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Identification of an ethylene-responsive region in the promoter of a fruit ripening gene

(plant hormone/gene regulation/transcription/*Lycopersicon esculentum*)

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ABSTRACT **Transcription of the E4 gene is controlled by an increase in ethylene concentration during tomato fruit ripening. To investigate the molecular basis for ethylene regulation, we have examined the E4 promoter to identify cis elements and trans-acting factors that are involved in E4 gene expression. In transgenic tomato plants a chimeric gene construct containing a 1.4-kilobase E4 promoter fused to a β-glucuronidase reporter gene is rapidly induced by ethylene in ripening fruit. Deletion of E4 promoter sequences to 193 base pairs reduces the level of GUS activity but does not affect ethylene induction. Transient expression of E4 promoter–luciferase chimeric gene constructs containing various deletions, introduced into tomato fruit pericarp by particle bombardment, indicates that a positive ethylene-responsive region is localized between nucleotides −161 and −85 relative to the transcription start site. DNase I footprint analysis shows that a nuclear factor in unripe fruit interacts specifically with sequences in this element, from −142 to −110, which are required for the ethylene response. The DNase I footprint of this factor is reduced in ethylene-treated unripe fruit and undetectable in ripe fruit. Based on the correlation of a nuclear factor binding site with promoter sequences required for ethylene induction, we propose that this *in vitro* DNA-binding activity may represent a factor that is involved in ethylene-regulated E4 gene expression.**

The plant hormone ethylene influences numerous aspects of plant growth and development (1), including the ripening of climacteric fruits such as tomato. The role of ethylene in tomato fruit ripening has been demonstrated unequivocally by inhibiting endogenous ethylene production through the expression of antisense RNAs that block the accumulation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, which catalyzes the rate-limiting step in ethylene biosynthesis (2). Fruit from plants transformed with the ACC synthase antisense gene evolve only very low levels of ethylene and do not ripen.

The effect of ethylene on fruit ripening and other developmental processes is due, at least in part, to changes in the transcription of specific genes (3–5). To understand how gene expression is regulated by ethylene during fruit ripening, a number of ethylene-responsive genes have been cloned and studied in this laboratory (6). One of these genes, E4, is transcriptionally activated at the onset of ripening, coincident with the increase in ethylene biosynthesis (4). Although its precise function is unknown, the E4 predicted polypeptide shows significant sequence identity with a peptide methionine sulfoxide reductase protein from *Escherichia coli* (7). Several lines of evidence suggest that transcription of the E4 gene is dependent upon the presence of elevated levels of ethylene (4, 6, 8). (*i*) Norbornadiene, a competitive inhibitor of ethylene action, strongly inhibits E4 gene expression during fruit ripening. (*ii*) E4 gene transcription is rapidly induced by treatment with exogenous ethylene in both unripe fruit and leaves. (*iii*) Expression of the E4 gene is sensitive to ethylene concentration and displays a narrow dose–response curve with threshold and half-maximum responses at 0.25 μl and 1.0 μl of ethylene per liter, respectively. Finally, inhibition of high-level ethylene biosynthesis by mutations that block fruit ripening suppresses E4 gene transcription (9). Treatment of such fruit with ethylene results in normal levels of E4 gene expression (8).

We have undertaken a study of the E4 gene to identify DNA sequences and nuclear factors that control ethylene-regulated gene expression. We previously described the isolation of the E4 gene, which is present as a single copy in the tomato genome (10). Here, we report that a chimeric gene construct containing 1421 base pairs (bp) of E4 5′-flanking DNA sequences is ethylene- and ripening-regulated in transgenic tomato plants. We have measured the expression of chimeric E4–reporter gene constructs containing E4 promoter deletions both in transgenic plants and in a transient gene expression system to identify cis-regulatory regions required for the ethylene response. In addition, we have used DNase I footprint analysis to determine that a fruit nuclear factor(s) interacts specifically with E4 DNA sequences located in the ethylene-responsive region. We speculate that this binding factor may be involved in ethylene-regulated E4 gene expression.

MATERIALS AND METHODS

Plant Material. Tomato plants were grown under standard greenhouse conditions. Fruit maturity stage was determined as described (6). To treat with ethylene, unripe fruit were placed in a 25-liter chamber and exposed for 6 hr to 4.5 liters of ethylene per minute (10 μl/liter) in humidified air.

Construction of Chimeric Genes. To construct an E4–β-glucuronidase (GUS) chimeric gene, a genomic DNA fragment containing E4 5′ flanking sequence from the *Eco*RI site (−1421) to the *Bam*HI site (+65) was ligated to the GUS coding sequence and nopaline synthase (NOS) 3′ poly(A)-addition sequence in pBI101.2 (11). The luciferase (LUC) reporter gene was obtained from pDO432 (12) and modified to remove a short open reading frame located in the 5′ RNA leader sequence. To construct an E4–LUC gene, the *Eco*RI–*Bam*HI E4 promoter fragment (−1421 to +65) was ligated to a *Bam*HI–*Sst* I LUC gene fragment plus an *Sst* I–*Eco*RI fragment containing the 3′ poly(A)-addition sequence from the NOS gene (from pBI101.2) and pUC119 digested with *Eco*RI. The E4 sequence was removed by digestion with *Bam*HI to obtain a promoterless LUC–NOS gene. E4 pro-

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; GUS, β-glucuronidase; LUC, luciferase; NOS, nopaline synthase; CaMV, cauliflower mosaic virus.

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moter deletion mutants were generated with exonuclease III as described (13).

Plant Transformation. Chimeric E4–GUS genes were subcloned into the *Eco*RI site of shuttle vector pMLJ1 by using *Eco*RI/*Hin*dIII adapters (New England Biolabs). Plant transformation was carried out using tomato (*Lycopersicon esculentum* cv. Ailsa Craig) cotyledons as described previously (14), and transgenic plants were selected by rooting in the presence of kanamycin (25 μg/ml).

Determination of GUS Activity. Protein extracts were isolated and GUS activity was determined with 4-methylumbelliferyl β-D-glucuronide (Clontech) as described (15), except that 20% methanol was included in the assay buffer (16). Protein concentration of extracts was measured with the bicinchoninic acid (BCA) protein assay reagent (Pierce).

Transient Gene Expression Assay. Tomato fruits (*L. esculentum* cv. VFNT cherry) were sterilized by stirring for 5 min in 20% (vol/vol) commercial chlorine bleach/0.02% Tween 80 and then rinsed three or four times with sterile water. The seeds and locular tissue were removed and the pericarp of each fruit was cut into eight relatively flat pieces. Pericarp pieces from unripe mature green fruit were vacuum infiltrated with 100 μM L-α-(2-aminoethoxyvinyl)glycine. Fruit pericarp pieces were placed epidermis-side up onto 0.8% water-agar plates and bombarded with 1.7-μm tungsten particles coated with E4–LUC plasmid DNA and control cauliflower mosaic virus (CaMV) 35S promoter–GUS plasmid DNA in a 2:1 molar ratio. The 35S–GUS gene was subcloned from pBI121 (11) into pUC119 by using *Hin*dIII and *Bam*HI sites. Plasmid DNAs were purified by CsCl gradient centrifugation and precipitated onto tungsten particles as described (17) in a total volume of 200 μl. Bombardments were performed with a particle gun using 22-caliber, gray, power-level-1 power loads for Remington stud drivers. The bombarded pericarp tissue was incubated for 40–48 hr in air or in air with ethylene (50 μl/liter), ground to a powder in liquid nitrogen, and homogenized in 100 mM KPO_4, pH 8.0/1 mM EDTA/10 mM dithiothreitol/5% (vol/vol) glycerol. LUC assays were performed immediately on a Flowtech luminometer, model 3010, in a cuvette containing 50 μl of protein extract mixed with 100 μl of LUC assay buffer (50 mM Hepes, pH 8.0/20 mM $MgCl_2$/10 mM ATP with bovine serum albumin at 0.5 mg/ml); 100 μl of 1 mM luciferin (Analytical Luminescence, San Diego) was injected to start the reaction. GUS activity and protein concentration were measured in the same protein extract as described above. LUC activity was corrected for background luminescence and normalized to GUS activity.

Nuclear Protein Isolation, Gel Electrophoresis Mobility-Shift Assays, and DNase I Footprinting Reactions. Nuclear protein extracts were prepared from tomato fruit (*L. esculentum* cv. VFNT cherry) pericarp as described (18), except that frozen tissue was ground in liquid nitrogen, the homogenization buffer was at pH 7.5, and the nuclear lysis buffer and dialysis buffer were at pH 8. Protein concentrations were determined with the BCA protein assay reagent (Pierce). Preparation of ^{32}P-end-labeled E4 promoter fragments, gel electrophoresis mobility-shift experiments, and DNase I protection (footprinting) reactions were carried out as described (18), except that binding reaction mixtures for the mobility-shift assays had a volume of 20 μl with 8 fmol of ^{32}P-labeled DNA fragment, 10 μg of poly(dI-dC), and 4 μg of nuclear protein, were incubated at room temperature for 20 min, and were analyzed by electrophoresis in a 5% polyacrylamide nondenaturing gel in 45 mM Tris/45 mM boric acid/1 mM EDTA. Footprinting reaction mixtures contained 2 fmol of ^{32}P-labeled DNA, 30 μg of nuclear protein, and DNase I at a final concentration of 1 μg/ml.

RESULTS

Effect of 5′ Deletions on Ethylene-Responsive E4–GUS Gene Expression. To identify regions of the E4 promoter that are required for ethylene-regulated gene expression, an E4–GUS chimeric gene containing 1421 bp of 5′ upstream sequence and 65 bp of E4 transcribed sequence was used to generate a series of 5′ promoter-deletion mutants. These E4–GUS chimeric gene constructs were introduced into tomato plants by using modified Ti-plasmid vectors from *Agrobacterium tumefaciens*. In plants transformed with an E4–GUS gene containing a 1421-bp promoter, GUS activity increased 22-fold in unripe fruit treated with ethylene and almost 1000-fold in ripe fruit, which produced high levels of ethylene (Fig. 1). Deletion of E4 nucleotides to −193 reduced the overall level of GUS activity in unripe fruit exposed to ethylene and in ripe fruit, whereas deletion to +13 resulted in control levels of GUS activity. These results indicate that 193 bp is sufficient to program ripening-regulated and ethylene-inducible E4–GUS gene expression in transgenic tomato plants.

The E4 Promoter Is Ethylene- and Ripening-Regulated in a Transient Gene Expression System. To enable more rapid identification of E4 regulatory regions, we adapted a microprojectile gene-transfer method to measure transient gene expression in tomato fruit pericarp. For these experiments we used a more sensitive reporter, the LUC gene, fused to the E4 promoter. To reduce the level of wound ethylene produced upon particle bombardment, unripe fruit pericarp pieces were first vacuum infiltrated with 100 μM L-α-(2-aminoethoxyvinyl)glycine, an inhibitor of ethylene biosynthesis (19).

Transcription of E4–LUC genes in the transient gene expression system (Fig. 2) was qualitatively similar to that observed with equivalent E4–GUS chimeric genes in transgenic tomato plants (Fig. 1). Bombardment with E4–LUC genes containing 1421, 511, or 193 bp of E4 5′ flanking sequences resulted in increased levels of relative LUC activity in ethylene-treated unripe fruit and in ripe fruit (Fig. 2), and LUC activity was not detected in control experiments with a promoterless LUC gene (data not shown). By contrast, a 1.6-kb CaMV-35S promoter fused to the LUC gene was expressed more highly in unripe fruit than in ripe fruit. It is possible that this 2-fold reduction in CaMV 35S–LUC gene expression reflects a decrease in the stability or activity of the

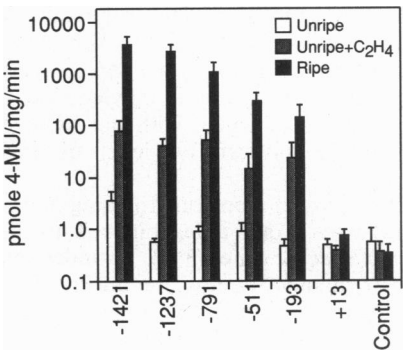

FIG. 1. Effect of promoter deletions on E4–GUS gene expression during fruit ripening and in ethylene-treated unripe fruit. GUS activity [pmol of 4-methylumbelliferone (4-MU) per mg of protein per minute] is expressed on a logarithmic scale to allow all the data to be presented on a single graph. E4 promoter deletion endpoints relative to the start of transcription are indicated in base pairs shown on the abscissa. Control, fruit from plants lacking a GUS transgene. Error bars indicate SEM. The number of independent transformants analyzed individually to determine the mean for each deletion was as follows: −1421, 9; −1237, 5; −791, 6; −511, 3; −193, 5; +13, 6; control, 3. Unripe, mature green fruit; Unripe + C_2H_4, mature green fruit exposed to ethylene (10 μl/liter) for 6 hr; Ripe, 50% red fruit.

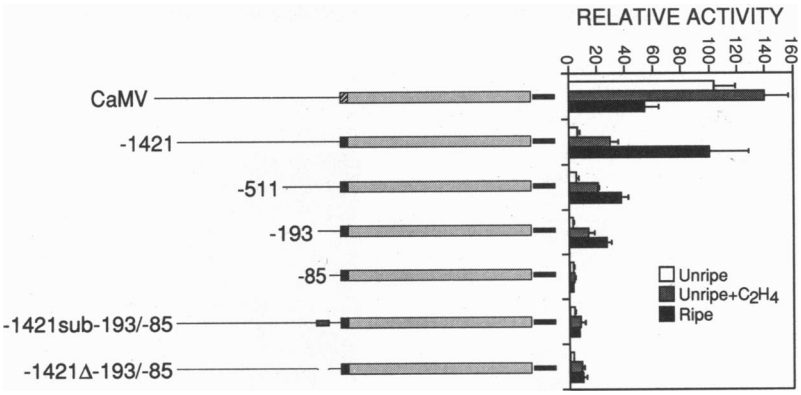

FIG. 2. Deletion analysis of the E4 promoter in a transient gene expression system. Schematic representations of E4 promoter–LUC chimeric genes with deletion endpoints in base pairs and a 1.6-kilobase-pair (kb) CaMV 35S promoter–LUC chimeric gene are shown on the left with corresponding relative LUC activities shown on the right. These chimeric genes were introduced into tomato fruit pericarp by particle bombardment. The pericarp pieces were kept in air or in air with ethylene and then assayed for LUC and GUS activities. LUC activity was corrected for the activity of a promoterless LUC gene, normalized to the expression of the 35S–GUS control plasmid in each extract and expressed relative to the 1421-bp E4–LUC activity in ripe fruit. Error bars indicate SEM. The data represent the average of at least four independent bombardments. In the diagrams at left, thin lines represent E4 or CaMV 35S 5′ flanking sequence; the small hatched box, 79 bp of transcribed polylinker sequence; the small black boxes, 65 bp of E4 transcribed sequence coding for 17 amino acids of the E4 polypeptide; and the thick lines, NOS 3′ sequence. Unripe, mature green fruit incubated in air; Unripe + C_2H_4, mature green fruit incubated with ethylene; Ripe, 50% red fruit incubated in air.

LUC protein in ripe-fruit extracts. This may indicate that the level of LUC activity in ripe-fruit extracts underestimates the amount of E4–LUC gene transcription in ripe fruit. Nevertheless, taken together, these results demonstrate that the transient gene expression system is a valid method to study the qualitative effects of promoter mutations on ethylene-inducible E4 gene transcription.

Identification of an Ethylene Regulatory Region in the E4 Promoter. To localize more precisely the regulatory sequences within the 193-bp E4 promoter that are involved in the ethylene response, an E4–LUC gene containing only 85 bp of E4 5′ flanking sequence was constructed (Fig. 2). Transcription of this gene was not induced by ethylene or ripening, indicating that DNA sequences between −193 and −85 are necessary for ethylene-inducible gene expression of the 193-bp E4 promoter.

To determine whether sequences between −193 and −85 are the only E4 promoter sequences that confer ethylene responsiveness, we deleted this region from an E4–LUC gene that contained 1421 bp of E4 5′ sequence, designated −1421Δ−193/−85 (Fig. 2). The effect of the deletion was to greatly decrease ethylene-inducible transcription, resulting in a 3- and 10-fold reduction in relative LUC activity in ethylene-treated unripe fruit and ripe fruit, respectively. To ensure that this reduced level of expression was not the result of altered spacing caused by the 108-bp deletion, E4 promoter sequences from −193 to −86 were replaced with a 108-bp plasmid DNA fragment. Expression of this construct, −1421sub−193/−85, was also decreased in ethylene-treated unripe and ripe fruit (Fig. 2). Thus, the region from −193 to −85 is required for high levels of ethylene-regulated E4 gene transcription.

Additional E4 promoter 5′ deletions with endpoints between −193 and −85 were constructed to delineate the sequences involved in the ethylene response (Fig. 3). Removal of nucleotides −193 to −161 had little effect on ethylene- and ripening-inducible E4–LUC gene expression (Fig. 3). However, deletion of sequence to −140, while not completely inactivating the ethylene response, reduced the level of E4–LUC gene expression in ripe fruit by a factor of 4. Internal deletion of nucleotides −140 to −85 within the context of the −193 E4 promoter abolished any ethylene- or ripening-regulated transcription (Fig. 3). These results demonstrate that the DNA sequence between −161 and −85 is necessary for full ethylene-responsive expression of the 193-bp E4 promoter and suggest that the 5′ boundary of an ethylene response region lies between −161 and −140.

Detection of a Fruit Nuclear Factor That Interacts with DNA Sequences Included in the Ethylene Response Region. Gel electrophoresis mobility-shift experiments were performed to determine whether sequences included in the ethylene regulatory region interact specifically with proteins in tomato fruit nuclei. To this end, nuclear protein extracts were isolated from unripe fruit, unripe fruit exposed to ethylene, and ripe fruit. A ^{32}P-end-labeled E4 promoter fragment from −193 to +13 interacted with factor(s) in an unripe-fruit extract (Fig. 4*A*). Binding activity was lower in extracts isolated from ethylene-treated unripe fruit or ripe fruit. Addition of a 10-fold molar excess of an unlabeled E4 promoter fragment from −193 to −85 eliminated binding to the labeled E4 fragment (−193 to +13). These data show that

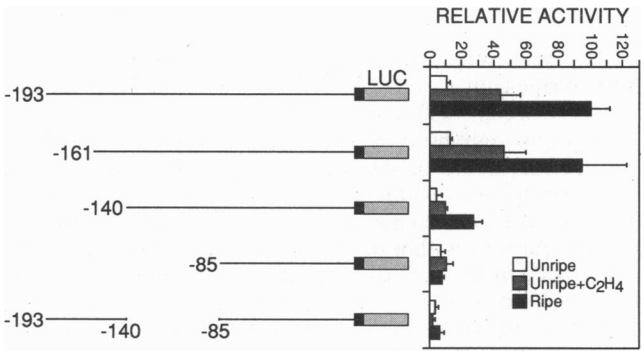

FIG. 3. Deletion analysis of the 193-bp E4 promoter identifies sequences required for the ethylene response. Schematic representations of E4 promoter–LUC chimeric genes with deletion endpoints in base pairs are shown on the left with corresponding relative LUC activities shown on the right. Transient expression of these chimeric genes introduced into tomato fruit pericarp by particle bombardment was determined as described for Fig. 2. Data are expressed relative to the 193-bp E4–LUC activity in ripe fruit and represent the average of at least four independent bombardments. Error bars indicate SEM. Lines indicate E4 5′ flanking sequence; small black boxes represent 65 bp of E4 transcribed sequence coding for 17 amino acids of the E4 polypeptide; Unripe, mature green fruit incubated in air; Unripe + C_2H_4, mature green fruit incubated with ethylene; Ripe, 50% red fruit incubated in air.

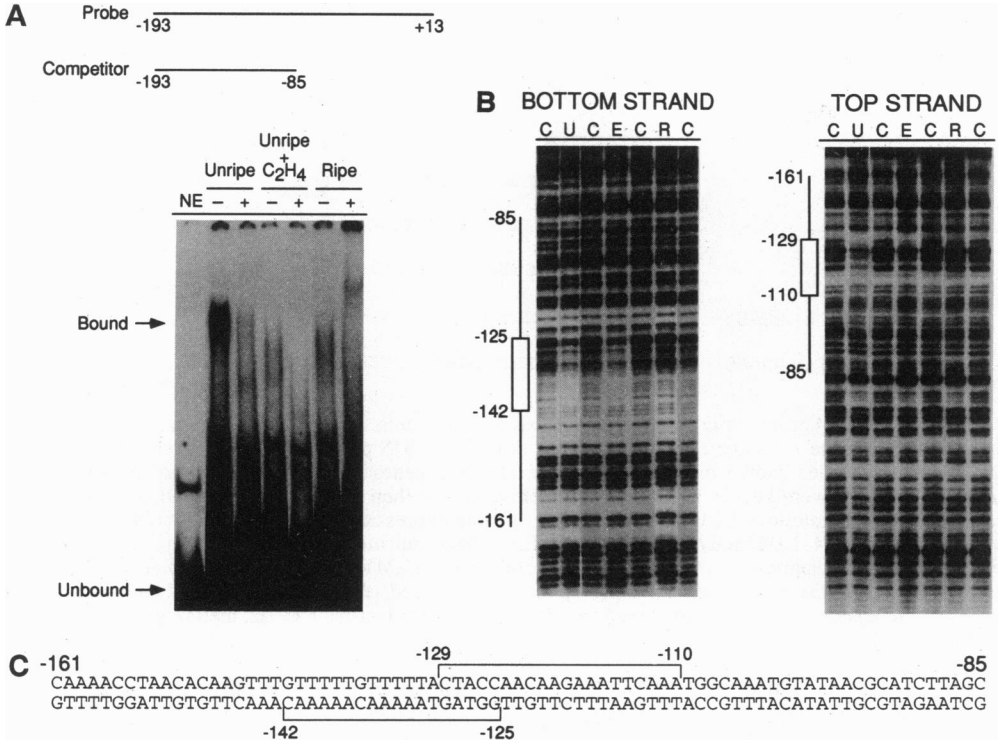

FIG. 4. Interaction of E4 promoter sequences with factors present in fruit nuclear protein extracts. (*A*) Gel electrophoresis mobility shift of an E4 promoter fragment by nuclear extracts isolated from unripe fruit exposed to air or ethylene and by extracts from ripe fruit. An end-labeled E4 promoter fragment (−193 to +13) was incubated with fruit nuclear proteins in the presence (+) or absence (−) of a 10-fold molar excess of unlabeled competitor E4 DNA (−193 to −85). Probe, ^{32}P-labeled E4 DNA fragment from −193 to +13; Competitor, unlabeled E4 DNA fragment from −193 to −85; NE, control reaction without nuclear extract; Unripe, nuclear extract from mature green stage 1 fruit; Unripe + C_2H_4, nuclear extract from ethylene-treated mature green stage 1 fruit; Ripe, nuclear extract from 30% red fruit. (*B*) DNase I footprint patterns defined by nuclear extracts isolated from unripe fruit exposed to air or ethylene and from ripe fruit. An E4 promoter fragment from the *Dra* I site (−193) to the *Mnl* I site (+13) was subcloned into the *Sma* I site of pUC119 and labeled at the +13 end (top strand) or the −193 end (bottom strand). DNase I digestion was carried out and analyzed by electrophoresis in an 8% polyacrylamide denaturing gel. All lanes contained equal amounts of ^{32}P-labeled DNA and nuclear protein. The ethylene response region is delineated by the solid lines on the left side of each autoradiogram, and the regions protected from DNase I digestion in the presence of nuclear protein are designated by the open boxes. Products of Maxam–Gilbert G and G+A sequencing reactions were electrophoresed in the same gel to determine the location of the protected regions (data not shown). Lanes: C, DNase I cleavage pattern without nuclear protein; U, nuclear extract from unripe (mature green stage 1) fruit; R, nuclear extract from ripe (30% red) fruit; E, nuclear extract from ethylene-treated unripe (mature green stage 1) fruit. (*C*) Delineation of the E4 promoter sequences protected by the fruit nuclear proteins in the footprint analysis. The DNA sequence of the ethylene response region from −161 to −85 is shown. Bracketed sequences on either the top or the bottom strand correspond to the regions of protection detected in *B*.

a DNA-binding factor that is more active in nuclear extracts from unripe fruit interacts specifically with the E4 promoter region between −193 and −85.

To define more precisely the E4 DNA sequences that bind the nuclear factor(s), we performed DNase I footprinting experiments. Incubation of the −193 to +13 E4 promoter fragment with the fruit nuclear extracts revealed protection of DNA sequences within the ethylene response region on the top strand, from −129 to −110, and on the bottom strand, from −142 to −125 (Fig. 4*B*). These sequences were differentially protected by the various fruit extracts, showing the strongest protection in unripe fruit, weaker protection in ethylene-treated unripe fruit, and no protection in ripe fruit. These data suggest that the reduced DNA-binding activity detected in ethylene-treated and ripe-fruit extracts in the gel mobility-shift experiments was due to a decrease in protection of E4 promoter sequences between −142 and −110. It is unlikely that the extracts from ethylene-treated unripe and ripe fruit were preferentially inactivated or degraded during isolation, because DNase I footprint analysis using these extracts revealed constitutive DNA-binding activities that interacted strongly with E4 promoter sequences 5′ to −161 and with the promoter of a tomato gene encoding the ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS* 3A promoter) as reported by Manzara *et al.* (18) (data not shown). Taken together, these data suggest that the decrease in DNase I protection of the E4 promoter between −142 and −110 is not the result of a nonspecific loss of DNA-binding activities in extracts from ethylene-treated unripe and ripe fruit.

DISCUSSION

The regulation of tomato fruit ripening by ethylene has been well established (20) and is thought to be mediated, at least in part, by ethylene-induced gene expression. To understand at the molecular level how ethylene controls gene expression during ripening, we have examined the cis elements and trans-acting factors that are involved in the ethylene-regulated expression of the E4 gene.

Functional Definition of DNA Sequences Involved in Ethylene-Responsive Gene Expression. Using both transgenic tomato plants and a transient gene expression system, we determined that 161 bp of E4 5′ flanking sequence are sufficient for ethylene-inducible gene expression during fruit ripening, and we localized the ethylene-responsive region to a 76-bp DNA segment from −161 to −85 (Fig. 3). In addition, at least one other positive regulatory element, between −1421 and −193, can independently mediate low-level ethylene-responsive gene transcription, and optimal expression

is obtained with the intact 1421-bp E4 promoter (Fig. 2). The sequences in the ethylene response region were compared with other ethylene-regulated promoters to determine whether similar regions mediated the ethylene response in different genes. No significant homologies were detected in ethylene-regulatory sequences of the tomato E8 gene (21), a bean chitinase gene (3), the carnation SR12 gene (22), or an avocado cellulase gene (23).

A Fruit Nuclear Factor Interacts with Sequences Within the Ethylene Response Region. Promoter sequences involved in the regulation of gene transcription generally function by interacting with nuclear factors which influence the formation of an active transcription complex (24). In this study, gel mobility-shift and DNase I footprinting experiments show that a fruit nuclear DNA-binding factor interacts specifically with nucleotides between −142 and −110, which are contained within the functionally defined ethylene response region. It is possible that this *in vitro* DNA-binding activity represents a trans-acting factor that is involved in ethylene-inducible gene expression. In support of this hypothesis, deletion of a DNA segment from −140 to −85, which includes almost all of the protected region, abolishes LUC expression from a 193-bp E4 promoter in response to both ethylene and ripening (Fig. 3). Therefore, DNA sequences protected by a fruit nuclear factor *in vitro* are included in the region necessary for ethylene-responsive E4 gene expression *in vivo*.

Comparison of the DNA-binding activity in mobility-shift experiments and the DNase I footprint patterns generated by the fruit nuclear extracts indicates that these sequences are most strongly protected by extracts from unripe fruit, where the E4 gene is not expressed, with little to no protection by extracts from ethylene-treated unripe and ripe fruit, where E4 is transcribed (Fig. 4). Thus, we have defined a positive ethylene response element which, paradoxically, interacts most strongly with a nuclear factor when ethylene levels are low. To explain these results, we propose that E4 transcription is stimulated in response to ethylene by a factor that binds the region from −142 to −110 but is not detected in ethylene-treated unripe and ripe fruit by DNase I footprint analysis. It is possible that the factor which binds the ethylene response region when E4 is not transcribed is modified when ethylene levels increase, so that it interacts differently with the DNA and activates transcription. For example, transcription of a phospho*enol*pyruvate carboxykinase gene is regulated by the relative abundance of the mammalian regulatory proteins Fos and Jun, which bind as homo- or heterodimers to the same sites in the gene's promoter (25). At low protein concentration, DNase I footprints can be detected with the Fos/Jun heterodimer, which does not activate transcription, but not with the Jun/Jun homodimer, which stimulates transcription. This is analogous to our observation that the DNase I footprint in the ethylene response element is detected in the unripe fruit extract but not in the ripe fruit extract. The ability of the ethylene response element-binding factor detected in unripe fruit to induce E4 gene expression may be modulated by ethylene through a signal transduction pathway that alters protein–protein interactions or modifies the binding factor. Regulation of the E4 gene through the binding of a factor whose transcriptional activity is determined by ethylene concentration would be consistent with the strict dependence of E4 transcription on elevated levels of ethylene.

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