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DEVELOPMENT OF ANIMAL CELL EXPRESSION SYSTEM WHICH CONFERS POSITION-INDEPENDENT AND ENHANCED FOREIGN GENE EXPRESSION

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In order to develop the novel gene expression system, we introduced new control elements which could influence the foreign gene expression in animal cells. When the foreign genes are introduced into the genome of higher eukaryotic cells, the expressions from these integrated genes are often low and can vary greatly depending on the positions of the integration sites due to the complex nature of the chromatin structures (1). First we screened the various DNA sequence elements which can function as an insulator of gene expression from these position effects and can cooperate with the SV40 enhancer/promoter. Among the several DNA elements from the various sources, we identified the particular DNA element which confers the increased frequency of the positive colonies, assayed by the reporter gene from stable selections indicating significantly reduced position effects. This element also showed the several fold-increased expression level as well as the copy-number dependent expression with host cell specificity. Second we modified the transcription termination element where we introduced the specific terminator in combination with SV40 polyA signal. This modified terminator showed the increased efficiency and the level of the gene expression. By combining these two elements, we made the animal cell expression system and tested successfully for the recombinant protein productions of TGF β-soluble receptor, Antithrombin III, and single chain Pro-Urokinase.
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Introduction

For certain type of recombinant proteins, the use of animal cells such as CHO (Chinese Hamster Ovary) and BHK (Baby Hamster Kidney) cells are unavoidable due to the proper foldings and the post-translational modifications, mainly glycosylations. For the production of the recombinant proteins, the main difficulties lies in the laborious selection processes due to the poor frequency of the desired cell clones and the low stability of the foreign genes during the cell cultivation. Even though the use of transient gene expression system is possible for the production of recombinant proteins, the generation of stably integrated and even gene amplified clones are the conventional methods. When the foreign gene is introduced into the chromosome, the expression of transgene is unpredictable and varies with the chromosomal site of integration (2). This phenomenon is, due to the position effect of neighboring chromosome contexts, depending on the transcriptional status of these sites (3). The eukaryotic chromosomes are organized into high order structure defining each domain of gene regulation and separating each unit as examplified by euchromatin vs. heterochromatin. These domains are thought to be constrained by the nuclear framework called matrix in interphase which serve as binding site for DNA element called nuclear matrix attachment region (MAR) (4). MARs are typically 70% A+T and contain sequences similar to topoisomerase II consensus sequences (5,6). The functions of MARs in vivo are largely unknown, but it has been demonstrated that the use of MAR element in conjugation with eukaryotic promoters provide the foreign gene with the enhanced- and position -independent expression in several systems (7,8).
In order to improve the efficiencies of achieving the positive recombinant clones as well as to

increase the level of the gene, we screened various DNA elements potential for serving as the positive MAR elements for the conventional promoter (SV40 promoter). We found that the DNA element from human β–globin region serves as positive element improving the efficiency and the relative expression level. We also tested the specific terminator sequence from human Gastrin gene whether these elements can improve the stability of the foreign gene compared with the conventional SV40 poly A signal sequences. From the site-directed mutagenesis, the new terminator (mutant gastrin terminator in combination with SV40 poly A signal) showed the increased expression level in reporter genes. After we introduced these two new elements into the animal cell expression system, we tested this vector system for the production of recombinant proteins such as TGF β–soluble receptor, Antithrombin III, and single chain pro-Urokinase gene as a reporter. The overall efficiencies were highly improved and the amount of recombinant protein were increased which is comparable to the previous reports.

Materials and methods

Culture and Tansfection of CHO DG44 Cells.
CHO DG 44 cells (DHFR -/-) were cultured in Minimum Essential Medium alpha Medium (Gibco BRL, 11900-024), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, BRL). CHO DG 44 cells were co-transfected with control pSV-β -galactosidase vector, or vectors containing DNA elements, each in combination with plasmid pSV2neo containing the neomycin resistance gene, using DOSPER (Boehringer Mannheim). Stable transfectant clones were selected by neomycin resistance in Minimum Essential Medium alpha Medium containing 10% heat-inactivated fetal bovine serum and G418 (500ug/ml) for 14 days. The gene amplification were performed with the gradual increase of MTX in nucleoside free MEM medium supplemented with dialyzed fetal bovine serum.

Isolation of Nuclei and Preparation of nuclear matrix.
A typical experiment started with $2x10^8$ CHO cells, cultured in DMEM containing 10% FBS. The cultured cells were washed once in PBS, trypsinized, collected by centrifugation. The detached cells were washed with PBS and 20ml of Cell Wash Buffer, subsequently. After centrifuged, the cells were suspended in 10ml CWB, passed through 21 gauge needle eight times, and 40ml CWBD (cell wash buffer with 0.1% Digitonin) was added to the cells. After suspended, the cells in CWBD were overlaid onto 25ml CWBD with glycerol cushion and centrifuged at 1200 rpm for 5 min. The supernatant was decanted and the pellet (nuclei) was washed in 10 ml of CWBD. It was suspended with 10 ml of Nuclear Solublizing Buffer and incubated at 37℃ for 20 min, and then 190 ml of LIS solution was added, rocked at RT for 10 min. It was centrifuged at 2700 xg for 20 min at RT. The nuclei were washed with Matrix Wash Buffer with Digitonin twice. They were suspended in 1.4 ml of buffer containing 100 mg/ml DNase I, rocked for 90 min at RT and centrifuged at 800 xg for 10 min. They were suspended in 700 ml RSBS and 700 ml of SETx2 was added to them. They were incubated at 0℃ for 4 min, centrifuged at 1500 xg for 10 min. The matrix was washed in 1.4 ml of SETB, 1.4ml of RSBSB, subsequently. They were suspended in 500 ml RSBSB and 500 ml of glycerol, stored at -20℃.

In vitro binding assay.
For binding assay, $6x10^6$ matrix were washed in buffer two times and suspended. Seven ml of 10 mg/ml competitor (sonicated E.coli genomic DNA) were added to them and they were rocked at 37℃ for 45 min. After centrifuged, they were suspended in 118 ml of DB and 1 ml of 200 mg/ml competitor and 2 ml of labeled DNA. After rocked at 37℃ for 15 hours, they were seperated into supernatant (matrix-unbound) and pellet (matrix-bound) fractions. After the pellet was washed with DB, treated with 90 ml of proteinase K buffer, 10 ml of 20 mg/ml

proteinase K and then incubated at 60 ℃ for 2 hours. The matrix-bound DNA fragments. which released from matrix, were obtained in supernatant by centrifugation. Both fractions were counted using the scintillation counter, the same counting DNA fractions were seperated on agarose gel, autoradiographed.

β–galactosidase assay.

Cells to be assayed for β -gal activity were harvested from exponentially growing cultures by trypsinization, washed with PBS and counted cell number using hemocytometer. Cell lysates were generated by lysis buffer treatment [0.1% NP40 in 0.25M Tris·HCl (p H8.0) and cellular debris was removed by centrifugation for 10min at 4℃. Protein determination was estimated using the BCA reagent with bovine serum albumin as a standard. The lysates was assayed for enzyme activity as described previously. For in situ assays with the X-gal, cells were fixed by incubating in PBS containing 2% formaldehyde, and 0.2% glutaraldehyde for 10 min at 4℃. The cells were rinsed wiht PBS and incubated at 37℃ in PBS containing 0.1M X-gal, 0.01M potassium ferrocyanide, 0.01M potassium ferricyanide, and 1mM MgCl2 for overnight. Measurements of the percent of blue stained cells and photographs were done under phase contrast optical microscopy. The method to measure total β -galactosidase activity was as described previously.

Results and Discussion

Enhancement effects of DNA elements on the foreign gene expression

To determine whether the various DNA elements could improve the foreign gene expression. we introduced the various DNA elements in the upstream of the complete SV40 promoter. The resulting activities are measured with the reporter gene (bacterial β–galactosidase). From the transient transfection assays, we found that DNA elements used have no effect on the function of SV40 promoter indicating these elements have no additional enhancer like activities. In contrast, the stably transfectant, selected with the additional neomycin resistance marker which were co-transfected with reporter gene demonstrated the different levels of the expression. Among those tested, the DNA sequence from the human β–globin upstream region enhanced the expression about seven fold. The frequency of positive colonies was analyzed by measuring the number of β–galactosidase expressing clones with G418 resistance by staining method. The same DNA element which showed the increased expression level also demonstrated that the dramatically enhanced frequency of positive colonies as 75% in comparison to the normal control vector as 30%.

Characterization of the sequence element from the human β–globin upstream region

From the analysis of DNA sequences, the human β–globin upstream region could be divided into three regions of Alu repeat, MAR consensus sequences and the rest of region. To elucidate the mechanisms underlying the above activity, the various deletion mutant were generated and tested for the effect on the gene expression as well as the position effect. All the deletion mutant demonstrated the greatly lessened activity, when compared with wild type. The DNA construct containing Alu element and MAR consensus region, which showed the highest activity of deletion mutants, still exhibited only about 60% of wild type activity. The deletion construct containing only Alu element showed rather a dramatic decrease in the β–galactosidase activity and the frequency of positive colony. These results suggest that the MAR consensus sequence itself is not enough to function as wild type and that other factors are involved in complex manners for proper gene expressions.

The effects on the stability of foreign gene expression

In order to meet the industrial purposes, the level for the recombinant protein productions should be high at least in the level of 10ug/10^6 cell/day. In general, the recombinant gene has to be amplified using the high amount of toxic selection drug such as methotrexate. We postulate that our vector system might provide the stability of foreign genes based on the stable interaction with a nuclear matrices. Therefore, we selected the initial positive colonies using the above element and challenged the prolonged culture in the presence and absence of the selection drug. After 40 days of culture, frequency of positive cells in two group was measured by β–gal staining and β–gal activity. The results showed that in case of control vector-clones, when selection force was removed, frequency β–gal expressing cells in individual positive clones was dramatically reduced, but in β–globin MAR vector-clones, frequency of β–galactosidase expressing cells in individual positive clones was steadily maintained indicating that this element enhanced stability of transgene. These features might be very useful for the production of recombinant protein for human uses.

The introduction of the specific terminator

In animal cell, the termination site of the transcription is not clearly defined, rather it is assumed that the transcription terminate in random locations which might contribute to the potential instability of mRNA. In order to improve the stability of mRNA, we introduced the specific terminator derived from human gastrin gene. With various combination and mutagenesis, we found that mutant gastrin terminator combining the SV40 polyA element gave the highest expression level for the foreign genes upto 150 %.

The expression of recombinant proteins using the combined system

Using this combined expression system, we applied the recombinant gene which requires the glycosylation for their proper functions. We cloned gene for TGF β soluble receptor (potentially useful for TGF β antagonist), Antithrombin (for thrombosis application), and single chain pro-Urokinase(for thrombosis). The clones using CHO DG44 (DHFR -/-) cell line, we were able to achieve the protein expression levels in the range of 10ug/10^6 cell/day. In addition, their biological activities are identical to the original proteins. It is also interesting that the CHO cell line containing the above expression system shows the improved tolerance to the selection drug, MTX compared to the control cell lines. The average level for the protein expression was greatly improved as well. Therefore we were able to modify the tedious conventional multi-step amplification and screening procedures into two step simple procedure which could be beneficial to the general users.

References

1. Bode, J., Schlake, T., Rios-Ramirez, M., Mielke, C., Stengert, M., Kay, V., and Klehr-Wirth, D. (1995) *Int Rev Cytol*, 389-454
2. Kalos, M., and Fournier, R. E. (1995) *Mol Cell Biol* **15**(1), 198-207
3. McKnight, R. A., Spencer, M., Wall, R. J., and Hennighausen, L. (1996) *Mol Reprod Dev* **44**(2), 179-84
4. Namciu, S. J., Blochlinger, K. B., and Fournier, R. E. (1998) *Mol Cell Biol* **18**(4), 2382-91
5. Phi-Van, L., von Kries, J. P., Ostertag, W., and Stratling, W. H. (1990) *Mol Cell Biol* **10**(5), 2302-7
6. Phi-Van, L., and Stratling, W. H. (1996) *Biochemistry* **35**(33), 10735-42
7. van der Geest, A. H., and Hall, T. C. (1997) *Plant Mol Biol* **33**(3), 553-7
8. Yu, J., Bock, J. H., Slightom, J. L., and Villeponteau, B. (1994) *Gene* **139**(2), 139-45