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Induction and Accumulation of Heat Shock-Specific Poly(A+) RNAs and Proteins in Soybean Seedlings during Arsenite and Cadmium Treatments[1]

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LEONARD EDELMAN*, EVA CZARNECKA[2], AND JOE L. KEY
Department of Botany; The University of Georgia, Athens, Georgia 30602

ABSTRACT

Northern blot hybridization analyzes revealed that poly(A+) RNAs homologous to eight heat shock (HS)-specific cDNA clones were induced by arsenite (As) or Cd treatments. The mRNAs accumulated slower, and maximum accumulations were consistently lower than HS-induced levels. Prolonged treatment with low concentrations (50–100 micromolar) of As for 6 hours, or Cd for 12 hours, resulted in decreased accumulations of HS-specific mRNAs. This response resembled the 'autoregulation' observed during continuous 40°C HS. However, no autoregulation was evident when soybean seedlings were exposed to high concentrations of As (250 micromolar) or Cd (1 millimolar) for 12 hours. The cDNA probe pCE54 detected a second higher molecular weight poly(A+) RNA following As or Cd treatments which accumulated concomitantly with the lower molecular weight HS-specific poly(A+) RNA. The patterns of low molecular weight HS polypeptides from *in vitro* translations induced by HS, As, and Cd, and analyzed by one-dimensional and two-dimensional SDS-PAGE, were similar but temporal differences were apparent. In addition to HS proteins, many control proteins were also detected in both *in vitro* and *in vivo* labeling patterns from As and, to a lesser extent, Cd treatments. The chemical agents used in this study apparently induced the accumulation and translation of HS messages *in vivo* but not in the selective manner as observed during HS treatment.

We have reported that the HS[3] response in soybean seedlings can be elicited to some extent by various chemicals (4). Treatment with As or Cd results in the accumulation of some HS-specific poly(A+) RNAs to levels similar to those induced by HS. The synthesis of HS proteins is induced by As treatment and correlates with the development of a certain degree of thermotolerance in the soybean seedlings (16). Additionally, As or Cd treatment causes the induction of a second higher mol wt poly(A+) RNA not present in control and barely detectable in HS tissue which hybridizes to the cDNA probe pCE54.

The addition of As or Cd to animal cell cultures can selectively enhance the synthesis of both specific poly(A+) RNAs and specific proteins which are similar, if not identical, to HS-induced poly(A+) RNAs and proteins (HS protein 70 being the most reproducibly and commonly elicited protein) (7, 15).

Drosophila hsp70 genes, which encode the 70 kDa HS proteins, are heat inducible after being introduced into COS monkey cells (18). Treatment with As also induces *Drosophila* hsp70 gene transcription in transformed COS monkey cells. This observation and others (11) prompted the suggestion that HS and As may have a common target, possibly a specific protein, involved in the repression or induction of the HS genes. The variety of stresses which induce the HS response in bacteria apparently all act through a single positive protein effector, htpR (22). In *Drosophila* the HS transcription factor is normally present in unstressed cells but is 'activated' and regulates gene expression during stress treatment (24, 28, 30).

Cd can be readily taken up and accumulated by soybean plants, and even at very low concentrations it has significant toxic effects on soybean growth, metabolism, and enzyme activities (8). Invariably, there is a positive correlation between the severity of toxicity and both the concentration of Cd and duration of exposure in the assayed plant part. At the molecular level, Cd-binding proteins, inducible by Cd and having metallothionein-like characteristics and mol wt less than 4 kDa, have been isolated from soybean plants (6).

This study describes the induction and accumulation of HS-specific poly(A+) RNAs during As or Cd treatment in more detail than in the earlier report (4). Investigations on both optimum concentrations and the time course of induction by As or Cd and subsequent accumulations of poly(A+) RNAs based on Northern blot hybridization analyses using eight HS cDNA clones are described and compared with HS results. Proteins synthesized *in vivo* during either HS or treatment with different concentrations of As or Cd for 2, 6, or 12 h, and *in vitro* translation products are analyzed by SDS-PAGE.

MATERIALS AND METHODS

Plant Material and Incubation Conditions. Soybean seedlings (*Glycine max* [L.] Merr. var Wayne) were grown in moist Chempak bags at 28°C ± 1°C in the dark for 48 h (length of seedlings was 3–4 cm). Intact seedlings were incubated in a 28 or 40°C shaking water bath in 250 ml Erlenmeyer flasks containing 1% sucrose and 1 mM KPO_4 phosphate buffer (pH 6.0) and $CdCl_2 \cdot 2\frac{1}{2} H_2O$ (Sigma) or $NaAsO_2$ (Sigma) was added at the indicated concentrations.

Preliminary studies were conducted to determine the time course and concentration dependence of the induction and accumulation of the poly(A+) RNAs homologous to the HS-specific cDNA clones. Seedlings were treated with 50 μM As for 3.5, 7.5, 15, and 30 min, 1, 2, 5, 9, and 12 h, or with 1 mM Cd for 10 and 30 min, 1, 2, 4, 6, 8, and 12 h. Subsequently, each of four As

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[2] Present address: Department of Microbiology and Cell Science, University of Florida, Gainesville, FL.

[3] Abbreviations: HS, heat shock; As, arsenite; 1-D or 2-D, one-dimensional or two-dimensional; SSC, 150 mM sodium chloride + 15 mM sodium citrate (pH 7.0); poly(A+) RNA, polyadenylated RNA; IEF, isoelectric focusing; Vh, volt hours.

concentrations (25, 50, 100, and 250 μM) or seven Cd concentrations (10, 50, 100, and 500 μM, 1, 5, and 10 μM) were applied to the seedlings for 2 h. Poly(A$^+$) RNAs were isolated following each treatment and analyzed by Northern blot hybridization utilizing five HS-specific cDNA clones for all time periods and concentrations, and three additional HS-specific cDNAs were used for three time periods and three concentrations of As or Cd.

Purification of Poly(A$^+$) RNA. Total RNA was extracted by the tri-isopropylnaphthalenesulfonate/*p*-aminosalicylate/phenol method (10) except that phenol was added to the extraction buffer after the plant material was homogenized. Poly(A$^+$) RNA was purified from the total RNA by oligo-(dT)-cellulose fractionation and quantitated for Northern blot hybridization analysis by ^{3}H-poly(U) hybridization (3).

Northern Blot Hybridization Analysis of Poly(A$^+$) RNA. Polyadenylated RNA samples (1 μg) were electrophoresed on 2% agarose, 6% formaldehyde gels and transferred overnight to nitrocellulose filters. Northern gel electrophoresis, high stringency hybridizations, fluorography, and nick-translation of plasmids were carried out as previously described (4, 25). High stringency filter washes were modified to three times in 2 × SSC and 0.1% SDS for 10 min each at room temperature, once in 2 × SSC and 0.1% SDS for 10 min at 60°C, and once in 0.2 × SSC and 0.1% SDS for 10 min at 60°C. Low stringency hybridization was carried out in 6 × SSC, 5 × Denhardt's, 50 μg/ml sheared salmon sperm DNA and 0.2% SDS at 60°C for 24 h. Low stringency filter washes were performed three times in 3 × SSC and 0.5% SDS for 30 min each at room temperature, and one time in 3 × SSC and 0.2% SDS for 30 min at 60°C (21). Dried filters were autoradiographed at −70°C for 4 h with Cronex lighting plus intensifying screens and Kodak XAR film. The appearance and intensity of signals on the autoradiographs were assessed visually to judge the relative amount of poly(A$^+$) RNA from different treatments hybridizing to individual HS-specific cDNA probes. Different probes were not compared with each other due to differences in size and base composition (*i.e.* specific activity).

***In Vitro* Translations of Poly(A$^+$) RNAs and *In Vivo* Labeling and Gel Electrophoresis of HS-, As-, and Cd-Induced Proteins.** *In vitro* translations were carried out in a cell-free S30 wheat germ lysate system as described by Key *et al.* (13). For isolating *in vivo* labeled proteins, 10 seedlings were pretreated in 7.0 ml of incubation solution and then labeled with 150 μCi of L-[4,5-^{3}H]leucine during the final 2 h of each indicated treatment period (*i.e.* 2, 6, or 12 h). Proteins were extracted, separated by electrophoresis and fluorographed as described by Key *et al.* (13) and Lin *et al.* (16). One-dimensional gel electrophoresis was performed using 10 to 20% (w/v) gradient SDS-PAGE and the buffer system of Laemmli (14). For 2-D gel electrophoresis proteins were precipitated, dried briefly, and resuspended in O'Farrell sample buffer (23). Samples were loaded in the acidic end of the tube gels and run for 5800 Vh. The effective range of the IEF gels was pH 4.0 to 7.0. Proteins were separated on the second dimension using SDS-PAGE on 10 to 20% (w/v) acrylamide gels (6).

RESULTS

As and Cd Induction of HS-Specific Poly(A$^+$) RNAs. Utilizing the Northern blot hybridization technique we were able to determine the concentrations of As or Cd necessary for induction and continued accumulation of poly(A$^+$) RNAs which hybridize to the eight HS-specific cDNA clones described in Table I. A comparison of the results for five of the HS-specific cDNA clones is summarized in Table II. Northern blot hybridization data for three time periods and three concentrations of both As and Cd are presented for all eight probes (Fig. 1). Since only three time points and three concentrations were determined for pHS70, pHS80, and pEV3, these three probes were not included in Table II.

The cDNA probes pCE53, pCE75, and pFS2005 each hybrid select/translate many of the HS-induced mRNAs coding for a group of 27 low mol wt HS proteins (Fig. 3K) in the 15 to 19 kD range (13, 25), and pCE54 hybrid select/translates mRNAs for five to six approximately 27 kD HS proteins (4). RNAs hybridizing to these four cDNA clones also showed a high level of induction and accumulation by both As and Cd following prolonged exposure to the highest concentrations tested (Fig. 1, A, B, and C, 6 h, lanes 5, 8). In particular, the maximum induction of poly(A$^+$) RNAs homologous to probe pCE54 (Fig. 1E) was at least equivalent to the level induced by HS.

The poly(A$^+$) RNAs hybridizing to the cDNA probes pCE54 and pHS80 are present constitutively at low, but detectable levels in untreated soybean tissue (4; J Roberts, personal communication). At 2 h after transferring the 2-d old seedlings to control incubation media we detected the accumulation of poly(A$^+$) RNAs which hybridized to pCE54 (Fig. 1E, lane 1) and pHS80 (Fig. 1C, lane 1, upper band) above the constitutive levels. This apparent induction was specific for these two probes, and differed both quantitatively and qualitatively from the results obtained during HS, As, and Cd treatments (Fig. 1, C and E, lanes 2–8). These observations may represent a mild stress response in the seedlings to removal from the moist Chem-paks after 2 d of undistributed germination and growth. The subsequent decreasing accumulations of these RNAs in control tissues at 6 and 12 h of continuous incubation may be the result of the seedlings' acclimation to the liquid media.

The soybean HS-specific cDNA pFS2033 hybrid select/translates mRNAs for three HS proteins in the 21 to 24 kDa range (25). These HS proteins are apparently induced by As treatment (Figs. 2A and 4A, lanes 3–5) and to a lesser extent by Cd treatment (Figs. 2A and 4A, lanes 6–8). The highest concentrations of As and Cd tested produced the maximum induction and accumulation of these mRNAs (Fig. 1D, lanes 5 and 8) and encoded proteins (Fig. 2, B and C, lanes 5 and 8; Fig. 3, F and I).

The cDNA probes pHS70 and pHS80 hybrid select/translate mRNAs coding for members of two high mol wt groups of HS proteins from soybean (J Roberts, personal communication). The Northern blot analyses using these probes indicated that the homologous poly(A$^+$) RNAs were induced by As or Cd treatment but not to maximal HS levels (Fig. 1, A and C, upper band). A similar situation was found with the high mol wt HS proteins which accumulate to higher levels during HS than during As or Cd treatment (Fig. 4). Figure 1C, 12 h, lane 8 shows there was an increase in the mol wt of the poly(A$^+$) RNAs hybridizing to pHS80. This result was unexpected but repeatable.

The last HS-specific cDNA which was used in this investigation, pEV3, hybrid select/translates nuclear-encoded mRNAs for two 27 kD polypeptides which localize in the chloroplast during HS as 22 kD polypeptides (26). The results of Northern blot hybridizations using this probe indicated that the strong induction following 2 h HS (Fig. 1F, 2 h, lane 2) was never matched during As (lanes 3–4) or Cd (lanes 6–8) treatments. The highest concentrations of As tested showed accumulation at 6 to 12 h, and the highest concentrations of Cd tested also produced some accumulation by 12 h, but those levels were far below that seen during a 2 h HS.

Relationship of As- and Cd-Induced Proteins to HS-Induced Proteins. When the growth temperature of soybean seedlings is shifted from 28°C to 40°C there is a rapid and dramatic change in the pattern of protein synthesis (13). The synthesis of most control proteins is decreased and a new set of proteins (HS proteins) is synthesized. When viewed on 1-D SDS-PAGE (Figs. 2 and 4, lane 2) soybean HS proteins may be grouped into abundant low mol wt groups (15–19, 21–23, and 26–27 kD HS pro-

Table I. *Summary of Published and Unpublished Data Indicating the Size (DNA Base Pairs) of Each HS-Specific cDNA Probe used in Northern Blot Hybridization Analysis (Fig. 1; Table II)*

Also listed are the number and size(s) of the proteins detected by 2-D SDS-PAGE analysis following hybrid/select translation of HS poly(A$^+$) RNAs using each of the probes.

Probe	Size	Hybrid Select/Translation Proteins		Ref.
	base pairs	*number detected*	*size(s) (kD)*	
pCE53	351	13	15–19	12
pHS70	2100	3	68–70	Unpublished
pFS2005	359	13	15–19	25
pCE75	722	5–8	15–16	12
pHS80	850	1	83–84	Unpublished
pFS2033	438	3	21–24	25
pCE54	537	5–6	26–27	4
pEV3	675	2	27[a]	26

[a] Processed to 22 kD protein in the chloroplast.

Table II. *Times of Initial Induction and Maximum Accumulation were Determined for Several HS-Specific poly(A$^+$) RNAs in Intact Soybean Seedlings Treated for Different Time Periods with 50 μM As or 1 mM Cd*

At various time points between 3.5 min and 12 h of treatment poly(A$^+$) RNAs were isolated and their hybridization with five labeled HS-specific cDNA clones (5×10^6 cpm per filter) was analyzed by the Northern blot hybridization technique. The effect of four different As concentrations (25–250 μM) and seven different Cd concentrations (10 μM–10 mM) during a 2 h treatment of intact soybean seedlings was determined by Northern blot analysis. Following a 4 h exposure, the earliest visible autoradiographic evidence of labeling was taken as initial induction, and the most visibly extensive (relative to other lanes) autoradiographic signal was taken as maximum accumulation. Comparable initial induction by 40°C HS occurs within 15 min, and maximum accumulation occurs after 2 to 4 h (25). The approximate %HS is a relative visual comparison of the maximum accumulation levels of each HS-specific poly(A$^+$) RNA detected after HS, As, or Cd treatments.

Probe	Treatment Times				Concentrations				Approximate % HS	
	Initial induction		Maximum accumulation		Initial induction		Maximum accumulation			
	As (50 μM)	Cd (1 mM)	As (50 μM)	Cd (1 mM)	As (2 h)	Cd (2 h)	As (2 h)	Cd (2 h)	As	Cd
	h		*h*		μM		μM			
pCE53	2	0.5	5	4–8	50	100	250	1	90	25
pFS2005	2	1	5	4–8	50	500	100	1	90	90
pCE75	2	1	5	4–8	25	500	100	1	75	75
pFS2033	2	4	5	4–8	25	1000	100	10	90	75
pCE54	1	0.16	2	8	50	250	50	1	100	100

teins) and less abundant high mol wt groups (70, 84, and 92 kD HS proteins). Figure 2 shows the *in vitro* translation products corresponding to poly(A$^+$) RNAs isolated after the indicated treatments. All the low mol wt HS proteins showed up very prominently following a 2 h HS (Figs. 2A and 4A, lane 2; Fig. 3A), but by 6 and 12 h of continuous HS they have decreased dramatically in abundance (Figs. 2, B and C; 4, B and C). This corresponds to the decreasing abundance of the respective RNAs from 2 to 12 h of continuous HS visualized in Figure 1, lane 2.

Following treatment with As (Fig. 2, lanes 3–5) we observed many of the same *in vitro* translation products as HS, but only at the highest As concentrations tested for the 2 h treatment (Fig. 2A, lane 5). The accumulation of HS-specific *in vitro* translation products from As-treated tissue appeared to peak following 6 h of treatment (Fig. 1, 6 h, lanes 3–5; Fig. 2B, lanes 3–5), and a high level of those translation products was still observed at 12 h of treatment with the highest As concentration (Fig. 1, 12 h, lane 5; Fig. 2C, lane 5). In general, the labeling patterns visible after Northern blot hybridization and SDS-PAGE of *in vitro* translation products indicated the accumulation of HS-specific mRNAs and, possibly, the corresponding proteins occurred more slowly during treatments with low and high concentrations of As than during HS. The decline in accumulation observed after more than 2 h of continuous HS is also apparent during continuous treatment with low concentrations of As. However, high levels of HS mRNA accumulation persist at 12 h of continuous treatment with high concentrations of As. A 1 mM Cd treatment for 2 h induced HS-specific poly(A$^+$) RNAs (Fig. 1, 2 h, lane 8) and we also detected the corresponding *in vitro* translation products (Fig. 2A, lane 8). However, in most cases a 6 h Cd treatment was required before the levels of accumulation of HS-specific poly(A$^+$) RNAs (Fig. 1, 6 h, lanes 6–8) or corresponding *in vitro* translation products (Fig. 2B, lanes 6–8) approached HS-induced levels. The highest accumulation of these RNAs and polypeptides was seen in all instances using the highest Cd concentrations applied for the longest times (Fig. 1, 12 h, lane 8; Fig. 2C, lane 8). Following As or Cd treatment the *in vivo* labeled low mol wt HS proteins (Fig. 4, lanes 3–7) did not appear as prominently as those synthesized during HS (Fig. 4, lane 2). Incorporation of label during long-term 1 mM Cd treatment was prohibitively low and consequently those samples were not included on the gel (Figs. 4B and C, lane 8).

There also appeared to be a set of poly(A$^+$) RNA *in vitro* translation products (Fig. 2, lanes 3–8) in the 40 to 50 kDa range which accumulated to high levels in As- and Cd-treated samples which were not apparent in the HS samples (Figs. 2 and 4, lane 2).

Despite the induction of mRNAs hybridizing to the cDNA

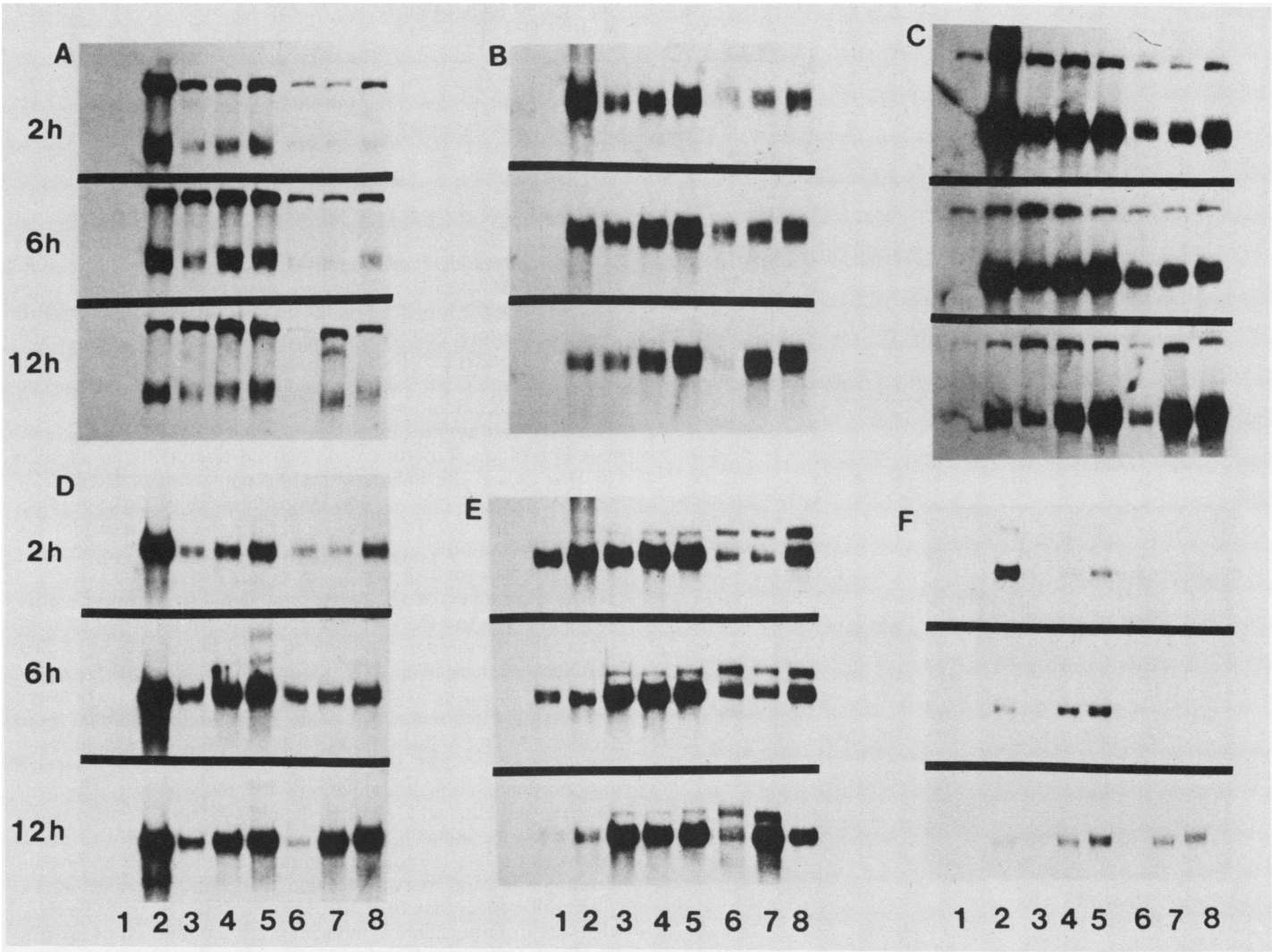

FIG. 1. Northern blot hybridization analysis of HS-specific poly(A^+) RNAs induced by various sodium As or Cd chloride concentrations for the 2, 6, or 12 h time periods indicated at the left of the figures. Treatments were 28°C (lane 1), 40°C (lane 2), or 28°C with $NaAsO_2$ added to the incubation medium: 50 μM (lane 3), 100 μM (lane 4), and 250 μM (lane 5); or $CdCl_2$ added to the incubation medium: 50 μM (lane 6), 100 μM (lane 7), and 1 mM (lane 8). Filters A and C were each hybridized with two heterologous probes. The HS-specific cDNA probes were: A, pCE53 (lower bands in each time period), and pHS70 (upper bands); B, pFS2005; C, pCE75 (lower bands), and pHS80 (upper bands); D, pFS2033; E, pCE54; F, pEV3.

probes pHS70 (Fig. 1A, upper band) and pHS80 (Fig. 1C, upper band), the corresponding high mol wt *in vitro* translation products were not very apparent in Figure 2. Based on our previous experience we believe this to be the result of relatively inefficient translation of large mRNAs by the wheat germ system. The induction of the high mol wt HS proteins is most prominent with *in vivo* labeling following HS (Fig. 4, A and B, lane 2), and to a lesser extent after Cd treatment (Fig. 4A, lanes 6–8).

The very complex group of twenty-seven 15 to 19 kD HS-specific *in vitro* translation products seen on 2-D gel autoradiographs (shown diagrammatically in Fig. 3K) was most prominent for the 2 h HS treatment (Fig. 3A). Continuous HS treatment beyond 2 h resulted in successively lower levels at 6 h (Fig. 3B) and 12 h (Fig. 3C) of treatment. After 2 h of Cd treatment this group of low mol wt HS polypeptides was barely detectable (Fig. 3G), and although the effect of As was apparent at 2 h (Fig. 3D) it was much lower than the corresponding 2 h HS level (Fig. 3A). Continuous exposure to the highest concentrations of As or Cd tested, resulted in increased accumulation at 6 h (Figs. 3E and 3H) and 12 h (Figs. 3F and 3I); these long-term treatments resulted in patterns which resembled 2 h HS levels both quantitatively (Fig. 3A) and qualitatively (Fig. 3K).

In addition to HS proteins many more control proteins were visible in the Cd-treated (Fig. 3, G–I) and, especially,the As-treated samples (Fig. 3, D–F) than in the HS samples (Fig. 3A). The data in Fig. 3F (As treatment, 12 h) apparently showed a 28°C pattern of control protein synthesis superimposed upon the HS protein pattern. The background or 28°C control 2-D SDS-PAGE pattern of *in vitro* translated polypeptides observed after 2 h (Fig. 3J) did not change after 6 or 12 h (data not shown).

In our previous study (4) when poly (A^+) RNA from Cd-treated soybeans was hybrid select/translated using HS-specific cDNA clone pCE54, two basic, low mol wt polypeptides were detected. They were not seen when poly(A^+) RNA from control or HS-treated soybeans were used. In this report we similarly found two basic, low mol wt *in vitro* translation products (<10 kD) (Fig. 3, G–I, lower right). The possible relationship between these Cd-specific polypeptides observed in 2-D gels and

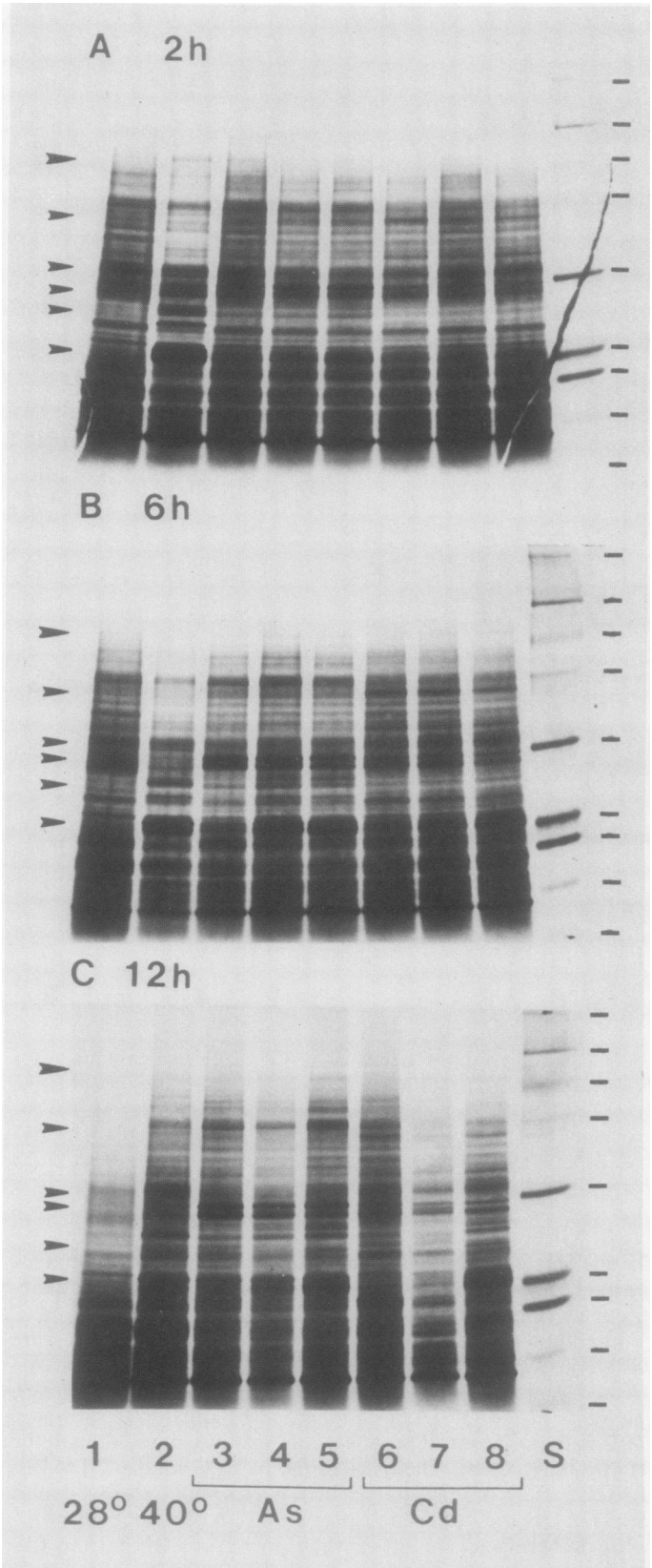

FIG. 2. 1-D SDS-PAGE analysis of *in vitro* translation products. Intact soybean seedlings were treated for 2 h (A), 6 h (B), or 12 h (C), at 28°C (lane 1), 40°C (lane 2), or at 28°C with $NaAsO_2$ added to the incubation medium: 50 μM (lane 3), 100 μM (lane 4), and 250 μM (lane 5); or at 28°C with $CdCl_2$ added to the incubation medium: 50 μM (lane 6), 100 μM (lane 7), or 1 mM (lane 8). One μg poly(A$^+$) RNA samples from each treatment were translated in a cell-free S30 wheat germ lysate system. The *in vitro* translation products were extracted, separated by SDS-PAGE (5×10^4 cpm incorporated L-[4,5-^{3}H]leucine/lane) and fluorographed as described by Key *et al.* (13). Bars to the right of the figures indicate protein standards (3.0, 6.2, 14.3, 18.4, 25.7, 43.0, 68.0, 92.5, and 200.0 kD). Arrows to the left of the figures indicate the low mol wt HS polypeptides (lower four arrows); the As- and Cd-specific polypeptides (fifth arrow from the bottom); and the high mol wt HS polypeptides (top arrow) referred to in the text.

the second higher mol wt RNA seen on Northern blots probed with pCE54 (Fig. 1E) is presently being investigated.

Finally, the low mol wt Cd-binding proteins reportedly detected in soybeans (6) were not apparent on any of our gels. However, this observation was not unexpected since similar proteins in other systems have a very low leucine content.

DISCUSSION

HS mRNAs for the Low Molecular Weight HS Proteins. In soybean seedlings the mRNAs for the low mol wt HS proteins are normally undetectable at 28°C, but within 5 min of a 40°C HS these HS-specific mRNAs are detectable (20). They accumulate during 2 h of continuous 40°C HS to represent 20% or more of the total poly(A$^+$) RNA population (25). Lower levels of the HS-specific mRNAs are detectable after 2 h at 32.5, or 37.5°C, but 45°C for 2 h is a lethal HS treatment and causes no significant accumulation of HS mRNAs (20). In the present study Northern blot hybridization, and 1-D and 2-D SDS-PAGE analysis of proteins showed that As and Cd treatments, in addition to HS, caused the induction and accumulation of HS-specific poly(A$^+$) RNAs (Fig. 1) and HS proteins (Figs. 2–4) in intact soybean seedlings. Table II enumerates the Northern blot hybridization data for lengths of time and concentrations of As or Cd needed to treat the soybeans before HS-specific poly(A$^+$) RNAs were detected and then accumulated to peak levels after detection. The five HS-specific cDNAs used in these experiments differed in conditions for induction and accumulation of HS-mRNAs but these results are not directly comparable due to the different specific activites of the probes. The differences between the As or Cd treatments and a 40°C HS treatment were far more significant. The induction times listed for As or Cd were much longer than for a 40°C HS; the concentrations of As or Cd which produced maximum accumulations were more toxic to the plants than the sublethal 40°C (growth data not shown); neither As nor Cd treatment induced accumulation of HS mRNA to levels equivalent to HS treatment. Assuming that heat is a faster penetrating and more pervasive agent than either chemical agent, these differences may be a function of uptake. It is also possible that one or both chemical inducers were sequestered by specific binding proteins and/or compartmentalized upon entering the cell. This might effectively halt the induction by isolating the inducer. Finally, there may be different pathways leading to the induction of HS mRNAs, and the As- or Cd-induction pathway is slower and less efficient than the HS pathway. Alternatively, a single pathway may have different components working in tandem or cooperatively to elicit a complete HS response (*i.e.* activation, transcription, processing, translation, modifications, and 'autoregulation'). If all or some of these components had different sensitivities to the different inducing agents, the resulting 'HS responses' would vary in induction kinetics and maximum response levels as was observed in these studies.

HS mRNAs for the High Molecular Weight HS Proteins. Treatment with As or Cd, like HS, did not induce high levels of poly(A$^+$) RNAs homologous to the pHS70 and pHS80 cDNA probes. These data emphasize that HS, As, and Cd have a far greater effect on the mRNAs coding for the low mol wt HS

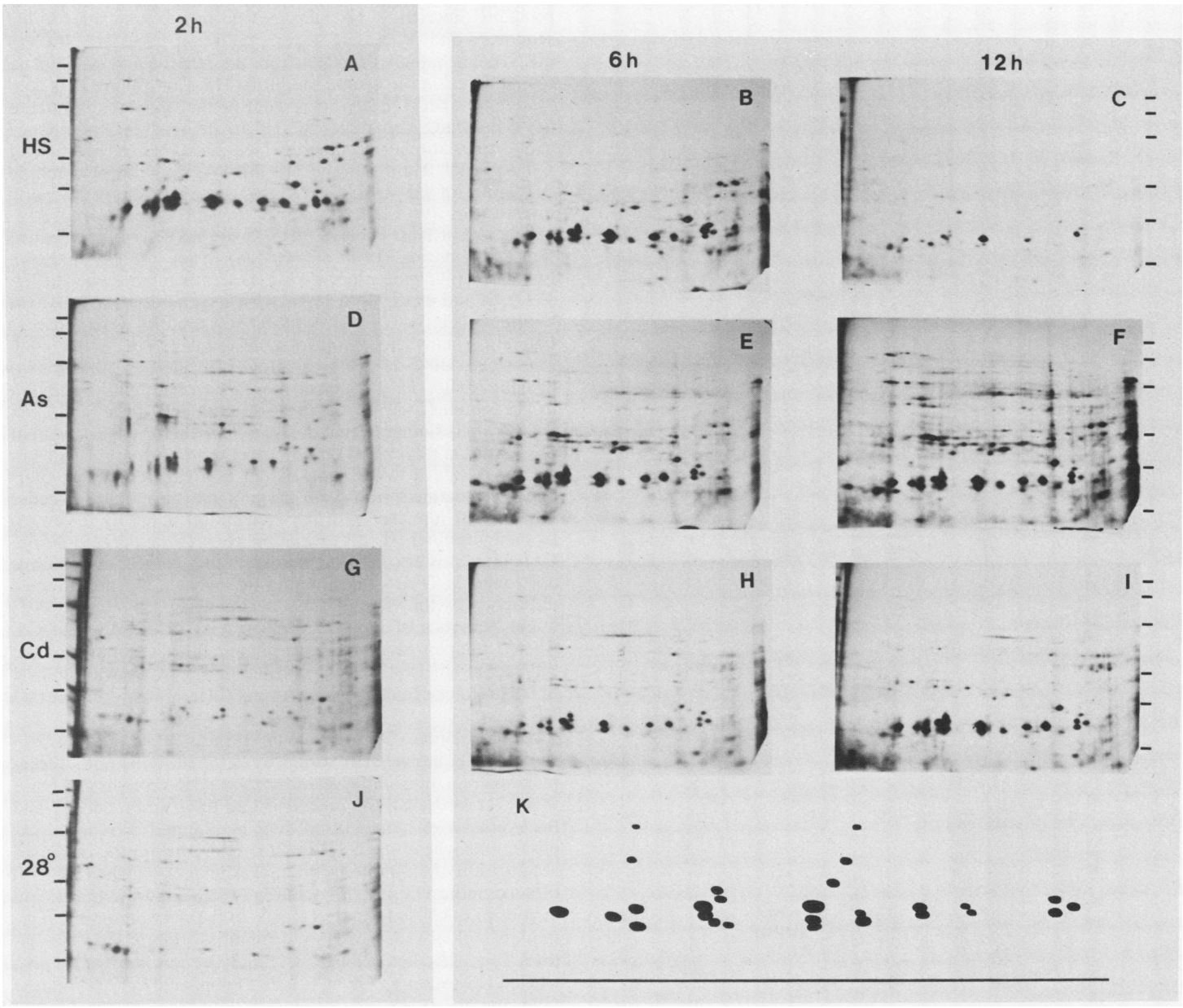

FIG. 3. 2-D gel analysis of *in vitro* translation products. Poly(A$^+$)RNA was extracted from intact soybean seedlings treated for the time periods indicated at the top of each column: 2 h (A, D, G, J), 6 h (B, E, H), and 12 h (C, F, I) at 28°C with 250 μM $NaAsO_2$ added to the incubation medium (D, E, F), or at 28°C with 1 mM $CdCl_2$ added to the incubation medium (G, H, I). One μg poly(A$^+$)RNA samples were translated in a cell-free S30 wheat germ lysate system. The *in vitro* translation products were extracted, run in the first dimension on IEF gels (1×10^6 cpm incorporated L-[4,5-^{3}H]leucine/gel), in the second dimension on SDS-PAGE gels (23) and visualized by fluorography (13) following a 3 d exposure. Figure 3K is an enlarged diagrammatic representation of the gel area containing the soybean-specific pattern of low mol wt HS proteins (15–19 kD) induced by stress treatments (*e.g.* 3, A, F, or I). Bars on the sides of the figures indicate protein standards (14.3, 18.4, 25.7, 43.0, 68.0, and 92.5 kD).

proteins in soybean. This contrasts with animal systems in which the higher mol wt HS proteins are the most prominently induced stress proteins.

In the present study there was an increase in the mol wt of the poly(A$^+$) RNA hybridizing to the high mol wt HS protein gene probe pHS80 after 12 h of treatment with 1 mM Cd. It has previously been reported that different size messages for the low mol wt proteins in soybean are detected at different HS temperatures (20). The HS mRNAs that accumulate at a supraoptimal temperature (42.5°C) are apparently larger than those which accumulate at the lower 'breakpoint' HS temperature (40°C). These unexpected but repeatable results may represent the use of more distal 3′ poly(A) addition sites resulting in longer poly(A) tails (20), or the read-through of normal transcription termination and/or incomplete processing of the HS message during supraoptimal temperature treatment or prolonged exposure to 1 mM Cd.

Control and HS Protein Synthesis. Regulation during the HS response also extends to mRNA processing and translation. Molecular analysis by Yost and Lindquist (29) confirmed that HS disrupts the processing of mRNA precursors in *Drosophila* and the protection of this process correlates with the presence of HS

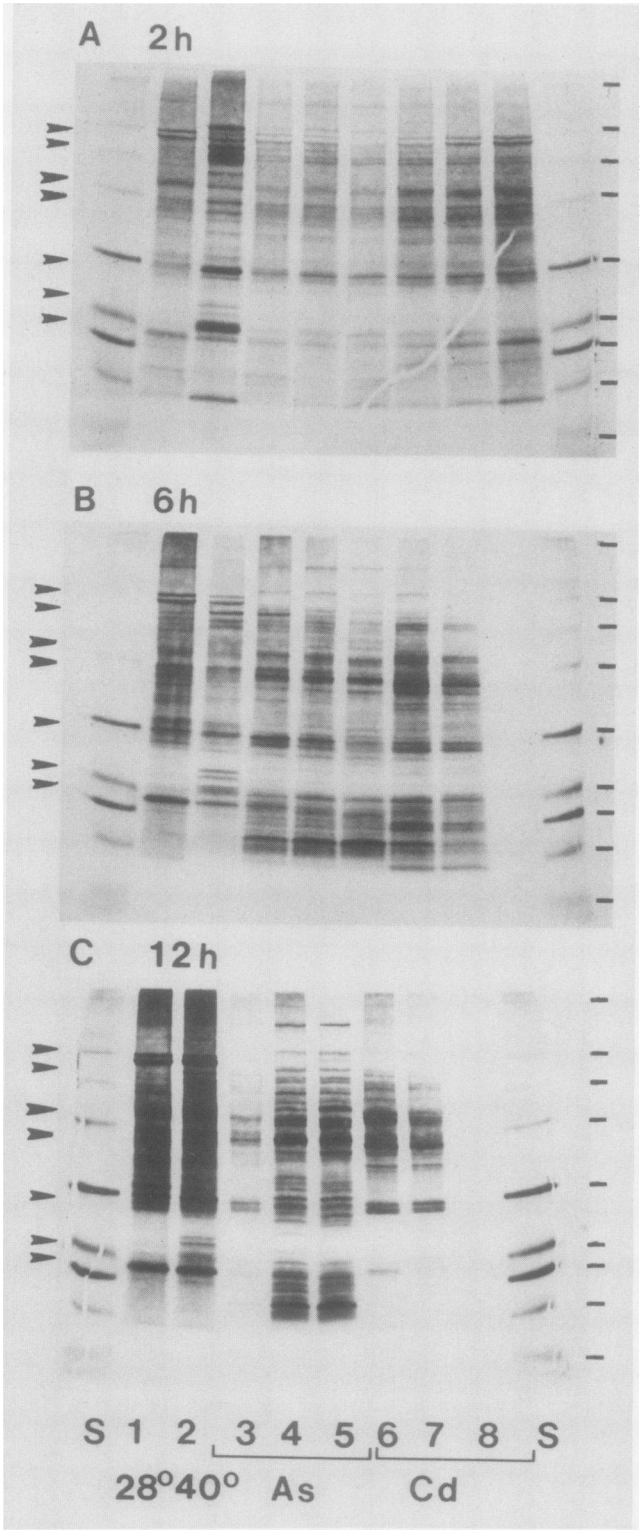

FIG. 4. 1-D SDS-PAGE analysis of *in vivo* labeled proteins. Intact soybean seedlings were incubated for various time periods: 2 h (A), 6 h (B), or 12 h (C), at 28°C (lane 1), 40°C (lane 2), or at 28°C with $NaAsO_2$ added to the incubation medium: 50 μM (lane 3), 100 μM (lane 4), and 250 μM (lane 5); or at 28°C with $CdCl_2$ added to the incubation medium: 50 μM (lane 6), 100 μM (lane 7), and 1 mM (lane 8). Proteins synthesized *in vivo* were labels with 150 μCi of L-[4,5-^{3}H]leucine during the final 2 h of each incubation period, extracted and 5 × 10^4 cpm were loaded per gel lane. The proteins were fractionated by SDS-PAGE and visualized by fluorography (13). Bars to the right of the figures indicate protein standards in lane S (3.0, 6.2, 14.3, 18.4, 25.7, 43.0, 69.0, 92.5, and 200.0 kD). Arrows to the left of the figures indicate the high mol wt HS proteins (top two arrows); the As- and Cd-specific proteins (third and fourth arrows from the top); and the low mol wt HS proteins (three bottom arrows) referred to in the text.

proteins. Since most HS messages do not contain intervening sequences and processing is apparently not necessary, the HS mRNAs are rapidly exported from the nucleus and accumulate to high levels in the cytoplasm during HS. Control mRNAs continue to remain in the cytoplasm during the stress period but are not translated. Apparently the HS treatment induces a change in the translational machinery which allows the recognition of some special feature of the HS mRNAs and the preferential translation of HS mRNAs over control mRNAs (20). Several investigators (9, 17) have shown the untranslated leader sequences of at least two HS mRNAs are necessary for selective translation during HS in *Drosophila*. In the present study we found that following either a toxic As treatment in soybean (Fig. 3, D, E, and especially F) and, to a lesser extent, a 1 mM Cd treatment (Fig. 3, G–I) HS-specific polypeptides were apparent but, also, many more control polypeptides were visible than following a 40°C HS (Fig. 3, A–C). In addition, the *in vivo* labeling results (Fig. 4) showed more control proteins in addition to HS proteins at 12 h of As or Cd treatment. If the regulation in soybean is similar to that in *Drosophila* then it is possible that the As or Cd treatments do not affect pre-mRNA processing in the same way or to the same extent as a HS-treatment. Alternatively, treatment with As or Cd may somehow alter the leader sequence or interfere with the recognition signal by the translational machinery thereby negating any preferential translation. Of course, it is also possible that still other control mechanisms not found in *Drosophila* operate in soybean and are affected differently by the various stress treatments.

The presence of more control proteins in the As- and Cd-treated samples also may have altered the distribution of the radioactive label and been a contributing factor to the lower level of HS proteins detected during As and Cd treatments than during HS.

Autoregulation. The repression of HS protein synthesis in *Drosophila* cells has been reported to be controlled by the accumulation of a threshold level of functional HS proteins (5). Once that level is reached the process is said to be autoregulated and the synthesis of more HS proteins is inhibited. Prolonged exposure of soybean seedlings to 40°C HS results in the increased accmulation of HS proteins for approximately 6 h when the synthesis of more HS proteins apparently stops and the translation of control mRNAs resumes (1, 20). In the data presented here, the low mol wt group of HS polypeptides from *in vitro* translations (Fig. 2A, lane 2; Fig. 3A) and the low mol wt *in vivo* labeled HS proteins (Fig. 4A, lane 2) appeared very prominent following a 2 h HS but declined in abundance as the time of continuous HS increased (Figs. 2, B and C; 3, B and C, and 4, B and C, lane 2). Thus, the so-called autoregulatory process described previously during continuous HS in soybean (1, 20) was also readily apparent in the present experiments.

Treatments with low concentrations of As (25–50 μM) or Cd (50–100 μM) for 2 h induced the synthesis of some of the HS-specific mRNAs (Table II). The accumulation of these HS mRNAs apparently peaked after 6 h (Fig. 1, A–F, lanes 3, 4, and 6) but at levels lower than the maximum levels seen after HS (Fig. 1, lane 2). Further treatment with low concentrations of As or Cd for 12 h resulted in a decline in the detectable level of these messages reminiscent of the autoregulation seen during continuous HS. However, the highest accumulated levels detected during As or Cd treatment were much lower than the level we

observed prior to the onset of autoregulation during HS. Therefore, if these peak levels of HS mRNAs and corresponding *in vitro* translation products paralleled the 'threshold' level(s) of HS proteins required to elicit autoregulation then As and Cd treatments must have a different requirement than HS to initiate the same regulation, or some other mechanism is responsible.

Exposing the seedlings to increased concentrations of As or Cd produced different results than with lower concentrations or a 40°C HS. During 12 h of treatment with high concentrations of As (250 μM, Fig. 1, lane 5) or Cd (1 mM, Fig. 1, lane 8) the HS mRNAs accumulated slowly to near HS-induced levels (*i.e.* much higher than the levels observed with low concentrations of As or Cd). Under these conditions the accumulation of HS proteins also approached HS levels (Figs. 2 and 4, lanes 5 and 8; Fig. 3, F and I). However, these As- and Cd-induced HS responses required toxic concentrations of the chemical inducers (*i.e.* more than 50% growth inhibiting) as well as longer times of treatment than was needed with nonlethal HS temperatures (Figs. 2, 3, and 4). The different HS mRNA levels observed during treatment with low *versus* high concentrations of As or Cd may be attributable to different sensitivites of transcriptional induction, and/or differences in the stabilities of the HS mRNAs. During continuous HS of soybean seedlings the level of HS mRNAs begins to decline but at a slower rate than if the seedlings are returned to control temperatures (20). This indicates that HS mRNAs are more stable at HS temperatures. It is possible that during continuous treatment with high, toxic concentrations of As or Cd the decay of HS mRNAs also slows down resulting in increased accumulations. Alternatively, the results from at least one other system lend support to an explanation involving different sensitivities of induction. In sea urchin embryos, two members of the metallothionein multigene family are both inducible by high levels of Cd but only one gene responds to low levels of the heavy metal ion (27). The primary response of the metallothionein genes to Cd is mediated through metal responsive elements (MREs) in the 5′ regulatory region of the genes (27). The presence of MRE-like sequences in addition to heat shock elements (HSEs) in the 5′ regulatory region of soybean HS genes suggests that the primary induction of HS gene expression by HS, and As or Cd may occur through separate regulatory sequences (20). If the HSEs are HS-specific and function as much stronger promoters of HS gene expression than As- or Cd-specific MREs then a compensatorily high threshold concentration of As or Cd, although toxic to the plant tissue, might be required to elicit a HS response equivalent to a 40°C-induced HS response.

One very conspicuous difference between the various stress treatments was the decreased accumulation of HS mRNAs and corresponding *in vitro* translation products with continuous HS or exposure to low concentrations of As or Cd, while exposure for the same times to high levels of As or Cd resulted in steadily increasing accumulations to high levels at 6 and 12 h for As, and 12 h for Cd. Therefore, the autoregulation of HS proteins and HS mRNAs observed with continuous 40°C HS or exposure to low concentrations of As or Cd was not apparent for 12 h of continuous exposure to high concentrations of As or Cd.

Inducing, Sustaining, and Repressing the HS Response. Based on these observations it seems possible that a high level of damaged and abnormal proteins resulting from the stress treatment may be the factor which sustains the HS response following initial induction. As the nonfunctional proteins are repaired and/or removed from the cell the HS response declines. It has been suggested previously that abnormal proteins trigger the HS response (2, 19). However, considering the very short time needed for induction (30) it is more plausible that the initial induction step involves an almost instantaneous reaction (*e.g.* a conformational change or phosphorylation in the plasma membrane or transcription factor) which occurs prior to cellular damage and is independent of protein synthesis. The subsequent damage inflicted upon the cell by the stress (*e.g.* denatured proteins) may act to sustain the HS response. This secondary response would be both dependent upon and accompanied by protein synthesis, especially HS protein synthesis. The physiological role of HS proteins in soybeans has not yet been elucidated, but there is evidence for a thermoprotective function (16) including the stabilization of soybean proteins against denaturation (CY Lin, personal communication). Once the damage is corrected the HS response would decline in a manner previously attributed to autoregulation by a high level of HS proteins (5). However, if the damage is unmanageable and the level of abnormal proteins remains high (*e.g.* with high, toxic concentrations of As or Cd), then the HS response would persist and the levels of HS mRNAs and HS proteins would remain high in an ongoing effort to effect repairs. Regression of the HS response would only occur if that effort was successful.

We cannot rule out the possibility of autoregulation occurring after 12 h; however, the high concentrations of As and Cd applied for long periods of time are ultimately lethal to soybean seedlings. The deterioration of the plant tissues by 12 h was deemed to be too pronounced for longer term experiments to be useful. It is also possible that the lack of repression of the HS response we have observed under these conditions is the result of regulatory mechanisms breaking down or some other specific characteristic of impending cell death. In addition, the lack of seedling vigor and/or uptake and transport of the chemical agents, especially when very toxic concentrations are used for long periods of time, may play an important role in producing the differential kinetics of induction and accumulation of the low mol wt HS mRNAs and proteins (*e.g.* the amount of total RNA synthesized during As stress has been reported to be much lower than during HS [18]).

In summary, the HS responses induced by HS, As, or Cd treatments are qualitatively similar, but there are distinct quantitative and temporal differences. In addition, the regulatory mechanisms controlling transcriptional induction, translational preference, and repression of the HS response were apparently affected differently by the various stress treatments, as well as by different concentrations of the chemical inducers. Therefore, we feel it is most appropriate to describe our observations during the current study as a HS response with common induction and/or sustentation but variable expression due to differential sensitivities and/or levels of cellular damage, rather than several different HS responses or some unified stress response.

Note Added in Proof. After submission of this manuscript, we have noted that a paper by E. Czarnecka *et al.* (1988 Mol Cell Biol 8: 1113–1122) was just published: analysis of a soybean genomic clone homologous to cDNA clone pCE54 revealed the presence of a single intron of 388 base pairs. The removal of this intron from pre-mRNA is preferentially inhibited by treatment of soybean seedlings with $CdCl_2$ and results in the second higher mol wt RNA we have described on Northern blots probed with pCE54.

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