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UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*

(cDNA clones/flavonoid biosynthesis/phytochrome control)

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ABSTRACT **DNAs complementary to poly(A)$^+$ mRNAs from UV-irradiated cell suspension cultures of parsley (*Petroselinum hortense*) were inserted into pBR322 and used to transform *Escherichia coli* strain RR1. A clone containing a DNA complementary to chalcone synthase mRNA was identified by hybrid-selected and hybrid-arrested translation. Large and rapid changes in the amount of chalcone synthase mRNA in response to irradiation of the cells was detected by RNA blot hybridization experiments. The pattern of changes coincided with that previously determined for the rate of chalcone synthase synthesis as measured either *in vivo* or with polyribosomal mRNA *in vitro*. The results are consistent with the hypothesis that induction of chalcone synthase by UV light is due to a transient increase in the rate of synthesis of chalcone synthase mRNA.**

Light plays an important role in the development of higher plants (1). One of the most extensively studied regulatory effects of light on specific metabolic pathways, besides plastid development, is the induction of flavonoid biosynthesis (2). Flavonoids constitute one of the most abundant classes of phenolic compounds in higher plants and serve important functions as flower pigments, antimicrobial agents ("phytoalexins"), and UV-protective compounds (2–6).

The biosynthesis of flavonoids has been elucidated to a large extent, particularly in parsley (6). Irradiation of dark-grown cell suspension cultures of parsley causes a coordinated and selective induction of about 16 enzymes, all of which are involved in the formation of several flavonoid glycosides which accumulate in irradiated cells (2). The induction has an absolute requirement for UV-B light, and the expression of the response is under phytochrome control (5, 7). The requirement for UV light may be related to the putative UV-protective function of the induced flavonoid products.

The key enzyme of the flavonoid glycoside pathway, chalcone synthase, is the most abundant of the light-induced enzymes and can be easily isolated in large quantities from irradiated parsley cells (8). It catalyzes the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to give naringenin chalcone (9). This chalcone is the central intermediate in the biosynthesis of flavones, flavonols, and various other flavonoids (6). Chalcone synthase from parsley consists of two M_r 42,000 subunits which are coded for by a mRNA of approximately 1,700 nucleotides (8). Chalcone synthase mRNA activity increases rapidly in irradiated cells for about 10–15 hr, depending on the length of the irradiation period (10). The mRNA activity then decreases to the preinduction level at a rate with an apparent half-life of about 5 hr. At maximal induction (10, 11), the rate of synthesis of chalcone synthase *in vivo* amounts to almost 2% of the total rate of protein synthesis, whereas no chalcone synthase synthesis or activity is detectable in dark-grown cells.

Similar rapid changes in mRNA activity have been observed for three other light-induced enzymes. In all cases, the observed changes in enzyme activity coincided with theoretical curves calculated from the changes in mRNA activity, with allowance for the differing rates of degradation or inactivation of the enzymes (10, 12, 13). This clear-cut relationship between mRNA and enzyme activities suggested that UV light acted primarily through mRNA induction. One major question, which had not been answered in our previous studies, was whether the changes in mRNA activity were due to increased rates of *de novo* synthesis or to the activation of preexisting inactive forms of the mRNAs. Answering this question requires a hybridization probe specific for UV-induced mRNA. We have identified a DNA complementary to chalcone synthase mRNA and have used it for studies of the induction kinetics of this mRNA.

MATERIALS AND METHODS

Cell Cultures. Cell suspension cultures of parsley (*Petroselinum hortense*) were propagated for 7 days in darkness or until the medium had reached a conductivity of about 2 mmho (13). Cells were irradiated with about 20,000 lx from fluorescent lamps (Philips TL 40 W/18) that emit sufficient UV light at wavelengths in the range 320–350 nm for mRNA induction (5, 10).

Cloning Procedure. Poly(A)$^+$ mRNA, either from cells irradiated for 10 hr or from dark-grown cells, was isolated as described (14). cDNA was prepared with 120 μg of mRNA from irradiated cells according to the method of Law *et al.* (15). Oligo(dT)$_{12\text{-}18}$ was used as primer for the first DNA strand. Synthesis of the second strand was carried out with the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) and 5 μg of single-stranded cDNA. The double-stranded cDNA (5 μg) was treated with S1 nuclease, and 1 μg (4 pmol of ends) was tailed with 40 μCi (1 Ci = 3.7×10^{10} Bq) of [5-^{3}H]dCTP and 10 units of terminal transferase (Bethesda Research Laboratories) (16). The average length of the oligo(dC) extensions was 18 nucleotides. The tailed double-stranded cDNA was annealed for 8 hr (60–30°C) with pBR322 which had been linearized with *Pst* I and tailed with dGTP (17). The annealed DNA was precipitated with ethanol and used for transformation of *E. coli* strain RR1 (18). Bacteria containing the plasmid were selected on medium containing tetracycline and resistant colonies were tested for the presence of recombinant plasmids on medium containing ampicillin (19).

Screening and Identification of Clones. Tetracycline resistant ampicillin-sensitive clones were tested by colony hybrid-

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ization (20) for the presence of DNA complementary to light-induced mRNA from parsley. Duplicate filters were hybridized with ^{32}P-labeled poly(A)$^+$ RNA (5×10^6 cpm per filter) either from dark-grown cells or from cells that had been irradiated for 10 hr. Nicked RNA (10 μg) was labeled with [γ-^{32}P]ATP as described by Sim *et al.* (21), except that the reaction was carried out for 5–7 min in 50 mM Tris·HCl (pH 7.9) at 90°C. Plasmids were isolated (22) from clones that hybridized with higher efficiency to RNA from irradiated than from dark-grown cells. The plasmids (200 μg each) were linearized with *Pst* I and bound to nitrocellulose filters (2.5 cm in diameter) in 20× standard saline citrate buffer (23). The filters were cut into halves which were hybridized with labeled RNA (5×10^5 cpm) from irradiated and dark-grown cells, respectively. Filters giving the highest ratios (irradiated/dark, >3) of bound radioactivity were further identified by hybrid-arrested (24) and hybrid-selected translation (25). Immunoprecipitation and gel electrophoretic analysis of chalcone synthase subunits were carried out as described (10).

Isolation of RNA. Total RNA for RNA blot hybridization experiments was isolated from 20 g of frozen cells which were thawed in a mixture of 20 ml of phenol and 20 ml of 50 mM Tris·HCl, pH 7.5/5 mM EDTA. The aqueous phase was extracted three times with phenol and once with chloroform. Nucleic acids were then precipitated with ethanol and washed twice with 20% ethanol. Poly(A)$^+$ mRNA for hybrid-arrested and hybrid-selected translation was prepared as described (14), except that the RNA solution was passed through oligo(dT)-cellulose only once.

RNA Blot Hybridization. RNA (40 μg) was denatured with glyoxal, separated by electrophoresis on an agarose gel, blotted onto nitrocellulose, and hybridized with the cDNA insert of pLF56 ^{32}P-labeled by nick-translation with DNA polymerase I (26, 27).

RESULTS

Approximately 1,000 colonies were first tested by the Grundstein–Hogness method and yielded about 100 colonies which gave a positive response also in the second, more reliable, screening procedure. From these 100, 10 clones were selected in the second screening as the most likely to contain plasmids with DNA inserts complementary to light-induced mRNAs. Plasmids isolated from three of these clones (pLF12, pLF15, and pLF56) gave positive results with a chalcone synthase-specific antiserum in hybrid-arrested and hybrid-selected translation assays. The translational product obtained with the mRNA released from filter-bound pLF56 is shown in lane C of Fig. 1. For comparison, lane A shows authentic chalcone synthase subunits immunoprecipitated from extracts of *in vivo* labeled parsley cells. A control experiment with a filter carrying pBR322 instead of pLF56 is shown in lane B. The highest ratio (about 13) for the binding of mRNA from irradiated and from dark-grown cells in the second screening experiment was obtained with the clone carrying pLF56.

The plasmid pLF56 contains an insert of about 1,300 base pairs at the *Pst* I site of the cloning vehicle, pBR322. DNA sequence data indicate that the insert comprises most of the coding sequence, all of the 3′ untranslated region, and a poly(A) segment of chalcone synthase mRNA (unpublished data). The cDNA insert of pLF56 contains one *Pst* I site; its position relative to the DNA sequence corresponding to the poly(A) segment of the mRNA is given in Fig. 2. The two insert fragments generated by restriction of pLF56 with *Pst* I were separated from the pBR322 cloning vehicle and used for the following experiments.

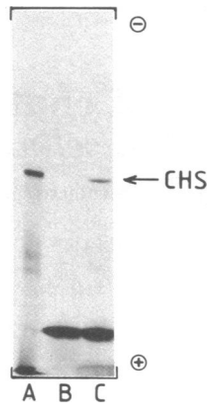

FIG. 1. Identification of the translational product obtained with hybrid-selected mRNA from nitrocellulose-bound pLF56 (lane C). Lanes: A, Chalcone synthase (CHS) subunits immunoprecipitated from crude extracts of parsley cells after irradiation of a culture for 10 hr and labeling of the proteins with L-[^{35}S]methionine during the last hour of the light treatment (10); B, control experiment using nitrocellulose-bound pBR322 instead of pLF56 for hybrid selection of mRNA. A micrococcal nuclease-treated rabbit reticulocyte lysate (28) was used for mRNA translation *in vitro* with [^{35}S]methionine as the labeled substrate. Products were separated by gel electrophoresis in the presence of $NaDodSO_4$ (10) and detected by fluorography (29).

For the determination of UV light-induced changes in the amount of chalcone synthase mRNA, total RNA was isolated from parsley cells at various times after irradiation of a culture for 2.5 hr. The amount of RNA, as determined by hybridization of ^{32}P-labeled cDNA at the expected position of chalcone synthase mRNA [about 16 S (8)], increased greatly from the 3rd to the 11th hour after induction (Fig. 3*A*). Much less hybridization occurred at 24 hr. The RNA from nonirradiated control cells at 0 hr showed no hybridization to the labeled cDNA.

Fig. 3*B* compares the present results with previous data (10) on the activity changes of chalcone synthase mRNA obtained under the same conditions of induction as used here. The timing of light-induced changes either in amount or in activity of chalcone synthase mRNA was essentially the same.

Results similar to those shown in Fig. 3*A* were obtained when continuous irradiation of the cells was used for induction (data not shown). Under these conditions, the peak for chalcone synthase mRNA activity was somewhat broader and the subsequent rate of decline was slower than after short-term irradiation (10). Accordingly, greater hybridization was observed at 24 hr after the onset of induction.

DISCUSSION

Our results demonstrate that light-induced changes in chalcone synthase mRNA activity in cultured parsley cells correspond to

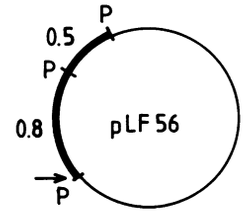

FIG. 2. Schematic representation of the plasmid pLF56, consisting of pBR322 with a DNA insert complementary to chalcone synthase mRNA (thick line) at the *Pst* I restriction site. The orientation of the insert is indicated by the arrow marking the sequence that is complementary to the poly(A) segment of the mRNA. The approximate lengths of the cDNA fragments generated with *Pst* I (P) are given in kilobases.

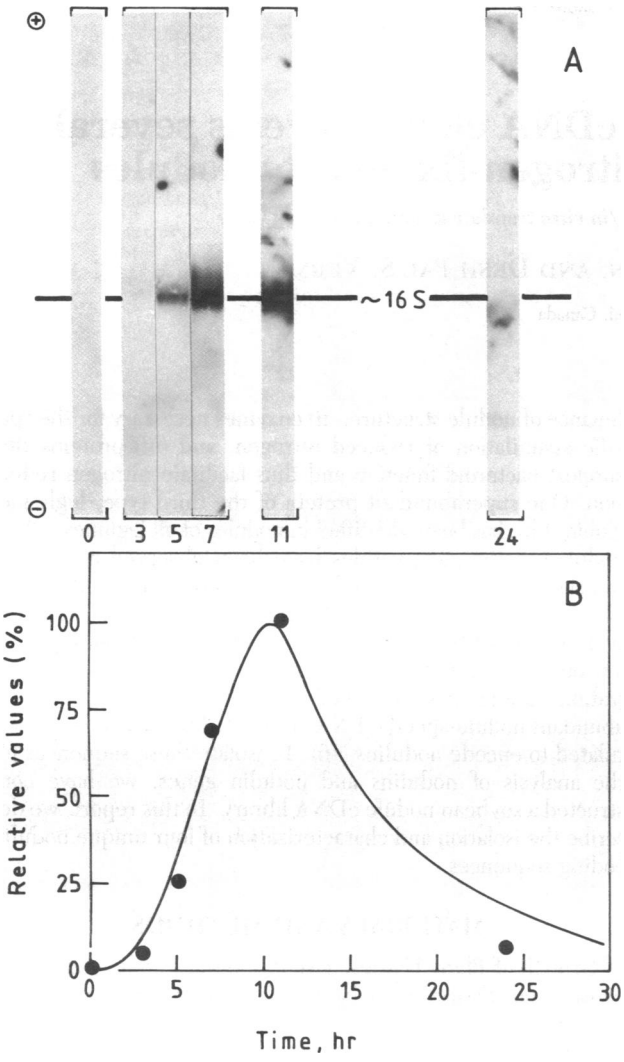

FIG. 3. Time courses of light-induced changes in amount and translation activity of chalcone synthase mRNA. (*A*) A cell culture was irradiated for 2.5 hr, and then samples were taken for extraction of total RNA at the times indicated in *B*. A RNA blotting experiment with ^{32}P-labeled DNA from pLF56 is shown. The expected position of chalcone synthase mRNA is indicated by the horizontal line. (*B*) Relative amounts of ^{32}P-labeled cDNA hybridized by chalcone synthase mRNA (●). The data were obtained by scanning the gels shown in *A* with an LKB laser densitometer scanner. The curve represents relative changes in polyribosomal chalcone synthase mRNA activity, as previously determined (10) under the same conditions of induction as used here.

changes in the amount of mRNA. Total cellular RNA was used in the hybridization experiments, ruling out the possibility that mRNA activity was not detected in the noninduced state due to sequestration of RNA from polyribosomes. The present data therefore suggest that light causes a true induction of chalcone synthase mRNA—i.e., a transient increase in the rate of transcription—although other mechanisms such as changes in the rates of mRNA processing or degradation are possible alternative explanations.

Using similar techniques, Bedbrook *et al.* (30) have shown that the amount of mRNA coding for the small subunit of RuP_2 carboxylase in *Pisum sativum* is also increased by light. White light was used for those experiments, and a specific wavelength dependence of the effect was not reported. However, more recent experiments by Tobin (31) with *Lemna gibba* have demonstrated that the induction of this mRNA activity requires red light and is a typical phytochrome-mediated response. In contrast, flavonoid synthesis in parsley is absolutely dependent on UV irradiation (5, 7).

The selective and coordinated changes in activity of chalcone synthase and the other enzymes of the flavonoid glycoside pathway (2) suggest that the same photoreceptor(s) and the same mechanisms of signal transmission are involved in the induction of all mRNAs of this pathway.

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1. Mohr, H. (1972) *Lectures on Photomorphogenesis* (Springer, Berlin).
2. Hahlbrock, K. & Grisebach, H. (1979) *Annu. Rev. Plant Physiol.* **30**, 105–130.
3. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids* (Academic, New York).
4. Grisebach, H. & Ebel, J. (1978) *Angew. Chem.* **90**, 668–681.
5. Wellmann, E. (1982) in *Encyclopedia of Plant Physiology*, New Series: Photomorphogenesis, eds. Shropshire, W., Jr., & Mohr, H. (Springer, Berlin), in press.
6. Hahlbrock, K. (1981) in *The Biochemistry of Plants*, eds. Stumpf, P. K. & Conn, E. E. (Academic, New York), Vol. 7, pp. 425–456.
7. Duell-Pfaff, N. & Wellmann, E. (1982) *Planta* **156**, 213–217.
8. Kreuzaler, F., Ragg, H., Heller, W., Tesch, R., Witt, I., Hammer, D. & Hahlbrock, K. (1979) *Eur. J. Biochem.* **99**, 89–96.
9. Heller, W. & Hahlbrock, K. (1980) *Arch. Biochem. Biophys.* **200**, 617–619.
10. Schröder, J., Kreuzaler, F., Schäfer, E. & Hahlbrock, K. (1979) *J. Biol. Chem.* **254**, 57–65.
11. Heller, W., Egin-Bühler, B., Gardiner, S., Knobloch, K. H., Matern, U., Ebel, J. & Hahlbrock, K. (1979) *Plant Physiol.* **64**, 371–373.
12. Gardiner, S., Schröder, J., Matern, U., Hammer, D. & Hahlbrock, K. (1980) *J. Biol. Chem.* **255**, 10752–10757.
13. Ragg, H., Kuhn, D. N. & Hahlbrock, K. (1981) *J. Biol. Chem.* **256**, 10061–10065.
14. Ragg, H. & Hahlbrock, K. (1980) *Eur. J. Biochem.* **103**, 323–330.
15. Law, S., Tomoaki, T., Kreuzaler, F. & Dugaiczyk, A. (1980) *Gene* **10**, 53–61.
16. Vogt, V. M. (1973) *Eur. J. Biochem.* **33**, 192–200.
17. Bollum, F. J. (1974) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 10, pp. 145–171.
18. Mandel, M. & Higa, A. (1970) *J. Mol. Biol.* **53**, 159–162.
19. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
20. Grundstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961–3965.
21. Sim, G. K., Kafatos, F. C., Jones, C. W., Koehler, M. D., Efstratiadis, A. & Maniatis, T. (1979) *Cell* **18**, 1303–1316.
22. Clewell, D. B. (1972) *J. Bacteriol.* **110**, 667–676.
23. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
24. Paterson, B. M., Roberts, B. E. & Kuff, E. L. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4370–4374.
25. Ricciardi, R. P., Miller, J. S. & Roberts, B. E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4927–4931.
26. Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1184–1188.
27. Alwine, J. C., Kemp, D. J. & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5350–5354.
28. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
29. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
30. Bedbrook, J. R., Smith, S. M. & Ellis, R. J. (1980) *Nature (London)* **287**, 692–697.
31. Tobin, E. M. (1981) *Plant Mol. Biol.* **1**, 35–51.