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Multiple transcription start sites of the carrot dihydrofolate reductase-thymidylate synthase gene, and sub-cellular localization of the bifunctional protein

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Abstract

The analysis of clones obtained by rapid amplification of the 5′ end and by primer extension of the mRNA for carrot bifunctional dihydrofolate reductase-thymidylate synthase showed transcripts of differing lengths that belonged to two sub-populations. The longer transcripts were found to contain a translation start site 147 nt upstream of, and in frame with, the one which is present in the shorter transcripts. The ORF that begins at this ATG codes for a protein of 64 714 Da, which is much larger than mature DHFR-TS subunit. The N-terminus region of this polypeptide shows features typical of plant transit peptides. Immunogold labelling studies and immunorecognition of the plastid-containing sub-cellular fraction suggested a plastidial localisation of the bifunctional protein. Although plant cells were shown to contain folate pools in plastids, in mitochondria and in the cytosol, few enzymes of the folate pathway have been associated with any sub-cellular compartment. Thus, this is the first indication for the presence of an enzyme of the folate biosynthetic pathway in plastids. The longer transcripts revealed the presence of a TC microsatellite at the 5′-untranslated end.

Introduction

One-carbon metabolism mediated by folates is highly compartmentalized in eukaryotic cells; in *Saccharomyces cerevisiae* and in animal cells, several folate-dependent enzymes are found both in mitochondria and in the cytosol [3, 48]. In plants, folate pools are found in plastids, mitochondria and the cytosol [12], but only a few folate-dependent enzymes have been associated with any cell compartment [13].

Dihydrofolate reductase (DHFR; EC 1.5.1.3) is the last enzyme of the pathway that leads to tetrahydrofolate, the precursor of folic cofactors. Thymidylate synthase (TS; EC 2.1.1.45) catalyses the synthesis of deoxythymidine monophosphate from deoxyuridine monophosphate and 5,10-methylenetetrahydrofolate. In this reaction, 5,10-methylenetetrahydrofolate acts both as a donor of the methyl group and as a reducing agent, and thus produces dihydrofolate. Consequently, TS is dependent on DHFR for regeneration of tetrahydrofolate, which is in turn necessary for the formation of 5,10-methylenetetrahydrofolate.

In bacteriophages, viruses and most organisms (bacteria, fungi, vertebrates and mammals) DHFR and TS occur as distinct monofunctional polypeptides, while Protozoa contain a bifunctional DHFR-TS which carries both activities on a single polypeptide [22]. For plants, both monofunctional and bifunctional forms have been described [9].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Z33383 (DRTSRACE).

In mammals, DHFR isoforms occur both in the cytosol and in mitochondria; however, cytosolic DHFR and TS appear to change sub-cellular compartment on the basis of the state of cell proliferation. It has been reported that in Chinese hamster embryo fibroblasts, TS is cytosolic in resting cells and nuclear in dividing cells [34, 37]. According to these authors, several enzymes involved in deoxyribonucleotide synthesis (thymidine kinase, deoxycytidylate kinase, nucleoside diphosphate kinase, ribonucleotide reductase, DHFR and TS) become associated with DNA replicating enzymes to form a multi-enzyme complex, which was named replitase, as cells enter the S-phase; in quiescent cells, the enzyme complex dissociates and components move back to the cytosol. This hypothesis has received support from the description of various systems [43] but the literature is not unanimous [38].

In carrot, experiment that used immunofluorescent techniques revealed no difference in DHFR-TS localization between resting and growing suspension-cultured cells; irrespective of the cell growth phase, the bifunctional protein appeared to be cytoplasmic. Moreover, a continuous cytoplasmic localization of DHFR-TS was observed in carrot cells synchronised with aphidicolin. In contrast, in MTX-resistant cells overproducing DHFR-TS, a nuclear or perinuclear localization of the bifunctional protein was observed in addition to the cytoplasmic one. In this case, too, no variation was observed between growing and resting cells, and this finding indicated that nuclear localization was linked to enzyme overproduction [7].

The isolation and sequencing of a carrot cDNA and two *Arabidopsis thaliana* genomic clones for the bifunctional protein has been reported [26, 29]; analysis of the nucleotide sequence of these clones revealed a unique ORF that corresponded to a protein of slightly larger size than that reported for carrot DHFR-TS [1].

This paper reports the results of molecular and immunochemical studies that show the presence of multiple *Dcdhfr-ts* transcripts which correspond to proteins of differing lengths; the paper also provides some evidence for the sub-cellular localization of DHFR-TS.

Materials and methods

Plant cell culture and fractionation

Carrot cell suspensions of *Daucus carota* cv. Lunga di Amsterdam (line E4) were cultured as previously described [29]. Extraction and purification of organelles were essentially performed as described [11]. Cytochrome-c-oxidase activity and the presence of starch and of DNA were used as subcellular fraction markers as described [11]. Somatic embryogenesis was induced by the dilution and transferral of suspension cells (line Hypo I) to hormoneless Gamborg's B5 medium.

Probes

Probes used for molecular hybridisation were as follows: 2 kb cDNA [29]; a genomic sequence of 870 bp containing the 5′ end of the first intron and the adjacent part of the region corresponding to the 5′ end of cDNA (obtained by digestion of the relevant genomic clone with *Eco*RI); a sequence corresponding to the 5′ end of the cDNA obtained by PCR with pDRTS [29] as template, and with the vector-specific primer (SP6) and the sequence-specific primer VVQY, 5′-CAACCTGATAAGTTCTTTGTG-3′ (complementary to 160–142 of the cDNA); a sequence corresponding to the region from −110 to −365 (see Fig. 2A). The latter was obtained by PCR with primers RACE 6 (5′-GAACAAGAGGAGACACAATTC-3′, corresponding to the region from −364 to −344) and RACE 7 (5′-AGAAGAGGAAATGTGGATGAA-3′, complementary to the region from −110 to −130); the reaction was performed in a 50 μl volume containing: 10 ng of carrot genomic DNA, 1× PCR buffer (Perkin Elmer Cetus), 0.2 mM of each dNTP, 500 ng of each primer, 2 units of *Taq* polymerase (Perkin Elmer Cetus). Cycling conditions were: 2 min at 94 °C followed by 30 cycles of 50 s at 94 °C, 50 s at 57 °C, 1 min at 72 °C. Probes were labelled with [^{32}P]-dCTP by means of the Prime-a-Gene System kit (Promega).

DNA extraction and Southern blot hybridization

Carrot genomic DNA was extracted as described [15] and purified by cesium chloride gradient centrifugation [40]. Hybridization conditions were as previously described for Southern blotting [29].

RNA isolation and northern blot hybridization

Total RNA was extracted from 5-day-old carrot suspension cells by means of Extract-A-Plant RNA isolation kit (Clontech) and in accordance with the manufacturer's instructions. Poly(A)$^+$ RNA was purified using PolyATract mRNA isolation kit II (Promega), and in accordance with the manufacturer's instructions.

Total RNA (15 μg aliquots) was separated by 1.0% agarose/formaldehyde gel as described [36], transferred by capillarity to Hybond N (Amersham) and cross-linked by UV irradiation. Hybridization was performed under standard conditions [40] with 50% formamide, at 42 °C for two days with the indicated probe. Filters were washed (2× SSC/0.1% SDS) twice at room temperature for 15 min and once at 65 °C for 15 min.

*Rapid amplification of 5′ cDNA end (*RACE*)*

The RACE technique [17, 16, 41] was performed with some modifications. The following gene-specific primers were used: EVTA, 5′-TTCGACAGTTGCAATATCA-3′ (complementary to 399–381 nt of the cDNA); VVQY, 5′-CAACCTGATAAGTTCTTTGTG-3′ (complementary to 160–142 of the cDNA); GNTP: 5′-GCGAATTCCATTAGTAGGATTTGCGA-3′ (complementary of 123–98 of the cDNA. C residues at positions 121 and 119 were substituted with G and A, respectively, to create an *Eco*R1 site at the 5′ end of the primer). Adapter primers $T_{17}R_IR_O$, R_O and R_I, synthesized as described by Frohman [16], were a gift of Dr C Mondello. We performed two independent RACE experiments with differing starting material under differing conditions. In one case, RACE was performed essentially as described [41]. cDNA synthesis was obtained with total carrot RNA; an aliquot of 1 μg was mixed with 2 pmol of the gene-specific primer EVTA (in a volume of 7 μl), the mixture was heated at 70 °C for 6 min and chilled on ice. This was followed by the addition of 8 μl of a 2.5 mM solution of the 4 dNTPs (final concentration was 1 mM) and of 4 μl of 5× reverse transcriptase buffer (Promega). After preincubation at 41 °C for 2 min, 200 units of M-ML V (RNaseH minus) reverse transcriptase (Promega) were added to the reaction mixture and incubated at 41 °C for 40 min. The sample was then equilibrated at 55 °C and after the addition of 2 units of RNaseH (Promega), further incubated for 10 min at this temperature. For the other RACE experiment, cDNA synthesis was obtained with poly(A)$^+$ RNA and in accordance with the protocol described above except that incubation at 41 °C was for 1 h, and the sample was not treated with RNaseH. Excess primers and salts were removed by means of the Qiaex kit (Qiagen), with 4.5 volumes of binding solution (Qiaex 1). Purified cDNA was eluted with 30 μl of distilled water. First-strand cDNA (12 μl), obtained from total RNA, was dA-tailed in PCR buffer (Perkin Elmer Cetus) as described [41]; the cDNA (10 μl) obtained from poly(A)$^+$ RNA was dA-tailed in tailing buffer (Promega) as described by Frohman [16]. In both cases, the first-strand cDNA (in a final volume of 20 μl) was mixed with 0.2 mM dATP, heated at 70 °C for 5 min and chilled on ice. After the addition of 10 units of TdT (Promega), the reaction proceeded at 37 °C for 5 min and finally at 65 °C for 10 min. Either PCR or tailing buffer can be used. However, if the starting material is limiting, or if the gene copy number is low, the PCR buffer appears to be a better choice in that tailed products can be used directly for PCR without further dilution.

A 5 μl aliquot of tailed cDNA (obtained from total RNA) was added to the PCR cocktail (final volume 50 μl) that contained 1× PCR buffer (Perkin Elmer Cetus), 10 nmol of each dNTP, 25 pmol of gene-specific primer VVQY, 2.5 pmoles of primer $T_{17}R_IR_O$ and 25 pmol of primer R_O. Samples were overlaid with two drops of light mineral oil and heated at 94 °C for 5 min and chilled on ice. After the addition of 2 units of Taq DNA polymerase (Perkin Elmer Cetus), the reaction proceeded as follows: 1 cycle of 2 min at 94 °C, 3 min at 50 °C and 3 min at 72 °C followed by 35 cycles of 45 s at 94 °C, 25 s at 50 °C, 2 min at 72 °C. A 10 min extension at 72 °C was added at the end of the PCR.

For cDNA obtained from the poly(A)$^+$ (tailed in tailing buffer), 5 μl of 1:20 dilution of tailed products were added to an identical PCR cocktail supplemented with 5% formamide as described [4]. Cycling conditions were as follows: 5 cycles of 1 min at 94 °C, 45 s at 45 °C, 1 min at 72 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C and 10 min extension at 72 °C.

Aliquots of 1:20 dilutions of the first round of amplification products (2 μl in the case of cDNA obtained from total RNA, or 5 μl in the case of cDNA obtained from poly(A)$^+$) were added to the following PCR cocktail (final volume of 50 μl): 1× PCR buffer, 10 nmoles of each dNTP, 25 pmol of primer R_I and 25 pmol of gene-specific primer GNTP. Cycling conditions were: 3 cycles of 1 min at 94 °C, 50 s at 57 °C, 1 min at 72 °C followed by 32 cycles of 1 min at 94 °C, 50 s at 54 °C, 1 min at 72 °C and 10-min extension at 72 °C. Due to the low annealing temperature used during cDNA synthesis, many non-specific products were formed and subsequently tailed. Thus, after the first amplification round, analysis of products gave a smear in the agarose gel. In the second amplification round, contrary to Frohman [16], we used unbalanced

primers; the gene-specific primer had higher T_m. Step PCR was performed with the first several cycles at a higher annealing temperature. This modification was designed to ensure linearity of increase in the amount of gene-specific products during the first cycles, and thus to improve the specificity of single-sided PCR. The same approach was successfully used for the isolation of the genomic sequence corresponding to the 5′ end of the *Dcdhfr-ts* cDNA [27]. In both experiments, the gene-specific primer was GNTP.

In order to evaluate specificity, we ran RACE products on a 1.5% agarose gel, transferred them to Hybond N^+ (Amershan) and hybridized them with the 5′ end of the *Dcdhfr-ts* cDNA; protocols were standard.

The products of the second round of amplification were purified by Microcon 100 (Amicon). The T-vector was prepared as described [31], except that pGEM-4Z vector (Promega) was linearized with *Sma*I before dT-tailing with TAQ polymerase (Perkin Elmer Cetus). The ligation mixture was used to transform DH5α *Escherichia coli* cells by electroporation (BioRad). The recombinant clones were identified as white colonies on LB agar plates containing 60 μg/ml ampicillin, IPTG and X-gal, as recommended by the manufacturer (Sigma).

The selection of positive clones was achieved by colony hybridisation for RACE products obtained with total RNA. Plates containing white and blue colonies were directly transferred onto Hybond N^+ (Amersham). Colonies were plate-printed directly onto the membrane. Depending on colony density and ratio of positive to total colonies, a second selection step might be necessary. However, plate-printing avoids the tedious transfer of single colonies to fresh plates and accelerates the screening of the minilibrary. Hybridization was performed as described above. For those products obtained with poly(A)$^+$ RNA, white colonies were used directly for DNA analysis.

It was found that probes for PCR product identification (or colony hybridization of PCR clones) could be a sequence containing either of the two PCR primers since, under the high stringency washing conditions used, no unspecific recognition was found to occur.

Primer extension

Primers YTTF (5′-ATATGTAGTAAACCGATGGC-3′, complementary to the region from -1 to -20 in Fig. 2B) and RACE 2 (5′-TTGACGGCAGAAAAGGT GTGCGGGTG-3′, complementary to the region from -180 to -205 in Fig. 2B) were end-labelled with [γ-^{32}P]ATP by T4 kinase by means of the dsDNA Cycle Sequencing System kit (Gibco-BRL) and in accordance with the manufacturer's instructions. An aliquot of 10 μg poly(A)$^+$ RNA and 1 pmol of labelled primer were resuspended in 20 mM of Pipes pH 6.4 containing 0.4 M NaCl, denatured at 80 °C for 5 min and then annealed for 3 h at 45 °C in the case of YTTF, and for 2 h at 70 °C prior to another 2 h at 50 °C in the case of RACE 2. The mRNA:primer complex was precipitated with ethanol, resuspended in 20 μl of 1× reverse transcriptase buffer containing 20 units of RNasin, 1 mM dNTP, 200 units of M-MLV reverse transcriptase (Promega) and incubated at 39 ° for 2 h. The reaction was stopped by the addition of 1 μl of 0.5 M EDTA (pH 8) and RNA was digested by RNase A and T1. The sample was extracted once with phenol/chloroform and the cDNA was precipitated with ethanol and resuspended in 8 μl of H_2O, to which 4 μl of the stop solution of dsDNA Cycle Sequencing System kit (Gibco-BRL) was added.

Nucleotide size markers were produced by sequencing of the genomic clone p312A2 [27] with the same labelled primer by means of the dsDNA Cycle Sequencing System kit (Gibco-BRL).

Primer extension and sequencing products were separated by 7% PAGE and exposed overnight to an autoradiographic film at -80 °C.

Nucleotide sequence analysis

DNA sequencing was performed with the T7 sequencing kit (Pharmacia) and ^{35}S-dATP. When needed, oligonucleotides (17–23-mers) were synthesised and used as sequencing primers. Sequence data were analyzed by MacDNASIS v3 (Hitachi).

Protein purification

DHFR-TS was purified from *D. carota* cells grown in suspension culture essentially as described [1], except that both DEAE-Sephacel peaks I and II were applied to MTX-Sepharose. The column was then washed with 50 volumes of 5 mM $(NH_4)_2CO_3$ pH 8.4 buffer containing 1 M KCl, and 10 volumes of 5 mM $(NH_4)_2CO_3$ pH 8.4; DHFR activity was eluted with 7 volumes of 5 mM $(NH_4)CO_3$ pH 8.4 containing 1 mM dihydrofolate.

Electrophoretic and immunochemical techniques

Electrophoretic and immunoblot analyses

Electrophoresis under denaturing conditions, electrophoretic transfer of polypeptides to nitrocellulose filters and immunochemical detection of DHFR-TS were performed as described [6]. Polyclonal antibodies against the bifunctional protein were raised in rabbit as already reported [6]. Monospecific antibodies were obtained by incubation of the immune serum with purified DHFR-TS bound to nitrocellulose.

Immunoblots were analysed with the ECL^{TM} Western blotting analysis system (Amersham).

Immunogold labelling

Carrot cells (13 days old) and somatic embryos (7 days old) were fixed for 2 h by immersion in 0.1 M phosphate buffer pH 7.4, containing 2% paraformaldehyde and 1% glutaraldehyde [25]. After fixation, cells were washed for 2 h with 0.1 M phosphate buffer pH 7.4 and left overnight in the same buffer. Cells were then treated with 0.5 M NH_4Cl in phosphate buffer for 2 h to block free aldehyde groups. All these operations were performed at 4 °C. Fixed cells were dehydrated through a graded series of dimethylformamide (DMF) in water (50% DMF for 15 min at 4 °C; 75% DMF for 15 min at −20 °C; 90% DMF for 60 min at −20 °C) and infiltrated with Lowicryl K4M (DMF: Lowicryl K4M, 2:1 for 15 min, 1:1 for 15 min, 1:2 for 15 min, Lowicryl 100% for 30 min; Lowicryl 100% for 60 min) [39]. Polymerisation at −20 °C was obtained by overnight ultraviolet irradiation [2]. Thin sections were cut out with glass or diamond knives and mounted on nickel grids. Sections on grids were incubated with TBS-T buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.3% Tween 20) for 15 min and in TBS-T buffer containing 10 mg/ml bovine serum albumin (TBS-TB) for 15 min. Grids were incubated overnight at 4 °C with primary antibodies diluted 1:200 or 1:100 in TBS-TB buffer for 15 min at room temperature [21]; they were then washed with TBS-TB buffer for 15 min, incubated with a 1:20 dilution of goat-anti-rabbit-gold (10 nm, Biocell Research Laboratories) or protein-A-gold (20 nm, Sigma) for 2 h and finally washed several times with TBS-TB buffer for 15 min. Control grids were incubated with pre-immune serum. Sections were post fixed in 1% glutaraldehyde and 0.5% OsO_4 and subsequently stained with uranyl acetate and lead citrate [14, 39]. Quantitative evaluation of immunogold particle distribution was performed on prints from at least 10 electron micrographs; the number of gold particles in a defined area was counted and the density of labelling expressed as number of grains per measured area [39].

Results

Carrot cells contain two dhfr-ts *transcript species*

The comparison of the sequence of the cDNA for carrot DHFR-TS [29] with that of the corresponding genomic region, which was obtained by single-sided PCR [27], showed a divergence for the last 6 nt of the 5′ end of the cDNA; the presence of an AG 3′ splicing consensus motif at the point of divergence suggested the occurrence of an intron. Moreover, this genomic region did not reveal a TATA box, even at several hundreds nucleotides upstream of ATG. In order to determine transcript length, we used the technique of rapid amplification of the 5′ cDNA end [17], modified as described in Fig. 1A. An experiment performed with total carrot RNA showed two broad bands on EtBr-stained agarose gel (not shown) and three bands after hybridization (Fig. 1B). Similar results were obtained when the RACE experiment was repeated with carrot poly(A)$^+$ RNA (not shown). In this second experiment, we allowed more time for the synthesis of the first cDNA strand, and added 5% formamide during the first round of amplification; formamide was shown to improve the yield of the specific product for 5′ RACE of CpG island-containing mRNA [4]. We took his precaution since it had been reported that mammalian monofunctional *dhfr* genes contain CpG islands at the 5′ region of mRNA [32, 42]. DNA sequencing was performed on 23 randomly selected RACE clones (13 obtained from total RNA and 10 obtained from poly(A)$^+$ RNA; in this second case, 5′-RACE products were size-selected and found to correspond to the larger hybridization band). Sequence data, which are shown in Fig. 2A, show that short transcripts (7 clones) possess a 5′-untranslated region of 111 nt (from the previously observed start site, indicated as ATG_1 in Fig. 2D); the length of the region from ATG_1 of long transcripts was 339 nt (2 clones), 361 nt (13 clones) and 365 nt (1 clone). Only the 361 nt transcript was found among RACE clones obtained from poly(A)$^+$ RNA. These results indicate that the larger and the smaller bands of Fig. 1B correspond to the 5′ ends of two transcripts, while the medium one appears to be the unclonable hybrid which likely forms during the annealing step, and gives rise to an unamplifiable

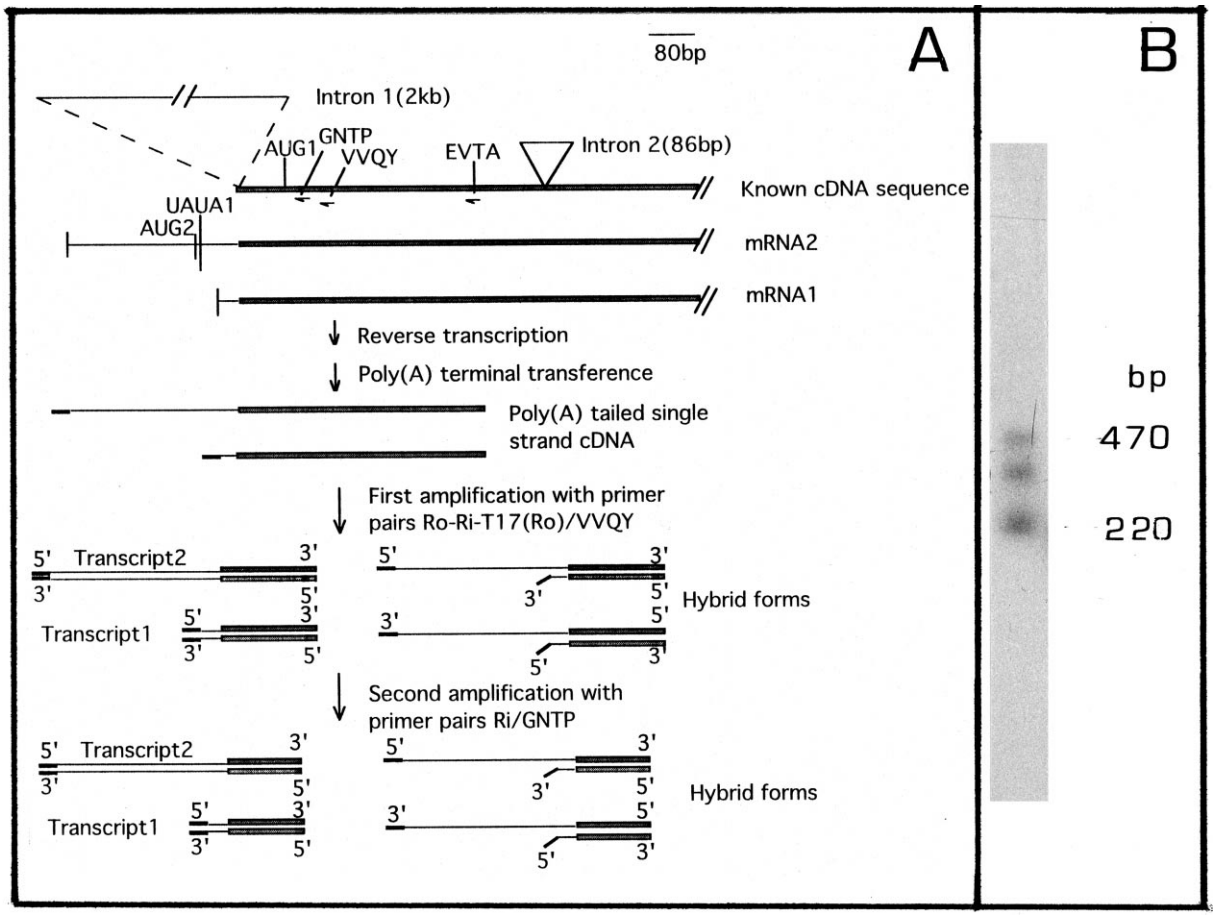

Figure 1. Rapid amplification of the 5′ end of *Dcdhfr-ts* cDNA. A. Flow diagram of RACE experiments. B. Southern blot analysis of amplification products obtained in the first round of amplification with the 5′ end of the *Dcdhfr-ts* cDNA as probe.

template. Identity at the 5′ end between cDNA and genomic DNA sequences [28] was found about 2 kb upstream of the site of divergence (Fig. 2B).

Although the shortness of RACE products could be conceivably due to an incompleteness of reverse transcription, we found a TATA box 27 nt upstream of the proximal transcription start point (in this region cDNA is colinear with the corresponding genomic sequence); we also found a good fit between the TATAAA and ATGGC sequences and the consensus sequences for plant genes [23, 30]. The results of primer extension analysis, which are reported in Fig. 3, confirm the occurrence of the short transcript as indicated by the extension products of 109–111 nt.

The genomic DNA sequence showed the presence of a second TATA box situated 25–51 nt (29 nt for the 361 nt type) upstream of the distal transcription start points (Fig. 2B).

Northern blot hybridisation analysis with a probe that was exclusively specific to the longer RACE products (from −110 nt to −365 nt, Fig. 2A) showed that the longer mRNA species was present in the mRNA population of suspension cultured carrot cells (Fig. 4).

A primer extension experiment that used primer RACE 2 confirmed the presence of an extension product of 201 nt corresponding to the longer transcripts (not shown).

The longer transcript species contains an ORF that codes for a pre-protein with a putative transit peptide

The longer transcripts contain a potential translation start site (ATG_2) which is situated 147 nt upstream of, and in frame with, ATG_1 (Fig. 2). ATG_2 gives rise to an ORF of 1731 nt corresponding to a protein of

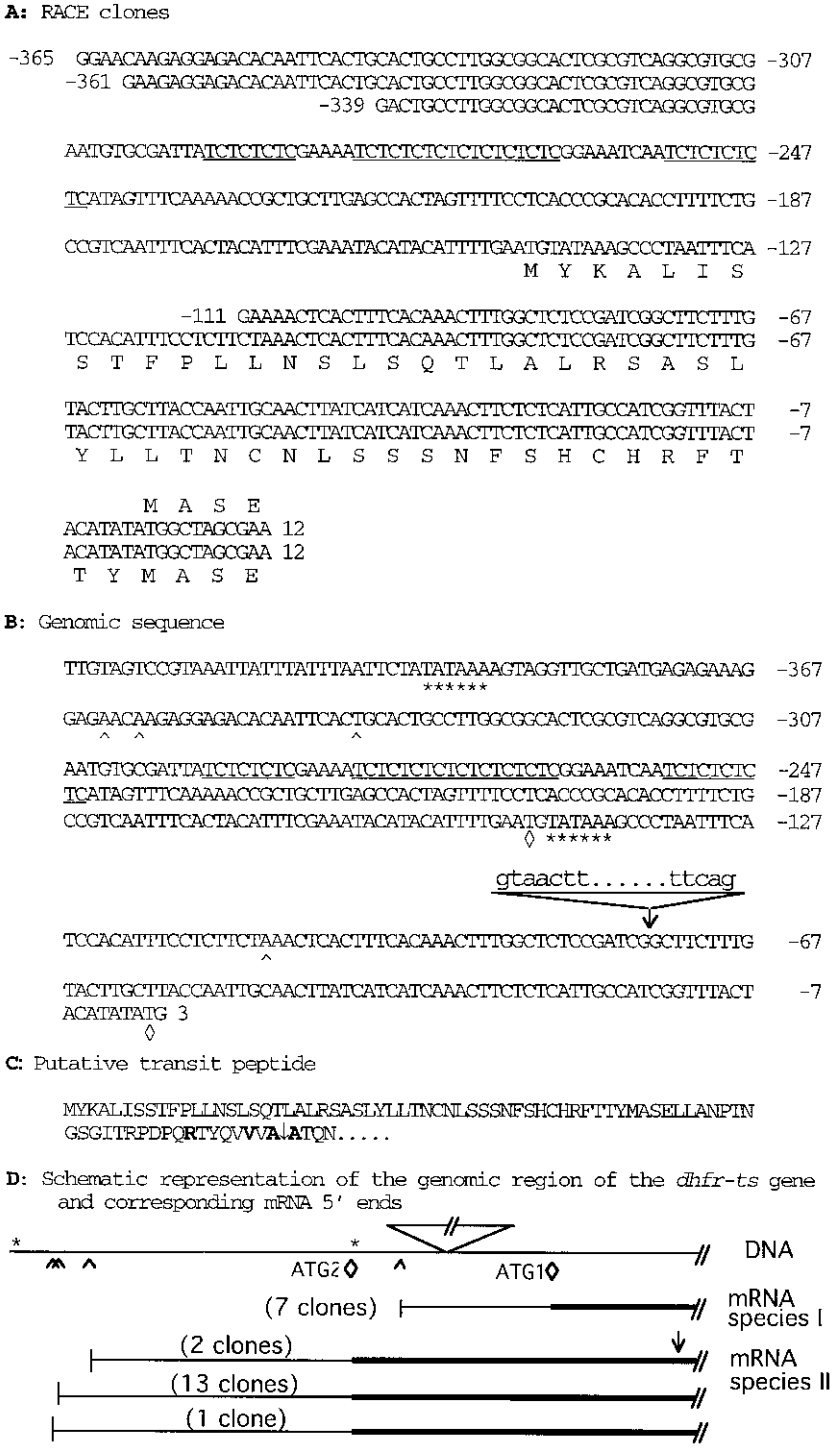

Figure 2. Sequence analysis of clones obtained by RACE. A. Sequences representative of the 23 clones analysed. Differences were observed only at the 5′ end. The solid line marks TC-rich streches. B. Genomic sequence [28] corresponding to the 5′ end of mRNAs. Asterisks indicate TATA boxes, while arrow heads and diamonds indicate respectively transcription and translation start sites. Position and partial sequence of ends of the first intron are also indicated. Numbers refer to nucleotide positions relative to ATG 1 (excluding the intron). C. Putative transit peptide. Bold characters and arrow indicate respectively consensus sequence and site of proteolytic cleavage. D: schematic representation of the genomic region and corresponding 5′ end of mRNA for carrot DHFR-TS. Symbols are as in B and C. Figures in parenthesis represent numbers of RACE clones analysed.

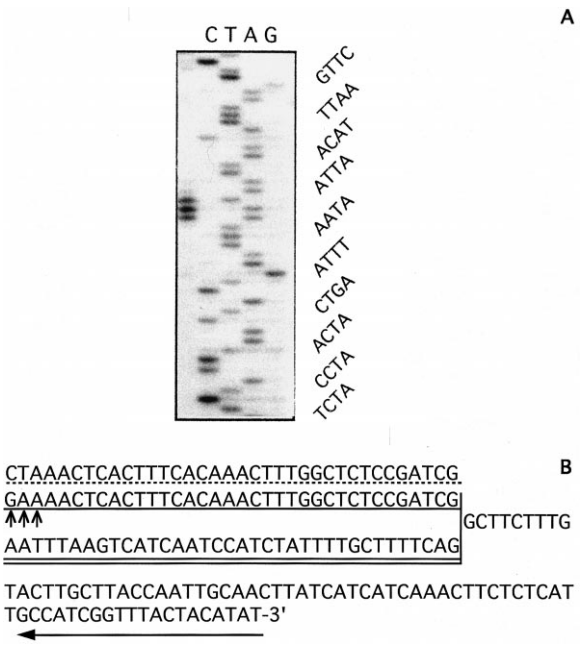

Figure 3. Primer extension experiment for the shorter transcripts. A. Primer extension products and the nucleotide size markers separated by 7% PAGE. The sequence is complementary to that of the genomic clone [28]. B. Sequence comparison. The horizontal arrow shows position and direction of the primer. The dashed line indicates the genomic sequence corresponding to the 5′ end of the shorter transcript species (Fig. 2). Single and double plain lines indicate the respective sequences of the 5′ end of the shorter transcript species and of the intron [28] near to the divergence point. Vertical arrows indicate the positions of primer extension products. The G and A residues at the 5′ cDNA and appear to be artifacts of reverse transcription, since they differ from the genomic counterpart.

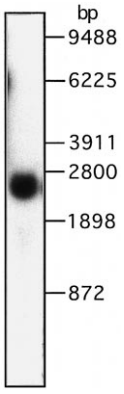

Figure 4. Northern blot analysis of total RNA extracted from 5-day-old carrot suspension cells. The RNA sample (15 μg) was hybridized with a sequence specific for the longer transcript species (from -110 nt to -365 nt, Fig. 2A).

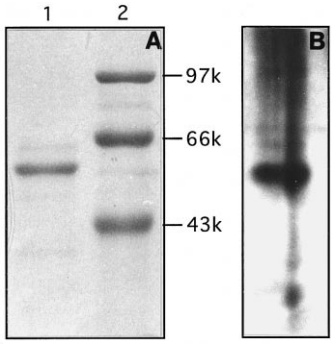

Figure 5. Electrophoresis under denaturing conditions and immunoblot analysis of carrot DHFR-TS purified by MTX-Sepharose. A. SDS-PAGE. Lane 1, DHFR-TS (4 μg); lane 2, mass relative protein markers. B. Immunoblot of DHFR-TS (200 ng) using antibodies against the major form of the bifunctional protein. Protein bands analysed in A and B were obtained in distinct electrophoretic runs.

577 amino acids with a deduced molecular mass of 64 714 Da. The latter value is much higher than that of 58 400 $\pm$ 1000 Da as determined by SDS-PAGE of the purified carrot DHFR-TS [1].

We purified carrot DHFR-TS by MTX-Sepharose affinity chromatography of both major and minor DEAE-Sephacel peaks [1], and submitted this fraction to SDS-PAGE analysis. This revealed a band of about 60 kDa along with the predominant lower size form (Fig. 5A). The relative amounts of the two bands showed a ratio of about 1 to 9 as judged by densitometry. In immunoblot experiments, only these two bands were recognised by antibodies against the 58 kDa form, while the co-purifying faint bands of lower size did not stain (Fig. 5B). This observation therefore suggests that ORF_2 codes for a pre-protein that contains a transit peptide whose removal gives rise to the major form of DHFR-TS. As reported in Fig. 2, the deduced amino acid sequence corresponding to the N-terminal region of the polypeptide starting from ATG_2 does indeed show features that are typical of transit peptides, such as the abundance of charged and hydroxylated amino acid, the presence of hydrophobic residues and a lack of acidic amino acids [5, 46]. The presence of a block prevented us from to determining the N-terminus amino acid of the major carrot DHFR-TS form [8], and attempts to remove this block both by mild HF hydrolysis and by a deacetylase treatment were unsuccessful.

It has been reported that V/I-X-A/C↓A is a conserved cleavage site motif in chloroplast transit peptides (cTP) with an arginine that is often found in pos-

Table I. Amino acid composition of the putative transit peptide and mature protein (values are in percent)

Amino acid	Putative transit peptide (78 aa)	Mature protein (499 aa)
Ala	7.69	5.01
Arg	5.12	4.80
Asn	7.69	4.80
Asp	1.21	6.01
Cys	2.56	2.20
Gln	3.84	3.00
Glu	1.28	5.81
Gly	2.56	7.41
His	2.56	2.40
Ile	2.56	6.81
Leu	15.38	8.41
Lys	1.08	5.21
Met	2.56	3.00
Phe	3.84	5.81
Pro	5.12	4.60
Ser	15.38	7.81
Thr	10.25	5.21
Trp	0	1.60
Tyr	5.12	3.20
Val	3.84	6.81

ition −6 to −10 relative to the cleavage point [20]. Such **R**TYQV**V**V**A**↓**A** motif is present from 71 to 79 residues of the deduced amino acid sequence of ORF 2 (Fig. 2C). Cleavage of the protein at this site would remove 78 amino acids (8595 Da) and give rise to a polypeptide of 499 amino acids with an estimated molecular mass of 56 187 Da. This latter value is very close to that of 56 500 Da calculated from amino acid analysis of the major DHFR-TS form [8]. The amino acid content analysis of this putative transit peptide, as compared to analogous analysis of the mature protein [29], revealed a high abundance of Ser, Thr and Ala, and a lower level of Glu, all of which are typical of cTP (Table 1). Moreover, a high level of Leu (15.38% vs 8.41% of the mature protein), which is typical of mitochondrial transit peptides, was observed.

The hydrophobicity profile of putative DHFR-TS TP, determined in accordance with Kyte and Doolittle, presents three domains as reported from the analysis of representative samples of chloroplast-targeting peptides [46], and shows a strong similarity to the hydrophobicity profile of the Rubisco SSU of *A. thaliana* [47] (not shown).

Immunocytochemical studies indicate a plastidial localization of carrot DHFR-TS

In order to investigate the sub-cellular localization of DHFR-TS, we undertook immunogold labelling on thin cell sections, using antibodies against the bifunctional protein. In the first experiment, we used early-stationary-phase suspension-cultured cells, since they were known to have the highest DHFR-specific activity during a growth cycle [1]. We quantified immunogold labelling by subtracting the number of gold particles present in a defined area of sections incubated with pre-immune serum from the number of particles present in areas of sections incubated with the immune serum. Amyloplasts and nucleoli were found to contain the highest number of gold particles (71 grains/μm^2 and 22 grains/μm^2 respectively), while in all other compartments analysed, the amount found was less than 10 grains/μm^2. However, in this experiment, a high background in section treated with pre-immune serum reduced the labelling density ratio (immune/preimmune). Nevertheless, amyloplasts and nucleoli were clearly recognized at a ratio of 2.74 and 3.6 respectively. Mitochondria and the cytosol, which in animal cells and yeast are known to contain distinct DHFR isoforms [3], showed a ratio of only 1.3.

However, the use of suspension cells did not prove to be an optimal experimental choice, since TEM preparation showed substantial debris. Therefore, we performed a second experiment, this time using somatic embryos (7 days after induction of embryogenesis, torpedo stage), which had been found to have a high expression of *dhfr-ts* genes, by means of *in situ* hybridization (M. Luo, L. Giorgetti, L. Pitto, R. Cella, unpublished data). The results of this experiment, which are shown in Fig. 6 and in Table 2, confirmed the presence of DHFR-TS in plastids. The background observed in sections treated with the pre-immune serum was generally much lower than it had been in the previous experiment. In the case of amyloplasts, the sections treated with the immune compared to the pre-immune serum gave a labelling density ratio of 23.75. In this experiment too, mitochondria and the cytosol showed a low labelling density ratio (0.77 and 0.78 respectively). Interestingly, nucleoli from somatic embryos did not show DHFR-TS.

To confirm the results of immunogold labelling, we performed an immunoblot analysis of proteins from sub-cellular fractions obtained by differential centrifugation of suspension cell homogenate [11] with monospecific antibodies against carrot DHFR-TS. The res-

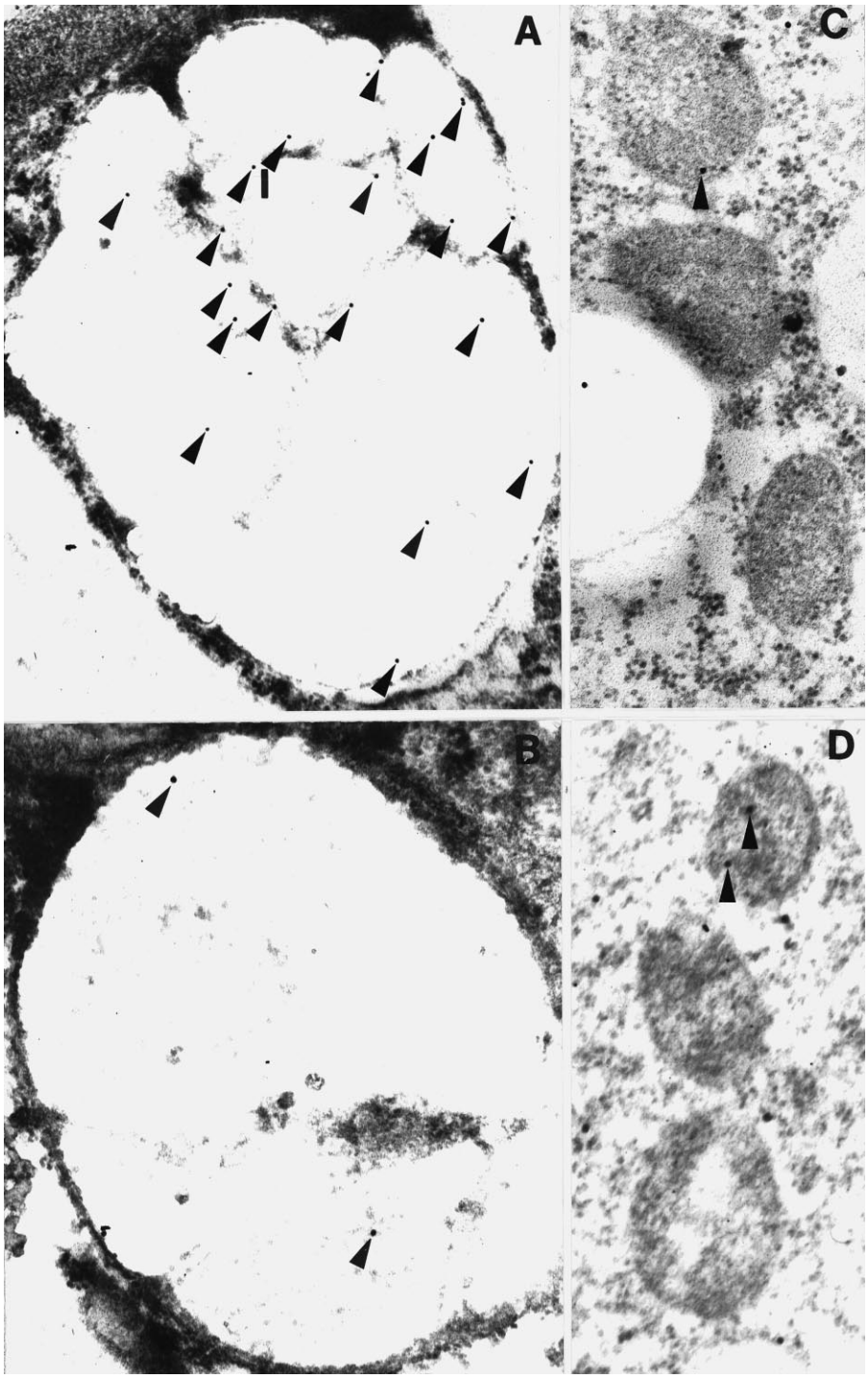

Figure 6. Immunogold localization of DHFR-TS in sections of carrot somatic embryos. A. Micrograph of an amyloplast in a section incubated with antibodies to DHFR-TS (×31 300). B. Micrograph of a plastid in a section incubated with the pre-immune serum (×40 900). C. Labelling density in mitochondria and surronding cytoplasm in a section treated with antibodies to DHFR-TS (×39 700). D. Labelling density in mitochondria in a section incubated with the pre-immune serum (×40 000). Gold particles are indicated by arrowheads.

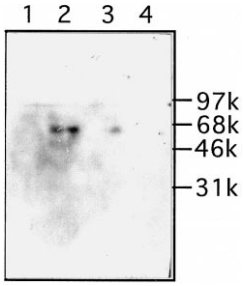

Figure 7. Immunoblot analysis of sub-cellular fractions obtained by fractionation of a homogenate obtained from carrot cells grown in suspension culture. Lane 1, supernatant resulting from centrifugation of the homogenate at 20 000 × *g*; lane 2, amyloplasts; lane 3, nuclei; lane 4, mitochondria. Each cellular fraction aliquot contained 30 μg of protein. Mass relative protein markers are indicated.

ults of this experiment (Fig. 7) show that DHFR-TS is present in the fraction that contains amyloplasts. This fraction was the only one to stain for starch, while its cytochrome-c-oxidase activity was about 9% of that of the mitochondrial fraction (data not shown).

Transcripts originate from the same gene

It had previously been shown that the higher content of DHFR-TS in carrot cells derived from the high number of copies of the relevant gene, a number which a reconstruction experiment estimated as at least 5 copies/haploid genome [29]. This finding suggested the possible occurrence of differing genes and of corresponding differences in transcript length.

In order to investigate this possibility, we digested genomic DNA with *Bam*HI, *Eco*RI, *Hin*dIII or double-digested with *Eco*RI and *Bam*HI or *Hin*dIII, and applied Southern blot analysis which used a genomic probe of 870 bp containing both the 5′ part of the first intron and the adjacent upstream region corresponding to the 5′ of the cDNA. The results of this experiment, which are shown in Fig. 8, showed only a single band in each digest. We obtained an identical pattern when using a cDNA corresponding exclusively to the 5′ end of longer transcripts as a probe (not shown). It is worth noting that only one band was recognized in Southern blot experiments that used the 0.7 kb fragment corresponding to the 5′ end of the cDNA as a probe [27]. Taken together these results indicate that differing transcripts originate from the same gene.

TC dinucleotide repeats of different lengths (4+9+5) were found in the untranslated sequence of longer transcripts (underlined in Fig. 2A). Sequence analysis of independently obtained RACE clones revealed in one of them a longer central repeat (10 TC dinucleotide instead of 9) and a C→T transition in the 5 dinucleotide repeat (Fig. 9). Although several *dhfr-ts* gene copies are present in the carrot genome, Southern blot analysis of genomic DNA digested with *Bam*HI, *Eco*RI and *Hin*dIII did not reveal a composite pattern [29]. Thus, these microsatellite sequence variations provide evidence for variability between *Ddhfr-ts* gene copies.

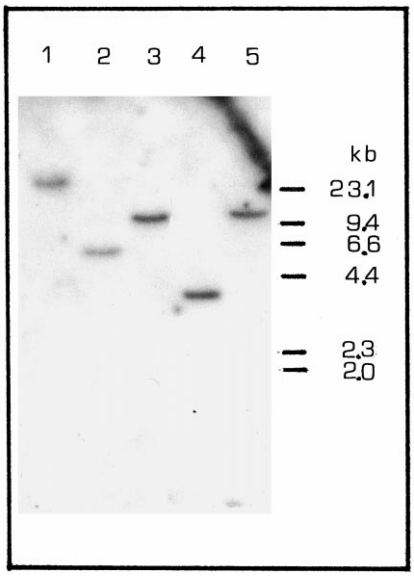

Figure 8. Southern blot hybridization analysis of carrot genomic DNA (10 μg aliquots) digested with *Bam*HI (lane 1), *Bam*HI/*Eco*RI (lane 2), *Eco*RI (lane 3), *Eco*RI/*Hin*dIII (lane 4) and *Hin*dIII (lane 5) using a genomic sequence of 870 bp containing the 5′ part of the first intron along with the adjacent upstream region of the cDNA as a probe.

Discussion

The results reported in this paper indicate a plastidial localisation of DHFR-TS. This explains why a previous immunocytochemical study both of resting and proliferating carrot cells grown in suspension culture, and of S- and G_2-phase cells synchronized with aphidicolin had observed a continuous cytoplasmic presence of the bifunctional enzyme [7]. The suggestion of an organelle localization of the bifunctional protein came from the sequence analysis of clones corresponding to the 5′ end of the DHFR-TS mRNA, obtained by RACE, which showed the presence of transcripts of differing lengths. Particularly, longer transcripts were found to contain a second translation start site (ATG_2) upstream

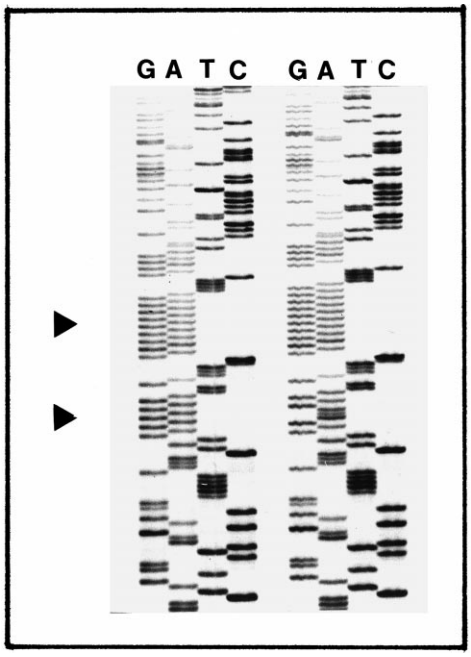

Figure 9. Sequences of clones obtained by RACE show microsatellite polymorphism, as indicated by arrows heads. Reported ladders refer to the sequencing of the complementary DNA strand with the GNTP primer (Fig. 1A).

of, and in frame with, the previously described one (ATG_1) [29]. The ORF beginning at ATG_2 (1731 nt) corresponds to a protein of 577 amino acids with a calculated molecular mass of 64 714 Da, a value much higher than that of 58 400 ± 1000 Da determined by SDS-PAGE of carrot DHFR-TS [1, 8]. This high value can be explained by the synthesis of a pre-protein that, upon removal of the transit peptide, gives rise to the mature polypeptide, whose actual end could not be determined because of a blocked N-terminus [8]. The N-terminus region of the polypeptide starting from ATG_2 is characterized by an amino acid composition which indeed differ from that of the mature protein; the given region showed a high content of hydroxylated amino acids, a low content of acidic ones and the presence of a conserved cleavage consensus motif of chloroplast transit peptides from residues 71 to 79 [20, 46].

Immunogold labelling and immunochemical evidence of plastidial localization of DHFR-TS provides the first direct indication of the presence of an enzyme of the folate biosynthetic pathway in plastids.

Our findings of two transcript species and of two ORFs contradict those reported for *A. thaliana dhfr-ts* genes (THY-1 and THY-2), where only one ORF was found; in this latter case primer extension and sequencing of genomic clones revealed neither a second ATG nor a TATA box even at as much 400 bp upstream of the unique observed translation start site [26]. However, all introns of *A. thaliana* THY-1 and THY-2 are present at the same positions in the *Dcdhfr-ts* gene ([28], M. Luo, L. Giorgetti, L. Pitto, R. Cella, unpublished data). Thus, the discrepancy at the 5′ end between carrot and *A. thaliana dhfr-ts* genes can be explained by the presence of the large intron (about 2 kb), which might have been overlooked in *A. thaliana*, and whose occurrence in carrot was revealed by the comparison of sequences of the cDNA (pDRTS), RACE products and the relevant genomic DNA [28]. The frequent presence of introns at the 5′ end of plant genes [35] indicates that particular attention is needed when primer extension analysis does not show a TATA box in the putative promoter region.

Interestingly, the comparison of nucleotide and amino acid-deduced sequences of *Dcdhfr-ts* ORF1 (from ATG_1) with the exons and amino acid-deduced sequences of the two bifunctional genes of *A. thaliana* [26] revealed a sequence conservation of about 70%. Moreover, the pI of carrot DHFR-TS and the product of *A. thaliana* THY-1 (calculated on the basis of the deduced amino acid sequence) were respectively found to be 5.86 and 5.85.

It is worth noting that most (if not all) genes coding for monofunctional DHFR (e.g. those of mouse, CHO and human) [32] and bifunctional DHFR-TS (such as that of *Leishmania major*) [24] contain multiple transcription start sites. Compared to mammals, what is striking in the case of carrot is that transcription products reach different sub-cellular compartments. However, a similar situation has been recently described in the case of the *A. thaliana* ATRBP33 protein [10].

Since several copies of the *dhfr-ts* gene exist in the carrot genome, it was possible that the two transcript species derived from differing genes. We used Southern blot hybridization to analyse genomic DNA digested with differing restriction enzymes, and with probes corresponding to the 5′ end of the gene, common to all transcripts of specific for the longer ones. In both cases, only one band was recognized, indicating that the transcripts originated from the same gene.

An issue of particular interest is the role of the bifunctional protein that corresponds to the shorter transcript (the one that lacks the transit peptide); according to immunogold labelling data from suspension cells, said protein shows a nucleolar localisation.

It appears unlikely that the presence of DHFR-TS in the nucleolus is to be considered as evidence for the cytosol-nucleus shuttle that is consistent with the replitase model [34, 37], since immunocytochemical studies used early stationary phase cells, which show a high DHFR-specific activity [1]. However, it is possible that some other forms of DHFR, which have been described in plants [9], may be part of the replitase complex; in this respect, it is worth noting that monofunctional DHFR and TS have been reported as occurring in wild carrot cells [45]. To date, nucleolar localization has not been observed in somatic embryos. However, differences in sub-cellular distribution that depend on cell type have been reported for other proteins; for instance, a variable distribution of calmodulin was observed between somatic and zygotic carrot embryos and between individual cells [44].

Further investigation is required to ascertain whether the presence of DHFR-TS in the nucleolus of suspension cells might be related to a possible regulatory or autoregulatory role of the protein, as observed in the case of *Lactobacillus casei* DHFR [18].

Microsatellites have been proposed as polymorphic genetic markers [19, 33]. The 5′-untranslated sequence of the bifunctional *Dcdhfr-ts* gene upstream of ATG_2 shows a TC dinucleotide microsatellite which, upon analysis of RACE clones, revealed some degree of polymorphism; thus, this provides evidence for some differences among *Dcdhfr-ts* gene copies. Interestingly, a stretch of two polydinucleotides, $(CT)_4$ followed by $(CA)_{20}$, has also been observed in the untranslated region of the longer transcript of *L. major* DHFR-TS. It remains to be established whether these repeats might play a functional role.

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Note added in proof

After submission of the manuscript for final consideration, in the literature appeared the article 'Mitochondria are the major site for folate and thymidylate synthesis in plants' (Neugebauer *et al.*, J Biol Chem 271: 9466–9472, 1996) that shows a prevalent mitochondrial localisation of DHFR-TS enzymatic activities. These results were obtained by analysing differentiated tissues while we used somatic embryos and suspension cells that, according to our unpublished data, show maximum *dhfr-ts* gene expression. Therefore, further studies will be necessary to verify a possible differing sub-cellular accumulation of DHFR-TS during plant development.

References

1. Albani D, Parisi B, Carbonera D, Cella R: Dihydrofolate reductase from *Daucus carota* cell suspension cultures: purification, molecular and kinetic characterization. Plant Mol Biol 5: 363–372 (1985).
2. Altman LG, Schneider BG, Papermaster DS: Rapid embedding of tissues in Lowicryl K4M for immunoelectron microscopy. J Histochem Cytochem 32: 1217–1223 (1984).
3. Appling DR: Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. FASEB J 5: 2645–2651 (1991).
4. Braghetti A, Piazzi G, Lanfranco L, Mondello C: Multiple DNA-protein interactions at the CpG island of the human pseudoautosomal gene MIC 2. Somat Cell Mol Genet 19(1): 51–63 (1993).
5. Caelles C, Ferrer A, Balcells L, Hegardt FG, Boronat A: Isolation and structural characterization of a cDNA encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Mol Biol 13: 627–638 (1989).
6. Cella R, Albani D, Carbonera D, Etteri L, Maestri E, Parisi B: Selection of methotrexate-resistant cell lines in *Daucus carota*: biochemical analysis and genetic characterization by protoplast fusion. J Plant Physiol 127: 135–146 (1987).
7. Cella R, Balestrazzi A, Carbonera D, Nano R, Rezzani R: Cellular localisation of dihydrofolate reductase-thymidylate synthase in carrot cells. Eur J Bas Appl Histochem 35: 61–71 (1991).
8. Cella R, Carbonera D, Orsi R, Ferri G, Iadarola: Proteolytic and partial sequencing studies of the bifunctional dihydrofolate reductase-thymidylate synthase from *Daucus carota*. Plant Mol Biol 16: 975–982 (1991).
9. Cella R, Parisi B: Dihydrofolate reductase and thymidylate synthase in plants: an open problem. Physiol Plant 88: 509–521 (1993).
10. Cheng S-H, Cline K, DeLisle AJ: An *Arabidopsis* chloroplast RNA-binding protein gene encodes multiple mRNAs with different 5′ ends. Plant Physiol 106: 303–311 (1994).
11. Chibbar RN, van Huystee R: Immunochemical localisation of peroxidase in cultured peanut cells. J Plant Physiol 123: 477–486 (1986).
12. Cossins EA: One carbon metabolism. In: Davies DD (ed) The Biochemistry of Plants, Vol. 2, pp. 365–418. Academic Press, New York, ISBN 0-12-675402-0, (1980).
13. Cossins EA: Folate biochemistry and the metabolism of one-carbon units. In: Davies DD (ed) The Biochemistry of Plants,

Vol. 11, pp. 317–353. Academic Press, San Diego, CA ISBN 0-12-675411-X (1987).
14. Craig S, Moore PJ, Dunahay TG: Immunogold localisation of intra- and extra-cellular protein and polysaccharides of plant cells. Scanning Microscopy 1: 1431–1437 (1987).
15. Della Porta SL, Woo JE, Hicks JB: A plant DNA minipreparation: version II. Plant Mol Biol Rep 2: 1–19 (1983).
16. Frohman MA: Amplifications: a forum for PCR users, no. 5, pp. 11–15. Perkin Elmer Cetus Corp. (1990).
17. Frohman MA, Dush MK, Martin GR: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85: 8998–9002 (1988).
18. Groneborn AM, Clore GM: Characterization of the DNA binding region recognized by dihydrofolate reductase from *Lactobacillus casei*. J Biol Chem 258: 11256–11259 (1983).
19. Hearne CM, Ghosh S, Todd JA: Microsatellites for linkage analysis of genetic traits. Trends Genet 8: 288–294 (1992).
20. Gavel I, von Heijne G: A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett 261: 455–458 (1990).
21. Horisberger M: Colloidal gold: a cytochemical marker for light and fluorescent microscopy and for transmission and scanning microscopy. Scann Electr Micr 2: 9–31 (1981).
22. Ivanetich KM, Santi DV: Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. FASEB J 4: 1591–1597 (1990).
23. Joshi CP: An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucl Acids Res 15: 6643–6653 (1987).
24. Kapler GM, Zhang K, Beverley SM: Sequence and S1 nuclease mapping of the 5′ region of the dihydrofolate reductase-thymidylate synthase gene of *Leishmania major*. Nucl Acids Res 15: 3369–3383 (1987).
25. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27: 137–138 (1965).
26. Lazar G, Zhang H, Goodman HM: The origin of the bifunctional dihydrofolate reductase-thymidylate synthase isogenes of *Arabidopsis thaliana*. Plant J 3: 657–668 (1993).
27. Luo M-Z, Cella R: A reliable amplification technique with single-sided specificity for the isolation of 5′ gene-regulating regions. Gene 140: 59–62 (1994).
28. Luo M-Z and Cella R: Analysis of the structure of the 5′ end of the gene coding for carrot dihydrofolate reductase-thymidylate synthase. In: Terzi M, Cella R, Falavigna A (eds) Current Issues in Plant Molecular and Cellular Biology, pp. 583–588. Kluwer Academic Publishers, Dordrecht (1995).
29. Luo M-Z, Piffanelli P, Rastelli L, Cella R: Molecular cloning and analysis of a cDNA coding for the bifunctional dihydrofolate reductase-thymidylate synthase of *Daucus carota*. Plant Mol Biol 22: 427–435 (1993).
30. Lutcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA: Selection of AUG initiation codons differs in plants and animals. EMBO J 6(1): 43–48 (1987).
31. Marchuk D, Drumm M, Saulino A, Collins FS: Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucl Acids Res 19: 1154 (1991).
32. Mitchell PJ, Carothers AM, Han JH, Harding JD, Kas E, Venolia L, Chasin LA: Multiple transcription start sites, DNase I-hypersensitive sites, and an opposite-strand exon in the 5′ region of the CHO *dhfr* gene. Mol Cell Biol 6: 425–440 (1986).
33. Morgante M, Olivieri AM: PCR-amplified microsatellites as markers in plant genetics. Plant J 3: 175–182 (1993).
34. Noguchi H, Reddy GPV, Pardee AB: Rapid incorporation of label from ribonucleotides diphosphates into DNA by cell-free high molecular weight fraction from animal cell nuclei. Cell 32: 443–451 (1983).
35. Norris SR, Meyer SE, Callis J: The intron of *Arabidopsis thaliana* polyubiquitin genes in conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol Biol 21: 895–906 (1993).
36. Ogden RC, Adams DA: Guide to molecular cloning. Meth Enzymol 152: 61–87 (1987).
37. Reddy GPV, Pardee AB: Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc Natl Acad Sci USA 77: 3312–3316 (1980).
38. Reichard P: Interaction between deoxyribonucleotide and DNA synthesis. Annu Rev Biochem 57: 349–374 (1988).
39. Roth J, Bendayan M, Carleman E, Villinger W, Garavito M: Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. J Histochem Cytochem 29: 663–671 (1981).
40. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
41. Schuster DM, Buchman JW, Rasthchian A: A simple and efficient method for amplification of cDNA ends using 5′ RACE. Focus 14(2): 46–52 (1992).
42. Stein R, Sciary-Galili N, Razin A, Cedar H: Pattern of methylation of two genes coding for housekeeping functions. Proc Natl Acad Sci USA 80: 2422–2426 (1983).
43. Taylor GR, Lagosky PA, Storms RK, Haynes RH: Molecular characterization of the cell cycle-regulated thymidylate synthase gene of *Saccharomyces cerevisiae*. J Biol Chem 262: 5298–5307 (1987).
44. Timmers ACJ, Kieft H, Schel JHN: An immunofluorescence study on calmodulin during somatic and zygotic embryogenesis of carrot (*Daucus carota* L.). Acta Bot Neerl 44: 19–32 (1995).
45. Toth I, Lazar G, Goodman HM: Purification and immunochemical characterization of a dihydrofolate reductase-thymidylate synthase enzyme complex from wild carrot cells. EMBO J 6: 1853–1858 (1987).
46. von Heijne G, Steppuhn J, Herrmann R: Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 180: 535–545 (1989).
47. von Heijne G, Hira T, Klogsen R-B, Steppuhn J, Bruce B, Keegstra K, Herrmann R: CHLPEP-A database of chloroplast transit peptides. Plant Mol Biol Rep 9: 104–126 (1991).
48. Zelikson R, Luzzatti M: Mithocondrial and cytoplasmic distribution in *Saccharomyces cerevisiae* of enzymes involved in folate-coenzyme-mediated one-carbon-group transfer. Eur J Biochem 79: 285–292 (1977).