where E_{rev} is the reversal potential; [D] and [M] are concentrations and P_D and P_M are the permeabilities of the divalent and monovalent ions, respectively; a_D and a_M are activity coefficients; R is the Boltzmann constant; T is temperature; and F is the Faraday constant.

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$$P_0 = \frac{\sum_{i=0}^{n} i \cdot A_i}{n \sum_{i=0}^{n} A_i} \quad (13)$$

where n is the number of channels in the patch, P_0 is the probability that any one channel is open, and A_i is the area under the peaks with i channels open (*14, 19*).

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Structure, Expression, and Antisense Inhibition of the Systemin Precursor Gene

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A gene that encodes systemin, a mobile 18–amino acid polypeptide inducer of proteinase inhibitor synthesis in tomato and potato leaves, has been isolated from tomato, *Lycopersicon esculentum*. Induction of proteinase inhibitors in plants is a response to insect or pathogen attacks. The gene has 10 introns and 11 exons, ten of which are organized as five homologous pairs with an unrelated sequence in the eleventh, encoding systemin. Systemin is proteolytically processed from a 200–amino acid precursor protein, prosystemin. Prosystemin messenger RNA was found in all organs of the plant except the roots and was systemically wound-inducible in leaves. Tomato plants transformed with an antisense prosystemin complementary DNA exhibited greatly suppressed systemic wound induction of proteinase Inhibitor I and II synthesis in leaves.

PLANTS HAVE INDUCIBLE DEFENSES in response to pathogen or herbivore attacks (*1–4*), including systemic synthesis and accumulation of serine proteinase inhibitors that inhibit the digestive proteinases of insects and microorganisms (*5–8*). The wound-induced synthesis of such inhibitors results from transcriptional activation of the inhibitor genes (*9*) and has been described in a variety of species including tomato (*10, 11*), potato (*12*), alfalfa (*13, 14*), cucurbits (*15*), and poplar trees (*16*). Wounding results in the rapid accumulation of inhibitors not only in wounded leaves but also in distal, unwounded leaves, indicating that a signal, or signals, released from the wound site travels throughout the plant. Proposed signals include pectic fragments derived from the plant cell wall (*17*), the lipid-derived molecule jasmonic acid (*18*), the plant hormone abscisic acid (*19*), electrical potentials (*20, 21*), and an 18–amino acid polypeptide called systemin (*22*). Systemin was isolated from the leaves of tomato plants where it induces the synthesis of two proteinase inhibitors. Radioactively labeled systemin, when applied to a wound site, was rapidly translocated to distal tissues. We now report the cloning and characterization of the cDNA and gene encoding systemin.

We isolated a systemin cDNA by screening a primary cDNA library synthesized from tomato leaf mRNA with an oligonucleotide corresponding to amino acids 12 through 18 of systemin (*23*). Approximately 50 positive clones were identified and rescreened with a second oligonucleotide corresponding to amino acids 1 to 6 of systemin. Of the initial positive clones, only one hybridized to the second probe; it was a partial cDNA encoding the systemin polypeptide within a larger protein, prosystemin.

This partial cDNA consisted of 839 bp, although Northern (RNA) blot analysis indicated that the systemin mRNA was 1 kb. We determined the complete prosystemin mRNA sequence by sequencing the prosystemin gene (*24*) and mapping the transcriptional start site (*25*). The open reading frame was 600 bp encoding a 200–amino acid prosystemin protein (Fig. 1). Identification of the initiating methionine codon was made on the basis of two criteria: multiple stop codons immediately 5′ to the methionine codon and an adjacent sequence similar to the plant consensus sequence for translational initiation (*26*).

Of the 200–amino acid prosystemin, amino acids 179 through 196 encode systemin. Prosystemin contains a high percentage of charged amino acids (aspartic acid, 10%; glutamic acid, 17%; lysine, 15%) but very few hydrophobic amino acids and is therefore quite hydrophilic. We did not find a hydrophobic region at the NH_2-terminus that resembled a leader peptide, and the posttranslational processing pathway and site of subcellular compartmentalization of prosystemin are undetermined. Neither the cDNA nor the deduced protein precursor sequences had homologs in either GenBank or the European Molecular Biology Laboratory data bank.

The putative processing sites bordering systemin did not conform to the consensus sequence for endoproteolytic processing sites flanking bioactive peptides in animal prohormone precursors (*27*). The animal consensus sequence was, however, found once in prosystemin at amino acid residues 183 through 188, which are part of the mature systemin polypeptide. The half-life of systemin may be regulated by further processing at this site. In animal systems prohormones are often processed to yield multiple bioactive peptides (*28, 29*), although we have no evidence to suggest that other bioactive polypeptides are derived from prosystemin.

Prosystemin is encoded by a single gene that consists of 11 exons and 10 introns (Fig. 2, A and B). The transcriptional start

```
  1  MGTPSYDIKNKGDDMQEEPKVKLHH
 26  EKGGDEKEKIIEKETPSQDINNKDT
 51  ISSYVLRDDTQEIPKMEHEEGGYVK
 76  EKIVEKETISQYIIKIEGDDDAQEK
101  LKVEYEEEEYEKEKIVEKETPSQDI
126  NNKGDDAQEKPKVEHEEGDDKETPS
151  QDIIKMEGEGALEITKVVCEKIIVR
176  EDLAVQSKPPSKRDPPKMQTDNNKL
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Fig. 1. Amino acid sequence of prosystemin (*38*). The systemin sequence is underscored with a double line. A polypeptide sequence element repeated five times is underscored with a single line. GenBank accession numbers for prosystemin cDNA and gene are M84800 and M84801, respectively.

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site was established by both primer extension and mung bean nuclease analysis. The first ten exons are organized as pairs, and the last (single) exon encodes systemin. The nucleotide sequence of each of the five exon pairs in prosystemin is homologous to that of every other pair, which suggests that they evolved by successive duplication or elongation events from a common ancestor. Consistent with the exon repeats, a 6– to 9–amino acid imperfect repeat occurred five times within the deduced polypeptide sequence (Fig. 1). The repeated polypeptide sequence is encoded within the first exon of each pair. In contrast, the 18–amino acid systemin sequence occurred only once within the precursor, close to the COOH-terminus, and the sequence of the exon encoding systemin is unrelated to those of the other exons.

To determine if prosystemin gene homologs are found in other plant species, we performed Southern (DNA) and Northern blot analysis on genomic DNA and total RNA from three species known to possess wound-inducible proteinase inhibitors—potato (*Solanum tuberosum*, var. Russett Burbank) (*12*), tobacco (*Nicotiana tabacum*, var. Xanthi) (*30*), and alfalfa (*Medicago sativa*, var. Vernema) (*13, 14*)—and from one species, *Arabidopsis thaliana*, var. Columbia, in which wound-inducible proteinase inhibitors have not been reported. Of the four, a homolog of the prosystemin gene was identified only in potato (Fig. 2C), the nearest relative to tomato. An mRNA species that hybridized to the prosystemin cDNA and comigrated with the tomato prosystemin mRNA was also identified in potato. We were unable to detect a homolog in the others even at greatly reduced hybridization and wash stringencies (*31*).

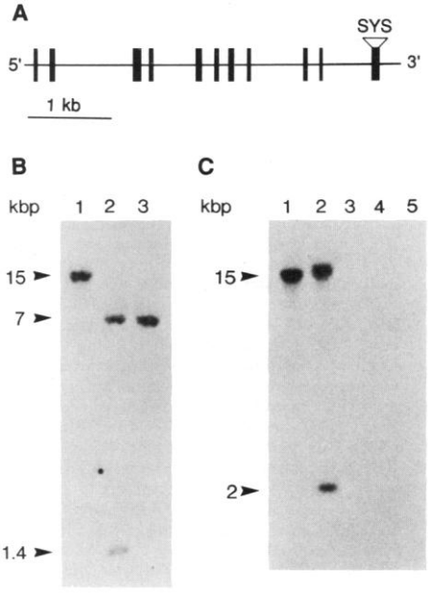

Fig. 2. (**A**) Structure of the prosystemin gene. The gene consists of a 104-bp 5′ untranslated region, a 4176-bp coding region composed of 11 exons (vertical bars) interrupted by 10 introns, and a 246-bp 3′ untranslated region. The position of systemin is indicated by SYS. (**B**) Southern blot analysis of the prosystemin gene. Tomato genomic DNA was isolated from leaves as described (*39*) and 5 μg were digested with either Eco RI (lane 1), Bgl II (lane 2), or Sca I (lane 3). Samples were separated by electrophoresis on a 0.8% agarose gel and then hybridized with nick-translated prosystemin cDNA (*37*). (**C**) Southern blot analysis of the species distribution of prosystemin gene homologs. Genomic DNA (5 μg) from tomato (lane 1), potato (lane 2), tobacco (lane 3), alfalfa (lane 4), and *Arabidopsis* (lane 5) was digested with Eco RI and separated by electrophoresis on a 0.8% agarose gel. The gel was blotted onto nitrocellulose and probed with nick-translated prosystemin cDNA as described (*37*), except that the blot was washed at 55°C.

Next, we investigated the possibility that the prosystemin gene, itself, might be wound-inducible. We used Northern blot analysis to examine the amount of prosystemin mRNA and Inhibitor I mRNA in leaves of wounded tomato plants (Fig. 3A). Prosystemin mRNA accumulated in the upper, unwounded leaves of young tomato plants that had been wounded on the lower leaves, demonstrating that prosystemin mRNA, like Inhibitor I mRNA, is systemically wound-inducible. Amounts of prosystemin mRNA were highest 3 to 4 hours after wounding, whereas Inhibitor I mRNA was most abundant 8 to 10 hours after wounding. Although Inhibitor I mRNA was absent from the leaves of unwounded tomato plants, a small amount of prosystemin mRNA was detected there (Fig. 3A), which may provide a continuous supply of systemin and allow the plant to respond immediately to wounding. Accumulation of prosystemin mRNA in unwounded as well as wounded tissue may amplify the ability of the plant to react to subsequent damage. Continued damage by insect attacks would therefore liberate more systemin from the newly synthesized precursor than initial wounds, resulting in increased synthesis of proteinase inhibitors as the attacks persist. Because the initial rate of accumulation of prosystemin mRNA was faster than that of Inhibitor I mRNA in response to wounding (Fig. 3A), some aspects of the signal transduction pathways activating the two genes may differ.

Because prosystemin mRNA was found throughout the plants except their roots (Fig. 3B), wounding of any aerial part would be expected to result in the systemic induction of proteinase inhibitor synthesis, although we have observed the induction of proteinase inhibitor synthesis in tomato leaves in response to wounding of their roots (*32*). It is possible that their roots either contain a small amount of prosystemin mRNA or use a different wound signal or signals to activate proteinase inhibitor genes in the leaves. The prosystemin mRNA signal in unwounded leaves is stronger in Fig. 3A than in Fig. 3B. This difference may reflect a higher constitutive prosystemin mRNA level in mature plants or, more likely, differences in the specific activities of the probes and in the exposure times of the two blots.

To determine if the prosystemin gene product is important in the signal transduction pathway leading to the expression of proteinase inhibitor genes in tomato leaves, we transformed tomato plants with a prosystemin antisense gene. The chimeric gene was composed of prosystemin cDNA, in the antisense orientation, under the con-

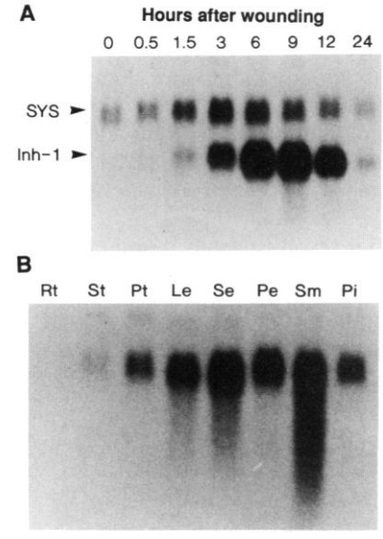

Fig. 3. (**A**) Northern blot analysis of the induction of prosystemin mRNA and Inhibitor I mRNA after wounding. Thirty-two young tomato plants were wounded 3 weeks after germination. The plants had an upper and a lower leaf and a small apical leaf. The lower leaf was wounded and mRNA was isolated from the upper, unwounded leaf at 0.5, 1.5, 3, 6, 9, 12, and 24 hours after wounding. Four plants were used at each time. Total RNA (5 μg) at each time was separated by electrophoresis on a 1.4% agarose-formaldehyde gel and blotted onto nitrocellulose. The blot was probed simultaneously with nick-translated prosystemin (SYS) and Inhibitor I (Inh-1) cDNAs (*37*). (**B**) Northern blot analysis of the distribution of prosystemin mRNA in various parts of an unwounded, fully grown tomato plant. Total RNA was extracted from the following parts: root (Rt); stem (St); petiole (Pt); leaf (Le); sepal (Se); petal (Pe); stamen (Sm); and pistil (Pi). Total RNA (5 μg) from each sample was separated by electrophoresis and blotted as described in (A). The blot was probed with nick-translated prosystemin cDNA (*37*).

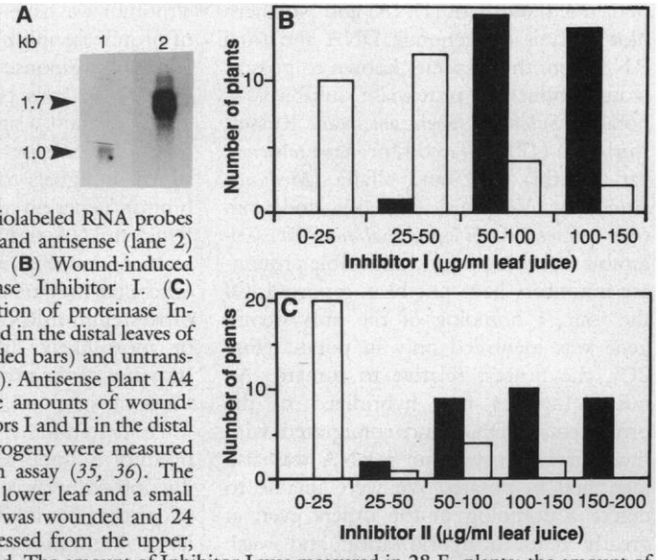

Fig. 4. (**A**) Northern blot analysis of total RNA extracted from antisense plant 1A4. Two samples of total RNA (5 μg) were separated by electrophoresis and blotted as described in Fig. 3. The samples were probed separately with radiolabeled RNA probes specific for sense (lane 1) and antisense (lane 2) prosystemin mRNA (*40*). (**B**) Wound-induced accumulation of proteinase Inhibitor I. (**C**) Wound-induced accumulation of proteinase Inhibitor II. Both accumulated in the distal leaves of F_1 antisense plants (unshaded bars) and untransformed controls (solid bars). Antisense plant 1A4 was self-fertilized, and the amounts of wound-induced proteinase Inhibitors I and II in the distal leaves of 3-week-old F_1 progeny were measured by radial immunodiffusion assay (*35, 36*). The plants had an upper and a lower leaf and a small apical leaf. The lower leaf was wounded and 24 hours later juice was expressed from the upper, unwounded leaf and assayed. The amount of Inhibitor I was measured in 28 F_1 plants; the amount of Inhibitor II was measured in 27 of the 28 F_1 plants. A control group of 30 untransformed tomato plants, var. Better Boy, was also wounded and the amounts of Inhibitors I and II measured. Inhibitor proteins were not detected in juice expressed from the leaves of six unwounded antisense plants and six unwounded control plants.

trol of the constitutive cauliflower mosaic virus 35S promoter incorporated within the binary vector pGA 643 (*33*). Tomato plants transformed with the binary vector alone served as controls, and 18 antisense plants and 21 controls were regenerated (*34*).

Three weeks after the transformed plants had been transferred to soil the lower leaves on each plant were extensively wounded; the amounts of wound-inducible proteinase Inhibitors I and II were determined in the expressed juice of upper leaves 24 hours later (*35, 36*). None of the plants produced either Inhibitor I or Inhibitor II in their upper leaves before wounding. Of the 18 plants with the antisense gene, 11 plants produced Inhibitor I at less than 40% of the mean control level of 126.7 ± 8.2 μg per milliliter of leaf juice and Inhibitor II at less than 30% of the mean control level of 164.7 ± 18.6 μg/ml.

One of the antisense plants, designated 1A4, was analyzed for wound-inducible Inhibitor I and II accumulation. When the plant's lower leaves were wounded, its upper, unwounded leaves expressed Inhibitor I at 42 μg/ml and Inhibitor II at 41 μg/ml. The antisense RNA appeared as a band of approximately 1.7 kb and the prosystemin mRNA as a 1-kb band. Southern blot analysis showed that plant 1A4 contained a single copy of the antisense construct, which we confirmed by self-fertilizing plant 1A4 and analyzing its 28 progeny by Southern blot analysis. Seven (one-quarter) of the F_1 progeny did not inherit the antisense construct.

To demonstrate that the antisense phenotype segregated with the antisense construct, we measured the amounts of Inhibitors I and II in the upper, unwounded leaves of the 28 F_1 plants 24 hours after wounding the lower leaves. Three-quarters of the plants inheriting the antisense construct responded more weakly to wounding than the control population of untransformed plants (Fig. 4, B and C). Plants not inheriting the construct produced amounts of proteinase inhibitors equal to those of the untransformed control plants. In six of the F_1 plants that inherited the antisense gene, Inhibitor I synthesis in the upper, unwounded leaves of plants wounded on the lower leaves was less than 15% of the mean control level of 97.2 ± 4.7 μg/ml while Inhibitor II synthesis was undetectable in the upper leaf (mean control level, 122.3 ± 7.2 μg/ml). Southern blot analysis of the six least responsive F_1 plants suggests that these plants inherited two copies of the antisense construct.

We have shown that systemin, perhaps the first polypeptide hormone found in plants, is derived from a larger propeptide, a feature commonly found in animal systems. The expression of a gene containing an antisense cDNA encoding prosystemin resulted in almost complete suppression of the systemic wound induction of proteinase inhibitors and suggests that systemin is an integral component of the signal transduction system that regulates the synthesis of proteinase inhibitor proteins in response to herbivore attack.

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31. Aliquots of genomic DNA (5 μg) from the five plant species described in Fig. 2C were digested with Xba I, Eco RI, and Sca I, separated by electrophoresis on a 0.8% agarose gel, and blotted onto nitrocellulose. The blot was hybridized as described (*37*) but without formamide. The blot was washed at 55°C in 1× SSC and 0.1% SDS.
32. B. McGurl and C. A. Ryan, unpublished observations.
33. The 747-bp fragment used as an antisense gene was excised from the prosystemin cDNA as a Bam HI–Hind III fragment. The antisense fragment thus duplicated the prosystemin mRNA sequence from the eighth amino acid through 154 bp of the 3′ untranslated region. The antisense construct was transformed into *Agrobacterium* strain LBA 4404 and the recombinant bacteria were used to transform tomato, var. Better Boy, as described (*34*).
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38. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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41. We thank C. Palm and S. Thompson for help with sequence analysis, G. Munske for synthesizing oligonucleotides, and G. Wichelns for growing tomato plants. Supported in part by NSF grants DCB-8702538 and DCB-8608594; Project 1791, College of Agriculture and Home Economics, Washington State University; and the McKnight Foundation.

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Transcription Factor Loading on the MMTV Promoter: A Bimodal Mechanism for Promoter Activation

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The mouse mammary tumor virus (MMTV) promoter attains a phased array of six nucleosomes when introduced into rodent cells. This architecture excludes nuclear factor 1/CCAAT transcription factor (NF1/CTF) from the promoter before glucocorticoid treatment and hormone-dependent access of nucleolytic agents to promoter DNA. In contrast, when the promoter was transiently introduced into cells, NF1/CTF was bound constitutively and nucleolytic attack was hormone-independent. Thus, induction at this promoter was a bimodal process involving receptor-dependent remodeling of chromatin that allows NF1/CTF loading and direct receptor-mediated recruitment of additional transcription factors.

EUKARYOTIC DNA IS PACKAGED into chromatin (*1–3*), which can confer selective access of regulatory factors to their DNA binding sites (*4–6*). Thus, the structure of eukaryotic DNA in chromatin may be important in its interaction with soluble transcription factors (*6–9*). When the MMTV promoter is stably introduced into cells, it acquires a series of six positioned nucleosomes (*3, 10*). One of these nucleosomes (nuc-B) is positioned over binding sites for a group of transcription factors involved in promoter activation, including the glucocorticoid receptor (GR) and NF1. Hormone induction of the promoter results in hypersensitivity around nuc-B to endonucleases, chemical agents, and restriction endonucleases (*3, 6*). In parallel with this structural transition, hormone-dependent loading of a transcription preinitiation complex including NF1 has been demonstrated in vivo (*5*).

The exclusion of NF1 from promoter DNA in noninduced cells was puzzling, given its high affinity for its binding site on purified MMTV DNA (*11*). Further, no alterations in NF1 nuclear concentration or intrinsic binding affinity accompany hormone activation (*12*). Thus, we tested whether NF1 was excluded from the template by nucleoprotein structure before the hormone-dependent remodeling of the chromatin. We compared the interaction of NF1 with MMTV DNA organized in stably replicated chromatin and with an identical promoter introduced into the cell by transient transfection of a nonreplicating plasmid. The MMTV promoter, driving a reporter gene, luciferase (LUC), was transiently introduced into cells that had a stably replicating copy of the identical MMTV long terminal repeat (LTR) driving a second reporter gene, chloramphenicol acetyl transferase (CAT). Expression from each of the two classes of MMTV promoter was examined by use of reporter-specific primers for primer extension and the respective enzyme assays. The assays demonstrated that both promoters are active and strongly hormone inducible (Fig. 1). The degree of induction from the stable promoter was three- to fourfold greater than that from the transient construct (*13*), raising the possibility that the transient promoter may be derepressed (*14, 15*).

To monitor NF1-template interactions, we performed in vivo exonuclease III footprinting in combination with linear amplification by Taq polymerase (*5, 16*). Nuclei were isolated from cells incubated in the presence or absence of dexamethasone and digested with a restriction enzyme whose cleavage site was in a nucleosomal linker (*6*). The nuclei were digested with Exo III, the DNA was purified, and positions at which Exo III digestion was blocked were detected by extension with gene-specific primers that hybridized downstream of the anticipated Exo III boundary. We confirmed the previously reported finding (*5*) that NF1 was excluded from its binding site in the absence of glucocorticoid treatment on stable chromatin templates (Fig. 2, lane 2). Administration of dexamethasone resulted in a hormone-dependent Exo III block at position −82 (Fig. 2, lane 4). Hormone-dependent occupancy of the NF1 site in vivo was confirmed by deoxyribonuclease I footprinting (*15*).

In contrast, NF1 was constitutively present on transient DNA templates (Fig. 2, lanes 5 and 6). Digestion by Exo III was blocked at the NF1 site in cells in the absence of hormone. Furthermore, hormone induction produces no increase in occupancy at the NF1 site. Thus, in nuclei isolated from the same cells, the MMTV promoter was organized into two distinct classes with respect to NF1 loading (Fig. 2, lanes 2 and 4 through 6).

We examined the status of factors downstream of the NF1 site with the use of an Exo III entry point at +107 of the promoter, a site that was specific for the transient template (*6*). Digestion with Exo III revealed a stop at position −56 (Fig. 2, lanes 7 and 8), which corresponded precisely to the 3′ Exo III boundary of NF1 determined in vitro (*5, 12*). Identification of the 3′ boundary of NF1 in vivo confirmed the

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