Effect of the Nuclear Factors EmBP1 and Viviparous1 on the Transcription of the *Em* Gene in HeLa Nuclear Extracts

Mona A. Razik and Ralph S. Quatrano¹

Department of Biology and Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280

Templates constructed from the wheat *Em* and maize *rab28* promoters are efficiently and accurately transcribed in the well-characterized cell-free transcription system prepared from HeLa nuclei. Deletion analysis of the *Em* promoter indicates that a G-box (CACGTG) element (Em1b) is required for transcription. USF, a Myc transcription factor in HeLa nuclear extracts, activates transcription by binding to Em1b, as shown by the ability of an antibody raised against USF to inhibit transcription and to interfere with Em1b complex formation in an electrophoretic mobility shift assay. The addition of the recombinant Viviparous1 protein from maize to HeLa nuclear extracts specifically stimulated transcription of the *Em* promoter but was dependent on the presence of USF in the extract. In USF-depleted extracts, the addition of recombinant EmBP1, a basic leucine zipper transcription factor from wheat, activated transcription through Em1b as well as from a similar G-box in the adenovirus major late promoter. Our study demonstrates that the basic transcriptional apparatus in HeLa nuclear extract supports transcription from plant promoters and can be used to assay the function of certain plant nuclear proteins, thereby helping to determine their effects on transcription.

INTRODUCTION

The *Em* gene encodes an abundant protein that is found in mature embryos of developing seeds of many plants. The phytohormone abscisic acid (ABA) and the regulatory locus *viviparous1* (*vp1*) in maize (and its homolog in ABA-insensitive *Arabidopsis* [*abi3*]) are required for the embryo-specific expression of the *Em* gene (Marcotte et al., 1988; McCarty et al., 1991; Parcy et al., 1994; Rock and Quatrano, 1995). The ABA response element in the wheat *Em* promoter has been localized to a 76-bp segment (region 1) by a transient assay using rice protoplasts (Marcotte et al., 1988). Deletion of region 1, which contains two G-box (CACGTG) elements (Em1a and Em1b), eliminates the ABA response in transient assays, whereas mutations in either Em1a or Em1b dramatically reduce this ABA response. Tetramers of either Em1a or Em1b in maize or rice protoplasts can confer ABA responsiveness to a nonresponsive cauliflower mosaic virus promoter (CaMV 35S) (Marcotte et al., 1989; Vasil et al., 1995).

Electrophoretic mobility shift assays have been used to identify the presence of DNA binding proteins in wheat and rice nuclear extracts that are specific for the G-boxes in region 1. A basic leucine zipper (bZIP)-type transcription factor, EmBP1, has been isolated by screening a wheat embryo cDNA library with an oligonucleotide probe containing region 1 (Guiltinan et al., 1990). Competition experiments using recombinant EmBP1 indicate that it preferentially binds

to Em1a. In addition, a mutation in the core CACGTG of the Em1a element not only eliminates binding by nuclear extracts and EmBP1 but also eliminates the ABA response in transient assays (Guiltinan et al., 1990; Vasil et al., 1995). Consequently, EmBP1 has been identified as a factor that binds to Em1a and is thus implicated in the regulation of the *Em* promoter (Guiltinan et al., 1990).

EmBP1 belongs to a family of at least seven closely related DNA binding proteins in wheat (DeVos et al., 1991). More than 20 bZIP proteins have been isolated from plant species, and all share the same core target sequence ACGT (Ehrlich et al., 1992; Katagiri et al., 1992; Kawata et al., 1992; Schindler et al., 1992a; Foley et al., 1993; Pysh et al., 1993; Zhang et al., 1993; Foster et al., 1994; Nantel and Quatrano, 1996). Of these bZIP proteins, a group has been identified that preferentially binds to the G-box sequence CACGTG. These are called G-box binding factors (GBFs) and include EmBP1 from wheat (Guiltinan et al., 1990), osZIP from rice (Nantel and Quatrano, 1996), TGA1a from tobacco (Katagiri et al., 1990), and the GBFs from *Arabidopsis* (Schindler et al., 1992b). These transcription factors have been shown to bind to the highly conserved G-box motif located on a variety of plant promoters that are inducible by such diverse signals as UV and red light (Giuliano et al., 1988; Weisshaar et al., 1991), drought, pathogen infections, and anaerobiosis regulation (Katagiri et al., 1989; Guiltinan et al., 1990; McKendree and Ferl, 1992). Schindler et al. (1992b) have shown that the proline-rich N-terminal domain of *Arabidopsis*

¹ To whom correspondence should be addressed. E-mail rsq@unc.edu; fax 919-962-6840.

GBF1, when fused to the yeast GAL4 binding domain, can stimulate transcription in plant protoplasts and mouse 3T3 cells.

Studies using ABA-insensitive mutants have shown that the regulatory locus *vp1* is required for *Em* expression (McCarty et al., 1991). In addition, several reports (McCarty et al., 1991; Hattori et al., 1994; Vasil et al., 1995; Hill et al., 1996) have demonstrated that overexpression of the VP1 protein in maize or rice protoplasts can transactivate the *Em* promoter at subthreshold levels of endogenous ABA. In the presence of exogenous ABA and overexpression of VP1 in these protoplasts, a synergistic activation of *Em* expression has been observed. The region in the *Em* promoter required for VP1 transactivation and the synergistic effect with ABA overlaps with region 1 (Hattori et al., 1995; Vasil et al., 1995). Similar to the ABA response, tetramers of either Em1a or Em1b were shown to be sufficient for the VP1 responses, whereas mutations in the G-boxes within region 1 of the *Em* promoter prevented VP1-mediated expression (Marcotte et al., 1989; Vasil et al., 1995). Hence, promoter elements required for ABA and VP1 to enhance expression of the *Em* gene reside within region 1 and correspond to the G-box(es).

Analyses of the VP1 sequence and functional assays indicate that the N-terminal domain of VP1 can serve as a transcriptional activator (McCarty et al., 1991). Electrophoretic mobility shift assays also indicate that a truncated VP1 without activation can enhance the binding of a variety of transcription factors to their target sequences (Hill et al., 1996), including EmBP1 to Em1a and Em1b. This nonspecific enhancement effect of VP1 in vitro may occur through a weak, nonspecific, and transient association of VP1 with DNA. However, the ability of VP1 to enhance the binding of sequence-specific transcription factors in vitro has not been correlated with its effect in transactivating the *Em* promoter in protoplasts and transgenic plants. Recently, Suzuki et al. (1997) showed that the conserved B3 domain of VP1 can specifically bind to the Sph element in the *C1* gene from maize. However, unlike the regulation of *C1*, neither the Sph element in the *Em* promoter nor the B3 domain of VP1 is required for *Em* expression (Hill et al., 1996).

One approach to determining the mechanism(s) by which EmBP1 and VP1 exert their effects on *Em* expression is to use a well-characterized cell-free transcription system. For example, can recombinant EmBP1 activate transcription in vitro and can VP1 enhance transcription through a sequence-specific factor as a coactivator? Some success has recently been reported for plant in vitro transcription systems (Yamagauchi et al., 1994; Sugiura, 1996). A wheat germ chromatin extract was developed that accurately transcribed from the initiator sequence in the CaMV 35S minimal promoter and was stimulated by the DNA binding protein TGA1a (Yamazaki et al., 1990; Schweizer and Mozing, 1994). Nuclear extracts from parsley (Frohnmeier et al., 1994) and tobacco cells (Fan and Sugiura, 1994) have also been shown to support cell-free transcription. Most recently,

a whole-cell extract from rice suspension cultures was reported to be transcriptionally competent and dependent on the TATA box, the initiator sequence, and specific spacing requirements between the initiator and the TATA box for accurate transcription. However, none of these plant systems has been used to analyze functional interactions between *cis* elements and added plant transcription factors, nor have they been reproducibly used with a variety of plant promoters (Zhu et al., 1995).

The basic transcription machinery appears to be highly conserved between plants and animals and may share a common mechanism of transcriptional activation (Haas and Feix, 1992; Mukumoto et al., 1993). For example, the yeast acidic transcription factor GAL4 was shown to activate transcription in plant (Ma et al., 1988), *Drosophila* (Fischer et al., 1988), and mammalian (Kakidani and Ptashne, 1988) cells. In fact, the plant transcription factor TGA1a from tobacco was shown to activate transcription from the CaMV 35S promoter in a human reconstituted in vitro transcription system (Katagiri et al., 1990). Given these results, we decided to test the ability of the well-characterized and widely used HeLa nuclear extract (HNE) not only to support transcription from the plant *Em* promoter but also to determine how the plant factors EmBP1 and VP1 might function with the basic transcriptional apparatus of HeLa cells.

We report that templates containing G-boxes from the wheat *Em* and maize *rab28* genes (Pla et al., 1993) are accurately and efficiently transcribed in the HNE. The conserved G-box element in the *Em* promoter is required and apparently is recognized by the mammalian Myc transcription factor USF, which is present in the HNE. VP1 without its transcriptional activation domain stimulated transcription in the HNE by using the *Em* template but was dependent on the presence of USF. We show that recombinant EmBP1 can activate not only the *Em* promoter but also the adenovirus major late (*AdML*) viral promoter in the HNE depleted of USF. These results indicate that the HNE can be used to accurately transcribe plant genes that contain *cis* elements that are recognized by HeLa factors. Furthermore, HNE can be used to test the effect of certain plant nuclear factors on transcription.

RESULTS

Transcription from the *Em* and the *rab28* Promoters Using the HNE

The 113kp plasmid, containing 642 bases of the *Em* promoter fused to a β -glucuronidase (*GUS*) reporter gene (Em113kp), was linearized with EcoRV and used as a template to generate run-off transcripts in the HNE. One major transcript (697 nucleotides) of the correct size (Figure 1A) and one minor transcript were detected in the in vitro transcription assay (Figure 2A, lane 2). The addition of RNase A

Figure 1. Diagram of Various Promoters and G-Box Sequences Used in This Study.

(A) Map of the *Em* promoter-*GUS* template and the position and length (in nucleotides) of the main transcript produced in the HNE.

(B) Comparison of the regions in the *Em* (Em1a and Em1b), *rab28*, and the *AdML* promoters containing G-boxes (underlined and boldface letters).

(C) Comparison of the *Em*, *rab28*, and the *AdML* promoters with respect to the distances (in bases) between the transcription initiation site (+1), TATA, and the G-box (crosshatched) closest to the TATA box.

to the transcription reaction digested the products (Figure 2B, lane 2), whereas incubation with α -amanitin inhibited accumulation of the products (Figure 2B, lane 3). The *rab28* promoter fused to the *GUS* reporter gene was used as another plant promoter similar to *Em* (Pla et al., 1993; see Figures 1B and 1C). The *rab28* plasmid was also digested with EcoRV and used as a template to generate run-off transcripts in the HNE. Lane 4 of Figure 2C shows that the *rab28* template generated a single transcript of the correct size (650 nucleotides). The addition of RNase A to the transcription reaction digested the products (Figure 2C, lane 2), whereas incubation with α -amanitin inhibited the accumulation of the products (Figure 2C, lane 3). These results indicate that the RNA products detected from the *Em* and *rab28* promoters in the HNE were RNA transcripts generated by RNA polymerase II activity.

The 5' start site of the main *Em* transcript (CCACC) was mapped on the *Em* promoter by a primer extension assay

and found to be 32 bases downstream from the TATA box (Figure 3), which corresponds to the *in vivo* start site and the observed 697-nucleotide transcript (Figures 1A and 2A). The start site of the minor transcript is 5' to the start of the major transcript but was not mapped.

Em1b, a G-Box Element in the *Em* Promoter, Is Sufficient for Transcription in the HNE

Deletions in the 5' regions of the Em113kp (Figure 4A) were used as templates to determine the regions required for transcription in the HNE. Transcription levels of templates 1 to 4 were equivalent, as shown in Figure 4B (lanes 2 to 5). However, without the region between nucleotides -57 to -38 (template 5), no transcripts were detected (Figure 4B, lane 6). Competition with an excess of the Em1b oligonucleotide inhibited transcription from template 4 (Figure 4C,

Figure 2. Transcription Reactions in the HNE Using the *Em* and the *rab28* Promoters.

(A) Lane 1 contains a λ PstI digest, with the base (b) size markers indicated at left. Lane 2 contains the transcripts generated from the *Em* promoter-*GUS* template (Em113kp; see Figure 4A).

(B) Lane 1 contains a λ PstI (λ Pst) marker (805 bases). Lanes 2 to 4 contain transcription reactions with the Em113kp (113kp) template and the HNE (100 to 150 μ g of protein). The reaction in lane 2 had been treated with 2 μ g of RNase A. The reaction in lane 3 included 1 μ g/mL α -amanitin (Aman). The two *Em* transcripts from the Em113kp template are visible in lane 4 only. The main transcript is 697 nucleotides long (see Figure 1A).

(C) Lane 1 contains a λ PstI marker (514 bases). Lanes 2 to 4 contain transcription reactions with the *rab28* template and the HNE (100 to 150 μ g of protein). The reaction in lane 2 had been treated with 2 μ g of RNase A. The reaction in lane 3 included 1 μ g/mL α -amanitin. A single transcript from the *rab28* template is visible in lane 4 only (650 nucleotides long).

lanes 2 and 3) compared with competition with the mutant Em1b oligonucleotide that lacks the CACGTG core sequence (Figure 4C, lanes 4 and 5). The Em1b oligonucleotide, when added to the transcription assay at the same molar excess, was a more effective inhibitor of transcription from template 4 than was the mutant Em1b oligonucleotide (in Figure 4C, compare lane 2 with lane 4 and lane 3 with lane 5). These results indicate that a single G-box closest to the TATA sequence (Figure 1A, Em1b) appeared to be sufficient for transcription in the HNE.

Experiments were also performed with the HNE to compare transcription rates from the *Em* promoter with two other promoters active in plants, that is, ubiquitin from maize (Christiansen et al., 1992) and CaMV 35S (Marcotte et al., 1989). There were no visible transcripts from the ubiquitin or the CaMV 35S promoters in the HNE when the conditions and exposure time were the same as those used for Em113kp (Figure 4B, lanes 7 and 8). Both the ubiquitin and CaMV 35S promoters lacked G-box sequences, although the CaMV 35S has a related Hex-1 sequence (TGACG), which is apparently responsible for the transcripts generated from the CaMV 35S promoter in the HNE, as reported by Katagiri

Figure 3. Primer Extension Analysis of the Major *Em* Transcript Generated in the HNE.

The RNA synthesized in an *in vitro* transcription assay was analyzed by primer extension. Deoxynucleotide sequencing reactions of the *Em* promoter and primer extension products were fractionated on an 8% denaturing polyacrylamide gel. DNA sequences immediately adjacent to the RNA start site (arrow) of the *Em* promoter are shown. The single major transcript (697 nucleotides) can be seen to the left of the sequencing lanes. The primer used was 5'-GGTGCTCGAC-CGCTAAGGTT-3'.

Figure 4. Region of the *Em* Promoter Required for Transcriptional Activity in the HNE.

(A) Full map of Em113kp and the deletions used as templates in the transcription reactions. Template 1 (1) contains the entire Em113kp construct; template 2 (2) lacks the AT-rich region. Template 3 (3) contains only region 1; template 4 (4) has only the Em1b G-box. Template 5 (5) lacks a G-box but has all elements of the *Em* promoter 3' of Em1b.

(B) Transcripts generated in the HNE from the templates shown in **(A)**. Lane 1 contains a λ PstI (λPst) marker (805 bases). Lanes 2 to 6 contain transcription reactions generated from templates 1 to 5, respectively. Lanes 7 and 8 contain transcription reactions generated using the ubiquitin (Ubiq) or the CaMV 35S (35S) promoter template, respectively.

(C) Transcription reactions using template 4 (4) in the HNE. Lane 1 is the control transcription reaction; lanes 2 and 3 contain transcription reactions in the presence of Em1b oligonucleotide competitor at 10-fold (10x) and 15-fold (15x) the molar concentration of template 4, respectively. Lanes 4 and 5 contain transcription reactions in the presence of a mutant (Mut) Em1b oligonucleotide competitor at 10-fold and 15-fold the molar concentration of template 4, respectively.

et al. (1990). However, the rate of transcription in the HNE of the CaMV 35S promoter was significantly less than that of the Em113kp under our conditions.

USF Appears to Be the Protein in the HNE That Binds to the Em1b Element

Electrophoretic mobility shift assays in the presence of saturating concentrations of poly(dI-dC) revealed the presence of a protein(s) in the HNE that specifically bound to the Em1b probe (Figure 5A, lane 4) and was used in a competition assay with an excess of the Em1b oligonucleotide (Figure 5A, lanes 5, 7, and 9). This protein(s) in the HNE was not competed with equivalent concentrations of the mutant Em1b oligonucleotide, as seen in lanes 6, 8, and 10 (Figure 5A). This was further indication that the HeLa protein(s) is specific to the G-box element Em1b.

The addition of the USF antibody (anti-USF) to this electrophoretic mobility shift assay resulted in a dose-dependent disappearance of the retarded band and a supershift to a slower moving position on the gel (Figure 5B, lanes 12 and 13). Preimmune sera had no effect (Figure 5B, lane 14). The effect of anti-USF on the HNE also influenced transcription. This effect on transcription by Em113kp (Figure 6, lane 2) was specific because the anti-USF had little effect on transcription when a template from the dihydrofolate reductase (*DHFR*) promoter lacking a G-box was used (Farnham and Means, 1990) (in Figure 6, compare lanes 1 and 2 with lanes 3 and 4). The antibody raised against the plant bZIP transcription factor EmBP1, which recognizes G-box elements in the *Em* promoter (Guilting et al., 1990), had no effect on transcription in the HNE (data not shown).

Recombinant EmBP1, VP1, and USF Stimulate Transcription Using the *Em* Promoter in the HNE

When the recombinant EmBP1 fusion protein was added to the HNE with Em113kp (Figure 4A, template 1), only the main transcript from the +1 start site (Figure 1A) was detected (Figure 7A, lane 3). This effect of EmBP1 is specific to the *Em* promoter because transcription with the *DHFR* promoter (which also has multiple transcription start sites) was unaffected by the addition of EmBP1 (Figure 7B, lanes 3 and 4). When the truncated VP1 fusion protein (lacking the transcriptional activation domain) or the purified USF protein was added to the HNE, transcription from the *Em* promoter was stimulated to approximately the same levels (Figure 7C). However, unlike the effect of EmBP1, both transcripts from Em113kp were detected when either VP1 or USF was added to the HNE. Stimulation of transcription using VP1 appeared to be specific to the *Em* promoter because no stimulation was observed when the *DHFR* promoter was used (Figure 7B, lane 5). A similar specificity

Figure 5. Electrophoretic Mobility Shift Assays Using the HNE and Em1b as Probe.

(A) Electrophoretic mobility shift assays in the absence (lane 1) and presence (lane 2) of the HNE and with the HNE in the presence of 1 μ g (lane 3) or 2 μ g (lanes 4 to 10) of poly(dI-dC) [(dI-dC)]. A 10-fold (10 $\times$), 20-fold (20 $\times$), and 50-fold (50 $\times$) molar excess of cold Em1b oligonucleotide competitor was included in the gel shift assays in lanes 5, 7, and 9, respectively. A 10-, 20-, and 50-fold molar excess of a mutant (Mut) Em1b oligonucleotide was included in the gel shift assays in lanes 6, 8, and 10, respectively. Each reaction contains 5 μ g of the HNE protein and 1 ng of the labeled Em1b probe.

(B) Electrophoretic mobility shift assays in the presence of the USF antibody. Lanes 11 to 14 are identical to lane 4 in **(A)** except that lanes 12 and 13 contain 1 and 2 μ L of the anti-USF (USF Ab), respectively, and lane 14 contains the preimmune control. The anti-USF and the preimmune control were preincubated with the HNE for 10 min before the addition of the labeled Em1b probe.

for the *Em* promoter was demonstrated when USF was used (data not shown).

AdML and *Em* Promoters Are Recognized by EmBP1 in the HNE Immunodepleted of USF

Different-sized transcripts were generated from the *AdML* and *Em* promoters in the HNE (Figure 8A). Transcription from Em113kp was drastically reduced in HNE immunodepleted of USF (Figure 8B, lane 2). When full-length recombinant EmBP1 was added to the immunodepleted HNE, we observed not only transcription from the main transcriptional start site but also stimulation of transcription (Figure 8B, lane 3). We have reproducibly observed a two- to threefold enhancement of the 697-nucleotide transcript when compared with the combined expression levels of the major and minor transcripts in the HNE. Truncated recombinant VP1 had no effect on transcription in immunodepleted HNE, either alone (Figure 8D, lane 3) or with EmBP1 or USF (data not shown).

The *AdML* promoter contained a single CACGTG site 23 bp upstream of the TATA sequence and was very similar in sequence and position to the *rab28* promoter and the Em1b element in the *Em* promoter (Figures 1B and 1C). When whole HNE was used to transcribe the *AdML* promoter, there was no detectable effect when EmBP1 was added (data not shown). However, when the HNE depleted of USF was used with *AdML*, the effects of EmBP1 were similar to that previously observed with the *Em* promoter, that is, a two- to threefold stimulation of transcription (Figure 8C, lane

Figure 6. Effects of the USF Antibody on Transcription from the *Em* and *DHFR* Promoter Templates in the HNE.

Control transcription reactions contain either the Em113kp (113kp) template (lane 1) or the *DHFR* template (lane 3). Transcription reactions preincubated with anti-USF (USF Ab) (1 μ L of anti-USF/15 μ L of the HNE) are shown in lane 2 (Em113kp template) and lane 4 (*DHFR* template).

Figure 7. Effects of the Recombinant Maltose-Binding Fusion Proteins EmBP1 and VP1 on Transcription from the *Em* and *DHFR* Promoter Templates in the HNE.

(A) Transcription reactions containing the Em113kp (113kp) template (lane 1) in the presence of 0.5 μ g of LacZ (lane 2) or 0.5 μ g of EmBP1 (lane 3).

(B) Transcription reactions containing the Em113kp (lanes 1 and 2) or the *DHFR* (lanes 3 to 5) templates in the presence of 0.5 μ g of EmBP1 (lanes 2 and 4) or 0.6 μ g of VP1 (lane 5).

(C) Transcription reactions containing the Em113kp template (lane 1) in the presence of 25 ng of recombinant USF (lane 2) or 0.6 μ g of VP1 (lane 3).

3). Therefore, it appears that USF and EmBP1 can interchangeably stimulate transcription from either the *Em* or *AdML* promoter through their respective CACGTG core elements.

DISCUSSION

Transcription Reaction

We report here the detection of RNA transcripts in the HNE by using templates composed of the wheat *Em* and maize *rab28* promoters fused to the *GUS* reporter gene as well as the *AdML* promoter. We have shown that transcription from the *Em* template resulted in a major transcript of 697 nucleotides that starts 32 bases downstream of the TATA box. The initiation site of the main transcript (+1) from the *Em* promoter in the HNE is CCACCATC, which is similar to that of the *AdML* promoter (CACTCTC). Both are in agreement with the consensus (CAYYYTC) reported by Roy et al. (1991) and Kozak (1984). Studies of other plant transcription start sites (Joshi, 1987; Zhu et al., 1995) and the prediction by Litts et al. (1991) for the *Em* initiation site confirm that CCACCATC is the correct site for the *Em* gene. We also have evidence that the site of the main transcript identified in the HNE is the likely start site in vivo. When we performed primer extension assays, one of the bands generated from wheat poly(A)⁺ RNA was identical in size to the major transcript from the HNE. Because we did not detect the minor size transcript in vivo, we believe that the larger minor transcript generated in the HNE is likely to be an artifact of in vitro transcription.

Although the HNE transcription system has been used previously to express two polymerase II genes from plants (Boston and Larkins, 1986; Katagiri et al., 1990), this study shows that both the *Em* and the *rab28* promoters are transcribed at much higher levels than either of the previously used promoters. We believe this is due primarily to the presence of a CACGTG target sequence (i.e., G-box) in both promoters (Litts et al., 1991; Pla et al., 1993). These same G-box elements in the *Em* promoter (i.e., Em1a and Em1b) have been shown to be responsive to both the ABA and VP1 signals to activate *Em* expression in transient assays (Vasil et al., 1995). Furthermore, the G-boxes in the *rab28* and *Em* promoters are identical to the binding site for USF (Figure 1), the endogenous transcriptional activator in the HNE, and the sequence in the *AdML* promoter that is recognized by USF (Sawadogo and Roeder, 1985). In addition, the flanking sequences on either side of the G-boxes in these promoters are conserved, for example, nine of 13 bases are the same in the Em1b and *AdML* elements (Figure 1B). The positions of the G-boxes relative to the TATA box are also very similar; in the *AdML* promoter, the G-box is positioned 23 bases upstream of the TATA, whereas in the *Em* promoter, Em1b is 38 bases upstream of the TATA sequence. Finally, these two

Figure 8. Effect of the Recombinant Maltose-Binding Fusion Protein EmBP1 on Transcription from the *Em* Promoter and the *AdML* Promoter Templates.

(A) Transcription reactions contain the *AdML* (lane 1) and the *Em*113kp (*Em*; lane 2) templates in the HNE.

(B) Lane 1 is the transcription reaction containing the *Em*113kp (113kp) template and HNE that was passed over a protein A-Sepharose column. A transcription reaction containing the *Em*113kp template and the HNE was passed over an anti-USF protein A-Sepharose column (immunodepleted [depl] of USF) (lanes 2 and 3) in the absence (lane 2) or presence (lane 3) of 0.5 μ g EmBP1.

(C) Lane 1 is the transcription reaction containing the *AdML* template and the HNE that was passed over a protein A-Sepharose column. A transcription reaction containing the *AdML* template and the HNE was immunodepleted of USF (lanes 2 and 3) in the absence (lane 2) or presence (lane 3) of 0.5 μ g EmBP1.

(D) Lane 1 is the transcription reaction containing the *Em*113kp template and the HNE that was passed over a protein A-Sepharose column. A transcription reaction containing the *Em*113kp template and the HNE was immunodepleted of USF (lanes 2 and 3) in the absence (lane 2) or presence (lane 3) of 0.6 μ g VP1.

promoters have identical TATA boxes (TATAAAA), which are very strong TATA binding protein sequences (Mukumoto et al., 1993), and similar initiators; the initiator is 55 bases from the G-box (CACTCTC) in *AdML* and 70 bases from the G-box *Em*1b (CACCATC) in the *Em* promoter (Figure 1C). Hence, the similarity between the response elements of these plant promoters and those recognized by endogenous factors in the HNE contributes to the high levels of expression from these plant templates.

Additional data support the conclusion that the USF protein in the HNE is the factor responsible for transcription from the *Em* promoter through the *Em*1b element. Using 5' deletions of the *Em* promoter, we found that *Em*1b is essential for transcription of the *Em* promoter in the HNE. Competition assays performed with an excess of the *Em*1b oligonucleotide inhibited transcription when the *Em* promoter was used and interfered with gel mobility shift assays when the HNE was used. Both of these results are similar to those obtained with other eukaryotic G-box-containing promoters that require USF, such as the *U6* gene in sea urchin (Li et al., 1994) and *AdML* (Sawadogo and Roeder, 1985). The addition of anti-USF to the gel retardation assay using the *Em* template resulted in a dose-dependent disappearance of the retarded band and a supershift at a higher position on the gel. Furthermore, we show that when USF was immunodepleted from the HNE, transcription from the *Em* or *AdML* templates was reduced dramatically. To determine whether the HNE could be used to assess the function of certain factors believed to interact with the *Em* promoter, we added recombinant EmBP1 and VP1 proteins to transcription reactions containing the *Em* template.

Effects of VP1 on Transcription

VP1 is required for *Em* expression (McCarty et al., 1991; Hattori et al., 1994; Vasil et al., 1995; Hill et al., 1996). Functional assays indicate that the N-terminal region (120 amino acids) of VP1 is a transcriptional activator (McCarty et al., 1991), but we have been unable to detect specific binding of VP1 to DNA (Hill et al., 1996). Our results indicate that a truncated VP1 (lacking an activation domain) dramatically enhances the binding of a variety of transcription factors to their target sequences, including EmBP1 to region 1 of the *Em* promoter (Hill et al., 1996). This in vitro enhancement effect of VP1 appears to be nonspecific and may occur through a weak and transient association of a highly conserved region of VP1 (BR2) with DNA (Hill et al., 1996). However, it is not clear from the work of Hill et al. (1996) whether the enhancement effect of VP1 in vitro is related to its ability in transient assays (Vasil et al., 1995) and in transgenic plants (Parcy et al., 1994) to activate transcription from the *Em* promoter.

In this study, our results indicate that when VP1 is added to the whole HNE, transcription from the *Em* template is stimulated. Purified USF also stimulated transcription, but

~20 times more VP1 was needed to achieve the same level of transcript. The most likely explanation is that the truncated VP1 lacked its transcriptional activation domain and as such affected transcription by interacting with endogenous transcription factors, whereas USF was the endogenous, sequence-specific transcription factor that affected transcription directly. We believe that VP1 enhancement of transcription requires not only USF but other essential components found in whole HNE, because in immunodepleted HNE, VP1 had no effect, either alone or when used in combination with EmBP1 or USF. These conclusions are consistent with our *in vitro* results showing that VP1 cannot bind DNA alone but rather facilitates the binding of a wide range of sequence-specific factors to their target sites, either directly or through interactions with other proteins localized to the transcriptional complex.

Our view that VP1 appears to be a partner of USF is similar to the conclusions of Timchenko et al. (1995), who showed that the C/EBP protein can autoregulate its own gene by enhancing the binding of USF to a G-box present on the C/EBP promoter. C/EBP does not bind to its own promoter, but its presence presumably stimulates transcription by enhancing the binding of USF to its target G-box. It is possible that VP1 affects USF in a similar manner, that is, by stimulating transcription as a coactivator. This would make it possible for VP1 to coactivate transcription through different transcription factors without being required for basal transcription (Chen et al., 1994). By VP1 interacting with a variety of transcription factors to activate a wide range of different genes in diverse metabolic and developmental pathways is consistent with its pleiotropic effects of VP1 in maize and its homolog *abi3* in Arabidopsis (see Hill et al., 1996). However, the effects of VP1 on expression of embryonic maturation genes are also very specific. For example, both the *rab28* and the catalase *cat1* genes in maize are regulated similar to Em, that is, they are responsive to ABA and are temporally expressed at the same time during embryo maturation, but unlike Em, neither *rab28* nor *cat1* requires VP1 for expression (Pla et al., 1991; Williamson and Scandalios, 1992). A model in which VP1 functions as a coactivator capable of activating specific genes with different DNA binding partners may achieve the required level of specificity.

Effects of EmBP1 on Transcription

Because activation of the *Em* promoter is significantly reduced in USF-depleted extracts, we used this extract to determine whether recombinant EmBP1 can stimulate transcription from the *Em* template. We found that when EmBP1 is added to the HNE depleted of USF, transcripts from both the *Em* and *AdML* templates accumulate. This provides evidence that EmBP1 can directly activate transcription from a G-box in the *Em* promoter. In addition, EmBP1 directs transcription from the *in vivo* start site only, resulting in a single 697-nucleotide transcript; no additional transcripts were de-

tected when EmBP1 was added to either the immunodepleted HNE or whole HNE. The effect of EmBP1 directing transcription from one site on the *Em* promoter can be compared to the effect of Sp1 and E2F directing transcription on the *DHFR* promoter in the HNE. Each of these cell cycle-regulated transcription factors directs transcription from a different start site in the *DHFR* promoter; the Sp1 site is used for basal transcription, whereas the E2F site is required for growth (Schilling and Farnham, 1995). Therefore, by using USF-depleted extracts of the HNE, one should be able to test the activation function of purified transcription factors that recognize CACGTG target sites as well as their interaction with the conserved basal transcriptional machinery found in the HNE. However, although VP1 stimulated transcription from whole HNE, we did not observe the same effect when we added purified USF (or EmBP1) in combination with VP1 to the USF-depleted extracts. Perhaps, components essential for the VP1 enhancement were removed in the preparation of USF-depleted extract.

In summary, use of the HNE to activate transcription from plant promoters will allow investigators to analyze a promoter structurally and study the effects of transacting factors on their target sites. It might also be possible to study transcription from plant or other promoters that have low levels of expression in the HNE (e.g., zein and CaMV 35S) by enhancing their basal transcription rate to detectable levels by introducing G-box sequences into the given template. Furthermore, we may determine those factors required for the specificity of VP1 action. We observed specificity in the VP1 enhancement of transcription in the HNE; that is, VP1 could discriminate between the *Em* template and USF (which resulted in enhanced transcription rates) and the *DHFR* template and its sequence-specific factor in the HNE (which resulted in no enhanced transcription rates). Finally, our results with recombinant EmBP1 and VP1 in HNE suggest that one can identify functional domains in EmBP1 and VP1 that specifically initiate and enhance transcription from a given template. In addition, using USF-depleted extracts as an assay, we may be able to identify and characterize components in whole HNE (or plant extracts) that support the specificity of VP1 enhancement.

METHODS

Preparation of the HeLa Nuclear Extract

The HeLa nuclear extract (HNE) was prepared from frozen HeLa nuclei (Cellex Biosciences Inc., Minneapolis, MN), according to the method of Dignam et al. (1983). The nuclei were thawed and extracted in buffer C (20 mM Hepes, pH 7.9, 0.2 mM EGTA, 2 mM DTT, 25% glycerol, 0.15 mM spermine, 0.75 mM spermidine, 1.0 mM phenylmethylsulfonyl fluoride, and 0.4 M NaCl). The extract was recovered by centrifugation at 49,000 rpm for 45 min and then dialyzed for 2 hr against buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 1.0 mM

phenylmethylsulfonyl fluoride, and 12.5 mM MgCl_2). The extract was aliquoted, frozen in liquid nitrogen, and stored at -80°C .

Transcription Assays

Transcription assays were performed as described by Manley et al. (1980) in a total volume of 25 μL consisting of 15 μL (100 to 150 μg of protein) of the HNE, 400 μM ATP, 400 μM CTP, 400 μM GTP, 50 μM ^{32}P -UTP (10 μCi , 3000 Ci/mM), and 140 μM EDTA. The reactions were initiated by the addition (1 μg) of linearized single template. The reaction was incubated at 30°C for 1 hr and was stopped by adding a buffer consisting of 8 M urea, 0.5% SDS, 10 mM EDTA, and 10 mM Tris, pH 8.0. The samples were extracted twice with phenol-chloroform. The RNA was ethanol precipitated and applied to an 8% polyacrylamide-7 M urea sequencing gel. The gels were electrophoresed in $1 \times$ Tris-borate-EDTA buffer at 1400 V for 5 to 6 hr, dried, and then exposed to XRP film (Kodak).

Transcription competition was performed using double-stranded Em1b and mutant Em1b 24mer oligonucleotides (Genosys Biotechnologies, Inc., Woodlands, TX), and they are as follows: Em1b, 5'-GCGCTCGAGCGCTGCACACGTGCC-3' and 5'-CGCGTCGACGAGGCGGCACGTGTG-3'; and mutant Em1b, 5'-GCGCTCGAGCGCTGAATCTAGGCC-3' and 5'-CGCGTCGACGAGGCGGCCTAG-ATG-3'. These oligonucleotides were annealed, filled in using the Klenow fragment of DNA polymerase I, and used to complete the G-box binding factor by adding excess molar concentrations (specified in the legend to Figure 4C) at the same time as the template. All transcription reactions described were repeated at least three times.

Immunodepletion of USF from the HNE

Immunodepletion of USF from the HNE was conducted by the method of Luo et al. (1992) (Figures 8B to 8D). One hundred microliters of protein A-Sepharose beads (for fast flow; Pharmacia) was washed several times with 20 mM Hepes and equilibrated with transcription dialysis buffer (mentioned above) and then packed into a 1-mL pipette tip blocked with glass wool at the bottom. HNE (150 μL) was incubated with the protein G-Sepharose-purified anti-USF (0.5 mg of protein per mL) for 1 hr with very gentle shaking on ice. This mixture of antibody and HNE was then passed over the protein A-Sepharose column prepared as given above. The control for this experiment was prepared in the same way but with the same amount of transcription dialysis buffer being added in place of the antibody. It was then passed through the column in the same manner.

Anti-USF was prepared in rabbits by using a β -galactosidase-sea urchin USF fusion protein. This antibody cross-reacts with human USF (Singh et al., 1994) and was a generous gift from M. Kozlowski (Massachusetts General Hospital, Charlestown, MA).

Electrophoretic Mobility Shift Assays

The electrophoretic mobility shift assay was performed in binding buffer consisting of 12 mM Tris, pH 7.9, 12% glycerol, 35 mM KCl, 0.07 mM EDTA, 1 mM DTT, and 7.5 mM MgCl_2 with 1 to 2 μg of poly(dI-dC) as a nonspecific competitor, as indicated in the legend to Figure 5. The HNE (5 μg of protein) was added to 1 to 2 ng of the labeled Em1b probe (10,000 to 15,000 cpm) and incubated for 15 min at room temperature. The Em1b oligonucleotide competitions were performed by using the Em1b and the Em1b mutant oligonucleotides

mentioned above at the molar fold concentrations indicated in the appropriate figure legend. These competitor oligonucleotides were added to the reactions at the same time as the labeled probe. The reactions were resolved on a 4% nondenaturing gel (40:1 acrylamide to bis ratio) in $1 \times$ Tris-borate-EDTA. The gel was dried and then visualized by autoradiography.

Preparation of Recombinant Proteins

Recombinant MBP-EmBP1 (full length) and MBP-DVP1 (amino acids 190 to 692) were prepared as described by Hill et al. (1996), except for a few minor changes. After purification on the maltose column, these proteins were dialyzed for 1 hr in transcription dialysis buffer D. The USF-purified recombinant protein was a gift from P. Pognonec (University of Nice, Nice, France).

DNA Templates

The plasmid pBM113 was described previously (Marcotte et al., 1988). This plasmid was linearized with EcoRV, resulting in two fragments of 5206 and 231 bp. The larger fragment was isolated from a TAE-agarose gel by dialysis and used as template 1. The 5' deletion series of the *Em* promoter (templates 2 to 5) was described by Marcotte et al. (1989). These deletions were treated in the same manner as described for template 1. The maize *rab28* plasmid, which contains the *rab28* promoter fused to the β -glucuronidase (*GUS*) gene, was obtained from M. Pagès (Department de Genetica Molecular, Centro de Investigacion y Desarrollo, Barcelona, Spain), linearized with EcoRV, and treated in the same manner as the *Em* plasmids. The cauliflower mosaic virus (CaMV) 35S promoter template was made by linearizing the pBM314 plasmid (Marcotte et al., 1988) with EcoRV. This plasmid contains the CaMV 35S promoter inserted into the pBM113 plasmid in place of the *Em* promoter so that the template is identical to template 1, except for the promoter region. The ubiquitin promoter (Christiansen et al., 1992) was in the form of plasmid pAHC27 (gift of P. Quail, U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA), in which the ubiquitin promoter was cloned upstream of a *GUS* reporter gene in the PUC18 plasmid. It was linearized with EcoRV and used for transcription.

The dihydrofolate reductase (*DHFR*) template is the *DHFR*-chloramphenicol acetyltransferase template plasmid described by Swick et al. (1989). It consists of PUC18 containing the *DHFR* promoter region cloned upstream of the chloramphenicol acetyltransferase (*CAT*) reporter gene. The plasmid was linearized with *ScaI* to generate a major transcript of 736 nucleotides and a minor transcript of 780 nucleotides. The adenovirus major late (*AdML*) template (*AdML* promoter cloned upstream of the *CAT* reporter gene) was also described by Swick et al. (1989). This plasmid was linearized with *NcoI* to generate a transcript of 642 nucleotides.

Primer Extension

Primer extension assay was performed according to the method of Sambrook et al. (1989), without any modifications. The primer used was 5'-GGTGCTCGACCGCTAAGGTT-3'. It was labeled with polynucleotide kinase and γ - ^{32}P -ATP. Dioxynucleotide sequencing reactions (Sanger et al., 1977) were performed using this same primer. The labeled products of the primer extension and the sequencing re-

actions were analyzed on an 8% denaturing gel, dried, and visualized by autoradiography.

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Effect of the nuclear factors EmBP1 and viviparous1 on the transcription of the Em gene in HeLa nuclear extracts.

M A Razik and R S Quatrano

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