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RNA polymerase II transcription is concentrated outside replication domains throughout S-phase

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SUMMARY

Transcription and replication are, like many other nuclear functions and components, concentrated in nuclear domains. Transcription domains and replication domains may play an important role in the coordination of gene expression and gene duplication in S-phase. We have investigated the spatial relationship between transcription and replication in S-phase nuclei after fluorescent labelling of nascent RNA and nascent DNA, using confocal immunofluorescence microscopy. Permeabilized human bladder carcinoma cells were labelled with 5-bromouridine 5'-triphosphate and digoxigenin-11-deoxyuridine 5'-triphosphate to visualize sites of RNA synthesis and DNA synthesis, respectively. Transcription by RNA polymerase II was localized in several hundreds of domains scattered throughout the nucleoplasm in all stages of S-phase. This distribution resembled that of nascent DNA in early S-phase. In contrast, replication patterns in late S-phase

consisted of fewer, larger replication domains. In double-labelling experiments we found that transcription domains did not colocalize with replication domains in late S-phase nuclei. This is in agreement with the notion that late replicating DNA is generally not actively transcribed. Also in early S-phase nuclei, transcription domains and replication domains did not colocalize. We conclude that nuclear domains exist, large enough to be resolved by light microscopy, that are characterized by a high activity of either transcription or replication, but never both at the same time. This probably means that as soon as the DNA in a nuclear domain is being replicated, transcription of that DNA essentially stops until replication in the entire domain is completed.

Key words: replication, transcription, RNA polymerase II, nucleus, S-phase, domain, localization, confocal microscopy, image analysis

INTRODUCTION

In each cell cycle the genome must be duplicated accurately during S-phase (reviewed by Laskey et al., 1989). It has become clear that this occurs in distinct replication domains in the nucleus (Nakamura et al., 1986; Mills et al., 1989; Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Cox and Laskey, 1991; Fox et al., 1991; Kill et al., 1991; Manders et al., 1992; Hassan and Cook, 1993). In the course of the S-phase different distributions of replication domains are observed, designated early (type I), middle (type II), and late (type III) (Nakamura et al., 1986; Nakayasu and Berezney, 1989). Recently, by carefully screening S-phase as many as five distinct replication patterns have been identified (O'Keefe et al., 1992). Replication patterns differ in number, size, and location of replication domains. In these domains several proteins that are involved in replication, like PCNA (Bravo and Macdonald-Bravo, 1987), DNA methyltransferase (Leonhardt et al., 1992), cyclin A (Cardoso et al., 1993; Sobczak-Thepot et al., 1993) and cdk2 (Cardoso et al., 1993), are concentrated. In early S-phase a replication domain probably corresponds to

one or more clusters of synchronously replicating adjacent replicons (see Edenberg and Huberman, 1975; Hand, 1978; Jackson, 1990). All DNA in a replication domain is duplicated in about one hour and thereafter remains spatially separated from DNA that is duplicated in the remainder of S-phase (Manders et al., 1992). These findings demonstrate that the S-phase nucleus is organized in distinct domains with respect to replication.

The timing of replication of a gene is related to its transcriptional activity. Generally, actively transcribed genes are replicated during early S-phase, whereas inactive genes are replicated in late S-phase (reviewed by Goldman, 1988). Transcription factors probably play an important role in replication (Herbomel, 1990; Wolffe, 1991; Heintz, 1992; DePamphilis, 1993) as well as in DNA repair (Bootsma and Hoeijmakers, 1993). Whether transcription causes genes to replicate early, or early replication is a prerequisite for transcriptionally competent chromatin, is not known. As an exception to the rule, some early replicated genes are not expressed. Also, the existence of late-replicating, transcriptionally active genes (e.g. see Taljanidisz et al., 1989; Benard et al., 1992) demon-

strates that the relationship between replication timing and transcriptional activity is complicated.

Recently, labelling nascent RNA with 5-bromouridine 5'-triphosphate (BrUTP) showed that transcription by RNA polymerase II (RPII) is, like replication, located in domains in the nucleus (Jackson et al., 1993; Wansink et al., 1993). Transcription domains, which may contain one or more actively transcribed genes, are found throughout the nucleoplasm and are not confined to the nuclear periphery or interior.

Clearly, compartmentalization is an important feature of nuclear architecture. Like replication and transcription, other nuclear functions and components are concentrated in domains (reviewed by van Driel et al., 1991; Spector, 1993). The dynamics of nuclear compartmentalization, and the functional and spatial relationship between different nuclear domains, particularly those involved in replication, transcription and nuclear RNA metabolism, are largely unknown (see e.g. Rosbash and Singer, 1993; Xing and Lawrence, 1993). Therefore, more knowledge of the relationship between nuclear domains is important for understanding the organization of the interphase nucleus.

In this study we investigated the spatial relationship between replication domains and transcription domains. We have visualized sites of replication and sites of transcription by incorporating simultaneously digoxigenin-11-dUTP (dig-dUTP) into nascent DNA and BrUTP into nascent RNA in permeabilized cells. Double-labelled nuclei were stained with two different fluorochromes and examined using confocal microscopy. The spatial relationship between replication and transcription patterns was analysed using image analysis techniques. We found that RNA synthesis occurs predominantly outside domains where replication takes place, irrespective of the particular stage of S-phase. This demonstrates that in S-phase light-microscopically resolvable nuclear domains exist that are characterized by a high activity of either replication or transcription, but they are not involved in both processes simultaneously. We conclude that during replication in a nuclear domain essentially all transcription in that domain is transiently interrupted until replication of the DNA in that particular domain has finished. This dynamic interplay between different activities in the same nuclear domain implies an important role for compartmentalization in the regulation of gene expression and gene duplication in the S-phase nucleus.

MATERIALS AND METHODS

Cell culture

T24 (human bladder carcinoma) cells were grown at 37°C under a 10% CO₂ atmosphere in DME (Gibco, Paisly, UK) supplemented with 10% (v/v) heat-inactivated FCS (Gibco), 2 mM L-glutamine (Gibco), 100 i.u./ml penicillin and 100 µg/ml streptomycin (Gibco).

BrUTP and dig-dUTP incorporation (run-on transcription and run-on replication)

The procedure for labelling nascent RNA and nascent DNA simultaneously *in vitro* is a modification of protocols described earlier (Nakayasu and Berezney, 1989; Wansink et al., 1993). We used dig-dUTP instead of biotin-dUTP to label nascent DNA, because biotin was used in a biotin-streptavidin enhancement step to detect incorporated BrUTP.

Cells were transferred to gelatin-coated glass coverslips and were

allowed to grow for 24–36 hours (less than 50% confluent). Subsequently, the coverslips were washed once with TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂) and once with glycerol buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 25% glycerol, 1 mM PMSF (Sigma Chemical Co., St Louis, MO), 0.5 mM EGTA). Then cells were permeabilized with glycerol buffer containing 0.05% Triton X-100 (Sigma Chemical Co.) and 10 units/ml RNasin ribonuclease inhibitor (Promega Co., Madison, WI) for 3–4 minutes at room temperature. The detergent-containing buffer was removed and synthesis buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.5 mM EGTA, 25% glycerol, 25 µM S-adenosyl-L-methionine (Boehringer Mannheim, Mannheim, Germany), 20 units/ml RNasin, and 1 mM PMSF) was added. In addition, for labelling of nascent RNA, synthesis buffer contained 0.5 mM of ATP, CTP, GTP and BrUTP (Sigma Chemical Co.). In case nascent DNA was labelled, the synthesis buffer was supplemented with 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 25 µM digoxigenin-11-dUTP (Boehringer), and 1.8 mM ATP. In double-labelling experiments all ribonucleotides and deoxyribonucleotides were present in the synthesis buffer in the concentrations mentioned above. RNA synthesis and DNA synthesis were performed at room temperature for 30 minutes. Then the coverslips were washed once with TBS containing 0.05% Triton X-100, 1 mM PMSF and 5 units/ml RNasin for 3 minutes, and once with TBS containing 1 mM PMSF and 5 units/ml RNasin for 3 minutes. Cells were fixed immediately afterwards (see below).

In control experiments α -amanitin (1 µg/ml, Sigma Chemical Co.), which blocks RPII activity (Roeder, 1976), or aphidicolin (20 µg/ml, Sigma Chemical Co.), which inhibits DNA polymerase activity (see Thömmes and Hübscher, 1990; Bambara and Jessee, 1991; and references therein), was included during run-on synthesis.

Fixation and immunocytochemistry

Cells were fixed in 2% (w/v) formaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) for 15 minutes at room temperature. The formaldehyde solution was freshly prepared from paraformaldehyde (Merck, Darmstadt, Germany) by depolymerization. Subsequently, the coverslips were incubated as follows: 2 × 5 minutes in PBS; 5 minutes in PBS containing 0.5% Triton X-100; 2 × 5 minutes in PBS; 10 minutes in PBS containing 100 mM glycine; 10 minutes in 10% BSA (Sigma Chemical Co.) in PBS; overnight at 4°C simultaneously with a rat mAb raised against BrdU (Sera-Lab, Crawley Down, UK) diluted 1:500 and a mouse mAb against digoxigenin (Boehringer) diluted 1:50 in PBG (PBS containing 0.5% (w/v) BSA and 0.05% (w/v) gelatin (from cold-water fish skin, Sigma Chemical Co.)); 4 × 5 minutes in PBG; 10 minutes in 25% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS; 1.5 hours simultaneously with biotin-conjugated donkey anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories) diluted 1:300, and FITC (DTAF)-conjugated donkey anti-mouse IgG(H+L) (Jackson ImmunoResearch Laboratories) diluted 1:200 in PBG; 4 × 5 minutes in PBG; 30 minutes with streptavidin-Texas Red conjugate (Amersham, 's-Hertogenbosch, The Netherlands) diluted 1:500 in PBG; 2 × 5 minutes in PBG; 2 × 5 minutes in PBS; 3 minutes in PBS containing 0.4 µg/ml Hoechst 33258 (Sigma Chemical Co.) to stain DNA; 5 minutes in PBS. Then coverslips were mounted in PBS (pH 8.0) containing 90% glycerol and 1 mg/ml *p*-phenylenediamine (Sigma Chemical Co.).

In a control experiment to obtain complete colocalization, nuclei labelled with dig-dUTP were incubated overnight with a mouse monoclonal antibody against digoxigenin, followed by 1.5 hours in a mixture of FITC(DTAF)-conjugated donkey anti-mouse IgG(H+L) and TRITC-conjugated donkey anti-mouse IgG(H+L) (Jackson ImmunoResearch Laboratories), diluted 1:200 and 1:300, respectively, in PBG. The samples were rinsed and mounted as described above.

Labelling nascent DNA with dig-dUTP does not require denaturation of double-stranded DNA before immunolabelling. Acid

treatment, often used to denature DNA, does not preserve BrUTP labelling of nascent RNA (D. G. Wansink et al., unpublished results).

Confocal scanning laser microscopy and image analysis

Three-dimensional images of doubly labelled nuclei were recorded by dual-channel confocal scanning laser microscopy (Carlsson, 1990). The Leica CSLM was equipped with an argon-krypton ion laser tuned at 488 nm and 568 nm to excite FITC and Texas Red simultaneously. A dual-band dichroic mirror reflected the laser beam to the object. The light emitted by the fluorochromes was first transmitted through this dual-band dichroic mirror and then separated by a 580 nm long-pass dichroic beam splitter. A 610 nm long-pass filter and a 530/40 nm band-pass filter in front of two detectors, respectively, were used to minimize optical crosstalk and to block scattered laser light. The fluorescence signals from both fluorochromes were recorded simultaneously during one scan. Data of digitized fluorescence signals were stored according to the Image Cytometry Standard (ICS) format as 8 bit memory arrays (Dean et al., 1990). The dimensions of a voxel were $s_x=s_y=0.061\ \mu\text{m}$ lateral and $s_z=0.195\ \mu\text{m}$ axial. The axial dimension was calculated by multiplying the displacement of the scanning table ($0.24\ \mu\text{m}$) with a correction factor. This factor is defined by the numerical aperture of the objective ($\text{NA}=1.3$) and the diffractive index of the embedding medium of the preparation ($n=1.443$) (Visser et al., 1992).

For image processing and analysis the software package SCIL-IMAGE (developed at the University of Amsterdam) was used on a Sun Sparc II workstation. This package has been extended with several 3-D routines developed by the Netherlands Project Team for Computer Science Research (SPIN). First, noise was reduced by applying a $3\times 3\times 1$ uniform filter on each colour component of an image. Subsequently, the 3-D images underwent three transformations to correct for: (i) optical shift between the two colour components caused by optical disalignment (Manders et al., unpublished data); (ii) background caused by 'dark current' of the photo multipliers; and (iii) optical crosstalk and histochemical crossreactivity (Carlsson and Mossberg, 1992). Subsequently, local background, partly caused by residual out-of-focus fluorescence and non-specific staining, was removed. Background was determined using a $51\times 51\times 7$ uniform filter (low pass) on the 3-D images. The resulting background images were subtracted from the original images; negative voxels of the new images were set to zero.

For all dual-colour images the intensities of both colours of all voxels of each nucleus were plotted in bivariate fluorescence intensity histograms. In such a histogram the number of voxels is represented as a function of their relative Texas Red and FITC fluorescence intensity visualized as contours with exponentially increasing values (1,2,4,8,...).

RESULTS

Recently, RNA synthesis in the nucleus was visualized by labelling nascent RNA in permeabilized cells (Wansink et al., 1993; Jackson et al., 1993). Earlier, Nakayasu and Berezney (1989) reported labelling of nascent DNA in vitro to study replication. These two techniques have enabled us to label nascent RNA and nascent DNA simultaneously in S-phase nuclei to investigate the spatial relationship between transcription domains and replication domains.

Replication and RPII transcription occur concentrated in nuclear domains

To visualize nascent RNA, human bladder carcinoma cells were grown on coverslips, permeabilized and labelled with 5-bromouridine 5'-triphosphate (BrUTP) during run-on transcription for 30 minutes (Wansink et al., 1993). Sites of BrUTP incorporation were visualized with a mAb against BrUTP followed by confocal fluorescence microscopy. A punctate labelling was observed, consisting of several hundreds of domains scattered throughout the nucleus, excluding the nucleolus (Fig. 1A shows an optical section). No staining was found when $1\ \mu\text{g/ml}$ α -amanitin was present during run-on transcription, indicating that BrUTP was incorporated into RNA newly synthesized by RPII (Wansink et al., 1993). No significant differences between BrUTP-labelled patterns in different stages of the interphase were observed. It is not yet known whether one domain reflects the activity of a single gene or the activity of a cluster of active genes. Interestingly,

Fig. 1. Transcription by RPII and replication are concentrated in nuclear domains. Three optical sections through the centre of nuclei of human bladder carcinoma cells labelled for transcription (A) or replication (B and C). Cells grown on coverslips were permeabilized and run-on synthesis was performed for 30 minutes in the presence of BrUTP (A) or digoxigenin-dUTP (B and C). Cells were fixed in 2% formaldehyde and sites of incorporation were detected by indirect immunofluorescence using monoclonal antibodies against BrUTP and digoxigenin, respectively. Labelled nuclei were examined by confocal laser scanning microscopy. Each panel shows one optical section of a processed image (see Materials and Methods). (A) A typical distribution of transcription domains in the interphase nucleus. (B) The distribution of replication domains in an early S-phase nucleus. (C) A replication pattern in a late S-phase nucleus. Bar, $5\ \mu\text{m}$.

the transcription pattern has a striking resemblance to early S-phase replication patterns.

To visualize nascent DNA permeabilized human bladder carcinoma cells were labelled with the dTTP analogue digoxigenin-dUTP (dig-dUTP). After run-on replication for 30 minutes cells were fixed and incorporated dig-dUTP was visualized using a mAb against digoxigenin followed by confocal immunofluorescence microscopy. About 40% of the nuclei were labelled, which agrees with an S-phase of 8-10 hours in these human bladder carcinoma cells (cell generation time is

20-24 hours). Several distinct replication patterns could be recognized and reflected successive periods in S-phase, as was described earlier (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Manders et al., 1992; O'Keefe et al., 1992). In this paper we will only distinguish between two types of replication patterns, early S-phase and late S-phase patterns. Early replication patterns consist of many, relatively small, replication domains dispersed throughout the nucleus (Fig. 1B, optical section). Replication patterns in late S-phase are characterized by fewer, relatively large, replication domains that

Fig. 2. Labelling transcription and replication simultaneously, in S-phase nuclei. Four optical sections through the centre of nuclei of human bladder carcinoma cells that have been doubly labelled for transcription and replication. Cells grown on coverslips were permeabilized and run-on transcription and run-on replication were performed in the presence of BrUTP and digoxigenin-dUTP for 30 minutes. Samples were prepared for fluorescence microscopy as described in Materials and Methods. Each panel shows one optical section of a processed image. Replication domains are shown in green. Transcription domains appear in red. (A and B) early S-phase nuclei. (C and D) late S-phase nuclei. Note that essentially no colocalization (i.e. complete overlap) between transcription domains and replication domains is observed. Bar, 2 μ m.

are located in the nuclear periphery and in the interior near the nucleolus (Fig. 1C, optical section). Labelling with dig-dUTP was sensitive to the DNA polymerase inhibitor aphidicolin (20 $\mu\text{g/ml}$), which indicates that the patterns represent newly synthesized DNA (data not shown). Summarizing, labelling nascent DNA with dig-dUTP revealed well-defined replication patterns in S-phase nuclei. The observed patterns are in agreement with results obtained by others, as observed *in vitro* or *in vivo* (e.g. see Nakamura et al., 1986; Nakayasu and Berezney, 1989; Manders et al., 1992; O'Keefe et al., 1992).

Simultaneously visualizing transcription and replication in S-phase nuclei

Transcription patterns appear very similar - in the number, size, and distribution of domains - to replication patterns in early S-phase (compare Fig. 1A and B). Here, a transcription (replication) domain is defined as a nuclear compartment that in the light microscope displays a relatively high transcriptional (replicational) activity. Usually, transcription and early S-phase replication domains have an apparent diameter less than 0.5 μm , whereas replication domains in late S-phase may be larger. To investigate the spatial relationship between transcription domains and replication domains throughout S-phase we have labelled nascent RNA and DNA simultaneously during run-on synthesis in permeabilized cells. Run-on transcription and run-on replication were carried out in permeabilized human bladder carcinoma cells in the presence of BrUTP and digoxigenin-dUTP. After detecting the incorporated nucleotide analogues with the appropriate antibodies, labelled nuclei were examined by confocal scanning laser microscopy. Confocal images of 23 randomly selected, doubly labelled nuclei were processed as described in Materials and Methods. Thirteen nuclei had the finely punctated replication patterns characteristic for early S-phase, whereas the other 10 nuclei had mid or late S-phase replication patterns. The latter are designated below late S-phase nuclei.

On visual inspection of doubly labelled, processed images it became clear that there was hardly any overlap between tran-

scription domains (red) and the relatively large replication domains (green) in late S-phase nuclei (Fig. 2C and D). Obviously, transcription domains did not colocalize with replication domains in late S-phase. The relationship between transcription and replication in early S-phase nuclei seemed more complex (Fig. 2A and B). Also in early S-phase transcription domains and replication domains did not colocalize. However, overlap was observed occasionally where transcription domains and replication domains seemed in contact, as indicated by yellow borderlines between transcription domains and replication domains. Some transcription domains appeared slightly orange instead of red, which is indicative of a low replicational activity in that domain. Similarly, a low transcriptional activity was observed in some replication domains.

In conclusion, transcription and replication can be labelled simultaneously in permeabilized cells. In late S-phase nuclei transcription domains did not colocalize with replication domains. This is expected, since most DNA that is replicated in late S-phase is not transcriptionally active, and consists predominantly of heterochromatin (Goldman, 1988; O'Keefe et al., 1992). In contrast, most actively transcribed genes are replicated in early S-phase. Remarkably, also in early S-phase little overlap was observed between transcription and replication on visual inspection of doubly labelled nuclei.

Transcription by RPII is concentrated outside replication domains throughout S-phase

To investigate the spatial relationship between transcription and replication in more detail the dual-colour images were analysed in bivariate histograms. The dual-colour image of each doubly labelled nucleus was split into two parts, a red component (transcription) and a green component (replication). Nuclei doubly labelled for replication were used as a control for complete colocalization. For each nucleus all voxels from the processed image were collected in a bivariate histogram with respect to their red and green intensity. In Fig. 3A we have depicted a histogram of the control nucleus, that was labelled red and green for replication. Fig. 3B and C show

Fig. 3. Bivariate histograms of doubly labelled nuclei. The dual-colour image of each nucleus was split into a red (TR) component and a green (FITC) component. All voxels of each nucleus were collected in a bivariate histogram. The histogram shows the frequency with which a voxel with a certain quantity of green and of red fluorescence occurs in the image. The axes show relative fluorescence intensities on a linear scale. Contour lines that indicate the frequency that a specific voxel occurs in the image are drawn at exponential intervals (1,2,4,8...) as is shown in A. Bivariate histograms belonging to: (A) a control nucleus, which was doubly labelled for replication with two fluorochromes (i.e. the red and green image fully colocalized); (B) an early S-phase nucleus; and (C) a late S-phase nucleus, which were both simultaneously labelled for replication (FITC) and transcription (TR). Note that in case of complete colocalization all voxels have almost equal intensities in red and green and are found spread around the diagonal (A).

histograms of an early S-phase nucleus and a late S-phase nucleus, respectively, that were labelled red (TR) for transcription and green (FITC) for replication. As expected for the control nucleus, in which both components fully colocalized, all voxels have almost equal intensities in red and green (Fig. 3A). In contrast, in Fig. 3B and C only few voxels are found around the diagonal; most voxels are found near the axes representing voxels with a relatively high intensity in either red or green, but not both. Similar histograms were found for all nuclei examined. Obviously, only few voxels in these nuclei were present that were highly active in transcription as well as in replication. The voxels observed in early S-phase nuclei that show little or no overlap must refer to sites in the nucleus where at least one of the signals is weak or absent. This analysis corroborates our conclusions, based on visual inspection of dual-colour images, that nuclear domains that are highly active in transcription do not colocalize with domains that are highly active in replication. This demonstrates that replication and transcription essentially do not occur simultaneously in the same nuclear domain in any stage of S-phase.

DISCUSSION

By labelling nascent RNA and nascent DNA simultaneously in permeabilized cells, we have been able to investigate the spatial relationship between transcription domains and replication domains in early and late S-phase. In these run-on experiments one most likely observes only the effects of RNA polymerase molecules and DNA polymerase molecules that are active at the time of cell permeabilization, because initiation of replication and transcription are unlikely under these *in vitro* conditions. This implies that only actively transcribed genes and actively replicated replicons at the time of permeabilization are visualized.

In both early and late S-phase nuclei punctate patterns of foci of replication and transcription activity, respectively, are observed. It has been argued before (Nakayasu and Berezney, 1989; Jackson, 1990; Hozák et al., 1993) that in each replication focus (here referred to as a replication domain) several replication forks are simultaneously active in adjacent replicons. Also, multiple RNA polymerases are probably active in each transcription focus, here referred to as a transcription domain. A transcription domain may contain several active genes.

We detected very little RNA synthesis in replication domains in late S-phase nuclei. This means that transcription and replication are spatially separated in late S-phase. This finding confirms earlier observations (Jackson et al., 1993). The absence of transcription in late-replicating chromatin is expected, since in late S-phase mostly inactive genes are replicated (Goldman, 1988). Furthermore, electron microscopic studies, correlating replication with nuclear ultrastructure, showed that in late S-phase predominantly heterochromatin, corresponding predominantly to transcriptionally inactive loci, is replicated (O'Keefe et al., 1992).

In early S-phase nuclei the spatial distribution of transcription domains relative to replication domains was more complex. The degree of overlap between the two activities was higher in early S-phase than in late S-phase. Nevertheless, we almost never observed colocalization on the level of individual

transcription and replication domains. By definition, two domains colocalize if one completely overlaps the other. Bivariate histograms of doubly labelled early S-phase nuclei show that there are only a few voxels that have a high activity in both replication and transcription (Fig. 3A). Moreover, the observed overlap was caused largely by voxels that had a low intensity in one or both of the two labels. The overlap between transcription and replication, analysed on the level of individual voxels as shown in Fig. 3, is an overestimate of the real overlap. This is caused by the broadening of the imaged structures due to the point spread function (van der Voort and Brakenhoff, 1990). Typically, the imaging process results in an increase in the lateral dimensions of about 0.1 μm . We conclude that transcription domains essentially do not colocalize with replication domains throughout S-phase and that there is also little overlap on the level of individual voxels.

Numerous reports have indicated that most actively transcribed genes are replicated in early S-phase (reviewed by Goldman, 1988). This agrees with the observation that most euchromatin is replicated in early S-phase (O'Keefe et al., 1992). Remarkably, we find that most transcription domains do not colocalize with replication domains in early S-phase nuclei. Three explanations will be considered for the nearly complete absence of colocalization of transcription domains and replication domains in early S-phase nuclei of human bladder carcinoma cells. The first, most straightforward, explanation is to assume a transient interruption of transcription in a domain, until all DNA in that domain has been duplicated.

The second possibility takes into account that at any moment in early S-phase only a subset of early S-phase replication domains is active. This is explained as follows. Since about 40% of the cells are labelled with digoxigenin-dUTP and the cell generation time is 20–24 hours, the S-phase lasts 8–10 hours. We estimated that approximately 60% of the labelled cells have a typical early S-phase replication pattern. This implies that early S-phase takes about 6 hours. Evidence has been presented that the replication time of a replication domain is about 1 hour (Nakamura et al., 1986; Manders et al., 1992). Hence, the subset of active replication domains in a particular cell at a certain moment comprises 1/6 of the total population of early S-phase replication domains. From this it can be estimated that, if replication and transcription occur simultaneously in the same domain, on average about 17% (i.e. 1/6) of the transcription domains in each early S-phase nucleus will colocalize with a replication domain, assuming that all active genes are replicated in the first 6 hours of S-phase. This is in contrast with what we observe, i.e. transcription domains are largely excluded from replication domains. It could be argued that the replication time of a domain with a high transcription activity is much shorter than 1 hour. This would result in a proportionally lower degree of colocalization. Since there is no evidence for such short-living replication domains, we consider this second possibility unlikely.

The third explanation takes into account the possibility that all active genes that are detectable by our fluorescent labelling technique (i.e. possibly the most highly active subset of all genes) are replicated in the same, very short, period in early S-phase without interruption of transcription. This would result in a high degree of colocalization of transcription and replication domains in a small subset of the population of early S-phase cells. The possibility exists that we have missed this

subset. Apart from the fact that the chance that this might have occurred is small, we consider this third explanation highly unlikely, since many highly active genes are known that are replicated in either the first or the second quarter of S-phase (Taljanidisz et al., 1989).

In conclusion, the most likely model to explain the absence of colocalization between transcription domains and replication domains is that transcription in a domain is transiently interrupted until the DNA in that domain has been replicated. This is in agreement with results obtained with electron microscopy of spread chromatin of the heavily transcribed rRNA genes of yeast in the process of replication (Saffer and Miller, 1986). Here, the tightly packed transcription complexes are removed in front of a replication fork, whereas there is hardly new initiation of transcription in a replication bubble.

We and others find that replication and transcription are concentrated in nuclear domains. Obviously, some higher-order structure of the nucleus must exist to achieve this. These domains may correspond to (clusters of) DNA loops attached to the nuclear matrix (Razin, 1987; Goldman, 1988). The interaction of DNA in transcription and replication domains with the nuclear matrix is mediated, among others, via transcription complexes and the replication machinery (Ciejek et al., 1983; Nakayasu and Berezney, 1989; Jackson et al., 1993; Wansink et al., 1993; Hozák et al., 1993).

In conclusion, our results strongly suggest the existence of nuclear domains, resolvable by light microscopy, that are either highly active in transcription or replication, but not in both at the same time. Much needs to be learned about the higher-order chromatin structure of these domains and their dynamics during the cell cycle.

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