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Features of Nuclear Architecture That Influence Gene Expression in Higher Eukaryotes: Confronting the Enigma of Epigenetics

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Abstract Complex mechanisms that influence gene expression in mammalian cells have been studied intensively over recent years. Genetic elements that control both the tissue specific patterns and levels of gene expression together with the proteins they bind have been characterised in detail and are clearly pivotal in activating pathways of gene expression. But it is also clear that the behaviour of these genetic elements is complicated by epigenetic factors, so that their introduction into cells with the necessary developmental history—and hence appropriate global concentrations of essential transcription factors—will not guarantee the desired levels of transcription. Recent experiments have reinforced this view and confirmed that apparently critical functions performed by defined genetic elements at certain chromosomal sites are not inevitably recapitulated at other chromosomal locations. Hence, a re-evaluation of the function of critical control elements is required using experimental systems that simplify the range of factors arising from local chromatin organisation. In this way, it should be possible to reveal the intricacies of gene expression that might eventually allow us to reproduce natural levels of expression from artificial gene constructs in human cells. J. Cell. Biochem. Suppl. 35:69–77, 2000. © 2001 Wiley-Liss, Inc.

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Over the past 20 years we have witnessed a revolution in molecular biology that has had a dramatic impact on our understanding of the fundamental mechanisms involved in the control of gene expression. In particular, these very powerful technologies have emphasised the importance of transcription factors and the critical role that they play in association with both gene specific and general genetic motifs that are now known to be pivotal in activating gene expression [Goodrich et al., 1996]. More recently, interest has focused on the role of chromatin in modulating the accessibility of transcription factors to their binding sites in DNA and the changes that occur in chromatin in response to gene activation and transcription [Workman and Kingston, 1998; Kadonaga, 1998]. However, while the role of transcription factors and modification of chromatin proteins in gene expression have been studied intensively—the fundamental principles are now defined—other approaches demonstrate that these features are not sufficient to define patterns and levels of gene expression in different tissues. Most notably, experiments investigating expression from particular promoter elements driving ectopic genes in transgenic animals [Wilson et al., 1990] confirm a surprising variability of gene expression from different genomic sites, so emphasising the possible influence of epigenetic factors on patterns and levels of gene expression. Within this hierarchy of layered control systems, such is the complexity of gene expression that even for the most intensively studied human gene loci—such as the globin loci—mechanisms needed to control levels of gene expression and the exquisitely controlled patterns of expression seen during development remain controversial and surprisingly elusive [Higgs, 1998; Engel and Tanimoto, 2000]

Activating Gene Expression

A variety of features contribute towards levels of gene expression in eukaryotic cells. DNA sequence motifs found within gene pro-

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moters [Goodrich et al., 1996] and enhancers [Blackwood and Kadonaga, 1998], in combination with elements such as locus control regions [LCRs; Engel and Tanimoto, 2000] and nuclear scaffold or matrix attachment regions [S/MARs; Bode et al., 2000a], are responsible for interactions between chromatin and the synthetic machinery—the RNA polymerase II holoenzyme—that serve to drive appropriate levels of gene expression. Combinations of these elements—together with insulators [Bell and Felsenfeld, 1999]—define the active status of each gene by allowing the correct chromatin state to be established and ensuring that the gene is maintained in a nuclear compartment that is permissive for RNA synthesis. For active genes, promoter structure and the association of appropriate transcription factors appear to determine the rate of initiation while enhancers control switching between active and inactive states. This idea has been extended by recent experiments that used site-specific recombination techniques to demonstrate that a functional enhancer antagonises gene silencing by preventing localisation of a gene close to centromeric heterochromatin [Francastel et al., 1999]. MAR elements might also influence levels of gene expression by targetting gene loci to specific sub-nuclear domains [Alvarez et al., 2000]. In addition, certain factors such as Ikaros, a transcription factor involved in hematopoietic cell differentiation, play a role in locating genes at transcriptionally inert nuclear sites [Brown et al., 1997].

Influence of Chromosomal Location

Early attempts to dissect the genetic elements required for controlled expression of specialised mammalian genes were often confounded by complexities seen in levels of gene expression when the constructs in question were expressed from different chromosomal locations. Profound variations in levels of gene expression were often seen in artificial gene constructs expressed from different ectopic chromosomal sites in cultured cells while complex tissue specific variation in expression were seen in transgenic animals [Wilson et al., 1990]. On the basis of these experiments it was clear that different chromosomal sites had quite different expressional capabilities, with some genomic positions apparently incapable of supporting expression. The obvious conclusion drawn from such analyses was that local chromatin structure influenced expression, presumably through modulating the accessibility of critical transcription factors to the binding sites involved in activating gene expression.

The influence of chromatin status on expressional capabilities was first characterised in *Drosophila* when it was observed that genes translocated close to heterochromatin were commonly switched off. The dominant suppressive properties of constitutive heterochromatin (e.g., centromeres) can spread over 1 Mbp or more; cell to cell variations result in 'position effect variegation'. In *Drosophila*, heterochromatin protein 1 (HP1) and the related Polycomb (Pc) proteins maintain a repressed chromatin state. The critical chromatin organization modifier (chromo) domain identified in HP1 and Pc is found in many proteins, including human homologues. Polycomb is found in large, multisubunit protein complexes (~5 MDa) and controls expression from homeotic genes during *Drosophila* development. Though chromatin status is clearly the target, polycomb-repressed chromatin is not resistant to digestion by restriction endonucleases and so is not subjected to generalised condensation. Modification of chromatin stability (stable chromatin prevents transcription factors access), sequestration into an inactive nuclear compartment and reduced chromatin flexibility may account for these observations [McCall and Bender, 1996; Breiling et al., 1999].

With the benefit of hindsight these observations are not surprising as it is clear that the major fraction of DNA—typically 90–95% in proliferative cells—is found in a relatively condensed state and is spatially isolated from the machinery that drives gene expression. Moreover, it now seems likely that special mechanisms have evolved to sequester genes whose function is no longer required into these inactive nuclear sites [Brown et al., 1997]. Much more detailed studies will be required before we fully appreciate the roles played by this form of nuclear 'position effect' and the complex interaction between heterchromatin and the families of chromatin remodelling machines that modulate chromatin status and consequently gene expression.

Locus Control Regions (LCRs)

Seminal experiments analysing the control sequences within human and mouse β-globin loci provided a significant advance in under-

standing the influence of chromatin status on gene expression [reviewed in Higgs, 1998; Engel and Tanimoto, 2000]. This work revealed that surprisingly remote control elements (i.e., ~50 kb from the relevant expression units) played a dominant role in controlling the developmental programme and levels of tissue specific gene expression. When this LCR and linked genes were first introduced into ectopic sites of appropriate cells the intact LCR was shown to establish an active chromatin configuration that allowed efficient, position-independent gene expression. The critical sequences were shown to lie within ~20 kbp DNA and contain four strong hypersensitive sites, 5′HS1-4, each composed of a complex array of transcription factor binding sites that together establish the correct expressional programme. Hence, the expression of different genes in the β-globin cluster is controlled by the dual action of specific repressors and activating sequences within a chromatin domain with a "locus control region" that directs expression across the locus [Grosveld et al., 1987; Ryan et al., 1989].

At the natural site, it is thought that the concerted action of different elements within the LCR, together with promoter-bound factors, fold to form a tertiary structure that then drives gene expression. Though capable of interactions that generate different stable complexes as a precursor to transcription, the appearance of cells with both embryonic and foetal transcripts, at appropriate stages of development, supports a "flip-flop" mechanism of gene activation [Wijgerde et al.,1995]. Such switching might infer that the activating LCR-promoter complex is dynamic, capable of sensing the status of promoters within the locus. These and other experiments support the view that LCR and promoter-bound complexes must interact to drive expression, though direct contact between the LCR and relevant promoter remains to be demonstrated *in vivo*.

Though the dominant behaviour of an LCR and specific function in establishing a permissive chromatin status across a gene locus has been described in some experiments, others suggest that the behaviour of LCRs is similar to that of very efficient enhancer elements. Critical to this controversy is a series of experiments that analyse LCR function from the natural chromosomal site using step-wise homologous recombination. Intriguingly, in the absence of the functional mouse LCR an open chromatin structure is established and maintained across the locus, as is the natural developmental profile of gene expression, though RNA synthesis is reduced to ~20% of the natural level. This implies that the LCR, much like an enhancer, contributes to the level of RNA synthesis but has no indispensable or dominant role over chromatin structure at the natural locus [Epner et al., 1998; Bender et al., 2000]. Whether this reflects technical or organism-based variations (remember that the human LCR is found within a region of the locus that is deleted in some thalasaemias) remains to be resolved.

In other situations, the classical dominant properties of an LCR can be subverted. For example, the human CD2-LCR coupled to immunoglobulin heavy chain enhancer showed wide variations in expression from different ectopic sites, consistent with the suppression of transcription in some cells [Elliott et al., 1995]. Here, the enhancer appears to influence chromatin configuration in a negative way. Importantly, mice homozygous for the integrated locus contained cells with both active and inactive alleles, implying that the commitment to expression occurs independently at different sites and is inherited by daughter cells. Moreover, recent experiments have confirmed that "disabled" or partial LCRs can give rise to variegated transgene expression as a consequence of gene location and the availability of heterochromatin proteins [Festenstein and Kioussis, 2000]. Clearly, genes introduced into different ectopic sites must experience a variety of environments that differ from the natural site and influence expression in unpredictable ways.

Analysing Nuclear Structure by Recombination

Analysing the role of genetic elements by comparing the behaviour of artificial constructs introduced into different chromosomal sites in cultured cells or transgenic animals is clearly complicated by the fact that these sites might experience quite different local environments. Various experiments confirm the complexities of expression from different genomic sites. For example, randomly integrated Myf5/*lacZ* transgenes rarely reproduce endogenous Myf5 expression pattern unless the construct is targeted to the natural locus [Tajbakhsh et al., 1996]. But interestingly, targeted introduction of novel sequences into a candidate locus might have unpredictable effects on the behaviour of local genes [Meyers et al., 1998].

Clearly the use of different recombination strategies [Fiering et al., 1999; Bode et al., 2000b] provides a powerful means of analysing expression from families of constructs integrated at the same chromosomal position in different clonal cell lines. For example, recombinase-mediated-cassette-exchange (RMCE) was used to show that β-globin LCR elements function to control both the probability of expression and the rate of transcription from a linked promoter [Bouhassira et al., 1997]. These same experiments also highlight another fascinating feature of gene expression in mammalian cells that concerns the interaction of adjacent genes in artificial gene constructs. In this case, the gene of interest (β-globin) and MCNeo gene required for drug selection interacted to generate intriguing patterns of transcriptional variegation. Again this demonstrates that epigenetic features can influence expression, even when a major variable such as gene location is controlled. Such experiments reinforce the contention that gene domains have evolved to allow the desired levels of gene expression from the natural chromosomal locus; such that levels of expression might be difficult to reproduce once this natural context is lost.

This argument is taken one step further by recent experiments that analysed the expression of gene constructs introduced in predetermined target sites by homologous recombination. Using mouse ES cells, target sites were selected following integration of the HPRT marker lying upstream of an Oct4/*lacZ* transgene. Using homologous recombination, four developmentally controlled promoters were introduced into the target locus of two selected clones and their ability to drive *lacZ* expression in transgeneic animals determined. Animals derived from one clone showed appropriate regulation of the four different promoters; this was designated a "neutral" integration site. However, animals derived from the second showed additional, unexpected patterns of ectopic expression [Wallace et al., 2000), confirming that a gene's location can have a profound effect on its expressional profile during development.

Chromatin Domains as Units of Gene Expression

The fact that genes expressed from artificial gene constructs behave unpredictably when expressed from different chromosomal locations raises the need to restrict the extent of this seemingly complex phenomenon when genes are expressed from their natural chromosomal position. One obvious means of achieving this is to place genes in chromatin domains that are somehow spatially isolated for local epigenetic factors. Clear evidence for the existence of domains of chromatin function comes from the ease with which chromatin is cut by nucleases [Workman and Kingston, 1998; Kadonaga 1998]; transcriptional status correlates with a generalised nuclease "sensitivity" that results from an open—10 nm—chromatin fibre. Moreover, chromatin modifications that correlate with gene activity—such as histone acetylation—play a role in maintaining chromatin status that can be perpetuated once the initial developmental transactivators are removed [Cavalli and Paro, 1999]. Hence chromatin modifications are capable of generating epigenetic signals that could play a role in the control of gene expression, providing this information can be reproduced when chromatin is duplicated [Taddei et al., 1999].

As chromatin remodelling accompanies transcription, RNA synthesis will play a role in maintaining chromatin status. However, it is well known that the general nuclease sensitivity at active loci is not restricted to genes; the analysis of histone variants found specifically in active chromatin shows that open chromatin will often extend over a continuous region that is many kb longer than the coding region it contains. Interestingly, recent experiments analysing expression of human β-globin gene constructs in transgenic mice have shown that the locus is composed of three functional subdomians which acquire an active chromatin status at the time of their expression, at the appropriate stage of development [Gribnau et al., 2000]. Intriguingly, the appearance of these nuclease-sensitive domains correlates with the activity of intergenic, non-globin transcripts that extend throughout the corresponding sub-domains. However, the maintenance of chromatin status does not demand continual transcription of the non-genic sequences as most synthesis is seen in the G1 phase of appropriate cells.

Another possibility for restricting the influence of epigenetic factors is to develop structurally independent gene domains. The best evidence for structural domains (revealed as chromatin loops) that apparently correspond to

units of gene expression comes from studies of lampbrush chromosomes of amphibian oocytes. However, somatic mammalian cells offer no prospect of visualising chromatin loops in situ, and the existence of such structures remains a matter of debate. Nevertheless, various cell extractions provide compelling evidence for supercoiled DNA loops or domains in mammalian cells. One classical approach uses hypertonic treatment of nuclease-treated nuclei to yield a nuclear matrix. This family of structures and related nuclear scaffolds have revealed numerous candidate matrix (MAR) and scaffold (SAR) attachment regions [Mishra and Karch, 1999; Bode et al., 2000a]; though it is still unclear how the organisation of these extracted structures reflects that existing inside the cell. Despite this reservation, there is ample evidence to suggest that S/MARs can play important roles in controlling gene expression. For example, recent experiments have shown that a MAR facilitates long-range chromatin remodelling around the immunoglobulin μ enhancer [Jenuwein et al., 1997] and that a well-characterised MAR-binding protein (SATB1) plays a critical role in orchestrating the temporal and spatial expression of many genes during T-cell development [Alvarez et al., 2000].

Domain Boundaries and Insulators

S/MAR elements can be shown to operate as putative domain boundaries, but do not do so in every case [Mishra and Karch, 1999]. Indeed, it is unresolved whether transitions in chromatin structure associated with gene activity show a universal correlation with the attachment sites needed to generate DNA loops or sequences that are commonly found to be associated with the nuclear matrix. Genetic experiments performed using the fruit fly, *Drosophila*, have provided the most convincing evidence for chromatin domains in eukaryotes. Initially, "specialised chromatin structures" (scs) flanking two heat shock genes (HSP70) were shown to correlate with the boundaries of an ~15kbp active domain—at 87A7 on the polytene chromosome—following heat treatment. These elements (*scs*/*scs′*) contained pairs of very strong DNase hypersensitive sites flanking a nuclease resistant sequence of ~300 bp. Different reporter genes were used to demonstrate that only when the genes were flanked by the scs elements could position effects at the site of integration be eliminated [Kellum and Schedl, 1992]. Characterised boundary element attachment factors (BEAF-32A and -32B) and their recognition motifs within the scs may be important determinants of chromosome structure [Zhao et al., 1995]. The probable role of the scs as a boundary element was confirmed by its ability to uncouple linked promoters and enhancers, presumably by "blocking" enhancer function.

This property of putative boundary elements supports their role as insulators "that act as a neutral barrier to the influence of neighbouring elements" [Bell and Felsenfeld, 1999; Udvardy, 1999]. Many elements that are capable of providing insulator activity have now been described in Drosophila [Bell and Felsenfeld, 1999; Mishra and Karch, 1999] and some of these have been shown to perform an analogous role in vertebrates. Moreover, recent studies have confirmed that sequence elements at the upstream border of the chicken β-globin LCR act as classical insulators [Bell et al., 1999]. A protein CTCF has been shown to bind to critical sequence motifs within this region and is likely to play a role in determining insulator function. Intriguingly, this same protein appears to play a role in the process of "genomic imprinting" that controls the differential expression of maternally and paternally inherited genes [Reik and Murrell, 2000].

Various mechanisms of insulator function have been proposed on the basis of different assay to disrupt gene expression. In the classical enhancer-blocking experiment a single element situated between a linked enhancer and promoter is usually sufficient to uncouple enhancer function and reduce expression [Bell et al., 1999]. In other cases, two insulator elements are required for optimal effect. From experiments reported to date [reviewed in Bell and Felsenfeld, 1999] a plausible general role for insulators is to antagonise the role of enhancers. This may serve to disturb the equilibrium of transcription factors associated at appropriate promoters and consequently the nuclear localisation and chromatin status of the gene in question. Whether characterised S/MAR, boundary and insulator elements have common properties is presently unclear [Namciu et al., 1998]; though these elements may turn out to be members of families of elements that play related roles in genome structures. However, confirming this may prove difficult as related elements might have functional

properties that are only relevant to there natural genomic location.

DNA Loops A Simplified View

Different experiments support the existence of DNA loops and domains in mammalian cells. But it is also the case that the mechanisms by which these structures are formed and any functions they might perform are controversial and enigmatic. The main reasons for this emanate from two general sources. First, experiments that demonstrated the existence of supercoiled DNA domains in mammalian cells were performed on hypertonically extracted cells and are often regarded as preparation artefacts. Second, loops are generally assumed to represent structural domains of gene expression, though few sequences have been described that support this view. Nevertheless, experiments performed on extracted cells under physiological conditions do support the existence of chromatin loops. In mammalian cells these have an average size of $\sim$85 kb, though many much smaller loops are found in transcribed loci [Cook, 1999]. Moreover, an analysis of the sequences responsible for loop formation suggests that most are a by-product of gene functions such as transcription or replication [Jackson, 1997; Cook, 1999]. This simple statement allows us to begin to develop a unified view of nuclear structure that requires just one more conceptual leap.

Specialised Sites of Nuclear Function in Mammalian Cells

Understanding the genesis and function of chromatin domains now hinges on the organisation of sites that perform critical nuclear functions. Though dynamic, it is now clear the eukaryotic nuclei are highly structured; this is perhaps best emphasised by the organisation of components involved in RNA metabolism [Jackson and Pombo, 1998; Misteli and Spector, 1998; Cook, 1999]. One critical—and most intriguing—aspect of this organisation is revealed by the fact that the active centres involved in different aspects of RNA metabolism maintain their spatial disposition when most chromatin, representing $\sim$50% of the nuclear mass, is removed. This implies that some "nucleoskeleton" is involved in these fundamental processes and suggests that RNA synthesis and processing is performed in association with an organisational "solid" phase. To extend this organisational theme, it has also been demonstrated that the nucleoskeleton-associated active sites are complex centres dedicated to the synthesis and maturation of RNA from groups of transcription units [Jackson, 1997; Cook 1999]. As almost all aspects of nuclear function have been shown to take place in such complex active centres it is no surprise that sequences associated with the nucleoskeleton (and nuclear matrix that form during hypertonic extraction) are a very complex collection of elements that reflect these functions.

The structure and complexity of dedicated sites of nuclear function then becomes the unifying theme that explains how chromatin loops are formed and why specific elements responsible for the generation of such loops have proved so difficult to define. It is then obvious that any sequence elements with significant affinity for the protein complexes found within these active centres will contribute towards the formation of DNA-protein interactions that generate DNA loops; any two adjacent points of association between chromatin and a local active centre will generate an intervening chromatin loop. In this very simplistic way, associations that reflect nuclear function generate the majority of DNA loops found in mammalian cells.

This type of arrangement could have profound consequences for different aspects of nuclear structure and function. For example, in proliferating cells a highly choreographed programme of DNA synthesis is required to ensure the scheduled completion of S-phase. Part of this process involves the activation of groups of replicons in "replicon clusters" that appear to be basic units of higher-order chromosome structure [Jackson and Pombo, 1998]. In proliferating cells, these replicon clusters correspond to replication foci that at the beginning of S-phase contain groups of active genes that might also be transcribed at a single transcription site. In this situation, the constellation of genes within any particular transcription centre will represent some equilibrium that is restrained by both the chromosomal sequence of genes and local nuclear organisation. However, in slowly dividing or non-proliferative cells an extended interphase might allow new equilibria to be established so that individual active centres contain genes located on different chromosomes. If this occurs, subsequent defects in DNA metabolism might explain the develop-

ment of chromosomal translocations that are commonly seen in cancer.

Dynamics of Nuclear Function

Mammalian nuclei are highly dynamic. This is inevitable, as proliferative cells polymerise some 2×10^8 bases/min into nascent RNA that must be processed, assembled with the appropriate proteins and transported to the cytoplasm [reviewed in Jackson et al., 2000]. Even chromosmal domains and nuclear structures such as coiled bodies and nucleoil can be shown to experience periodical bouts of reorganisation. It is perhaps somewhat more surprising that a transcription factor such as the glucocorticoid receptor can also be shown to be highly dynamic, even in its association with binding sites in a promoter [McNally et al., 2000]; though it should be remembered that in vitro competition experiments have shown that many components of a transcriptional pre-initiation complex maintain a stable association within promoters. These observations imply that at least part of the system required to activate gene expression might be set up to continually test the status of critical control elements so allowing the very sophisticated fine tuning of gene expression. In fact, this dynamic behaviour of active genes was also demonstrated by a global analysis of nuclear structure which showed that basal factors required for gene expression were associated with the nucleoskeleton while the specialised protein factors bound to promoters and enhancers associated only transiently [Kimura et al., 1999]. Hence, promoters in chromatin that is competent for gene expression appear to be in dynamic equilibrium with local active centres, so that the balance of this equilibrium will determine levels of gene expression. Importantly, this dynamic contribution to gene expression could be influenced by sequences, such as enhancers or S/MARs that might target a chromatin domain to the active centre. Moreover, as active centres transcribe groups of expression units, events occurring within one domain might also be influenced by interaction occurring in adjacent domains. This type of organisation, in combination with dynamic changes in chromatin status generated by local chromatin remodelling machinery, begins to explain the complexities of gene expression and why natural levels of gene expression might not be reproduced when a gene is introduced at random into ectopic chromosomal sites.

CONCLUSION

In mammalian cells, different layers of organisation contribute to the complex processes required for gene expression. Simple recognition motifs in chromatin first bind transcription factors and set the activation process in motion. During this process, the constellation of factors assembled on promoters is likely to be in a continual state of flux until some dynamic equilibrium is established that allows transcription to proceed. The fact that transcription is activated by arrays of factor—many of which bind rather weakly to their recognition motifs—which function co-operatively to activate gene expression explains some of the complexities of transcriptional control.

In vivo, additional features can clearly have a profound effect on gene expression. This is clear from many experiments that demonstrate that different chromosomal locations have quite different expressional capabilities. A priori, we might assume that genes expressed from their natural chromosomal sites would operate with the required efficiency. It is now obvious that the combination of genetic factors determined by the organisation of DNA sequence elements and different epigenetic factors ensure that the expression of any particular gene is a very complex process. Moreover, the natural combination of factors is unlikely to be reproduced when genes are introduced at random into ectopic chromosomal sites. This obviously compromises our efforts to understand the behaviour of specific genetic elements using classical techniques to introduce artificial gene constructs into mammalian cells. In view of this, it seems that the only reliable techniques for analysing gene function will involve recombination technologies to either manipulate a gene at its natural chromosomal locus or alternatively select a "neutral" locus for recombination where ectopic genes are least likely to be exposed to complex epigenetic factors.

It is now clear that a complex interplay between genetic elements, local and even remote chromatin and the local nuclear environment determines levels of gene expression. But in addition to establishing the desired level of synthesis, this nuclear "set-up" ensures that the nascent transcript engages a pathway that

couples synthesis to the desired combination of post-synthetic events—RNA processing, export and perhaps even cytoplasmic location and function. The organisation and structure of active sites of RNA synthesis is likely to be critical to this process. Moreover, the idea that products from many transcription units are synthesised and processed within dedicated compartments of gene expression or "transcription factories"—explains how a gene's chromosomal position might influence its activity. Finally, this ordered view of nuclear structure also provides a unified theme to explain the generation of DNA loops and chromatin domains. A detailed analysis of these concepts of nuclear structure and function should greatly increase our prospects of achieving controlled expression of mammalian genes from ectopic chromosomal sites; and might eventually allow the development of robust systems to perform gene therapy on human cells.

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