Differential large-scale chromatin compaction and intranuclear positioning of transcribed versus non-transcribed transgene arrays containing β-globin regulatory sequences

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Summary

Previous work has demonstrated a more decondensed large-scale chromatin structure and a more internal nuclear position for gene-rich versus gene-poor chromosome regions. Here, we show that large-scale chromatin opening and changes in intranuclear positioning of chromosome regions can be induced by normal levels of endogenous transcription factors acting on mammalian regulatory sequences. We transfected mouse erythroleukemia cells with a 15 kbp plasmid containing a lac operator repeat plus β-globin regulatory sequences driving a β-galactosidase reporter gene. After green-fluorescent-protein/lac-repressor fusion-protein binding or after fluorescence in situ hybridization, the volume and location of the transgene array signal were measured. With both detection methods, we found that the volume was severalfold larger when transcription was on. While silent transgene arrays were located close to the nuclear membrane, we observed a significantly more internal position for the transcriptionally active state. Our results indicate that both large-scale chromatin decondensation and changes in nuclear positioning as observed for large, complex gene-rich chromosome regions can be reproduced by endogenous regulatory sequences acting within simple repetitive transgene arrays.

Key words: Large-scale chromatin organization, Nuclear architecture, Gene expression, Mouse erythroleukemia cells, β-Globin gene

Introduction

Over the past several decades, it has become abundantly clear that regulation of gene expression is closely tied to changes in chromatin structure (for a review, see van Driel et al., 2003). Most attention has been devoted to changes in local chromatin structure, involving histone modifications and nucleosome positioning in the vicinity of regulatory regions such as promoters and enhancers (for reviews, see Becker and Hörz, 2002; Fischle et al., 2003; Lachner et al., 2003; Sims et al., 2003; Turner, 2002; Vermaak et al., 2003). Yet several long-standing observations have suggested that regulation of gene expression might also involve changes in higher levels of chromatin structure. These include puffing of transcriptionally active regions of polytene chromosomes (e.g. Lewis et al., 1975) and increased nuclease sensitivity of large DNA regions flanking active genes (Weintraub and Groudine, 1976). Newer cytological methods suggest that these changes in higher levels of chromatin structure associated with transcriptional activity might also involve architectural features of interphase chromosomes, including the folding of 10-30 nm chromatin fibers into large-scale chromatin domains, the arrangement of these domains relative to chromosome territories, and the intranuclear positioning of these domains relative to other subnuclear compartments (for reviews, see Belmont et al., 1999; Cremer and Cremer, 2001; Spector, 2003).

In several cell types, gene-rich chromosome territories have been reported to be located preferentially towards the nuclear center, with gene-poor territories located more peripherally (Boyle et al., 2001; Cremer et al., 2001; Croft et al., 1999; Habermann et al., 2001; Sun et al., 2000). Although conflicting results were reported for flat fibroblast nuclei, the studies cited above generally agree about the described distribution in spherical nuclei (e.g. in lymphocytes). This architectural motif is evolutionary conserved (Tanabe et al., 2002). A similar behavior has been demonstrated for chromosomal subregions (Kozubek et al., 2002; Nogami et al., 2000; Rens et al., 2003; Weierich et al., 2003). Likewise, gene-poor chromosome regions were observed to be more condensed than gene-rich chromosome regions (Croft et al., 1999). On a smaller scale, several active genes have been observed to be more peripheral in their respective chromosome territory than inactive genes (Dietzel et al., 1999; Kurz et al., 1996), although active genes were also observed inside chromosome territories (Mahy et al., 2002a). Transcriptionally active gene clusters several Mbp in size have been found to sometimes extend some distance away from the chromosome territory (Mahy et al. 2002b; Volpi et al., 2000; Williams et al., 2002).

Many of the above studies relied on fluorescent in situ hybridization (FISH), in which questions arise of whether observed differences in condensation between active and

inactive chromatin reflect in vivo differences or a different susceptibility to certain fixation methods and denaturation during the FISH procedure. More importantly, in these studies, the behavior of complex gene loci or chromatin domains with DNA contents of hundreds to thousands of kbp were studied, making it difficult to identify the actual cis and trans factors determining the observed chromatin behaviors.

Both of these limitations have been bypassed recently by in vivo observations of engineered chromosome regions using lac operator repeats as a tag for in vivo detection by fusion proteins of green fluorescent protein (GFP) and the lac repressor (Robinett et al., 1996). This approach has allowed analysis of the effects of single transcription factors on large-scale chromatin structure. Targeting the VP16 acidic activation domain to a peripheral chromosome site via a lac-repressor fusion protein resulted in a repositioning of this site towards the nuclear interior (Tumbar and Belmont, 2001). Similar targeting of the VP16 acidic activation domain to a large, heterochromatic chromosome region produced dramatic uncoiling of large-scale chromatin fibers (Tumbar et al., 1999). Decondensation of large-scale chromatin structure also was produced using this approach by estrogen receptor (Nye et al., 2002) and BRCA1 transcriptional activation domains (Ye et al., 2001). Thus, changes in large-scale chromatin structure and intranuclear chromosome positioning, similar to those observed for complex active gene clusters and large gene-rich chromosome regions, can be produced by targeting a single transcription factor to engineered chromosome regions, creating a simplified model system to study these phenomenon. A legitimate question concerning this approach, however, concerns its physiological relevance given the large number of lac-operator binding sites present in the engineered chromosome sites, although similar large-scale chromatin decondensation has been observed in another type of transgene array when glucocorticoid receptor bound to glucocorticoid response elements in viral MMTV promoters (Müller et al., 2001).

Here, we address whether endogenous gene regulatory sequences can produce similar changes in large-scale chromatin structure and chromosome intranuclear positioning, using the β-globin locus as a model system. The β-globin locus has a locus control region (LCR) with several DNAse-I-hypersensitive sites (HS), each containing many binding sites for specific transcription factors (for reviews, see Harju et al., 2002; Levings and Bungert, 2002). It was shown that constructs with four fragments of the LCR containing the first four HS (Forrester et al., 1989) were sufficient to confer tissue-specific regulation in transgenic mice (Robertson et al., 1996). We combined such a 2.5-kbp β-globin μLCR with the β-globin promoter driving a β-galactosidase reporter gene (*LacZ*), and 64 lac-operator repeats. Examining large transgene arrays produced in mouse erythroleukemia (MEL) cells, we observed metastable transcriptionally on or off states. Transcriptionally active transgene arrays were significantly more decondensed than inactive arrays. Moreover, transcriptionally active transgene arrays showed a more interior distribution within the cell nucleus than inactive arrays.

Materials and Methods

Molecular cloning

pSV2-neo was obtained from the American Type Culture Collection (http://www.atcc.org/; plasmid 37149). A polylinker was designed and ordered as two 69-mers with four overhanging bases [(AATT)AA-TTAAATCGATGGGGGAATTCGTCGACAAGGAAGCGGCCGC-CTCGAGCCGGGTACCGGCGCGCC(TTAA); Operon Technologies, Alameda, CA]. It contained the restriction sites for *Pac*I, *Cla*I, *Eco*RI, *Sal*I, *Not*I, *Xho*I, *Kpn*I and *Asc*I. The overhanging ends were used to clone this linker into the single *Eco*RI site of pSV2-neo (at position 0=5729 bp) between the *amp* gene (*Pst*I at position 4977 bp) and the SV40 poly-A sites (*Bam*H1 at position 752 bp), resulting in the loss of this *Eco*RI site. The two resulting plasmids contained the polylinker either in the *amp*/*Pac*I/*Asc*I/SV40-poly-A site direction (pPA) or in the opposite direction (pAP). pPA was used for further cloning. The μLCR was taken as a *Cla*I-*Eco*RI fragment from the μLARβ plasmid (gift from A. Telling and M. Groudine, Seattle, WA) (Forrester et al., 1989) and cloned into pPA, resulting in pPAL. The β-globin-promoter-*LacZ* construct was cut out as an *Xho*I-*Not*I fragment from the miniLCRβlacZ plasmid (gift from E. Whitelaw, Sydney, Australia) (Robertson et al., 1996) and cloned into the *Sal*I and *Not*I sites of pPAL, resulting in pPALZ. The 64 repeats of the lac operator were cut out as a *Sal*I-*Kpn*I fragment from pPS8.8 (Robinett et al., 1996) and cloned into the *Xho*I and *Kpn*I sites of pPALZ, resulting in pPALZ8.8 (Fig. 1A).

Cell culture and transfection

To ease microscopic observation, we chose a MEL cell line that was described as semi-adherent in the literature [the adenine-phosphoribosyltransferase-negative line (Charnay et al., 1984; Forrester et al., 1989; Miller et al., 1988)]; in our hands, however, the

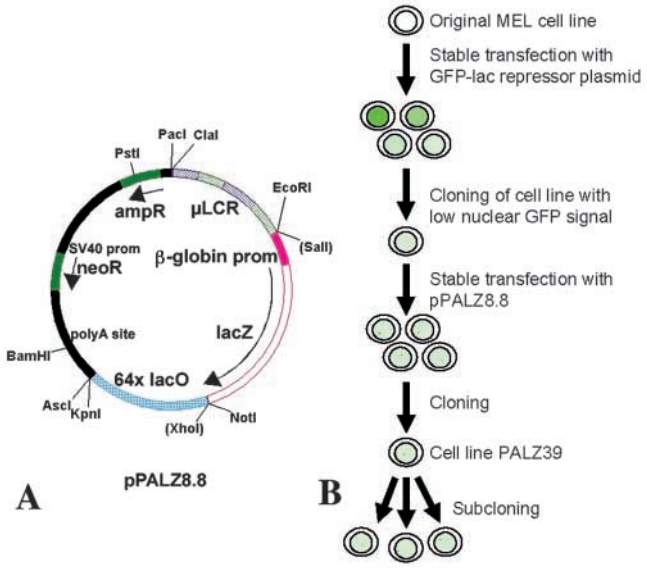


Fig. 1. Isolation of MEL cells with large transgene arrays, tagged with lac operator repeats, and carrying β-globin regulatory sequences. (A) The pPALZ8.8 plasmid was created by cloning several parts into pSV2-neo (black and dark green). Dotted blue and green boxes (flanked by *Cla*I and *Eco*RI restriction sites) indicate the hypersensitive sites HS1-HS4 of the human β-globin locus which form the μLCR. The human β-globin promoter (filled pink) controls a β-galactosidase gene (*LacZ*, hollow pink). The light blue box indicates 64 repeats of the lac operator, the binding site for the GFP/lac-repressor fusion protein. Arrows indicate open reading frames of the genes involved. Total size of plasmid is 15 kb. Restriction sites mentioned in the Materials and Methods section are indicated. Sites in brackets had been present in the polylinker and were lost during cloning. (B) Cloning scheme for PALZ39 subclones.

cells were rarely adherent. The karyotype was near diploid (data not shown). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum under 5-10% CO_2. For induction, HMBA (catalog number 22,423-5; Aldrich, Taufkirchen, Germany) was added to a final concentration of 4 mM (Richon et al., 1988).

For transfection of p3′SS-EGFPdimer lac repressor (Tumbar et al., 1999) we used FuGene [Boehringer Mannheim (now Roche Applied Science), Indianapolis, IN]. Two days after transfection, an appropriate dilution of cells was transferred to 9 ml selection medium containing 750 μg ml^{-1} Hygromycin B (catalog number 400051; Calbiochem, La Jolla, CA). 1 ml 3% soft agar (catalog number SeaPlaque #50111; FMC Bioproducts, Rockland, ME) was added and the mixture was transferred to 10 ml Petri dishes. After 30 minutes at 4°C, dishes were transferred to the incubator for a week. Resistant cells had then formed colonies in the soft agar, and these were individually transferred to cell culture flasks. A stable subclone with barely detectable nuclear GFP staining was selected (Fig. 1B) and subjected to a second round of stable transfection with pPALZ8.8, this time using Tfx50 (Promega, Madison, WI) as described previously (Elnitski and Hardison, 1999). Colonies were obtained as before, using 1 mg ml^{-1} G418 sulfate (catalog number 345810; Calbiochem) for selection. Circular DNA was used for both transfections. After passaging the clone PALZ39 12 times, PALZ39E was subcloned. The PALZ39M line was obtained by dilution of PALZ39, leaving several cells as founders of the population.

Fixation, X-Gal staining and FISH

Metaphase spreads were prepared according to standard protocols. For three-dimensional (3D) preparations, glass cover slips (22×22 mm, 170 μm thick) were coated with poly-L-lysine (molecular weight 300,000; Sigma, Deisenhofen, Germany) by incubation with a 0.1 mg ml^{-1} solution for 40 minutes, washes with water and air drying. Cell suspensions were incubated for 40 minutes or longer to allow cell attachment. Fixation was with 2% formaldehyde (freshly made from paraformaldehyde) in PBS (Dernburg and Sedat, 1998) for 15 minutes.

X-Gal staining was carried out according to Cheng et al. (Cheng et al., 1999) but using only 30 mM ferrocyanide and 30 mM ferricyanide. Controls showed that, although the intensity of staining increased, the proportion of positive cells was the same after 1 hour and after overnight incubations (data not shown).

FISH was performed according to Solovei et al. (Solovei et al., 2002). Briefly, for 3D FISH, cells were pretreated for 5 minutes in 0.5% Triton X-100, >30 minutes in 20% glycerol, dipping five times in liquid nitrogen with intervening thawing, 5 minutes 0.1 M HCl and incubation in 50% formamide in 2× SSC buffer until use. Air drying was carefully avoided at all times. pPALZ8.8 was labeled with digoxigenin or biotin by nick translation. The hybridization mix contained 50% formamide, 10% dextran sulfate, 10 ng μl^{-1} pPALZ8.8 and 25 ng μl^{-1} pancentromeric probe kindly provided by A. Brero (Ludwig-Maximilians-Universität München, Munich, Germany). This pancentromeric probe was generated and Cy3 labeled by PCR amplification of 170 bp of the 234 bp of the major satellite repeat sequence from genomic DNA (Weierich et al., 2003). Simultaneous denaturation of probe and target was at 75°C for 2 minutes. Detection of pPALZ8.8 was with mouse anti-digoxygenin-Cy3 antibodies or avidin-Alexa488. TO-PRO-3 (1 μM; Molecular Probes, Eugene, OR) was used as a DNA counterstain. Chromosome paint probes were kindly provided by N. Carter (Wellcome Trust Sanger Institute, Cambridge, UK) (Rabbitts et al., 1995).

Confocal microscopy and image analysis

3D images were acquired on Leica TCS 4D and Leica TCS SP confocal microscopes with 100×, NA 1.4 Plan Apo objectives. Voxel size was 0.08×0.08×0.24 μm. For measurements of chromosome-12 paint-probe signal intensity on metaphase chromosomes, confocal images were opened in Adobe Photoshop. Regions of interest (ROI) were interactively marked and the histogram tool was used to determine the average intensity of the signal in each ROI. For 3D volume measurements, image stacks were opened in ImageJ (http://rsb.info.nih.gov/ij/index.html) and the plugin Voxel Counter provided with ImageJ used to determine the volume of interactively thresholded signals. Projections were made for visualization. Figures for this article were assembled in Adobe Photoshop. For radial distribution determination, the chromatic aberration in the *z* direction was corrected. Then, a program was used that was kindly provided by J. von Hase and C. Cremer (Universität Heidelberg, Germany) and is described in detail elsewhere (Cremer et al., 2001). Briefly, this program segments each nucleus into 25 'shells'. The outermost shell is fitted to the surface of the segmented nucleus and inner shells are adapted accordingly. On any ray from the nuclear center to the surface, each shell has the same width, resulting in increasing volumes for outer shells. The proportion of a given signal in each shell is calculated. Owing to the limited resolution of light microscopy and Gaussian filtering, the edge of the nucleus in the processed images is not a sharp border but is blurred. The threshold-based segmentation of the border of the nuclei includes some of this blurred region leading to decreasing amounts of DNA signal in the outer shells of the segmented volume.

Results

Transgenic cell lines generated for this study showed unexpected cytogenetic and epigenetic instability. In the following three sections, we therefore describe the generation and characterization of the cell lines. The sections thereafter deal with the large-scale chromatin condensation state and the nuclear positioning of inactive versus active transgene arrays.

MEL cells with μLCR transgene arrays

MEL cells were first stably transfected with a plasmid coding for the GFP/lac-repressor/nuclear-localization-signal fusion protein. A cell line with low-level nuclear GFP expression was chosen for further experiments (Fig. 1B). We next stably transfected a plasmid containing the μLCR, the β-globin promoter regulating a β-galactosidase gene and 64 lac operator repeats (pPALZ8.8, Fig. 1A). Several clones were obtained that displayed large, distinct GFP signals in the nucleus, indicating the position of the lac-tagged transgenes. The size of the signals in these clones suggested that many plasmids had integrated at particular sites in the host genome. A clone (PALZ39) with a relatively large, bright GFP signal was selected for further subcloning.

Two-color FISH on metaphase preparations of the PALZ39E subclone using the plasmid pPALZ8.8 as probe together with a chromosome paint probe for chromosome 12 revealed an integration site on a chromosome-12 derivative (Fig. 2G-J). Length measurements on metaphase spreads yielded a ~50 Mbp transgene array size estimate (40-50% the 114 Mbp size of the normal chromosome 12; http://www.ensembl.org/). A close inspection of FISH signals revealed intermingling of chromosome 12 and plasmid sequences along the transgene array (Fig. 2G-I). The chromosome-12 library signal in the transgene array was on average 68% as bright as more proximal chromosome regions containing only chromosome 12 sequences. A chromosome

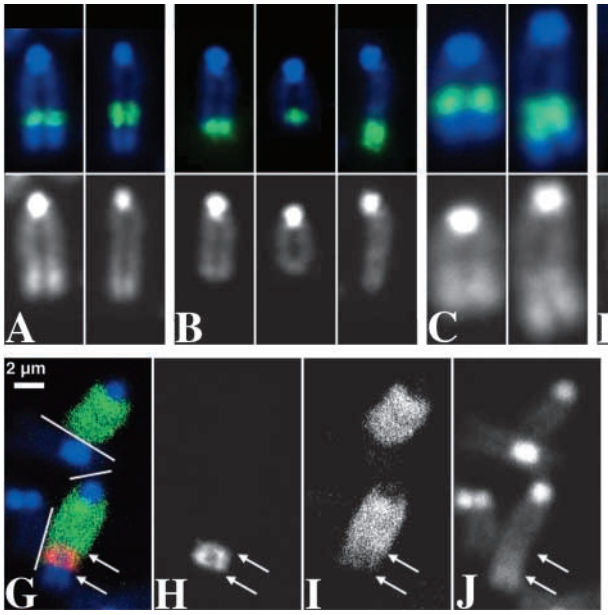


Fig. 2. Analysis of chromosomes carrying transgene arrays in different MEL subclones reveals a large transgene insertion and cytogenetic instability. FISH using the plasmid pPALZ8.8 (green in A-F, top; red in G) was carried out on metaphase spreads and counterstained for DNA (blue in A-F, top, and G; white in A-F, bottom, and J). (A) The predominant chromosome in PALZ39E cells. (B) Other forms found in PALZ39E. (C) The predominant chromosome in the PALZ39E subclone A9. (D) Other forms found in the A9 subclone. (E) Original PALZ39M chromosome. (F) PALZ39M chromosome found after more than 25 cell culture passages. (G-J) Part of a metaphase plate from the cell line PALZ39E showing the predominant transgene-carrying chromosome type (bottom) and a normal chromosome 12 (top). Arrows indicate the borders of the transgene array. (G) Multicolor overlay with transgene array in red, paint probe for mouse chromosome 12 in green and DNA counterstain in blue. White lines in the overlay separate individual chromosomes. The gray-scale images for the transgene array (H), the chromosome 12 paint (I) and the counterstain (J) are also shown.

paint probe does not paint all sequences of a chromosome equally well. Repetitive sequences are actively suppressed to avoid cross-hybridization to other chromosomes. Individual single-copy sequences may get lost or enriched during probe preparation. However, assuming similar chromosome-12 sequence representation within the transgene array to that found within the normal chromosome 12, about two-thirds of the transgene-array DNA corresponds to chromosome 12 material, leaving an upper limit of ~17 Mbp of plasmid DNA (~1100 plasmid copies). Distal to the transgene array is a region stained somewhat more strongly by the DNA counterstain (Fig. 2A,J). This is one of four regions in the karyotype of this cell line containing Y-chromosome DNA. This material does not spread into the 39E transgene array (data not shown). In another subclone, PALZ39M, the chromosome carrying the transgene array showed uniform DNA counterstaining, apart from the centromere (Fig. 2E). The region between centromere and transgene array was also chromosome-12 material (data not shown). The transgene array was too small, however, to perform a size estimation or to determine colocalization of chromosome 12 material.

Transgene hosting chromosomes are cytogenetically unstable

Metaphase preparations from PALZ39E revealed that the karyotype of this tumor cell line had multiple rearrangements compared with the normal mouse karyotype (data not shown). As described above, the predominant chromosome carrying the transgene array was acrocentric, with the transgene array taking up approximately the third quarter of the q-arm (Fig. 2A,G-J). Eight of 40 metaphases, however, had a different-looking transgene-carrying chromosome, often with the transgene array near the telomere (Fig. 2B).

To obtain a more homogeneous population, we subjected the cell line to another round of subcloning. One new line, dubbed A9, was selected for preparing metaphase spreads seven passages after subcloning. 96 chromosomes with transgene FISH signals were recorded, 89 of which were in accordance with the predominant chromosome from the mother cell line (Fig. 2C). Seven, however, showed aberrations similar to the ones found in the mother cell line (Fig. 2D), suggesting a cytogenetic instability in the transgene-carrying chromosome that induces breakage with a frequency that makes generation of a homogeneous population impossible.

In PALZ39M cells, only a single chromosomal form (Fig. 2E) was detected during the period when transgene volume measurements were performed (see below). When the cells were cultivated for another 15 passages, however, most metaphase spreads showed a different chromosome, with the transgene now near the telomere (Fig. 2F).

Transgene expression and inducibility are unstable in PALZ39 subclones

PALZ39 subclones showed different proportions of cells with β-galactosidase expression. In some clones, this proportion changed as a function of passage number and growth conditions. For instance, when culturing subclone PALZ39E for prolonged periods, we noted changes in the ratio of β-galactosidase-positive and -negative cells. In one instance, the number of positive cells in uninduced cultures increased from 5% to up to 95% over several months. We also found that, in cultures with a high proportion of X-Gal-positive cells, the proportion of positive cells could be reduced from ~90% to ~50% by not passaging the culture for 7-9 days or more. After this period, cells were diluted 1:40 with fresh medium and grown for some days so that enough cells were obtained for X-Gal staining. Another round of dense growing did not lead to a further decrease of positive cells.

MEL cells can be chemically induced by HMBA to undergo an erythroid-like differentiation, becoming post-mitotic and upregulating hemoglobin expression. However, the ability of MEL cells to undergo this chemically induced differentiation is also unstable, with many MEL cell cultures losing inducibility. We purposely selected a parent MEL cell clone

that retained this ability to upregulate hemoglobin expression after HMBA treatment. However, most PALZ39 subclone cultures did not show a change in the proportion of X-Gal-positive cells upon HMBA addition, although the cells still became post-mitotic. In one culture of PALZ39E, however, we did observe activation. Although the untreated culture had ~10% X-Gal-positive cells, this number increased to 56% by 3 days after HMBA induction and to 90% after 5 days. When we tried to reproduce this effect some weeks later, we found that, during cultivation, the behavior of the culture had changed, with the cells responding to HMBA by growth arrest but not by increased transgene expression.

Severalfold increase in transgene array volume after transcriptional activation

Using an inducible culture of the subclone PALZ39E, we compared the size of the transgene array before and after induction of transgene expression. The size of the GFP signal in uninduced, X-Gal-negative cells (Fig. 3A) was compared with the signal in induced, X-Gal-positive cells (Fig. 3B) using confocal microscopy. The GFP signal over the transgene array was small and frequently located near the nuclear periphery (Fig. 3A). A striking severalfold increase in transgene array volume was found in induced cells (Fig. 3B). In cells in which the transgene array localized near the nuclear periphery, the decondensed array appeared to extend towards the cell interior (Fig. 3B, top). Volume measurements showed a clear threefold increase in transgene array size in induced cells (P<0.001; Fig. 4A, Table 1).

In these experiments, we reduced the attenuation of GFP fluorescence by the blue X-Gal precipitate by minimizing the X-Gal staining time. Otherwise, accumulation of large X-Gal precipitates in expressing cells blocked the GFP fluorescent signal. Thus, some weakly β-galactosidase-positive cells might have gone unstained. We therefore recorded only clearly positive (blue) cells from an induced preparation and negative (white) cells from an uninduced preparation. Still, we were not able to observe the transgene array size in the most highly expressing cells, in which the GFP signal was blocked.

Owing to their generally brighter fluorescence, FISH signals of the transgene were less affected by X-Gal attenuation than were GFP signals. We therefore repeated measurements of transgene array size using 3D-FISH preparations with the plasmid pPALZ8.8 as a probe (Fig. 5). Transgene arrays visualized by FISH were noticeably larger than GFP signals. Consistent with the GFP measurements, however, there was a clear severalfold increase in transgene array size associated with transcriptional activity. Volume measurements of 3D-FISH signals showed a clear difference between β-galactosidase-positive (mean 6.4 μm^3, n=68) and -negative (1.7 μm^3, n=90) cells, confirming that transcribed signals are more extended than nontranscribed ones (Fig. 4B, Table 1). This difference was highly significant (P<0.001) in both HMBA-induced and uninduced cultures.

We next compared the sizes of transgene arrays in transgene-expressing cells with those in nonexpressing cells from different uninduced cultures. In growing PALZ39E and PALZ39M cultures, a substantial proportion of cells was usually positive. Volume measurements of the FISH transgene signals in cultures of PALZ39E cells, which were not inducible (see above), also showed a highly significant difference (P<0.001) between β-galactosidase-expressing cells (mean 6.2 μm^3) and non-expressing cells (mean 3.3 μm^3) (Fig. 4C).

Table 1. Mean values of transgene signal sizes in different cultures

	β-Galactosidase positive	β-Galactosidase negative
Inducible PALZ39E, +HMBA*	2.2 μm^3, n=43	n.d.
Inducible PALZ39E, no HMBA*	n.d.	0.6 μm^3, n=26
Inducible PALZ39E, +HMBA‡	6.4 μm^3, n=50	1.5 μm^3, n=20
Inducible PALZ39E, no HMBA‡	6.4 μm^3, n=18	1.8 μm^3, n=70
Non-inducible PALZ39E‡	6.2 μm^3, n=24	3.3 μm^3, n=22
PALZ39M‡	4.7 μm^3, n=23	1.1 μm^3, n=49

*Signal volumes are from GFP signals.
‡Signal volumes are from 3D-FISH experiment.
n.d., not determined.

In cells from the subclone PALZ39M (Fig. 5B), the transgene signals were generally smaller than in PALZ39E cells. Only a very few cells were β-galactosidase positive (1-3%), many of which were polynuclear. When we compared the volume of transgene signals in mononuclear β-galactosidase-positive cells with those in negative cells (Fig. 4D), we again found a highly significant difference (P<0.001), with mean values of 4.7 μm^3 for expressing cells and 1.1 μm^3 for non-expressing cells.

In the previous two sections, we have documented that both the karyotype of these MEL cells and the expression patterns of the transgene arrays are highly unstable. A trivial explanation of the size differences in expressing and non-expressing transgene arrays would be that both the relative size and the transcriptional activity are consequences of particular chromosome rearrangements. However, this explanation is incompatible with the increased array size observed after HMBA induction of reporter gene expression in the inducible PALZ39E cells.

Radial distribution of active and inactive signals

In several human and chicken cell types, it has been demonstrated that gene-rich chromosomes and chromosomal subregions preferentially locate towards the nuclear center, whereas gene-poor chromosomes preferentially locate towards the nuclear periphery. We considered that radial positioning might be a function of a transcriptional chromatin state rather than gene content per se. If so, active transgene arrays should be more centrally located than inactive ones.

We determined the radial position of transgene arrays in GFP- and FISH-labeled preparations from inducible PALZ39E cultures (Fig. 6A,B). Transgene array location showed a considerable shift from a narrow peripheral distribution in the inactive state (peak at 85-90% of the radius) to a broader distribution skewed towards more interior areas in the active state (peak around 75%). Distributions for both inactive and active transgene arrays were both clearly distinct from the general DNA counterstain distribution. Relative to the DNA-counterstain distribution, both active and inactive transgene arrays showed a depletion from the nuclear center area less than 50% the nuclear radius, which was particularly pronounced for the inactive transgenes. Distribution patterns in

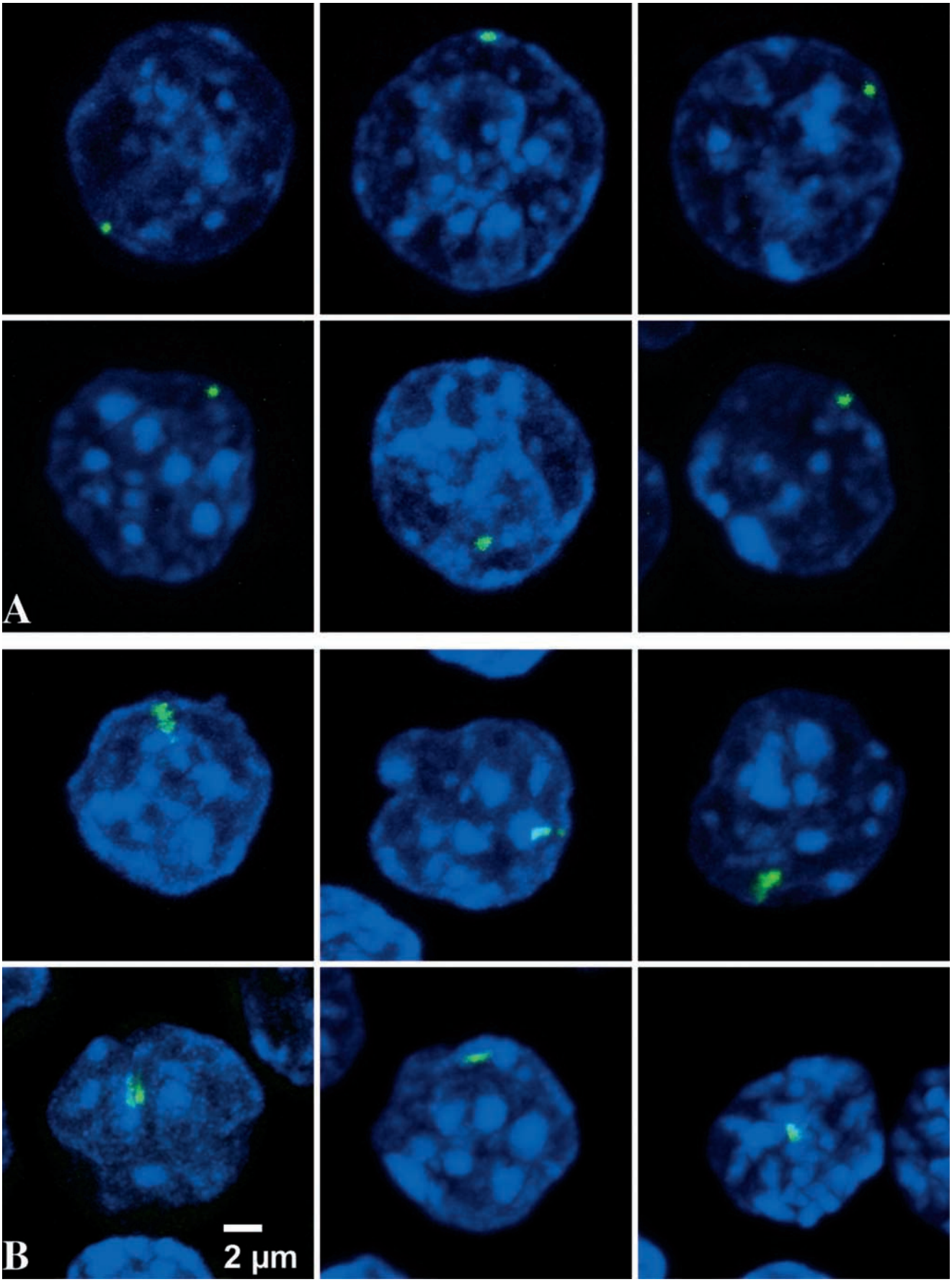

A
B
2 μm

Fig. 3. Transcriptional activation is associated with a severalfold increase in transgene array volume. GFP/lac-repressor signals from transgene arrays in PALZ39E cells from an inducible culture. Projections of confocal image stacks are shown. (A) Uninduced X-Gal-negative cells. (B) Induced X-Gal-positive cells. DNA counterstain in blue. Scale bar is for all images.

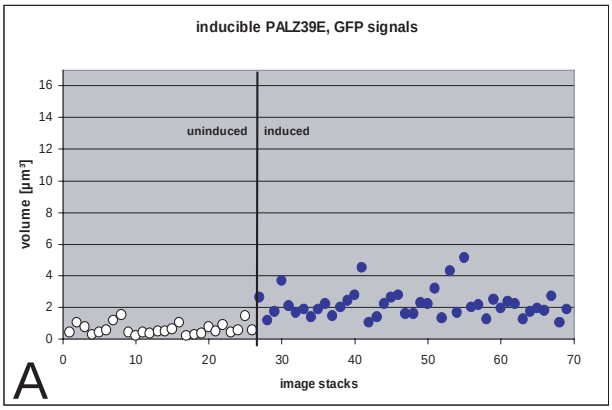
PALZ39M cells confirmed the more internal position of transcribed transgene arrays (Fig. 6C).

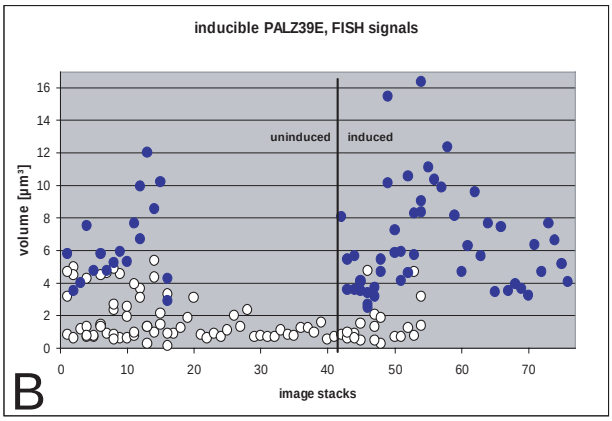
Transgene arrays do not colocalize with centromeres

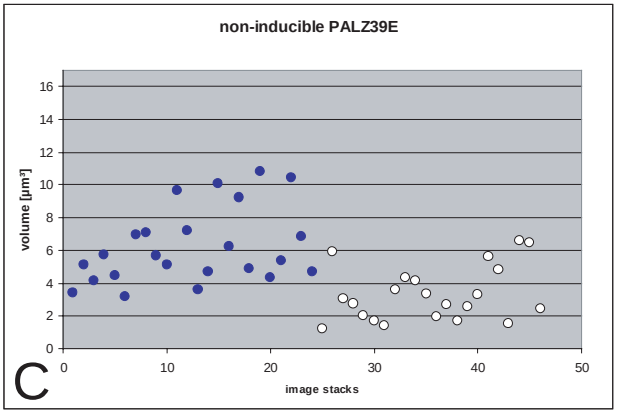
Centromeres in mouse cells cluster to a high extent, forming chromocenters. Francastel et al. (Francastel et al., 2001) described the relocation of the inactive endogenous β-globin locus in MEL cells away from the DAPI-stained chromocenters when activated after induction. The transgene investigated in the present study is controlled by β-globin locus elements. We therefore tested whether the transgene arrays also locate to chromocenters when inactive. In the inducible PALZ39E culture, we co-hybridized a pancentromeric probe with the transgene plasmid probe to label both chromocenters and transgene arrays (Fig. 5A,C-F). As expected, the pancentromeric probe signal colocalized with regions of intense DNA counterstain. We classified the transgene signal into one of four categories by visual inspection according to their position relative to the chromocenters – no contact, touching, partly overlapping and complete colocalization (Table 2). We never found a transgene signal with complete colocalization (i.e. embedded in a chromocenter). Most signals had no contact. In contrast to the initial hypothesis, we found expressing transgene arrays more often touching or partly overlapping with chromocenters than inactive ones.

Transgene arrays in the inducible culture could be in a transcriptionally poised state preventing localization to chromocenters. Therefore, we next investigated the cell line PALZ39M, which was not inducible and in which only 1-3% of cells were β-galactosidase positive. In this cell line, we used the TO-PRO-3 DNA counterstain alone to detect chromocenters. Again, most cells showed no contact of the transgene signal with chromocenters. And, again, the proportion of cells with contact was higher in β-galactosidase-positive cells than in negative cells (Table 2). Because expressing transgene arrays are larger than inactive ones, we assume that the higher contact rate is a consequence of their larger size.

Neither cell line had a pronounced DNA staining at the site of the transgene array that would indicate heterochromatinization, as has been described for previously







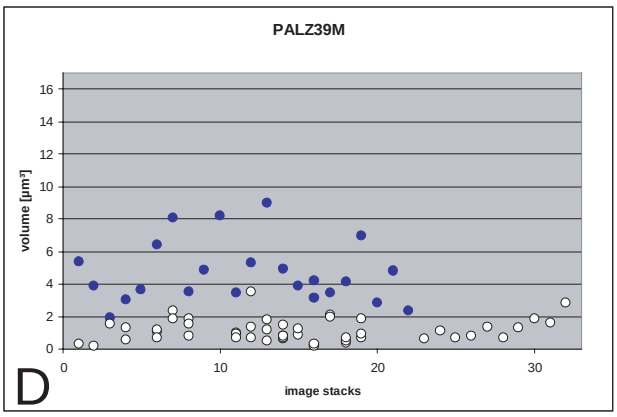


Fig. 4. Volume measurements of transgene arrays in β-galactosidase-expressing (blue dots) and non-expressing (white dots) cells. Each dot represents one nucleus. Dots above each other represent nuclei from the same image stack, thus excluding differences in local specimen conditions or image recording between these nuclei. (A) GFP/lac-repressor signals in PALZ39E cells from an inducible culture. Signals were recorded either from uninduced, non-expressing or induced, expressing cells. (B) FISH signals from an inducible PALZ39E culture that was either not induced (left) or induced with HMBA (right). (C) Uninducible PALZ39E cells. (D) PALZ39M cells.

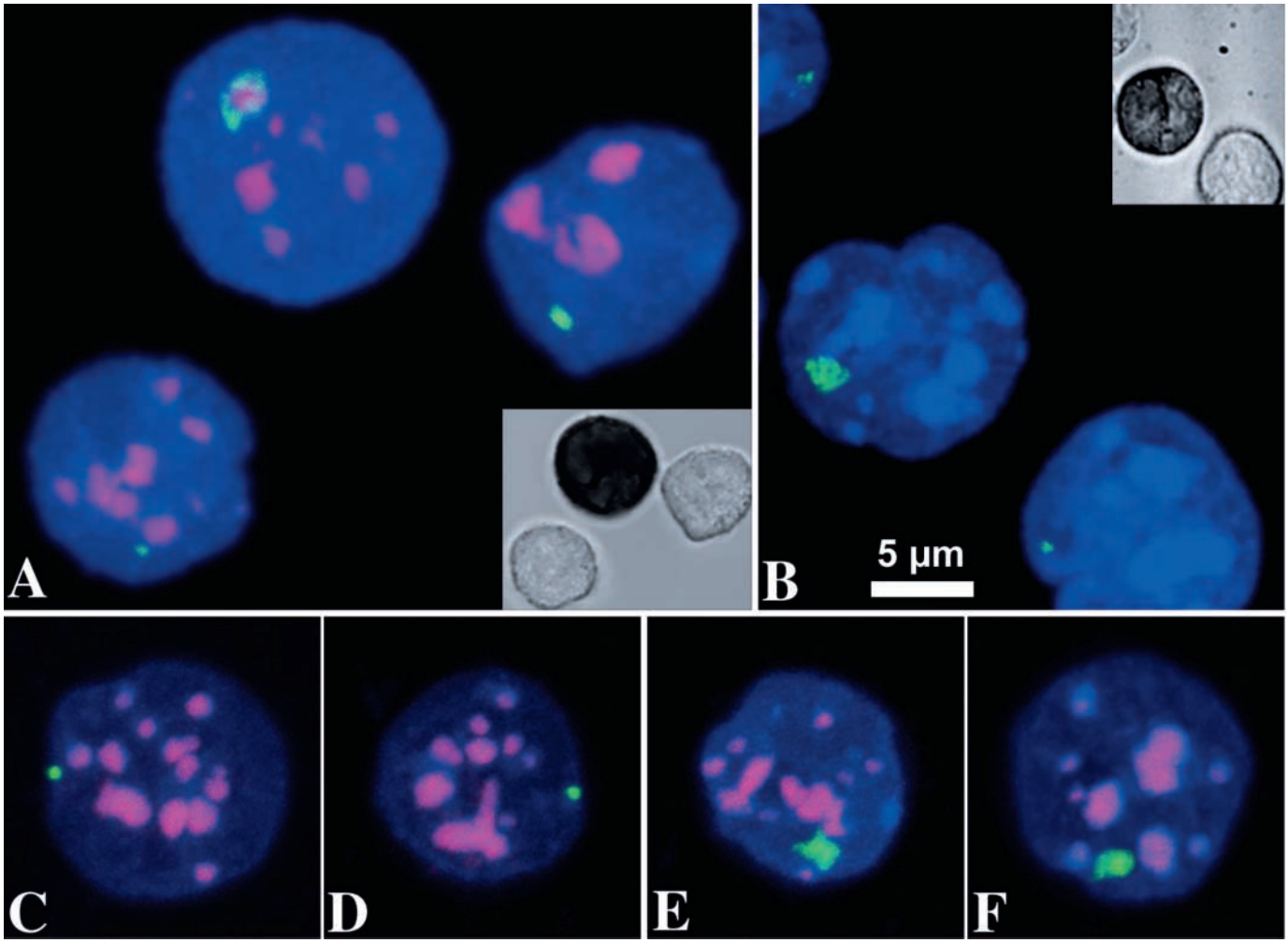


Fig. 5. Increase in transgene array volume associated with transcriptional activation is also observed using FISH. (A) PALZ39E nuclei with FISH signals for the transgene (green) and the centromeres (red). DNA counterstain in blue. Inset shows corresponding X-Gal staining. (B) PALZ39M nuclei with FISH signals for the transgene (green) in PALZ39M cells. Inset shows X-Gal staining. (C-F) Nuclei from PALZ39E cells without induction and X-Gal-negative (C,D) or with induction and X-Gal-positive (E,F). Colors as in (A). Fluorescence images are projections of confocal sections. Scale bar is for all fluorescence images.

characterized heterochromatic transgene arrays (Li et al., 1998).

Discussion

Here, we have demonstrated that transcriptional activation associated with β-globin regulatory sequences can induce both large-scale chromatin decondensation and changes in intranuclear positioning of large transgene arrays in MEL cells. Previously, after our demonstration of large-scale chromatin decondensation induced by VP16 targeting, we proposed a 'relay' model in which gene-rich chromosome regions might maintain an open chromatin chromosome region through long-range chromatin decondensation propagating from individual gene loci (Tumbar et al., 1999). Gene-rich chromosome regions were subsequently demonstrated to be less condensed and more centrally located than gene-poor chromosome regions (see Introduction), whereas movement of a multicopy

Table 2. Positions of transgene arrays relative to chromocenters (centromere clusters)

	A*	B*	C*	D*	Sample size
Inducible PALZ39E, no HMBA, X-Gal positive	50%	33%	17%	0%	18
Inducible PALZ39E, no HMBA, X-Gal negative	93%	2%	4%	0%	45
Inducible PALZ39E, with HMBA, X-Gal positive	81%	15%	4%	0%	26
Inducible PALZ39E, with HMBA, X-Gal negative	89%	11%	0%	0%	19
PALZ39M, X-Gal positive	78%	17%	4%	0%	23
PALZ39M, X-Gal negative	98%	2%	0%	0%	49

*A, no contact; B, touching; C, partly overlapping; D, complete colocalization.

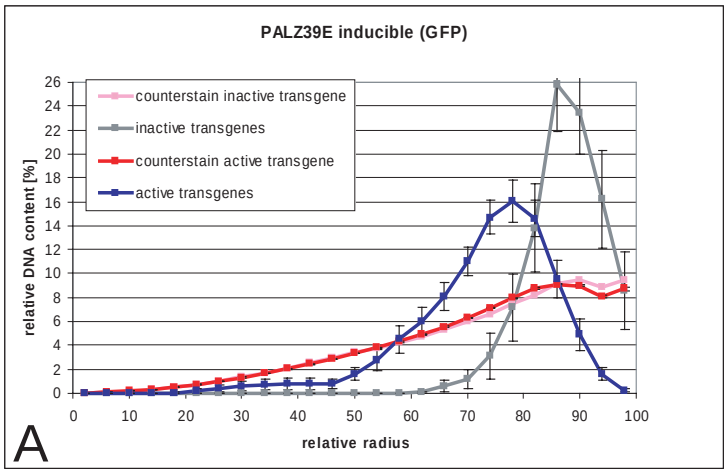
transgene array away from the nuclear periphery was demonstrated after VP16 targeting (Tumbar and Belmont, 2001). Our present results indicate that large-scale chromatin decondensation and changes in nuclear positioning as observed for large, complex gene-rich chromosome regions can be induced by endogenous regulatory sequences within transgene arrays. Therefore, a high density of transcriptionally active genes, even in the form of repetitive transgene arrays, might be sufficient to reproduce the behavior of complex chromosome regions. These transgene arrays therefore might represent a greatly simplified system to dissect the biochemical mechanisms underlying these phenomena.

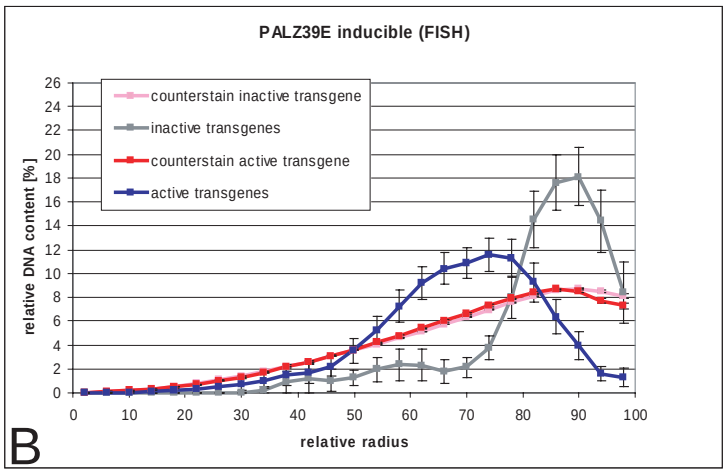
Endogenous transcriptional activators can cause large-scale chromatin unfolding

FISH studies on several strongly transcribed chromosomal regions have shown a disposition for looping out from their respective chromosome territories (Mahy et al., 2002b; Volpi et al., 2000; Williams et al., 2002), suggesting a large-scale chromatin decondensation reminiscent of results obtained by targeting transcription factors to transgene arrays. In the first of these targeting studies chromatin decondensation was induced by the viral transcriptional VP16 acidic activation domain. Targeting was achieved within the context of large transgene arrays containing multiple-copy plasmid integrations; each plasmid carried direct repeats of 256 (Tumbar et al., 1999) or 96 (Tsukamoto et al., 2000) operator binding sites for fusion proteins between the lac or tet repressor and VP16. Despite the large opening activity observed, the biological relevance of these observations hinges on the actual physiological relevance of the experimental system. In particular, there are three obvious concerns.

First, the viral VP16 acidic activation domain might represent an unusually potent transcription factor yielding a much larger large-scale chromatin opening activity than endogenous transcription factors. However, significant large-scale chromatin opening was reproduced with other lac-repressor fusion proteins, including fusions with the estrogen receptor (Nye et al., 2002), BRCA1 (Ye et al., 2001) and other endogenous acidic activation domains (Ye et al., 2001) (A. E. Carpenter and A.S.B., unpublished). Moreover, a similar opening of large-scale chromatin structure was observed with a transgene array consisting of the MMTV viral promoter driving the *Ras* gene (Müller et al., 2001). In this case, endogenous transcription factors, including glucocorticoid receptor, were acting on the viral promoter to induce transcriptional activation and large-scale chromatin opening. Here, we extend these results by showing that endogenous factors acting on mammalian regulatory sequences can produce similar effects.

Second, the unusual high number of targeted





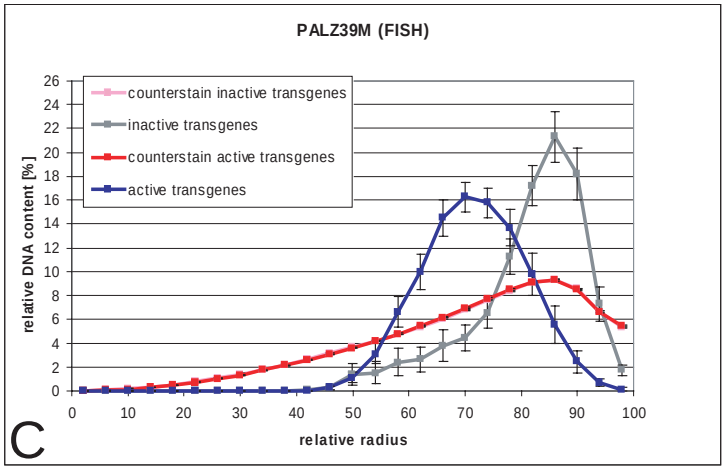


Fig. 6. Different radial distribution of active (blue) and inactive (gray) transgene arrays within interphase nuclei. Each nucleus was divided into 25 shells of equal thickness. 25 points on each curve show the percentage of the signal (transgene or counterstain) within each shell. The *x*-axis indicates the position of the center of the shell relative to the nuclear radius (which is defined as 100 units). Detection of the transgene in inducible PALZ39E cells was with GFP/lac-repressor (A; *n*=40 for expressing cells, *n*=12 for non-expressing cells) or by FISH (B; *n*=22 for expressing cells, *n*=25 for non-expressing cells). Signals in PALZ39M cells (C; *n*=22 for expressing cells, *n*=40 for non-expressing cells) were detected by FISH. Distribution of nuclear DNA is shown in red (from nuclei with active transgenes) and pink (from nuclei with inactive transgenes) for comparison. Red and pink curves are very similar, in (C), they completely overlap, so that the latter is not visible. Error bars indicate the standard deviation of the mean.

transcriptional factors might produce an opening activity much larger than would normally be present over an endogenous promoter. In the original experimental design, a direct repeat of 256 lac operators was used to target a lac-repressor/VP16-acidic-activation-domain fusion protein (Tumbar et al., 1999). Additional experiments with different transcription factor domains replaced the lac-repressor/VP16-acidic-activation-domain fusion protein with fusions of lac repressor to other activation domains (Nye et al., 2002; Ye et al., 2001). Although unpublished data (T. Tumbar and A.S.B., unpublished) suggest that the occupancy of the lac operator direct repeat is quite low, this value has never been directly measured. Again, however, the observation of similar large-scale chromatin opening with a multicopy plasmid integration of a transgene driven by the viral MMTV promoter indicates that similar large-scale chromatin opening can be observed with naturally occurring numbers of transcription factor binding sites (Müller et al., 2001). In this case, one might argue that the viral promoter has evolved an unusual, synergistic number of transcription-factor binding sites, leading again to a higher chromatin opening activity than would normally be present over an endogenous promoter. Our current results with β-globin regulatory regions now further extend these previous results by demonstrating that comparable large-scale chromatin opening can be produced by regulatory sequences from nonviral, developmentally regulated mammalian genes.

Third, the nature of the transgene array produces a higher density of transcription units than is usually found in mammalian chromosomes. This might produce an appearance of long-range decondensation of large-scale chromatin structure in these transgene arrays that might not be present over endogenous gene loci if changes in chromatin structure were confined to several kbp regions surrounding the enhancer/promoter of the endogenous genes. We currently cannot directly address this concern experimentally, because our present experimental design relies on plasmid transgene arrays.

In the original VP16-acidic-activation-domain study (Tumbar et al., 1999), the large-scale chromatin decondensation appeared to propagate across co-amplified blocks of genomic DNA estimated to average 1000 kbp. In the case of large-scale chromatin decondensation observed from the MMTV promoter, the transgene arrays consisted of at least 200 repeats of a 9 kbp plasmid. In the present study using β-globin regulatory sequences, the transgene arrays consist of copies of a 15 kbp vector. However, as in the VP16 study, significant amounts of genomic DNA are interspersed among the plasmid insertions. Based on the strength of the chromosome-12 paint signal within the transgene arrays, this genomic DNA is estimated to compose about two-thirds of the transgene-array sequence content.

We have observed unexpected behavior of the transgene arrays in this study, specifically the loss of inducibility of the reporter gene driven by β-globin regulatory sequences. However, β-globin expression can occur in uninduced MEL cells at low levels (Bender et al., 1988; Miller et al., 1988), although it was not always detected (Forrester et al., 1989). Therefore, in cells carrying our multiple copy transgene array, it might not be surprising that we find considerable proportions of X-Gal-positive cells in uninduced cultures. However, the absence of increased reporter expression after induction in most cultures indicates perturbed regulation of transgene expression. In earlier studies, transgenes with the same regulatory sequences present in fewer copies (<10) have shown appropriate regulation (Forrester et al., 1989; Robertson et al., 1996). Therefore, the size of the transgene arrays in our cell lines appears to interfere with proper reporter gene regulation for unknown reasons.

This loss of inducibility might be related to titration of β-globin-specific transcription factors and coactivators by the high transgene copy number. However, this does not explain the considerable proportion of expressing cells in uninduced cultures, the changing proportions of these expressing cells as a function of cell cultivation or the PALZ39E subclone that was initially inducible but then upon lost this inducibility further growth. Interestingly, the Felsenfeld laboratory (Pikaart et al., 1998) analysed epigenetic silencing of large transgene arrays and described an all-or-none silencing of these arrays in which gene silencing of reporter genes and accompanying alterations in chromatin structure and DNA methylation appeared to occur co-operatively within transgene arrays; specifically, cells underwent transitions between expressing and nonexpressing with no intermediate expression levels observed (Pikaart et al., 1998). We speculate that a similar phenomenon might have occurred in our study. Future work will need to move away from the use of these large transgene arrays to study more physiological aspects of β-globin gene regulation.

Intranuclear positioning of transcriptionally active versus inactive transgene arrays

Inconsistent data have been published concerning the colocalization of β-globin loci with centromeric heterochromatin. Brown et al. (Brown et al., 2001) described a tethering to centromeric heterochromatin in cycling lymphocytes but a euchromatic localization in primary human pronormoblasts, in which this gene is expressed. The Groudine lab investigated the endogenous mouse β-globin locus in MEL cells. They described an association with DAPI-stained chromocenters in 60% of uninduced cells, decreasing to 10% after chemical induction of transcriptional activation associated with differentiation. In uninduced cells, an additional 14% associated with the nuclear periphery, another target site for heterochromatin, decreasing to 5% after differentiation (Francastel et al., 2001). When the same group recently investigated the human β-globin locus in uninduced MEL hybrid cells carrying the complete human chromosome 11, however, they found an association with chromocenters in only 16% of the cells (Ragoczy et al., 2003). In the same study (Ragoczy et al., 2003), they describe a looping out of this β-globin locus on human chromosomes in the uninduced MEL cells. Upon induced differentiation of the cells with accompanying expression of β-globin, the looping frequency decreased. In the present study, we found only an 11% association of inactive transgene signals with chromocenters, with this proportion increasing in cells with active transgene arrays, possibly as the result of the increased size of the transgene array.

Gene-rich chromatin is preferentially located in central areas of the nucleus, whereas heterochromatin is often found at the nuclear rim, leading to a polar orientation of chromosome territories (Ferreira et al., 1997; Sadoni et al., 1999; Skalníková

et al., 2000). Some studies have looked at individual loci in activated and silent states but did not find a redistribution that would correlate with the change in transcription (Bártová et al., 2002; Parreira et al., 1997). However, a redistribution of lac operator transgenes by a lac-repressor/VP16 fusion protein from the nuclear rim to an internal position has been described (Tumbar and Belmont, 2001). Here, we show an example of such a relocation to a more internal position in transcriptionally active loci without viral activators. Possibly, a movement to a more internal position in the nucleus does not occur when a single gene locus is activated but only when the transcriptional activity of a large region changes, because of positional effects from neighboring chromatin.

Size difference between GFP and FISH signals

Transgene arrays detected by GFP/lac-repressor had a significantly smaller volume than those detected by FISH. In X-Gal-positive cells, this might be partly caused by X-Gal staining absorbing excitation and emission light. However, in X-Gal-negative cells, we also found smaller volumes for GFP signals than for FISH signals. We thus exclude the possibility that the size differences are an attenuation artifact. Previously, we compared the GFP signal over an amplified chromosome region in live cells with signals after the FISH procedure in the same CHO cells (Robinett et al., 1996). A noticeable increase in size was observed after the FISH procedure, although the effect was much smaller than observed in this study. However, in this previous study, a 3 hour paraformaldehyde fixation was used, compared with the 15 minute fixation used in the present experiments. The 3 hour fixation was chosen after previous FISH experiments indicating larger structural perturbation produced with shorter fixation times (beyond 3 hours fixation, the FISH signal could no longer be detected) (Robinett et al., 1996) (A.S.B., unpublished). We therefore conclude that the applied FISH procedure produced an artefactual increase in size of the labeled transgene array. We thus provide a quantitative demonstration of the alterations in structure that can be induced by FISH. This effect is likely to depend on fixation times and might vary in different cell types and possibly at different chromosomal locations. Significantly, however, the relative size differences between active and inactive transgene arrays was similar as measured from both the GFP or FISH signals.

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