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Review Article

Recombinant Protein Expression Optimizing: A Review of S/MAR, STAR, and UCOE, as a Chromatin-Modifying Element

Fateme Hasheminejad and Amir Amiri-Yekta*

Department of Genetics, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

Abstract

The rapid growth of the global biopharmaceutical market in recent years indicates its importance in the biotechnology industry. The production of these medicinal products is one of the fastest-growing industries in the patient treatment field. Since 1980, processes have been developed to optimize the production of recombinant protein products upstream and downstream. In this regard, one promising approach is the engineering of expression vectors based on combinations of DNA regulatory elements found in euchromatin regions. This approach aims to achieve proper gene integration and facilitate its expression in the target cell, as epigenetic mechanisms can lead to instability of the desired gene in long-term cell cultures and gene silencing. To address this issue, genetically engineered vectors have been produced, which include components such as Matrix Attachment Regions (MARs), Scaffold Attachment Regions (SARs), Stabilizing Anti-Repressor Elements (STAREs), and Ubiquitous Chromatin Opening Elements (UCOE). These components can modify the chromatin environment to minimize gene silencing and enhance higher, more stable, and biologically active expression of recombinant molecules. In this review, we focus on different approaches and developments in the technology of expression vector engineering and their impact on increasing the production of recombinant proteins.

Keywords: recombinant protein, biopharmaceuticals, chromatin-modifying elements, UCOE, S/MAR, STAR

Corresponding Author: Amir Amiri-Yekta; email: amir.amiriyekta@royaninstitute.org

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1. Introduction

Behind the production of human recombinant insulin as the first biopharmaceutical in 1982, the landscape of pharmaceutical production underwent a significant transformation, opening a new gateway for the emergence of millions of new treatments for patients worldwide [1,2]. Biopharmaceuticals bring in multimillion-dollar sales and are projected to grow from $149 billion in 2012 to over $389 billion in 2024, with an annual growth rate of 8.59% [3]. The success of biopharmaceutical products is attributed to their targeted, specific, and disease-modifying nature. Although the production and development of these molecules are more complex and challenging compared to small chemical molecules and drugs, biologics offer more satisfactory performance, fewer non-specific side effects, and a higher probability of treatment success. In 2019, approximately 50% of approved biological products were monoclonal antibodies and vaccines [4]. The higher therapeutic potential of these drugs has led to significant acceptance of biopharmaceuticals. Additionally, increased strategic collaborations between biopharmaceutical companies and global acceptance of biologics contribute to market growth. The mammalian cell lines are the dominant platforms for producing recombinant protein drugs. These cell lines function similarly to human natural proteins due to their ability to perform post-translational modifications, such as glycosylation and disulfide bonding [5, 6]. However, it should be noted that compared to microbial cells, the productivity of mammalian cell culture is much lower, and their cultivation is laborious, costly, and complex [7, 8]. The biologic manufacturing industry is experiencing a growing trend and creates opportunities for those who recognize the gradual nature of this industry. The major challenge in the biopharmaceutical industry is the variable expression of recombinant proteins by mammalian cell lines. Due to the rising demand for recombinant proteins, there is a growing interest in mammalian cell culture, particularly Chinese Hamster Ovary (CHO) cells. This surge in interest is accompanied by a drive to enhance cell lines to achieve higher yields and improved product quality. There are several methods of gene integration into the genome of host cells for producing recombinant proteins, site-specific integration, homologous recombination, CRISPR-Cas9-Mediated Integration, and random integration are some commonly used techniques [9, 10]. These methods provide different strategies for integrating genes into the genome of host cells to produce recombinant proteins. The choice of method depends on factors such as the specific goals of the study, the host cell type, and the desired level of control and stability in gene expression. Site-specific integration speeds up the cell line improvement by focusing on the integration of transgenes to predetermined sites in the host cell genome and can support long-term and steady expression [11]. Homologous recombination involves the precise replacement or insertion of a gene into a specific genomic locus by exploiting the cell's natural DNA repair mechanisms. This technique requires the design of a DNA construct that contains regions of homology to the target genomic locus. The construct is then introduced into the host cell, where it undergoes recombination with the target locus, leading to the integration of the desired gene (12). The CRISPR-Cas9 system, a powerful gene editing tool, can also be utilized for gene integration. The Cas9 protein, guided by a specifically designed guide RNA (gRNA), introduces double-strand breaks at the target genomic locus. The desired gene, along with homology arms, is then introduced into the cell, and the cell's DNA repair machinery incorporates

the gene into the break site through homology-directed repair. Heretofore, noteworthy endeavors have been made around the designing of CHO cells utilizing hereditary building strategies such as the CRISPR-Cas framework (13). Traditionally, cell line development procedures relied on random integration and gene amplification. Random integration is a relatively straightforward method compared to other gene integration techniques. It does not require the identification of specific target sites or the use of complex genetic engineering tools and this method often results in a high efficiency of gene transfer and integration into the host cell genome. This means that a significant proportion of the introduced genes successfully integrate and are expressed in the host cells (14). Despite these advantages, it is important to note that random integration also has some limitations. These include the potential for variable expression levels, genetic instability, and heterogeneity in the resulting cell population. Additionally, the random integration of genes may lead to insertions in undesirable genomic locations, such as heterochromatin regions, which can impact gene expression and stability (9, 15, 16). The purpose of this comprehensive review is to elucidate the efficacy and application of chromatin-modifying elements in mitigating gene silencing, thereby facilitating the production of high-quality recombinant proteins and biosimilars, thus contributing to the advancement of biopharmaceutical manufacturing.

2. Random Insertion Sites: Chromatin-Modifying Elements as Problem Solvers

Random genome insertion is a simple and widely used method for introducing a desired gene fragment into the host cell genome. In this method, gene-carrying vectors are randomly inserted into the host genome. These vectors can be circular or linear. This method has proven to be highly efficient, especially in the therapeutic proteins and monoclonal antibody production in (CHO) cell lines. However, the integration of genes at random sites, particularly in heterochromatic regions that are not controllable, poses drawbacks such as instability, variable expression levels, and heterogeneity of the resulting cell clones [11, 15]. The site of cassette integration into the chromosome is one of the critical factors affecting the transcription rate of the recombinant gene, stability, and protein production levels. Random insertion of the gene cassette into heterochromatin regions leads to reduced productivity and increased risk of gene silencing compared to insertion into euchromatin. Various approaches have been proposed to overcome the limitations of random insertion in heterochromatin regions and the risk of gene silencing (9, 17). One of the most important approaches to overcome this issue is the use of chromatin-modifying elements such as Scaffold/Matrix Attachment Regions (S/MAR), Stabilizing Anti-Repressor Elements (STAR), and Ubiquitous Chromatin-Opening Elements (UCOE) in the cassette structure. These elements can impact heterochromatin regions, activate chromatin, prepare it for transcription, and ultimately lead to the expression of the desired gene, even in centromeric regions (Figure **1**) (18, 19).

Chromatin-modifying elements can prevent the influence of surrounding chromatin on the inserted gene. Therefore, they increase the expression level and stability of the recombinant protein. Currently, several chromatin-modifying elements are used in mammalian cell systems, including Scaffold/Matrix Attachment Regions (S/MAR) and Ubiquitous Chromatin Opening Elements (UCOEs) (19). S/MARs can prevent the spread of heterochromatin into euchromatin regions with chromosomal

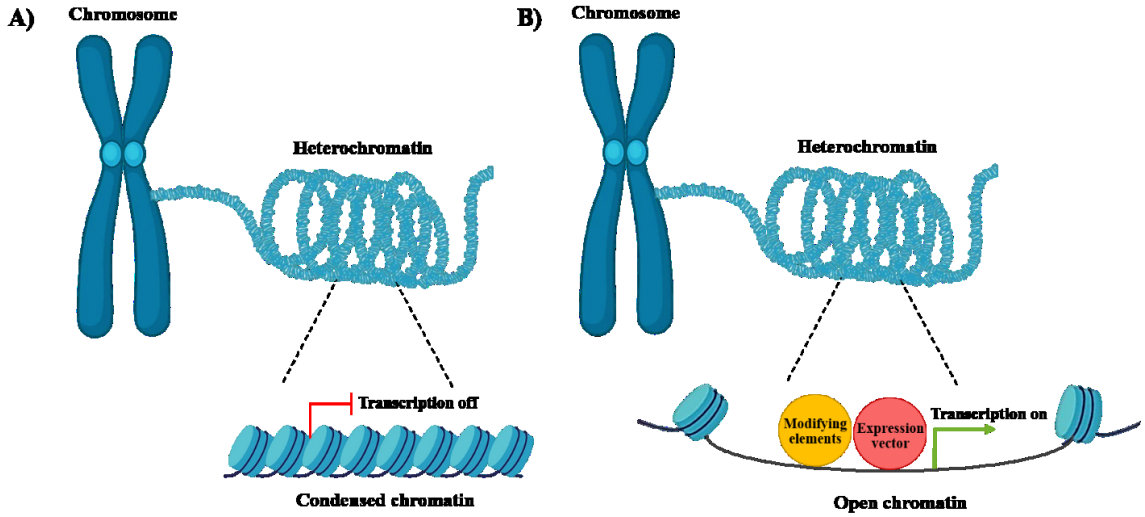

Figure 1: Random insertion of the expression vector containing the target gene. A; Random integration of an expression vector without chromatin-modifying elements into the host cell genome is affected by the adjacent heterochromatin region and the transfected gene expression is turned off. B; The attendance of chromatin-modifying elements in the expression vectors, remodel the condense region of heterochromatin into euchromatin and leads to better stability and higher expression of the transfected gene.

domain boundaries. UCOEs can actively maintain chromatin in a transcriptionally permissive state. These elements can be placed in various positions within the expression vector, such as around the cassette, upstream of the promoter, or downstream of the polyadenylation signal. Alternatively, they can be placed downstream of the target gene. In general, these elements can be located wherever they play a functional or regulatory role in the expression of the recombinant protein (Figure **2**) (19).

2.1. Matrix attachment regions and Scaffold attachment regions

Matrix attachment regions (MARs) and Scaffold attachment regions (SARs) have similar functions and are among the most widely used chromatin opening elements. These elements anchor chromatin loops to the nuclear matrix or core scaffold. Over 65% of the sequences of these elements are composed of AT base pairs and range from 300 base pairs to several thousand base pairs in length [20, 21]. These regions can serve as initiation points for DNA replication and regulate gene transcription. Utilizing these elements in an expression vector is a simple method to enhance the expression of recombinant proteins. Additionally, some studies have shown a correlation between protein expression and the copy number of S/MAR elements [6, 22]. In a study, the presence of these elements resulted in 2.6–3.9-fold higher protein expression compared to the control cell line lacking them [23]. Furthermore, two copies of S/MAR can exceptionally increase the number of clones with high expression. However, three copies of S/MAR did not show higher expression or better stability compared to using two copies [24].

2.2. Stabilizing anti-repressor element

The STAR element was initially discovered through screening random and diverse genomic fragments of the human genome, ranging from 500 to 1,200 base pairs. STAR can counteract with the chromatin (25). Ten types of STAR elements can have similar anti-suppressive activity against

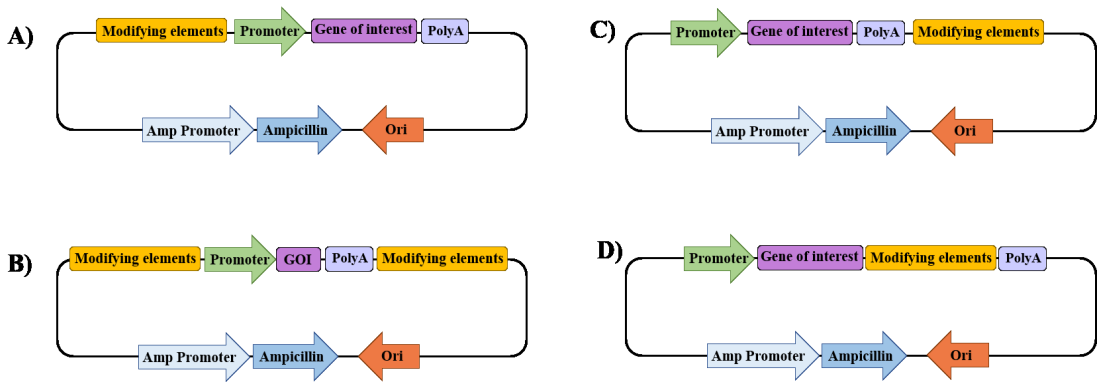

Figure 2: Possible locations of chromatin modifying elements in expression vector. A; The chromatin-modifying elements can be located upstream of the promoter. B; The chromatin-modifying elements can be located on both sides of the expression vector. C; The chromatin-modifying elements can be located downstream of the poly A signal. D; The chromatin- modifying elements can be located downstream gene of interest and upstream of the poly A signal.

chromatin in human and CHO cells. These elements can increase the number of positive clones and the expression of recombinant proteins [18, 25]. The STAR7 and STAR40 sequences also have similar anti-suppressive power, particularly in CHO cells, and have shown increased expression of reporter proteins dependent on gene copy number. However, another study showed the limited effect of the STAR40 element on producing a recombinant antibody in CHO cells [17, 19]. STAR elements enhance cell viability and gene transfer in suspension and serum-free growth conditions (17, 26). Although the STAR elements initially appeared very potent and promising in early reports, they have not yet been translated into large-scale industrial applications.

3. Ubiquitous Chromatin Opening Elements

Ubiquitous Chromatin Opening Elements (UCOE) is another commonly used chromosomal element consisting of CpG islands without methylation that extends at the transcription start site [27, 28]. UCOEs provide a high level of gene expression in CHO and BHK21 cells and maintain protein expression stability for over 100 generations [27, 29–31]. In a study, the UCOE effects on the recombinant protein expression stability and productivity were evaluated in CHO DG44 cells. They observed that cell lines containing UCOE exhibited superior growth characteristics, higher transcriptional activity in each gene copy, and less clonal diversity compared to non-UCOE cell lines [31–33]. When this upstream element is located in the promoter region, it can significantly protect the promoter from epigenetic silencing, effectively increasing the level and stability of recombinant protein expression. In 2002, a type of UCOE called A2UCOE was cloned into an expression vector and transfected into CHO-S cells. The presence of this element led to the production of recombinant antibodies in large quantities, up to 200 milligrams per liter (32). The UCOE elements are widely used for the production of recombinant monoclonal antibodies [33–36]. In another study, the presence of A2UCOE in the expression vector not only increased the number of stably transfected clones in CHO-S cell lines but also enhanced antibody production levels up to 180–230 milligrams per liter [29].

4. Discussion

Over the past 20 years, we have witnessed remarkable progress in recombinant cell line

development and biotechnological production technologies. Preventing gene silencing is a crucial functional concern for many mammalians cell-based biotechnology applications. For example, in industrial-adapted cell lines such as (CHO) or human embryonic kidney cells, the biopharmaceutical yields in long-term are reduced by gene silencing [37, 38]. Therefore, looking for suitable solutions to prevent the silencing of the target gene in the host cell genome is critical. In this review, we tried to describe the DNA modifying elements and their ability to enhance the stable insertion and higher expression of a gene in cell lines that produce biologics. All these elements show enhancing roles in the expression of the target gene, which is highly beneficial for the production of recombinant proteins in mammalian cells (19). In a study, the expression of monoclonal antibodies in CHO cell pools was examined in the presence of MAR, SAR, STAR, and UCOE elements. The enhancing effect of the UCOE element was highly evident. Its presence resulted in a six-fold increase in monoclonal antibody expression compared to the other three elements [39]. There is evidence that UCOEs provide an optimal epigenetic environment devoid of DNA methylation. Integrating UCOEs into vectors could be beneficial and generate a more homogeneous population of expressing cells and a higher number of high-expression clones. Thus, UCOE-based gene expression systems offer an attractive strategy for reducing the time and costs associated with upstream bioproduction stages. Generally, incorporating chromatin-modifying elements into expression vectors can increase the levels of recombinant protein production and stability in mammalian cell lines. It seems engineering expression vectors may play a crucial role in enhancing recombinant protein expression. The elements of the vector, particularly the promoters and even the physical distances between different segments have critical impacts. The chromatin-opening elements combination in an expression cassette can significantly improve gene expression, but several parameters need to be optimized to achieve the full benefits of this technology. It is anticipated that chromatin-modifying elements usage will continue, and further advancements will be made to increase stability and expression (24).

5. Conclusion

In addressing the challenge of variable recombinant protein expression in mammalian cell lines, cutting-edge engineering approaches have emerged, aiming to boost gene expression and stability. This review has highlighted the effectiveness of chromatin-modifying elements in combating gene silencing, thereby improving the production of top-tier recombinant proteins and biosimilars. Notably, elements like Scaffold/Matrix Attachment Regions (S/MAR), Stabilizing Anti-Repressor Elements (STAR), and Ubiquitous Chromatin-Opening Elements (UCOE) have shown immense promise in elevating gene expression levels and ensuring stability. By shielding inserted genes from the effects of neighboring chromatin, these elements enable enhanced productivity and uniformity in cell populations. The integration of these elements within expression vectors presents a compelling avenue for advancing biopharmaceutical manufacturing. Looking forward, the ongoing refinement and utilization of chromatin-modifying elements hold the potential to revolutionize biopharmaceutical production, guaranteeing the consistent delivery of safe, efficacious, and affordable therapies to patients worldwide.

Author contributions

Conceptualization, supervision: Amiri-Yekta; writing—draft preparation: Hasheminejad; writing—review and editing: Amiri-Yekta. All authors have read and agreed to the published version of the manuscript.

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