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THE EFFECT OF A PUTATIVE CHROMATIN-OPENING ELEMENT ON THE METHOTREXATE AMPLIFICATION OF CHO CELLS

A Major Qualifying Project Report

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ABSTRACT

Previous work at Abbott Bioresearch Center on a putative chromatin-opening sequence inserted into a plasmid dubbed “genomic vector”, suggested that the vector caused an increase in the rate of methotrexate-induced amplification in Chinese Hamster Ovary cells. This project supported the hypothesis that the genomic sequence may cause an increase in amplification effects; however, the increase was not statistically significant, nor was the increase in expression of antibody in CHO cells when compared to the parental vector.

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BACKGROUND

Introduction

Until recently, it was thought that the probability of a specific gene being transcribed was based largely on the sequence of its regulatory domains, either through promoters, terminators, and operators, or through the expression of regulatory proteins that then could act on the chromosome that encoded them. However, the study of epigenetics and a renewed focus on the nuclear matrix has revealed that the shape and positioning of a segment of DNA can affect its expression just as much as its DNA sequence (Frasier and Bickmore, 2007). This adds another level of depth to science's increasing, but limited, understanding of the genetic code. In this project, a putative genomic opening sequence was analyzed for its ability to increase in the rate of methotrexate-induced amplification of CHO cells, and increase the production of an antibody expressed by the cells.

Gene Expression and Nuclear Organization

Except during replication, DNA is wound around a tetramer of proteins called histones in structures named nucleosomes that in turn supercoil to form chromosomes (Marko and Siggia, 1994). During transcription, DNA is unwound to let the RNA polymerase bind to the template strand. However, DNA is not wound with the same tightness in every sequence or gene. For example, centromeric DNA, from the regions in the center of the chromosomes to which spindles attach during replication, is very tightly wound, diminishing its transcription (Pidoux and Allshire, 2005). This transcriptional

silencing effect, a process called variegation, has a tendency to spread across the genome, blocking the replication of any gene that does not have an innate mechanism to keep it open (Girod et al., 2005). This is one potential mechanism that can cause a transgene to express well when it is first introduced into a genome but become silenced over time.

However, sequences and regulatory proteins exist that modify the histones, causing them to loosen, allowing greater accessibility to the genes. Covalent modifications on the N-termini of histones include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, ADP-ribosylation, ubiquitination, and sumolation (Zhang, 2007). Modification of the histones causes the histones to either bind more tightly or to loosen, depending on the modification.

Regulation of transcription by changes in conformation of chromatin is just one facet of how the three-dimensional structure of DNA affects the expression of individual genes. Genes whose sequences reside far apart on the chromosome, or are on different chromosomes entirely, may actually lie within close geometrical distance to each other in the dynamic environment of the nucleus. Chromosomes observed in their natural nuclear environment have shown a dramatic change in expression of a particular gene at the same time the section of the chromosome the gene resides on relocates to a different position, which could be described as a shift from a favorable position for transcription to a non-favorable, vice-versa, or that the act of transcription itself causes the relocation of the DNA (Frasier and Bickmore, 2007).

One model based on the position of actively transcribed genes states that the nucleus is spotted with dynamic pockets of higher concentrations of RNA polymerase and other proteins involved with transcription. According to this model, all active genes

can be expressed, but genes that are geographically located near these "transcription factories" have a higher chance of expression. This model suggests that the chromatin-opening elements act not only to better enable protein interaction in a two-dimensional stretch of DNA, but also by allowing greater freedom of the section of the chromosome to approach one of the active pockets. The model also includes a method for gene silencing effects, using X-chromosome inactivation in females as an example (Figure-1). During this process, *Xist* proteins are produced which then bind to the inactive X chromosome, winding it tightly to itself and sequestering it away from the transcription factories (Frasier and Bickmore, 2007).

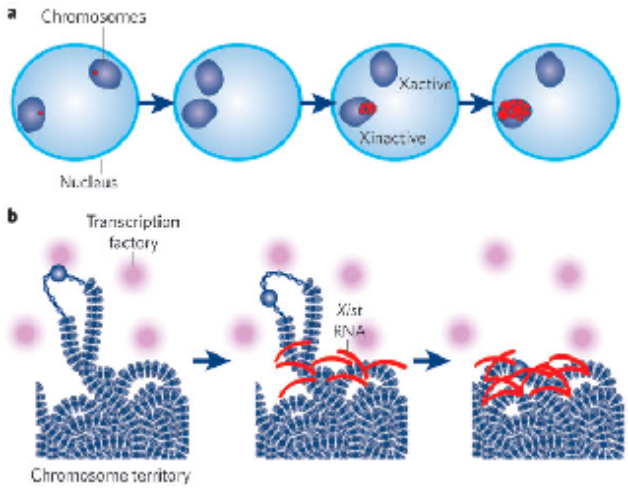

Figure 1: Inactivation of the X Chromosome by *Xist* RNA. Panel-A: X-inactivation factors upregulate *Xist RNA* (red), which binds to the inactive X chromosome. Panel-B: once Xist has bound the DNA, it can no longer undergo positional changes to bring it closer to the transcription factories (pink) (Frasier and Bickmore, 2007).

Chromosomes that cluster around the same transcription factory are expressed simultaneously (Figure-2). It is still unknown how much of this clustering phenomenon is due to the functional relationship of the proteins being simultaneously expressed, or whether the mRNAs from the same factory all undergo similar post-transcriptional

processing before exiting the nucleus. However, it is likely that there is some benefit to gene co-expression in this way (Frasier and Bickmore, 2007). This model also provides insight into the difficulty involved in maintaining the activity of an inserted gene, since many of the spatial mechanisms that regulate the gene in its cell of origin are changed or simply not present in its new environment.

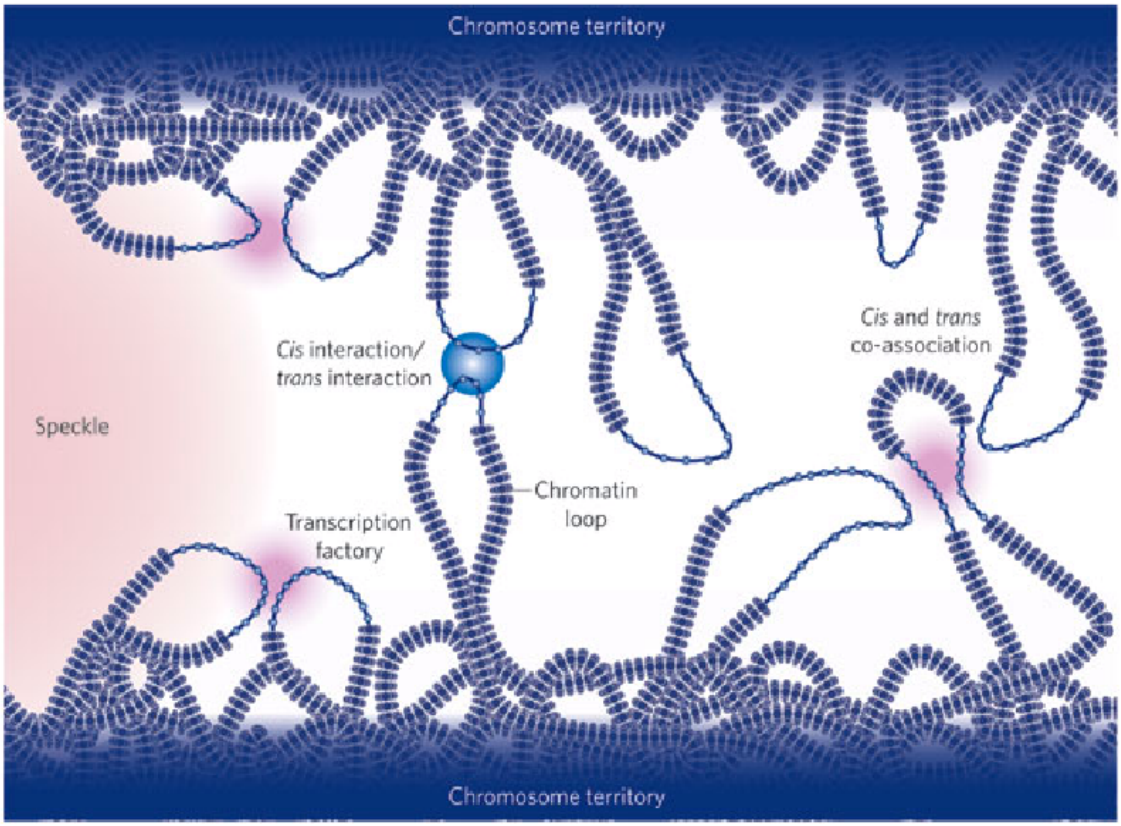

Figure 2: Co-localization Model of Gene Expression. Chromatin can loop outwards from the chromosome territory and interact with splicing-factor rich speckles (pale pink), polymerase II rich transcription factories (dark pink) or co-regulatory proteins (blue). Nonadjacent genes may simultaneously loop out to co-transcribe at the same transcription factory (Frasier and Bickmore 2007).

This model illustrates the importance of finding genomic sequences that can near-universally increase the expression of a transgene, regardless of the origin of either of the co-transfected sequences, and regardless of the recipient host cell. Chromatin-opening

elements have the potential to facilitate our ability to take advantage of positioning effects without analyzing the effect of each gene on the dynamic environment of the host cell nucleus.

Chromatin-Opening Elements

Multiple sequences have been identified that affect chromatin packing. A number of elements have been identified, although there is limited knowledge of the mechanisms by which they affect expression levels. The most common elements identified are Scaffold/Matrix Attachment Regions (SARs or MARs), STAR elements, Ubiquitous Chromatin Opening Elements (UCOEs), and Locus Control Regions (LCRs). Matrix attachment regions are defined as genomic sequences where chromatin binds nuclear matrix proteins during interphase, blocking the closing effects of variegation (Girod et al., 2005). STAR elements have been found to counteract transcriptional suppression effects when flanking a gene of interest, both by modifying the histones nearby and by facilitating the recruitment of regulator proteins to the gene (US Patent Application 10/190,312, 2002). UCOEs maintain the open state of the chromatin by modifying histone domains (like STARs), allowing increased gene expression even for genes located in centromeres, an area almost completely consisting of closed DNA (International Patent WO 00/05393, 2000). LCRs can increase or reduce gene expression levels by affecting the packing of the DNA in close proximity to the LCR, and some LCRs can both increase or reduce transcription of the gene it regulates (Feng et al., 2005). These chromatin-opening elements are differentiated from enhancers by the fact

that they usually do not have an effect in transient transfections. Enhancers are also *trans*-acting elements requiring the binding of regulatory proteins, whereas chromatin-opening elements usually act in *cis* (Schübeler et al., 1996).

The chicken lysozyme 5' MAR was discovered during a study to observe how chromatin structure affects expression (Girod et al., 2005). This sequence was used to enhance the expression of human immunoglobulin from Chinese hamster ovary (CHO) cell lines. Adding the MAR element in *cis* to the immunoglobulin gene caused a significant increase in IgG production, even more than adding the element in *trans* on a separate plasmid. It also decreased the number of low-producing clones in the transfection (Girod et al., 2005). This study supported the idea that MAR elements and other chromatin opening elements work in close spatial proximity to the transgene, and provided evidence that an element could have an effect on any transgene, even if isolated from a different species than the opening element.

Another study involving CHO lines (Kim et al., 2004) used the human β-globin sequence, including the human beta-globin MAR to measure expression levels, compared to the DHFR intron SAR element from CHO cells, the human HPRT MAR, the human CSP-B SAR, and the chicken alpha-globin MAR. Of the four tested, the human β-globin MAR was the most effective, increasing the frequency of positive colonies as well as the level of gene expression. After further investigation, it was shown that the MAR-positive cells had a higher transgene copy number and an increased level of beta-globin expression per copy of the gene. The study also showed that traditional transfection techniques often result in the MAR or SAR element integrating in a position where it could not affect the transgene. Moreover, some MARs and SARs integrated downstream

of the promoter and were transcribed as a part of the gene sequence, while others integrated upstream of the promoter and were not transcribed (Kim et al., 2004).

The human interferon-β S/MAR region was used in a study to determine the effects of distance on the enhancing abilities of the element (Schübeler et al., 1996). The S/MAR insert was placed at different locations relative to the promoter region of the reporter gene SEAP in a pM5SEPA retroviral vector. Increased expression levels correlated to the shorter distance ranges, about 4 kb (Schübeler et al., 1996). Several chromatin-opening elements would have to be tested for distance effects to confirm these findings, however, the data suggest that each element has a specific distance from the gene at which it will optimally enhance expression. It is unclear whether the element operates at a separate optimal spot, or if a wide variety of chromatin-opening elements operate at a similar distance from the gene(s) they affect.

Much of the literature on chromatin-opening elements has focused on their function in blocking variegation, however, some elements have been studied in the context of a more regulatory role. Among these are elements that have been shown to decrease as well as increase expression. The human β-globin control locus, a MAR element, has also been studied as the basis for an even more complicated model of *cis* regulation and chromatin modification, the locus control region (Feng et al., 2005). During early development, β-globin expression is very tightly controlled to ensure that the proper expression at the appropriate time. This involves the expression and silencing of two different β-globin-like genes in precise succession: ε-globin and γ-globin. These are then both silenced in favor of β-globin and δ-globin around the time of birth. The β-globin LCR has been shown to be responsible for the regulation of all five of these genes.

The study showed that the LCR causes transcription initiation in the highly repetitive flanking sequences around the gene, which interferes with the ability of the RNA polymerase to transcribe the gene itself. This silencing effect was correlated with late replication of the genes involved, which might indicate a specific stimulus signature of the progress of development, signaling the LCR to silence the early β-globin-like genes (Feng et al., 2005). It is unclear if the same sequences of the β-globin LCR are involved in both activation and silencing.

CHO cells are often used to develop therapeutic lines for the production of antibodies for treating diseases. The CHO strains pre-transfection are *dhfr*-negative, and the plasmid containing the genes for the produced antibody also contain *dhfr*. This allows methotrexate (MTX) to be used as a selection pressure. However, MTX also represses dhfr protein. In response, the CHO cells overexpress *dhfr*, and any constitutive genes. Increasing the MTX concentration will either kill the cells, or will cause them to grow faster and produce more antibodies. Cell lines that amplify faster in MTX can be characterized quicker, causing the process from laboratory to therapy to go much faster.

Previous Abbott Research on the "Genomic Vector"

The proprietary genomic sequences analyzed in this MQP were originally identified as part of a phage library of a particularly high expressing CHO clone at Abbott Bioresearch Center. Two genomic flanking "arms" were isolated and inserted into one of Abbott's proprietary plasmids, to determine whether the sequences could increase gene expression of a usually low-expressing antibody, and to determine whether the genomic sequences would work in multiple vectors. The vector was created by

separately cloning two genomic DNA arms that were then ligated together to form the genomic vector.

A previous WPI MQP in partnership with Abbott Bioresearch Center on this vector was performed in 2005, to determine whether the new "genomic" vector would confer higher reporter gene expression levels in CHO cells than control plasmid. The reporter gene for Enhanced Green Fluorescent Protein (EGFP) was cloned into both plasmids. The vectors were then transfected into CHO cells that were tested for GFP fluorescence using FACS analysis. The experiments showed a significant increase in the percentage of successful expressing clones, and a small increase in expression levels (Kedves, 2005). The "genomic vector" was then used in a series of experiments with CHO cell lines that express therapeutic antibodies.

PROJECT PURPOSE

Previous work at Abbott Bioresearch Center identified a genomic DNA sequence in 2000, which was subsequently analyzed (Kedves, 2005) for its effects on GFP reporter gene expression in CHO cell lines. During experiments to compare cell line antibody expression levels between this "genomic vector" and the parental vector, Abbott researchers observed a possible faster cell growth during the methotrexate amplification step in cell lines using the "genomic vector" than the control cell vector lacking the genomic element. This finding was extended in this MQP to determine whether the genomic sequence causes an increase in the rate of cell amplification as well as increased reporter (IgG) expression levels. Parallel transfections were performed with parental and "genomic" vectors. During each transfection step, the cell growth was recorded using first daily cell counting, then later by tracking split ratios. A vector that increases gene expression is useful for the potential development of therapeutic antibody-expressing cell lines. A cell line that amplifies faster in methotrexate can be developed, characterized, and optimized faster, decreasing the jump from concept to scale-up in an industry setting.

METHODS

Transfection of CHO Cells

One vial of *dhfr*-B3.2 CHO cell stock was thawed from -140˚C storage in PF CHO 2Y medium made in-house + 1% fetal bovine serum (FBS) (Invitrogen) +1x HT supplement (Gibco) to establish a spinner flask. In five days, the CHO culture reached a viable cell density of 1.96 x 10^6 cells/mL. 10 mL of α-minimum essential medium (αMEM) (Invitrogen) + 5% FBS + 1x HT was added to each of seven 100mm culture dishes. 510 µL of CHO culture (10^6 cells) was pipetted into each dish, which were then incubated at 37˚C overnight.

After two days, the medium was aspirated and replaced with 9 mL of F-12 medium (JRH Biosciences). Cultures were incubated at 37˚C for 2 hours. 100 µg of parental plasmid or genomic-plasmid DNA containing the *dhfr* selection gene was added to 200 µL of $CaCl_2$ and brought to a final volume of 2 mL with H_20. Each sample was added dropwise to 2 mL aliquots of 2x HEBES buffer (1.64 g NaCl, 1.19 g HEPES acid (Sigma Aldrich), 0.021 g Na_2HPO_4, pH to 7.05 with NaOH) (bubble HEBES with 1 mL pipette while adding with the pipetor) and incubated at room temperature for 10 minutes. 1 mL of genomic-plasmid DNA solution was layered onto three of the seven dishes, which were agitated to distribute the DNA precipitate, then incubated at 37˚C for 4 hours. 1 mL of parental DNA solution was layered onto four of the seven dishes, which were agitated to distribute the DNA precipitate and then incubated at 37˚C for 4 hours. Following the incubation, each dish was aspirated and overlaid with 2 mL 10% sterile dimethyl sulfoxide (DMSO Sigma-Aldrich) in F12 media for 1 minute. All of the dishes

were aspirated and rinsed once in 5 mL of Dulbecco's phosphate buffered saline (dPBS) and twice in 10 mL of DPBS. 10 mL of αMEM + 5% FBS + 1x HT supplement was added to each of the seven dishes, which were then incubated at 37˚C overnight.

Seeding and Screening of 96-well Plates

2000 mL αMEM + 5% FBS were filter sterilized (0.22 μm cellulose acetate filter). The three genomic dishes were aspirated, washed in 10 mL DPBS, and trypsinized for 5 minutes with 1 mL of trypsin. Cells were pooled into a final volume of 10 mL αMEM + 5% FBS. A 1.0 mL aliquot of this was removed and counted on the Cedex (cell-counting device from Innovatis). The viable cell density was 0.93×10^6 cells/mL. The four parental dishes were aspirated, washed in 10 mL PBS, and trypsinized for 5 minutes with 1 mL of trypsin. Cells were pooled into a final volume of 10 mL αMEM + 5% FBS. 1 mL of this was removed for counting on the Cedex. The viable cell density was 0.90×10^6 cells/mL. 1.86 mL of genomic culture was added each to two of the four 500 ml media aliquots. 1.80 mL of parental culture was added each to the other two containers. This brought the concentration of each container to 1000 cells/mL. 200 μL/well was added from the genomic containers to 96-well plates, 48 plates total. 200 μL/well was added from the parental containers to 96-well plates, 48 plates total. All the plates were incubated at 37˚C for six days.

The plates were aspirated and fed with 100 μL of αMEM + 5% FBS and put back into the incubator. Four days later, the culture dishes were aspirated and fed with 100 μL of αMEM + 5% FBS per well. During this process, 4 of the parental plates were accidently dropped and were thus discarded. A 96-well plate for every transfection plate

was filled with 200 µL of PF CHO 2Y medium per well in preparation for the ELISA screening. 48 hours after feeding, 5 µL of supernatant from each well was transferred from the transfection plates to the corresponding well in the screening plates to make a 1:40 dilution. The screening plates were taken to the in-house assay lab for ELISA. The 30 highest expressing colonies from each transfection were aspirated, trypsinized, and transferred to a well in a 12-well plate containing 2 mL of αMEM + 5% FBS. Each one was designated with a number.

Cell Density Monitoring

Three days after colony picking, the confluent clones were each fed1.5 mL of αMEM + 5% FBS. 24 hours after feeding, 500 µL of supernatant was removed for ELISA. Each of these clones were then aspirated, washed with 1 mL PBS, trypsinized with 100 µL of trypsin for 5 minutes, and resuspended in 400 µL of αMEM + 5% FBS. 50 µL of this was then pipetted into a new well containing 2 mL of αMEM + 5% FBS. Two days later, all wells were aspirated, trypsinized, and split into two new wells in a dilution to reach confluency after the weekend.

In order to measure cell density over a period of 4 days, each clone was seeded 0.1×10^6 cells/well into four wells of a 12-well plate, with a well for 24 hours after seeding, 48 hours, 72 hours, and 96 hours. For the clones that did not have enough cells to seed four wells at this density, as many wells as possible were seeded at that density, designated them the later time periods. So if only 3 wells could be seeded, they were designated 48 hours, 72 hours, and 96 hours. If only two wells, then 72 hours and 96 hours. For the clones that could only seed one well, only one was designated at 96 hours.

At each 24-hour period, the cell density of the corresponding well from each clone was measured on the Cedex. The 96-hour wells were also fed 1.5 mL of αMEM + 5% FBS at the 72-hour period, to be sampled for ELISA at the 96-hour period.

The fourth well from each clone was then trypsinized and resuspended into two wells of 1 mL of αMEM + 5% FBS + 20 nM MTX. Five clones did not survive in 0 nM MTX and could not be advanced into 20 nM MTX. One well from each was trypsinized and resuspended in 1 mL of freezing medium (αMEM + 50% FBS + 10% DMSO) and frozen in a vial at -80˚C. The other well from each was then used to seed four new wells (or fewer) at 0.1 x 10^6 cells/well, which were measured for cell density every 24 hours as before. The fourth well was again fed at the 72-hour time period, this time with 1.5 mL of αMEM + 5% FBS + 20 nM MTX, and sampled for ELISA at the 96-hour time period. The fourth well of each clone was trypsinized and split with to reach confluency after 48 hours. At this time, all of the wells were cloudy with contamination and measurements could not continue.

Thawing and Cell Passage Recording

All of the clones except for the five that did not survive in 0 nM MTX were thawed from the frozen stocks. Each freeze vial was quickly thawed in the 37˚C waterbath, pipetted into a centrifuge tube containing 10 ml medium, and spun down. The supernatant of each was pipetted off and the pellet was resuspended in 2 mL of αMEM + 5% FBS. Each sample was pipetted into a well in a 12-well plate and incubated at 37˚C.

The clones were allowed to grow independently, while their growth times were recorded. As each well came to 100% confluence, it was fed with 1.5 mL of medium of

the MTX concentration it was in at the time. Twenty-four hours later, 500 μL of supernatant was pipetted into an eppendorf tube for later ELISA. The well was then split by a dilution of 1:10 into the next passage. On the last day of the week, all of the wells were split so that they would be confluent at the beginning of the next week.

The clones spent one passage in 0 nM MTX, four passages in 20 nM MTX, and four passages in 100 nM MTX. Six of the clones grew fast enough to be split into two passages in 500 nM MTX. Each time a clone was split from one MTX concentration to the next, the remaining cells in the well after the split were resuspended in 1 mL of freezing medium and frozen at -80˚C. Every cell that came to 100% confluence in the fourth passage of 100 nM MTX were sampled for IgG levels by ELISA like before, and then trypsinized and frozen in 1 mL of freezing medium.

RESULTS

The purpose of this project was to determine if the "genomic vector" previously identified by Abbott Bioresearch Center, containing a putative chromatin opening element flanking an inserted IgG gene, causes an increase in the rate of methotrexate cell amplification as well as an increase in antibody expression levels. This was accomplished by two methods. The first approach involved daily cell counting to determine the viable cell density of each clone over several passages. The second approach involved observing the well confluency over a larger number of passages of amplification. This data was then compared to levels of a proprietary IgG secreted into the conditioned medium as quantitated by ELISA.

After the initial parallel transfections, 14 plates from each transfection were counted for colonies. The genomic transfection (i.e. the transfection performed with the "genomic vector") averaged 44.1 colonies/plate, with an estimated total colony count of 2117. The parental (i.e. the transfection performed with the parental plasmid containing the IgG gene but not containing the genomic element) average was 15.8 colonies/plate and the estimated total was 758, as is typical for transfections with this parental plasmid. Thus, the initial genomic transfection produced an approximate 2-3 fold greater number of colonies than the initial parental transfection.

The clones from each transfection were seeded into 4 wells in a 12-well plate with 0 nM MTX medium (Figure-3). Each well was measured for growth by cell density at 24, 48, 72, and 96 hours, respectively. MTX was used as a selection pressure to identify cells containing the *dhfr* gene during transfections and cell cloning. Clones were seeded at the same initial viable cell density of 0.1×10^6 cells/well. The viable cell density after

96 hours was used as a comparison for the growth rates of each clone. The average viable cell density of the genomic transfection was 0.116 x 10^6 cells/mL versus 0.090 x 106 for the parental transfection (Figure-3). A two-tailed t-test with a 95% confidence interval was performed, resulting in a p-value of 0.122, indicating no significant difference. Although the average growth rates of the clones in the genomic and parental groups were not statistically different, the observed number of clones in the genomic group with a growth rate ≥ 0.1 x 10^6 cells/mL was 2 fold more than the number of clones observed in the parental group.

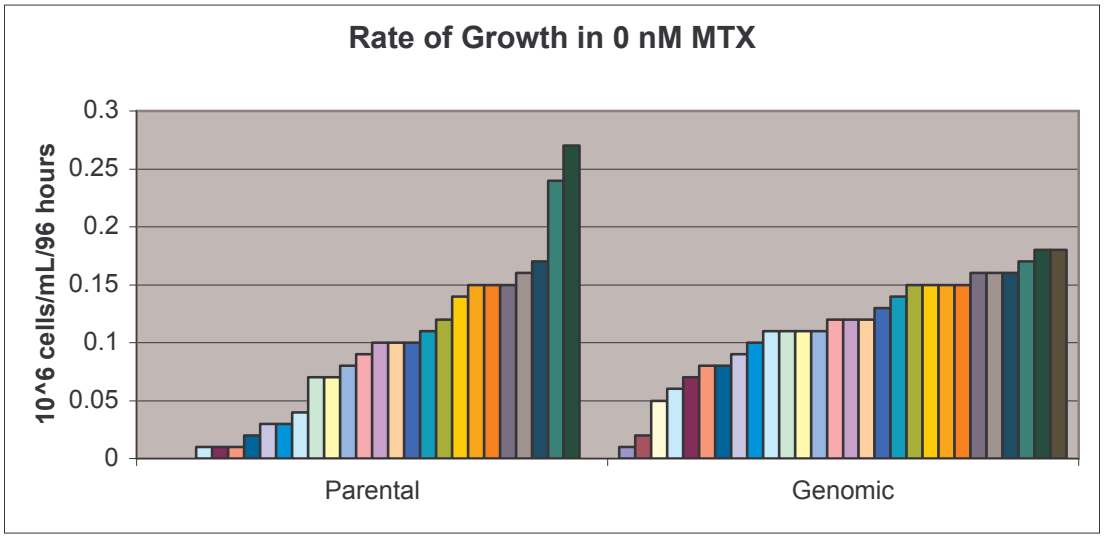

Figure 3: Viable Cell Density of Each Surviving Clone After 4 Days Growth in 0 nM MTX. The average viable cell density of the parental transfection was 0.090 x 10^6 cells/mL. The average viable cell density of the genomic transfection was 0.116 x 10^6 cells/mL.

The 96-hr well of the 0 nM MTX experiment was split and used to seed another 4 or fewer wells at 0.1 x 10^6 cells/well, in 20 nM MTX similar to the 0 nM MTX time point assessments. The average viable cell density of the genomic transfection was 0.059 x 10^6 cells/mL, and the parental transfection was statistically equivalent at 0.044 x 10^6 (p value = 0.257; Figure-4). The average growth rate in 20 nM MTX decreased approximately 2

fold for each transfected group, in comparison to the average values observed with each 0 nM MTX, respectively. This may be due to adaptation of the cells to the selection pressure of the MTX. After the first passage of the clones in 20 nM MTX, there was a contamination event, so new frozen vials of each clone were thawed and grown in 0 nM MTX medium until confluence, approximately one week.

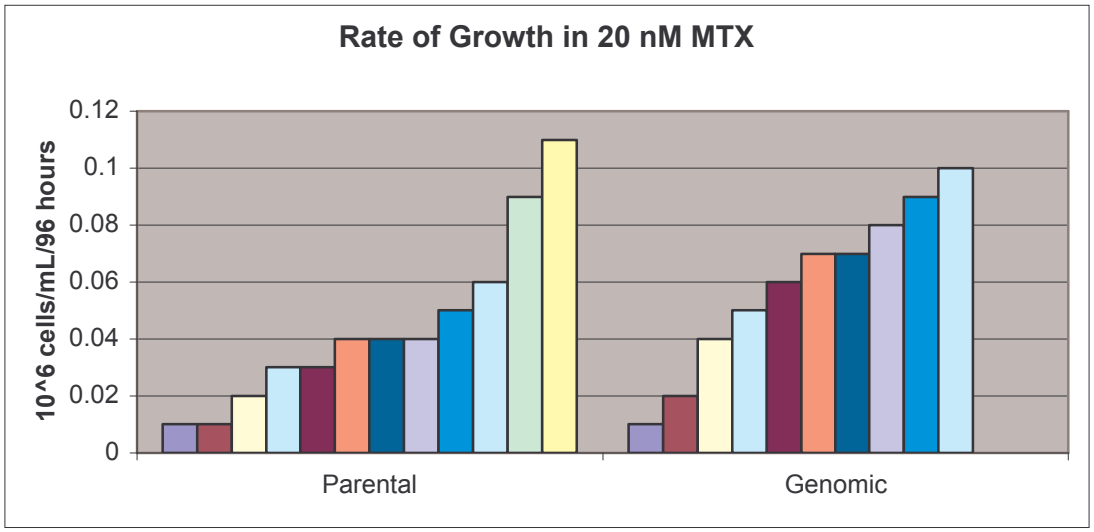

Figure 4: Viable Cell Density of Each Surviving Clone After 4 Days Growth in 20 nM MTX. The average viable cell density of the parental transfection was 0.044 x 10^6 cells/mL. The average viable cell density of the genomic transfection was 0.059 x 10^6 cells/mL.

Due to the low-throughput nature of the previously described counting approach, a confluence method was employed to test multiple passages of clones at 20 nM and 100 nM MTX concentrations. One hundred percent confluence was defined as the state at which the cells fully covered the bottom of the well, with no visible empty space. The clones were passaged in 0 nM MTX once, 20 nM MTX four times, and in 100 nM MTX four times. The growth rates for 20 nM and 100 nM were measured as an average of the growth rates for the four passages in that concentration (Figure–5). As plotted in Figure-5, there appeared to be a difference in time to confluence between the genomic and the

parental groups observed across the four passages of 100 nM MTX. Thus the data shown this way indicate that the genomic element may facilitate the amplification of the clones in the presence of increasing concentrations of MTX.

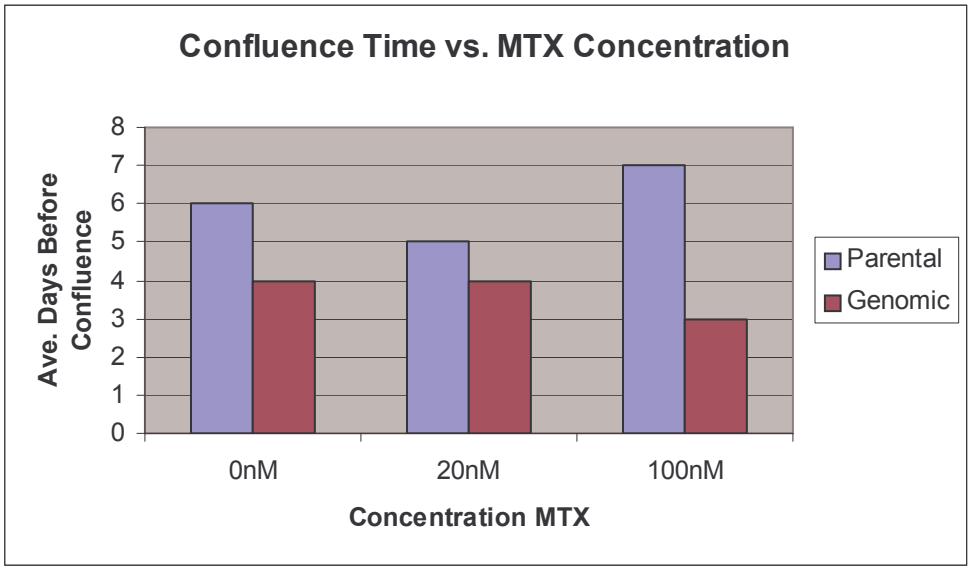

Figure 5: Average Days Before 100% Cell Confluence at Various MTX Concentrations. The average in 0 nM was measured from one passage. The averages of 20 nM and 100 nM were calculated from the individual averages of each passage in the respective concentrations of MTX.

Despite this intial subjective appearance of faster growth in the presence of the genomic element, a more thorough statistical analysis (Figure-6) indicated that the two transfections were not statistically different in terms of growth time. In 0 nM MTX, the p-value was 0.060. In 20 nM MTX, the first passage had a p-value of 0.123, the second passage, 0.649, the third passage, 0.339, and the fourth passage, 0.286. In 100 nM MTX, the p-value of the first passage was 0.582, the second passage, 0.463, the third passage, 0.394, and the fourth passage, 0.533. Thus, although the genomic passage was initially observed to grow faster, the times to confluency of the two transfections from each passage were not significantly different from each other in any of the passages recorded.

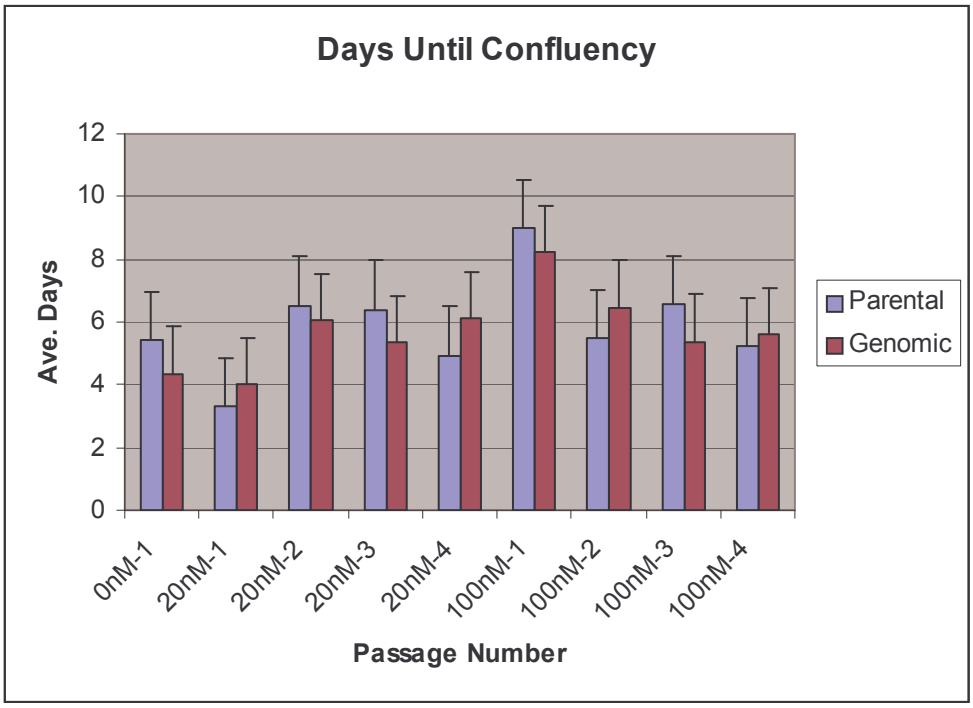

Figure 6: Averate days before confluence in each passage. The average days for each individual passage, and the standard deviation. Data from any clones which were discarded or contaminated before the fourth passage of 100 nM was not included.

Effect of the Genomic Element on Secreted IgG Levels

ELISA values to quantitate IgG levels secreted into the medium were taken at the first passage of 0 nM MTX, the fourth passage in 20 nM MTX, and the fourth passage in 100 nM MTX. Every time a cell clone came to confluence, a sample of medium (spent over a 24-hr period) was removed for IgG determination by ELISA. The ELISA readings were analyzed and sorted in ascending order within the parental and genomic groups for each MTX concentration. IgG levels are reported as "Units per mL" for proprietary reasons.

In 0 nM MTX, the average antibody titer for the genomic sample was 0.319 units/mL and the parental average was statistically identical at 0.243 units/mL, with a p-value of 0.408 (Figure 7). Although the average titers from each transfection were not

significantly different (p value = 0.408), specific clones in the genomic group had much higher than the average IgG titers.

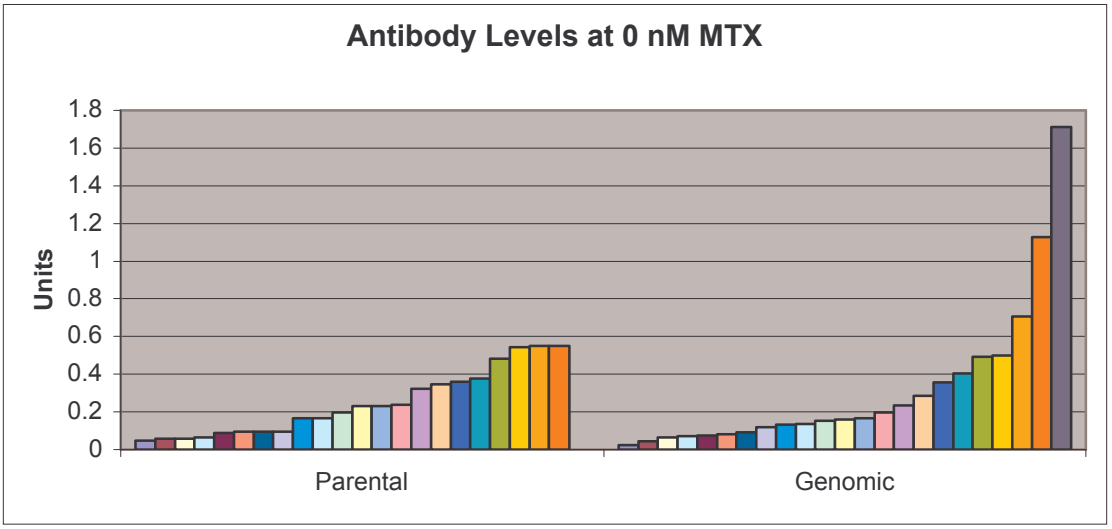

Figure 7: Antibody Production in the Spent Medium of Both Transfections in 0 nM MTX. The average IgG units per mL for the parental transfection were 0.243 and the average units per mL for the genomic was 0.319.

Clones were transferred into 20 nM MTX for four passages (Figure-8). The IgG titers of the fourth passage in 20 nM MTX were sorted in ascending order of units per mL by each transfection. In this passage, the average antibody titer for the genomic group was 5.64 units/mL, approximately 2 fold greater than that of the parental average of 2.61 units/mL (p-value of 0.170). Similar to the 0 nM MTX data, the average titers were not significantly different, although select clones with substantially higher IgG units/mL than average for their transfection group were observed in the genomic group.

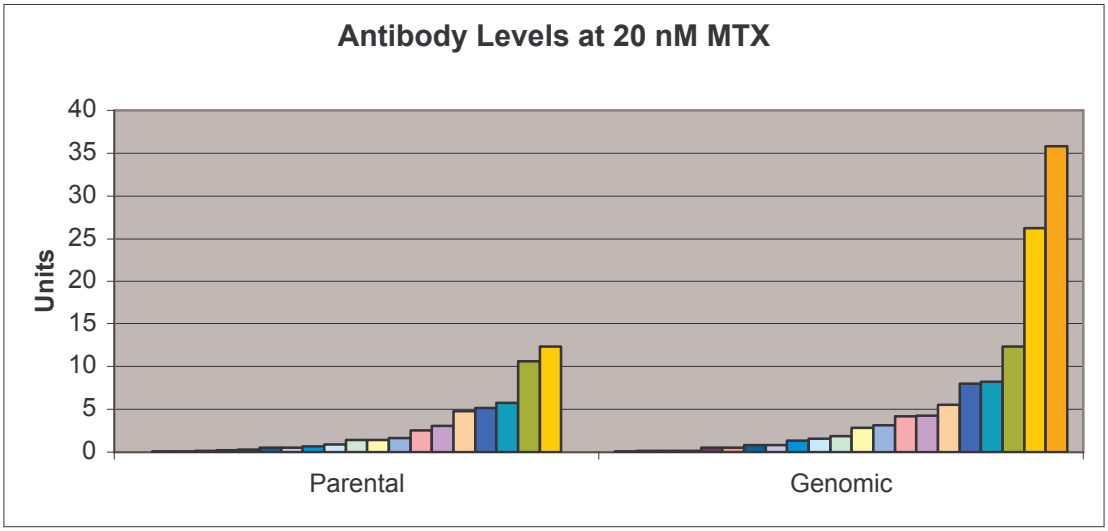

Figure 8: Antibody Production in the Spent Medium of Both Transfections in 20 nM MTX. The average units per mL for the parental transfection were 2.61 and the average units per mL for the genomic was 5.64.

Clones were transferred into 100 nM MTX for four passages (Figure-9). The IgG titers of the fourth passage in 100 nM MTX were sorted in ascending order by each transfection. In this passage, the genomic average antibody titer was 5.64 units/mL, approximately 2 fold higher than that of the parental average of 2.61 units/mL, with a p-value of 0.194. Although the averages were not significantly different, specific clones with IgG units/mL appreciably higher than average for their transfection group were observed in the genomic group. An upward trend in IgG titers was observed in earlier passages at 100 nM MTX, however, in the fourth passage, there was a decrease in IgG levels (data not shown). The averages of both groups are similar from both concentrations of MTX. Neither group expressed more antibody in 100 nM MTX than in 20 nM MTX.

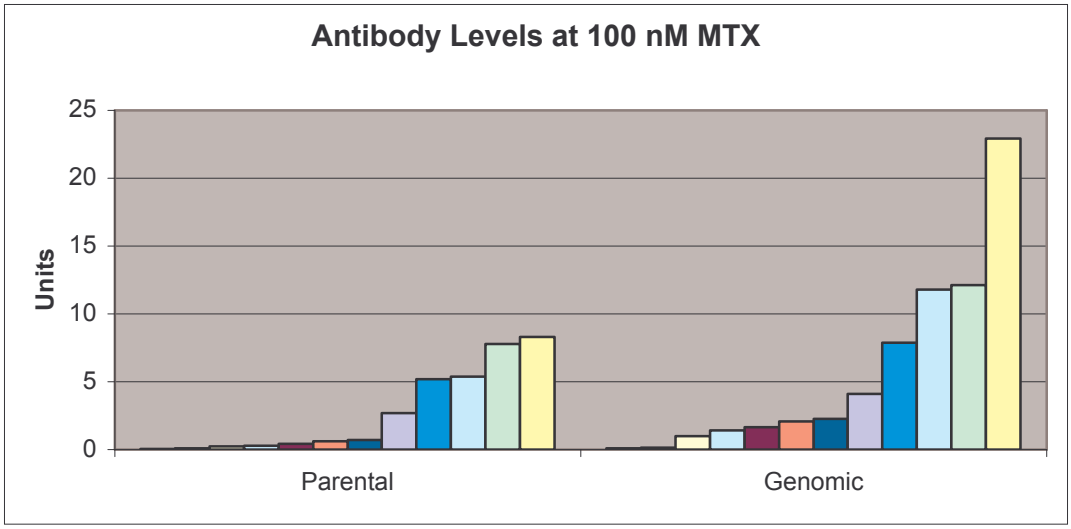

Figure 9: Antibody Production in the Spent Medium of Both Transfections in 100 nM MTX. The average units per mL for the parental transfection were 2.64 and the average units per mL for the genomic was 5.62.

DISCUSSION

Previous work at Abbott Bioresearch Center on a putative chromatin-opening sequence inserted into an IgG secretion plasmid dubbed "genomic vector", suggested that the vector caused an increase in the rate of methotrexate-induced amplification in Chinese Hamster Ovary (CHO) cells. To determine the effect of the "genomic vector" on transfected CHO cells, the cell growth and secreted antibody levels of the "genomic" transfection were compared to those of cells transfected with the parental plasmid containing the IgG gene but not containing the genomic element (parental transfection). The cell-counting method to determine growth rates (cell density over 96-hrs) of comparing the two transfections resulted in similar average cell densities in both 0 nM MTX and 20 nM MTX, however, the genomic group exhibited select clones with faster growth rates. Growth rate data for 20 nM MTX was taken in the first passage. It is not uncommon, for the first cell passage to show limited initial growth due to adaptation to selection pressures such as MTX, which may be indicated by the decrease in growth rate observed in 20 nM MTX. The confluence method, a higher throughput format, was used to assess clones at four passages each of 20 nM MTX and 100 nM MTX. Initially (Figure-5), the genomic clones appeared to reach confluency faster that the parental clones in all concentrations of MTX. However, after the clones that did not survive 20 nM MTX were removed from the data set, the remaining data (Figure-6) showed no statistical difference between the genomic averages and the parental averages in any individual passage.

A comparison of the ELISA titers of secreted IgG for each of the MTX concentrations revealed appreciable increases in the IgG levels of select clones within the genomic group, although the average titers were not statistically different. Both types of transfection showed cell amplification in 20 nM MTX as compared to 0 nM MTX, however, the levels of expression for both transfections plateaued in 100 nM MTX. These clones represent pools of cells that may not have homogeneous growth and production characteristics. It is probable that these pools are comprised of both faster-growing, lower-producing cells and slower-growing, higher-producers. In a clonal pool of this type, faster-growing cells could overtake slower growers that are potentially higher-producers, which can lead to decreases in IgG titer over time. Future research should use a subcloning technique to better characterize individual clones from the genomic and parental transfections to further the current research.

If the genomic sequence tested in this project is a chromatin-opening element or other related sequence, it would be expected that the only gene actively affected by its presence would be the inserted gene nearby the sequences, in this case the antibody gene. The effects of MAR elements and SAR elements in particular, and most chromatin-opening elements in general, are highly sensitive to the element's position relative to the gene of interest (Schübeler et al., 1996). However, any observed increase in cell growth under multiple passages in increasing concentrations of MTX would suggest that the *dhfr* gene present on the plasmid also inserted as part of both the parental and the genomic vectors, and this *dhfr* gene received some sort of positive effect from the presence of the genomic sequences, despite its position. This is possibly due to the element's potential chromatin-opening effect, which would potentially affect any gene located in three

dimensions near the element. The area in the optimal position (most commonly flanking) feels the strongest effect, but this does not exclude the possibility that genes outside of that optimal position can be influenced as well. So in this case, the element might have some weaker chromatin-opening effects on the *dhfr* gene as well, resulting in a stronger response to the MTX amplification than would otherwise be expected.

The strongest motivation into researching various chromatin-opening elements is the possibility that a sequence will be found that can be transfected with any target gene into any cell line that will increase the expression of the target gene. Generally, chromatin-opening elements can operate in a sequence non-specific manner (US Patent 7,129,062 2006). However, each individual element will most likely have the strongest effect for its own cell type, at a relative position from the gene that it naturally regulates (Frasier and Bickmore, 2007). However, it is also possible that a sequence that evolved in the DNA of one species can produce unpredictably strong effects in another species. CHO cells are the most common antibody producing cell lines, so research on elements for production purposes would be well-spent comparing elements for efficacy in CHO (Kim et al., 2004). However, sequence-independence does not mean position – independence. If each element does have an optimal distance at which its chromatin opening effects are strongest, it might be worth the painstaking research to determine the optimal location for the genomic sequences relative to the target expression gene.

If the element does cause a faster rate of amplification, that means that any cell line containing the element in the transfection plasmid should amplify faster, facilitating initial development, scale-up, and production. This results in a decrease in the time a cell

line goes from concept to clinic, and therefore would be useful as a way for decreasing costs and increasing the number of valuable antibody-based therapeutics.

BIBLIOGRAPHY

Feng YQ, Warin R, Li T, Olivier E, Besse A, Lobell A, Fu H, Lin C, Aladjem M, Bouhassira E (2005) The human beta-globin locus control region can silence as well as activate gene expression. *Molecular and Cellular Biology* **25**: 3864-3874.

Frasier P, and Bickmore W (2007) Nuclear organization of the genome and the potential for gene regulation. *Nature* **447**: 413-417.

Girod PA, Zahn-Zabal M, Mermod N (2005) Use of the chicken lysozyme 5' matrix attachment region to generate high producer CHO cell lines. *Biotechnology and Bioengineering* **91**: 1-11.

International Patent WO 00/05393 (2000) A Polynucleotide Comprising a Ubiquitous Chromatin Opening Element (UCOE). Millipore.

Kedves A (2005) Analysis of a Matrix Attachment Region in an Experimental Vector, pA205. WPI Major Qualifying Project, Submitted March, 2005.

Kim JM, Kim JS, Park DH, Kang HS, Yoon J, Baek K, Yoon Y (2004) Improved recombinant gene expression in CHO cells using matrix attachment regions. *Journal of Biotechnology* **107**: 95-105.

Marko JF and Siggia ED (1994) Fluctuations and supercoiling of DNA. *Science* **265**: 506-508.

Pidoux A, and Allshire R (2005) The role of heterochromatin in centromere function. *Philosophical Transactions of the Royal Society: Biological Sciences* **360**: 569-579.

Schübeler D, Mielke C, Maass K, Bode J (1996) Scaffold/matrix-attached regions act upon transcription in a context-dependant manner. *Biochemistry* **35**: 11160-11169.

US Patent Application 10/190,312 (2002) Nucleic Acid Sequences Having Gene Transcription Regulatory Qualities. Crucell.

US Patent 7,129,062 (2006) Matrix Attachment Regions and Methods For Use Thereof. Selexis.

Zhang Y (2007) Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes and Development* **17**: 2733-2740.