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Use of the Chicken Lysozyme 5′ Matrix Attachment Region to Generate High Producer CHO Cell Lines

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Abstract: Scaffold or matrix attachment region (S/MAR) genetic elements have previously been proposed to insulate transgenes from repressive effects linked to their site of integration within the host cell genome. We have evaluated their use in various stable transfection settings to increase the production of recombinant proteins such as monoclonal antibodies from Chinese hamster ovary (CHO) cell lines. Using the green fluorescent protein coding sequence, we show that S/MAR elements mediate a dual effect on the population of transfected cells. First, S/MAR elements almost fully abolish the occurrence of cell clones that express little transgene that may result from transgene integration in an unfavorable chromosomal environment. Second, they increase the overall expression of the transgene over the whole range of expression levels, allowing the detection of cells with significantly higher levels of transgene expression. An optimal setting was identified as the addition of a S/MAR element both in *cis* (on the transgene expression vector) and in *trans* (co-transfected on a separate plasmid). When used to express immunoglobulins, the S/MAR element enabled cell clones with high and stable levels of expression to be isolated following the analysis of a few cell lines generated without transgene amplification procedures.

Keywords: matrix attachment region; transfection; gene expression; monoclonal antibodies; CHO cells

INTRODUCTION

One of the major impediments to the generation of mammalian cell lines that stably produce recombinant proteins is the high degree of variability of transgene expression among independent transformants. This usually results in relatively low levels of recombinant protein synthesis by the majority of the transduced cell lines. In effect, only a small fraction (<1%) of the clones initially isolated stably expresses high amounts of the desired protein product. Therefore, lengthy selection and screening procedures are usually required to identify the prized clone that possesses the productivity, growth properties, and product specifications required for large-scale production.

In Chinese hamster ovary (CHO) cells, a classical approach to achieve high expression involves the selection of subclones that display an increased number of transgene copies, referred to as transgene amplification (Kaufman, 2000). This relies on a gradual increase in the selection pressure over several months for a co-transfected selection gene product such as dihydrofolate reductase, and on mutant cells that require the expression of the selection gene for growth (Kaufman and Sharp, 1982; Schimke et al., 1982). More recent approaches to address the problem have included the isolation and integration in vectors of CHO cell sequences such as endogenous promoters and favorable integration site sequences, the identification of rare sites on the chromosome with high transcriptional activity combined with targeted transgene integration at one of these sites, the construction of artificial chromosomes, the improvement of the cell line selection and screening procedures, and the metabolic engineering of the recipient cells (Brezinsky et al., 2003; Csonka et al., 2000; Fussenegger et al., 1998, 1999; Koduri et al., 2001). While all of these approaches have been used with success, they are often tedious and time-consuming, and they do not always guarantee that stable recombinant protein synthesis will occur at the production phase.

The variability in transgene expression is usually attributed to the number of transgene copies integrated and to the site of transgene integration within the host genome, which differ in each primary clone (Kalos and Fournier, 1995; Recillas-Targa et al., 2002). Thus, transgene expression may be influenced by the fortuitous presence of regulatory

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elements at the integration site. In addition, transgene expression will also be submitted to the influence of particular chromatin structures that stem from adjacent chromosomal domains. Some of these effects may not be noticed in the initial cell clone screening, but may only become apparent after prolonged culture and analysis. For instance, the expression of the transgene may gradually be silenced if its integration site lies close to a repressive heterochromatic portion of the chromosome, in a process called variegation (Bell and Felsenfeld, 1999; Henikoff, 1992).

A simple and rapid approach to overcome position effects and long screening procedures relies on the use of chromosomal elements that insulate the transgene from the effects of neighboring chromatin and/or regulatory elements (Bell and Felsenfeld, 1999). Recently, the use of these boundary or insulator elements for the production of recombinant proteins has raised considerable interest. Several studies demonstrated the advantage of using them to increase recombinant protein synthesis in mammalian cell lines of biotechnological interest (Kim et al., 2004; Zahn-Zabal et al., 2001), or for transgenesis and gene therapies in vivo (Agarwal et al., 1998; Allen et al., 1996; Castilla et al., 1998). For instance, scaffold or matrix attachment regions (SARs or MARs, collectively referred to as S/MAR elements) were shown to improve the probability of isolating a clone exhibiting the desired level of expression for the production of a recombinant protein and/or to increase the stability of production. Consequently, the time spent screening clones can be considerably reduced, and more reliable candidate cell lines may enter process development to select cells with favorable growth and scale-up properties (Bode et al., 2003a; Girod and Mermod, 2003).

How elements such as the MARs control transgene expression is not fully understood. MARs were first discovered on the basis of their association with a relatively insoluble proteinaceous fraction of the cell nucleus called the nuclear matrix or scaffold (Bode et al., 2003b; Hart and Laemmli, 1998). They were therefore proposed to act as structural components that anchor the chromosomes to the matrix and partition chromosomes into loop structures. It was hypothesized that S/MAR may form genetic boundaries between chromosomal domains that independently organize into structures permissive or non-permissive for gene expression, referred to as euchromatin and heterochromatin domains, respectively. A transgene flanked by S/MAR elements may therefore constitute an autonomous chromatin domain whose expression would remain independent of the adjacent chromosomal environment. Consistent with this view, S/MARs have been shown to increase the expression of adjacent transgenes when co-inserted into a chromosomal environment (Kalos and Fournier, 1995; Klehr et al., 1991; Phi-Van et al., 1990; Poljak et al., 1994).

An alternative, but not mutually exclusive model proposes that S/MARs directly recruit activities involved in gene expression. For instance, S/MARs act as entry sites for regulatory proteins like histone acetyl transferases that modify the chromatin structure towards expression-permissive states (Recillas-Targa et al., 2002; Yasui et al., 2002). Accordingly, S/MARs have been shown to mediate long-range histone hyperacetylation and DNA hypomethylation effects in synergy with enhancers, and may thereby activate the expression of nearby genes (Fernandez et al., 2001; Forrester et al., 1999).

Overall, many of the S/MAR-associating proteins that constitute the nuclear matrix proteins are part of the transcription and RNA maturation machineries (Hart and Laemmli, 1998; Pederson, 1998). Thus, components of the transcription machinery may concentrate in areas of the cell nucleus that would be favorable to gene expression. S/MARs and the proteins interacting with them may therefore regulate gene expression by acting on the topology of chromosomes, by controlling the position and partitioning of chromosomal domains between expression-permissive and inhibitory areas of the cell nucleus (Cai et al., 2003; Helbig and Fackelmayer, 2003).

The chicken lysozyme 5′ MAR was identified as one of the most active sequence in a previous exploratory study that compared the effect of various chromatin structure regulatory elements on transgene expression (Zahn-Zabal et al., 2001). This element was shown to increase the levels of regulated or constitutive transgene expression in various mammalian cell lines. In the present study, we have investigated and compared various processes that make use of this MAR element to generate CHO cell lines producing a recombinant immunoglobulin with therapeutic perspectives. This study demonstrates that stable and high specific productivities can be achieved by screening a limited number of cell clones transfected with the MAR element, obviating the need for lengthy procedures such as transgene copy number amplification.

MATERIALS AND METHODS

Plasmids

The complete 2,960 base pair chicken lysozyme 5′ MAR sequence was cloned into the BamHI and XbaI restriction sites of the pUC19, after PCR amplification from total chicken genomic DNA, to generate pPAG1. The pSV40EGFP control GFP expression vector contains the SV40 enhancer, early promoter and vector backbone from pGL3 (Promega, Inc., Madison, WI) driving the expression of the eGFP coding sequence from pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA). The MAR element of pPAG1 was inserted in pSV40EGFPcontrol to generate pPAG01SV40EGFP, thus containing successively the MAR element, the SV40 promoter, the eGFP coding sequence, and the SV40 enhancer. The immunoglobulin heavy and light chain expression vectors driven by the SV40 early promoter/enhancer alone (pMZ37, pMZ59) or by the human CMV promoter (pMZ36, pMZ57) are as described elsewhere (Miescher et al., 2000; Zahn-Zabal et al., 2001). Derivatives containing one MAR inserted upstream of the SV40 enhancer/promoter were created by cloning the BamH1-Xba I MAR fragment in plasmids pMZ59 and pMZ37

linearized with EcoRI and BamHI respectively, after first blunting the XbaI site of the MAR fragment and EcoRI site of pMZ59 and pMZ37 with Pfu. To synthesize the MAR and CMV promoter-driven vectors, pMZ57 and pMZ36 were first digested with AvaI and KpnI, respectively, blunted with T4DNA polymerase and then cut with BamHI. The XbaI-filled BamHI MAR fragment described above was cloned in the latter vectors.

CHO Cell Culture and Transfection

The CHO DG44 cell line (Urlaub et al., 1983) was cultivated in DMEM:F12 (Invitrogen Corp., Carlsbad, CA) supplemented with HT (Gibco-BRL) and either 2% or 10% FBS (Gibco-BRL).

Pools of stable CHO cells expressing GFP or anti-Rhesus D immunoglobulin were obtained by transfection with polyethyleneimine (PEI) as described in Boussif et al. (1995) and Zahn-Zabal et al. (2001), or with LipofectAMINE 2000 (Invitrogen, Inc., Basel, Switzerland), as recommended by the manufacturer. Cells were seeded in 6-well plates at 500–750,000 cells/well and allowed to attach overnight. The GFP expression vector was co-transfected with pSV2neo (CLONTECH Laboratories, Inc.) in a 10:1 molar ratio. Stable CHO clones expressing human anti-Rhesus D IgG1 antibody were obtained by co-transfecting the light chain and the heavy chain vectors and either the MAR-bearing plasmid, or pUC18 as a control, as indicated in the figure legends. The light and heavy chain expression vectors bear the DHFR and neomycin resistance selection genes, respectively. A total of 2.5 μg of DNA per well, with either a 1:1.75:11 or 1:1.75:27.5 molar ratio of light chain vector:heavy chain vector:MAR-containing (or control pUC18) vector was used, corresponding to a 4:1 or 10:1 MAR:antibody plasmid molar ratio respectively. All plasmids were linearized with PvuI before transfection and total DNA concentration was kept constant in transfection mixes with the addition of either pUC19 or pBluescript. After 48 h, cells were washed with PBS, trypsinized and replated in DMEM medium supplemented with 700 μg/mL geneticin for the GFP expression vector, or with 500 μg/mL geneticin and 5 μM methotrexate for the IgG expression vectors. Pools of clones were assayed after 13 and 15 days of selection. Individual clones were generated by limited dilutions of cell suspensions which were seeded into 96-well plates 24 h after transfection. Monoclonal cell populations were maintained in selective medium prior to analysis, unless otherwise noted in the figure legend. Individual clones were adapted to growth in suspension by passaging in serum-free synthetic ProCHO5 medium (Cambrex Corporation, Walkersville, MD) with agitation of the culture dish over a period of 2–3 weeks, after which they were propagated in spinner culture flasks.

GFP and IgG Productivity Assays

Cells co-transfected with the GFP and pSV2neo expression vectors were selected by growth in the presence of G418 for 2 weeks, as described in the previous section. At the end of the selection period, the polyclonal cell populations were harvested in trypsin-EDTA, pooled, rinsed with PBS and resuspended in fresh medium without serum. The expression of EGFP in suspended cell samples was directly determined using a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, NJ). Over one hundred thousand fluorescent events were acquired using a 530/15 bandpass filter for the green fluorescence protein signal obtained with a fluorescence emission centered at 530 nm.

Human immunoglobulin secreted into the medium was measured by a sandwich ELISA, with unconjugated goat anti-human kappa light chain antibody and alkaline phosphatase-conjugated goat anti-human IgGγ as capture and detection antibodies respectively (both from BioSource, Worcester, MA).

RESULTS

Stable GFP Expression in CHO Cells With the MAR

The chicken lysozyme MAR element has been reported to mediate elevated transgene expression when introduced in *cis* into the expression vector (Phi-Van et al., 1990; Stief et al., 1989), or when co-transfected in *trans* on a distinct plasmid (Zahn-Zabal et al., 2001). In this study, we systematically analyzed the use of the MAR element in such experimental settings, with the perspective of producing recombinant proteins using CHO cell lines. To analyze the effect of MARs over the entire population of live transfected cells, we first used the green fluorescent protein (GFP) as a marker for transgene expression. DG44 CHO cells were co-transfected with various combinations of GFP expression vectors and MAR elements, as depicted in Figure 1A, together with an antibiotic resistance plasmid. Transfected cells maintaining stable transgene expression were selected by culture in the presence of antibiotic for 2 weeks, and the average GFP fluorescence for the cell population was determined (Fig. 1B). Addition of MARs, either in *cis* or in *trans*, increased transgene expression in the entire polyclonal population of transfected cells (Fig. 1B). A three to fourfold increase in the average expression levels was observed when the MAR element was added in *cis*. The co-transfection of the MAR-bearing plasmid at a twofold molar excess over the GFP expression vector yielded a two to threefold increase of fluorescence. The effect of adding the MAR element in *trans* was generally found to vary depending on the transfection reagent and on the MAR-transgene ratio. It was often less than the effect observed when one MAR element was present in *cis* (Fig. 1B and data not shown).

It has been shown that the addition of more than one MAR element per transgene copy further improves transgene expression (Stief et al., 1989; Zahn-Zabal et al., 2001). However, given the large size of these elements, usually of several kilobases, the insertion of multiple MAR copies into large expression vector remains cumbersome. Therefore, we assessed whether the two approaches of adding MARs in

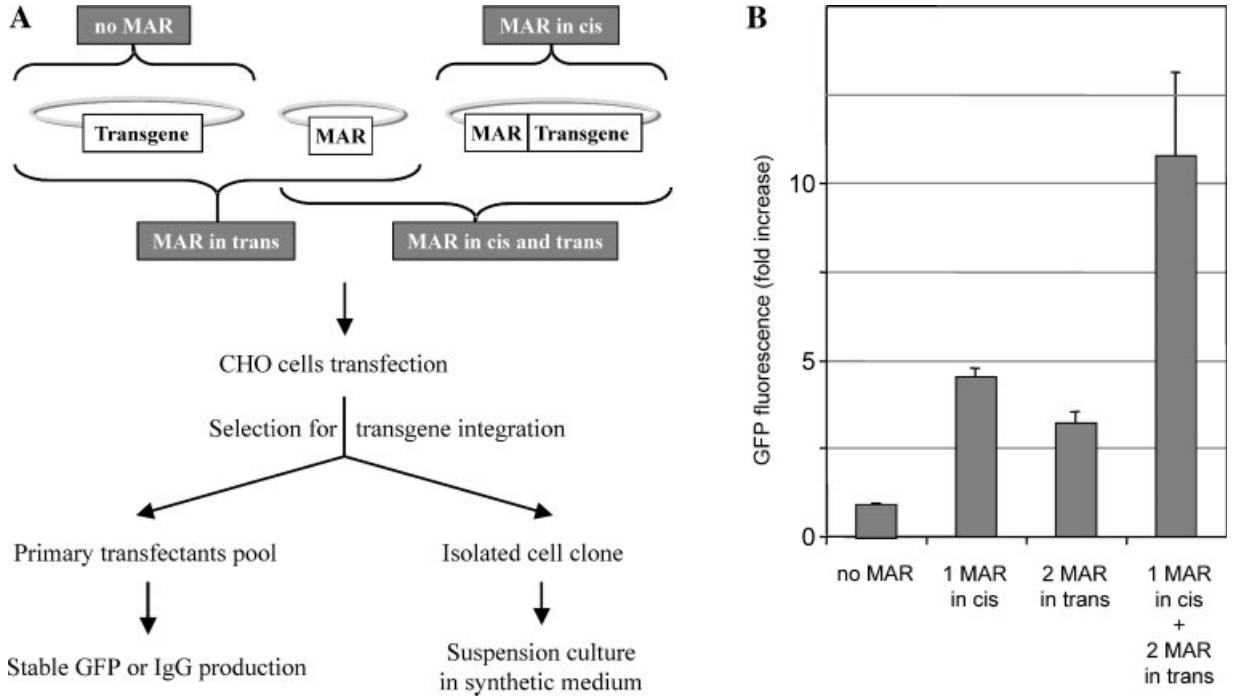

Figure 1. Comparison of the stable GFP expression obtained using various transfection schemes. **A**: Schematic representation of the transfections. The transgene expression vector was either transfected alone or it was co-transfected with another plasmid containing the chicken lysozyme MAR element (MAR in *trans*) in Chinese hamster ovary (CHO) cells. Alternatively, one MAR element was introduced upstream of the promoter and enhancer sequences and transfected alone (MAR in *cis*), or co-transfected with the MAR containing plasmid (MAR in *cis* and in *trans*). In all of the above transfection experiments, an additional expression plasmid for a selection antibiotic-resistance gene was added to the transfection mix, as described in the "Materials and Methods." After selection for resistant cells, the analysis was performed on either the polyclonal population of all transfected cell clones, or alternatively, from cell clones isolated from the polyclonal population and adapted to growth in suspension in synthetic medium. **B**: Total GFP fluorescence from polyclonal cell populations. Cells were co-transfected with the GFP expression pSV40EGFPcontrol vector without MAR, or with pPAG01SV40EGFP containing the MAR, together with either the control pUC18 or MAR-bearing pPAG1 plasmid as described in part (A). When present, the MAR-bearing plasmid is present at a twofold molar excess over the GFP expression vector. The polyclonal populations of transfected cells were selected for stable chromosomal integration of the transgenes by 2 weeks of culture in the presence of G418 and analyzed for total GFP fluorescence.

cis or in *trans* might be combined. Surprisingly, the average GFP expression was significantly increased when the MAR element was added both in *cis*, by cloning in the expression vector, and in *trans*, by co-transfection on a separate plasmid (e.g., *cis* and *trans* condition, Fig. 1A).

The MAR effect might result from a general increase in the GFP level of the entire cell population, or it might be limited to a sub-population of cells, such as those with low productivity levels. To distinguish between these possibilities, cell populations stably transfected with or without the MAR element were analyzed with a fluorescence activated cell sorter (FACS®) to assess the GFP level of individual cells. In the absence of MAR element, a biphasic distribution for GFP expression was obtained (Fig. 2A, left-hand side panel). A portion of the cells displayed slightly higher fluorescence than the profile of non-transfected cells, as indicated by the dashed line. Therefore, these cells express the GFP and antibiotic resistance transgenes at low levels, but nevertheless remain resistant to the antibiotic used to select for transfected cells. Another broad peak was also observed, corresponding to the cells that express medium or high levels of GFP, spanning two orders of magnitude of fluorescence levels.

We reasoned that the peak of low producer cells might stem from integration events within chromosomal domains that would be non-permissive for high level GFP expression, such as the heterochromatic regions of the chromosomes known to be prone to epigenetic gene silencing (Fig. 2B, left hand-side panel). In contrast, the peak of high expressor cells would correspond to transgene integration at euchromatic chromosomal portions known to be more permissive for expression. In addition to this phenomenon, other effects such as variations in the number of integrated transgenes, and/or the fortuitous presence of regulatory elements at the integration site such as enhancers, would account for the heterogeneity of the expression levels in each of the two subpopulations.

As permissive and non-permissive chromosomal regions are thought to be separated by genetic boundaries such as MAR elements, the above model was tested by analyzing populations of cells transfected with the MAR-containing vector. Two noticeable effects were reproducibly obtained in the presence of the MAR. First, the ratio of low producer to medium/high producer cells was shifted in favor of the latter, with few cells remaining in the low producer peak (Fig. 2A, right-hand side panel). This effect is consistent with the proposed chromatin domain boundary effect of the MAR, resulting in the shielding or protection of the transgene from silencing effects stemming from adjacent heterochromatin

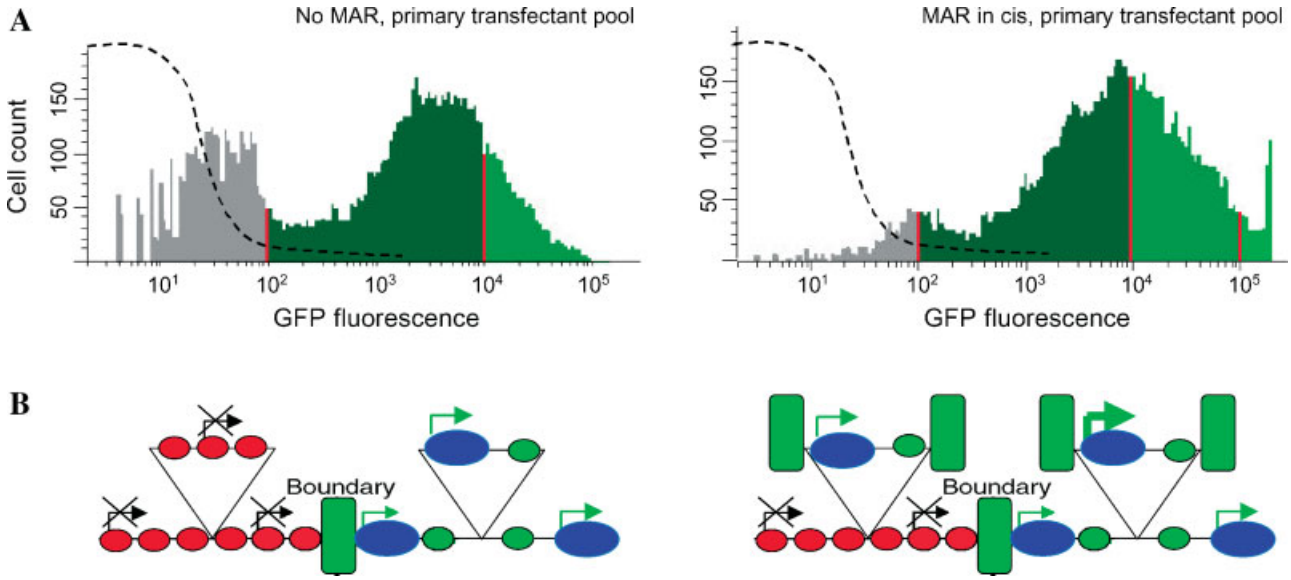

Figure 2. Analysis of the fluorescence distribution in a polyclonal cell population of GFP-expressing cells. **A**: Populations of cells expressing GFP generated as shown in Figure 1B were analyzed by a fluorescence-activated cell sorter (FACS®), and typical profiles are shown for populations of cells generated in the absence of the MAR element (left-hand side panel), or with one MAR element in *cis* (right-hand side panel). The profiles display the cell number counts as a function of the GFP fluorescence level, and low, medium, high and very high GFP expressor cells are indicated by various shades of gray. The dashed line indicates the profile of endogenous low level fluorescence measured in non-transfected cells. **B**: Interpretation of the cell population fluorescence profiles. Cellular chromosome domains are schematically represented as relatively non-permissive chromatin structures and as chromatin structures more permissive for gene expression (dense and sparse arrays of nucleosomes, represented by small ellipses, respectively). Permissive and non-permissive domains are thought to be bordered by genetic boundary elements such as MARS, represented as rectangles. In the absence of MAR elements, transgenes inserting in permissive chromosomal domains will be transcribed at higher levels than those integrating in non-permissive regions (broken vs. crossed broken arrows), consistent with the bimodal distribution of GFP expression shown in the **left panel** of part (A). While just one copy of the transgene is schematized for simplicity, it is known that multiple tandem copies of the co-transfected plasmids often co-integrate at the same chromosomal locus. Thus, in the presence of MAR elements (**right panel**), transgenes may be effectively bracketed by MAR elements and thereby shielded from adopting a repressive structure, even if they have integrated in repressive domains. In addition, expression may be generally increased for all clones, which is shown by the thick broken arrow, as suggested by the right-hand side expression profile in part (A).

(Fig. 2B, right-hand side panel). However, other interpretation such as or nuclear position-mediated effects of the MAR on transgene expression are also possible. Second, a shift of both the low and high expressor cell peaks was noted towards higher levels of GFP fluorescence. The appearance of a new subpopulation of cells with very high expression levels was also noted, corresponding to approximately 2%–5% of the total cell population.

Overall, these results indicate that the co-transfection of the MAR mediates two types of effects that are superposed in the resulting profile. One of the effects mediated by the MAR is to specifically decrease the occurrence of low producer clones, while the other results in a general increase of transgene expression over the whole range of GFP levels.

Use of the MAR Element for Stable IgG Expression in CHO Cells

Next, the relevance of the results obtained with GFP was evaluated by extending our analysis to the expression of Rhesus D-specific IgG immunoglobulins under consideration for the treatment of the hemolytic anemia of the newborn (Miescher et al., 2000). Expression vectors were constructed using commonly available elements, such as the simian virus SV40 early promoter and polyadenylation signals, to drive the expression of engineered IgG heavy and light chain genes (Fig. 3A). Alternatively, the SV40 promoter was replaced by the cytomegalovirus (CMV) promoter. CHO cells were transfected with both the light and heavy chain vectors, with or without the MAR element in *cis* and/or in *trans*. The titers of IgGs secreted in the supernatant of culture dish were determined for each of the primary polyclonal populations of stably transfected cells. As observed with GFP, the highest expression levels were obtained with the MAR element both in *cis* and in *trans*, with either the SV40 or the CMV promoters (Fig. 3B and data not shown).

To analyze the effect of the MAR on monoclonal populations, cell clones were randomly picked and expanded for analysis. From a first transfection, 12–24 clones were picked for each of the settings with the SV40 promoter (i.e., no MAR, MAR in *cis*, Mar in *trans*, MAR in *cis* and *trans*), while 42 clones were tested with the CMV promoter using the MAR both in *cis* and *trans*. Figure 4 displays the profile of the IgG titers of clones ranked in decreasing order of productivity. Consistent with the GFP assays, clones in which the SV40 promoter drives IgG expression secreted on average higher levels of IgG when transfected in presence of the MAR in *cis* and *trans*. When comparing the SV40- and CMV-based vectors, both with MARs in *cis* and in *trans*, higher titers were obtained using the CMV promoter when comparing the low producer clones. This is consistent with the CMV promoter being somewhat more efficient than

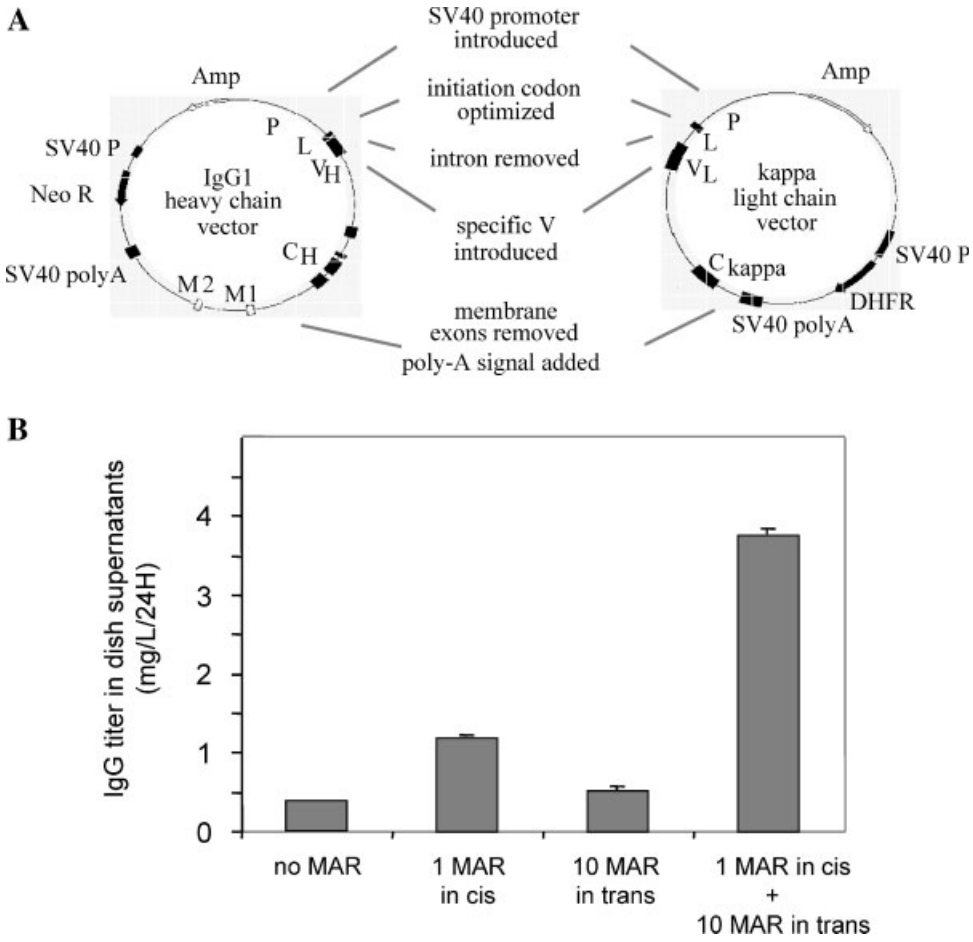

Figure 3. MAR-driven expression of IgG in stable CHO populations. **A**: Maps of the basic IgG expression vectors. Expression of IgG1 heavy chain and kappa light chain is driven by the SV40 early promoter, an optimized initiation codon and leader peptide sequence, followed directly by the genomic sequence starting with the variable chain-coding exon. Exons encoding the constant IgG motifs are followed by the SV40 polyadenylation signal. When inserted in *cis*, the MAR element was introduced upstream of the expression cassette, adjacent to the SV40 enhancer sequence. **B**: Anti-Rhesus D IgG1 antibody expression in polyclonal cell populations. CHO cells were co-transfected as described in Figure 1 with the light and heavy chain SV40 promoter-driven expression pMZ59 and pMZ37 vectors, or with their MAR-containing counterparts, with or without co-transfection with the indicated molar ratios of either control pUC18 or MAR-bearing plasmid. After selection of primary transfectant cells, antibody titers in the supernatant of polyclonal cell populations grown on the surface of culture dishes were determined.

the SV40 promoter in transiently transfected CHO cells. However, the two lines crossed for clones with higher titers, and the most productive clones were obtained using the SV40 promoter, despite the fact that more clones were assayed from the population of cells transfected with the CMV vectors.

A second set of transfections was performed similarly, but with the SV40 promoter-driven vectors only, and 294 stable clones altogether were isolated and expanded. The concentration of secreted IgG was estimated from the supernatant of culture dishes for all clones as before. Some of the clones displaying the highest titers, from either the first or the second transfection, are listed in Table I. As for the first transfection, higher titers were obtained when the expression vector was co-transfected with the MAR element both in *cis* and in *trans*.

These cell clones were adapted for growth in suspension in a synthetic serum-free growth medium, and cell doubling time and specific productivities were determined. Of the 16 cell clones analyzed, 6 had productivities in the range spanning 15–55 picograms per cell per day of IgG. All of the high producer clones were derived from the transfections that included the MAR element in both *cis* and *trans*. Interestingly, the cell doubling time often appeared to be directly correlated with the specific productivity. Thus, it is likely that very high specific productivity levels represent a metabolic burden to the cells at this very early stage, that is after the transfer to the fully synthetic medium but before the selection of fast growing cells and further process development steps have occurred.

Characterization of IgG-Producing CHO Cell Lines

To determine the effect of transgene copy number on productivity, the proportion of light and heavy chain sequences with respect to the total genomic DNA were determined by Southern analysis for several clones. Overall, some correlation was noted between IgG titers and the heavy chain transgene copy number (Fig. 5A). The clones with the higher titers and copy numbers were those obtained by

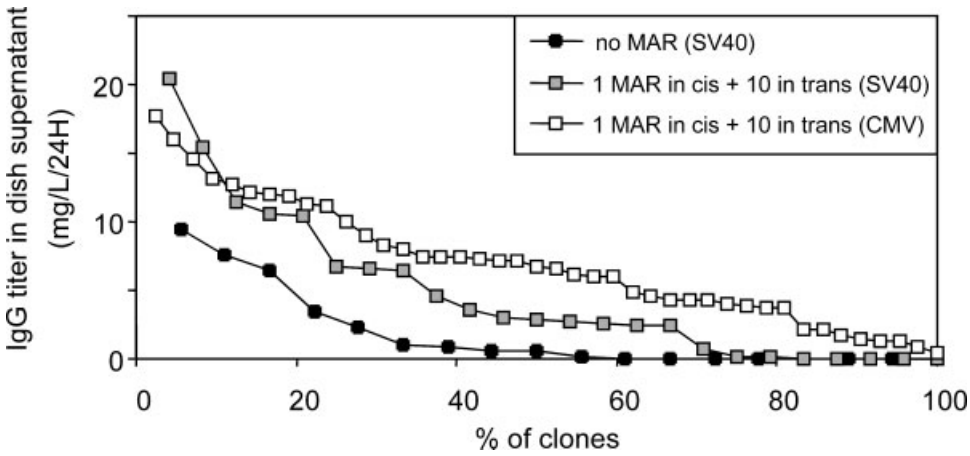

Figure 4. Analysis of IgG synthesis by clones generated with or without MAR element. Clones were generated from a population of CHO cells transfected with vectors with the SV40 promoter driving IgG heavy and light chain expression without MAR (no MAR), or with the MAR element added in *cis* and in *trans*, as in Figure 3. Alternatively, cells were co-transfected with vectors in which the SV40 promoter has been replaced by the CMV promoter/enhancer region, again with the MAR element added in *cis* and/or in *trans*. After selection of stable transfectant cells, IgG titers were measured in the supernatants of culture dishes containing equivalent cell numbers. Each clone analyzed is represented by a square symbol, and clones are ordered from highest to lowest expression level.

transfecting the MAR element both in *cis* and in *trans* (Fig. 5A and data not shown). Interestingly, light chain coding sequences were often over-represented in the higher-producing clones, despite the transfection of a twofold higher molar concentration of the heavy chain vector. The reason for this phenomenon is unclear, as differences in the transfection and/or integration efficiency might result from the different size of each vector, or from the distinct selection marker present on each of the vectors. This suggested that IgG productivity might be limited in some clones by the synthesis of lower amounts of the heavy chain.

To probe transgene association with the nuclear matrix, cell nuclei were extracted from two of the clones, and association of the MAR or IgG light chain DNA with the nuclear matrix was probed by restriction enzyme cleavage and quantitative PCR. This indicated that linkage of the MAR to the IgG sequence results in increased association of the latter with the nuclear matrix (Supplemental Fig. 1 and Table I), as known for the MARs (Bode et al., 2003b; Hart and Laemmli, 1998). Consequently, the MAR sequences are co-integrated with the transgene and mediate its association with the nuclear matrix.

Table I. Specific productivity of Chinese hamster ovary (CHO) clones grown in suspension.

Clone	Promoter	MAR element	IgG titer (mg/L/day)	Doubling time (h)	Productivity (pg/cell/day)	Cell count (×1,000)
I/2-1	SV40	In *cis*	1.7	164	12.8	300
I/2-3	SV40	In *cis*	1.8	76	4.7	480
I/4-3	SV40	In *cis* and *trans*	11.6	42	12.9	994
I/4-4	SV40	In *cis* and *trans*	10.4	38	28.0	1,166
I/9-1	CMV	In *cis* and *trans*	6.1	56	8.0	654
I/9-16	CMV	In *cis* and *trans*	6.2	41	8.7	1,014
I/9-17	CMV	In *cis* and *trans*	7.4	35	6.2	1,340
I/9-27	CMV	In *cis* and *trans*	10.2	164	43.4	300
II/3-1	SV40	In *trans*	0.9	43	0.9	942
II/4-1	SV40	In *cis* and *trans*	10.2	42	20.3	972
II/4-10	SV40	in *cis* and *trans*	14.6	68	28.4	532
II/4-11	SV40	In *cis* and *trans*	9.2	50	7.2	760
II/4-12	SV40	In *cis* and *trans*	4.7	28	2.8	2,200
II/4-25	SV40	In *cis* and *trans*	14.8	47	13.7	812
II/4-26	SV40	In *cis* and *trans*	16.1	43	16.8	944
II/4-28	SV40	In *cis* and *trans*	13.6	164	54.7	300
Control clone	SV40	No MAR	ND	28	0.6	

Clones were picked from two independent transfections performed using IgG expression vectors with the promoter and MAR elements indicated. IgG titers were determined from 0.6 mL of culture supernatants of cells grown to confluence in 24-well dishes. Clones with high titers were adapted to growth in synthetic medium in spinner cultures for 2 weeks, after which cell number doubling time and specific productivity were assayed. ND, not determined.

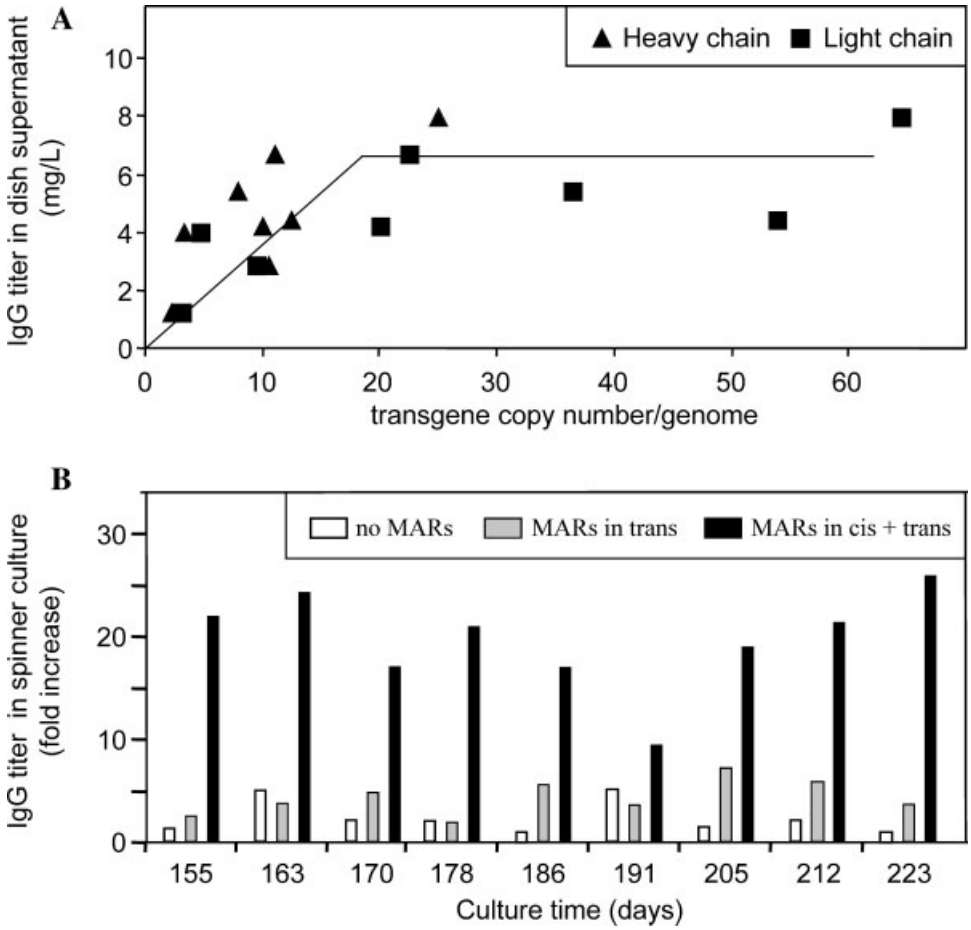

Figure 5. Characterization of clones with high IgG specific productivities. **A**: Determination of the relationship between production titers and transgene copy numbers. The copy number of the heavy and light chain coding sequences was determined by Southern and quantitative PCR analysis from cultures of clones described in Table I. The IgG titers, as determined in culture dish supernatants, are represented as a function of the transgene copy number per genome equivalent for each of the heavy and light chain coding sequences. **B**: Characterization of the production titer stability in suspension cultures. Clone II/4-1 is compared to another clone generated with the same vector, but generated by adding the MAR in *trans* only, and to a third clone generated with no MAR at all. Clones were grown in serum-free synthetic medium in spinner culture, without applying a selection for transgene expression. Cultures were maintained by regular dilution in fresh medium at suspension numbers ranging between 10^6 and 10^7 cells per liter and the IgG titer in the culture supernatants was assayed at the time indicated. Relative titer values are normalized to the lowest titer obtained from the clone generated without the MAR element, which was set to a value of one.

To determine productivity at the RNA level, a cDNA library was prepared from polyA-containing mRNAs isolated from the II/4-1 clone, which contains approximately 12 copies of the heavy chain coding sequence and 54 copies of the light chain sequence. A total of 110 cDNAs were picked randomly from the library of expressed sequence tags and sequenced to estimate the frequency of transgene mRNAs relative to CHO cell gene products. The most represented mRNAs correspond to the IgG light chain expressed from the transfected DNA, followed by the heavy chain sequence (Table II). Overall, mRNAs generated from the transgenes amount to 15% of all the transcripts, a number that can only be rivaled by the total occurrence of all ribosomal protein mRNAs taken together.

As expected from the transgene copy number, the IgG light chain mRNA appeared more frequently than that corresponding to the heavy chain. To assess whether this is limiting IgG secretion, cell clones displaying an unbalance in mRNA ratio in Figure 5A were re-transfected with the vector encoding the heavy chain together with the MAR element in both *cis* and *trans*, along with a puromycin selection gene. After selection of the secondary transfectants, secreted IgG titers were found to be on average $40\% \pm 12\%$ higher than those of the primary clones. This indicates that the IgG productivity was indeed limited by the synthesis of lower amounts of the heavy chain in the primary clones, and that the MAR elements can be used to correct such limitations.

Given the metabolic burden that may result from very high specific productivities in terms of energy balance, mRNA and protein levels (Tables I and II), the stability of the specific productivity was assessed for one of the clones showing favorable properties. The primary clone II/4-1 was cultured as a cell suspension in fully synthetic medium in the absence of antibiotic selection, and IgG titers were estimated at regular intervals. As can be seen from Figure 5B, titers and growth properties remained stable in the spinner culture of

Table II. Total mRNA sequence analysis from the II/4-1 MAR-transfected clone.

mRNA class	mRNA identity	Number of occurrence
Unidentified mRNA sequences	—	15
mRNAs of unknown function	—	7
mRNAs with known function (total)[a]	—	88
Transgene products (total)	—	14
	IgG light chain	8
	IgG heavy chain	5
	Neomycin R	1
Ribosomal proteins (total)	—	14
	Ribosomal S19	3
	Ribosomal S12	2
	Ribosomal L4	2
	Ribosomal L6	1
	Six others	One occurrence each
Enzymes, metabolism (total)	—	20
	GAPDH	2
	Cytochrome C oxid.	2
	Ribophorin II	2
	14 others	One occurrence each

[a]Only the most frequently occurring mRNA species are indicated here, as obtained from the sequence determination of 110 randomly picked cDNA EST. mRNAs with known function were grouped in functional sub-classes as indicated. mRNAs from other sub-classes all correspond to single occurrence (complete list available upon request).

II/4-1 over a period of over 200 days of continuous culture. In addition, IgG titers were maintained at levels significantly superior to those obtained from a cell line generated previously using the MAR element in *trans* only (Zahn-Zabal et al., 2001), or from a cell line generated without the MAR element and which was used as a control and cultured in parallel. This demonstrates that the higher productivity obtained from the clone generated with the MAR element added in *cis* and in *trans* can be stably maintained when cultured over prolonged periods in the absence of selection pressure.

DISCUSSION

In this study, we investigated the advantage of using the chicken lysozyme MAR genetic element to generate cell lines producing recombinant proteins. We found that the optimal setting consists of adding the MAR element both in *trans* and in *cis*, as compared to solely in *trans* or in *cis*. This implies that the co-integration of more copies of the MAR element with the transgene results in higher expression. This conclusion is consistent with earlier studies that indicated that addition of the MAR element in *trans* increased transgene expression in a dose-dependent fashion (Zahn-Zabal et al., 2001). Addition of the MAR in *cis* and in *trans* resulted in a 5- to 10-fold increase in IgG or GFP expression on average, which is significantly better than the 2- to 5-fold increase obtained by adding the MAR in *cis* or in *trans* only. Adding multiple MAR elements in *cis* on the same expression vector is another possible approach, for instance when using relatively short reporter sequences such as the GFP or luciferase cDNAs (Zahn-Zabal et al., 2001). However, the length of MAR elements (usually 3–4 kb) makes it difficult to combine two MAR elements with the larger and relatively more efficient genomic sequences that are often used to express immunoglobulins. In such case, the large size of the resulting plasmids and possible recombination events between two repeated MAR elements inserted in the same vector renders plasmid construction and transfection unpractical. The *cis* and *trans* setting is therefore an alternative that is both faster and easier to implement, while allowing high productivity gains.

These results clearly show that cell lines with specific productivities around or above 40 pg/cell/day can be easily identified from the analysis of a few candidate clones, when using the MAR and widely available vector elements such as the SV40 promoter. This strategy may therefore render the tedious analysis of large numbers of clones and/or the lengthy amplification of transgenes unnecessary in order to reach significant productivities. Furthermore, cell clones generated with this strategy were easily adapted to growth in suspension in synthetic medium, and high specific productivities and stable titers of secreted IgGs were maintained. Overall, the use of S/MARs may therefore shorten significantly the time devoted to the identification of cell lines with the required specific productivities and may concomitantly increase the number of candidate clones that can enter further process development and scale-up in the bioreactor.

A number of mechanisms have been evoked to explain the mode of action of genetic elements such as S/MARs. As S/MARs have little effect in transient transfections, they are believed to regulate chromatin structure. S/MAR elements and the proteins associated with them may mediate the formation of boundaries between chromosomal loops or subnuclear domains (Bode et al., 2003b; Hart and Laemmli, 1998) or chromatin modifications such as histone tail hyperacetylation (Recillas-Targa et al., 2002; Yasui et al., 2002). Alternatively, one of the S/MARs was shown to mediate episomal replication of transfected DNA (Jenke et al., 2004; Piechaczek et al., 1999). Our analysis of GFP expressing cells revealed a bimodal distribution of expression, with the occurrence of cells that express the transgene at low levels, as would be expected from its integration at non-permissive heterochromatic loci. The most striking effect of the MAR is the almost complete reduction of this population and the concomitant increase in cells belonging to the population with higher expression levels. This effect is consistent with the inhibition of the propagation of heterochromatin along the chromosomal domain by the boundary formed by an interposed MAR element, thereby preventing the silencing of the transgene. Such a mechanism has also been proposed for mammalian DNA binding proteins acting as boundary elements in lower eukaryotic cells (Fourel et al., 2001). Alternatively, the MAR may actively reconfigure chromatin around its chromosomal integration site and thereby prevent transgene silencing, for instance by mediating histone modifications or changes in subnuclear localization.

Our results imply that higher productivities can be obtained with the SV40 promoter than with the CMV promoter. Clones with the SV40 promoter driving IgG expression in presence of the MAR element exhibit stable IgG secretion, even when cultured for a prolonged period in the absence of antibiotic selection. In contrast, IgG secretion was often unstable when using the CMV promoter-based expression vectors in CHO cells, irrespective of whether the MAR element was added or not (unpublished results). We attribute this silencing effect to the presence of a CpG island within the CMV promoter. As this CpG island is located between the transgene and the MAR element, the latter would not protect the transgene from silencing effects that originate from within the expression cassette itself.

The proposed chromatin modification and/or boundary activity of MAR satisfactorily accounts for its effect on low producer cells. However, it does not necessarily explain the general increase in transgene expression noted for the high expressor cell class, when the transgenes may be integrated within a permissive chromatin structure. Several potential explanations for the general increase in expression may be given. For instance, transgenes may be submitted to inhibitory chromatin-related effects even in the more permissive chromosomal loci, which would be counteracted by the MAR. Alternatively, MAR elements may increase transcription initiation, either directly or indirectly, by increasing the potency of the transgene promoter or enhancer. Finally, the MAR elements might favor integration at permissive loci within the chromosome, or they may increase the number of transgene copies integrated. With regard to the latter possibility, Kim et al. (2004) have observed higher transgene copy numbers when these are co-transfected for the β-globin MAR. In the present study, higher transgene copy numbers were also observed on average in the clones obtained by transfecting the transgene with the MAR in *cis* and in *trans* when compared to clones transfected with MAR in *cis* or *trans* only, which in turn had more transgene copies than the clones generated without the MAR element. Thus, in addition to its boundary effect, the MAR element may act by increasing the copy number of integrated transgenes. However, analysis of a larger number of clones would be required to determine if these effects fully account for the increased productivities observed when the chicken lysozyme MAR element is used.

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