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Functional analysis of sequences required for transcriptional activation of a soybean heat shock gene in transgenic tobacco plants

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The 5′ DNA sequences involved in the thermal inducibility of the soybean heat shock gene *hs6871* were analysed in transgenic tobacco plants. The transcriptional activity of various *in vitro* generated deletion mutants was examined by Northern blot analysis, S1 nuclease mapping and dot-blot hybridization. At least 181 bp upstream from the translational start site are sufficient for thermal induction at 40°C and correct initiation of transcripts. Full promoter activity with the induction of wild-type levels of transcripts requires additional upstream sequences contained within 439 bp 5′ to the coding sequence. Our results suggest that faithful regulation and the generation of high levels of *hs6871*-specific mRNA depend on the presence of sequences which show homology to the 14-bp heat shock consensus element of *Drosophila* and, in addition, on as yet unidentified enhancer-like upstream sequences.

Key words: heat shock/plant promoter/soybean/transgenic tobacco

Introduction

In plants the effects of heat shock (hs) on specific gene activation have been studied predominantly in soybean (*Glycine max*) (Key *et al.*, 1984; Schöffl *et al.*, 1984a). A comparison between the much investigated *Drosophila* and the soybean system reveals hs-proteins of similar sizes induced in *Drosophila* at 37°C and in soybean at 40°C (Key *et al.*, 1981). However, there are also marked differences especially in the appearance of an extra set of hs-proteins in soybean in the low molecular mass range 15–18 kd. The high abundance and complexity of proteins in this group is typical for plants, suggesting a common role of these low molecular mass hs-proteins in the plant hs-response (Key *et al.*, 1983). The biological importance is indicated by the conservation of the corresponding genes (Schöffl *et al.*, 1984b), which are organized in soybean in several multigene families (Schöffl and Key, 1982, 1983; Schöffl *et al.*, 1986).

The expression of these genes is primarily controlled at the level of transcription (Schöffl *et al.*, 1987). This property is structurally related to the presence of several copies of an element in the 5′ promoter region which is homologous to the consensus hs-element (HSE) from *Drosophila* (Schöffl *et al.*, 1984b, 1986; Nagao *et al.*, 1985). The occurrence of multiple copies of HSE-like sequences in the promoter is also found in hs-genes of various other organisms e.g. *Dictyostelium*, *Xenopus* and *Drosophila* (for a review, see Pelham, 1985). The *Drosophila* HSE is necessary and sufficient to confer heat inducibility upon an adjacent TATA box (Pelham, 1982) and is the binding site for a transcription factor that is activated in response to hs (Dudler and Travers, 1984; Parker and Topol, 1984; Wu, 1984, 1985; Topol *et al.*, 1985). The DNA sequences involved in the hs response have been strongly conserved during evolution and the *Drosophila* hsp 70 promoter is appropriately regulated in mammalian (Corces *et al.*, 1981) and plant cells (Spena *et al.*, 1985).

In this study we used the soybean hs-gene *hs6871*, synonymous with *Gmhsp17.3-B*, a member of a multigene family encoding a hs-protein of 17.3 kd. The 5′ promoter upstream region of *hs6871* contains a number of HSE-like sequences at positions −163, −173, −225, −234, −245 and −276 with respect to the translational start site (Schöffl *et al.*, 1984b). Previously, we showed that the soybean hs-gene *hs6871* is accurately initiated in sunflower tumour tissue (Schöffl and Baumann, 1985) and in transformed tobacco plants (Schöffl *et al.*, 1986) in a heat-regulated fashion.

In the present study we introduced a series of deletions into the promoter region of *hs6871* and tested their effects on heat-regulated transcription of the native gene in transgenic tobacco plants. The results suggest a requirement of two separate *cis*-active regions, one for correct thermoinducible initiation and one for maximal enhancement of transcription.

Results

Isolation of promoter deletions and construction of vector plasmids

The starting material to construct deletions was the recombinant plasmid phs6823 containing the entire *hs6871* gene with 5′ and 3′ flanking sequences of 1 kb and 0.7 kb respectively.

After linearization of phs6823 with *Hin*dIII, the plasmid was partially digested with *Bal*31-nuclease, flush-ended with the Klenow fragment of DNA polymerase I and cut with *Pst*I. Deleted fragments were cloned into the *Sma*I/*Pst*I-digested pUC8 plasmid, the precise endpoints of the deletions were mapped by DNA sequence analysis (Figure 1). We also exploited two convenient restriction sites, one for *Sca*I cutting at position −154 and one for *Nsi*I cutting at position −181 respectively, to construct additional deletions. The designations of the deletions refer to their distance in bp from the ATG start codon in *hs6871*.

The three deletions *hs6871*−439, −407 and −298 still contain all the HSE-like elements (Figure 1), the deletion *hs6871*−242 lacks the element with the highest homology (90%) with the *Drosophila* HSE-consensus sequence. The deletion *hs6871*−181 still contains the two overlapping HSE-like elements in front of the TATA box. The endpoint of *hs6871*−154 maps just 5′ from the TATA box sequence and the endpoint of *hs6871*−115 is in between the TATA box and the transcriptional start site.

The various promoter deletions were cloned into the binary vector *BIN19* (Bevan, 1984), using *Eco*RI/*Hin*dIII (Figure 1). A further mutant, designated *hs6871*−181/*inv*−407 was constructed by inverting the sequence between the positions −407 and −181 of *hs6871*−407 and fusing it in this opposite orientation in front of *hs6871*−181 (Figure 1). This was done by ligating

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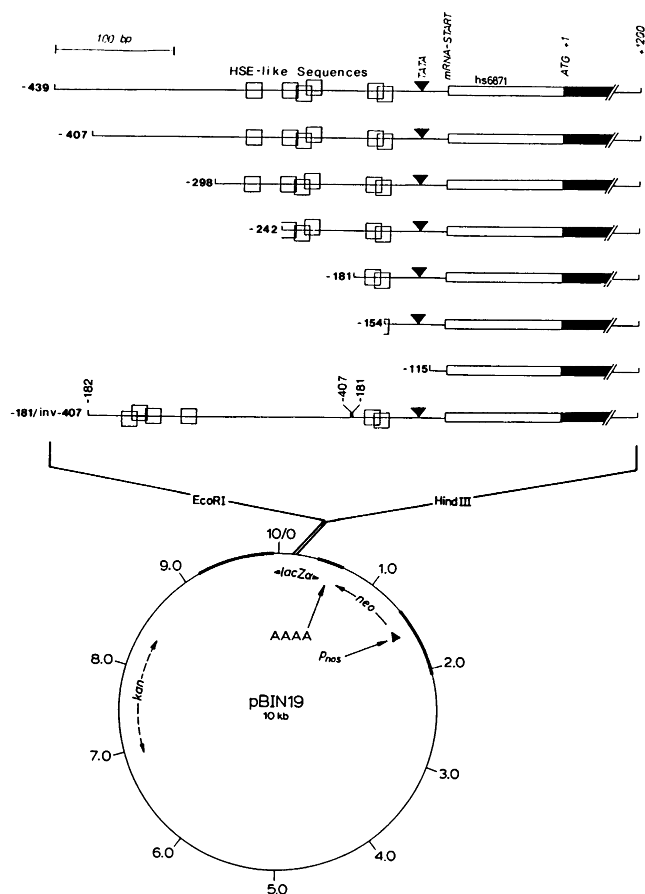

Fig. 1. Diagram of the 5′ upstream promoter deletions of *hs6871*, cloned into the plant vector *BIN19* and used for transformation of tobacco. The designation of the deletions refers to the distances in nucleotides from the translation start site of the gene which is taken as +1. The positions of HSE-like sequences are marked by open squares, the TATA box sequence by filled triangles, open bars represent the 5′ untranslated sequence, the solid portions of the bars indicate the coding region of *hs6871*. Selected characteristics of the plasmid BIN19 (Bevan, 1984) are the lacZα sequence (*lacZα*), the nopalin synthase promoter (*Pnos*), the neomycin phosphotransferase gene (*neo*), the *nos* polyadenylation site (AAAA) and the kanamycin resistance gene (*kan*). The relative map positions on the plasmid are given in kb.

the *Eco*RI/*Nsi*I fragment from *hs6871*−407 to an *Eco*RI/*Pst*I linker fragment derived from the multiple cloning site of pUC8 and cloning the resultant fragment into the compatible *Pst*I site of *BIN19*−*hs6871*−181.

Recombinants were identified by restriction mapping and the proper constructions were mobilized into *Agrobacterium tumefaciens* strain LBA4404 using *Escherichia coli* HB101 harbouring pRK2013 as described by Bevan (1984). The integrity of the transferred *BIN19*−*hs6871* deletions was tested by Southern blot hybridization analysis of total *Agrobacterium* DNA.

Transformation of tobacco and regeneration of plants

The various promoter deletions of *hs6871* were introduced into the genome of tobacco cells via *BIN19* by using the leaf disc transformation method as described by Horsch *et al.* (1985). Morphologically normal shoots were rooted and maintained on hormone-free medium containing kanamycin (100 μg/ml). The presence of the transformed genes, as full-length copies, was tested by Southern blot analysis using total DNA isolated from independent, kanamycin-resistant plants, digested with *Eco*RI and *Hin*dIII and hybridized after blotting with a *hs6871*-specific DNA probe. Out of 67 plants tested, 61 (91%) revealed the correct restriction pattern, as predicted for *Eco*RI/*Hin*dIII digests of the *hs6871* deletion mutants (data not shown). Genomic DNAs digested separately with *Eco*RI, cutting at a unique site in the various deletion constructs, showed 1−3 hybridizing bands as plant/T-DNA junction fragments which is in accordance with 1−3 copies of the introduced DNA, being integrated independently in the genomic DNA. The strong correlation between the ability to form roots in the presence of kanamycin and correct transformation is in agreement with the observation of other groups, that ~90% of the shoots regenerated in the presence of kanamycin were also positive for the expression of a co-transferred non-selective marker (De Block *et al.*, 1984; Bevan *et al.*, 1985).

The induction of hs6871-specific mRNA in transgenic plants

To test the ability of promoter deletions to direct hs-specific transcription of *hs6871*, RNA was isolated from transgenic plants before and after a hs (2 h, 40°C). Seven or eight independently transformed plants were tested for each deletion to mediate for possible position effects.

The RNAs were fractionated in denaturing agarose gels and blots were hybridized with a *hs6871*-specific DNA probe, a 208-bp *Taq*I fragment (see Figure 4C), which does not cross-hybridize with endogenous hs-specific RNA from tobacco (Schöffl *et al.*, 1986). The presence of *hs6871* transcripts in transgenic tobacco plants is indicated by hybridization bands which have identical electrophoretic mobilities to hs-mRNA isolated from soybean (~900 nucleotides). The variation in the level of transcripts of the same deletion in different transgenic plants does not correlate with different copy numbers of the transformed genes. The average level of transcripts per deletion, however, declines in a non-linear fashion with progressing deletion of the 5′ upstream promoter sequences.

Plants containing the deletions *hs6871*−439 and −407 synthesize high levels of *hs6871*-specific RNA. The deletion of sequences from position −407 to −298 causes a large reduction of *hs6871*-specific transcripts, which is further reduced in plants of *hs6871*−242 and −181.

The deletion mutant *hs6871*−154, which still contains the TATA box sequence, and the deletion mutant *hs6871*−115, which does not, failed to induce transcrips at a detectable level. This is also true for all transgenic plants when incubated at normal temperature of 25°C (see Figure 2, lane 2). The effect of the polarity of regulatory upstream sequences on the levels of hs-induced transcription was tested by the inversion mutant *hs6871*−181/*inv*−407. Transgenic plants containing *hs6871*−181/*inv*−407 seem to generate an intermediate level of transcripts between those in *hs6871*−407 and in *hs6871*−181 transformed plants (Figure 2).

Quantification of mRNA levels

Since independently isolated transgenic plants containing the same deletion of *hs6871* show a large clonal variation in their levels of expression, a semi-quantitative evaluation of the average mRNA levels was obtained by dot-blot hybridization.

Equal amounts of RNA from seven or eight independent plants per deletion (as used in Northern blot hybridization) were pooled and poly(A) RNA was isolated. Serial dilutions of these RNAs were bound to nylon filters using a slot-blot device and probed with the same *hs6871*-specific fragment as used for the Northern blot analysis.

A comparison of the resulting signal intensities in the autoradiograph suggests a high level of steady-state hs-RNA for the

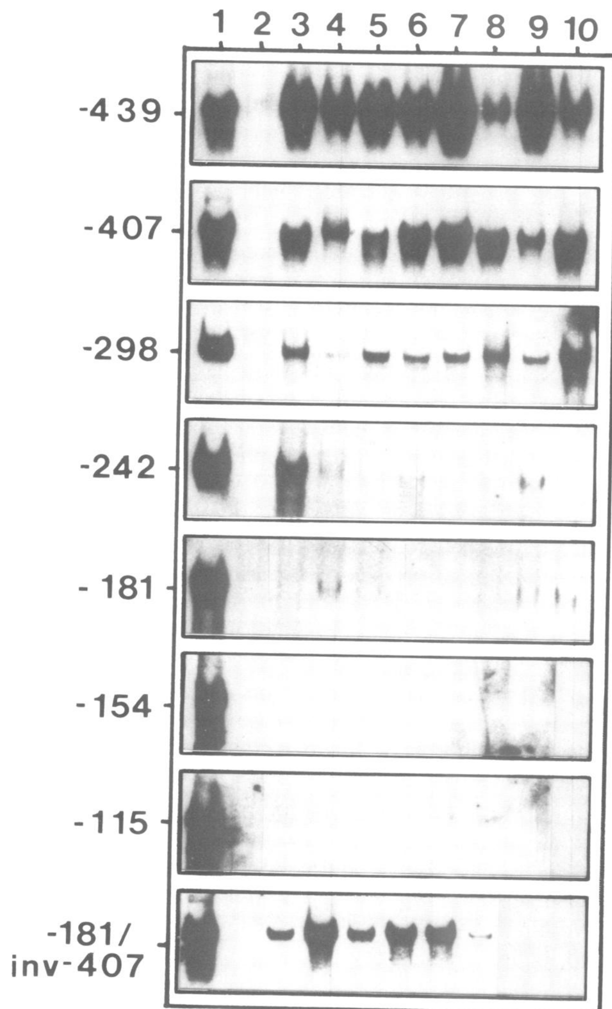

Fig. 2. Cellular levels of *hs6871*-specific RNA in transgenic tobacco plants tested by Northern blot hybridization, using a gene specific probe as diagrammed in Figure 4C. 3μg total RNA isolated from hs-induced, independently transformed plants containing the respective *hs6871* deletions as given in Figure 1 were loaded onto **lanes 3–10** and the same amount of total hs-RNAs from soybean onto **lane 1**, from the untreated (25°C) transgenic tobacco plants used in **lane 3** onto **lane 2**. The **lane 10** in panels *hs6871*−154 and *hs6871*−115 and **lanes 9** and **10** in *hs6871*−181/inv−407 are not occupied.

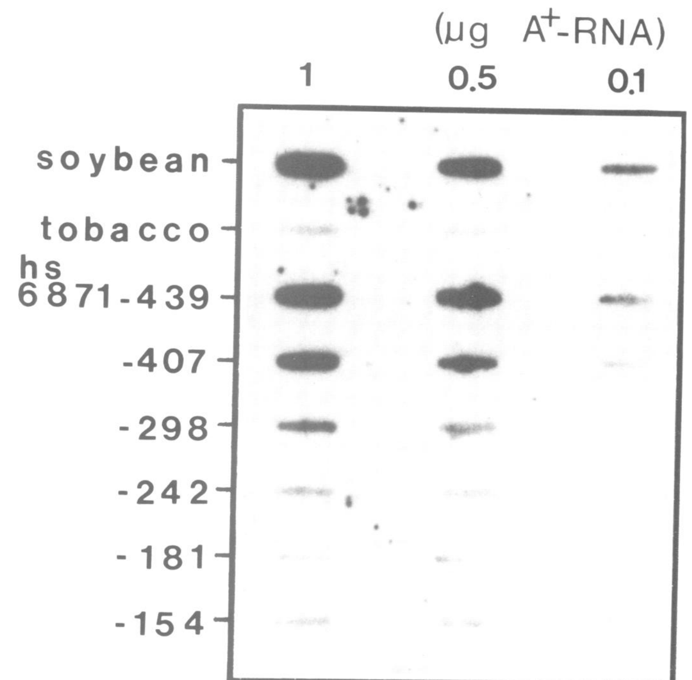

Fig. 3. Quantification of *hs6871*-specific RNA present after hs in soybean and tobacco by dot-blot hybridization.

deletion mutant *hs6871*−439 that is indistinguishable from the level of this hs-RNA in soybean (Figure 3). Hence, no further regulatory sequences upstream of position −439 in *hs6871* are required to induce a wild-type level of transcription in the heterologous tobacco plant. The RNA level in *hs6871*−407 plants is reduced to ~50% and the level in *hs6871*−298 plants to ~10%. The RNA levels in the other deletion mutants are further reduced but could not be quantified by this assay due to the resolution limits of the applied method.

Faithful initiation of transcripts in tobacco

The start of transcription of *hs6871* in soybean (Schöffl *et al.*, 1984b) and in sunflower (Schöffl and Baumann, 1985) was previously established by S1 nuclease mapping to be at position −103. This technique was also used to determine the precise start site for transcript–initiation of the deletion mutants in tobacco.

RNA isolated from transformed tobacco before and after a hs was hybridized to the *hs6871*-specific, 5′ end-labelled DNA probe as described in Figure 4C. The RNA/DNA hybrids were digested with an excess of S1 nuclease and electrophoresed on a sequencing gel. The resulting autoradiographs show that individual plants transformed with one of the following deletions: *hs6871*−439, −407, −298, −242 and −181, contain hs-specific RNAs which protect the same major fragment, up to position −150, as the hs-mRNA from soybean (Figure 4A, B). The result suggests faithful initiation of the gene in transgenic tobacco. No hs-specific transcripts were identified in plants transformed with *hs6871*−154 and in transformed tissue incubated at normal temperature (25°C).

Discussion

The experiments described here show that the hs-transcription system is conserved between soybean and tobacco. The signal sequences in the promoter of the soybean hs-gene *hs6871* are correctly recognized in tobacco, probably by *trans*-acting factors. Various soybean hs-promoter deletions were introduced into the genome of tobacco via T-DNA transfer and analysed or heat-inducible transcription.

At least two different functions can be assigned to distinct parts in the promoter region of *hs6871*. The basic sequences for hs-inducible transcription seem to be located in between the positions −181 (the first positive deletion upstream from the TATA box) and −154 (the negative deletion proximally adjacent to the TATA box). The structural feature within this region is an overlapping HSE-like doublet (Figure 5). Most other soybean hs-genes sequenced to date contain a similar arrangement of HSEs with a rather strict spacing (15–20 bp) upstream from the TATA box sequences (Schöffl *et al.*, 1986).

The organization of the other upstream HSE-like sequences in *hs6871* is further dissected by the deletions *hs6871*−242 and −298 which result in a modest modulation of transcription. The high level of thermoinduced transcripts generated with the promoter inversion *hs6871*−181/inv−407 suggests a bidirectional function of the respective promoter elements.

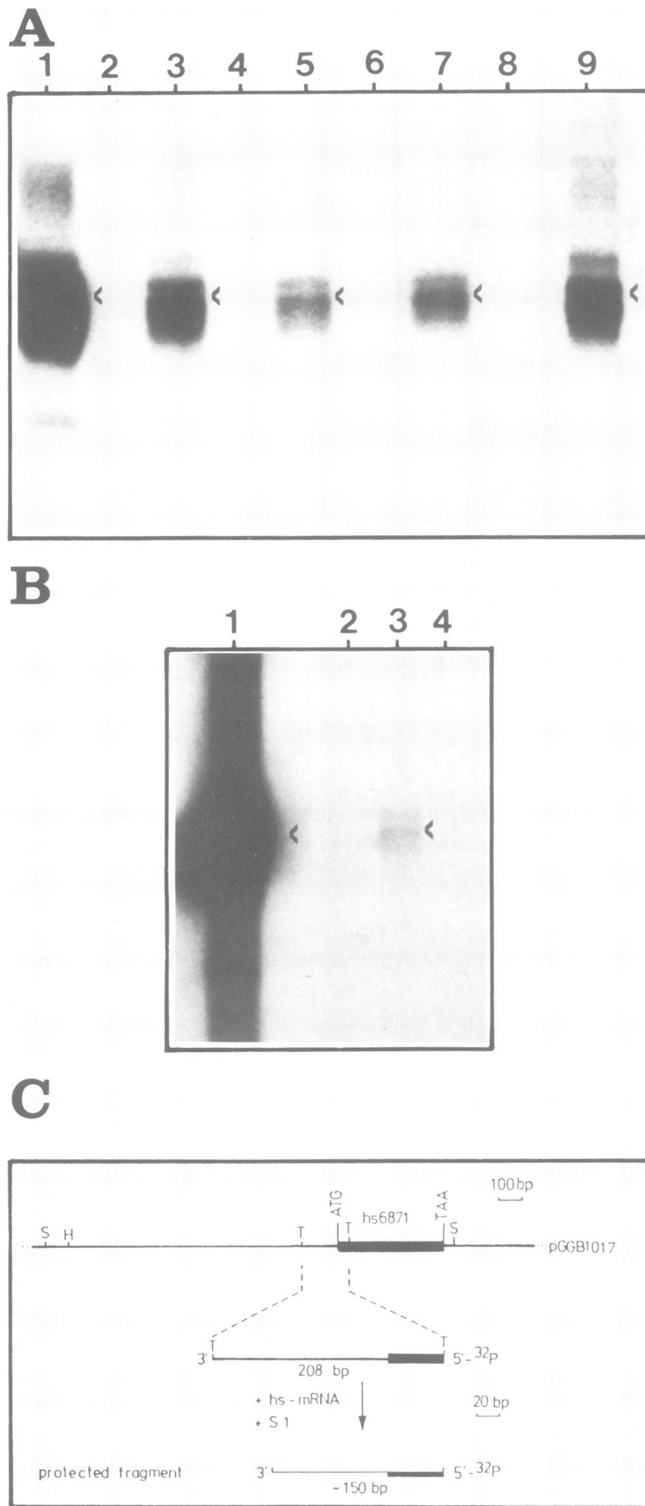

Fig. 4. S1 nuclease mapping of the 5′ start site of *hs6871* transcripts in soybean and transgenic tobacco plants. **(A)** 30 μg of total RNA isolated from heat-shocked (odd lanes) and untreated (25°C) (even lanes) transgenic tobacco plants containing the deletions *hs6871*−439 **(lanes 1, 2)** *hs6871*−407 **(lanes 3, 4)**, *hs6871*−298 **(lanes 5, 6)** and *hs6871*−242 **(lanes 7, 8)** or 1 μg poly(A) RNA from heat-shocked soybean **(lane 9)** were tested by this assay for the protection of the indicated 150 nucleotides fragment as outlined in **(C)**. **(B)** For each lane 1.5 μg poly(A) RNA were used in the S1 nuclease protection assay, isolated from soybean after hs **(lane 1)**, *hs6871*−181-transformed plant, 25°C **(lane 2)** and after hs **(lane 3)** and *hs6871*−154-transformed plant after hs **(lane 4)**.

The organization of the *hs6871* promoter with its various HSE-like elements resembled that of the hs-gene promoters in *Drosophila*. Multiple HSEs in the promoter of the *Drosophila hsp70* gene contribute in a co-operative way to the formation of an active transcription complex, whereby molecules of the hs transcription factor bind co-operatively to the adjacent HSEs and may increase the affinity of RNA polymerase II for this promoter (Topol *et al.*, 1985).

Assuming a similar interaction between HSE-like elements of *hs6871* and hs-transcription factors in tobacco, one finds a positive correlation between the number of HSE-like elements and the average level of transcripts induced by the different deletions between −181 and −298. Individual HSE-like elements may only have a little effect but accumulation of these elements and appropriate spacing between them may potentiate promoter activity and govern fine tuning of transcription. A different function is suggested for the additional regulatory sequences located upstream from the cluster of HSE-like elements, a region delimited by the deletions *hs6871*−439 and −298. This region is obviously required for maximal transcription and increases the specific mRNA level by a factor of 10. Such regulatory upstream sequences, known as enhancers, can function in a bidirectional manner as shown for genes in animals and yeast (Gluzman and Shenk, 1984). The expression of genes in plants may also be regulated by enhancer-like elements as shown for light-regulated genes from pea in tobacco (Timko *et al.*, 1985; Fluhr *et al.*, 1986; Simpson *et al.*, 1986).

There is no preferential homology in the putative enhancer region of *hs6871* with the SV40-enhancer core sequence 5′TGTGG(A/T)(A/T)AG (Herr and Gluzman, 1985; Sassone-Corsi and Borrelli, 1986) which is a characteristically repeated motif within many enhancer sequences. Computer searching for direct or indirect repeats revealed an imperfect hyphenated dyad symmetry of 34 bp with its centre at position −407 (see Figure 5). A functional significance of this sequence is supported by the fact that the level of transcripts in *hs6871*−407 is reduced to 50% of the level in *hs6871*−439. The region between −407 and −298 contains another structural feature which is a run of 14 As between positions −357 and −371 (see Figure 5). Similar 'simple sequences' have been reported to function as upstream enhancer elements in yeast (Struhl, 1985) and the cohabition of nuclear scaffold attachment regions (SARs, containing also runs of As or Ts) and transcriptional enhancers in *Drosophila* (Gasser and Laemmli, 1986, 1987) suggests a functional interrelationship of structural and gene regulatory sequences. Simple sequences are also present in the proximity of 5′ non-transcribed regions of other soybean hs genes (E.Raschke, G.Baumann and F.Schöffl, in preparation).

The results of our investigation suggests that the high level of transcription and faithful regulation by a plant hs-promoter sequence depends on the presence of HSE-like elements, possibly with an appropriate spacing, upstream from the TATA box sequence and on the additional function of other as yet unidentified enhancer-like sequences that augment promoter activity. The enhancer function is confined to a total DNA sequence of 141 bp (between −439 and −298) as defined by our deletion analysis and will further facilitate detailed studies on the specificity of regulation, when joined to a heterologous promoter (Fluhr *et al.*, 1986).

To date, hs-genes of different origins have been tested in heterologous plant systems for correct and efficient activation. The *hsp70* promoter when fused to the *Npt*II reporter gene shows only a 5- to 50-fold stimulation of the gene in response to hs

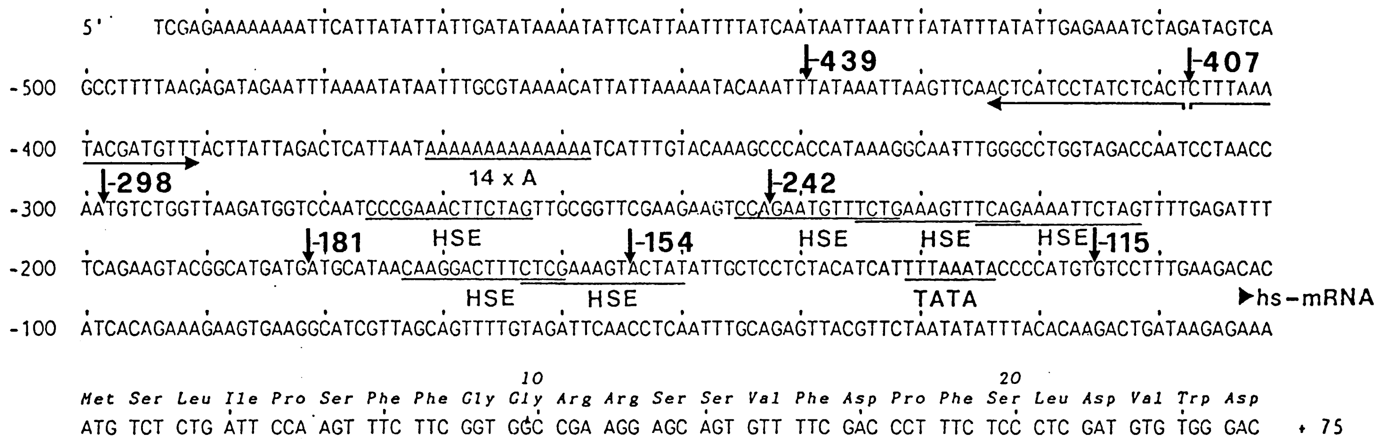

Fig. 5. The nucleotide sequence of the 5′ flanking region of *hs6871*. Consensus sequences (TATA box, HSE) are underlined. The 5′ endpoints of the promoter deletions are marked by vertical arrows. The imperfect (34/19), indirect repeat with its centre at position −407 is underlined by horizontal arrows. The transcriptional start site is indicated (redrawn from Schöffl *et al.*, 1984b).

(Spena *et al.*, 1985). The level of hs-induced transcripts for a gene fusion of two maize *hsp70* genes in transgenic petunia is also greatly reduced compared with maize (Rochester *et al.*, 1986). In neither of the two systems was the activity of the native hs-promoter indicated. However, this has been demonstrated for the soybean *hs6871* gene in transgenic tobacco plants containing the original gene and ~1 kb (Schöffl *et al.*, 1986) or 439 bp of 5′ upstream sequences (this paper). This high activity may be due to the close proximity of the enhancer-like sequences to the other *cis*-acting HSE-like elements and/or to the otherwise strictly unmanipulated gene and its native genomic context.

Materials and methods

DNA manipulations, plasmids and bacterial strains

All DNA manipulations were performed using standard DNA techniques as described by Maniatis *et al.* (1982) or according to the supplier's instructions (Boehringer Mannheim).

The *hs6871* gene was isolated from phs6871 (Schöffl and Key, 1983) by subcloning the *Hin*dIII/*Pst*I fragment into the respective sites of pUC8 (Vieira and Messing, 1982).

The *Bal*31 deletions were subcloned into pUC8 and digested with *Eco*RI/*Pst*I. The fragments of interest, containing the endpoints of deletion at the *Eco*RI site, were purified after digestion by gel-elution and end-labelling by a 3′ filling-in reaction with the Klenow fragment of polymerase I. The endpoints of the deletions were determined by DNA sequencing according to Maxam and Gilbert (1980). The sequencing reactions were separated on 0.3-mm 5% polyacrylamide gels using the LKB Macrophor gel system.

The *Agrobacterium* binary vector *BIN19* was utilized essentially as described by Bevan (1984).

Plant transformation

Sterile leaf discs from *Nicotiana tabacum* var. Samsun were incubated for 30 s with the *A. tumefaciens* LBA4404-strains carrying the respective *hs6871* deletions. Plant regeneration was carried out as described by Horsch *et al.* (1985) but without using nurse culture plates at the initial incubation step. After rooting in plant medium, containing kanamycin (100 μg/ml) and cefotaxime (500 μg/ml), regenerated tobacco plants were grown under greenhouse conditions.

Heat shock treatment

A heat shock temperature of 40°C was applied for 2 h to tobacco and soybean leaves according to Key *et al.* (1981) described for soybean seedlings.

DNA and RNA isolation

Total DNA from *A. tumefaciens* was isolated as described by Meade *et al.* (1982). High mol. wt DNA from tobacco leaves was prepared using the procedure of Dellaporta *et al.* (1983).

Total RNA was isolated from soybean and tobacco tissue immediately after incubation at the respective temperatures. The plant tissue was frozen and homogenized in liquid nitrogen, incubated in extraction buffer [100 mM Tris−HCl, pH 8.0, 100 mM NaCl, 50 mM EDTA, proteinase K (100 μg/ml), 1% SDS 0.1% β-mercaptoethanol] and then extracted three times with phenol/chloroform. The nucleic acids were precipitated from the aqueous phase with isopropanol, dissolved in TE (pH 7.6) and reprecipitated by a 4-h incubation at 0°C with lithium chloride added to a final concentration of 2 M. The RNA pellet was resuspended in TE (pH 7.6).

Poly(A) RNA was isolated by passage over messenger affinity paper (mAP) as described by Werner *et al.* (1984).

Southern, Northern and slot-blot hybridization

Southern and Northern blot hybridizations were performed according to Schöffl and Baumann (1985). Evaluations of specific RNAs were obtained using a slot-blot device (Schleicher and Schüll). Dilution series of poly(A) RNA from leaf tissue were denatured in 6.15 M formaldehyde, 10 × SSC at 65°C for 15 min and loaded onto the slot-blotter. A sheet of Hybond N (Amersham) rinsed in 10 × SSC was placed beneath the slots. After addition of the RNA samples the filters were dried and the RNA was immobilized by irradiation on a standard u.v. transilluminator for 5 min. Prehybridization, hybridization and washing conditions were as used in Northern blot hybridization previously described by Schöffl and Baumann (1985).

S1 nuclease mapping

Thirty microgrammes of total RNA or 1.5 μg poly(A) RNA were hybridized for 18 h at 43°C with the 5′ ^{32}P-end-labelled DNA fragment and then treated with S1 nuclease (100 U/ml, 30 min, 37°C) followed by electrophoresis and autoradiography of the protected fragments on a sequencing gel (Schöffl *et al.*, 1984b). The fragment sizes were determined by a sequencing standard run on the same gel.

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