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Transcriptional activity is inducible in the cauliflower mosaic virus 35 S promoter engineered with the heat shock consensus sequence

Maciej Pietrzak*, Markus Burri*, Juan-Jose Herrero° and Klaus Mosbach*+

*Institute of Biotechnology, ETH-Hönggerberg, CH 8093 Zürich, °Friedrich Miescher Institut, CH 4002 Basel, Switzerland and +Department of Pure and Applied Biochemistry, Chemical Center, University of Lund, S 221 00 Lund, Sweden

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Transcriptional activity of a strong constitutive plant promoter, the cauliflower mosaic virus (CaMV) 35 S promoter, could be modulated by addition of multiple synthetic oligonucleotides, carrying the heat shock promoter consensus sequence. The activity of this modified promoter increased up to 3-fold under heat shock conditions. A similar construction using enhancerless 35 S promoter resulted in an engineered promoter, which showed an increased activity under both normal and heat conditions, without a significant induction by the heat shock. We did not observe a silencer effect of the heat shock elements in combination with either complete or enhancerless 35 S promoter, using transient expression assay to measure the transcription activity.

Ca M V 35 S promoter; Heat shock promoter; Consensus sequence; Transient expression; Plant promoter; Protoplast

1. INTRODUCTION

The cauliflower mosaic virus (CaMV) 35 S RNA promoter [1] is interesting from the biotechnological point of view because it is presently the strongest known plant promoter [2]. The 35 S promoter used as a part of an expression plasmid directs constitutive transcription in transgenic plants or in protoplasts used in transient expression experiments in dicotyledonous and monocotyledonous plants [3–5]. In this paper we report on the imposition of a transcriptional control on the 35 S promoter by incorporation of a synthetic oligonucleotide containing the heat shock promoter consensus sequence element CTxGAAxxTTCxAG [6,7].

Correspondence address: K. Mosbach, Institute of Biotechnology, ETH-Hönggerberg, CH 8093 Zürich, Switzerland

2. MATERIALS AND METHODS

2.1. *Materials*

The *Orychophragmus violaceus* O.E. Schultz suspension culture was established in Dr C. Matsui's laboratory at the Nagoya University and was obtained through Dr T. Hohn of the Friedrich Miescher Institut. Protoplasts from the suspension culture were isolated as described in [8]. An oligonucleotide carrying the heat shock genes consensus sequence CTAGAAGCTT was synthesized in a commercial DNA synthesizer. All enzymes, biochemicals and other oligonucleotides were obtained from commercial sources.

2.2. *Genetic engineering techniques used in plasmid construction and analysis*

Restriction enzyme digestion, DNA ligation, *E. coli* transformation, agarose and polyacrylamide DNA electrophoresis, DNA sequencing and CsCl plasmid isolation were essentially as described in [9]. The construction of modified expression plasmids is shown in fig.2.

2.3. *Labelling of proteins with [^{35}S]methionine in electroporated, heat shock treated* O. violaceus *protoplasts*

Protoplasts were isolated as described in [8] and a sample of 0.75 ml was electroporated, without DNA, as described below. After electroporation the protoplasts were incubated in 6 ml of protoplast A medium [10] without amino acids, for 18 h at

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26°C and then for 30 min at 40°C. Radioactive [^{35}S]methionine (1 mCi) was added at this point and the sample was left for another 3 h at 40°C. After this time the protoplasts were collected and dissolved in sample buffer and the sample was analysed by SDS-polyacrylamide gel electrophoresis according to [11]. The gel was impregnated with 'Amplify' (Amersham) and autoradiographed. As control samples, protoplasts without heat treatment at 40°C and whole suspension culture cells with and without heat treatment were used.

2.4. *Electroporation of* O. violaceus *protoplasts and chloramphenicol acetyltransferase assay*

Electroporation was essentially made following the procedure of Potter et al. [12] and Fromm et al. [4]. Plasmid DNA (50 μg) and carrier calf thymus DNA (300 μg) were placed on ice in the electroporation cuvette in 100 μl of water. The protoplast sample (0.8 ml) was added to the cuvette and 820 μF capacitor charged to 200 V was discharged through the protoplast suspension. After 10 min on ice protoplasts were transferred into 10 ml of the protoplast A medium [10] and left 24 h at 26°C in the dark. On the second day, the protoplasts were divided into two samples: one control sample was left at 26°C and the second sample was set at 40°C for 3 h. After the heat shock the sample was left for a further 24 h at 26°C, as were the control samples.

For the CAT assay, protoplasts were collected by centrifugation, suspended in 0.5 ml of 0.1 M Tris, pH 7.6, and sonicated for 5 s to open the cells. The extract was centrifuged for 8 min in an Eppendorf centrifuge, the supernatant heated for 30 min at 68°C, centrifuged again, and a supernatant aliquot taken for the CAT assay according to [13] and [4]. The same amount of protein was taken from each sample for the assay.

After autoradiography the plastic backed, silica thin layer chromatography plates were aligned with the X-ray films and the spots containing acetylated [^{14}C]chloramphenicol were cut out for liquid scintillation counting.

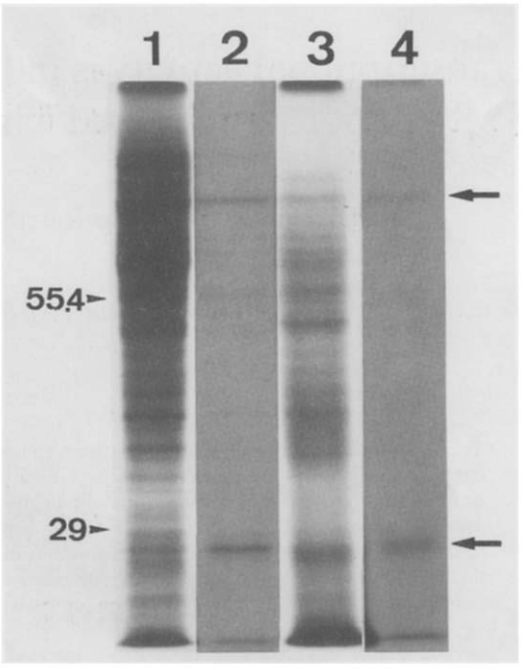

Fig.1. Heat shock protein synthesis in electroporated *Orychophragmus violaceus* protoplasts. Electroporated protoplasts and whole suspension culture cells were heat treated and labelled with [^{35}S]methionine as described in section 2. Total cell protein was separated on 10% SDS-polyacrylamide gel according to Laemmli [11] and visualized by fluorography. Numbers on the left mark positions of the molecular mass standards. Two arrows on the right side show positions of the major heat shocked proteins. Lanes: 1, control protoplasts incubated at 26°C; 2, heat shocked protoplasts (3.5 h at 40°C); 3, control suspension culture cells; 4, heat shocked suspension culture cells.

3. RESULTS

3.1. *Heat shock proteins in electroporated protoplasts*

Fig.1 shows the results of the protein labelling experiment for electroporated protoplasts exposed to heat shock conditions (lane 2) or control samples without heat shock (lane 1). Protoplasts exposed to the heat shock respond in the same way as the whole cells, synthesizing a set of specific heat shock proteins (compare fig.1, lanes 2 and 4). On the other hand, control protoplasts produce approximately the same pattern of protein bands on the polyacrylamide gel as non-treated whole cells (lanes 1 and 3).

This result suggests that the isolation of protoplasts and electroporation do not interfere with the normal heat shock response of these plant cells. However, applying the heat shock immediately after an electroporation resulted in protoplast death.

3.2. *Modification of the 35 S promoter with the heat shock consensus sequence oligonucleotide in plant expression plasmids*

Two series of the CaMV 35 S promoter based expression plasmids have been constructed for the transient expression experiments. All of these plasmids contained the 'core promoter' [14], comprising 'CCAAT' and 'TATA' boxes, and had the *Eco*RV restriction site, which is located at the junction of the enhancer region and the core promoter, converted to an *Xba*I site. The two series of constructed expression plasmids differed in the presence (the HKS series) or absence (the HKN

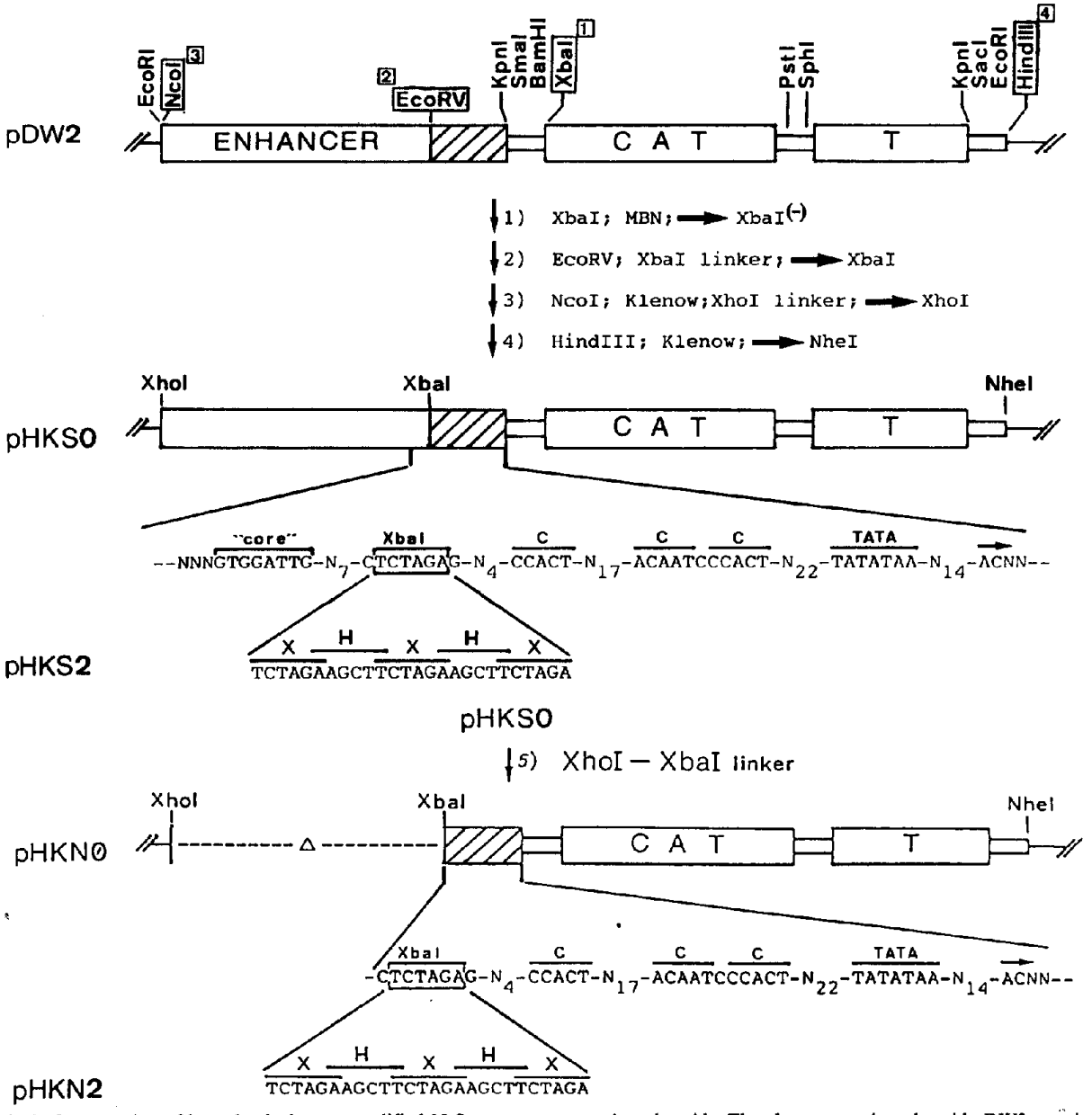

Fig.2. Construction of heat shock element modified 35 S promoter expression plasmids. The plant expression plasmid pDW2 carrying the unmodified 35 S promoter has been described in detail [5]. Hatched box between *Eco*RV and *Kpn*I restriction sites represents the 'core promoter' containing CCAAT boxes and the TATA box. Abbreviations 'CAT' and 'T' represent chloramphenicol acetyltransferase and CaMV transcription terminator, respectively. A new *Xba*I restriction site, which is located in this plasmid between the enhancer and the 'core promoter', has been used for insertion of the heat shock element oligonucleotide CTAGAAGCTT. The sequence below the pHKS0 shows details of the modified 35 S promoter; 'core' corresponds to the enhancer 'core' sequence [3], letters 'C' mark three sequences in the 'core promoter' with 80% homology to the CCAAT box consensus sequence and the arrow, right of the TATA box, shows start of transcription. Plasmid pHKS2 has two units of the oligonucleotide inserted into *Xba*I site as shown underneath. X and H above the insert sequence mark *Xba*I and *Hin*dIII restriction sites, respectively. Other plasmids of the pHKS series (pHKS3–pHKS5) have the number of oligonucleotide units in the *Xba*I site that correspond to the number of the plasmid in the series. In the pHKN plasmid series, the enhancer region (*Xho*I-*Xba*I fragment) has been deleted from the plasmid pHKS0, with the restoration of both *Xho*I and *Xba*I restriction sites to produce plasmid pHKN0. The number of each plasmid in this series also corresponds to the number of insert units. MBN, mung bean nuclease.

series) of the enhancer region of the CaMV 35 S promoter [3,14].

3.3. *Transient expression in* O. violaceus *protoplasts*

Results of a transient expression experiment using the complete, modified 35 S promoter (pHKS series), are shown in fig.3. Samples corresponding to plasmids with the heat shock consensus sequence (pHKS2–pHKS5) show an induction of the CAT activity in protoplasts after heat shock. This increase in activity was on average 1.6-, 2.1-, 3.1- and 2.2-fold for pHKS2 (1.8, 1.6), pHKS3 (2.1), pHKS4 (2.4, 3.2, 3.6) and pHKS5 (2.5, 1.8), respectively (numbers in parentheses represent data from independent experiments). The level of activity in the control sample containing the pHKS0 in non-induced protoplasts was similar to the activity in non-induced protoplasts containing heat shock element modified plasmids (pHKS2–pHKS5), and did not change under heat shock conditions (fig.3, lanes 1 and 2).

The second series of expression plasmids (pHKN series) in which the enhancer region has been deleted from the modified 35 S promoter, showed a different expression control in plant protoplasts.

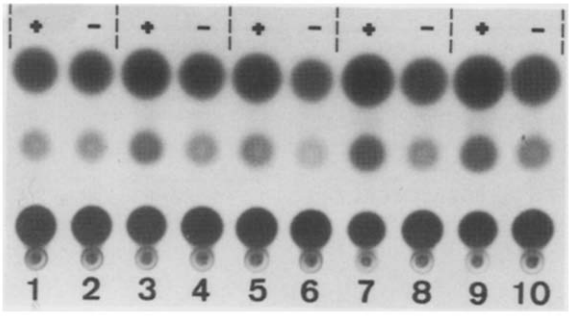

Fig.3. Transient expression of the chloramphenicol acetyltransferase gene in *O. violaceus* protoplasts electroporated with the pHKS series plasmids. Protoplasts were isolated, electroporated and incubated as described in section 2. The three rows of radioactive spots, from the bottom (the origin) to the top, correspond to unreacted [^{14}C]chloramphenicol, 1′-acetylchloramphenicol and 3′-acetylchloramphenicol, respectively. '+', heat shocked samples; '−', control samples. Radioactivity recovered after a 30 min reaction from the 3′-acetylchloramphenicol spot (see section 2) is given in parentheses for each sample as cpm × 10^{-3}. Lanes: 1 (52) and 2 (48), pHKS0; 3 (102) and 4 (55), pHKS2; 5 (76) and 6 (36), pHKS3; 7 (142) and 8 (60), pHKS4; 9 (140) and 10 (77), pHKS5.

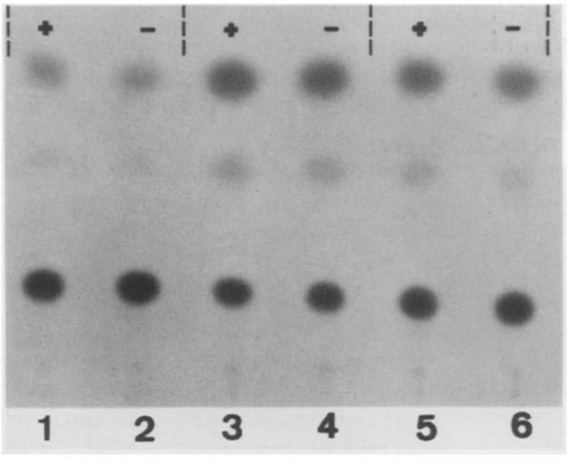

Fig.4. Transient expression of the chloramphenicol acetyltransferase gene in *O. violaceus* protoplasts electroporated with the pHKN series plasmids. For details see legend to fig.3. Radioactivity recovered after a 2 h reaction from the 3′-acetylchloramphenicol spot (see section 2) is given in parentheses for each sample as cpm × 10^{-3}. Lanes: 1 (26) and 2 (18), pHKN0; 3 (59) and 4 (38), pHKN2; 5 (78) and 6 (47), pHKN4.

As can be seen in fig.4, plasmids pHKN2 and pHKN4, with 2 and 4 heat shock elements, respectively, direct apparently higher expression of the CAT gene in both control and heat shocked protoplasts as compared to the control plasmid pHKN0. We have found this difference to be 2–3-fold at 26°C. On the other hand, heat shock induction is much less pronounced in the case of this enhancerless plasmid series as compared to the previous pHKS series (see figs 3 and 4). On average the increase of the CAT activity after heat shock induction in protoplasts electroporated with pHKN2 (1.2, 1.1) and pHKN4 (1.4, 1.2, 1.3, 1.1) was about 1.3-fold (numbers in parentheses represent data from independent experiments).

4. DISCUSSION

Earlier results [15–18] from animal and plant systems suggested that the synthetic sequence carrying the heat shock promoter consensus sequence GTxGAAxxTTCxAG [6] would also be active in plant cells. The objective of this work was rather to study the interaction between a constitutive plant promoter containing a very strong enhancer and a synthetic promoter regulatory element and not simply to show that this synthetic sequence is

functional in plants. We have chosen a transient expression system to assay the activity of the modified promoter because it is fast, and even more important, it overcomes problems connected with the position effect of the 35 S promoter, reported in transgenic plants [3].

Results of the transient expression using plasmids of the pHKS series (see fig.3 and section 3) show clearly the modulation effect of the 35 S promoter activity by the synthetic heat shock element. We can define this modulation as a positive effect only, it means that we observe an induction under the heat shock, but no silencer effect under normal growth conditions. The situation which we have created in the case of the pHKS series of engineered 35 S promoter may be comparable with some heat shock genes [19–21] which besides being inducible, are also expressed constitutively or in a tissue-specific manner. In addition to the heat shock enhancer, promoters of these genes also contain other upstream controlling elements responsible for this expression.

Removing of the enhancer in the heat shock modified 35 S promoter (pHKN series) changes the pattern of expression, as can be seen on fig.4. Surprisingly, a truncated promoter with inserted heat shock elements (pHKN2 or pHKN4) shows higher activity than the control promoter (pHKN0) in both induced and non-induced protoplasts. Heat shock induction of transcriptional activity in this case was only about 1.3-fold, apparently lower than for the pHKS series. It is possible that the heat shock element interacts with the CAAT boxes at the normal temperature in the absence of the original enhancer. Bienz [20] has also found that the heat shock element was necessary for full expression of the *Xenopus hsp70* promoter under non-induced conditions.

The results presented in this paper show the possibility of modulating the activity of a very strong constitutive plant promoter and to increase its transcriptional strength several times, simply by insertion of a short synthetic controlling element. This information may be important for the further development of plant expression systems.

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