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Upstream Sequences Required for Efficient Expression of a Soybean Heat Shock Gene†

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A soybean gene (*Gmhsp17.5-E*) encoding a small heat shock protein was introduced into primary sunflower tumors via T-DNA-mediated transformation. RNA blot hybridizations and S1-nuclease hybrid protection studies indicated that the heat shock gene containing 3.25 kilobases of 5′-flanking sequences was strongly transcribed in a thermoinducible (40°C) manner. Transcriptional induction also occurred to a lesser extent upon treatment of whole tumors with sodium arsenite and $CdCl_2$. Basal (26°C) transcription was not detected in soybean seedlings, but it was quite evident in transformed tumor tissue. A 5′ deletion to −1,175 base pairs with respect to the CAP site had no effect on the levels of thermoinducible transcription, but it resulted in a large increase in basal transcription. Further removal of DNA sequences (including the TATA-distal heat shock consensus element) to −95 base pairs reduced thermoinducible transcription by 95% and also greatly decreased basal transcription. The termini of the *Gmhsp17.5-E* RNA in the tumor were generally the same as those present in soybean RNA, with the exception of several additional 3′ termini.

The exposure of higher plants to elevated temperatures elicits a strong response at both the translational (1, 4, 5, 12, 20, 24) and transcriptional (14, 35) levels. The heat shock (HS) response of plants is in general similar to that observed in many higher organisms including *Drosophila melanogaster*, in which it is best characterized (2). In soybean plants, thermal stress results in the rapid loss of polysomes and in a decreased synthesis of many normally abundant proteins (24). A new set of RNAs also rapidly appears which is translated into several classes of HS-specific proteins (22, 23, 35). The low-molecular-weight group is predominantly expressed and contains from 30 to 50 members (20, 24). This complex group of low-molecular-weight heat shock proteins (HSPs) shows a similarity in hydropathy profiles and amino acid sequences (15, 21, 29, 36) to the small HSPs of *D. melanogaster* (39), *Xenopus laevis* (7), and *Caenorhabditis elegans* (33).

In addition to similarities in the protein structure of HSPs among diverse groups of eucaryotes, the regulatory mechanisms responsible for thermally inducible transcription also appear to be highly conserved. A direct demonstration of the universal aspects in HS promoter design and function is provided by examples of heterologous expression of the *Drosophila HSP70* promoter in mammalian (10, 13, 28, 32) and amphibian cells (44). Deletion studies (31) and the insertion of synthetic DNA oligomers (32) identified a region 13 to 28 bases upstream from the TATA motif that is essential for the thermoinducibility of *Drosophila HSP70* transcription. In *Drosophila* HS genes, this TATA-proximal element forms the consensus sequence CT-GAA--TTC-AG (31) and lies within a region that interacts with a HS-specific transcription factor (30, 46). Indirect evidence that higher plants possess analogous regulatory mechanisms for heat-inducible transcription is based on the presence of the HS consensus (80 to 90% homology) sequence 17 to 18 bases upstream from the TATA-like region in several soybean HS genes (15, 36).

This study represents the first step in the definition and characterization of DNA sequence elements that contribute to the regulation of thermally inducible genes in higher plants. We inserted a small soybean HSP gene (*Gmhsp17.5-E*) into sunflower tumor cells by using a Ti plasmid-based vector system, and we characterized transcriptional expression of the introduced soybean gene in primary tumors. With this system, we tested the effect of two 5′-deletion mutants on basal (26°C) and thermoinducible (40°C) transcription.

MATERIALS AND METHODS

Introduction of *Gmhsp17.5-E* into *Agrobacterium tumefaciens*. A 4.25-kilobase (kb) *Bgl*II restriction fragment containing the soybean HS gene *Gmhsp17.5-E* was transferred into sunflower tumors. This fragment contains a little over 3 kb of 5′-flanking sequences and extends through the protein-coding region to include the portion of the gene corresponding to the 3′-untranslated tail of the transcript. The downstream *Bgl*II site is positioned 35 nucleotides beyond the 3′ terminus of the longest transcript present in the soybean gene (15). Thus, this restriction fragment probably contains all major components of the promoter essential for thermoinducible transcription and sufficient sequences downstream to ensure proper polyadenylylation.

The *Bgl*II fragment containing the *Gmhsp17.5-E* gene was ligated into a pBR322-based shuttle plasmid for subsequent transfer into *Agrobacterium tumefaciens*. The shuttle plasmid was derived from p233G kindly provided by Dennis Sutton (Agrigenetics Advanced Research Division, Madison, Wis.). This plasmid consists of a 7.25-kb portion of pTi-15955 T-left substituted for the small *Eco*RI-*Bam*HI fragment of pBR322 (9). The T-left fragment allows formation of a cointegrate in the T region of the Ti plasmid after transfer to *A. tumefaciens*. In p233G, the T-DNA *Sma*I site (11,207 base pairs [bp] [3]) was changed to a *Bgl*II site by linker addition (D. Sutton, personal communication) and served as a convenient site for insertion of the HS gene. The

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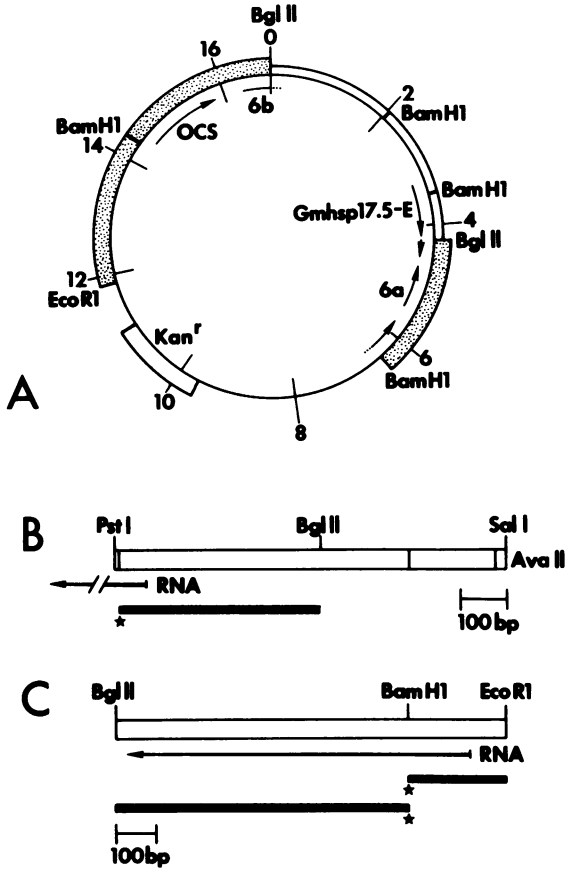

FIG. 1. Restriction maps of the vector plasmid and genes mapped by S1-nuclease hybrid protection (19). (A) Shuttle vector pHSE(−3250) comprised of pBR322 (thin line), the kamamycin gene from pUC4K (*Pst*I) (43), a portion of T left (stippled; *Bam*HI, 9,062 bp to *Eco*RI 12,823 bp [3]), and a 4.25-kb *Bgl*II fragment containing the *Gmhsp17.5-E* gene. Arrows represent potential transcripts (15, 45). Numbered lines delineate kilobases. (B) T-right gene transcribed in *A. tumefaciens*. The 5′-end-labeled DNA hybridization probe used for S1-nuclease mapping (solid line) extended from the *Ava*II to *Bgl*II sites (22,465 to 22,930 bp [3]). (C) Soybean HS gene *Gmhsp17.5-E*. The end-labeled DNA probes (solid lines) used for determination of the 5′-(*Bam*HI-*Eco*RI) and 3′-(*Bam*HI-*Bgl*II) termini of the RNA are indicated; ★, [^{32}P]-end label.

final construction resulted in the intermediate vector pHSE (−3250) shown diagrammatically in Fig. 1A.

Deletions of the 5′-flanking region were constructed by removal of the appropriate upstream restriction fragments, by the addition of *Bgl*II linkers, and by subsequent subcloning of the deleted gene into pUC8 containing a *Bgl*II linker at the *Bam*HI site. The deleted genes were then inserted into the *Bgl*II site of the intermediate vector and screened for the orientation depicted in Fig. 1A. The intermediate shuttle vector used for the 5′ deletions consisted of the T-left fragment from p233G inserted into the *Bam*HI site of pACYC184 (11) instead of pBR322. The first deletion [pHSE(−1175)] contains 1,175 bp of 5′-flanking sequences and was constructed by removal of all sequences upstream of an *Ava*II site at position −1175. The deletion pHSE(−95) was constructed similarly by removal of all sequences upstream from an *Eco*RI site at −94 bp. Addition of the *Bgl*II linkers in the construction of pHSE(−95) and pHSE(−1175) reconstituted the guanine residues at positions −95 and −1175.

The shuttle plasmids were mobilized into *A. tumefaciens* by tripartite mating (11, 17) with *Escherichia coli* LE392 containing the mobilization plasmid pRK2013 (17), *E. coli* SK1590 or LE392 containing one of the three shuttle plasmids, and *A. tumefaciens* 15955 (Str^r). The resultant *Agrobacterium* strains containing cointegrate Ti plasmids incorporating shuttle plasmids pHSE(−3250), pHSE (−1175), and pHSE(−95) were designated strains AtHS-1, AtHS-2, and AtHS-3, respectively.

Induction of *Gmhsp17.5-E* transcription in primary tumors. Primary tumors incited on sunflower seedlings (*Helianthus annuus* cv. Large Grey) were used to study expression of the soybean HS gene in heterologous cells. Seedlings were grown under fluorescent lighting (18-h light cycle); 7 to 10 days after planting the seedlings were inoculated by syringe with a drop of an overnight culture of *A. tumefaciens*. After 10 to 14 days of growth, tumors incited by *Agrobacterium* strains containing the HS gene (heat shock tumor [HST]) and tumors incited by strain 15955 (Str^r) (control tumor [CT]) were excised and placed in ice-cold water until harvesting was completed. Induction of the HS gene was performed by shaking excised tumors in flasks containing incubation medium (15 to 20 g of tumor material per 30 ml of medium) consisting of 1 mM potassium phosphate buffer (pH 6.0), 1% sucrose, 10 μg of 2,4-dichlorophenoxy acetic acid ml^{-1}, 50 μg of chloramphenicol ml^{-1}, and 100 μg of rifampin ml^{-1}. Tumors were barely covered with incubation medium and shaken continuously for 3 to 5 h. Thermoinduction was analyzed by comparing poly(A) RNA isolated from tumors incubated at temperatures ranging from 28 to 45°C. Induction of the *Gmhsp17.5-E* gene by other stresses was performed by incubation of tumors at 28°C in incubation medium adjusted to either 100 μM sodium arsenite or 1 mM $CdCl_2$.

Tumor RNA purification and RNA blot hybridization. Total RNA purification and fractionation of poly(A)$^+$ from HSTs and CTs was performed as previously described (14), except that precipitation of total RNA with 3.0 M NaCl was replaced by two 2.0 M LiCl precipitations. Poly(A)$^+$ RNA samples were electrophoresed on denaturing gels (2% agarose, 6% formaldehyde) and transferred overnight to nitrocellulose filters. The filter-immobilized RNA was hybridized overnight to nick-translated purified insert from cDNA clone pFS2019 (predominantly 3′-untranslated region) according to Baulcombe and Key (6). The blots were washed and autoradiographed as described by Czarnecka et al. (14).

Purification of RNA from *A. tumefaciens* species. Four 250-ml cultures of *A. tumefaciens* AtHS-1 were grown overnight (optical density at 560 nm was ca. 1.0) in Luria broth. Carbenicillin (500 μg ml^{-1}) was added to each flask to facilitate lysis during RNA extraction. The cultures were then grown at either 28 or 40°C with or without 100 μg of rifampin ml^{-1}. After 4 h, the cells were pelleted at 8,000 rpm (7,740 × *g*) for 10 min and suspended in buffer A (0.05 M sodium acetate, 0.1 M NaCl, 1 mM disodium EDTA). After two washings with buffer A, cells were first suspended in 25 ml of buffer A and then lysed by the addition of 6 ml of 20% N-lauroyl sarcosine (Sigma Chemical Co.). Lysis was complete after incubation of the mixture at 68°C for 5 min. Proteins were removed by three successive phenol-chloroform-isoamyl alcohol (25:24:1) extractions. DNA was removed by spooling and LiCl (2 M) precipitation.

Transcription analysis by S1-nuclease hybrid protection

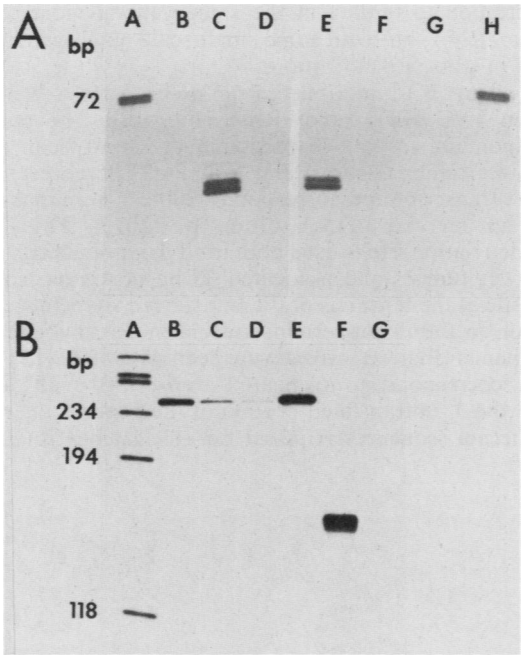

FIG. 2. Analysis of *Agrobacterium* AtHS-1 transcription by S1-nuclease mapping. (A) 5′ terminus of the T-right gene (see Fig. 1B). Lanes: A and H, ΦX174 DNA *Hae*III-generated marker; B through E, S1 protection with 12 μg of total RNA from *A. tumefaciens* AtHS-1; F through G, S1 protection with 1 μg of poly(A) RNA from soybean hypocotyls. Treatments: B and C, 40°C, with and without rifampin, respectively; D and E, 28°C, with and without rifampin, respectively; F, 40°C; G, 28°C. S1-nuclease-protected bands are seen at 59 to 61 bp. (B) 5′ terminus of the *Gmhsp17.5-E* gene (see Fig. 1C). Band at 250 bp represents intact probe. S1-nuclease-protected band is seen at 158 bp. Lanes: A, ΦX174 DNA *Hae*III-generated marker; B through E, 12 μg of total RNA from *A. tumefaciens* AtHS-1; F through G, 1 μg of poly(A)$^+$ RNA from 40°C soybean hypocotyls. Treatments: B, rifampin at 40°C; C, 40°C; D, rifampin at 28°C; E, 28°C; F, 40°C; G, 28°C.

mapping. A *Bam*HI site located within the protein coding region of the HS gene *Gmshp17.5-E* was used as a reference point to map the 5′ and 3′ termini of the corresponding transcripts in HST poly(A) RNA and strain AtHS-1 total RNA. Appropriate restriction fragments (Fig. 1C) for 5′- and 3′-end analysis were end labeled at the *Bam*HI site and used in S1-nuclease hybrid protection studies by the procedure of Favaloro et al. (19).

The sensitivity of *Agrobacterium* transcription to rifampin and high temperature (40°C) was assessed by monitoring the activity of a bacterial promoter (W. B. Gurley, unpublished results) located within the agropine synthetase gene (38). Transcriptional expression from this promoter was assayed by S1-nuclease hybrid protection (19). A 465-bp nucleotide, *Ava*II to *Bgl*II fragment (Fig. 1B) of T-DNA, was 5′-end labeled at the *Ava*II site and used as a hybridization probe with 12 μg of strain AtHS-1 total RNA.

RESULTS

HS gene *Gmhsp17.5-E* not expressed in *A. tumefaciens*. Rifampin and chloramphenicol were included in the incubation medium to inhibit transcriptional and translational expression of the *Gmhsp17.5-E* gene in *A. tumefaciens* that may have been present in freshly excised tumors. To evaluate the effectiveness of rifampin at 28 and 40°C, we assayed the transcriptional activity of a bacterial promoter located within the T-DNA gene encoding agropine synthetase. S1-nuclease hybrid protection mapping (Fig. 2A) of the 5′ terminus of the bacterial transcript showed that RNA homologous to the T-DNA gene was detectable in bacteria grown for 3 h at both 28 and 40°C. It is clear that rifampin (100 μg ml^{-1}) abolished transcription from the bacterial promoter at both temperatures. Transcriptional expression of the soybean HS gene [pHSE(−3250)] in *A. tumefaciens* AtHS-1 was examined in a similar manner with a 5′-end-labeled restriction fragment of the *Gmhsp17.5-E* gene as a hybridization probe. This type of analysis failed to detect transcription of the *Gmhsp17.5-E* gene at 28 or 40°C, with or without rifampin (Fig. 2B). These studies with AtHS-1 RNA indicate that rifampin was an effective inhibitor of transcription in *A. tumefaciens* and that the HS gene was not expressed under any conditions tested in *Agrobacterium* cultures. Therefore, any expression of the *Gmhsp17.5E* gene observed in freshly excised HSTs could not be attributed to *A. tumefaciens*.

RNA blot analysis of tumor RNA. Total poly(A)$^+$ RNA from HSTs and CTs was fractionated by agarose gel electrophoresis, immobilized on nitrocellulose, and probed with the nick-translated cDNA insert of pFS2019 (Fig. 3A and B). This cloned cDNA probe contains the 3′-untranslated tail and extends 19 bases into the protein-coding region of the HS gene (15). Hybrid-selection studies have shown cDNA pFS2019 to be specific for RNA homologous to the *Gmhsp17.5-E* gene in soybean RNA (35). Further evidence of its specificity is the lack of cross-hybridization with CT RNA (Fig. 3A and B). Hybridization of 40°C HST RNA with this probe resulted in a prominent autoradiographic band indicating strong, thermally induced expression of the soybean HS gene in sunflower tumors. It is clear from Fig. 3 that most HST RNA homologous to the *Gmhsp17.5-E* gene was similar, if not identical, in size to that present in 40°C soybean seedlings. The slight expression seen at 28°C in HSTs may have been caused by the increased temperature sensitivity, based upon amino acid incorporation studies (data not shown), of tumor tissue compared with soybean seedlings. Alternatively, this basal expression may be a vector artifact resulting from the relatively close spacing of neighboring genes within T-DNA.

The soybean HS gene was also induced by sodium arsenite and $CdCl_2$ at 28°C (Fig. 3B). Since the soybean HS gene shows some activity in tumors at 28°C, transcriptional induction by sodium arsenite and $CdCl_2$ can be seen by comparison with HST RNA from untreated tumors incubated at 28 and 40°C (Fig. 3B, lanes E through H).

Poly(A)$^+$ tumor RNA was used in parallel RNA blot hybridizations (Fig. 3C) to obtain a general indication of the relative activity of the HS promoter compared with neighboring T-DNA promoters. The hybridization probe was a cloned T-DNA restriction fragment (*Pst*I, 10,069 bp, to *Eco*RI, 12,323 bp) homologous to transcripts 6A and 6B (0.9-kb RNA) and the 3′ portion of the octopine synthetase gene (1.4-kb RNA) (45). The specific activity of the probes, the amount of poly(A) RNA per lane, and the autoradiography exposure conditions were approximately equal for blots probed with T-DNA and for those probed with the *Gmhsp17.5-E* gene. It is evident from Fig. 3C that autoradiographic bands corresponding to T-DNA transcripts are much weaker than those homologous to the HS gene. In addition, the abundance of the T-DNA transcripts in both HST and CT RNA decreased upon treatment at elevated temperatures. These results indicate that thermal induction of the *Gmhsp17.5-E* gene was restricted to the HS gene and

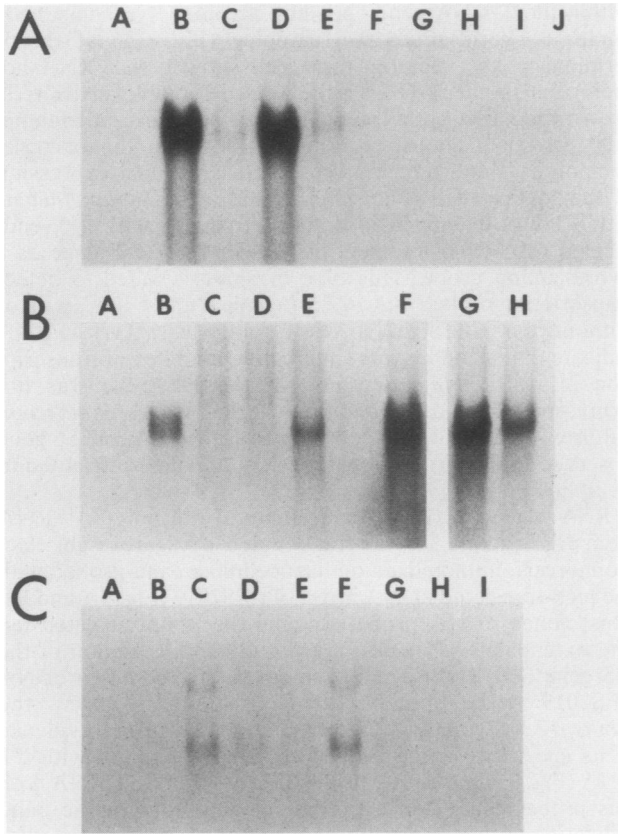

FIG. 3. RNA blot hybridizations of sunflower tumor RNA. All lanes contain 1 μg of poly(A) RNA except lanes A and B of panel B which contain 0.25 μg of soybean poly(A) RNA. (A) Thermoinduction of the *Gmhsp17.5-E* gene. The hybridization probe was cDNA pFS2019. Lanes: A, soybean hypocotyl at 28°C; B, soybean hypocotyl at 40°C; C, HST at 28°C; D, HST at 40°C; E, HST at 42.5°C; F, HST at 45°C; G, CT at 28°C; H, CT at 40°C; I, CT at 42.5°C; J, CT at 45°C. Bands are from 0.9 to 1.0 kb. (B) Induction of the *Gmhsp17.5-E* gene by heat, sodium arsenite, and $CdCl_2$. The hybridization probe was cDNA pFS2019. Lanes: A, soybean hypocotyl at 28°C; B, soybean hypocotyl at 40°C; C, CT at 28°C; D, CT at 40°C; E, HST at 28°C; F, HST at 40°C; G, HST at 28°C, 100 μM sodium arsenite; H, HST at 28°C, 1 mM $CdCl_2$. (C) Transcripts from T-left. The T-left hybridization probe was a *Bam*HI-*Eco*RI subfragment of *Bam*HI-17a. The upper band represents RNA homologous to the octopine synthetase gene (1.4 kb), and the lower band (0.9 kb) represents transcripts 6A and 6B (29). Lanes: A, soybean hypocotyl at 28°C; B, soybean hypocotyl at 40°C; C, HST at 28°C; D, HST at 40°C; E, HST at 42.5°C; F, CT at 28°C; G, CT at 40°C; H, CT at 42.5°C; I, CT at 45°C.

did not result in a general increase in the activity of non-HS genes in the immediate vicinity.

Determination of the 5′ and 3′ termini by S1-nuclease mapping. The 5′ terminus of heat-induced HST RNA homologous to the *Gmhsp17.5-E* gene was determined (Fig. 4A) with nucleotide-level precision by using S1-nuclease hybrid protection with a DNA sequence ladder of the end-labeled hybridization probe as a size marker. The end-labeled probe (Fig. 1C) was specific for *Gmhsp17.5-E* transcripts in sunflower cells since CT RNA showed no S1-protected bands (Fig. 4A, lanes G and H). Previous work established the start of transcription in soybean RNA to be 24 bases downstream from the TATA motif within the sequence TCGTCA (15). Analysis of *Gmhsp17.5-E* expression in tumors showed transcription to initiate in the same general vicinity as in soybean RNA, but with some small-scale heterogeneity (±2 bases) in start site utilization.

All of the 3′ termini present in soybean RNA were also seen in 40°C HST tumor RNA (Fig. 4B). The terminus corresponding to band b predominates in soybean and in sunflower tumor tissue. Band a in Fig. 4B represents the longest transcript and corresponds to the 3′ terminus of the soybean-derived cDNA clone pFS2019. This distal polyadenylation site is used both in soybean seedlings and in sunflower tumors and is located 35 bp upstream from the *Bgl*II site at the terminus of the transferred soybean gene. In addition to the major termini present in the soybean gene, other bands (Fig. 4B, arrows) are seen with 40°C HST RNA which correspond to truncated transcripts with termini within the 3′-untranslated portion of the gene.

Upstream sequences required for efficient heat induction.

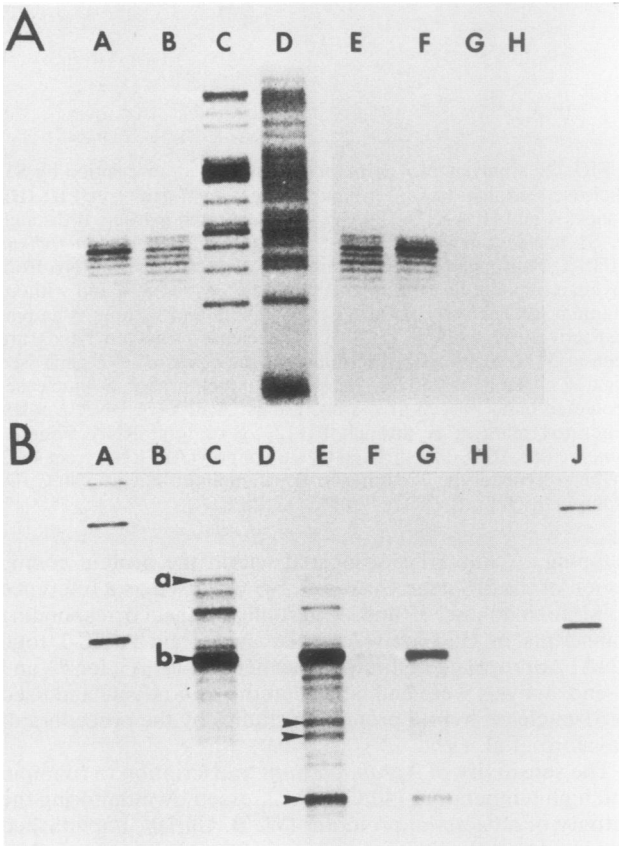

FIG. 4. S1-nuclease mapping of the 5′ and 3′ termini of transcripts homologous to the *Gmhsp17.5-E* gene. (A) 5′ termini. The hybridization probe is shown in Fig. 1C. Lanes: A and F, soybean hypocotyl at 40°C; B and E, HST at 40°C from tumors inoculated in separate experiments; C and D, DNA sequence ladder resulting from chemical cleavage (26) of the 5′-end-labeled hybridization probe; C, G; D, A-G; G, CT at 40°C; H, CT at 28°C. Lanes A, B, and E through H contained 1 μg of S1-nuclease digested poly(A)$^+$ RNA. (B) 3′ termini. The hybridization probe is shown in Fig. 1C. Lanes: A and J, ΦX174 DNA *Hae*III-generated marker; B, soybean hypocotyl at 28°C; C, soybean hypocotyl at 40°C; D, HST at 28°C; E, HST at 40°C; F, 0.5 μg of HST at 28°C; G, 0.5 μg of HST at 40°C; H, CT at 40°C; I, CT at 28°C. All lanes except F and G contained 1 μg of S1-nuclease-digested poly(A)$^+$ RNA. RNA in lanes F and G was extracted from tumors inoculated in a separate experiment. Arrows are explained in the text.

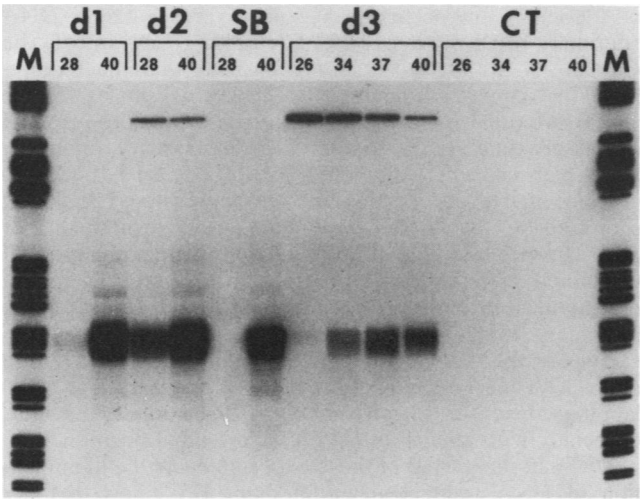

FIG. 5. S1-nuclease mapping of the 5′ terminus of *Gmhsp17.5-E* deletion mutants. All lanes contained 1 μg of poly(A) RNA hybridized to an internal 5′-end-labeled *Bam*HI fragment of the HS gene. The molecular weight marker (M) was *Hae*III-digested lambda DNA. The temperatures (°C) of tissue incubation before RNA extraction are indicated immediately above the lanes. RNA samples derived from primary sunflower tumors and soybean seedlings are designated as follows: d1, tumors inoculated with an *Agrobacterium* strain containing the *Gmhsp17.5-E* gene deleted in the 5′-flanking region to −3,250 bp; d2, 5′ deletion to −1,175 bp; d3, 5′ deletion to −95 bp; SB, soybean seedlings, CT, control tumors. The high molecular weight band represents an intact probe (250 bp) presumably protected due in part to read-through transcription from T DNA. The lower band (158 bp) reflects initiation at the HS gene promoter. The relative intensity of bands in the d3 lanes is exaggerated due to overexposure of the photograph to aid visualization of basal transcription.

The transcriptional activity of two 5′ deletions of the *Gmhsp17.5-E* gene were analyzed to identify regions of the gene that are involved in thermoinducibility. The S1-protection results are shown in Fig. 5. Gene constructions 3,250 and −1,175 bp of DNA upstream from the transcription start site were strongly expressed under HS conditions to levels similar to those found in soybean seedlings. In contrast, a 5′ deletion to −95 bp sharply curtailed heat-inducible transcription to roughly 5% of the fully inducible level. In all cases the initiation site for tumor transcription corresponded to that found in soybean RNA.

The basal level of transcription was also affected by the deletion of upstream sequences. In soybean RNA the basal level is not normally seen in RNA blot or S1-protection analyses. In our transgenic expression experiments, however, transcripts derived from the *Gmhsp17.5-E* gene were detected in all assays at control temperatures, which indicated a loss of basal repression in all three of our gene constructions. This apparent derepression of basal transcription was most evident with the −1,175-bp deletion mutant. Further exposure (not shown) of the autoradiograph shown in Fig. 5 indicated that basal transcription of the −3,250- and −95-bp deletion mutants was roughly equal.

DISCUSSION

The transcriptional activity of three different constructions of the HS gene containing from 3,250 to 95 bp of 5′-flanking sequences was analyzed by S1 protection. In each case the soybean gene initiated transcription in a thermally inducible manner under the direction of its natural promoter. The two constructions containing at least −1,175 bp of upstream flanking sequences were strongly induced by elevated temperatures (40°C). The −3,250-bp construction was also induced by sodium arsenite treatment and very weakly induced by $CdCl_2$.

The absolute level of thermally induced transcription of the *Gmhsp17.5-E* gene can only be approximated by these studies, but a visual comparison of the autoradiographic intensities of hybrid-protected bands suggests a roughly equal abundance of the thermally induced transcripts derived from the −3,250- and −1,175-bp constructions expressed in sunflower tumors compared with expression in soybean seedlings. Since RNA isolated from primary tumors is derived from a mixture of transformed and nontransformed cells (42), the introduced soybean gene appears to be at least equally active on a per copy basis and possibly more active in sunflower tumors compared with soybean seedlings.

There is a growing body of evidence suggesting that multiple HS consensus elements are required for efficient thermal induction of HS promoters (8, 18, 37). The *Drosophila HSP70* gene contains two HS elements in the

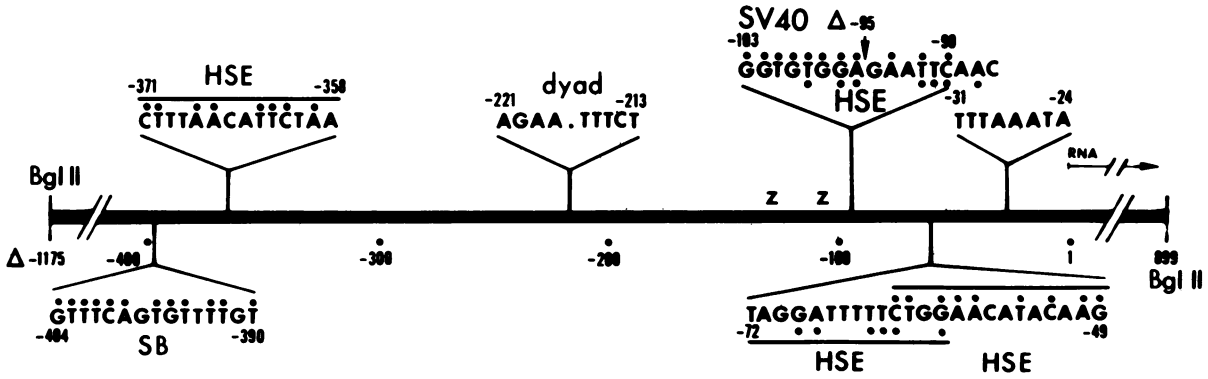

FIG. 6. Schematic representation of the *Gmhsp17.5-E* gene, showing the position of the −1,175 and −95 bp 5′ deletions (Δ). Numeric designations denote base pairs from the start of transcription. Regions of consensus homology with sequences present in other eucaryotic genes are shown. Dots indicate positions of nucleotide homology. Abbreviations: HSE, heat shock consensus element (31); dyad, pentameric palindrome (39); SB, steroid-binding site in a *Drosophila* HS gene (8); Z, potential Z DNA, eight uninterrupted alternating purine-pyrimidine pairs; SV40, simian virus 40 enhancer core (25).

near vicinity of the TATA box and two more HS elements further upstream. A 5′ deletion (−68 bp) terminating in the second HS element from TATA reduces the thermal inducibility of this gene in *D. melanogaster* by 50- to 100-fold when present on a low-copy-number P-element vector (18). Further evidence supporting the modulatory role of secondary HS elements is supplied by deletion studies with the *Xenopus HSP70* gene, where removal of the TATA-distal HS element decreases the level of heat-inducible transcription severalfold in monkey cells (COS-1) cells (8).

Our results indicate that the soybean HS gene *Gmhsp17.5-E* requires upstream sequences in addition to the TATA-proximal HS element to provide efficient induction. In the *Gmhsp17.5-E* gene (Fig. 6) the TATA-proximal HS element shows a 9 of 10 homology to the consensus core and overlaps a weak homology element (6 of 10) immediately upstream. This overlapping arrangement of a weak upstream element with a stronger TATA-proximal HS element is present in four of the soybean HS genes corresponding to the low-molecular-weight proteins (15, 29, 36). This combination of elements near the TATA region appears to be sufficient for low-level thermal induction, but sequences located between −95 and −1,175 bp are necessary for full transcriptional activity under HS conditions.

A redundant HS consensus element (HSE) located from −358 to −371 bp (CTttAAcaTTCtAa) is removed by the 5′ deletion to −95 bp. This upstream consensus element contains an 8 of 10 match with the *Drosophila* HS element and is located 296 bp upstream from the TATA-proximal HS element. Another possible HSE is centered near −93 bp (Fig. 6). This sequence has a 7 of 10 homology to the consensus core and is disrupted by the −95-bp deletion. In view of the evidence suggesting the requirement of upstream HS elements for the strong expression of animal HS genes (8, 18, 37), it seems likely that one or both of these redundant HS elements of the *Gmhsp17.5E* gene are involved in a similar fashion in the modulation of heat-inducible transcription.

Other interesting homologies (Fig. 6) deleted or disrupted by the −95-bp deletion construction that may play a role in the modulation of transcriptional expression include several potential sites for Z-DNA formation, a region similar to the pentameric palindrome found in several *Drosophila* genes (39), the SV40 enhancer core (11 of 14 bases) (25), and the steroid-binding site (G-T/A-T/A-T/A-C-A-C/A-T/A-C/G-T-G-T-T/C-C-T) (8) of an ecdysone-inducible *Drosophila* gene. The functional significance of these sequences in the 5′-flanking region of the soybean HS gene is only speculative until further results of mutagenesis studies are available.

Schoffl and Baumann (34) have introduced a soybean HS gene into sunflower tumors via the T-DNA of the nopaline-type plasmid pTiC58. In contrast to the *Gmhsp17.5-E* gene, the level of transcriptional expression of *hs6871* in excised tumors at 40°C is much less than that in 40°C soybean hypocotyl tissue. This large variation in the transcriptional efficiency between these closely related genes is surprising since their promoter structures appear by inspection to be similar. For example, the *hs6871* gene (36) also has an overlapping double HSE proximal to TATA, with 80% homology for the TATA-proximal consensus and 70% homology for the more distal element. In the *hs6871* gene the poorer homology (80 versus 90% for the *Gmhsp17.5-E* gene) to the HSE at the TATA-proximal position appears to be only partially compensated by multiple HSEs (70 to 90% homology to the consensus) further upstream, for a total of six HSEs within 280 bp of the start of transcription.

A comparison of the transgenic expression and DNA sequence structure of the 5′-flanking region of the *Gmhsp17.5-E* and *hs6871* genes suggests that the requirement for sequence homology to the HS consensus for the TATA-proximal element is sharply defined. Although the TATA-proximal HSE confers heat inducibility upon the gene, high consensus homology (≧90%) alone is not sufficient for high levels of transcriptional expression. It has been proposed (8, 18, 37) that redundant HSEs located further upstream are required for the full upward modulation of thermoinducible activity. Apparently the degree of homology to the HS consensus and the precise location of the redundant HSEs shows greater functional latitude than exists for the TATA-proximal HS element. The deletion study with the *Gmhsp17.5-E* gene also suggests that overlapping consensus regions act as a single functional unit, especially with regard to the TATA-proximal position.

The long-range goal of these experiments is the identification of DNA sequences within plant HS genes that are involved in the regulation of transcription. We are interested not only in the mechanisms operative in large-scale activation, but also in secondary modulation that may occur in response to different types or combinations of stress. Dudler and Travers (18) have discussed the possibility that high-copy-number vectors may not allow detection of fine-scale modulation since excessive template may saturate various transcription factors that potentially interact in a cooperative manner. In this regard, the relatively low number of stable T-left insertions per genome (16, 27, 40, 41) renders the T-DNA of *A. tumefaciens* well suited as an expression vector for studying the molecular aspects of the fine-scale modulation of plant genes.

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