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ORIGINAL ARTICLE

The Action of Cytomegalovirus (CMV) Promoter on Expression of Genetically Engineered Insulin in Rat Hepatocytes

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ABSTRACT

Background: Background: Insulin is hormone production in the pancreas that stimulates glucose uptake from blood to enter the body's cells, where it is converted into energy needed by muscles and tissues to function. The pancreatic cells are responsible for the secretion of insulin. In diabetes, the body cannot produce enough insulin or cannot use insulin effectively.

Objectives: In this study, we attempted to activate the hepatic cells to secrete insulin instead of the pancreatic cells.

Methodology: This was done by gene therapy; an excellent strategy to treat diabetes by supplying the correct wild type copy of a furin cleavable sites preproinsulin. The preproinsulin was extracted from the rat DNA, cloned and mutated to generate the two furin cleavable sites responsible for the removal of C-peptide to form the two chains (A and B) for mature insulin production. This mutated insulin was derived by Cytomegalovirus (CMV) promoter to express insulin by its transfection inside the primary rat hepatocytes using a non-viral vehicle to keep the hepatic cells healthier against the transfection. In vitro, the rat hepatocytes could not divide well as in vivo, but special hormones like insulin and dexamethasone lived longer and kept their function.

Results: The CMV promoter is strong and lead to overexpression of mature insulin inside rat hepatocytes leading to deterioration, the toxicity of hepatocytes and finally cell death. Hepatocytes in vitro were more fragile and needed some modification to adapt to the secretion of insulin.

Conclusion: Glucokinase or glucose transporter promoters were much more perfect than CMV. They can activate the hepatocytes to modulate the glucose level and so limiting the amount of insulin secreted. These promoters are weaker than CMV but much more perfect for hepatocytes.

Keywords	*Address of Correspondence	Article info.
Cloning, CMV promoter, Gene therapy, Hepatocytes, Mutation, Non-viral Vector, Preproinsulin, Transfection.	*gehadelsayedkg@gmail.com*	*Received: September 15, 2020* *Accepted: August 16, 2020*

Cite this article: *Mahmoud GE. The Action of Cytomegalovirus (CMV) Promoter on Expression of Genetically Engineered Insulin in Rat Hepatocytes. RADS J Biol Res Appl Sci. 2020; 11(1):1-8.*

Funding Source: *Nil*
Conflict of Interest: *Nil*

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INTRODUCTION

The intra-cellular conversion for all known mammalian proinsulin to insulin involves cleavage at two paired basic sites present at either end of the C-peptide[1]. In both rat and human proinsulins, these are Arg31-Arg32 at the B-chain/C-peptide junction and Lys64-Arg65 at the C-peptide/A-chain junction. The cleavage is carried out by the endopeptidases PC2 and PC3/1[2-4].

Most cell engineering approaches to target the non-neuroendocrine cells that lack the specific endopeptidases (PC2 and PC3/1) required to process proinsulin into active mature insulin. To overcome this

problem, many researchers used site-directed mutations to engineer proinsulin to be a substrate for furin[5-7]. The enzyme Paired Basic Amino Acid cleaving enzyme also known as (PACE) is a Golgi-associated propeptide endoprotease that is present in the constitutive secretory pathway of virtually all cells[8].

The most chosen target cells for insulin gene therapy are hepatocytes. Although hepatocytes do not have the machinery to store insulin within secretory vesicles and secrete it in a regulated fashion, hepatocytes are attractive targets for insulin expression because they are closely related to the pancreatic β cells developmentally, play a very important role in glucose homeostasis, and are relatively easy to target[9-11].

MATERIALS AND METHODS

Amplification of the Preproinsulin Gene from the Rat Spleen

We extracted the rat genomic DNA from the rat spleen by genomic extraction kit. Amplification of the preproinsulin gene by PCR was performed using the designed primers:

F: 5'CATGGCCCTGTGGATGCGCTTCCTGCCCCTG3'

R: 5'GAGTTGCAGTAGTTCTCCAGTTGGTAGAGGA3'

The PCR cycle step was done as: initial denaturation (94˚C, 3min, and 1cycle), denaturation (94˚C, 30sec), annealing (55˚C, 30sec), extension (72˚C, 1min, 35 cycles), final extension (72˚C, 5min, 1cycle). The amplified gene was desalted and purified, then cloned into a cloning NEB® PCR cloning vector (Cat. No. E1202). Further, the gene was mutated by substitution using site directed mutagenesis (Cat. No. E0554S) to substitute lysine instead of glutamic acid in A chain, arginine instead of valine in the connected C peptide, and arginine instead of glutamine in the B chain.

Translated protein

MALWMRFLPLLALLVLWEPKPAQAFVKQHLCGPHLVEA LYLVCGERGFFYTPKSR**R**EVEDPQVPQLELGGGPEAG DLQTLALEVARQ**K**RGIVDQCCTSICSLYQLENYCN**Stop**

C-peptide

Wild type Insulin R
EVEDPQVPQLELGGGPEAGDLQTLALEVARQ K

Mutant type R
KREDPQVPQLELGGGPEAGDLQTLALEVARR K

C- peptide with Tetrabasic furin cleavage site

According to the NEBase Changer, the primers are **at the B-chain/C-peptide:**

F:5'CAAGTCCCGTCGTAAACGGGAGGACCCGCAAG3'
R: 5'GGTGTGTAGAAGAAACCACGTTCCC3'

At the C-peptide/A-chain:
F:5'GAGGTTGCCCGGCGGAAGCGTGGCATTG3'

R:5'CAGTGCCAAGGTCTGAAG A TCCC3'

The vector used for mutation now contains the preproinsulin with the new three amino acids. After mutation, transformation into bacteria and isolation of the pure vector has proceeded. Then digested the vector by one restriction enzyme (NruI), amplify the gene of interest by:

F:TTGGATCCACCATGGCCCTGTGGATGCGCTTCCTG CCCCTG,

R:GTGAATTCGTTGCAGTAGTTCTCCAGTTGGTAGAG GGA

Insertion of Preproinsulin Gene into the Expressing Plasmid

The expressing plasmid (#13031) with CMV promoter was received from the Addgene, isolated, purified, and digested by the two restriction enzymes (ECORI & BamHI), then purified and desalted by using purification kit (extraction kit Cat. No. 20021), then by the same restriction enzymes we digested the amplified preproinsulin gene, the ligation step was later proceeded for the digested plasmid and digested gene.

Digested expressing vector (0.3µg/ul)	1.5µl
Digested purified preproinsulin gene (1µg/ul)	3µl
Ligase buffer	2µl
Distilled H_2O	13µl
(Mix well)	
Ligase	1µl

Culture of Hepatocytes

The culture of hepatocytes was performed according to the protocol by Ling *et al.,* with some modifications[12]. Rat hepatic cells were purchased from cell biologics, shipped in suspension (Cat. No. RA-6224F), centrifuged, then

suspended in 30ml warm William's complete medium (Add the following to Williams' Medium E: L-glutamine 2mM, fetal calf serum (FCS) to 5%, dexamethasone to 100nM, cadmium chloride to 9µg/l, Dimethyl sulfoxide (DMSO) to 0.5%, penicillin to 100IU/ml and streptomycin to 100mg/ml), and then centrifuged and resuspended again in 20ml warm William's complete medium. Cells were counted within the cell suspension using a hemocytometer and cell viability was determined by trypan blue staining.

Cells were plated in collagen coated plates, cultured at 37°C in 95% air and 5% CO_2. After 4hrs of culture, the cells were replaced with another medium that is serum free to maintain the morphology of the cells. After 24hrs of culture images, every 2hrs on the first day of culture and every 2 days was captured by Olympus inverted microscope. After optimization the cell culture condition, transfection takes place using 1.5µl TurboFect transfection reagent (Cat. No. R0531) in 1ml culture medium after 24hrs of culture.

Examination of the Released Green Fluorescent Protein

The expressed green fluorescent protein was detected under the inverted green fluorescent microscope.

Measurement of the Amount of Insulin Released

The insulin release was measured according to the concentration of glucose added by adding different concentrations of glucose from 5 to 25mM glucose to the media of the transfected cells. We measured the amount of insulin released according to the concentration of glucose added by the Cohesion rat insulin ELISA kit (Cat. No. CEK1622).

Sequencing

The designing plasmid containing the genetically engineered insulin was forward and reverse sequenced using the ABI prism Big Dye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Germany).

RESULTS

To get the rat preproinsulin gene, we extracted the genomic DNA from the rat spleen. Then we amplified it by PCR amplification technique using the specific primer designed for the rat preproinsulin gene.

The amplified preproinsulin gene was inserted into the cloning vector, transformed to get high yield and isolated by midiprep kit. The mutation proceeded in the two chains A & B. Then gene of interest was amplified by digesting the vector and inserted into the expressing plasmid. So, we obtained a vector containing the preproinsulin gene with sites of cleavage derived by the CMV promoter. This vector was measured for its concentration to detect its purity (Table **1**) and sequenced for both forward and reverse sequence to be sure from the location of mutated preproinsulin gene (Fig. **1 & 2**).

Table 1. Measurement of the Concentration and Purity of the Extracted Plasmid using UV Spectrophotometer. (The dilution factor was X300).

S. No.	Plasmid	A260	A280	Concentration (µg/ml)	Ratio
01	Designing CMV expressing plasmid	0.270	0.140	4050	1.92

Fig 1. The forward sequence for designing CMV expressing plasmid showing the area between CMV promoter and the Enhanced green fluorescent protein (EGFP).

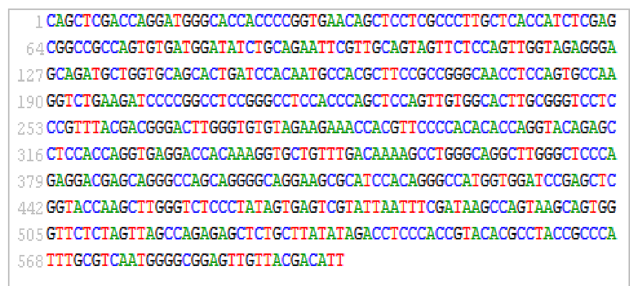

Fig 2. The reverse sequence for designing CMV expressing plasmid showing the area between EGFP and CMV promoter.

The expressing plasmid was now ready to transfect into the primary rat hepatic cells. Primary rat hepatocytes were washed, diluted, counted (Table **2**), then cultured in 6 well coated collagen plates with complete Williams' E medium in a humified atmosphere incubator for 2hrs and after that demonstrate the morphology of the cells (Fig. **3**).

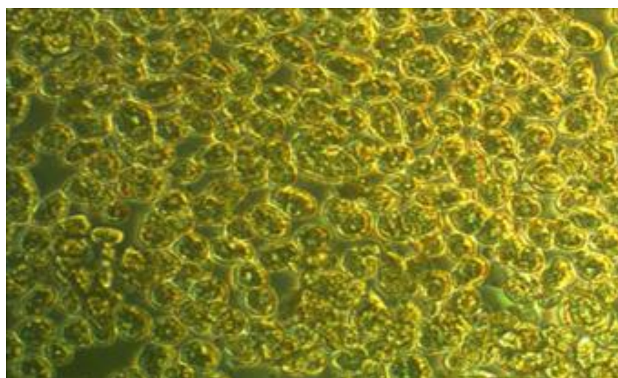

Fig 3. The rat hepatocytes immediately after plating on 6 well collagen coated plate with Williams' E complete medium under an inverted microscope. Shows the cells X200.

Table 2. The Count of Live and Dead Cells on Haemocytometer to Detect the Viability and Cell Density of the Rat Hepatic Cells.

S. No.	No. of squares	Live cells	Dead cells	Total count
01	1	71	8	79
02	2	52	0	52
03	3	85	4	89
04	4	68	6	74
05	Average	69	5	74

Viable cells = 69 X 10^4 x 2 = 138 x 10^4 cells/ml.

Total count = 74 X10^4 x 2 = 148 x10^4 cells/ml.

Viability % = viable/total =138/148 = 93%

Cell density = Total count

The rat hepatic cells were investigated after plating under the inverted microscope every 48hrs to detect the cell viability, division, and morphology (Fig. **4**). The cells show high viability already after plating, the viable cells count decreases day after day to reach about 32% at day 19 after plating.

After optimization of the cell culture condition, transfection takes place using 1.5µl TurboFect transfection reagent in 1ml culture medium after 24hrs of culture, in a 12 well collagen coated culture plate. Then transgenic expression was analyzed 24-48hrs later. We will transfect the cells with the prepared expressing plasmid then examine the cell morphology and viability after transfection (Fig. **5**).

The rat hepatocytes after transfection with the construct containing CMV promoter showed a high change in the morphology and viability, hepatocytes cannot stand more than 5 or 6 days, the viability decreases fast and day after day to reach about 5% on day 5 from transfection. The morphology high changed; the cells appeared very tired. The CMV promoter is strong which resulted overexpression for the gene controlled by it, the rat hepatocytes are fragile and cannot divide well *in vitro* to stand with this over expression.

Under the inverted fluorescent microscope, we examined the fluorescent cells which released the fluorescent fused insulin-EGFP (Fig. **6**). The media around the hepatocytes showed no fluorescent even under addition different concentrations of glucose, but the cells show high fluorescent from the first day but, this fluorescence decreases every day till reach day 6 in which almost no fluorescence found.

The over expression of the fused protein trapped inside the cells lead to the failure of hepatocytes to stand.

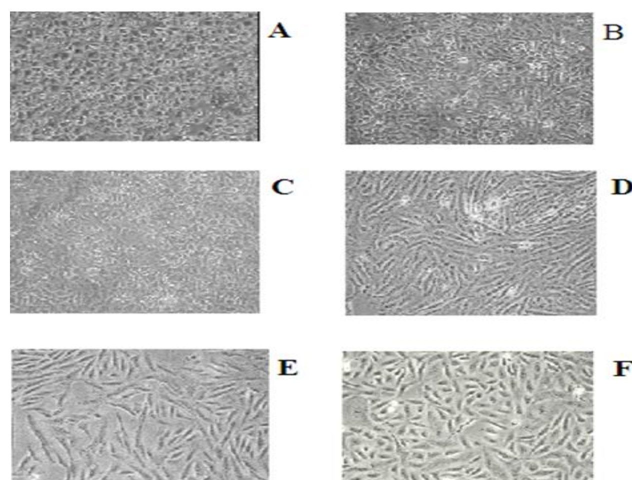

Fig 4. X100 rat hepatic cells after plating. (A) After 24hrs of plating. (B) After 5 days of plating. (C) After 7 days of plating. (D) After 11 days of plating. (E) After 17 days of plating. (F) After 19 days of plating.

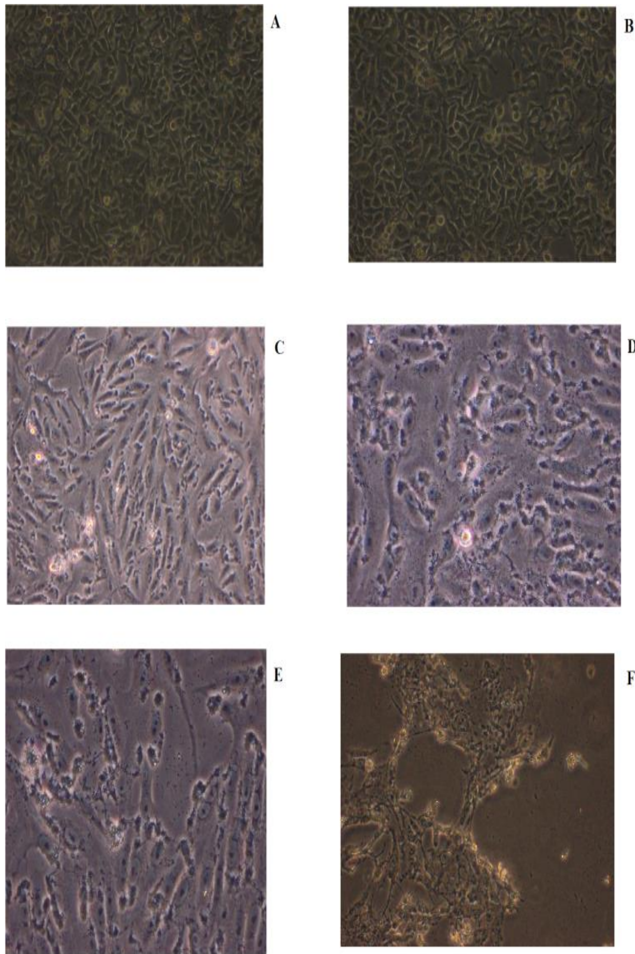

Fig 5. The rat hepatic cells after the transfection with the designing plasmid with CMV promoter X100. (A) The rat hepatocytes after 7hrs from transfection. (B) The rat hepatocytes after 24hrs from transfection. (C) The rat hepatocytes after 2 days from transfection. (D) The rat hepatocytes after 3 days from transfection. (E) The rat hepatocytes after 4 days from transfection. (F) The rat hepatocytes after 5 days from transfection.

CMV promoter has not the ability to make balance out and in the cells, has not the ability to transport glucose from media to cells and vice versa. So, the insulin still trapped inside.

Glucose was measured in cell lysates and cell media of transfected culture cells 3 times by colorimetric glucose assay (Table **3**), after the addition of different concentrations of glucose (0-25mM) to the media and obtained the average.

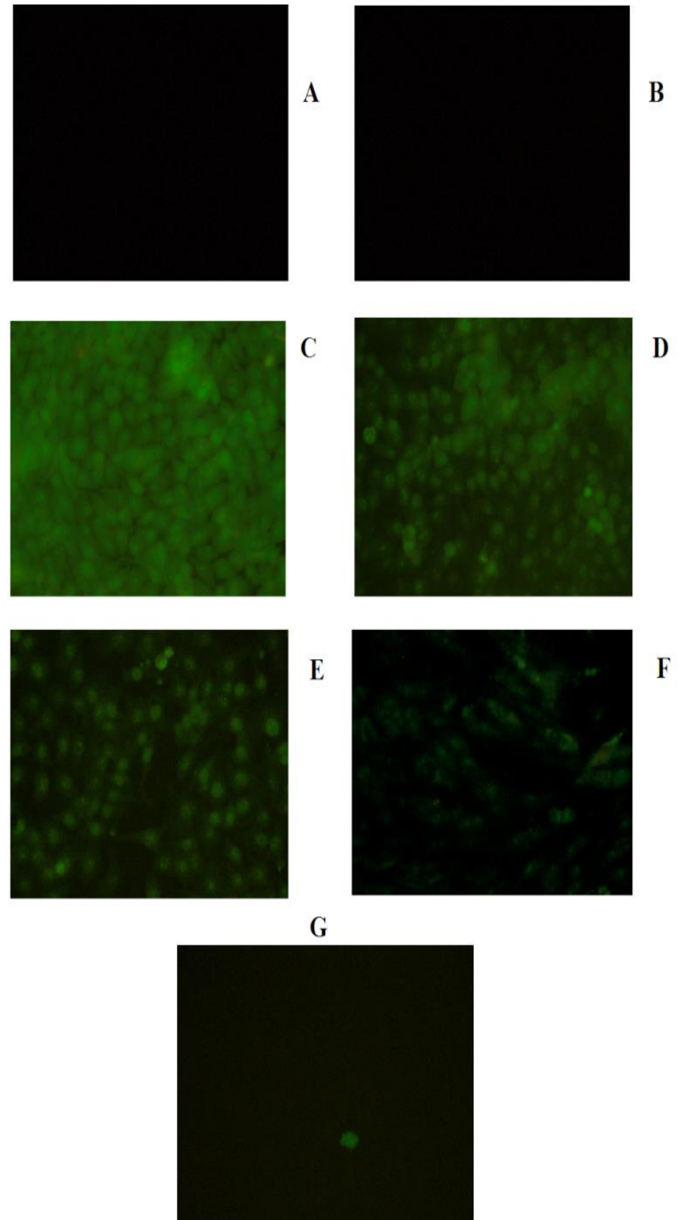

Fig 6. The transfected rat hepatocytes with the designing plasmid with CMV promoter under the green fluorescent microscope X200. (A) The media after stimulation with 25mM glucose. (B) The media of free glucose. (C) The transfected hepatocytes after 24hrs from transfection. (D) The transfected hepatocytes after 2 days from transfection. (E) The transfected hepatocytes after 3 days from transfection. (F) The transfected hepatocytes after 5 days from transfection. (G) The transfected hepatocytes after 6 days from transfection.

Table 3. The Colorimetric Method for the Detection of the Amount of Glucose.

S. No.	Parameters		Calorimetric glucose assay in mg/dl after 24hrs from the addition of different doses of glucose in the transfected primary hepatocyte culture medium				
01	Different doses of glucose added in mM		Free glucose	5mM	10mM	15mM	25mM
02	The designing plasmid with CMV promoter	In media	ND	81	210	240	301
		In Cell lysate	ND	ND	ND	ND	ND

Note: Present in both cell media & lysates after the addition of different amount concentration of glucose to the media of transfected cells with (the designing plasmid with cmv promoter) no detected amount of glucose can be found inside the cells, as the cmv promoter has not the ability to transport glucose in and out the cells.

Due to the amount of mature insulin released from the designing plasmid with CMV promoter, we measured it 3 times by cohesion rat insulin kit Cat. No. CEK1622). and get the standard curve for rat insulin (Fig. **7**). From this standard curve, we can obtain the different amounts of insulin released from the construct in both cell lysate and media in pg/ml from its relative O.D. And so make a relation between the concentration of glucose in mM and the concentration of insulin in pg/ml.

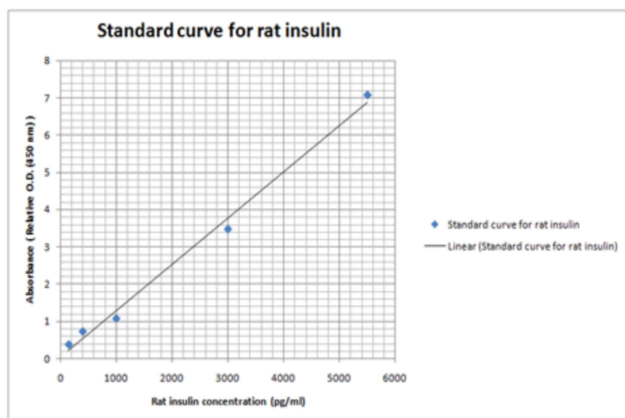

Fig 7. Standard curve for rat insulin.

For the designing of a plasmid with CMV promoter, this construct releases overdose for insulin only inside the cells not outside the medium, as this promoter has not the ability to transport the glucose in and out the cell and the insulin remain trapped inside the cells and not out to the media. So, the relation between the glucose and insulin inside the cell be constant and overexpression of insulin found in case of low dose or high dose glucose, and outside the cells (in media), no insulin found to make a relation **(**Fig. **8**).

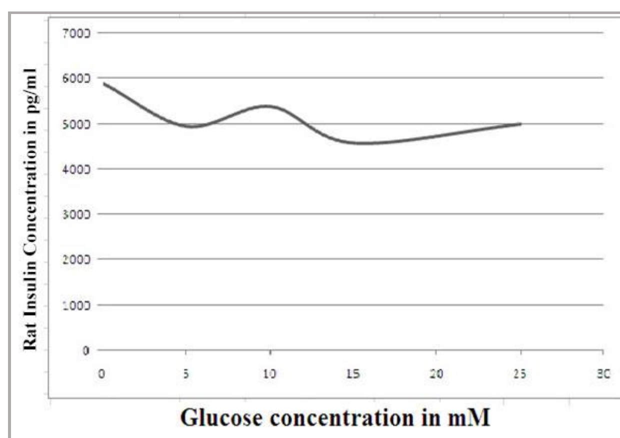

Fig. 8. The relation between the concentrations of insulin released corresponding to different doses of glucose in cell lysate using the designing plasmid with CMV promoter. No response to glucose appeared even in high expression of insulin.

DISCUSSION

Many alternative approaches to treat Type 1 Diabetes mellitus (T1DM) using the correct type of gene or genetically engineered gene, without using pancreas transplantation, have been attempted. Examples of such attempts include insulin production from various native cells, such as liver cells[13-15]. In our study, we engineered insulin to be correctly secreted from the liver cells.

The mammalian CMV promoter is strong, constitutive transgene expression and would drive consistent, high-level expression of insulin even during using low concentration of glucose. Thus, a weaker promoter must be used to maintain low levels of insulin production to gain a successful insulin gene therapy in treating T1DM[16].

CONCLUSION

Our current study aimed to express insulin *in vitro* in rat hepatocytes, a strategy to try to adapt hepatic cells to secrete insulin instead of pancreatic cells, using a construct containing the furin cleavable sites preproinsulin derived by a CMV promoter. The mature insulin secreted successfully but the usage of this promoter was a bad choice, as the CMV promoter lead the insulin trapped inside the cells and no passage for the glucose in and out the media. The rat hepatocytes *in vitro* are fragile and not divide to stand with this insulin overexpression. The usage of the weaker promoter will work better like glucose transporter 2 promoter (GLuT2) or glucokinase promoter which can transport glucose in and out the cells.

ACKNOWLEDGEMENTS

I am gratefully acknowledged Mr Karim Morsi, Eng. Mohammed Abdalla, Dr. Necolas and The Research and Medical Technology Institute, Smouha, Alexandria, Egypt and the research lab of Faculty of Science, Alexandria, Egypt.

LIST OF ABBREVIATIONS

CMV	Cytomegalovirus
DMSO	Dimethyl sulfoxide
FCS	Fetal Calf Serum
GLuT2	Glucose Transporter 2
PACE	Paired basic Amino acid Cleaving Enzyme

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