

# Chromatin loops are selectively anchored using scaffold/matrix-attachment regions

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Accepted 00976

Journal of Cell Science 117, 999-1008 Published by The Company of Biologists 2004  
doi:10.1242/jcs.00976

## Summary

The biological significance of nuclear scaffold/matrix-attachment regions (S/MARs) remains a topic of long-standing interest. The key to understanding S/MAR behavior relies on determining the physical attributes of *in vivo* S/MARs and whether they serve as rigid or flexible chromatin loop anchors. To analyze S/MAR behavior, single and multiple copies of the S/MAR-containing constructs were introduced into various host genomes of transgenic mice and transfected cell lines. These *in vivo* integration events provided a system to study the association and integration patterns of each introduced S/MAR. By utilizing FISH to visualize directly the localization of S/MARs on the nuclear matrix or chromatin loop, we were able to assign specific attributes to the S/MAR. Surprisingly, when multiple-copy S/MARs were introduced they were selected and used as nuclear matrix

anchors in a discriminatory manner, even though they all contained identical primary sequences. This selection process was probably mediated by S/MAR availability including binding strength and copy number, as reflected by the expression profiles and association of multi-copy tandem inserted constructs. Whereas S/MARs functioned as the mediators of loop attachment, they were used in a selective and dynamic fashion. Consequently, S/MAR anchors were necessary but not sufficient for chromatin loops to form. These observations reconcile many seemingly contradictory attributes previously associated with S/MARs.

Key words: Chromatin loop, Loop anchor, S/MAR, FISH, Gene expression, Chromosome structure

## Introduction

Increasing evidence supports the view that eukaryotic chromatin is organized as independent loops (Heng et al., 2001). Following histone extraction, these loops can be visualized as a DNA halo anchored to the densely stained nuclear matrix or chromosomal scaffold (Vogelstein et al., 1980; Gerdes et al., 1994; Bickmore and Oghene, 1996). As a basic unit, the loop is essential for DNA replication, transcription regulation and chromosomal packaging (Gasser and Laemmli, 1987; Berezney et al., 1995; Bode et al., 1995; Nickerson et al., 1995; Razin et al., 1995; Jackson, 1997; Stein et al., 1999; Sumer et al., 2003). The formation of each loop is dependent on a specific chromatin segment that must function as an anchor to the nuclear matrix. Sequences that attach to the nuclear scaffold or matrix have been termed either 'SARs' or 'MARs' (collectively termed S/MARs) as characterized by their preparation from metaphase or interphase cells respectively (Mirkovitch et al., 1984; Izauralde et al., 1988; Craig et al., 1997; Hart and Laemmli, 1998). The recent description of mobile nuclear proteins has raised the issue of whether all S/MAR sequences serve as anchors and whether these anchors are rigid or flexible (Hancock, 2000; Pederson, 2000), but this now appears to be reconciled (Nickerson, 2001; Jackson, 2003).

Despite increasing acceptance of the concept of S/MARs, one issue of concern is related to the procedure used to release loops and whether or not the protein extraction method will alter the nature of native chromatin loop domains. Recent evidence supporting the use of released loops for the study of S/MARs showed that sperm nuclear halos did transform into normal chromosomes after injection into oocytes (Mohar et al., 2002). These interesting results suggest that sperm nuclear halos retain all the information necessary for normal chromosomal organization, and the procedure to prepare the nuclear matrix does not change the basic biological features of nuclear chromatin. By monitoring the loop size of different chromatin domains along the nuclear matrix, our recent data also demonstrated that the chromosome territory information is well maintained in this experimental system (H.H.Q.H. et al., unpublished). Another significant development linking structural elements (S/MARs) with chromatin remodeling and high-order folding has recently been achieved by the characterization of SATB1 (for 'special AT-rich binding protein 1'). SATB1 is one of the best-characterized S/MAR-binding proteins and it is preferentially expressed in thymocytes (Dickinson et al., 1992). SATB1-deficient mice die soon after birth as they do not develop T cells (Alvarez et al., 2000). SATB1-binding S/MAR sequences are located at the base of chromatin loops functioning as loop anchors on the nuclear matrix in cells expressing SATB1 and are

located on the loop portion in inactive cells. This showed that, in vivo, the cell-type-specific factor SATB1 binds to the base of the chromatin loop and is integral to T-cell-specific gene regulation (deBelle et al., 1998; Cai and Kohwi-Shigematsu, 1999). Related properties have been demonstrated for a ubiquitous protein, SAF-A (for 'scaffold-attachment factor A, otherwise known as hnRNP-U) (Bode et al., 1995). Recent data have now established that SATB1 recruits histone deacetylase to specific sequences and mediates the specific deacetylation of histones to remodel a large chromatin domain (Yasui et al., 2002). SATB1 is necessary for folding chromatin by tethering specialized DNA sequences onto its network. The binding between SATB1 and its sequences establishes specific histone modifications over a region containing genes whose expression is SATB1 dependent. SATB1 clearly serves as a docking site for specialized DNA sequences and enzymes that modify chromatin activity (Cai et al., 2003).

The question thus arises: do S/MARs universally act as loop anchors that are utilized in a dynamic manner as suggested in the case of SATB1? A direct approach that created a loop 'anchor' using defined S/MARs was implemented to address this question. Both well-characterized S/MAR and non-S/MAR sequences were introduced into mouse and human cells. The comparison of the patterns of integrated sequences with respect to their location on the nuclear matrix and chromatin loop shows that a selected subset of identical S/MAR sequences do function as anchors. Interestingly, not all S/MARs are used as anchors when introduced into the host genome as tandem arrays, since many copies of the inserted S/MARs were detected as part of the chromatin loop (Heng et al., 2000). Combined with the observation that even a single-copy integrated MAR can be detected on the loop portion of certain halos from the same preparation, our data support the dynamic nature of the anchor. Interestingly, the gene expression profile was directly correlated with the number of transgenes associated with the nuclear matrix but not with the total number of transgenes integrated. Thus, this system permitted us effectively to compare the structural and behavioral characteristics of endogenous and 'foreign' S/MARs to identify specific features of gene regulation caused by varying the copy number of integration. This selective and dynamic feature of the 'foreign' (i.e. introduced) S/MARs is in accordance with the behavior of endogenous S/MARs. S/MARs provide the landing platform for several chromatin-remodeling enzymes (Yasui et al., 2002) facilitating chromatin loop movement as each region is pulled through the transcriptional machinery (Cook, 1999; Lemon and Tjian, 2000; Bode et al., 2003). The results presented in this study reconcile many apparent and previously documented contradictions and an interactive model is proposed.

## Materials and Methods

### Transgenic mice

To introduce a defined human S/MAR into the mouse genome, the human protamine genomic locus was selected. A 40 kb fragment (Hp 3.1) of protamine sequence (HSU15422) was used for mouse pronuclear microinjection. This genomic fragment consists of a linear array of three genes: protamine 1 (PRM1), protamine 2 (PRM2) and transition protein 2 (TNP2). A single human somatic S/MAR sequence was identified within this 40 kb fragment 3' of the TNP2 in demarcating a second locus containing the SOCS-1 gene (Kramer et

al., 1998). Southern blot and FISH analysis showed that this construct was integrated into the mouse genome. Several varied copy number transgenic lines were created. The 40 kb transgenes were arranged in a head-to-tail tandem array in a single locus (Choudhary et al., 1995; Kramer and Krawetz, 1996; Stewart et al., 1999).

### Transfected stable cell lines

A 36 kb fragment encompassing the human interferon (IFN)- $\beta$  (*hulIFNB1*) gene that contained the endogenous 5' S/MAR element (Bode and Maass, 1988) was stably transfected into Ltk cells. Ltk cell lines containing a varied number of copies of the *hulIFNB1* gene were generated by calcium phosphate-mediated transfection of 1-5  $\mu$ g of DNA per 1E6 cells. The low-copy-number cell lines were derived from the transfer of low concentrations of linearized DNA (using the unique *Mlu*I site within this region). The high-copy-number cell lines were similarly created by using a higher concentration of supercoiled cosmid.

### Halo preparation

Nuclear halos were prepared essentially as described (Gerdes et al., 1994; Cai and Kohwi-Shigematsu, 1999). In brief, cultured mouse spleen cells and transfected mouse cells were harvested; then immersed in 4°C CSK buffer containing 0.5% Triton X-100 for 10 minutes. After washing at room temperature with PBS, the nuclei were loaded onto the slides using a cystospin centrifuge (800 rpm for 5 minutes). Nuclei were then treated with a 2 M NaCl buffer (2 M NaCl, 10 mM Pipes, pH 6.8, 10 mM EDTA, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1.2 mM phenylmethylsulfonyl fluoride) for 4 minutes at 4°C to extract the histones and soluble non-histone proteins. This released the DNA loops from the nuclei. The slides were then rinsed by a series of 10 $\times$ , 5 $\times$ , 2 $\times$  and 1 $\times$  PBS gentle washes. This was followed by another series of graded rinses with 10%, 30%, 50%, 70% and 95% ethanol. The slides were then air dried at room temperature for 30 minutes. The nuclear halos were fixed by baking the slides at 70°C for 2 hours using a slide warmer.

### Nuclease digestion of the chromatin loop

The transfected cells were digested in situ with nuclease, essentially as described (Cai and Kohwi-Shigematsu, 1999). The slides containing the transfected cells were treated with 0.5% Triton X-100 in CSK for 5 minutes at 4°C to remove the membrane and soluble proteins. The nuclei were then treated with extraction buffer (42.5 mM Tris-HCl, pH 8.3, 8.5 mM MgCl<sub>2</sub>, 2.6 mM MgCl<sub>2</sub>, 1.2 mM phenylmethylsulfonyl fluoride, 1.0% Tween 40, 0.5% deoxycholic acid) for 5 minutes at 4°C to remove cytoskeleton proteins. The slides were then transferred to a humid chamber and 100  $\mu$ l digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 1.2 mM phenylmethylsulfonyl fluoride) containing 100  $\mu$ g/ml DNase I was overlaid onto the nuclei on each slide. The slides were then incubated at 37°C for 1 hour. Ammonium sulfate was then added to a final concentration of 0.25 M and the resulting mixture incubated at 4°C for 5 minutes to remove the chromatin fraction (DNA and histones) from the nuclei. The slides were then rinsed in ice-cold PBS prior to a 3 minute -20°C fixation in methanol then acetone. Following fixation the slides were allowed to air dry.

### FISH

Various DNA probes were used for FISH detection to study the distribution of integrated DNA along the nuclear halo/matrix. Transgenes of human protamine locus were detected using a 5 kb fragment from the 3' region of Hp3.1 encompassing the SOCS-1 MAR. The *hulIFNB1* gene locus within the stably transfected cell lines (clone 25, clone 40, clone 9 and clone 24) was detected using the

endogenous S/MAR sequences from the human IFN region as a probe (Goetze et al., 2003). Briefly, after RNase treatment, the halo slides were denatured with 70% formamide in 2×SSC for 2 minutes at 70°C, followed by dehydration with ethanol. Biotinylated DNA probe was prepared using the BioNick DNA labeling kit (Gibco BRL), denatured at 70°C for 5 minutes in a hybridization mixture consisting of 50% formamide and 10% dextran sulfate, and then incubated on halo spreads. After overnight incubation, the post-hybridization wash and FISH detection were carried out using published protocols (Heng et al., 1992; Heng and Tsui, 1993).

Two-color FISH was performed as described (Heng et al., 1994a; Heng and Tsui, 1994). The endogenous S/MAR sequences and the human IFN sequences were labeled by biotin and digoxigenin, respectively. To verify their distribution separately, the color was switched and the S/MAR were labeled with digoxigenin and the *hIFNBI* gene was labeled with biotin. Two adjacent BAC probes from human chromosome 8 were labeled with biotin and digoxigenin to monitor the configuration of the two probes on the nuclear matrix to facilitate the analysis of loop movement. The FITC-avidin and rhodamin-anti-digoxigenin (Dig) were used to detect both biotin-labeled and Dig-labeled probes.

### RNA isolation and northern analysis

To monitor the expression of the transgenes, RNA was isolated from human, non-transgenic mouse and transgenic mouse testes using the single-step guanidine-based method. The RNAs were resolved on 1.5% agarose gels containing 2% formaldehyde and then transferred by electroblotting onto positively charged nylon membranes according to standard methods. Random primed [ $\alpha$ -<sup>32</sup>P]dCTP-labeled PCR products specific to the human PRM1, PRM2 and mouse  $\beta$ -actin transcripts served as probes (PRM1, GCAGATATTACC-GCCAGAGAC + CAGGAGTTTGGTGGATGTGCT; PRM2, CTGCCACCCAGAGTCCCT + CACTTGGTGTTCGGGCGA-CCT; Actin, AACACCCAGCCATGTACG + ATGTCACGCA-CGATTTCCC). The membranes were hybridized at 50°C overnight in a 5× SSPE buffer containing: 50% formamide, 5× Denhardt's reagent, 0.5% SDS, 0.1 mg/ml sheared salmon sperm DNA, 0.1 mg/ml yeast t-RNA, 10% PEG and 10<sup>6</sup> dpm/ml of [ $\alpha$ -<sup>32</sup>P]dCTP-labeled probe. Following hybridization, the membranes were washed at room temperature in 2× SSPE containing 0.1% SDS for 30 minutes. They were then transferred to a circulating bath at 50°C containing 0.1× SSPE and 0.1% SDS for 5-15 minutes. Subsequent to washing, the hybridized signal was visualized by autoradiography. Autoradiographic images were quantitated using the Millepore 60S version 3.0 Whole Band image analysis system.

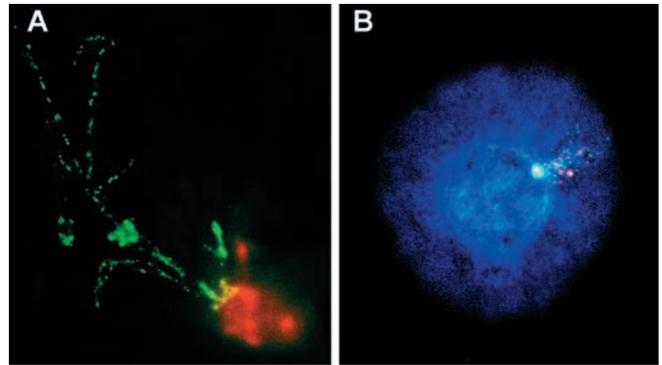
### IFN production measurement

To monitor the IFN production for the transfected cell lines, aliquots of 2×10<sup>5</sup> cells of clone 25, clone 40, clone 9 and clone 24 were induced with Sendai virus (10 plaque-forming units/ml) or mock induced. Induction was carried out as previously described (Nourbakhsh et al., 2001). Human IFN was measured using an antiviral assay on FS4 cells (Nourbakhsh et al., 1993).

## Results

### Integrated S/MARs associate with the nuclear matrix region

It is known that DNA fragments containing S/MAR sequences bind to the nuclear matrix whereas non-S/MAR sequences are detected to a greater extent on the loop portion (Kramer et al., 1998; Kramer and Krawetz, 1996; Schmid et al., 2001; Nickerson, 2001). Accordingly, it has been suggested that these S/MARs are anchored on the nuclear matrix (Kramer and



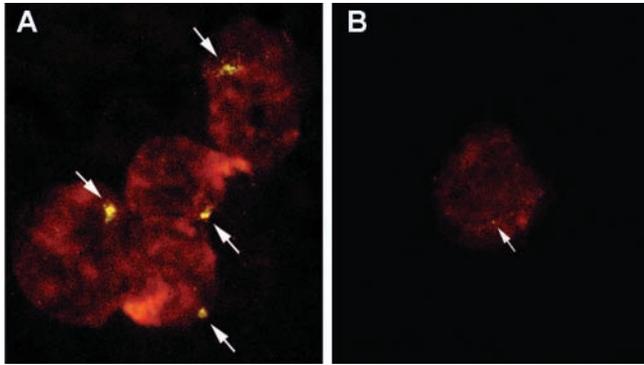
**Fig. 1.** The loop formation patterns of the lambda phage insert and the S/MAR-*IFNBI* insert. Integrated lambda loops (A) and the integrated *IFNBI* construct loops (B) visualized with FISH probes to compare the loop size. (A) The lambda phage insertion (an example of non-S/MAR integrated construct) is shown as a green color that illustrates a large extended loop; the red color distinguishes the nuclear matrix. The integrated lambda loop detected as large released loops seems to lack endogenous MARs, and the fortuitous genomic MARs adjacent to the integrated sites are probably serving as anchoring sites. (B) The S/MAR-containing *IFNBI* construct is shown as red and green signals (two-color FISH) in loop patterns closely resembling the endogenous loop pattern. The darker DAPI staining region in (B) represents the nuclear matrix and the lighter blue surrounding area illuminates the loop region. The *IFNBI* construct displayed considerably smaller loop sizes than that of the lambda phage insert even though the total size of the insert was the same as the lambda insert. This is most probably due to the availability of S/MAR sequences within the *IFNBI* construct.

Krawetz, 1996; Heng et al., 2001). To test this tenet specifically, comparisons between integrated S/MARs and non-S/MARs were undertaken using a direct visualization approach.

We have previously shown that the 40-copy tandemly inserted lambda phage DNA formed a large irregular chromatin loop along meiotic chromosomal cores (Heng et al., 1994b). This represents an example of a lack of anchors necessary for regular loop formation. The large size of the phage DNA loop is thought to be due to the absence of S/MAR anchors. To examine whether a phage insertion forms a similar pattern of irregular loops in mitotic nuclear matrices, somatic halos were prepared from the same transgenic mouse line harboring the lambda phage. As expected, the lambda inserts formed irregular large loops (Fig. 1A). In most cases, the length of the loop varied between the different halos. These irregular loops were significantly larger than the endogenous loops that defined the DNA halo. The insertion of phage DNA into the host genome without S/MAR sequences probably reflects the fortuitous use of a limited number of endogenous S/MARs that are adjacent to the site of integration. In comparison, stable transfected cell lines of similar sized S/MAR-containing *IFNBI* constructs formed normal-sized regular loops indicating that the S/MARs were used as anchors (Fig. 1B). This supports the view that S/MARs are necessary for the formation of loops and that loop size is dependent on the distribution of the anchor sequences.

### Integrated S/MAR fragments are tightly anchored on the nuclear matrix

To verify that the S/MAR sequences detected on the nuclear

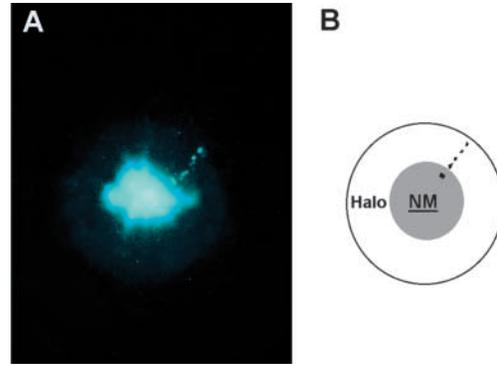


**Fig. 2.** FISH detection of inserted S/MARs (from the human IFN- $\beta$  locus) following DNase I digestion of the halo, demonstrating different nuclear matrix association patterns. The yellow FISH signals of the S/MAR anchor sequences show that they are tightly bound to the nuclear matrix and are therefore protected from nuclease digestion. (A) A 100-copy S/MAR-containing insert following DNase I digestion that displays multiple FISH signals in a pattern of many yellow dots. Because the halo or loop portion has been digested away, this pattern indicates that many but not all the S/MAR copies were tightly bound and were positioned to serve as loop anchors (indicated by arrows). (B) The FISH signal of a single-copy insert following DNase I digestion displays a single yellow spot localized to the nuclear matrix (indicated by the arrow). The great majority of single-copy S/MAR-containing inserts were detected on the nuclear matrix, whereas multiple-copy S/MAR-containing inserts all had a portion of the S/MARs associated with the nuclear matrix as well as detected on the loop. This illustrates that, regardless of the number of copies, those sequences that are anchored to the nuclear matrix are indeed strongly anchored and resistant to DNase I digestion.

matrix were tightly associated with the nuclear matrix and functioned as anchors, halo preparations of each stable transfected cell line were subjected to mild digestion with DNase I (deBelle et al., 1998; Cai and Kohwi-Shigematsu, 1999). In this manner, the free portion of the loop was degraded whereas the portion of DNA that was tightly bound to the nuclear matrix would essentially remain intact. The results presented in Fig. 2B shows that single-copy S/MAR sequences were tightly bound to the nuclear matrix and protected from DNase I digestion as they appeared as a tiny spot following FISH detection of the S/MAR sequences. This is consistent with their use as anchors, indicating that most single-copy, anchor-containing insertions strongly associate with the nuclear matrix. When nuclei containing multi-copy S/MAR constructs were digested with DNase I, many S/MARs were tightly bound to the nuclear matrix and protected from digestion as they appeared as a cluster of spots (Fig. 2A) on the nuclear matrix. There were approximately 10-20 copies as judged by the area and the intensity of the FISH signals. Compared with FISH signals from the interphase nuclei of the same cell line without halo preparation and digestion, the FISH signals were significantly reduced because a large portion of the inserts was digested away.

Not every copy of a S/MAR is used as an anchor or participates in expression

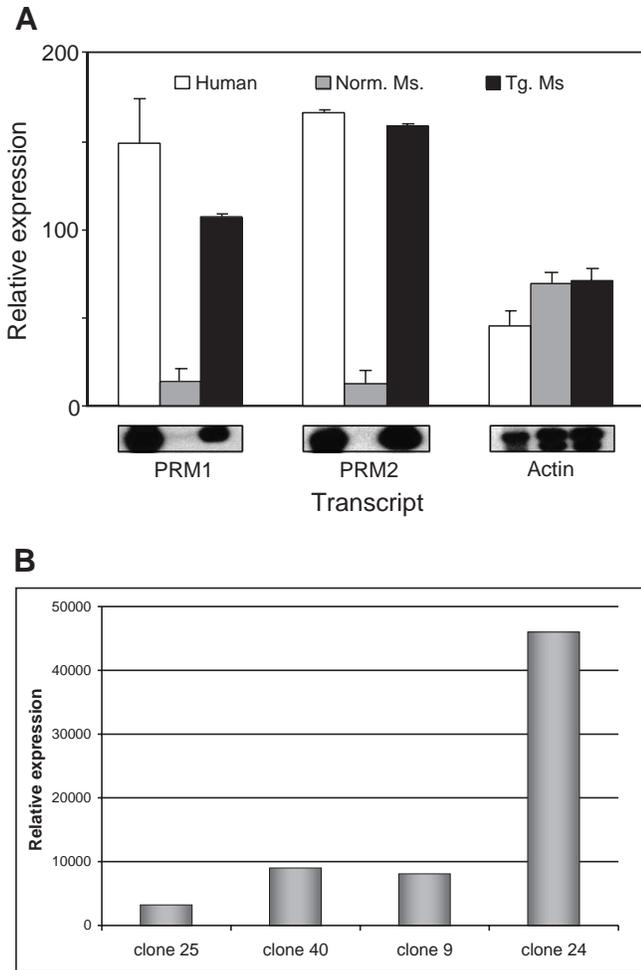
If S/MAR sequences associate with the nuclear matrix and act



**Fig. 3.** The distribution of a multi-copy S/MAR-containing transgene on somatic loops as detected by FISH. (A) The majority of green FISH signals representing non-anchoring S/MAR sequences are located on the chromatin loop and not the nuclear matrix (NM) region. There is only one FISH signal that appears to be attached to the nuclear matrix. If all inserted S/MARs anchor on the nuclear matrix, all the FISH signals should be restricted to the nuclear matrix region. (B) Diagram of (A) illustrating the position of the nuclear matrix, the chromatin loops and the FISH signals. FISH analysis clearly demonstrated that the transgenic S/MARs were present in both the loop and nuclear matrix regions demonstrating that, when multiple S/MAR copies are available in tandem fashion, they do not all associate with the nuclear matrix.

as anchors causing loops to form then, in principle, each S/MAR could serve as an anchor and form a new loop. We have effectively tested this premise by analyzing the distribution of human S/MARs with respect to the nuclear matrix that we have inserted into the mouse genome. Several varied-copy-number lines of a head-to-tail tandemly arrayed transgene of a 40 kb segment of human chromosome 16 containing the multigenic PRM1 $\rightarrow$ PRM2 $\rightarrow$ TNP2 locus were created (Choudhary et al., 1995; Stewart et al., 1999). Each 40 kb segment contained two haploid specific S/MARs bounding the ends of a haploid expressed multigenic domain. The 3' end of the domain was further flanked by a single somatic S/MAR of an independently linked constitutively expressed domain. The head-to-tail array provided at least nine 40 kb regions that were bounded on each side by a somatic S/MAR (Choudhary et al., 1995; Kramer and Krawetz, 1996). Thus, if each of the somatic S/MARs served as an anchor, then every copy of this 40 kb segment should form a 40 kb loop, bounded by a pair of regions attached to the nuclear matrix. This should be observed as a number of small 40 kb loops where each copy of the somatic S/MARs should be detected on the nuclear matrix.

Surprisingly, FISH analysis (Fig. 3) showed that the transgenic somatic S/MARs were present in both the loop and nuclear matrix regions. Most of the inserted somatic S/MARs appeared on the loop and were not anchored to the nuclear matrix. However, at any given time, a subset of the S/MARs-encompassed regions was anchored. This showed that biochemically and biophysically identical S/MAR sequences do not equally participate in the formation of a chromatin loop *in vivo* even if sufficient spacing is provided for a chromatin loop to form. A similar pattern was also observed from sperm halos of the same transgenic mouse. This tenet was confirmed when the relative expression of the multi-copy transgenic PRM1 $\rightarrow$ PRM2 $\rightarrow$ TNP2 locus was assessed by northern



**Fig. 4.** (A) Expression results of the transgene by northern analysis: RNA was isolated from human, non-transgenic and transgenic mouse testes analyzed by northern analysis using PCR products specific to the human PRM1, PRM2 and conserved  $\beta$ -actin transcripts as probes. Autoradiographic images were quantitated and the mean integrated intensities standardized to the 28S ribosomal RNA. The relative level of each of the PRM1, PRM2 transcripts in the transgenic mice were similar to that observed for human testes, indicating that only one copy of the transgene was being expressed.  $\beta$ -actin, which hybridizes to both the mouse and human transcripts, served as the control. (B) Comparison of relative IFN- $\beta$  production and the copy number of transgenes. The IFN- $\beta$  protein level was determined by an IFN test. Four transfected cell lines with 2, 2-4, 20 and 100 copies of transgenes respectively, showing the non-linear relationship between the expression level and the number of insertions.

hybridization analysis using RNA isolated from human, non-transgenic and transgenic mouse testes. These results are summarized in Fig. 4A. As shown, the relative levels of each of the human PRM1 and PRM2 transcripts in the transgenic mice were similar to that observed in human testes. When multiple MARs are present, only one copy of the transgene is expressed when the construct has been passed for several generations. Thus, it is apparent that identical sequences can either be used as anchors or exist on the loop portion as non-anchors.

This apparent transformation among anchors or even

**Table 1. Patterns of distribution of inserted S/MARs detected by FISH**

Cell line analysis	Copy number of integrated S/MARs	Distribution pattern/frequency (NM only) (NM & loop) (loop only)		
		(NM only)	(NM & loop)	(loop only)
Clone 25	2	90 $\pm$ 2.8		10 $\pm$ 2.8
Clone 40	2-4	66 $\pm$ 5.7	19 $\pm$ 1.4	15 $\pm$ 7.1
Clone 9	~20	28 $\pm$ 8.5	68.5 $\pm$ 7.8	3.5 $\pm$ 0.7
Clone 24	~100		100	

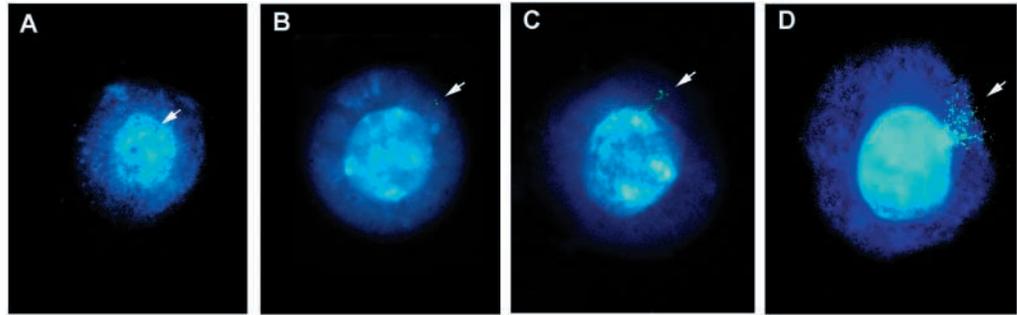
The table shows the distribution patterns and frequency of different integrated constructs. For single copy integrated S/MAR constructs (1-2 copies), the majority of the cells (90%) had insertions detected on the nuclear matrix, while in the remaining cells (10%) the insertions were detected on the loop. Integrated tandem arrays with multiple copies (20 and 100) showed that few inserts were detected exclusively on the matrix or loop as seen with single copy inserts. These arrays were typically seen distributed on both the nuclear matrix and the loop. These data suggest that the S/MARs are selectively used regardless of the copy number. For single integration, not every copy was detected as an anchor; for multiple integration, not all copies were detected as anchors.

200 halos were scored for each of the four lines. The actual images depicted in the distribution pattern/frequency column referred to as 'NM only', 'NM & loop', and 'loop only' can be located in Fig. 5A, C and D, and B, respectively. NM, nuclear matrix.

between anchor and non-anchor sequences of S/MARs would then appear to be dependent on functional requirements. These observations strongly suggested that a regulatory system exists within the cell to direct the manner in which S/MAR sequences are used. This might be S/MAR mediated or require a yet-to-be described trans- or cis-element like SATB1 (Choudhary et al., 1995; Kramer and Krawetz, 1996; Alvarez et al., 2000).

To elucidate the principal components underlying this interesting observation, a systematic analysis of the effect of S/MAR copy number on stably integrated constructs was undertaken. Cell lines with single- and multiple-copy insertions of the well-characterized S/MAR-*IFNB1* region were constructed (Bode and Maass, 1988). Both non-tandem, inserted, single-copy and tandem, inserted, multiple copies were included to compare the anchor association patterns. DNA halos were then prepared from the 1-2, 2-4, ~20 and ~100 S/MAR copy lines (Table 1), then probed with a single copy of the construct. As shown in Fig. 5, different patterns were observed, reflective of the number of copies of the inserted S/MAR. For example, when two copies of the S/MAR-*IFNB1* construct were independently integrated, the majority of the S/MAR-specific FISH signals were closely associated with the nuclear matrix of the host cell. This indicates that, at low copy number, the insertion is primarily located in the nuclear matrix. As summarized in Table 1, the majority (i.e. >90%) of all halos examined use the available S/MARs as specific nuclear matrix anchors. However, in approximately 10% of the cases examined, the S/MARs were not associated with the nuclear matrix but appeared in the loop as defined by the halo. This indicates that, at any given stage, while the majority of single integrated S/MARs were associated with the nuclear matrix, these same integrated S/MARs were able to disassociate from the nuclear matrix.

**Fig. 5.** Distribution patterns of the S/MAR-*IFN $\beta$*  insert detected by FISH. (A) Two independently integrated copies are clearly visible by FISH detection as two light spots within the nuclear matrix (indicated by the arrow). (B) Two independently integrated copies are clearly visible by FISH detection as two light spots on the loop portion (indicated by the arrow). (A,B) Even though the majority of single-copy integrations were detected on the nuclear matrix, some (a minority) were detected on the loop. (C) Approximately 20 integrated copies visualized by FISH detection as a large cluster of light blue spots located at the nuclear matrix and loop regions (indicated by the arrow). (D) Approximately 100 integrated copies visualized by FISH detection. The FISH signals of the S/MAR in the 100-copy construct were localized to both the nuclear matrix region (large light blue spot) and the loop region (multiple light spots). (C,D) Many multiple copy integrations serve as anchors (matrix location) whereas others do not (loop location). These data suggest that S/MARs are selectively used as anchors.



In comparison, when multiple copies of the S/MAR construct were tandemly integrated, FISH signals were detected on both the nuclear matrix and the loop in the same nucleus. This shows that some, but not all, copies of the identically inserted S/MARs serve as anchors attached to the nuclear matrix (Table 1, Fig. 5). The visualization of the 100-copy S/MAR construct indicated that significantly more copies were associated with the nuclear matrix than with the single-copy integrant (Fig. 5D). A much lower percentage of the available S/MARs were actually used in accord with the above observations for the PRM1→PRM2→TNP2 domain. Interestingly, among the halos, the length of the loops was constant and the number of loops was fewer than 100. As above, the loop size was much larger than would have been expected if every S/MAR of the 100-copy construct had been used as an anchor. This indicated that one S/MAR was anchored on the nuclear matrix for every few copies of S/MAR inserted.

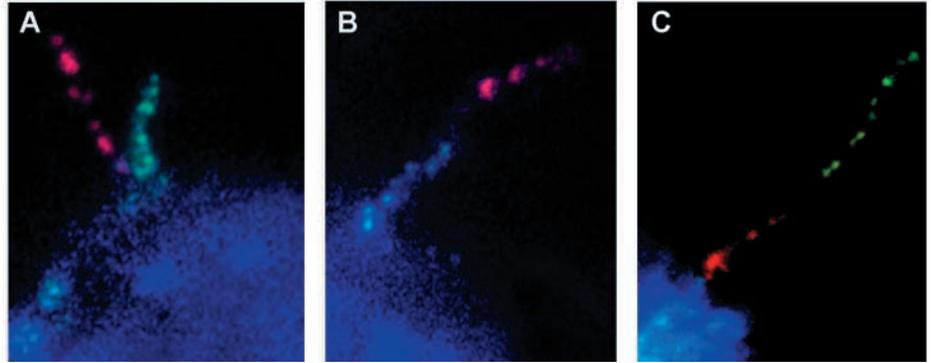
This observation was confirmed using simultaneous two-color FISH (Fig. 1B) by differentially labeling various segments of the construct with a unique color (Heng et al., 1994a; Heng and Tsui, 1994). The 'red' and 'green' FISH signals correspond to different regions of the construct (S/MAR - *IFN $\beta$* ). If one loop consisted of only one copy of the construct, each loop would have a uniform color concentrated on either the matrix or the loop. However, each color was detected on the nuclear matrix as well as the loop. The alternating mixed pattern shows that the loop might contain several identical tandem copies of the construct. Clearly, those S/MAR sequences detected on the loop do not serve as anchors. The same conclusion can be reached by the DNase I digestion experiment with nuclei halos containing multiple copies of S/MAR constructs (Fig. 2A). For the line with 100 inserted copies, approximately 10-20 copies remain after digestion. This indicates that a small fraction of anchors are associated with the nuclear matrix at any given time when large numbers of multiple copy transgenes are tandemly inserted. Utilization in this manner was also supported by our analysis of many unrelated transgenic lines with genomic fragments. In general, when only one copy was inserted, it was usually detected within the nuclear matrix region. When multiple copies were inserted in a tandem fashion, only a proportion of these fragments were detected on the nuclear matrix region. These observations indicate that, when multiple

copies of S/MARs are present, the S/MARs are used in a selective and possibly discriminatory manner. A large-scale study is in progress to test whether this is a universal phenomenon.

The correlation between the copy number of integration and expression level was also investigated by testing relative IFN expression using these cell lines with various copy insertions of the S/MAR-*IFN $\beta$*  region (Table 1). Following infection with Sendai Virus, the IFN- $\beta$  protein level was measured and standardized via a protein standard. As shown in Fig. 4B, the integration number and the level of expression were not in a linear relationship. For example, with 20 copies of inserts, the level of IFN production for clone 9 was similar to clone 40 with 2-4 copies. The 100-copy clone 24 produced IFN- $\beta$  only 5-6 times higher than both the 2-4-copy and 20-copy constructs, suggesting that the majority of inserts were not expressed when tandemly integrated. Interestingly, the expression level of these cell lines correlated well with the anchoring patterns demonstrated by halo-FISH analysis. As shown in Fig. 5C, for the 20-copy cell line, there were only a few copies detected on the nuclear matrix, therefore they had a similar expression to clone 40, which had 2-4 copies detected on the nuclear matrix. For the 100-copy line, since there were only 10-20 copies detected on the nuclear matrix while the remaining copies were detected on loops (Fig. 2A, Fig. 5D), they exhibited only a 5-6-fold increased level of expression compared with clone 9 and clone 40, and only a 10-fold increase in expression when compared to clone 25, which contained only 2 copies.

#### Endogenous chromatin loops move along the nuclear matrix

The multi-copy integrant that contained multiple sets of S/MARs in a reasonably sized insertion can be considered as recapitulating the endogenous condition. As shown above, some but not all of the copies were always associated with the nuclear matrix. The observation that the inserted S/MAR copies did not behave in an equivalent manner might be indicative of the adaptability of S/MAR sequences to serve as anchors when needed. Alternatively, this may simply reflect the spatial limitations of S/MAR-binding sites, since the tandem multiple copies present a locally higher density of S/MARs than is



**Fig. 6.** Two-color FISH showing the loop configuration changes of a 300 kb genomic region. (A) The V-shaped configuration of both red and green color probes were anchored on the nuclear matrix. (B,C) The linear-shaped configuration with only one probe (red or green) anchored on the nuclear matrix whereas the adjacent probe was not anchored on the matrix. The explanation for the configuration changes is that the anchor was not fixed.

typically encountered in the genome. Thus, it could be argued that the potential binding sites are saturated when the S/MARs are arranged in tandem. These alternatives were resolved by determining whether the endogenous anchors were selectively utilized and whether loop regions move along the nuclear matrix.

We examined a large contiguous 300 kb genomic region from human chromosome 8. It was reasonable to expect that several endogenous S/MARs would be contained within the region because, on average, one S/MAR is present at every 60-90 kb of genomic DNA (Boulikas, 1995). If the loops are fixed, then loops from different nuclei should display the same configuration when the same sequence on each loop was painted. By contrast, if the loop is a dynamic structure, with the ability to use alternative attachment sites, then one should observe different color configurations for each nucleus. Two adjacent BACs from human chromosome 8 that spanned this region were randomly selected and used as probes. Two-color FISH analysis was used to visualize the configurations of two probes on the chromatin loops. Two probes were biotin and digoxigenin labeled respectively and detected by green (FITC) and red (rhodamin) color. If the loop was fixed on the nuclear matrix, then the same color configuration should be preserved. If the color configuration changes, then this is indicative and consistent with dynamic loop movement. As shown in Fig. 6, the color configuration of these two BAC probes varied among nuclei. The majority of signals (53%) are displayed as V-shaped configuration FISH signals (Fig. 6A), where both green and red signals form the two portions of the loop. Two other configurations can also be detected (Fig. 6B,C). In one case, the green signal is located proximally whereas the red signal is distal. In the other case, the red signal is located proximally and the green signal is distal. These cases respectively represent 20 and 27% of the total of analyzed FISH images. This suggests that the endogenous chromatin loops of this genomic locus are not stationary with respect to the nuclear matrix. By contrast, it has been shown that some sequences (e.g. centromeric and telomeric sequences) and sperm MAR-containing sequences are tightly anchored on their nuclear matrix (Schmid et al., 2001; Ratsch et al., 2002; Sumer et al., 2003). In addition, in transgenic animal models, most (i.e. over 97%) of the insertions are detected on the nuclear matrix. This is consistent with a 'fixed-anchor' characteristic. In accord with the above, it is reasonable to postulate that some S/MAR

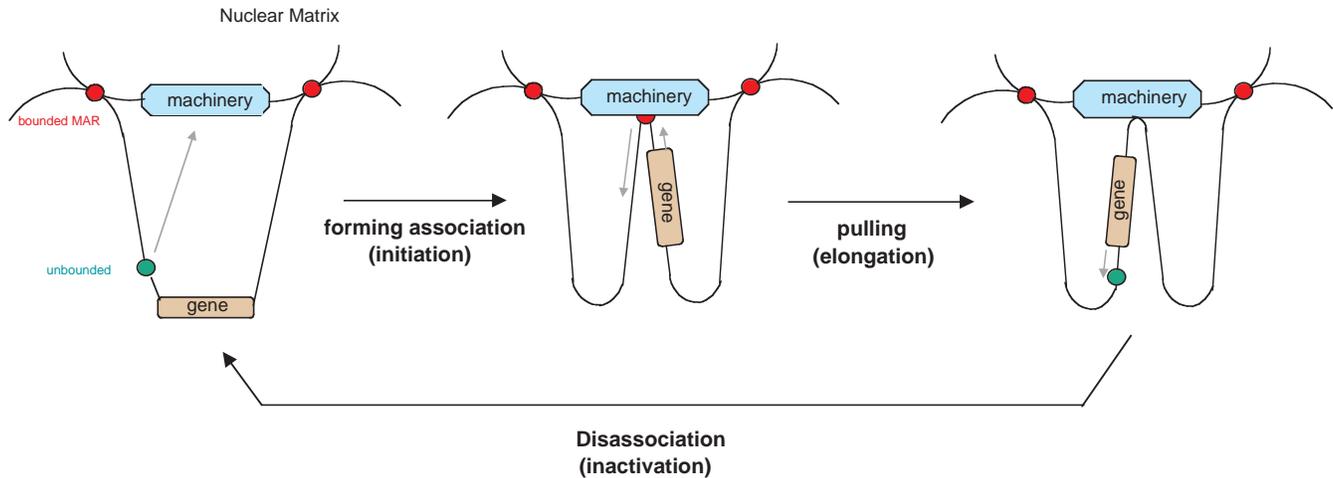
anchors are 'fixed', representing the structural anchors, whereas others are transmutable.

## Discussion

S/MARs are necessary but not sufficient to anchor a loop

The transgenic and transfection studies reported above are consistent with the view that S/MARs are necessary for loop formation for both meiotic and mitotic chromatin domains, but are selectively used. The correlation of the abnormal size of chromatin loops in the absence of S/MARs has been previously documented in meiotic chromosomes (Heng et al., 1994). One could argue that the observation that not every inserted S/MAR copy serves as an anchor simply reflects the 'out-titration' of the 100,000 endogenous binding sites. However, this is very unlikely considering that single-copy integrants that were examined by FISH revealed that 10% of the inserted S/MARs were not associated with the nuclear matrix but were associated with the loop portion. It is clear that a cell has to be tolerant to at least a twofold increase in the number of attachments during the natural course of the cell cycle.

We have previously shown that loop size is constrained according to the chromosomal region (Heng et al., 1996). When the same DNA fragments were integrated into different regions of the chromosome, the loop size was significantly different. Smaller loops were observed towards the telomere, but were observed as larger loops when integrated away from the telomeric position. This indicates that not every anchor was used in the formation of the larger loops. These preliminary observations further showed that, for a given genomic fragment, meiotic loops are longer than interphase mitotic loops, yet loops from metaphase chromosomes were smaller than interphase loops (H.H.Q.H. et al., unpublished). It is apparent that these changes in size require a change of anchors. This loop movement indicates that a switching or changing occurs among the S/MARs, with some S/MARs being selected for attachment to the nuclear matrix whereas others are not. It does not simply reflect the limitation in the number of available S/MAR-binding sites and must be related to the structural and functional requirements of the cell. It is thus reasonable to conclude that S/MARs are necessary to form chromatin loops using S/MARs as anchors but are not sufficient for loop formation; that is, their formation is contingent upon both cellular requirements and the presence of the S/MARs in a



**Fig. 7.** A proposed model for the selective use of S/MAR for transcription/replication regulation. The left panel shows a gene located on the loop with a S/MAR in close proximity. When functional demands require the specific association of this gene with the transcriptional machinery located on the nuclear matrix, the S/MAR moves the gene to the nuclear matrix, thereby initiating gene expression (center panel). Following initiation, the gene is pulled in through the transcriptional machinery, thus completing the process (right panel). There are two types of S/MARs. Functional S/MARs serve as mediators to bring genes onto the nuclear matrix. Structural S/MARs serve as anchors, which are less dynamic compared with functional S/MARs.

given region. The correlation between the number of anchored S/MARs and the gene expression level illustrates the importance of direct interaction with the nuclear matrix. It seems that such anchor-mediated interaction is essential for gene expression.

#### The selective use of S/MARs

The selective use of S/MARs reconciles seemingly contradictory properties attributed to them. Various biochemical studies have shown that in some cases even under 'physiological' conditions, high-affinity matrix attachment sequences were not attached in all populations of cells (Jackson et al., 1996). For example, S/MARs can be electrophoresed from embedded nuclei after DNase I digestion (Eggert and Jack, 1991; Hempel and Stratling, 1996). These results appear contrary to the specificity of S/MARs since one would have expected that high-affinity S/MAR sequences would be exclusively and tightly bound to the nuclear matrix region. As our single-copy S/MAR integrant study has documented, these observations can now be reconciled as reflecting selective and conditional S/MAR specificity.

Interestingly, when the concept of the S/MAR was first introduced it was considered a dynamic structure (Berezney, 1979). However, subsequent debate led to a favoring of the fixed-anchor S/MAR model as early biochemical studies concluded that the attachment sites were the same for both metaphase and interphase cells. This was supported by a series of related observations that the same S/MAR attachment sites were used in different *Drosophila* developmental stages and that the attachment sites remained constant for the Hsp70 heat-shock gene cluster in the control and in the heat-shocked cells (Mirkovitch et al., 1984; Mirkovitch et al., 1988). Together, these observations supported the view that the chromatin fiber is segregated into a series of discrete and topologically independent loop domains that are constrained at their base by their interaction with the nuclear matrix or scaffold.

The dynamic nature of the S/MAR and mobile loops has again re-emerged from recent exciting observations of the *Xenopus* rDNA, Myc and somatic 5S genes (Vassetzky et al., 2000). Using this model system, it has been clearly shown that the rDNA, Myc and somatic 5S genic domains anchor specifically to the nuclear matrix when the chromatin domains are actively transcribed. Similarly, following T-cell activation, specific DNA sequences become anchored on the nuclear matrix, altering the chromatin loop structure (deBelle et al., 1998; Cai and Kohwi-Shigematsu, 1999). Recently, the molecular characterization of chromatin insulators reveals the involvement of insulators in loop domain formation (Gerasimova et al., 2000). For example, the gypsy insulator in *Drosophila* was found to cause two sequences located in different regions of the nucleus to come together at a single site positioned peripherally in the nuclei, presumably associated with the nuclear membrane and involved in anchoring loops (Gerasimova et al., 2000).

By experimentally inserting S/MARs in tandem to create new loops, we have now demonstrated that some but not all S/MARs serve as anchors even though all of the S/MARs that were inserted were identical at the primary structural level. They possessed the same biochemical and biophysical properties, as well as identical *in vitro* binding activity to a host of nuclear matrix proteins. These observations strongly suggest that a regulatory system exists within the cell to manage how S/MAR sequences are used. It should be emphasized that our transgenic mouse and transfected cell line models represent a case of tandem repeats and additionally the state of the native sequence. Short tandem repeated arrays of transgene have been reported to form heterochromatin and silence gene expression (Henikoff, 1998; Garrick et al., 1998). Caution is needed when the tandem repeats are used to study the genome structure. According to expression data and the chromosomal morphology analysis, the heterochromatinization is not the case for our tandem inserted 40 kb transgene. Evidence for

the mechanism revealed by this study has recently been provided for the human  $\beta$ -globin gene cluster (Ostermeier et al., 2003).

#### A dynamic chromatin domain model using a S/MAR-mediated mechanism

The chromatin loop domain is an integral component of the transcriptional regulatory mechanism associated with the nuclear matrix (Gasser and Laemmli, 1987; Berezney et al., 1995; Jackson et al., 1996; Nardozza et al., 1996; Jackson, 1997; Hart and Laemmli, 1998; Stein et al., 1999; Razin, 2001; Heng et al., 2001). The selective use of S/MARs appears to be directly linked to the movement of the loops. This provides a key component for the functional mechanism of chromatin packaging and gene regulation. On the basis of the specific, yet flexible, nature of some chromatin loops, as well as the correlation between the number of S/MAR anchors and gene expression profiles, we present the following model (Fig. 7). Similar to the pulling model (Cook, 1999; Lemon and Tjian, 2000; Heng et al., 2001; Bode et al., 2003), immediately prior to transcription or replication, genes destined for transcription or replication located on the loop must move towards the nuclear matrix. This is facilitated by the S/MAR anchor sequence that promotes the association of genes with the nuclear matrix as requisite to initiate transcription or replication. Elongation then occurs and the loop continues to be pulled through matrix-associated RNA polymerase II complexes until the process is complete. This is consistent with the observation that the induction of the *IFNB1* gene coincides with an increased level of binding of a weak S/MAR immediately downstream from the gene (Bode et al., 1995). At the same time, the coding region becomes DNase I sensitive and the flanking S/MARs become tightly bound and thus resistant to DNase I digestion, indicating their strong association with the nuclear matrix (Bode et al., 1995; Kramer and Krawetz, 1996).

It should be noted that, for the purposes of communicating a simple presentation, the long extended loop structure has been drawn in the model shown here. Even though such an extended chromatin loop can typically be observed within nuclear halos after the release of DNA loops, these changes might be difficult to visualize in vivo owing to high-order packaging. The model presented utilizes two essential elements: the dynamic physical association and the duration of the dynamic association. Similar to locus control region (LCR) function based on studies of the globin loci, the length of time that MAR remains bound to the mitotic machinery could determine the 'strength' of MAR binding, which would certainly have an impact on gene expression.

As summarized in the above molecular cytological observations and in accord with previous biochemical observations, a few integrated copies (i.e. one or two) can be transcribed as efficiently as 20 to 40 copies. Although the multi-copy integrants are stably expressed because the matrix anchors select the sequences for transcription, multiple-copy integration does not necessarily lead to higher expression levels (Wirth et al., 1988; Bode et al., 1998; Bode et al., 2000). This was further confirmed when the expression of the multi-copy human PRM1 $\rightarrow$ PRM2 $\rightarrow$ TNP2 transgenic locus was assessed. Even though there are multiple copies of transgenes present within the transgenic mouse, only one copy was expressed

(Fig. 4A). The same conclusion was reached by expression profile analysis using transfected cell lines (Fig. 4B). Thus, as long as the usage of anchoring sites on the matrix is selective and not every inserted copy will be used, such multiple-copy integration does not necessarily lead to higher expression levels corresponding to copy number even though the integrants are stably expressed. This clearly suggests strategies to control and obtain optimized gene transfer/integration procedures for stable high-level and long-term expression.

This work was supported by the start-up fund from the CMMG, Wayne State University School of Medicine to H.H.Q.H.; by the NIH grant HD36512 to S.A.K.; by the Deutsche Forschungsgemeinschaft grant Bo 419/6-2 to J.B.; by Deutscher Akademischer Austauschdienst grant (DAAD) to S.G.; and by the research and development fund from SeeDNA Biotechnology for C.Y. Appreciation is conveyed to the editorial reviewers of *J. Cell Sci.* for suggestions.

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