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Conserved function in *Nicotiana tabacum* of a single *Drosophila hsp70* promoter heat shock element when fused to a minimal T-DNA promoter

David Wing*, Csaba Koncz, and Jeff Schell
Max-Planck-Institut für Züchtungsforschung, Egelspfad, D-5000 Köln 30, Federal Republic of Germany

Summary. To demonstrate the extent of evolutionary conservation in the mechanism of induction of heat shock genes between plants and animals, the minimal sequence from the *Drosophila hsp70* promoter sufficient to confer heat shock inducible transcription in tobacco was determined. Segments of the *hsp70* promoter were fused to a minimal promoter of the T-DNA indole-3-acetamide hydrolase (*iaaH*) gene, in a chimaeric gene fusion to a neomycin phosphotransferase (*NPT II*) reporter gene. Sequences bearing one or more heat shock elements (HSEs) rendered the minimal promoter heat shock inducible, with a 37 bp fragment containing a single complete HSE sufficing. The induced *NPT II* mRNA peaked during the heat shock period, but the maximal level of NPT II activity was not observed until 4 h later in the recovery phase, showing that the translation of the *NPT II* mRNA was shifted from the heat shock period of the recovery phase. That similar sequences containing a single HSE of the *Drosophila hsp70* promoter could function in both flies and tobacco indicates the high degree of homology between the two heat shock gene induction systems.

Key words: Heat shock – Plant gene transfer vector – *Drosophila hsp70* – Tobacco – Minimal promoter

Introduction

A sudden increase in temperature, or heat shock, induces a rapid change in gene expression of all organisms so far examined (Schlesinger et al. 1982). During the initial stages of the heat shock response the rate of transcription of a set of genes, termed the heat shock genes, is increased dramatically (Ashburner and Bonner 1979). These new mRNAs are preferentially translated during the heat shock period (McKenzie et al. 1975; Lindquist 1981) and encode the heat shock proteins which are thought to protect the cell from the thermal stress (Schlesinger et al. 1982).

Biochemical and genetic evidence illustrates the critical role played by a 14 bp consensus sequence CTnGAAnnTTCnAG, termed the heat shock element (HSE), to mediate the transcriptional activation of the heat shock genes. It is observed, often in multiple copies, in the 5′ upstream regions of the heat shock genes of all eukaryotes (Pelham 1985). A heat shock transcription factor (HSTF) isolated from *Drosophila* or yeast cells can bind specifically in vitro to HSEs of *hsp70* promoters of either organism (Wiederrecht et al. 1987). Probing of the chromatin structure of the *Drosophila hsp70* gene after heat shock shows that the HSEs are complexed with protein, presumably with HSTF (Wu 1984). DNA binding and in vivo heat shock activation experiments using the cloned yeast HSTF gene confirm its role in heat shock gene regulation, which is to bind to the HSE and mediate the heat shock induced increase in transcription of the heat shock gene (Sorger and Pelham 1988; Wiederrecht et al. 1988).

Promoter deletion analysis of the *Drosophila hsp70* gene assayed in stably transformed *Drosophila* cells has demonstrated that a single HSE is required for partial heat shock inducible transcription, although promoters with two HSEs more closely approached the level of induction of an intact promoter (Dudler and Travers 1984; Xiao and Lis 1988). Furthermore, in DNA transfection experiments, a single synthetic HSE oligonucleotide placed 5′ of a thymidine kinase gene promoter of herpes virus is heat shock inducible in COS monkey cells (Pelham and Bienz 1982). As a demonstration of how well conserved is the function of the HSE throughout the animal kingdom, two constructions using the *Drosophila hsp70* promoter, one carrying two HSEs on the sequence from −108 to −37 added to a truncated *Xenopus hsp70* promoter (Bienz and Pelham 1986) and a second leaving only one HSE on the 5′ deletion to −67, are both heat shock inducible in monkey COS cells (Mirault et al. 1982; Pelham 1982).

The structure and function of the HSE have been found to be conserved in the plant kingdom as well. HSEs have been observed in the promoter regions of heat shock genes of soybean and maize (Schöffl et al. 1984; Czarnecka et al. 1985; Rochester et al. 1986). A further indication of how well the function of the HSE is maintained throughout all eukaryotes is shown by the ability of a 5′ deletion to −250 of the *Drosophila hsp70* promoter to confer heat shock regulation to a reporter gene in tobacco (Spena et al. 1985). Promoter deletion analysis of the soybean gene has demonstrated that an overlapping dimer of the HSEs, a structure not present in the *Drosophila hsp70* promoter, functions in a regulated manner in tobacco (Baumann et al. 1987) and that this overlapping dimer also confers heat shock inducibility to a truncated CaMV 35S RNA promoter (Strittmatter and Chua 1987).

In order to determine whether and to what degree the function of the HSE is conserved between the animal and plant kingdoms, the *Drosophila hsp70* promoter was ana-

* *Present address:* National Institute of Agrobiological Resources, 2-1-2, Kannondai, Tsukuba, Ibaraki, 305 Japan

Offprint requests to: C. Koncz

lysed in tobacco callus to delimit the minimal sequence required for heat shock inducible gene activation. Using a pair of vectors constructed for assaying positively regulated enhancers, segments of the *hsp70* promoter were linked to a heterologous minimal promoter derived from the T_L-DNA indole-3-acetamide hydrolase (*iaaH*) gene and transferred on a plant reporter gene binary vector by *Agrobacterium*-mediated DNA transfer to tobacco cells. A fragment bearing a single complete HSE from the *Drosophila hsp70* promoter was sufficient for heat shock induction in tobacco cells.

Materials and methods

Bacterial strains and media. Escherichia coli strain DH1 (Hanahan 1983) was transformed with plasmid DNA constructions (Maniatis et al. 1982) and served as recipient for back-mating with *Agrobacterium tumefaciens* (Koncz and Schell 1986). *E. coli* BMH71-18 (Yanisch-Perron et al. 1985) was transformed with *lacZ-HPT* fusion constructions. *E. coli* S17-1 (Simon et al. 1983) was transformed with the binary vectors, which were subsequently transfered into *A. tumefaciens* pGV3101 (pMP90RK) by bacterial conjugation (Koncz and Schell 1986). *E. coli* and *A. tumefaciens* cultures were grown respectively in liquid or on agar-containing LB and YEB medium (Miller 1972). The antibiotic concentrations used for selection of *E. coli* were 50 μg/ml ampicillin or 10 μg/ml gentamycin and of *A. tumefaciens*, 100 μg/ml rifampicin, 100 μg/ml carbinicillin or 25 μg/ml gentamycin.

Transformation and maintenance of plant tissues. Nicotiana tabacum W38 leaf-discs were transformed by *A. tumefaciens*-mediated DNA transfer (Horsch et al. 1985). For isolation and growth of transformed tobacco calli, the inoculated leaf-discs were placed on callus induction medium [LS medium supplemented with 1 mg/l naphthyl-1-acetic acid (NAA) and 0.2 mg/l 6-benzyl aminopurine (BAP), Linsmaier and Skoog 1965]. To regenerate shoots, the inoculated leaf-discs were placed on LS medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BAP. Shoots were rooted and propagated on MS medium without growth factors (Murashige and Skoog 1962). Claforan (500 μg/ml) was present in all media except during the inoculation of leaf-discs. Hygromycin at 15 μg/ml was used to select for transformed tissues.

In vitro DNA recombination. Standard recombinant DNA techniques were used for DNA isolation, restriction and modification, DNA transformation of DH 1 and S17-1 cells and identification of bacterial colonies containing the correct DNA plasmids (Maniatis et al. 1982).

Construction of DOVE. A *Bam*HI fragment from pGV0153 (De Vos et al. 1981) containing the *iaaH* gene of the octopine Ti plasmid pTiAch5 was digested at the *Taq*I (−417) and *Hae*III sites (+8, the A of the initiation codon of the *iaaH* gene is referred to as position 1) and subcloned into pBR322 which had filled-in *Bam*HI and *Acc*I (position 2246) ends. This resulted in plasmid p6M3. The *iaaH* promoter (P*iaaH*) was then isolated from p6M3 DNA as a *Bam*HI – *Hin*cII fragment and inserted into pUC9 DNA (Vieira and Messing 1982) with *Bam*HI and filled-in *Eco*RI ends. The *Sal*I, *Pst*I and *Hin*dIII sites of pUC9 3′ of P*iaaH* were removed by restriction endonuclease digestion followed by S1 nuclease treatment, resulting in plasmid pUC9-pro/b.

In the final step, a modified pUC19 (Yanisch-Perron et al. 1985) polylinker sequence, containing a *Bam*HI site filled-in and religated to generate a *Cla*I site (plasmid pUC19*), was placed 5′ of the P*iaaH* sequence. pUC19* DNA was digested with *Hin*dIII and the ends were filled-in. The DNA was then partially digested with *Pvu*I to yield a fragment with the 5′ half of the β-lactamase gene and the polylinker. This was ligated to pUC9-pro/b DNA, digested with *Eco*RI, filled-in and digested with *Pvu*I leaving the 3′ half of the β-lactamase gene. Figure 2a shows the resulting plasmid, DOVE (DOmain VEctor), which served as the vehicle for cloning positively regulated enhancers situated 5′ of a minimal P*iaaH*.

Mutagenesis of the hygromycin phosphotransferase (HPT) *gene.* A *Bam*HI fragment bearing the *HPT* gene (Gritz and Davies 1983) from plasmid pVU1011 (kindly provided by P. Van den Elzen) was subcloned into the *Bam*HI site of an *Eco*RI site deficient pUC19 vector to create a *lacZ-HPT* fusion (plasmid pUC-hyg) conferring hygromycin resistance to *E. coli* strain BMH71-18. The cells were treated with hydroxylamine (Miller 1972) and *Eco*RI site deficient plasmids were enriched for by four repetitions of *Eco*RI digestion of reisolated pUC-hyg DNA followed by retransformation of *E. coli* BMH71-18 with the mixed population of linear and supercoiled DNAs (Vieira and Messing 1982). Sequencing of an isolated *Eco*RI site deficient pUC-hyg (plasmid pGDW11) revealed an A to C transversion (GAATTC to GCATTC), resulting in a glutamic acid to alanine substitution in the amino acid sequence of the HPT protein. Enzyme kinetics of HPT in crude bacterial extracts showed no change in the K_M of the mutated enzyme for hygromycin (Gritz and Davies 1983).

*Construction of a plant gene transfer vector carrying the Eco*RI *site deficient HPT gene.* The *Eco*RI site deficient *HPT* gene was cloned as a *Bam*HI fragment into the *Bcl*I site of pCV5013 DNA (C. Koncz, unpublished result), between the nopaline synthase gene promoter and the polyadenylation sites from T-DNA gene *4*. This was moved to binary vector pPCV002 (Koncz and Schell 1986) as a *Hin*dIII-*Sph*I fragment resulting in plasmid pGDW31. To create pGDW32, the T_L-DNA gene *5* promoter near the left border was deleted and the multiple cloning sites were replaced with those of pUC19* from the *Eco*RI through the *Sal*I sites.

Construction of the DOVE promoter-reporter gene fusion vectors. pGDW44 was based on the binary vector pGDW31. The polyadenylation signal from the *iaaH* gene was added 3′ of the neomycin phosphotransferase (*NPT II*) gene on pKM109/9 (Reiss et al. 1984a) by inserting into the *Aha*II and *Sma*I sites, a *Cla*I (+1307) and filled-in *Hin*dIII (666 bp downstream of the stop codon) digested fragment from pGV0153 DNA resulting in plasmid pKM109/9-pAd. To this the minimal P*iaaH* from DOVE was added as an *Eco*RI + *Bam*HI digested fragment. The P*iaaH-NPT II* gene with the *iaaH* gene polyadenylation site was then cloned onto pGDW31 as an *Eco*RI-*Sal*I fragment resulting in plasmid pGDW44 shown in Fig. 2.

Construction of the hsp70-*P*iaaH *heterologous promoters.* pHS-neo (Spena et al. 1985), a subclone of the *Drosophila*

hsp70 promoter provided the *hsp70* sequences. The following fragments were cloned into DOVE to give the heterologous promoters listed: pD811 is the *Eco*RI to *Hpa*I fragment in the *Eco*RI and *Cla*I sites; pD821 is the *Hae*III to *Hpa*I fragment in the *Sma*I and *Cla*I sites; pD831 is the *Taq*I (position −72) to *Hpa*I fragment in the *Cla*I site; pD841 is the *Taq*I (position −58) to *Hpa*I fragment in the *Cla*I site.

DNA sequencing. DNA sequences were determined by the dideoxynucleotide chain termination method (Sanger et al. 1977). One guanidine was lost from the ligation junction between the *Hae*III end of the *iaaH* sequence and the filled-in *Bam*HI site of pBR322.

NPT II assay. 50 to 100 mg of callus tissue was ground in 100 µl of extraction buffer 0.0625 M TRIS-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.01% SDS, 0.01% bromphenol blue). 50 µg of protein, determined by the Bradford (1976) assay, was loaded onto polyacrylamide gels for native protein separation. An in situ assay was used to detect the NPT II activity (Reiss et al. 1984b).

RNA isolation. RNA was isolated from frozen pulverized calli by grinding the tissue in a guanidium thiocyanate solution followed by centrifugation of the resulting homogenate over a 5.7 M CsCl cushion (Chirgwin et al. 1979). RNA pellets were redissolved in water. Polyadenylated RNA was purified by two passages through oligo(dT) cellulose (Aviv and Leder 1972). RNA concentrations were determined by absorbance at 260 nm.

DNA isolation. Total plant cell DNA was isolated as a mini-preparation (Dellaporta 1983) and further purified by digestion with RNase and then proteinase K, extraction with phenol, phenol:chloroform and chloroform, precipitation with isopropanol and washing with 70% EtOH. DNA concentrations were determined by absorbance at 260 nm.

Nucleic acid transfer to and detection on nitrocellulose filters. Transfer of DNA or RNA after size resolution on agarose gels was done according to standard procedures. DNA hybridization was done in 6 × SSC solution at 65° C. RNA hybridization was done in 50% formamide solution at 42° C using an *NPT II* probe and at 39° C using the soybean actin probe (kindly provided by R. Meahger) (Maniatis 1982). Radioactive DNA probes were generated by "random primer" synthesis with [^{32}P]dCTP to specific activities of at least 5×10^7 cpm/µg DNA (Feinberg and Vogelstein 1984). Autoradiographs were taken using Kodak X-OMAT films.

Results

Characteristics of the minimal PiaaH *applied towards assaying for positively regulated enhancers using vectors DOVE and pGDW44*

DOVE is a 2.7 kb vector containing multiple cloning sites located 5′ of a truncated P*iaaH* (Fig. 2). The vector is half of a system designed to facilitate the testing of putative enhancer sequences placed upstream of a heterologous minimal promoter. The basis of the minimal promoter is a truncated P*iaaH* from the octopine Ti plasmid pTiAch5 (Gielen et al. 1984). The full promoter drives a very low level of mRNA transcription, representing about 0.00005% to 0.0001% of the total mRNA population in T-DNA transformed cells (Willmitzer et al. 1981). This reduced level of transcriptional activity was chosen to provide a low basal level of expression, thereby increasing the likelihood of detecting positively regulated enhancers. Figure 2 shows the minimal P*iaaH* consisting of the sequences begining at the "CCAAT" box, through the "TATA" box of the P*iaaH* and ending at position +8, preserving the 5′ untranslated leader sequence and the ATG of the *iaaH* gene. Suspected positively regulated enhancer sequences can be simply cloned into one of the multiple cloning sites placed 5′ of the minimal P*iaaH*, and then shuttled as an heterologous promoter into the *Eco*RI and *Bam*HI sites on the heterologous promoter-plant reporter gene transfer vector pGDW44 shown in Fig. 2.

pGDW44 is based upon the binary vector pPCV002 for *Agrobacterium*-mediated DNA transfer to plant cells (Koncz and Schell 1986). pPCV002 was first modified by exchanging the kanamycin resistance plant selectable marker for a hygromycin resistance marker (Van den Elzen et al. 1985) consisting of a mutated *HPT* gene lacking the *Eco*RI site under the control of the nopaline synthase promoter and the T_L-DNA gene *4* polyadenylation signals, to give an all-purpose plant gene transfer vector pGDW31 (Fig. 1). Further modification of pGDW31 removed the gene *5* promoter and replaced the multiple cloning sites

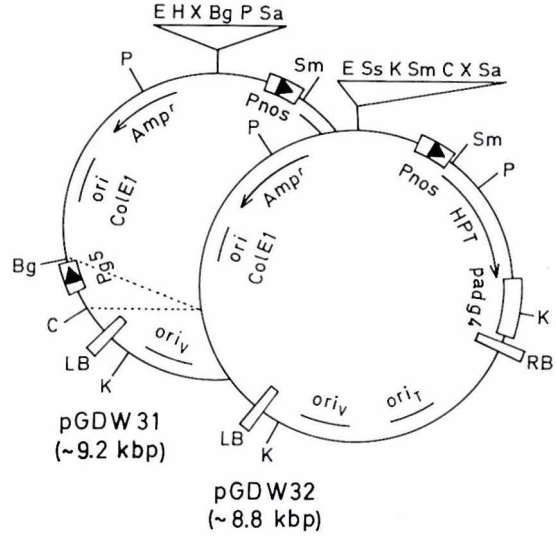

Fig. 1. General plant gene transfer vectors pGDW31 and pGDW32. pGDW31 (background) and pGDW32 (foreground) have all of the functions necessary for use as binary vectors for *Agrobacterium*-mediated gene transfer to plant cells. pGDW32 differs from pGDW31 by the lack of Pg5 and the substitution of a different set of multiple cloning sites. Only sites present in the multiple cloning site region and where they occur elsewhere on the plasmid are represented. The following abbreviations are used in all the plasmid diagrams. Pg5, T_L-DNA gene *5* promoter; Pnos, nopaline synthase promoter; pad g4, polyadenylation signals of T_L-DNA gene *4*; ori, Col E1 origin of replication; ori_V, RK2 origin of replication; ori_T, RK2 origin of conjugational transfer; RB and LB, right and left T-DNA border sequences; HPT, hygromycin phosphotransferase; Amp^r, ampicillin resistance marker. Restriction endonuclease sites: Bg, *Bgl*I; C, *Cla*I; E, *Eco*RI H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xba*I

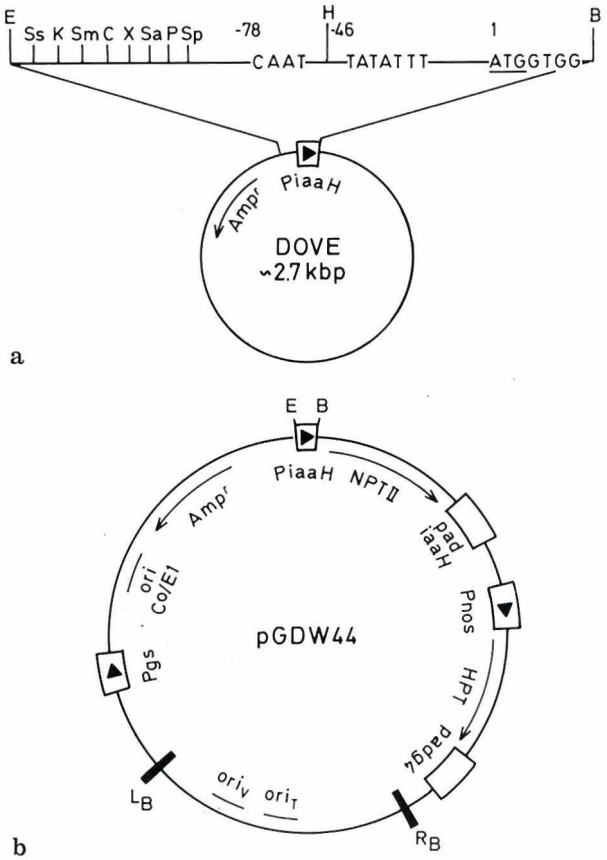

Fig. 2a and b. Representations of the vectors DOVE and pGDW44. **a** DOVE is an insertion of the multiple cloning sites 5′ of the minimal P*iaaH* (expanded region) into pUC9 at the *Bam*HI and *Eco*RI sites. The ATG at position 1 is the initiation codon for *iaaH* and the last G presented is the first G of the *Bam*HI recognition site. **b** pGDW44 is the minimal P*iaaH* fused to an *NPT II* gene with termination signals from the *iaaH* gene cloned into pGDW31. All heterologous promoters constructed on DOVE can be rapidly recloned into pGDW44 as *Eco*RI-*Bam*HI fragments. Abbreviations: B, *Bam*HI site; Sp, *Sph*I site; P*iaaH*, *iaaH* promoter; pad *iaaH*, polyadenylation sites of the *iaaH* gene; *NPT II*, neomycin phosphotransferase gene. Other abbreviations as in Fig. 1

with a pUC19-related set of sites to give pGDW32 shown in Fig. 1.

A second chimaeric gene fusing the minimal P*iaaH* to the *NPT II* reporter gene terminated by the polyadenylation signals from the *iaaH* gene was added to pGDW31. This resulted in vector pGDW44 (Fig. 2). The fusion of the minimal promoter sequence and the *NPT II* gene of pKm109/9 (Reiss et al. 1984a) created a 17 amino acid addition to the amino-terminal end of the NPT II protein and apparently did not adversely affect the enzyme activity.

When pGDW44 carrying the minimal P*iaaH* alone fused to the *NPT II* gene was tested for the level of NPT II expression in transformed tobacco callus tissue, the basal level of expression was consistently low (Fig. 3), approximately 100- to 200-fold lower than with an intact 1′ promoter (Velten et al. 1984, data not shown). Regenerated tobacco plants had nearly undetectable levels of NPT II activity in roots, stem and leaves (data not shown). This reduced level of expression also showed that the T_L-DNA gene *5* promoter located 2.5 kb upstream of the minimal P*iaaH* had a minor effect on the level of expression. When the

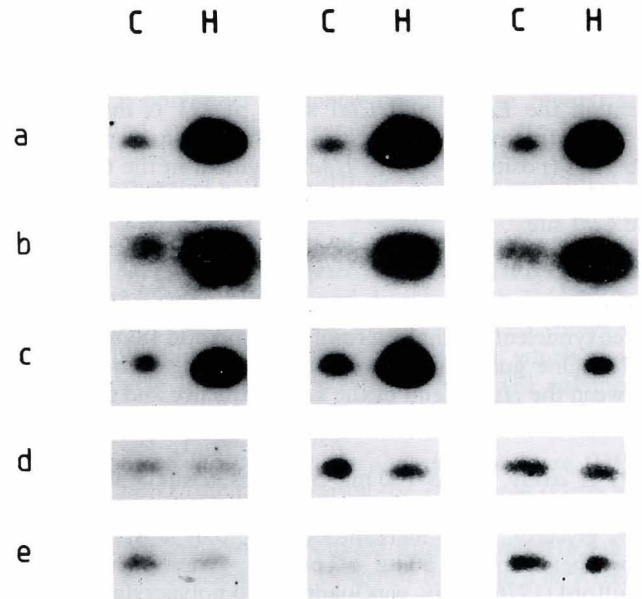

Fig. 3a–e. NPT II activity from tobacco callus lines before and after heat shock. Three representative callus lines containing pGDW811 **a**, pGDW821 **b**, pGDW831 **c**, pGDW841 **d** and pGDW44 **e** were kept at 22° C or heat shocked and allowed to recover for 2 h. NPT II activity was assayed from each sample as described in Materials and methods. Column heading C indicates the control samples and H indicates the heat-shocked samples

same chimaeric gene was transfered by the co-integrate plant vector pGV3851 (Zambryski et al. 1984) into tobacco, the expression in five independent transformants was comparable to that seen in callus tissue; thus the truncated P*iaaH* was unaffected by the high cytokinin levels present in the teratoma tissue (data not shown). Together these results indicated the suitability of the minimal P*iaaH* for use as a non-tissue-specific and minimal promoter for testing transcriptional regulatory sequences in plants.

Heat shock induced NPT II *expression by heterologous promoters with* hsp70 *5′ upstream sequences*

Four DNA fragments from the *Drosophila hsp70* promoter beginning at the 5′ positions −252, −175, −72 and −57 and all ending at position −35, just 5 bp upstream of the *hsp70* TATA box were cloned into DOVE such that the 3′ end of each *hsp70* sequence was 79 bp from the P*iaaH* TATA box (Fig. 4). The heterologous promoters were then fused to the reporter gene *NPT II* on pGDW44 giving plasmids pGDW811, pGDW821, pGDW831 and pGDW841 carrying respectively three, two, one and a fraction of the HSEs.

Transformed tobacco calli containing one to three copies of the chimaeric gene (data not shown) were propagated and analysed individually to test for heat shock induction of the *NPT II* gene. Uninduced calli were first screened for NPT II activity and two lines transformed with pGDW811 were tested for heat shock inducible activity before and after heat shock at 40° C for 1 h, and throughout the recovery period at 22° C.

The graph depicted in Fig. 5 shows the NPT II activity levels of the two callus lines measured by scintillation counting of the [^{32}P]kanamycin assayed in duplicate samples at different times. Both callus lines displayed a similar pattern

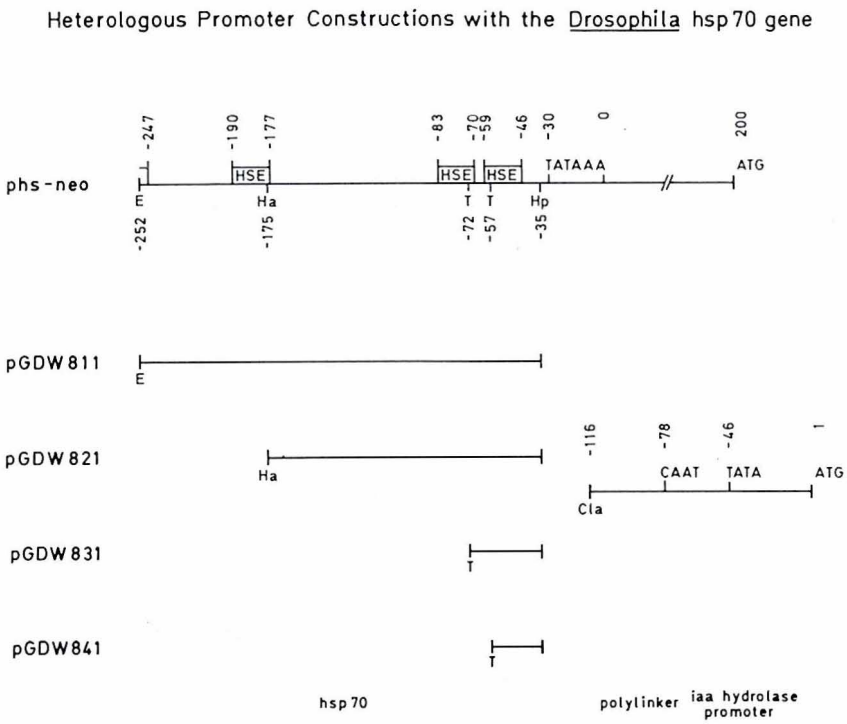

Fig. 4. The regions of the *Drosophila hsp70* gene promoter subcloned into DOVE. A *Drosophila hsp70* subclone, pHS-neo, was cleaved at the restriction endonuclease site positions (indicated below the line), the fragments were cloned into DOVE and the heterologous promoters were moved to pGDW44. The heat shock elements (HSEs) are indicated by the *labelled boxes* and their nucleotide positions are indicated above the line representing pHS-neo

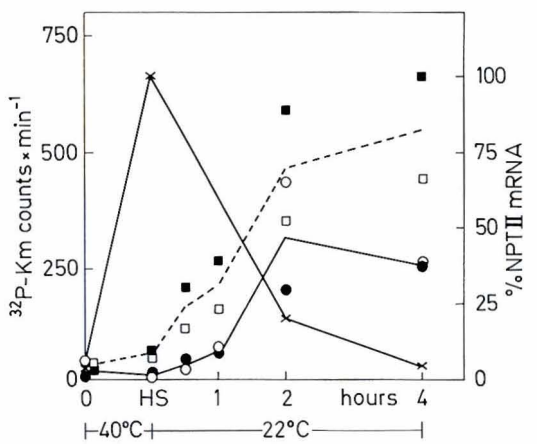

Fig. 5. Kinetics of accumulation of *NPT II* mRNA and NPT II activity. NPT II assays were done for duplicate samples from two callus lines transformed with pGDW811 before (O) and after (HS) heat shock and during the recovery period at 0.5, 1, 2 and 4 h (*abscissa*). The [^{32}P]kanamycin (^{32}P-Km) spots were cut from the P81 paper and counted in scintillation fluid to determine the NPT II activity levels (*ordinate*). The lines from the average of the duplicate points indicate NPT II activity from callus line 1f (*solid line* between *circles*) and callus line 1a (*broken line* between *squares*). The *NPT II* mRNA level (×——×) was determined from densitometer tracings of the results from Northern RNA blotting experiments using callus line 1f and is expressed as a percentage of the *NPT II* mRNA isolated from callus immediately after heat shock (*right ordinate*)

of inducible NPT II activity, where instead of appearing during the heat shock period along with the heat shock proteins, the increase in NPT II activity was delayed until the recovery period. The calli had a baseline of activity in the uninduced state which remained relatively unchanged after the heat shock period. During the recovery period, the NPT II activity increased in a linear fashion until 2 h after heat shock, followed by an additional slight increase during the next 2 h, after which the level of NPT II activity remained constant for up to 14 h. The same test was also performed at heat shock temperatures of 37° and 45° C and the same pattern of NPT II expression was observed, but the final levels of induction were reduced, confirming the optimal temperature for heat shock induction in tobacco to be 40° C (Barnett et al. 1980). Calli containing constructs pGDW821 and pGDW831 also accumulated NPT II at an identical rate, demonstrating that the delayed NPT II accumulation was independent of the promoter construction and probably a function of the induced mRNA and a post-transcriptional control.

Comparison of the levels of NPT II *induction with different* hsp70 *promoter fragments*

The optimal conditions for assaying and comparing the level of heat shock inducible activity of the various promoter deletions were judged to be a heat shock treatment for 1 h at 40° C followed by a recovery period of 2 h at 22° C. Representative examples of each promoter construction are shown in Fig. 3 and the results from all the callus lines are compiled in Table 1. None of 18 callus lines containing the minimal promoter construction pGDW44, not of 5 lines transformed with pGDW841 which has 13/14 bp of the HSE closest to the *hsp70* TATA box (HSE1), had increased NPT II activity after heat shock treatment (Fig. 3 and Table 1) even though they all exhibited baseline levels of NPT II expression. Of the NPT II expressing callus lines transformed with the other 3 constructs containing intact HSEs, all but 2 had heat-shock inducible NPT II activity (Table 1). The longest segment conferred the greatest level of induction upon the minimal promoter of between 5- to 30-fold. The segments begining at −175 and −72 were heat shock inducible by 5- to 8-fold. These results indicated that pGDW831, which has the sequence from −72 to −35

Table 1. The level of heat shock inducible *NPT II* activity of the transformed tobacco callus lines

Construction	*NPT II*$^+$/ transformants	Heat shock inducible/ *NPT II*[a]	Range of induction[a]	Average induction
pGDW811	13/15	13/13	5–30	13
pGDW821	5/7	4/5	5–7	6
pGDW831	5/5	4/5	6–8	7
pGDW841	5/5	0/5	–	–
pGDW44	16/19	0/16	–	–

[a] Induction level is [^{32}P]kanamycin cpm of heat shock sample/ control sample. The range is the lowest and highest level observed among the induced samples

containing the complete HSE1 and half of the second upstream HSE (HSE2), contains enough information to confer heat shock induction to minimal P*iaaH*. The 5′ end of the minimal *hsp70* sequence required for the heat shock induction must therefore lie between −72 and −58 (pGDW841).

Determination of the steady-state level of NPT II *mRNA*

To prove directly that the induction of NPT II activity was at the transcriptional level during the heat shock period and to examine the stability of the transcript and correlate it with the level of NPT II activity, the amount of *NPT II* mRNA was determined for a representative line from each inducible construction. Poly(A$^+$) RNA (1 μg) isolated from the callus lines before and after heat shock, and during the recovery period was analysed by probing for *NPT II* mRNA on Northern blots. All three lines show clear heat shock induction of *NPT II* mRNA of the predicted size of 1100 bases (Fig. 6). When comparing the hybridization signals with those of the DNA standards included on the blot, the induced level of *NPT II* mRNA from the pGDW811 line represented between 0.02 to 0.04 ng of poly(A$^+$) RNA (Fig. 6a). Longer exposures of the blot revealed uninduced *NPT II* transcript corresponding to less than 0.002 ng of RNA. This suggested a 10- to 20-fold induction of the *NPT II* mRNA, a value similar to he 16-fold induction of NPT II for this line. The lines transformed with pGDW821 (Fig. 6b) and pGDW831 (Fig. 6c) both had lower levels of induced *NPT II* mRNA, also comparable to what was observed with the NPT II measurements. Thus, the induced NPT II activity reflected the induction of *NPT II* mRNA and showed that the *hsp70* promoter segments containing one or more complete HSEs conferred heat shock inducibility to the minimal P*iaaH* in tobacco cells.

Upon examination of the steady-state level of *NPT II* mRNA from a callus line transformed with pGDW831 during the recovery period, a decrease was observed to about 20% of the heat shock induced *NPT II* mRNA after 2 h, and to nearly undetectable after 4 h of recovery (Fig. 6c). The non-heat-shock inducible actin mRNA (Nagao et al. 1986), was measured as a control for the mRNA levels present and was found to be reasonably constant (Fig. 6d). The same experiment performed with line 1f from pGDW811 transformed callus, resulting in a graph based on values from densitometer tracings, is shown in Fig. 5.

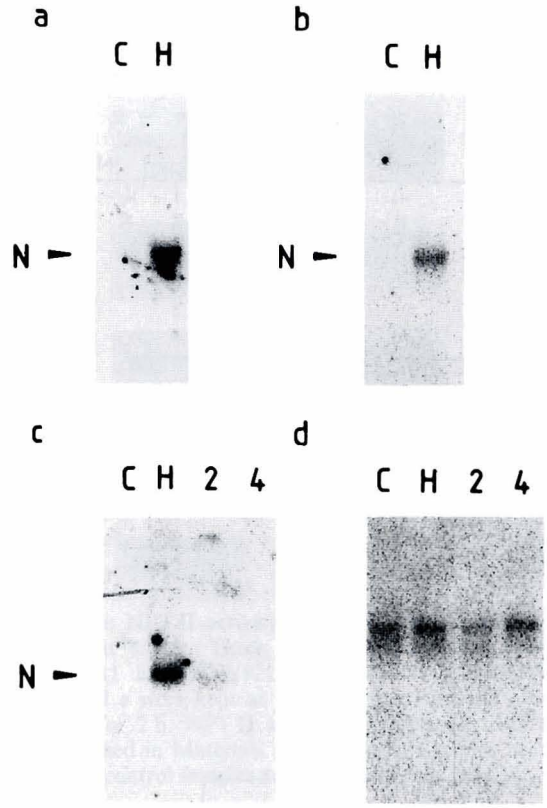

Fig. 6a–d. Steady-state levels of *NPT II* mRNA. A Northern blot of 1 μg of poly(A) RNA from control (C) and heat shock induced (H) callus lines transformed with pGDW811 **a** pGDW821 **b** and pGDW831 **c** was hybridized with an *NPT II* gene fragment. **c** The loss of total *NPT II* mRNA during the recovery period at 2 and 4 h after heat shock is also shown (lanes 2 and 4, respectively). The correct position of *NPT II* mRNA is indicated by N. **d** The result of reprobing the blot of **c** with soybean actin sequences

Superimposition of the rates of accumulation of *NPT II* mRNA and activity, showing the delay in the increase in enzyme activity until after the mRNA level has peaked, suggests post-transcriptional control of the *NPT II* mRNA and shows the suitablity of this chimaeric mRNA structure for post-heat-shock expression in tobacco.

Discussion

Sequences from the *Drosophila hsp70* promoter were able to confer heat shock inducibility to the minimal promoter from the *iaaH* gene of T-DNA, affirming the suitability of the vectors DOVE and pGDW44 for resolving positively regulated enhancers. The following changes in the wild-type promoter structure of *Drosophila hsp70* could possibly affect the level of induction observed: the introduction of a 79 bp gap including a CCAAT box between the HSE and the TATA box. The distance is enough to overcome any possible periodic effects of the DNA tertiary structure (Cohen and Meselson 1988) and little effect of increased spacing has been noted with other heat shock promoter constructions (Pelham and Bienz 1982; Strittmatter and Chua 1987). Second, most heat shock promoters do not have a CCAAT box, but have instead an HSE within the first 50 bp upstream of the TATA box (Pelham 1982). In the constructions reported here, the P*iaaH* CCAAT box

did not prevent the heat shock response, consistent with an example from the *Xenopus hsp70* gene which contains a CCAAT box found necessary for heat shock inducible transcription (Bienz and Pelham 1986).

The profile of induction of *NPT II* mRNA and enzyme activity presented a clear demonstration of the role of post-transcriptional regulation in gene expression. The *NPT II* mRNA, while properly induced by heat shock, was very inefficiently translated, if at all, during the heat shock phase, as little or no corresponding increase in the level of NPT II could be detected then. It is unlikely that the NPT II is synthesized and then rapidly inactivated or degraded in tobacco, since callus lines expressing the same *NPT II* fusion constitutively had no change in levels of NPT II after heat shock (unpublished results). Thus the translation of the chimaeric mRNA is most probably repressed.

This is observed for many of the mRNAs translated at normal growth temperatures in *Drosophila* (McKenzie et al. 1975) and plant cells (Key et al. 1981; Barnett et al. 1980), where heat shock represses the translation of the mRNAs and upon recovery the cell resumes synthesizing the proteins from pre-existing mRNAs (Storti et al. 1980; Lindquist 1981; Key et al. 1981). In *Drosophila* cells, the first 95 nucleotides of the 5′ untranslated region of the *hsp70* mRNA can shift the translation of a non-heat-shock mRNA to the heat shock period, demonstrating that a heat shock 5′ mRNA leader has a particular structure which allows heat shock mRNAs to be translated during heat stress (Klemenz et al. 1985). Thus plant heat shock mRNAs may also require a specific leader sequence to bestow translatability on an mRNA during heat shock. These sequences are probably different from those of *Drosophila hsp70*, because the *NPT II* mRNA with the *Drosophila* 5′ leader is also poorly translated during heat shock (Spena et al. 1985). This translational control represents one divergent feature of heat shock regulation in plants and animals where a similar function is now seemingly directed by a different structure.

The 37 bp *hsp70* sequence containing a complete HSE1, as similarly observed in stably transformed *Drosophila* cells (Dudler and Travers 1984; Xiao and Lis 1988), confers heat shock inducibility to a gene. Examination of a number of synthetic promoters structured after the *hsp70* promoter showing the importance of positions bordering the HSE 14 bp dyad, led to a proposed expanded consensus sequence which is a dimer of a 10 bp unit centred over the original HSE (Xiao and Lis 1988). As evidence for the 10-mer basic unit, a synthesized sequence containing one and a half 14 bp HSEs, or in other terms a trimer of the 10 bp unit, is partially heat shock inducible. That sequence corresponds in structure to the 37 bp segment used in pGDW831 which contains the 3′ half of HSE2 and the complete HSE1 and is similarly partially heat shock inducible. In comparison, the two overlapping HSEs in the 36 bp sequence of the soybean *hs6871* gene promoter which can-render genes heat shock inducible in tobacco (Baumann et al. 1987; Strittmatter and Chua 1987) also represent one and a half HSEs. However, when viewed in terms of a 10 bp element, as suggested on observing overlapping HSE structures (Xiao and Lis 1988), the 36 bp soybean sequence is a trimer of that element. The structural similarity between the *Drosophila* and soybean sequences which are able to confer partial heat shock inducibility to a gene is better described as a trimer of the 10 bp basic unit and thus, as in *Drosophila*, the dimer of the 10 bp unit may better describe an HSE in tobacco.

Unlike the situation in stably transformed *Drosophila* though, where two complete HSEs can confer full heat shock inducibility to a gene (Dudler and Travers 1984; Xiao and Lis 1988), the 120 bp sequence with both HSE1 and HSE2 on pGDW821 results in no further increase in the level of induction in tobacco. Models for activation of the *hsp70* promoter based on the genetic evidence and the stronger affinity of HSTF for HSE1 than for HSE2, hypothesize that the two HSEs work cooperatively to mediate full heat shock induction (Topol et al. 1985). This discrepancy suggests possible nuances in the structure, interaction and roles of the HSE and HSTF in the mechanism of heat shock induced gene transcription of tobacco and *Drosophila*. Indeed, the analysis of the soybean heat shock promoter points out the need for an upstream non-HSE enhancer-like element for maximal heat shock induction in tobacco (Baumann et al. 1987).

The ability of the one and a half HSE sequence from the *Drosophila hsp70* promoter to confer partial heat shock inducible transcription to a gene in both *Drosophila* and tobacco, corroborates the high degree of conservation of function of the HSE between the plant and animal kingdoms. This conservation of structure and function most likely extends to the HSTF as well, since the *Drosophila* HSE is presumably acting as the binding site for the tobacco HSTF. The eventual isolation of the tobacco HSTF should further demonstrate the extent of the conservation and clarify possible differences between *Drosophila* and tobacco heat shock induced gene transcription.

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