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Synthesis, transport and localization of a nuclear coded 22-kd heat-shock protein in the chloroplast membranes of peas and *Chlamydomonas reinhardi*

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The synthesis, transport and localization of a nuclear coded 22-kd heat-shock protein (HSP) in the chloroplast membranes was studied in pea plants and *Chlamydomonas reinhardi*. HSPs were detected in both systems by *in vivo* labeling and *in vitro* translation of poly(A)$^+$RNA, using the wheat-germ and reticulocyte lysate systems. Heat-shock treatment of pea plants for 2 h at 42 – 45°C induces the expression of ~10 nuclear coded proteins, among which several (18 kd, 19 kd, 22 kd) are predominant. A 22-kd protein is synthesized as a 26-kd precursor protein and is localized in a chloroplast membrane fraction *in vivo*. Following post-translational transport into intact chloroplasts *in vitro* of the 26-kd precursor, the protein is processed but the resulting 22-kd mature protein is localized in the chloroplast stroma. If, however, the *in vitro* transport is carried out with chloroplasts from heat-shocked plants, the 22-kd protein is preferentially transported to the chloroplast membrane fraction. In *C. reinhardi* the synthesis of poly(A)$^+$RNAs coding for several HSPs is progressively and sequentially induced when raising the temperature for 1.5 h from 36°C to 42°C, while that of several preexisting RNAs is reduced. Various pre-existing poly(A)$^+$RNAs endure in the cells at 42°C up to 5 h but are no longer translated *in vivo*, whereas some poly(A)$^-$RNAs persist and are translated. As in pea, a poly(A)$^+$RNA coded 22-kd HSP is localized in the chloroplast membranes *in vivo*, although it is translated as a 22-kd protein *in vitro*. The *in vitro* translated protein is not transported in isolated pea chloroplast which, however, processes and transports other nuclear coded chloroplast proteins of *Chlamydomonas*. The poly(A)$^+$RNA coding for the 22-kd HSP appears after 1 h at 36°C. Its synthesis increases with the temperature of incubation up to 42°C, although it decreases after ~2 h of heat treatment and the already synthesized RNA is rapidly degraded. The degradation is faster upon return of the cells to 26°C. None of the heat-induced proteins is identical to the light-inducible proteins of the chloroplast membranes.

Key words: Chlamydomonas/pea/chloroplast membranes/heat-shock/*in vivo* and *in vitro* protein synthesis/post-translational transport

Introduction

Following a sub-lethal heat shock several nuclear genes are expressed, which code for a set of proteins ranging in molecular mass from 18 to 94 kd. These heat-shock proteins (HSPs), have been detected in every organism so far investigated (Kelly and Schlesinger, 1978), including higher plants (Altschuler and Mascarenhas, 1982; Cooper and Ho, 1983) and algae (Kloppstech and Ohad, in preparation). No function has yet been established for these proteins, however, it is widely assumed that they confer, at least temporarily, protection against heat damage (Key *et al.*, 1982). Information has been gained on the intracellular localization of heat-shock proteins, their transfer into and from the nucleus (Velazquez and Lindquist, 1984) and their association with the cytoskeleton (Lim *et al.*, 1984).

An early light-induced protein (ELIP) of 17 kd, derived from a precursor of 24 kd, is located in chloroplast thylakoids; ELIP resembles a stress protein in the kinetics of rise and decay of its mRNA, and in its turnover and failure to accumulate (Meyer and Klopstech, 1984). The ELIP is localized in photosystem II particles prepared according to Steinback *et al.* (1981) (Mayer and Kloppstech, unpublished); however, its function is unknown. A protein of similar mol. wt. induced by heat shock has been described by Cooper and Ho (1983) and Cooper *et al.* (1984). This protein appears and turns over with similar kinetics to the ELIP. The light-inducible light-harvesting (LHC II) mRNA is also expressed in the dark after the transfer of the dark grown *Chlamydomonas* y-1 mutant cells to 38°C (Hoober *et al.*, 1982), which, according to our findings, is already a moderate heat-shock treatment in *Chlamydomonas*. This situation is further complicated by the fact that other stresses such as water, salt, light, oxygen and even culture stress (Wolffe *et al.*, 1984) affect or induce synthesis of proteins that are either identical or closely related to the heat-induced proteins.

Chlamydomonas offers some advantage for the study of the heat-shock phenomenon. It can be grown under defined nutrient and CO_2 concentrations in the light or in the dark (Ohad *et al.*, 1967), and it is not susceptible to water stress during heat-shock. Furthermore, it is a suitable photosynthetic organism for the study of *in vivo* labeling and turnover of proteins (Wettern and Ohad, 1984; Ohad *et al.*, 1984). However, the isolation of intact subcellular organelles from *Chlamydomonas* presents difficulties which are not encountered when using higher plants.

We have analyzed the expression, transport and localization of heat-shock proteins in *Chlamydomonas* and pea, and compared them with the light-inducible genes. We found that ELIP and the 18-kd HSP are not identical. Nevertheless, in both organisms we detected a nuclear coded, heat-inducible chloroplast protein of 22-kd which is localized in a chloroplast membrane fraction *in vivo*, and *in vitro* in pea after post-translational transport into chloroplasts isolated from heat-shocked plants.

Results

Induction of HSPs as a function of temperature in Chlamydomonas

Several poly(A)$^+$mRNAs are induced by heat in *Chlamydomonas*, as analyzed by translation in the wheat-germ and reticulocyte cell-free systems. Figure 1 shows the induction of heat-shock poly(A)$^+$RNAs as a function of temperature *in vivo* and their detection by *in vitro* translation. The predominant heat-induced proteins are indicated by arrowheads. Figure 1 demonstrates an

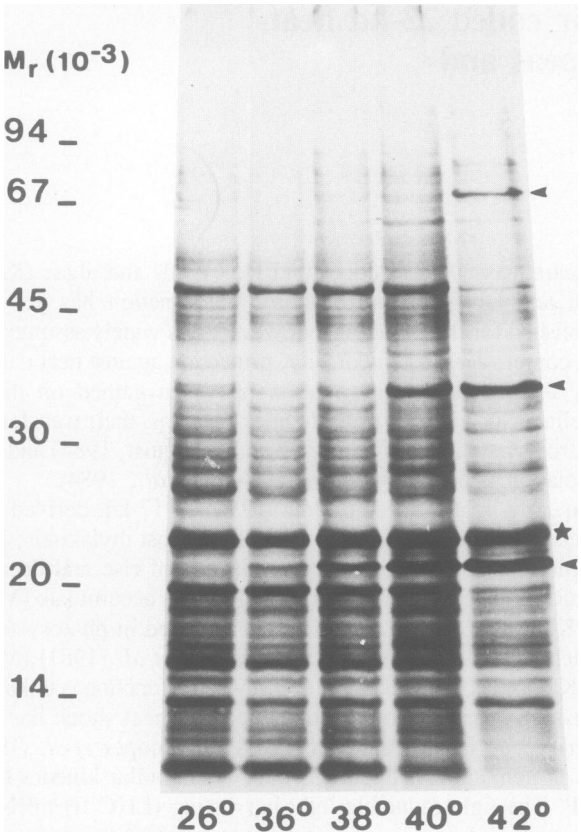

Fig. 1. Temperature of induction of the HSPs in *Chlamydomonas*. *Chlamydomonas* cell suspensions (200 ml) were incubated for 90 min at the indicated temperatures. Cells were harvested, poly(A)$^+$RNAs extracted and limiting amounts translated in the wheat-germ system. The arrowheads point to the predominant heat-inducible protein bands. The asterisk indicates the position of the precursor to the small subunit of RuBPCase.

interesting property of the induction process. The synthesis of the poly(A)$^+$RNA for the 22-kd protein is slightly induced even at 36°C, whereas the mRNA for a 36-kd protein appears at 40°C and that for a 70-kd protein is induced only above 40°C. This implies the existence of a temperature-controlled sequence of induction of individual mRNAs for HSPs. Furthermore, the induction of the heat-shock mRNAs is not an 'all or none' process. Since we used limiting amounts of mRNA for translation, the radioactivity incorporated in a specific protein is indicative of the relative amounts of the corresponding mRNAs present in the translation system. Under these experimental conditions, the intensity of the 22-kd protein band increases with the temperature of the heat-shock. From the *in vitro* translation pattern, it can also be concluded that the mRNAs for many of the pre-existing proteins persist throughout the investigated spectrum of temperatures, while the mRNAs of a few other proteins are no longer expressed and are most probably no longer present after heat-shock for 90 min at 42°C. However, the poly(A)$^+$RNA coding for the small subunit of the RuBPCase and a protein of ~14-kd seem to persist throughout the heat-shock process (Figure 1).

Comparison of Chlamydomonas HSPs synthesized in vivo and in vitro

In Figure 2a and b, the electrophoretic pattern of HSPs synthesized *in vitro* (Figure 2a, lane 7) is compared with that of proteins obtained after pulse-labeling *in vivo* with [^{35}S]SO_4^{2-} of cells pre-incubated at 42°C for up to 5 h (Figure 2a, lanes 8–13). Already during the first hour of heat treatment at 42°C, the incorporation of [^{35}S]SO_4^{2-} is greatly reduced, compared with controls kept at 26°C (lane 13). In heat-treated cells, a large proportion of the label can be found in several prominent protein bands, some of them co-migrating with the *in vitro* synthesized HSPs. However, in the *in vitro* wheat-germ system, the translation of the high molecular mass heat-induced proteins is considerably reduced as compared with labeling *in vivo*.

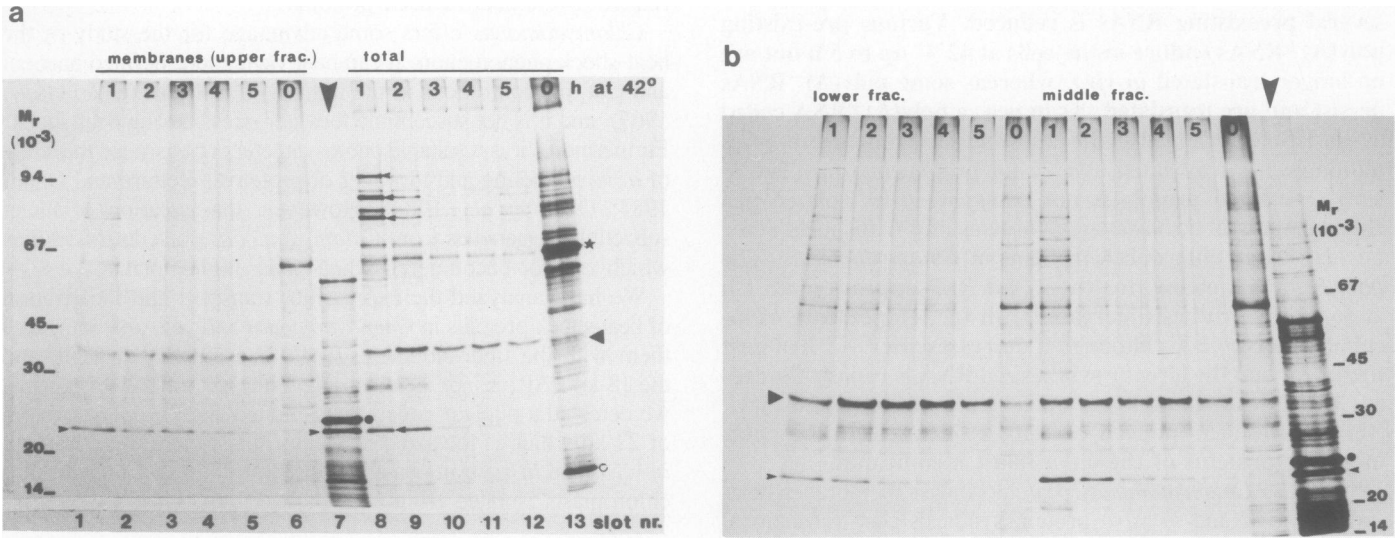

Fig. 2. Pulse-labeling *in vivo* and localization of *Chlamydomonas* HSP. *Chlamydomonas* cell suspensions (30 ml) were heat-shocked at 42°C for 1–5 h and pulse-labeled with $^{35}SO_4^{2-}$ during the last hour, after transfer to 42°C. Control cells were incubated for 4 h and labeled for 1 h at 26°C. Total proteins and membranes were isolated as described in Materials and methods. **(a) Slots 1–5**: thylakoid membranes banding at 0.5 M sucrose; **slot 6:** control kept at 26°C; **slot 7:** *in vitro* translation products of poly(A)$^+$ RNA isolated from heat-shocked cells; **slot 8–12:** total proteins from pulse-labeled heat-shocked cells; **slot 13:** total proteins from control cells. **(b) Slots 1–6:** thylakoid membrane fraction banding at 1.5 M sucrose, and **slots 7–12,** thylakoid membrane fraction banding at 2.0 M sucrose; conditions were the same as for **slots 1–6** in **(a)** above. **Slot 13:** *in vitro* translation products of heat-shock poly(A)$^+$ RNA. Asterisk: large subunit of RuBPCase. Precursor (●) and mature protein of small subunit RuBPCase (○), respectively. ◀, 32-kd herbicide-binding protein. ◀, HSPs; numbers on top of Figure 2b, hours of incubation at 42°C. ▼ (lane 7), *in vitro* translation products.

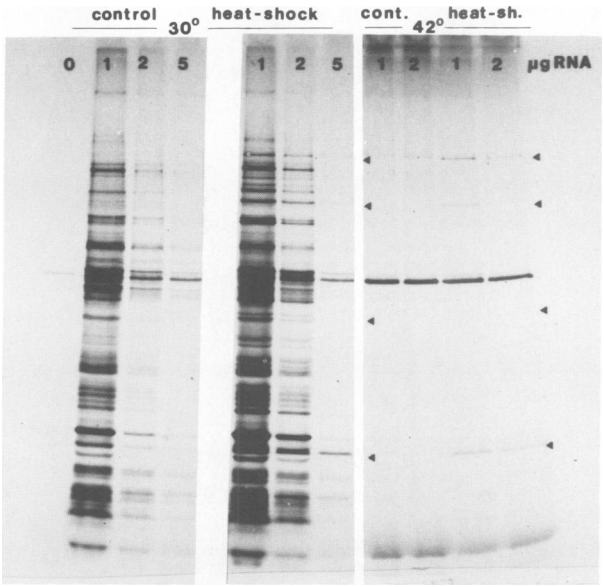

Fig. 3. Translation of poly(A)$^+$RNA from control and heat-shocked *Chlamydomonas* in the reticulocyte lysate system. The indicated amounts of control and heat-shock poly(A)$^+$ RNA were translated in 20 µl reticulocyte lysate at 30°C and at 42°C. The arrowheads point to HSPs of 22, 40, 70 and 94 kd. The band migrating at ~50 kd is apparently a translation artifact specific to the reticulocyte lysate system and is also present in the control lane.

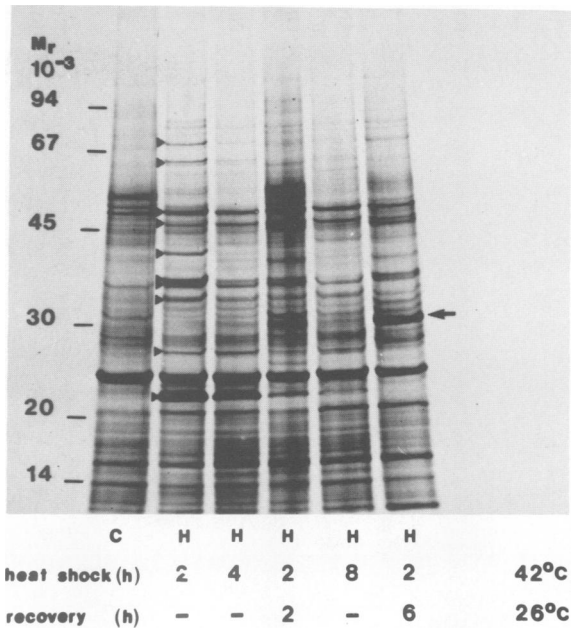

Fig. 4. Extended heat-shock and recovery in *Chlamydomonas*. *Chlamydomonas* cells were treated at 42°C for 2, 4 and 8 h (H); part of the cells were returned after 2 h of heat-shock to normal conditions (26°C) and incubated for another 2 or 6 h. Cells were collected by centrifugation, frozen and the poly(A)$^+$RNA extracted. Limiting amounts of RNA were translated. Translation products of poly(A)$^+$RNA from control cells are shown in the left lane (C); ←, 30 kd protein.

Since in the reticulocyte lysate system high mol. wt. proteins are more efficiently translated than in the wheat-germ system, heat-shock mRNAs were also translated in the former system (Figure 3). HSPs of higher mol. wt. are expressed to a greater extent in this case but, even so, the translation patterns obtained by initiation *in vitro* differ from those obtained *in vivo* or by run-off translation of isolated polyribosomes (Kloppstech and Ohad, in preparation).

Translation of increasing amounts of heat-induced mRNAs in the reticulocyte lysate gives similar results to those obtained in the wheat-germ system (Kloppstech and Ohad, in preparation), i.e., leading to a decrease of incorporation of amino acids. Under these conditions, the 22-kd heat-shock mRNA is preferentially translated. Moreover, when translation is carried out at 42°C instead of 30°C while using non-saturating amounts of RNA, HSPs are preferentially expressed (Figure 3).

Persistence of heat-shock mRNAs at high temperature and after return to normal temperature (recovery) in Chlamydomonas

The dramatic decrease in the translation of pre-existing messages during heat-shock in *Chlamydomonas* is not a simple 'switch-off' mechanism affecting equally all of the pre-existing RNAs. The synthesis of some proteins such as the small subunit of RuBP-Case, whose messenger is still present (Figure 1), is almost completely turned off during the first hour, while other proteins continue to be expressed. The latter category includes the large subunit of RuBPCase and the herbicide-binding 32-kd protein (Figure 2a, b), both of which are coded by chloroplast DNA (Bedbrook *et al.*, 1978; Coen *et al.*, 1977) and thus are translated from poly(A)$^-$RNA. The data in Figure 2a demonstrate that following heat-shock, many of the poly(A)$^+$RNA-coded proteins are no longer labeled *in vivo*, although their mRNAs are still present (compare with Figure 1).

The heat-induced proteins themselves are expressed to a large extent only during the first 1 or 2 h of heat-shock. Thereafter their synthesis declines rapidly (Figure 2a, lanes 8–12). The transient expression of HSPs of *Chlamydomonas* appears to be regulated by reduction in the level of the corresponding mRNAs (Figure 4). After 4 h at 42°C, some of the mRNAs of the high molecular mass HSPs are no longer present, while others, including the 22-kd protein, are greatly reduced. As heat-shock proceeds, loss of heat-shock RNA continues, and the 22-kd protein, which is predominant in the *in vitro* translation pattern of poly(A)$^+$RNA isolated after 2 h of heat-shock, is hardly detectable after 8 h of heat-shock. When returned to normal temperature (25°C), the disappearance of heat-shock mRNAs is substantially enhanced. This indicates that the synthesis of heat-shock mRNAs is rapidly turned-off after return to 25°C and that some of the heat-shock mRNAs, including that of the 22-kd protein, have a very short half-life (Figure 4). It is rather surprising that, after return of heat-treated cells to the normal growth temperature, an mRNA appears which codes for a 30-kd protein that is not present to the same extent in control cells.

Comparison of in vivo and in vitro heat-induced proteins of pea

The induction of several HSPs is also observed in pea both by *in vivo* labeling with [^{35}S]methionine and by *in vitro* translation of isolated poly(A)$^+$RNA (Figure 5). The HSPs synthesized *in vivo* and *in vitro* (Figure 5) are very similar. As in *Chlamydomonas*, it is, however, evident from Figure 5 that the translation of the lower mol. wt. bands in the wheat-germ system is favored relative to the higher mol. wt. polypeptides which, in turn, are preferentially expressed *in vivo*. Preferential translation of lower mol. wt. polypeptides, although not to the same extent, has also been observed after translation of heat-shock mRNAs in the reticulocyte lysate system (Figure 5). The heat-shock polypeptides of pea shown in Figures 5 and 6 have the same apparent

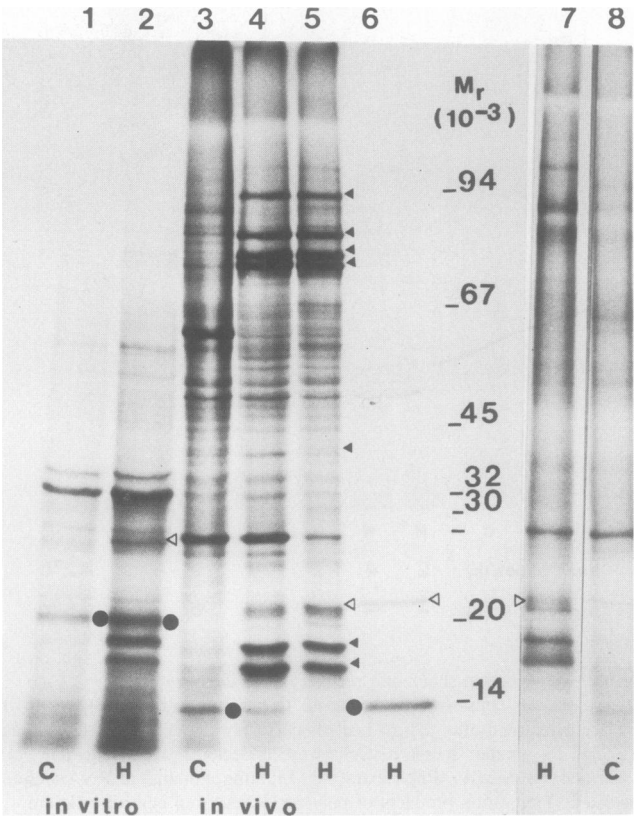

Fig. 5. Comparison of pea HSPs synthesized *in vivo* and *in vitro*. Control (C) and heat shocked pea poly(A)$^+$ RNA (H) were translated and compared in **lanes 1** and **2**. Total protein was isolated after *in vivo* labeling from control (C, **lane 3**) and heat-shocked (H) plants after 2 h **(lane 4)** and 6 h **(lane 5)** of labeling, as described in Materials and methods. **Lane 6** shows a stroma fraction after transport of *in vitro* translation products of heat-shock poly(A)$^+$ RNA. **Lanes 7** and **8** represent an *in vivo* labeled, membrane-enriched fraction from 6 h heat-shock (H) and control cells (C). The samples were from the same experiment as those in **lanes 3–5** but from a different gel. The Mr values refer to the left panel. ◀, Heat-induced protein of the same approximate mol. wt. *in vivo* and *in vitro*; ◁, heat-induced band showing a different Mr between the *in vivo* and *in vitro* patterns; ●, small subunit RuBPCase precursor synthesized *in vitro* **(lanes 1** and **2)** and product after processing both *in vivo* **(lane 3)** or *in vitro* **(lane 6)**.

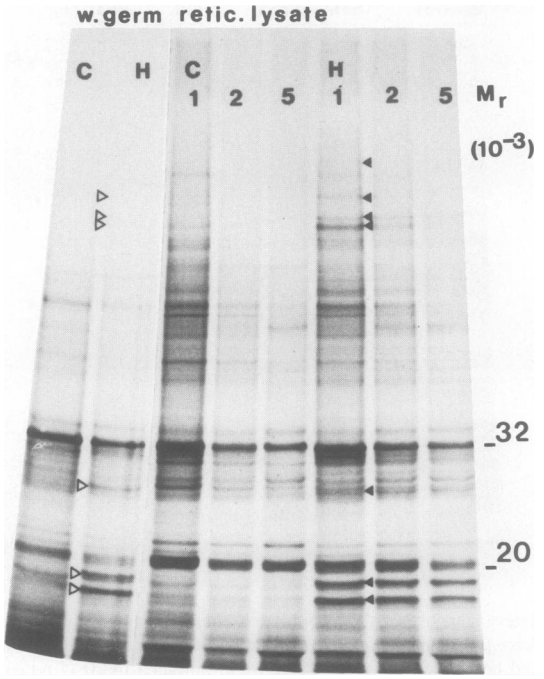

Fig. 6. Comparison of *in vitro* translation products of control and heat-shock poly(A)$^+$ RNA of pea in wheat-germ and reticulocyte lysate systems. Control (C) and heat-shock (H) pea poly(A)$^+$ RNAs were translated in the wheat-germ and reticulocyte lysate systems, and the translation products were compared by both electrophoresis and autoradiography. Equal amounts of counts were loaded on the slots. The numbers indicate translation of 1-, 2- and 5-fold saturating amounts of mRNA. The heat-shock polypeptide bands, ▷, wheat germ, ◀, reticulocyte lysate, and also most of the other proteins are almost identical in both translation systems.

mol. wts., whether translated *in vivo* or *in vitro*, with one important exception: a band of 26 kd obtained *in vitro* has no counterpart *in vivo* (Figure 5). The reverse is true for a 22-kd polypeptide synthesized *in vivo*, suggesting a precursor product relationship between the two proteins in peas.

Localization of the poly(A)$^+$RNA coded 22-kd HSP in the chloroplast membranes of Chlamydomonas and pea

The 22-kd protein in *Chlamydomonas* is apparently located in the chloroplast membranes, since it is present in three thylakoid membrane fractions sedimenting at different sucrose densities (Figure 2). The herbicide-binding 32-kd polypeptide is a typical thylakoid component (Steinback *et al.*, 1982), and can therefore be used as a marker for these membranes. Among the various membrane fractions obtained by sucrose gradient centrifugation, the 22-kd HSP is enriched in the fraction banding at 1.5 M sucrose, relative to the 32-kd herbicide-binding polypeptide. This fraction consists almost exclusively of large thylakoid vesicles (De Petrocelis *et al.*, 1970). The 22-kd protein which resides in the thylakoid membranes appears to be coded by nuclear DNA and is identical to that translated *in vitro* by heat-shock poly(A)$^+$RNA.

This conclusion is supported by the observation that *in vivo*, the synthesis of the 22-kd protein can be inhibited by cycloheximide in *Chlamydomonas*, whereas the synthesis of the chloroplast-coded 32-kd herbicide-binding protein is not affected by this inhibitor (Figure 7). However, chloramphenicol, an inhibitor of translation on 70S ribosomes, blocks the synthesis of the 32-kd protein but not that of the 22-kd protein. Comparing the labeling pattern of heat-shock-induced proteins *in vivo* and *in vitro*, indicates that beside the poly(A)$^+$RNA-coded 22-kd protein, no additional HSP is found in the chloroplast membranes of *Chlamydomonas*. As mentioned above, in contrast to *Chlamydomonas*, a 22-kd HSP is found in the chloroplast membranes of pea only *in vivo*, while a 26-kd protein is obtained following translation of pea heat-shock poly(A)$^+$RNA *in vitro*.

Numerous nuclear coded proteins are processed while being transported into isolated organelles such as mitochondria (Neupert and Schatz, 1981) or chloroplasts (Chua and Schmidt, 1979). To test whether the pea 26-kd protein might be a precursor to a chloroplast protein, the translation mixtures of heat-shock and control RNAs were added to intact chloroplasts, as described by Grossman *et al.* (1982). From the comparison of the total translation products of heat-shock pea mRNA with those remaining in the supernatant after removal of the chloroplasts following transport (Figure 8), it is evident that the amount of the 26-kd heat-shock polypeptide band is reduced, at least partially, from the supernatant, whereas all the other heat-shock bands remain un-

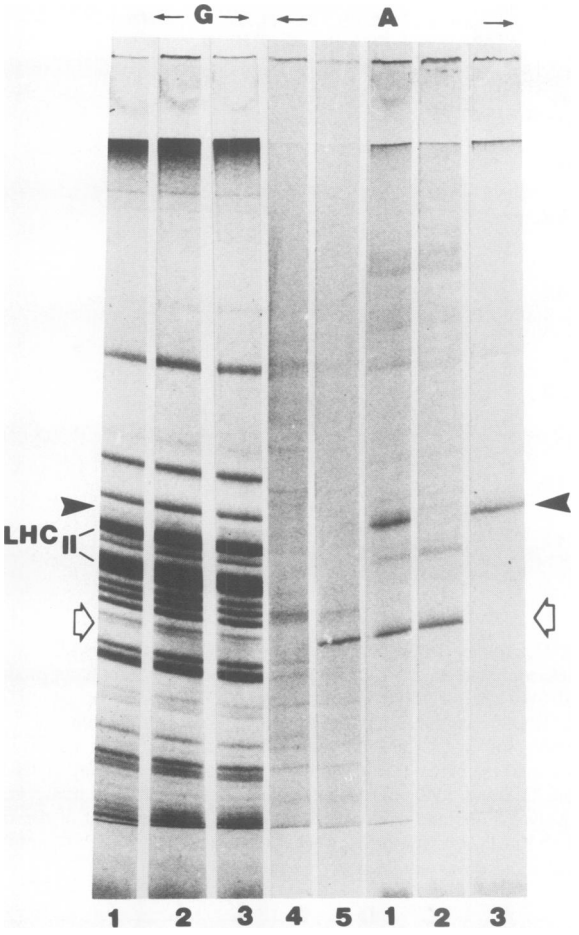

Fig. 7. Effect of inhibitors of translation on the synthesis *in vivo* of the 22-kd HSP in *Chlamydomonas*. *Chlamydomonas* cells were transferred to 42°C and incubated for 1 h, following which $^{35}SO_4^{2-}$ was added and the incubation continued for another hour. The inhibitors were added 15 min prior to the addition of the isotope. **Lanes G 1–3**, thylakoid membrane pattern of control cells **(1)** and cells treated with either chloramphenicol (200 μg/ml) **(2)** or cycloheximide (1.5 μg/ml) **(3)**. **Lanes A 1–3**, autoradiograph of **lanes G 1–3; lanes 4** and **5** show separated polypeptides obtained by *in vitro* translation of poly(A)$^+$RNA from control and heat-shock cells, respectively. ►, 32-kd herbicide-binding protein; ⇨ 22-kd HSP.

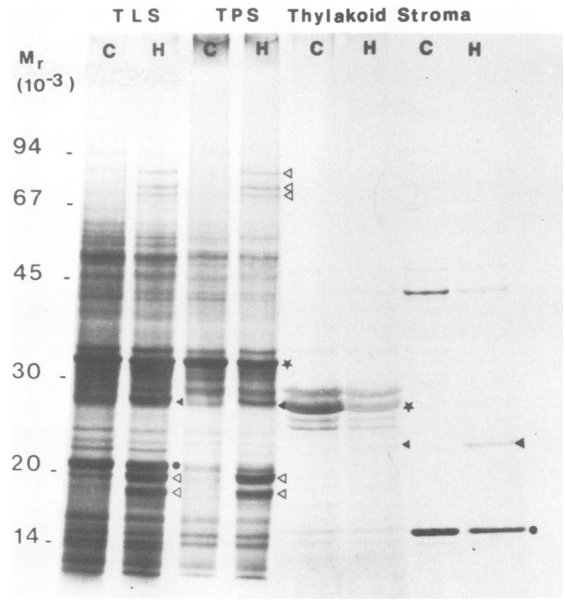

Fig. 8. Post-translational transport of translation products of control and heat-shocked pea poly(A)$^+$ RNA into isolated chloroplasts of pea. Control (C) and heat-shock (H) pea poly(A)$^+$ RNAs were translated and transported into intact chloroplasts isolated from plants grown at 26°C. 100 μl of translation mixture (2.5–5 x 10^8 d.p.m./min) were added to the chloroplast suspension (400 μg chlorophyll). TLS, translation products before transport (over-exposed to show the high mol. wt. heat-shock bands) and TPS, translation supernatant recovered after transport; ◀, transported and ◁, non-transported HSPs. The arrowhead in the thylakoid (H) and stroma fractions (H) point to the 22-kd transported protein which stays mostly in the stroma fraction. Precursor and transported products of LHC II (*) and small subunit RuBPCase (●) respectively.

changed. On the other hand, as indicated by the black arrowheads in Figure 8, a band of 22-kd appears predominantly in the chloroplast stroma fraction and, to a lesser extent, in the membrane fraction. The distribution of the 22-kd protein in this experiment differs from that obtained *in vivo*, because *in vivo* the 22-kd polypeptide is integrated specifically into a chloroplast membrane fraction (Figure 5). When decreasing amounts of translation supernatant were transported into a constant amount of chloroplasts, the relative distribution of the processed 22-kd protein between the membrane and the stroma fraction did not change, indicating that availability of the membrane binding sites was not a limiting factor (data not shown).

We have previously observed considerable diurnal differences *in vivo* in the level of poly(A) mRNA-coded polypeptides processed and transported into the chloroplast (Kloppstech, in preparation). Since the chloroplast used for *in vitro* transport experiments were isolated at various times during the day, while heat-shock was carried out always in the early morning, there might also be diurnal differences in the capability of chloroplasts to transport proteins or to integrate them into the appropriate compartment. Using chloroplasts obtained from peas at the beginning of the light phase or at the end of the same day, no difference in either the capacity for transport or in the fidelity of the transport was observed. In this experiment, most of the heat-shock 22-kd protein was again localized in the stroma (data not shown). Thus, the discrepancy between the results obtained *in vitro* and *in vivo* is not due to the illumination regime of plants prior to the preparation of chloroplasts.

Transport of the pea 22-kd HSP into chloroplasts isolated from heat-shocked pea plants

The difference between the results obtained *in vivo* and *in vitro* could be accounted for, if we consider that the chloroplasts used for transport were obtained from plants grown at 26°C, whereas transport of the protein *in vivo* occurs in plants that have been heat-shocked. To mimic the *in vivo* conditions, we tried to perform the transport using chloroplasts from heat-shocked pea plants. This proved to be difficult. The amount of intact chloroplasts which could be obtained from heat-shocked peas declined dramatically during the heat-shock and, after 4 h at 43°C, no intact chloroplasts could be obtained at all. In this case, chloroplasts were found only in the upper fraction of the Percoll gradient (Douce and Joyard, 1979), which contains mostly broken chloroplasts. It was, however, possible to isolate sufficient amounts of chloroplasts from 10–12 day old plants which were heat-shocked only for 2 h. Under these conditions, the yield was ~50% of the untreated controls. As can be seen in Figure 9, chloroplasts from heat-shocked pea plants were able to transport the pea proteins and to integrate them with the same fidelity as

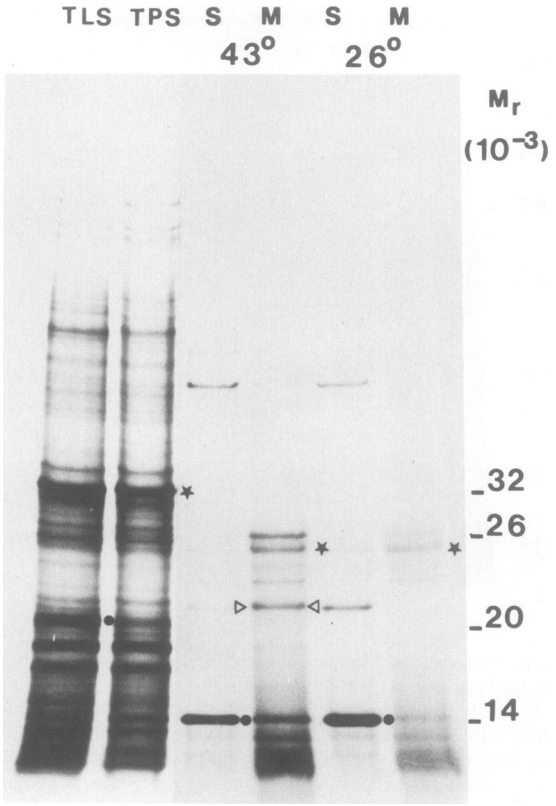

Fig. 9. Post-translational transport into isolated chloroplasts from heat-shocked pea plants. Same experimental conditions as in Figure 7, except that only pea heat-shock poly(A)$^+$ RNA was used. Chloroplasts were isolated from control and heat-shocked pea plants for 2 h at 42°C. The open triangles indicate the localization of the 22-kd polypeptide in the membrane fraction of chloroplasts from heat pre-treated plants (26°C). TLS and TPS, translation supernatants before and after transport, respectively. S, stroma; M, membrane fraction; (*) precursors and transported products of LHC II and (●) small subunit RuBPCase.

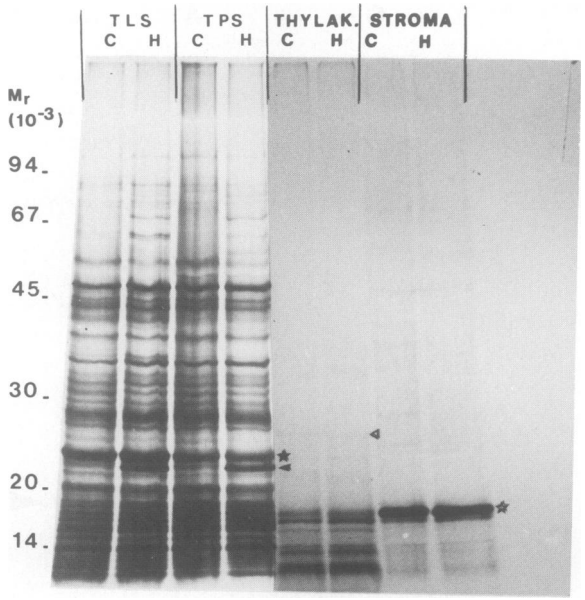

Fig. 10. Heterologous transport of *in vitro* translation products of *Chlamydomonas* poly(A)$^+$ RNA into pea chloroplasts. Translation products obtained from control (C) and heat-shock (H) *Chlamydomonas* poly(A)$^+$RNA were transported into isolated, intact pea chloroplasts. Chloroplasts still intact were recovered after transport, treated with proteases, and thereafter fractionated into stroma and membrane thylakoid fractions. TLS and TPS, translation products before and after transport, respectively. Closed and open asterisks, precursor and product of the small subunit of RuBPCase, respectively. ◀, position of 22-kd HSP; ◁, protein of 26 kd in the thylakoid fraction.

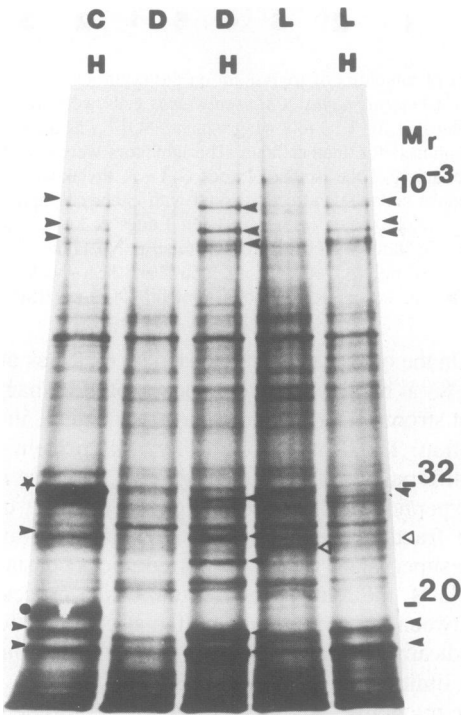

Fig. 11. Induction of HSPs in etiolated and greening pea plants. Etiolated plants (5 days old) were either taken as controls (D) and heated for 2 h in the dark (DH) or illuminated for 2 h without (L) or with heat-shock treatment (LH). The plants were frozen in liquid nitrogen, poly(A)$^+$ RNA extracted, and limiting amounts of RNA translated in the wheat-germ system. Poly(A)$^+$ RNA from heat-shocked green plants served as control (CH). ◀, HSPs; ◁, ELIP. The precursors of LHCP (*) and ssRuBPCase (●) are also indicated.

do control chloroplasts. This is demonstrated by the presence of LHC II in the thylakoid fraction and the small subunit RuPBCase in the stroma fraction (Figure 9). Furthermore, in these chloroplasts from heat-treated plants, the pea 22-kd HSP resided predominantly in the membrane fraction. Attempts to perform a post-translational transport *in vitro* at 40°C, using chloroplasts from both control or heat-shocked pea plants, were unsuccessful.

Based on the data presented thus far, we concluded that the 22-kd HSP of *Chlamydomonas* must also pass the chloroplast envelope. Hence, it was of interest to find out whether chloroplasts from heat-shocked pea would also be able to recognize the *Chlamydomonas* protein which possesses the same electrophoretic mobility *in vivo* and *in vitro*.

Earlier attempts (Chua and Schmidt, 1979) to transport *Chlamydomonas* proteins into spinach chloroplasts were unsuccessful, and they concluded that *Chlamydomonas* chloroplast proteins are not recognized by these heterologous envelope receptors. However, the transport of proteins is possible in the pea heterologous system (Figure 10). After transport, the amount of precursor to the small subunit of RuBPCase is partially reduced in the translation supernatant and the processed protein appears in the stroma fraction of the pea chloroplasts, while other polypeptides, one of which matches the apparent mol. wt. of LHC II, are transported into the membrane fraction. Since the *Chlamydomonas* 22-kd band is quite predominant among the *in vitro* translation products, its transport should be easily detec-

table. However, no indication for its transport could be detected even after over-exposure of the autoradiograms.

Induction of HSPs in etiolated pea plants

The heat-shock response varies between different tissues (Cooper *et al.*, 1984), it was of interest, therefore, to compare the synthesis of the chloroplast-localized, HSP in etiolated and greening pea plants. Five day old etiolated pea plants were kept in the dark or transferred to light for 2 h, and either heat-shocked or kept at 26°C. The plants were then frozen in liquid nitrogen, and the poly(A)$^+$ was isolated, translated and the polypeptide patterns of the translation mixture analyzed (Figure 11). The results of this experiment should also show whether any of the light-inducible protein precursors, e.g., ELIP (Meyer and Kloppstech, 1984), might have the same apparent mol. wt. as one of the HSPs. Figure 11 shows that the precursors of ELIP and the chloroplast HSP have a different electrophoretic mobility. Surprisingly, several bands of low mol. wt. (18, 19, 24 and 30-kd as well as the 26-kd HSP) were induced by heat in etiolated pea plants to a higher extent than in the heat-shocked green plants. On the other hand, the latter band was enhanced by heat-shock in the plants illuminated for 2 h (Figure 11).

Discussion

Although several plant HSPs have been cloned and sequenced (Schöffl *et al.*, 1984), little is known about their function and intracellular localization. In animal cell cultures a 68-kd HSP distributes between the lamina of the nucleus and the microfilaments of the cytosol during heat-shock (La Thangue, 1984). The 70-kd HSP, which might be identical to the 68-kd protein mentioned above, resembles one of the microtubule-associated proteins and binds to these structures (Lim *et al.*, 1984). Based on autoradiography studies, Neumann *et al.* (1984) have described an association of HSPs with the nucleus and the cytoskeleton in plants.

In this study, the expression of HSPs of *Chlamydomonas* and pea plants has been compared by *in vitro* translation and *in vivo* labeling. Within the limits of the methods, which primarily affects the comparison of the high mol. wt. bands, it has been found that all of the *in vivo* labeled proteins can also be detected in the pattern of *in vivo* translation of poly(A)$^+$RNAs. Thus, our results indicate that all the HSPs of *Chlamydomonas* and peas are coded by the nuclear DNA. HSPs also occur in prokaryotic organisms (Yamamori *et al.*, 1978); therefore, during evolution, the corresponding prokaryotic heat-shock genes have either been lost or transferred from the prokaryotic genome to the nucleus.

In *Chlamydomonas*, the 22-kd HSP synthesized *in vivo* comigrates in a sucrose gradient with various fractions of chloroplast membranes and is enriched in the thylakoid fraction. This is a strong indication for its localization within the *Chlamydomonas* chloroplast. However, as opposed to *Chlamydomonas*, the poly(A)$^+$RNA coding for the chloroplast heat-shock 22-kd protein in pea, gives rise to a protein of 26-kd. A precursor product relationship between the 26-kd polypeptide band obtained after *in vitro* translation of poly(A) RNA and the 22-kd membrane protein in pea is deduced from the following observations. The 22-kd band can be found only *in vivo* or after *in vitro* transport of translation products of heat-shock poly(A)$^+$RNA, and therefore it is a heat-induced protein. After transport *in vitro* into isolated chloroplasts, the 22-kd processed band is inaccessible to degrading proteases and thus must reside within or beyond the inner side of the chloroplast envelope. These data support the conclusion that only one of the nuclear-coded, heat-induced proteins is a chloroplast protein. In a recent abstract, Vierling *et al.* (1984) report that a similar 22-kd HSP is localized in the chloroplast stroma. In contrast to this report, we conclude that the pea and *Chlamydomonas* 22-kd proteins are membrane proteins.

It is interesting that the final localization of the nuclear-coded pea 22-kd HSP *in vitro* is affected by the previous history of the receiving organelle. Isolated chloroplasts obtained from heat-shocked plants process, transport and localize several nuclear-coded proteins such as the LHC II and the small subunit RuBP-Case in the same manner as *in vivo*, or as in chloroplasts isolated from control plants. Both types of chloroplasts process and internalize the 26-kd pea protein in its mature form of 22 kd. These data demonstrate that the processing and transport sites on the chloroplast envelope are not affected by heat-shock treatment. However, only chloroplasts isolated from heat-shocked plants localize the pea 22-kd protein in a chloroplast membrane fraction, as is also the case *in vivo*. Hence, the state of organization of this receiving membrane is altered by heat-shock treatment in a specific fashion which is not reversed after chloroplast isolation and further incubation in ice and at 25°C for up to 2 – 3 h. This indicates that the change induced by the heat-shock is not merely a transient, temperature-dependent alteration of the fluidity of the membrane.

Attempts to perform the *in vitro* transport at elevated temperatures (40°C) have been unsuccessful because, after 1 h at this temperature, the chloroplasts were completely broken. This effect has not yet been thoroughly studied either with respect to critical time and temperature or with regard to protection of the isolated chloroplasts against heat effect. The response to heat is more dramatic in etiolated pea seedlings, in which the plastids are not yet fully differentiated, than in light-grown plants. The mRNAs for the low mol. wt. HSPs in these plants are expressed to a higher degree and in a different proportion to each other. The etiolated seedlings were killed faster after exposure to light and 42°C as compared with light-grown plants (data not shown).

The identical mol. wt. and chloroplast membrane localization shared by these HSPs in pea and *Chlamydomonas* suggest that both proteins are homologous. Nevertheless, one observation seems to be in disagreement with the postulated homology of the 22-kd HSP of pea and *Chlamydomonas*. We have not been able to transport this *Chlamydomonas* protein into pea chloroplasts, while at least some of the *Chlamydomonas* proteins were recognized by the pea envelope receptor.

In contrast to pea, no precursor for the 22-kd protein has been found after *in vitro* translation of *Chlamydomonas* heat-shock poly(A)$^+$RNA in the two cell-free systems used. In this connection, it is worth mentioning that the purified mRNA of the 22-kd *Chlamydomonas* HSP migrates with 14S, i.e., faster than the mRNA of 12S coding for the 24-kd precursor of the small subunit RuBPCase (Kloppstech and Ohad, in preparation). Thus, it could be assumed that the *Chlamydomonas* mRNA indeed codes for a larger precursor protein but that *in vitro* a first initiation site might not be recognized, or, alternatively, that under *in vitro* conditions, the precursor leading sequence might be cleaved off. Cleavage of precusors to chloroplast proteins has occasionally been observed in the wheat-germ system (Pfisterer *et al.*, 1982). However, it seems unlikely that this proteolytic activity should be present in both *in vitro* systems used. The answer to these questions awaits molecular cloning and sequencing of the *Chlamydomonas* HSP.

HSPs are rather conservative proteins (Kelly and Schlesinger, 1982). Surprisingly, the comparison between the *Chlamydomonas*

and pea heat-induced proteins reveals that not all the heat-induced proteins in pea are represented in *Chlamydomonas*. The coinciding bands include those of 70 kd, 36 kd and 22 kd. In particular, the prominent 18- and 19-kd heat-shock bands observed in plants (Key *et al.*, 1982) have no counterpart in *Chlamydomonas*. It, therefore, seems that besides the very conservative proteins such as the 70-kd protein, a group of less conserved HSPs is present in vascular plants and algae.

Most pre-existing mRNAs are not translated under heat-shock conditions (Ballinger and Pardue, 1983). This is apparently also true for most of the *Chlamydomonas* proteins coded by the nucleus. However, proteins coded by chloroplast DNA, especially the 32-kd herbicide-binding protein and the large subunit of RuBPCase, are synthesized throughout the entire 5 h period of heat-shock. This finding might indicate that translation on 70S ribosomes is less impaired by heat treatment than the translation on the cytosolic 80S ribosomes. The results presented in this work suggest that the chloroplast might play a role in the plant response to heat stress. Work is now being carried out to understand the role of the 22-kd chloroplast-located HSP.

Materials and methods

Growth of pea plants and Chlamydomonas cells

Pea seeds (cv *Rosa Krone* and in some experiments cv *Dean*) were soaked overnight in tap water and grown on vermiculite at 3000 lux in a 12:12 h light:dark cycle and 26:21 °C temperature day:night cycle. *C. reinhardi* y-1 mutant was grown at 26 °C in a mineral medium supplemented with sodium acetate as a carbon source, as previously described (Ohad *et al.*, 1967).

Heat-shock

Plants were watered and thereafter shocked in a pre-heated incubator at 42 °C (*Rosa Krone*) or at 43 – 45 °C (*Dean*) for 2 – 6 h, either in the light or dark, and then quickly harvested, and either frozen in liquid nitrogen for RNA extraction or immediately used for chloroplast isolation.

For heat-shock treatment of *Chlamydomonas*, cells at the end of the logarithmic phase of growth were harvested by centrifugation (5000 *g*/min) and resuspended in fresh growth medium to a density of 10^7 cells/ml (non-dividing conditions; Ohad *et al.*, 1967). The cell suspension was transferred to water baths adjusted to the indicated temperatures and incubated with stirring for 1 – 5 h. Light intensity was adjusted to 10 – 30 W/m^2. After heat-shock, cells were again harvested and washed by centrifugation in 0.01 M Tris-HCl, pH 7.4. The pellet, usually 1 g fresh weight from 10^9 cells, was either directly frozen or suspended in 1 ml of the above solution and frozen in droplets in liquid nitrogen. The frozen material was stored at −80 °C until further processing.

Labeling in vivo and isolation of chloroplast membranes

In vivo labeling of 7-day-old pea plants was as described before (Meyer and Kloppstech, 1984) at 26 °C or 42 °C (heat shock). The apices of three plants were homogenized in 2 ml buffer (0.05 M Tris-HCl, pH 9.0; 0.01 M EDTA; 0.1 M NaCl; 3% SDS) and heated for 5 min at 65 °C. The homogenate was clarified by filtration over Miracloth, followed by centrifugation at 15 000 *g* for 1 min, and the proteins were precipitated by the addition of 4 volumes of acetone at −20 °C. The precipitate was washed twice with cold 80% acetone (−20 °C) by centrifugation for 2 min at 15 000 *g* and once with ether, dried and dissolved in 400 μl sample buffer (6% lithium dodecyl sulfate, 0.15 M tricine, pH 7.8, 0.5 M dithiothreitol, 0.02% bromophenol blue and 30% glycerol). For isolation of membranes, 20 apices were homogenized with a Waring blender in 20 ml of the chloroplast isolation medium (Grossman *et al.*, 1982), filtered over Miracloth, and broken chloroplasts were isolated at 5000 *g* for 5 min. To the chloroplast pellet, 2 ml distilled water were added and the membranes were obtained again as described above in the protocol for transport.

Labeling of *Chlamydomonas* cells *in vivo* was carried out under conditions similar to those described above, except that the cells were washed and resuspended in growth medium from which sulfate was omitted. At the indicated times, [^{35}S]-Na_2SO_4 was added (400 μCi/μmol, 10 μM) and incubation continued for 1 h. The cells were then harvested by centrifugation, washed once in 0.01 M Tris-HCl, pH 7.4, buffer and further processed for isolation of chloroplast membranes as described earlier (Owens and Ohad, 1981), except that the discontinuous sucrose gradient consisted of 2 M, 1.5 M and 0.5 M sucrose layers. The membranes were collected, washed by centrifugation in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM KCl and 5 mM $MgCl_2$, and the labeled membrane polypeptides were identified by autoradiography following electrophoresis on polyacrylamide gradient slab gels in the presence of LDS and 4 M urea (Kyle *et al.*, 1984). Total incorporation was assayed in aliquots of intact cells. The cells were washed as above, extracted with 80% acetone chilled to −20 °C, and the pellet dissolved in the electrophoresis sample buffer. The 32-kd herbicide-binding polypeptide was identified in the gel slabs from its fast light-dependent turnover and affinity labeling with [^{14}C]atrazine (Kyle *et al.*, 1984).

Extraction of poly(A)$^+$ RNA

Polyadenylated RNA of pea plants was extracted by a slight modification of the published procedure (Apel and Kloppstech, 1978). The same protocol was used for *Chlamydomonas*. Frozen cells (1 – 3 g) were transferred to 30 ml extraction buffer (0.05 M Tris-HCl, pH 9.0, 0.1 M NaCl, 0.01 M EDTA and 3% SDS) and sonicated four times for 15 s at room temperature. Equal volumes of 80% phenol and 99:1 chloroform/isoamyl alcohol (v/v) were added, and the extraction was continued as previously described (Spradling *et al.*, 1977). Two phenol extraction steps were found to be sufficient.

Translation in vitro

In vitro translation, using the wheat-germ system, was performed according to Roberts and Paterson (1973) with the inclusion of 0.5 mM EDTA and 25 mM CTP. The optimal RNA concentration was checked for every RNA preparation, assaying different amounts of poly(A)$^+$RNA. Unless otherwise specified, limiting amounts of RNA were used for translation.

Translation of poly(A)$^+$RNA in the reticulocyte lysate, kindly provided by Dr. M. Edelman of The Weizmann Institute of Science, Rehovot, Israel, was performed according to Pelham and Jackson (1976). The same mRNA concentration as in the wheat-germ system was used.

If temperatures above 26 °C were used for *in vitro* translation, the system was first incubated for 2 min, and translation was started by the addition of either mRNA or methionine.

Post-translational transport

This procedure followed the protocol of Grossman *et al.* (1982) with the modifications described by Meyer and Kloppstech (1984). For isolation of chloroplasts from heat-shocked plants, the pea was grown for 12 instead of 7 days, the plants were shocked for 2 h at 42 °C, rapidly chilled in the extraction medium and further processed as described above. The light intensity used for transport was 600 W/m^2 and was provided by a Tungsten-Halogen lamp.

Heterologous in vitro transport

Polypeptides obtained by *in vitro* translation of *Chlamydomonas* control or heat-shock poly(A)$^+$RNAs (200 μl of wheat-germ assay containing ~ 10^8 d.p.m.) were added to intact chloroplasts (400 μg chlorophyll) isolated from pea, and transported at a light intensity of 600 W/m^2 and at 25 °C. The transport protocol was that of Grossman *et al.* (1982) with the modifications described by Meyer and Kloppstech (1984).

Gel electrophoresis

Analysis of translation products was according to Neville (1971) but using 6% lithium dodecyl sulfate, 0.15 M Tricine, pH 7.8, 0.15 M dithiothreitol, 0.02% bromophenol blue and 30% glycerol as sample buffer. Detection of labeled products was facilitated by fluorography (Bonner and Laskey, 1974).

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