Differential nuclear scaffold/matrix attachment marks expressed genes

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It is well established that nuclear architecture plays a key role in poising regions of the genome for transcription. This may be achieved using scaffold/matrix attachment regions (S/MARs) that establish loop domains. However, the relationship between changes in the physical structure of the genome as mediated by attachment to the nuclear scaffold/matrix and gene expression is not clearly understood. To define the role of S/MARs in organizing our genome and to resolve the often contradictory loci-specific studies, we have surveyed the S/MARs in HeLa S3 cells on human chromosomes 14–18 by array comparative genomic hybridization. Comparison of LIS (lithium 3,5-diiodosalicylate) extraction to identify SARs and 2 M NaCl extraction to identify MARs revealed that approximately one-half of the sites were in common. The results presented in this study suggest that SARs 5′ of a gene are associated with transcript presence whereas MARs contained within a gene are associated with silenced genes. The varied functions of the S/MARs as revealed by the different extraction methods highlights their unique functional contribution.

INTRODUCTION

Within the nucleus chromosomes occupy distinct territories (1) from which actively transcribing genes may extend and loop into structurally distinct interchromatin compartments (2–4). Considerable evidence has accumulated to suggest that the topological constraints required for looping are provided through the association of discrete regions of the genome with the nuclear scaffold/matrix (5) at scaffold/matrix attachment regions, or S/MARs. The nuclear scaffold/matrix provides an anchor for higher order genome structure that is more than a simple mechanical organizer. Trans-factors including topoisomerases (6) are often found in association with the nuclear scaffold/matrix. Working in conjunction with other chromatin modifiers they may actively promote chromatin restructuring to reduce torsional stress and activate processes, or conversely, condense and silence various chromosomal segments (7). In this manner, the nuclear scaffold/matrix is dynamically modified during the cell cycle to serve a continuously changing role.

Regions of the genome attach to the nuclear scaffold/matrix in both a cell type and cell cycle context specific manner (8,9). Although the precise mechanism(s) await determination, S/MARs exhibit varied functions that include augmenting transcription (5,10), insulating genic domains (11–13) and facilitating replication (14,15). For example, the positions of MARs of the human β-globin locus are arranged to specifically facilitate developmentally ordered transcription/repression (16). Induction of gene expression at the mouse T-helper 2 cytokine locus is correlated with a local increase in the total number of MARs as they form a series of small loops (17). These and other single-gene locus association studies hint at the importance of the nuclear scaffold/matrix in domain remodeling to permit transcription. However, several loci focused studies of S/MARs on chromosome 16 (6,10,18,19), as well as extended mammalian studies of megabase size genomic regions (20) have yet to reach a consensus.

Transcriptionally active regions as well as regions undergoing replication (21) are segregated into 50–200 kb looped domains through their dynamic association with the nuclear scaffold/matrix. The varied functions of the S/MARs as revealed by the different extraction methods highlights their unique functional contribution.

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scaffold/matrix (22–25). Since attachment of the genome to the nuclear scaffold/matrix is dynamic and contextually dependent, the exact number and locations of genomic attachment sites remains contentious. It has been estimated that ~64 000 S/MARs divide the somatic genome into a series of ~100 kb domains, given that each domain is bounded by an S/MAR at each end. However, only a subset of potential S/MARs may be active in a cell at any given time. Changes in the activity of these sites may provide a means to modulate phenotype (8). To date, few S/MARs have been identified and this has hampered efforts to develop meaningful biological models. Attempts to identify S/MARs in silico are largely refractory to sequence analysis often yielding over predictive models (reviewed in 26).

Several protocols have been engineered to isolate DNA tethered to the nuclear scaffold/matrix away from freely extended loop DNA. The most widely used methods rely on LIS (lithium 3,5-diiodosalicylate) or 2 M NaCl to isolate what are viewed as different types of attachment sites. LIS appears to disrupt binding mediated through transcription complexes (27), yielding the nuclear scaffold, whereas 2 M NaCl has been suggested to isolate a nuclear matrix interwoven with newly synthesized RNA (28). Accordingly, distinct groups of SARs or MARs should be identified by each method.

The literature contains many small-scale S/MAR studies using different isolation methods as well as a variety of cell types. These have utilized both in vivo analysis and in vitro reassociation to study scaffold/matrix association potential. Although in vitro studies have shown that LIS and 2 M NaCl isolate similar attachment sites congruent with structural analyses (29), the differences in attachment in vivo suggest that the nuclear environment plays a larger role than binding potential alone. Indeed, small-scale studies comparing NaCl and LIS extraction-based protocols have been shown to isolate different regions of attachment (30).

To establish the genomic differences between isolation methods and their potential role in gene expression, we have mapped, at the chromosomal level, HeLa S3 MARs and methods and their potential role in gene expression, we have isolate different regions of attachment (30).

The results for chromosome 16 are highlighted as they generalize to chromosomes 14, 15, 17 and 18 (see Supplementary Materials). Nuclear matrices prepared with 2 M NaCl were primarily associated with intergenic gene-poor regions and genes that were attached to the nuclear matrix were silent. Conversely, LIS-isolated SARs were more closely associated with genes with many overlapping the genes themselves. Interestingly, SARs residing within the 5′ proximal region of genes were coupled with higher transcript levels. This first chromosomal-wide survey suggests that SARs and MARs work in concert to mediate genome organization and facilitate expression.

**RESULTS**

S/MARs were isolated by either 2 M NaCl or 25 mM LIS to remove histones thereby enabling unconstrained DNA to diffuse away from the nuclear scaffold/matrix forming a peripheral halo (Fig. 1A). The halo of unconstrained loop DNA was released from the scaffold/matrix-bound DNA by EcoRI restriction enzyme digestion then separated by sedimentation. The total amount of DNA recovered after restriction digestion in both the loop and scaffold/matrix fractions was similar. Subsequent DNase I digestion can reduce the scaffold/matrix fraction to 10% of the estimated total genomic DNA (data not shown). The differential hybridization of the loop and scaffold/matrix fractions were compared using the Nimblegen Systems human whole-genome CGH array system (CGAR0150-WHG8CGH array 7) yielding chromosome-wide profiles of nuclear scaffold and nuclear matrix association.

**MAR-mediated chromosomal looping correlates with gene-dense regions**

Normalized signal ratios of the loop to scaffold/matrix signal from all aCGH probes for each extraction method from duplicate independent experiments were calculated. Replicates maintained high correlation coefficients (HeLa LIS: $r = 0.859$ and HeLa NaCl: $r = 0.610$) compared with randomly permuted signal ratios (LIS: median: $r = 0.020$, SD = 0.009; NaCl: median: $r = 0.024$, SD = 0.008). To moderate the impact of residual variance, probe signals for the LIS and NaCl extractions were averaged between replicates then assessed as a function of their position along the chromosome (Fig. 1B). LIS scaffold and NaCl matrix association are indicated by negative signal ratios while positive values indicate loop enrichment. The chromosomal organization with respect to the nuclear scaffold/matrix was compared with G-banding and gene density. At the chromosomal level, the positive correlation of gene density with loop enrichment relative to the NaCl prepared nuclear matrix is clear and accentuated at the telomeric regions. In contrast, the AT rich, gene poor, G banded regions are matrix enriched. Analysis of the LIS nuclear scaffold showed no significant relationship with gene density.

**Identification and global characterization of S/MARss**

Distinct sites of scaffold/matrix attachment were identified using a three tier process to minimize type 1 false-positive error as verified by permutation analysis. The signal was not normally distributed; hence a non-parametric statistic that segregated data analogous to 2 SD above the mean was adopted. Accordingly, only probes exhibiting a signal ratio ($\log_{2}[\text{loop/matrix}]$ and $\log_{2}[\text{loop/scaffold}]$) in the extreme 2.5% of all signals were considered. This level of signal is analogous to an ~70% enrichment or higher in the scaffold/matrix fraction that should resolve a robust and stable set of attachments. Dynamic or transient attachments were also expected to occur, but only in a subset of cells. These regions would likely resolve within the approximately equal portions of loop and scaffold/matrix, represented by signal ratios near zero. They would be difficult to resolve within this background and as expected they show significant variability with respect to PCR validation and were not considered further. To filter spurious outlier signals, additional pairs of probes with a similar signal distribution within 3 kb either
side of the extreme probe were required. Finally, the average signal across each entire restriction fragment was required to be concordant with the primary observation. Upon meeting these criteria the independent biological replicates were compared, resolving 1016 SARs and 775 MARs along chromosome 16. A total of 403 regions of attachment were shared between both extraction protocols (Supplementary Material, Tables S1 and S2 for complete list along with other chromosomes assayed). The veracity of this strategy for identifying regions of attachment from each extraction method was then validated by quantitative real-time PCR as described (31,32). This revealed a high level of concordance between the scaffold/matrix enrichment assessed on the arrays and that measured by PCR (Supplementary Material, Table S3).

Relative distance between each S/MAR can be used to estimate loop size. The uneven spacing of S/MARs encompassing a range of loop sizes across chromosome 16 is illustrated in Figure 2 (see Supplementary Material, Fig. S1 for the other chromosomes examined). Both SARs and MARs show a bimodal distribution consistent with two ranges of loop sizes that may serve different functions as predicted by the interchromosomal network model that also proposes various classes of nuclear attachment (33). The first peak contained S/MARs that were spaced from 87 to 2217 bp apart, yielding a 762 bp average MAR loop and 558 bp average SAR loop. This represented ~17% of all S/MAR bounded loop domains. S/MARs creating these small loops were distributed across the entire length of the chromosome with no preference for either gene-dense or gene-poor regions. The larger peak contained ~78% of the loop domains created by S/MARs that were spaced from ~3.3 kb to ~970 kb apart. This subset yielded an average MAR loop of ~94 kb and SAR loop of ~88 kb.

S/MAR sequence properties

Regions of nuclear scaffold/matrix attachment were examined using RegionMiner (Genomatix Software GmbH, Munich, Germany) to determine general S/MAR sequence characteristics. This analysis included the mapping of S/MARs relative to genes and a host of trans-factor binding sites as well as assessing conservation within the regions identified. Significance of enrichment within the S/MARs relative to the loop-enriched regions (compare Supplementary Material, Tables S1 and S2) was calculated by chi-square at 95% CI (1 df).

MARs tend to be located in intergenic regions (439 of 775 MARs are intergenic) while SARs tend to overlap genes (560 of 1016 MARs are genic). When compared with the loop-enriched regions, the genic/intergenic distributions are statistically significant for both MARs and SARs (P < 0.001). The majority of MARs and SARs contain at least one conserved region of at least 50 nucleotides (576 of 775 MARs and 767 of 1016 SARs). Their frequency is not significantly different from that observed in the loop-enriched regions. Approxi-
mately 51% of the regions that are common between the MAR and SAR data sets overlap genes (207 of 403 regions are genic). All other parameters queried including the AT distribution within the S/MAR fractions were unremarkable.

The distribution of the intergenic S/MARs relative to the nearest gene is summarized in Figure 3 (see Supplementary Material, Fig. S2 for other chromosomes examined). Both intergenic MARs and SARs are similarly distributed from the 5' and 3' ends of all genes reflective of the chromosomal distribution of signal observed in Figure 1. Only a subset of the intergenic S/MARs is located immediately proximal to genes. The median distance to the nearest 5' end MAR was \( \sim 207 \) kb, whereas the 3' end MAR was located \( \sim 126 \) kb away. In comparison, the median distance to the nearest 5' end SAR was \( \sim 169 \) kb, whereas the 3' end SAR was located \( \sim 113 \) kb away. These similarities were reiterated between extraction methods on the various chromosomes analyzed (Supplementary Material, Fig. S2).

Candidate trans-factor S/MAR associations

Lamin proteins are a part of the nuclear matrix (34) and MARs specifically bind to matrix components of the nuclear lamina such as lamin B1 (35). Comparison of S/MARs identified in this study with lamin B1-associated domains, LADs (36), revealed significant but varied overlap. For example, \( \sim 64\% \) of the MARs and \( \sim 51\% \) of the SARs overlap LADs on chromosome 16. However, the level of S/MAR/LAD overlap varies throughout the genome, i.e. chr14: 52/44%; chr15: 41/34%; chr17: 41/26% and chr18: 52/42%. Throughout these regions of overlap, there appear to be clusters of S/MARs within LADs.

The distribution of several known nuclear scaffold/matrix trans-factors or associated families within both the MAR and SAR regions was assessed in silico and compared with that observed in the loop fraction. These included sites for AT-binding factor, CTCF, p53, SWI/SNF-related nucleophosphoproteins, SATB, SOX/SRY-sex/testis determining and related HMG box factors, GC box factors SP1/GC, STAT, Y-box binding transcription factors and YY1. Interestingly, these binding sites were not enriched within the S/MAR fraction when compared with the loop fraction as might have been expected. Of note, both SARs and MARs have a significantly reduced number of CTCF binding sites when compared with loop-enriched regions \( (P \leq 0.001) \), suggesting that CTCF is not a key player in S/MAR function within HeLa cells as reported in other cases (12).

S/MAR-mediated organization and gene expression

Small-scale studies have suggested that NaCl extracted nuclear matrix preparations will identify MARs that are associated with transcriptionally active regions (28). Similarly, since LIS disrupts transcription complexes (27), the SARs thus isolated were expected to support an indirect function, such as potentiation, to spatially poise rather than direct the interaction of the nuclear scaffold with the transcription factory (37). To test these relationships, the analysis of nuclear scaffold/matrix profiles relative to expressed and silent genes was undertaken.

Transcript profiles were established using the Illumina WG8 v2.0 RefSeq bead array system. This interrogated 2885 genes that were assayed by aCGH on chromosomes 14–18, of which 364 were found to be present at a level above the lowest signal value for the Illumina spike-in controls \( (S_{min} > 3000) \).

As illustrated in Figure 4, gene-dense regions contain both expressed, e.g. MAPK and XTP3TPA, and silent genes, whereas gene-poor regions contain relatively few expressed genes and many silenced genes such as CDH8. The blue chromosomal-wide nuclear scaffold/matrix and green loop
profiles are shown in the upper panel. Black bar regions in the middle and lower panels identify S/MARs relative to the orange genes. In gene-dense regions (middle, the 30–31 Mb region), where many SARs are identified, regions of nuclear matrix attachment are often absent. In contrast, in gene-poor regions (lower, the 60–61 Mb region), where genes are often silenced, NaCl extraction reveals a multitude of nuclear matrix binding sites, whereas SARs are less prevalent.

To assess the local effect of S/MARs on transcript levels, the LIS and NaCl binding profiles from all attachments within as well as immediately 5' and 3' ends of all genes were analyzed by chi-square at 95% CI (1 df) with Yate’s correction for continuity. This estimated the extent to which the measured parameters of S/MAR presence or absence at each 5' and 3' end region could also predict transcript presence. As summarized in Figure 5, correlative transcript level differences with SARs and MARs were revealed. A 5' end SAR located at a distance of up to 10 kb from a gene correlates with expression of that gene, unlike MARs that show no significant correlation when located upstream of a gene. In contrast, the presence of a MAR within a gene correlates with a lack of transcripts, consistent with nuclear matrix attachment-induced silencing. Together, this data suggests that the different types of attachment, as revealed by either LIS or NaCl extraction, work in concert with other factors to modulate expression.

DISCUSSION

Recently, Wang et al. (2008) identified a histone modification module consisting of a combination of 17 modifications that are overrepresented at the promoters of genes that tend to be highly expressed in human CD4+ T cells (38). However, it was cautiously noted that although the genes associated with the module tend to have higher expression, the histone modifications do not uniquely determine gene expression. This supports the notion that the modifications may not be the sole nucleating event in the chromatin remodeling that is seen in preparation for gene expression. There may be other events that coordinately act to regulate structure and poise a gene/locus for transcription including chromosomal looping and scaffold/matrix attachment (6). The distribution of S/MARs identified in this study relative to genes, and the differences observed between those isolated by the different extraction methods, suggests that these interactions can be defined and their effects predicted.

We have shown that gene-dense regions tend to loop away from a 2 M NaCl-prepared nuclear matrix and that this is accentuated at the telomeric regions. In comparison, the AT-rich, gene-poor, G-banded regions are matrix enriched. This is consistent with previous observations of an inverse correlation of matrix attachment with gene density (39) as well as recent genomic analysis of MARs at the human MHC locus (40). This general pattern of organization is reminiscent of gene ridges (41) and open/closed chromatin fibers (42). It appears that at least three classes of interaction consistent with functional classes of attachment have been resolved (8). MARs appeared as peaks of enrichment that were biased towards intergenic regions, whereas SARs exhibited a more even distribution across the chromosome as expected from the global profile of scaffold enrichment (Fig. 1). The subset of SAR and MAR regions that were in common was not biased towards either genic or intergenic regions.

It is expected that restriction digestion will preferentially remove the apex of each loop, leaving the S/MAR attached along with immediately proximal DNA. Estimation of loop size based on the spacing between neighboring MARs and SARs revealed that the majority of MAR and SAR spacing...
is similar to the 86 kb loop size identified using a physiological extraction protocol (25). Of particular interest is the subset of both SARs and MARs that are clustered to create loops in the range of 87 to 2217 bp. The median restriction fragment size is 4.6 kb. However, the sizes of restriction fragments range from 100 bp to 10 kb consistent with the limits observed. Although the subset of small loop domains appears to be evenly distributed along the entire length of each chromosome, there are several regions on the chromosome where two small loops are separated by a single large loop. This organization is consistent with the clustering of S/MARs at the boundaries of a single large loop that would effectively isolate the components of that loop from neighboring DNA.

The validation of some, but not all regions of attachment at 16q21 to a LIS-extracted HeLa cell nuclear scaffold that had previously been identified (19) requires consideration. Interestingly, loss of heterozygosity of chromosome 16 at q21 is a genomic characteristic of many breast cancers. Differences in attachment could indicate inherent instability of this region. SKY analysis of HeLa cells used in this study and aCGH hybridization comparing these cells to a normal human female reference genome (Promega, Madison, WI, USA) revealed chromosome 16 aneuploidy (data not shown) and showed that other chromosomes exhibited aneuploidy and recombination. In accord with the instability of the genome in cell lines that have been extensively passaged (43), the cells are still viable, yet the karyotype differs (44).

Figure 4. LIS and NaCl extractions reveal different profiles relative to gene density/expression. The nuclear scaffold/matrix aCGH profiles in a 30–31 Mb, 16p11.2 gene-rich and 60–61 Mb, 16q21 gene-poor region are shown Log2 loop enrichment is indicated in green and scaffold/matrix association in blue. Regions identified as S/MARs (black bars) were compared with orange: silent and gray: expressed genes (indicated by asterisks). Gene-dense regions show little, if any nuclear matrix attachment and rather are bound to a LIS extracted nuclear scaffold. Gene-poor regions show significant nuclear matrix attachment with fewer-attachment sites to the LIS-isolated nuclear scaffold.
Together, these observations support the view that genome-wide instability resolving as a cell line/culture-specific spatial disruption of the nuclear scaffold/matrix presents as differences in their locations when various studies are compared. This tenet will be addressed when the nuclear scaffold/matrix binding profiles in primary cell lines are determined where genomic instability and prolonged cellular life in long-term cultures are not factors.

The presence of nuclear lamins supporting the nuclear envelope as well as dispersed throughout the nucleus suggests that these proteins may play a major structural role as a part of the nuclear scaffold/matrix. For example, the presence of lamin B1 throughout the nucleus as a part of the nucleoskeleton was recently shown to be necessary for RNA synthesis (45). The specific overlaps observed in this study between S/MARs and lamin B1-associated domains (36) may provide insight into the role of lamin proteins in the expression of our genome. However, the average length of the 37 LADs on chromosome 16 is 1.26 Mb, impeding the direct interpretation of the mechanistic significance.

We have shown that SARs located 5′ end of a gene, within and extending through the proximal promoter region, correlate with gene expression and may have a profound effect on whether a gene is transcribed. In contrast, MARs are generally located in gene-poor regions and at larger distances from expressed genes than SARs. However, the subset of MARs located within genes correlates with their silencing. This suggests that SARs may spatially poise a region of the genome for transcription and/or recruit factors necessary for genomic remodeling in preparation for transcription while attachment to a nuclear matrix as revealed by NaCl extraction may provide a means to restrict transcription.

The elucidation of the chromosome-wide distribution of S/MARs and their correlation with gene expression in HeLa cells has suggested a model of organizational architecture in which SARs and MARs are complementary predictors of whether a gene lies in a silenced or potentiated chromatin state. This supports a model of organization that functions with other architectural elements to bring regions of the genome into intimate contact with the factors that control expression. It is clear that at least structurally, attachment to the nuclear scaffold/matrix contributes to the modulation of gene expression. The S/MARs biologically delineated in this study begin to provide the extended sequence evidence that has frequently been called for and until now, not been available to develop a robust in silico model.

**MATERIALS AND METHODS**

**Isolation and purification of S/MARs and loop regions**

S/MARs and loop regions were prepared by either NaCl (46) or LIS (47) extraction. The optimal extraction time to remove histones and non-matrix nuclear proteins with 2 mM NaCl was first determined as described (46). Nuclear halos were then prepared in solution from isolated HeLa nuclei with either the optimal timed exposure to 2 mM NaCl as determined earlier or by dounce homogenization in the presence of 25 mM LIS as described (47) using ~1 \times 10^7 cells. After extraction, the halos were pelleted at 1000 \( g \) for 5 min at 4°C then washed gently in REact\textsuperscript{3} restriction buffer (Invitrogen, Carlsbad, CA, USA) on a rocker platform for 20 min at room temperature then centrifuged at 1000 \( g \) for 5 min at 4°C then the supernatant discarded. This washing procedure was repeated an additional two times. After the third wash, the halos were resuspended in restriction buffer and the loops separated from the nuclear matrix/scaffold-associated DNA by digestion with 400 U of EcoRI (Invitrogen) at 37°C for 3 h. Subsequent to restriction digestion, the matrix/scaffold fractions were pelleted by centrifugation at 16,000 \( g \) for 5 min at 4°C and the loop containing supernatants were removed and placed in separate tubes. The matrix/scaffold fractions were resuspended, and then washed in restriction buffer, immediately pelleted at 16,000 \( g \) for 5 min at 4°C and supernatant discarded. The nuclear matrix/scaffold containing pellet fraction was washed an additional two times. Both loop and matrix/scaffold restriction fragments were then freed from any nuclear proteins by overnight digestion at 55°C with 50 \( \mu \)g/ml of proteinase K buffered with 50 mM Tris–HCl buffer, pH 8.0, containing 50 mM NaCl, 25 mM EDTA and 0.5% SDS. DNA was recovered and purified using a Quantum-prep matrix (BioRad, Hercules, CA, USA) then resuspended in deionized water.

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**Significance of S/MAR Association with Gene Expression**

|          | 20kb | 10kb | 5kb | 2kb | 1kb | Within Gene | 1kb | 2kb | 5kb | 10kb | 20kb |
|----------|------|------|-----|-----|-----|------------|-----|-----|-----|------|------|
| SAR      | 0.197| 0.008| <0.001| 0.027| 0.013| 0.736| 1   | 0.777| 0.519| 0.914| 0.325|
| MAR      | 0.867| 0.848| 0.356| 0.729| 0.859| <0.001| 0.139| 0.147| 0.954| 0.97 | 0.89 |

**Figure 5.** SAR and MAR correlations with gene expression. The presence of S/MARs encompassing 20 kb region 5′ and 3′ of a gene, as well as within genes was compared with transcript presence using chi-square analysis (95% CI, 1 df with Yate’s correction). The resulting \( P \)-values are displayed for each measurement with significance <0.05 indicated in bold. A SAR within 10 kb of the 5′ end region of a gene significantly correlates with gene expression. In contrast a MAR within a gene correlates with silencing of that gene. Direction of transcription is indicated by the arrow.
Verification of fractionation and CGH hybridization

The separation of loop and matrix/scaffold DNA was assessed by real-time PCR analysis as described (16). Regions previously shown to be matrix-associated or loop-enriched, including the human β-globin HS3 (16) and protamine 2 (48) regions, respectively, were initially amplified in triplicate from each fraction. Upon verification of expected fractionation, the remaining portions of the samples were utilized to identify loop-enriched and matrix/scaffold-attached fragments along the chromosomes. Purified DNA from each nuclear matrix/scaffold and loop fraction was analyzed using the array containing human chromosomes 14, 15, 16, 17 and 18 (Array 7 of the 8 array set) from the Nimblegen Systems CGAR0150-WHG8 CGH isothermal oligonucleotide array system (Nimblegen Systems, Inc., Madison, WI, USA). These arrays offer a median probe spacing of 713 bp, however, the experiments are limited to the ~4.6 kb median length of Eco R1 restriction fragments. All experiments were replicated in independent preparations.

Identification and confirmation of S/MARs

CGH array data was initially viewed using Nimblescan version 1.9 (Nimblegen Systems, Inc.) for broad validation of similarity between replicates. Dual-channel array data were q spline normalized log2 using the NimbleGen data analysis suite. The normalized ratios of the loop and matrix/scaffold signals were not found to be simple symmetric distributions that could be readily transformed for parametric statistics (i.e. Kolmogorov–Smirnov test for normality failed for all datasets). Hence, rank comparisons were undertaken to assess concordance between biological replicates and non-parametric statistics were employed to establish peaks of enrichment.

Initial PCR validation of the array data suggested that similar signal distribution of additional nearby probes to the signal peaks was able to denote real signal and thus eliminate the effect of false hybridization to single probes and minimize the false-positive rate. Furthermore, the binning of probe signals was found to increase the correlation between replicates in a non-linear manner whereby a significant increase in correlation coefficients is observed up to a bin of 3 kb but levels off thereafter. One might expect that correlation would increase until the point at which the average restriction fragment size is met, as is the case here. By first analyzing the data solely on the basis of neighboring probe similarity as opposed to signal averages within EcoR1 fragments, the effect of outliers within restriction fragments that contain only a single array probe is ablated. Therefore, the regions of significance were identified initially as those probes with a signal ratio in the top 2.5% of the ranked signal. Probes located within 3 kb on either side of the top probes were then analyzed for similar signal distribution with a minimum requirement that at least two were present for inclusion of the region. Restriction fragments containing probes meeting these criteria were then analyzed for consistent signal across the entire fragment. Upon validation of consistent signal for each replicate, consistency was validated by comparing the two independent biological replicates. Regions identified in this manner by both replicate experiments are presented. All S/MAR locations identified are available in Supplementary Material, Table S1. Loop regions for significance comparisons were identified in an analogous manner and are available in Supplementary Material, Table S2.

Eighteen regions of chromosome 16 were randomly selected to ensure an unbiased representation of loop or scaffold/matrix regions for real-time PCR verification as described (16). These regions represented both genic and intergenic segments across the chromosome and included a sampling of both loop-enriched regions and S/MARs as identified by aCGH. All PCRs were performed in triplicate starting with the same concentration of loop or matrix DNA. Initial template was calculated by the KLab PCR algorithm and ratios compared to array data as described (32). The percent enrichment of either loop or matrix relative to the total loop plus matrix template was then calculated. This was compared with the analogous percent total calculated from the independent loop or matrix array signal channel relative to the sum of the loop and matrix signals. For both datasets there was significant concordance between array identification and PCR validation. Those showed discordance between the array data and PCR validation displayed log2 signal ratios near zero, indicating that they are approximately equal parts loop and matrix. All primer sequences and ratios are available in Supplementary Material, Table S3.

Expression analysis

The expression profile of the HeLa cells used for aCGH analysis was determined. Total RNA was isolated using RNeasy (Qiagen Inc., Valencia, CA, USA). The RNA was then amplified using the Illumina RNA amplification system (Ambion, Austin, TX, USA) and 750 ng was used for hybridization to Illumina Sentrix Human-8 v2 Expression BeadChip arrays. Data was analyzed using the Illumina Bead Studio software suite. The average signal for each reporter was cubic spline normalized between chips to derive a standardized expression value. Expressed genes were identified by signal values higher than internal spike-in controls for expression ($S_{min} > 3000$).

Data analysis

Correlation of S/MARs between extraction methods as well as with expressed and silent genes was carried out using several tools including Suite 16 (49) and RegionMiner (Genomatix Software GmbH). When trends were detected, statistical significance was assessed using Sigmap Stat (http://www.ysstat.com) and SPSS (http://www.spss.com). Chi-square analysis was conducted using Sigma Stat to establish the significance of any relationship between a propensity for genes to be expressed and the scaffold/matrix binding evidenced around them. Gene expression status was assigned a value of 1, where maximum expression over the possible reporters for each gene was expressed ($S_{min} > 3000$). All other genes for which expression was below this threshold level were assigned a value of zero. The scaffold/matrix association state of DNA between gene model 5’ end and 3’ end limits was used as the first variable where detection of one or more S/MARs assigned this variable a value of 1 (scaffold/matrix-associated) otherwise...
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SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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