted with phenol/chloroform and ethanol precipitated. Protected fragments were analyzed by gel electrophoresis on 6% polyacrylamide-7 M urea gels using labeled Hinf I-digested pBR322 and synthesized labeled RNA probes (from Promega Biotech and generated from appropriate subclones) as standards, followed by autoradiography.

S1 Nuclease Analysis

For S1 nuclease analysis of gelsolin mRNA, hybrid genomic DNA–cDNA constructs were prepared. The cytoplasmic exon-specific construct (CEC)¹ is a hybrid consisting of bases 380–642 of exon 1 (Fig. 4 *a*) and bases 4–211 of cytoplasmic cDNA (Fig. 2, *a* and *c*). The plasma-specific construct (PEC2) consists of bases 1–386 of exon 3 (Fig. 4 *c*) and bases 15–224 of plasma cDNA (Fig. 2, *b* and *c*; plasma cDNA numbering). The plasmids were digested with Sau3A, phosphatased with calf intestinal phosphatase (Boehringer-Mannheim Diagnostics, Inc.), end-labeled with γ³²P ATP with polynucleotide kinase, digested with Ban I and Msp I, respectively, and size-fractionated by PAGE. 10,000 cpm of isolated probe was hybridized to cellular RNA in a solution of 80% formamide, 0.05% SDS, 1 mM EDTA, 10 mM Pipes pH 6.4, 0.4 M NaCl by heating at 90°C for 5 min, then 65°C for 30 min, and then incubated at 42°C for 8–12 h. S1 nuclease (3–100 U; New England Nuclear) was then added in 0.3 M NaCl, 30 mM sodium acetate pH 4.5, 3 mM ZnSO₄, and digestion proceeded for 1 h at 22°C. Digestion products were analyzed on 7 M urea–6% polyacrylamide-sequencing gels. Probes were also sequenced by the Maxam and Gilbert procedure (1977), and run on the same gel.

Results

Comparison of Cytoplasmic and Plasma Gelsolin cDNA

We have previously shown that HepG2 synthesizes primarily plasma gelsolin (Yin et al., 1984), and the predominance of this message form in these cells is indicated by the fact that many plasma but no cytoplasmic gelsolin cDNA clones were isolated from our HepG2 library (Kwiatkowski et al., 1986). In contrast, human macrophages and dermal fibroblasts synthesize primarily cytoplasmic gelsolin and secrete a small amount of plasma gelsolin (Fig. 1). As shown previously, cytoplasmic gelsolin migrates on SDS–polyacrylamide gels as a 90-kD polypeptide, while secreted gelsolin has an apparent molecular mass of 93 kD. Using a 400-bp 5' fragment of the plasma gelsolin cDNA as probe, six cytoplasmic gelsolin clones were isolated from a human macrophage cDNA library out of 500,000 plaques screened. The clone with largest insert is 2.6 kb long, and contains 154 bp of sequence upstream of the first codon (val) of cytoplasmic gelsolin (Fig. 2 *a* and *c*). This codon is immediately preceded by an ATG/met codon with flanking sequences matching the consensus for initiation (Kozak, 1984) at 7–9 bases. Since there are no other initiator methionines in this 5' region, nascent cytoplasmic gelsolin is most likely translated with an NH₂-terminal met (position –1) which is subsequently cleaved to generate the mature protein.

As would be expected for cytoplasmic gelsolin message, the macrophage cDNA is distinct from the previously reported HepG2 plasma gelsolin cDNA (Fig. 2 *b*). It does not encode the 27 amino acid signal peptide nor the unique 25 amino acid sequence at the amino terminus of plasma gel-

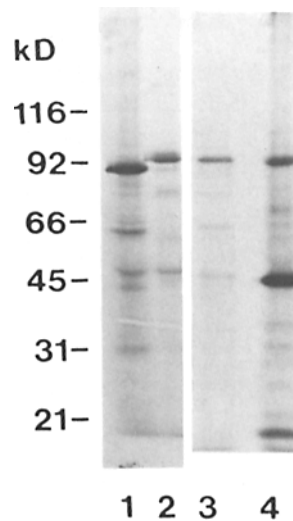


Figure 1. Immunoprecipitation of secreted and intracellular gelsolin from human macrophages and fibroblasts. Day-7 human macrophages and passage-7 foreskin fibroblasts were labeled with [³⁵S]-methionine, and culture supernatants and cellular lysates were immunoprecipitated with anti-gelsolin–Sepharose beads. Radio-labeled bands were detected by autoradiography. Lane 1, macrophage lysate; lane 2, macrophage supernatant; lane 3, fibroblast supernatant; and lane 4, fibroblast lysate. Molecular mass standards in kilodaltons are indicated. The prominent 42-kD band in the fibroblast lysate most likely is actin bound to gelsolin.

solin (plasma extension) (Kwiatkowski et al., 1986). There are 145 bases of unique macrophage cDNA sequence and 158 bases of unique plasma cDNA sequence. Comparison with the corresponding protein sequence indicates that the divergence occurs at residue 21 of the 25 residue plasma gelsolin extension, and is upstream of the initiation met of cytoplasmic gelsolin (Fig. 2 *d*). In contrast, the remaining 2.44 kb of the macrophage cDNA, encompassing 10 bases of 5' untranslated region, the entire coding region of cytoplasmic gelsolin, and the 3' untranslated region is identical to that of plasma gelsolin. Thus, the calculated molecular mass of cytoplasmic gelsolin is 80.5 kD (vs. 83.0 kD for plasma gelsolin).

Mapping the Genomic Structure of Gelsolin

The perfect sequence identity of the 3' 2.44 kb of the cytoplasmic and plasma gelsolin message strongly suggested that they are products of a single gene, in accord with previous indirect evidence based upon Southern blot analyses (Kwiatkowski et al., 1986). The specific differences in their 5' regions were therefore likely to be generated by alternative exon splicing. To determine the genomic structure of gelsolin and the pattern of splicing involved, seven distinct overlapping clones were isolated from a human genomic library prepared in EMBL3 (Fig. 3) by screening with the labeled cDNAs as probes and chromosome walking in the 5' region of the gene. Mapping studies show that they encompass the entire gelsolin gene, spanning a length of 70 kb. There are at least 14 distinct exons, and the first exon that is common to plasma and cytoplasmic message is actually exon 4, located in the middle of the gene.

Genomic fragments containing the first 5 exons within the first 40 kb of the gene were subcloned and sequenced to permit their precise localization (Fig. 4).¹ The unique 145-bp 5' cytoplasmic untranslated cDNA sequence is spliced together from two different exons separated by 13 kb. The first 58 bp of the cDNA sequence is derived from exon 1, while bases 59–145 are derived from exon 2. The contiguous cDNA se-

1. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00817.

a 50
 AGCTGAGCGCAGCTGGACCCAGCAGCCGCTGTCTCCAGTGCCGCGAGCAGAGGTAGTGCTCATAGCTCTCTTTGTCCAGTGCTTCGGCCCT
 100
 TGGTCCAGCGCCTTCCCACGGAGCAGCACTCTTACCCTGCACAGCCTTGTTAG

b 50
 GCCGTGTCGCCACCATGGCTCCGCACCGCCCCGCGCCCGCGTGTCTTTCGCGCTGTCCCTGGCGCTGTGCGCGCTGTGCGCTGCCCGTCC
 100 150
 GCGCGGCCACTGCGTTCGCGGGGGGCGTCCCAGGCGGGGGCGCCCCAGGGGCGGGTGCCTCGAGGCGCGG

c 150 200
 CCCAACAGCATGGTGGTGGAAACACCCCGAGTTCCTCAAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGT
 (M) V V E H P E F L K A G K E P G L Q I W R

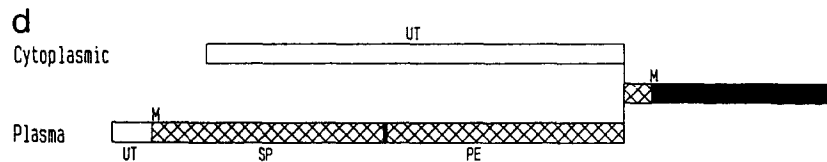


Figure 2. Comparison of 5' sequences of macrophage and plasma cDNA clones. (a) 145 bases of sequence unique to macrophage cDNA clones. (b) 158 bases of sequence unique to plasma gelsolin cDNA clones (Kwiatkowski et al., 1986). (c) Sequence common to both groups of cDNA clones. The deduced sequence of human macrophage gelsolin is shown in the single letter code. The first ATG present in the macrophage sequence is shown in boldface. Numbering is according to the macrophage cDNA sequence. (d) Diagram of unique and common regions of these cDNA clones. (Open box; UT) untranslated; (cross-hatched box) translated in plasma gelsolin only; SP, signal peptide; PE, plasma extension; (solid box) translated in both messages; (M) methionine.

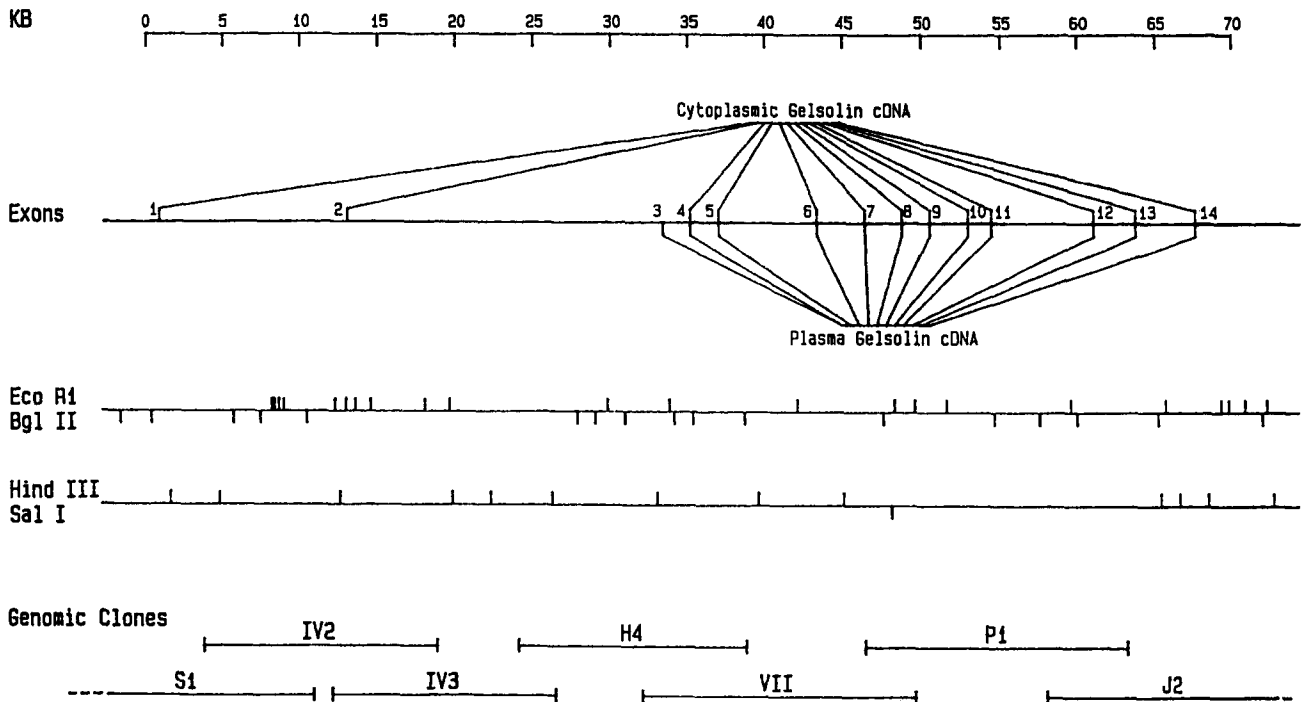


Figure 3. Map of gelsolin gene. DNA size markers are shown at top in kilobases. Overlapping genomic clones are shown at bottom. Eco RI, Bgl II, Hind III, and Sal I restriction enzyme sites are as indicated. Exons and splicing patterns to generate plasma and cytoplasmic gelsolin message are as indicated. Positions of exons 1-5 are localized precisely by DNA sequence while exons 6-14 are localized only to within restriction fragments by blotting studies.

exon 1

1 GAGCTCGCTTGCCCAAAGTTACAGGACTAGTCGATGTCTGGGGTTC... 100
101 AGGGATGCACTTGGCATTTCGTTACTTTCATTTCGGAATGAGCTGGGAATCGCTTTCCTCCTTATTC... 200
201 TGCAGCCTATGCTACTTTCGAGGGCTTTGGCTCCTCGAAGTCCGTTCTCTCCAGGGCTGAAATCTGCCTCCGCTGGTGTAGTTGAGCATTGCTCGCTTG 300
301 CCCGTCCCATCTGAGCTCTTTGGCCCCAGCCAGCTCAGGAGCTGCCCTGGGTCTCCTCCCCAGCCCTAGGCCCTTCAGATCTCCCTCCGTGCTCCGG 400
401 GCCTCAGTTTCTCCAGGGGAAAGCAGAGGGCTTGGTTGGTGCTTCTCTCGAGKTGAAGCGAGGGTCCCCCTGGACGAAGGCGAGCTTGGAGCCACC 500
501 GCCCAGCAGCCGAGGGAGGGCTGGCGCTCTCCCCACCCGCTGCCCGGCCCGCCCGCCGAGGGCGGCTCCCGCCCGCCCTGCCACCCCGGC 600
601 CGCGCGCaccAACGCCCCCGCCCGCCCGCCGGAACAGCTGAGCGCAGCTGGACCAGCAGCCGCTCTCCAGTGCAGCAGCAGGTAGGGAG 700
701 GCGCGCGCCCCGGCCCCGGGCATCTGGGGGTCCCGGGCTGATTGAGTCTTCTCCTTCTTCCGGTCTATCTCGCCCGCCTGGAGCTC 798

exon 2

1 AGGTAGTGAGTGCGGTGTTGCTGGATGTGTGTGCAAGTACTGATTGTGTGACCCCTGGTGGAGGGGAGGAAAGGGGTTGAAACATCAAATGGGGATTG 100
101 CCAGATGACCTTGAACCTCCCTCGAGGCTGACATTCTGTGCCCTTGTTTACAGGTAGTGTCTATAGCTCTCTTTGTCCAGTGTTCGGCTTGGTCCCA 200
201 GCGCCTTCCACGGAGCAGCACTCTTACCCTGCACAGCCTGTTAGGTAGGTAGAGCAATACAGACACCTGTTGTCTCTTTAACCCGCTTGCTGCC 300
301 AGGGAAGCCTGGAGGGGACTTCAGTGGTGAAGCAGCCGCTGTAGCCACAGTGGATTCAAGTGGAGTCCCTGAGGT 376

exon 3

1 AAGCTTTCAAATAATGTTAGTTCATGTTATTTTTTTGTGCGAGGTGTTAGGTGCGGAGAGGCGAGGGGGCTCGCGTGCCTCGCAGGAGGCTCAGCTGGGC 100
101 TCGCCGCGCTCGTGCCTGCGCCATTTAGTGTGCACACAGCTAGCGCTCGCGTATGTGAGGCTGGTGTGGGTCTCCGCCCGGAGCTGGGGTGCA 200
201 GGGTGCCTCGCGCCCTGTCGGGTGATCCGGGTGGGAACCCAGATGTCTCAAGATCCGAGACAGATCCCCGCCCGCGCCCTCCCTGGGGGGCGGTCCC 300
301 GGCTTGGGCGGATGGGCGGGCGGCTACTTAAGGTGCGCGACCCGAGGCGCGGctGCCGACTGGGTCCCTGCGGTGTCCGACCATTGGCTCGCACCG 400
401 CCCCGCGCCGCTGCTTTGCGCGCTGTCCCTGGCGCTGTGCGCGCTGTGCGTCCCGTCCGCGCGGCCACTGCGTCCGGGGGGCGTCCCAGGCGGG 500
501 GCGCCCCAGGGGCGGGTGCCTGAGGCGGGGTGAGTCCCGGGGGCCCCGGGGTCCCGGAGTAACTCTTATTGTAAGTCTTTCGAGATACAGCGCTA 600
601 GGAAGAGGGGAGTAATTCAGGTCTAGAATGGAATAACTGTTTTGTGCTTGTGTAAGTATCTCTGTGCTTCCGGGGCTCTGGCCGAGACGAGGGTGG 700
701 GAGCCTCGGGGCCCTGGCTGTTCTTCCAGGAAGCTAACCTCACCTTGGCCGATTCTTTTCCCTGTCTCCTCTAACATCTTACCTCTGTCTCT 800
801 GAATTCCTCTTCTCTGATTTTTCTCCCTCTGCTCACTTCTCTCTCTCTCTCTCTGATGTCTTCTCCATTTACGATTCCTTGTGTTCTCATTT 900
901 GATCTCTCTCTTCTCAATATTTCTTCTTCTTGTATGACCTCGCCTGTCTGCTTATGGTCATTATTATCATTTCTTCTCTCTCTCTCTCTCT 1000
1001 CTGCCCTGTCTGGTTTCGTGTTTTCCACCCTTCTGCTGAGTCTCCCCACCTTGTCTTCTGACTTCTCTCTCTGATTTTTACTCCACCCAGATCTC 1100
1101 TTCTCCGGCCAGAGGTGACCTGCACCTGCTGATTTTTGTCTCGACTCCAGCCCTAACCCCTCTGCAACCTCCCGGGCCCCAGCCCCAGTGCATGTGTAAT 1200
1201 AGCCTCCCCATTTTAAACCCTTCACTCTCAACAGCCTCCTGAAGACCTGGTGATCTCTTCTCTCTTCAAGAAATGAAATTTGGGGTGGTGTGTT 1300
1301 GTGGGGCAGGAGGAGGAGGAGCAGCAGCGCAGGAGGCCACTGGACTCAGCCATTTGGACCTTCTTGGGGGTATGATGGGGATGGGTACAGAGAGGAGA 1400
1401 GGGGCTCCTGTGGA

exon 4

1 TTGCACTCCAGACTGGACAACAGTGCAGACTCTGTCTCAAAAAAAAAAAAAAAAAAAGAAAAAAAAAGAAAGAAAGAAAGAAAGAAAGAGCAGGGAGGTAGAG 100
101 CCCTCTGTACCCTCCATCACCAGAAAAGCCTGAAGAGGGGCTGAGTAGGAGGACAGATGCTGGCCAGGGCACAGAGGTTTGAAGCATAAAACCTTTG 200
201 CCCTGTTGTGCTGACTCGTTGAGACAGGGTCCCCAGAGGGGATAGACTTCCCTGGGGCTGGGGAGAGCAGGAGGCTCAAGTGAGATGCTCTTGGTGCTA 300
301 GAAACCGCCCTCCCTCATGCTGGGGTCTCTCCCTGCCAGGACCTGCCCGCTTAGGCTCTGCCCTGTCTCATCCAGCCCAACAGCATGGTGGTGGAA 400
401 CACCCCGAGTTCCTCAAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGTGGGAAGTTGATCTGGTCCCGTCCCAACCTTTATGGAGACT 500
501 TCTTACAGGGCGACGCTACGTCATCTGAAGACAGTGCAGCTGAGGAACGGAATCTGCAGTATGACTCCACTACTGGCTGGGTGAGGCTGGCCCTGC 600
601 CCAACCCCTTGGCCAGCCCCATTTCTGAACAGTGCAGACCTTTGGGGCATGGTCCCAGGGAGGGAAGTATTATGAGCACCTAGTATGTGCTGGGC 700
701 CCTTGACTGGTGGTTCATGCCCTGAGACGCTAGAGCCAGCCTGGCCAAGTTCACACCCCACTCTGCCCTAACCCATGTTCTGTCTTTGGCAAGTAC 800
801 TTAGTGTCCCTGTGGAGTGGACATGATGATAGCTCTGCACTACCTCACAGGAATGTTGTGAGCGTTAAACACCTTACTGTGACTCAGAACTGTGGGTG 900
901 AATTGGAGACCTGGACCTCTCCAAACTTCCCTGGCCAGCTCCAGGCACTCCCAGTTG

Figure 4. Genomic sequences of regions containing exons 1-4. Exon sequence, as identified by comparison with cDNA sequence, is shown in boldface. Sequences present upstream of exons 1 and 3 matching the Spl consensus (GGGCGG), in either orientation, are underlined. Transcription initiation sites in exons 1 and 3 are in lowercase, bases 608-612 and 354-356, respectively.

quence (bases 146-350) is found another 23 kb downstream in exon 4, which also codes for bases 159-363 of the plasma gelsolin cDNA. The entire unique 5' plasma gelsolin sequence, bases 1-158, is found in a single exon, exon 3, located 2.3 kb upstream from exon 4. Clearly exon 3, as well as the large adjacent intervening sequence, must be spliced out of the macrophage gelsolin message RNA during processing of the primary transcript.

RNAse Protection Analysis of Gelsolin Message

The identification of specific plasma and cytoplasmic gelsolin exons and the genomic structure presented above are not sufficient to define transcriptional initiation site(s) of the two message forms. In addition, it remains possible, based on the

data presented thus far, that one or both of the macrophage exons are present in the 5' region of plasma gelsolin message, but are missing from the plasma gelsolin cDNAs due to incomplete reverse transcription during cDNA synthesis. This is, however, unlikely because Northern blots probed with gelsolin cDNA indicated that HepG2 and macrophage messages are of equal size (2.7 kb; data not shown), and only slightly larger than the cDNA clones. To examine specifically the 5' ends of plasma and cytoplasmic gelsolin message, RNase protection analysis was performed using subcloned 5' fragments of the plasma and cytoplasmic gelsolin cDNAs (Fig. 5). In each tissue or cell type examined, there was full-length protection (apart from 36 bp of vector sequence) of the plasma and cytoplasmic cDNA-derived labeled RNA

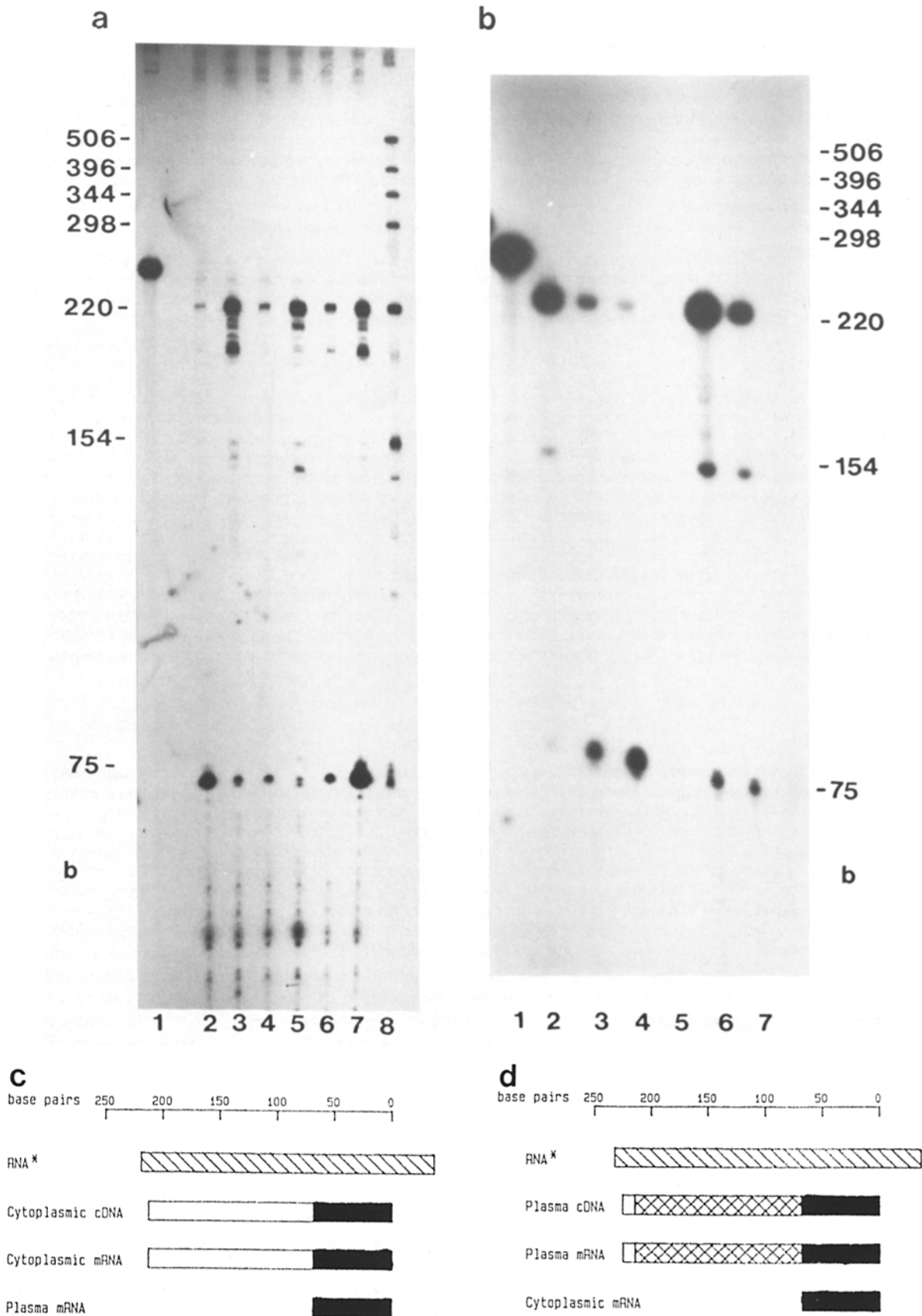


Figure 5. cDNA-derived RNase protection experiment. Uniformly labeled antisense RNA corresponding to macrophage cDNA (bases 1-212, Fig. 2, *a* and *c*) and plasma cDNA (bases 1-225, Fig. 2, *b* and *c*) were hybridized with 10 μ g total RNA. Protected fragments were separated on sequencing gels and detected by autoradiography. (*a*) Macrophage cDNA-derived RNase protection. Lane 1, undigested mac-

probes, suggesting that the cells express both message forms. However, the intensity of the ~230-base fully protected band varies with the cell type. The plasma cDNA protection results (Fig. 5 *b*) indicate that HepG2 and uterus contain abundant plasma gelsolin message, HT29 has an intermediate amount, and macrophages and fibroblasts have much less. Because the plasma probe also contains, in addition to 157 bases of unique plasma cDNA sequence, 67 bases of common sequence (Fig. 5 *d*), it is partially protected from RNase digestion when hybridized to cytoplasmic gelsolin message. Accordingly, a ~73-base protected band is observed in each case, corresponding to that predicted (plus a few bases of incompletely digested overhang). By comparing the relative intensities of the fully with partially protected bands (and allowing for the decreased amount of radioactivity of the shortened probe), we conclude that HepG2, uterus, and HT29 contain more plasma than cytoplasmic gelsolin message, while the reverse is true for fibroblasts and macrophages. The plasma gelsolin message levels correlate with protein biosynthetic data presented previously (Yin et al., 1984) and shown above (Fig. 1). In Fig. 5 *b*, lanes 2, 6, and 7, a ~150-base protected fragment was also observed and its intensity appears to be proportional to that of the fully protected band. It was not seen consistently and may be derived from partial digestion of the 230-base fragment. As expected, no protection was observed with yeast RNA (Fig. 5 *b*, lane 5).

Parallel RNase protection analysis with the cytoplasmic cDNA-derived RNA probe confirms the authenticity of the macrophage cDNA sequence and the relative abundance of the two message forms (Fig. 5 *a*). Major bands of ~220 (cytoplasmic) and ~73 bp (plasma) are seen. Furthermore, these experiments exclude the possibility that exons 1 and 2 are present at the 5' end of plasma gelsolin message, because the corresponding protected fragments are not found (58, 87, or 145 bp for exon 1, exon 2, or both, respectively). Therefore, exons 1 and 2 are contained only in cytoplasmic gelsolin message. In Fig. 5 *a*, lanes 2, 4, and 6 a series of bands representing fragments of 190–200 bases are present. These were seen inconsistently and, as above, appear to be partial digestion products derived from full-length hybridization.

Transcription Initiates at Different Sites for Plasma and Cytoplasmic Gelsolin

Having demonstrated that cytoplasmic and plasma gelsolin message contain distinct 5' exons, we sought to define their transcription initiation site(s) by primer extension and S1 nuclease protection analysis. To define the transcription site of plasma gelsolin message, a hybrid exon 3–plasma cDNA

construct (PEC2) was used in S1 nuclease analysis of uterine poly A⁺ RNA (Fig. 6, *left*). At low concentrations of S1 (Fig. 6, *left*, lanes 3 and 4), five protected bands were seen. The upper bands disappeared with increasing S1 concentration, but the lower three bands remained through the highest concentration used, which in comparison with the Maxam–Gilbert sequence reactions (Fig. 6, *left*, lanes 1 and 2), indicate that plasma gelsolin message extends to bases 354–356 of exon 3 (Fig. 4 *c*). Primer extension products of uterine and HepG2 poly A⁺ RNA using an oligonucleotide primer (plasma cDNA, bases 191–215), are seen in Fig. 6, *left*, lanes 7 and 8, respectively. Although the oligonucleotide primer used will hybridize to both plasma and cytoplasmic gelsolin message, uterine and HepG2 poly A⁺ RNA have mostly plasma message (Fig. 5), so the extension products seen are derived from plasma gelsolin message (see also below). Since the extension products are 9 bases shorter than the S1 nuclease protected fragments, and the S1 nuclease probe begins 9 bases 5' of the oligonucleotide primer, we conclude that transcription of plasma message initiates in exon 3 at bases 354–356. The heterogeneity of bands seen in the S1 nuclease analysis is also seen, to a lesser degree, in the primer extension analysis and suggests that initiation of transcription occurs at all three of these residues (G, C, T) for plasma gelsolin message.

The region directly upstream of this initiation site contains multiple copies of the Sp1 promoter sequence (GGGCGG) (Fig. 4 *c*) (Dyban and Tjian, 1985) in both orientations and has extremely high GC content (76% in bases 38–571). It also contains a "TATA" boxlike sequence (Breathnach and Chambon, 1981) at the appropriate distance (bases 326–332) upstream of the plasma gelsolin mRNA start site. However, this sequence does not fit the consensus perfectly, and no "CAAT" box sequence is seen.

The transcription initiation site for cytoplasmic gelsolin has not been located precisely. Primer extension analysis of tissues rich in cytoplasmic gelsolin message with the oligonucleotide primer described above and a second oligonucleotide derived from exon 1 sequence (Fig. 2 *a*, bases 7–23) failed to give a clear result. With the first primer, multiple extension products that are shorter than the defined length of cytoplasmic gelsolin mRNA from the isolated cDNA are seen (data not shown); no extension was detected with the second oligonucleotide. S1 nuclease analysis of macrophage RNA (Fig. 6 *b*), using a hybrid exon 1–cytoplasmic cDNA construct (CEC), indicated that transcription within exon 1 extends to base ~610 (Fig. 4 *c*). The region upstream of this site contains multiple copies of the Sp1 consensus sequence and has 74% GC content, (bases 301–798, Fig. 4 *a*), but also does not contain a classic TATA or CAAT box.

rophage cDNA probe; lanes 2–7, respectively, HepG2, fibroblasts, A431, macrophage, HT29, and uterus RNA hybridized with the macrophage cDNA probe. The size of labeled DNA standards, loaded in lane 8, are indicated on the left. (*b*) Plasma cDNA-derived RNase protection; lane 1, undigested plasma gelsolin cDNA probe; lanes 2–7, respectively, HepG2, fibroblast, macrophage, yeast, uterus, and HT29 RNA hybridized with the plasma cDNA probe. DNA standards are indicated on the right. (*c*) Diagram illustrating macrophage cDNA-derived RNase protection. (*Top to bottom*) RNA probe including vector sequence; cytoplasmic cDNA; protected fragment resulting from probe hybridization to cytoplasmic message; protected fragment resulting from hybridization to plasma gelsolin message. (*Open box*) Cytoplasmic-specific sequence; (*solid box*) common sequence. (*d*) Diagram illustrating plasma cDNA-derived RNase protection. (*Top to bottom*) RNA probe including vector sequence; plasma gelsolin cDNA; protected fragment resulting from probe hybridization to plasma message; protected fragment resulting from hybridization to cytoplasmic message. (*Open box*) Plasma 5' untranslated sequence; (*cross-hatched box*) plasma specific-coding sequence; (*solid box*) common sequence.

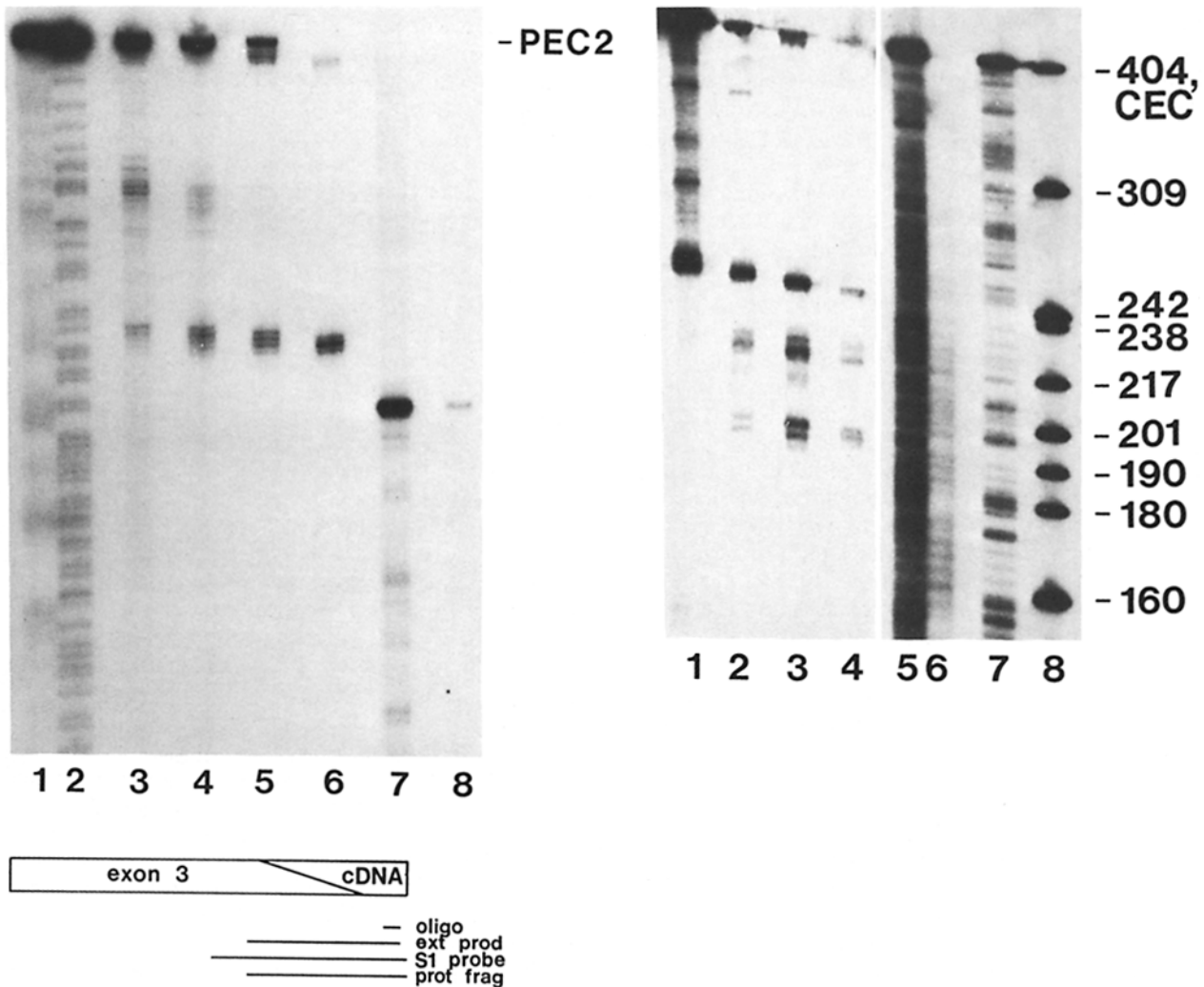


Figure 6. Determination of plasma gelsolin transcription initiation site, and S1 nuclease analysis of cytoplasmic gelsolin message. (*Left*) S1 nuclease and primer extension analyses of plasma gelsolin mRNA. The end-labeled hybrid exon 3-plasma gelsolin cDNA fragment (*PEC2*) was used for S1 nuclease analysis (see Materials and Methods). Lanes 1 and 2, Maxam-Gilbert sequencing reactions of *PEC2* (A > C, GA, respectively). Lanes 3-6, S1 nuclease analysis of 0.5 μ g poly A⁺ human uterus RNA; after hybridization 3, 10, 30, and 100 U S1 nuclease was added to the samples loaded in lanes 3, 4, 5, and 6, respectively. Lanes 7 and 8, primer extension using a 25-base oligonucleotide complementary in sequence to bases 191-215 of the plasma cDNA, of uterine poly A⁺ (1.0 μ g) and HepG2 poly A⁺ (0.5 μ g), respectively. (*Right*) S1 nuclease analysis of cytoplasmic gelsolin mRNA. The end-labeled hybrid exon 1-cytoplasmic gelsolin cDNA fragment (*CEC*) was used (see Materials and Methods). Lanes 1-4, S1 nuclease analysis of 10 μ g human macrophage RNA; after hybridization 3, 10, 30, and 100 U S1 nuclease was added to the samples loaded in lanes 1, 2, 3, and 4, respectively. Lanes 5-7, Maxam-Gilbert sequencing reactions of *CEC* (GA, CT, A > C, respectively). Lane 8, labeled *MspI*-digested pBR322 standards, with size in bases as indicated. Lanes 1-4 and 5-8 were contiguous on the same gel but autoradiograph exposures were 4 and 1 d, respectively.

Discussion

We demonstrate that human plasma and cytoplasmic gelsolins are derived from a single gene through the use of unique transcriptional initiation sites and that the plasma specific exon (exon 3) is excluded from mature cytoplasmic gelsolin message during message processing. The final protein products are identical except at their amino termini. In addition, we show by biosynthetic labeling and RNase protection studies that a wide variety of cell types synthesize both forms of gelsolin in varying amounts. The simultaneous production of an intrinsic cytoplasmic protein and a secreted protein is unique among vertebrate cells. However, a similar situation has been described for the yeast sucrose-hydrolyzing en-

zyme, invertase (Perlman and Halvorson, 1981; Carlson et al., 1983). *Saccharomyces cerevisiae* produce both a constant unregulated amount of intracellular invertase and a secreted invertase, which is highly regulatable in response to reduced extracellular glucose. The two proteins are encoded by two message forms transcribed from a single gene through the use of two distinct transcriptional initiation sites. The start site for secreted invertase is \sim 100 bases upstream of that for the intracellular form (Carlson et al., 1983). The organization of the gelsolin gene is similar but remarkable for the presence of large introns between the plasma and cytoplasmic exons, the wide separation of transcriptional initiation sites, and the use of exon skipping to generate the cytoplas-

mic message. Exons comprising the first 400 bp of gelsolin message RNA are derived from over 35 kb of the genome and transcription initiation sites for the two message forms are separated by ≥ 32 kb. The plasma specific 5' exon must be removed during cytoplasmic message RNA processing, since it is downstream of the cytoplasmic 5' exons. This arrangement implies that the splicing event that joins exons 2 and 4 must be favored over that joining exons 3 and 4, such that cytoplasmic gelsolin message is generated in high yield when transcription initiates at exon 1. Similar alternative splicing mechanisms are well-recognized among mammalian genes (Breitbart et al., 1987), but how choices are made among alternative pathways is poorly understood.

Translation of the cytoplasmic RNA begins at met-1 relative to the mature sequence and this methionine is proteolytically excised during processing. The amino-terminal amino acid sequences of pig plasma and pig platelet gelsolin have been recently reported (Weeds et al., 1986) and are interesting to compare with these data. Pig plasma gelsolin has only a 9 residue extension (vs. 25 in human plasma gelsolin) before matching the pig platelet (and rabbit macrophage) sequence. The last residue of the pig plasma extension is also a methionine, suggesting a similar genomic organization. Chicken plasma gelsolin does not contain a plasma extension, and its amino-terminal sequence is identical to human cytoplasmic gelsolin at 9 of 11 residues (Nodes et al., 1987), indicating that gelsolin is highly conserved among both mammalian and avian species.

We showed that, both at the protein and message levels, plasma and cytoplasmic gelsolins are present in a wide variety of tissues but in highly variable amounts and ratios. Use of unique transcriptional initiation sites is one obvious method for providing for independent regulation of synthesis of the two protein forms. Both S1 nuclease protection and primer extension analysis indicate that plasma gelsolin message transcription initiates within the plasma specific exon 3, at 1 of 3 bases (Figs. 3, 4, and 6). The region upstream of this transcription initiation site is somewhat unusual in that neither a CAAT homology nor clear TATA homology (Breathnach and Chambon, 1981) is seen. There is, however, a region of some resemblance to the TATA element (bases 326-332) as well as multiple copies in both orientations of the Sp1 promoter consensus GGGCGG (Dyan and Tjian, 1985). TATA and CAAT elements, although common, are not universally present in the 5' untranslated regions of mammalian genes. The hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase gene (Osborne et al., 1985), for example, contains neither TATA nor CAAT elements. We were unable to obtain reproducible primer extension results using tissues rich in cytoplasmic gelsolin message but S1 nuclease analysis and the high GC content of the surrounding region suggests transcription initiates within exon 1. As for plasma gelsolin message, the region upstream of exon 1 contains multiple copies of the Sp1 promoter consensus. We predict these Sp1 sequences are essential for transcription of plasma and cytoplasmic gelsolin message. Clearly other, as yet unidentified, distinct enhancer elements must be present near exons 1 and 3 to account for the independent transcriptional regulation of the two message forms. The large separation between transcription initiation sites may be essential to permit this regulation. The relatively large size of the gelsolin gene (70 kb)

is in sharp contrast to β -actin which has only five small introns and an overall size of 2.8 kb (Ng et al., 1985).

The presence of plasma gelsolin message in a variety of cell types, and its abundance in uterus, were unexpected. This result is in agreement with the recent report that chicken gizzard, a smooth muscle, devotes 1% of its total protein synthetic output to the production of chicken plasma gelsolin (Nodes et al., 1987). By comparison, chicken liver makes relatively little plasma gelsolin. A more comprehensive and quantitative analysis will be required to define major contributory sites of plasma gelsolin synthesis in man. Nonetheless, these observations indicate that secreted gelsolin is synthesized in a variety of tissues and allow us to speculate that secretion of gelsolin may be locally regulated in response to external stimuli. In view of the hypothesized role of gelsolin in the clearance of actin released from dying cells, it may be advantageous to have gelsolin secreted directly at sites where high levels of cell death occur, exposing actin cytoskeletons. Our demonstration that cytoplasmic gelsolin message is present in all tissues examined confirms that gelsolin is widely distributed and that it may participate in the restructuring of the cytoskeleton and initiate motile responses in a variety of cells after receptor-mediated signaling.

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