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SPECIAL ARTICLE

The Localization of Sites Containing Nascent RNA and Splicing Factors

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Sites of transcription in the HeLa nucleus are not diffusely spread throughout euchromatin but are concentrated in ~2000 discrete sites or "transcription factories." These sites can be immunolabeled after allowing permeabilized cells to elongate nascent RNA chains in the presence of BrUTP. Splicing factors are also concentrated in a few tens of nuclear domains known as "speckles"; they are also more diffusely spread throughout euchromatin. As there has been some controversy whether the speckles are sites of transcription, we investigated the relative distributions of transcription factories and the speckles (detected using Sm autoimmune antibodies). We used conditions that minimize the redistribution of both the nascent RNA and the antigens during permeabilization and fixation and imaged the sites with high resolution using cryosections and a "confocal" microscope. The speckles contained little—if any—nascent RNA and so were usually not transcriptionally active, but they were often surrounded by a few transcription sites. Whether they are active sites of processing of RNA or merely sites where the machinery is stored remains to be established.

SITES OF TRANSCRIPTION

The distribution of nascent transcripts has been studied by autoradiography after incubating living cells in [^{3}H]uridine for several minutes; label was initially found in perichromatin fibrils and subsequently within interchromatin granule clusters (for review see [1]). However, as transcripts grow at ~1.5 kb/min, many transcripts are completed during the incubation period and they may move away from the transcription sites to accumulate at later bottlenecks in the pathway to the cytoplasm. Therefore incorporation times of a few seconds are needed *in vivo* to ensure that only sites of transcription become labeled. Recently, nascent transcripts have been localized after permeabilizing cells, washing away the endogenous pools of triphosphates, and then incubating the cells in BrUTP and the three other nucleotide triphosphates; nascent RNA chains then grow by only a few tens of bp/min, which increases the chances that the incorporated Br marks the synthetic site and not more distant processing sites [2, 3]. Fortuitously, it seems that *in vitro* Br-RNA does not travel down the processing pathway, so that the modified transcripts do indeed remain at—or close to—the synthetic sites [4].

Approximately 2000 discrete sites of transcription with average diameters of ~70 nm were identified using this approach [4]. If each HeLa nucleus contains ~40,000 active polymerases, then each transcription site would contain ~20 active polymerases. These sites were christened "factories" as so many active polymerases are concentrated in each one. As there is good evidence that some splicing occurs cotranscriptionally [5–9], these factories are also likely to be sites where RNA is processed.

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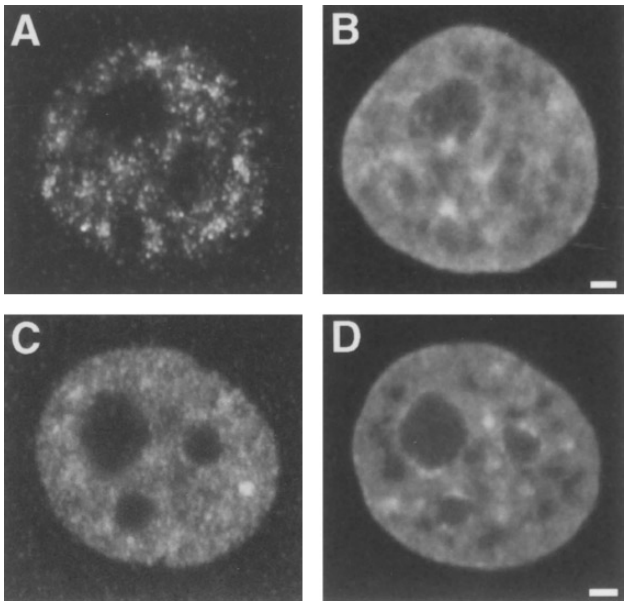

FIG. 1. Distributions of SC35 and Sm antigen imaged in optical sections of unpermeabilized HeLa cells. HeLa cells on coverslips were fixed (4% paraformaldehyde in 250 m*M* Hepes, pH 7.4, 10 min, 4°C, then with 8% paraformaldehyde in 250 m*M* Hepes, pH 7.4, 50 min, 20°C), permeabilized (0.5% Triton X-100 in PBS), and indirectly immunolabeled (first antibodies were the mouse monoclonal antibody SC35 or human Sm autoimmune antibodies, second antibodies were raised in donkey anti-mouse IgG conjugated with indocarbocyanine or anti-human IgG conjugated with fluorescein isothiocyanate, respectively; nucleic acids were stained with TOTO-3), and images were obtained sequentially using a Bio-Rad 1000 confocal microscope (63X oil-immersion objective with NA 1.4; iris aperture 0.7 mm, minimum laser power giving occupancy of the whole gray scale in the low scan/low signal mode; no bleedthrough was detected). Bars, 2 μm. (A, B) Distributions of SC35 antigen (A) and nucleic acids (B) in the same optical section of a nucleus. (C, D) Distributions of Sm antigens (C) and nucleic acids (D) in the same optical section of a nucleus.

SITES OF SPLICING

Splicing factors are concentrated in a few tens of nuclear domains known as "speckles"; they are also more diffusely spread throughout euchromatin (for review see [10]). For example, a non-snRNP splicing factor—SC35 [11]—is concentrated in fine dots in interchromatin spaces, which stain poorly with TOTO-3; clusters of these dots give rise to bright speckles (Figs. 1A and 1B). These bright speckles contain poly(A)$^+$ RNA and correspond to the interchromatin granule clusters seen by electron microscopy [12, 13]. Other splicing factors recognized by the Sm autoimmune sera are more diffusely spread, but concentrations also appear as speckles (Figs. 1C and 1D).

The function of both the diffusely spread sites and the brighter speckles is a matter of debate; the speckles could be sites of both transcription and splicing, or they might be sites without any transcriptional activity where completed transcripts accumulate and are processed.

DO SPLICING FACTORS COLOCALIZE WITH NASCENT TRANSCRIPTS?

Therefore we investigated the relative distributions of transcription factories (labeled with BrUTP) and concentrations of Sm antigen. We took special care to use conditions that minimize the redistribution of both the nascent RNA and the antigens during permeabilization and fixation, and we also imaged the sites in optical sections of whole cells using a "confocal" microscope. Nascent RNA did not not colocalize with the speckles (Figs. 2A–2D).

Unfortunately, the confocal microscope cannot resolve below ~200 nm in the *x* and *y* axes or ~700 nm in the *z* axis. This poses severe problems when imaging small structures like transcription sites and speckles. Consider an optical section with a thickness of 700 nm taken through a HeLa nucleus (diameter ~9 μm) containing ~2000 transcription sites (diameter ~70 nm). It contains ~270 sites, each with an apparent diameter of ~400 nm, and the 270 sites appear to occupy half the area of the two-dimensional image. Therefore, in an experiment like that illustrated in Figs. 2A–2D it is difficult to assess the true degree of colocalization, as one kind of site may lie underneath the other, without any true colocalization. Therefore, we prepared cryosections of ~150 nm and imaged them in the confocal microscope. Although sites still appear aberrantly large, there is less overlap between sites. Background levels of fluorescence are also considerably reduced, as there is no out-of-focus "flare" from sites above and below the optical section. Now both kinds of sites are imaged with excellent resolution (Figs. 2E–2H). The transcription sites do not usually colocalize with the bright speckles, but they often surround them. However, they do overlap the fainter foci distributed more diffusely throughout the nucleus. This result is consistent with both results obtained initially by autoradiography and—more recently—results using high-resolution immunoelectron microscopy and an approach analogous to that used here [1, 4]. Therefore most speckles are not directly involved in transcription. Whether they are active sites of processing of RNA or merely sites where the machinery is stored remains to be established (for review see [14]).

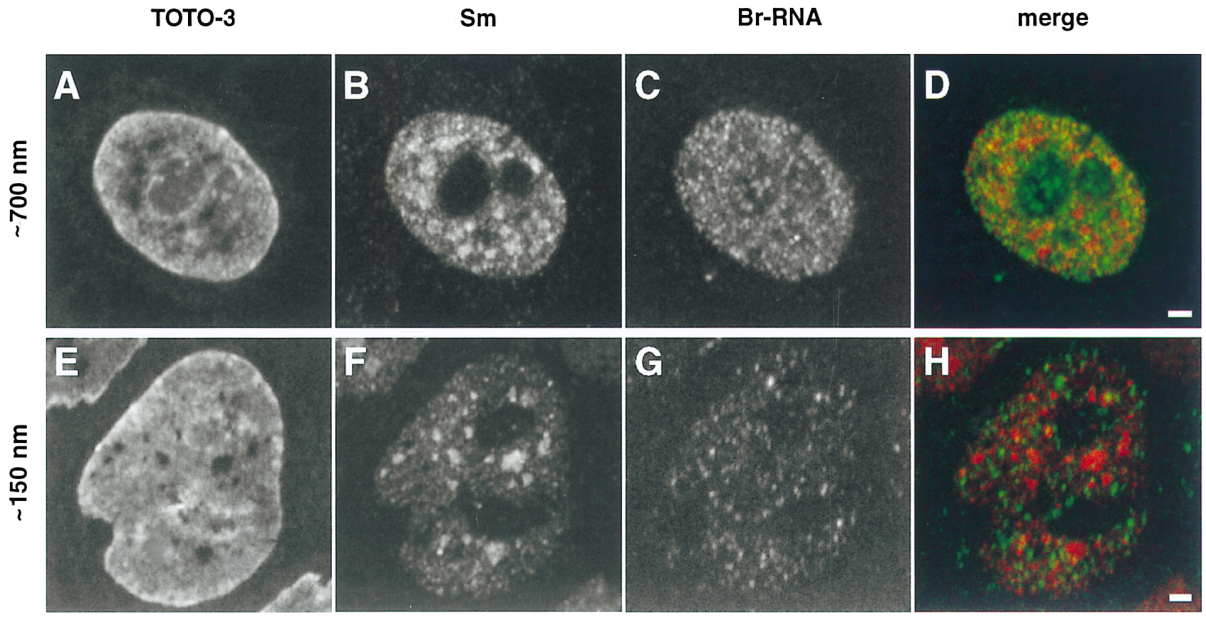

FIG. 2. Distribution of Sm antigens and nascent RNA imaged in an optical section (A–D) or a cryosection (E–H) of permeabilized HeLa cells. HeLa cells were permeabilized with saponin in a "physiological buffer," incubated with BrUTP for 15 min ([2, 4] and A. Pombo and P. R. Cook, unpublished), and fixed. (A–D) Cells were repermeabilized (as described in the legend to Fig. 1), Sm antigens and Br-RNA were indirectly immunolabeled (first antibodies were human Sm autoimmune antibodies and mouse anti-Br-DNA antibodies; second antibodies were donkey anti-human IgG conjugated with lissamine rhodamine sulfonyl chloride and donkey anti-mouse IgG conjugated with fluorescein isothiocyanate; nucleic acids were stained with TOTO-3), and images were collected as described in the legend to Fig. 1. (E–H) Cells were embedded in sucrose and frozen in liquid nitrogen, and cryosections (~150 nm thick) were prepared; next the sections were treated with Triton X-100, stained, and then imaged as above (Pombo *et al.*, in preparation). Bars, 2 μm. Separate views of the nucleic acids (TOTO; A, E), Sm antigens (Sm; B, F), and nascent RNA (Br-RNA; C, G) in one cell or cryosection. The colored images (merge; D, H) were made by merging the Sm image (red) with the Br-RNA image (green); sites containing Sm antigens and Br-RNA appear yellow.

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REFERENCES

1. Fakan, S., and Puvion, E. (1980) *Int. Rev. Cytol.* **65,** 255–99.
2. Jackson, D. A., Hassan, A. B., Errington, R. J., and Cook, P. R. (1993) *EMBO J.* **12,** 1059–1065.
3. Wansink, D. G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993) *J. Cell Biol.* **122,** 283–293.
4. Iborra, F. J., Pombo, A., Jackson, D. A., and Cook, P. R. (1996) *J. Cell Sci.* **109,** 1427–1436.
5. Osheim, Y. N., Miller, O. L., and Beyer, A. L. (1985) *Cell* **43,** 143–151.
6. Le Maire, M. F., and Thummel, C. S. (1990) *Mol. Cell. Biol.* **10,** 6059–6063.
7. Baurén, G., and Wieslander, L. (1994) *Cell* **76,** 183–192.
8. Wuarin, J., and Schibler, U. (1994) *Mol. Cell. Biol.* **14,** 7219–7225.
9. Tennyson, C. N., Klamut, H. J., and Worton, R. G. (1995) *Nature Genet.* **9,** 184–190.
10. Carmo-Fonseca, M., and Ferreira, J. (1995) *in* Pre-mRNA Processing (Lamond, A. I., Ed.), pp. 163–172, Molecular Biology Intelligence Unit, Springer-Verlag, Heidelberg.
11. Fu, X.-D., and Maniatis, T. (1990) *Nature* **343,** 437–441.
12. Carter, K. C., Taneja, K. L., and Lawrence, J. B. (1991) *J. Cell Biol.* **115,** 1191–1202.
13. Spector, D. L., O'Keefe, R. T., and Jiménez-Garcia, L. F. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 799–805.
14. Spector, D. L. (1993) *Annu. Rev. Cell Biol.* **9,** 265–315.

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