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Mammalian genome organization and its implications for the development of gene therapy vectors

Review Article

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Summary

The transcription of mammalian genes and transgenes integrated into mammalian genomes is regulated at three different levels: the molecular level (comprising the interaction of transcription factors with specific DNA elements), the level of chromatin structure, and the level of nuclear architecture. Transcriptional regulation of integrating gene therapy vectors is only well investigated at the molecular level, few data exist regarding the involvement of chromatin structure, and virtually nothing is known about the involvement of nuclear chromosome- and genome architecture. Therefore, it is not surprising that the expressional behavior of gene therapy vectors after integration is often unpredictable and difficult to improve. This review will outline, after giving an overview of recent results and concepts concerning mammalian genome architecture, how this level of organization might be involved in the transcriptional regulation of integrating vectors. First results will be presented and the implications for future vector development will be discussed.

I. Introduction

The transcription of mammalian genes is regulated at three different levels: the molecular level, the level of chromatin structure, and the level of nuclear architecture. The first level, comprising the interaction of positive and negative regulatory transcription factors with specific DNA elements flanking a gene, has been investigated for many years now and multiple regulatory processes are understood in detail. During the nineties research concentrated also on the second level of gene regulation, i.e. chromatin structure. Gene regulation at this level concerns a plenitude of structures and processes involved in chromatin packaging, ranging from nucleosome-DNA interactions and histone modifications to only rarely understood forms of higher-order packaging of chromatin. Although gene regulation at this level is less well understood, many details, in particular regarding nucleosome-DNA interactions and their dynamic regulation as well as the influences of histone modifications and specific forms of higher-order packaging on gene regulation, were intensively studied and are in the focus of research activities (for reviews see e.g. (Henikoff, 1990; Imhof & Wolffe, 1998; Strahl & Allis, 2000; Varga-Weisz & Becker, 1998)).

In contrast, not very much is known about the question how mammalian nuclear architecture and in particular nuclear chromosome- and genome architecture is involved in transcriptional regulation. Detailed knowledge is still missing. Nevertheless, recent studies gave for the first time clear evidence for a specific mammalian chromosome- and genome architecture and its involvement in functional processes like replication or transcription. In the following paragraphs we will describe the present state of knowledge. The review will concentrate on results obtained with mammalian cells. (Although this review focuses on mammalian nuclear

architecture and a comparison between different eukaryotic taxa would go far beyond the necessary limitations of this review it should be noted that important concepts regarding functional nuclear architecture have been developed in model organisms like yeast and Drosophila (see e.g. (Cockell & Gasser, 1999) and citations therein).

As until recently no clear evidence existed for a specific functional genome architecture in mammals, it was also difficult to investigate its involvement in gene regulation. There is not only a remarkable lack of knowledge in this regard concerning the regulation of endogenous genes. It is even less understood how integrated exogenous sequences, as proviruses or retroviral gene therapy vectors are regulated in the context of functional genome architecture. As transcriptional regulation of gene therapy vectors is only well investigated at the molecular level, few data exist regarding involvement of chromatin structure, and nothing is known about the involvement of nuclear chromosome- and genome architecture it is not surprising that the expressional behavior of gene therapy vectors after integration is often unpredictable and difficult to improve. In the following paragraphs we will outline, after giving an overview about the recent results concerning mammalian genome architecture, how this level of organization might be involved in the transcriptional regulation of integrating vectors. First results will be presented and the implications for future vector development will be discussed.

II. Mammalian chromosome- and genome architecture

Since the end of the eighties it became more and more clear that the banding patterns of mammalian mitotic chromosomes are closely related to mammalian genome organization (Bickmore & Sumner, 1989; Craig & Bickmore, 1993). For example, DNA sequence composition differs between the so-called R- and G- or C-bands. While R-bands are GC-rich, G- and C-bands (the latter contain heterochromatic repeats while G- and R-bands belong to the euchromatin) are AT-rich. Interestingly, the about 10^5 copies of AT-rich LINE-elements within the human genome are mainly found in the AT-rich G-bands, while the about 10^6 copies of GC-rich Alu-repeats are predominantly integrated into the GC-rich R-bands (Dunham, 1999; Hattori et al., 2000; Korenberg & Rykowski, 1988).

More important concerning functional chromosome- and genome architecture is the finding that the bulk of genes localizes to R-bands (Craig & Bickmore, 1993; Hattori et al., 2000). Only about 20% of all human genes are found within G-bands. Strikingly, housekeeping genes are found almost exclusively within R-bands. Correspondingly, R-bands are rich in CpG islands (Craig & Bickmore, 1994). Therefore, one would expect transcriptional activity mainly confined to R-band chromatin during interphase. This is consistent with the estimation that about 97% of the mostly cell type specific G-band genes are inactive in a given cell type (Goldman et al., 1984). It was also well documented that chromosomal banding patterns are related to another interphase function, namely the process of replication. While R-bands harbor early replicating chromatin, G- and C-bands replicate late during S-phase (Dutrillaux et al., 1976;) Camargo & Cervenka, 1982).

Although these findings indicated that the organization of mitotic chromosomes into alternating distinct bands might be closely related to functional chromosome and genome architecture during interphase, clarification of this point was a major problem. Favored models, like the random-walk/giant-loop model, did not predict that interphase chromosome organization is related to the structure of mitotic chromosomes (Sachs et al., 1995; Yokota et al., 1995). Other favored models like the inter-chromosomal domain compartment (ICD)-model (Cremer et al., 1995; Cremer et al., 1993) also did not make clear suggestions regarding this relationship,. Thus, for more than ten years after the discovery that during interphase chromosomes occupy individual territories (Lichter et al., 1988; Schardin et al., 1985), the relationship between the organization of mitotic and interphase chromosomes remained unclear as well as the internal structure of interphase chromosomes and their contribution to a presumable higher-order genome architecture within cell nuclei.

Interestingly, the major impulse that led to recent advances in understanding nuclear genome architecture came from replication labeling studies. Nakayasu and Berezney could show in 1989 (Nakayasu & Berezney, 1989) for the first time that DNA synthesized at specific temporal stages during S-phase localizes to specific nuclear sub-regions. Therefore, pulse labeling with nucleotide analogs at specific S-phase stages results in typical nuclear patterns. During early S-phase, hundreds of small so-called replication foci occupy the nuclear interior. At later stages of S-phase the replication activity within the nuclear interior ceases and replication foci concentrate at the nuclear and nucleolar peripheries. During late S-phase, replication activity is only found within a few large foci, which locate at the nuclear periphery as well as within the nuclear interior. It was mainly believed that these patterns reflect a specific S-phase arrangement of replication proteins and DNA and therefore patterns of pulse-labeled DNA were predominantly investigated during S-phase (from the extensive literature on S-phase replication patterns see e.g. Aten et al., 1992; Aten et al., 1993; Berezney et al., 1995; Manders et al., 1992; O´Keefe et al., 1992).

However, eight years after the first description of these patterns it was published in 1997 that the typical

patterns of DNA pulse-labeled at specific S-phase stages are maintained at other cell cycle stages (Ferreira et al., 1997). This finding indicated that DNA with a specific replication timing occupies specific nuclear positions not only during S-phase. The results further indicated that DNA with a defined replication timing locates to its typical nuclear positions already at telophase/early G1. Therefore, the data implied that a defined higher-order architecture of chromatin with a specific replication timing exists within mammalian cell nuclei independent of the replication process. Furthermore, it was shown that this higher-order organization is related to the banding patterns of mitotic chromosomes. It was concluded that chromosomes re-arrange their banding patterns at interphase into clusters of early- or later replicating chromatin and that alignment of these interphase chromosomes with a particular sub-structure gives rise to the observed higher-order nuclear architecture of chromatin. However, a direct prove for a particular substructure of interphase chromosomes was missing as well as its involvement in other nuclear functions like, for example, transcription.

Double-pulse labeling experiments directly demonstrated for chromosomes 13 and 15 that R- and G-/C-bands are maintained during interphase as distinct domains but are rearranged in cycling cells in a way that R-bands cluster in one part of the interphase chromosome while G-/C-bands cluster in another part, thereby giving rise to a polar sub-structure of the chromosome territory (Zink et al., 1999) (**Fig**. **1**).

Additional double-pulse labeling experiments demonstrated that a polar substructure is generally adopted by interphase chromosomes and that the alignment of the polar chromosomes generates the higher-order nuclear arrangement of chromatin into specific compartments that can be distinguished by their replication timing (Sadoni et al., 1999) (**Fig**. **2**). Furthermore, it was shown that this specific higher-order compartmentalization organizes chromatin not only with regard to the process of replication but also with regard to the process of transcription. Transcriptional activity is confined to the nuclear interior (interior compartment), which is occupied by early replicating R-band chromatin (Sadoni et al., 1999)(**Fig**. **3**). In contrast, no obvious transcriptional activity is found at the nuclear and nucleolar peripheries (peripheral compartments) and minor internal compartments (late replicating compartments) which are occupied by later replicating G-/C-band chromatin. The relationships between mitotic chromosome structure, polar interphase chromosome structure, and the typical nuclear higher-order genome compartments established by the alignment of polar interphase chromosomes are summarized in **Fig**. **4**. The figure also outlines the functional characteristics of the distinct nuclear higher-order compartments regarding the

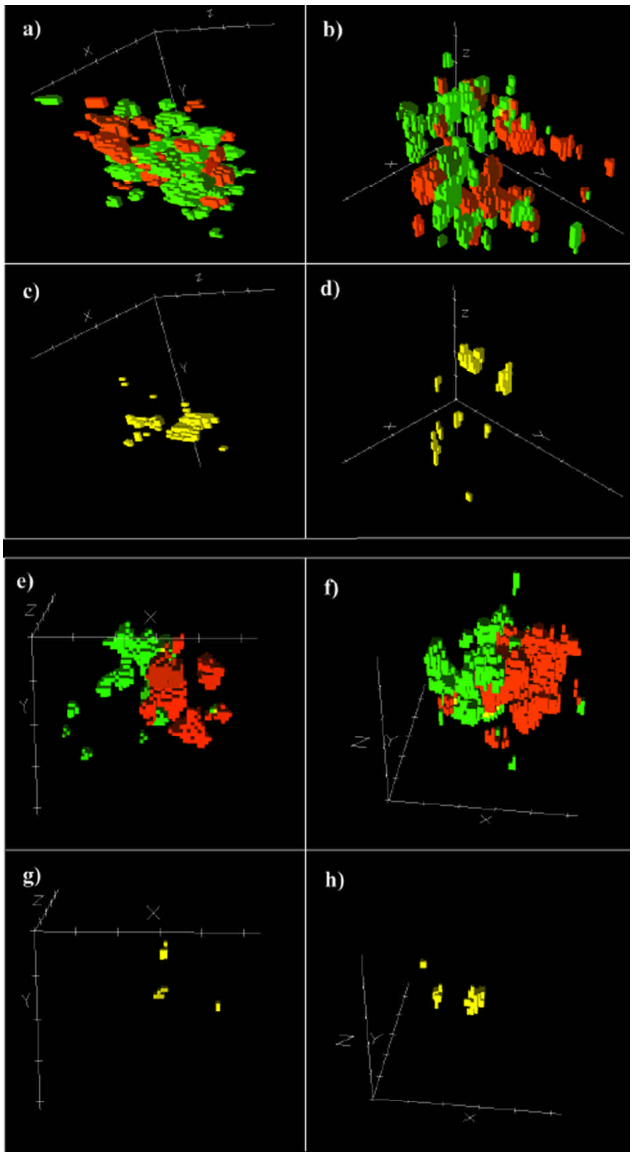

Figure 1: **Three-dimensional structure of chromosome 15 territories**. Early replicating R-band DNA (red) and late replicating G-/C-band DNA (green) of chromosome 15 territories was labeled by double-pulse replication labeling several cell cycles before fixation (for exact procedures see (Zink et al., 1999)). After immunodetection, chromosome territories were scanned by confocal microscopy, segmented and three-dimensionally reconstructed. The squares from which the reconstructed territories are build correspond to the voxels of the original image stacks. The axes correspond to those of the confocal microscope and the ticks denote distances of 1 μm. The figure shows pseudo three-dimensional visualizations of two chromosome 15 territories from quiescent cells (a,b) and two chromosome 15 territories from cycling G1 cells (e,f). The overlap between red R-band domains and green G-/C-band domains is shown in yellow and exclusively (c,d,g,h) below each corresponding panel. Note that R- and G-/C-band chromatin occupies exclusive domains in G0 as well as in G1 chromosome territories. R- and G-/C-band domains display a polar organization (clustering within different parts of the territory) only in G1 chromosome territories (e,f). The figure was reproduced with permission from (Zink et al., 1999).

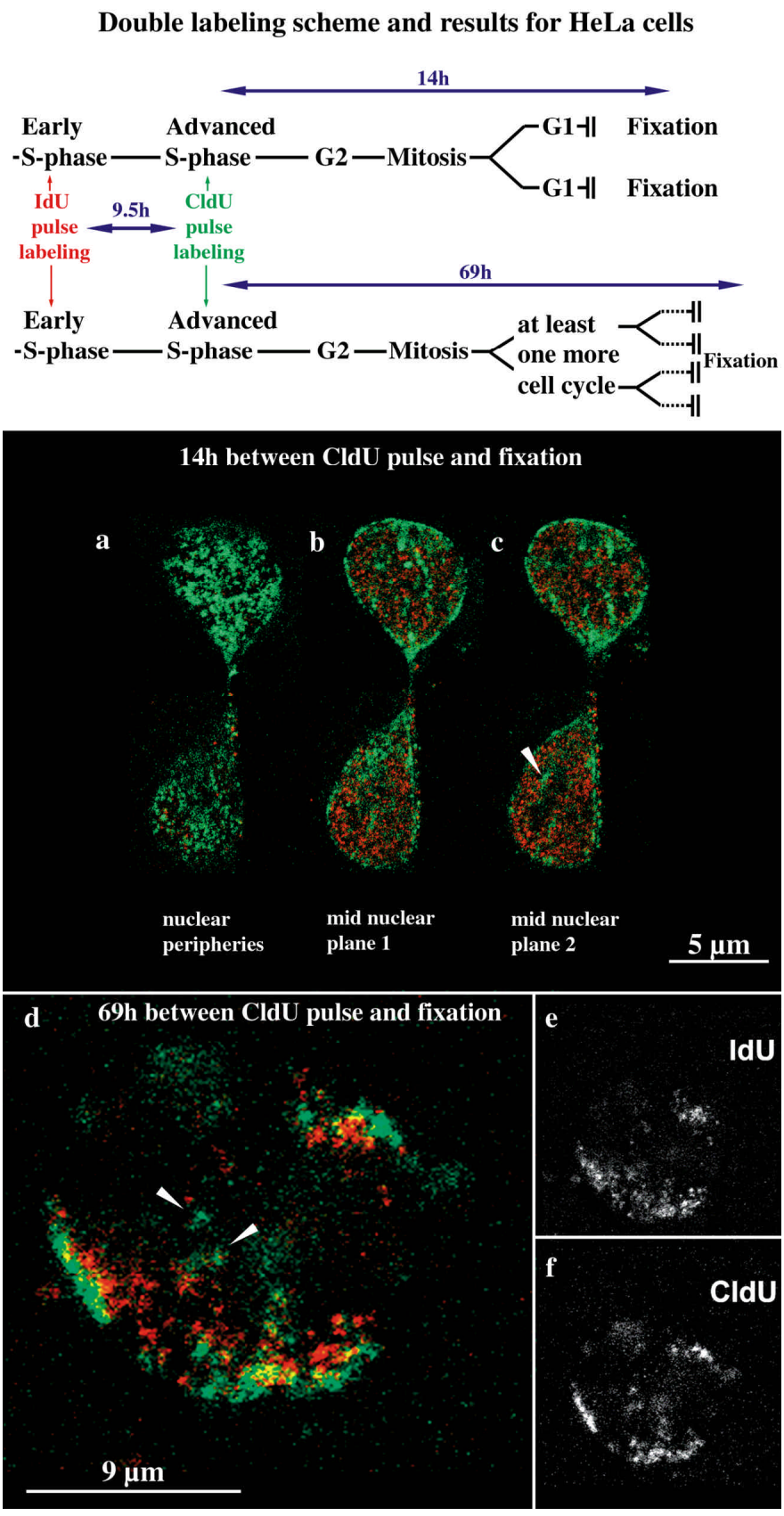

Figure 2: **Establishment of higher-order compartments after cell division and contribution of single chromosome territories to higher-order genome organization.** Synchronized HeLa cells were double-pulse labeled with two different thymidine analogs (Iododesoxy-uridine, IdU, red and Chlorodesoxy-uridine, CldU, green) according to the labeling scheme shown at the top. This double-pulse labeling scheme results in simultaneous labeling of the interior compartment containing early replicating R-band chromatin (IdU, red) and the peripheral compartments containing later replicating G-/C-band chromatin (CldU, green). After 14h the labeled cells went through mitosis and panels a,b,c show three different nuclear planes of two early G1 daughter cells. These early G1 cells already established the higher-order genome organization typical for cycling cells. IdU labeled early replicating chromatin fills the nuclear interior while CldU labeled late replicating chromatin occupies the nuclear peripheries (exclusively visible in the peripheral nuclear planes, a) as well as the nucleolar peripheries (e.g. arrowhead in c). 69h after double-pulse labeling the labeled cells went through at least two cell divisions. Therefore, the nuclei contain a mixture of labeled and unlabeled (not visible) chromosomes (Zink et al., 1999; Zink et al., 1998). Panel d shows a mid-nuclear plane of one cell nucleus with a few labeled chromosome territories (double-labeled "patches"). The polar structure of the single chromosomes is visible and the parts of the territories occupied by CldU-labeld chromatin are oriented towards the nuclear or nucleolar (arrowheads) peripheries while those parts occupied by IdU labeled chromatin localize to the nuclear interior between these peripheries (IdU and CldU label is exclusively shown in e and f). The alignment of these polar chromosome territories with a defined nuclear orientation leads to the higher-order chromatin organization as shown in panels a,b and c. This figure was reproduced from "The Journal of Cell Biology", Sadoni et al.,1999, Vol. 146(6), pp. 1211-1226, by copyright permission of "The Rockefeller University Press".

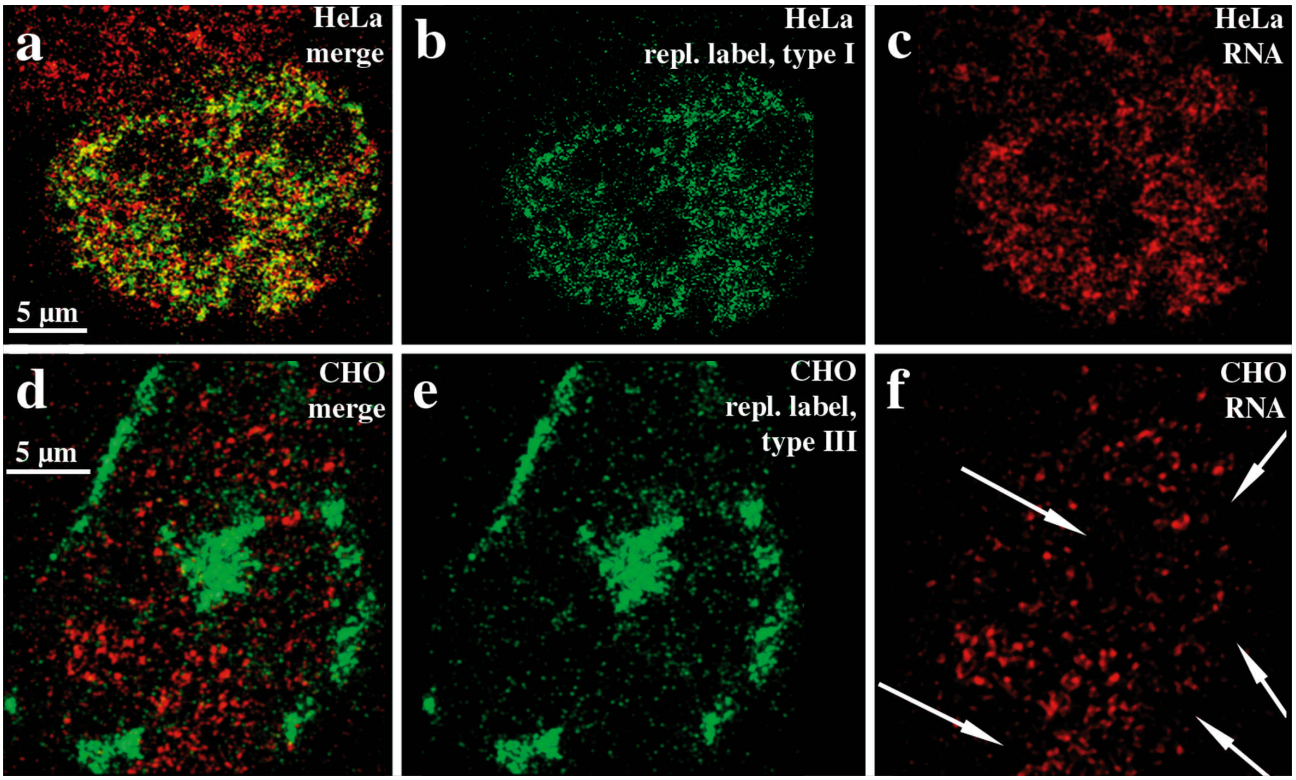

Figure 3: **RNA synthesis is confined to the early replicating interior compartment**. Higher-order genome compartments of HeLa cells (a,b,c) and CHO cells (d,e,f) were marked by replicational pulse labeling (green). A so-called type I pattern (b) highlights the interior compartment containing the early replicating R-bands while a so-called type III pattern (e) labels the peripheral compartments containing the later replicating G- and C-bands (for the distinct labeling patterns and their relationship to chromosomal bands see (Sadoni et al., 1999)). Nascent RNA was labeled with short pulses of BrUTP (red, c,f). The merge of replication labeling patterns and BrUTP labeling patterns shows that nascent RNA synthesis is confined to the interior nuclear compartment (a) and exclusded from the peripheral compartments (d). Arrows in f show that sites occupied by the replication label are indeed devoid of BrUTP label. The nascent RNA label in the left upper corner in a and c stems from an adjacent nucleus which displays no replication label. This figure was reproduced from "The Journal of Cell Biology", Sadoni et al.,1999, Vol. 146(6), pp. 1211-1226, by copyright permission of "The Rockefeller University Press".

processes of replication and transcription and the distribution of highly acetylated isoforms of histone H4.

It was shown that this specific functional higher-order genome architecture, which seems to be highly conserved in mammals, is present during all interphase stages (Sadoni et al., 1999). An elegant study by Dimitrova and Gilbert (Dimitrova & Gilbert, 1999) recently demonstrated that higher-order compartments are established in early G1 in parallel with the determination of the replication timing of comprised chromatin. In addition, it was shown that higher-order chromatin architecture is clonally inherited (Sadoni et al., 1999).

In agreement with these data it was demonstrated for different human cell types that the gene-poor (high proportion of G-bands) chromosome 18 occupies more peripheral positions in cell nuclei while the gene-rich (high proportion of R-bands) chromosome 19 occupies more central positions (Croft et al., 1999). Although it was hypothesized that at least in Drosophila heterochromatic sequences play an important role in nuclear chromatin architecture (Csink & Henikoff, 1996), data obtained with translocations between chromosomes 18 and 19 indicated that the centromeric heterochromatin does not play an outstanding role in chromosome positioning. Rather, sub-regions of the euchromatic chromosome arms seem to localize to corresponding nuclear sub-regions independent from the rest of the chromosome (Croft et al., 1999).

III. How is nuclear architecture integrated with the other levels of gene regulation?

The results described so far raise the question what mediates the nuclear positioning of chromosomes and chromosomal sub-regions. The establishment of the typical mammalian genome compartments (occupying

specific nuclear positions) shortly after mitosis in early G1, the clonal inheritance of this form of genome compartmentalizaton as well as the results obtained with translocation chromosomes (see above) imply that chromosomal sub-regions must contain positional information that mediates their correct nuclear localization. The close relationship between chromosome banding patterns during mitosis, interphase chromosome organization, and nuclear higher-order genome architecture suggests that positional information might be specific for chromosome bands or sub-bands.

Regarding the nature of the positional information it lies unlikely in the DNA sequence as the active and inactive X chromosomes of female mammals adopt nuclear positions corresponding to their functional states (Belmont et al., 1986) although their DNA sequences are similar. This implies that an answer to the question about the positional information might lie in the functional regulation. Indeed, it has been demonstrated that replication timing, transcriptional regulation, corresponding changes of chromatin structure, and nuclear localization are closely related to each other. This applies to the functionally distinct X chromosomes of female mammals, to chromosomal bands and sub-bands in the size range of mega-basepairs (see above) as well as to single gene loci (Dhar et al., 1989; Dimitrova & Gilbert, 1999; Forrester et al., 1990; Hatton et al., 1988; Imhof and Wolffe, 1998). Although the findings show that the different levels of gene regulation are closely linked to each other, the question remains how they are integrated and which is the cause and the consequence of what.

Regarding the question, how gene regulation at the molecular level, chromatin structure and nuclear positioning are linked to each other, one possibility might be provided by regulatory proteins like Ikaros. This protein is a transcription factor in activated lymphocytes and binds to specific regulatory DNA sequences (Lo et al., 1991). Target sequences have been described within many lymphoid-associated genes (Hambor et al., 1993; Molnar & Georgopoulos, 1994). Strikingly, Ikaros localizes to clusters of centromeric satellite DNA (included into the silenced, late replicating compartments described above) in activated or cycling mouse lymphocytes (Brown et al., 1999; Brown et al., 1997). Inactive gene loci associate with these clusters and it was suggested that Ikaros is involved in recruiting genes upon inactivation to these clusters (Brown et al., 1999; Brown et al., 1997). Thus, Ikaros might be involved in both, regulation at the molecular level and nuclear positioning and therefore provides a link between these two levels of gene regulation.

Another way of integrating information between the different levels of gene regulation might be provided by modifications of the chromatin structure. Specific modifications of chromatin structure are closely linked to the molecular level of gene regulation and it is, for example, well established that transcription factors target enzymes to specific gene loci that alter the histone-acetylation status (Grant et al., 1998; Imhof & Wolffe, 1998; Utley et al., 1998). The once established histone-acetylation status is maintained during mitosis and it has been suggested that the histone-acetylation status serves as a cellular "memory" in order to transmit information concerning the activity of gene loci from one interphase into the next (Jeppesen, 1996). Strikingly, histone acetylation patterns also follow the chromosomal banding patterns during mitosis (Jeppesen & Turner, 1993) and are specific for the corresponding higher-order genome compartments during interphase (Sadoni et al., 1999) (see **Fig**. **4**). It is tempting to speculate that, as the positional information carried through mitosis is likely not transferred via the DNA sequence, it is provided by chromosme band and gene locus specific chromatin modifications like histone acetylation patterns. Thus, histone-acetylation patterns and other chromatin modifications might play a multiple role in i) creating specialized structures at the chromatin level suitable for transcription or silencing, ii) conveying cellular memory regarding gene activity and iii) serve as a "tag" for specific nuclear positions. Regarding the latter point, this would imply that the connection between gene regulation at the molecular level and higher-order nuclear architecture is mediated via the chromatin structure.

A close link between chromatin structure and nuclear positioning has been demonstrated with regard to the human β-globin locus (Schübeler et al., 2000). Here, general histone H3/H4 acetylation correlates with a general nuclease sensitivity of the β-globin locus, which is typical for the "open state" of active genes. As studies with mutated loci did show, these changes of chromatin structure (histone H3/H4 acetylation, nuclease sensitivity) occur independent from a functional locus control region (LCR) and correlate with a re-localization of the locus away from the centromeric heterochromatin (included into the inactive, late replicating compartments described above). As in the absence of a functional LCR the β-globin locus is transcriptionally inactive these data show that transcriptional activity is not a prerequisite for re-localization of the locus in a specific nuclear compartment. However, in this case it is not clear whether localization into a specific nuclear compartment is a prerequisite for propagation of an open or closed chromatin structure and histone acetylation or a consequence thereof.

Concerning the relationship between the three levels of gene regulation, they might not reflect a strict hierarchy. As suggested by Schübeler et al. (2000) the different levels might interact in a multi-step process. According to this model, specific *cis*-acting elements in the β-globin locus other than the LCR are responsible for establishing an open/acetylated chromatin configuration in conjunction with the specific nuclear localization. This

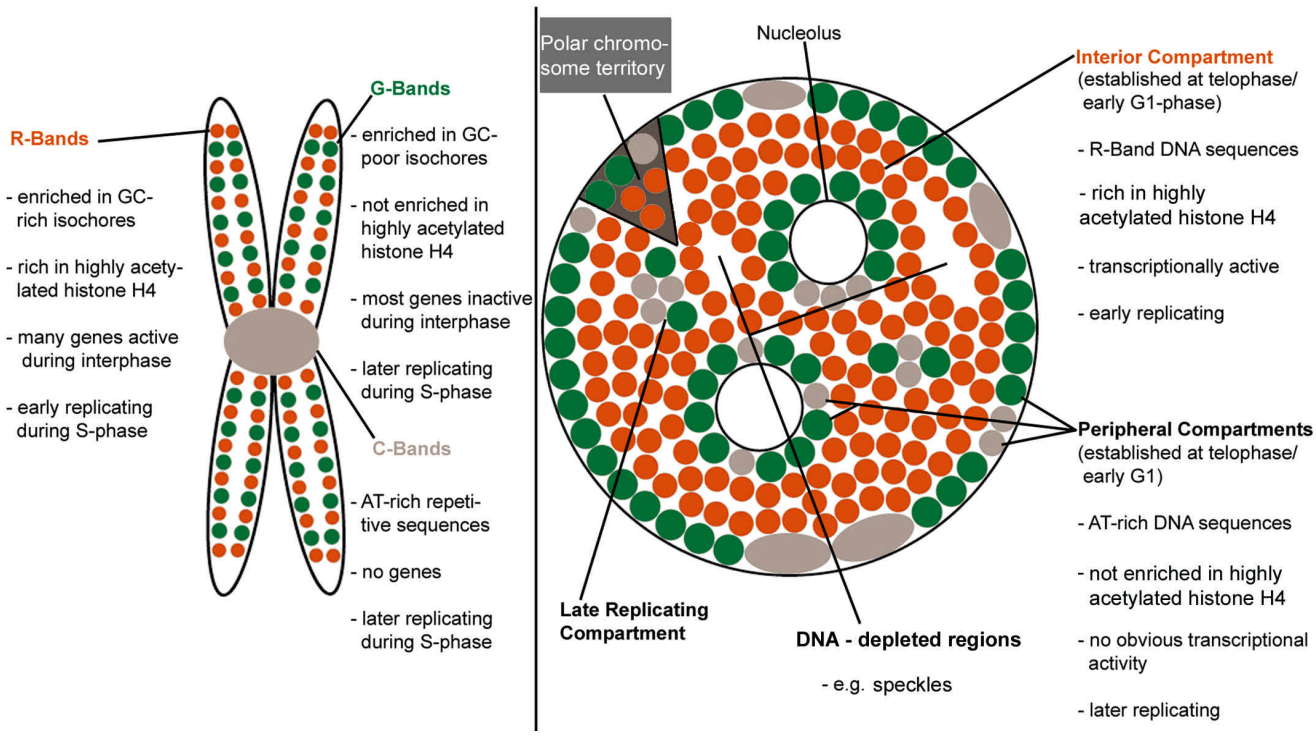

Fig. 4: The scheme depicts the higher-order organization of mammalian genomes during mitosis and interphase. The well characterized bands of mitotic chromosomes give rise to distinct higher-order functional compartments within the cell nucleus. Distinct bands of mitotic chromosomes differ in a variety of features as isochore composition and corresponding DNA sequence composition (Bernardi, 1995; Bickmore & Sumner, 1989; Craig & Bickmore, 1993), gene content (Bernardi, 1995; Bickmore & Sumner, 1989; Craig & Bickmore, 1993; Cross et al., 1997), acetylation levels of histone H4 (Jeppesen & Turner, 1993), transcriptional activity of genes (Craig & Bickmore, 1993; Craig & Bickmore, 1994) and replication timing during interphase (Camargo & Cervenka, 1982; Dutrillaux et al., 1976). Differences in DNA sequence composition ((Sadoni et al., 1999) (Manuelidis & Borden, 1988; O´Keefe et al., 1992; Rae & Franke, 1972)), acetylation levels of histone H4 (Sadoni et al., 1999), transcriptional activity (see Fig. 3) and replication timing (Ferreira et al., 1997; Sadoni et al., 1999) of chromatin targeted to distinct nuclear compartments demonstrate the functional features of these compartments and their relation to genome organization revealed by banding patterns of mitotic chromosomes. R-band sequences (symbolized by red dots) localize to the interior compartment while G- and C-band sequences localize to the peripheral and late replicating compartments (symbolized by green and grey dots). Higher order nuclear compartments are build up by chromosome territories displaying a polar distribution of R-band DNA and G-/C-band DNA (see Figs. 1 and 2). Speckles occupy chromatin-depleted regions within the interior compartment (see (Sadoni et al., in press) and Fig. 6). This figure was reproduced with minor changes from "The Journal of Cell Biology", Sadoni et al.,1999, Vol. 146(6), pp. 1211-1226, by copyright permission of "The Rockefeller University Press".

LCR independent pre-activation step would be followed by a LCR dependent local change in chromatin structure and gene activation.

In addition, it is tempting to speculate whether gene regulation at the molecular level, chromatin structure and nuclear architecture are not only required at specific points of an integrative multi-step process but also play specific roles in the establishment and maintenance of defined functional states. In this sense, chromatin structure and nuclear positioning might rather play a role in "freezing" and maintaining established states, while transcription factors might impose flexibility on the system if necessary. In this regard it is interesting to note that nuclear re-positioning of silenced genes in murine lymphocytes occurs only if silencing becomes heritable (Brown et al., 1999). Also an intriguing study by Francastel et al. (1999) supports this view. Here, the authors integrated a reporter construct under the control of the 5'HS2 enhancer into different sites of the genome of K 562 erythroleukemia cells. They found in accordance with earlier studies that an intact enhancer counteracts position dependent silencing. In accordance with the studies described above (Brown et al., 1999; Brown et al., 1997; Schübeler et al., 2000) active constructs localize away

from centromeric heterochromatin. Mutational analysis demonstrated that the same enhancer motifs were required for both suppression of transgene silencing and localization of the transgene away from centromeric heterochromatin. In addition, binding of transcription factors to core enhancer sequences increased the stability of an active chromatin structure as assessed by DNase I and methylation analysis. Interestingly, this study revealed that transcriptional activity per se does not influence the localization of the transgenes but that the nuclear position corresponds to the stability of the expressional status. These data support the notion that nuclear positioning might be involved in "freezing" an established state while the interaction of transcription factors with corresponding DNA elements can serve to establish specific functional states and to switch them (high levels of expression at normally repressive loci).

In addition, the study by Francastel et al. (1999) revealed that the chromosomal integration sites possess nuclear addresses that are related to their ability to suppress transcriptional activity. For example, an integration site where the transgene was also stably active in the absence of a functional enhancer was always at a position distant from centromeric DNA, suggesting that this was its "default address". In contrast, more repressive loci moved closer to the centromeric DNA in the absence of a functional enhancer. In the light of these results it is obvious that a promising strategy for obtaining transcriptionally active transgenes is to integrate them into chromosomal sites that possess a "default address" for the active nuclear compartment (see chapter IX). From the previous chapter it is clear that these are the chromosomal R-bands.

IV. Involvement of nuclear organization in functions specific for cycling cells

Providing broader chromosomal regions where genes with similar states of activity cluster (as for example the R-bands where the constitutively active housekeeping genes cluster) with a default "address" for the suitable nuclear compartment might help to efficiently organize the genome after mitosis. It should be considered that in the case that each single gene locus must be sorted out individually after mitosis the cell nucleus would have the difficult task to sort about hundred-thousand loci (diploid nucleus) in a short time period (after the highly energy consuming process of mitosis during which a cell cannot exert its functional duties a cell should be ready for function again as soon as possible). Individual sorting of each locus would also be sterically difficult and might lead to an entangling of DNA fibers that would be challenging to handle during the next mitosis. Therefore, providing broader chromosomal regions harboring many genes with a "default address" and thereby facilitating genome dynamics associated with cell division might be one reason why chromosomal bands evolved. Indeed, so far no good explanation exists for the fact that on mammalian chromosomes specific genes (and other specific sequences) cluster within chromosomal bands. A default sorting mechanism for the bulk of genes does not exclude, of course, that under special conditions single gene loci within a band might switch their "address" by mechanisms discussed above.

Indeed, it should be considered that gene expression is only one of the manifold genome-associated functions and that higher-order architecture might also play an important role in other processes. The results described above demonstrated that higher-order genome architecture is closely linked to the process of replication and chromosome organization during mitosis. Replication and mitosis do not occur in quiescent and senescent cells. Strikingly, higher-order genome architecture seems to be "relaxed" in quiescent and/or senescent cells: R- and G-/C -bands do not show a polar organization within chromosome territories (Zink et al., 1999) (**Fig**. **1**), the nuclear positions of chromosome territories 18 and 19 become similar and chromosome 18 is located at more central positions (Bridger et al., 2000), accordingly, late replicating chromatin occupies also central nuclear positions (**Fig**. **5**) and silenced genes do no longer contact clusters of centromeric DNA (Brown et al., 1999). These differences to cycling cells suggest that higher-order genome architecture plays a prominent role in functions specifically associated with the cell cycle.

Remarkably, although overall nuclear higher-order architecture is relaxed in non-cycling cells, the distinct band domains of chromosomes are maintained (**Fig**. **1**)(Zink et al., 1999). This result demonstrates that expressed and silenced gene loci, which cluster in the distinct chromosomal bands do not intermingle in quiescent cells but are still organized into distinct higher-order domains. As from the genome-associated functions mentioned above only transcription is maintained in non-cycling cells, chromosomal band domains might play a role in creating suitable environments for transcription or silencing. It should be kept in mind that chromosomal bands are in the size range of mega-basepairs and that the nuclear domains created by these bands display diameters in the size range of several hundred nanometers and are well resolvable by light microscopy. Therefore, as these domains are already of considerable size, it might only for transcriptional regulation not be necessary to build up from these domains the huge higher-order compartments typical for cycling cells (**Fig**. **4**). As stated above, this even higher level of genome organization might become important if the number and complexity of associated processes increases.

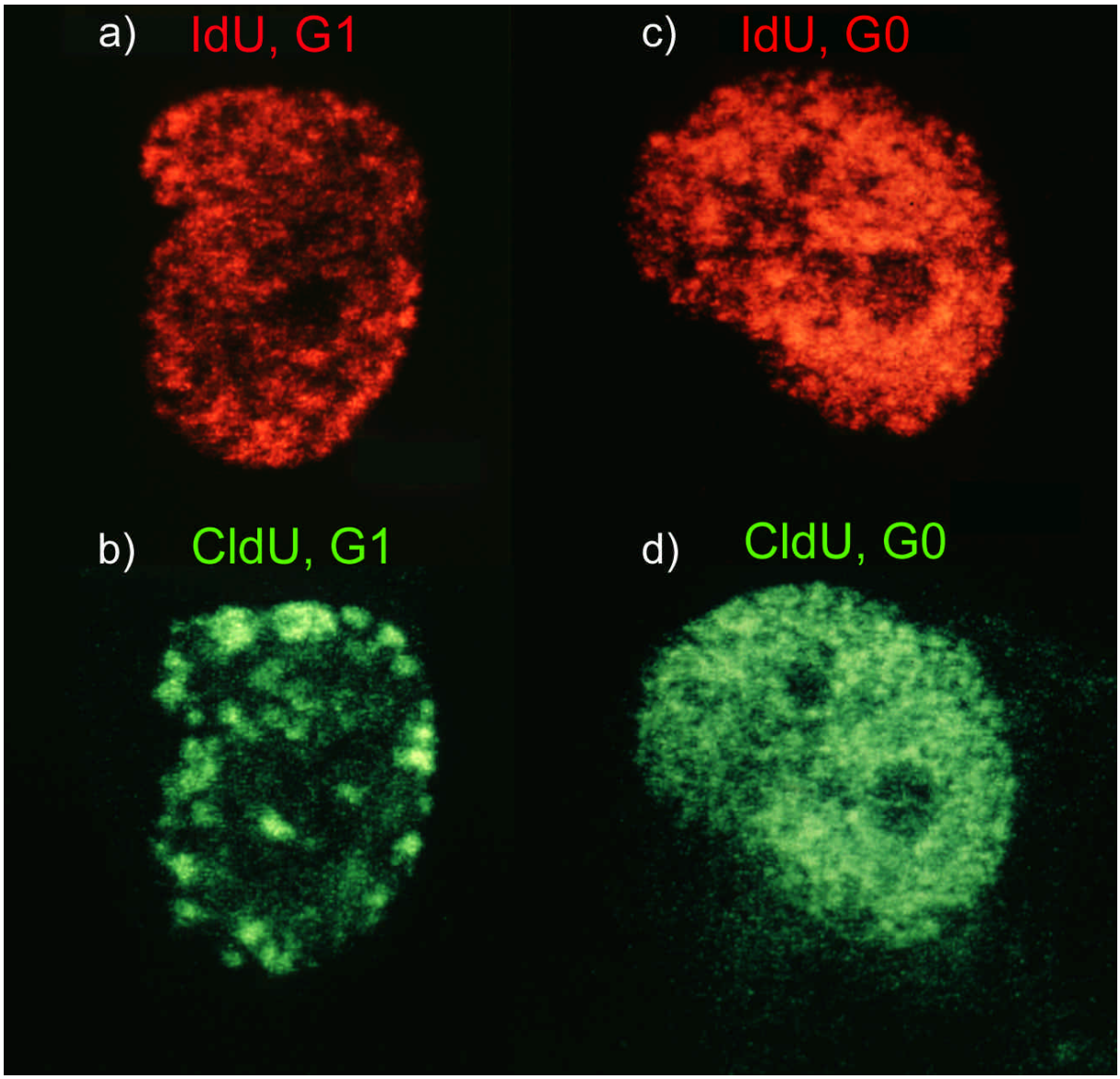

Figure 5: Nuclear genome organization is "relaxed" in quiescent cells. Early R-band and later replicating G-/C-band DNA of human diploid fibroblasts was labeled with two 30 min pulses of IdU (red, first pulse) and CldU (green, second pulse) with a chase period of 6h between the two pulses. The cells were examined after one mitosis in G1 (a,b) or G0 (c,d). Note that the later replicating chromatin (b,d) is no more confined to the peripheral compartments in quiescent cells but is distributed throughout the nuclear volume (d). This is in accordance with the fact that chromosome territories loose their polar structure in G0 (compare Fig. 1). Nevertheless, although the huge higher-order compartments are disorganized in quiescent cells, the chromatin is still confined to distinct band domains (compare Fig. 1).

V. How dynamic is the architecture of mammalian genomes?

Regarding cycling cells, the finding that the nuclear higher-order architecture established in early G1 is maintained during all stages of interphase (Sadoni et al., 1999) implies that mammalian genomes are not very dynamically organized. The most dynamic process associated with mammalian genomes is mitosis. Nevertheless, recent data obtained with live cell microscopy suggest that dramatic rearrangements of chromosomal domains do not occur during mitotic prophase (Manders et al., 1999). This is in accordance with the finding that R- and G- or C-bands, that alternate on mitotic chromosomes, display a polar organization during interphase. As G- and C-bands are already attached

to the nuclear lamina during interphase, R-bands only have to be retracted into interstitial positions in order to create a mitotic chromosome, which indeed seems to be the case (D. Zink, unpublished observations). These findings also support the idea that the maintenance of band domains and their polar organization in cycling cells facilitates mitosis (see above). Accordingly, also no complicated rearrangements seem to take place after mitosis. Time-lapse series of centromeric domains show that they are redistributed at telophase/G1 transition by uniform isometric expansion with little evidence for directed motion of individual centromeres (Sullivan & Shelby, 1999).

Another process, which was expected to be associated with considerable chromatin dynamics is the process of replication. However, also the process of replication does not involve large-scale movements of chromatin. Chromatin occupies already shortly after mitosis those nuclear compartments where it will replicate during S-phase (Dimitrova & Gilbert, 1999; Ferreira et al., 1997; Sadoni et al., 1999). In these compartments the replication factories appear at the appropriate time points of S-phase, locally replicate the chromatin without performing large-scale movements and leave the newly synthesized chromatin in these compartments after performing their task (Leonhardt et al., 2000). Remarkably, the replication factories also do not perform large-scale movements in order to "visit" the distinct genome compartments during S-phase progression. The changes in higher-order nuclear patterns of replication factories during S-phase are due to an asynchronous and constant process of assembly and reassembly of these factories (Leonhardt et al., 2000).

Also time-lapse microscopy of single interphase chromosomes or pericentromeric domains in live cells revealed that large-scale movements are exceptional (Shelby et al., 1996; Sullivan & Shelby, 1999; Zink et al., 1998). Nevertheless, some large-scale movements have been observed and are also suggested by fixed cell studies (the latter are reviewed in (De Boni, 1994)). However, this does not contradict the finding that the overall higher-order nuclear architecture is maintained. If chromosome domains move within a particular compartment or between functionally equivalent compartments (e.g. if a pericentromeric C-band domain moves from the nuclear to the nucleolar periphery) the overall higher-order nuclear architecture would be maintained.

Together the data obtained with cycling cells suggest that large-scale movements of chromosomes or chromosomal sub-domains in cells, which do not change their functional state are rare and slow (for a more detailed discussion see also (Zink & Cremer, 1998)). The kinetics of the movements observed are compatible with the idea that diffusion is the driving force (Bornfleth et al., 1999; Zink & Cremer, 1998) and so far (except for the phenomenon of nuclear rotation in neuronal cells) no chromatin dynamics have been described that could only be explained by the involvement of motor proteins.

So far, no comprehensive data exist regarding the extent of re-arrangements taking place if a cell switches its functional state. Re-localization of single activated and silenced genes is linked to B-cell differentiation (Brown et al., 1997). Whether this occurs during interphase or in association with mitosis has to be determined. At least for quiescent lymphocytes it was shown that the repositioning of gene loci in response to stimulation occurs before cell division (Brown et al., 1999). The kinetics of these movements are not known but these movements seem to take place during a time period of several hours and therefore might also be slow. In contrast, re-positioning of chromosome 18 to the nuclear periphery after stimulation of quiescent cells does not take place before the first mitosis. As re-positioning occurs during G1 after the first mitosis nuclear movements of the chromosome seem to be involved (Bridger et al., 2000).

Regarding the extent of re-arrangements it should be kept in mind that no gross differences could be detected in the interphase higher-order genome architecture of different cell types (Ferreira et al., 1997; Sadoni et al., 1999). A relatively constant genome architecture in functionally different cells is also indicated by the fact that replication patterns are highly conserved (from the extensive literature on S-phase replication patterns see e.g. (Aten et al., 1992; Aten et al., 1993; Berezney et al., 1995; Manders et al., 1992; Nakayasu & Berezney, 1989; O´Keefe et al., 1992)). Therefore, at least with regard to cycling cells that switch their functional state one would expect that this does not lead to gross alterations in overall nuclear genome architecture but that re-positioning rather affects single gene loci with altered functional states.

VI. Anchoring of chromosome domains

The finding that chromosomal domains are predominantly stably positioned raises the question whether there are specific interactions between defined chromosomal domains and other nuclear components that anchor chromosomal domains at specific nuclear regions. This seems to be the case with regard to the nuclear lamina. In mammalian cells the nuclear lamina consists of A- and B-type lamins (lamin C belongs to the A-type lamins) that form a meshwork underlying the inner nuclear membrane (INM) (Stuurman et al., 1998). While B-type lamins are found in all nucleated somatic cells, the expression of A-type lamins is developmentally regulated. In addition to the lamins, a variety of lamin-binding proteins are associated with the inner nuclear membrane (Gant & Wilson, 1997; Gerace & Foisner, 1994). These include the lamin B receptor (LBR) and different isoforms of the lamina-associated polypeptide-1 (LAP 1) and LAP 2. Two proteins related to LAP 2, named emerin and

MAN, also reside at the INM. Loss of emerin causes Emery-Dreifuss muscular dystrophy (EDMD) (Bione et al., 1994).

The INM proteins seem to interact with DNA and chromatin in multiple ways: for example, biochemical studies suggest that the tail domains of lamins bind to DNA and core histones (Burke, 1990; Glass et al., 1993; Taniura et al., 1995). LBR and LAP2 both bind to chromatin in vitro. Particularly interesting is the chromatin-binding partner of LBR, which are the mammalian HP1-type proteins (Ye et al., 1997; Ye & Worman, 1996). These proteins are associated with repressed chromatin and the LBR seems to play a role in targeting membranes to repressed G- and C-band chromatin (Pyrpasopoulou et al., 1996). Therefore, the LBR might be involved in establishing the silenced compartment at the nuclear periphery after mitosis. This might be an alternative mechanism of chromatin positioning in addition to the mechanisms discussed above.

In addition to the LBR, A-type lamins seem to play a role in anchoring the silenced chromatin at the nuclear periphery. Obvious discontinuities in this chromatin layer are present in cells derived from A-type lamin knockout mice (Sullivan et al., 1999). However, it is not clear whether this is due to a direct interaction of peripheral chromatin with A-type lamins or due to the interaction of the peripheral chromatin with other factors, which are mis-localized in A-type lamin mutants. The latter possibility is supported by the following findings: emerin is mis-localized in A-type lamin mutants and an EDMD-like phenotype occurs in mice after ablation of A-type lamin expression (Sullivan et al., 1999). Interestingly, human EDMD in most cases maps to the emerin locus on the X-chromosome (Bione et al., 1994). However, a human autosomal dominant variant of EDMD maps to the lamin A/C gene (Bonne et al., 1999). How defects at the nuclear lamina can cause the EDMD phenotype is still unclear.

Another principle for anchoring chromatin domains within the cell nucleus might be the interaction of so-called scaffold- or matrix-attached regions (S/MARs) with a skeleton of protein cross-ties called nuclear matrix (interphase) or nuclear scaffold (metaphase). It is now generally accepted that the DNA within a mammalian nucleus can be organized into about 60 000 chromatin loops, each representing an independent regulatory unit. The domain organization is brought about by the anchorage of the loop bases to the nuclear matrix via S/MARs. As the properties of S/MARs and the nuclear matrix are extensively reviewed elsewhere (see e.g. (Berezney et al., 1995; van Driel et al., 1995; Bode et al., 1995; Boulikas, 1995; Pederson, 2000)) and as this review focuses on genome architecture above the level of chromatin loops these topics will not be further discussed here.

VII. Interactions between the genome and compartments involved in genome-associated functions

Given the findings that mammalian genomes are not very dynamically organized and that nuclear proteins or macromolecular complexes in many cases localize to confined nuclear compartments (for reviews see (de Jong et al., 1996; van Driel et al., 1995)) the question arises how DNA sequences and other nuclear factors come together in order to interact with each other. One example is already discussed above. In case of replication the replication factors move to the stably localized DNA sequences. However, not the whole factories move but rather their single components assemble into the large complexes called factories at those places where they are needed (Leonhardt et al., 2000). An unresolved question is still how the single proteins "know" where to assemble when.

Regarding splicing factors it is known that they concentrate within the so-called speckles. Although the function of speckles is not clear a favored hypothesis is that they supply sites of active transcription with splicing factors (Huang & Spector, 1996; Misteli et al., 1997; Pombo et al., 1994). Interesting in this regard is the finding that speckles are embedded into the transcriptionally active chromatin within the interior compartment (Sadoni et al., in press). Therefore, transcriptionally active chromatin and speckles are already in close contact. This is in agreement with the finding that transcriptionally active gene loci are in intimate contact with speckles (Xing et al., 1995). Also, the mainly positionally stable speckles can move to sites where transcriptional activity is induced (Misteli et al., 1997).

However, it has also been shown that there is a fraction of active gene loci, which is not in contact with speckles (see (Xing et al., 1995) and citations therein). Furthermore, splicing factors only concentrate into speckles and there is a substantial fraction of these factors, which is more uniformly distributed within the nucleus (see e.g. (Misteli et al., 1997)). The diffusely distributed factors might be sufficient to provide at least a part of the active loci with splicing factors and a closer contact to speckles with a higher concentration of these factors might only be necessary in cases of high transcriptional activity.

Recent studies indicated that the splicing factor ASF/SF2 fused to GFP that is also concentrated in speckles moves through the nucleus with high mobility (Kruhlak et al., 2000; Phair & Misteli, 2000). Regarding the diffusion coefficient of 0.24 $\mu m^2 s^{-1}$ there seems to be no difference between ASF-GFP molecules enriched in speckles and those more diffusely distributed. Although the rate of diffusion is about hundred times slower than expected there seems to be a rapid movement of the proteins through the nucleus that should allow them to

reach every gene locus within a short time period (with the given diffusion coefficient it would take about one minute to move half-way across the nucleus).

A prominent model of nuclear architecture, the so-called inter-chromosomal domain compartment (ICD)-model proposed that proteins and other nuclear components do not move freely through the nucleus but are confined to a network of channels between chromatin domains (the so-called ICD-space) (Cremer et al., 1995; Cremer et al., 1993; Zirbel et al., 1993). As proteins and enzyme complexes would be confined to the ICD-space a consequence would be that DNA sequences determined to interact with these proteins (e.g. transcription factors, splicing factors, replication factors) have to be exposed at the surfaces of chromatin domains. Therefore, surface exposure of DNA-sequences would play a fundamental role in their regulation.

In contrast to the predictions of this model the recent investigations concerning the diffusional motion of proteins did not reveal that the motion of even large protein complexes (about 500 nm in diameter) is hindered by chromatin domains (Kruhlak et al., 2000). Also, studies investigating a general surface exposure of transcriptionally active sequences lead to conflicting results (Abranches et al., 1998; Verschure et al., 1999). As described above, transcriptionally competent or active sequences are organized into specific sub-chromosomal regions but show within these regions no specific exposure to any known surfaces (Sadoni et al., 1999; Zink et al., 1999). The same is true for the organization of DNA sequences with a defined replication timing (Sadoni et al., 1999; Zink et al., 1999). Therefore, the functionally significant predictions of the ICD-model that the interaction between proteins and DNA sequences is facilitated and regulated by channeled diffusion of the former and specific surface exposure of the latter so far have not been confirmed. Rather, the experimental results argue against this scenario.

Also, in living cells no chromatin-free channel system can be observed (data from fixed and/or stained cells should be carefully interpreted as various standard procedures easily can induce or suggest an artificial "space" between chromatin domains (Sadoni et al., in press)). Some chromatin-depleted regions can be observed, which are occupied by nucleoli, speckles or nuclear bodies ((Sadoni et al., in press), see also **Fig**. **6** and **Tables 1 and 2**). Although these regions were regarded as the equivalents of the ICD-space (Cremer et al., in press) the findings might be due to the simple fact that DNA is not a component of all nuclear structures. When using the term ICD-space one should keep in mind that the ICD-space according to its definition is not simply a chromatin-depleted region but that the ICD-space should be involved in specific functional and architectural characteristics of the cell nucleus as predicted by the ICD-model. As described above, recent experimental results suggest that the nucleus might not be organized as predicted by the ICD-model.

In summary, recent data suggest that proteins that are involved in the formation of structures like speckles or replication factories diffuse rapidly and not hindered by chromatin domains through the whole nucleus. Therefore, the relatively static arrangement of DNA sequences should not be a problem for dynamic interactions. The enrichment of specific proteins in defined compartments might facilitate and coordinate the interaction of proteins and the assembly of larger complexes, as well as the temporal control of processes like replication.

VIII. The nuclear localization of integrated viral genomes and gene therapy vectors

Given the findings described above, it is expected that the genomic and nuclear positions of proviruses influence their transcriptional regulation. Therefore, it might be possible that viruses have evolved mechanisms to either preferentially integrate at advantageous genomic and nuclear positions or to modulate the nuclear organization. Evidence for the former was observed for adeno-associated virus (AAV), which preferentially integrates at a position on the q-arm of chromosome 19 (Kotin et al., 1991; Kotin et al., 1990; Samulski et al., 1991). Chromosome 19 is a very gene rich chromosome with a high proportion of R-bands. In accordance, the site of integration was GC-rich and contained an open reading frame expressed at low levels in some tissues (Kotin et al., 1992). The nuclear localization of the integration site did not seem to be important, since transferring the target-site to an extrachromosomally-replicating vector also allowed site-specific integration (Giraud & Berns, 1994). However, it remains unclear, whether site-specific integration indeed provides any selective advantage for the virus over other integration sites.

For retroviruses including the lentiviruses, a large number of different integration sites have been observed (Carteau et al., 1998; Pryciak & Varmus, 1992; Stevens & Griffith, 1996; Withers-Ward et al., 1994), leading to the popular notion that retroviruses integrate randomly into the genome of the host cell. In vitro studies revealed preferential integration at specific positions of nucleosomes (Brown, 1997). In intact cells, frequent integration of murine leukemia virus in the vicinity of DNase-hypersensitive sites (Rohdewohld et al., 1987; Vijaya & Robinson, 1986), CpG islands (Scherdin & Breindl, 1990) or transcribed regions (Mooslehner & Harbers, 1990) suggested preferential integration into the transcriptionally active regions of the genome. Although an initial study provided evidence for highly preferred regions of integration for Rous Sarcoma Virus (RSV) (Shih et al., 1988), a second study concluded that integration of RSV into all genomic regions tested

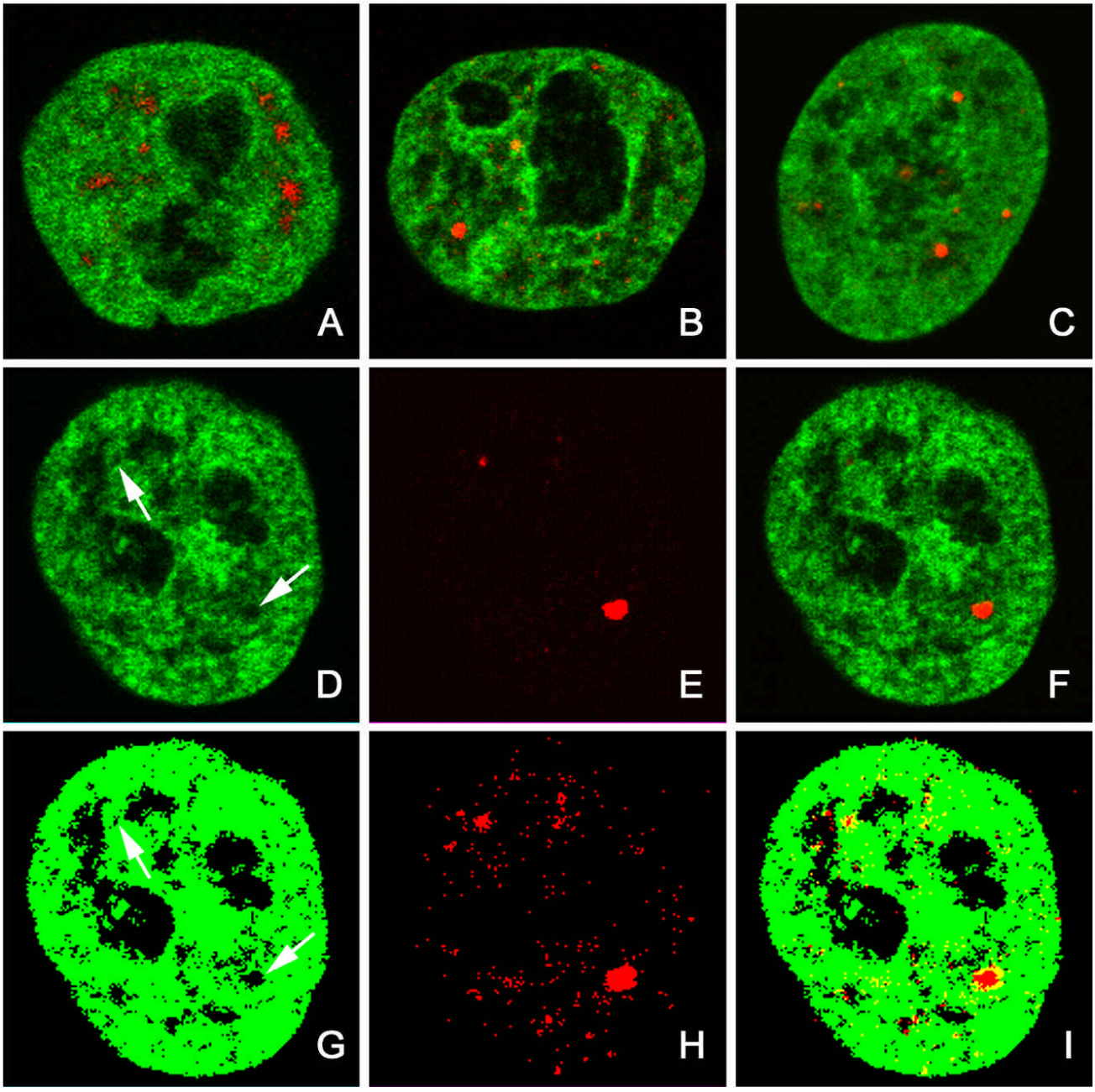

Figure 6: Speckles and nuclear bodies occupy chromatin-depleted areas. Speckles (**A**), coiled bodies (**B, E, F**) and PML bodies (C) were immunostained (formaldehyde fixation, all immunostained structures are shown in red) in HeLa cell nuclei with histone H2B-GFP labeled chromatin (green). Panels **A, B** and **C** show mid-nuclear planes of three different nuclei. The corresponding light optical sections displaying the immunostained speckles or nuclear bodies (red) or the H2B-GFP fluorescence (green) were merged (overlapping red and green appears yellow). The large chromatin depleted regions correspond to nucleoli. Panels **D-F** show light optical sections from an identical mid-nuclear plane displaying the histone H2B-GFP fluorescence (**D**) and the immunostained coiled bodies (**E**). The merge of D and E is shown in **F**. **Panels G-I** show binary images corresponding to the images shown in D-F that were used to determine the degree of overlap for the quantitative evaluation presented in tables 1 and 2. Both coiled bodies detected here were localized in chromatin-depleted regions (arrows in D and G) and displayed peripheral overlap with chromatin (yellow rim around the red coiled bodies in I).

Table 1

A

coiled bodies	>500nm			400-500nm		
nucleus	complete overlap	peripheral overlap	no overlap	complete overlap	peripheral overlap	no overlap
1	0	2	0	6	7	4
2	1	1	0	3	1	0
3	1	1	0	3	2	0
4	1	2	1	4	0	0
5	0	2	0	0	2	2
6	0	4	0	1	2	1
7	0	3	0	0	7	2
8	0	1	1	0	4	6
8	0	0	0	2	5	5
10	0	1	0	1	3	4
11	0	2	2	2	1	1
12	0	4	2	0	1	1
total	3	23	6	22	35	26
total in %	9	72	19	27	42	31

B

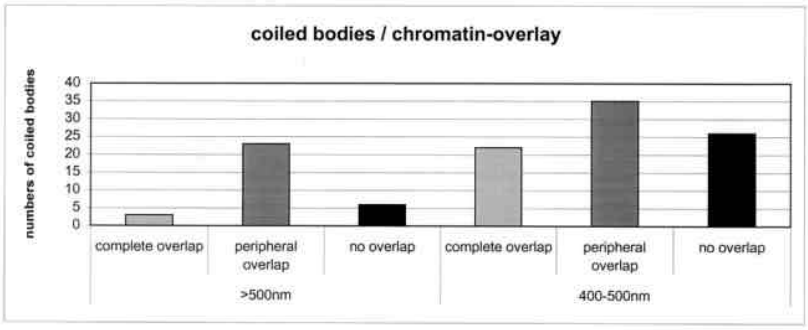

Table 1: A) The table shows the numbers of coiled bodies counted in 12 nuclei (115 in total) that show complete overlap, peripheral overlap or no overlap with chromatin. Coiled bodies were classified according to their sizes: one group contained only coiled bodies with diameters in the x,y plane above 500nm while a second group contained only coiled bodies with diameters in the x,y plane between 400nm and 500nm. **B**) Graphical representation of the data shown in A). The numbers of coiled bodies that show complete overlap (light grey bars), peripheral overlap (dark grey bars) or no overlap (black bars) with chromatin are shown. The higher degree of overlap observed in the group of coiled bodies with diameters between 400nm-500nm as well as the frequently observed peripheral overlap is likely due to the limited resolution of light microscopy.

Table 2

A

PML bodies	>500nm			400-500nm		
nucleus	complete overlap	peripheral overlap	no overlap	complete overlap	peripheral overlap	no overlap
1	0	4	0	0	3	1
2	0	2	2	0	0	1
3	0	2	3	0	5	1
4	0	0	9	0	0	2
5	0	1	4	0	3	3
6	0	1	1	2	1	0
7	1	4	0	2	7	0
8	0	2	0	2	1	0
9	0	2	0	0	0	2
10	0	1	1	1	1	0
11	0	1	3	0	0	1
12	0	3	1	0	2	1
total	1	23	24	7	23	12
total in %	2	48	50	16	55	29

B

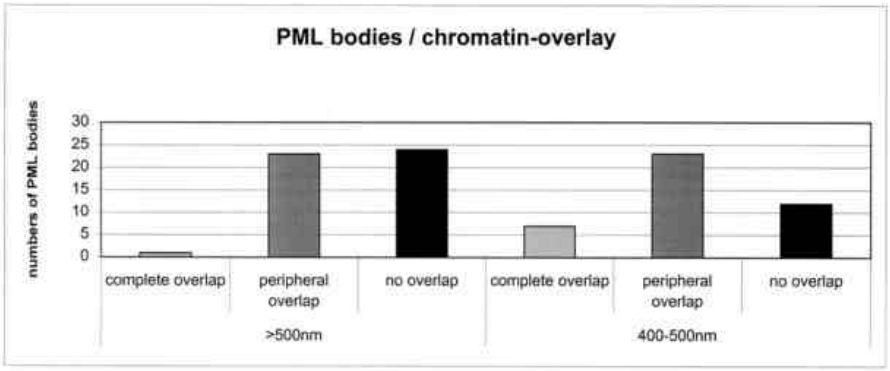

Table 2: A) The table shows the numbers of PML bodies counted in 12 nuclei (90 in total) that show complete overlap, peripheral overlap or no overlap with chromatin. PML bodies were classified according to their sizes: one group contained only PML bodies with diameters in the x,y plane above 500nm while a second group contained only PML bodies with diameters in the x,y plane between 400nm and 500nm. **B**) Graphical representation of the data shown in A). The numbers of PML bodies that show complete overlap (light grey bars), peripheral overlap (dark grey bars) or no overlap (black bars) with chromatin are shown. The higher degree of overlap observed in the group containing PML bodies with diameters between 400nm-500nm as well as the frequently observed peripheral overlap is likely due to the limited resolution of light microscopy.

occurred with a frequency close to that expected for random integration (Withers-Ward et al., 1994). In agreement with the *in vitro* data, the frequency of integration site usage varied considerably within the regions (Withers-Ward et al., 1994). To determine the effect of transcriptional activity on integration site usage, a transcription factor activating a target gene was expressed. In the 1.3 kb target DNA analysed, the integration frequency seemed to be reduced rather than enhanced by expression of the transcription factor (Weidhaas et al., 2000).

For human immunodeficiency virus type 1 (HIV-1), sequence analysis of a number of integration sites suggested preferential integration in the vicinity of topoisomerase cleavage sites (Howard, 1993), LINE elements (Stevens SW, 1994) or Alu islands (Stevens & Griffith, 1996). A second study revealed that centromeric alphoid repeats were disfavored target sites, but could not confirm preferential integration close to LINE or Alu repetitive elements (Carteau et al., 1998). Most of these studies only analysed the sites of integration within approximately 1000 bp. With the exception of one study (Weidhaas et al., 2000) the transcriptional activity of the sites of integration at the time of integration could not be assessed. In addition, little is known about the association of integration sites with the functional compartments of the nucleus.

Some hints on the association of proviruses with functional compartments could be obtained from studies reporting that proviruses of different mammalian species reside predominantly at genomic sites where the AT-content of the host DNA sequence corresponds to their own sequence composition (Glukhova et al., 1999; Kettmann et al., 1979; Rynditch et al., 1991; Salinas et al., 1987; Zoubak et al., 1992, 1994). Furthermore, expression of proviral sequences seems to be dependent on the AT-content of host DNA sequences at the integration site. Again, a comparable AT-content of the host and the proviral sequence favors expression (Bernardi, 1995; Zoubak et al., 1994; Zoubak et al., 1992). However, since stable cell lines were used in these studies, the preferential location of proviruses at genomic sites with an AT-content similar to the provirus could also be due to selective processes after integration rather than during integration. Therefore, it is still unclear whether retroviruses preferentially integrate into defined functional compartments of the nucleus or not.

Whereas the knowledge about nuclear positions of wild-type proviruses is at the moment not a problem that might have a direct impact on therapeutic applications, the situation is different with regard to corresponding retroviral, lentiviral, and other integrating vectors. One of the most serious drawbacks in the use of integrating vectors in gene therapy is the problem that an efficient and stable long-term expression is difficult to achieve. This seems to be a particular problem, if undifferentiated cells such as bone marrow stem cells are first infected with a retroviral vector and then undergo differentiation. Methylation-dependent mechanisms seemed to be involved in silencing of integrated retroviruses during differentiation (Jaenisch et al., 1985); Laker et al., 1998). Since inhibition of methylation only rescued a fraction of silenced cells, methylation independent mechanisms were postulated (Cherry et al., 2000). It was frequently suggested that silencing of retroviral vectors might be at least partially due to changes in chromatin structure (see e.g. (Naldini et al., 1996)). However, as discussed above, chromatin structure and nuclear localization seem to be intimately linked with each other and both seem to be involved in the stable maintenance of an active or silenced state. As in particular the maintenance of an active state is a major problem it would be advisable to investigate the nuclear localization of integrating vectors and to optimize them for localization into the active genome compartment.

So far, it was difficult to investigate the localization of gene-therapy vectors with regard to the distinct genome compartments. On the one hand, there was a lack of knowledge regarding nuclear genome architecture and, one the other hand, tools to visualize integrated vectors within distinct genome compartments were not developed. This situation has changed. Distinct genome compartments can be easily visualized now with a variety of methods, for example, replication labeling or immunostaining with antibodies against specific histone isoforms (Sadoni et al., 1999). In addition, the in situ hybridization techniques are now sensitive enough to detect reliably DNA sequences of only a few kb in length (typical length of vectors used), also in combination with the detection of genome compartments.

To demonstrate the reliability of the techniques available we show in **Figs**. **7-10** the methods applied to the endogenous Masp 2 gene we used as a control. This housekeeping-gene has been mapped to the chromosomal region 1p36 at the distal tip of the short arm of chromosome 1 (**Fig**. **7**) (Stover et al., 1999). This is a very gene-rich region mostly occupied by R-bands. Accordingly, the gene localizes to the active nuclear compartment as shown in **Fig**. **8** (active compartment detected by replication labeling) and **Fig**. **10** (active compartment detected by immunostaining against highly acetylated isoforms of histone H4). Clearly, the gene loci are excluded from the silenced peripheral and late replicating compartments as shown in **Fig**. **9**. (Although this is generally believed, it should be noted that in humans loci close to the telomere do not predominantly localize to the nuclear periphery as in yeast or Drosophila. This is in accordance with the fact that terminal chromosome bands in humans are in most cases very gene-rich R-bands). The Masp 2 gene was detected in the experiments outlined in **figures 7-10** with a DNA probe of only 2.5 kb in length and the detection procedure (which was compatible with both, replication labeling and

immunostaining) involved amplification of the signal with biotinyl-tyramide (commercially available kit, (Kappelsberger, 1999)).

How this technique can be used to investigate the nuclear localization of vectors in relation to their transcriptional behavior is shown in **Fig**. **11**. Here, a simian immunodeficiency virus derived vector (Schnell et al., 2000) was detected within the active nuclear compartment. Accordingly, the GFP reporter gene of the vector was expressed. These results demonstrate that the tools are at hand now to investigate the transcriptional behavior of gene therapy vectors in relation to their nuclear localization.

IX. Strategies for targeting vectors to specific genome compartments

Integration of a vector into a chromosomal locus permissive for high expression levels is advantageous to obtain a stable and efficient long-term expression. The data discussed above suggest that these loci are predominantly those, which posses a “default address” for the active nuclear genome compartment. These are the chromosomal R-bands.

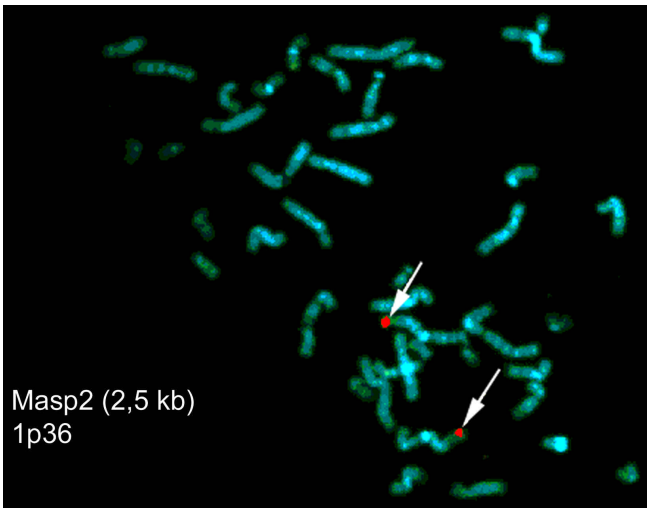

Fig. 7: The Masp2 gene localizes to 1p36 at the tip of the short arm of chromosome 1. The Masp2 gene was mapped on human mitotic chromosomes (blue) with a 2.5 kb probe. Also with this short probe clear signals (red, arrowheads) could be obtained.

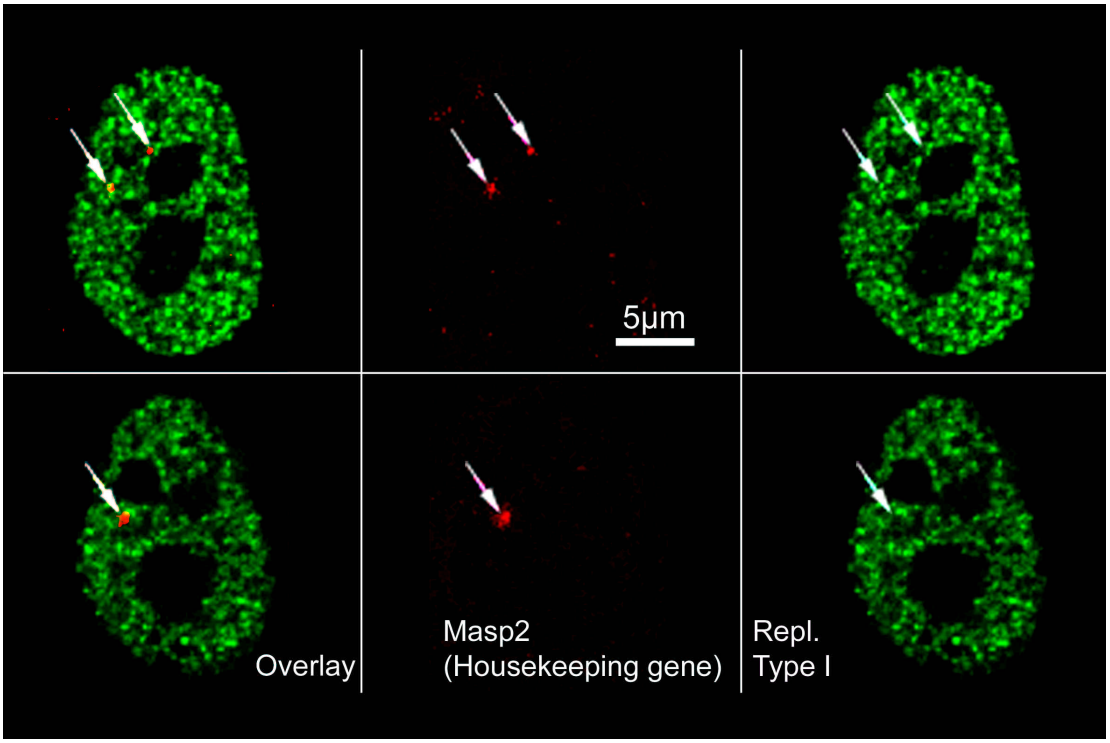

Fig. 8: The Masp2 gene localizes to the interior early-replicating compartment. The nucleus of a human neuroblastoma cell was labeled with Cy3-dUTP (green) in order to detect the early replicating interior R-band compartment (type I replication pattern). After replication labeling, the Masp2 gene was detected by in situ hybridization with a 2.5 kb DNA probe (red, arrows). The upper and the lower rows of images represent two different nuclear planes. Only in the plane shown in the upper row both signals are present. Only the replication label is shown in the right panels while the panels in the middle of each row show only the in situ hybridization. The merges of both are shown on the left panels.

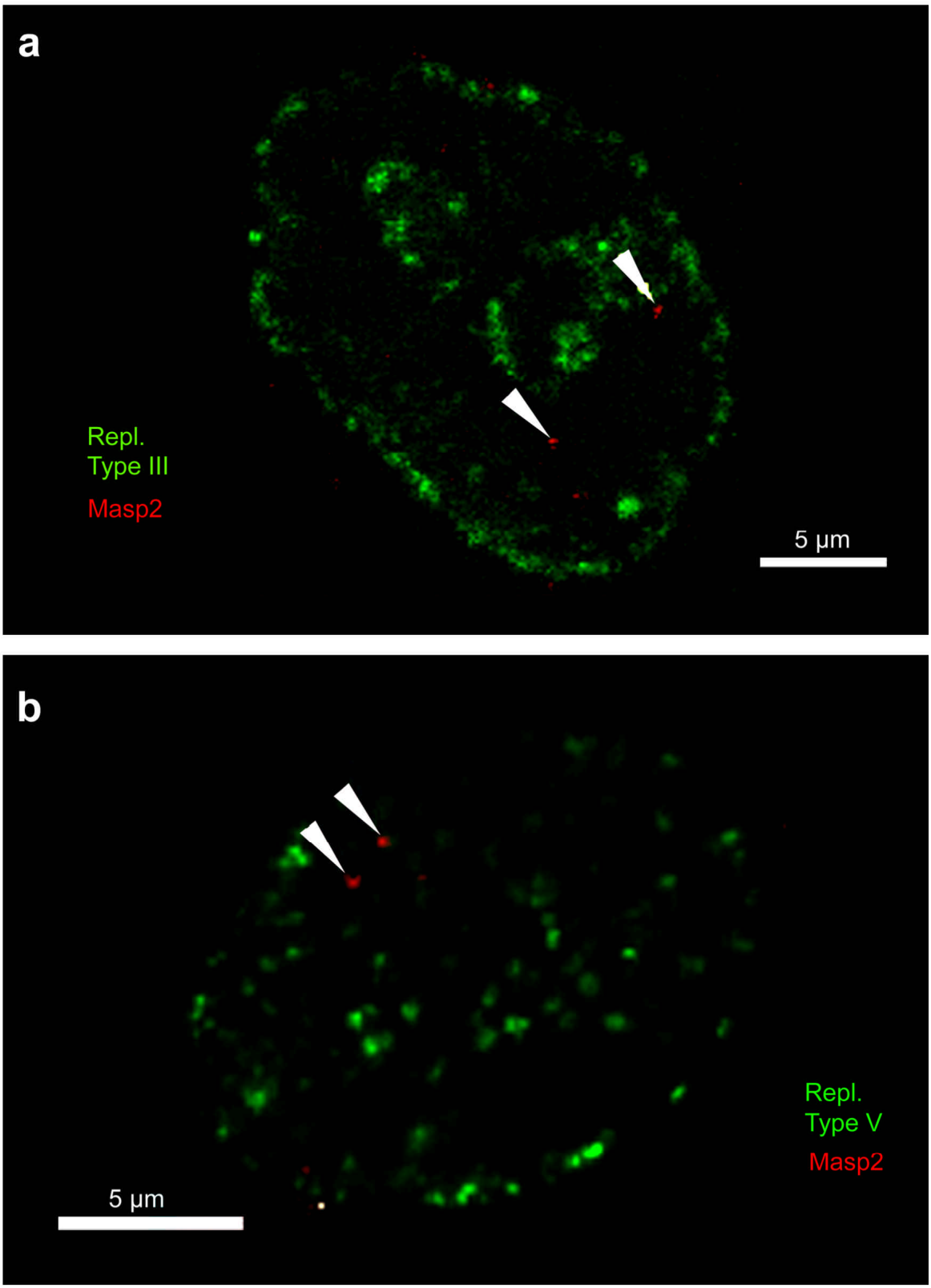

Fig. 9: The Masp2 gene is excluded from the silenced peripheral and late replicating compartments. Nuclei of human neuroblastoma cells were labeled with Cy3-dUTP (green) in order to detect the silenced peripheral (a, type III replication pattern) and late replicating (b, type V replication pattern) compartments. After replication labeling, the Masp2 gene was detected by in situ hybridization with a 2.5 kb DNA probe (red, arrowheads).

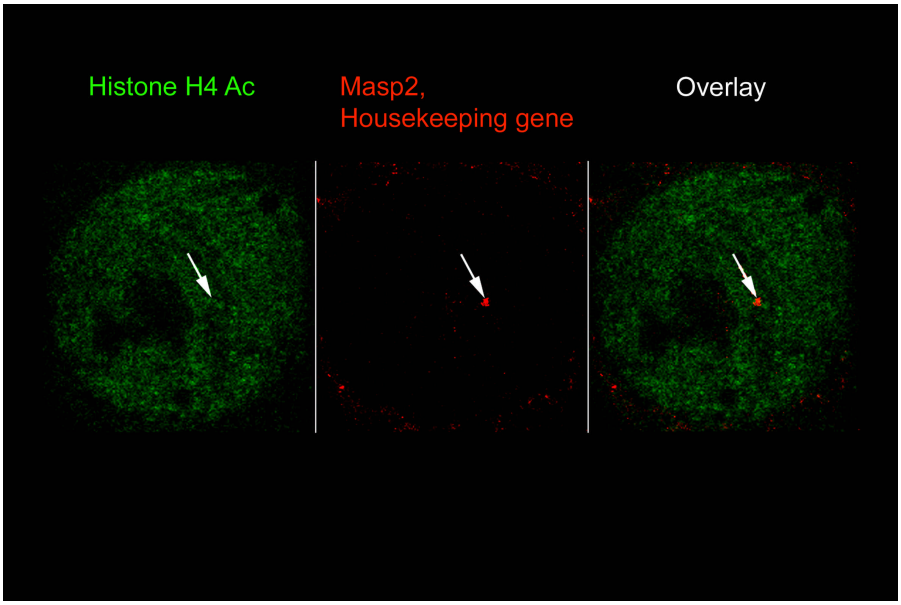

Fig. 10: An alternative way of detecting the Masp2 gene within the transcriptionally active interior compartment. The interior compartment was labeled with an antibody against highly acetylated isoforms of histone H4 (green, left) (Sadoni et al., 1999). The Masp2 gene was detected by in situ hybridization with a 2.5 kb DNA probe (red, middle, arrow). The overlay (right) of the immunostaining and the in situ hybridization shows the localization of the gene to the active, interior compartment. In the nuclear plane shown only one of the two copies of the gene is present.

As stable localization of an integrating vector into the active genome compartment consisting of R-band sequences might be crucial to obtain a stable and efficient long-term expression the question arises how this might be achieved. If the preferred integration site of AAV localizes to the R-band sequences, AAV based vectors that maintain the site-specific integration property of the parental virus could be developed. However, until now this requires the transfer of the rep-gene, which might be not feasible in a clinical setting. An interesting naturally occurring example for targeted integration comes from Ty3 retrotransposons. By interaction of the Ty3 integration machinery with basal transcription factors the retrotransposon is believed to be targeted close to the start sites of RNA polymerase III transcription units (Chalker & Sandmeyer, 1990; Chalker DL, 1993; Chalker DL, 1992; Kirchner J, 1995). By fusion of the integrase of HIV-1 to a DNA-binding protein the integration could be targeted at least in vitro to the respective binding site (Bushman, 1994). Another possible route for targeting integration to favourable genomic regions might be the direct integration into R-band sequences by homologous recombination. Recent results indicated that integration of transgene containing vectors via homologous recombination seems to be a frequent event in mammalian cells (McCreath et al., 2000). The frequency should increase if the regions of homology are present in multiple copies in the genome. Therefore, the R-band specific Alu-repeat family with about 10^6 copies per genome might be an ideal target for this approach.

Another route might involve the use of specific regulatory elements. Although mechanisms like methylation of viral sequences seem to play a role in silencing (Jaenisch et al., 1985; Laker et al., 1998), silencing of constructs lacking any viral sequences has also been observed. Therefore, it is yet unclear if these epigenetic inactivation events are triggered by particular sequences or if certain enhancer-promoter combinations have different abilities to overcome the negative effects of an integration site and its corresponding nuclear compartment.

The latter possibility is supported by the results discussed above, which demonstrated that regulatory elements as enhancer elements are able to switch the chromatin structure as well as the nuclear “address” of transgenes (Francastel et al., 1999). Strikingly, the nuclear localization of transgenes does not seem to be correlated with their actual transcriptional status but is rather correlated with the ability to stably maintain a given level of expression (Francastel et al., 1999). Therefore, by investigating how regulatory elements influence the nuclear localization of integrated vectors one might not only be able to improve the levels of expression. In addition, to screen regulatory elements for their ability to mediate localization into the actively transcribed compartment likely provides information about their

ability to sustain efficient long-term expression (as the nuclear localization correlates with the stable maintenance of given expression levels). A corresponding screening approach for suitable regulatory elements (using, for example, experimental procedures as those outlined in **figures 7-11**) will be much cheaper and faster then the approaches used so far to identify suitable regulatory elements.

Apart from classical enhancer elements, regulatory elements like locus control regions (LCRs) or S/MARs came recently into the focus of interest. LCRs mediate at ectopic sites expression levels independent of the integration locus (Grosveld et al., 1987; Talbot et al., 1989). This makes them of course particularly attractive for gene therapy approaches although the function of the most intensively studied LCR from the β-globin locus is mainly restricted to erythroid cells. Although it is still an open question how LCRs work experimental data involving an enhancer element derived from the β-globin LCR strongly suggest that at least at ectopic sites this LCR or its sub-regions act also on the level of nuclear positioning (Francastel et al., 1999). Therefore, LCRs or LCR sub-regions might be useful elements for localizing gene therapy vectors into the transcriptionally active nuclear compartments.

S/MARs are thought to act on the level of chromatin structure (Zhao et al., 1993) and/or by targeting transgenes to the nuclear matrix (Boulikas, 1995). In addition, they might function as domain borders shielding chromatin domains from the influences of neighboring regulatory elements (Bode et al., 1995). Whatever their mode of action is, they have been shown to improve expression from retroviral vectors (Agarwal et al., 1998; Schübeler et al., 1996). Strikingly, they mediate mitotic stability of episomal vectors and this function correlates with targeting these vectors to the nuclear matrix (Baiker et al., 2000; Piechaczek et al., 1999). Whether S/MARs in addition might be useful for localizing a transgene into specific higher-order nuclear compartments remains to be shown.

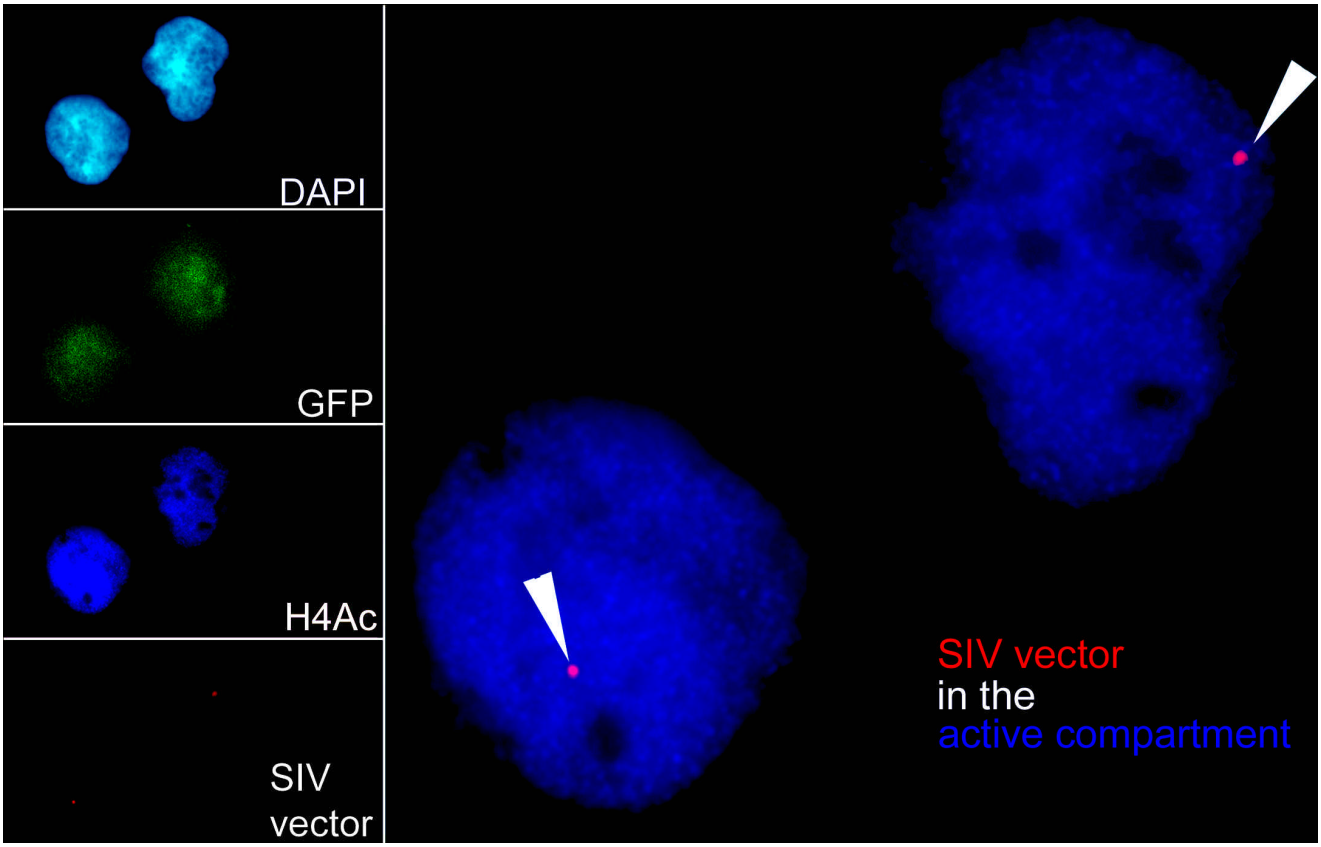

Fig. 11: The localization of a simian immunodeficiency virus (SIV) derived vector to the active nuclear compartment and its transcriptional activity. Human lung epithelial cells were infected with a SIV-derived vector, which was detected by in situ hybridization (red, arrowheads). The active genome compartment was stained with an antibody against highly acetylated isoforms of histone H4 (H4 Ac) (dark blue, "holes" in the nuclear interior result from the exclusion of nucleoli and transcriptionally inactive chromatin). Clearly, the integrated vector localizes to the active nuclear compartment. The small panels on the left show from top to bottom the DAPI counterstain, the GFP-fluorescence (shows that the GFP reporter gene of the vector is expressed), only the anti H4Ac staining and only the in situ hybridization signals.

In summary, the data imply that position effects, which often lead to problems in transgene expression are not only due to the local chromatin environment which might be permissive or repressive for transcription. In addition, specific chromosomal sites are targeted to defined nuclear higher-order compartments, which are transcriptionally active or silenced. The targeting to nuclear higher-order compartments correlates with the stability of expression levels. A variety of possible routes to prevent sequestration of a transgene into a silenced higher-order compartment and to localize it into an actively transcribed compartment can be tested now experimentally. This might not only lead to new strategies to improve expression from gene therapy vectors but might also help to develop fast and efficient screening methods for suitable vector constructs.

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