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Coding and 3′ non-coding nucleotide sequence of chalcone synthase mRNA and assignment of amino acid sequence of the enzyme

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The nucleotide sequence of an almost complete cDNA copy of chalcone synthase mRNA from cultured parsley cells (*Petroselinum hortense*) has been determined. The cDNA copy comprised the complete coding sequence for chalcone synthase, a short A-rich stretch of the 5′ non-coding region and the complete 3′ non-coding region including a poly(A) tail. The amino acid sequence deduced from the nucleotide sequence of the cDNA is consistent with a partial N-terminal sequence analysis, the total amino acid composition, the cyanogen bromide cleavage pattern, and the apparent mol. wt. of the subunit of the purified enzyme.

Key words: complementary DNA/nucleotide sequence/chalcone synthase/amino acid sequence/flavonoids

Introduction

Chalcone synthase is the key enzyme of flavonoid biosynthesis and catalyzes the formation of the central intermediate in this pathway, naringenin chalcone, from 4-coumaroyl-CoA and three molecules of malonyl-CoA (Hahlbrock, 1981). This enzyme is characteristic of higher plants and has several interesting features. It is inducible under certain defined conditions in cultured parsley cells as well as in various other systems (Hahlbrock, 1981), it resembles closely the condensing enzyme of type-II fatty acid synthesis with respect to several of its catalytic and structural properties (Schüz *et al.*, 1983), and is the only enzyme which is specifically involved in the synthesis of all classes of flavonoids, such as chalcones, flavanones, flavones, flavonols, anthocyanins and isoflavones. Flavonoids occur widely in higher plants and serve a variety of important functions as flower pigments, u.v.-protective agents and antimicrobial substances (phytoalexins).

The enzymes involved in the biosynthesis of several structurally related flavone and flavonol glycosides have been studied extensively in cultured parsley cells (Ebel and Hahlbrock, 1982), and cloned cDNA copies of some of the mRNAs encoding these enzymes have been prepared (Kreuzaler *et al.*, 1983; Kuhn *et al.*, 1983). The parsley cell system is particularly suitable for studies of the regulation of this pathway, since the enzymes can be induced coordinately and selectively by u.v. light (Hahlbrock *et al.*, 1976). Transient high enzyme activities lead to the rapid accumulation of large amounts of flavonoids in the vacuoles of the cultured cells (Matern *et al.*, 1983).

Chalcone synthase is the most abundant of all enzymes of the flavonoid glycoside pathway (Kreuzaler *et al.*, 1979). A few hours after the onset of induction, the rate of chalcone synthase synthesis temporarily increases to ~2% of the total rate of cellular protein synthesis (Schröder *et al.*, 1979). Concomitant with the rapid light-induced increase and subsequent decrease in the rate of synthesis of the enzyme *in vivo*, chalcone synthase mRNA changes with the same kinetics in both total cellular amount (Kreuzaler *et al.*, 1983) and translational activity *in vitro* (Schröder *et al.*, 1979).

Some of these results were obtained by RNA blot hybridization with cloned chalcone synthase-specific cDNA (Kreuzaler *et al.*, 1983). We report here the nucleotide sequence of this cDNA and the deduced amino acid sequence of the encoded polypeptide, together with some related properties of the authentic protein, to provide a basis for further studies of both the structure of the chalcone synthase gene(s) and the catalytic function of the enzyme.

Results and Discussion

The nucleotide sequence of cloned cDNA

Three plasmids, pLF12, pLF15 and pLF56, have previously been shown to contain cDNA inserts which are specific for chalcone synthase mRNA by the criteria of hybrid-arrested and hybrid-selected translations (Kreuzaler *et al.*, 1983). The cDNAs had been cloned into the *Pst*I site of pBR322 and were now re-isolated from the plasmids with this restriction endonuclease. The three inserts varied in length from ~400 to 1500 base pairs, with the two larger ones, derived from pLF15 and pLF56, containing one internal *Pst*I site and thus giving rise to two fragments each upon cleavage of the plasmids with this enzyme (Figure 1).

The *Pst*I fragments and an 110-bp fragment generated with *Hha*I were used as starting material for further cleavage with various restriction endonucleases (Figure 1) and determination of the nucleotide sequences by the method of Maxam and Gilbert (1980). The result is summarized in Figure 2, which shows the nucleotide sequence of the largest of the three inserts, the one derived from pLF15. As indicated by the thin arrows in Figure 1B, this nucleotide sequence was almost completely established from both DNA strands. It represents an mRNA fragment which possesses an open reading frame with a coding capacity for a protein of 397 amino acids (1194 bases) and contains a short, A-rich piece of the 5′ non-coding region (15 bases) as well as a 3′ non-coding region of 222 bases. Short oligo(C) or oligo(G) ends of the cDNA originate from the cloning procedure. A schematic representation of this result is given in Figure 1A.

With the exception of only one position, all nucleotide sequences determined were identical for the three cDNAs. The exception is marked with an arrow in Figure 1 and represents a one-base difference in the 3′ non-coding region between the cDNA from pLF12 and the two other cDNAs.

The mRNA corresponding to the nucleotide sequence shown in Figure 2 has several interesting features. Although we cannot exclude the possibility of an unfaithful transcription during the cloning procedure, particularly for DNA se-

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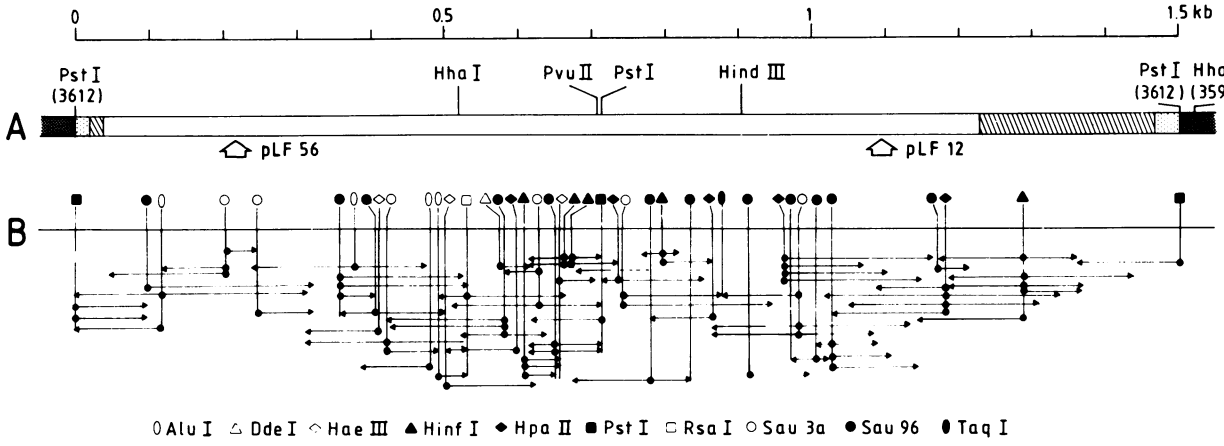

Fig. 1. Restriction map of chalcone synthase cDNA and sequencing strategy. **(A)** Physical map of the cDNA inserts of pLF15 in pBR322. Large open arrows indicate the 5′ ends of pLF56 and pLF12 cDNAs. Their 3′ ends coincide with the end of pLF15 cDNA. The locations of pBR322 (dark area), G/C tailing (stippled area) and noncoding sequences (vertical stripes) are shown. The external *PstI* and *HhaI* sites are numbered according to the nucleotide sequence of pBR322 (Sutcliffe, 1978). **(B)** Restriction map and sequencing strategy of pLF15 cDNA. Sites determined by restriction mapping were confirmed by computer analysis (Kröger and Kröger-Block, 1982) of the sequence shown in Figure 2. The sequencing strategy is shown below the restriction map. Horizontal arrows indicate direction and extent of sequence determinations from 5′ end-labelled DNA fragments according to the technique of Maxam and Gilbert (1980).

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   2 GGGGGGGGGGGGGGGGGGGGTATAAAAAAAAAAAAAAATGGCAAATCATCATAATGCAGAAATTGAGGAAATCAGGAACAGGCAGAGAGCCCAAGGTCCT
                                                 MetAlaAsnHisHisAsnAlaGluIleGluGluIleArgAsnArgGlnArgAlaGlnGlyPro

 101 GCCAATATACTAGCTATTGGCACTGCCACTCCTTCCAACTGTGTCTACCAGGCTGATTATCCTGATTACTACTTTCGTATTACCAACTCCGAACACATGA
      AlaAsnIleLeuAlaIleGlyThrAlaThrProSerAsnCysValTyrGlnAlaAspTyrProAspTyrTyrPheArgIleThrAsnSerGluHisMetThr

 201 CCGATCTCAAACTCAAATTCAAGCGAATGTGTGAAAAATCAATGATCAGAAAGCGTTACATGCATATAACAGAGGAATATCTGAAAGAAAACCCCAATGT
       AspLeuLysLeuLysPheLysArgMetCysGluLysSerMetIleArgLysArgTyrMetHisIleThrGluGluTyrLeuLysGluAsnProAsnVal

 301 ATGCGCATACGAGGCACCCTCACTGGACGCTCGCCAAGACCTGGTTGTGGTGGAGGTCCCAAGGCTAGGCAAAGAAGCTGCATCCAAAGCCATCAAAGAG
      CysAlaTyrGluAlaProSerLeuAspAlaArgGlnAspLeuValValValGluValProArgLeuGlyLysGluAlaAlaSerLysAlaIleLysGlu

 401 TGGGGCCAACCTAAATCCAAGATCACTCACCTCATTTTCTGCACCACTTCTGGTGTGGACATGCCTGGCGCTGACTACCAGCTCACCAAGCTCCTTGGCC
      TrpGlyGlnProLysSerLysIleThrHisLeuIlePheCysThrThrSerGlyValAspMetProGlyAlaAspTyrGlnLeuThrLysLeuLeuGlyLeu

 501 TCCGTCCCTCTGTCAAGCGCTTCATGATGTACCAACAGGGTTGCTTTGCTGGCGGCACTGTCCTCCGTCTGGCTAAGGACCTCGCCGAGAACAATGCCGG
       ArgProSerValLysArgPheMetMetTyrGlnGlnGlyCysPheAlaGlyGlyThrValLeuArgLeuAlaLysAspLeuAlaGluAsnAsnAlaGly

 601 TGCACGAGTCCTCGTTGTCTGCTCCGAGATCACTGCCGTCACTTTCCGTGGGCCCTTCTGACTCTCACCTTGATTCCCTAGTTGGTCAGGCACTTTTTGGT
      AlaArgValLeuValValCysSerGluIleThrAlaValThrPheArgGlyProSerAspSerHisLeuAspSerLeuValGlyGlnAlaLeuPheGly

 701 GATGGTGCAGCTGCAGTCATTCTCGGGTCAGATCCGGATCTATCCGTGGAGCGTCCACTTTTTCAGCTCATATCCGCGGCCCAAACAATTTTACCCGACT
      AspGlyAlaAlaAlaValIleLeuGlySerAspProAspLeuSerValGluArgProLeuPheGlnLeuIleSerAlaAlaGlnThrIleLeuProAspSer

 801 CTGACGGGGCGATTGACGGGCATCTCCGTGAAGTGGGCCTTACCTTCCATCTTCTTAAAGATGTACCGGGCTTAATCTCGAAAAATATAGAAAAGTCGTT
       AspGlyAlaIleAspGlyHisLeuArgGluValGlyLeuThrPheHisLeuLeuLysAspValProGlyLeuIleSerLysAsnIleGluLysSerLeu

 901 GAAGGAAGCTTTTGGGCCTATAGGCATATCAGACTGGAACTCTTTATTTTGGATAGCCCATCCGGGTGGCCCAGCTATTTTGGATCAGGTAGAACTGAAG
      LysGluAlaPheGlyProIleGlyIleSerAspTrpAsnSerLeuPheTrpIleAlaHisProGlyGlyProAlaIleLeuAspGlnValGluLeuLys

1001 TTGGGCCTTAAAGAAGAAAAAATGCGGGCCACCCGACAGGTGTTGAGTGATTATGGAAACATGTCAAGTGCATGTGTGTTGTTTATTTTAGATGAAATGA
      LeuGlyLeuLysGluGluLysMetArgAlaThrArgGlnValLeuSerAspTyrGlyAsnMetSerSerAlaCysValLeuPheIleLeuAspGluMetArg

1101 GAAAGAAGTCTATTGAAGAAGGGAAAGCAACAACTGGAGAAGGTTTGGATTGGGGTGTTCTTTTCGGGTTCGGGCCGGGTCTTACTGTGGAAACCGTTGT
       LysLysSerIleGluGluGlyLysAlaThrThrGlyGluGlyLeuAspTrpGlyValLeuPheGlyPheGlyProGlyLeuThrValGluThrValVal

1201 GTTGCATAGCGTACCCGCTACTTTTACTCACTGAAGTTGTTCCCGATAAATTGTGTCGGTTATATGGTCTAAATTTAAGGCTGTGGGGAGTCTATGTTGT
      LeuHisSerValProAlaThrPheThrHis

1301 CTTAAGTACATGGTGTGTGCACTCTTTGTTAGTTTTAAGTTGGTTCTGGTTTGCTTTGCTTTGCTCATGCATCTCATGTTTTAGTTAATTTACCCACTTT

1401 TCTATATGAATTTGTCGGTTTAATGTCTTCAATAAAGATATTCTTTTCCCTTTAAAAAAAAAAAAAAAAAAAACCCCCCCCCCCCCCCCCCCCCCCCC

1501 CCC
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Fig. 2. Nucleotide sequence of the cDNA insert of pLF15 and the deduced amino acid sequence of chalcone synthase. The strand homologous to the mRNA is shown. Sequences in boxes are oligo(G) and oligo(C) tails from the cloning procedure. Underlined sequences represent two translation termination codons and a potential polyadenylation signal. The arrow at nucleotide position 1334 indicates the single difference found between the sequences determined for pLF12 and the two other plasmids (A in pLF12).

Table I. Comparison of amino acid composition of purified chalcone synthase with that deduced from the nucleotide sequence (Figure 2)

Amino acid	Number of residues from	
	Nucleotide sequence	Protein analysis
Lys	24	27 ± 3
His	11	11 ± 1
Arg	19	18 ± 1
Met	1 + 10	10
Cys	7	8
Asn	13 } 35	38 ± 3
Asp	22 }	
Thr	21	22 ± 1
Ser	25	27 ± 4
Gln	13 } 39	38 ± 5
Glu	26 }	
Pro	19	20 ± 4
Gly	33	34 ± 3
Ala	34	33 ± 1
Val	26	23 ± 3
Ile	23	20 ± 3
Leu	42	38 ± 4
Tyr	10	9 ± 1
Phe	15	15 ± 1
Trp	4	n.d.

The data given for authentic chalcone synthase are mean values from seven independent determinations, with the exception of methionine (four determinations) and cysteine (two determinations). Standard deviations are given where seven determinations were carried out. n.d., not determined.

quences close to the borders of the insert, the unusual sequence of 12 adenosine residues immediately preceding the AUG translation start codon is very likely to be significant and might well be related to the mechanism and rate of translation initiation. Furthermore, the nucleotides flanking the start codon on both sides are in agreement with a consensus sequence, XXAUGG, postulated for eukaryotic translation initiation sites (Kozak, 1981). Another consensus sequence, AAUAAA, was found 17 nucleotides upstream from the poly(A) tail. This sequence is thought to function as a poly(A) addition signal (Benoist *et al.*, 1980).

The occurrence of a second translation termination codon shortly after the first one is in line with many similar observations with other mRNAs. The codon usage is non-random, as shown in Table I. This is in agreement with similar results obtained with other mRNAs whose nucleotide sequences have been determined (Grantham *et al.*, 1980).

The amino acid sequence deduced from the nucleotide sequence

The amino acid sequence deduced from the nucleotide sequence of the cDNA (Figure 2) gives a protein of mol. wt. 43 682. This value agrees well with previous estimations of ~42 000 for the chalcone synthase subunit after gel electrophoresis under denaturing conditions (Kreuzaler *et al.*, 1979). Three additional criteria were used to prove that the nucleotide sequence shown in Figure 2 is complementary to chalcone synthase mRNA.

Table II. Frequency of codon usage in chalcone synthase mRNA, deduced from the nucleotide sequence shown in Figure 2

	U		C		A		G		
U	Phe	8	Ser	7	Tyr	3	Cys	3	U
	Phe	7	Ser	8	Tyr	7	Cys	4	C
	Leu	4	Ser	5	Ochre	0	Opal	1	A
	Leu	7	Ser	2	Amber	0	Trp	4	G
C	Leu	10	Pro	7	His	7	Arg	7	U
	Leu	12	Pro	5	His	4	Arg	2	C
	Leu	4	Pro	3	Gln	5	Arg	3	A
	Leu	5	Pro	4	Gln	8	Arg	1	G
A	Ile	10	Thr	11	Asn	6	Ser	2	U
	Ile	6	Thr	7	Asn	7	Ser	1	C
	Ile	7	Thr	3	Lys	13	Arg	3	A
	Met	1 + 10	Thr	0	Lys	11	Arg	3	G
G	Val	5	Ala	11	Asp	12	Gly	11	U
	Val	8	Ala	10	Asp	10	Gly	13	C
	Val	4	Ala	11	Glu	18	Gly	2	A
	Val	9	Ala	2	Glu	8	Gly	7	G

Left: first nucleotide, top: second nucleotide, right: third nucleotide. Ochre, amber and opal are translation stop codons.

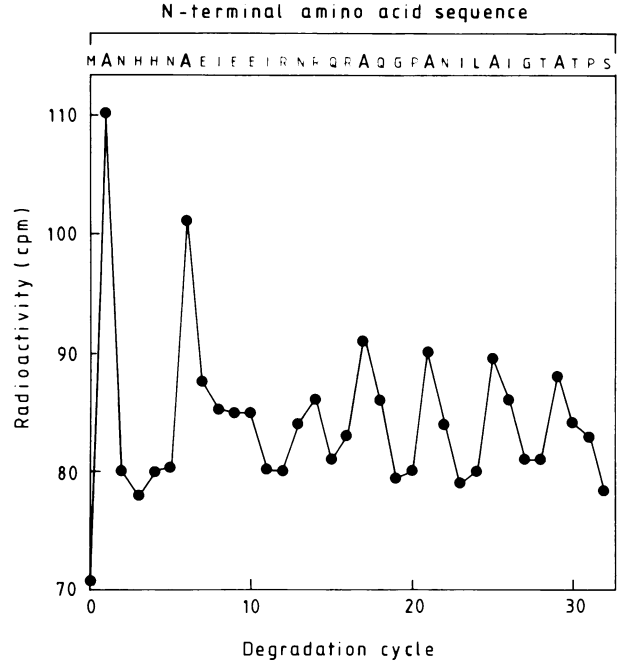

Fig. 3. N-terminal distribution of radioactivity in [^{3}H]alanine-labelled chalcone synthase. The enzyme was synthesized *in vitro* by translation of the poly(A)-rich fraction of polyribosomal RNA from light-induced parsley cells and prepared as described in Materials and methods. The amino acid sequence deduced from the cDNA sequence (Figure 2) is shown in an alignment allowing for the post-translational removal of the first methionine residue. See text for further explanations.

The first line of evidence is shown in Table II. Within the limits of experimental error, the amino acid compositions are the same for the protein deduced from the nucleotide se-

quence and purified chalcone synthase, as determined in seven independent experiments.

The second supporting result obtained with the purified enzyme was an unusual pattern of the cyanogen bromide cleavage products. Although the occurrence of 10 methionine residues in the chalcone synthase subunit indicates an average size of the cleavage products around mol. wt. ~4000, one large peptide of ~16 000 was found. This is in agreement with the amino acid sequence shown in Figure 2, which contains a long, methionine-free segment of 164 amino acids in the central part of the protein.

Finally, the positions of alanine residues within the first 32 N-terminal amino acids of the chalcone synthase subunit were determined. This experiment was carried out with immunoprecipitated protein which had been synthesized *in vitro* in the presence of ^{3}H-labelled alanine. The labelling pattern (Figure 3) is in agreement with the occurrence of alanine residues at positions 1, 6, 17, 21, 25 and 29 of the amino acid sequence deduced from the cDNA, with allowance for a post-translational removal of the methionine residue encoded by the AUG translation initiation codon. The removal of this methionine residue is very likely to occur both *in vivo* and *in vitro*, not only because it is a common event, but also because the native chalcone synthase protein is N-terminally blocked (Kreuzaler *et al.*, 1979), probably by an acetyl group, which is preferentially found at alanine residues. It was therefore necessary to use conditions for the *in vitro* synthesis under which acetylation of the protein was prevented (Palmiter, 1977). The fact that an unblocked protein was obtained only under these conditions strongly suggests that the previously undefined blockage group is an acetyl residue.

Taken together, our results prove that all three plasmids used as starting material for nucleotide sequence determinations contain cDNA inserts complementary to chalcone synthase mRNA. Despite the one-base difference between the cDNA inserts from pLF12 and the two other plasmids, it is likely that all three cDNAs are copies of the same mRNA species. Furthermore, the great similarity between the amino acid compositions of authentic chalcone synthase and the protein deduced from the nucleotide sequence suggests that the proposed two subunits of the enzyme are identical not only with respect to size (Kreuzaler *et al.*, 1979), but also with respect to amino acid composition. It is therefore possible that both subunits have the same primary structure.

At present, the question as to how many genes code for chalcone synthase remains open. In any case, the nucleotide and amino acid sequences presented here might be useful for further studies of this enzyme and its induced expression in higher plants. Recent interest in chalcone synthase has mainly focussed on the mechanism of action and evolutionary origin [both in connection with some striking similarities between chalcone synthase and 3-oxoacyl-[acyl-carrier-protein] synthase (Schüz *et al.*, 1983)], on the regulation of chalcone synthase mRNA activity in response to defined treatments of various plant tissues (Schröder *et al.*, 1979; Lawton *et al.*, 1983; Kreuzaler *et al.*, 1983) and on the structure of the gene(s) coding for chalcone synthase mRNA, particularly in relation to mutations by transposable elements (Wienand *et al.*, 1982).

Materials and methods

Enzyme purification

Propagation, irradiation and harvest of cell suspension cultures of parsley *(Petroselinum hortense)* were carried out as described by Hahlbrock *et al.* (1976). Chalcone synthase was purified according to the methods of Kreuzaler *et al.* (1979) and Heller and Hahlbrock (1980).

Analysis of cyanogen bromide fragments

Lyophilised protein was cleaved in 70% (v/v) aqueous formic acid with a 5-fold excess of CNBr over protein (w/w) for 24 h at 4°C, dried under a stream of nitrogen, and analyzed by SDS-polyacrylamide gel electrophoresis.

Amino acid analysis

The lyophilised enzyme was hydrolysed for 24 h in boiling HCl. Amino acid analysis was performed with a Biotronic Amino Acid Analyser Model LC 6000 E or Durrum Amino Acid Analyser Model D 500.

Translation of mRNA in vitro

Isolation of poly(A)$^{+}$ mRNA from light-induced parsley cells and translation and immunoprecipitation of chalcone synthase protein with specific antibodies were carried out in a manner similar to that described by Ragg and Hahlbrock (1980) and Schröder *et al.* (1979). The following modifications were used. Translation in the wheat germ system followed the method of Davies *et al.* (1977) using 5 μCi L-[3-^{3}H]alanine (82.7 Ci/mmol) per assay (50 μl). Translation in the presence of citrate synthase and oxaloacetate was performed as described by Palmiter (1977).

Amino acid sequence analysis

The N-terminal sequence of the *in vitro* synthesized, [^{3}H]alanine-labelled and immunoprecipitated chalcone synthase protein was analysed with a Beckman 890 B updated sequence analyser using a program adapted from Dildrop *et al.* (1982).

Determination of nucleotide sequence

The nucleotide sequence of cloned DNA (Kreuzaler *et al.*, 1983) was determined according to the method of Maxam and Gilbert (1980). All isolated fragments were purified by butanol extraction (Langridge *et al.*, 1980) prior to the kinase reaction and chemical degradation steps.

Computer analysis

Computer-assisted evaluation of nucleotide sequences used the program developed by Kröger and Kröger-Block (1982).

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