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Efficient functioning of plant promoters and poly(A) sites in *Xenopus* oocytes

Nurit Ballas, Shimshon Broido, Hermona Soreq and Abraham Loyter*

Department of Biological Chemistry, The Life Sciences Institute, The Hebrew University, Jerusalem 91904, Israel

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ABSTRACT

Mature Xenopus oocytes were challenged with DNA constructs including plant regulatory elements, namely, the Cauliflower mosaic virus (CaMV) 35S promoter as well as the nopaline synthase (NOS) promoter and polyadenylation signal. The bacterial chloramphenicol acetyl transferase (CAT) was used as a reporter gene. When microinjected into these cells, the plant-derived DNA constructs effectively promoted CAT synthesis in a manner dependent on the presence of the plant promoters and probably also on the polyadenylation signals. Structural studies revealed that the supercoiled structures of the above DNA plasmids were much more active in supporting CAT synthesis in microinjected oocytes than their linear forms, with clear correlation between efficient gene expression and DNA topology. In contrast, the linear forms of these plasmids were considerably more active than the supercoiled ones in tranfected plant protoplasts. These findings demonstrate, for the first time, the activity of regulatory elements from plant genes in Xenopus oocytes and shed new light on the specific rules applicable for gene expression in plant and animal cells.

INTRODUCTION

Transfer of isolated genes into eukaryotic cells has been established as an informative approach to study questions related to control of gene expression by the use of specific DNA sequences and transcription signals (1). Indeed, using such systems it has been demonstrated that gene expression in animal cells depends on the efficiency of initiation (2), transcription processes (3) and RNA stability (3).

Recently, attempts have been made to elucidate the specific regulatory signals which control gene expression in plant cells and to compare them to those required in animal cells (4). One approach was to examine the ability of DNA sequences and regulatory elements derived from animal origins to function

appropriately in plant cells (5-7). In these studies, it has been demonstrated that plant cells were unable to correctly synthesize, splice or polyadenylate RNA transcripts from transfected DNA constructs composed of genes such as yeast alcohol dehydrogenase (8), mammalian interferon (9), rabbit ß-globin (10) and various genes under the control of the Simian virus 40 (SV40) promoter (9,11). In other studies, genes for ovalbumin and α-actin from chicken were introduced into the Agrobacterium Ti-plasmid as a vehicle (5). Tobacco plant tissues were found to be unable to transcribe the chicken α-actin genes in these constructs. In contrast, transcription of the ovalbumin gene was observed, however, with a different initiation sequence from that used in the chicken oviduct (5).

The failure of animal genes to be effectively expressed in plant cells was generally considered to reflect species specificity of regulatory elements as well as of recognition and interaction processes in plant versus animal cells (5-7). Should this be the case, it might inversely imply that DNA sequences and regulatory elements of plant origin would be inactive when introduced into animal cells. To examine this hypothesis, we therefore microinjected DNA constructs containing plant regulatory elements and a reporter chloramphenicol acetyl transferase (CAT) gene into Xenopus oocytes. Results obtained demonstrate that promoters and probably polyadenylation sites of plant origin can efficiently and correctly function in the oocyte environment.

MATERIALS AND METHODS

Plasmids

The plasmids pUC8CaMVCAT and pNOSCAT were a generous gift from V. Walbot (Department of Biological Sciences, Stanford University). Both plasmids consist of the CAT coding region, the nopaline synthase (NOS) polyadenylation region and either the 35S promoter from the Cauliflower mosaic virus (CaMV) (pUC8CaMVCAT) (Fig. 1) or the NOS promoter (pNOSCAT) (12). The plasmid pSV2CAT (13) contains the SV40 early gen s promoter, the CAT coding

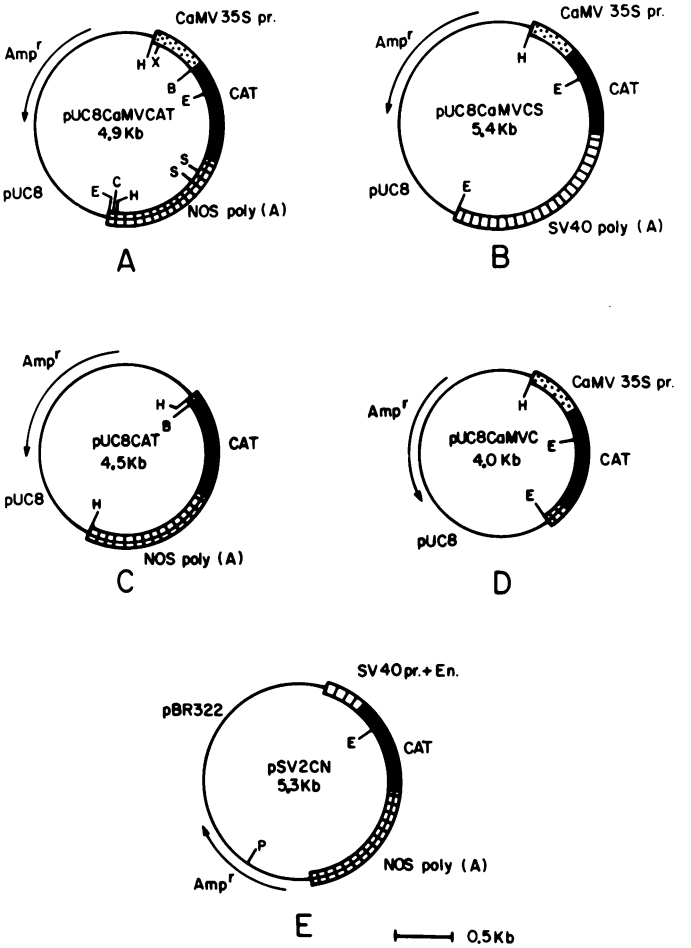

Figure 1. Plasmids containing plant (CaMV 35S, NOS) or animal (SV40) promoters and plant (NOS) or animal (SV40) polyadenylation sites: schematic presentation.

The construction procedures of the plasmids pUC8CaMVCS, pUC8CAT, pU8CaMVC and pSV2CN, were described in Materials and Methods.

The source of each fragment is indicated by different backgrounds: stippled - cauliflower mosaic virus (CaMV) 35S promoter; black - chloramphenicol acetyl transferase (CAT); cross-hatched - nopaline synthase (NOS) promoter and polyadenylation region; hatched - SV40 promoter and polyadenylation region. Restriction sites relevant for construction of the plasmids are indicated: B, BamHI; C, ClaI; E, EcoRI; H, HindIII; P, PvuI; S, SphI; X, XbaI.

region and the SV40 polyadenylation region.

Plasmid construction

pUC8CaMVS(Fig. 1) was constructed by excising the 1.65 kb EcoRI fragment of pUC8CaMVCAT and inserting the 2.15 kb EcoRI fragment of pSV2CAT containing the SV40 polyadenylation region and the remaining region of the CAT gene. The plasmid pUC8CAT (Fig. 1) was prepared by deleting the 0.4 kb XbaI/BamHI fragment of pUC8CaMVCAT containing the CaMV 35S promoter. pUC8CaMVC (Fig. 1) was obtained by deleting the 0.9 kb fragment restricted with SphI/ClaI of pUC8CaMVCAT carrying the NOS polyadenylation signal. pSV2CN (Fig. 1) was obtained by ligating the 3.85 kb EcoRI fragment of pSV2CAT containing the SV40 promoter and a part of the CAT gene with the 1.65 kb EcoRI fragment of pUC8CaMVCAT containing the NOS polyadenylation region and the remaining region of the CAT gene. Cloning methods were performed using standard procedures (14).

Injection into oocytes

Stage 6 oocytes from adult Xenopus laevis females were microinjected each with 50 nl of plasmid DNA (1 mg/ml) according to established procedures reviewed in (15). Injected oocytes were incubated at 19°C in Barth medium (16).

Transfection of plant protoplasts

Protoplasts were prepared from Petunia hybrida cell suspension line 3704, as previously described (17). Isolation and transfection of protoplasts using polyethylene glycol transfection method were as detailed elsewhere (18,19).

CAT assay

Oocytes and protoplasts were sonicated in solution containing 0.125 M Tris-HCl pH 7.8, 0.5 M EDTA, 0.25 mM phenylmethyl sulfonyl fluoride (PMSF). Following centrifugation (10,000 x g for 5 min) the supernatants were heated at 60°C for 10 min and assayed for CAT activity (13). Briefly, assay mixtures (containing 0.37 M Tris HCl pH 7.8, 150 µl of protoplast or oocytes extracts, 0.6 µCi [^{14}C]chloramphenicol (53 mCi/mmol), 0.5 mM acetyl-CoA in a final volume of 350 µl) were incubated for 30 min at 37 C. Reaction products were separated by thin-layer chromatography (TLC) and autoradiographed.

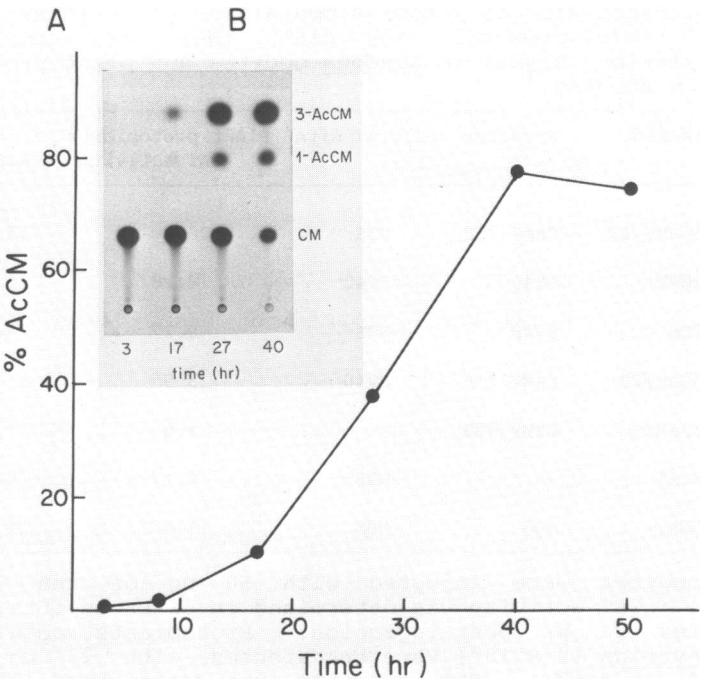

Figure 2. Kinetics of CAT activity accumulation in Xenopus oocytes microinjected with the pUC8CaMVCAT plasmid DNA.

Xenopus oocytes were injected with 50 ng of pUC8CaMVCAT. At each indicated time, extracts from 30 oocytes were assayed for CAT activity as described in Materials and Methods.

A. The percentage of the acetylated chloramphenicol (AcCM) (out of total chloramphenicol (CM added) was obtained by cutting the spots in the thin layer chromatography (TLC) plate, and counting the radioactivity (19).

Inset (B): AcCM as detected by TLC and autoradiography.

DNA extraction from Xenopus oocytes and blot analysis

Oocytes were homogenized in a solution which contained 20 mM Tris-HCl pH 7.4, 10 mM NaCl, 25 mM EDTA, 1% SDS and 0.5 mg/ml proteinase K (20). Following incubation for 2 hr at 37°C, 5 M NaCl (1/10 volume of the oocyte homogenate) was added and the DNA was gently extracted by phenol: chloroform. The purified DNA was electrophoresed on 0.9% agarose gel and blotted onto nitrocellulose filter. The [^{32}P]-labelled HindIII fragment of pUC8CaMVCAT containing the CAT gene served as a probe for these hybridizations, which were performed as previously detailed (14).

Table 1. Expression of plasmids containing plant (CaMV 35S, NOS) or animal (SV40) promoters and plant (NOS) or animal (SV40) polyadenylation signal in Xenopus oocytes and plant protoplasts: comparison studies.

Plasmid	Promoter	poly(A) site	Plant protoplasts CAT activity (% AcCM)	X. oocytes CAT activity (% AcCM)
1. pUC8CaMVCAT	CaMV 35S	NOS	20.0	23.8
2. pSV2CAT	SV40	SV40	0.28	26.0
3. pSV2CN	SV40	NOS	0.27	21.6
4. pUC8CaMVCS	CaMV 35S	SV40	3.90	21.7
5. pUC8CaMVC	CaMV 35S	--	1.0	1.0
6. pUC8CAT	--	NOS	0.17	0.15
7. pNOSCAT	NOS	NOS	11.0	1.7

Xenopus oocytes were injected with 50 ng of the indicated plasmids. CAT activity was determined in extracts obtained from 30 oocytes 24 hr post-injection. Protoplasts obtained from Petunia hybrida (4×10^6) were transfected with 25 µg of the indicated plasmids. CAT activity was determined 20 hr post transfection as described in Materials and Methods.
CAT activity is given as percentage of the acetylated chloramphenicol (AcCM), out of the total chloramphenicol (CM) added to the reaction mixture.

RESULTS

Plant promoters are efficiently active in microinjected oocytes

Microinjection of the plasmid pUC8CaMVCAT (containing the CaMV 35S promoter, the CAT gene as a reporter and the NOS polyadenylation site) into Xenopus oocytes promoted synthesis of the CAT enzyme. This was inferred from the appearance of CAT activity in DNA-microinjected, but not in sham-injected control oocytes. Considerable CAT activity could be observed as early as 8 hours following microinjection of pUC8CaMVCAT into the oocytes. CAT activity increased linearly between 20-40 hrs post-injection and reached a plateau level by 50 hours (Fig. 2). The level of CAT activity observed in oocytes injected with pUC8CaMVCAT was almost identical to the level of CAT activity observed in oocytes injected with the plasmid pSV2CAT, which carries the SV40 early genes promoter as well as its polyadenylation site (Table 1).

A second plasmid, the pNOSCAT, carrying the NOS promoter and polyadenylation signal was also able to promote CAT gene expression in the oocytes. However, this promoter induced CAT activity in Xenopus oocytes at a much lower efficiency than that induced by the CaMV 35S promoter or the SV40 promoter (Table 1).

Plasmids combining animal and plant regulatory elements function in oocytes and plant protoplasts.

In order to evaluate the contribution of particular DNA elements towards the efficient expression of the CAT gene in the microinjected oocytes, several new plasmids were constructed (see Fig. 1 and Materials and Methods). The promoter-deficient plasmid pUC8CAT composed of the bacterial CAT gene and the NOS polyadenylation site, was incapable of promoting CAT activity in the oocytes, demonstrating that the plant promoter is a necessary pre-requisite for the expression of the bacterial CAT gene in this system (Table 1). Similarly, very low CAT activity appeared in oocytes which were microinjected with the pUC8CaMVC plasmid, containing the CaMV 35S promoter and the CAT gene, but lacking the NOS polyadenylation site (Table 1). In addition, DNA constructs containing the plant regulatory elements in conjunction with the appropriate animal regulatory elements were able to operate in the oocytes (Table 1). The pUC8CaMVCS plasmid which contains the CaMV 35S promoter, the bacterial CAT gene and the SV40 polyadenylation site promoted CAT activity in microinjection oocytes at a level that was close to or identical with the activity induced by the original pUC8CaMVCAT or pSV2CAT plasmids (Table 1). Similar results were obtained following the microinjection of the pSV2CN plasmid, which possess the SV40 early genes promoter, the CAT gene and the NOS polyadenylation site (Fig. 1, Table 1). In reciprocal experiments, petunia protoplasts were challenged with the DNA constructs containing animal regulatory elements. Replacement of the NOS polyadenylation site by the SV40 polyadenylation site in plasmids containing the CaMV 35S promoter resulted in a considerable reduction of CAT gene activity in transfected protoplasts although this did not happen in microinjected oocytes (compare system No. 1 to No. 4 in Table 1). Furthermore, the pSV2CAT plasmid which contains the SV40 promoter, the CAT reporter gene

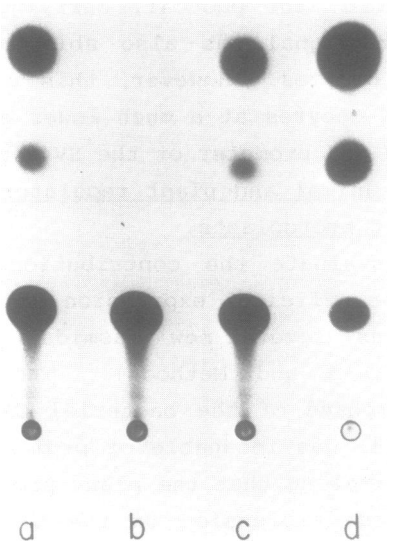

Figure 3. CAT activity in microinjected Xenopus oocytes or in transfected petunia protoplasts: Effect of DNA topology.
pUC8CaMVCAT was linearized by ClaI. Xenopus oocytes were injected with 50 ng of supercoiled (a) or linear (b) pUC8CaMVCAT each. Oocyte extracts were assayed for CAT activity 24 hr after injection.
Petunia protoplasts (4 x 10^6) were transfected with 25 µg of supercoiled (c) or linear (d) pUC8CaMVCAT as described in Materials and Methods. Protoplasts were assayed for CAT activity 15 hr after transfection.

and the SV40 polyadenylation signal was not expressed at all in transfected plant protoplasts. Even upon replacement of the SV40 polyadenylation site by the NOS polyadenylation site, as was done in the pSV2CN plasmid (Fig. 1), CAT production could not be detected in plant protoplasts transfected with DNA constructs containing the SV40 promoter (Table 1). Altogether, these results confirm and extend previous reports (5-11) demonstrating that animal regulatory elements are not recognized by plant cells. This implies that within plant protoplasts, plant regulatory elements can function well only in conjunction with each other, whereas in microinjected oocytes their expression is relatively independent of the origin of the counterpart signal.

The linear form of the pUC8CaMVCAT plasmid is expressed in plant protoplasts but not in microinjected oocytes

The linear forms of the DNA constructs carrying plant

promoters and polyadenylation sites, among them pUC8CaMVCAT, support gene expression in transfected plant protoplasts much better than their corresponding supercoiled forms (19). Indeed, the linear form of the pUC8CaMVCAT plasmid was 10-fold more active than the supercoiled one in transfected petunia protoplasts (Fig. 3). In contrast, the supercoiled structure of DNA constructs is known to be required for efficient gene expression in recipient animal culture cells and oocytes (21-23). To examine whether the topological state of DNA constructs carrying plant regulatory elements plays a role in their level of expression in the animal oocytes, CAT activities were measured following microinjection of linear or supercoiled DNA forms of the pUC8CaMVCAT plasmid. Fig. 3 demonstrates that in microinjected oocytes, as opoposed to transfected protoplasts, the linear form of the pUC8CaMVCAT plasmid DNA was by far less active than its supercoiled structure. These results are in complete agreement with previous observations (21-23) that supercoiled plasmids were the only ones to be efficiently expressed in microinjected Xenopus oocytes and in transfected cultured animal cells

DNA blot hybridization of oocyte-extracted plasmid DNAs revealed that both linear and supercoiled structures of the pUC8CaMVCAT plasmid were present within microinjected oocytes at 6 and 20 hours post-injection (Fig. 4), with the linear form being more susceptible to intracellular degradation processes than the supercoiled template. In addition, a significant proportion of the linear injected DNA appeared in a higher molecular weight form within 20 hr post-injection.

DISCUSSION

Transient gene expression in microinjected Xenopus oocytes clearly showed that two promoters known to function in plant systems, namely the CaMV 35S and the NOS promoters, are able to direct expression of the CAT gene in animal cells as well. The CaMV promoter was as efficient as the SV40 promoter in directing CAT synthesis, whereas the NOS promoter was considerably less efficient in oocytes, in agreement with parallel studies in transfected plant protoplasts (12,24). Expression of the CAT gene

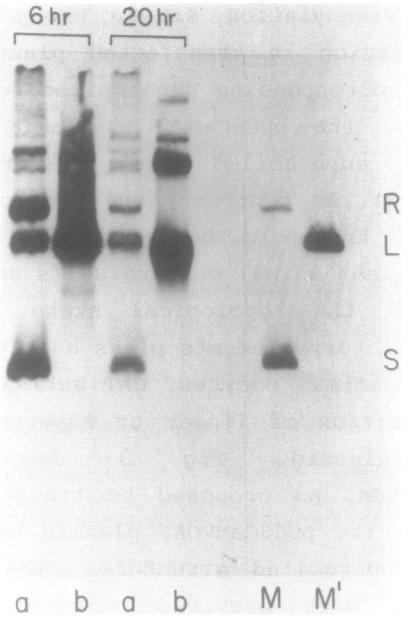

Figure 4. Fate of supercoiled and linear pUC8CaMVCAT in microinjected Xenopus oocytes.

Xenopus oocytes were injected with 50 ng of supercoiled (a) or linear (b) pUC8CaMVCAT. The plasmid was linearized by ClaI. At the indicated times, DNA was extracted from 30 oocytes as described in Materials and Methods. The DNA (20 µg) was electrophoresed on 0.9% agarose gel in the presence of ethidium bromide. The gel was blotted onto nitrocellulose filter and hybridized with ^{32}P-labeled HindIII fragment of pUC8CaMVCAT containing the CAT gene. M and M' represent the supercoiled and the linear preparations of the plasmid, respectively. R, relaxed; L, linear; S, supercoiled.

in Xenopus oocytes was also dependent on the presence of a polyadenylation site in the injected plasmids. This was inferred from our experiments showing that very little or no CAT activity was detected following microinjection of the pUC8CaMVC plasmid which contains the CaMV 35S promoter, but lacks a polyadenylation signal. However, the same level of CAT activity was observed when the injected plasmid possessed a polyadenylation signal from an animal virus (SV40) or from a plant-specific gene such as NOS. Attempts to isolate specific CAT mRNA transcripts from microinjected oocytes gave inconclusive results, probably due to the low amounts of such transcripts and the presence of

contaminating complementary DNA sequences. However, it is conceivable that CAT mRNA transcripts without their poly(A) tails are unstable, getting rapidly degraded, similar to the poly(A) deprived globin mRNA (15,25). The ability of the NOS polyadenylation signal to support CAT expression in Xenopus oocytes is of particular interest, since this polyadenylation site does not possess the AAUAAA sequence which characterizes most of the animal polyadenylation signals (25).

Expression of exogenous genes in prokaryotic as well as in animal cells was shown to be dependent on the topology of the transfected gene, supercoiled forms being generally more active than their corresponding linear structures (21-23). Recently (19), we have shown that the linear DNA forms of the DNA constructs employed in the present work were more active in supporting transient gene expression in tranfected plant protoplasts than their corresponding supercoiled structures. The observation seen in animal cells were hence in discrepancy with those in the trasfected plant cells. However, this topological regulation appeared not to be specific to the DNA sequences that were used. Rather, it most probably reflected different control mechanisms in plant as opposed to animal cells. Results of the present experiments as well as of previous observations (21-23) show that the linear forms of plasmids are significantly less active in oocytes than the supercoiled structures, the latter being more stable in microinjected oocytes (22). The high activity of the linear form of these plasmids in plant cells could hence indicate that trans-regulatory elements and transcription factors of plant origin act differently than those of animal origin and may better recognize linear DNA forms than supercoiled structures.

Our results demonstrate that promoters and probably polyadenylation signals of plant origin may each independently and efficiently function in microinjected oocytes, and that in the oocytes these signals obey the general rules for topological organization of expressible DNA elements in animal cells. Hence, the topological state of plant genes appears to affect their expression in a manner that is directly dependent on the host system.

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*To whom correspondence should be addressed

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