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Interchromosomal clustering of active genes at the nuclear pore complex

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Genomes are spatially organized on many levels and the positioning of genes within the nucleus contributes to their proper expression. This positioning can also result in the clustering of genes with similar expression patterns, a phenomenon sometimes called "gene kissing." We have found that yeast genes are targeted to the nuclear periphery through interaction of the nuclear pore complex with small, cis-acting "DNA zip codes" in their promoters. Our recent study demonstrated that genes with the same zip codes cluster together at the nuclear periphery. The zip codes were necessary and sufficient to induce interchromosomal clustering. Finally, we identified a transcription factor (Put3) that binds to the GRS I zip code. Put3 binds to GRS I and is required for both GRS I-dependent positioning at the nuclear periphery and interchromosomal clustering of GRS I-targeted genes. We speculate that our findings might provide insight into other types of gene kissing, some of which also require cis-acting DNA sequences and trans-acting proteins.

Keywords: nuclear pore complex, transcription, nuclear organization, gene kissing, nuclear body

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Introduction

DNA within the nucleus is spatially organized. Folded chromosomes occupy discrete "territories" within the nucleus in interphase metazoan cells.[1,2] Chromosome folding, inter- and intra-chromosomal interactions and the association of chromosomes with subnuclear structures have been suggested to create subnuclear environments that both reflect and facilitate different expression states.[3] Although the spatial organization of chromosomes and the positioning of individual genes are stereotyped within differentiated cells, they are dynamic and can change during differentiation or in response to an environmental cue.[1,4] For example, many genes that are induced during differentiation move from the nuclear periphery (where they associate with the nuclear lamina) to a more nucleoplasmic position, sometimes in association with "transcription factories."[5] Disruption of the spatial organization of genes is also associated with disease. For example, the spatial arrangement of genes is disrupted in breast cancer cells.[6,7] Thus, the spatial positioning of individual genes is dynamic and either reflects their expression state or contributes to their regulation.

Gene clustering. The clustering of co-regulated genes is a common theme in nuclear architecture.[3] Techniques such as fluorescent in situ hybridization (FISH), chromosome painting and variations of the chromosomal conformation capture (3C) technique have illuminated a large number of intra-chromosomal and inter-chromosomal interactions in many organisms. For example, in yeast, FISH revealed that many tRNA genes from throughout the genome cluster together in the nucleolus,[8] centromeres cluster near the spindle pole body[9,10] and telomeres cluster at the nuclear envelope.[11] Data from 3C experiments in yeast reveals the clustering of tRNA genes, genes near early firing origins and genes with Gal4 binding sites.[10,12] Polycomb-repressed Hox genes cluster during embryogenesis in Drosophila.[13,14] In mouse erythrocytes, during hematopoiesis, the globin genes

cluster with each other and with many erythrocyte-specific genes.[15] These co-regulated genes cluster in close proximity to foci of active RNA polymerase II called "transcription factories".[15-18] Therefore, interchromosomal clustering may represent an additional layer of transcriptional regulation that may either reinforce signals or allow better coordinated expression of co-regulated genes.

Gene targeting to the nuclear pore complex. As a model for these phenomena, we have studied the movement of inducible yeast genes from the nucleoplasm to the nuclear periphery upon activation.[19,20] Within the yeast nucleus, there are three major zones: the nucleoplasm, the nuclear periphery and the nucleolus. ChIP experiments using nuclear pore proteins suggests that hundreds of active yeast genes interact with the nuclear pore complex (NPC).[19,21] Likewise, thousands of active genes interact with nuclear pore proteins in Drosophila.[22,23] However, in Drosophila, most of these genes interact with nuclear pore proteins away from the NPC, in the nucleoplasm.[22,23] This suggests that in metazoans, nuclear pore proteins can interact with genes both in the nucleoplasm and at the pore. Inducible yeast genes such as *INO1, TSA2, GAL1*, *HSP104* and *SUC2* localize to the nuclear periphery when active. Movement from the nucleoplasm to the nuclear periphery is rapid[24] and requires several nuclear pore proteins, mRNA export factors, NPC-associated Mlp proteins and the SAGA histone acetyltransferase.[25-28] Targeting of *INO1* to the nuclear periphery and interaction of *GAL1* with the NPC does not require active transcription, suggesting that it is not mediated by nascent mRNA.[24,29] Small cis-acting DNA elements called gene recruitment sequences (GRSs) in the *INO1* and *TSA2* promoter are necessary for targeting to the nuclear periphery and interaction with the NPC.[25] These DNA elements function as DNA zip codes: when inserted at a locus that normally localizes in the nucleoplasm, they are sufficient to induce repositioning to the nuclear periphery.[25,30,31]

The interaction of active genes with the NPC has been proposed to promote transcription,[25] to function as a chromatin boundary,[32-34] to facilitate epigenetic transcriptional memory[24,30,35] and to provide negative feedback.[36] Mutations in the GRSs of *INO1* and *TSA2* that block interaction with the NPC lead to a defect in transcription.[25] Likewise, knockdown of nuclear pore proteins in Drosophila results in decreased expression of many of the genes that bind to nuclear pore proteins.[22,23] However, currently it is not clear how interaction with nuclear pore proteins or positioning at the nuclear periphery impacts transcription.

Results

To ask if DNA zip codes play a role in interchromosomal clustering of genes at the nuclear periphery, we adapted a chromatin localization assay to allow simultaneous localization of two genes. An array of 112 Tet repressor binding sites was integrated beside *INO1* on chromosome X and an array of 128 Lac repressor binding sites was integrated at several other loci. These strains expressed GFP-TetR and RFP-LacI. The position of the two loci, one red dot and one green dot, with respect to the nuclear membrane and with respect to each other, could be measured. Alternatively, we also utilized strains with either 128 or 256 Lac repressor binding sites at two loci, expressing GFP-LacI. These strains exhibited discernably different-sized green dots. These modifications allowed us to study the behavior of two loci with overlapping or distinct mechanisms of targeting in the same cell.

Gene-specific clustering at the nuclear periphery. To analyze clustering of genes, we measured the distances between the loci in the population. When we compared a gene at the nuclear periphery (*INO1* or *HSP104*) and a gene in the nucleoplasm (*URA3*), we did not observe significant clustering: the distributions of distances were normal with means of 0.8–1.0 μm (approximately the radius of the yeast nucleus) and the two loci were ≤ 0.5 μm apart in less than 20% of the cells. Similarly, repressed *INO1*, which localizes in the nucleoplasm, did not cluster with itself in diploid cells (1.0 ± 0.47 μm; 20% ≤ 0.5 μm). However, when active, the two alleles of *INO1* clustered together (0.60 ± 0.33 μm, 52% ≤ 0.5 μm; $p < 0.0001$).

To confirm that this clustering was due to targeting and not related to homology between chromosomes, we inserted an ectopic copy of *INO1* at the *URA3* locus in a haploid strain. This hybrid locus, *URA3:INO1*, is targeted to the nuclear periphery normally when *INO1* is induced.[25,30] When active, but not when repressed, *INO1* clustered with *URA3:INO1*. Thus, the information necessary for both targeting to the nuclear periphery and for interchromosomal clustering is contained within the sequence inserted at *URA3*.

To confirm that the clustering was gene-specific, we compared the positioning of several genes known to localize to the nuclear periphery. We performed pairwise comparisons between *INO1*, *GAL1*, *HSP104* and *GAL2* and we found that, although these genes all localize to the nuclear periphery, they did not cluster with each other. Therefore, recruitment to the nuclear periphery is not sufficient to cause genes to cluster, even for genes on the same chromosome: *HSP104* and *GAL2* are ~290 kb apart on opposite sides of the centromere of chromosome XII. The *GAL2* locus is close to the rDNA locus and is positioned within or adjacent to the nucleolus.[37,38] This example further highlights how chromosome structure can create micro-domains or sub-compartments of the nucleus.

***INO1* clustering requires interaction with the nuclear pore complex.** We also examined the role of the NPC on the clustering of genes at the nuclear periphery. *INO1* recruitment to the nuclear periphery is blocked in strains lacking Nup2, one of the proteins that make up the nuclear basket-like structure on the nucleoplasmic face of the NPC.[24] In strains lacking Nup2, *INO1* did not cluster with *URA3:INO1*. This suggests that targeting to the nuclear pore is required for clustering.

When genes are targeted to the nuclear periphery, they are still mobile and the targeting is not uniform. In a typical experiment, a gene will colocalize with the nuclear periphery in ~65% of the cells in the population (compared with ~30% for a nucleoplasmic locus). This reflects both the continuous motion of genes in living cells[9,26,37] and the negative regulation of peripheral targeting during S-phase.[39] This

allowed us to more rigorously test the idea that clustering only occurs at the nuclear periphery. To do this, we examined the clustering of *INO1* and *URA3:INO1* separately in three different classes of cells: (1) cells in which either both genes were at the nuclear periphery, (2) cells in which both genes were in the nucleoplasm or (3) cells in which one gene was at the nuclear periphery and the other was in the nucleoplasm. As we expected, when both genes were at the nuclear periphery, they were clustered (72% ≤ 0.5 μm) and when one gene was at the periphery and the other was in the nucleoplasm, they were not (12.5% ≤ 0.5 μm). However, we were surprised to find that, when both genes were nucleoplasmic, they were also clustered (59% ≤ 0.5 μm). Therefore, although clustering required Nup2, it could also be maintained in the nucleoplasm. This raised the possibility that targeting to the nuclear pore complex might be a pre-requisite for clustering. To test this idea, we treated the cells with hydroxyurea to arrest them in S-phase, a moment in the cell cycle in which peripheral targeting is blocked.[39] We asked (1) if clustering could be maintained in cells arrested in S-phase and (2) if clustering could be established in cells arrested in S-phase. In cells in which *INO1* is expressed and targeted to the nuclear periphery prior to arresting in S-phase, *INO1* and *URA3:INO1* remained clustered in the nucleoplasm after arrest. Therefore, clustering was maintained in the nucleoplasm in cells arrested in S-phase. However, in cells arrested in S-phase and then shifted to medium to induce *INO1*, *INO1* and *URA3:INO1* did not cluster. Therefore, clustering could not be established in the nucleoplasm in cells arrested in S-phase. Together, these results suggest that targeting to the nuclear periphery is a pre-requisite for clustering, but that clustering can persist in the nucleoplasm.

DNA zip codes control interchromosomal clustering. We previously identified two DNA zip codes in the promoter of *INO1* (GRS I and GRS II) that are necessary and sufficient for gene targeting to the nuclear periphery.[25] We explored the role of these elements in controlling *INO1* clustering. Insertion of either GRS I or GRS II beside *URA3* is sufficient to reposition *URA3* to the nuclear periphery.[25] Because *URA3:INO1* possesses GRS I but not GRS II,[25] we hypothesized that GRS I controls clustering. Indeed, insertion of GRS I, but not GRS II, was sufficient to induce clustering with the endogenous *INO1* gene. Furthermore, disruption of GRS I, but not GRS II, within the endogenous *INO1* promoter blocked clustering with *URA3:INO1*. Therefore, the GRS I zip code controls both gene targeting to the nuclear periphery and gene clustering.

The GRS I sequence appears in the promoter of 94 genes, including the stress-inducible *TSA2*.[25] Therefore, we asked if *TSA2* clustered with *INO1*. Uninduced *TSA2* did not cluster with *INO1* (0.83 ± 0.41 μm; 25% ≤ 0.55 μm). But when both genes were activated, *INO1* and *TSA2* genes clustered (0.58 ± 0.38 μm; 55% ≤ 0.55 μm; $p < 0.0001$). When the GRS I in the *INO1* promoter was disrupted, clustering was lost (0.91 ± 0.42 μm; 25% ≤ 0.55μm). Therefore, genes from different chromosomes cluster based on a small targeting determinant found in their promoters.

To expand on these results, we asked if other genes cluster at the nuclear periphery and if clustering is similarly controlled by DNA zip codes. *HSP104* is recruited to the nuclear periphery upon heat shock or in ethanol.[28] We identified a DNA zip code in the *HSP104* promoter which we have designated GRS III. Like other zip codes, GRS III is sufficient to target *URA3* to the nuclear periphery. Furthermore, active *HSP104* clusters with *URA3:GRS III* at the nuclear periphery. Therefore, DNA zip codes play a general role in targeting to the nuclear periphery and in promoting inter-chromosomal clustering.

The Put3 transcription factor mediates GRS I-dependent gene targeting and clustering. Because the GRS I zip code is critical for gene localization and interchomosomal clustering, we identified the protein that recognizes the GRS I. Using a combination of electrophoretic mobility shift assays (EMSA) and affinity chromatography followed by mass-spec analysis, we identified Put3. Put3 from yeast extracts interacted with the GRS I sequence in EMSA experiments. In strains lacking Put3, GRS I-dependent targeting to the nuclear periphery was blocked. Put3 is a Zn_6-Cys_6 zinc-finger transcription factor that regulates the expression of genes involved in proline metabolism.[40] Put3 binds to *PUT1* and *PUT2* via the UAS_{PUT} element (CGG-N_{10}-GCC) that has no obvious similarity to GRS I (GGG TTG GA). However, using chromatin immunoprecipitation we showed that Put3 binds to the GRS I in vivo and that Put3 is required for the interaction between the GRS I and the nuclear pore. Loss of Put3, like loss of GRS I, leads to a defect in *INO1* and *TSA2* transcription. Finally, clustering of *INO1* and *URA3:INO1* requires Put3. Therefore, recognition of a DNA zip code by a transcription factor mediates targeting to the nuclear periphery, interaction with the nuclear pore complex and inter-chromosomal clustering.

Conclusions

The work summarized here showed that, in yeast, genes that are targeted to the nuclear pore complex upon activation can cluster together. Clustering is mediated by DNA zip codes. We showed that two different zip codes, when inserted at the *URA3* locus, induce both targeting to the nuclear periphery and clustering with endogenous genes having the same zip code. In other words, *URA3* can be directed to the nuclear periphery and can cluster with two distinct sets of genes when the right zip code is placed at this locus. The focus of our work has been genes that are targeted to the nuclear periphery and cluster in a regulated manner. It remains to be seen whether these lessons will apply to housekeeping genes or repressed genes that cluster together.

Outlook

A full understanding of the molecular mechanism by which gene clustering can be achieved will require the identification of additional proteins involved in the process and a better understanding of the phenomenon in living cells. However, we would like to close with a brief discussion of three other important questions. First, to what extent are these lessons generalizable, either within the yeast genome or in other genomes. Second, how does gene

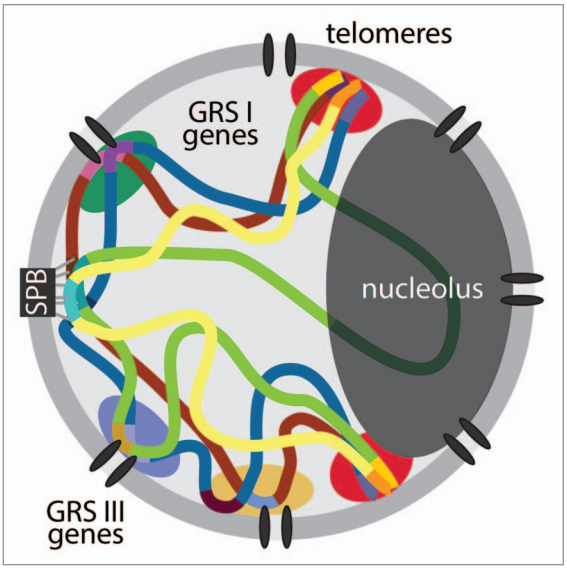

Figure 1. Model for the clustering of co-regulated genes at the nuclear periphery in yeast. Throughout the cell cycle, yeast centromeres remain stably associated with the spindle pole body (SPB), which is embedded in the nuclear envelope. The rDNA locus is positioned within the nucleolus at the opposite pole. Telomeres cluster together at the nuclear periphery and concentrate proteins involved in transcriptional silencing (red clouds). Different sets of genes that are targeted to the nuclear pore complex by different DNA zip codes (GRS I and GRS III) cluster together, potentially resulting in a heterogeneous distribution of factors that promote their expression (green, blue and orange clouds).

clustering affect the spatial organization of the genome as a whole? And third, how does gene localization at the nuclear periphery, interaction with nuclear pore proteins or clustering with co-regulated genes impact gene expression? Although we cannot answer any of these questions here, we will briefly summarize our thoughts about each of them.

Generalizability. Hundreds of yeast genes interact with the NPC.[19] We have tested a handful of genes (*INO1*, *HSP104*, *GAL1*, *ACT1* and *GAL2*) and we were able to identify cis-acting promoter elements that were capable of targeting *URA3* to the nuclear periphery for all but one of these genes (data not shown). Genes with a GRS I zip code in their promoters are over-represented among genes that interact with the NPC.[25] Although this set of genes may not be representative and there may be other ways that genes are targeted to the NPC, it is reasonable to propose cis-acting DNA zip codes play an important general role in controlling interaction with the NPC.

Not all zip codes are capable of inducing interchromosomal clustering. Both the GRS III element from the *HSP104* promoter and the GRS I element from the *INO1* promoter, when inserted at *URA3*, induced clustering of *URA3* with *HSP104* and *INO1*, respectively. However, the GRS II element from the *INO1* promoter did not induce clustering and was not required for clustering of *INO1* with *URA3:INO1*. Therefore, we conclude that some, but not all, DNA zip codes induce interchromosomal clustering.

Do these lessons provide insight into the spatial organization of the genomes of other organisms, such as metazoans? Do cis-acting DNA elements and transcription factors control gene positioning and interchromosomal clustering? The positioning of metazoan genes often reflects their expression and can change when genes are induced or repressed. There are several well-documented patterns of gene positioning in metazoan nuclei. Many repressed or silenced genes physically interact with nuclear lamina but, if they are induced, they move to a more internal site.[41-43] Such genes can also cluster together with co-regulated genes in close association with transcription factories.[15] Likewise, Polycomb-repressed loci in flies colocalize with each other at Polycomb bodies.[14,44] Both the localization of genes at the nuclear lamina and the clustering of co-regulated genes require cis-acting DNA sequences or *trans*-acting transcription factors.[15,45,46] The interaction of genes with nuclear pore proteins in metazoans can occur both at the NPC and in the nucleoplasm.[23,47,48] It is not known if metazoan genes that interact with nuclear pore proteins exhibit interchromosomal clustering or if the interaction of nuclear pore proteins with these genes is mediated by cis-acting DNA elements or trans-acting factors. However, these genes are enriched for certain transcription factor binding sites.[48] Therefore, it is plausible that our understanding of NPC-gene interactions in the yeast system will illuminate similar phenomena in multicellular organisms.

The effect of gene clustering on the global organization of the yeast genome. Our observations suggest that yeast genes that share the same mechanism of targeting to the nuclear pore complex cluster together. The most extreme interpretation of this conclusion is that the DNA zip codes encode targeting to a particular portion of the nuclear envelope. Although our data are consistent with this idea, it is equally likely that targeting to the nuclear periphery, interaction between genes and a gene's position along the chromosome provide sufficient constraints to limit the fraction of the nuclear periphery that it will visit (**Fig. 1**). Because yeast centromeres are stably associated with the spindle pole body during interphase, the two-dimensional distance between genes and the centromere will likely impact their mobility and their interchromosomal interactions (**Fig. 1**).

If the default position of genes with respect to each other were influenced by their position along the chromosome arm, we would expect the 3D distance between two genes to be influenced by the similarity of their 2D distance from the centromere. The genes that we have examined in detail (*INO1*, *TSA2*, *URA3* and *HSP104*) are ~300 kb, 385 kb, 34 kb and 60 kb from their centromeres, respectively. Under conditions where these genes are not targeted to the nuclear periphery (i.e., default positioning), the 3D distances between them are somewhat reflective of the similarity of their 2D distance to the centromere:

repressed *INO1* and *URA3* were further apart (300 kb vs. 34 kb; 1.08 ± 0.43 μm) than repressed *INO1* and *TSA2* (300 kb vs 385 kb; 0.83 ± 0.41 μm). However, when genes are targeted to the nuclear periphery, we observe clustering between *INO1* and *TSA2* (300 kb vs. 385kb), *INO1* and *URA3:GRS I* (300 vs. 34kb) and *HSP104* and *URA3:GRS III* (60 kb vs. 34 kb). This suggests that, although the distance between a gene and the centromere constrains spatial positioning, DNA zip codes can provide an additional, dominant input. Therefore, we speculate that the clustering of genes at the nuclear periphery may affect the folding and disposition of chromosomes in the yeast nucleus.

Gene clustering and expression. The clustering of *INO1* and *TSA2* at the nuclear pore complex is required for maximal expression of these genes.[25,31] How gene positioning impacts expression is poorly understood. The assumption is that genes move to exploit the heterogeneous nuclear distribution of factors important for transcription or post-transcriptional regulation. For example, the clustering of activated genes during hematopoiesis leads to their co-localization with active RNA polymerase II transcription factories. However, several of the nuclear "bodies" that are observed in mammalian cells can form de novo in association with genes.[49-51] Therefore, it is not always necessary to change gene position to establish contact between a gene and a nuclear body.

In yeast, factors that promote transcription or chromatin remodeling do not, in general, exhibit heterogeneous nuclear staining.[52] We speculate that genes that share zip codes also share requirements for factors involved in their transcription. If so, then the clustering of co-regulated genes, like the clustering of telomeres at the nuclear envelope,[53] might itself serve to compartmentalize the open environment of the nucleoplasm by concentrating these factors and enhancing the efficiency of transcription (**Fig. 1**). This compartmentalization would be dynamic and the compartments would not necessarily outlive the clusters. Such a model would be consistent with both the functional importance of clustering and the ability of nuclear bodies to form de novo.

The inter- and intra-chromosomal clustering of co-regulated genes is a widespread and fundamental aspect of genome organization. Understanding the molecular basis for this phenomenon will provide new and important insight into how long-range interactions impact gene expression and global organization of the genome.

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