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Induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in cultured plant cells by UV light or fungal elicitor

(two-dimensional gels/cloned cDNAs/RNA blot hybridization/coordinated gene expression/*Petroselinum hortense*)

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ABSTRACT **The mRNAs encoding two enzymes of phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and 4-coumarate:CoA ligase (4CL; EC 6.2.1.12), were induced in cultured parsley cells (*Petroselinum hortense*) either by irradiation with UV light or by treatment with elicitor, a cell-wall fraction of the fungus *Phytophthora megasperma* f. sp. *glycinea*. Two-dimensional gel electrophoresis of the encoded PAL and 4CL proteins revealed that the mRNAs induced by either treatment were very similar if not identical. RNA blot hybridization with cDNAs complementary to these mRNAs was used to measure changes in the mRNA amounts at various times after either treatment. Total cellular PAL and 4CL mRNA amounts increased coordinately after UV irradiation to a maximum at 7 hr and then decreased to uninduced levels by 30 hr with the same kinetics as observed previously for the changes in the translational activities. Treatment with the fungal elicitor also caused coordinated, but more rapid, changes in PAL and 4CL mRNA translational activities, with a sharp peak occurring 3 hr after the addition of elicitor. Corresponding changes in mRNA amounts were observed only for 4CL, whereas the amount of PAL mRNA continued to increase at least up to 20 hr after elicitor addition. Our results suggest that parsley cells respond to UV irradiation or addition of fungal elicitor by increased rates of transcription of genes involved in the synthesis of compounds related to UV or disease resistance, respectively.**

Cell suspension cultures of parsley (*Petroselinum hortense*) produce and accumulate either flavonoids, when treated with UV light (1), or furanocoumarins, when treated with a fungal elicitor preparation (2). Flavonoids may be involved in protecting a plant from excess UV irradiation (3). Furanocoumarins are the putative phytoalexins (antimicrobial substances) of parsley (2) and could protect the plant from fungal attack. Neither flavonoids nor furanocoumarins are found in uninduced cells, and activities of the enzymes involved in flavonoid or furanocoumarin production are low or undetectable (1, 4). Teleologically, these inductions are similar in that the cells react to an exogenous, potentially harmful, signal by increasing the activity of certain groups of enzymes to produce specific products.

When parsley cells are irradiated with UV light, flavonoids are first observed in the cells after 5–10 hr (1). Rapid increases in the activities of two related groups of enzymes (group I and group II, see Fig. 1) can account for the flavonoid production. Under defined conditions of irradiation (5–7), group I enzyme activities reach a maximum 12 hr after induction, several hours before the peak in group II enzyme activities. This difference in maximal enzyme activities is one criterion used to place enzymes into either group I or group II (1).

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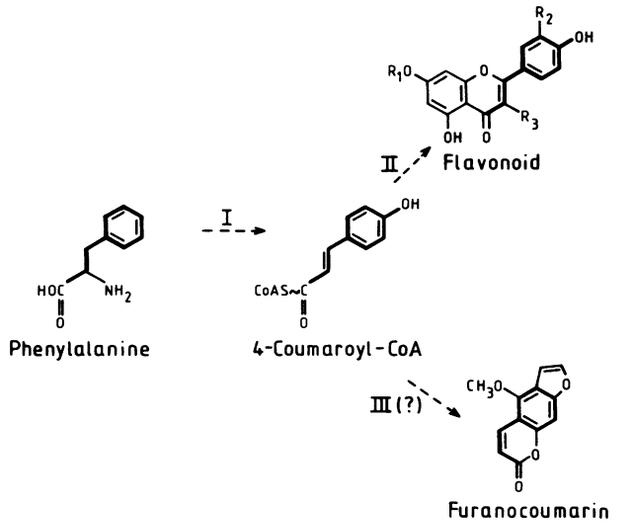

FIG. 1. Proposed metabolic pathways for the biosynthesis of flavonoids and furanocoumarins. Three enzymes of general phenylpropanoid metabolism (group I), including phenylalanine ammonia-lyase and 4-coumarate:CoA ligase, catalyze the formation of 4-coumaroyl-CoA, the common substrate for both classes of phenylpropanoid compounds (3). Group II consists of 13 enzymes of the flavonoid glycoside pathway, including chalcone synthase. Group III is still hypothetical (2, 3). In cultured parsley cells, UV irradiation induces group I and group II; treatment with elicitor induces group I and probably group III.

The UV light-induced changes in the rates of synthesis of two enzymes of group I, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and 4-coumarate:CoA ligase (4CL; EC 6.2.1.12), and of two enzymes of group II, chalcone synthase (CHS) and UDP-apiose synthase (UAS), were similar when measured *in vivo* and *in vitro*. Synthesis of group I enzymes reached a maximum after approximately 7 hr (5, 6), whereas the synthesis of group II enzymes was maximal at 10–11 hr after induction (5, 7). Thus, the separation in time of the maxima of the group I and group II enzyme activities is preserved at the level of the rates of synthesis, and the increases in enzyme activities are accounted for most simply by the *de novo* synthesis of the enzymes.

Increases in PAL and 4CL enzyme activities have also been observed when cultured parsley cells are incubated

Abbreviations: PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase.

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with an elicitor preparation from the cell wall of the fungus *Phythophthora megasperma* f. sp. *glycinea* (4). These enzyme activities may be involved in the early steps of furanocoumarin biosythesis (Fig. 1), and, as in the induction with UV light, the changes in enzyme activities can be accounted for by corresponding changes in the rates of *de novo* synthesis of the enzymes (4, 6). Similar studies of the effect of an elicitor from *Colletotrichum lindemuthianum* on suspension-cultured bean cells (*Phaseolus vulgaris*) have shown a transient increase in the translational activity for PAL mRNA preceding an increase in the enzyme activity (8).

We extend here our previous observations that PAL and 4CL mRNAs can be induced in cultured parsley cells by two different methods, irradiation with UV light or treatment with fungal elicitor, and we compare the translational products obtained with the differentially induced mRNAs. We also compare by RNA blot hybridization the kinetics of PAL and 4CL mRNA induction, using recently identified cDNAs (9, 10). A representative of group II, CHS mRNA, whose induction kinetics in UV-irradiated parsley cells has previously been reported (9), was included as a reference for possible variations of the induction in different batches of cells.

MATERIALS AND METHODS

Cell Cultures. Cell suspension cultures of parsley (*Petroselinum hortense*) were grown in the dark as described (6). Culture growth was monitored by measuring the conductivity of the medium. Cultures, approximately 7 days after subculturing, with medium conductivity between 1.5 and 2.0 mS were used for the experiments.

Plasmids. cDNA was prepared by reverse transcription of poly(A)$^+$ RNA isolated from either UV-irradiated or elicitor-treated parsley cells as described by Kreuzaler *et al.* (9). These collections of cDNA were inserted into the *Pst* I site in pBR322 and the recombinant plasmids were used to transform *Escherichia coli* strain RR1. pcPAL, pc4CL, and pcCHS [previously designated as pLF15 (9, 11)] were identified by hybrid-selection translation, followed by two-dimensional gel electrophoresis or immunoprecipitation of the products, as plasmids containing cDNA inserts complementary to PAL, 4CL, and CHS mRNAs, respectively (9–11). Plasmids were isolated from cleared lysates (12) according to the method of McMasters *et al.* (13).

UV Irradiation and Elicitor Treatment. Cultured parsley cells were irradiated for 2.5 hr under eight fluorescent lamps (Philips TL W/18) at a distance of 30 cm from the flask at 25°C with continuous shaking. Cultures were then returned to the dark. Heat-released elicitor from *Phytophthora megasperma* f. sp. *glycinea* was a gift from J. Ebel (Freiburg) and was prepared by the method of Ayers *et al.* (14). Cultures were induced by adding 40–80 μg (dry weight) of elicitor per ml of medium. Incubation was in the dark, with shaking at 25°C.

RNA Isolation. Total RNA, used for gel electrophoresis and *in vitro* translation, was isolated as described by Langridge *et al.* (15). Five grams (frozen weight) of parsley cells was ground to a fine powder in a mortar containing liquid nitrogen. The powder was transferred to another mortar at room temperature and the frozen powder was ground to a slush in 10 ml of 20% sucrose/0.1 M magnesium acetate/0.1 M KCl/0.1 M Tris·HCl, pH 8.5. The brei was centrifuged briefly, $NaDodSO_4$ was added to 0.5%, and the supernatant was extracted twice with an equal volume of phenol/chloroform (1:1, vol/vol). RNA in the aqueous phase was precipitated twice with ethanol. The final precipitate was dried and dissolved in sterile H_2O and the concentration of nucleic acid was determined by measuring absorbance at 260 nm. For hybrid-selection experiments, polyribosomal RNA and poly(A)$^+$ RNA were isolated as described (9).

RNA Electrophoresis. RNA was denatured and separated on formaldehyde/agarose gels (16). Twenty micrograms of total RNA was denaturated by incubation at 65°C in 50% (vol/vol) formamide/6% (vol/vol) formaldehyde/10 mM 3-morpholinopropanesulfonic acid (Mops), pH 7.0, for 15 min. The sample was cooled, and 1/5th vol of 50% formamide/1% bromophenol blue/40% (vol/vol) glycerol was added. Electrophoresis was at 100 V for 4–5 hr at room temperature in a gel containing 1.2% agarose/6% formaldehyde/10 mM Mops, pH 7.0, with 10 mM Mops, pH 7.0/1 mM EDTA as the running buffer. Gels were washed for 20 min in 25 mM sodium phosphate, pH 6.5, and transferred to GeneScreen (New England Nuclear) in the same buffer. *E. coli* rRNA and *Hin*dIII-digested λ phage DNA were coelectrophoresed as molecular weight standards. These channels were cut from the gel before transfer, stained in 0.5% toluidine blue in 40% (vol/vol) ethanol, and destained in 20% ethanol.

RNA Blot Hybridization, Autoradiography, and Densitometry. For use as hybridization probes for specific parsley mRNAs, pcPAL and pcCHS were digested with *Pst* I, and the inserted cDNA (approximately 1700 and 1500 base pairs, respectively) was isolated by electrophoresis on 1.5% agarose gels followed by electroelution of the desired DNA fragment from the gel and labeling by nick-translation (17). pc4CL had lost one *Pst* I site, and the inserted cDNA (about 700 base pairs) plus 700 base pairs of pBR322 were excised by digestion with *Pst* I and *Eco*RI. This fragment was isolated and labeled as above.

Blotted RNA was hybridized to the various ^{32}P-labeled cDNAs in 50% formamide/0.6 M NaCl/60 mM trisodium citrate, pH 7.0/0.1% $NaDodSO_4$/250 μg of denatured herring sperm DNA per ml/0.02% each of bovine serum albumin, polyvinylpyrrolidone 25, and Ficoll 400 for 16 hr at 42°C with continuous shaking. Blots were washed, air dried, and autoradiographed at −70°C with Kodak AR-5 film and intensifying screens for 48–72 hr. Autoradiograms were scanned with an LKB laser scanning densitometer and scanning data were integrated with a Hewlett–Packard integrator. Relative film darkness was normalized with the darkest band being 1. The amount of RNA was assumed to be directly related to film darkness.

Labeling of Protein *in Vivo*. Protein was labeled by incubating cells in [^{35}S]methionine (>600 Ci/mol, Amersham; 1 Ci = 37 GBq) at 10 μCi/ml of medium for 1.5 hr. For light-induced cells, the labeling was started 4.5 hr after the beginning of the induction. Elicitor-induced cells were labeled starting at 1.5 hr after the addition of elicitor. Cells were extracted and PAL and 4CL were immunoprecipitated with specific antisera (6). Immunoprecipitates were dissolved in 50 μl of 9.5 M urea/2% (wt/vol) Nonidet P-40 (Sigma)/2% Ampholines (LKB; pH 3.5–10) for two-dimensional gel electrophoresis.

***In Vitro* Translation.** RNA was translated *in vitro* as described by Pelham and Jackson (18) with the following modifications. RNA to be translated was precipitated with ethanol and centrifuged, and the pellet was dried under reduced pressure. Twenty-five microliters of the complete translation mix containing 5–10 μCi of [^{35}S]methionine was used to dissolve the dried RNA, and the mixture was incubated for 90 min at 30°C. For antibody precipitation of proteins labeled *in vitro*, the translation mix was first diluted 1:10 with 0.1 M NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA and then treated identically as the proteins labeled *in vivo*.

Hybrid Selection. RNAs homologous to immobilized cDNAs were selected as described by Ricciardi *et al.* (19) with the following modifications. pcPAL, pc4CL, or pcCHS was linearized by digestion with *Eco*RI and 40 μg was immobilized on a 1-cm circle of diazobenzyloxymethyl paper (20). These filters were hybridized with 35 μg of poly(A)$^+$ RNA and 35 μg of polysomal RNA from cultured parsley cells in a

total volume of 100 μl for 16 hr at 37°C. Filters were washed three times with 0.3 M NaCl/30 mM trisodium citrate/0.5% $NaDodSO_4$ at 65°C and twice in 0.15 M NaCl/15 mM trisodium citrate at room temperature. RNA was released by boiling the filter in water, precipitated, and translated *in vitro*. For two-dimensional gel electrophoresis, either 4 μl of the translation products was added to 50 μl of the Ampholine solution described above (total products) or the translation products were first immunoprecipitated.

Two-Dimensional Gel Electrophoresis. Proteins labeled *in vivo* or *in vitro* were characterized by two-dimensional polyacrylamide gel electrophoresis according to the method of O'Farrell (21) with the following modifications. The first-dimension gel was 3% polyacrylamide and contained a 1:1 mixture of pH 3.5–10 and 5–7 Ampholines. The second dimension was a 10–18% polyacrylamide gradient. Fluorography was as described by Bonner and Laskey (22) and films were exposed for 5–7 days at −70°C.

mRNA Translation. Seventy-five micrograms of total RNA from each time point was translated *in vitro* and the translation products were immunoprecipitated with antisera as above. Precipitates were electrophoresed on a 10% polyacrylamide gel and PAL and 4CL bands were identified by their molecular weight. Fluorographs were scanned with an LKB scanning densitometer and relative film darkness at the PAL and 4CL bands was used as the measure of translational activity.

Enzyme Assays. PAL and 4CL activities were measured as in ref. 6.

RESULTS

Analysis of Induced Enzyme Proteins. Four and one-half to 6 hr after treatment of parsley cell cultures with UV light or 1.5 to 3 hr after addition of elicitor, total proteins were labeled either *in vivo* or by translation of the mRNAs *in vitro*. Total poly(A)$^+$ RNA or RNA that had been selected by hybridization with pcPAL or pc4CL was used for translation *in vitro*. The proteins were then immunoprecipitated with PAL-specific or 4CL-specific antisera and analyzed on two-dimensional gels.

Fig. 2 shows the sections of the gels containing the immunoprecipitates. PAL migrated as a set of multiple proteins around M_r = 76,000 between pH 5.8 and pH 7.8 (Fig. 2 *A–F*), and 4CL migrated as at least two proteins at $M_r \approx 60{,}000$ between pH 4.9 and pH 5.4 (Fig. 2 *G–L*). The PAL proteins as well as the 4CL proteins migrated to similar positions in all cases. The differences seen here in the relative intensities of the PAL spots between light and elicitor treatment were observed in several independent experiments. In agreement with earlier observations (6), no 4CL immunoprecipitate was obtained from crude extracts of light-induced cells (Fig. 2*G*).

Kinetics of mRNA Induction. Total cellular RNA was isolated at various times from UV-irradiated parsley cells and used for RNA blot hybridization with pcPAL, pc4CL, and pcCHS. The time courses of changes in the hybridizable amounts of the corresponding mRNAs are shown in Fig. 3 *A–C* and compared graphically in Fig. 3*D* to the curves reported previously for the mRNA translational activities (5, 6). PAL and 4CL mRNAs showed the same maximum around 7 hr after induction, whereas the peak for CHS mRNA occurred at about 10 hr. In all three cases, the maximal amount of mRNA corresponded to the peak in mRNA translational activity. Hybridization with pcCHS served as an internal reference, and the present data are in close agreement with results reported earlier (9).

Cells from the same batch were treated in a parallel experiment with elicitor, and total RNA was isolated at various times for both translation and RNA blot hybridization. PAL and 4CL mRNA translational activities increased to a sharp peak around 3 hr and returned to very low levels by about 9 hr (Fig. 4). The kinetics of the relative changes in PAL and 4CL mRNA activities were identical within experimental error and coincided with previously determined changes in the rates of PAL synthesis *in vivo*, as measured by immunoprecipitation from extracts of pulse-labeled cells (4). The observed rapid changes in mRNA activity could account for the slower changes in PAL and 4CL enzyme activities (Fig. 4).

While the increase and decrease in the amount of 4CL mRNA coincided with the changes in 4CL mRNA translational activity (Fig. 5 *B* and *C*), the amount of PAL mRNA

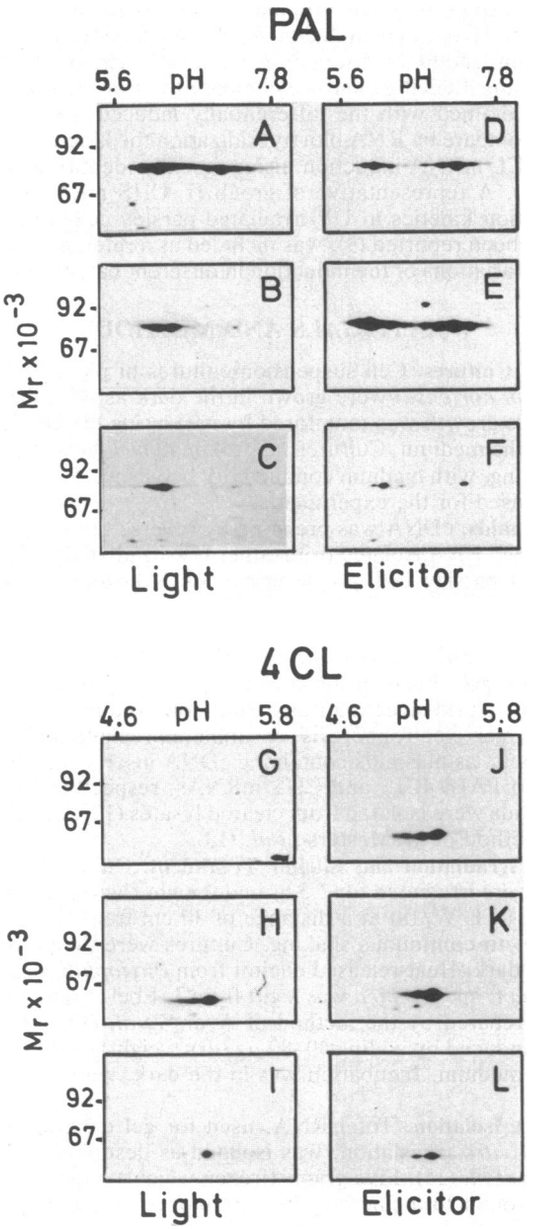

FIG. 2. Comparison of UV light-induced and elicitor-induced enzyme subunits obtained with PAL-specific and 4CL-specific antisera. Corresponding sections from fluorograms of two-dimensional gels are shown in each panel. Different absolute amounts of radioactivity were applied to each gel, depending on the availability of labeled material. Proteins were labeled *in vivo* (*A*, *D*, *G*, *J*) or *in vitro*, using either total poly(A)$^+$ mRNA (*B*, *E*, *H*, *K*) or RNA that had been hybrid-selected with pcPAL or pc4CL (*C*, *F*, *I*, *L*). The complete series of experiments is shown, although no 4CL immunoprecipitate (*G*) can be obtained from crude extracts of light-induced cells (6), and the only spot seen here is due to a contamination. Samples were taken 6 hr (light) and 3 hr (elicitor) after induction.

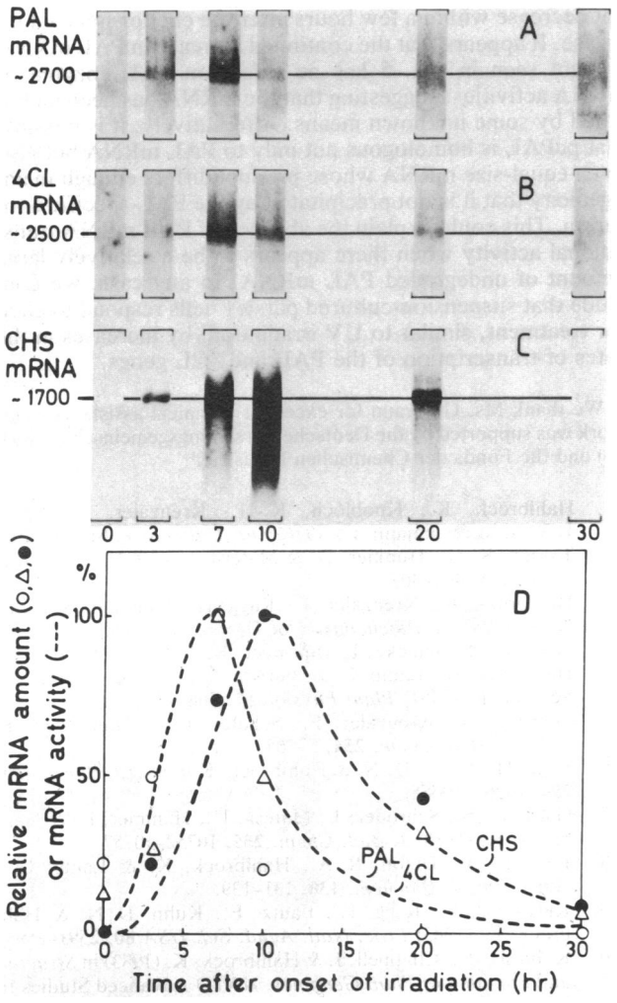

FIG. 3. Time courses of UV light-induced changes in amounts and translational activities of PAL, 4CL, and CHS mRNAs. Total RNA (25 μg), isolated at various times from cells irradiated from 0 to 2.5 hr (9), was electrophoresed on formaldehyde-agarose gels, blotted to nitrocellulose, and hybridized with ^{32}P-labeled cDNA from pcPAL (*A*), pc4CL (*B*), and pcCHS (*C*). The approximate sizes (number of nucleotides) and expected positions of the mRNAs on the gels (horizontal lines) are indicated. The changes in relative amounts of mRNAs, measured by scanning densitometry of the autoradiograms shown in *A–C*, are compared in *D* with the previously determined (5, 6) changes in the mRNA translational activities (broken lines). Data are plotted relative to the highest value for each mRNA. ○, PAL; △, 4CL; ●, CHS.

continued to increase beyond the time of its maximal translational activity (Fig. 5 *A* and *C*). These same results were obtained in three independent experiments. Even 20 hr after elicitor addition, a full-size PAL mRNA could be detected by hybridization with pcPAL. However, no PAL was immunoprecipitated when pcPAL was used to select PAL mRNA from polyribosomal or poly(A)$^+$ RNA isolated at this late stage after addition of elicitor. Furthermore, the total translational activity *in vitro* of polyribosomal or poly(A)$^+$ RNA from cells treated for 17 hr with elicitor was only about 10–30% of that obtained with RNA from untreated cells or from cells treated with elicitor for 3 hr. Mixing RNA from cells treated with elicitor for 3 hr and 17 hr reduced the expected translational activity of the RNA by 40%.

CHS mRNA was not detected by hybridization of pcCHS to RNA from elicitor-treated cells (data not shown). This result is in agreement with the earlier observation that CHS is not induced in elicitor-treated cells (4).

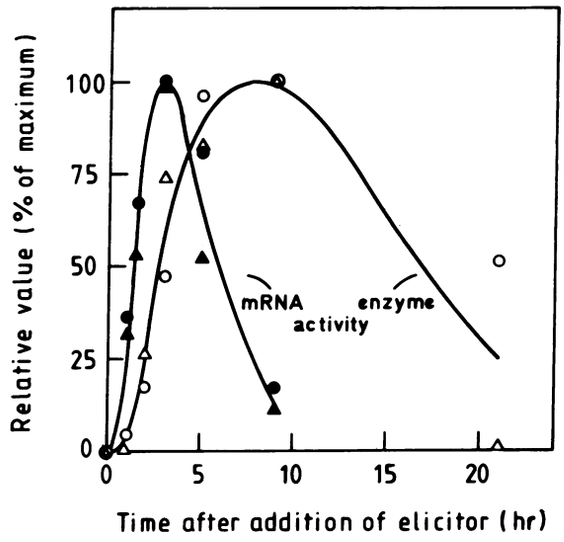

FIG. 4. Elicitor-induced changes in enzyme and mRNA translational activities *in vitro* for PAL (○, ●) and 4CL (△, ▲). Cells were harvested at the indicated times and activities were measured as described in the text. Data are plotted relative to the highest value for each mRNA or enzyme activity. Highest absolute enzyme activities (open symbols) were 53 μkat/kg (PAL) and 167 μkat/kg (4CL), corrected for activity levels in untreated cells (0 μkat/kg, and 135 μkat/kg, respectively) (1 kat = 1 mol/sec).

The sizes of the mRNAs were estimated by using rRNA from *E. coli* and *Hin*dIII restriction fragments of λ DNA as molecular weight standards. The mean values obtained in four separate determinations were approximately $M_r = 1.0 \times 10^6$ or 2700 base pairs for PAL mRNA; $M_r = 0.9 \times 10^6$ or 2500 base pairs for 4CL mRNA, and $M_r = 0.7 \times 10^6$ or 1700 base pairs for CHS mRNA. These data are in close agreement with those determined previously by sucrose density gradient centrifugation for PAL mRNA [$M_r \approx 1.05 \times 10^6$ (23)] and CHS mRNA [$M_r \approx 0.62 \times 10^6$ (24)].

DISCUSSION

We have shown that the PAL and 4CL proteins induced by UV light or elicitor were very similar with regard to their pattern on two-dimensional gels. Translation of total poly(A)$^+$ mRNAs or of the mRNAs selected by pcPAL and pc4CL produced the same PAL and 4CL protein patterns as seen with the proteins labeled *in vivo*. Therefore, identical mRNAs are probably induced by both stimuli, albeit at different ratios. Assuming that no processing or protein modification takes place after translation in the rabbit reticulocyte lysate, we conclude that more than one mRNA exists for PAL and for 4CL. This suggests that more than one gene is present for each enzyme, that more than one allele of each gene is expressed, or a combination of both. These possibilities can be distinguished by isolating the genes for PAL and 4CL and determining the number of copies present per haploid genome.

The large and rapid increases in the activities of the two enzymes of group I, PAL and 4CL, in UV-irradiated or elicitor-treated parsley cells were associated with increases in the amounts of the PAL and 4CL mRNAs. In the case of UV irradiation, we have demonstrated that the different induction kinetics for group I and group II enzyme activities (1) were maintained not only at the levels of enzyme synthesis *in vivo* and mRNA translational activities *in vitro* (5–7) but also at the level of RNA amounts. Furthermore, coordinated changes were observed for the two mRNAs of group I.

On the basis of our present data together with previous results (3, 5–7), we conclude that the major control point in

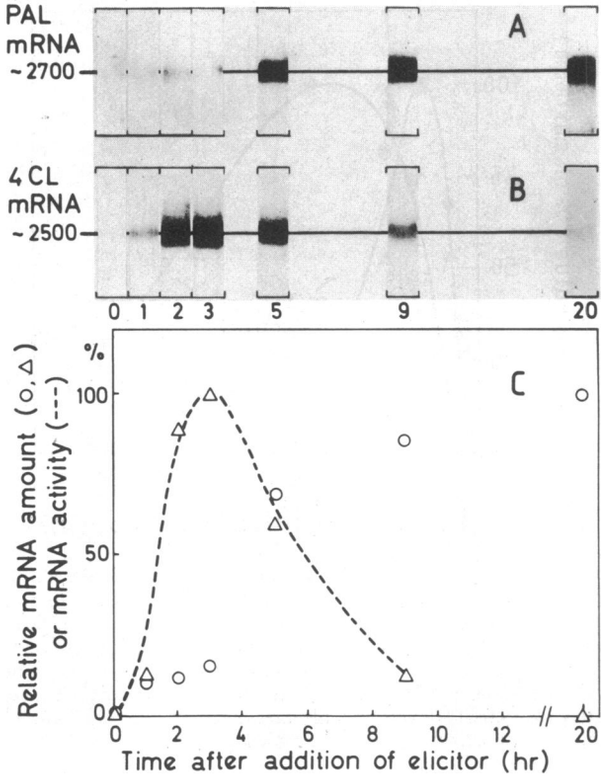

FIG. 5. Time courses of elicitor-induced changes in the amounts of PAL and 4CL mRNAs. Total RNA (25 μg) from cells treated with elicitor at 0 hr was isolated, electrophoresed, and hybridized with ^{32}P-labeled cDNA from pcPAL (*A*) or pc4CL (*B*). The changes in relative amounts of the mRNAs, measured by scanning densitometry of the autoradiograms, are compared in *C* with the changes in mRNA translational activities (broken curve, taken from Fig. 4). ○, PAL; △, 4CL.

UV-light-stimulated flavonoid glycoside accumulation is the transient increase in the amounts of mRNAs coding for the enzymes involved in flavonoid biosynthesis. Preliminary evidence suggests that these changes in mRNA amounts are due primarily to increased rates of transcription of the genes encoding these enzymes (unpublished results).

Differences in the relative maxima of mRNA amounts for group I and group II enzymes do not necessarily mean that the two groups are sequentially induced. From the studies of cells treated with elicitor (4) or transferred to fresh medium (25), it is clear that the induction of group II enzymes is not always linked to, or caused by, the induction of group I enzymes.

The induction of PAL and 4CL mRNAs by elicitor cannot be explained as simply as UV light induction of these same mRNAs. While the changes in amount and translational activity of 4CL mRNA coincided in both cases, the apparent accumulation of PAL mRNA in elicitor-treated cells was unexpected. Unfortunately, we do not have cDNA probes for an mRNA of group III, so we do not know how the induction of group I and group III mRNAs by elicitor compares to the induction of group I and group II mRNAs by UV light. However, two cDNAs for small acidic proteins that appear in parsley cells only after elicitor induction were used to measure relative changes in the mRNAs for these proteins (unpublished data). The amounts of these mRNAs were induced with kinetics similar to 4CL mRNA, with a maximum between 3 and 5 hr after elicitor addition. Therefore, PAL mRNA is the only mRNA we have so far measured that does not decrease within a few hours after the elicitor-induced increase. It appears that the continued increase in PAL mRNA amount seen in Fig. 5 has no effect on PAL enzyme or mRNA activities, suggesting that the mRNA has been inactivated by some unknown means. Alternatively, it is possible that pcPAL is homologous not only to PAL mRNA but also to an equal-size mRNA whose product differs enough in antigenicity that it is not precipitated by the PAL-specific antiserum. This could explain the absence of PAL mRNA translational activity when there appears to be a relatively large amount of undegraded PAL mRNA. In any case, we conclude that suspension-cultured parsley cells respond to elicitor treatment, similar to UV irradiation, by increases in the rates of transcription of the PAL and 4CL genes.

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