Interaction of a Developmentally Regulated DNA-Binding Factor with Sites Flanking Two Different Fruit-Ripening Genes from Tomato

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To investigate mechanisms that control fruit development, we have begun experiments to identify proteins that control gene expression during tomato fruit ripening. We focused on the regulation of two different genes, E4 and E8, whose transcription is coordinately activated at the onset of fruit ripening. We report here that a DNA-binding protein specifically reacts with similar sequences flanking the E4 and E8 genes. The E4 binding site is at position -34 to -18 and, therefore, overlaps the region (TATA box) that in many eukaryotic genes serves to determine the efficiency and initiation site of transcription. In contrast, the E8 binding site is distal, located at -936 to -920 relative to the start of E8 gene transcription. Gel electrophoresis mobility retardation experiments indicate that the DNA binding activity that interacts with these two sites increases at the onset of fruit ripening. Taken together, these results suggest that this DNA-binding protein may function to coordinate E4 and E8 gene expression during fruit ripening.

INTRODUCTION

At the onset of tomato fruit ripening, many changes in metabolism occur, including increased respiration and ethylene production, chlorophyll degradation, elevated carotenoid synthesis, production of essential oils and flavor components, and increased activity of cell wall-degrading enzymes (for review see Brady, McGlasson, and Speirs, 1987). Coincident with the promotion of these metabolic pathways is the activation of gene expression. Analysis of pericarp protein extracts revealed the appearance of newly synthesized polypeptides at the onset of ripening (Biggs, Harriman, and Handa, 1986), and mRNAs that increase in concentration during tomato fruit ripening have been identified and cloned (Grierson, 1985; Mansson, Hsu, and Stalker, 1985; DellaPenna, Alexander, and Bennett, 1986; Lincoln et al., 1987). These results support the model that fruit ripening is controlled, at least in part, by the activation of gene expression resulting in the production of enzymes that catalyze diverse ripening-related processes (Brady, McGlasson, and Speirs, 1987).

To understand how gene expression is regulated during fruit ripening, we have studied two different fruit-ripening genes of unknown function, designated E4 and E8. We have found that the E4 and E8 genes are expressed similarly during plant development (Lincoln et al., 1987; Lincoln and Fischer, 1988a, 1988b; DellaPenna et al., 1989). First, both the E4 and E8 mRNAs are abundant in ripening fruit and are not detected in leaf, root, stem, or unripe fruit. Second, both E4 and E8 gene expression is activated by ethylene, a hormone that plays an important role in controlling fruit ripening (Rhodes, 1980; Biale and Young, 1981; Yang, 1985). Third, the rin (ripening inhibited) mutation that blocks many aspects of ripening, including softening, ethylene production, and color development (Tigchelaar, McGlasson, and Buescher, 1978; Giovannoni et al., 1989), reduces the concentration of E4 and E8 mRNA by greater than 10-fold and threefold, respectively. Fourth, nuclear run-on transcription experiments indicate that the E4 and E8 genes are both regulated at the transcriptional level. However, it is important to note that certain differences in the patterns of E4 and E8 gene expression have also been detected. For example, exposing whole plants to exogenous ethylene results in E4 gene expression in many plant organs, whereas E8 gene expression is induced only in fruit (Lincoln and Fischer, 1988a). Taken together, these results suggest that there may be common factors involved in the control of E4 and E8 gene expression.

To investigate the mechanisms that coordinate gene expression during fruit ripening, we have initiated experiments to identify DNA sequences and nuclear factors that control E4 and E8 gene transcription. Previous results suggested that a developmentally regulated DNA-binding activity reacts with a restriction fragment flanking the E8 gene (Deikman and Fischer, 1988). We report here that...
the same DNA-binding activity reacts with DNA sequences flanking the coordinately expressed E4 gene. Methylation interference experiments indicate that the E4 binding site overlaps the TATA box region at position −34 to −18 relative to the start of transcription. In contrast, the E8 gene has a distal binding site located at −936 to −920 relative to the start of E8 gene transcription. The two sites determine the DNA-binding activity that interacts with these two sites increases at the onset of fruit ripening. Taken together, these results suggest that the DNA-binding protein may function to coordinate E4 and E8 gene expression during fruit ripening.

RESULTS

E4 Gene Structure and Organization

Previously, we described the isolation of a cDNA clone, pE4, representing a 0.9-kb mRNA that accumulates during tomato fruit ripening and when unripe fruit are exposed to ethylene (Lincoln et al., 1987). To analyze the E4 gene, a bacteriophage λ library of tomato leaf nuclear DNA was screened by hybridizing plaques with labeled pE4 DNA, a genomic clone was isolated, and a map of its restriction endonuclease sites was determined (Figure 1A). To investigate E4 gene organization further, labeled pE4 DNA was hybridized with restriction endonuclease-digested tomato genomic DNA that had been transferred to nitrocellulose. The molecular mass of the hybridizing genomic restriction fragments was consistent with the restriction map of the tomato genome.

To investigate the structure of the E4 gene, we determined the DNA sequence of both genomic and cDNA clones. The 3.8-kb EcoRI restriction fragment that hybridized with pE4 was subcloned (designated pE4RR3.8, Figure 1B), and the location of 2797 bp (EcoRI to Ncol site) of the cDNA clone shown in Figure 1A (data not shown), suggesting that there is one E4 gene present per haploid tomato genome.

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Figure 1. E4 Gene Structure.

(A) Restriction endonuclease site map of E4-3. B, BamHI; Bg, BglII; H, HindIII; K, KpnI; N, Ncol; P, PstI; R, EcoRI; X, XbaI.

(B) Strategy for determining DNA sequences. pE4RR3.8 was subcloned from hE4-3. Horizontal arrows indicate the extent and direction of DNA sequence determinations. Structure of E4 transcript: filled box, untranslated exon sequences; open, translated exon sequences; cross-hatched, intron sequences.

(C) DNA sequence of the E4 gene and predicted amino acid sequence of the polypeptide encoded by cDNA clone pE4FL. Amino acid sequences are in single-letter code.

The molecular mass of the hybridizing genomic restriction fragments was consistent with the restriction map of the tomato genome. Gel electrophoresis mobility retardation experiments indicate that the DNA-binding activity that interacts with these two sites increases at the onset of fruit ripening. Taken together, these results suggest that the DNA-binding protein may function to coordinate E4 and E8 gene expression during fruit ripening.

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are present 28 bp and 87 bp, respectively, 5’ to the transcription start site.

The E4 gene and predicted polypeptide sequences were compared with sequences compiled in the National Institutes of Health GenBank, the European Molecular Biology Laboratory Nucleotide Sequence Library, and the National Biomedical Research Foundation Protein Identification Resource. As shown in Figure 2, the homology search indicated a distant relationship with the 29-kD polypeptide encoded by the EIP28/29 gene from Drosophila melanogaster (Cherbas et al., 1986; Schulz, Cherbas, and Cherbas, 1986). Optimal alignment of 141 amino acids revealed 37% amino acid sequence identity with only three gaps. In addition, 35% of the nonidentical amino acids represented conservative substitutions. Expression of the EIP28/29 gene has been shown to be rapidly induced by the steroid hormone ecdysone in Drosophila cell lines (Savakis, Koehler, and Cherbas, 1984). However, the function of the EIP28/29 and E4 genes remains to be elucidated.

In Vitro Binding of Nuclear Proteins from Tomato Fruit to E4 and E8 5’-Flanking Sequences

Previously, we isolated a cDNA clone, pE8, representing a 1.4-kb mRNA that accumulates during fruit ripening (Lincoln et al., 1987) and showed that multiple DNA-binding factors react in vitro with end-labeled restriction fragments flanking the E8 gene (Deikman and Fischer, 1988). In particular, an activity that increased at the onset of fruit ripening reacted specifically with the E8-1 restriction fragment (see Figures 3A and 3B for restriction fragments flanking the E4 and E8 genes, respectively). However, adding molar excess quantities of unlabeled competitor E4-4 restriction fragment abolished the interaction between the factor and end-labeled E8-1 DNA, suggesting that the same DNA-binding activity reacted with the DNA sequences flanking the E4 and E8 genes.

To localize the E4 DNA binding site, nuclear proteins were extracted from ripening fruit and reacted with DNA sequences flanking the E4 gene. As described in Methods, all reactions included poly(dl-dC)-poly(dl-dC) duplex DNA to eliminate nonspecific protein-DNA interactions. The presence of DNA-binding factors was assayed by the DNA gel electrophoresis mobility retardation assay (Singh et al., 1986). As shown in Figure 3C, the mobility of the E4-4b restriction fragment was retarded when reacted with nuclear extracts isolated from ripening tomato fruit. To analyze the specificity of binding, molar excess quantities of unlabeled competitor DNAs were added to the reactions.
Whereas unlabeled fragment E4-4b eliminated the binding of protein to end-labeled E4-4b DNA, addition of pUC18 plasmid DNA, an adjacent restriction fragment E4-4a, or 5′-flanking sequences (−463 to +61) of a light-regulated tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase failed to abolish binding (Figure 3C). However, addition of molar excess quantities of unlabeled E8-1 prevented the formation of the more slowly migrating complex with end-labeled E4-4b. These results are consistent with those observed in analogous binding competition experiments using end-labeled E8-1 DNA (Deikman and Fischer, 1988), and suggest that a factor extracted from ripening tomato fruit nuclei forms a specific complex with the E4-4b and E8-1 restriction fragments flanking the E4 and E8 genes.

To identify specific guanine residues involved in the binding reaction, methylation interference experiments were performed. Methylation of guanine residues in the major groove has been shown to disrupt the interaction of factors with DNA either by steric hindrance or by disrupting the contact between specific guanine residues and the binding factor (Otwinowski et al., 1988). To this end, the E4-4b and E8-1 restriction fragments were end-labeled, partially methylated, and reacted with nuclear protein extracts isolated from ripening tomato fruit. The resulting DNA-protein complexes were isolated, cleaved at methylated guanine sites, and fractionated by gel electrophoresis as described in Methods. As shown in Figure 4, analysis of restriction fragment E4-4b indicated that methylation of guanine residues at position −21 of the noncoding strand and position −30 of the coding strand interfered with binding. As shown in Figure 5, analysis of restriction fragment E8-1 revealed that methylation at position −923 of the noncoding strand and position −931 and −932 of the coding strand interfered with binding. These results show that an essential component of both binding sites is guanine residues separated by 8 bp on opposite DNA strands.

The DNA sequences surrounding the guanine residues that interfere with binding are identical at 11 of 17 positions (Figure 6). To determine whether these regions are sufficient for binding to nuclear factors, complementary oligonucleotides 27 residues in length and spanning a presumptive binding site, E4 (−38 to −12) and E8 (−940 to −914), were chemically synthesized, annealed, and ligated as described in Kadonaga and Tjian (1986). Each was inserted into pUC119, and recombinant plasmids containing three adjacent binding sites were identified. The subcloned binding sites were then isolated and end-labeled. As shown in Figure 7, gel electrophoresis mobility retardation experiments indicate that each binding site formed a DNA-protein complex when reacted with nuclear extracts isolated from ripening tomato fruit. This result indicates that the E4 and E8 oligonucleotides contain sufficient sequence information for binding factors in nuclear extracts isolated from ripening tomato fruit.

To determine whether the E4 and E8 binding sites compete for the same factors, molar excess quantities of unlabeled oligonucleotides were added to the binding reactions. As shown in Figure 7, addition of the unlabeled E4 binding site E4 (−38 to −12) abolished the interaction between factors and the end-labeled E8 binding site E8 (−940 to −914). Conversely, adding unlabeled E8 binding site E8 (−940 to −914) eliminated binding to the end-labeled E4 binding site E4 (−38 to −12). In contrast, addition of excess unlabeled control oligonucleotide (see Methods for DNA sequence of the control oligonucleotide)
see Methods for description of stages). To determine whether binding and gene transcription coordinately increase, oligonucleotide probes representing the E4 and E8 binding sites were reacted with nuclear protein isolated from fruit at the mature green 1 and 4 stages. As shown in Figure 8, gel electrophoresis mobility shift experiments indicate that DNA-binding activity was significantly less in unripe mature green stage 1 fruit than in ripening mature green stage 4 fruit. It is unlikely that the unripe mature green 1 stage fruit extract was inactivated or degraded during isolation because equal levels of a DNA-binding protein activity were detected when mature green 1 and 4 stage fruit extracts were reacted with 5'-flanking sequences from another fruit-ripening gene, polygalacturonase (J.R. Montgomery and R.L. Fischer, unpublished results). We conclude from these results that, at the onset of ripening, DNA-binding activity and gene transcription of the E4 and E8 genes coordinately increase.

**DISCUSSION**

Comparing the E4 (Figure 1) and E8 (Deikman and Fischer, 1988) genes reveals little DNA sequence identity in their amino acid coding regions, as well as in their respective 5'-flanking regions. However, transcription of these two genes is coordinately activated at the onset of tomato fruit ripening in response to ethylene (Lincoln et al., 1987; Lincoln and Fischer, 1988a). To elucidate mechanisms that may coordinate gene expression during fruit ripening, we have begun to analyze DNA-binding proteins that specifically react with the E4 and E8 genes.

**Figure 6.** Comparison of Binding Sites Flanking the E4 and E8 Genes.

DNA sequences are numbered relative to the transcription start of the E4 and E8 genes. Underlined G represents guanine residues that interfere with binding when methylated (see Figures 4 and 5 and text). Overlined sequence represents putative E4 gene TATA box. Vertical lines, DNA sequences identical in E4 and E8; —, DNA sequences not identical in E4 and E8; *, DNA sequences identical in E4, E8, and C (control) oligonucleotide.
DNA sequence and preparation of oligonucleotides are described in Methods. End-labeled oligonucleotides are indicated below the lanes. The specific unlabeled competitor oligonucleotide and the molar ratio of unlabeled to labeled DNAs in the binding reaction are indicated above the lanes. E4, E4 (-38 to -12) oligonucleotide; E8, E8 (-940 to -914) oligonucleotide; C, control oligonucleotide; N, control reaction without addition of protein; 0, no unlabeled oligonucleotide competitor DNA added; F, free DNA that is not bound to protein; B, DNA bound to factor(s).

**Delineation of Shared Binding Sites**

Ripening tomato fruit nuclei contain several DNA-binding activities that interact in vitro with sites flanking the E4 and E8 genes. Some of these activities are unique to the E4 gene (data not shown) and to the E8 gene (Deikman and Fischer, 1988). However, binding experiments in the presence of unlabeled competitor DNAs indicated that a DNA-binding protein reacts with restriction fragments E4-4b and E8-1 flanking the E4 and E8 genes, respectively (Figure 3C; Deikman and Fischer, 1988). The specificity of the interaction was demonstrated in control experiments, where the factor was shown not to bind with pUC118 plasmid DNA, 5'-flanking sequences of the polygalacturonase fruit-ripening gene, or 5'-flanking sequences of the light-regulated tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. (Figure 3C; Deikman and Fischer, 1988; J.R. Montgomery and R.L. Fischer, unpublished results).

Methylation interference experiments localized binding to sites flanking the E4 (Figure 4) and E8 (Figure 5) genes, respectively, that share DNA sequence similarity (Figure 6). Several lines of evidence indicate that these are the correct binding sites. First, the factor binds directly to chemically synthesized oligonucleotides spanning the regions delineated by the methylation interference experiments (Figures 7 and 8). Second, addition of a molar excess of chemically synthesized unlabeled E4 binding site E4 (-38 to -12) abolished the interaction between the factor and the end-labeled restriction fragments E4-4b and E8-1 (data not shown). These results suggest that the binding of this factor to the E4-4b and E8-1 restriction fragments occurs only at the sites delineated by the methylation interference experiments.

**Common Binding Sites Have Similar DNA Sequences**

Comparison of the E4 and E8 binding sites revealed several conserved features. First, the binding sites share DNA sequence identity at 11 of 17 positions, with similar DNA sequences flanking a nonconserved AT-rich core. Second, methylation of guanine residues on opposite DNA strands in each binding site interferes with binding. Because they are separated by 8 bp, the two guanines are on the same face of the DNA helix and within one helical turn of each other in each binding site.

That these conserved features are sufficient for binding of the same factor was shown by competition experiments (Figure 7). Addition of a molar excess of the unlabeled E8 binding site abolished the interaction with the factor and the end-labeled E4 binding site, whereas adding unlabeled E4 binding site interfered with factor binding to end-labeled E8 binding site. Taken together, these results indicate that the chemically synthesized E4 and E8 binding sites react with the same factor(s) present in ripening tomato nuclei. The factor did not bind to a control oligonucleotide (Figure 8).

![Figure 7](image-url)

*Figure 7.* In Vitro Interaction of Factors with Oligonucleotide Binding Sites.

DNA sequence and preparation of oligonucleotides are described in Methods. Binding reactions were carried out with nuclear proteins isolated from 50% red tomato fruit. End-labeled oligonucleotides are indicated below the lanes. The specific unlabeled competitor oligonucleotide and the molar ratio of unlabeled to labeled DNAs in the binding reaction are indicated above the lanes. E4, E4 (-38 to -12) oligonucleotide; E8, E8 (-940 to -914) oligonucleotide; C, control oligonucleotide; N, control reaction without addition of protein; 0, no unlabeled oligonucleotide competitor DNA added; F, free DNA that is not bound to protein; B, DNA bound to factor(s).

![Figure 8](image-url)

*Figure 8.* Regulation of a DNA-Binding Activity during Fruit Development.

DNA sequence and preparation of oligonucleotides are described in Methods. End-labeled oligonucleotides used in binding reactions are indicated below the lanes. E4, E4 (-38 to -12) oligonucleotide; E8, E8 (-940 to -914) oligonucleotide; F, free DNA that is not bound to protein; B, DNA bound to factor(s). Lane N, control reaction without addition of protein. Nuclear proteins isolated from the following tomato fruit cultivars at the indicated stage of development: lane 1, mature green stage 1 fruit; lane 2, mature green stage 4 fruit.
7) containing 6 of the 11 nucleotides conserved in the E4 and E8 sites, including the guanine residues separated by 8 bp (Figure 6). This result suggests that binding involves more than the interaction between the factor and the guanine residues in the configuration described above, and that the additional conserved DNA sequences in the E4 and E8 sites are also important for binding site recognition.

Placement of E4 and E8 Binding Sites Is not Conserved

The position of binding sites relative to the start of E4 and E8 gene transcription differs significantly. The E8 site is far upstream from the E8 transcription initiation site, at position −936 to −920. Whether the factor binding to the E8 site regulates E8 gene transcription is unknown. However, its distal placement, and the positive correlation between factor binding and E8 gene transcription (Figure 8; Deikman and Fischer, 1988), are consistent with the hypothesis that the E8 site is an enhancer-like region for high-level gene expression. In contrast, the E4 site is at position −34 to −18 and spans the presumptive TATA box region (−29 to −23; Joshi, 1987). Studies in vitro (Grosveld et al., 1981; Hu and Manley, 1981; Mathis and Chambon, 1981; Sasylyk and Chambon, 1981; Zarucki-Schulz et al., 1982; Concino et al., 1984) and in vivo (Benoist and Chambon, 1981; Grosveld et al., 1982; McKnight and Kingsbury, 1982; Dierks et al., 1983; Charnay, Mellon, and Maniatis, 1985) studies have shown that for many genes, although not all, the TATA motif is critical for both promoter activity and for determining the precise site of transcription initiation. The TFIID protein (also known as BTF1 and DB) interacts specifically with the TATA motif. In conjunction with two other constitutive factors (TFIIB, TFIIE) and proteins that bind to specific distal enhancer sequences, it directs the initiation of transcription approximately 30 bp downstream (Sawadogo and Roeder, 1985; Buratowski et al., 1988; Horikoshi et al., 1988a, 1988b; Nakajima, Horikoshi, and Roeder, 1988). However, several lines of evidence suggest that the factor reacting with the E4 TATA region (−34 to −18) is not a ubiquitous TATA-binding factor. First, we do not detect this DNA-binding activity in unripe mature green stage 1 fruit (Figure 8), when presumably many thousands of genes (Goldberg, 1986) are undergoing TFIID-mediated transcription. Second, the factor we observe binding to the E4 TATA region does not react with the TATA region of other tomato fruit-ripening genes, e.g., E8 and polygalacturonase, or the light-regulated gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (Deikman and Fischer, 1988; data not shown). In this regard, it is important to note that most of the DNA sequences that are conserved between the E4 and E8 sites flank the sequence TATATAA (Figure 6), suggesting that the TATA motif may not play an important role in the in vitro binding observed.

Recent studies suggest that DNA-binding proteins that react with distal sites can functionally replace proximal TATA-binding factors such as TFIID. In yeast the GCN4 protein stimulates transcription of amino acid biosynthetic genes by binding to enhancer sequences upstream from TATA elements (Hope and Struhl, 1985; Arndt and Fink, 1986). However, it has recently been shown that replacing a TATA element with a GCN4 binding site permits GCN4-mediated gene transcription (Chen and Struhl, 1989). In addition, a naturally occurring GCN4-responsive gene, TRP3, has a GCN4 binding site instead of TATA sequences (Aebi et al., 1984; Zalkin et al., 1984). These results suggest that DNA-binding proteins that act at distal sites can in some cases also functionally interact with the RNA polymerase II transcription complex at a proximal site, approximately 30 bp upstream from the start of transcription. We speculate that, in an analogous fashion, factor binding to both the distal E8 site (−936 to −920) and to the proximal E4 TATA region (−34 to −18) might play a role in the coordinate activation of E4 and E8 gene transcription at the onset of fruit ripening. In this regard it is interesting to note that gel retardation and footprinting experiments suggest that the factor may also react with a more distal E4 binding site at position −749 to −728 relative to the start of E4 gene transcription (data not shown) that has DNA sequence similarity with the binding sites shown in Figure 6. However, competition experiments suggest that another class of factors uniquely binds the distal E4 site as well (data not shown). Experiments designed to test the significance of factor binding in vivo are in progress that involve mutagenesis of binding sites, followed by analysis of promoter function in transgenic tomato plants.

METHODS

Plant Material

Tomato plants were grown under standard greenhouse conditions. For studies on the regulation of DNA-binding activity during fruit development, nuclear proteins were isolated from tomato fruit (Lycopersicon esculentum cv VFNT Cherry). Fruit maturity stage and ethylene evolution rates were determined as described previously (Lincoln et al., 1987). Mature green stage 1 fruit were full size, and evolved ethylene at a low level (0.6 ± 0.2 nL/g/hr). In mature green stage 4 fruit carotenoid pigments were observed in the interior of the fruit, and elevated ethylene levels were observed (3.5 ± 1.0 nL/g/hr).

Isolation of Clones

A library of tomato (L. esculentum cv VFNT Cherry) genomic DNA in the Charon 35 vector was screened by plaque hybridization with labeled pE4 to obtain a genomic clone, pE4-3, containing an E4 gene. Endpoints for subclones were generated by digesting
DNA with either restriction endonucleases or with exonuclease III (Henikoff, 1984). The name of each subclone indicates its flanking restriction endonuclease or exonuclease III site and molecular mass. A cDNA library enriched for full-length cDNA clones of tomato ripe fruit mRNAs (DeltaPenna, Alexander, and Bennett, 1986) was screened by colony hybridization with labeled pE4 to obtain a full-length E4 cDNA clone, pE4FL.

Analysis of Nucleic Acids

The 3.8-kb EcoRI restriction fragment from \( \lambda E4-3 \) that spans the E4 gene was subcloned in both orientations into pUC118 to enable both sense and antisense single-strand template preparation (Vieira and Messing, 1987). Deletions were generated using exonuclease III (Henikoff, 1984). Nucleotide sequences were determined using the dideoxy chain-termination method (Sanger, Nicklen, and Coulson, 1977). DNA sequence analysis and searches of the NIH GenBank, EMBL Nucleotide Sequence Library, and NBRF Protein Identification Resource were performed using the Bionet National Computer Resource for Molecular Biology. S1-nuclease protection assays and primer extension analysis were performed as described in Deikman and Fischer (1988).

Preparation of Nuclei and Nuclear Extracts

Nuclei were isolated as described by Walling, Drews, and Goldberg (1986). Proteins were extracted from the nuclei by the procedure of Miskimins et al. (1985). To inhibit protease activities, 0.8 mM phenylmethylsulfonyl fluoride and 10 \( \mu \)M leupeptin were added to all solutions.

Preparation of Oligonucleotide Templates

Oligonucleotides that span presumptive binding sites E4 (~38 to –12), ATCCATTCTATATAAAGAAACATACA; E6 (~940 to ~914), TTATTATCCCAACATAAGAAGTCTTG; a control oligonucleotide designated C, GTACATTTAAAGTACATCGTCAC; and their respective complementary sequences were chemically synthesized, annealed, and ligated as described in Kadonaga and Tjian (1986). Each was inserted into pUC119, and recombinant plasmids containing three adjacent binding sites were identified.

DNA Gel Electrophoresis Mobility Retardation Assay

End-labeled DNA restriction fragments used in the binding reactions were isolated from low-melting-point agarose gels. Binding was carried out in 15-\( \mu \)L reactions containing 0.25 ng of end-labeled DNA restriction fragment, 1.5 \( \mu \)g of nuclear protein, 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, at 30°C for 30 min. In addition, 4.4 \( \mu \)g of poly(dI-dC)-poly(dI-dC) duplex DNA was included in all reactions. Titration experiments with end-labeled E4 restriction fragments indicated that this amount of poly(dI-dC)-poly(dI-dC) was sufficient to eliminate nonspecific protein-DNA interactions. The protein-DNA complex was separated from unbound DNA by electrophoresis on nondenaturing 4% acrylamide gels as described by Singh et al. (1986). Following electrophoresis, the gel was dried and exposed to x-ray film with an intensifying screen for 12 hr to 24 hr.

Methylation Interference Analysis

End-labeled DNA was partially methylated with dimethyl sulfate (Maxam and Gilbert, 1980) and reacted with nuclear proteins as described for the DNA gel electrophoresis mobility retardation assay, except that the binding reactions were 10-fold larger. The DNA-protein complex and unbound DNA were separated by electrophoresis on nondenaturing 4% acrylamide gels (Singh et al., 1986), eluted from the gel, cleaved at the methylated G residues with piperidine, and analyzed on denaturing 8% polyacrylamide gels (Maxam and Gilbert, 1980). DNA methylation and piperidine cleavage reactions were carried out with reagents and procedures supplied by Clontech (Palo Alto, CA).

ACKNOWLEDGMENTS

We express our gratitude to Dr. Alan Bennett for a cDNA library representing ripe fruit mRNAs, Dr. Wilhelm Grussem for the tomato RuBP carboxylase gene, and to Alex Federman for contributions to this work. We also thank Barbara Rotz and John Franklin for providing excellent greenhouse services. This research was supported by a National Institutes of Health grant (GM38856). Computer resources used to carry out our studies were provided by the National Institutes of Health-sponsored BIONET National Computer Resource for Molecular Biology.

Received August 21, 1989; revised August 30, 1989.

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*Plant Cell* 1989;1;1025-1034

DOI 10.1105/tpc.1.10.1025

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