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Auxin-Induced Changes in the Level of Translatable Ribosomal Protein Messenger Ribonucleic Acids in Soybean Hypocotyl†

J. Stephen Gantt* and Joe L. Key

ABSTRACT: Two-dimensional polyacrylamide gel electrophoretic maps of ribosomal proteins isolated from soybean seedlings were constructed prior to the investigation of mRNA levels. Polysomes and ribosomal subunits were isolated from the apical hooks of 3-day-old soybean seedlings. A total of 40 and 51 basic proteins, associated with the 40S and 60S subunits, respectively, was resolved on the four different two-dimensional gel systems used in this study. The effect of the synthetic auxin (2,4-dichlorophenoxy)acetic acid (2,4-D) on ribosomal protein mRNA levels was investigated by in vitro translation of RNA isolated from 2,4-D-treated and nontreated hypocotyls. The in vitro synthesized proteins were extracted and separated by two-dimensional polyacrylamide gel electrophoresis. Stained protein spots that corresponded to ribosomal proteins were excised from the gel, and the in vitro incorporation of [^{35}S]methionine was determined. Results indicate that auxin induces an 8-fold increase in the relative amount of translatable ribosomal protein mRNAs.

Auxins are a class of naturally occurring plant growth regulators that are required for both cell division and expansion (Thimann, 1969; Meins, 1977). There is a great deal of information that supports the hypothesis that the physiological effects of auxin result from altered gene expression. Some 10–12 h after the application of auxin to young soybean hypocotyls, cell division commences in this normally quiescent tissue (Key et al., 1966). However, prior to cell proliferation, there is a massive accumulation of protein and RNA. Early studies used specific inhibitors of protein and RNA metabolism to link the influence of auxin to changes in gene expression. Inhibition of RNA synthesis by actinomycin or of protein synthesis by cycloheximide results in an inhibition of auxin-induced cell elongation (Key & Ingle, 1964, 1967; Key et al., 1967). Hybridization analyses have shown that the levels of few of the approximately 40 000 poly(A)-containing RNA species are significantly altered by auxin (Baulcombe et al., 1980, 1981). In vitro translation of poly(A)-containing RNA followed by two-dimensional gel electrophoresis reveals that auxin significantly alters only 5–10% of the abundant mRNA species (Baulcombe et al., 1980). Zurfluh & Guilfoyle (1982a) have shown that auxin induces changes in the levels of about ten translatable mRNAs in the elongating section of soybean hypocotyl tissue. It also alters the levels of approximately 20 translatable mRNAs in both excised basal sections and intact soybean hypocotyl (Zurfluh & Guilfoyle, 1982b). Theologis & Ray (1982) have made similar observations using poly(A)-containing RNA isolated from excised pea epicotyl tissue. Using cloned cDNA, Walker & Key (1982) have directly demonstrated that auxin induces changes in the level of two mRNAs found at high levels in the elongating section of the soybean hypocotyl. One mRNA increases 3–5-fold while the other increases 5–8-fold. These mRNAs are quickly induced, which suggests that auxin directly affects their expression.

Auxin treatment of soybean hypocotyl results in large increases in rRNA production (Key et al., 1966). Olszewski & Guilfoyle (1980) have shown that by 24 h auxin-treated hypocotyls have 9-fold more template-engaged RNA polymerase I activity per amount of DNA than does control hypocotyls. This change in the rate of rRNA synthesis is the result of an increase in the rate of both initiation and elongation by RNA polymerase I. In artichoke explants, enhanced RNA polymerase I activity and an increased conservation of rRNA sequences during pre-rRNA processing account for the observed increase in rRNA synthesis (Melanson & Ingle, 1978).

The increase in rRNA production and the concomitant accumulation of ribosomes following auxin treatment suggests that ribosomal proteins may also be produced at an elevated rate. Two mechanisms have been described that account for increased amount of ribosomal protein accumulation in higher eukaryotes. Nabeshima & Ogata (1980) demonstrated that the in vivo rate of ribosomal protein synthesis in regenerating rat liver was selectively increased by a factor of 3 compared to that in normal rat liver. Furthermore, in a cell-free translation system, the elevated rates of synthesis were shown

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to be caused by an increase in the amount of translatable ribosomal protein mRNA. Studies measuring ribosomal mRNA content with cloned ribosomal protein cDNAs have confirmed a 2-fold increase in regenerating rat liver (Faliks & Meyuhas, 1982).

DePhilip et al. (1980) have demonstrated that addition of insulin to the growth medium stimulates the synthesis of ribosomal proteins in confluent cultured chicken embryo fibroblasts. The increased rate of synthesis was very rapid, reaching half its maximum value within 20 min. The addition of actinomycin D did not prevent this increase, which suggests that the synthesis of neither poly(A)-containing RNA nor rRNA was necessary for an increase in ribosomal protein synthesis. Furthermore, it was shown in these insulin-deprived fibroblasts that the efficiency of translational initiation of ribosomal protein mRNAs was low compared to that of growing cells. It is likely that these changes in the translational efficiency of the mRNAs account for the decrease in ribosomal protein synthesis.

Similarly, Tushinski & Warner (1982) have demonstrated that upon addition of serum to serum-starved mouse 3T3 cells, the rate of synthesis of ribosomal proteins increased about 3-fold. However, in related experiments using ribosomal protein cDNAs, Geyer et al. (1982) have shown that there is little change in the content of ribosomal protein mRNAs in serum-stimulated mouse 3T6 cells. This group also presented evidence that the increased rate of ribosomal protein synthesis resulted from an alteration in the efficiency of ribosomal protein mRNA translation.

In the present study, we have mapped soybean ribosomal proteins on four different two-dimensional polyacrylamide gel systems (Madjar et al., 1979a). Additionally, we have examined the effects of auxin on the amount of translatable ribosomal protein mRNA. RNA was extracted from 4-day-old etiolated soybean hypocotyls that had been treated with the synthetic auxin (2,4-dichlorophenoxy)acetic acid. Cell-free translation products of these RNAs were separated on two-dimensional gels. mRNA extracted from auxin-treated hypocotyls was approximately 8-fold more efficient at directing the in vitro synthesis of ribosomal proteins than was control mRNA. These data indicate that auxin induces an 8-fold increase in the relative amount of ribosomal protein mRNA.

Materials and Methods

Preparation of Ribosomes and Ribosomal Subunits. The apical hooks of 3-day-old etiolated soybean seedlings (Glycine max, variety Wayne), grown in moist vermiculite at 27 °C, were excised and immediately placed on ice. Two volumes of buffer A (50 mM Tris,[1] pH 7.5, 5 mM $MgCl_2$, 20 mM KCl, 0.1% β-mercaptoethanol) containing 250 mM sucrose were added to the hooks and homogenized with a Brinkmann polytron at high speed for 15–20 s. Just prior to homogenization, PMSF was added to 1 mM. The homogenate was centrifuged at 5000 rpm for 10 min in a Sorvall SS34 rotor and then strained through cheesecloth. Triton X-100 was added to 1%, and the mixture was centrifuged at 15 000 rpm for 10 min. The supernatant was then filtered through miracloth and layered over 7 mL of buffer A containing 1.5 M sucrose. The preparation was then centrifuged at 54 000 rpm for 2 h in a Spinco 60 Ti rotor. The resulting pellet was resuspended in buffer A for ribosomal protein isolation or in buffer B (50 Tris, pH 7.5, 500 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 250 mM sucrose) for the isolation of ribosomal subunits.

Ribosomal subunits were separated by the addition of puromycin to a final concentration of 1 mM (Blobel & Sabatini, 1971). The sample was left on ice for 15 min, warmed at 30 °C for 20 min, and then left on ice for 2 h. The sample was then either frozen or used directly. Puromycin-treated ribosomes (30 A_{260} units/gradient) were applied to 20–40% linear sucrose gradients that contained buffer B. The loaded gradients were centrifuged in a Spinco SW 27 rotor at 25 000 rpm for 15 h. The gradients were fractionated, and the isolated ribosomal subunits were then concentrated by centrifugation for 5 h at 54 000 rpm and resuspended in buffer A.

Preparation of Ribosomal Proteins. Total polysomal or ribosomal subunit proteins were extracted with acetic acid (Waller & Harris, 1961) as described by Madjar et al. (1979b). After dialysis against 1 M acetic acid–0.1% β-mercaptoethanol, the dialyzate was dried in vacuo and either analyzed directly or alkylated (Madjar & Traut, 1980).

In Vivo Labeling of Ribosomal Proteins. Seedlings were germinated in moist paper rolls. After 2 days, 6 g of seedlings was incubated in 30 mL of incubation medium containing 500 μCi of [^{3}H]leucine (140 Ci/mmol) and 500 μCi of [^{3}H]valine (33 Ci/mmol) for 8 h (Key et al., 1981). Polysomal proteins were prepared as described above.

Isolation of RNA. Seeds were germinated in the dark in moist vermiculite at 27 °C for 3 days, sprayed with 2.5 mM 2,4-D, and harvested 24 h later. Control tissue was treated identically except it was not treated with 2,4-D. RNA was extracted as described by Baulcombe & Key (1980) and Silflow et al. (1979). Poly(A)-containing RNA was isolated as described by Silflow et al. (1979). The amount of poly(A)-containing RNA was determined by hybridization to poly([^{3}H]U) (Bishop et al., 1974).

In Vitro Translation of RNA. RNA was translated in vitro with a wheat germ S30 preparation (Roberts & Paterson, 1973). The translation mixture contained 22 mM Hepes, pH 7.6, 1.9 mM magnesium acetate, 71 mM potassium acetate, 940 μM ATP, 370 μM GTP, 7.5 mM creatine phosphate, 1.9 mM DTT, 40 μM spermine, 25 μM of each amino acid except methionine, 100 μM PMSF, 2 mCi/mL [^{35}S]methionine (1200 Ci/mmol), and 30 A_{260} units of wheat germ S30 preparation per milliliter. In all translations, RNA was present in subsaturating amounts. Incubation was at 25 °C for 3 h. All translations were centrifuged at 100000g for 30 min prior to determination of radioactive incorporation.

Gel Electrophoresis. Polysomal 40S and 60S subunit proteins were analyzed on four different two-dimensional gel systems: acidic–$NaDodSO_4$ (system I), basic–$NaDodSO_4$ (system II), basic–acidic (system III), and acidic–acidic (system IV) (Madjar et al., 1979a; Madjar & Traut, 1980). Briefly, the four dimensions used are as follows: (acidic first dimension) the 10-cm gel is 4% acrylamide, pH 5.5, electrophoresis is for 5 h at 150 V; (basic first dimension) the 10-cm gel is 4% acrylamide, pH 8.6, electrophoresis is for 7 h at 150 V; (acidic second dimension) the 14-cm gel is 18% acrylamide, pH 4.2, electrophoresis is for 15 h at 130 V; ($NaDodSO_4$ second dimension) the 12-cm separation gel is 15% acrylamide, pH 6.75, electrophoresis is for 5 h at 5 W/gel. Systems I and III are similar to those described by Mets & Bogorad (1974) and Kaltschmidt & Wittmann (1970), respectively.

Determination of [^{35}S]Methionine Incorporation into Ribosomal Proteins in Vitro. In all experiments 145 000 cpm

[1] Abbreviations: 2,4-D, (2,4-dichlorophenoxy)acetic acid; cDNA, complementary deoxyribonucleic acid; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

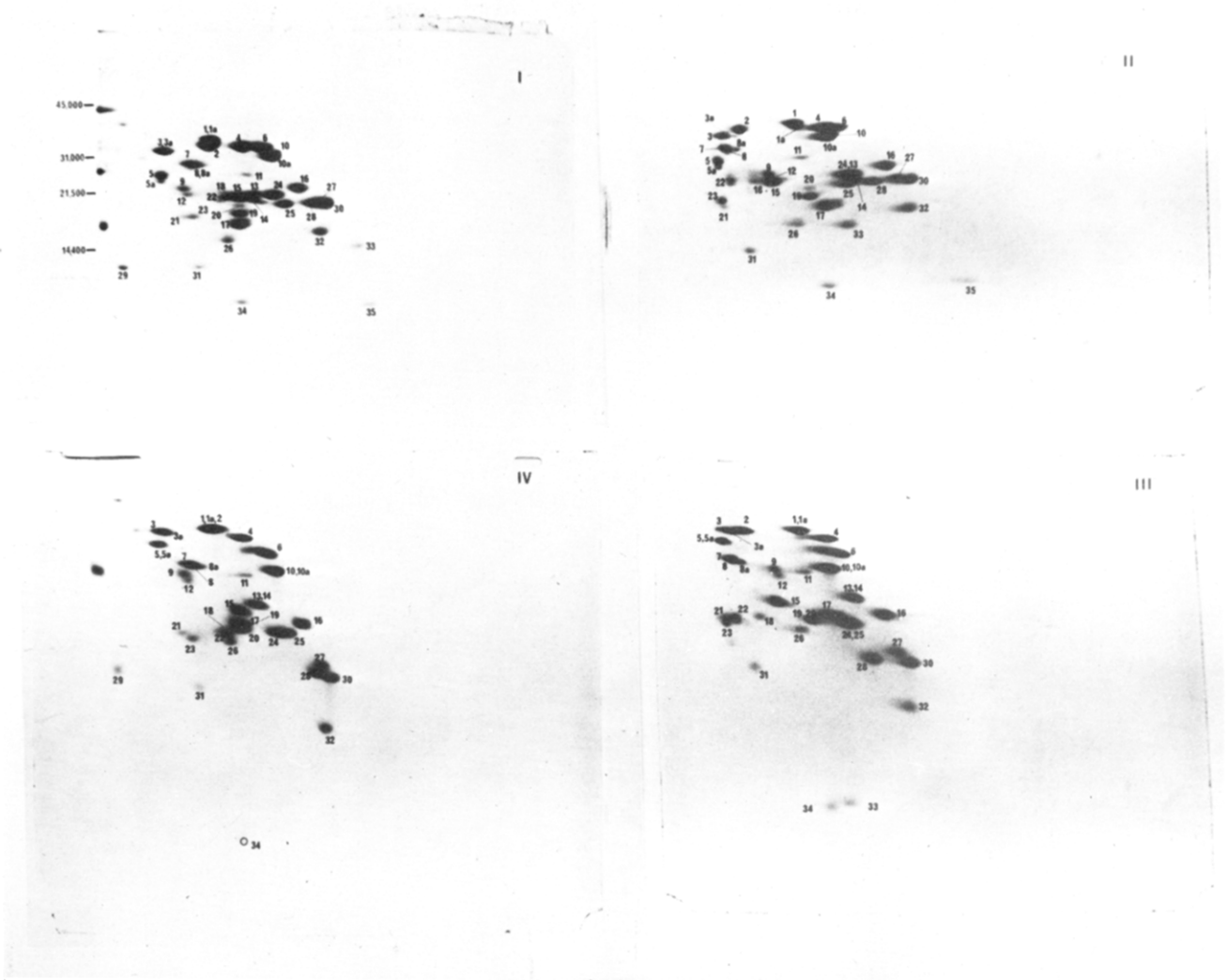

FIGURE 1: Map of proteins extracted from 40S subunit. Electrophoretic separations were performed according to the procedures outlined under Materials and Methods. Approximately 40 μg of protein was loaded onto the first-dimension gels. Faint spots are indicated by open circles.

of in vivo labeled ribosomal proteins were added to 2 000 000 cpm of in vitro synthesized proteins. This mixture was then extracted with acetic acid as described above followed by acetone precipitation (Ramagopal & Ennis, 1980). Following electrophoresis on either system I or III, the gels were stained, destained, and dried. Spots corresponding to ribosomal proteins were cut out and digested (Warner, 1977). Radioactive counting was performed under conditions in which less than 4% of the ^{35}S disintegrations were counted in the tritium window and less than 0.5% of the tritium disintegrations were counted in the ^{35}S window. The counting efficiencies of ^{3}H and ^{35}S were approximately 15 and 60%, respectively. A_i is the ratio of ^{35}S dpm to ^{3}H dpm for the protein.

Results

Two-Dimensional Electrophoretic Maps of Ribosomal Proteins. Four separate gel systems were used in this study. Systems I and IV and systems II and III have common first dimensions, while systems I and II and systems III and IV have common second dimensions. When gels are arranged as in Figure 1 each protein spot in systems I and II has a corresponding spot in systems IV and III that is on the same vertical line. Spots found in systems I and IV have corresponding spots in systems II and III that lie on a horizontal line. Thus, a protein identified on system I can be found on system III by the "four-corner method" (Madjar et al., 1979a).

From system I, horizontal and vertical lines can be drawn to systems II and IV, and from these, vertical and horizontal lines are drawn to system III, completing the rectangle. Systems I and III use conditions similar to those originally described by Mets & Bogorad (1974) and Kaltschmidt & Wittmann (1970), respectively. Use of the method of four corners allows identification of proteins on both of these systems (which have no common dimension) without purifying individual proteins. Thus, proteins that are not resolved on one system may be distinguished on the other. It is unlikely that two different proteins will migrate identically through all four different electrophoretic systems.

The gel systems used in this study resolve only basic ribosomal proteins. No attempt has been made to account for the relatively few acidic ribosomal proteins found in other studies (Gualerzi et al., 1974). Protein spots that are consistantly observed on gels displaying proteins derived from polysomes and from salt-washed, isolated 40S and 60S ribsomal subunits are considered to be ribosomal proteins.

The 40S ribosomal proteins are displayed in Figure 1. Nomenclature for both large and small subunit proteins is based on their migration in system III. Proteins are numbered consecutively from top to bottom and left to right. The four electrophoretic gel systems resolve 40 proteins stably associated

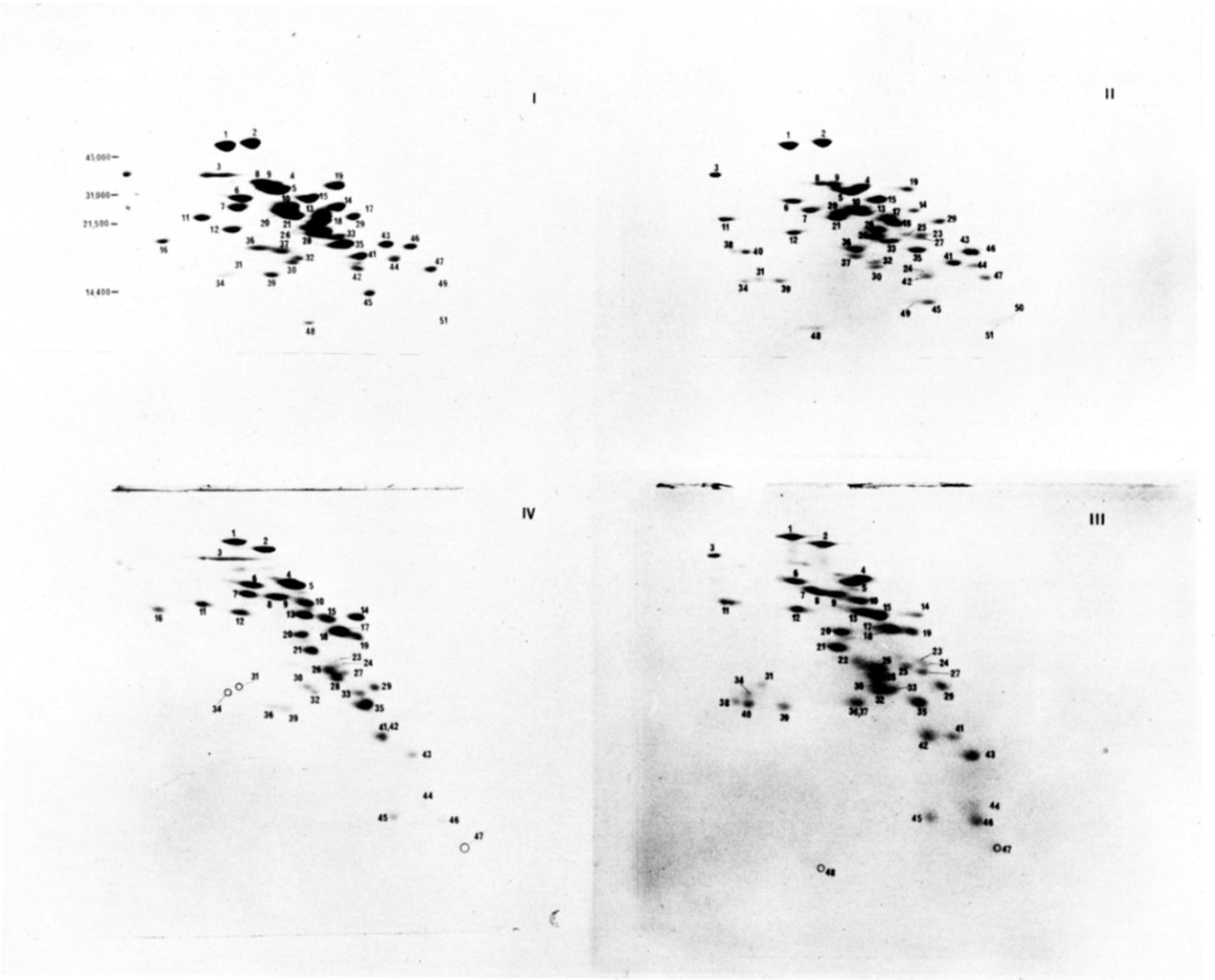

FIGURE 2: Map of proteins extracted from 60S subunit. Electrophoretic separations were performed according to the procedures outlined under Materials and Methods. Approximately 60 μg of protein was loaded onto the first-dimension gels. Faint spots are indicated by open circles.

with the 40S subunit. Some proteins migrate peculiarly. The pairs of proteins S1 and S1a, S5 and S5a, and S10 and S10a and the series of spots labeled S22 (as seen on system II, Figure 1) all have the curious property of migrating together in both acidic and basic first dimensions and being resolved on the $NaDodSO_4$ second dimension yet apparently migrate as a single protein spot in the acidic second dimension. Interestingly, in vitro synthesized S10 and S10a and S5 and S5a are seen in system I autoradiograms. This reduces the chance that these pairs of proteins result from some posttranslational modification. Further work is needed to determine the relationship among these proteins. S17 appears as a doublet in systems I and II (Figure 1); however, this is not seen when total polysomal proteins are displayed (Figure 3). The doublet may represent a degradation product or some other artifact of the subunit isolation procedure. Protein S11 is relatively faint in Figure 1, yet in Figure 3, it stains intensely. This suggests that S11 is loosely bound to the 40S subunit. S29 apparently does not enter the basic first-dimension gels, and S35 is probably lost to the cathode buffer in systems III and IV.

Figure 2 shows the electrophoretic migration of 60S subunit proteins. It is apparent that the 60S subunit preparation is slightly contaminated with 40S subunits (Figure 2, system III). This is probably the result of 40S dimers cosedimenting with 60S subunits. A total of 51 proteins is observed to be associated with the 60S subunit, none of which comigrate with a 40S subunit protein in all four systems. Protein L3 migrates as a smear through the acidic first-dimension gel. However, when ribosomal proteins are directly isolated from polysomes, migration of L3 is much more typical (Figure 3). The reason for this is unknown. L16 apparently does not enter the basic first-dimension gels, suggesting that its pI is less than 8.6.

Several of the faint 60S protein spots seen on system III were not identified on the other gel systems even though it is unlikely that they did not enter the other types of gels or migrated completely through them. With the exception of L14, which was not found in some 60S subunit preparations while in others it stained intensely, all of the identified ribosomal proteins were present in constant amount and migrated identically in different protein preparations and on gels run at different times.

The gels in Figure 3 display acid-soluble polysomal proteins. All of the proteins that were identified as being associated with 40S and 60S subunits are seen in these gels.

Molecular Weights of Soybean Ribosomal Proteins. The approximate molecular weights of the ribosomal proteins are given in Table I. The average molecular weight of 40S and 60S subunit proteins is 24 000 and 23 000, respectively. These values are similar to those reported for a variety of other

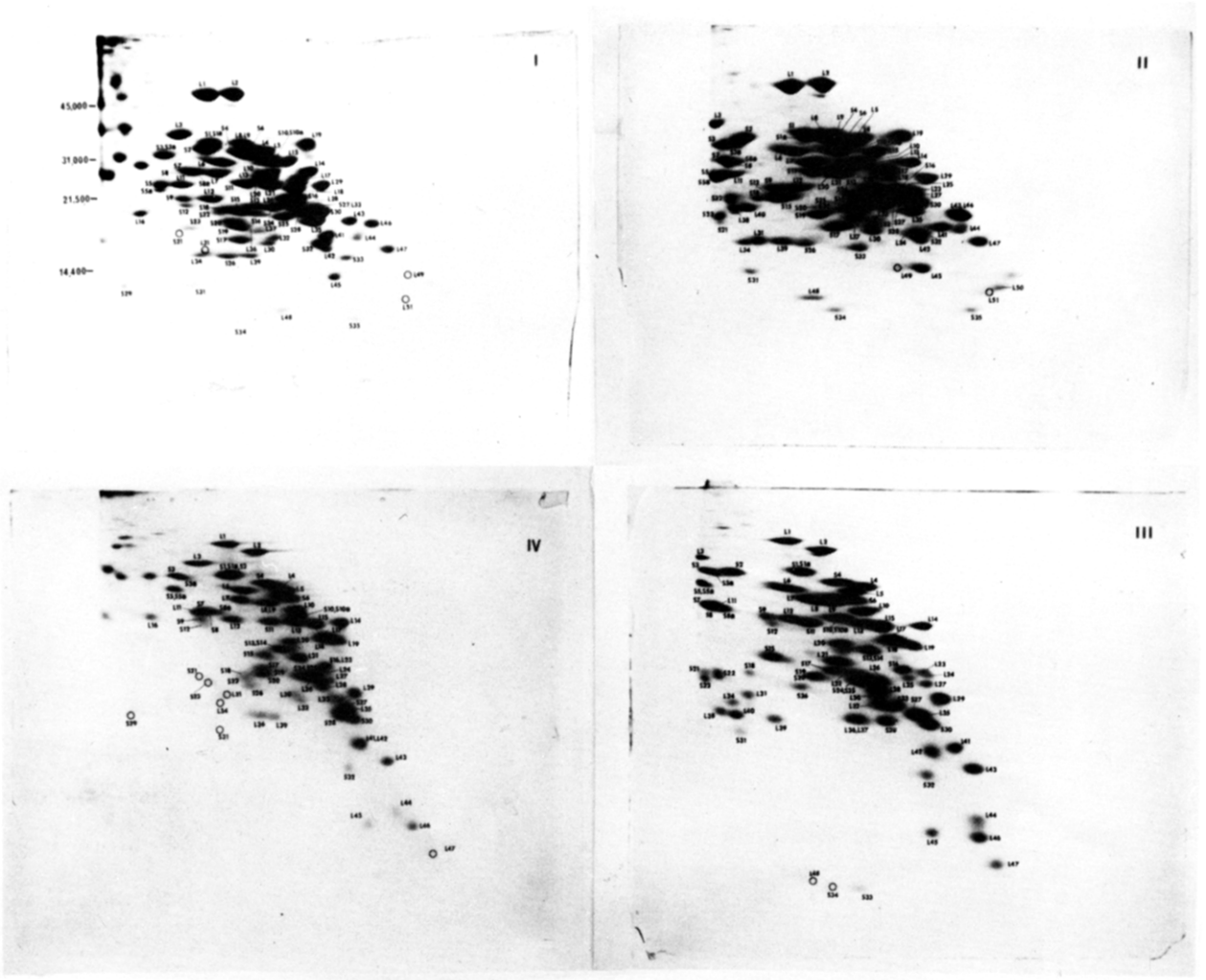

FIGURE 3: Map of proteins extracted from polysomes. Electrophoretic separations were performed according to the procedures outlined under Materials and Methods. Approximately 100 μg of protein was loaded onto the first-dimension gels. Faint spots are indicated by open circles. The approximate positions of molecular weight markers are indicated. Proteins prefixed S and L are derived from 40S and 60S subunits, respectively.

eukaryotic organisms [see, for example, Ramagopal & Ennis (1980) and Capel & Bourque (1982)].

In Vitro Synthesis of Ribosomal Proteins. Auxin enhances the activity of RNA polymerase I in soybean hypocotyls. After 24 h of treatment with 2,4-D, nucleoli have increased in diameter by 140% (Chen et al., 1975), and the amount of RNA polymerase I engaged in transcription is up some 9-fold (Olszewski & Guilfoyle, 1980). The dramatic increase in rRNA synthesis and accumulation suggests that the rate of synthesis of ribosomal proteins may also be elevated. To test this hypothesis, we compared the ability of RNA isolated from auxin-treated and nontreated hypocotyls to direct the synthesis of ribosomal proteins in vitro.

RNA was isolated from 4-day-old etiolated soybean hypocotyls. To examine the influence of auxin on the amount of ribosomal protein mRNA, seedlings were sprayed with 2.5 mM 2,4-D 24 h prior to harvest. Approximately 2.5 times more total RNA per gram of fresh tissue was obtained from the auxin-treated hypocotyls.

Figure 4 shows autoradiograms of [^{35}S]methionine-labeled proteins that were electrophoresed on system I. Panels A and B of Figure 4 display the in vitro translation products of total RNA that was extracted from control and auxin-treated hypocotyls, respectively. From a comparison of Figure 4B with system I of Figure 3, many of the spots in the autoradiograph can be identified as specific ribosomal proteins. Almost all of these spots comigrated with authentic ribosomal proteins. In this experiment, equal amounts of ^{35}S-labeled protein were loaded onto the first-dimension gels. A comparison of panels A and B of Figure 4 reveals that in vitro translation of RNA isolated from auxin-treated hypocotyls is considerably more effective at directing the synthesis of ribosomal proteins than is control RNA. Thus, relative to total in vitro synthesized protein, the proportion that is ribosomal protein is greater when RNA extracted from auxin-treat tissue is used. This indicates that ribosomal protein mRNA is more abundant in the auxin-treated tissue than in the control tissue relative to other mRNAs.

To more accurately determine the degree to which auxin treatment enhances the ability of RNA to direct the in vitro synthesis of ribosomal proteins, dual-label experiments were performed. Following translation of RNA isolated from control and auxin-treated hypocotyls, 145 000 cpm of tritiated ribosomal proteins were added to 2 000 000 cpm of ^{35}S-labeled, in vitro synthesized protein. Following acetic acid extraction, this mixture was electrophoresed on either system I or III. These gels were then stained for protein and dried. The ribosomal protein spots were excised with a razor blade and digested, and the ratios of ^{35}S dpm to ^{3}H dpm were determined. Adding a constant amount of tritiated ribosomal protein corrects for possible differences in the recovery of individual proteins. These differences may arise during the acetic acid

Table I: Molecular Weights of Ribosomal Proteins[a]

40S ribosomal proteins		60S ribosomal proteins	
protein	M_r ($\times 10^{-3}$)	protein	M_r ($\times 10^{-3}$)
S1	35.5	L1	49
S1a	34.5	L2	51
S2	34	L3	38
S3	32	L4	35
S3a	32	L6	31
S4	33	L7	28.5
S5	26.5	L8	37
S5a	25.5	L9	37
S6	34	L10	29
S7	30	L11	26.5
S8	29	L12	24
S8a	29	L13	28
S9	23.5	L14	28
S10	33	L15	31
S10a	31	L16	19.5
S11	27	L17	27
S12	23	L18	25.5
S13	23	L19	34
S14	22	L20	27.5
S15	22	L21	26
S16	24	L22	
S17	17.5	L23	23
S18	23	L24	17
S19	19.5	L25	23
S20	20	L26	24
S21	19	L27	22.5
S22	22	L28	22
S23	19	L29	25.5
S24	22	L30	17
S25	21	L31	16
S26	15.5	L32	18
S27	21	L33	21.5
S28	20	L34	15.5
S29	12	L35	19.5
S30	21	L36	20.5
S31	12	L37	19
S32	16.5	L38	20
S33	15	L39	15.5
S34	<10	L40	20
S35	<10	L41	17.5
		L42	16
		L43	20
		L44	17.5
		L45	12.5
		L46	19.5
		L47	15.5
		L48	<10
		L49	12.5
		L50	11
		L51	10

[a] Molecular weights are the average of two determinations. First-dimension gels were cut in half, and molecular weight standards were placed between the two halves and at either end to ensure close approximation of molecular weights across the entire gel.

extraction, because of unequal entry of protein into the gel or inconsistent excision of the protein from the gel.

Table II summarizes the results of the in vitro translation experiments. Well-resolved ribosomal proteins that migrated away from the bulk of the cytoplasmic proteins were chosen for analysis. Of these, ribosomal proteins that contained sufficient ^{35}S and ^{3}H for accurate determination are listed in this table. The amount of tritium recovered from the gels was, as expected, independent of the type of RNA used in the translations. These data show that auxin treatment resulted in a 4.6–13.3-fold (average of about 8-fold) increase in the amount of translatable ribosomal protein mRNAs.

As Figure 4 illustrates, a significant amount of ^{35}S-labeled material enters the first dimension. Any amount of nonribosomal protein that comigrates with ribosomal proteins will

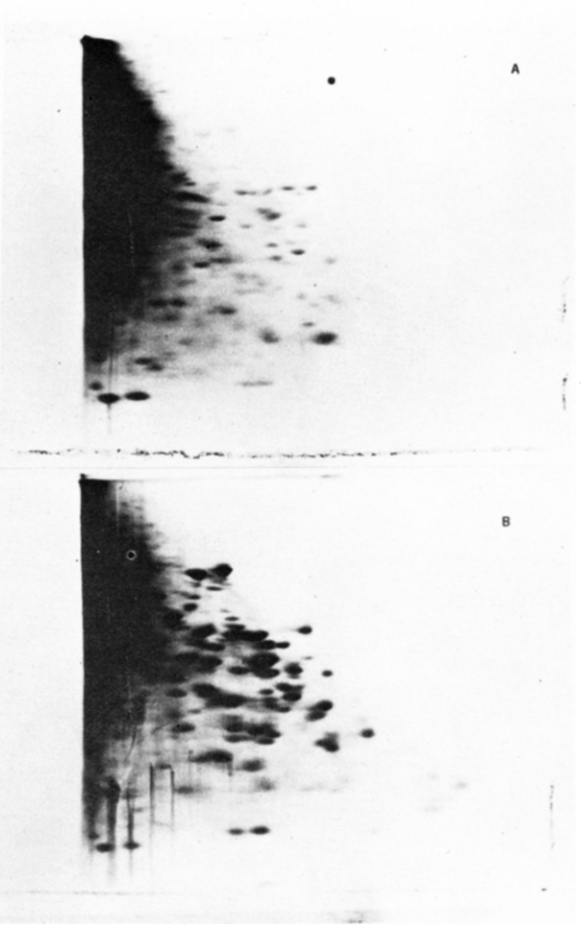

FIGURE 4: Analysis of in vitro synthesized ribosomal proteins. Total cytoplasmic RNA was translated as described under Materials and Methods. 2 000 000 cpm of TCA-insoluble protein was extracted with acetic acid and electrophoresed on system I. The gels were dried, and the proteins were detected by direct autoradiography (6-day exposure). Control RNA and RNA isolated from auxin-treated hypocotyls were used to direct in vitro protein synthesis in panels A and B, respectively. The smearing in the lower left corner of panel B is a gel artifact of unknown cause.

tend to reduce the calculated enhancement by auxin of ribosomal protein mRNA template activity. This presented less of a concern when system III was used, although the problem was not completely eliminated.

In this study, no significant difference was found in the A_i values of individual proteins when total RNA or poly(A)-containing RNA was translated in vitro (data not shown). This indicates that plant ribosomal protein mRNAs, like those of yeast (Warner & Gorenstein, 1977), fruit fly (Vaslet et al., 1980), and mouse (Meyuhas & Perry, 1980), are polyadenylated.

Discussion

As a prelude to the study of cytoplasmic ribosomal protein gene expression in higher plants, we have identified by two-dimensional gel electrophoresis 91 basic proteins stably associated with soybean cytoplasmic ribosomes. The staining intensity of each spot varied more than would be expected on the basis of differences in molecular weight alone. This is not an unusual finding and may result from differences in the intrinsic dye binding properties of the proteins, from differential recovery of the protein from the isolation or extraction of polysomes and subunits, or from differences in the efficiency of fixing the protein following electrophoresis (Steck et al., 1980). We estimate the number of independently migrating

Table II: Incorporation of [^{35}S]Methionine into in Vitro Synthesized Ribosomal Proteins[a]

	no auxin		auxin		
protein	^{35}S/^{3}H (dpm)	A_i (mean ± 90% confidence level)	^{35}S/^{3}H (dpm)	A_i (mean ± 90% confidence level)	A_i(auxin)/A_i(no auxin)
L1	327/1054	0.31 ± 0.05	2797/1093	2.56 ± 0.20	8.3
L2	198/761	0.26 ± 0.07	1874/781	2.40 ± 0.32	9.2
L4	99/342	0.29 ± 0.11	826/359	2.30 ± 0.53	7.9
L5	135/613	0.22 ± 0.06	817/541	1.51 ± 0.19	6.9
L6	189/401	0.47 ± 0.07	1105/404	2.73 ± 0.30	5.8
L7	426/600	0.71 ± 0.13	3162/591	5.35 ± 0.76	7.5
L10	184/461	0.40 ± 0.10	1561/459	3.40 ± 0.43	8.5
L12	404/339	1.19 ± 0.06	2304/350	6.58 ± 0.63	5.5[b]
L14	65/405	0.16 ± 0.04	582/407	1.43 ± 0.27	8.9
L15	114/476	0.24 ± 0.06	963/512	1.88 ± 0.32	7.8
L18	76/423	0.18 ± 0.09	757/462	1.64 ± 0.12	9.1
L19	260/189	1.38 ± 0.42	2739/244	11.2 ± 4.3	8.1
L20	80/187	0.43 ± 0.11	709/187	3.80 ± 0.68	8.8
L21	142/316	0.45 ± 0.16	1462/323	4.53 ± 0.57	10.1
L28	213/434	0.49 ± 0.09	2936/450	6.53 ± 1.41	13.3[b]
L29	86/120	0.71 ± 0.29	731/139	5.26 ± 1.56	7.4
L35	204/621	0.33 ± 0.05	2028/535	3.79 ± 0.87	11.5
L39	236/150	1.58 ± 0.58	863/102	8.42 ± 2.44	5.3
L41	60/263	0.23 ± 0.05	276/260	1.06 ± 0.18	4.6[b]
S1	92/612	0.15 ± 0.04	481/579	0.83 ± 0.28	5.5[b]
S4	161/447	0.36 ± 0.11	1905/576	3.31 ± 0.14	9.2
S11	150/334	0.45 ± 0.07	1189/325	3.66 ± 0.53	8.1
S13	99/152	0.65 ± 0.20	489/121	4.12 ± 0.72	6.3
S16	46/117	0.39 ± 0.06	378/90	4.22 ± 0.94	10.8
S17	100/145	0.98 ± 0.19	697/141	5.18 ± 0.42	5.3[b]
S24	167/315	0.53 ± 0.19	961/329	2.92 ± 0.57	5.5
S25	140/311	0.45 ± 0.07	807/300	2.69 ± 0.39	6.0
S26	243/150	1.62 ± 0.26	1747/163	10.7 ± 2.4	6.6
S30	128/178	0.69 ± 0.39	1376/212	6.48 ± 1.06	9.4

[a] 145 000 cpm of ^{3}H-labeled polysomal proteins was added to 2 000 000 cpm of ^{35}S-labeled, in vitro synthesized proteins. Following acetic acid extraction and acetone precipitation, samples were electrophoresed on either system I or III. After staining, spots were excised from the gel and digested, and the amount of radioactivity was determined. A_i values are the mean of 5–10 determinations. [b] These values differ significantly (<0.01) from the mean A_i(auxin)/A_i(no auxin) value.

40S and 60S subunit proteins to be 40 and 51, respectively. Gualerzi et al. (1974) reported finding similar numbers of proteins in the cytoplasmic ribosomes of pea, corn, and white clover, and Capel & Bourque (1982) have reported the tobacco cytoplasmic ribosome contains approximately 80 proteins.

No protein was associated with both large and small subunits, and all of the proteins found with the isolated subunits were also seen on gels displaying acid-soluble polysomal proteins (Figure 3). With the exception of S11, L3, and L14, all proteins migrated and stained the same whether they were isolated from subunits or polysomes. We do not assume that all of the identified proteins are of unique origin. Immunological and sequence data have traditionally been used to definitively identify ribosomal proteins of independent genetic origin. Nevertheless, the total number, molecular weights, and migration patterns of the proteins reported here are comparable to other, more thoroughly studied, proteins of higher eukaryotic ribosomes.

The concentration of 2,4-D used in these studies is sufficient to induce both cell expansion and division and is required for maximal stimulation of RNA accumulation in the basal section of the hypocotyl, a normally quiescent tissue (Key et al., 1966). However, this concentration of 2,4-D also leads to a cessation of cell division and elongation in the meristemic region and eventually leads to the death of the plant. Although the mechanism of auxin action is not known, its effects in the basal section of the hypocotyl are associated with increased DNA, RNA, and protein synthesis (Key et al., 1966). The activity of RNA polymerase I following auxin stimulation has been studied in some detail (Zurfluh & Guilfoyle, 1981; Guilfoyle et al., 1975, 1980). Fundamental to the present study is the observed increase in RNA polymerase I activity. In order to investigate changes in the amount of ribosomal protein mRNA levels that may accompany the increase in rRNA production, we have compared the ability of RNA isolated from auxin-treated and nontreated tissue to synthesize, in vitro, ribosomal proteins. These results indicate that the relative amount of translatable ribosomal protein mRNA is elevated approximately 8-fold following treatment with 2,4-D.

Assuming that the amount of translatable mRNA reflects the abundance of that mRNA, these results suggest that increased ribosome formation is accompanied by an increase in the level of ribosomal protein mRNAs. This is similar to the results found in rat liver where there is a 2–3-fold increase in the level of ribosomal protein mRNAs, as measured by in vitro translation and cDNA hybridization, following partial hepatectomy (Nabeshima & Ogata, 1980; Faliks & Meyuhas, 1982).

One copy of each ribosomal protein is apparently contained as a necessary part of a functional ribosome. Thus, for efficient use of the plant's resources, it is expected that ribosomal proteins are synthesized in equimolar amounts. This appears to be the case in a number of different systems (Gorenstein & Warner, 1976, 1977; Warner, 1977; DePhilip et al., 1980; Kief & Warner, 1981). In the present study, there appears to be some differential stimulation of mRNA induction. As Table II shows, the fold induction of translatable mRNA for proteins L12, L41, S1, and S17 is significantly smaller than the average, and the amount induced for protein L28 is significantly greater than the average induction of translatable ribosomal protein mRNA. There are several possible explanations for these observations. A trivial explanation is that

the ribosomal proteins may comigrate with proteins that respond to auxin to a different degree. Alternatively, even if the stimulus to increase ribosomal protein mRNAs was simultaneously given, differences in message half-lives would cause the induction to occur at different rates. Those mRNAs that are the least stable would accumulate most rapidly, while those mRNAs that have the longest half-lives would accumulate slowly. Another possibility is that the cell can "fine tune" ribosomal protein synthesis by the in vivo regulation of translational efficiency. It has been shown that the efficiency of ribosomal protein mRNA translation is altered when chick embryo fibroblasts incubated in insulin-deficient medium are stimulated with insulin (Ignotz et al., 1981). Geyer et al. (1982) have shown that while the rate of ribosomal protein synthesis is elevated in growth-stimulated mouse fibroblasts, there is little detectable increase in the amount of mRNA for these proteins as measured by cDNA hybridizations. While these studies have primarily concentrated on ribosomal proteins as a group, some discrepences were noted among the various proteins, suggesting that each mRNA may be translated with its own particular efficiency during each stage of growth. However, conformation of these data by cDNA hybridization studies is needed before a thorough investigation of possible differential stimulation of mRNA is warrented.

The data presented here suggest that the synthesis of rRNA and ribosomal protein mRNAs are coordinated. How closely these are coupled is unknown. In this regard, several other higher eukaryotic systems have been studied (Warner et al., 1979). For example, an early event in the serum stimulation of 3T3 cells is an increase in pre-rRNA synthesis (Grummt et al., 1977). This has been found to be accompanied by a corresponding increase in the rate of ribosomal protein synthesis (Tushinski & Warner, 1982). In contrast, Krauter et al. (1979) have shown that as the rat myoblast L6E9 cell line differentiates, there is a 10-fold reduction in the number of ribosomes and a 5–10-fold decrease in rRNA synthesis. However, the rate of ribosomal protein synthesis in the myotubes is the same as that in the myoblasts (Krauter et al., 1980). Clearly, rRNA and ribosomal protein synthesis are not coupled in this developmental system.

The effects of auxin on gene expression are not well understood. A study of ribosomal protein gene expression may provide some insight into how auxin alters gene expressin. Connected with this are the mechanisms coordinating the expression of many different ribosomal protein genes with the synthesis of rRNA and activity of RNA polymerase I. The ease with which RNA polymerase I activity and ribosomal protein mRNA levels can be modulated over a relatively large range is a unique advantage of this system.

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Mollusk Shell Formation: Isolation of Two Organic Matrix Proteins Associated with Calcite Deposition in the Bivalve *Mytilus californianus*†

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ABSTRACT: The role of the organic matrix during shell formation is investigated by comparing the soluble protein constituents in two shell layers of the bivalve *Mytilus californianus*. The two shell layers differ primarily with respect to ultrastructure and mineralogy. The proteins are separated by using ion-exchange chromatography followed by high-performance liquid chromatography. They are further characterized by an analysis of their mild acid hydrolysis cleavage products which reveals information on aspartic acid containing amino acid sequences present in the proteins. Of the approximately 40 matrix constituents separated, only two proteins present in the outer prismatic (calcite) layer contain the amino acid sequence -(Asp-Pro-Thr-Asp)-. These proteins, which have been purified to homogeneity, may in part be responsible for determining the particular type of calcium carbonate polymorph deposited in the outer shell layer.

Mollusk shells are among the many skeletal hard parts that are formed by the so-called "organic matrix mediated" process (Lowenstam, 1981). Cells of the mantle epithelium synthesize an extracellular structural framework composed primarily of proteins and carbohydrates. The mineral crystals, usually either aragonite or calcite, nucleate and grow within this preformed framework. The precise manner in which the organic matrix influences crystal growth is not known. X-ray diffraction studies of the septal nacreous layer of *Nautilus repertus* show that the bulk of the matrix protein polypeptide chains is aligned parallel to the *b* axis of the associated mineral, aragonite (Weiner & Traub, 1980). Similar results have been obtained for the nacreous layer of the gastropod shell by using electron diffraction (unpublished data). These observations are consistent with the notion that crystal growth in mollusks occurs by an epitaxial process upon a matrix template. Yet little is known about the macromolecules responsible for this process or the precise manner in which crystal growth occurs.

Many attempts have been made to study the functions of matrix molecules in vitro (Nawrot et al., 1976; Blumenthal et al., 1979; Termine et al., 1980; Krampitz et al., 1976; Wheeler et al., 1981). Although these experiments undoubtedly provide important guidelines as to possible functions that these macromolecules may perform, they differ significantly from the in vivo situation in which the matrix constituents together constitute a macro-three-dimensional framework which is assembled prior to the onset of crystal growth. One approach is to reintroduce various components of the framework into the in vitro system. This has been done successfully by Termine et al. (1981), who demonstrated that a particular acidic bone matrix protein, called osteonectin, when complexed with insolubilized type I collagen, is capable of binding synthetic apatite crystals and free calcium ions. Biosynthesis studies of mineralized tissues indicate that certain of the matrix molecules do migrate rapidly to the mineralization front (Weinstock & Leblond, 1973; Dimuzio & Veis, 1978) or are introduced into the extracellular space only after the onset of mineralization (Price et al., 1981). These experiments, together with the large body of biochemical in-

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