intron, as well as HeLa cell culture, microinjection, SC35 immunolabelling and microscopy were performed according to Melčák et al. (2001).

In order to determine the effect of energy depletion on RNA localization, an ATP depletion by microinjected apyrase was performed. In order to reach this aim, consecutive microinjections were performed. Cells were microinjected with 500 U/ml of apyrase in 5 mM Tris-acetate, pH 6.95, and incubated for 5 min at 37°C. The same cells were then microinjected with RNA, washed with fresh prewarmed medium and incubated for 15 min at 37°C. The control cells were, instead of apyrase, microinjected with buffer only.

Four series of experiments were perfomed and 30 to 40 cells were microinjected in each experiment. However, not all microinjected cells could be subsequently investigated because a few cells either detached from the support or were disrupted. Also for this reason the described fluorescence pattern after apyrase microinjection (Fig. 1) was observed in 75, 85, 89 and 90% of microinjected cells.

Results and Discussion

The control experiment was found to be compatible with the findings in previous studies (Wang et al., 1991; Melčák et al., 2001) and documented the rapid movement of microinjected splicing-competent Ad1 pre-mRNA into the speckles. The RNA was localized within the speckles, which were depicted by SC35 labelling (Fig. 1A).

A striking change in the RNA distribution was seen when the RNA microinjection was preceded by apyrase microinjection. RNA was also targeted towards the speckles, but remained accumulated outside this nuclear compartment. It formed dots of accumulated RNA adjacent to the speckles (Fig. 1B). Moreover, the speckles rounded up. As the ATP depletion necessarily led to transcription inhibition, the rounding up of the speckles was in agreement with previous results (e.g. O'Keefe et al., 1994; Melčák et al., 2000) documenting that the normal pattern of speckles is converted into round speckles in transcriptionally inactive cells.

The fluorescence pattern testified to the existence of an ATP-dependent step, which allowed for RNA accumulation within the speckles. Importantly, the fluorescence pattern was compatible with the experiment in which microinjected HeLa cells with Ad1 RNA were kept at 4°C, and strikingly resembled the fluorescence pattern observed with several microinjected mutant RNAs (Melčák et al., 2001). The mutant RNAs allow for the generation of pre-spliceosomal complexes, but only of such complexes which do not require ATP for their formation in *in vitro* experiments (Seraphin and Rosbach, 1989; Bennett et al., 1992, Michaud and Reed, 1993; Query et al., 1997; see Melčák et al. (2001) for detailed discussion).

In our previous study (Melčák et al., 2001), it was suggested that the targeting of microinjected splicing-competent RNA into speckles consisted of two interdependent steps, namely the movement of RNA towards the speckles, probably as a diffusion process, followed by the energy-dependent translocation of RNA into the speckles. The result of the performed ATP depletion experiment, which resulted, on the one hand, in the blocking of accumulation of the splicing-competent RNAs in the speckles, but, on the other, in their accumulation in the fluorescent dots adjacent to the speckles, demonstrates the existence of these two targeting steps, and supports the concept that nuclear speckles are involved in splicing.

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References

Baurén, G., Wieslander, L. (1994) Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. *Cell* **76**, 183-192.

Bennett, M., Michaud, S., Kingston, J., Reed, R. (1992) Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes Dev.* **6**, 1986-2000.

Beyer, A. L., Osheim, Y. N. (1988) Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.* **2**, 754-765.

Corden, J. L., Patturajan, M. (1997) A CTD function linking transcription to splicing. *Trends Biochem. Sci.* **22**, 413-416.

Custodio, N., Carmo-Fonseca, M., Geraghty, F., Pereira, H., Grosveld, F., Antoniou, M. (1999) Inefficient processing impairs release of RNA from the site of transcription. *EMBO J.* **18**, 2855-2866.

Dirks, R. W., de Pauw, E. S. D., 97a Splicing factors associate with nuclear HCMV-IE trancripts after trancriptional activation of the gene, but dissociate upon transcription inhibition: evidence for a dynamic organization of splicing factors. *J. Cell Sci.* **110**, 505-513.

Fakan, S. (1994) Perichromatin fibrils are in situ forms of nascent transcripts. *Trends Cell. Biol.* **4**, 86-90.

Fu, X. D., Maniatis, T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature* **343**, 437-441.

Graessmann, M., Graessmann, A. (1982) Simian virus 40 cRNA is processed into functional mRNA in microinjected monkey cells. *EMBO J.* **1**, 1081-1088.

Huang, S., Spector, D. L. (1996) Intron-dependent recruitment of pre-mRNA splicing factors to sites of transcription. *J. Cell Biol.* **133**, 719-732.

Ishov, A. M., Stenberg, R. M., Maul, G. G. (1997) Human cytomegalovirus immediate early interaction with host nuclear structures: definition of an immediate transcript environment. *J. Cell Biol.* **138**, 5-16.

Johnson, C., Primorac, D., McKinstry, M., McNeil, J., Rowe, D., Lawrence, J. B. (2000) Tracking COL1A1 RNA in osteogenesis imperfecta: splice-defective transcripts initiate transport from the gene but are retained within the SC35 domain. *J. Cell Biol.* **150**, 417-431.

Jolly, C., Vourc'h, C., Robert-Nicoud, M., Morimoto, R. I. (1999). Intron-dependent association of splicing factors with active genes. *J. Cell Biol.* **145**, 1133-1143

Krämer, A. (1996) The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.* **65**, 367-409.

Lamm, G. M., Lamond, A. I. (1993) Non-snRNP protein splicing factors. *Biochim. Biophys. Acta* **1173**, 247-265.

McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., Bentley, D. L. (1997) The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. *Nature* **385**, 357-361.

Melčák, I., Raška, I. (1996) Structural organization of the pre-mRNA splicing commitment. A hypothesis. *J. Struct. Biol.* **117**, 189-194.

Melčák, I., Cermanová, Š., Jirsová, K., Koberna, K., Malínský, J., Raška, I. (2000) Nuclear pre-mRNA compartmentalization: trafficking of released transcripts to splicing factor reservoirs. *Mol. Biol. Cell* **11**, 497-510.

Melčák, I., Melčáková, Š., Kopský, V., Večeřová, J., Raška, I. (2001) Prespliceosomal assembly on microinjected precursor mRNA takes place in nuclear speckles. *Mol. Biol. Cell* **12**, 393-406.

Michaud, S., Reed, R. (1993) A functional association between the 5' and 3' splice site is established in the earliest prespliceosome complex (E) in mammals. *Genes Dev.* **7**, 1008-1020.

Minvielle-Sebastia, L., Keller, W. (1999) mRNA polyadenylation and its coupling to other RNA processing reactions and to trancription. *Curr. Opin. Cell Biol.* **11**, 352-357.

Misteli, T. (2000) Cell biology of transcription and pre-mRNA splicing: nuclear architecture meets nuclear function. *J. Cell Sci.* **113**, 1841-1849.

Misteli, T., Spector, D. L. (1998) RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. *Mol. Cell* **3**, 697-705.

Misteli, T., Cáceres, J. F., Spector, D. L. (1997) The dynamics of a pre-mRNA splicing factor in living cells. *Nature* **387**, 523-527.

Moore, M. J., Query, C. C., Sharp, P. A. (1993) Splicing of precursors to messenger RNAs by the spliceosome. In: *The RNA World,* eds. Gesteland, R. F., Atkins, J. F., pp. 303-357, CSHL Press, Cold Spring Harbor.

Neugebauer, K. M., Roth, M. B. (1997) Distribution of pre-mRNA splicing factors at sites of RNA polymerase II transcription. *Genes Dev.* **11**, 1148-1159.

Newman, A. J. (1994) Small nuclear RNAs and pre-mRNA splicing. *Curr. Opin. Cell Biol.* **6**, 360-367.

O'Keefe, R. T., Mayeda, A., Sadowski, C. L., Krainer, A. R., Spector, R. L. (1994) Disruption of pre-mRNA splicing in vivo results in reorganization of splicing factors. *J. Cell. Biol.* **124**, 249-260.

Query, C. C., McCaw, P. S., Sharp, P. A. (1997) A minimal spliceosomal complex A recognizes the branch site and polypyrimidine tract. *Mol. Cell. Biol.* **17**, 2944-2953.

Seraphin, B., Rosbash, M. (1989) Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell* **59**, 349-358.

Smith, K. P., Moen, P. T., Wydner, K. L., Coleman, J. R., Lawrence, J. B. (1999) Processing of endogenous pre-mRNAs in association with SC-35 domains is gene specific. *J. Cell Biol.* **144**, 617-629.

Snaar, S. P., Vincent, M., Dirks, R. W. (1999) RNA polymerase II localizes at sites of human cytomegalovirus immediate-early RNA synthesis and processing. *J. Histochem. Cytochem.* **47**, 245-254.

Spector, D. L. (1990) Higher order nuclear organization: three-dimensional distribution of small nuclear ribonucleoprotein particles. *Proc. Natl. Acad. Sci. USA* **87**, 147-151.

Steinmetz, E. J. (1997) Pre-mRNA processing and the CTD of RNA polymerase II: the tail that wags the dog? *Cell* **89**, 491-494.

Steitz, J. A., Black, D. A., Gerke, V., Parker, K. A., Krämer, A., Frendewey, D., Keller, W. (1988) Functions of the abundant U-snRNPs. In: *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles,* ed. Birnstiel, M. L., pp. 71-79, Springer-Verlag, Berlin.

Wang, J., Cao, L. G., Wang, Y. L., Pederson, T. (1991) Localization of pre-messenger RNA at discrete nuclear sites. *Proc. Natl. Acad. Sci. USA* **88**, 7391-7395.

Wuarin, J., Schibler, U. (1994) Physical isolation of nascent RNA chains transcribed by RNA polymerase II: evidence for cotranscriptional splicing. *Mol. Cell. Biol.* **14**, 7219-7225.

Xing, Y., Johnson, C. V., Dobner, P., Lawrence J. B. (1993) Higher level organization of individual gene transcription and RNA splicing: integration of nuclear structure and function. *Science* **259**, 1326-1330.

Xing, Y., Johnson, C. V., Moen, P. T. Jr., McNeil, J. A., Lawrence, J. B. (1995) Nonrandom gene organization: structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. *J. Cell Biol.* **131**, 1635-1647.

Zachar, Z., Kramer, J., Mims, I. P., Bingham, P. M. (1993) Evidence for channeled diffusion of pre-mRNAs during nuclear RNA transport in metazoans. *J. Cell Biol.* **121**, 729-742.

Zhang, G., Taneja, K. L., Singer, R. H., Green, M. R. (1994) Localization of pre-mRNA splicing in mammalian nuclei. *Nature* **372**, 809-812.