addition, it will be vital to establish universal standards of data quality, storage, analysis and presentation. The 'minimum information about a microarray experiment' (MIAME) standards ([17]; http://www.mged.org/Workgroups/MIAME/miame_checklist.html) go some way towards this but do not cover the basics of what data can be considered reliable. How to assess the quality of data is another problem left to the individual researcher.

Concluding remarks

Overall, what can be learned from this approach? As used by Clark *et al.* to analyse deletions of genes that are not essential for cell viability, the intron microarray offers a rapid method to investigate the functions of candidate new splicing factors; for example, those identified through a physical or genetic interaction with a known splicing factor. The genome-wide approach is ideal for non-essential genes, which are the most difficult to characterize by other approaches because, by definition, they are not essential for the splicing of all pre-mRNAs, and their targets might be difficult to identify. This study has also demonstrated the drawbacks of the conventional approach of analysing the effects of mutations on a limited number of substrates, which could result in misleading conclusions. The microarray approach also permits a global analysis under many conditions of growth, cell cycle progression and, in more complex organisms, different tissues and different stages of development or of disease.

Applied to higher organisms, this type of analysis offers huge potential to investigate the regulation of splicing. For example, overexpressing splicing factor genes, or transiently blocking their expression, allows their role in the development of an organism to be evaluated, as shown by Longman *et al.* [18], using RNA-mediated interference (RNAi) on members of a family of SR and SR-related splicing factors in the nematode, *Caenorhabditis elegans*. Combined with microarray analysis of splicing events, this could provide an extremely powerful means of investigating which differential splicing events affect development, how they are regulated and how these processes might be perturbed in disease.

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Transcription and the territory: the ins and outs of gene positioning

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When cells exit mitosis, the neat rod-like chromosomes decondense into their interphase state. However, the chromatin threads are not randomly dispersed throughout the nucleoplasm. Rather, individual chromosomes appear to be organized into discrete, non-overlapping 'territories'. Current studies attempt to unravel how gene loci are organized within these territories, whether their subterritorial positions are dependent on transcription, and the extent to which the loci can move.

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Box 1. Chromosome territories

- A chromosome territory is defined as the distinct physical form of a chromosome in the interphase nucleus.
- Territories occupy a particular fraction of the nuclear volume as opposed to being fully decondensed throughout.
- Neighbouring territories are not thought to intermingle (although see last point).
- The surface of the territory (or territory subdomain) is a loose term to describe the non-physical boundary between chromatin and non-chromatin space.
- Non-chromatin space (originally called the ICD but now the IC) is thought to be home to splicing speckles, transcription machinery and several nuclear bodies including PML bodies, Cajal bodies etc.
- Territories can be visualized by fluorescence *in situ* hybridization (FISH) using probes covering the entire chromosome of interest. This is termed chromosome painting, and territory dynamics can now be observed by painting in living cells.
- Whole territories display only a small degree of movement during interphase.
- Particular chromosome territories have reproducible positions within the nucleus. This non-random relative positioning of chromosomes is thought to explain why certain chromosomes have apparently favoured partners in translocation events.

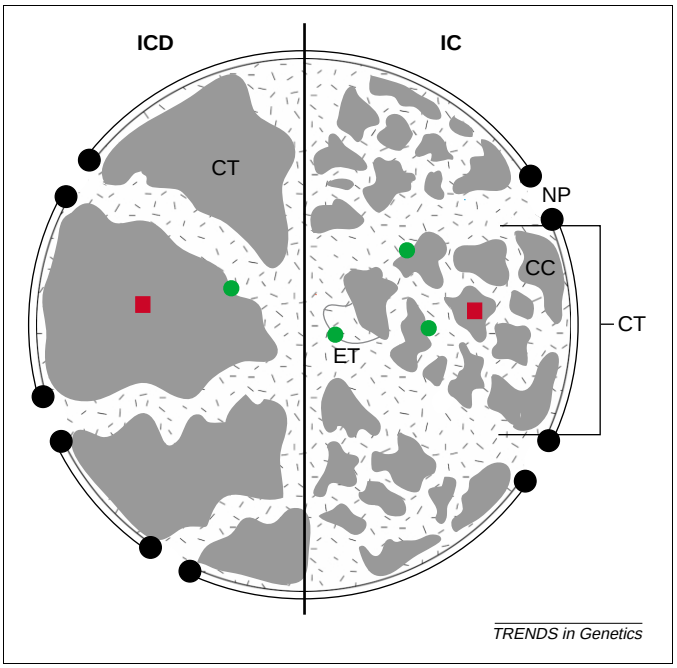

Fig. 1. Comparison of the interchromosome domain model (ICD) and the interchromatin compartment model (IC) [1]. The ICD model predicted that active genes (green circles would be located at the surface of chromosome territories (CT) where they would be accessible to the transcription/splicing factors of the ICD (dashed area). Transcripts could then be easily transported to the nuclear pores (NP) for export. Silent genes (red squares) or intergenic loci might instead be found on the interior of chromosome territories and be inaccessible to factors of the ICD. The IC model was proposed following observations that the non-chromatin space (dashed area), and factors thereof, extended through the interior of the territory. Active genes would be found on the surface of the territory or on the surface of the condensed chromatin subdomains (CC), whereas silent genes or intergenic loci could be found within the condensed chromatin domains. It has been observed that certain active loci can also reside 'extraterritorially' (ET) on large (megabase) chromatin loops extending from the territory surface.

Thanks to the genome projects, the position of genes along chromosomes is now known. However, little is known about how this linear arrangement relates to the organization of chromosomes in the nucleus. Evidence suggests that individual interphase chromosomes occupy a discrete nuclear space, the 'territory' (Box 1). The use of fluorescence *in situ* hybridization (FISH) to detect entire chromosome territories, genomic probes and RNA has provided an effective tool for investigators to research interphase chromosome organization. Particular emphasis has been placed on the question of whether transcriptional activity of genes can affect their territorial positioning and, indeed, whether active genes can leave the territory altogether. Two recent reports from Wendy Bickmore's laboratory challenge the view of interphase chromosome organization, from the inner territory structure to the outermost chromatin extrusions [1,2].

The ins...

The discovery, by combined immunofluorescence and FISH, that small nuclear ribonucleoproteins (snRNPs) and RNA transcripts (from an integrated human papilloma virus) were located at the boundary of painted chromosome territories (but excluded from territory interiors) [3], led to the proposal that transcription and splicing machinery might be confined to a system of channels that run between the chromosome territories and link to the nuclear pores. This interchromosome domain (ICD) model (Fig. 1) predicted that actively transcribed genes would be located at the surface of chromosome territories where they would be accessible to the transcription and splicing factors and where RNA transcripts could be released directly into the ICD for transportation to the nuclear pores. Conversely, the chromatin organization of interior of chromosome territories would be impenetrable to protein factors and thus repressive to transcriptional activity (reviewed in Ref. [4]).

Support for this model came from the discovery by Kurz *et al.* that in muscle cells, fibroblasts and HeLa cells, the *DMD*, *HBB* and *MYH7* genes (but not noncoding regions) reside at the surface of their respective chromosome territories [5]. Extending this analysis, Dietzel *et al.* showed that the *ANT2* gene (located at Xq24–25 and subject to X inactivation) resides at the periphery of the active X territory, but on the interior of the inactive X territory (where it is silenced). By contrast, *ANT3* (located at the pseudoautosomal region, Xp22.3) escapes inactivation and is peripheral in both X territories [6]. More recently, it has been reported that some transcriptionally active gene-dense loci can apparently stretch large distances 'outside' of the chromosome territory (possibly into the ICD) [2,7,8].

Although the observations described above seem to support the hypothesis of the ICD model, growing evidence has led to the need for refinement. A study in wheat nuclei revealed that sites of transcription initiation, visualized by BrUTP incorporation were present in the interior of chromosome territories [9]. In addition, immunolabelling of nascent BrdU-labelled RNA together with chromosome territory painting in

human fibroblasts revealed that RNA transcripts are found throughout the interior of chromosome territories, although notably, in regions of decondensed chromatin and not in regions of condensed chromatin [10]. An electron microscopy study of the substructure of individual BrdU-labelled chromosomes in hamster fibroblasts revealed that territories are composed of a meandering structure of condensed chromatin domains and that non-chromatin space does not appear restricted to channels running between chromosome territories but also could be seen running through the territory in regions of decondensed chromatin [11].

These observations led to the revised interchromatin compartment (IC) model (Fig. 1) (reviewed in Ref. [12]), which predicts that active genes might be found not only on the surface of the territory, but also within the territory on the surface of the condensed chromatin subdomains that line the invaginating interchromatin channels. Until recently, however, validation of this prediction has been elusive.

A recent paper from the Bickmore laboratory now provides the first evidence that active genes can indeed be found within the territory [1]. Mahy *et al.* found, by FISH analysis, that the WAGR locus at 11p13 (associated with Wilm's tumour aniridia, genitourinary anomalies, mental retardation syndrome and containing the genes *WT1*, *RCN*, *PAX6* and *PAXNEB*) is located well within the painted chromosome 11 territory and, more importantly, that transcriptional activation of these genes did not result in their relocation to the territory surface.

The *RCN* gene was found to reside on the surface of a condensed chromatin subdomain, whereas an intergenic (noncoding) probe from the WAGR region was located within a condensed subdomain. These relative positions support the prediction of the IC model (Fig. 1); however, the authors suggested (unpublished data) that the *PAX6* and *WT1* genes might be transcriptionally active from within a condensed chromatin subdomain. This hints that, contrary to the IC model, even the interior of condensed chromatin subdomains might not be refractory to transcription. A resolution to this controversial postulate is eagerly awaited, particularly in the light of the recent paper from Roel van Driel's laboratory that reports that both TFIIH and RNA polymerase II (transcription factors) and hnRNP-U (splicing factor) are excluded from condensed chromatin subdomains [13] (as are transcripts [10]). Definitive proof might be sought through combined DNA and RNA FISH together with territory painting. This would then enable position, relative to condensed chromatin, and activity status of loci to be established simultaneously.

… and outs

As well as active genes being located within the chromosome territory, it was discovered in Denise Sheer's laboratory that active genes can also be observed 'outside' the painted territory [7]. Volpi and colleagues found that the major histocompatibility (MHC) region at 6p21 is able to extend out from the chromosome 6 territory on large loops, in response to activation of MHC genes. A significantly increased proportion of nuclei displaying these loops were observed in B-lymphoblastoid cells and in interferon-γ (INF-γ)-treated fibroblasts, which express the MHC genes, compared with untreated fibroblasts [7]. In addition, at 1q21 the epidermal differentiation complex (EDC), a cluster of >40 genes that are expressed coordinately during keratinocyte differentiation, adopts an 'extraterritorial' position in keratinocytes, where the genes are highly expressed, more often than in lymphoblasts where the genes are silent (Fig. 2) [8].

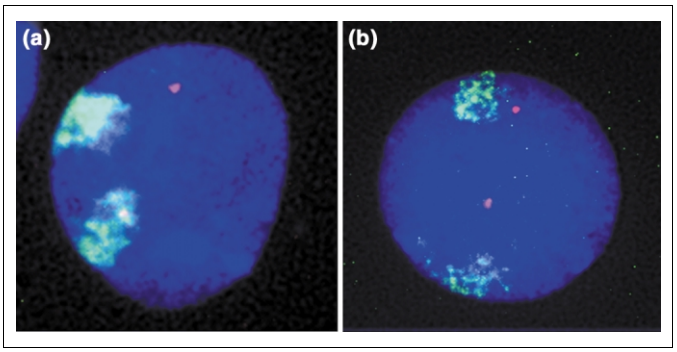

Fig. 2. Extraterritorial positioning of (a) the epidermal differentiation complex (EDC) from chromosome 1 in keratinocytes and (b) the major histocompatibility (MHC) region from chromosome 6 in lymphoblasts. Green, chromosome territory signal; red, EDC or MHC region probes.

Given that the MHC and the EDC are both gene dense complexes containing genes of related function and coordinated expression pattern, it is possible that the phenomenon of extraterritorial positioning is unique to such complexes. Another recent paper by Bickmore and colleagues suggests that, in fact, such positioning is more widespread, being correlated to gene density and transcriptional activity in general [2]. The authors looked at several genomic loci including 11p15.5 (a region of high gene density and generally high levels of transcription in many cell types) and several probes from chromosomes 21 and 22 for which the relative gene densities were known. They found that 11p15.5 was frequently observed outside of the chromosome 11 territory in both fibroblasts and lymphoblasts and that, in general, there was a strong correlation between gene density and extraterritorial positioning.

Inhibiting transcription by ActinomycinD (ActD) and 5,6-dichloro-b-ribofuranosylbenzimidazole (DRB) treatment caused a reduction in the number of extraterritorial signals scored at each locus observed, however it was not a dramatic reduction because many signals from 11p15.5, for example, were still observed extraterritorially. By contrast, both the MHC and EDC have a significantly reduced number of extraterritorial signals in cell types where the genes are not expressed. Furthermore, the frequency with which the MHC is scored as external more than doubles following 10 mins of treatment with IFN-γ (and triples after 16 hours of treatment) [7,8]. It could be that extraterritorial

positioning at these loci is more closely correlated to transcription levels than at regions such as 11p15.5. Alternatively, it could be that ongoing transcription facilitates the formation of loops, but is not required to maintain them [2]. With this in mind, it would be interesting to observe the effect of ActD or DRB at the MHC and EDC loci in cells where they are transcriptionally active.

What remains to be established is whether the looping out of DNA is a requirement for transcription at these particular loci; that is, are genes at the MHC, EDC or 11p15.5 only active when exposed on chromatin loops? Also, where are the loci stretching to – particular nuclear domains (reviewed in Ref. [14]), neighbouring chromosome territories, or somewhere else? Electron microscopy reveals that although the borders between chromosomes are generally separated by interchromatin space, decondensed chromatin from neighbouring territories can be seen in contact in limited regions [11].

The finding that many loci can be observed outside the territory might also imply that current chromosome paints are falling short of revealing the full extent of the territories.

Chromatin in motion

The use of FISH has the unfortunate limitation of providing only snapshots of what is going on. To circumvent this, investigators photograph and score large numbers of nuclei to gain statistically meaningful results. Such analyses reveal that positions of gene loci, with respect to the territory, are not absolute but represent a statistically significant percentage (a trend). For example, in the case of extraterritorial positioning, it is noted that none of the loci described are always observed outside of the territory [2,7,8]. Furthermore, homologues in the same nucleus are frequently observed in different positions. In fact, the variation in positioning between homologues within the same nucleus is no different than that between nuclei [1]. These observations might suggest that the loci are dynamic.

Work carried out in John Sedat's laboratory on yeast and *Drosophila* [15,16], using lac-repressor protein fused to green-fluorescent protein (GFP) to visualize randomly integrated *lac* operator repeat arrays in live cells, established that chromatin at interphase is continually moving. It does not, however, roam freely around the nucleus, but instead exhibits constraint; for example, in *Drosophila* a locus was estimated to be confined to a territory-sized region, with an average maximum 'radius of confinement' of 3 μm (over a 30–60 min period) [16].

A similar study of the human genome, confirmed that the movement of chromatin by constrained random diffusion is also a feature of mammalian cells. Interestingly, the authors report that different genomic regions display different degrees of constraint [17]. So one might postulate that certain loci, such as the MHC, EDC and 11p15.5, which can be observed extraterritorially, are subject to less territorial constraint than regions such as the WAGR locus, located deep within the territory (Fig. 3). It might be possible to test this by specifically targeting *lac*-operator constructs to such loci. The particular dynamics, size and position of their radius of confinement could then be compared, both with each other and among different cell types and transcriptional states. However, the integration of genetic elements could significantly alter the behaviour of surrounding chromatin, as has been displayed recently through the ectopic integration of a *cenH* element in the yeast genome [18]. It will be important, therefore, to confirm the unaltered expression pattern of genes at targeted loci.

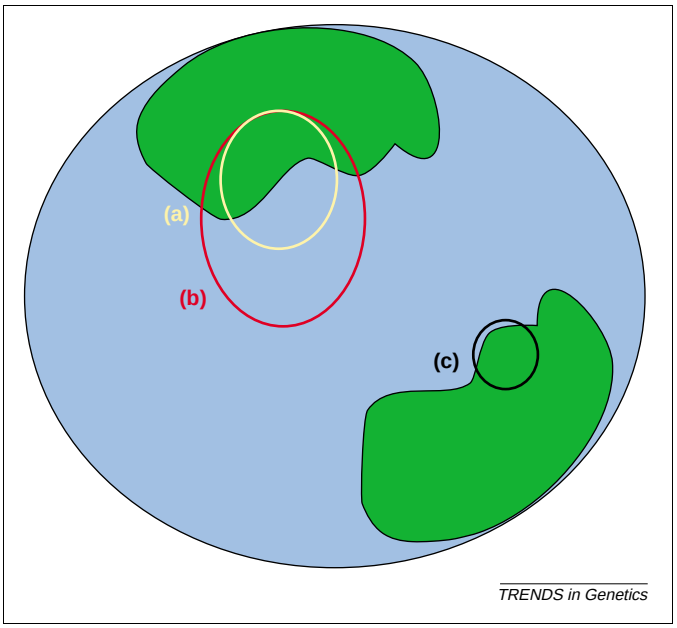

Fig. 3. The discovery that chromatin moves by random diffusion indicates that the particular position of a locus relative to its territory (green), as determined by fluorescence *in situ* hybridization (FISH) analysis, represents the particular position of its radius of confinement (circles) (defined by the position or affinity of chromatin tethers). Thus, FISH datasets represent a set of random positions within a non-random radius of confinement. Regions such as the epidermal differentiation complex (EDC) or the major histocompatibility complex (MHC) might have different territorial constraints when their genes are downregulated (a) and when expressed at high levels (b) compared with other loci (c) that display tighter constraint.

The constraints on chromatin motion are thought to be a result of 'tethering' of the chromosome by protein complexes at particular points. In agreement with this, it is noted that the territory as a whole, viewed by live-cell painting (fluorescent nucleotide incorporation at S phase and subsequent passage) does not move to any great extent during interphase [19]. The change in subterritorial positioning of a locus correlated with transcription (e.g. the looping out of a locus) could represent a change in the affinity or position of such tethers. Currently, the molecular nature of tethers is not known, but candidates include components of the nuclear envelope and lamina, nuclear pore complexes, insulator bodies and nucleolus (reviewed in Refs [20,21]). The yeast telomere-associated protein yKu70, for example, associates with nuclear pore protein Mlp2 and a mutation of yKu70 prevents telomere tethering to the nuclear pore [22]. Similarly, the *Drosophila gypsy* element (a retrotransposon and insulator) associates with the nuclear envelope through binding to Su(hw) protein, but it becomes detached from the envelope and looses insulator function if Su(hw) is

mutated [23]. Insulator elements, such as *gypsy*, are regulatory sequences that are thought to organize independent genomic regions of transcriptional activity (or repression) [21] and thus point towards a functional link between a tether site and transcription control.

So far, equivalent mechanisms have not been identified in mammalian cells; however, Chubb *et al.* observed in human cells that genomic regions that were associated with the nuclear envelope and nucleolus had reduced radii of confinement compared to more nucleoplasmic regions [17].

Chromatin is also thought to have both permanent and temporary attachments to a proteinaceous substructure know as the nuclear matrix. The permanent attachments are thought to provide structural integrity to the chromosome and are associated with non-transcribed DNA, whereas the transient attachments are determined by associations with transcription and replication machinery [24–26]. The existence of a nuclear matrix has recently been disputed with the suggestion that proteins aggregate together artificially under the conditions used for matrix preparation [27]. However, there is much evidence that argues for its existence (reviewed in [28]).

Concluding remarks

Mahy and colleagues have now provided the proof of principle that, as predicted by the revised IC model, genes can indeed be actively transcribed from within the territory (at the WAGR locus at least) [1]. However, the authors' suggestion that genes may even be expressed from within the condensed chromatin subdomains, (shown to be devoid of RNA transcripts, transcription factors and splicing factors [10,13]) clearly deserves further investigation.

Mahy *et al.* have now also shown that the phenomenon of extraterritorial positioning is not unique to gene dense coordinately regulated complexes such as the MHC and EDC, but instead appears to be correlated to both gene density and transcriptional activity in general [2]. It remains to be determined what the biological meaning of such protrusions might be: are the loci stretching towards a particular nuclear domain or are they a more passive result of active transcription? The data presented so far, although convincing, are still correlative and seek a functional explanation.

A functional angle might be gleaned from discovering the identity of the protein elements and DNA sequences involved in tethering chromatin. This could provide explanations not only of why some loci are tightly confined within the painted region territory (such as the WAGR locus) and other loci seem to have a larger radius of confinement (such as the MHC, EDC and 11p15.5), but also of how and why the degree of movement of a locus might be affected by cell type and transcriptional activity.

Complexes such as the EDC, which is coordinately upregulated during skin differentiation, might then prove to be useful model regions for studying how the positioning and affinity of tethers alters during differentiation down a particular cell lineage.

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