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Stimulatory effect of the maize HMGa protein on reporter gene expression in maize protoplasts

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The high mobility group (HMG) proteins represent a class of chromosomal non-histone proteins with an assumed influence on transcription. In this context, the effect of the maize HMGa protein on reporter gene expression was examined. Transient co-transformation experiments in maize protoplasts with plasmid constructs directing the synthesis of the maize HMGa protein and with a luciferase reporter plasmid demonstrated a stimulatory effect of the HMGa protein on the reporter gene expression. Additional experiments with HMGa deletion constructs indicated that the HMG-Box DNA-binding motif is important for the observed effect, while the acidic carboxy-terminal domain of the HMGa protein appears to be dispensable.

Zea mays; Chromosomal protein; High mobility group protein; Gene expression; Transient transformation

1. INTRODUCTION

High mobility group (HMG) proteins represent a family of small and abundant non-histone proteins associated with eukaryotic chromatin, which have been implicated in replication and transcription, although their exact biological function is still unclear [1]. In plants, HMG proteins have been described from wheat [2], barley [3], maize [4], soybean [5], pea [6] and *Arabidopsis thaliana* [7]. In the case of wheat and barley, HMG proteins have been shown to be associated with transcriptionally active chromatin [8,9]. Furthermore, an in vitro binding of several plant HMG proteins to A/T-rich stretches of the 5′ flanking region of various genes has been demonstrated [5,10–12] together with a preferential binding of the maize HMG proteins a and b to the CCAAT- and TATA-Boxes of the zein storage protein gene, pMS1 [13]. The amino acid sequence of the maize HMGa protein, the largest of the four major maize HMG proteins, was deduced from the respective cDNA and displays structural features of the vertebrate HMG1 family, such as an HMG-Box DNA-binding domain and a highly acidic carboxy-terminal region [14].

Towards an elucidation of a possible in vivo function of this maize HMGa protein, we analyzed its effect on gene expression in transient transformation assays performed in maize protoplasts. Constructs directing the expression of HMGa were co-transformed with a luciferase reporter plasmid, resulting in an approximately 2-fold stimulation of luciferase expression.

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2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

DNA manipulations were carried out by standard procedures [15]. The first step towards obtaining the HMGa-expressing effector constructs was the preparation of the expression vector, pEx-SK/KS, by deleting the luciferase coding region from the plasmid, plucA (a derivative of plasmid pDW2 [16] in which the chloramphenicol acetyltransferase gene has been replaced by the luciferase gene) by cutting with *Hin*dIII/*Bam*HI, making blunt-ends and inserting the blunt-ended *Sac*I/*Kpn*I multiple cloning site (MCS) of the plasmid pBluescript-SK (Stratagene) in both orientations leading to the expression vectors, pEx-SK and pEx-KS, with the MCS between the CaMV 35S promoter and the 35S terminator. HMGa- cDNA constructs were then excised from pBluescript-SK [14] and inserted into the MCS of pEx-SK/KS. The complete HMGa coding region was excised with *Bam*HI/*Apa*I and the construct with the deletion of the acidic carboxy-terminus with *Pst*I. In another construct the HMG-Box was deleted using the internal *Dde*I sites at positions 162 and 391 [14], and the remaining fragments were re-ligated in frame. The resulting fragment was cut with *Bam*HI/*Apa*I and also inserted into pEx-SK. The deletion constructs were sequenced. The reporter construct, pSP1176+1088 Luc (assigned here psh-Luc) (a derivative of plasmid pRT 101 Luc [17] in which the CaMV 35S promoter was replaced by a fragment (position −1,176 to +1,088) of the maize shrunken-1 gene containing the promoter region) with the luciferase coding region under control of the maize shrunken (sh) promoter was obtained from Dr. W. Werr, Universität Köln, Germany. All plasmids used for transient transformation assays were prepared using Maxiprep columns (Promega) according to the manufacturer.

2.2. *Preparation of protoplasts*

The BMS maize embryo cell suspension culture (derived from the variety 'black mexican sweet') was maintained on MS medium [18] supplemented with 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)

and 30 g/l sucrose as described previously [19]. 80 ml of gravity-packed cells were washed in P_i buffer (50 mM $CaCl_2$, 10 mM sodium acetate, 0.48 M sorbitol, pH 5.6), and resuspended in 200 ml P_i buffer containing 2% (w/v) cellulase Onozuka RS (Yakult Honsha, Tokyo) and 0.01% (w/v) pectolyase Y23 (Seishin Pharmaceutical, Tokyo) and incubated in plastic Petri dishes at 28°C and 40 rpm for 2–3 h. After sequentially filtering through 250 μm and 60 μm nylon screens, protoplasts were collected by centrifugation at 50 × *g* for 5 min and washed in P_i buffer without enzymes. After repeating the centrifugation the protoplasts were washed in HBS electroporation buffer (10 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM $CaCl_2$, 0.36 M sorbitol) and counted with a haemocytometer. After another centrifugation the protoplasts were resuspended to 2×10^6/ml HBS.

2.3. *Electroporation of protoplasts*

The electroporation of the protoplasts was performed basically as described previously [20]. 1 ml of the protoplast suspension (2×10^6 protoplasts) was added to a 3 ml plastic cuvette (d = 1 cm) and cooled on ice. After adding 10 pmol of the test plasmids adjusted to 70 μg total DNA with pUC19 (50 μl total volume), the protoplasts were electroporated by discharging a 1,000 μF capacitor loaded to 350 V using platinum mesh electrodes. The protoplasts were left on ice for 3 min after which they were added to 9 ml of MS medium supplemented with 0.2 mg/l 2,4-D, 30 g/l sucrose and 0.38 M sorbitol and incubated at 28°C for 20 h.

2.4. *Luciferase assays*

The gene expression assays were performed essentially as described previously [19]. Briefly, transformed protoplasts were centrifuged 5 min at 60 × *g*, resuspended in 1 ml of ice-cold 0.1 M phosphate buffer, pH 7.8, containing 1 mM dithiothreitol, and broken by 5 strokes in a Dounce homogenizer The extracts were centrifuged 5 min at 10,000 × *g* and 0.1 ml of the supernatants were assayed for luciferase activity with a luminometer (Berthold, Wildbad, Germany) by sequentially injecting 0.15 ml of 1 mM ATP and 0 15 ml of 0.5 mM d-luciferin, both dissolved in 14 mM $MgCl_2$, 14 mM glycylglycine, pH 7.8 [21], and integration of the luminescent output over 10 s.

3. RESULTS

Transient transformation assays in plant protoplasts represent a frequently used system for investigating the influence of different parameters on reporter gene expression (e.g. [22,23]). In the case of co-transformation assays the influence of a concomittantly synthesized gene product on reporter gene expression can be studied. Such an approach was used here in order to study the effect of the maize HMGa protein on the in vivo expression of the luciferase reporter gene carried on another plasmid. Accordingly, the HMGa effector constructs and the luciferase reporter construct were simultaneously transformed by electroporation into protoplasts derived from a maize BMS (black mexican sweet) suspension cell line [19] and the luciferase activity was determined 20 h later.

The structure of the maize HMGa protein displaying an HMG-Box DNA-binding region and a highly acidic carboxy-terminal domain is shown schematically in Fig. 1A, while the structure of the various effector constructs containing HMGa–cDNA sequences are depicted in Fig. 1B. In these constructs, the full length HMGa–cDNA [14] was placed under the control of the CaMV 35S promoter [24] in sense (pEx HMGa+) or antisense (pEx HMGa−) orientation or in truncated forms after deletion of the HMG-Box DNA-binding region (pEx HMGa-D) or the acidic carboxy-terminal domain (pEx HMGa-P). The CaMV 35S promoter used in these constructs containing the enhancer and the TATA-Box [24] has proved to be a strong promoter in this system and, therefore, was appropriate to direct synthesis of the respective proteins. In the reporter construct, the luciferase gene was placed under the control of the maize shrunken (sh) promoter of the sucrose synthase gene [25] as shown in Fig. 1C. This promoter is active in kernels, roots and shoots of the maize plant [25].

As shown in Fig. 2 co-transformations of the reporter construct with the HMGa effector constructs contain-

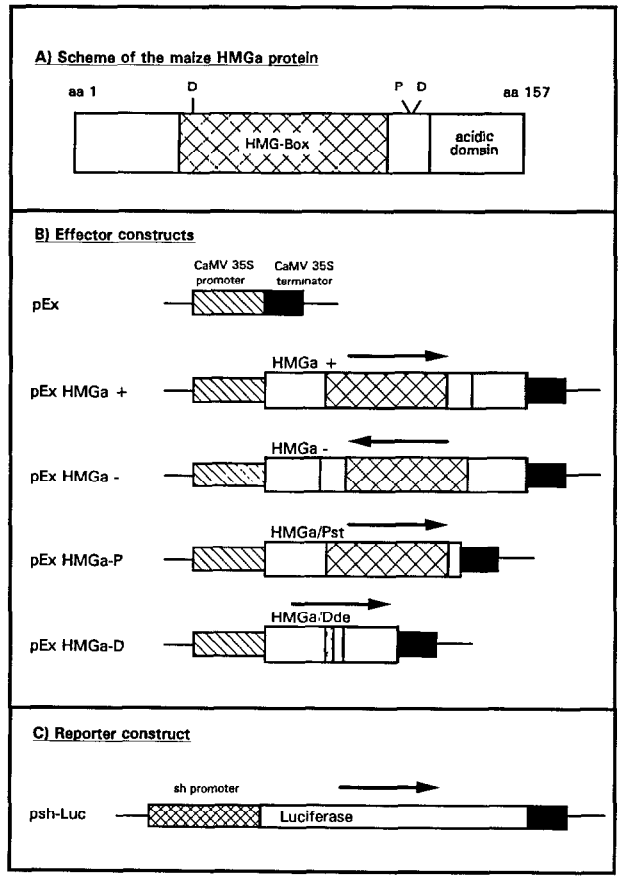

Fig. 1. Structure of the HMGa protein and schemes of effector- and reporter plasmid constructs. (A) Schematic representation of the structure of the maize HMGa protein, including the localization of the restriction enzyme sites used at the cDNA level for generating the construct with the deletion of the acidic domain (P = *PstI*) and the deletion of the HMG-Box (D = *DdeI*). (B) Schematic representation of the effector plasmid constructs. The expression vector pEx has the complete CaMV 35 S promoter and 35 S terminator. pEx HMGa+ additionally has the HMGa–cDNA in the sense and pEx HMGa− in the antisense orientation. pEx HMGa-P has the deletion of the acidic domain, and pEx HMGa-D has the deletion of the HMG-Box. (C) Schematic representation of the reporter construct. Within the reporter plasmid, psh-Luc (= pSP1176+1088), the firefly luciferase gene is placed under the control of the maize shrunken (sh) promoter The arrows indicate the orientation of the HMGa–cDNA elements. Plasmid constructs in B and C are not drawn to scale.

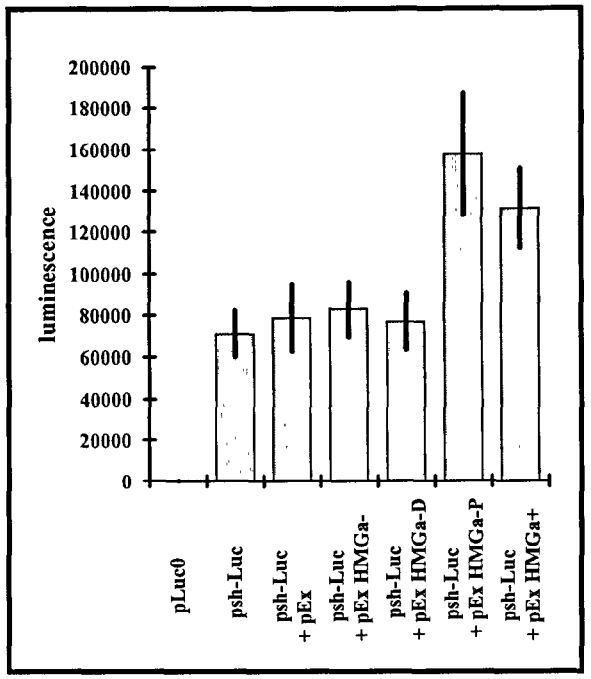

Fig. 2 Luciferase expression in transient co-transformation assays. The bars of the histogram represent the mean luminescence of 10 transformations with the indicated standard deviation. pLuc0 represents transformations with the promoterless luciferase control construct, psh-Luc represents transformations of the reporter construct alone, while the other experiments are psh-Luc co-transformations, as indicated.

ing the full-length cDNA in the sense orientation led to an about 2-fold higher luciferase expression (psh-Luc + pEx HMGa+) in comparison to the control co-transformations with the insertless expression vector (psh-Luc + pEx). A comparable result was obtained in co-transformation experiments with the effector construct missing the acidic domain (psh-Luc + pEx HMG-P); however, the effector construct without the HMG-Box DNA-binding region (psh-Luc + pEx HMG-D) does not reveal an influence on luciferase activity, which was also the case with the antisense construct (psh-Luc + pEx HMG−). Further control experiments with the promoterless reporter construct (pLuc0) or with the reporter construct transformed alone without co-transfection with an effector plasmid (psh-Luc) showed the expected behavior. It should be mentioned that every transformation set comprising all constructs was repeated three times with independent protoplast preparations, and that ten transformations were measured of each of these assays.

4. DISCUSSION

The maize HMGa protein belongs to the vertebrate HMG1 family which is conserved among various eukaryotic organisms such as plants, insects or yeast [26]. Several in vitro transcription experiments indicate a stimulatory role of these proteins in gene transcription [27,28]. In yeast cells, however, HMG protein fusion constructs did not elevate reporter gene expression in vivo [29]. In contrast to the vertebrate HMG1/2 proteins, which bind through their HMG-Box domains preferentially to single-stranded or cruciform DNA [30,31], several plant HMG proteins, including the maize HMGa protein, have been shown to bind in vitro to distinct A/T-rich stretches of double-stranded DNA occurring in the 5′-flanking region of plant genes [5,10–13]. We now extended these studies by an vivo analysis of the influence of the maize HMGa protein on gene expression, taking advantage of the commonly used transient co-transformation assays.

Despite high endogenous levels of the HMGa protein an about 2-fold stimulation of luciferase gene expression could be observed in the presence of additionally synthesized HMGa protein. In the case of the related vertebrate HMG1/2 proteins the occurrence of about 10^6 molecules per cell has been estimated leading to approximately one HMG1 or HMG2 protein for every 15 nucleosomes, assuming equal distribution of the proteins [32]. The observed stimulatory effect of the intact HMGa protein on luciferase expression appears to be strictly dependent on the presence of the HMG-Box DNA-binding motif as demonstrated by the experiments using the construct missing this domain, while, interestingly, the acidic carboxy-terminal domain is without influence in these experiments. There is no inhibition of luciferase gene expression by the HMGa antisense construct which may be due to the potential stability of the endogenous HMGa protein during the 20 h of the transformation assays, which would be consistent with the half-life of about 55 or 90 h observed for vertebrate HMG1 proteins [33,34].

Based on the presented data we cannot decide conclusively what level of gene expression is influenced by the HMGa protein. The observation of the binding of this protein to A/T-rich stretches within the 5′ flanking region of a zein storage protein gene [10,13], together with the inability of the HMGa constructs lacking the HMG-Box DNA-binding domain to stimulate luciferase gene expression in the transient co-transformation assays presented here, may indicate a transcriptional effect. While being in agreement with in vitro data obtained with vertebrate HMG1/2 proteins [27,28], more work is clearly needed to further substantiate these findings.

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REFERENCES

[1] Bustin, M., Lehn, D.A. and Landsman, D. (1990) Biochim. Biophys Acta 1049, 231–243.
[2] Spiker, S. (1984) J. Biol. Chem. 259, 12007–12013.
[3] Vincentz, M. and Gigot, C. (1985) Plant Mol. Biol. 4, 161–168
[4] Grasser, K.D., Wurz, A. and Feix, G. (1991) Planta 185, 350–355.
[5] Jacobsen, K., Laursen, N.B., Jensen, E.O., Marcker, A., Poulsen, C. and Marcker, K.A. (1990) Plant Cell 2, 85–94.
[6] Ull, M.A., Herrero, M.E. and Franco, L. (1991) Plant Sci. 75, 55–62.
[7] Moehs, C.P., McElwain, E.F. and Spiker, S. (1988) Plant Mol Biol. 11, 507–515.
[8] Spiker, S., Murray, M.G. and Thompson, W.F. (1983) Proc Natl. Acad. Sci. USA 80, 815–819.
[9] Mithieux, G. and Roux, B (1984) Biochim. Biophys. Acta 781, 286–293.
[10] Maier, U.-G , Grasser, K D., Haass, M.M. and Feix, G. (1990) Mol. Gen. Genet. 221, 164–170.
[11] Pedersen, T.J., Arwood, L.J., Spiker, S., Guiltinan, M.J. and Thompson, W.F (1991) Plant Mol Biol. 16, 95–104.
[12] Czarnecka, E., Ingersoll, J C. and Gurley, W.B (1992) Plant Mol. Biol 19, 985–1000.
[13] Grasser, K D., Maier, U.-G., Haass, M.M. and Feix, G (1990) J. Biol. Chem. 265, 4185–4188.
[14] Grasser, K.D and Feix, G. (1991) Nucleic Acids Res. 19, 2573–2577.
[15] Sambrook, J , Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[16] Pietrzak, M., Shillito, R.D., Hohn, T. and Potrykus, I. (1986) Nucleic Acids Res. 14, 5857–5868.
[17] Maas, C., Laufs, J., Grant, S , Korfhage, C. and Werr, W. (1991) Plant Mol. Biol. 16, 199–207.
[18] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473–497.
[19] Quayle, T.J.A., Hetz, W. and Feix, G (1991) Plant Cell Rep. 9, 544–548.
[20] Schwall, M. and Feix, G. (1988) Plant Sci. 56, 161–166.
[21] DeLuca, M and McElroy, W.D. (1984) Biochem. Biophys. Res. Commun 123, 764–770.
[22] Maas, C., Schaal, S and Werr, W. (1990) EMBO J. 9, 3447–3452.
[23] McCarthy, D.R., Hattori, T., Carson, C.B , Vasil, V , Lazar, M. and Vasil, I.K. (1991) Cell 66, 895–905.
[24] Benfey, P.N. and Chua, N.-H. (1990) Science 250, 959–966.
[25] Springer, B , Werr, W., Starlinger, P , Bennert, D C., Ziokolica, M. and Freeling, M. (1986) Mol. Gen. Genet. 205, 461–468.
[26] Griess, E.A , Rensing, S A., Grasser, K.D., Maier, U.-G and Feix, G (1993) J Mol. Evol. 37, in press
[27] Tremethick, D J. and Molloy, P.L. (1986) J. Biol. Chem 261, 6986–6992.
[28] Singh, J. and Dixon, G.H (1990) Biochemistry 29, 6295–6302.
[29] Landsman, D. and Bustin, M. (1991) Mol. Cell Biol 11, 4483–4489.
[30] Isackson, P.J., Fishback, J.L., Bidney, D.L. and Reeck, G.R. (1979) J. Biol. Chem. 254, 5569–5572.
[31] Bianchi, M.E., Beltrame, M. and Paonessa, G. (1989) Science 243, 1056–1059.
[32] Goodwin, G.H and Mathew, C.G.P. (1982) in. The HMG Chromosomal Proteins (Johns, E.W. ed.) pp. 193–222, Academic Press, London.
[33] Rechsteiner, M. and Kuehl, L. (1979) Cell 16, 901–908.
[34] Begum, N , Pash, J.M and Bhorjee, J.S. (1990) J Biol. Chem. 264, 11936–11941