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Expression of mRNA electroporated into plant and animal cells

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ABSTRACT

A general method to introduce RNA molecules into plant protoplasts and animal cells is described. This technique utilizes the ability of electric pulses of high field strength to form pores in biomembranes. RNA molecules containing the coding region for the bacterial enzyme chloramphenicol acetyltransferase (CAT) were used as a model system. The presence of CAT activity as a result of the in vivo translation of the introduced RNA is entirely dependent on the presence of a 5' cap and greatly increased by the presence of a poly A tail at the 3' end. The introduction of RNA into eukaryotic cells has broad applicability both as an assay for the uptake of nucleic acids into cells independent of transcriptional activity and as a tool to study eukaryotic mRNA translation.

INTRODUCTION

A eukaryotic cell achieves control over the rate of appearance and steady state levels of a gene product by regulating synthetic or degradative processes controlling that particular gene product. Investigations in vivo of the regulation of two steps, the translatability and stability of mRNA encoding that gene product, have been limited by the lack of a general technique to introduce RNA directly into cells. Therefore, most of our knowledge about the translational requirements of eukaryotic mRNAs have been derived from studies utilizing heterologous systems; cell free translation (1-3) or translation of RNAs microinjected into Xenopus oocytes (4-7). A few investigations have used in vivo systems, either microinjection directly into cultured cells (8-11), complexing RNA with DEAE-dextran or $CaCl_2$ and incubation with cells (12,13) or liposome encapsulation of RNA and subsequent fusion with cells (14-17). We have investigated the use of another technique, electroporation, to introduce mRNAs into eukaryotic cells. Electroporation is the application of an electric field to reversibly permeabilize biomembranes (18) and has been used to introduce and monitor the expression of DNA into bacteria (19), animal cells (20,21) and plant protoplasts (22- 26). Others have detected Tobacco Mosaic Virus (TMV) coat protein after the introduction of TMV RNA into plant protoplasts using electric fields (27-29). The replication of TMV RNA to high copy number greatly facilitates the detection of its expression. It has not been previously determined whether electroporation-mediated transfer of a non-replicating RNA would result in readily detectable levels of protein.

Studies using in vitro translation and/or microinjection of RNA into Xenopus oocytes have

indicated that the cap present at the 5' end of cellular and many viral mRNA molecules as a 5' to 5' ^{7}m GpppN linkage is important both for RNA stability and translation initiation, although there are naturally occurring uncapped viral mRNAs (30-33). The role of the poly adenylate residues at the 3' end (poly A tail) of mRNAs is less understood (34, 35). For example, the presence of a poly A tail has been observed to increase the functional stability of rabbit globin mRNAs microinjected into Xenopus oocytes (36), but polyadenylation does not affect the stability of human interferons in the same assay (37). Because the behavior of mRNA in vitro and in Xenopus oocytes may not be representative of mRNA in other differentiated cells from other organisms, we tested the effects of 5' and 3' modifications on RNA activity in plant protoplasts and animal fibroblasts using electroporation-mediated RNA transfer. RNA molecules containing the coding region for the bacterial enzyme chloramphenicol acetyl transferase (CAT, ref 38) were used as a model mRNA because the enzymatic assay for CAT activity is convenient and quantitative.

MATERIAL AND METHODS

Structures of the CAT mRNAs

A 773 bp Taq1 fragment from pBR325 containing the chloramphenicol acetyl transferase gene (38) was cloned into a modified pUC8 plasmid that contains the poly linker from the plasmid πAN7 to form pUCπCAT. A BamHI-BglII fragment from pUCπCAT was cloned into the plasmid SP65 to generate the DNA template (pSP65CAT) for the poly A^{-} RNA shown in Fig. 1A. To generate the DNA templates for the poly A^{+} mRNA (pSP65CAT-A^{+}, Fig. 1B), pUCπCAT was linearized with PstI, adenylate residues were added at the 3' end with dATP and terminal transferase and then cut with BamHI which cuts 5' of the CAT coding region. The CAT containing fragment was annealed and ligated to a pSP65 plasmid that had been linearized with PstI, and tailed with thymidylate residues by terminal transferase and dTTP and then cut with BamHI. Plasmids were purified by the alkaline lysis method (39), linearized with HindIII, transcribed in vitro and the resulting RNA purified as described by Melton (40). An aliquot of the RNAs was capped in vitro by guanylyltransferase (BRL, ref 41), phenol extracted and ethanol precipitated. All RNAs were stored in dH_2O at -80 C.

Introduction of RNA into plant protoplasts

Maize and carrot protoplasts were prepared and the electroporation solutions used were as described previously (22) with the addition of an extra wash prior to electroporation. Protoplasts (1-2 x 10^{6}/ml) were given a 625 V/cm electric pulse from the discharge of a 490 μF capacitor in solutions (22) containing 5 μg/ml CAT mRNA or 10 μg/ml DNA controls. After 10 minute incubation on ice and 10 minute incubation at room temperature, 8 ml of media (22) was added to the protoplasts and they were incubated 6 hours in the dark at room temperature. The protoplasts were then spun down (300xg), resuspended in 540 μl 100 mM Tris pH 7.9, 1 mM EDTA, sonicated for 30 seconds, the debris removed by centrifugation, and 180 μl of the supernatant

assayed for CAT activity (42). The ^{14}C acetylated products were separated from the substrate, ^{14}C-chloramphenicol, by chromatography on thin layer silica gel plates and located by autoradiography (42). The resulting autoradiograms were quantitated by scanning densitometry. The variability between experiments was two-fold.

Introduction of CAT mRNA into cultured mammalian cells

In vitro synthesized RNA was introduced into cultured monkey fibroblast COS cells (43) based on a previously described procedure (21). Cells were trypsinized, washed two times with PBS to remove the trypsin and resuspended in PBS at a concentration of 10^6 cells/ml and kept on ice. The electroporation of the COS cells was as described above for plant protoplasts except a pulse of 500 V/cm from a 490 μF capacitor was used and the cells were incubated for 6 hours after electroporation in Dulbecco's Modified Eagles Medium (the DNA control was incubated for 18 hours). The cells were concentrated by centrifugation, lysed by sonication and the supernatant assayed for CAT activity as described above.

In vitro translation of mRNA

100 nanograms of each of the CAT mRNAs were incubated with a rabbit reticulocyte lysate (BRL) for 1 hour at room temperature. Conditions used were as recommended by the manufacturer; the lysate concentration of added potassium acetate was 87 mM. An aliquot was removed and assayed for CAT activity as described above. A titration curve was performed to ensure that the CAT signal was proportional to the amount of RNA added.

RESULTS

The two different DNA templates used for the in vitro transcriptions contain the CAT coding region from pBR325 in either a SP65 plasmid (pSP65CAT) or in a modified SP65 plasmid (pSP65CATA$^+$) containing a downstream poly A region approximately 40 nucleotides long. The

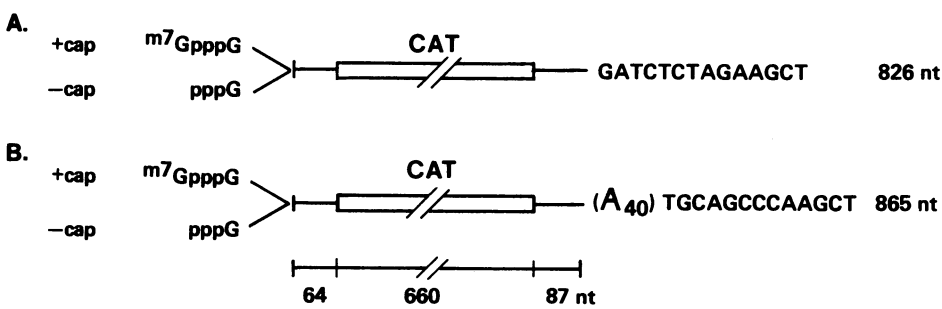

Figure 1. Structures of the CAT mRNAS.
Poly A$^-$ (A) and poly A$^+$ (B) mRNAs were produced by in vitro transcription of pSP65CAT and pSP65CATA$^+$, respectively (Material and Methods). Capping of these RNAS with guanylyltransferase (41) generates the 5' structures shown. The size, in nucleotides, of the CAT mRNA 5' region, coding region and 3' untranslated region are shown below the RNAs.

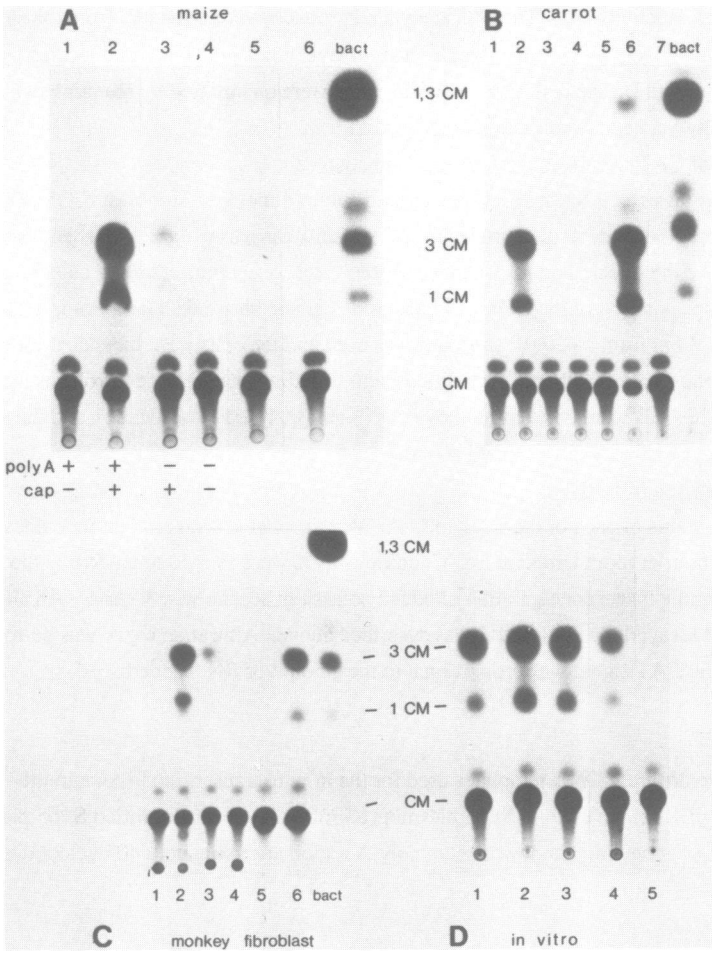

Figure 2. CAT activity as a result of in vivo and in vitro translation of introduced mRNA.

The CAT RNAs shown in Figure 1 were electroporated into maize protoplasts (A), carrot protoplasts (B) and monkey fibroblast cells (C) and resulting extracts were assayed for CAT activity (Methods). The same RNAs were also translated in vitro and the lysate assayed for CAT activity (D). For all panels, lanes 1-4 represent the results from the introduction of the following CAT mRNAs; poly A^+ and uncapped (lane 1), poly A^+ and capped (lane 2), poly A^- and capped (lane 3), poly A^- and uncapped (lane 4), respectively. The lanes marked "bact" are the CAT activity produced from an E.coli extract containing pBR325 and serve to designate the migration of acetylated chloramphenicol (1,3 CM; 3 CM; 1 CM), products of CAT enzymatic activity. For panels A-C, lane 5 represents the CAT activity produced after the introduction of 10 μg/ ml of the DNA pSP65CATA$^+$. In (A) lane 6, substrate alone; (B) lane 6, 10 μg/ml pNOSCAT DNA (22); lane 7, substrate alone; (C) lane 6, 10 μg/ml pSV2CAT (39); (D) lane 5, no added RNA. Each experiment was repeated twice with similar results.

transcription of these DNA templates, after linearization with HindIII, produced either poly A^- (Fig. 1A) or poly A^+ (Fig. 1B) CAT mRNA. The poly A tract does not terminate the poly A^+ mRNA; 3' of the poly A tract are 13 nucleotides, 5 of which comprise the HindIII site necessary for the linearization of the template DNA. Subsequent capping of an aliquot of the poly A^+ and A^- RNAs in vitro with Vaccinia virus guanylyltransferase produced the set of four RNAs shown in Figure 1.

Two plant species, maize and carrot, were chosen for study as representative species from the two highly diverged subclasses of angiosperms, the monocotyledoneae and the dicotyledoneae, respectively. Electroporation was used to introduce the four different RNAs into protoplasts from maize (Zea mays L., Fig. 2A) and carrot (Daucus carota L., Fig. 2B) cultured cells. After a 6 hour incubation, extracts were prepared and assayed for the presence of CAT activity (39). Eighteen hour incubations produced amounts of CAT activity equivalent to a 6 hour incubation (data not shown). Both maize (Fig. 2A, lane 2) and carrot (Fig. 2B, lane 2) protoplasts have CAT activity after introduction of capped polyadenylated RNAs. The absence of a 3' poly A tract on capped RNAs decreases the CAT activity by 150 fold for maize (Fig. 2A, lane 3) and 60 fold for carrot (Fig. 2B, lane 3). The difference in CAT expression between capped poly A^+ and capped poly A^- RNA is not a result of differential capping efficiencies because both are capped to approximately the same extent as measured by ^{32}P GTP incorporation (data not shown).

The presence of a 5' cap is absolutely required for CAT activity; the introduction of poly A^+ RNAs without a 5' cap into maize (Fig. 2A, lane 1) and carrot (Fig. 2B, lane 1) cells results in no detectable CAT activity. Similarly, maize (Fig. 2A, lane 4) and carrot (Fig. 2B, lane 4) protoplasts have no detectable CAT activity when electroporated in the presence of poly A^- RNAs lacking a 5' cap.

The amount of CAT activity in extracts from cells electroporated in the presence of pSP65CATA$^+$, the DNA template, for both maize (Fig. 2A, lane 5) and carrot (Fig. 2B, lane 5) is equivalent to that found in extracts from control protoplasts (no nucleic acid present) and equivalent to the amount present in the ^{14}C-chloramphenicol label without incubation with plant extracts (Fig. 2A, lane 6; Fig. 2B, lane 7). Therefore, any CAT activity produced depends on the introduced RNA, and not on transcription and translation from any DNA remaining after treatment of the RNA with DNase I during its purification (37). For carrot protoplasts, electroporation with pNOSCAT DNA (Fig. 2B, lane 6) serves as a positive control for electroporation-mediated nucleic acid transfer because expression from pNOSCAT has been observed in these cells (22).

The four CAT mRNAs (Figure 1) were also electroporated into a cultured monkey fibroblast cell line (Fig. 2C) and extracts analyzed for their resulting CAT activity. As observed for plant protoplasts, capped poly A^+ RNA (Fig. 2C, lane 2) gave maximal CAT activity with a 70 fold

increase over capped, poly A^- RNA (Fig. 2C, lane 3). Poly A^+ (Fig. 2C, lane 1) and poly A^- (Fig. 2C, lane 4) RNAs without a 5' cap did not produce detectable CAT activity when electroporated into these animal cells, again indicating that the presence of a cap is absolutely required for RNA activity in vivo. Electroporation with the DNA template, pSP65CATA^+, (Fig. 2C, lane 5) does not produce any CAT activity. The presence of CAT activity after the transfer of pSV2CAT (Fig. 2C, lane 6), a construct known to express CAT in monkey cells (42), indicates that nucleic acid transfer was occurring under these electroporation conditions.

We determined that each of the four CAT mRNA samples was intact and capable of producing CAT protein by analyzing the RNA on a 1% agarose gel (data not shown) and by translating the RNA in a rabbit reticulocyte extract and assaying the extract for CAT activity (Fig 2D). In marked contrast to the situation in vivo, each RNA directed the synthesis of comparable amounts of CAT protein, indicating that the RNAs lacking a 5' cap were functional templates for the synthesis of active CAT protein. The in vitro translation of the poly A^+, capped CAT mRNAs (Fig 2D, lane 2) resulted in the highest level of CAT activity, although CAT levels were only approximately 2 fold lower for capped, poly A^- RNAs (Fig 2D, lane 3). The lack of a 5' cap reduced the level of CAT activity another 3-6 fold for both poly A^+ (Fig. 2D, lane 1) and poly A^- (Fig. 2D, lane 4) RNAs. These differences are much smaller than observed in vivo (see above).

DISCUSSION

Our results extend previous work which demonstrated that mRNAs produced by in vitro transcription were functional mRNAs in Xenopus oocytes and in wheat germ extracts (44, 45). We show that the technique of electroporation can introduce in vitro synthesized mRNAs in plant protoplasts and animal cells where they are translated, producing detectable levels of CAT activity. We have examined the requirements in vivo for a 5' cap and a 3' poly A tail on CAT mRNA. This work shows that the in vivo structural requirements of mRNA resulting in maximal translation observed in Xenopus oocytes also holds true for plant and animal cells. The requirement for a 5' cap observed here is similar to that found for mRNAs injected into oocytes (30, 32, 44). However, RNA expression in both plant and animal cultured cells shows a much greater dependence on the presence of a poly A tail than has been previously described in Xenopus oocytes (36). Our results are quantitatively more similar to those of Huez (11) and Drummond (45) who observed no product synthesized after microinjection of either globin or lysozyme poly A^- RNAs, respectively, into cultured cells. Indeed, our ability to detect CAT activity from the translation of the capped poly A^- mRNA probably results from high sensitivity of the CAT enzyme assay. Although we consider it unlikely that the strong dependence on the presence of a poly A tail observed here is specific for the CAT coding region, we have not yet formally eliminated this possibility. Future analysis of other RNAs in vivo will determine the generality of this dependence. Also, we have not distinguished whether the differences in CAT activity observed in this work between the presence and absence of a 5' cap and a poly A tail result from differences in the stability or the translatability of the introduced RNA.

These results also demonstrate the disparity between the requirements for RNA activity in vivo and in vitro. There is at least a 500 fold effect on the presence of a 5' cap in vivo for both plant protoplasts and animal cells, but only a 6 fold effect in vitro. The presence of a poly A tail has only a 2 fold effect in vitro, but a 60-150 fold effect in vivo. The magnitude of the in vitro effects of a 5' cap observed here are comparable to previous investigations using either wheat germ extract or reticulocyte lysate. The relative in vitro translational efficiencies of capped and decapped TMV RNA (46), reovirus and vesicular stomatitis (VS) virus RNA (47) are dependent on the amount of added RNA template and K^+ ions. At their relative optima, translation of capped and uncapped VS virus RNAs differed only 3 fold (47). The effect of a poly A tail on mRNA activity in vitro has also been observed to be much smaller than that observed in vivo. Deadenylated globin mRNA translated in vitro had either no significant difference (44,48) or only a 50% reduction (49) in globin synthesis as compared to poly A^+ globin mRNA. Thus, an accurate assessment of the effects of modification of cellular RNA structure and/or sequence in vivo benefits from in vivo analysis.

Electroporation offers several advantages over other RNA transfer techniques. In contrast to microinjection, electroporation can introduce RNA into 10^6 cells simultaneously. Electroporation, unlike liposome encapsidation and cell fusion, is a simple technique and uses no agents such as polyethylene glycol that are toxic to some species. Because of the universality of the response of biomembranes to electric fields (18), it is probable that RNA can be introduced into the cells of any organism or differentiated tissue. Thus, the stability and translatability of an RNA can probably be studied in the cell normally expressing it. The availability of bacterial plasmids for the transcription of cloned DNA templates (40) allow the production of microgram quantities of a single RNA species. Using in vitro transcription, poly A^+ and poly A^- RNAs are produced without using chemical reagents and exonucleases, ensuring a pure population of RNAs with defined 5' and 3' ends.

The expression of RNA introduced via electroporation does not require transcription. Thus, the ability of coding regions to be translated and detected in cells can be analyzed without the uncertainties of promoter expression. Because electroporation conditions for RNA and DNA transfer are similar (unpublished data), RNA can be used for organisms or cell types lacking functionally defined promoters to establish that marker genes can be detected and that successful electroporation-mediated nucleic acid transfer is occurring. The development of a DNA transformation system for maize (22,50) benefited from the introduction of CAT mRNA to verify electroporation parameters. Thus, the electroporation of mRNAs into eukaryotic cells provides a rapid and convenient method for analyzing the effects of RNA structure on activity in vivo and for monitoring nucleic acid transfer.

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