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Effect of Viral Enhancers on the Tissue-Specific Expression of Bovine Growth Hormone Gene

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소성장호르몬 유전자의 조직 특이성 발현에 미치는 바이러스 *enhancer*의 영향

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ABSTRACT: The effect of SV40 and murine cytomegalovirus (MCMV) enhancers on the general and tissue-specific gene expression was investigated. Recombinant plasmids containing these transcriptional enhancers downstream of a structural gene for chloramphenicol acetyl transferase (CAT) were constructed. The transient expression of CAT gene from these plasmids was measured in monkey (CV1PD) and HeLa cells. Both SV40 and MCMV enhancers activated the expression of CAT gene by more than 20 and 150 folds, respectively, compared with enhancerless plasmids. When the SV40 promoter, upstream of CAT gene, was replaced with 2.2 kbp of promoter regulatory region of bovine growth hormone (bGH) gene, there was no expression of CAT even in the presence of enhancers, reflecting the tissue-specific expression of bGH genes. However, when the bGH regulatory region was shortened to 230 bp, the expression level increased dramatically in the presence of SV40 enhancers. In contrast, the expression from the shortened promoter was only marginally activated by the stronger MCMV enhancer.

KEY WORDS□Enhancers, SV40, MCMV, bovine growth hormone, tissue-specific expression, CAT assay

The transcriptional enhancers, which increase the level of transcription *in cis* in position - and orientation - independent manner, are found in many viral and cellular genes. The mechanisms of the action of enhancers as well as the enhancer-binding proteins are getting revealed by many researchers (See Atchinson (1988) for a recent review). Apart from these enhancer elements, eukaryotic transcription is regulated both positively and negatively by many cis-acting elements. The regulation is usually mediated by binding specific proteins to these elements (Jones *et al.*, 1988; Horikoshi et al., 1988; Briggs *et al.*, 1986; Santoro *et al.*, 1988; Lebowitz *et al.*, 1988). Some of these regulatory elements exert their effect in many different cells, whereas some exhibit strict tissue-specificity as well as temporal specificity (Cereghini *et al.*, 1987; Franza *et al.*, 1987; Boulet *et al.*, 1986; Maniatis *et al.*, 1987).

The expression of bovine growth hormone (bGH) gene is restricted *in vivo* to somatotrophic cells in pituitary anterior lobes. *In vitro*, bGH-specific expression is repressed in various non-somatotrophic cell lines tested (Ramabhadran *et al.*, 1985). When the 5'-flanking sequences of bGH gene, about 250 bps of which were sequenced, were compared with those of human and rat growth hormone genes, many homologous regions were located (Woychick *et al.*, 1982). In rat and human

genes, a trans-acting factor which binds to a region between −100 and −130 nucleotides upstream from transcriptional initiation site was found to be responsible for tissue-specific expression of these genes (Bodner and Karin, 1987; Bodner *et al.*, 1988; Ingraham *et al.*, 1988). In addition to that, the expression of rat growth hormone was reported to be regulated negatively in non-somatotrophic cells, by cis-acting DNA elements located between −554 and −237 nucleotides upstream from the major cap site (Larsen *et al.*, 1986). This observation, however, was refuted by contradicting results by Nelson *et al.* (1986) and Ye *et al.* (1988), and hence needs to be clarified.

Not much is known about the regulation of expression of bGH gene, partly because of the lack of primary culture and/or permanent cell line from bovine pituitary cells. In order to find out the effect of heterologous enhancers on the expression of bGH in non-somatotrophic cell lines, we constructed various recombinant plasmids containing 3' enhancers and chloramphenicol acetyltransferase (CAT) gene as a reporter. SV40 and murine cytomegalovirus (MCMV) enhancers were chosen for this purpose, since these are known to be both strong, and active in a wide variety of tissues and species (Moreau *et al.*, 1981; Laimins *et al.*, 1982; de Villers *et al.*, 1982; Sasson-Corsi *et al.*, 1984; Wasylyk *et al.*, 1984; Dorsch-Häsler *et al.*, 1985). The transient expression of CAT in HeLa and CV1PD cells was assayed, following transfection of cells with various recombinant DNAs containing bGH regulatory region and/or viral enhancers.

METERIALS AND METHODS

Construction of recombinant plasmids

Standard methods as described by Maniatis *et al.*, (1982) were used. CAT expressing vector, pSV2-CAT, was provided from the laboratory of Dr. P. Berg at Stanford University. SV40 enhancer fragments were obtained by digesting pSV2-CAT with PvuII and NcoI. Plasmid containing MCMV enhancer (p MCMVP) was provided by S. Shin in Dr. H.S. Kang's laboratory at Seoul National University. The genomic bGH gene cloned as a 4.3 kbp EcoRI fragment in pBR322 was a kind gift from Dr. Rottman at Case Western Reserve University. pCH110, used for normalizing the transfection efficiency, was purchased from PL Biochemical Co.. *E. coli* HB101 cells were used for transformation and amplification of recombinant DNAs. Restriction enzymes were purchased from KOSCO, and NEB. All the chemicals used were of reagent grade.

Purification of plasmid DNAs.

Following alkaline lysis of cells containing plasmids, PEG (MW 8,000) and NaCl were added to the supernatant to final concentrations of 6.5% (w/v) and 1 M, respectively, and incubated at 4°C for 12-15 hours, as described by Lis (1980). The precipitates were collected by spinning for 20 minutes in a microcentrifuge, and washed with 70% ethanol. The dried pellet was dissolved in TE and further purified through 3 cycles of phenol/chloroform extraction. The aqueous phase was precipitated with ethanol and the pellet was redissolved in TE. Plasmids prepared in this way were good substrates for restriction enzymes, and ensured high effeciency of transfection.

Transfection of cells

We employed the calcium phosphate method, following the procedure by Graham and Eb (1973). CV1PD and HeLa cells were grown to about 60% confluency in DMEM (Dulbecco's modified Eagle's medium) containing 5% fetal bovine serum. 10-20 μg of plasmid DNA was dissolved in 450 μl of distilled water and mixed with 50 μl of 2.5 M $CaCl_2$. A drop-by drop mixing of DNA/$CaCl_2$ solution with 0.5 m*l* of 2× HBS (Hepes-buffered saline) produces fine precipitates. This DNA solution was applied to healthy cells and mixed well with the media. Following 12-16 hours of incubation in 5% CO_2 incubator at 37°C, the cells were washed 3 times with PBS (phosphate-buffered saline) and incubated further by providing with DMEM containing 5% serum and antibiotics, for 40-48 hours.

CAT assay

The procedure developed by Gorman *et al.* (1982) was employed. The transfected cells were washed 3 times with PBS, and 1 m*l* of TEN (40 mM Tris, pH 7.4, 1 mM EDTA, 0.15 M NaCl) was added to

each plate. After harvesting cells, the cell pellet was resuspended in 10 μl of 0.25 M Tris (pH 7.8) and underwent 3 cycles of freezing (in dry ice-ethanol bath) and thawing (in 37°C incubator). The cell debris was removed by centrifugation, and the supernatant was saved in a clean tube. Portions of the cell extracts were assayed for β-galactosidase as described by Miller (1972). Cell extracts containing same amounts of β-galactosidase activity were mixed with the following reagents; 4μl ^{14}C-chloramphenicol (54 mCl/mmole), 70μl of 1 M Tris (pH 7.8), 20 μl of 4 mM acetyl Co-A, and distilled water to a final volume of 150 μl. Following incubation at 37°C for 1 hour, chloramphenicol was extracted with 1 ml of ethyl acetate. The upper phase was transferred to a new tube, and ethyl acetate was evaporated in a speed-vac concentrator. The radioactive pellet was redissolved in 30 ul of ethyl acetate and applied to a TLC plate (Macherey-Nagel). The plate was developed for 15 min in chloroform: methanol (95:5) and was air dried. Following autoradiography, the positions on TLC plate which gave signals were excised and counted in liquid scintillation counter for quantification.

RESULTS

Activity of 3' viral enhancers

We constructed recombinant plasmids containing CAT gene driven by enhancerless SV40 promoter and either SV40 or MCMV enhancer. Since we intended to measure the transcriptional activity of bGH regulatory region in the presence of heterologous enhancers, we cloned viral enhancers at the 3' end of the reporter gene (Fig. 1). The parent plasmid used for this construction was pSV2-CAT which expresses high levels of CAT in various animal cells (Gorman *et al.*, 1982). This plasmid contains SV40 enhancer and promoter upstream of CAT gene, and we replaced the upstream region with enhancerless SV40 promoter fragment excised from plasmid pXS-13 (Fromm and Berg, 1982). The resulting plasmid, p ΔE-CAT, was then used for inserting viral enhancers into the BamHI site 3' to the CAT gene. The activities of SV40 and

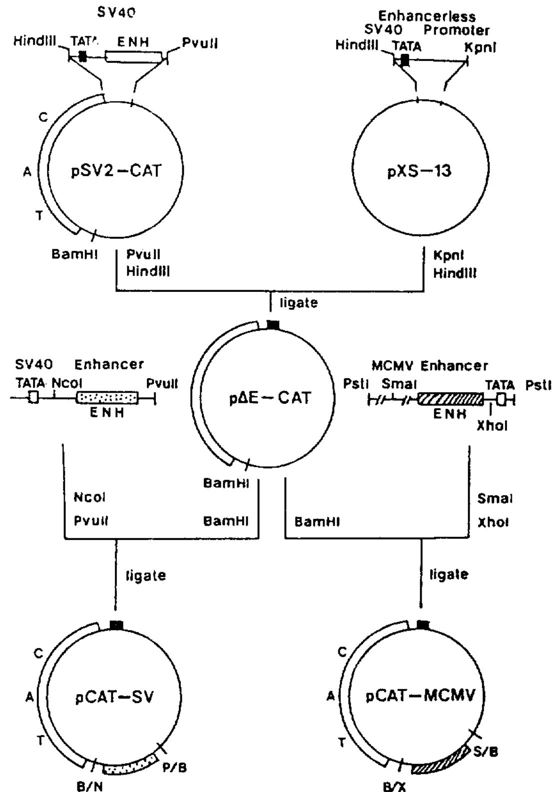

Fig. 1. *Construction of recombinant plasmids containing viral enhancers at the 3' end of CAT gene.* Enhancerless plasmid containing CAT gene (pΔE-CAT) was made by replacing SV40 promoter and enhancer region of pSV2-CAT with enhancerless SV40 promoter from pXS-13. Promoterless SV40 enhancer (NcoI-PvuII fragment) and MCMV enhancer (SmaI-XhoI fragment) were then inserted into the BamHI site of pΔE-CAT. The resulting recombinant plasmids, pCAT-SV and pCAT-MCMV, express CAT gene from SV40 promoter.

MCMV enhancers in activating transcription from the upstream gene were measured by determining the level of CAT in HeLa and CV1PD cells transfected with appropriate plasmid DNAs.

In order to have control over the variable efficiency of DNA-mediated transfection we cotransfected equal amount of expression vector (pCH110), which produces high levels of β-galactosidase. Portions of cell extracts from transfected cells were assayed for β-galactosidase activity, and cell extracts containing same amount of β-galactosidase were then assayed for CAT. In this way, we could normalize the variability in

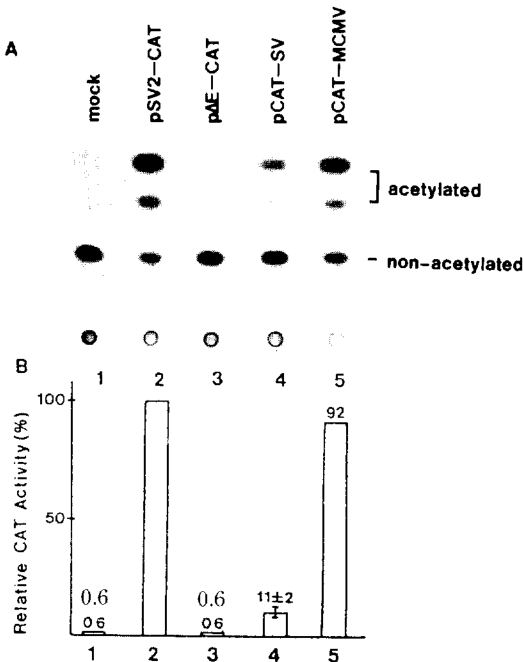

Fig. 2. *Effect of 3' enhancers on CAT gene expression.* Recombinant plasmid DNAs described in Fig. 1 were purified and introduced into HeLa cells via calcium phosphate precipitation. Panel A: The transient expression of CAT in HeLa cells were assayed and detected by autoradiography as described in Materials and Methods. Panel B: The level of expression was quantitated for each sample. The % conversion was calculated as described in Materials and Methods. The sample which rendered more than 50% conversion were appropriately diluted and re-assayed to ensure linearity of the assay. The relative CAT activities were determined by taking the % conversion by pSV2-CAT as 100%. Each sample was normalized for variances in transfection efficiency and cell lysis by assaying amounts of equal β-galactosidase activities rendered from the co-transfected plasmid, pCH110.

transfection efficiency, as well as in cell lysis. When cell extracts, each containing 10 units of β-galactosidase, was assayed for CAT activity, we obtained results in Fig. 2 (panel A) from the autoradiogram of thin layer chromatograph (TLC) plates. The enhancerless plasmid gave actually no detectable CAT activity, similar to the background level with no plasmid. Both SV40 and MCMV enhancers increased the CAT expression significantly. When CV1PD cells were transfected with the same plasmid DNAs, we obtained similar results (data not shown). In order to quantify the amount of CAT in each sample, we excised parts from TLC plates, corresponding to acetylated and non-acetylated chloramphenicol, and counted the radioactivity in liquid scintillation counter. The % conversion was then calculated by dividing cpms from acetylated form by cpms from the total chloramphenicol. The results obtained through this type of quantitation were represented in panel B, Fig. 2. The data was an average from two separate experiments. We estimated that SV40 and MCMV increased the CAT expression by more than 18 - and 150 - fold, respectively. This indicates that MCMV is about 7 times stronger than SV40 in its effect on activation of transcription from SV40 promoter.

The transcription from bGH regulatory region / Effect of viral enhancers

In order to study the bGH-specific gene expression, we replaced the SV40 promoter in the above plasmids used with 2.2 kbp long bGH regulatory region containing TATA and transcriptional initiation site. Schematic diagram for constucting these plasmids were shown in Fig. 3. Ramabhadran *et al.* (1985) had shown previously that bGH gene containing 2.2 kbp long regulatory region was not expressed in monkey cells, reflecting its *in vivo* tissue specificity. We tried to see whether the strong viral enhancers could override this silence in non-expressing cells. When HeLa cells were transfected with plasmid DNAs containing 2.2 kbp bGH regulatory region (pbGH (L)-series; Fig. 3), we observed nearly no activity for CAT. (Fig. 4). This suggested that even 150 fold activation in transcription by MCMV enhancer was not sufficient to express bGH gene in HeLa cells. Reports by Larsen *et al.* (1986) suggested that there were cis-acting regulatory element upstream of -230 nucleotides from the major cap site in rat growth hormone gene, which represses gene expression in non-expressing cells. In an effort to test the existence of such negatively regulating site in bGH gene, we replaced the long bGH regulatory region with 230 bp fragment, and constructed another set of recombinant plasmids (pbGH (S)-series). (See Fig. 3). HeLa cells were then transfected with these plasmid DNAs

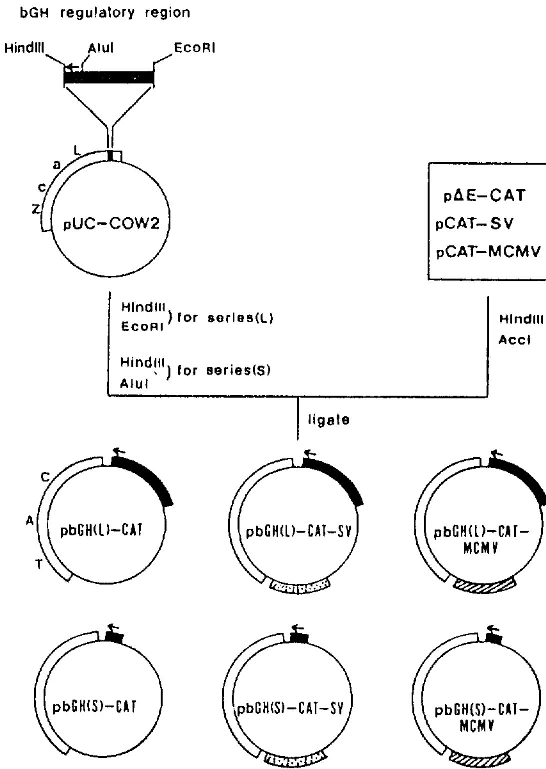

Fig. 3. *Construction of recombinant plasmids containing bGH regulatory region and 3' enhancers.*
The long (HindIII-EcoRI fragment) and short (HindIII-AluI fragment) bGH regulatory region were cloned in front of CAT gene, thereby replacing SV40 promoter, in plasmids pΔE-CAT, pCAT-SV, and pCAT-MCMV cut with HindIII and AccI. The direction of bGH promoter was marked with an arrow.

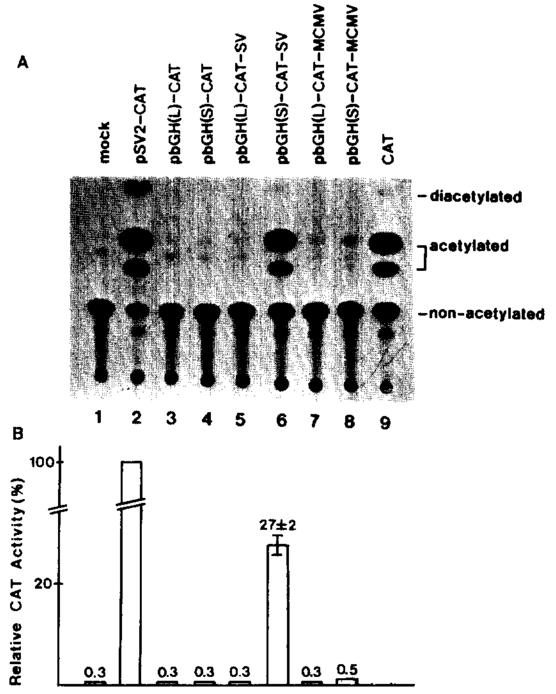

Fig. 4. *Expression of bGH-specific CAT gene in the presence and absence of enhancers.*
Plasmid DNAs described in Fig. 3 were purified and introduced into HeLa cells. Panel A : Cell extracts were assayed for CAT activities on TLC plates. The activity in lane 9 is from commercial enzyme. Panel B: The CAT activities were quantitated as described in Fig. 2 and Materials and Methods.

and assayed for CAT activities. The results shown in Fig. 4 demonstrated that shorter bGH regulatory fragment, alone, was not enough to induce detectable CAT expression. However, when SV40 enhancer was present, the level increased by more than 90-fold, indicating that there is indeed a negatively regulating DNA elements upstream of -230 nucleotide residue in bGH gene. In contrast, MCMV enhancer, which is stronger than SV40 in activating SV40 promoter-driven transcription, was only marginally active on bGH promoter driven expression.

DISCUSSION

The activity of transcriptional enhancers is rather insensitive to their position and orientation relative to the structural gene, even though this insensitivity is not absolute. The activity of SV40 enhacer was reduced almost 10-fold when it was placed to the 3' end of the structural gene from just upstream of promoter (Compare pCAT-SV with pSV2-CAT in Fig. 2). However, this reduced level was still significantly greater than that of enhancerless expression by about 20-fold. The MCMV enhancer, which contains several 18 bp and 19 bp repeats (Dörsch-Häsler *et al.*, 1985) on 1.3kbp long SmaI-XhoI fragment, was more active than SV40

enhancer by about 7-fold. These strong enhancers were not sufficient, however, in activating bGH specific CAT expression in HeLa cells to a detectable level. The removal of the bulk of bGH regulatory region dramatically increased the CAT expression in the presence of SV40 enhancer, suggesting that there are negatively regulating DNA elements upstream of bGH gene. However, the inactivity of MCMV enhancer on the shorter bGH promoter suggests that there are specific interactions between the bGH promoter and the viral enhancers. Depending on the nature of the promoter and the enhancer involved, some interaction might activate transcription whereas some might not. On the other hand, there are preliminary reports that MCMV enhancer fragment used in this study contained yet another negatively regulating element (Yu, S., personal communications). The existence of negative elements in MCMV enhancer, therfore, could have nullified the effect of the removal of similar elements in bGH gene. In order to figure out more clearly about the interaction between the distantly located promoter and enhancer, we need to define precisely the putative negative DNA elements on bGH gene as well as various enhancer elements.

적 요

조직특이적 및 일반적 유전자 발현에 미치는 SV 40 와 murine cytomegalovirus (MCMV) enhancer 의 영향을 조사하였다. 이를 위하여 chloramphenicol acetyl transferase (CAT) 유전자의 아래쪽에 이들 enhancer 들을 삽입한 재조합 플라스미드들을 제조하였다. 원숭이세포 (CV1PD)와 HeLa 세포에 이들 플라스미드들을 이입시킨 후, CAT 유전자가 발현되는 정도를 조사하였다. Enhancer 가 없는 플라스미드에 비해 SV 40 와 MCMV enhancer 는 CAT 의 발현을 각각 20 배와 150 배로 증가시켰다. CAT 유전자의 앞에 있는 SV 40 프로모터를 2.2 kbp 의 소성장호르몬 (bGH) 유전자의 조절부위로 치환한 경우는 enhancer 가 있어도 전혀 CAT 의 발현이 검출되지 않았다. 조절부위를 230 bp 로 짧게 하여 치환한 경우는, SV 40 enhancer 가 있을 때, CAT 의 발현이 매우 증가하였다. 이와는 대조적으로 더 강한 MCMV enhancer 는 bGH 특이적인 발현을 별로 증가시키지 못하였다.

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