The Plant Cell, Vol. 2, 457–467, May 1990 © 1990 American Society of Plant Physiologists

Interaction of Nuclear Factors with Upstream Sequences of a Lipid Body Membrane Protein Gene from Carrot

Polydefkis Hatzopoulos,[1] Gerald Franz, Lisa Choy,[2] and Renee Z. Sung[3]

Department of Plant Biology, University of California, Berkeley, California 94720

To study the regulation of gene expression during embryo development, we isolated a gene, DC 59, expressed in embryos but not in mature carrot plants. Sequence and S1 analysis showed that the gene was composed of one exon encoding a polypeptide of 19 kilodaltons and was highly homologous to the lipid body membrane protein gene L3 from maize. The plant hormone abscisic acid regulated the accumulation of DC 59 mRNA. To understand the mechanism of embryo-specific and hormonal regulation of DC 59, 5′ DNA fragments were incubated with nuclear proteins. Two adjacent regions (from −706 to −235) interacted with nuclear extracts from embryos, resulting in the formation of four complexes (C1, C2, C3, and C4). Factors involved in the formation of the C3 and C4 complexes could be competed with sequences upstream of DC 8, a gene that is coordinately expressed with DC 59 during embryo development. DNase I footprinting analysis revealed that nuclear extracts from embryos bound to four AT-rich sequences, and the protected motifs within fragment V were located in the highly homologous upstream regions of DC 59 and DC 8 genes.

INTRODUCTION

Organ- and tissue-specific genes are useful in studying the mechanisms of developmental regulation. We have isolated cDNA clones that detect embryo-specific RNAs to investigate embryonic gene regulation (Choi et al., 1987). The expression patterns of the embryo-specific RNAs are similar during somatic and zygotic embryogenesis (Borkird et al., 1988). In this paper, we report the molecular characterization of a gene, DC 59, that corresponds to one of the cDNA clones, cDNA 59. We found that DC 59 encodes a lipid body membrane protein gene similar to that of L3 in maize (Vance and Huang, 1987). Like many late embryogenesis abundant genes, Lea (Galau and Hughes, 1987; Dure et al., 1989) DC 59 RNA level is regulated by abscisic acid (ABA). However, ABA can only induce DC 59 RNA accumulation in embryonic, not in adult, organs. Thus, we have investigated the interaction of nuclear proteins with upstream sequences of the DC 59 gene to characterize the mechanism of hormonal and embryonic regulation. Results from DC 59 were compared with that of a similarly regulated gene, DC 8 (Franz et al., 1989), to study the possible mechanism of coordinate gene regulation.

[1] Current address: Faculty of Sciences, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece.

[2] Current address: Harvard University, Division of Medical Science, Boston, MA 02139.

[3] To whom correspondence should be addressed.

RESULTS

Isolation and Genomic Organization of Gene DC 59

The cDNA 59 was initially selected with embryo-enriched antibodies from a cDNA expression library constructed in λgt11 with poly A^+ RNA isolated from somatic embryos of the carrot cell line W001C (Liu et al., 1985). Figure 1 shows the cDNA 59 hybridizing genomic clone, λ59/3112, isolated by screening a genomic library in λEMBL3 made from partially Sau3AI-digested W001C DNA. Restriction enzyme analysis followed by DNA gel blot hybridization showed that the region complementary to the cDNA lies in the middle of the isolated genomic clone within the 2.7-kb EcoRI fragment (Figure 1).

To determine the number of genes homologous to cDNA 59, W001C genomic DNA was digested with EcoRI restriction endonuclease and electrophoresed on an agarose gel in parallel with EcoRI-digested λ59/3112 recombinant phage DNA. Figure 2A shows an autoradiogram of this DNA gel blot hybridized to cDNA 59. The cDNA 59 hybridized strongly to three restriction fragments of 8.7 kb, 5.6 kb, and 2.7 kb in length and, to a far lesser extent, to 15 additional fragments. One of the strongly hybridizing fragments (2.7 kb) co-migrated with the EcoRI fragment from λ59/3112 (Figure 2A). The gene within this cloned 2.7-kb EcoRI fragment was designated as DC 59. From the intensity of the diluted λ59/3112 phage DNA, it was evident that the gene was present once per haploid genome. The overall hybridization pattern was the same in callus

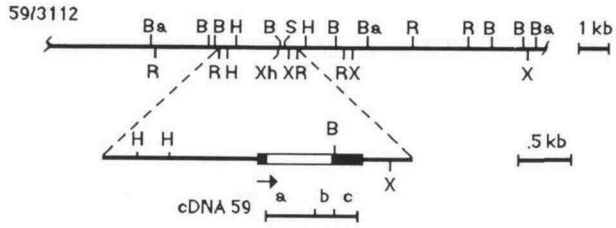

Figure 1. Restriction Map of the Genomic Region Containing the Gene DC 59.

The top line represents the chromosomal region cloned in λEMBL3 (λ59/3112). Filled and open boxes indicate 5′ and 3′ nontranslated and coding regions, respectively. Arrow indicates the transcriptional orientation. The homologous cDNA 59 that was cloned into λgt11 with EcoRI linkers is shown below. Fragments a (EcoRI-SpII), b (SpII-BgIII), and c (BgIII-EcoRI) from cDNA 59 were subcloned in pUC 19. The restriction map was constructed with the following enzymes: EcoRI (R), BamHI (Ba), BgIII (B), HindIII (H), SalI (S), XbaI (X), and XhoI (Xh).

and embryo DNA, indicating that there are no detectable rearrangements between callus and embryo cultures (Figure 2A). In genomic DNA isolated from the two haploid cell lines HA and J1, only one prominent (2.2 kb) and several fainter hybridizing fragments were detected. The hybridization intensity of the 2.2 kb is about twice that of the W001C 2.7-kb EcoRI fragment (Figure 2A). This is additional evidence that the DC 59 gene was present once per haploid carrot genome. The possibility of partial digestion of the genomic DNA was excluded when the same filter was hybridized to the gene DC 8 (data not shown). Even though the hybridization pattern indicated a gene family when the insert of cDNA 59 was used to hybridize digested genomic DNA at higher stringency (70°C), Figure 2B shows that only the three fragments (8.7 kb, 5.6 kb, and 2.7 kb) in W001C and one (2.2 kb) in HA DNA were detected. This result supports the conclusion that the gene was present once per haploid carrot genome and that W001C contained two alleles with different restriction enzyme patterns (Figure 2B).

Three small non-overlapping fragments from cDNA 59 (Figure 1, a, b, and c) were subcloned and used as probes to hybridize to genomic DNA. Figure 2C shows that only fragment a resembled the hybridization pattern of cDNA 59. When the same probes were used to hybridize poly A^+ RNA isolated from different tissues, b and c hybridized to DC 59 mRNA, whereas fragment a hybridized to two additional mRNA species, one about 880 nucleotides and a second about 1300 nucleotides in length. cDNA 59 also hybridized to these mRNAs to a lesser extent.

Figure 2D shows that all three mRNAs were only detectable in embryos, not in mature organs. Tubulin hybridization to the same RNA gel blot showed no RNA degradation (data not shown).

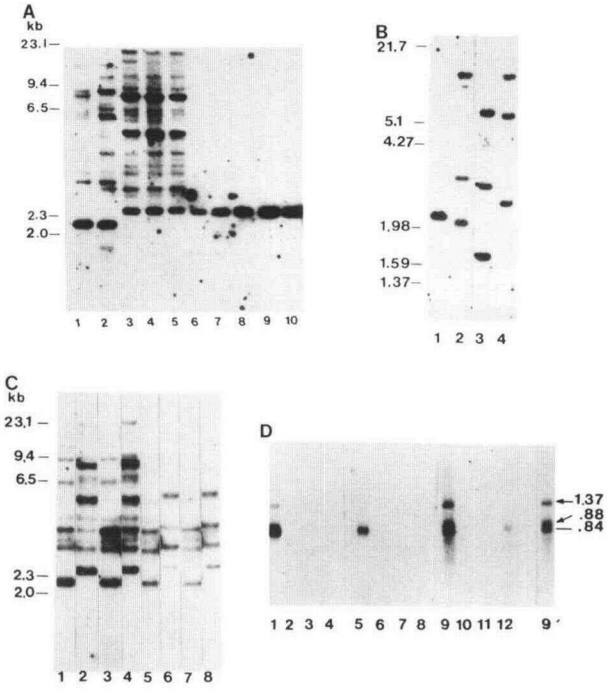

Figure 2. Representation of DC 59 in the Carrot Genome.

Carrot genomic and λ59/3112 phage DNAs were digested, electrophoresed on 0.9% agarose gels, and hybridized to cDNA 59. Numbers represent molecular weight standards in kilobases.

(A) Genomic DNAs (lanes 1 to 5) were digested with EcoRI. Lane 1 has 5 μg of genomic DNA isolated from J1 callus; lane 2, 5 μg of HA callus; lane 3, 5 μg of W001C callus; and lanes 4 and 5, 5 μg and 2.5 μg of W001C embryo, respectively. Parallel lanes (6 to 10) were loaded with EcoRI digests of λ59/3112 phage DNA. Lane 6 has 0.5 times equivalent of the content of the carrot genome (lane 4), and lanes 7, 8, 9, and 10, 1 time, 2 times, 4 times, and 8 times, respectively.

(B) DNA gel blot analysis at high stringency, 70°C, was carried out with genomic DNA from HA callus digested with EcoRI (lane 1), or DNA from W001C embryo digested with BgIII (lane 2), HindIII (lane 3), or EcoRI (lane 4). All lanes have 5 μg of DNA.

(C) DNA gel blot of EcoRI digests of genomic DNAs isolated from HA callus (lanes 1, 3, 5, and 7) and W001C embryos (lanes 2, 4, 6, and 8) hybridized to cDNA 59 (lanes 1 and 2); fragment a from cDNA 59 (lanes 3 and 4), fragment b (lanes 5 and 6), and fragment c (lanes 7 and 8). Numbers represent molecular weight standards in kilobases.

(D) Poly A^+ RNA isolated from somatic W001C embryos (lanes 1, 5, and 9), from roots (lanes 2, 6, and 10), leaves (lanes 3, 7, and 11), and from flowers (lanes 4, 8, and 12) from *D. carota* var Juwarot was electrophoresed on formaldehyde-agarose gels and hybridized to cDNA 59 (lanes 1 to 4), fragments b and c (lanes 5 to 8), and fragment a (lanes 9 to 12). Lane 9′ is a lower exposure of 9. Arrows indicate the two mRNA species that cross-hybridize to cDNA 59 and to fragment a.

```
AAGCTTGCGGTCTATATATATGAAGTGAAACAAGCATGGTGGATATTACATGTAA
GAATGATGTTTTCGTTGTCAGATTTTATATTTTCCTTTTGATGCTAGGAGGGTCA -1108
AGAGCCCTGACAAGAAAATGCAGCGTTCTTGTATACTGCAAGAAAATCTATGATC
ATGGTCCAGCTTTGCAGAGAAATTTTTGCTGTGGTGATGGGCGGACAAATTGGGA -898
ATCGGAACTAACATGTTGATCCATCAATTCAGTATTTATGGTAGATTTTTTTGAT
AAATAGAAGATTGTAACTAAATCGGTAATTGATAAATTGATAAAACATGTTTTAA -788
AAATTAATCGTGGTTTTTTGAATAGCAAAATAATGTGAGGTGCAGCGTTCTTGTA
TATACTAGAATAAATCTATGCGTTGAGCTTTCTTTCTAATAAATCAAGTCTCTTA -678
AGAAAAAGATTGTTTCAATTTGTTTATTCTATGAAACTGAGTTGTGGATATGTGT
ATACTACTCGAAATTAAAAATGGTACTCCCTTCGGTACCTTTTCAGTTATCACAT -568
TTCTATTTTTCTTGTCCAATTGATCCAAATTTTGACCAAAGTTTATAAGTCACCC
CTTTTATTATTTTAAAAAACTGAAAATTACATATTAAGTAGATTAAAAGTAATTT -458
CCGATGACATATTTTTTTAATTGATAAAATAATAATAAATTTCAGTCAAATTTTG
GTCAATTTGACCGGCACAATACAAATGTGACCAACTAAAAAGGGACGGAGGAGT -348
ACAAGAAAATTATTTAAAAAACTATTCTGAAAATAGAAAAACGAAAATATTTTGGA
ACTCGGAAGGAAGGAGAGATTACACTATTAACGTGGACATCCAGGTTCCTGTTT -238
GTGAATCAATGTATCGGCCCATGAAAACAGGACCCATAAAAAATCACCAAAAAGC
CCAAACTAAAAGAAGTAATGATGATCCATTCCAGACAGAGCCAAACACACAGAAA -128
GGTGGCAGAGTGGCAGAGACACATGTACAACATGCAAGAACTCAAAATGACAAGT
GACATGTCACTCCCACGCGTCACATCCTCTATCAACCTCCCCTTTCTTTATAACC -18

                    ┌Start
CCCTTCACAACCCCCATATCAACACCATCCTATACACACACAATACACACAAACA
CATAACTCTTAAGTTCTCAACAATGGCAGAACGAGGCACTTATGCTCATCAAGTC   93
                    M  A  E  R  G  T  Y  A  H  Q  V
CAAGTCCACCCCCAACAAACCGCCAACCAACCGGCGGCGGTCAAATCGCTCCTCC
 Q  V  H  P  Q  Q  T  A  N  Q  P  G  G  V  K  S  L  L
CCAAAAACAGCCCCTCCACCTCCCAGGTCCTCGCGGTCGTCACCCTCCTCCCGT  203
P  K  N  S  P  S  T  S  Q  V  L  A  V  V  T  L  L  P  V
CGGCGGCAGCCTCCTCTTCCTCGCCGGCATCACCCTCGTCGGCACCCTCATCGGC
  G  G  T  L  L  F  L  A  G  I  T  L  V  G  T  L  I  G
CTCGCCGTCGCCACCCCGCTCTTCCTCCTCTTCAGCCCGTCCTCGTCCCGCCG  313
 L  A  V  A  T  P  L  F  L  L  F  S  P  V  L  V  P  A
CCCTCACCATCGGCCTCGCCGTCACCGGATTCCTCGGCTCCGGCGCTTTCGGGCT
A  L  T  I  G  L  A  V  T  G  F  L  G  S  G  A  F  G  L
GACGGGACTCTCGTCTCTCTCATGGGTTCTGAGCTACTTCCGGCAGGCGAGCCAG  423
  T  G  L  S  S  L  S  W  V  L  S  Y  F  R  Q  A  S  Q
AGAGTGCCGGACCAGATCGAGCTGGCGAAGAAGAGGGCTCAGGAGATGGCGGCGT
 R  V  P  D  Q  I  E  L  A  K  K  R  A  Q  E  M  A  A
ACGCGGGACAGAAGACCAAGGAAGTGGGGGACACCATTCAGAGCAAGGCGGCTCA  533
Y  A  G  Q  K  T  K  E  V  G  D  T  I  Q  S  K  A  A  Q
GGCTCAGGACACAACGGCGACCACCGGAAGGGACACTCGTAGCACTGCTCGAGAC
  A  Q  D  T  T  A  T  T  G  R  D  T  R  S  T  A  R  D
AGTTCAAGGACTTGAAGCCTCCGTGGAGACGGGGGCGAGATCTGGCCGCTTCATC  643
  T  S  R  T

GTGTCTTAATAATAATGTTATGTGTTTTGTTGTGTTAGAGTGTGAATTGTGTTCT
GTGTTATGTTTGTCTGTGTTTGTTTTAGCCGTTTCGGGGTCGACTCCTGTCTAGT  753
TTCGTTGTACCTGTTTGTTGGTGGTTAATGTGCTTTGTAATCGAACGAATGTGGT
TTAATGCAGGTGAAGTTTTGTTTTGGTTGTATATGTTTATGTACTCATTTAATGT  863
TTCTTTGTTTTGTTTTTACTTAATAATACCGAAATTTTAAGTTCGCTTAATTTGA
AATTAGAGATATA                                             931
```

Figure 3. DNA Sequence of DC 59 and Its Flanking Regions.

The DNA sequence of DC 59 present in λ59/3112 and the predicted amino acid sequence are shown. TATA box, plant −80 region, and the two poly A^+ addition consensus sequences are underlined. Start indicates the position of the initiation of transcription as determined by S1 protection analysis. Amino acids are shown in single-letter code. Dot represents the stop codon.

Structural Analysis of Gene DC 59

The RNA of DC 59 is 840 nucleotides in length and can encode a polypeptide chain of about 22 kD. S1 analysis was carried out to determine the exon/intron structure of the gene DC 59. By using overlapping subfragments from the 2.7-kb EcoRI fragment only, one contiguous protected segment was detected, indicating that the gene was composed of one exon extending from 640 bp upstream to about 180 bp downstream of the BglII site (data not shown).

To investigate further the structure of DC 59, we have determined the DNA sequence of the genomic as well as of several cDNA clones. (Additional cDNA clones were isolated from the λgt11 cDNA library; data not shown.) The 2.1-kb HindIII/EcoRI was used to subclone overlapping fragments and the sequence was determined, as shown in Figure 3. A comparison of cDNA and genomic sequences revealed that the gene was composed of one exon. There are two potential polyadenylation signals downstream of the coding region (Proudfoot and Brownlee, 1976). The longest cDNA analyzed ends 176 bp downstream of the first polyadenylation site. According to the S1 analysis, transcription started approximately 60 bases upstream of the ATG, where the sequence shows similarity to the consensus for the initiation of transcription in plants. Upstream of the CAP site, a TATA box was found at position −24. A sequence homologous to the −80 plant box (Heidecker and Messing, 1986) was present at position −84.

Starting with the ATG at position +61, the open reading frame encoded a polypeptide of 18.8 kD that is in agreement with the polypeptide mass estimated on protein gel blots (Choi et al., 1987). Analysis of the hydropathicity revealed that the DC 59 protein could be divided into three distinct domains, as illustrated in Figure 4. The central core was composed mainly of hydrophobic amino acids, whereas the NH_2 and the COOH terminals were mostly hydrophilic. The polypeptide contained 17 basic and 9 acidic amino acids, resulting in an average isoelectric point (pI) of 10.59. All basic amino acids were found either in the NH_2 or COOH domains, which give them a pI of 10.28 and 10.46, respectively, whereas the central domain had a pI of 6.12.

Function of the DC 59 Gene Product

To determine the function of the DC 59 polypeptide, we searched the current version of the Genbank DNA sequence and the PIR protein libraries (version 16) with the DC 59 sequence using the FASTN and FASTP programs, respectively (Lipman and Pearson, 1985). A high degree of similarity was detected with the L3 protein from maize (Vance and Huang, 1987). This protein was present in

Figure 4. Hydropathicity Plot of the Deduced DC 59 Amino Acid Sequence.

An average hydropathy was determined over a moving window of 11 amino acids and plotted against the polypeptide sequence. Regions above the dotted line are hydrophobic; those below are hydrophilic.

```
40  L A V V T L L P V G G T L L F L A G I T L V G T L I   DC 59
    : . . : . . : : . . : : . . : . . : : . . : : . :
32  L K A A T A A T F G G S M L V L S G L I L A G T V I   L 3

66  G L A V A T P L F L L F S P V L V P A A L T I G L A   DC 59
    . : . : : : . . . . : : : : : : : : . . . . : .
58  A L T V A T P V L V I F S P V L V P A A I A L A L M   L 3

92  V T G F L G S G A F G L T G L S S L S W               DC 59
    . . : : . . : : . . : . . . : : . . : :
84  A A G F V T S G G L G V A A L S V F S W               L 3
```

Figure 5. Homology between the DC 59 and L3 Hydrophobic Domains.

The sequences were aligned with the FASTP program. Numbers on the left represent the amino acid position within the polypeptide chain. Amino acids are shown in single-letter code. One or two dots mark evolutionary related or identical amino acids, respectively. The two unrelated amino acids are underlined.

embryonic tissues as a structural component of the lipid body membrane. Computer analysis predicted that the DC 59 gene product would also be a *trans*-membrane protein. Figure 5 shows the alignment of the hydrophobic domain found in both proteins. The homology in this region was quite high; nearly 95% of the amino acids were homologous, and of those, 49% were identical. Only 2 amino acids, Ala^{37} and Ala^{38} of L3 and Leu^{44} and Leu^{45} of DC 59, were unrelated, but all were nonpolar. Besides the high degree of homology within the hydrophobic domain, additional features were shared by the two proteins. The profile of the hydropathic curve of both proteins and the size of the central hydrophobic domain were very similar (72 amino acids in L3 and 76 in DC 59). The NH_2 and COOH ends of both proteins were hydrophilic, had about the same length, and were highly basic. Regions in the COOH terminus of the DC 59 gene product can form amphipathic α helices (as in L3) because hydrophobic and hydrophilic amino acids are located on opposite sides of the helix (data not shown). These results and the fact that both proteins were present only in embryonic tissues indicated that the DC 59 gene product was a structural component of the lipid body membrane.

Effect of ABA on DC 59 Gene Expression

The plant hormone ABA has been implicated in the regulation of gene expression during embryo development (Bewly and Black, 1986). To investigate the effect of ABA on DC 59 gene expression, RNA was extracted from somatic embryos grown under various conditions. The accumulation of DC 59 mRNA in ABA-grown somatic embryos as shown in Figure 6A (lane 3) was higher than that grown as control (lane 2). Fluridone, an effective inhibitor of carotenoid biosynthesis and ABA accumulation (Fong et al., 1983), drastically decreased the steady-state level of DC 59 mRNA (lane 4). The effect of fluridone alone was reversible because the addition of both ABA and fluridone restored the accumulation of DC 59 mRNA to levels found in control cultures (lane 5). To determine the mechanism of ABA-regulated RNA accumulation, we extracted RNA from young somatic embryos (8 days old). The expression of the DC 59 gene is very low in young somatic embryos (Borkird et al., 1988), but the addition of ABA had a dramatic effect (Figure 6B). An increase of 50-fold to 100-fold of DC 59 mRNA accumulation was observed in 15 min (lane 2). Longer exposures of the somatic embryos to ABA did not increase mRNA level further (lanes 3 and 4), indicating that the ABA effect was at least in part at the transcriptional level.

Nuclear Factors from Somatic Embryos Bind to 5′-Flanking Sequences

To characterize *trans*-acting factors and *cis*-elements involved in the regulation of DC 59, nuclear proteins were extracted from somatic embryos and calli and reacted with

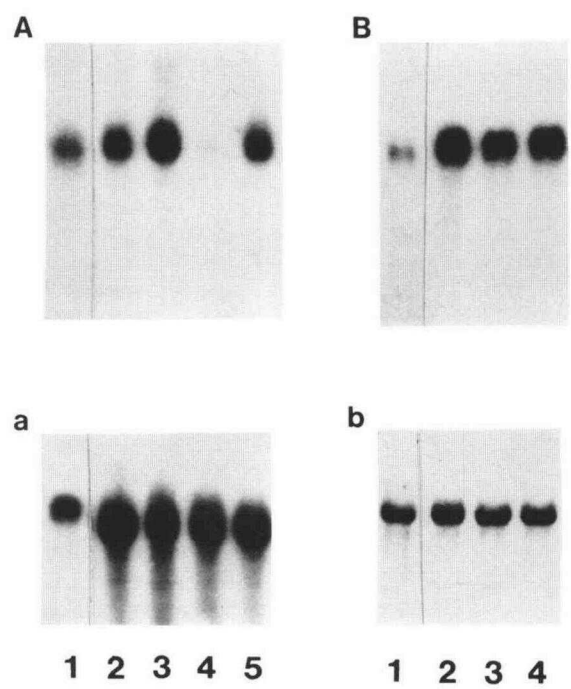

Figure 6. ABA Effect on the Expression and mRNA Accumulation of the DC 59 Gene.

Total RNA was isolated from somatic embryos and hybridized to DC 59 gene [**(A)** and **(B)**] or to tubulin [**(a)** and **(b)**].
(A) Each lane contains 20 μg of total RNA except lane 1, which contains 1 μg of poly A^+ RNA from somatic embryos [**(A)** and **(a)**]. Lane 2, RNA from 13-day-old embryos; lane 3, RNA from 13-day-old embryos grown in the presence of ABA and fluridone.
(B) Total RNA from 8-day-old young embryos (lane 1). Then ABA was added to the medium and the cultures were harvested after 15 min (lane 2), after 1 hr (lane 3), and after 3 hr (lane 4).

labeled DNA fragments from −1118 bp up to +580 bp. Incubation of these extracts with fragments I (−1118 to −899), II (−899 to −706), VI (−235 to +340), and VII (+373 to +580) showed no formation of protein-DNA complexes, as shown in Figure 7. Fragment VI, containing the TATA box, reacted with nuclear extracts from embryos only when 5 to 10 times more nuclear extracts were used, and the appearance and quantity of a slower migrating complex was variable from experiment to experiment (data not shown). Three fragments, however, interacted with nuclear extracts from embryos forming slower migrating complexes. Fragment III (−706 to −547) reacted with nuclear extracts isolated from embryos, resulting in two distinct complexes, C1 and C2 (Figure 7). However, callus nuclear extracts reacted with fragment III forming only one slower migrating complex, which co-migrates with C1, indicating that the DNA-binding factor involved in the C1 complex could be present, although at lower levels, in callus (see Figure 8). Fragment IV (−623 to −547), equivalent to the 3′ 76 bp of fragment III, reacted with embryo nuclear extracts forming only one of the two complexes (Figure 7). Because this complex was not detectable with callus nuclear extract (data not shown), we concluded that fragment IV formed only C2. This complex was rather faint with both fragments, indicating either that C2 was not as stable as C1 or that this DNA-binding factor was present in lower concentration. Fragment V (−547 to −235) also formed two complexes with nuclear extracts from embryos designated C3 and C4 (Figure 7). These complexes were not detected when fragment V was incubated with nuclear extracts from callus (Figure 8). Because the C1 complex was formed, it is unlikely that this result was due to inactivation of callus of nuclear proteins during isolation. Furthermore, SDS-PAGE showed that proteins from callus nuclear extracts were not degraded (data not shown). Thus, higher levels of DC 59 mRNA accumulation during embryo development were correlated to either the de novo synthesis or the activation of nuclear proteins that bind in vitro to the 5′-flanking region and result in the formation of C2, C3, and C4 complexes in embryos.

To determine the specificity of the complexes formed between the fragments and the DNA-binding factors, competition analysis was performed. Figure 8 shows that the interaction between *cis*-elements and DNA-binding factors was highly specific. Whereas unlabeled fragment III eliminated the binding of nuclear factors to the homologous labeled fragment, addition of linear pUC 19 DNA had no effect on binding (Figure 8, top). The same was observed with fragment V (Figure 8, bottom). To distinguish whether fragments III and V contained two related *cis*-elements that reacted with the same DNA-binding factor(s), unlabeled DNA fragments (III, IV, and V) were incubated in the reactions with labeled III and V fragments in all combinations (Figure 8). It was evident that fragments III and IV did not compete with fragment V and vice versa, suggesting that the DNA-binding factors involved in formation of C1 and C2 were different from those resulting in C3 and C4 complexes (Figure 8). From the molar quantities required in the homologous competition experiments, the DNA-binding factor(s) resulting in the C1 complex had high affinity to fragment III, whereas the second group, consisting of the factors forming C2, C3, and C4, had less affinity with the corresponding fragments III and V, respectively. Table 1 summarizes the results on DNA-protein interaction and the competition analysis.

To investigate whether any of the DNA-binding proteins associated with the 5′-flanking region of DC 59 reacted with DNA from a gene coordinately expressed during embryo development, we added an unlabeled fragment of DC 8 as competitor DNA to the reactions. A DC 8 gene fragment, extending from −1800 bp to +200 bp relative to the site of transcriptional initiation (Franz et al., 1989), did not compete with labeled fragment III (Figure 8, top). However, this DC 8 gene region did compete with the labeled fragment V for binding and resulted in the release of the C3 and C4 complexes (Figure 8, bottom). The degree of competition of the DC 8 5′-flanking sequences to the labeled fragment V resembled the competition detected when unlabeled fragment V was used, indicating a

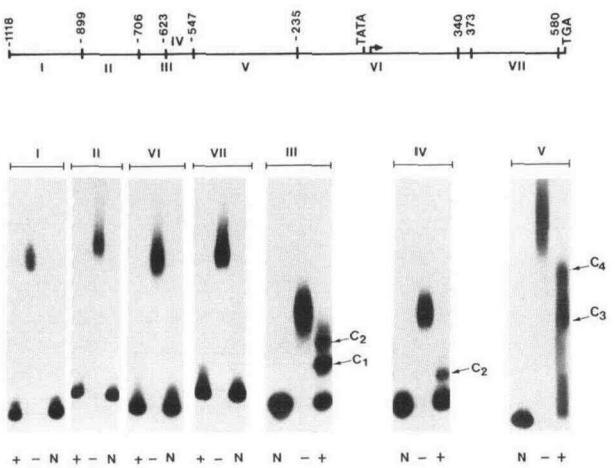

Figure 7. Binding Activities of Embryo Nuclear Extracts to DC 59.

Diagram at the top is a schematic representation of the 5′-flanking and transcribed region of DC 59. Fragments indicated with Roman numerals represent DNA segments used in protein-DNA binding reactions shown in the panel below. Arabic numbers represent the coordinates of each fragment used in the reactions. The TATA box and the transcriptional stop codon are shown. Arrow indicates the start of gene transcription. N, control reaction without proteins; −, control reaction with embryo nuclear extracts but without IC (polydIC·polydIC); +, reaction with embryo nuclear extracts and IC. C1, C2, C3, and C4 refer to complexes formed with embryo nuclear extracts bound to fragment III (C1 and C2), fragment IV (C2), and fragment V (C3 and C4).

Table 1. Competition Analysis of Labeled Fragments III and V with Unlabeled Homologous and Heterologous Sequences

			Competition													
			Fragment III			Fragment IV			Fragment V			puc		Fragment DC 8		
	Embryo NE	Callus NE	10	30	100	10	30	100	10	30	100	10	100	10	30	100
Fragment III	C1	C1	+	++	+++	−	−	+	−	−	−	−	−	−	−	−
	C2	ND	+	++	+++	+	++	+++	−	−	−	−	−	−	−	−
Fragment V	C3	ND	−	−	−	−	−	−	+	++	+++	−	−	+	++	+++
	C4	ND	−	−	−	−	−	−	+	++	+++	−	−	+	++	+++

Complexes C1 and C2 were formed between fragment III and nuclear extracts from embryos (Embryo NE). Complexes C3 and C4 were formed between fragment V and nuclear extracts from embryos. The C1 complex was formed between fragment III and nuclear extracts from callus (Callus NE), whereas all the other complexes were not detected (ND) with nuclear extracts from callus. Nuclear extracts from embryos were used during the competition analysis. The degree of competition is shown by +, and − shows no competition. The numbers 10, 30, and 100 below the unlabeled fragments used for competition refer to molar ratio of unlabeled to labeled DNA. PUC is linearized puc DNA and fragment DC 8 is 5′-flanking and transcribed DC 8 gene sequences (−1800 to +200).

high affinity of the DC 8 sequences for the same DNA-binding factor(s) (Figure 8, bottom).

DNase I Footprinting of DNA-Protein Complexes

DNase I footprinting analysis was used to elucidate the sequences of fragments III, IV, and V that reacted with nuclear extracts. After treatment with DNase I, the products of enzymatic digestion were electrophoresed on a urea/polyacrylamide gel together with Maxam and Gilbert (1980) ladders of the same fragments (G and G+A). Figure 9 shows that four different sequences exhibited varying degrees of protection from DNase I digestion. The protected sequence of fragment IV was also protected when fragment III was used (C1 complex) and was located at −613 to −600. The other region of fragment III that was protected from DNase I digestion was distal to the previous one (−655 to −643). Another protected region was located near −560. Furthermore, the other two protected sequences within fragment V were located at −474 to −457 and −446 to −425. All protected sequences were rich in AT; the latter was almost an entirely AT region. It is interesting to note that the protected sequences of fragment V were located within the upstream region of the DC 59 gene that was highly homologous to the upstream region of the DC 8 gene, as detailed in Figure 10. Recent results (P. Hatzopoulos, unpublished data) showed that these two protected sequences of the DC 59 gene overlap with the protected sequences of the DC 8 gene within this homologous region.

DISCUSSION

We have isolated a gene designated DC 59. The transcription of DC 59 is under strict developmental control, and both mRNA and protein are detected only in embryonic tissues (Borkird et al., 1988). Structural analysis showed that the gene was composed of a single exon resulting in an mRNA and a polypeptide with sizes that are in good correlation with previously published results (Choi et al., 1987). Sequence analysis showed that the gene had the features characteristic of a standard plant transcription unit with a TATA box, followed after 24 bp by a sequence showing similarity to the transcriptional initiation site. It also contained two polyadenylation signal sequences downstream of the stop codon. Combined results from genomic DNA digests of haploid and diploid cell lines indicated that DC 59 was a single copy gene. Nevertheless, there were sequences in the coding region that were related to other genomic fragments. Only the 5′ part of the transcribed region contained sequences homologous to other genes encoding two mRNA species present only in embryonic tissues. Their size, 880 and 1300 nucleotides, may result in polypeptides of about 20 kD and 40 kD, respectively.

The translational product of DC 59 was a basic polypeptide, and the analysis of the hydropathicity indicated that the protein could be divided into three distinct domains. A search of the PIR protein bank showed that DC 59 is highly homologous to the L3 membrane-associated protein of lipid bodies from maize (Vance and Huang, 1987). This homology is restricted to the hydrophobic regions found in both proteins. Alignment of the amino acid sequence of the L3 and DC 59 polypeptides showed that 49% of the amino acids in this region were identical and an additional 46% were related through evolution. Both polypeptides had the following features in common: (1) They were small—about 19 kD—with a very similar hydropathicity profile. (2) DC 59 and L3 were composed of three domains, two hydrophilic (NH_2 and COOH) and a central hydrophobic domain. (3) The hydrophobic domain had nearly the same length, 76 amino acids in DC 59 and 72 in L3. The other two domains, NH_2 and COOH, were also of similar size. (4) NH_2 and COOH domains of DC 59 were basic with an average pI of 10.5. The same was found for the equivalent

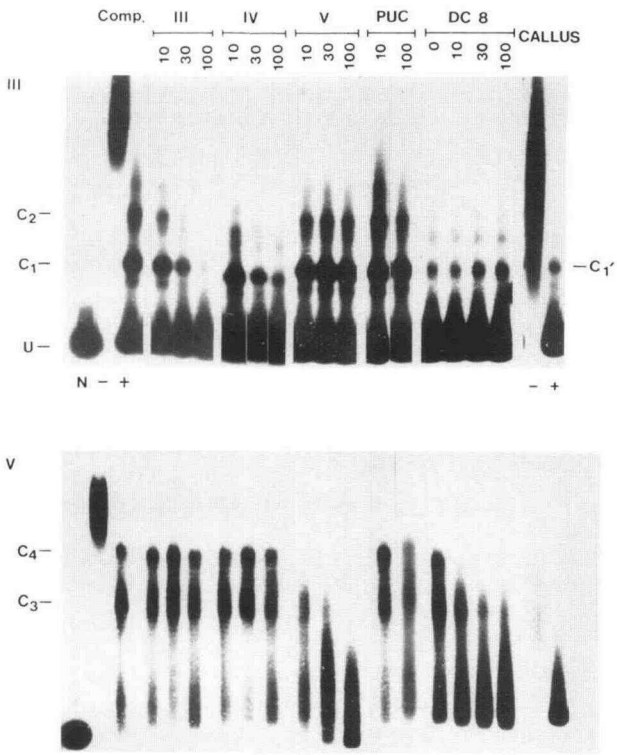

Figure 8. Competition Analysis of Labeled Fragments III and V with Unlabeled Homologous and Heterologous Sequences.

The first three lanes in each panel represent N, control; −, without IC; +, with IC, as in Figure 7. The last two lanes in each panel represent binding activities of callus nuclear extracts to the fragments in the absence (−) or presence (+) of IC. Lanes marked with III, IV, and V are the three unlabeled fragments, as in Figure 7, used to compete labeled fragment III (top) and fragment V (bottom). PUC lanes are reactions in the presence of unlabeled linearized pUC 19 plasmid DNA. DC 8 lanes are reactions in the presence of unlabeled 5′-flanking and transcribed DC 8 gene sequences (−1800 to +200). Numbers above the lanes refer to molar ratio of unlabeled to labeled DNA. C1, C2, C3, and C4 are as in Figure 7. C1′ is the complex formed between fragment III and callus nuclear extracts. U is the unbound DNA fragment.

regions of the L3 protein. (5) The COOH end of the DC 59 translation product had two potential α helix-forming regions, one of 18 and the second of 10 amino acids. These two helices have amphipathic properties and could be partially embedded in the phospholipid layer. In the L3 protein, the COOH end also contains a potential amphipathic α helix-forming region of 18 amino acids (Vance and Huang, 1987). All of these common properties, in addition to the fact that both genes show the same temporal and spatial expression (Vance and Huang, 1988), led us to the conclusion that the DC 59 polypeptide was one of the membrane proteins found in the lipid bodies. The function of this protein is unknown but it may serve as a rigid component to stabilize the structure of the lipid body. It is known from maize that the membrane of lipid bodies contains four major polypeptides, one of 45 kD called H, and L1, L2, and L3 of about 16 kD to 20 kD (Vance and Huang, 1987). The part of DC 59 encoding the hydrophobic domain cross-hybridized to two additional mRNAs present only in embryonic tissues. A possible explanation is that this region (the membrane-spanning region) shares homologies to other lipid body membrane proteins leading to the observed cross-hybridization.

The plant hormone ABA played an important role in the regulation of DC 59 gene expression. When exogenous ABA was supplied, the DC 59 mRNA level increased. This accumulation was more profound when young embryos were used. Because fluridone decreased the DC 59 mRNA concentration and because fluridone inhibits ABA accumulation, it is possible that ABA controls, at least in part, DC 59 mRNA stability. However, the fact that the ABA effect was very drastic and rapid—almost maximum accumulation in 15 min in young embryos that otherwise exhibited very low DC 59 mRNA accumulation—indicated that ABA also regulated the DC 59 gene expression.

To elucidate the mechanism that coordinates gene expression during embryo development, we began studies to identify *cis*-elements and *trans*-acting factors involved in the regulation of DC 59. Gel retardation analysis showed that nuclear extracts from embryos contain DNA-binding factors that interact in vitro with two adjacent 5′-flanking regions of DC 59 forming four complexes, designated C1, C2, C3, and C4. Competition experiments showed that the DNA fragments involved in the formation of the C1 and C2 complexes could not be competed with those forming C3 and C4 complexes and vice versa. Because only C1 was found in callus nuclear extracts, one can conclude that C1 and C2 contain different DNA-binding proteins. This indicates that there must be at least three different factors present in the nuclear extracts isolated from embryos interacting with DC 59 upstream sequences. It is unknown whether all these factors regulate the expression of the DC 59 gene in a complex manner. However, the following three results suggest that they may play a role in the coordination of DC 59 gene expression: (1) The level of the C1 complex was correlated with the accumulation of DC 59 mRNA in callus and in embryos. DC 59 is expressed, although at lower levels, in callus (Borkird et al., 1988). (2) Three of the four complexes could be detected only in embryos where the gene is highly active. (3) Complexes C3 and C4 formed between embryo nuclear extracts and 5′ *cis*-sequences of DC 59 could be competed with the unlabeled 5′ region of DC 8. These two genes were coordinately expressed during embryo development but otherwise unrelated. DC 59 is a membrane-spanning protein, whereas DC 8 is highly hydrophilic and probably belongs to the class of late embryogeny abundant (Lea) genes (Franz et al., 1989). Therefore, it is possible that the C3 and C4 complexes are formed with *trans*-

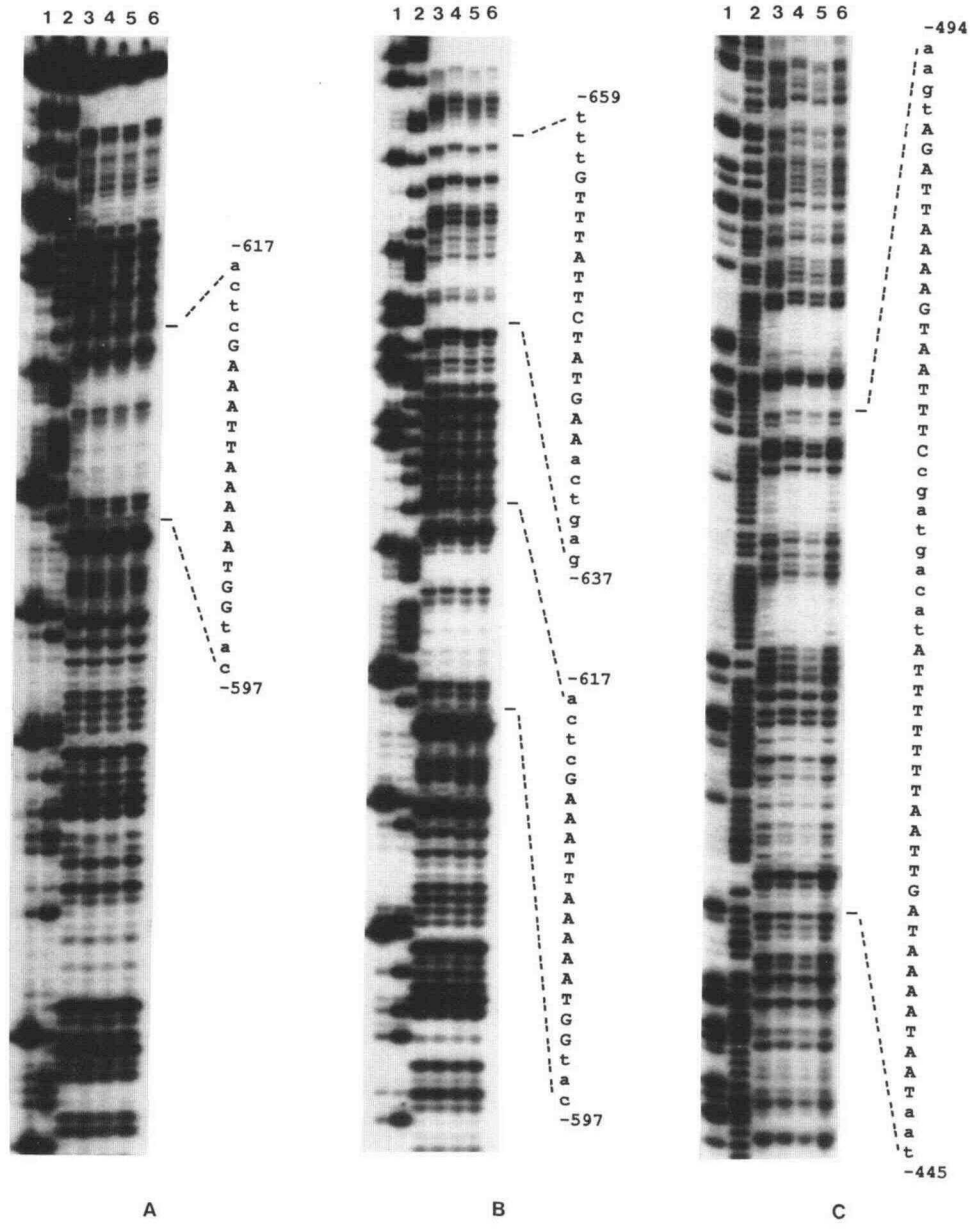

Figure 9. DNase I Footprinting of Fragments III, IV, and V.

Nuclear extracts from embryos 35 μg (lane 4), 70 μg (lane 5), without (lane 3) or with (lane 6) BSA were incubated with the fragments as shown and then digested with DNase I and electrophoresed on urea/polyacrylamide gels. Lanes 1 and 2 are G and G+A Maxam and Gilbert (1980) sequencing reactions, respectively. The sequences protected from DNase I digestion are indicated by capital letters. Only the coding strand is written. Numbers indicate distances from initiation of transcription.

(A) Fragment IV.

(B) Fragment III.

(C) Fragment V.

acting factor(s) that specifically coordinate genes expressed during embryo development. The comparison of the DNA sequences of fragment V with the DC 8 5′-flanking sequence (Franz et al., 1989) revealed several regions of homology located in both genes at approximately the same distance from the transcriptional start site

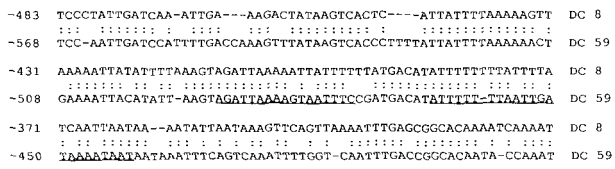

Figure 10. Homology between DC 59 and DC 8 Upstream Regions.

The sequences were aligned and gaps were introduced for maximum homology. Numbers represent distances from the initiation of transcription. Underlined are the sequences of the *DC 59* upstream region that are protected from DNase I digestion.

(data not shown). In contrast, the other two complexes (C1 and C2) were formed with DNA-binding factors that may control the expression of DC 59 alone or coordinate a subset of genes encoding proteins with related functions.

DNase I footprinting analysis showed that the protected sequences of fragment V were located within the upstream region of the DC 59 gene that is highly homologous to the upstream region of the DC 8 gene. These two protected sequences overlapped with the sequences protected from DNase I digestion of the DC 8 gene located within this homologous region. All four DNase I-protected sequences are AT-rich regions, as reported for other plant genes (Bustos et al., 1989; Jordano et al., 1989). The protected sequences were clustered into two groups and the members of each group were 10 to 30 nucleotides apart. Although the DNase I-protected sequences of fragment V were somehow homologous to each other, the ones present within fragment III were highly distinctive, indicating that they interacted with different DNA binding proteins, resulting in two different complexes, C1 and C2.

The DC 59 5′-flanking sequences that interacted with nuclear factors were relatively distant from the initiation of transcription. This has also been found in other plant systems (Maier et al., 1987; Deikman and Fisher, 1988). Recently, it has been shown that multiple *cis*-elements result in several complexes with nuclear factors in animal (Babiss et al., 1987; Celeghini et al., 1987; Lichtsteiner et al., 1987; Monaci et al., 1988) as well as in plant systems (Green et al., 1987; Deikman and Fischer, 1988), and it has been proposed that enhancer-like elements modulate transcription although they are located relatively far upstream of their respective genes. It is interesting to note that the region of fragment III responsible for the formation of the C1 complex contains the sequence TGTGGATATG, which is similar to a motif found not only in many viral enhancers (Odell et al., 1985; Sassone-Corsi and Borrelli, 1986) but also upstream of several plant genes (Timko et al., 1985; Kaulen et al., 1986; Maier et al., 1987).

METHODS

Plant Material and Tissue Culture Conditions

Carrot (*Daucus carota* var Juwarot) plants were grown under standard greenhouse conditions. Tissue samples from flowering plants were collected, frozen immediately in liquid nitrogen, and stored at −80°C. Three carrot cell lines were used in this study. W001C is a diploid cell line derived in 1970 from a wild carrot (*D. carota*, Queen Anne's Lace) grown in England (Sung, 1976). HA and J1, both haploid cell lines, were derived from *D. carota* var Juwarot, via the twin seedling method (HA; Smith et al., 1981) and anther culture, respectively (J1; Franz et al., 1989).

W001C has been maintained in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.1 mg/L 2,4-D, whereas HA and J1 have been maintained in Gamborg (B5) medium (Gamborg et al., 1968) with 1 mg/L 2,4-D. Liquid suspension cultures were subcultured at 8×10^5 cells/mL (high cell density). Under this condition, cells propagate as unorganized cell clumps. Somatic embryos were produced from 10-day-old to 15-day-old liquid suspension cultures. W001C and HA cultures were filtered through 200-μm or 500-μm nylon mesh, respectively. The filtrates were centrifuged, washed with the same media without 2,4-D, and resuspended in medium without 2,4-D at low cell density of 2×10^4 cells/mL for W001C or 1 to 2×10^5 cells/mL for HA. Freshly prepared abscisic acid (±2-*cis*-ABA) was filter sterilized and added to a final concentration of 10^{-5} M in the culture medium. Fluridone [1-methyl-3-phenyl-5(3-trifluoromethylphenyl)-4 (1H)-pyridone, Eli Lilly Co.] was added from stock solutions (2.5 mg/mL acetone) to a final concentration of 50 μg/mL.

RNA Preparations

Total RNA from different plant tissues and somatic embryos or calli was isolated according to Franz et al. (1989). Poly A^+ RNA was purified using oligo(dT)-cellulose (Aviv and Leder, 1972). One microgram to 5 μg of poly A^+ RNA was loaded onto a denaturing formaldehyde-agarose gel (1.4%) and blotted without further treatment onto nitrocellulose paper. Nick-translated probes were prepared from recombinant plasmids or isolated fragments according to Rigby et al. (1977). RNA blot filter hybridization and washing conditions were performed as described by Church and Gilbert (1984).

Molecular Techniques

Molecular procedures were performed according to Maniatis et al. (1982). Genomic DNA was isolated as previously described (Franz et al., 1989). DNA blot filter hybridization and washings were performed at 65°C according to Church and Gilbert (1984). For S1 analysis essentially the method described by Berk and Sharp (1977) was followed. The DNA fragments were co-precipitated with up to 20 μg of poly A^+ RNA and 20 μg of yeast tRNA, and dissolved in 30 μL of hybridization solution consisting of 40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% recrystalized formamide. Hybridization was at 65°C overnight. Then 300 μL of ice-cold S1 buffer (0.28 M NaCl, 0.05 M sodium acetate, pH 4.8, 4.5 mM $ZnSO_4$, 20 μg denatured salmon sperm DNA) containing

1000 units/mL S1 nuclease were added, incubated at 37°C for 1 hr, and electrophoresed on alkaline agarose gels (McDonnel et al., 1977).

Preparation of templates for sequencing was as described in Franz et al. (1989) and Chen and Seeburg (1985). The sequencing was done according to Sanger et al. (1977). DNA sequence was edited and analyzed with the IBI-Pustell (Pustell and Kafatos, 1984) program. Protein (PIR version 16) and DNA (GenBank version 57) data bases were searched with FASTP and FASTN programs (Lipman and Pearson, 1985), respectively. PC gene from Intelligenetics was used for protein structural analysis.

Nuclear Extracts, Gel Retardation Assays, and DNase I Footprinting

Embryo and callus nuclear extracts were isolated from somatic embryo and callus cultures according to Green et al. (1987). End-labeled and competitor DNA fragments were isolated by phenol extractions after electrophoresis in low-melting-point agarose. Binding reactions (10 μL to 30 μL) contained 0.2 ng of end-labeled DNA probe, 3.5 μg of poly(dIdC)·poly(dIdC), 45 mM KCl, 25 mM Hepes, pH 7.6, 1.1 mM EDTA, 0.5 mM DTT, 5% v/v glycerol, and competitor DNAs in molar quantities as indicated in the figure legends. Reactions were started by the addition of nuclear extracts (2 μg of protein per 10-μL reaction for embryos and 1.6 μg to 3.2 μg of protein per 30-μL reaction for callus), followed by incubation at room temperature for 15 min to 20 min. The samples were electrophoresed on 1% agarose gels (in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) with constant buffer recirculation. For DNase I footprinting, appropriate fragments were end labeled and reacted with nuclear extracts using the same buffer and reagents in a total volume of 50 μL for 30 minutes. At the end, DNase I (2 μg/mL) was added and the reaction was stopped after 4 min to 8 min. The products were electrophoresed on urea/polyacrylamide gels.

ACKNOWLEDGMENTS

The authors thank Robert Chorneau for the preparation of carrot cultures and plants and Rania Hatzopoulos for the preparation of the figures. This work was supported by grants from the National Science Foundation (DMB 850914) and the Department of Energy (DE-FG03-87ER13698) and by an unrestricted gift from Charter Venture, Mountain View, CA, to Z.R.S.

Received October 4, 1989; revised February 27, 1990.

REFERENCES

Aviv, H., and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidilic acid-cellulose. Proc. Natl. Acad. Sci. USA **69,** 1408–1412.

Babiss, L.E., Herbst, R.S., Bennett, A.L., and Darnell, J.E. (1987). Factors that interact with the rat albumin promoter are present both in hepatocytes and other cell types. Genes Dev. **1,** 256–267.

Berk, A.J., and Sharp, P.A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell **12,** 721–732.

Bewley, J.D., and Black, M. (1986). Seeds: Physiology of Development and Germination. (New York: Plenum Press), pp. 29–83.

Borkird, C., Choi, J.H., Jin, Z.H., Franz, G., Hatzopoulos, P., Chorneau, R., Bonas, U., Pelegri, F., and Sung, R.Z. (1988). Developmental regulation of embryogenic genes in plants. Proc. Natl. Acad. Sci. USA **85,** 6399–6403.

Bustos, M.M., Guiltinan, M.J., Jordano, J., Begum, D., Kalman, F.A., and Hall, T.C. (1989). Regulation of β-glucuronidase expression in transgenic tobacco plants by an A/T-rich, *cis*-acting sequence found upstream of a French bean β-phaseolin gene. Plant Cell **1,** 839–853.

Celeghini, S., Raymondjean, M., Carranca, A.G., Herbomel, P., and Yaniv, M. (1987). Factors involved in control of tissue-specific expression of albumin gene. Cell **50,** 627–638.

Chen, E.Y., and Seeburg, P.H. (1985). Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. DNA **4,** 165–170.

Choi, J.H., Liu, L.-S., Borkird, C., and Sung, R.Z. (1987). Cloning of genes developmentally regulated during plant embryogenesis. Proc. Natl. Acad. Sci. USA **84,** 1906–1910.

Church, G., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Deikman, J., and Fischer, R.L. (1988). Interaction of a DNA binding factor with the 5′-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J. **7,** 3315–3320.

Dure, L., III, Crouch, M., Harada, J., Ho, T.H.O., Mundy, J., Quatrano, R., Thomas, T., and Sung, R.Z. (1989). Common amino acid sequence domains among Lea proteins of higher plants. Plant Mol. Biol. **12,** 475–486.

Fong, F., Smith, J.D., and Koehler, D.E. (1983). Early events in maize seed development: Fluridone induction of vivipary. Plant Physiol. **73,** 899–901.

Franz, G., Hatzopoulos, P., Jones, T.J., Krauss, M., and Sung, R.Z. (1989). Molecular and genetic analysis of an embryonic gene, DC 8, from *Daucus carota* L. Mol. Gen. Genet. **218,** 143–151.

Galau, G.A., and Hughes, D.W. (1987). Coordinate accumulation of homologous transcripts of seven cotton Lea gene families during embryogenesis and germination. Dev. Biol. **123,** 213–221.

Gamborg, O.L., Miller, R.A., and Ohuama, K. (1968). Nutritional requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50,** 151–158.

Green, P.J., Kay, S.A., and Chua, N.-H. (1987). Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rbcS-3A* gene. EMBO J. **6,** 2543–2549.

Heidecker, G., and Messing, J. (1986). Structural analysis of plant genes. Annu. Rev. Plant Physiol. **37,** 439–466.

Jordano, J., Almoguera, C., and Thomas, T.L. (1989). A sunflower helianthinin gene upstream sequence ensemble contains

an enhancer and sites of nuclear protein interaction. Plant Cell **1,** 855–866.

Kaulen, H., Schell, J., and Kreuzaler, F. (1986). Light-induced expression of the chimeric chalcone synthase-NPTII gene in tobacco cells. EMBO J. **5,** 1–8.

Lichtsteiner, S., Wuarin, J., and Schilber, U. (1987). The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. Cell **51,** 963–973.

Lipman, D.J., and Pearson, W.R. (1985). Rapid and sensitive protein similarity searches. Science **227,** 1435–1441.

Liu, L., Borkird, C., and Sung, R.Z. (1985). Molecular cloning of cDNAs complementary to poly(A)$^+$ mRNA differentially expressed during somatic embryogenesis. In Proceedings of a Workshop on Somatic Embryogenesis, M. Terzi, L. Pitto, and R.Z. Sung, eds (Rome: IPRA), pp. 70–76.

Maier, U.-G., Brown, J.W.S., Toloczyki, C., and Feix, G. (1987). Binding of a nuclear factor to a consensus sequence in the 5′ flanking region of zein genes from maize. EMBO J. **6,** 17–22.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Maxam, A., and Gilbert, W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65,** 499–560.

McDonnel, M.W., Simon, M.N., and Studier, F.W. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110,** 119–127.

Monaci, P., Nicosia, A., and Cortese, R. (1988). Two different liver-specific factors stimulate in vitro transcription from the human α1-antitrypsin promoter. EMBO J. **7,** 2075–2087.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Odell, J.T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313,** 810–812.

Proudfoot, N.J., and Brownlee, G.G. (1976). 3′ Non-coding region sequence in eukaryotic messenger RNA. Nature **263,** 211–214.

Pustell, J., and Kafatos, F.C. (1984). A convenient and adaptable package of computer programs of DNA and protein sequence management, analysis and homology determination. Nucl. Acids Res. **12,** 643–655.

Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977). Labeling of deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113,** 237–251.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Sassone-Corsi, P., and Borrelli, E. (1986). Transcriptional regulation by *trans*-acting factors. Trends Genet. **2,** 215–219.

Smith, J., Furner, I., and Sung, R.Z. (1981). Nutritional and karyotypic characterization of a haploid cell culture of *Daucus carota*. In Vitro **17,** 315–321.

Sung, R.Z. (1976). Turbidimetric determination of plant cell culture growth. Plant Physiol. **57,** 460–462.

Timko, M.P., Kausch, A.P., Castresana, C., Fassler, J., Herrera-Estrella, L., Van den Broeck, G., Van Montagu, M., Schell, J., and Cashmore, A.R. (1985). Light regulation of plant gene expression by an upstream enhancer-like element. Nature **318,** 579–582.

Vance, V.B., and Huang, A.H.C. (1987). The major protein from lipid bodies of maize. J. Biol. Chem. **262,** 11275–11279.

Vance, V.B., and Huang, A.H.C. (1988). Expression of lipid body protein gene during maize seed development. J. Biol. Chem. **263,** 1476–1481.