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Regional and temporal specialization in the nucleus: a transcriptionally-active nuclear domain rich in PTF, Oct1 and PIKA antigens associates with specific chromosomes early in the cell cycle

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PTF (PSE-binding transcription factor) activates transcription of snRNA and related genes. We investigated its distribution in HeLa nuclei by immunofluorescence, and found it spread throughout the nucleoplasm in small foci. In some cells, PTF is also concentrated in one, or very few, discrete regions (diameter ~1.3 μm) that appear during G_1 phase and disappear in S phase. Oct1, a transcription factor that interacts with PTF, is also enriched in these domains; RNA polymerase II, TBP and Sp1 are also present. Each domain typically contains 2 or 3 transcription 'factories' where Br-UTP is incorporated into nascent transcripts. Accordingly, we have christened this region the Oct1/PTF/transcription (OPT) domain. It colocalizes with some, but not all, PIKA domains. It is distinct from other nuclear domains, including coiled bodies, gemini bodies, PML bodies and the perinucleolar compartment. A small region on chromosome 6 (band 6p21) containing only ~30 Mbp DNA, and chromosomes 6 and 7, associate with the domain significantly more than other chromosomes. The domains may act like nucleoli to bring particular genes on specific chromosomes together to a region where the appropriate transcription and processing factors are concentrated, thereby facilitating the expression of those genes.

Keywords: Br-UTP/CLSM/FISH/Oct1/PTF

Introduction

Mammalian nuclei are complex organelles containing different sub-compartments. For example, the vital process of replication takes place in specific domains, and many splicing components are found in discrete 'speckles' (Spector, 1993; de Jong *et al.*, 1996; Jackson, 1997). RNA polymerases and their associated transcripts are also concentrated in defined areas. The nucleolus is the best-characterized of these regions (Hozák *et al.*, 1994a; Shaw and Jordan, 1995). It contains RNA polymerase I, and it is dedicated to the transcription of 45S rRNA. Each nucleolar transcription site contains >100 engaged polymerases and nascent transcripts. Nucleoli are usually able to bring together some, but not necessarily all, chromosomes carrying rDNA genes, and to organize them into a transcriptionally-active domain. Active RNA polymerase II, the enzyme responsible for transcribing most genes, is found with its associated transcripts in several thousand nucleoplasmic foci; each focus typically has a diameter of ~80 nm and contains tens of active RNA polymerases and transcripts (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Iborra *et al.*, 1996a; Fay *et al.*, 1997; D.A.Jackson, F.J.Iborra, E.M.M.Manders and P.R.Cook, in preparation). Nascent transcripts generated by RNA polymerase III are also concentrated in several hundred extranucleolar sites (A.Pombo, D.A.Jackson and P.R.Cook, in preparation). As each of these foci contains many engaged polymerases and associated transcripts, they have been called transcription 'factories' (reviewed by Iborra *et al.*, 1996b).

These results suggest that different polymerases are concentrated in factories that specialize in the transcription of particular classes of genes. We have investigated whether factories also contain specific transcription factors. Previous studies have shown that transcription factors are generally concentrated in discrete foci spread throughout the nucleoplasm (e.g. van Steensel *et al.*, 1995; Grande *et al.*, 1997). However, the highest concentrations rarely colocalize with sites rich in nascent RNA or RNA polymerases, and they probably mark inactive stores rather than active sites of transcription. Moreover, each transcription factor often has its own characteristic distribution that does not overlap that of other factors. We have also screened different factors to see if they were concentrated in transcription factories, and we can confirm that the highest concentrations are rarely associated with nascent RNA (A.Pombo and P.R.Cook, unpublished). So far, then, there are no examples (other than nucleoli) of unambiguous colocalization between high concentrations of transcription factors and active transcription. We now describe two factors that are present in high concentrations at transcription sites.

The transcription factors, PTF and Oct1, activate the transcription of genes encoding snRNAs and other 'processing RNAs'; they bind to proximal and distal sequence elements (PSEs and DSEs) within the promoters and activate transcription by RNA polymerases II or III (for reviews, see Hernandez, 1992; Lobo and Hernandez, 1994). PTF (PSE-binding transcription factor) is also known as PBP (PSE-binding protein; Waldschmidt *et al.*, 1991) and SNAPc (snRNA-activating protein complex; Sadowski *et al.*, 1993). It is a tetramer of α, β, γ and δ subunits that interacts with TBP and Oct1 (Murphy *et al.*, 1992; Henry *et al.*, 1995; Yoon *et al.*, 1995; Bai *et al.*, 1996; Sadowski *et al.*, 1996; Yoon and Roeder, 1996). No

PTF-dependent transcripts are known to be spliced. We have immunolocalized PTFα and δ relative to transcription factories using confocal laser scanning microscopy (CLSM). We find that PTFα, PTFδ and Oct1 are concentrated in discrete domains at a certain stage of the cell cycle, and that each domain contains a number of transcription factories. These domains colocalize with some, but not all, PIKA domains. They also contain various transcription factors and polymerases, but not detectable concentrations of splicing factors. Therefore, they are more like the nucleolus than any other known nuclear domain. This raised the possibility that the domain might bring together specific chromosomes, so that genes activated by PTF and/or Oct1 could be transcribed there. Therefore, we investigated whether any chromosomes associated preferentially with the domain, and found two that did. Our results suggest that the PTF domain acts like the nucleolus to bring particular genes on different chromosomes to a region of the nucleus where the appropriate factors are concentrated, thereby facilitating expression of those genes.

Results

PTF is concentrated in discrete domains in a proportion of cells

PTFα was localized in HeLa nuclei by indirect immunofluorescence using CLSM. In an unsynchronized population, 25–30% cells contain one or a few domains where PTFα is concentrated (Figure 1A, arrows). PTFα is not uniformly spread throughout the domain, but enriched in certain regions (Figure 1B). All nuclei also contain local concentrations distributed throughout the nucleoplasm (Figure 1B). PTFδ is also found both in domains and nucleoplasmic foci (Figure 1C). No domains are found with pre-immune sera, which give some background staining over the whole cell (Figure 1D and E). Similar domains are found in other human cell lines including MSU (Figure 1F), T24 and CaCo (bladder carcinoma and colon cells respectively; data not shown). In HeLa cells, they typically have an average diameter of 1.3 μm (range 0.4–3.4 μm), and often lie next to nucleoli (e.g. Figure 1B). In cells which contain domains, 81, 16 and 3% contained 1, 2 or ⩾3 domains, respectively.

Double-labelling shows that PTFα and δ are concentrated in the same domains. As both antisera are derived from the same species, PTFδ was first indirectly immunolabelled with FITC, before PTFα was indirectly immunolabelled with Cy3 using anti-PTFα antibodies conjugated with digoxigenin; then, α and δ domains were found to colocalize in all cells (Figure 1G and H; arrows). The same result was obtained when the labelling procedure was reversed (i.e. PTFα was indirectly immunolabelled with FITC, before PTFδ was labelled with an anti-PTFδ antibody conjugate; data not shown). In both cases, the second primary antibody stained the domain less intensely than the first, and gave a more punctate pattern within the domain (compare Figure 1G with H). This is probably because the first primary antibody hinders access of the second to certain regions in the domain. This effect was seen whenever two primary rabbit antibodies were used in this way (e.g. Figures 4J–L and 5S–U).

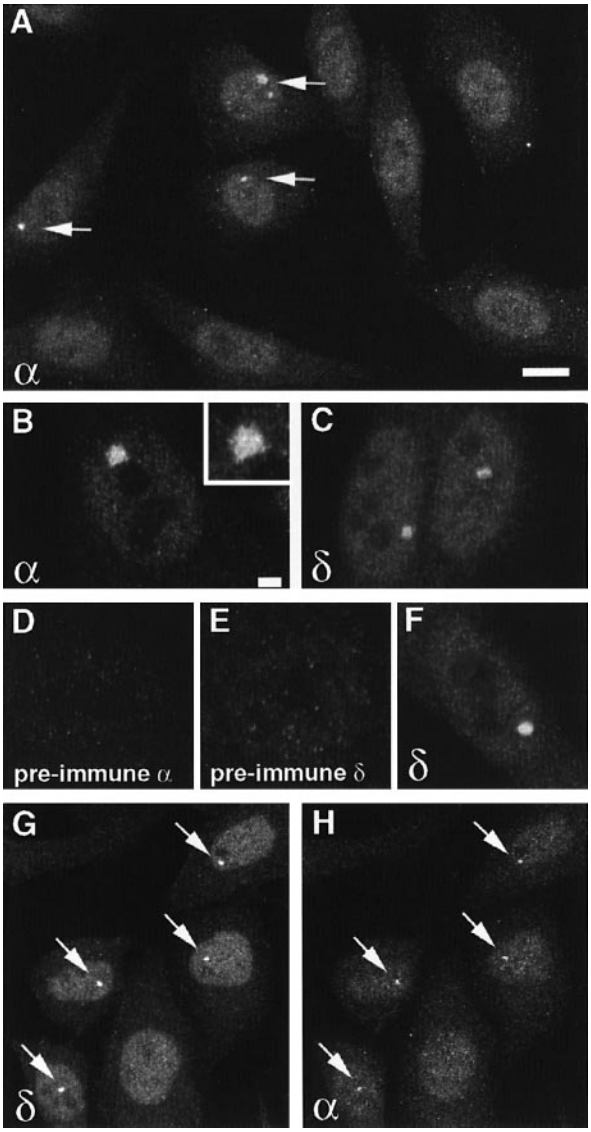


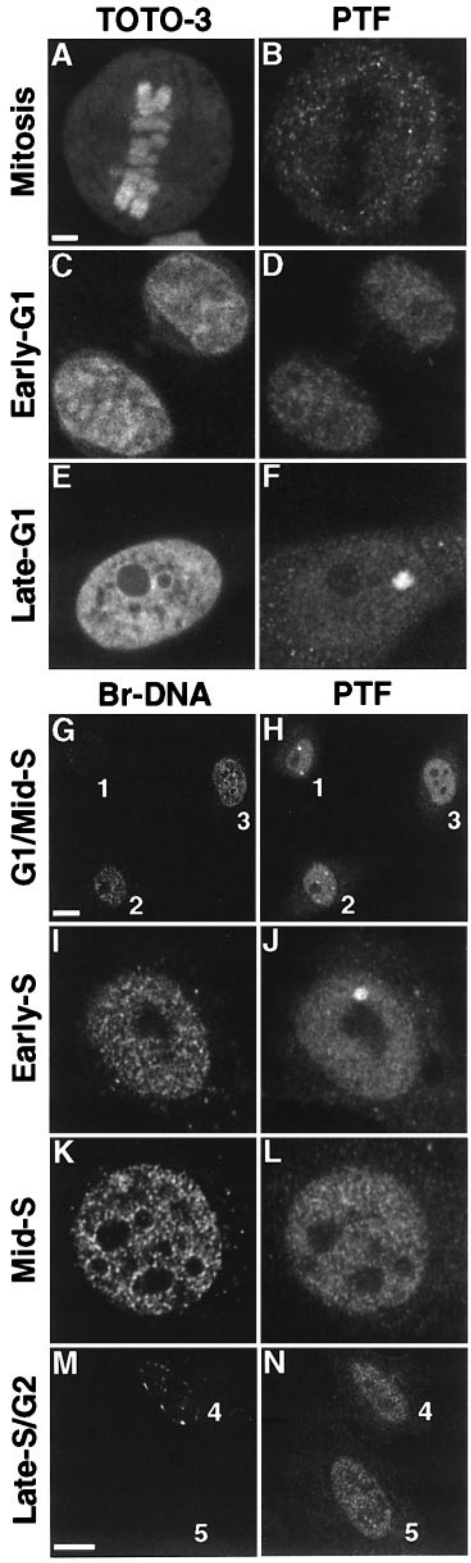
Fig. 1. PTF is concentrated in discrete nuclear domains in a proportion of cells. PTF was localized by indirect immunofluorescence using unsynchronized HeLa (A–F and H) or MSU 1.1 (G) cells and rabbit antisera raised against PTFα or δ. The antibody used is indicated in each panel. Images were collected by CLSM. (A, G and H): projections of serial sections collected at 500 nm intervals (bar: 10 μm). (B–F): single equatorial sections (bar: 2.5 μm). (**A**) Low-power view showing that PTFα is concentrated in nuclear domains (arrows) in some cells. (**B**) High-power view shows one PTFα domain abutting a nucleolus; the domain appears heterogeneous (see text). (**C**) PTFδ is concentrated in similar domains. (**D** and **E**) Domains are not seen with pre-immune sera, which give some background labelling. (**F**) The domain is also found in MSU cells. (**G** and **H**) Two views of the same field after labelling PTFδ then α; domains always colocalize (arrows).

PTF domains appear and disappear during the cell cycle

As PTF domains are found in a fraction of the population, we investigated their cell cycle dependence. HeLa cells were synchronized by mitotic 'shake-off' (with or without nocodazole treatment), and grown for different periods; cells in mitosis and the different periods of S phase were then identified after TOTO-3 staining and Br-dU incorporation respectively. No detectable PTF is associated

with mitotic chromosomes (Figure 2A and B), but it enters nuclei when they reform (Figure 2C and D). However, no domains are visible during the first hours of G_1 phase. Initially small, these grow to become most prominent late during G_1 phase (Figure 2E and F), before disappearing during S phase. Twelve hours after mitosis, the population contains some cells in late G_1 phase with prominent domains, others in early S phase with shrinking domains, and still others in mid S phase with none (Figure 2G and H; cells 1, 2 and 3 respectively). Figure 2I illustrates a cell with the replication pattern typical of an early S phase cell (e.g. Nakayasu and Berezney, 1989; Hozák *et al.*, 1994b), which has a domain (Figure 2J). By mid-S phase, no domains can be seen (Figure 2K and L). The solid line in Figure 3 illustrates the kinetics of appearance of the PTFα domain as cells progress through the cycle. PTFδ domains appear and disappear similarly (data not shown). As ~30% cells in an unsynchronized population contain ⩾1 PTF domain (see above), and ~25% of this fraction of cells with domains are in early S phase (data not shown), the PTF domain must exist for 4–5 h in G_1 phase and ~2 h in S phase. In contrast, the punctate distribution of PTF in the nucleoplasm persists through interphase (e.g. Figure 2M and N; cells 4 and 5).

Short exposures to moderate concentrations of the transcriptional inhibitor, α-amanitin, do not affect the appearance or disappearance of the PTF domain. Thus, when an unsynchronized population of HeLa cells was grown for 3 h in 50 μg/ml α-amanitin, a concentration that completely inhibits RNA polymerase II *in vitro* (Weinmann *et al.*, 1975), 34% cells possessed ⩾1 domain, like untreated controls (data not shown). However, a different inhibitor, dichlororibofuranosylbenzimidazole (DRB; Granick, 1975; Chodosh *et al.*, 1989), prevents the



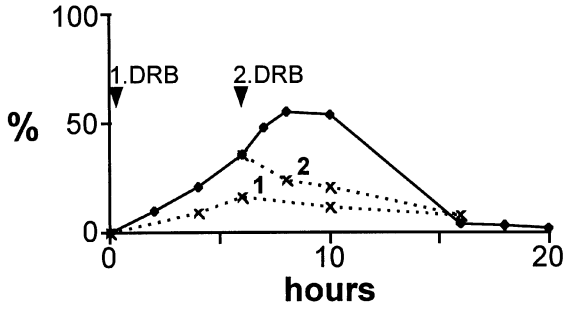


Fig. 3. DRB inhibits the formation of the PTF domain and hastens its disappearance. HeLa cells were released from mitosis, grown for different periods, and fixed; then, PTFα was indirectly immunolabelled, and the percentage of cells containing at least 1 PTF domain determined. The domain forms soon after mitosis and disassembles during S phase (solid line). Sometimes, cells were grown in 100 μM DRB from 0.5 or 6 h after mitosis (curves 1 and 2, respectively); the drug inhibits the formation of the domain, and hastens its disappearance. Br-dU labelling showed that 28% cells had reached S phase after 6 h.

Fig 2. PTF domains appear and disappear during the cell cycle. HeLa cells were released from mitosis, grown for the times indicated, then for 15 min in Br-dU, and fixed; PTFα and Br-DNA were indirectly immunolabelled, cells stained with TOTO-3, and equatorial optical sections collected. Two views of the same plane through each cell are shown. Cells in mitosis and different periods of S phase were identified from the patterns of TOTO-3 staining and Br-DNA. Bars are 2.5 μm (A–F and I–L) and 10 μm (G, H, M and N).
(**A** and **B**) During mitosis (t=0 h), PTF is excluded from the chromosomes. (**C** and **D**) PTF has entered the nucleus (t=1 h). (**E** and **F**) A PTF domain has formed (t=6 h). (**G** and **H**) Cells at different stages can be distinguished by their Br-DNA patterns (t=12 h). Cell 1 (2 domains) has not incorporated Br-dU, and so is still in G_1 phase. Cell 2 (4 small domains) has the replication pattern typical of early-S phase. Cell 3 (no domain) is in mid S phase. (**I** and **J**) A typical cell in early S phase (t=12 h). (**K** and **L**) A cell in mid S phase with no domain (t=12 h). (**M** and **N**) Cell 4 (no domain) is in late S phase; cell 5 (no domain) is in G_2 phase (t=22 h).

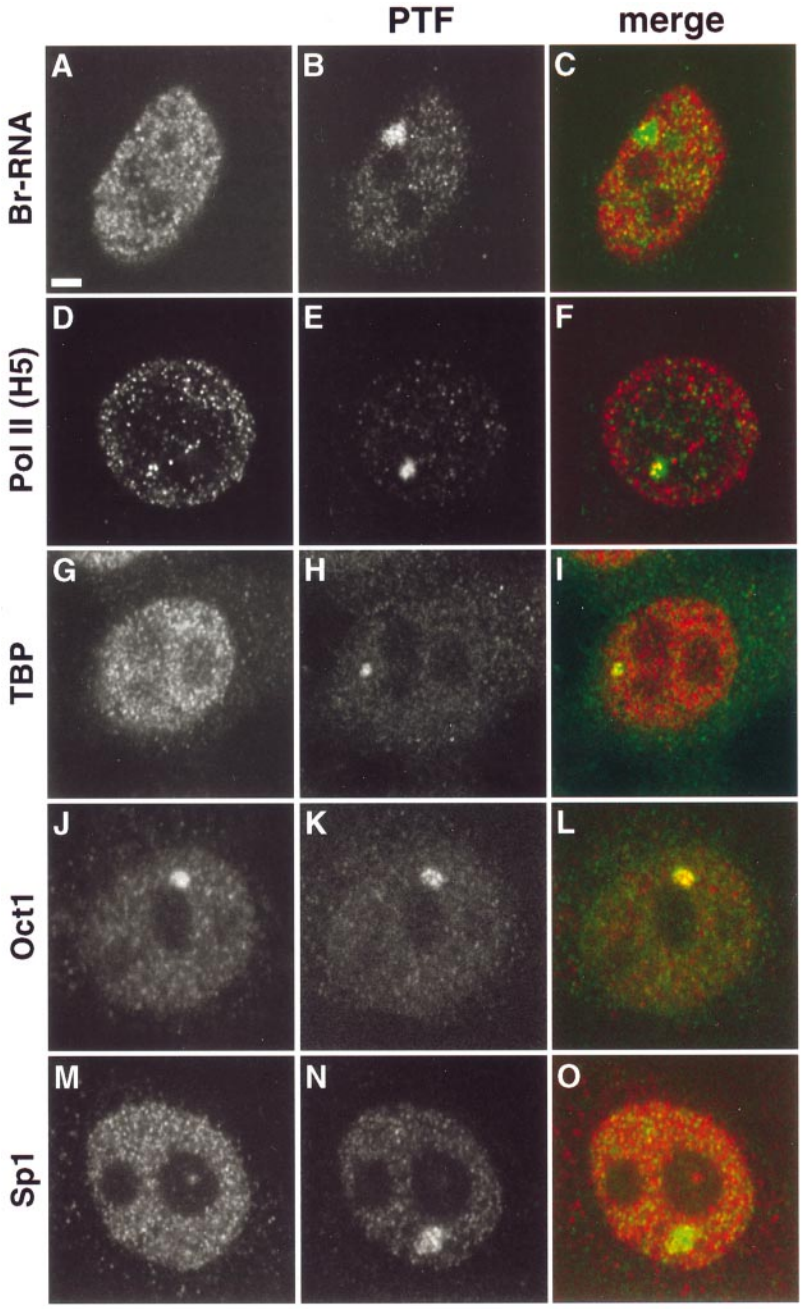


Fig. 4. PTF domains are transcriptionally active, and contain RNA polymerase and various transcription factors. HeLa cells were doubly labelled with antibodies against PTFα and different markers in turn (i.e. nascent Br-RNA, the hyperphosphorylated form of the large subunit of RNA polymerase II recognized by the H5 antibody, TBP, Oct1 and Sp1). Series of confocal sections were collected through each cell, and an equatorial section through a PTF domain is shown. The distribution of a marker in the same section is illustrated, and a coloured image made by merging pseudocoloured images of the marker and PTF; where PTF domains (green) overlap local concentrations of a marker (red), yellow is seen. In each case, markers are found within the domain, and elsewhere in the nucleus. Bar: 2.5 μm.

appearance of domains. For example, growth in 10 or 100 μM DRB for 3 h reduces the number of cells with ⩾1 domain to 27 and 21% respectively (data not shown). Moreover, if cells are released from mitosis into media containing DRB, fewer domains form (Figure 3, curve 1), and if DRB is added when many have appeared, they disappear more rapidly (Figure 3, curve 2). These results indicate that the formation and maintenance of the domain depend on some DRB-sensitive activity.

PTF domains are transcriptionally active, and contain RNA polymerase and various transcription factors

Next we investigated whether PTF domains were transcriptionally active. Cells were permeabilized and allowed to extend nascent transcripts in Br-UTP (Jackson *et al.*, 1993); then, domains were localized relative to sites containing nascent Br-RNA by CLSM. Figure 4A illustrates the distribution of Br-RNA in a single optical

section, and Figure 4B shows the PTF domain in the same section; several bright transcription 'factories' lie within the domain. This overlap is obvious in the 'merge' illustrated in Figure 4C, where yellow marks overlap between Br-RNA (red) and PTF (green). Inspection of adjacent optical sections confirms that peaks of intensity due to Br-RNA lay within the PTF domain in the same section (see Materials and methods). Note that PTF domains do not necessarily contain a higher density of factories than the surrounding nucleoplasm, and that the factories they contain are as active as those outside the domain. We typically detect 2 or 3 (range 1–5) transcription factories in ~90% domains, indicating that the domains are usually transcriptionally active. The minority of inactive domains tend to be smaller, perhaps because they are growing or shrinking. These factories contain transcripts generated by both polymerases II and III. This was shown by allowing nascent transcripts to elongate in 1 μg/ml α-amanitin (to inhibit polymerase II); then ~60% of PTF domains contain 1 or 2 (polymerase III) factories (A.Pombo and P.R.Cook, in preparation).

PTF domains also contain concentrations of RNA polymerase II detected using the monoclonal antibody H5 that recognizes the hyperphosphorylated C-terminal domain of the large subunit, a form implicated in active transcription (Bregman *et al.*, 1995; Kim *et al.*, 1997). Some foci rich in this subunit are found in the domain (Figure 4D–F). As PTF interacts directly with TBP and Oct1 (see Introduction), and many PSE-containing genes also contain binding sites for TBP, Oct1 and Sp1 (Hernandez, 1992), we investigated whether these factors were also found in the domain. TBP is found in foci spread throughout nuclei, and several of these lie within PTF domains (Figure 4G–I; see also Jordan *et al.*, 1996). Oct1 is also found in nucleoplasmic foci, and in some cells it is also concentrated in discrete domains with diameters of ~1 μm (Grande *et al.*, 1997). The PTF domain turns out to be identical to this Oct1-rich domain identified previously (Figure 4J and K), even though transcriptional activity was not detected before (see Materials and methods). Some foci containing Sp1 lie within PTF domains (Figure 4M–O; see also Saffer *et al.*, 1990), just like some of those containing Br-RNA, polymerase II and TBP.

These results indicate that PTF domains are transcriptionally active, and that they contain local concentrations of RNA polymerase II, TBP, Oct1 and Sp1. This activity contrasts with the inactivity of other domains (e.g. PML and coiled bodies; Weis *et al.*, 1994; Schul *et al.*, 1996), including those rich in transcription factors (e.g. GATA domains; Elefanty *et al.*, 1996). We also screened to see if the PTF domain contained any factors involved in RNA processing; however, none of those tested (i.e. SC35, Sm antigens, hnRNP I and L, CstF 64) were detected within the domain (see below and data not shown).

The relationship of the PTF domain to other nuclear domains

Various nuclear bodies and distinct compartments within nuclei have been described, including nucleoli, 'speckles', coiled bodies, gemini bodies, perinucleolar compartments, PML bodies, PIKA domains, and replication 'foci' or 'factories'. We investigated the relationship between such regions and the PTF domains. The PTF domain often lies next to nucleoli, stained with a nucleolar marker, p120 (Freeman *et al.*, 1988; Ochs *et al.*, 1988), but never colocalizes with it (data not shown). Little, if any, SC35, a marker used to define 'speckles' (Fu and Maniatis, 1990), is found within the PTF domain (Figure 5A–C). Coiled bodies—which are marked by coilin and are transcriptionally inactive (Schul *et al.*, 1996)—never completely overlap the PTF domain (Figure 5D–F). Although both structures often lie near nucleoli, the two only appear to touch in 6% cells (analysis of cells with ⩾1 PTF domain; data not shown). Gemini bodies are found in approximately similar numbers to coiled bodies and contain the SMN protein (Liu and Dreyfuss, 1996); they too do not colocalize with PTF domains (Figure 5G–I). The perinucleolar compartment (PNC) contains hnRNP I/PTB as well as Y RNAs, RNase P, RNase MRP (Ghetti *et al.*, 1992; Matera *et al.*, 1995; Lee *et al.*, 1996), and it does not coincide with the PTF domain (Figure 5J–L), like other compartments containing hnRNP L (Figure 5M–O; Piñol-Roma *et al.*, 1989). HeLa cells contain 5–10 PML bodies (Weis *et al.*, 1994), and one of these often abuts a PTF domain (Figure 5P–R). Polymorphic interphase karyosomal association (PIKA) domains are heterogeneous in number and size, and have been classified into types 1–6 (Saunders *et al.*, 1991). PTF domains always coincided with some PIKA domains (i.e. types 1 and 2; Figure 5S–U), although the many smaller PIKA domains (i.e. types 3–5) rarely contained any detectable PTF (Figure 5V–X). Finally, PTF domains contain a few of the small replication 'factories' seen at the very beginning of S phase (e.g. Nakayasu and Berezney, 1989; Hozák *et al.*, 1994b), but no more than elsewhere in the nucleus (data not shown).

Certain chromosomes tend to associate with PTF domains

The transcriptional activity and large size of the PTF domain prompted us to see whether it acted like a nucleolus to bring particular genes on different chromosomes to a region where specific factors were concentrated, facilitating expression of those genes. Therefore, we screened whole chromosomes to see if any associated preferentially with the domain, before going on to test a sub-chromosomal region (Table I). We analyzed chromosomes 1, 6, 7 and 17, since they encode genes regulated by PTF and Oct1; the sub-chromosomal region 6p21 was also tested since it contains the large histone cluster and many Oct1 sites. As controls, we chose chromosome 2 (the biggest encoding no candidate genes known to us), chromosome 13 (the largest carrying a nucleolar organizing region, or NOR) and chromosome 11 (for its average size). PTF domains were immunolabelled, chromosomes or bands 'painted', and overlap between the two determined by inspection of stacks of optical sections. The procedure preserved the distribution of PTF in both the domain and the numerous nucleoplasmic foci, and overlap was defined using stringent criteria (see Materials and methods).

Typical examples of this approach are given in Figure 6, and results are summarized in Table I. Aneuploid HeLa cells typically contain three copies of each chromosome. For the data shown in Table I, all cells analyzed contained three copies of the territory indicated, and ⩾95% contained only 1 PTF domain. In some cases, part(s) of one or more

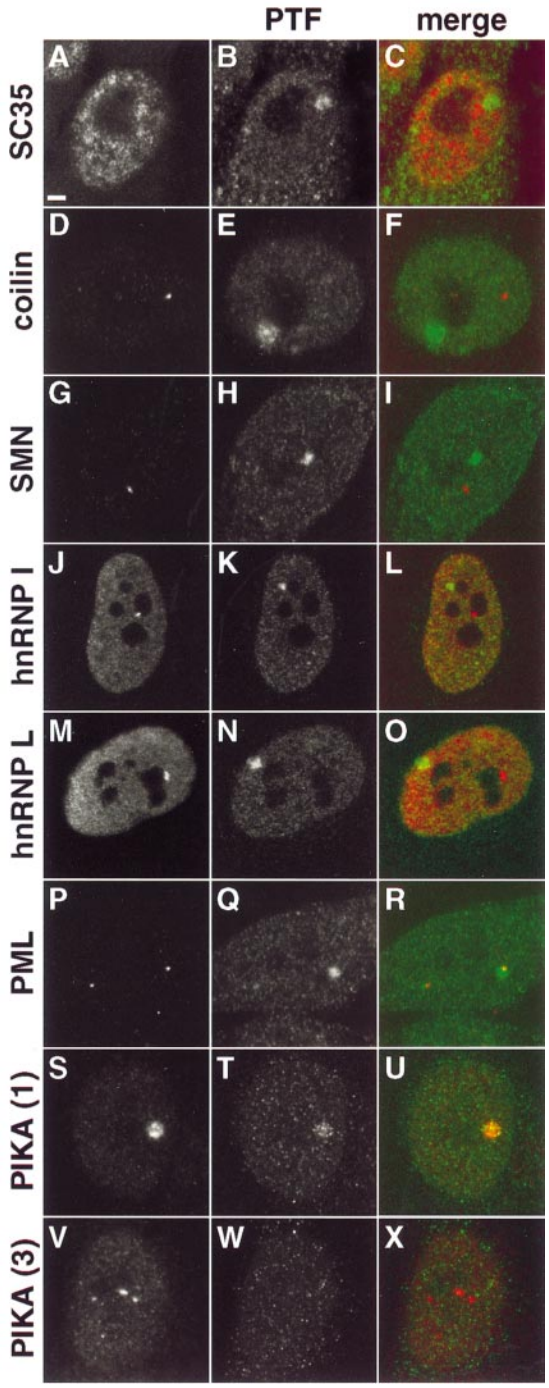


Fig. 5. The relationship of the PTF domain to other nuclear domains. HeLa cells were doubly labelled with antibodies against PTF and different markers. Series of confocal sections were collected through each cell at 400 nm intervals; one equatorial section through a PTF domain is shown in (A–U), and a projection of three consecutive sections in (V–X) (to illustrate several PIKA domains). The distribution of a marker in the same section is illustrated, and a coloured image made by merging images of the marker and PTF (pseudocoloured red and green respectively). SC35 (**A**–**C**), coilin (**D**–**F**), SMN (**G**–**I**), hnRNP I (**J**–**L**), hnRNP L (**M**–**O**) are concentrated in domains that do not overlap the PTF domain (and so appear red). One of the many PML bodies in a cell often lies next to the PTF domain (and also appears red; **P**–**R**). A (type 1) PIKA domain (**S**–**U**) found late in G_1 colocalizes with the PTF domain (and so appears yellow). However, type 3 PIKA domains contain no detectable PTF (**V**–**X**). Bar: 2.5 μm.

of the three chromosomal territories overlapped a PTF domain, in others none did, while a minority lay nearby (Figure 6); these three categories are labelled +, – and ± in Table I. Associations of chromosomal territories with the domain do not correlate with chromosome length, and so volume; for example, band 6p21 was associated more often than chromosome 1, which is 8-fold longer (Table I). Moreover, positive overlap is not explained by proximity of PTF domains to nucleoli, as chromosome 13 (which carries a NOR) is not particularly associated with the domain. Chromosomes 2, 6 and 7 and band 6p21, were associated with an OPT domain in >50% cells. A χ^2 test then showed that we can be reasonably confident that the associations of 6p21, chromosome 7 and perhaps 2, were significantly higher than those of other chromosomes, including the largest (Table I, legend). As summed associations are >100%, one domain must associate with several chromosomes at any one time, and the association of chromosome 7 and 6p21 with one PTF domain was confirmed by multiple labelling (data not shown).

Only a fraction of PTF- and Oct1-dependent genes have probably been identified, and probes long enough to perform FISH are available for only some of these. We screened those that were available to see if any associated preferentially with the domain (data not shown). Tested genes encoded U1 and U2 snRNA (containing both PTF and Oct1 sites, transcribed by polymerase II), 7SK and hY RNA (containing both PTF and Oct1 sites, transcribed by polymerase III) and histones from the large cluster at 6p21 (containing Oct1 sites, transcribed by polymerase II). As a control, we also screened 5S rRNAs genes; these lack PTF- and Oct1-binding sites and are transcribed by polymerase III. Perhaps unsurprisingly, none colocalized with the domain. Although we cannot eliminate a role for the domain in the transcription of these genes, any association must be transient, with transcription occurring outside the domain. [Note that genes encoding U1 and U2 snRNAs, and histones, associate with coiled bodies (Frey and Matera, 1995; Smith *et al.*, 1995; W.Schul, R.van Driel and L.de Jong, in preparation).] However, it remains a possibility that other, so far unidentified, genes regulated by PTF/Oct1, and whose transcripts are not spliced or polyadenylated, associate with the domain.

Discussion

The 'OPT' domain is transcriptionally active

Active RNA polymerases and nascent RNA are concentrated in only a tiny fraction of the volume of a mammalian nucleus (reviewed by Jackson, 1997). For example, nascent transcripts, which can be immunolabelled after allowing engaged polymerases to incorporate a precursor like Br-UTP, are found in several thousand 'foci' or 'factories' that typically have diameters of ~80 nm (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Hozák *et al.*, 1994a; Iborra *et al.*, 1996a; Fay *et al.*, 1997). RNA polymerase II is also found in these factories (e.g. Bregman *et al.*, 1995; Iborra *et al.*, 1996a; Kim *et al.*, 1997), and we might expect many transcription factors also to be concentrated in these sites. However, the highest concentrations are usually found elsewhere, apparently clustered in inactive stores (van Steensel *et al.*, 1995; Grande *et al.*, 1997; A.Pombo and P.R.Cook, unpublished). We now describe

Table I. Frequency of association of specific chromosomes/band with PTF domain

Chromosome (length in Mbp)	Candidate genes	Association with domain (% cells)			
		+	–	±	*n*
1 (~263)	U1 snRNA, histone, 5S rRNA	44	44	12	27
2 (~255)		61[a]	27	12	33
6 (~183)	7SK snRNA, histone	64[b]	23	13	31
7 (~171)	hY1, 3,4 and 5 RNAs	53[c]	42	5	53
11 (~144)		30[d]	57	13	23
13 (~98)	45S rRNA	34	54	12	35
17 (~92)	U2 snRNA, U3 snRNA	36[e]	58	6	45
6p21 (~30)	histone	57[f,g,h]	28	15	46

PTF domains and interphase territories were labelled, optical sections through whole nuclei collected at 300 nm intervals, and overlap between the PTF domain and at least one chromosome was scored as +, – or ±. A rough estimate of the length of each chromosome (Morton, 1991) or band (Guan *et al*., 1995), and some PTF- or Oct1-activated and rRNA genes that they encode, are indicated. The number of cells (*n*) analyzed was sufficient to ensure that progressive means given by the last ⩾13% (i.e. ⩾6) cells analyzed always lay within ± 5% of the final mean. The probabilities that associations were the same (calculated using the χ^2 test, and dividing ± associations equally amongst the + and the – associations) were: [a]0.13, [b]0.09, [c]0.003 and [h]0.01 (all compared with chromosome 1); [d]0.0002 (11 with 7), [e,f]0.3 (17 with 13, and 6p21 with 7), and [g]0.02 (6p21 with 17). Therefore, these statistics provide reasonable confidence that chromosome 7 and band 6p21, and perhaps chromosomes 6 and 2, associate with the domain more than others (e.g. 1).

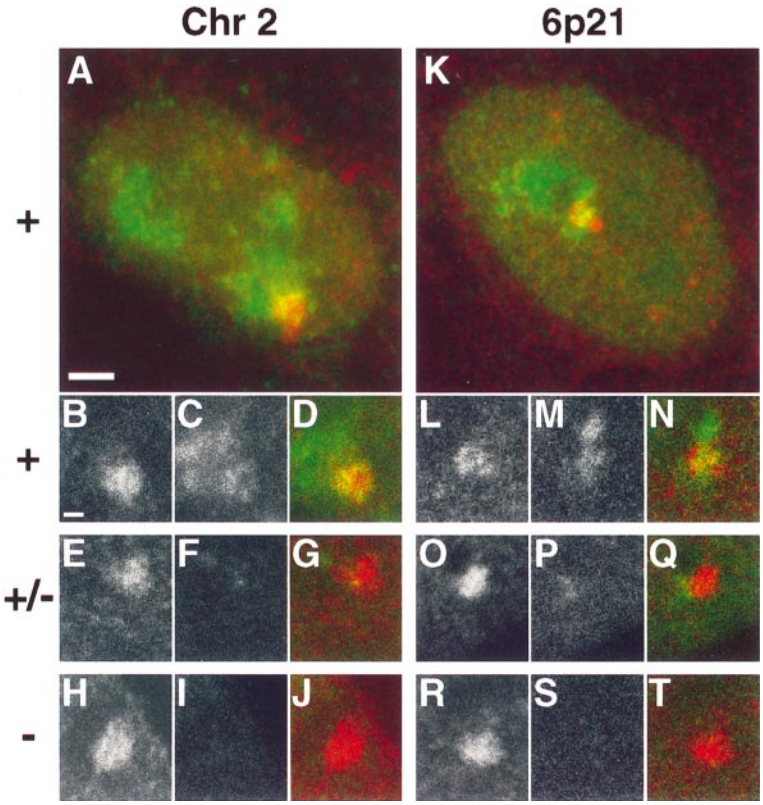


Fig. 6. Certain chromosomes tend to associate with PTF domains. PTF domains in interphase HeLa cells were immunolabelled ('red'), chromosome 2 or band 6p21 'painted' ('green'), and a *z*-axis series of optical sections collected through individual cells. (B–J) and (L–T) are 'raw' images. Bars: 2.5 μm (A and K) and 1 μm (B–J and L–T). (**A**–**D**) Positive (+) association with chromosome 2. Two complete series of images of one cell were projected onto a plane and 'merged'; one chromosomal territory (pseudocoloured green) appears to overlap the PTF domain (pseudocoloured red), giving yellow (A). Overlap was tested by examining each section in the series in turn. Three high-power views of the PTF (red; B), chromosome (green; C) and 'merged' channels (D) of one such section are shown. (**E**–**G**) Doubtful association (±). Three views of a single optical section [like those in (B–D)]. The PTF domain (E) overlaps signal that could be from a diffuse chromosomal 'territory' (F). (**H**–**J**) Negative (–) association seen in three views of a section [like those in (B–D)]. (**K**–**N**) Positive association with band 6p21. Images were prepared as for (A–D); the narrow chromosomal 'thread' runs through the PTF domain and this overlap is observed in single optical sections across the domain (L–N). (**O**–**Q**) Doubtful association seen in three views of one section; the band touches, but does not overlap, the PTF domain. (**R**–**T**) Negative association.

the first example of a domain that contains a high concentration of two interacting transcription factors, PTF and Oct1, in addition to TBP and RNA polymerase. Like nucleoli, but unlike all other domains described to date, this one is transcriptionally active.

PTF (which is also known as PBP and SNAPc) is

essential for transcription of snRNA and related genes; it is a tetramer of α, β, γ and δ subunits, and it interacts with TBP and Oct1 (see Introduction). Immunofluorescence using rabbit antisera directed against the α and δ subunits shows that PTF is concentrated in several hundred foci spread throughout the nucleoplasm (Figure 1A); such a distribution is typical of many transcription factors (e.g. Grande *et al.*, 1997). In some cells, PTF is also concentrated in a few, usually one, discrete domain(s) (diameter ~1.3 μm). Oct1 has also been found in a domain of similar size in a fraction of cells (Grande *et al.*, 1997); it colocalizes with the PTF domain (Figure 4J–L). This PTF/Oct1 domain typically contains several transcription factories that incorporate Br-UTP into nascent transcripts (Figure 4A–C), as well as local concentrations of RNA polymerase II, TBP and Sp1 (Figure 4). It contains little, if any, of some components involved in RNA processing (i.e. SC35, Sm antigens, hnRNP I and L, CstF 64; Figure 5A–C and data not shown; see also Saunders *et al.*, 1991). Therefore, we have christened this region the Oct1/PTF/transcription (OPT) domain.

OPT domains are distinct from other nuclear domains, including 'speckles', coiled bodies, gemini bodies, PML bodies, and the perinucleolar compartment (Figure 5). However, they always coincided with a large PIKA (Figure 5S–U), although the many smaller PIKAs rarely contain much detectable PTF (Figure 5V–X). PIKAs are visible in the phase-contrast microscope; they are heterogeneous in number and size. At the ultrastructural level, they have a lower electron density than their surroundings, and they are devoid of interchromatin granules (Saunders *et al.*, 1991).

The OPT domain assembles in G_1 to disappear after the onset of S phase

OPT domains assemble during G_1 phase, and disappear early during S phase (Figures 2 and 3). It remains to be shown how they might do so, although reversible phosphorylation of specific components is an obvious possibility (see Roberts *et al.*, 1991). The domain also seems to exclude factors involved in RNA processing, despite its transcriptional activity (see also Saunders *et al.*, 1991). Taken together, these results raise the possibility that specific genes (regulated by PTF/Oct1, and perhaps encoding transcripts that are not polyadenylated or processed by the common splicing factors), may be transcribed in the domain at a specific stage of the cell cycle. Interestingly, transcripts regulated by PTF and, in some cases, by Oct1 are not spliced or polyadenylated.

Chromosomes 6 and 7 tend to associate with the OPT domain

The nucleolus is a transcriptionally-active nuclear domain that specifically associates with chromosomes encoding rDNA. Therefore, we determined whether the OPT domain also associated with particular chromosomes. If chromosomes were arranged randomly, we would expect the largest chromosome territory to be associated more often than any other, and the frequency to correlate with size. However, the largest chromosome (i.e. 1) associated less than three smaller ones (i.e. 2, 6 and 7), and frequencies did not correlate with size (Table I). Moreover, a small region on chromosome 6 (i.e. band 6p21) containing only ~30 Mbp DNA, and chromosomes 2, 6 and 7, were associated with an OPT domain in >50% cells, with the associations of 6p21 and chromosome 7 being particularly significant (Table I, footnote). This suggests that genes on these chromosomes may associate preferentially with the PTF domain. When the sequence of 6p21 becomes available, it will be possible to screen genes containing Oct1 and PTF sites to see if any associate with the domain.

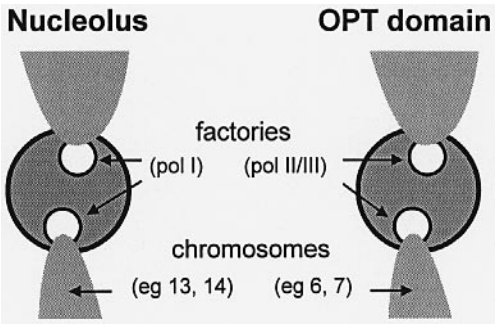


Fig. 7. Similarities between nucleoli and OPT domains. A nucleolus contains several (polymerase I) transcription factories; each nucleolus can associate with rDNA genes carried on several chromosomes. The OPT domain may be an analogous region containing transcription factories rich in polymerases II and/or III, and which tends to associate with specific chromosomes. Although OPT domains often lie next to nucleoli, they never overlap them.

The appearance and disappearance of the OPT domain

The nucleolus is the best-characterized domain (Hozák *et al.*, 1994a; Shaw and Jordan, 1995); it provides a precedent for the way the OPT domain might form and influence chromosome position (Figure 7). The nucleolus is dedicated to the transcription of 45S rRNA and the production of ribosomes. The genes for 45S rRNA are encoded by nucleolar organizing regions (NORs) carried on several chromosomes (e.g. human chromosomes 13, 14, 15, 21 and 22). When chromosomes decondense after mitosis, one or more NORs nucleate the formation of a nucleolus containing several transcription factories. These factories contain the transcription machinery (e.g. RNA polymerase I, UBF and TBP), and nascent RNA. However, only some NORs are incorporated into nucleoli and so are transcribed; others remain inactive (Roussel *et al.*, 1996). As a result, only some chromosomes carrying NORs associate with a nucleolus. Therefore, the OPT domain resembles the nucleolus; it contains the necessary polymerases and transcription factors organized into active factories, and it associates with specific regions on some, but not all, copies of a specific chromosome (Figure 6A). This raises the possibility that chromosomes 7 and 6, and perhaps 2, carry 'OPT organizing regions' that seed the formation of OPT domains. Alternatively, the OPT domain could form before associating with chromosomes, and then, only when random chromosome movement generated chance associations, might certain chromosomes stably associate with it.

General conclusions

We have shown that the OPT domain (i) is transcriptionally active, (ii) contains few, if any, of some components involved in RNA processing, (iii) appears during G_1 phase

to disappear during S phase, and (iv) seems to organize chromosomes within the nucleus, much like the nucleolus. Whatever the role of this mysterious domain proves to be, our results reinforce the idea that nuclear architecture is more dynamic than has been imagined, and that different regions of the nucleus specialize in different functions (e.g. Jackson, 1997; Misteli *et al.*, 1997). Our results also show that high concentrations of transcription factors are not always inactive stores, and they raise the possibility that other transcription factors will be found in analogous domains surrounding other transcription factories. (For example, might these include some of the smaller PIKA domains?) This, in turn, raises the possibility that those factories might specialize in the transcription of certain groups of genes, just like nucleolar factories.

Materials and methods

Cells

HeLa or MSU-1.1 monolayers (Morgan *et al.*, 1991) were grown in DMEM or Ham's F10 medium respectively, supplemented with 10% fetal calf serum. HeLa cells synchronized at various stages of the cycle after growth in medium supplemented with 10 mM HEPES (pH 7.4) for 7 d, and blocking in mitosis with nocodazole (2–4 h; 40 ng/ml; Aldrich); then, mitotic cells were shaken-off, washed, regrown (1 h), plated on coverslips coated with bovine fibronectin (40 μg/ml; Sigma), and medium replaced after 2 h. Synchrony was monitored by DNA staining (to visualize mitotic cells), and by Br-dU incorporation and immunostaining (to score the percentage of S phase cells; see below). Cells synchronized without nocodazole gave similar results.

Antibodies

The following primary antibodies were used. Rabbit antibodies against: (i) PTFα and PTFδ, and corresponding pre-immune sera (dilution 1/500; Yoon and Roeder, 1996); (ii) Oct1 (1/100; Zwilling *et al.*, 1994); and (iii) PIKA (1/1000; Saunders *et al.*, 1991). Mouse monoclonals directed against: (i) digoxigenin (0.2 μg/ml; clone 1.71.256; Boehringer); (ii) bromodeoxyuridine (Br-dU; 2 μg/ml; clone BMC9318; Boehringer); (iii) hyperphosphorylated C-terminal domain of large subunit of RNA polymerase II (1/1000; clone H5; Bregman *et al.*, 1995); (iv) TBP (1/100; clone SL35–2–556; Lobo *et al.*, 1992); (v) Sp1 (2 μg/ml; clone 1C6; Santa Cruz Biotechnology); (vi) SC35 (1/50; Fu and Maniatis, 1990); (vii) coilin (1/200; clone 1D4-δ; Rebelo *et al.*, 1996); (viii) SMN (1/2000; clone 2B1; Liu and Dreyfuss, 1996); (ix) hnRNP I (1/200; 7G12; Ghetti *et al.*, 1992); (x) hnRNP L (1/5000; clone 4D11; Piñol-Roma *et al.*, 1989); (xi) PML (5 μg/ml; clone PG-M3; Santa Cruz Biotechnology); (xii) nucleolar protein p120 (1/50; Freeman *et al.*, 1988); and (xiii) CstF 64 (1/200; Takagaki *et al.*, 1990). Human autoimmune serum: Sm (1/2000; AF-CDC ANA reference lab; Tan *et al.*, 1982). Various secondary antibodies conjugated with FITC or Cy3 were obtained from Jackson Immunoresearch Labs (0.5–1 μg/ml; multiple-labelling grade).

Immunolabelling, general procedures

Cells were generally fixed (4°C; 10 min) in 4% paraformaldehyde, 0.1% Triton X-100, 250 mM HEPES (pH 7.4), and then in 8% paraformaldehyde, 250 mM HEPES (pH 7.4) for 30 min. The PTF domain was also seen after fixation in: (i) 4% paraformaldehyde in HEPES (4°C; 10 min), and then in 8% paraformaldehyde in HEPES (4°C; 50 min); (ii) 4% paraformaldehyde in HEPES (4°C; 20 min); (iii) 2% paraformaldehyde in PBS (15 min); (iv) methanol (–20°C; 20 min). After fixation, cells were incubated (20 min) in 25 mM glycine in PBS, permeabilized (20 min) with 0.1% Triton X-100 in PBS, washed 5× over 20 min in PBS, blocked (20 min) with PBS+ (PBS plus 1% BSA, 0.2% fish skin gelatin; pH 7.4), incubated (1 h) with rabbit antibodies against PTFα or δ (in PBS+), washed 5× over 30 min in PBS+, incubated (1 h) with donkey antibodies against rabbit IgG conjugated with FITC (in PBS+), washed 5× over 30 min in PBS+, rinsed 3× in PBS, incubated (20 min) with TOTO-3 (20 μM in 0.1% Tween 20 in PBS; Molecular Probes), washed successively in 0.1% Tween 20 in PBS and then PBS, before coverslips were mounted in VectaShield (Vector Labs).

Double-labelling PTF and other nuclear proteins

Three protocols were used. (i) When both primary antibodies were from rabbits (i.e. against PTF, Oct1 or PIKA), anti-PTFα was conjugated with digoxigenin using the DIG protein labelling kit (Boehringer). Cells were fixed, permeabilized, blocked, incubated with anti-PTFδ (or anti-Oct1, or anti-PIKA), washed, and incubated with donkey anti-rabbit IgG conjugated with FITC. After blocking (30 min) with normal rabbit serum (10% in PBS+; Jackson Immunoresearch Labs), cells were incubated with anti-PTFα conjugated with digoxigenin, washed, incubated with mouse anti-digoxigenin, washed, and incubated with donkey anti-mouse IgG conjugated with Cy3, before washing, staining with TOTO and mounting. (ii) If the two primary antibodies were from different species, cells were incubated simultaneously with both antibodies, washed, and incubated simultaneously with both fluorescently-labelled secondary antibodies. (iii) When labelling PTF and other proteins (especially the large subunit of RNA polymerase II), procedure (ii) labelled the other protein within the domain weakly, probably because bound anti-PTF inhibited access of the other primary antibody. Such blocking by one antibody of another probably explains why Br-RNA and polymerase II were not detected in the Oct1 domain by Grande *et al.* (1997). In such cases, cells were incubated with a primary antibody against a marker (not PTF), then a mixture of the secondary antibody against the marker plus anti-PTF antibodies, and finally the secondary antibody against anti-PTF.

Double-labelling PTF and replication sites

Cells on coverslips were grown (10 min) in 150 μM BrdU, fixed (4°C; 20 min) in 4% paraformaldehyde in 250 mM HEPES (pH 7.4), stored in PBS, PTFα immunolabelled as above, and Br-DNA was detected as follows. Cells were rinsed 3× in PBS, fixed (10 min) in 4% paraformaldehyde in 250 mM HEPES (pH 7.4) and then in 8% paraformaldehyde in 250 mM HEPES (pH 7.4) for 15 min, incubated (15 min) in 4 M HCl, rinsed 6× in PBS, before Br-DNA was immunolabelled using the mouse monoclonal antibody against bromodeoxyuridine and donkey anti-mouse IgG conjugated with Cy3.

Double-labelling transcription sites and PTF

Cells on coverslips were washed in PBS, and permeabilized (4°C; 5 min) with 100 μg/ml saponin in a 'physiological' buffer (Jackson *et al.*, 1993) supplemented with BSA (PB-BSA). Both nuclear structure and transcriptional activity are well preserved in this buffer (A.Pombo, D.A.Jackson and P.R.Cook, in preparation), which contains 100 mM potassium acetate, 30 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM Na_2ATP (Sigma Grade I), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride, human placental ribonuclease inhibitor (10 units/ml; Amersham), and 100 μg/ml BSA (pH 7.4). [As the acidity of ATP batches can vary, 100 mM KH_2PO_4 (usually 1/100th volume) is sometimes added to adjust the pH.] After washing in ice-cold PB-BSA, engaged RNA polymerases were allowed to extend nascent transcripts (33°C; 15 min) in PB-BSA plus 0.1 mM ATP, CTP, GTP and Br-UTP. After rinsing in ice-cold PB-BSA, cells were fixed (4°C; 10 min) in 4% paraformaldehyde in 250 mM HEPES (pH 7.4) and then in 8% paraformaldehyde in 250 mM HEPES (pH 7.4) for 50 min. Next, cells were incubated (20 min) in glycine-PBS, permeabilized (30 min) with 0.5% Triton X-100 in PBS, washed 5× over 20 min in PBS, and Br-RNA immunolabelled using procedure (iii).

Double-labelling PTF and chromosomes

DNA sequences were localized by FISH using a modification of the method described by Kurz *et al.* (1996) and (i) digoxigenin-conjugated whole-chromosome paints (Oncor Appligene), (ii) a rhodamine(110)-conjugated paint for band 6p21 (Biovation, Aberdeen, Scotland; Guan *et al.* 1995), or (iii) biotin-DNA made by 'nick-translating' (BioNick kit; Boehringer) DNA encoding 5S rRNA (plasmid ph5s8544; Little and Braaten, 1989), U1 snRNA (plasmid cosD21; Bernstein *et al.*, 1985), U2 snRNA (plasmid pTP18; Pavelitz *et al.*, 1995), 7SK snRNA (Murphy *et al.*, 1986), histones (BAC clone 227-P–14 from Research Genetics that maps within the histone gene cluster on human chromosome 6p21; A.G.Matera, personal communication), and hY1,3,4, and 5 RNA (clones yWSS1020 and 1476; Maraia *et al.*, 1996).

As PTF domains could not be immunolabelled after FISH, they were labelled first. Cells were fixed (4°C; 30 min) in 0.1% Triton X-100 in 4% paraformaldehyde, 250 mM HEPES, (pH 7.4), and then in 8% paraformaldehyde, 250 mM HEPES (pH 7.4) for 10 min. Fixed cells were incubated (20 min) with glycine-PBS, permeabilized (1 h with gentle rocking) in PBS plus 0.5% Triton X-100 and 0.5% saponin, washed 4× in PBS, blocked (30 min) with PBS+, and indirectly

immunolabelled with anti-PTFα and donkey anti-rabbit IgG conjugated with Cy3 (as above). After antibodies were fixed (30 min) in 4% paraformaldehyde, 250 mM HEPES (pH 7.4), the fixed cells were incubated (30 min) in glycine-PBS, treated (50 min with gentle rocking) with 0.5% Triton X-100, 0.5% saponin in PBS, washed (8× over 20 min) in PBS, treated (20 min) with 0.1 M HCl, and washed successively in PBS (6×) and 20% glycerol in PBS (3× over 20 min). Then, cells in glycerol were frozen in liquid N_2, thawed, washed 6× in PBS, 3× in 10 mM Tris–HCl (pH 7.5), incubated (2 h; 37°C) with 200 µg/ml RNase A (Sigma) in 10 mM Tris–HCl (pH 7.5), washed in 10 mM Tris–HCl (pH 7.5), and then 4× in 2×SSC. Hybridizations were then carried out exactly as described by Kurz *et al.* (1996). After this procedure, the PTF domains and nucleoplasmic foci seemed unchanged. Digoxigenin-labelled probes bound to DNA were detected using mouse antibodies against digoxigenin, and then donkey anti-mouse IgG conjugated with FITC. Biotin-labelled probes were detected using avidin DN conjugated with FITC (10 µg/ml, in 1% BSA, 0.4% gelatine, 4× SSC, 0.1% Tween 20; pH 8.0; Vector Labs), then biotinylated goat anti-avidin (2.5 µg/ml in 1% BSA, 0.4% gelatine, 4×SSC, 0.1% Tween 20, pH 7.5; Vector Labs.), and finally avidin DN conjugated with FITC (as above). No signal was obtained from the chromosome paint if the denaturation step was omitted, or if samples were treated with DNase I (200 units/ml; 1 h; 37°C; Boehringer).

Confocal microscopy

Images were obtained using a Bio-Rad MRC1000 confocal laser scanning microscope (running under Comos 7.0a) equipped with an Argon/Krypton laser and coupled to a Nikon Diaphot 200 inverted microscope (60× PlanApo oil-immersion objective; numerical aperture 1.4). Kalman-filtered images (N=6–10) were collected with minimum iris aperture (0.7 mm) and the minimum laser power that filled the whole grey scale in the low scan/low signal mode. For multiple labelling, sequential series were obtained by alternating the different channels to minimize *z* axis changes during collection; then, no 'bleedthrough' was detected between channels. Images were printed from Adobe Photoshop 3.0 using a Tektronix Phaser 440 printer at 300 d.p.i. resolution.

Overlap between PTF and other markers (e.g. Br-RNA, the large subunit of RNA polymerase II) was determined as follows. The confocal microscope has a resolution of ~200 nm in the *x* and *y* axes, but only ~600 nm in the *z* axis; therefore, a marker (e.g. transcription site) might lie over or under a PTF domain (diameter ~1.3 µm) and so appear to overlap it. Sophisticated algorithms are available to improve *z* axis resolution, but they rely on assumptions (e.g. concerning thresholds) that are difficult to justify when analyzing structures with many different intensities. As the human eye and brain are so well adapted to analyzing differences in intensities in the grey scale (Russ, 1990), we used the following procedure. (i) Collect optical sections every 200 nm in the *z* axis; images were 'contrast-stretched' but not filtered in any way. (ii) Merge images, and analyze grey-scale images using Adobe Photoshop. (iii) Identify sections containing PTF domains, and draw limits around domains. (iv) Change to the other channel, and score whether foci are present; if found, check through the optical stack to identify the section that contains the peak intensity of that focus (determining the position of peak intensity greatly improves resolution). (v) Switch back to the first channel to check that it lies within the PTF domain limits. (vi) Repeat steps 3–5 for each section containing a PTF domain, and each marker focus in that domain. A similar procedure was used to detect overlap (association) between chromosome territories and PTF domains, using optical sections collected every 300 nm in the *z* axis. Sometimes it was difficult to define the limits of FISH signals (e.g. Figure 6E–G and L–N), so overlaps were scored as 'doubtful' (i.e. ±) in Table I; even so, three different individuals agreed in 80% cases.

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