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Ribonucleic Acid and Protein Metabolism in Pea Epicotyls[1]

II. RESPONSE TO WOUNDING IN AGED TISSUE

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ABSTRACT

Aged pea *Pisum sativum* L. var Alaska epicotyl tissue was wounded by excising the apical 10 or 20 millimeters and incubating the excised segments upright in buffer. Wounding induced a very rapid formation of polysomes which was accompanied by minor increases in ribosomes, mRNA, and poly(A) and by a doubling of the *in vivo* protein synthesizing capacity. This increase in protein synthesis *in vivo* was matched by a similar increase in polypeptide synthesis *in vitro* in wheat germ reactions primed by polysomes. However, *in vitro* reactions primed by total and polysomal RNA from wounded tissue were affected much less.

Two-dimensional gel patterns of silver-stained proteins accumulated *in vivo* were almost unchanged, even after 6 hours of wounding, since only two spots decreased in intensity and none increased. In contrast, two-dimensional gel fluorographs of polypeptides generated *in vitro* by both total RNA and polysomal RNA showed numerous changes within 3 hours of wounding. Of the more than 200 spots visualized by fluorography, 17 decreased and 26 increased when total RNA from wounded tissue was used; 15 decreased and 10 increased when polysomal RNA was used. Those polypeptides that decreased after wounding were generally of lower molecular weight; those which increased were of higher molecular weight.

Although wounding must be affecting transcription insofar as different mRNAs must be present to encode different polypeptides, its major effect appears to be on translation, presumably through formation of ribosomes with greater translational efficiency.

Aging of apical pea stem tissue results in decreased protein synthesizing activity and lowered amounts of protein, RNA, and ribosomes (10). When tissue aged for 3 to 4 d is wounded by excision (or other means), there is a reactivation of several processes, particularly characterized by the formation of polyribosomes (3). The response is very rapid and stable, since polysome formation is detectable within 30 min after wounding and it endures for up to 24 h.

The term 'aging' is also used when referring to 'activation' of plant storage tissue which occurs after excision and incubation. This response is more aptly described as a wounding process (5), and has been extensively studied in different plant storage tissues. Several reports have shown there is an increase in polysome formation and protein synthesis following wounding (aging) of such tissues, including Jerusalem artichoke (12), potato tubers (4), and carrot roots (6).

Although non-storage tissues, including stems, roots, and leaves, are frequently excised (*i.e.* wounded) as part of the basic experimental protocol, few studies other than those on wound-induced ethylene biosynthesis (14) have been aimed at elucidating the specific response to wounding. Even less frequent are reports of wound responses in aged or mature tissues. However, Travis *et al.* (13) showed that basal (non-growing) soybean hypocotyl tissue, after excision and incubation, exhibited increased levels of polysomes and an increased capacity of the monosomes to support polypeptide synthesis *in vitro*, but they saw no effect on segments excised from elongating tissue. We have also shown that wounding has little effect on polysome formation in actively growing tissues, but suggested that this is because the polysome content is already very high in such tissue (3). In contrast, in aged tissue the polysome content is relatively low and the effect of wounding is readily observable.

The questions posed in this study are: (a) to what extent are various components involved in protein synthesis affected during wounding; (b) what factors contribute to increased polysome formation and protein synthesis; (c) are specific proteins made (and accumulated) or degraded in response to wounding; and (d) does wounding affect the composition of the mRNA within the whole tissue extract and within the polysomes?

MATERIALS AND METHODS

Eight-d-old etiolated pea seedlings were aged as described in Schuster and Davies (10). Apical segments were harvested 3 to 4 d after the aging process was initiated and used as the control. Wounding was performed by excising tissue 10 or 20 mm from the apex and then incubating the excised segments upright in 20 mM phosphate buffer (pH 6.0) for various lengths of time.

All of the other materials and methods were identical to those described previously (10).

RESULTS AND DISCUSSION

Increase in Components Involved in Protein Synthesis after Wounding. We have shown previously (3) that wounding causes an increase in polysomes and decrease in monosomes that is detectable within 15 min, maximal within 3 to 6 h, and endures for up to 24 h.

Table I summarizes the effects of wounding on the level of individual components involved in protein synthesis, and on protein synthesis *in vitro* of tissue 1 and 3 h after wounding. One h after wounding, there was a minor increase (12% or less) in rRNA, total ribosomes, polysomal mRNA, and polysomal poly(A) per segment, whereas there was a 28% increase in the amount of polysomes and more than a 120% increase in polysome-primed protein synthesis. Three h after wounding, the

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Table I. *RNA Content and Protein Synthesizing Capacity Increase at Different Rates following Wounding*

Values are expressed as a percentage of the control ± SD.

Time after Wounding	Parameter Measured per Segment					
	I rRNA[a]	II Total ribosomes[b]	III Polysomes[c]	IV Polysomal mRNA[d]	V Polysomal poly(A)[e]	VI Protein synthesis[f]
h						
1	110.9 ± 8.9	98.8 ± 20.0	128.0 ± 14.4	110.7 ± 1.4	112.0 ± 7.8	224.1 ± 9.4
3	120.8 ± 5.9	112.5 ± 17.5	146.7 ± 23.2	121.7 ± 2.5	137.0 ± 18.8	270.1 ± 11.1

[a] Values were derived from GPS gradient profiles of rRNA recalculated from Davies and Schuster (3).

[b] The amount of ribosomal material was determined spectrophotometrically. Values are the average of six experiments.

[c] Area of polysomes (excluding subunits and monosomes) was determined from polysome profiles. Values are the average of three experiments.

[d] Relative numbers of mRNA per polysome profile were determined according to Davies and Larkins (2), where the area of the dimer was divided by 2, the area of the trimer by 3, etc. Values are the average of three experiments.

[e] Poly(A) content was determined by [^{3}H]poly(U) hybridization (3). Zero time value was 978 ± 32 cpm.

[f] *In vitro* protein synthesizing capacity was determined by [^{3}H]leucine incorporation (3). Zero time value was 3410 ± 190 cpm.

Table II. *Magnitude of the Increase in Protein Synthesizing Capacity In Vitro after Wounding Depends upon the Source of Primer*

Results are averages of three experiments ± SD for [^{35}S]methionine incorporation.

Basis for Measurement	Protein Synthesis at 3 h and Primed by		
	Total ribosomes[a]	Polysomal RNA[b]	Total RNA[c]
	% of control		
I, per A_{260}[d] unit	211.5 ± 29.6	113.8 ± 16.7	134.5 ± 9.8
II, per segment	235.5 ± 46.7	130.0 ± 0.9	177.3 ± 0.3

[a] Total ribosomes refers to subunits + monosomes + polysomes. Control values (cpm × 10^5) were: 0.10, 0.28, 0.52 per segment; 1.34, 4.06, 5.29 per A_{260} unit.

[b] Polysomal RNA refers to phenol-extracted RNA from ribosomal pellets (subunits + monosomes + polysomes). Control values (cpm × 10^5) were: 3.37, 4.57, 2.51 per segment; 88.7, 120.3, 66.2 per A_{260} unit.

[c] Total RNA refers to phenol-extracted RNA from whole tissue extracts. Control values (cpm × 10^5) were: 5.25, 5.70, 4.36 per segment; 91.3, 99.2, 75.9 per A_{260} unit.

[d] The amount of RNA or ribosomal material was determined spectrophotometrically.

ribosome content had increased by 13%, rRNA by 20%, polysomal mRNA by 22%, polysomal poly(A) by 37%, whereas the amount of polysomes had increased almost 50% and polysome-primed protein synthesis by 170% compared with the control.

Increase in the *In Vitro* Protein Synthesizing Capacity after Wounding. The magnitude of this wound-induced increase in protein synthesizing activity differs depending upon the type of RNA used to prime the wheat germ cell-free system. Table II shows the value at 3 h after wounding as a percentage of the control value. When the data are expressed on a unit RNA basis, the polysomal RNA showed the least effect, increasing 13%, the total RNA showed a somewhat greater increase (35%), and the total ribosomes were affected the most, increasing 112% (row I). When the data are expressed on a segment basis, a similar trend was observed, insofar as the protein synthesizing capacity of the polysomal RNA, total RNA, and ribosomal material increased 30, 77, and 136% respectively, over the control values.

Table III demonstrates the change in polysomal mRNA content within the first 30 min after wounding. There was little or no increase in the total amount of message present in the polysomes and, at most, a slight increase in rRNA during this time period. There was, however, a definite shift in the proportion of the messages present in small polysomes to ones present in larger polysomes.

In the first 60 min after wounding, most components of the translation apparatus increase less than 12%, the level of polysomes increases almost 30%, whereas the priming capacity for polysomes *in vitro* rises 120% (Table I). The priming capacity *in vitro* for polysomal RNA and total RNA are much less, being only 14% and 35%, respectively (Table II). Furthermore, the

Table III. *Wound-Induced Conversion of Small Polysomes into Large Polysomes*

Ten 20-mm apical segments excised from 3-d aged pea epicotyls were incubated upright in buffer for the designated periods.

Time after Excision	Relative mRNA Content in Each Size-Class with Following No. of Ribosomes/Polysome (*n*)							
	2	3	4	5	6	7	8	9+
min	A_{254}/n[a]							
0	21.0	15.8	14.4	10.7	8.0	6.4	4.3	19.4
10	20.5	14.8	14.3	10.8	7.9	6.5	5.0	20.2
15	17.9	14.3	13.9	11.0	8.5	6.9	5.3	22.2
20	12.9	11.0	12.7	10.6	9.9	7.3	5.4	30.3
25	9.7	11.0	12.7	12.4	10.7	7.4	5.8	30.5
30	9.0	10.9	12.5	11.8	10.5	7.5	6.1	31.6

[a] Areas under the dimer were divided by 2, trimer by 3, etc., nine-mers and greater divided by 10 as in Davies and Larkins (2). Values were adjusted to constant mRNA units (100) per profile. Actual values, which indicate the variability in the total amount of polysomes extracted, were: 0 min, 95.3; 10 min, 105.8; 15 min, 105.3; 20 min, 94.7; 25 min, 93.9; 30 min, 111.7. Quantitation of ribosomal RNA from similar tissues showed increases of less than 5% over the 30-min time course. Values are the means from three experiments.

amount of mRNA associated with polysomes remains almost constant during the first 30 min after wounding even though it shifts from being associated primarily with smaller polysomes to being associated with large polysomes (Table III).

Viewed as a whole, these data suggest that an early effect of wounding is to enhance translation by increasing the number of pre-existing ribosomes attached to pre-existing polysomes, thereby increasing not only the average polysome size-class, but also the rate of protein synthesis *in vivo* (3) as well as the rate of polysome-primed protein synthesis *in vitro*. The increase in ribosome recruitment onto mRNA may be the result of increased initiation activity, perhaps of the ribosomes themselves. Although the initial effect of wounding seems to be at the translational level, we chose to examine possible effects of wounding at the transcriptional level by analyzing two-dimensional gel patterns of proteins accumulated *in vivo* and polypeptides generated *in vitro*.

Patterns of Unlabeled Proteins Accumulated *In Vivo* in Control and Wounded Tissue. Although there was only a slight increase in the total, soluble protein content per segment (4% by 6 h after wounding; Davies and Roy, unpublished data) it was possible that the protein composition was altered by wounding. Attempts using *in vivo* labeling to analyze proteins synthesized in response to wounding were unsuccessful. Intact plants did not take up and incorporate sufficient amounts of the radiolabeled precursors. If the tissue was excised and incubated in a solution containing the labeled amino acids, the wound response was generated, and short term labeling studies to minimize this unavoidable wound effect did not generate protein labeled to a sufficient specific activity for analysis by gel electrophoresis. Therefore, unlabeled protein was extracted from control and wounded tissue, electrophoresed, and the patterns visualized by silver staining (Fig. 1). The most notable feature about the gels is that the patterns are nearly identical. Out of more than 500 proteins resolved, none increased and only two appeared to decrease slightly in abundance after wounding (*cf.* Fig. 1b and Fig. 1a). Patterns of proteins isolated from tissue wounded for 1 or 3 h showed similar results (data not shown). This suggests that during the first 6 h, wounding has little or no effect on the accumulated amounts of proteins resolved by this two-dimensional gel system.

Patterns of Labeled Polypeptides Synthesized *In Vitro* by RNA from Control and Wounded Tissue. To determine whether wounding affected the mRNA population within the tissue, RNA was isolated from whole tissue extracts as well as from ribosomal pellets and used to prime the wheat germ cell-free system and the products analyzed by two-dimensional gel electrophoresis and fluorography. Attempts were made to compare products generated by polysomal RNA and postribosomal RNA directly, but the postribosomal RNA from control tissue generated insufficient products for analysis by fluorography (data not shown).

Total RNA from 3-h wounded tissue encoded a different pattern of products from that of the control (Fig. 2). Those which increased in intensity (26/200+) were typically higher mol wt products ranging from about 30,000 to 75,000 D. Those which decreased in intensity (17/200+) after wounding were primarily lower mol wt and ranged between 15,000 and 38,000 D. The products encoded by polysomal RNA from control and 3-h wounded tissue also showed some differences (Fig. 3). Those polypeptides which decreased in intensity (15/200+) after wounding were heterogeneous in mol wt (20,000–50,000 D), whereas those which increased in intensity (10/200+) were generally higher mol wt products (30,000–50,000 D).

Relation to Other Wounding Studies. The vast majority of work performed on wounded tissues has employed plant storage organs where the wound response is frequently called aging (see 5). Some of the research with storage organs most relevant to that discussed here is the work of Lin *et al.* (7), Setterfield *et al.* (12, and references therein), and Ishizuba *et al.* (4).

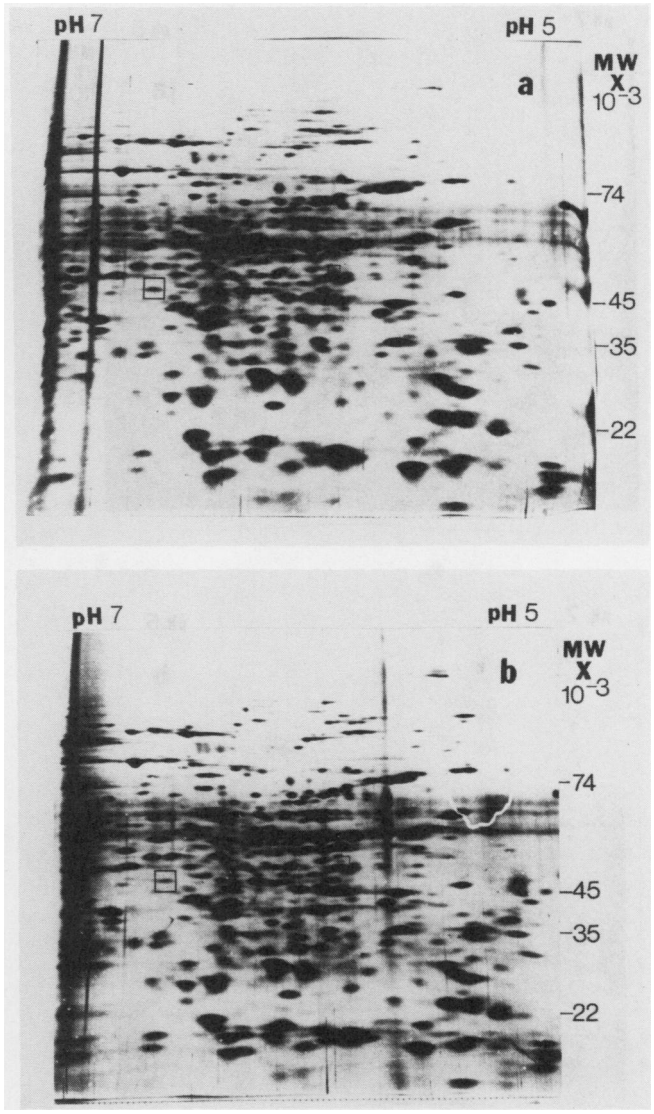

FIG. 1. Relative abundancies of endogenous proteins change little following wounding treatment. Apical 10-mm segments from 4-d aged control tissue (a) and 6-h wounded tissue (b) were homogenized and total, unlabeled protein was extracted. Fifty μg protein were loaded onto the isoelectric focusing dimension, electrophoresed, and silver stained after the second dimension. Two spots decreased in abundancy following wounding (□). Photographs represent results from one of at least three independent experiments, and only those changes which were reproducible are indicated here.

Lin *et al.* (7) showed that carrot tissue, when excised and incubated ('aged') for 5 h, exhibited increased ribosomal activity compared to the nonexcised tissue. They also demonstrated the presence of two new ribosomal proteins associated with the ribosomes from excised tissue, which they suggest may play a role in the increased activity of the ribosomes. Setterfield *et al.* (12), using Jerusalem artichoke tissue slices, showed that polyribosome formation and protein synthesis are stimulated almost immediately following excision. This is followed by mRNA synthesis (some poly(A)+, some poly(A)−) and a turnover of the ribosomal RNA. Ishizuka *et al.* (4) also showed that increased polysome formation and protein synthesis resulted following excision and incubation of potato tuber slices. They suggested

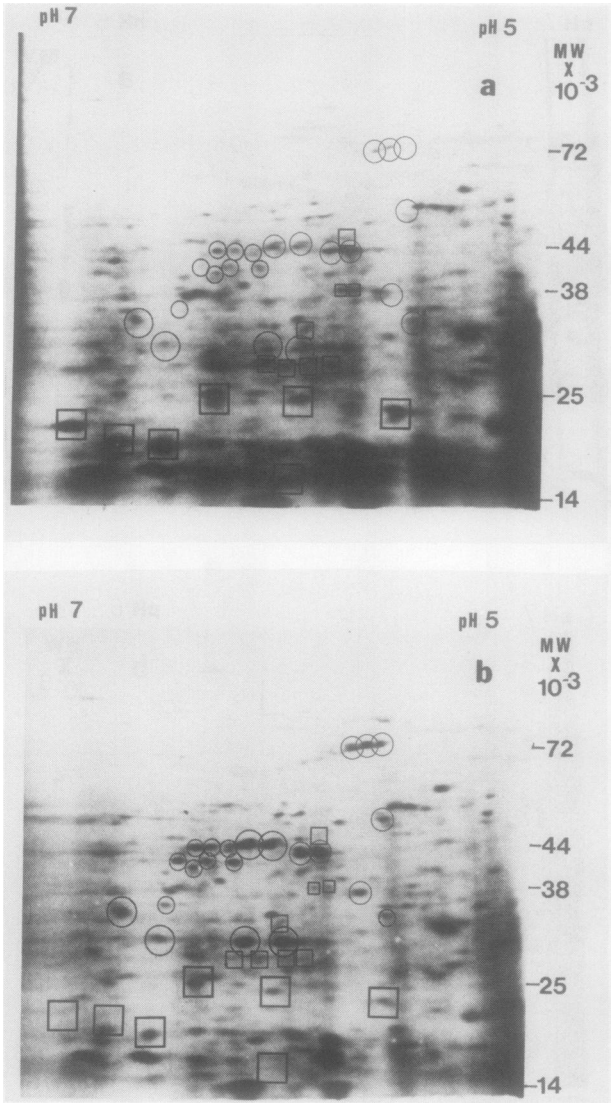

FIG. 2. Total RNA phenol-extracted from wounded tissue evokes the synthesis of polypeptides different from those synthesized by RNA from control tissue. Total RNA was extracted and used for *in vitro* translation with the resulting polypeptides subjected to electrophoresis. Radioactivity equivalent to 750,000 cpm was applied to each isoelectric focusing gel. The second dimension gels were stained with Coomassie brilliant blue R to provide internal markers, processed for fluorography, and exposed to x-ray film for 144 h. (□), Polypeptides which are more intense in the control sample; (○), polypeptides which are more intense in the wounded sample. Fluorographs correspond to patterns generated by RNA extracted from the equivalent of: a, 1.5 segments of 96-h aged (control) tissue; b, 0.8 segments of 3-h wounded tissue. Photographs are representative of fluorographs from three experiments. Only those changes which were reproducible are indicated here.

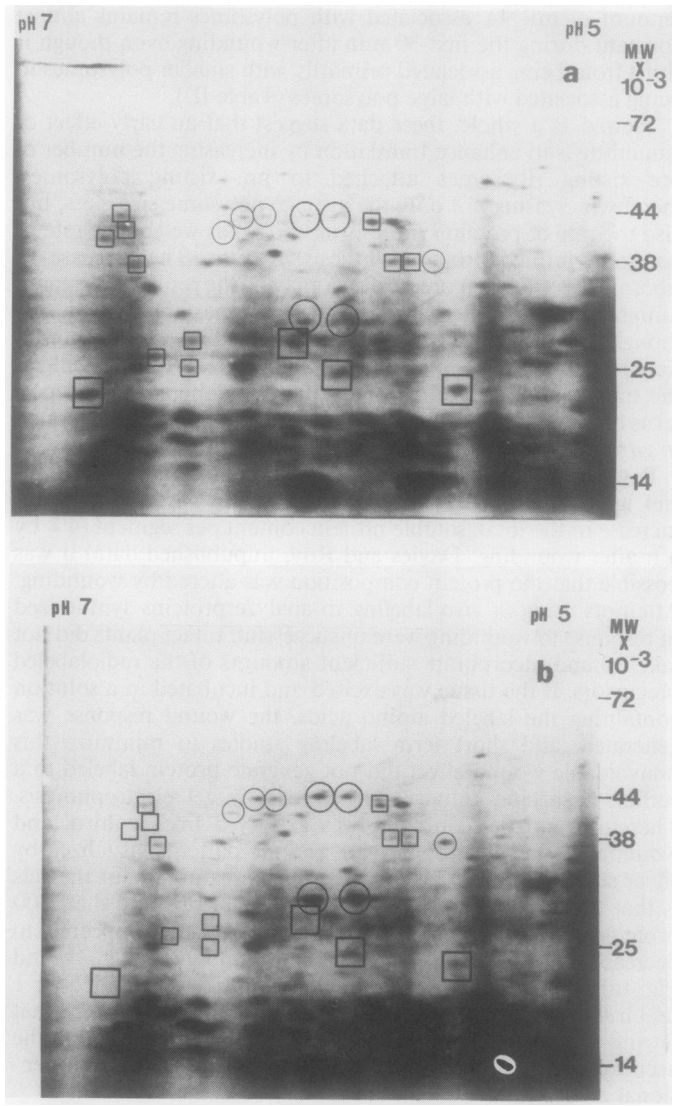

FIG. 3. Polysomal RNA extracted from wounded tissue evokes the synthesis of polypeptides different from those synthesized by RNA from control tissue. The protocol was identical to that described in Figure 2 except that polysomal RNA was used as a template for *in vitro* protein synthesis. (□), Polypeptides which are more intense in the control sample; (○), polypeptides which are more intense in the wounded sample. Fluorographs correspond to patterns generated by RNA extracted from the equivalent of: a, 2.3 segments of 96-h aged (control) tissue; b, 1.7 segments of 3-h wounded tissue. Photographs are representative of fluorographs from three experiments. Only those changes which are reproducible are indicated here.

that the increase in translational capacity is the result of newly synthesized or activated mRNA. This message, isolated at various times after excision, generated polypeptides *in vitro* which yielded one-dimensional gel patterns exhibiting a decrease in larger mol wt products and an increase in smaller mol wt products.

While studying the effect of auxin and ethylene on excised soybean hypocotyl tissue, Zurfluh and Guilfoyle (16, 17) showed that poly(A) RNA from intact elongating and basal tissue generates significantly different patterns of polypeptides *in vitro* compared to poly(A) RNA from excised segments. In contrast, Baszczynski *et al.* (1), in studies on the effects of heat shock on corn roots and shoots, showed no changes between the complements of *in vivo* labeled proteins from intact and excised tissues as visualized by one-dimensional gel electrophoresis and fluorography.

At present, the identity or functions of the polypeptides encoded by RNA from control (aged) or wounded apical pea stem tissue are unknown. However, several proteins have been shown to be synthesized following wounding, including ribosomal proteins (7), RNase (8), and 1-aminocyclopropane-1-carboxylic acid synthase, the regulatory enzyme in ethylene biosynthesis (15). These are likely candidates for newly synthesized proteins in this wounded system since ribosome activity increases dramatically, perhaps as a result of synthesis of ribosomal protein; RNase

activity is apparent (since wounded tissue showed a loss of specific mRNAs); and ethylene has been shown to increase after wounding in pea epicotyls (9).

Nevertheless, although some changes do occur in the spectrum of proteins synthesized after wounding, a much greater stimulation is seen in the amount of protein synthesized. This, together with the massive recruitment of monosomes into polysomes (Table III) suggests that wounding has a more rapid and more marked effect on translation than on transcription.

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