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An Allele of the *Prot* Locus in Maize Is a Variant for the Site of Protein Processing

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An allele of the Prot *locus, which encodes a major globulin of the maize scutellum, is a variant for a site of protein processing. Segregation analysis and recombination mapping indicate that the variant is an allele of the* Prot *locus. Designated* Prot-V, *this allele specifies three polypeptides, V1, V2, and V3. The V1 polypeptide is incompletely processed during the proteolytic processing step catalyzed by the product of the* Mep *locus. Cyanogen bromide cleavage studies support the precursor–product relationship between V1 and V2. The V1 product is shortened with respect to other PROT′ proteins and it is postulated that the normal site of MEP processing has been removed by this foreshortening.*

KEY WORDS: protein processing; globulins; embryo storage protein; *Zea mays*.

INTRODUCTION

Many proteins are processed by the removal of a portion of the molecule to reach the mature state. Examples in plants of such processing include the storage proteins napin (Crouch *et al.*, 1983) and vicilin (Spencer *et al.*, 1983). The product of the *Prot* locus in maize also undergoes cleavage, maturing from a prePROT′ to PROT′ to PROT. The second step is catalyzed by the product of the *Mep* locus (Schwartz, 1979; Kriz and Schwartz, 1986).

The *Prot* locus was identified by Schwartz (1979) as producing a major

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protein of the scutellum. The product has subsequently been characterized as a globulin (Kriz and Schwartz, 1986) which may function as a storage protein for the scutellum (Dierks-Ventling, 1982; Khavkin *et al.*, 1978). The gene is polymorphic for alleles that produce proteins of different apparent molecular weights as well as for CRM^- null alleles. The products of these alleles can be readily distinguished on one-dimensional (1-D) slab sodium dodecyl sulfate (SDS)–polyacrylamide gels. The polypeptide produced by each allele is subjected to at least two modifications that alter the electrophoretic mobility on SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The conversion of PROT′ to PROT is mediated by the product of the *Mep* locus, and *mep/mep* embryos accumulated PROT′. prePROT′ is converted to PROT′ by an unknown mechanism but the process involves modification to the amino and carboxy termini.

In an examination of lines for variants in *Prot* expression, a new pattern was found with three polypeptide bands that appeared to be cosegregating. Genetic and biochemical analyses have shown that this pattern can be attributed to an allele of the *Prot* locus. In keeping with the size designation of the *Prot* alleles, this allele has been designated *Prot-V* for *V*ery small, indicating that it specifies smaller polypeptides than the *Prot-S* allele. This report provides evidence that the *Prot-V* allele is a variant for the site of the protein processing pathway determined by the *Mep* locus.

MATERIALS AND METHODS

The lines used in these crosses were derived from lines inbred for *Adh1* alleles. The source of the *Prot-V* allele was a Central American line; the original material has been lost (Dlouhy, personal communication). The *Prot-O* allele was from Black Beauty pop obtained from the Maize Genetics Coop Stock Center.

Partial protein purification from scutellar material was performed by either of two methods. To obtain material for molecular weight determinations and cyanogen bromide cleavages, kernels were soaked overnight in distilled water at 30°C and the scutella were excised. The pooled scutella were homogenized in 5 mM phosphate buffer, *p*H 7.5, using a mortar and pestle. Ammonium sulfate cuts of 60 and 90% were done, and the precipitate from each was resuspended in 1 M NaCl and centrifuged to clear the solution. The protein solution was then dialyzed against two changes of distilled water over a 6-hr period. The dialysate was centrifuged to recover the precipitated protein. This resulted in a preparation that is greatly enriched for the globulins.

For handling individual scutella the ammonium sulfate precipitations were omitted and the excised scutella were homogenized in 1 ml of 1 M NaCl

using a mortar and pestle. The homogenate was centrifuged in 1.5-ml microfuge tubes for 5 min and the supernatant was transferred to dialysis bags. Dialysis was against 1 liter of distilled water, with two changes over a 3-hr period. The dialysate was centrifuged in microfuge tubes and the pellet was resuspended in sample buffer (0.0625 M Tris–HCl, *p*H 6.8, 10% glycerol, 2.3% SDS, and 5% 2-mercaptoethanol). This procedure enriches for the globulin fraction of the proteins and facilitates the interpretation of the SDS-PAGE protein profiles.

Polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). Protein molecular weight was estimated by comparison with the migration of marker proteins purchased from Sigma Chemical Company. Electrophoresis of ADH isozymes was performed using starch gels as described by Schwartz and Endo (1966).

For cyanogen bromide cleavage analysis, samples containing 100 μg of total protein were fractionated by SDS-PAGE on gels containing 10% acrylamide. Following staining with Coomassie brilliant blue R-250, slices containing the proteins to be studied were excised from the gel with a razor blade. One and one-half slice was subjected to cyanogen bromide cleavage, whereas one-half slice, to be used as the uncleaved control, was equilibrated with sample buffer. Slices to be treated were placed in the bottom of glass 12-ml conical centrifuge tubes and soaked with 1 ml of 88% formic acid; the tube was occasionally shaken during the 1-hr incubation at room temperature. The formic acid was removed and 0.2 ml of fresh formic acid was added to cover the gel slices. The slices were treated with 10 mg of cyanogen bromide in a fume hood for 20 hr and the solutions were neutralized by the addition of 600 μl of concentrated ammonium hydroxide followed by the addition of 20 μl of 0.1% bromphenol blue; additional ammonium hydroxide was added if necessary to generate a blue color. The samples were incubated at room temperature on a shaker for 4 hr and additional ammonium hydroxide was added if necessary to maintain the blue color. The slices were incubated in four changes of denaturing solution over a 2-day period to equilibrate fully, and the treated and control slices were loaded onto a 12.5% acrylamide gel for fractionation. Following electrophoresis, the gel was silver-stained by the procedure of Morrissey (1981).

RESULTS

The electrophoretic fractionation of the saline soluble proteins from *Prot-S* and *Prot-V* homozygotes maize scutella is shown in Fig. 1. These results were obtained using a 10% acrylamide gel in the absence of urea. Some attempts were made to fractionate these proteins in the presence of 6 M urea, but the bands were markedly less distinct and were poorly resolved. Molecular

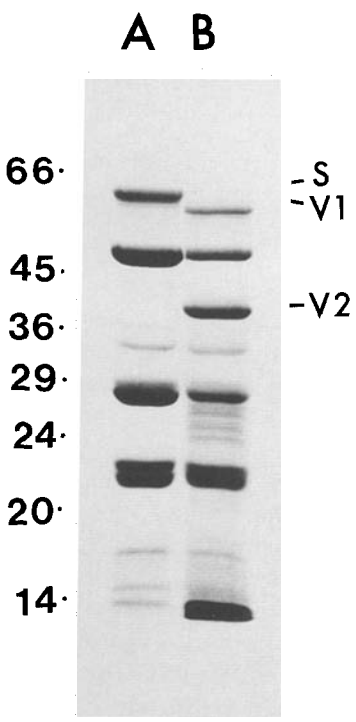

Fig. 1. Molecular weight determinations of the *Prot-V* proteins. The partially purified globulin fraction from scutellum was subject to SDS–polyacrylamide gel electrophoresis using a 10% polyacrylamide gel. Lane A is the globulin fraction from a *Prot-S* homozygote. Lane B is the globulin fraction from a *Prot-V* homozygote. Molecular weight (kD) is indicated on the side.

weights of the proteins were estimated by electrophoretic migration on 11 separate determinations involving three different extracts of *Prot-S* and *Prot-V* homozygotes. The molecular weight estimates were as follows: PROT-S, 61,000; PROT-V1, 58,000; and PROT-V2, 39,000. The standard deviation for these values was less than 2000. An additional difference between the *Prot-S* and the *Prot-V* samples was the appearance of an additional protein with an approximate molecular weight of 13,500 in the *Prot-V* material. The appearance of this polypeptide, designated PROT-V3, was sporadic. Following loading of the samples shown on this gel, the sample in denaturing solution was stored at 4°C for 1 week and analyzed again by SDS-PAGE. On this second gel, the 13,500 protein was not evident, but there was a light smear of staining material in the 10,000 to 20,000 fractionation range of the gel. The inability to fractionate this polypeptide consistently may be due to its stability in the denaturing buffer, but this has not been investigated further.

The *Prot-V* allele was shown to be allelic to the *Prot* locus by genetic analysis. *Prot-V* and *Prot-S* homozygotes were crossed to produce an F_1. The F_1 kernels showed the polypeptides expressed by both *Prot-S* and *Prot-V* homozygotes. When F_1 plants were self-pollinated, three types of progeny were detected: those containing *Prot-S* proteins alone, both *Prot-S* and *Prot-V*

Table I. Results of Segregation Analysis[a]

P: *Prot-S/Prot-S* × *Prot-V/Prot-V*
F_1: *Prot-S/Prot-V*

	Prot-S/Prot-S	*Prot-S/Prot-V*	*Prot-V/Prot-V*	Total
Self	90	186	102	378
Backcross by *Prot-S/Prot-S*	67	61		128
Backcross by *Prot-V/Prot-V*		96	88	184
Cross by *Prot-O/Prot-O*	48		47	95

[a]Numbers given are of kernels exhibiting the given genotype.

proteins, and *Prot-V* protein alone. These kernels occurred at the ratio of 1:2:1 (Table I). When an F_1 plant was backcrossed to the *Prot-S* parent two types of progeny were detected: The *Prot-S* homozygote and the *Prot-S/Prot-V* heterozygote. In the backcross to the *Prot-V* parent the two progeny types were the *Prot-V* homozygote and the heterozygote. In both backcrosses the ratios of the progeny types are 1:1 (Table I). The F_1 was also crossed by a plant homozygous for a null allele for the *Prot* locus, *Prot-O*. This fails to produce any PROT protein (Schwartz, 1979). The progeny of this cross were tested and only two types of progeny were seen: either *Prot-S* or *Prot-V* (Table I). Thus, the PROT-V polypeptides behave as if specified by an allele of the *Prot* locus.

A second test to show the allelic relationship of the *Prot-V* allele was a check of the linkage relationships. Schwartz (1979) had shown that the *Prot* gene was linked to the proximal side of *Adh1* with 13.1% recombination. *Prot-V* homozygotes, which were also homozygous for *Bz2* and *Adh1-F*, were crossed to plants homozygous for *bz2*, *Prot-S*, and *Adh1-C*. The F_1 was

Table II. Results of a Three-Point Cross to Determine Linkage Relationships

Cross: $\frac{Bz2\ Prot\text{-}V\ Adh1\text{-}F}{bz2\ Prot\text{-}S\ Adh1\text{-}C} \times \frac{bz2\ Prot\text{-}S\ Adh1\text{-}C}{bz2\ Prot\text{-}S\ Adh1\text{-}C}$

Genotypes			Individuals
Bz2/bz2	*Prot-V/Prot-S*	*Adh1-F/Adh1-C*	165
		Adh1-C/Adh1-C	32
	Prot-S/Prot-S	*Adh1-F/Adh1-C*	2
		Adh1-C/Adh1-C	33
bz2/bz2	*Prot-V/Prot-S*	*Adh1-F/Adh1-C*	31
		Adh1-C/Adh1-C	3
	Prot-S/Prot-S	*Adh1-F/Adh1-C*	29
		Adh1-C/Adh1-C	173

backcrossed by the *bz2 Prot-S Adh1-C* parent. Kernels were classified as bronze (*bz2/bz2*) or purple (*Bz2/bz2*). Each kernel was then split in half, and the scutellar proteins were extracted from one half for analysis on SDS-PAGE and the other half was analyzed by starch gel electrophoresis to determine the *Adh* genotype. The results are presented in Table II. A recombination frequency of 14.7% was observed between *Bz2* and *Prot* and a frequency of 14.1% between *Prot* and *Adh1*. This agrees with the results obtained by Schwartz (1979) of 15.5 and 13.1%, respectively.

To test the effect of the *Mep* gene on the products of the *Prot-V* allele, plants homozygous for the recessive allele *mep* were crossed by *Prot-V* homozygotes and the resulting F_1 was self-pollinated. The *mep* allele is linked to the *pr* allele by 13 map units (Schwartz, 1979) and the *Prot-V* line carries the dominant allele *Pr*. In the F_2, 76% of the red kernels will be homozygous for *mep*, while only 8% of the purple will be recessive homozygotes. Thus kernels were classified as red (*pr/pr*) or purple (*Pr/−*). After the seeds were sorted by color, individual scutella were excised and processed as described above. Examples of each class are shown in Fig. 2.

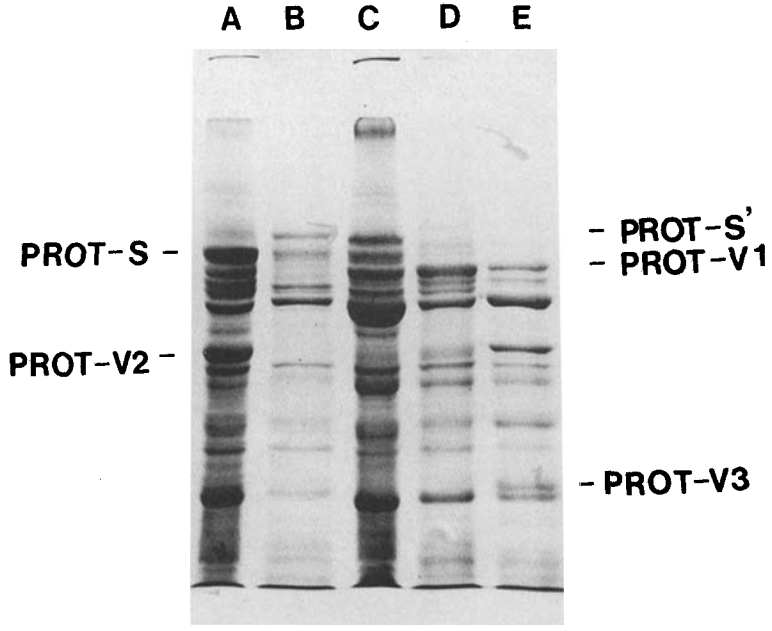

Fig. 2. Effect of *Mep* processing on PROT-V. Extracts from individual kernels were processed to enrich for globulins. The resulting extracts were separated by SDS-PAGE on a 10% polyacrylamide gel. The genotypes are as follows: (A) *Prot-S/Prot-V; Mep/−;* (B) *Prot-S/Prot-S;mep/mep;* (C) *Prot-S/Prot-V;mep/mep;* (D) *Prot-V/Prot-V;mep/mep;* (E) *Prot-V/Prot-V;Mep/−*.

The red class of kernels was segregating for *Prot-S* and *Prot-V*. Of the 52 kernels examined, 15 were homozygous for *Prot-S*, 16 were homozygous for *Prot-V*, and 21 were heterozygous. Eleven of the fifteen *Prot-S* homozygotes had a reduced amount of PROT-S and an elevated amount of PROT′-S, which is the phenotype expected for *mep/mep* kernels. Of the 16 *Prot-V* homozygotes, 10 had decreased or lost the V2 polypeptide as well as the 13-kD V3 polypeptide. It is unclear whether the V1 polypeptide is increased or remains unaffected in these *mep/mep* scutella. Sixteen of the twenty-one heterozygotes showed the shift of PROT-S to PROT′-S. This confirms that these kernels are *mep/mep*. These kernels also show the loss of the 39-kD V2 polypeptide and the 13-kD V3 polypeptide but still retain the V1 polypeptide. The molecular weight of these two polypeptides is consistent with a processing step catalyzed by the product of the *Mep* locus which cleaves the 58-kD polypeptide into the 39- and 13-kD polypeptides. Anomalous migration of the 13-kD polypeptide could result in an inaccurate molecular weight determination, which would account for the apparent discrepancy in the sizes. These results indicate that the 58-kD polypeptide is the PROT′ protein form for the *Prot-V* allele.

To examine further the relationship of the *Prot-V* and *Prot-S* specific proteins, the polypeptides were subjected to cyanogen bromide cleavage. Kriz and Schwartz (1986) have shown that the size difference between PROT and PROT′ is evident in a large fragment (40–45 kD), while differences among the size variants are associated with a small fragment (25–30 kD). Partially purified extracts from *Prot-V* and *Prot-S* kernels were fractionated by SDS-PAGE. The 58- and 39-kD polypeptides of *Prot-V* and the 61-kD PROT-S polypeptide were excised from the gel, incubated with cyanogen bromide, and analyzed by SDS-PAGE (Fig. 3). The approximate sizes of the major fragments observed following cyanogen bromide treatment are shown in Table III. All cleavages appear to be partial since each treated sample contains a polypeptide of the same size as the corresponding untreated control sample.

The cyanogen bromide cleavage patterns of the *Prot-V* proteins differ significantly from the *Prot-S* protein. Cleavage of the *Prot-S* protein produces a pattern with a protein band at 40-kD and an apparent doublet at 27–28 kD. This pattern suggests that there is at least one internal methionyl residue. Kriz and Schwartz (1986) interpret the apparent doublet as a single band. Close examination of their results suggests that the band could be a doublet, which is consistent with the results presented here. The doublet could be the result of two methionyl residues in close proximity. An alternative explanation is that the polypeptide is capable of forming secondary structures which would alter the electrophoretic mobility. There must be some secondary interactions in this region of the protein. In lane H in Fig. 3 the uncleaved control for V2 has

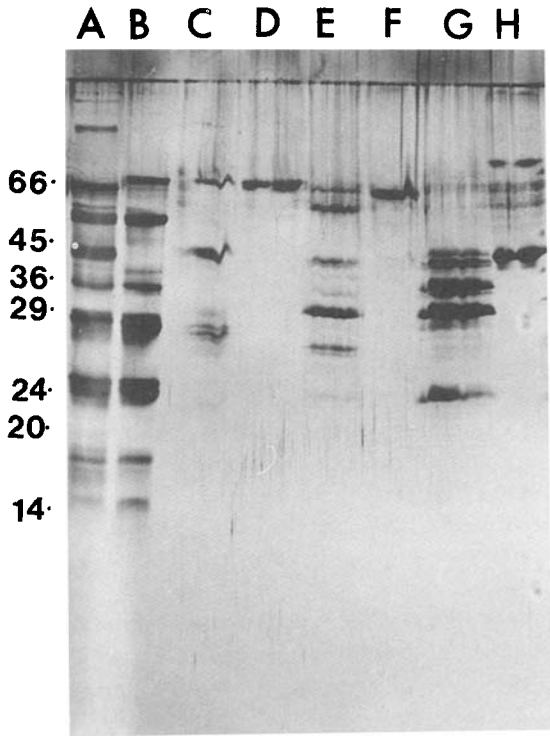

Fig. 3. Cyanogen bromide cleavage patterns of PROT proteins. The partially purified globulins were fractionated on a 10% polyacrylamide–SDS gel. The protein bands were excised from the gel. One-fourth of each sample was left untreated as an uncleaved control. The remainder of each sample was incubated with cyanogen bromide. The samples were fractionated on a 12.5% polyacrylamide gel. (A) Globulin fraction from *Prot-V* homozygote. (B) Globulin fraction from *Prot-S* homozygote. (C) Cyanogen bromide cleavage of PROT-S. (D) Untreated PROT-S. (E) Cyanogen bromide cleavage of PROT-V1. (F) Untreated PROT-V1. (G) Cyanogen bromide cleavage of PROT-V2. (H) Untreated PROT-V2. Molecular weight (kD) is indicated on the side.

an 80-kD protein which must be a dimer of V2. Many of the polypeptides in lane G also appear to migrate as doublets. Both V1 and V2 are cleaved into multiple polypeptides. These patterns suggest that there are at least four internal methionyl residues present in V1 and three in V2.

It is possible to construct a map for the methionyl residues that is consistent with the polypeptides produced by cyanogen bromide cleavage (Fig. 4). The positions in this map are expressed as kilodaltons, with the end of

Table III. Polypeptide Molecular Weights Resulting From Cyanogen Bromide Cleavage[a]

Polypeptide	MW	Cleavage products
S	61	40, 28, 27
V1	58	52, 38, 29, 26
V2	39	33, 29, 21

[a]All sizes are given as kilodaltons.

the molecule opposite to the site for *Mep*-specified protein processing assigned a position of 0 kD. There are methionyl residues located in the *Prot-V1* polypeptide at positions 6, 18, 29, and 44 kD. All the observed cleavage products can be accounted for as the result of partial digestion by cyanogen bromide. The 29-kD polypeptide is the result of cleavage at the position 29-kD residue in both V1 and V2. The fourth methionyl residue at position 44 kD in V1 is 5-kD distal to the MEP cleavage site. When this residue and the residues at either position 6 kD or position 18 kD are cleaved, the 38-kD polypeptide or the 26-kD polypeptide, respectively, is generated. These polypeptides are 5-kD smaller in the V2 digest since the MEP cleavage has removed the distal methionyl site. Other fragments are generated by the cyanogen bromide cleavage but are of too low a molecular weight to be resolved by this system.

DISCUSSION

The results demonstrate that *Prot-V* is an allele of the *Prot* locus. The variant segregates from the *Prot-S* allele in all crosses. These results cannot rule out the possibility that these are two closely linked loci; however, such loci would have to be less than one map unit apart and each line used would have to carry

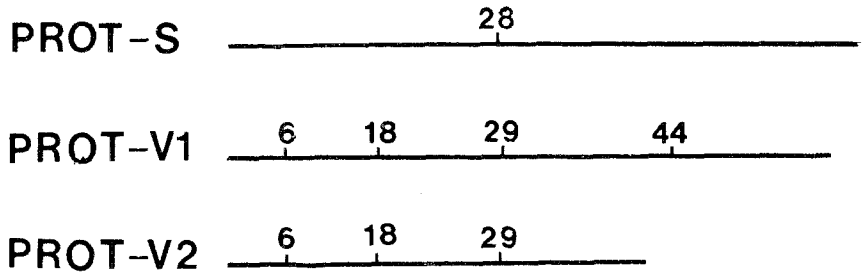

Fig. 4. Map of methionyl sites in PROT proteins. The proteins are mapped in terms of kilodaltons of protein. Numbering starts at the N terminus. The methionyl residues are indicated by numbers marking the distance in kilodaltons from the C terminus.

a null allele for the other gene. *Prot-V* maps in the same position as other *Prot* alleles (Schwartz, 1979). Since the recombination frequency between *Prot-V* and the flanking markers is similar to that observed for other *Prot* alleles, there is no major chromosomal rearrangement associated with the *Prot-V* allele. The products of the *Prot-V* allele have solubility properties similar to those of products of the other *Prot* alleles, as they can be isolated by the same purification scheme. The product of the *Prot-V* allele is processed by the *Mep*-specific proteolytic activity to generate V2 from V1. Thus, the V1 protein is the PROT′ form and V2 is the PROT. This relationship was established by the absence of V2 in the *mep* homozygote. Further support for a structural relationship is provided by the cyanogen bromide cleavage patterns. These results show that V1 and V2 have structural homology in that a map of the methionyl residues can be constructed that is consistent with both cleavage patterns.

The protein products that are produced from the *Prot-V* allele appear to be different from the other allelic products of *Prot* in terms of the number of internal methionyl residues. It is possible to make a model that accounts for all the observations. Since V1 and V2 exhibit the PROT′ and PROT relationship of the standard alleles, there should be a small polypeptide that is the same molecular weight in each digest. Kriz and Schwartz (1986) demonstrated that cyanogen bromide cleavage generates two fragments: the smaller fragment is of a constant molecular weight for each PROT′ and PROT pair, while the larger fragment, which contains the site for *Mep*-specific processing, varies in size for the pair. In the case of the *Prot-V* product, the small fragment would be the 29-kD polypeptide. In the other *Prot* products the size of this small polypeptide differs among the size alleles (Kriz and Schwartz, 1986). This polypeptide is larger in the *Prot-V*-specific proteins than in the *Prot-S* protein. In the size series of alleles such a molecular weight would be expected from a *Prot-I* protein which is larger than that specified by the *Prot-S* allele. The *Prot-V* allele could have been derived from a *Prot-I* progenitor by a nonsense mutation, which would produce a shortened polypeptide.

Creation of a nonsense mutation in a *Prot-I* progenitor to generate the *Prot-V* allele would result in the loss of the C terminus of the protein. I am proposing that the site of processing by the *Mep*-specified reaction is contained within this region of the protein. The V1 protein is shortened by about 9 kD with respect to PROT′-I. The normal MEP cleavage removes about 5 kD from the PROT′ form. Thus, the principle site for MEP processing is not present in the V1 protein. Since the *Prot-V* product is still processed, the loss of the C terminus must have uncovered a new site that was previously inaccessible. Such alternative processing has been observed in an invariant chain of class II histocompatibility antigens in which the presence of a potentially cleavable signal sequence was revealed by deletion of the N-

terminal amino acids (Lipp and Dobberstein, 1986). In the case of PROT′ processing, the structure of the protein could block access of the MEP processing to this cryptic site. An alternative explanation is that the cryptic site is a low-affinity site that is not recognized by the MEP processing because the sequence is not a perfect match for the normal processing site. In either case, it appears that a second site capable of being cleaved by the MEP reaction has been exposed by the loss of the carboxy terminus.

There are alternative positions for the site of protein processing. One such site would be processing in the N-terminal portion of the molecule. This would require that the *Prot-V* allele had lost the normal translation initiation codon via mutation and now was translated starting at the next suitable initiation codon, which would be about 5 kD from the former start of translation. The normal site for processing would be located between the original translation start and the new start site of the *Prot-V* allele. Another possibility is that the mutation resulted in alternative intron splicing. RNA processing would remove a part of the transcript that encodes the 5-kD section of the protein sequence with the recognition sequence for the *Mep* processing step. In this model the 5-kD section of protein could be found anywhere in the molecule.

The presence of additional methionyl sites in the *Prot-V* protein requires that the *Prot* locus be polymorphic for these sites. There must be three additional methionyl residues present in the *V1* protein as compared to an *I* or *S* protein. One explanation is that the sample size of allelic proteins that have been examined is very small. Only four different alleles have been examined and the range of variation in number of methionyl residues is unknown. The present sample could represent the extremes of variation. However, there does not appear to be intense selection pressure for the molecule to maintain a particular sequence, as there is polymorphism for size as well as for CRM^- nulls (Schwartz, 1979). In addition, the proposed model would suggest that the *Prot-V* allele was derived from a *Prot-I* allele by a nonsense mutation. It is not unreasonable to expect variation in the number or distribution of methionyl residues.

The model for the *Prot* product processing is that two separate processing events occur during maturation from a prePROT′ to PROT′ to PROT. The latter reaction is catalyzed by the MEP product (Kriz and Schwartz, 1986). Such double cleavage processing has been demonstrated to occur in plant proteins such as napin (Crouch *et al.*, 1983) and vicilin (Spencer *et al.*, 1983). These results suggest that the *Mep*-controlled processing occurs at the C terminus of the protein. The *Prot-V* allele provides a tool for analysis of the *Mep* processing event since it indicates that a second site exists in the protein that is subject to this cleavage. The amino acid sequence of the *Prot*-specific products will have to be established before these results can be confirmed.

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