**Point Mutations Distal to the Processing Site Affect Drosophila Pre-5S RNA Processing**

LONG RANGE COOPERATION AND A BREATHING MODEL*

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*Drosophila* pre-5S RNA, which consists of five conserved stem-loop domains and a 15-nucleotide 3' tail, is 3'-end processed to 120 nucleotide mature 5S RNA before ribosomal assembly. Large deletions in stems II and III, all of stems IV and V, and loop C prohibit *Drosophila* 5S RNA processing; deletion of stem IV and half of V does not (Preiser, P. K., and Levinger, L. (1991a) J. Biol. Chem. 266, 7509-7516). Several point mutations in stem I reduce, while certain neighboring sequence changes stimulate, processing (Levinger, L., Vasisht, V., Greene, V., and Arjun, I. (1992) J. Biol. Chem. 267, 23683-23687). Herein we extend this 5S RNA fine structure analysis to regions farther from the processing site.

Most point mutations in loop B, stem III, and loop C severely inhibit processing. One loop C substitution stimulates processing; when combined with stimulatory sequence changes in stem I and loop A, these dispersed mutations improve processing manifolds, perhaps by stabilizing a required conformation or strengthening a protein-binding site.

Central stem II sequence changes inhibit processing; several adjacent sequence substitutions which weaken base pairing improve processing. Combining these results with earlier work from stem I and loop A, we hypothesize that slight reduction in base pairing may improve groove access of polypeptide chains to essential contact positions.

Most eukaryotic RNAs are post-transcriptionally processed before utilization in their mature form (for a review, see Glover and Hames, 1988). 5 S RNA is a 120-nucleotide essential conserved RNA of the 60 S ribosomal subunit (Garrett et al., 1981). *Drosophila* 5S RNA is processed by removal of 15 nt from its 3'-end (Rubin and Hogness, 1975; Jacy et al., 1977; Sharp et al., 1984; Preiser and Levinger, 1991a; Levinger et al., 1992), and a shorter length is removed from the 3'-ends of mammalian, yeast, amphibian, and protozoan 5 S RNAs (Hamada et al., 1979; Tokamp et al., 1980; Xing and Worchel, 1989; Imboden et al., 1992). The recognition and processing mechanism has not been completely established for any of these organisms.

We are investigating the internal sequence and structure requirements for *Drosophila* 5 S RNA processing. Pre-5S RNA consists of five conserved stem-loops (Delihas and Anderson, 1982) and the single-stranded 3'-end (Fig. 1). Stem IV and half of stem V are dispensable for in vitro processing, whereas the base of stem V and stem III are required (Preiser and Levinger, 1991a). Point mutations surrounding the processing site at 120 nt severely inhibit processing (Preiser and Levinger, 1991b); further from the processing site in stem I, changes in the 7/112 GU pair and 13A at the loop A-stem II boundary consistently reduce relative processing rates (Levinger et al., 1992). On either side of the 7/112 pair, certain sequence changes stimulate processing.

We have extended this fine structure analysis to stem II and the base of stem V, loop B, stem III, and loop C (regions distant from the processing site, enclosed in Fig. 1). Stem transversions, transitions, insertions, and deletions were all made and assayed in sets of three. A single mutation (which would be predicted to disrupt base pairing) was introduced into one or the other strand of a putative stem, or the double mutation was made to restore base pairing with a sequence change. More distal positions in stem V, loop E, stem IV, and loop D were not analyzed because deletion of this region does not prevent processing (Preiser and Levinger, 1991a).

Most mutations distal to the processing site inhibit processing. Changes in several well-separated positions improve processing, and when combined, such distant changes cooperate to produce a manifolds processing improvement.

Clustered sequence changes in stems II and V stimulate processing. In stem II, changes which weaken base pairing improve processing. At the base of stem V, on the other hand, processing improvement is consistently linked with maintaining or strengthening base pairing.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Extract Preparation—*Drosophila melanogaster* Kc cells were propagated in serum-free D22 medium (Bachiller and Ohashian, 1970; Price et al., 1987), and S100 extracts were prepared (Dingermann et al., 1981) and stored in aliquots at -70 °C as previously described (Preiser and Levinger, 1991a) except for omission of the ATP regeneration system.

Site-directed Mutagenesis—Mutants were obtained by the Kunkel procedure (Kunkel, 1985) as previously described (Preiser and Levinger, 1991a, 1991b). Mutant DNAs were confirmed by supercoiled DNA sequencing (Levinger et al., 1994) with United States Biochemical Corp. Sequenase 2.0 using the chain termination method (Sanger et al., 1977) and [α-32P]dATP (DuPont-New England Nuclear). Double mutations were made with two mismatched oligonucleotides annealed to the template simultaneously. Mutagenesis efficiencies were ~75% for single and ~50% for double mutations.

T7 RNA Polymerase Transcription and 5 S RNA Processing—Wild type and mutant T7/5 S minirep DNAs (Promega) were digested with EcoRI and Drai, transcribed with T7 RNA polymerase, labeled internally with [α-32P]UTP (DuPont-New England Nuclear), and gel purified (Preiser and Levinger, 1991a). Processing reactions were performed at 28 °C in a volume of 250 µl, sampled at 0, 15, 30, 60, and 120 min (Fig. 2, panel 1, lanes 1-5), deproteinized, recovered by ethanol precipitation,
Fig. 1. 5 S RNA secondary structure. Stem II, the base of stem V, loop B, stem III, and loop C analyzed by mutagenesis/processing in this report are outlined. The stem mutagenesis strategy is to make single and paired transversions and transitions and selected single and double insertions and deletions. For loop C, we made all possible single nucleotide substitutions and selected additions and deletions. Loop B analysis was similar to that for loop C but not as exhaustive.

and electrophoresed on 6% denaturing polyacrylamide sequencing gels. After the xylene cyanol marker migrated to 4 cm from the bottom, analysis was similar to that for loop C but not as exhaustive.

Choice of Mutations for Analysis—Stem mutations were made singly and in pairs by separately changing the nucleotide in one or the other strand of a putative stem, or by simultaneously changing both nucleotides to restore base pairing with a sequence change. Insertions and deletions were prepared by adding/removing a nucleotide to/from one, the other, or both positions. The nucleotides inserted were the same as those proximal to the processing site. The processing analysis was performed on all three members of each set; a wild type RNA was included in every experiment as a positive control for day-to-day variations.

Quantitation—Relative processing rates were determined by microdensitometry using an LKB XL microdensitometer with GSXL software as previously described (Preiser and Levinger, 1991a). All mutant processing rate constants were evaluated at least twice.

RESULTS

We analyzed substitutions, insertions, and deletions for stem II base pairs 14/65 through 21/57, stem V base pairs 66/109 through 70/105, loop B nucleotides 22–27 and 50–56, stem III base pairs 28/49 through 32/45, and loop C nucleotides 33–44 (regions enclosed by dashed lines in Fig. 1). Data panels illustrate general trends or unusual processing values.

Effects of Stem II Substitutions on Processing—Most single substitutions throughout stem II inhibit processing, but less than those in stem I (Figs. 2 and 3, cf. Levinger et al., 1992). Single transversions on both strands of stem II reduce processing similarly; nucleotides 14–21 are less sensitive than nucleotides 65–57 (Fig. 3, A and B). Several stem II double substitutions consistently reduce processing; the 17/61 and 18/60 transversions are processed at 40 and 35% of the wild type rate, and the corresponding transitions are processed at 71 and 68% (Fig. 3C). The 21/57 double transversion and transition at the stem II/loop B boundary are processed at 23 and 25% of the wild type rate, respectively.

Several stem II transitions are processed at greater than or equal to the wild type rate (in Fig. 3), including C14U/G65A (128% of the wild type rate), C15U/G64A (104%), A16G (103%), C19U/G59A (189%), G59A (166%), and A58G (150%; Fig. 2, panels 6D, 8D, and data not shown). Interestingly, the single substitutions which stimulate processing are those which result in AU → GU pairings, with one AC apposition (G59A); the double substitutions are all from CG → UA except for the 19/59 CG → GC transversion (165%).

Stem II Insertion and Deletion Effects—We analyzed four sets of insertions and deletions spaced throughout stem II.

Fig. 2. Stem II substitutions. Selected stem II substitutions are illustrated in sets of three. Panels 1–4, the transversions C14G/G65C, C17G/G61C, G18C/C60G, and C19G/G59C, respectively. Panels 5–8, the transitions C14U/G65A, C17U/G61A, G18A/C60G, and C19U/G59A, respectively. A, wild type; B, mutations in nucleotides in the "top" strand as depicted in Figs. 1 and 3; C, "bottom" strand mutants; D, double mutations which restore base pairing with a sequence change. Incubations were for 1, 0; 2, 15; 3, 30; 4, 60; and 5, 120 min. Samples were recovered and electrophoresed on 6% sequencing gels. M, labeled nucleic acid markers. Numbers at right are marker nucleotide lengths. p at left, wild type or mutant pre-5 S RNA. m, mature 5 S RNA.

Fig. 3. Relative processing efficiency of stem II mutant 5 S RNAs are plotted as a percentage of wild type processing. A, only nucleotides in positions 14–21 were substituted. B, only nucleotides in positions 65–57 were substituted. C, double substitutions. D, single and double transversions. E, single and double transitions. * values which exceed wild type.
Pre-5S RNA Processing

Fig. 4. Selected stem II addition and deletion mutants. Experimental design is the same as in Fig. 2. Additions in both strands were repeats of the nucleotides proximal to the processing site at the position of the addition. Panel I, 20.21+U/57.58+A; 2, A14C/A65G; 3, AA"/AU"; 4, mutations in the bulged nucleotide C63. A, wild type; B, AC63; C, C63G; D, C63U; E, C63A.

Most stem II double insertions prevent processing, suggesting that stem II length must be strictly conserved (Fig. 4 and data not shown). 20.21+U is the only insertion which is processed well, while 57.58+A inhibits processing; the double insertion is processed at an intermediate rate (Fig. 4, ID; cf B and C).

All stem II deletions are processed at <10% of the wild type rate except for AU6* (Fig. 4, 3C). Processing inhibition is more severe with deletion of C63 than for G65 in the the 14/65 pair (Fig. 4, panels 2B and C).

Bulged nucleotide C63 is relatively unimportant (see “Discussion”); only the C63G transversion severely reduces processing (Fig. 4, panel 4C). ΔC63 and C63A are indistinguishable from wild type in processing rate, and C63U is reduced approximately 3-fold.

Stem V Substitution Processing Effects—Sequence changes in the base of stem V generally inhibit processing less than those in stem II (Figs. 5 and 6; cf. Fig. 3). A gradient of increasing processing accompanying single substitutions is established by moving distally down one strand from the stem V loop.

A boundary (Fig. 6B, nt 108 – 105); a similar gradient, although not quite as smooth, occurs on the other strand of the stem (Fig. 6A, nt 67 – 70).

Single transitions in nucleotides 66 – 70 and 109 – 105 are better tolerated than the corresponding transversions (Fig. 6, A and B). 66 and 109 single transitions are processed slightly better than wild type (110 and 118%, respectively); these two substitutions replace a GU pair with a Watson-Crick AU or GC pair. Single transversions at 66/109 (Fig. 6, A and B) produce GU and GA appositions which inhibit processing (16 and 44%); double substitutions at 66/109 which produce AC appositions are also weakly processed (23 and 26%; Fig. 6C). The stem V substitutions G66U/U109G, which allow base pairing with nucleotides C14 and A13 in loop A, increasing stem I and stem I1 length at the expense of loop A and stem V (Baudin et al., 1991), reduce processing to ~10% of the wild type rate (data not shown).

Stem V double transversions at 67/108 – 69/106 exceed the wild type processing rate (149, 131, and 154%, respectively; s in Fig. 6C). The stem V substitutions in positions 66/109 – 69/106 are thus those which improve base pairing (66/109) or retain GC pairing (67/108 – 69/106).

Stem V Insertion and Deletion Effects—Three sets each of insertions and deletions were made and analyzed, evenly spaced through the base of stem V (Fig. 5, panels 7 and 8).

Double deletions are generally tolerated, while the processing ability of single deletions varies depending on position. The double deletion at position 68/107 is processed at greater than wild type rate (153%; Fig. 5, panel 7D) and so is the double transversion at this position (Fig. 6C). On moving closer to the stem V loop E boundary, the insertion/deletion processing rates continue to equal or exceed wild type.

Stem V Insertion, Insertion, and Deletion Mutations Inhibit Processing—All eight loop B substitutions, insertions, and deletions analyzed severely reduce processing (Fig. 7). Some of
A49U transversion (101% of the wild type rate; Fig. 9B). Double substitutions in the first two stem III positions severely inhibit processing (Fig. 9C); the 28/49 transversion and transition gave values of 2 and 5% of wild type processing rate and 29/48 gave 7 and 6%, respectively. The 30/47 double transversion shows a high processing rate (102% of wild type), and the rate constants for double transversions 31/46 and 32/45 are also close to wild type.

Three stem III insertion and deletion sets at proximal, central, and distal positions were analyzed. Most stem III insertions and deletions prevent processing, suggesting the necessity for length as well as sequence conservation. The only exception is the AG30/AC47 double deletion, which is processed at or above wild type rate (Fig. 8, panel 3C). The 30/47 double transversion and double deletion (Fig. 8, panels 1D and 3D) both inhibit processing less than changes elsewhere in stem III (Fig. 8, panels 2D and 4D, and Fig. 9C).

Loop C Mutations Inhibit Processing Except for U35C—Loop C was analyzed using all three possible substitutions at each position and also with four evenly spaced insertions and deletions. The results of loop C single transitions are presented in Fig. 10; effects of the transversions, transitions, and “other” transversions are illustrated graphically in Fig. 11. Transversions are better tolerated than transitions; most single nucleotide changes greatly reduce the processing rate (Fig. 11) except for U35C, which is processed at 149% of the wild type rate (Fig. 10, panel 1D, and Fig. 11).

Long Range Cooperation—Several sequence changes stimulate processing, including the stem I double transversion A4/U, U115A and the addition 9,10+G at the stem I/loop A boundary (Levinger et al., 1992), stem II sequence changes (8s in Fig. 3), stem V double transversions, insertions, and deletions (s in Figs. 5 and 6), and the U35C loop C transition (Fig. 10, panel 1D; * in Fig. 11). Combinations of the 4/115 stem I double transversion, the 9,10+G loop A addition, and the U35C loop C transition stimulate processing up to 9-fold (Fig. 12).

We also tested short range cooperation by combining stem I and loop A mutations which individually stimulate processing (C9U/G110A, U111C; C9U/G110A, 9,10+G; 9,10+G/U111C; C9U/G110A, 9,10+G, U111C; see Levinger et al., 1992). Processing increases like those illustrated in Fig. 12 were not observed; most of these combinations severely inhibit processing.2

DISCUSSION

Point Mutations Distal to the Processing Site Inhibit Processing—We have nearly saturated stem II, the base of stem V, loop B, stem III, and loop C (regions enclosed in Fig. 1) of Drosophila 5 S RNA with single nucleotide and paired nucleotide mutations and tested the effects of these sequence changes on 3′-end processing. Most single substitutions inhibit

FIG. 6. Processing efficiency of stem V mutant 5 S RNAs relative to wild type processing, similar to Fig. 3. A, nucleotides from position 66-70; B, 109-105; C, double substitutions which restore base pairing with a sequence change. O, single and double transversions. □, single and double transitions. * values which exceed wild type.

Fig. 7. Loop B mutations. Mutations were produced and analyzed as in Fig. 2. Panel 1A, wild type; B, A22C; C, A27U; D, A50U; E, G56U. Panel 2A, wild type; B, ΔA30; C, U52C; D, 53,54+U; E, A55G.

the substitutions were chosen to allow loop B to close up, extending the length of either stem II or stem III. A22C, for example, which forms a CG pair with G54, completely prevents processing (Fig. 7, panel 1B).

Bands which appear between pre-5 S and mature 5 S RNA are more pronounced with certain mutant substrates (Fig. 7, panel 1B-E; Preiser and Levinger, 1991b; Levinger et al., 1992; Preiser et al., 1993) than with wild type (Fig. 7, panel 1A). These bands thus suggest abnormal products of endogenous ribonuclease activity and not processing intermediates (see “Discussion”).

Effects of Stem III Mutations on Processing—Stem III mutations generally inhibit processing more than those in stems I, II, and V (Figs. 8 and 9, cf. Levinger et al., 1992 and Figs. 2–6). Single substitutions severely inhibit processing except for the
processing. Several stem II double transversions and most stem III double substitutions do not restore processing to the wild type rate (positions 14/65 -> 18/60 and 21/57 in Figs. 3C and 9C). Some stem single substitutions improve processing (s's in Fig. 3, A and B; see below). We conclude that stem sequence as well as structure can influence processing. In striking contrast, Xenopus 5 S RNA processing depends solely on stem I base pairing (Xing and Worcel, 1989).

**Bulged Nucleotide C** Is Not a Major Processing Determinant—Bulged nucleotides sometimes specify binding contacts with the amino acid side chains of proteins, as in the HIV-1 TAR-TAR interaction (Weeks and Crothers, 1991; Puglisi et al., 1992); bulged nucleotide C does not appear to have much influence on TFIIIA binding to 5 S RNA (Baudin and Romaniuk, 1989; Theunissen et al., 1992). Only C63G severely reduces processing (Fig. 4, panel 4C). This substitution could pair with C14, causing G64 to pair with C14 and pushing G6' into the base of loop A.

**Aberrant Breakdown of Mutant 5 S RNAs Which Cannot be Processed**—Loop B is sensitive to both sequence and length changes (Fig. 7). Those mutants which cannot be processed (see Fig. 7, B-E, for prominent examples) are most susceptible to production of artifactual bands. The band previously designated m' which appears at approximately 127 nt (Sharp et al., 1984) is probably not a processing intermediate since many mutants which cannot be processed produce m'. Furthermore, inclusion of an ATP regeneration system (Dingermann et al., 1981) with wild type substrate inhibits processing and increases m' (data not shown), in contrast to the ATP requirement for faithful Acanthameba 5 S RNA processing (Imboden et al., 1992).

**Internal Loop C Structure**—The conservation of loop C sequence at positions 35, 36, 38, 41, and 42 (Delhia and Andersen, 1982), nucleotides sensitive and mutagenesis studies (Brunel et al., 1990), and a clustered pattern of loop C nucleotides to the bulky reagent Rh(phen),phi3+ (Chow et al., 1992) suggest that loop C is highly structured. The processing inhibition for all three types of substitutions (Fig. 11) closely tracks loop C sequence conservation except for U35C, which is processed better than wild type. One loop C model (Brunel et al., 1990) includes a trans-Hoogstein base pair between U and A and a Watson-Crick base pair between G and C. We made several 35/42 and 36/41 double transversions and transversions, and so far have not found any compensatory double substitutions (data not shown).

**Long Range Cooperation between Dispersed Stimulatory Sites**—Combination of mutations at disparate locations which separately stimulate processing (A4U/U115A and 9,10+G; Levinger et al., 1992 and U35C; Figs. 10 and 11) led to up to a nine times processing improvement (Fig. 12), suggesting long range cooperation. A structural protein in addition to the nuclease may be required for processing, and local improvements in contacts between this protein and RNA at separated sites could accumulate when the sequence changes are combined.

Interestingly, the three-way combination A4U/U115A, 9,10+G, U35C (Fig. 7, panels 2E and 3) is not processed more efficiently than the two-way combinations (Fig. 7, panels 2B-D and 3). Perhaps structural protein binding is the rate-limiting step in a wild type 5 S RNA processing reaction. With improved binding substrates, a later step in the reaction, such as catalysis or product release, could become the new rate-limiting step.

**Cis Effects and Protein Recognition**—Processing effects probably do not result strictly from major RNA structural rearrangements because numerous widespread single nucleotide changes inhibit processing. We attempted a renaturation pro-
Determination of processing rate constants or change in nonspecific degradation can explain the sequence dependence of processing. Direct footprinting should await purification of processing factor(s); footprinting with an S100 as the source of proteins would be inconclusive and potentially misleading.

The effect of pairwise and triple substitutions locally reduce processing rate, and GC pairing appears to be required only for helical structure independent of sequence, while stems I and II have stringent sequence requirements (Fig. 13). A more detailed model must await characterization of protein(s) with the ascribed processing function and binding specificity.

**Possible Tertiary Interactions**—Free 5 S RNA probably does not possess long range interactions leading to a stable tRNA-like tertiary structure (Zhang et al., 1989; Brunel et al., 1990; Chow et al., 1992). Loop C-loop D and loop B-loop E interactions observed in higher plant chloroplast 5 S RNAs (Romby et al., 1988; Joachimiak et al., 1990) are unlikely to be processing determinants in Drosophila because stem IV and half of stem V, including loops D and E, can be deleted without preventing processing (Preiser and Levinger, 1991a).

**Base Pair-specific Contacts and Helix Breathing**—Subtle reductions in stem I and stem II helix stability improve processing (Levinger et al., 1992; Fig. 3). Base pair-specific contacts can be postulated when single and double substitutions locally reduce processing relative to neighboring positions, as in the stem I 7/112 GU pair (Levinger et al., 1992). The center of stem II (base pairs 17/61 and 18/60) is similarly less tolerant of sequence changes than its surroundings (Fig. 9). Inhibitory sites in stems I and II may be flanked on either side by positions where sequence changes stimulate processing (Fig. 13). Mutations from GC to AU or non-canonical GU and AC appositions reduce helix stability, perhaps improving groove access for direct readout (Weeks and Crothers, 1993) or partial unwinding by the amino acid side chains of a polypeptide (Roul et al., 1989).

According to this model, the central stem II base pairs 17/61 and 18/60 would be the contact positions to which access is improved by breathing in the surrounding neighborhood (Fig. 13). Our previous analysis of stem I and loop A (Levinger et al., 1992) showed processing improvement accompanying similar substitutions clustered at the stem I-loop A boundary, close to the 7/112 GU pair which least tolerates substitutions.

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**5 S RNA-binding Proteins**—The stem IV-V domains which probably does not possess long range interactions leading to a stable tRNA-like tertiary structure (Zhang et al., 1989; Brunel et al., 1990; Chow et al., 1992). Loop C-loop D and loop B-loop E interactions observed in higher plant chloroplast 5 S RNAs (Romby et al., 1988; Joachimiak et al., 1990) are unlikely to be processing determinants in Drosophila because stem IV and half of stem V, including loops D and E, can be deleted without preventing processing (Preiser and Levinger, 1991a).

**5 S RNA-binding Proteins**—The stem IV-V domains which can be deleted without preventing processing (Preiser and Levinger, 1991a) further led us to discount the known eukaryotic 5 S RNA-binding proteins L5 and TFIIIA as possible processing factors. Ribosomal protein L5 forms a complex with 5 S RNA (Blobel, 1971) as a precursor to large subunit assembly (Steitz et al., 1989) and requires stems IV and V for 5 S RNA binding (Huber and Wool, 1986a; Wormington, 1990). TFIIIA similarly binds stem V (Huber and Wool, 1986b), but the footprint extends to stems I, II, and III and the bottom of loops B and C.
We have attempted fractionation of the Sl00 by column chromatography methods, Brow (Brow, 1987; Brow and Geiduschek, 1987) for advice on quantitation.

Vertebrate La protein binds the 3'-U, tail of RNA polymerase III transcripts (Rinke and Steitz, 1982; Stefano, 1984); this sequence inhibits processing (Preiser and Levinger, 1991a; Maraini et al., 1994). We recently found a Drosophila 3'-exonuclease inhibitor which could be a La homolog (Preiser et al., 1993).

Eukaryotic L5 (YL3 in yeast) has been suggested to be a fusion of three smaller prokaryotic large subunit ribosomal proteins (L5, L18, and L25 in Escherichia coli; Nazar and Wildeman, 1983; Chan et al., 1987) which bind specifically to 5 S RNA. Their binding sites (E. coli L25 binds stem I, L18 binds stem III, and L5 binds stem V; rat L5 binds mainly stem V) do not entirely support this suggestion, however. A Bacillus subtilis ribosomal protein, BL16 (equivalent to E. coli L18), binds specifically to 5 S RNA to mutations distal to the processing site (Stahl et al., 1984), making it the best available model for the sensitivity of Drosophila 5 S RNA to mutations distal to the processing site. Processing results with 5 S RNA mutants direct us to seek one or more proteins with binding sites resembling those of E. coli L25 and L18, with a processing function similar to that of B. subtilis BL16.

Search for a Processing Nuclease and Required Factors—Using a yeast 5 S RNA processing mutant and two-dimensional gel methods, Brow (Brow, 1987; Brow and Geiduschek, 1987) suggested regulatory functions for YL3 and a yeast La homolog. We have attempted fractionation of the S100 by column chromatography and reconstitution of processing activity. Preliminary results (not shown) suggest that the processing machinery can be separated into at least two components. Combinatorial mutants with improved processing (Fig. 12) may be useful in protein binding and fractionation/reconstitution studies.

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