Plant Molecular Biology **21**: 823–834, 1993.

Transient gene expression of foreign genes in preheated protoplasts: stimulation of expression of transfected genes lacking heat shock elements

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Received 17 August 1992; accepted in revised form 7 December 1992

Key words: chloramphenicol acetyltransferase, β-glucuronidase, heat shock, plant protoplasts, single-stranded DNA, transient expression

Abstract

Transfection of preheated petunia protoplasts with several biologically active DNA constructs resulted in a significantly higher gene expression than that observed in transfected unheated protoplasts. It was observed with supercoiled, linearized and single-stranded DNA structures that stimulation of transient gene expression in preheated protoplasts was neither dependent on the reporter gene nor on the regulatory elements used. Heat treatment at 42 °C also increased expression in protoplasts transfected with a plasmid bearing the tobacco mosaic virus (TMV) translational enhancer, Ω. Northern blot analysis revealed that heat treatment of protoplasts before the transfection event greatly increased the amount of the newly synthesized transcripts. Preheating of protoplasts did not affect the transfection efficiency, namely the number of transfected cells in the population, nor the amount of DNA in transfected nuclei, as was inferred from histochemical staining and Southern blot analysis, respectively. The possible mechanism by which heat treatment stimulates transient gene expression of genes lacking obvious heat shock elements is offered. The relevance of the present findings to transient gene expression in plants in general and to viral gene expression in particular is discussed.

Introduction

Studies during the past few years have shown that a specific set of genes which received the term 'heat shock' (HS) genes are greatly stimulated when the optimal temperature of cell growth and development is elevated [reviewed in 11, 7, 25]. The increase in the transcription rate of eucaryotic HS genes in response to an upwards temperature shift is attributed to the binding of specific HS transcription factors (HSTFs) to a short, highly conserved DNA sequence designated the 'heat shock element' (HSE) present in the promoter of such genes [reviewed in 7, 25]. In contrast, transcription of most of the chromosome-associated non-HS genes as well as the translation of the corresponding mRNAs is greatly inhibited at high temperatures [25, 31].

The consensus HSE sequence was first identified in the *Drosophila* heat shock genes [27] as

a 14 bp palindromic sequence, 5′-CTnGAAnnT-TCnAG-3′. Recently the HSE has been redefined as composed of a repeated critical 5 bp component sequence nGAAn or its complement nTTCn [3, 37]. For high-affinity binding of HSF *in vitro* there is a need for at least 2 basic nGAAn units arranged either head to head or tail to tail [29].

The existence of a specific HSE in the regulatory sequence of HS genes allowed the construction and use of heat shock-inducible expression vectors as was first demonstrated by Pelham and Bienz [26]. Such vectors include specific HS-inducible promoters in front of reporter genes, the expression of which is greatly stimulated at high temperatures. DNA constructs containing a specific HSE have been used in plant cells and stimulated, under HS conditions, either transient [1, 8] or stable [6, 32] gene expression.

In plants, however, heat shock may affect processes which are not directly correlated with a genomic HSE and expression of chromosomal HS genes. For example, synthesis of TMV proteins, in contrast to cellular proteins, was not affected by an upwards temperature shift probably due to stabilization of the TMV mRNA at high temperatures [14]. In developing soybean seedlings, translation of many normal non-HS proteins persists under HS conditions [22] and storage protein synthesis is even stimulated [2]. In another study [32], upstream sequences of a heat shock promoter from soybean were fused to the TATA box sequence of the cauliflower mosaic virus (CaMV) 35S promoter in front of the chloramphenicol acetyltransferase (CAT) coding sequence. Even after deleting most of the upstream sequences of the heat shock promoter, the construct bearing the CaMV TATA box sequence was still able to induce, under HS conditions, transcription and translation of the CAT gene.

The view that stimulation of HS genes and inhibition of non-HS genes is not the only way by which an increase in the environmental temperature may affect gene expression can also be inferred from previous experiments which showed that transformation of plant protoplasts could be stimulated 5–20 fold if such protoplasts were preheated before the transfection event [33]. None of the DNA constructs used in the transformation experiments described above [33] contained HSEs and therefore the increase in the number of the transformants should be due to a thermal effect either on the recombination process which is required for insertion of the added genes into the recipient chromosome or on the assembly of the cellular chromatin with the foreign genes.

In the present work we have studied the effect of heat shock on transient expression of several foreign genes, which are driven by viral and non-viral promoters, in transfected petunia protoplasts. Our results show that increase of the optimal cells' growth temperature has a pronounced stimulatory effect on transient expression of four different transfected reporter genes. All the DNA constructs used for the transfection experiments lack obvious HS consensus sequences. The application of the present findings for viral infection and expression of viral genes at high temperatures is discussed.

Materials and methods

Preparation of petunia protoplasts

Petunia hybrida protoplasts (line 3704) were obtained by incubating exponentially growing cells (25% v/v), in cpw solution [15] supplemented with 10% (w/v) manitol, 2% Cellulase R-10, 0.3% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Industry, Nishinomiya, Japan) and 0.01% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Chiba, Japan) for 15 h at 26 °C in the dark.

Plasmids

pUC8CAMVCAT and pNOSCAT [4, 5] are plasmids carrying a coding region of a bacterial CAT gene and a polyadenylation signal of a nopaline synthase (NOS) gene at the 3′ end. At the 5′ end the CAT sequence is either linked to a

CaMV 35S promoter (pUC8CaMVCAT [4]) or to a NOS promoter (pNOSCAT [5]). (Both plasmids are a generous gift from Dr V. Walbot, Stanford University.) The plasmid pDO432 is a generous gift from Dr S.H. Howell, Cornell University and contains a firefly luciferase-coding region linked to a CaMV 35S promoter at the 5′ end and to a NOS polyadenylation region at the 3′ end [26]. pDO432 was linearized with *Nde* I (New England Biolabs).

pBI221 (Clontech) is a plasmid in which a 3 kb *Hin*d III-*Eco* RI fragment of pBI121 [21] containing a CaMV 35S promoter, a β-glucuronidase (GUS) gene and a NOS polyadenylation site have been cloned into pUC19.

The plasmid pJD330 (a generous gift from Dr D.R. Gallie) is in principle identical to pBI221, namely bears a bacterial GUS gene driven by a 35S CaMV promoter and linked to a NOS polyadenylation sequence but contains an Ω fragment [16] downstream to the CaMV promoter (D.R. Gallie, personal communication).

The plasmid pAct1-D (a generous gift from Dr R. Wu, Cornell University) contains a portion of a 5′ flanking and 5′ transcribed sequence of a rice Act 1 gene fused to a sequence containing a GUS-coding region and 3′ NOS transcription terminator [24].

The expression unit of the single-stranded (ss) plasmid pUC118CaMVCATb [20], includes a CaMV35S promoter, a CAT gene and a NOS polyadenylation sequence.

Transfection of protoplasts with DNA constructs

Transfection of petunia protoplasts with DNA molecules was performed by a modification of previously described procedures [23]. Briefly, protoplasts were washed twice by centrifugation ($300 \times g$, 5 min) in MS '150' medium [34] and resuspended in solution T (40 mM $CaCl_2$, 13% (w/v) mannitol) to give a concentration of 4×10^6 protoplasts/ml and then incubated as follows.

Procedure A. If not otherwise stated, supercoiled plasmids and carrier DNA (sonicated calf thymus DNA) were added sequentially to give a total amount of 110 μg DNA per 4×10^6 protoplasts in a final volume of 1 ml solution T. A volume of 0.4 ml PEG 1500 (40% w/v in solution T) was added immediately and the suspension obtained was then mixed and incubated for 30 min at 25 °C with gentle shaking. Following the addition of 50 ml cold mannitol (13% w/v), the protoplasts were incubated again for 15–30 min at 4 °C, centrifuged ($300 \times g$, 5 min), suspended in 9 ml of MS '150' medium and then left in the dark at 26 °C, if not otherwise stated, for about 24 h. At the end of the incubation period the efficiency of transfection was estimated.

Procedure B. Supercoiled plasmids and sonicated carrier DNA were added sequentially to give a total amount of 50 μg DNA per 8×10^5 protoplasts in a final volume of about 0.2 ml of solution T. A volume of 0.3 ml PEG (40% w/v) in solution T was added as above and, after 5 min incubation at room temperature (25 °C) with gentle shaking, a volume of 15 ml cold mannitol (13% w/v) was added and the suspension obtained was incubated for 15–30 min in the cold. The protoplasts were centrifuged ($300 \times g$, 5 min) and the pellet obtained was resuspended in 3 ml of MS '150' medium and incubated as above (procedure A) in the dark.

Protoplasts were transfected with ss-DNA construct as described for double–stranded (ds) plasmids in procedure A.

Heat treatment of petunia protoplasts

If not otherwise stated, 4×10^6 protoplasts in 1 ml of solution T were incubated at 42 °C for 45 min. At the end of the incubation period the protoplasts were cooled to reach room temperature and transfected with DNA as described above.

Determination of reporter genes' activities

CAT activity in transfected protoplasts was determined as described [4, 5]. For luciferase activity, transfected protoplasts were harvested and

assayed for 24 h after transfection as described before [5]. The light produced (light units, LU) by sonicated extracts of 4×10^5 transfected protoplasts during the first 10 s of the enzymatic reaction was measured by LKB Luminometer.

For assaying GUS activity, cells were harvested 24 hours after transfection and suspended in a 50 mM sodium phosphate buffer, pH 7.4, containing 10 mM EDTA, 0.1% Triton x-100, 0.1% *N*-lauryl sarcosine and 10 mM 2-mercaptoethanol and kept at −70 °C. GUS activity in sonicated cell extracts (appearance of 4-methylumbelliferone (4-MU)) was estimated 24 h after transfection as described before [21].

Isolation of DNA from nuclei of transfected protoplasts and Southern blot analysis

Several samples each containing 4×10^6 protoplasts in 1 ml of solution T were heat-treated in parallel for 45 min at 42 °C. The heated as well as the unheated protoplasts were transfected with 25 μg of pUC8CaMVCAT as described in procedure A (see Transfection of protoplasts above).

For each point, few samples were combined to give a total of 1.2×10^7 protoplasts from which nuclei were isolated exactly as described [38]. DNA was extracted from the isolated nuclei 2.5 h and 20 h after transfection. The isolated nuclei were incubated in a buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl, 25 mM EDTA) containing 1% SDS and 0.5 mg/ml proteinase K, for 2 h at 37 °C. At the end of the incubation period, 5 M NaCl (1/10 volume of the DNA solution) was added and the DNA was gently extracted first by phenol/chloroform and then by chloroform.

The purified DNA was electrophoresed on 0.9% agarose gel, and after blotting to a nitrocellulose filter it was probed with [α-^{32}P]-labeled *Hin*d III fragment of pUC8CaMVCAT. Labeling was performed by the random-primed DNA labeling kit (Boehringer-Mannheim, FRG), using 3000 Ci/nmol [α^{32}P]dCTP (Amersham, England).

RNA isolation and northern blot analysis

Several samples each containing 4×10^6 protoplasts in 1 ml of solution T were incubated in parallel as described above for isolation of DNA. Heat-treated and untreated protoplasts were transfected with 35 μg DNA (pUC8CaMVCAT) per 4×10^6 protoplasts as described in procedure A above.

Samples of transfected protoplasts were combined to give a total number of 1.2×10^7 and RNA was extracted 10 and 20 h after transfection by lysis in guanidinium thiocyanate and centrifugation through a cesium chloride gradient [9]. Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography [18] and then 1 μg was electrophoresed on 1.2% agarose containing 6% formaldehyde gel. Following electrophoresis the poly(A)$^+$ RNA was transferred to a nitrocellulose filter and hybridized with ^{32}P-labeled *Hin*d III fragment of pUC8CaMVCAT which contains the CAT gene.

Western blot analysis

Extracts obtained from protoplasts transfected with either pBI221 or pJD330 (transfected as described in procedure B) were electrophoresed on tricine-SDS-PAGE [30]. Western blotting was performed as previously described [35]. The blots were probed with rabbit anti-GUS antibodies (Clontech) and were then visualized using ^{125}I-protein A and autoradiography.

Results

Heat treatment of protoplasts stimulates transient expression of foreign genes lacking heat shock consensus sequences

The results shown in Table 1 demonstrate that when petunia protoplasts were treated at 42 °C before the transfection event, the expression of three different transfected foreign genes, namely the bacterial CAT and GUS as well as the fire-

Table 1. Transient expression of foreign genes in transfected petunia protoplasts: effect of heat treatment.

Experiment	DNA constructs used	Heat treatment	AcCM (% of total)
I	pUC8CaMVCAT	–	17.2
		+	82.5
	pNOSCAT	–	9.3
		+	58.3
			LU $\times 10^4/4 \times 10^5$ cells
II	pDO432 (S)	–	1.5
	(S)	+	45
	pDO432 (L)	–	8.3
	(L)	+	40.8
			nmol 4-MU per mg protein per min
III	pBI221	–	3.7
		+	28.1
	pJD330	–	366
		+	783
	pAct1-D	–	0.8
		+	6.2

Exp. I. Petunia protoplasts (4×10^6) were transfected with 25 μg of either pUC8CaMVCAT or pNOSCAT and 85 μg of carrier DNA, as described in procedure A in Materials and methods.
Exp. II. Protoplasts (4×10^6) were transfected with 25 μg of supercoiled (S) and linearized (L) pDO432 and 85 μg of carrier DNA and as described in procedure A in Materials and methods. The plasmid pDO432 was linearized with *Nde* I (New England Biolabs).
Exp III. Petunia protoplasts (8×10^5) were transfected with 20 μg of pBI221, pJD330 and pAct1-D as well as with 30 μg of sonicated carrier DNA as described in procedure B in Materials and methods.
Estimation of all the gene products (CAT, Luciferase and GUS activities) in transfected protoplasts was performed as described in Materials and methods.

fly luciferase genes, were highly stimulated. Also, three different promoters, namely the CaMV, the NOS as well as the Act1D to which the above reporter genes were linked, were susceptible to stimulation in heat-treated protoplasts (Table 1). It appears, therefore, that heat-induced increase in transient gene expression was neither dependent on the kind of the promoter present nor on the reporter gene used.

Increase of transient gene expression in heat-treated protoplasts was also observed when the reporter gene (CAT) was linked to the polyadenylation signals of the CaMV or of the NOS (not shown). The stimulation obtained in heat-treated protoplasts using the different plasmids varied and was 4–30-fold above the expression observed in untreated transfected protoplasts (Table 1).

Recently it has been demonstrated that when the TMV translational enhancer, Ω, is present at the 5′ end of β-glucuronidase mRNA, it increases its translation efficiency [17]. In our experiments, transfection of petunia protoplasts with a DNA construct (pJD330, a generous gift from Dr D.R. Gallie), which contains the Ω fragment upstream of the GUS gene, resulted in about 100-fold stimulation of GUS gene expression than in those transfected with pBI221 (Table 1). Furthermore, GUS activity of heat-treated protoplasts transfected with pJD330 was about twice of that of untreated protoplasts and reached the high value of 783 nmol per mg protein per minute (Table 1).

Only heat-treatment before the transfection event resulted in stimulation of the transfected

Table 2. Stimulation of gene expression of heat treatment: timing of the HS treatment.

Incubation with PEG (min)	Treatment of protoplasts at 42 °C		
	none	before transfection	after transfection
	Gus activity (nmol 4-MU per mg protein per min)		
2′	0.51	12.50	1.10
5′	1.70	10.60	2.30
15′	1.70	5.70	1.00

Protoplasts were transfected as described in Materials and methods procedure B except that 14 μg of pBI221 and 8 μg of carrier DNA were used. Heat treatment before transfection was performed as described in Materials and methods procedure B. Heat treatment after transfection was performed by collecting the cells (which had been suspended in MS '150') 4 h after transfection, incubating them for 45 min at 42 °C and then again at 26 °C in the dark. All the treated protoplasts were finally collected 24 h after transfection.

GUS gene (Table 2) while the same treatment immediately (not shown) or 4 h after transfection caused very little or no change in GUS activity.

Heat treatment of petunia protoplasts under the present experimental conditions stimulated the synthesis of heat shock proteins as was shown before [8] (data not shown).

Transient expression of the CAT gene in heat-treated protoplasts: experimental conditions and level of regulation

Estimation of CAT activity in transfected petunia protoplasts (transfected with pUC8CaMVCAT [4]; see Materials and methods) revealed that the level of gene expression was highly dependent on the temperature at which the protoplasts were incubated before the transfection event (Fig. 1A). Maximum stimulation of CAT activity was observed when the protoplasts were preheated at 45 °C (Fig. 1A). However, the high stimulation observed at this temperature, as well as at 48 °C, was somewhat unreproducible probably due to the high killing effect observed under these conditions. Preheating of protoplasts at 42 °C also stimulated gene expression as is revealed by estimation of CAT activity. Such preheated protoplasts expressed high levels of this enzyme at least for 60–70 h (Fig. 1B). In our experiments, prep-

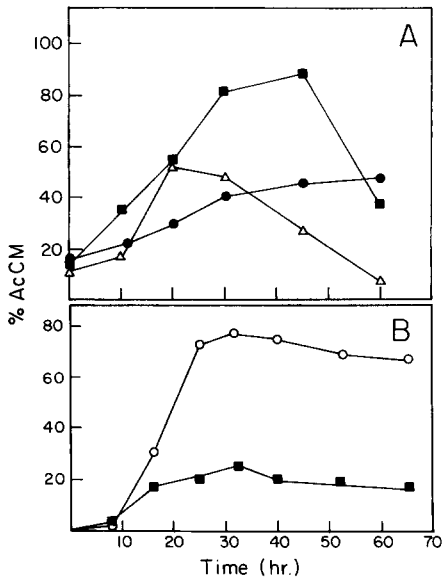

Fig. 1. Transient expression of the CAT gene in heat-treated protoplasts: effect of temperature and kinetic studies. A. Effect of temperature: protoplasts (4×10^6) were incubated at 42 °C (●), 45 °C (■) and 48 °C (△) for the times indicated in the figure. At the end of the incubation period the protoplasts were brought back to room temperature and then transfected with 25 μg of pUC8CaMVCAT as described in procedure A in Materials and methods. CAT activity was determined 24 hrs after transfection as described in Materials and methods. B. Kinetic studies: heated (42 °C, 45 min) (○) and unheated (■) protoplasts (4×10^6) were transfected with 25 μg of pUC8CaMVCAT as described in procedure A in Materials and methods. CAT activity was determined at the times indicated in the figure.

aration of the protoplasts caused 10–15% death as was inferred from their permeability to Phenosafranin. Heating at 48 °C caused an increase of about 10–15% of cell death above the background, while heating at 42 °C increased the killing degree only by 2–4%. Since incubation at this temperature (42 °C) caused a relatively low level of protoplast death, most of the experiments reported in this work were performed at this temperature.

It is possible that preheating of protoplasts may cause an increase in the intranuclear amount of the transfected DNA and consequently stimulate gene expression. However, Southern blot analysis did not reveal any significant difference between the amount of foreign DNA present in nuclei of heat-treated and control/untreated protoplasts (Fig. 2A). As can be seen (Fig. 2A) the supercoiled DNA was partially digested within the transfected nuclei and was converted to its relaxed and linear forms, thus confirming previous observations [5].

Heat-induced increase in CAT gene expression was neither dependent on DNA topology (Fig. 3A) nor on its structure (Fig. 3B). Preheating of protoplasts, prior to their transfection, stimulated expression of the CAT gene carried by either supercoiled (Fig. 3A, a, b) or linearized (Fig. 3A, a′ b′) plasmids. In agreement with our previous observations [5] the present results show (Fig. 3) that transfection with linearized forms of DNA constructs results in higher expression than with supercoiled structures.

The results in Fig. 3 show that transfection of preheated protoplasts with ss-DNA constructs carrying the CAT gene resembles transfection with ds-DNA and results in stimulation of gene expression. Previously we [20] as well as others [36] have shown that transfection of protoplasts with ss plasmids results in the expression of the reporter genes carried by them.

The stimulation of gene expression observed in preheated transfected protoplasts results from an increase in the amount of the newly synthesized transcripts, as is evident from the results depicted

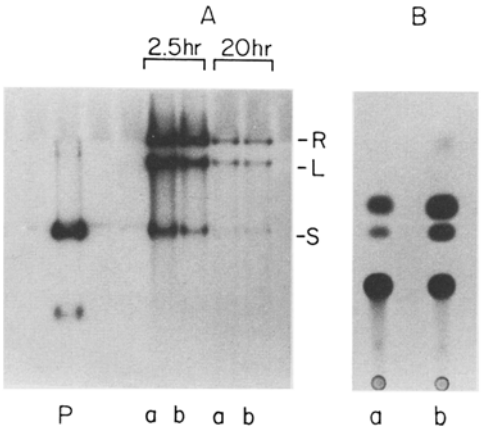

Fig. 2. The presence of transfected DNA constructs in nuclei of heat-treated and unheated protoplasts. A. DNA was extracted 2.5 h and 20 h after transfection from control (a) and heat-treated (b) protoplasts. Transfection conditions and Southern blot analysis were as described in Materials and methods. P, original plasmid (pUC8CaMVCAT); R, relaxed; L, linear; S, supercoiled. B. CAT activity was determined in untreated (a) or heat-treated (b) protoplasts as described in Materials and methods. CAT activity was estimated on the same protoplasts from which the DNA was isolated for the Southern blot analysis.

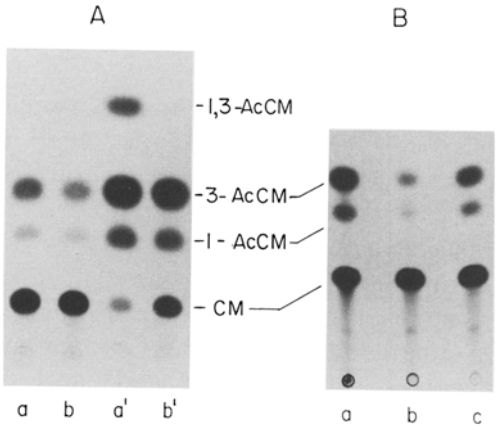

Fig. 3. Expression of transfected CAT gene in heat-treated protoplasts: effect of DNA topology and structure. Heat-treated (42 °C, 45 min) (a′, b′ in A and c in B) and unheated (a, b in A and b in B) protoplasts (4×10^6) were transfected (procedure A in Materials and methods) with 40 μg of linearized (a, a′ in A) Supercoiled (b, b′ in A) and ss (single-stranded) (b, c in B) forms of pUC8CaMVCAT. pUC8CaMVCAT was linearized by *Cla* I as described before [5]. CAT activity was determined in extracts obtained from transfected protoplasts 22 h after the transfection event as described in Materials and methods. a in B: CAT activity in extracted *Anabaena* cells. CM, chloramphenicol; AcCM, acetyl chloramphenicol.

Fig. 4. Effect of heat treatment on the transcription efficiency in transfected protoplasts: northern blot analysis. Poly(A)$^+$ RNA was extracted from unheated (a) and heat-treated (b) protoplasts 10 h and 20 h after transfection. All other conditions of protoplast transfection (procedure A), RNA isolation and northern blot analysis are as described in Materials and methods.

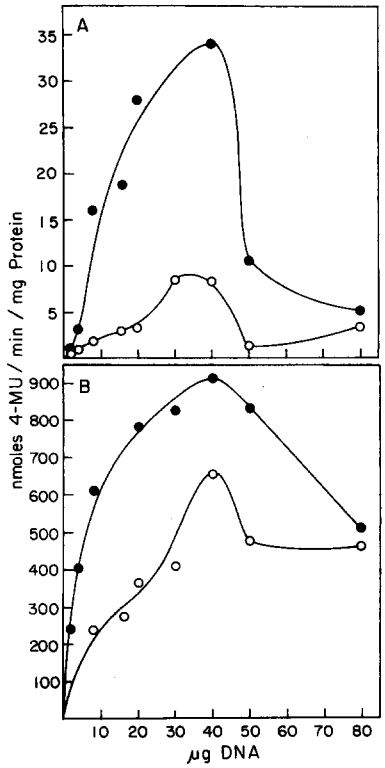

Fig. 5. Heat induced stimulation of GUS gene expression: effects of DNA concentrations. Untreated (○) or heat-treated (●) protoplasts were transfected with either pBI221 (A) or pJD330 (B) as described in procedure B in Materials and methods, except that no carrier DNA was added when 50 µg or 80 µg of plasmid were added. GUS activity was determined 28 h after transfection as described in Materials and methods.

in Fig. 4. A significant increase in the amount of specific CAT-poly(A)$^+$ RNA was found 10 to 20 h after transfection in heat-treated transfected protoplasts while at the same period practically no change was observed in the CAT poly(A)$^+$ RNA in untreated protoplasts (Fig. 4). It appears, therefore, that heat treatment may cause either an increase in the rate of transcripts' synthesis or a decrease in the rate of their degradation.

Heat-induced stimulation of GUS gene expression

The results in Fig. 5 show that GUS activity in transfected protoplasts is highly dependent on the amount of DNA added to the system. Transfection with either pBI221 or pJD330 yielded, essentially, the same pattern of dependency on DNA concentration. A maximum of GUS activity was observed with about 40 µg of plasmid per 8×10^5 protoplasts while a sharp decrease in its activity was observed when the DNA concentrations were increased above this value. It is evident (Fig. 5) that GUS activity in protoplasts transfected with the plasmid pJD330 (which contains the Ω regulatory element [16]) was about 80–100-fold higher than that obtained in protoplasts transfected with a plasmid lacking this element (pBI221) but containing the same promoter and polyadenylation signal. A specific activity as high as 660 nmol per mg protein per minute was estimated following the use of pJD330 as compared to 8 nmol per mg protein per minute when pBI221 was used.

Regardless of the amount of DNA added per system, transfection of heat-treated protoplasts always yielded higher GUS activity than that of

untreated protoplasts. Up to 8-fold stimulation was observed in heat-treated protoplasts which were transfected with pBI221 while 2–3-fold stimulation was observed in those transfected with pJD330 (Fig. 5A and B). The increase observed in heat-treated protoplasts cannot be explained by an increase in the transfection yield, namely in the number of the protoplasts which express the GUS gene. Histochemical staining of protoplasts transfected with the plasmid pJD330 using X-Gluc as a substrate [21] revealed that about 50% of the population appeared blue, namely showed GUS activity (not shown). Essentially, the same results were obtained with either untreated or pretreated protoplasts transfected with pBI221 (not shown). The only difference between the various systems was the time required for the appearance of the blue color which reflects GUS activity. While a relatively short period of time was required to stain control or preheated protoplasts transfected with pJD330 (5–10 min) as well as preheated protoplasts transfected with pBI221 (20–30 min), about 1–2 h were required for appearance of a blue color in untreated protoplasts transfected with the latter plasmid.

Western blot analysis revealed that both the presence of the Ω element as well as heat treatment induced a significant increase in the amount of the GUS polypeptides (Fig. 6). The difference in the amount of GUS polypeptides between non-treated and heat-treated transfected protoplasts was, as expected, much more pronounced after transfection with pBI221 than with pJD330 which carries the Ω element (Fig. 6).

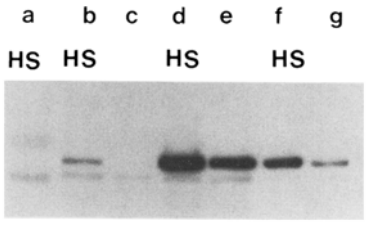

Fig. 6. Western blot analysis of protoplasts expressing the GUS gene. Each lane contains either 80 μg protein (lanes a–e) or 12 μg protein (lanes f, g). Extracts from heat-treated protoplasts (HS) lanes (a, b, d, f) are compared to untreated protoplasts (lanes c, e, g). a, mocked transfected protoplasts; b, c, protoplasts transfected with 8 μg pBI221; d–g, protoplasts transfected with 8 μg pJD330.

By the use of known amounts of highly purified β-glucuronidase (Clontech) as a standard, rabbit anti GUS antibody and ^{125}I-Protein A, we could estimate the amount of GUS which was synthesized by transfected protoplasts (not shown).

Our calculation shows that about 0.1 μg of GUS was synthesized by 10^5 protoplasts following their transfection with 5 μg of pJD330. This amount represents about 0.125% of the total protein present in such protoplasts.

Discussion

The results of the present work show that introduction of several biologically active DNA constructs (which lack known HSEs) into preheated petunia protoplasts resulted in much higher transient gene expression than that into unheated protoplasts. Expression of several transfected genes was found 4–30-fold higher in preheated protoplasts than in unheated protoplasts. Such stimulation was neither dependent on the reporter genes nor on the promoters used. Furthermore, the increase in expression was observed with genes carried by either supercoiled or linearized DNA structures, indicating that the effect of heat shock is not dependent on DNA topology.

Preheated protoplasts also stimulated the expression of the CAT gene carried by a ss-DNA expression vector. It has been shown before that in order to be expressed, transfected ss-DNA plasmids have to be converted into double stranded DNA structures [10]. This certainly is the case after infection of plants by ss-DNA viruses like those belonging to the Geminivirus group [12]. Our findings suggest that the preheating process did not interfere with the synthesis and the formation of the putative ds-DNA structures. In the light of our results it will be of interest to study the effect of heat shocks and increase in the environmental temperatures on the expression of specific viral genes as well as synthesis of second viral DNA strands after infection of plants by DNA viruses.

The view that HSEs are not involved in the response observed in the present work is strengthened by the results showing that stimulation of gene expression was observed only when protoplasts were heated before the transfection event. Similar results were reported previously when plant protoplasts were stably transformed with foreign DNA constructs [33]. This may indicate that the increase in the incubation temperature affected an early event in the process of control of gene expression such as the association of the transfected naked DNA with transcription factors or a preferential assembly into active chromatin structures.

From preliminary experiments it appears that the stimulatory effect in preheated protoplasts persists for at least up to 2 h (not shown). Currently, experiments are performed in our laboratory to study the various quantitative aspects of this system.

Heat treatment did not increase the transfection yield, as is evident from histochemical staining, nor the susceptibility of the protoplasts to the added DNA. The same amount of foreign DNA molecules was found in the nuclei of preheated and unheated transfected protoplasts.

It is interesting to note that the dependence of gene expression on the amount of the added DNA was not linear and showed a maximum expression at certain concentrations (40 μg DNA per 8×10^5 cells) above which the addition of DNA caused a decline in gene expression. However, even at high concentrations of added DNA, preheated protoplasts showed a higher expression of the transfected gene than untreated protoplasts when plasmids, with or without the Ω element, were used. It is conceivable that the inhibition observed at relatively high concentrations of DNA may be due to the saturation of limiting amounts of transcriptional or other regulatory factors by the multiple copies of the foreign DNA molecules. These DNA molecules probably compete with the chromosomal regulatory sequences for the available amounts of transcription factors. In this regard it should be mentioned that assembly of active minichromosomes in *Xenopus* oocytes was avoided by injecting DNA molecules which probably titrated specific factor(s) needed for the assembly process [18]. Preheating of plant protoplasts as performed here may increase the availability of *trans*-acting factors by either stimulating their dissociation from the chromosomal DNA or increasing their association with the foreign DNA. Our results showing that the dependence of gene expression on DNA concentration had exactly the same pattern in control and in preheated protoplasts may further indicate that heat treatment stimulates transcription by increasing the activity of transacting elements and not by increasing their amounts.

Infection of plants by viruses, especially those containing DNA, resembles in many of its features transfection by transiently expressed foreign genes. In both cases the invading DNA molecules have to reach the intranuclear space and exist as episomal DNA structures [19]. It is conceivable that any foreign DNA molecules (viral, bacterial or fungal) which are introduced into recipient cells' nuclei have to interact with various *trans*-acting factors and eventually assemble into minichromosomes, as has been demonstrated for foreign DNA injected into *Xenopus* oocytes [18].

Our results may suggest that under heat shock conditions, assembly of transcriptionally active nucleosomes is enhanced and consequently gene expression is stimulated, regardless of the presence of a HSE. It would be interesting to study whether heat treatment of plants may similarly affect the expression of plant viral genes and hence stimulate viral infection and distribution.

Acknowledgements

This work was supported by a grant from the Yeshaya Horowitz Association, the Hebrew University, Jerusalem, Israel.

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