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Expression of *GRP78 Enhancer-CAT* Fusion Constructs Microinjected into *Xenopus laevis* Oocytes

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Microinjection of genes into *Xenopus laevis* oocytes in highly useful in the analysis of gene regulation, since a large number of oocytes can be injected in a relatively short time. The GRP78 enhancer has been identified to a 291-bp fragment that spans a region of GRP78 promoter between -378 and -87 (Lin *et al.*, 1986; Kim and Lee, 1989). We examined whether this GRP78 enhancer is effective in directing expression of heterologous gene in *Xenopus laevis* oocytes. The chloramphenicol acetyltransferase (CAT) fusion constructs containing the GRP78 promoter and the SV40 early promoter were constructed and were injected into nuclei of *Xenopus laevis* oocytes. The recipient oocytes were then assayed for CAT activity. The fusion constructs exhibited higher activity as compared to SV40 promoter tested here. The GRP78 enhancer showed 8.5- to 9.2-fold enhancement over that of the SV40 promoter. The orientation of GRP78 enhancer with respect to the direction of CAT transcription unit had no significant effect. Thus, the GRP78 enhancer is a viable candidate for the construction of expression system for use in *Xenopus laevis* oocytes and will be important for the study of a gene expression throughout development.

KEY WORDS: microinjection, *Xenopus oocytes*, Glucose Regulated-Protein.

Since the *Xenopus* oocytes are much larger (1. 2 mm in diameter) than oocytes of other such as sea urchin (80 to 180 um), a large number of *Xenopus* oocytes can be injected with DNA in a relatively short time. Usually, a large number of oocytes are required to be analyzed for an accurate statistical evaluation. The *Xenopus* oocytes have proven to be highly useful in the analysis of gene regulation (Etkin, 1982; Etkin *et al.*, 1983). Microinjection of genes into *Xenopus* oocytes has been used to study the transcription. The injection of genes with mutated promoter region has enabled the identification of regulatory elements required for transcription (Groschedl and Birnstiel, 1980). The *Xenopus* oocyte system has also been used successfully to identify a factor capable of stimulating sea urchin early H2b gene transcription (Mous *et al.*, 1985).

Rat gene encoding GRP78, has been isolated and sequenced (Wooden *et al.*, 1988). GRP78 shares a common peptide domain near the N-terminal region with four proteins of the 70-kDa heat shock protein (hsp70) family (Chappell *et al.*, 1986). The GRP78 is identical to the immunoglobulin heavy chain binding protein (BiP). Since the GRP78 and BiP have been localized to the endoplasmic reticulum (ER) (Zala *et al.*, 1988; Bole *et al.*, 1986), they may function to prevent aggregation of immunoglobulin heavy chain in pre-B cells. Thus, GRP78 may serve a more general role in the assembly and stabilization of secreted and membrane-bound proteins in the ER

of many different cell types.

An enhancer element has been localized in viral and cellular genes at positions 5′ or 3′ to the promoter, and within intron sequences (Khoury and Gruss, 1983; Mercoia *et al.*, 1983; Serfling *et al.*, 1985). It functions in an orientation- and position- independent manner to stimulate the transcriptional activities of promoters. When *trans*-acting factors bind to enhancer sequence which is *cis*-acting element, transcriptional regulation may be stimulated actively (Ephrussi *et al.*, 1985). The GRP enhancer has been identified to a 291-bp fragment between positions -87 and -375 within the GRP78 promoter (Lin *et al.*, 1986; Kim and Lee, 1989). In this study, we further examined the function of the GRP78 enhancer using *Xenopus oocyte* system by fusing it with SV40 early promoter which is linked to CAT transcription unit.

Materials and Methods

Preparation of oocytes

Xenopus females were anesthetized by hypothermia, and oocytes were surgically removed. The oocytes were teased apart into group of around 10 and rinsed three times in Barth's solution containing streptomycin (50 mg/*l*) and penicillin (50 mg/*l*).

Plasmids

The plasmid pSV1BCAT is equivalent to the pSVXCAT previously described (Celander and Haseltine, 1984), with the *Xho* I site changed to a *Bgl* II site. Its unique *Bgl* II and *Bam* HI sites were used for 5′ insertions. A 291-bp fragment extending from the *Stu* I site at position –87 to *Sma* I site at position –375 within the GRP promoter was subcloned into the *Bgl* II site of pSV1BCAT in the same orientation [pSV1B(291r) CAT] and in the reverse orientation [pSV1B(291w) CAT] as the CAT-transcriptional unit. The plasmid pE43 was generated by the *BAL* 31 digestion of PI10 as described (Chang *et al.*, 1987). This plasmid contains the –480 to –37 fragment from the GRP78 promoter subcloned into a CAT vector in the same orientation.

Oocytes culture and Microinjection

Microinjection was performed as described (Etkin and Maxson, 1980: Colin, 1986). Twenty nl of 200 μg/ml plasmids were injected into the nuclei of *Xenopus* oocytes. Recipient oocytes were then incubated for 20 hours in Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.5), 0.82 mM $MgSO_4.7H_2O$, 0.33 mM $Ca(NO_3)_2.4H_2O$, 0.41 mM $CaCl_2.6H_2O$.

Protein extracts

Protein was prepared as described (Maxson *et al.*, 1988). Oocytes injected were lysed with low-salt buffer and ammonium sulphate [$(NH_4)_2SO_4$] was added to a final concentration of 0.36 M. Lysed oocytes were homogenized and centrifuged at 10,000 ×g for 5 min. the protein was precipitated by further addition of 55% $(NH_4)_2SO_4$ to the supernatant and the precipitated protein was collected by centrifugation. The pellet was then suspended in solution containing 10mM HEPES (pH7.0), 0.1 mM EDTA, 0.1 mM KCl, 20% glycerol, 0.5 mM PMSF and 1 mM DTT. Protein was dialyzed for 16 hour at 4°C against the same medium. Protein concentration were determined by Bio-Rad.

CAT assay

Equal amounts (100 μg) of protein extracted from *Xenopus* oocytes were used in each CAT assay. Each CAT assay was repeated five times. About 4×10^5 cpm of [^{14}C] Chloramphenicol (Cm) was used for each assay, which was analyzed on TLC plates (Whatman) as described (Gorman *et al.*, 1982). The TLC plates were exposed to Kodak XO-mat films to detect the acetylated [^{14}C] Cm. For quantitation of percent conversion, the spots corresponding to the acetylated and non-acetylated Cm were excised from the TLC plates and counted in a liquid scintillation counter. The background conversion level was about 60 cpm in mock samples.

Results and Discussion

Microinjection of genes and nuclei has been used to study transcription in amphibian oocytes and eggs (Gurdon *et al.*, 1971). The *Xenopus* oocyte system has been used to identify a regulatory element required for transcription by injection of genes with mutated region (Grosschedl *et al.*, 1980). It would be useful, therefore, to study for developmental switch in the rate of transcription which is caused by change in the synthesis of transcription factor during the development (Maxson *et al.*, 1988). Previously, it has been shown that the GRP enhancer stimulates the expression of heterologous gene after its gene was transfected into mouse embryonic cells (Kim *et al.*, 1990). In this study, we examined for the first time whether heterologous gene under the direction of a strong promoter is highly expressed in *Xenopus laevis* oocytes.

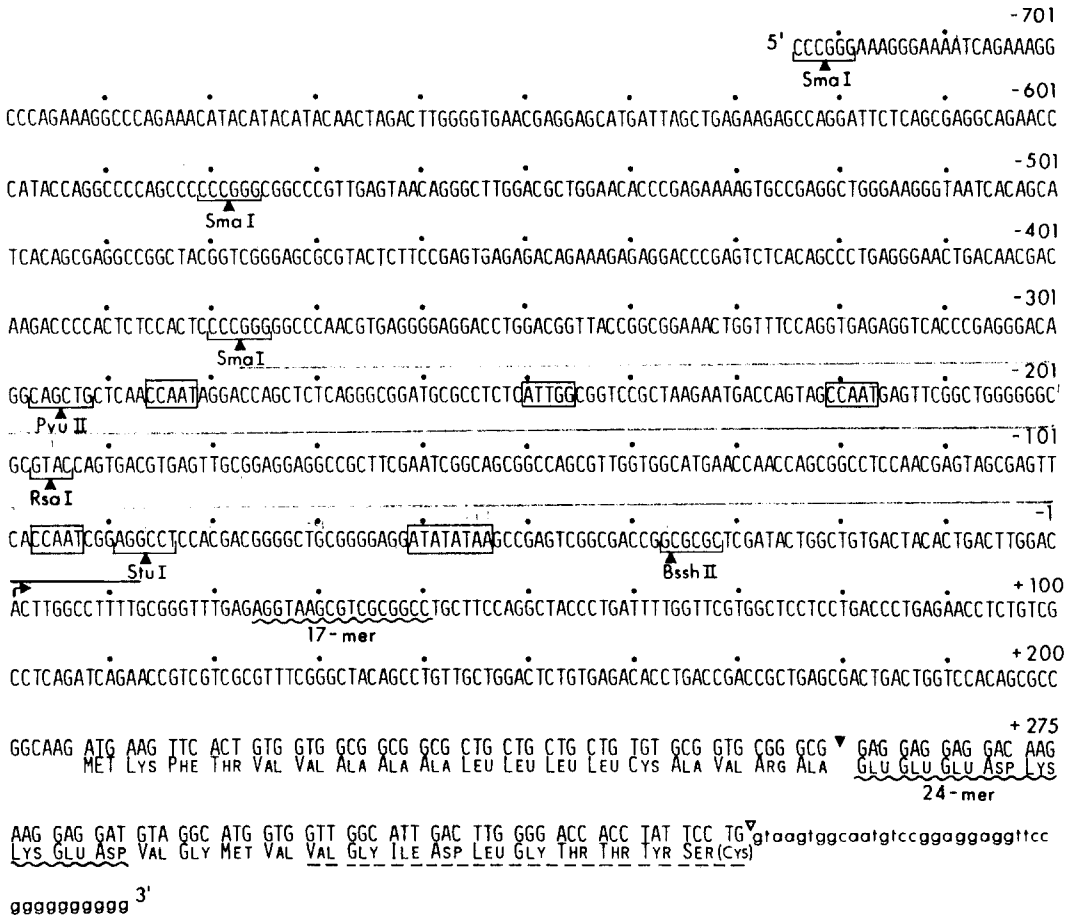

Fig. 1. Sequence of the GRP78 promoter and its first exon. The sequence is numbered starting from the major mRNA cat site (—) as +1. Bases downstream of this site are numbered positively and bases upstream are numbered negatively. The TATAA and CCAAT box are boxed. The GRP78 enhancer region between −378 and −87 is underlined. The wave line show the 17-mer and 24-mer used in primer extension experiment. The black triangle (▼) indicates the proteolytic cleavage site that excises the 18 amino acid leader sequence. The open triangle (▽) indicates the 5' splice site of the first exon/intron junction. The sites of restriction enzyme are noted. Dashed line shows the conserved peptide shared other stress-induced protein.

The GRP78 promoter region and the first exon of the rat GRP78 gene are shown in Fig. 1. Besides having strong promoter activity, the 291-bp *SmaI-StuI* fragment immediately 5′ to the TATA sequence can act as an enhancer to increase the transcriptional activities of heterologous promoter (Lin *et al.*, 1986). This GRP78 enhancer has been localized to 291-bp fragment extending between positions −87 to −375 within the GRP78 promoter (Kim *et al.*, 1989). This enhancer contains four copies of the CCAAT motif, one of which exists in a reverse orientation. A sequence (GCCGCT TCGAAT CGGC) which is highly conserved within the GRP78 promoters from different species, is found at positions −169 to −154, and has a interesting feature that it is palindromic (Redendez *et al.*, 1988). In addition, the sequence (GGCTG GGGGG) resembling that of the binding site for the transcription activation factor AP2 (Imagawa *et al.*, 1987) is found. Detailed analysis of the nucleotide sequence also revealed sequence homologies to other viral and cellular core enhancers and an abundance of repeat elements (Lin *et al.*, 1986).

The GRP78 enhancer that spans −378 to −87 was cloned into *Bgl* II site of the SV40-CAT plasmid (pSV401BCAT) to create the fusion gene in same orientation [pSV40(291r)CAT] or in an opposite orientation [pSV40(291w)CAT] with respect to CAT transcription unit. The plasmid, pE43, contains 443-bp region spanning from −480 to −37 that included the GRP78 enhancer, a associated upstream sequence of additional GRP78 promoter and downstream sequence of own TATA box together (Fig. 2). We have attempted to determine whether inserted GRP78 enhancer is effective in direct expression of a heterologous gene in *Xenopus* oocytes. To test these, the plasmid constructs were injected into nuclei of *Xenopus laevis* oocytes and assayed for CAT activity. For comparison, the percentages of conversion of the chloramphenicol into its acetylated form were quantitated in each sample, and the enhancer-less parental plasmid (pSV1BCAT) was set to unity. The average values from the experiments were summarized in Table 1. Most eggs survived 20 hrs incubation, after injection with Barth's solution and plasmids. Smaller (2 μm internal diameter) injection pipets were used for this experiment as they may improve the rate of egg survival. *Xenopus laevis* oocytes after microinjectin were used to assay the CAT activity (Fig. 3). The CAT activities were not observed in control eggs injected with Barth's solution (Fig. 1B; 1a, 2a, 3a, and 4a). Since the frog egg can be readily activated by pricking the egg cortex with a needle or micropipette (Wolf,

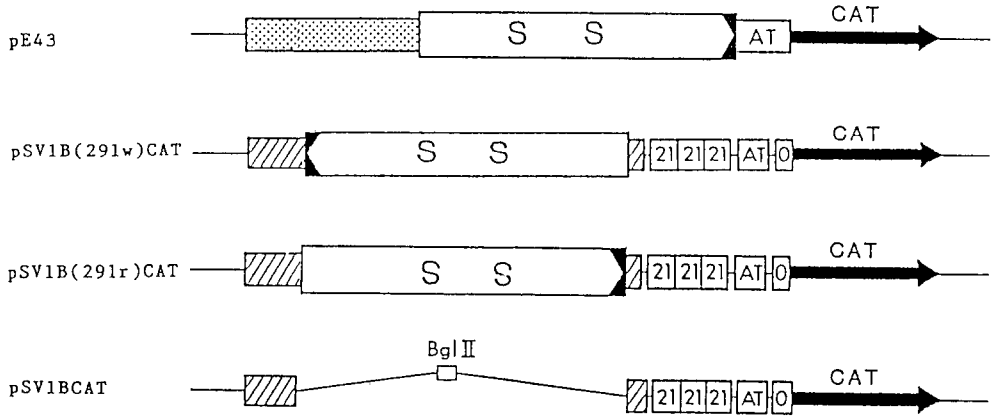

Fig. 2. Structure of CAT fusion constructs. Open bar, GRP78 enhancer sequence (S; *Sma* I or *Stu* I site within the GRP78 promoter sequence, open bar arrow; orientation of insertion) Shaded bar, upstream sequence of 291-bp of GRP78 enhancer sequence. Deviant bar, SV40 early promoter sequence. Darked Bar, CAT sequence. AT, TATA region within the SV40 early promoter and GRP78 promoter sequence. 21, a nearly perfect triple tandem of a highly G+C-rich 21-bp. O, origin of replication. *Bgl* II, cloning site for GRP78 enhancer. lines, prokaryotic vector sequence.

Table 1. Results of injecting plasmids into *Xenopus laevis* oocytes

Oocytes injected with Barth's sol. and plasmids	No. of experiments	No. of oocytes	Survivors	No. of assay	CAT activity	
					No. of oocytes per assay	relative activity (Cm %)
Barth's sol.	4	59	55	3	10	1
pSV1BCAT	6	78	73	5	10	1
Barth's sol.	5	88	82	3	10	1
pSV1B(29lr)CAT	6	120	115	5	10	8.7
Barth's sol.	5	72	67	3	10	1
pSV1B(29lw)CAT	7	102	98	5	10	8.5
Barth's sol.	3	55	53	3	10	1
pE43	5	83	81	5	10	9.2

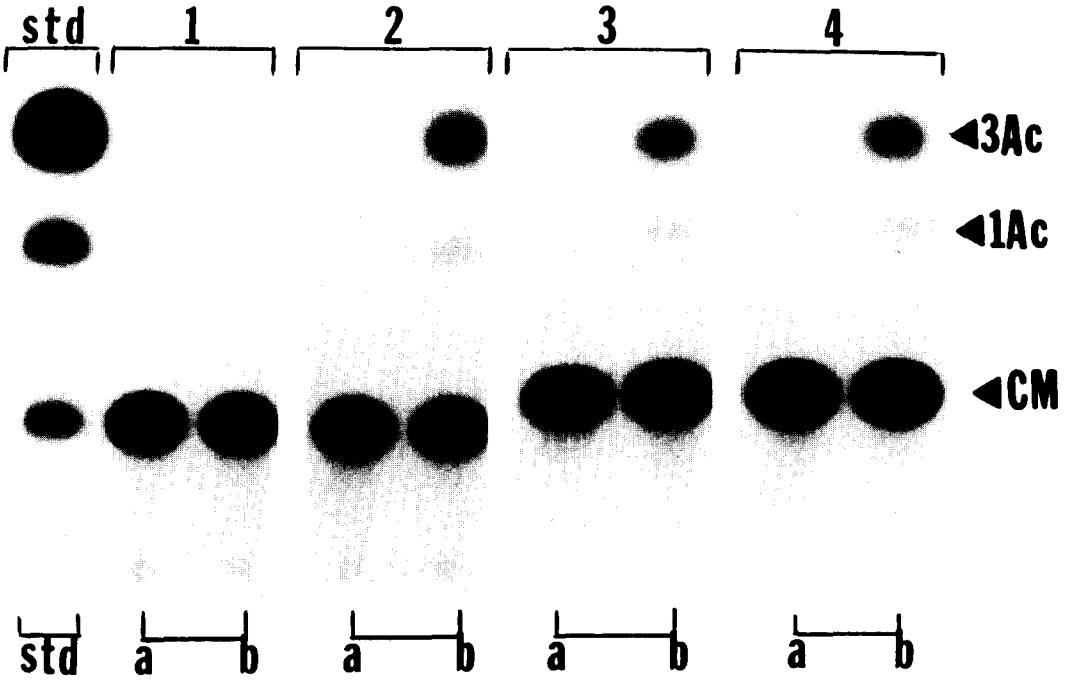

Fig. 3. Promoter activity of CAT fusion consturcted in *Xenopus laevis*. *Xenopus* oocytes were injected with the plasmids indicated. Equal amounts of protein from the *Xenopus* oocytes injected with plasmids were assayed for CAT activity. The position of chloramphenicol (CM) and its acetylated form (1 Ac, 3 Ac) are shwon.

1974), we injected with Barth's solution to eliminate the possibility that activation of egg after microinjection of plasmids may contribute to the CAT activity. When eggs were injected with pSV1BCAT, no CAT activities were observed (Table 1 and Fig. 1B;1b). The commonly used SV40 early promoter is active in cells derived from a wide variety of tissues and species (Gorman *et al.*, 1982). However, in *Xenopus* oocytes the CAT activity under the control of SV40 promoter was not observed, indicating that a negative regulatory factor may be present in oocytes. In embryonic cell, the SB40 promoter also does not stimulate transcription unit to which it is linked,

since embryonic cell such as F9 contains a negative regulatory factor that acts on viral enhancers to prevent expression of transcription units to which they are linked (Arizumi *et al.*, 1989; Gorman *et al.*, 1985). Insertion of 291-bp GRP78 enhancer resulted in increses in the CAT activities. It showed 8.5- to 9.2- fold enhancement over that of the SV early promoter (Table 1 and Fig. 3B; 3b, 4b). The orientation of GRP78 enhancer with respect to the direction CAT transcription had no significant effect (Table 1 and Fig. 3B; 2b, 3b). This result is similar to that observed in hamster fibroblast cells (Kim and Lee, 1989). Therefore, the GRP78 enhancer is a viable candidate for the construction of an expression system for use in embryonic cells and in *Xenopus* oocytes. The GRP78 enhancer will also be useful for the study of gene expression during development.

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Xenopus 난자에 미세주입된 ***GRP78 Enhancer-CAT*** 이형접합자의 발현

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Xenopus 난자는 크기가 커서 짧은 시간내에 쉽게 미세주입 할 수 있기 때문에 유전자를 난자에 주입하여 유전자 조절 연구에 대단히 유용하다. GRP78 enhancer는 GRP78 유전자의 Promoter −378과 −87 사이의 291 염기쌍으로 확인된 바 있다(Lin *et al.*, 1986; Kim and Lee, 1989).

본 연구는 이 enhancer의 효과를 연구하기 위해 CAT 전사단위와 연결한 이형접합을 만들어 이를 *Xenopus* 난자의 핵에 주입하였다. 주입된 난자의 CAT 효소활성을 측정한 결과 GRP78 enhancer 조절하의 CAT 활성은 GRP78 enhancer가 없는 것보다 8.3 배에서 9.2 배가 높았다. 또한 GRP78 enhancer의 CAT 전사단위에 정방향과 반대방향의 위치에 대한 효과의 차이는 인정되지 않았다. GRP78 enhancer는 *Xenopus*에 있어서 expression vector로서 중요하며 *Xenopus* 발생과정에서 유전자를 연구하는데도 중요할 것으로 사료된다.