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The Promoter for Tomato 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Gene 2 Has Unusual Regulatory Elements That Direct High-Level Expression[1]

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The promoter region of tomato (*Lycopersicon esculentum*) 3-hydroxy-3-methylglutaryl coenzyme A reductase gene 2 (HMG2) has been analyzed using the transient expression of HMG2-luciferase fusions in red fruit pericarp. The mRNA for HMG2 accumulates to high levels during fruit ripening, in a pattern that coincides with the synthesis of the carotenoid lycopene. Unlike most promoters, the region that is upstream of the HMG2 TATA element is not required for high-level expression. The 180-bp region containing the TATA element, the 5′ untranslated region, and the translation start site are comparable in strength to the full-length 35S cauliflower mosaic virus promoter. Pyrimidine-rich sequences present in the 5′ untranslated leader are important in regulating expression. Also, the ATG start region has been found to increase translation efficiency by a factor of 4 to 10. An alternative hairpin secondary structure has been identified surrounding the HMG2 initiator ATG, which could participate in the translational regulation of this locus. HMG2 appears to be a novel class of strong plant promoters that incorporate unusual, positive regulators of gene expression.

Isoprenoids are the essential components of more than a dozen classes of end products in both plants and animals. These include cholesterol and phytosterols, which are required for membrane biosynthesis; heme A, chlorophyll, and quinone side chains, which are involved in electron transport; dolichol, which is required for glycoprotein synthesis; and hormones such as auxins and cytokinins in plants and steroid hormones in animals (Sabine, 1983; Priess, 1985; Bach, 1987; Bach et al., 1991). Two pathway intermediates, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are covalently attached to key cell-cycle regulators and structural proteins such as nuclear lamins, thus integrating the isoprenoid metabolism with the regulation of cell division (Omer and Gibbs, 1994). The accumulation of abundant end products of this pathway is rate-limited by the first committed step in isoprenoid biosynthesis. In this reaction the enzyme HMGR catalyzes the NADPH-dependent reduction of HMG-CoA into mevalonate. Mevalonate is decarboxylated and phosphorylated to yield five-carbon isopentenyl pyrophosphate, which serves as the building block of all isoprenoids. Because of its role in regulating cholesterol accumulation, the single-copy mammalian HMGR gene has been extensively studied, whereas the regulation of the multi-copy plant HMGRs genes is not well understood.

The first HMGR cDNA was isolated from Chinese hamster ovary cells by amplifying the gene in challenges with the specific inhibitor compactin (Chin et al., 1982). The unique HMGR gene of animal genomes is extensively regulated in its expression. Comprehensive analysis of the animal HMGR promoter has led to the identification of positive and negative steroid-responsive elements, which regulate activity of the promoter (Gil et al., 1988). In addition, it has been shown that a complicated array of post-transcriptional events, such as mRNA processing (Simonet and Ness, 1989; Goldstein and Brown, 1990), stability (Edwards et al., 1977), and translation efficiency (Verdi and Campagnoni, 1990), as well as protein activity (Edwards et al., 1977; Beg et al., 1987; Zammit and Easom, 1987) and degradation (Edwards et al., 1983), modulate the expression of this gene. Apparently, animal cells utilize this sophisticated regulation of HMGR expression to modulate the response of a single gene to requirements for different isoprenoid end products.

Recent studies indicate that, unlike animal systems, most plant genomes contain multiple copies of HMGR. There are two genes in Arabidopsis (Caelles et al., 1989), at least two genes in *Hevea brasiliensis* (Chye et al., 1991, 1992), at least three genes in potato (Oba et al., 1985; Yang et al., 1991), and two genes in wheat (Aoyagi et al., 1993). In most plants HMGR genes are differentially expressed in organs, tissues, or during normal stages of development (Caelles et al., 1989; Burnett et al., 1993). Additionally, in potato and tomato (*Lycopersicon esculentum*) the HMGR genes are differentially expressed in response to a pathogen attack (Yang et al., 1991; Choi et al., 1992; Park et al., 1992). These observations may reflect specialization of the different members of the HMGR gene family. In contrast to a single animal HMGR gene, which is regulated in response to a wide variety of extracellular and intracellular signals, the expression of the individual members of the HMGR gene

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Abbreviations: 5′UTR, 5′ untranslated leader; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HMGR 1/2/3/4, individual tomato HMGR genes (four total); Luc, firefly luciferase; 35S, 35S cauliflower mosaic virus promoter.

family in plants may be modulated by distinct stimuli (Chappell, 1995).

The HMGR gene family in tomato consists of four genes (Park et al., 1992), the most highly expressed of which are HMG1 and HMG2. Expression of these two genes is regulated during tomato development (see "Results"). The initial focus of our work has been on HMG2, since its pattern of activation does not require cell division, is developmentally regulated, and can be induced. Our hypothesis is that HMG2 is a late-evolving gene with a specialized function in tomato. Therefore, the induction of this gene may be more direct and tractable than that of other HMGR genes, in which their function is essential for viability.

In this study the regions of the HMG2 promoter that are responsible for high-level expression are defined in particle-bombardment experiments in ripe tomato fruits. Luc reporter constructs containing deletion or substitution derivatives of the HMG2 promoter are used to define the regions that are controlling expression. Both transcriptional and translational fusions are tested. The elements that are crucial for high-level HMG2 gene expression are located within a 180-bp region that extends from the TATA box to the translation start site. Sequences more than 20 bp upstream from the TATA element do not play a significant role in high-level expression. Surprisingly, the 5′ untranslated region and coding sequence of HMG2 contain elements and potential secondary structures that are essential for high-level gene expression.

MATERIALS AND METHODS

Plasmids

The plasmids 35S-Luc and pLuc have been previously described (Ow et al., 1986; Montgomery et al., 1993). pLuc was altered by modifying the *Bam*HI site to form a *Sma*I site, which was accomplished by inserting a 12-mer linker (5′-GATCTCCCGGGA-3′) into a *Bam* HI site. The plasmid HMG2-Luc was constructed by cloning the 4.5-kb HMG2 *Hin*dIII-*Bgl*II promoter fragment into the *Hin*dIII-*Sma*I sites of modified pLuc. The integrity of the open reading frame was verified by sequencing. The HMG2-Luc construct was modified to contain *Spe*I and *Pst*I sites upstream of the promoter region by inserting a double-stranded 18-mer linker (5′-AGCTACTAGTAGGCCTGC-3′) into the *Hin*dIII site.

The 5′ deletions of the 4.5-kb HMG2 promoter fragment were produced by exonuclease III treatment (Ausubel et al., 1992) of the *Pst*I- and *Acc*65I-digested HMG2-Luc construct. Plasmids with deletions of the 5′ untranslated regions (−900-5′Δ and −58-5′Δ) were constructed by amplification of the −900 to +13 (with reverse and 5′ PCR primers) and −58 to +13 (with −20T and 5′ PCR primers) HMG2 promoter regions and subsequent cloning into *Pst*I- and *Age*I-digested HMG2-Luc. The 5′ PCR primer sequence is: 5′-CCCCACCGGTGAGTGATGGGATGTG-3′. The sequence of the −20T primer is: 5′-CCCCTGCAGCGCGGCAACCGGGTACCTC-3′. In the construct −900-5′Sub the 90-bp substitution fragment was amplified from 35S-Luc (3463–3553) by *Xma*I-containing primers (5′Sub1: 5′-CTCGGTACCCGGGGATC-3′; 5′Sub2: 5′-GCAACCTTCCCGGGGTTCGT-3′) and cloned into the *Age*I site of −900-5′Δ. The sequence of the substitution fragment is: CCGGG GATCCAGGGGAATTCGATCCAACACTTACGTTTGCAACGTCCAAGAGCAAATAGACCACGAAC.

The 900Trx-Luc transcriptional fusion was constructed by amplifying the −900 to +13 region of the HMG2 promoter and cloning it into the *Spe*I and *Sma*I sites of pLuc. 900Trx-Luc was subsequently modified to contain a *Pst*I site upstream from the promoter sequence by the insertion of a 14-mer linker (5′-CTAGCCCTGCAGGG-3′) into the *Spe*I site.

The 5′ deletions of the 900-bp promoter fragment in the 900Trx-Luc construct were produced by the exonuclease III treatment (Ausubel et al., 1992) of the *Pst*I and *Acc*I digested 900Trx-Luc construct.

35STnl-Luc was constructed by amplifying the 1.5-kb 35S promoter sequence from 35S-Luc with reverse and *Xma*I-containing (TCCAAGCCCGGGTCCTCTCC) primers and then cloned into the *Age*I-*Spe*I sites of the modified HMG2-Luc.

pH2ΔCT is derived from the 900-bp exonuclease III deletion of the HMG2-Luc so that the region from +59 to +81 is substituted with *Bam*HI by oligo-directed mutagenesis (5′-TAAGTCTGCCGCCGGTGGATCCTGTTGTTTGTGGG-3′).

T7-Luc and T7-82H.Luc were constructed by cloning *Bam*HI-*Sst*I fragments of pLuc and pH2ΔCT in the pGEM-4Z vector (Promega). All constructs were verified by sequencing using reverse or Luc (5′-CGGCGCCATTCTATCCTCTAGAGG-3′) sequencing primers.

Plant Material and Cell Bombardments

The tomato (*Lycopersicon esculentum*) cv VFNT (LA1221) was used in particle-bombardment experiments as described previously (Manzara et al., 1994), and Luc activity was assayed in bombarded tissue after 20 h of incubation at 20°C. Each construct was used in at least three independent experiments; and each experiment included three replica bombardments. The mean of those experiments and the SD were calculated and are presented in the figures.

Enzyme Assay

Luc was assayed in cell extracts using the Luciferase Assay System (Promega), and its activity was expressed in terms of the number of arbitrary light units detected in 10 s at 25°C using a luminometer (model TD-20e, Turner, Sunnyvale, CA) as described previously (Manzara et al., 1994).

RNA Isolation and RNA Gel Blot Hybridization

RNA was isolated from young (0.6–0.8 cm) and ripe tomato fruit pericarp according to standard methods (Ausubel et al., 1992). Five micrograms of total RNA was separated on Mops/formaldehyde agarose gels, blotted onto Hybond-N filters (Amersham), and hybridized following standard procedures (Narita and Gruissem, 1989). Oligonucleotides that were used as probes were 5′-end radiolabeled using T4 polynucleotide kinase (GIBCO/BRL). The

HMG1-specific oligonucleotide (5′-CAACAGTCTTGCATTTGAACCTGGTGCATCTCTATTTGCACCTTTAACTC-3′) and the HMG2-specific oligonucleotide (5′-CAAGAG-CCTTGCATTTGACCCTGGTGCCTGTCTGTTGGCACCTTTC-ACTC-3′) were from the homologous regions of exon 4. The HMGR universal oligonucleotide (5′-ATATGGAGGTCTT-TGCCATCATTTACAGCCTCCATCATAGTGATGCAGTG-3′) was complementary to a conserved region of exon 3.

In Vitro Transcription and Cell-Free Translation of the Luc mRNA

In vitro transcription of the *Luc* gene was performed using T7 polymerase (Ambion, Austin, TX) in a 100-μL reaction according to the standard protocol (Ausubel et al., 1992). RNA was precipitated from the reaction by 8 M LiCl and dissolved in water.

One microgram of Luc RNA was translated in wheat germ extract (Promega) for 20 min according to the protocol provided in a 25-μL reaction. Five-microliter aliquots were assayed for Luc activity as described above.

RESULTS

Tomato HMG1 and HMG2 Genes Are Differentially Expressed in Young and Ripe Fruit

To analyze the pattern of HMG1 and HMG2 gene expression in tomato fruit, the mRNA from young and ripe fruit was hybridized with HMG1- and HMG2-specific probes. The results are shown in Figure 1. HMG1 mRNA accumulates to high levels exclusively in the young fruit, whereas HMG2 mRNA accumulates only during the last stages of tomato fruit ripening. The presence of developmentally regulated HMGR isoforms in tomato plants appears to accommodate the changing needs for diverse cellular isoprenoids during growth and development of the fruit. Specifically, expression of HMG1 appears to be required for normal membrane biogenesis in the actively dividing cells of young fruit, whereas HMG2 gene expression may be responsible for carotenoid accumulation during the last stages of tomato fruit ripening. To understand why two HMGR genes are highly expressed in fruit requires a better comprehension of what controls their activation.

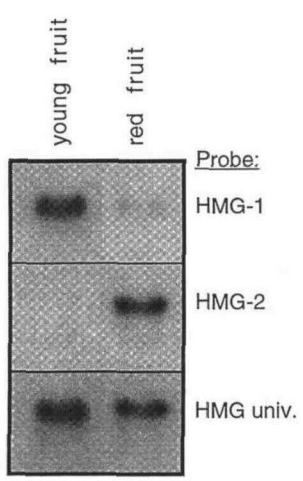

Figure 1. The HMG1 and HMG2 genes are differentially expressed during tomato fruit development. Northern blot analyses were performed with total RNA isolated from young (0.6–0.8 cm) and ripe tomato fruit. Radiolabeled HMG1- and HMG2-specific oligonucleotides and an oligonucleotide from a region conserved between both HMGR genes (HMGR univ.) were used as probes.

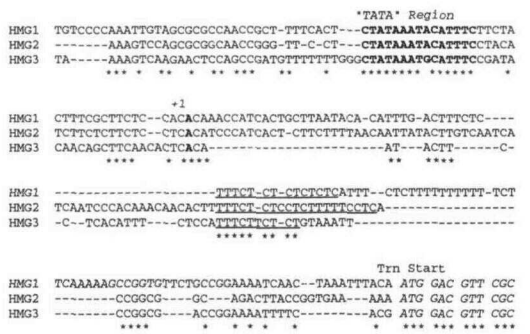

Figure 2. Alignment of the HMG1, HMG2, and HMG3 promoter regions. The TATA box region is shown in boldface. The first transcribed nucleotide is marked +1 and is also shown in boldface. The coding sequence of each gene is presented in italics, and the pyrimidine-rich stretches within the 5′ untranslated region of each gene are underlined. Positions that are conserved in all three genes are indicated by asterisks (*).

Tomato HMGR Genes Contain Several Conserved Elements

Figure 2 shows the alignment of the promoter regions of the HMG1, HMG2, and HMG3 genes. The upstream promoter regions are not homologous (data not shown). In contrast, the TATA element is highly conserved and unusually long. No obvious CCAAT box can be identified in any of the HMGR promoters. The transcription start site has been mapped for *HMG1* using RNase protection (J.O. Narita, unpublished observation) and appears to be conserved in all four HMGR genes. The 5′ untranslated region does not show any extensive homology except for the presence of pyrimidine-rich stretches (underlined) in all three genes. HMG1 and HMG2 genes are more homologous to each other than to HMG3. The region of homology extends from −60 to +1 and includes the TATA box.

The coding sequences of the HMGR gene family are highly homologous in the region encoding the catalytic domain of the enzyme. In contrast, the first exon, which is believed to encode the membrane-spanning domain (Campos and Boronat, 1995), differs significantly among these three genes. Despite these differences, the sequence corresponding to the first six amino acids is almost identical among the genes. It is intriguing that the sequence around the initiator ATG of HMG2 is capable of forming two alternative hairpin structures. This feature is unique to HMG2 and is not found in the other genes, even though the participating coding sequence is identical in the three cases. This hypothetical structure could be involved in regulating gene expression, and this possibility will be discussed in more detail below.

Expression of 5′ Deleted HMG2 Translational Fusions

Reporter gene expression from an introduced promoter can be accomplished using either transcriptional or translational fusions. The use of both types of reporter constructs can facilitate the identification of positive or negative transcriptional elements that may be masked by sequences controlling translation efficiency. Both types of fusions were tested for HMG2, since the absence of a CCAAT box and the presence of a long, 108-bp untranslated leader made this an unusual plant promoter. The first set of constructs that was tested was the translational fusions of the HMG2 promoter with the Luc reporter gene. The initial HMG2-Luc construct contained a 4.5-kb promoter region (−4500 to +129) including the 5′UTR, the initiator ATG, and the first seven amino acid residues from HMG2 fused in-frame with a Luc coding sequence. Hence, the N terminus of the Luc was modified by addition of the first seven amino acids of the HMG2 gene and the conversion of the Luc 5′ untranslated region into a coding sequence (Fig. 3A). The terminator sequence from the nopaline synthase gene flanks the 3′ end of the Luc gene. This construct was tested using a transient expression system based on particle bombardment of ripe tomato fruit. The activity of the HMG2 promoter was comparable to that of a strong plant promoter, 35S (Fig. 3B).

To determine the 5′ boundary of the region that is important for the high-level activity of the HMG2-Luc fusion,

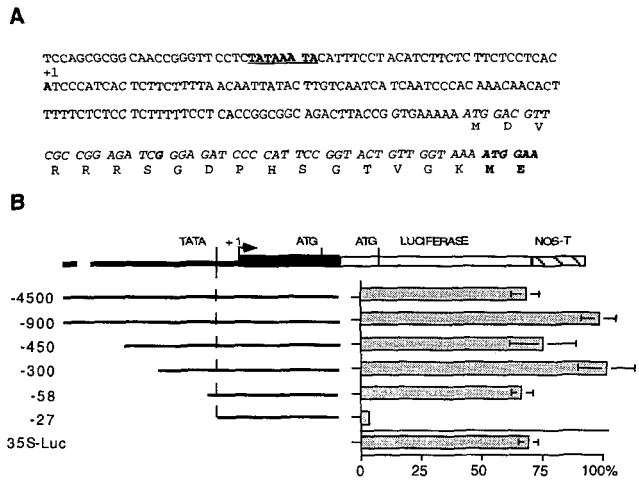

Figure 3. Analysis of the HMG2-Luc translational fusions. A, The structure and sequence of the HMG2-Luc translational fusion region. The first seven amino acids of the HMG2 coding region (italics) are fused in-frame with the Luc coding sequence (boldface italics). As a result, the Luc 5′UTR (starting with the boldface italic G) became a 10-amino acid insertion between the HMG2 and Luc coding sequences. B, Expression of the 5′ promoter deletions of the HMG2-Luc fusion. The end points of each construct are shown on the left, and the level of Luc expression is shown on the right. Luc expression for each construct is shown as a percentage of the −900 HMG2-Luc fusion, which has the maximum level of expression. The vertical line in the promoter denotes the position of the TATA element. Note that the −58 construct retains the TATA, whereas the −27 constructs have lost this region (refer to the sequence in A). The level of Luc expression driven by the strong viral 35S promoter is given for comparison.

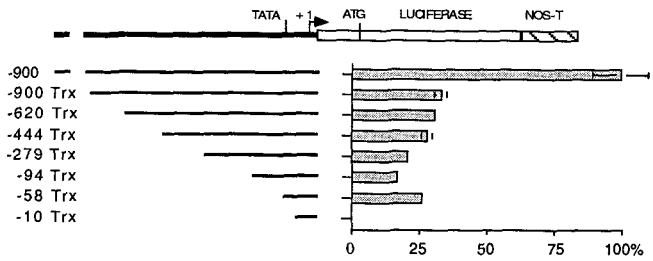

Figure 4. Expression of the HMG2-Luc transcriptional fusions. The end points of each transcriptional fusion are shown on the left and are comparable to those shown in Figure 2. The level of Luc expression for each deletion is shown as a percentage of the −900 HMG2-Luc translational fusion expression level. NOS-T, Nopaline synthase gene 3′ end.

a series of 5′ deletions of the 4.5-kb HMG2 promoter was constructed. The constructs were derived from HMG2-Luc and contained a 5′ deleted HMG2 promoter fragment (5′ end point from −4500 to −28 relative to the transcription start site). Luc activity from the constructs was measured in transient gene expression assays, following the introduction of the plasmids into ripe tomato fruit by microprojectile bombardment. The activity for the 5′ promoter deletions relative to the activity of the −900 construct, which showed maximum activity, is shown in Figure 3B. Relative activities from the constructs with 5′ deletion end points between −900 and −58 were essentially equal to the full-length promoter. For the shortest truncated version of the HMG2 promoter (−28) lacking the TATA box (located −29 to −36), relative activity of 1% or less was observed.

Thus, in the translational fusion, the upstream portion of the HMG2 promoter seems to be unimportant for the high level of expression, but the presence of the TATA box is essential. The −58 HMG2-Luc fusion is as active as the strong viral 35S promoter. This finding suggests that either the HMG2 TATA region itself defines a very strong promoter, or that additional positive regulatory elements involved in expression are located downstream in the 5′UTR region.

Expression of 5′ Deleted HMG2 Transcriptional Fusions

To complement the preliminary analysis, a transcriptional fusion of the 900-bp HMG2 promoter fragment to the Luc gene was constructed, which contained the −900 to +13 region of the HMG2 promoter driving the Luc gene (Fig. 4). A series of 5′ deletions of this construct was generated, with the 5′ end ranging from −900 to −58. All constructs were tested by bombardment-mediated transient gene expression in ripe tomato fruit; the result of this experiment is presented in Figure 4. The transcriptional fusions were all 3- to 4-fold lower in activity than the −900 translational fusion. However, the overall pattern of Luc activity for the sequential 5′ end deletion of the 900 Trx-Luc nearly reproduces the patterns seen in the 5′ deletion of the translational fusions. In both transcriptional and translational fusions the region upstream from the TATA box appears to be unimportant for high-level expression, whereas the presence of the TATA box is absolutely nec-

essary. These results support the hypothesis that positive regulatory elements are located downstream from the TATA region, presumably within the 5′ untranslated region of the HMG2 gene.

Role of the HMG2 5′ Untranslated Region in HMG2 Gene Expression

The data described previously suggest that there are transcriptional and translational components regulating HMG2 gene expression. The elements modulating the level of HMG2 mRNA translation presumably are located within the 5′UTR and/or the first seven amino acids of the HMG2 coding region. To test whether the 5′ untranslated region of the HMG2 gene is important for high-level expression, plasmids were constructed representing the translational fusions of the 900-bp HMG2 promoter; the 5′ untranslated region of the HMG2 mRNA was deleted (−900-5′Δ). To exclude the possibility that the length of the 5′UTR alone can affect the level of Luc expression, this region was substituted with an unrelated vector sequence of the same length (−900-5′Sub). Both constructs were tested in ripe tomato fruit by the particle-bombardment assay. Relative activities of 7% (−900-5′Δ) and 13% (−900-5′Sub) were observed (Fig. 5). The same reduction in the level of Luc expression was observed when the 5′ untranslated region was removed from the shortest version of the translational HMG2 promoter-Luc fusion (−57-5′Δ). Therefore, we concluded that the presence of the 5′UTR of HMG2 is critical for efficient expression from the HMG2 locus. It remains unclear, however, whether the elements contained within the 5′UTR modulate the expression of HMG2 on a transcriptional or posttranscriptional level.

Role of the Translational Start Site in the Regulation of HMG2

To investigate the role of the translation start site and coding region in the expression of the Luc fusions, a construct was made in which the 35S promoter drives the translational fusion of the first seven amino acids of the HMG2 gene and Luc coding region. The structure and the sequence of the fusion is exactly the same as in HMG2-Luc starting from position +82. The activity of this construct was compared with the standard 35S-Luc fusion, described above. The results from this experiment are presented in Figure 6A. Inclusion of the 5′ end of the HMG2 coding sequence in front of the Luc coding sequence was shown to enhance the level of expression by approximately 3-fold.

The role of the translational start site was further analyzed by in vitro translation. For this, two constructs were made: T7-Luc, containing the Luc gene with its own 5′UTR under the T7 promoter, and T7-82H.Luc, wherein fusion of the HMG2 translation start site and first seven amino acids and Luc (structurally identical to HMG2-Luc fusion) were cloned under the T7 promoter. Both messages were transcribed in vitro and translated using wheat germ extract. The efficiency of translation was determined by assaying an aliquot of the reaction for Luc activity. The results are shown in Figure 6B. In agreement with the experiment shown in Figure 6A, a fusion of the HMG2 translation start site to the Luc gene results in a 10-fold higher translation initiation rate. The same two constructs were also tested in reactions with rabbit reticulocyte lysates (Fig. 6C). In the animal system Luc and 82H.Luc transcripts do not show a difference in translatable Luc activities. This result suggests that the additional amino acids do not affect Luc activity.

DISCUSSION

High-level expression from the HMG2 promoter appears to be controlled by at least two positive regulatory components. Surprisingly, the region upstream from the HMG2 TATA element is unimportant for high-level expression in transient assays. This result sets the HMG2 promoter apart from the majority of the analyzed promoters, in which the

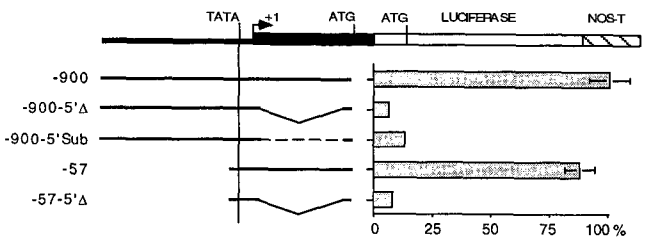

Figure 5. Expression of the HMG2-Luc translational fusions with the deleted 5′ untranslated regions. Deleted regions are indicated by the V-shaped line. The 90-bp vector sequence substituted for 5′UTR is shown with a dashed line. The level of expression for each construct is shown as a percentage of the −900 HMG2-Luc expression level. NOS-T, Nopaline synthase gene 3′ end.

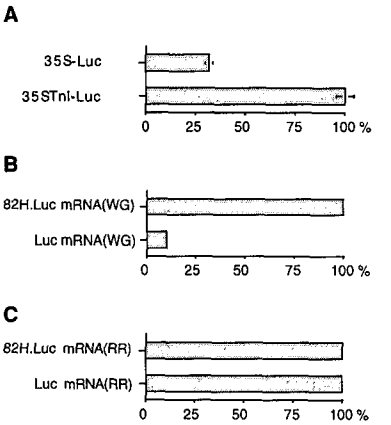

Figure 6. The HMG2 translation start region enhances translational efficiency. A, Effect of the HMG2 translation start site on the expression of the Luc driven by 35S. The level of Luc expression of 35S-Luc is given as a percentage of 35S-HMG2-Luc fusion. B, Luc expression in the wheat germ cell-free translation system. Luc mRNA and in-frame fusion of the Luc mRNA with the HMG2 translation start region (82H.Luc) were translated in the wheat germ extract (WG). Luc activity in a reaction was assayed, and results are given as a percentage of 82H.Luc expression level. C, Luc expression in the rabbit reticulocyte cell-free translation system. Luc mRNA and in-frame fusion of the Luc mRNA with the HMG2 translation start region (82H.Luc) were translated in rabbit reticulocyte (RR) extract. Luc activity in a reaction was assayed. Results are given as a percentage of 82H.Luc expression level.

presence of positive and/or negative upstream regulatory elements is known to be necessary for the modulation of activity. Deletion of the 5′ untranslated region from the HMG2 promoter reduced the level of expression by a factor of 10, which indicates that a positive regulatory element is located within 5′UTR of the HMG2. However, the deleted 5′UTR construct still retained a level of expression at least 10 times higher than the background level that was found with a TATA-less construct. Apparently, the TATA element of the HMG2 gene is capable of providing transcription at a significant level. For comparison, deletion of the CCAAT box from the 35S-Luc construct resulted in a 100-fold decrease in the Luc activity in the transient assays (data not shown). Notably, the −57 to +1 region of HMG1 and HMG2, which are both highly expressed genes, are very homologous, but differ significantly from the corresponding region of the HMG3 gene, which is expressed at a much lower level. Conceivably, the HMG2 TATA element may possess a high affinity for the components of the basal transcription complex. Experiments are underway to identify a protein(s) that interacts with this region.

As mentioned above, the 5′ untranslated region of the HMG2 gene contains element(s) responsible for the high-level expression of the gene. The presence of a regulatory element within the 5′ untranslated region is unusual, but not unprecedented. Sequence analysis of this region reveals homology between the pyrimidine-rich stretch and a CT box identified within the 5′UTR of the nuclear-encoded spinach chloroplast genes PsaF and PetH (Bolle et al., 1994). This CT box region has a positive effect on the transcription of a glucuronidase reporter construct in transgenic plants. Preliminary footprint and DNA-fragment gel-retardation experiments indicate that proteins specifically recognize and bind to the tomato pyrimidine-rich sequences (N.D. Daraselia, D. Rebatchouk, T. Manzara, and J.O. Narita, unpublished observations).

The region surrounding the HMG2 initiator ATG (+82 to +129) is capable of increasing the overall expression of a Luc reporter in fusions driven by HMG2 and 35S promoters, as well as from an in vitro-transcribed message. Furthermore, the results from the wheat germ and rabbit reticulocyte in-vitro translations show that the HMG2 translation start has a positive effect on protein production in the plant but not in the animal extract. This result suggests that the factors necessary for enhancing translation from the HMG2 leader may be present in the wheat germ extract but absent from the animal extract. The immediate context of the HMG2 initiator ATG (AAAAATGG) is almost identical to the context of the Luc ATG and differs only at position −4, which is T for Luc. Thus, the possibility exists that the difference in translation efficiency of Luc and HMG2-Luc messages is determined by this single base mismatch in the ATG context. It is interesting that the AAAAATGG context was demonstrated to enhance translation of the GUS mRNA by about 10-fold (Helliwel and Gray, 1995). Alternatively, the effect of the translation enhancement in HMG2-Luc fusion can be explained by the presence of more complex *cis*-acting elements. The alignment of the HMGR gene coding sequences revealed two

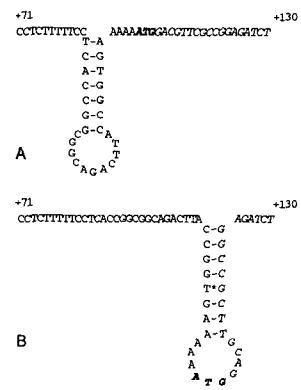

Figure 7. Two alternative hairpin structures that can potentially form around the HMG2 initiator ATG. The first hairpin (A) is formed upstream from ATG and consists of a perfect 7-bp stem and an 11-bp loop. The second hairpin (B) includes ATG in the center of a 12-bp loop and contains one G-T pair within the 7-bp stem.

interesting features. First, despite the fact that the first exon of three tomato HMGR genes is highly heterologous, the first 20 bases immediately downstream from the ATG reveal high homology. Second, structural analysis indicates that the sequence surrounding the initiator ATG of HMG2 can potentially form two alternative hairpin structures (Fig. 7), with the free energy of −9.1 kcal/mol for the first and −9.5 kcal/mol for the second. HMG1 and HMG3 sequences are not able to form either hairpin. The pyrimidine-rich stretch, which was shown to have a positive effect on the overall expression level (Bolle et al., 1994), is adjacent to the first potential hairpin, which can be formed immediately upstream of the ATG codon. The proximity of these two potential regulatory elements is intriguing and may provide the basis for a regulatory mechanism. The simplest mechanism would involve a protein that specifically binds to the CT-rich element and stabilizes or inhibits formation of one of the hairpins, which affects the translation efficiency. Further analysis is necessary to investigate the role of each alternative secondary structure in HMG2 expression regulation.

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