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ARTICLES

Reorganization of Nuclear Factors During Myeloid Differentiation

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Abstract Differentiation in several stem cell systems is associated with major morphological changes in global nuclear shape. We studied the fate of inner-nuclear structures, splicing factor-rich foci and Cajal (coiled) bodies in differentiating hemopoietic, testis and skin tissues. Using antibodies to the splicing factors PSF, $U2AF^{65}$ and snRNPs we find that these proteins localize in foci throughout the nuclei of immature bone marrow cells. Yet, when granulocytic cells differentiate and their nuclei condense and become segmented, the staining localizes in a unique compact and thread-like structure. The splicing factor-rich foci concentrate in the interior of these nuclei while the nuclear periphery and areas of highly compact chromatin remain devoid of these molecules. Differentiated myeloid cells do not stain for p80 coilin, the marker for Cajal bodies. Immature myeloid cells contain Cajal bodies although these usually do not coloclaize with PSF-rich foci. Following complete inhibition of transcription in myeloid cells, the threaded PSF pattern becomes localized in several foci in the different lobes of mature granulocytes while in human HL-60 immature myeloid leukemia cells PSF is found in the perinucleolar compartment. Studies of other differentiating stem cell systems show that PSF staining disappears completely in differentiated, transcriptionally inactive sperm cells, is scarce as cells migrate from the inner skin layers outward and is lost as cells of the hair follicle mature. We conclude that the formation and distribution of splicing factor-rich foci in the nucleus during differentiation of various cell lineages is dependent on the levels of chromatin condensation and the differentiation status of the cell. J. Cell. Biochem. 81:379–392, 2001.

Key words: splicing factors; nuclear proteins; PSF; hemopoiesis; spermatogenesis; differentiation; transcription

Structure/function relationships in the organization of regulatory molecules within the cell nucleus received much attention in recent years. The nucleus is now viewed as a highly organized and compartmentalized organelle, consisting of functional specific domains; hetero- and euchromatin are intertwined with replication/transcription/splicing domains, nucleoli and various speckle-like foci. All these structures are enveloped by nuclear lamins and a nuclear membrane containing a specific trafficking mechanism via nuclear pores [for reviews, see Spector, 1997; Berezney and Wei, 1998; Lamond and Earnshaw, 1998; Schul et al., 1998; Matera, 1999; Park and De Boni, 1999]. These domains are dynamic and thus RNAs and proteins are constantly in motion, shuttling between the different nucleoplasmic regions.

Domains of replication and transcription within the nucleus are well characterized [Wei et al., 1998], yet, pin pointing areas of pre-mRNA splicing remains somewhat controversial [Smith et al., 1999; Wei et al., 1999]. It is agreed upon, that the interchromatin granules (IGs) are multi-protein complexes consisting of more than 70 proteins, predominantly pre-mRNA processing factors [Mintz et al., 1999]. The IGs are the most prominent structures, besides the nucleolus, observed in the mammalian cell nucleus and appear interconnected during interphase suggesting a dynamic structure, as recently observed in vivo, using GFP-tagged proteins [Misteli et al., 1997]. Labeling of nuclei with antibodies to splicing factors produces a speckled pattern of IGs and diffuse labeling of perichromatin fibrils [Carmo-Fonseca et al., 1991b; Spector et al., 1991]. Perichromatin fibrils are believed to contain, besides other RNA molecules, also pre-mRNA [Fakan and Puvion, 1980] and are considered sites, around and in-between IG clusters, in which splicing takes place either co-transcriptonally,

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post-transcriptionally or both [de Jong et al., 1996; Cmarko et al., 1999]. The clusters of IGs have been suggested to play a role in the regeneration and storage of splicing factors [Jimenez-Garcia and Spector, 1993] while some speckles act in splicing factor recruitment to active sites of transcription [Huang and Spector, 1996; Zeng et al., 1997]. On the other hand, it has been shown that nascent mRNA transcripts and transcription sites are closely associated with all speckles and it has been thus proposed that speckles can act coordinately in both transcription and splicing [Clemson and Lawrence, 1996; Wei et al., 1999]. Recently, a dual mechanism has been proposed in which, on the one hand, splicing factors are recruited to transcription sites, while on the other certain released transcripts associate with splicing factor reserviors [Melcak et al., 2000].

In addition to the intranuclear dynamics of the various nuclear factors, the nucleus as a whole has a certain degree of plasticity. Nuclear morphogenesis is observed especially in differentiating systems and depends on the cell type and state of differentiation [for review, see Georgatos, 1994]. One such system that produces a variety of nuclear structures is the hemopoietic tissue. The bone marrow contains pluripotent stem cells giving rise to all the different cells of the blood system. The most pronounced case of nuclear restructuring occurs during myeloid differentiation as the round-shaped nucleus ends up segmented and condensed in mature polymorphonuclear granulocytes. Other cases are lobulation of giant nuclei, as seen in megakaryocytes, nuclear condensation in differentiating lymphocytes, and nuclear condensation during terminal erythroid differentiation ending in picnosis and enucliation. The distribution of interchromatin granules and pre-mRNA processing factors in the process of differentiation such as in spermatogenesis [Biggiogera et al., 1993; Richler et al., 1994; Kamma et al., 1995, 1999; Elliott et al., 1998] and in the neuronal system [Sahlas et al., 1993; Santama et al., 1996; Ulfig and Briese, 1999] indicate major stage-specific differences in the distribution of these factors. An in vitro study on the organization of snRNPs in the Friend erythroleukemia cells points to differentiation-related aggregation of these splicing factors [Antoniou et al., 1993].

The detailed organization of splicing factors during in vivo differentiation of the hemopoietic stem cell system has not been studied thus far. In this study, we analyzed nuclear organization in the hemopoietic and additional stem cell based differentiation systems. We describe here a unique organization of splicing factor-rich foci in differentiated hemopoietic cells and propose that the dramatic changes that occur in the organization of several nuclear regulatory proteins during nuclear restructuring correlate with stage of differentiation and transcriptional status of the cells.

MATERIALS AND METHODS

Organs and Cell Lines

Bone marrow cells were obtained from 6-week-old male or female BALB/c mice (Harlan-Olac, Weizmann Institute, Israel) and dissociated to single cell suspensions in cold phosphate buffered saline (PBS). HL-60 and HeLa cell lines were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Antibodies

Secondary antibodies used for immunoflourescence were donkey anti-mouse-FITC, goat anti-rabbit-PE (Jackson, West Grove, PA). Anti-Sm (clone Y12) and anti-p80 coilin (clone 204.5) were provided by Dr. A.I. Lamond (University of Dundee, Dundee, UK), anti-hnRNP A1 (clone 9H10) by Dr. G. Dreyfuss (University of Pennsylvania School of Medicine, Philadelphia, PA), anti-U2AF^{65} (clone MC3) by Dr. M. Carmo-Fonseca (University of Lisbon, Lisbon, Portugal) and anti-Gr-1 rat monoclonal antibody was provided by Dr. Alpha Peled (Weizmann Institute). Anti-PSF B92 monoclonal antibody (MAb) was prepared as previously described [Lee et al., 1996]. Polyclonal anti-PSF 1121 antibody was prepared as previously described [Shav-Tal et al., 2000].

Mouse Tissue Preparation

Tissues were removed from BALB/c mice and frozen in isopentane on liquid nitrogen. Serial cryosections were cut at 5 μm, fixed with 4% paraformaldehyde (PFA) in PBS for 10 min and then with 0.5% Triton X-100 in PFA for 3 min. After blocking with 5% BSA and 1% normal sheep serum cells were stained as below.

Immnuofluorescence

For BM, HL-60 and HeLa cells, fixation conditions included 2 min in 4% PFA with 0.5% Triton

X-100 and an additional 20 min in 4% PFA, and blocking in 5% BSA. Tissues and cells were stained with the indicated antibody for 45 min, washed twice and then with the appropriate fluorophore antibody for 45 min and counter stained with Hoechst. Immunofluorescence was determined in a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using × 40 or × 100 objectives and a 35-mm Zeiss camera.

Transcriptional Inhibition and Nuclease Digestion

For drug treatment, 5 μg/mL actinomycin D (Sigma, Rehovot, Israel) or 100 μM 5,6-dichlorobenzimidazole riboside (DRB, Sigma) were added to plates 2 h before fixation. For nuclease digestion, HeLa cells were grown on coverslips and treated in 0.5% Tx-100 in PBS for 2 min and digested with either RNase A (100 μg/mL in PBS + 3 mM $MgCl_2$, Sigma) for 45 min at room temperature or DNase I (100 μg/ml + 5 mM $MgCl_2$) for 1 h and then washed with 0.25 M ammonium sulfate for 10 min for removal of chromatin.

Western Blotting

Cell lines were lysed for 2 h on ice in 50 mM Tris–HCl pH 8, containing 1% Nonidet P-40 (NP-40), 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methylsulfonyl fluoride (PMSF), 3 μg/mL aprotinin, 20 μg/mL leupeptin, 10 mM iodoactetate, 1 mM soduim orthovanadate, and 10 mM sodium fluoride. BM cells were lysed by boiling immediately after addition of SDS-sample buffer (5% glycerol, 2% SDS, 62.5 mM Tris–HCl pH 6.8, 2% 2-mercaptoethanol, 0.01% bromophenol blue). Equal amounts of cellular proteins from each sample were loaded and fractionated by PAGE. Immunoblot analysis of PSF in cell lysates (50 μg/lane) was performed using the B92 antibody and specific binding was detected with horseradish peroxidase (HRP)-coupled antibodies and enhanced chemiluminescense (ECL) reagents.

Separation of Bone Marrow Sub-populations

BM cells were labeled with anti Gr-1, myeloid cell specific antibody (1:300) followed by goat anti-rat IgG microbeads and were separated on a LS^+ magnetic column (Miltenyi Biotec, Bergish Gladbach, Germany). The cells were then fractionated on a Percoll (Sigma) gradient (20% to 80%); cell smears were prepared in a cytocentrifuge (Shandon, London, UK) and were fixed and stained with May-Grunwald (Reidel-deHaen, Seelze, Germany) and Gurr's Giemsa solutions (Fluka, Buchs, Switzerland). The remainder of cells underwent protein extraction. Differential identification of myeloblasts, myelocytes, metamyelocytes and polymorphonuclear (segmented) cells was performed in stained cell populations by standard cytological criteria. In Hoechst stained BM populations metamyelocytes and segmented cells were identified by their unique nuclear structure.

RESULTS

Splicing Factors Undergo Nuclear Reorganization During Mouse Myeloid Cell Differentiation

Splicing factors aggregate in regions called speckles commonly observed in immunofluorescent staining of cell nuclei. We have previously described a monoclonal antibody (B92) [Lee et al., 1996], and recently we prepared a polyclonal antiserum (1121) [Shav-Tal et al., 2000] that identifies the polypyrimidine tract-binding protein (PTB)-associated splicing factor, PSF [Patton et al., 1993]. The B92 antibody detected nuclear foci that were delicate in hemopoietic stem cells, intense in more mature progenitors and hardly detectable in mature granulocytes [Lee et al., 1996]. These observations and the ease in morphological identification of differentiation stages in the hemopoietic system prompted us to study nuclear organization during blood cell differentiation. In immunofluorescent staining, both the polyclonal (not shown) and monoclonal anti-PSF antibodies detected the protein in extra-nucleolar foci in HL-60 human myeloid leukemia cells (Fig. 1A) and in normal bone marrow cells (Fig. 2A). DNase I and RNase A treatment indicated that PSF is mainly associated with RNA (Fig. 3). Antibodies to the splicing factors $U2AF^{65}$ and snRNPs produced granular nuclear staining in mouse bone marrow cells (Fig. 2B, C) while in the case of hnRNP A1 a diffuse pattern was seen (Fig. 2D).

Cells with relatively mature phenotype often seemed to have a markedly reduced staining in comparison to immature cells (compare the round or slightly bent immature nuclei (asterisks) to doughnut-shaped mature nuclei (arrowheads) (Fig. 2A,E and 2C,G). This was not due to a reduction of PSF protein levels in mature cells as shown in Western blotting of protein extracts from separated immature and mature myeloid cells of the bone marrow (Fig. 4); the

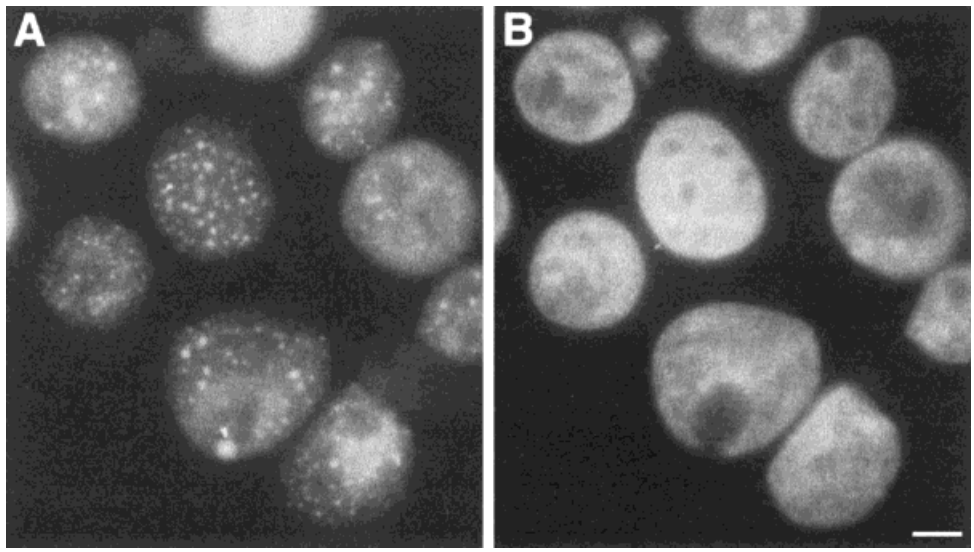

Fig. 1. Nuclear localization of PSF in HL-60 myeloid leukemia cells. **A:** PSF nuclear foci detected by the anti-PSF B92 MAb. **B:** Hoechst DNA counterstain. Scale bars shown here and in all other figures represent 5 microns.

equal intensity of the PSF band observed in immature and mature cells indicated that PSF levels remain as abundant during myeloid differentiation. As cells of the myeloid lineage mature their nuclear structure changes from a typical rounded shape of immature myeloblasts, through the intermediate myelocyte stages in which the nucleus starts to modify until reaching the doughnut-shaped metamyelocytic stage. The nucleus then becomes highly condensed and is lobulated in fully differentiated granulocytes. When carefully observed, these differentiating cells were found to have different staining patterns for PSF. Earlier cells, with less condensed nuclei, had regularly dispersed foci (Fig. 5A) while the staining in condensed nuclei of more mature cells resembled "beads on a string" running through the core of the circular nucleus (Fig. 5G,J). We further noted that these condensed threads filled only the inner volume of the nucleus while the nuclear periphery and its condensed appendages (Fig. 5H,K, arrowheads) did not contain any staining. This is emphasized by computer generated images in Figure 5 (C, F, I, L). In the cell shown in Figure 5E highly condensed areas are indicated (arrowheads). PSF staining was absent from these zones, although PSF was abundantly found throughout the rest of the nucleus (Fig. 5D). The compact organization of the nuclear foci was also observed with antibodies to $U2AF^{65}$ and snRNP's but not for hnRNP A1 (Fig. 2B,C,D).

Nuclear Redistribution of the Splicing Factor PSF Following Transcriptional Inhibition in Myeloid Cells

The compact distribution of splicing factors in granulocytes might correlate to the transcriptional status of these cells. During terminal differentiation of myeloid cells into mature granulocytes, chromatin condenses into heterochromatin and most of the gene activity is shut down [Aquiles Sanchez and Wangh, 1999]. In conjunction, cells from the metamyelocyic stage and onwards belong to the post-mitotic compartment. Addition of transcriptional inhibitors to cells have been shown to cause alterations in the distribution of splicing factors [Spector, 1993]. 5,6-dichlorobenzimidazole riboside (DRB) is an adenosine analog that inhibits transcriptional elongation by RNA polymerase II [Chodosh et al., 1989]. In human HL-60 undifferentiated myeloid leukemia cells, massive peri-nucleolar staining was seen 2 h following DRB transcriptional inhibition (Fig. 6B). Similarly, actinomycin D, which inhibits all RNA synthesis [Carmo-Fonseca et al., 1992], caused delicate petal-like redistribution of PSF around the nucleoli (Fig. 6C). Despite this change in distribution and staining intensity no reduction in PSF protein levels was observed in tran-

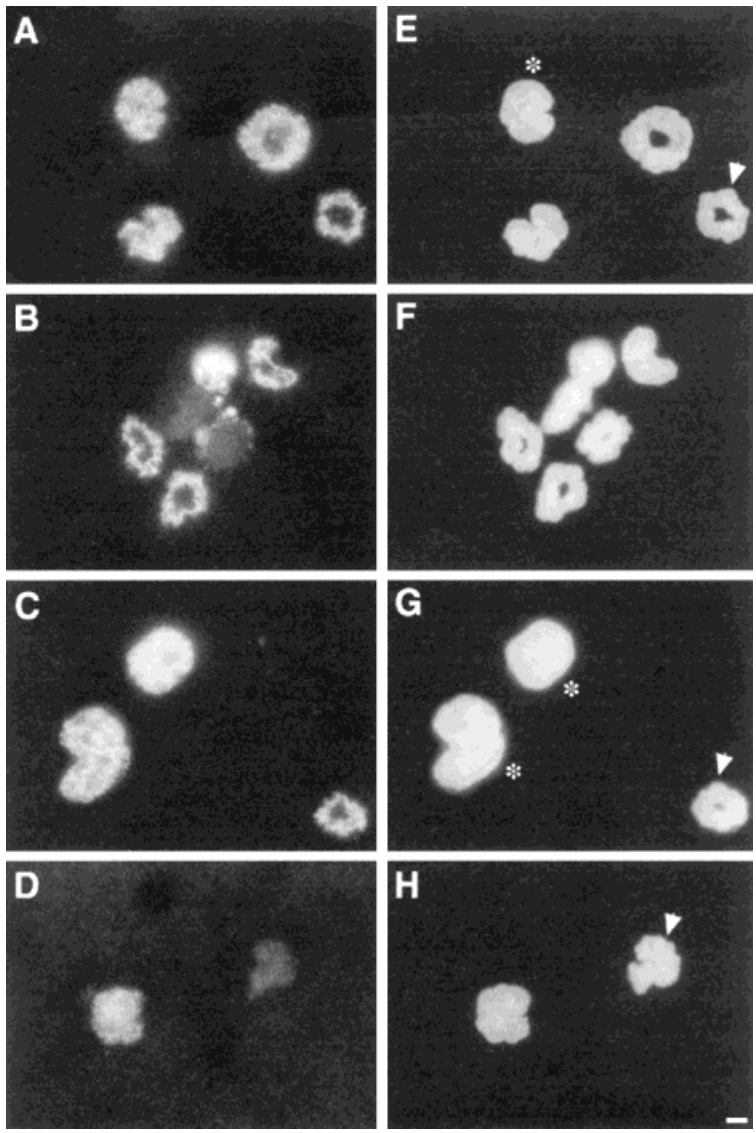

Fig. 2. Nuclear localization of splicing factors in freshly isolated mouse bone marrow cells. Granular nuclear labeling is detected in mouse BM cells following staining with antibodies to **A:** PSF (B92 MAb), **B:** U2AF^{65}, **C:** snRNP's (anti-Sm), **D:** hnRNP A1. **E–H:** The image on the right-hand side of each antibody staining is the corresponding DNA Hoechst counterstain. Asterisks point to immature cells and arrowheads to mature cells.

scriptionally inhibited cells (Fig. 7). Differentiated myeloid cells do not have nucleoli and when transcription was inhibited in such primary cells from normal mouse bone marrow, by the addition of DRB, the threaded pattern was disrupted and PSF foci localized symmetrically in each lobe of the segmented granulocytes (Fig. 6E). Interestingly, in immature cells PSF also localized in a symmetric fashion rather than around nucleoli (Fig. 6D). Thus, we conclude that complete transciptional inhibition results in the disruption of PSF-rich foci and in the compacting of PSF protein either in peri-nucleoalr complexes or in highly compact foci.

Cajal Bodies do Not Colocalize With PSF and are Absent From Mature Myeloid Cells

The differentiation state of cells is also known to affect Cajal bodies [for review, see Sleeman and Lamond, 1999a]. Cajal bodies are nuclear domains containing snRNP's and the autoantigen p80 coilin [Carmo-Fonseca et al., 1991b;

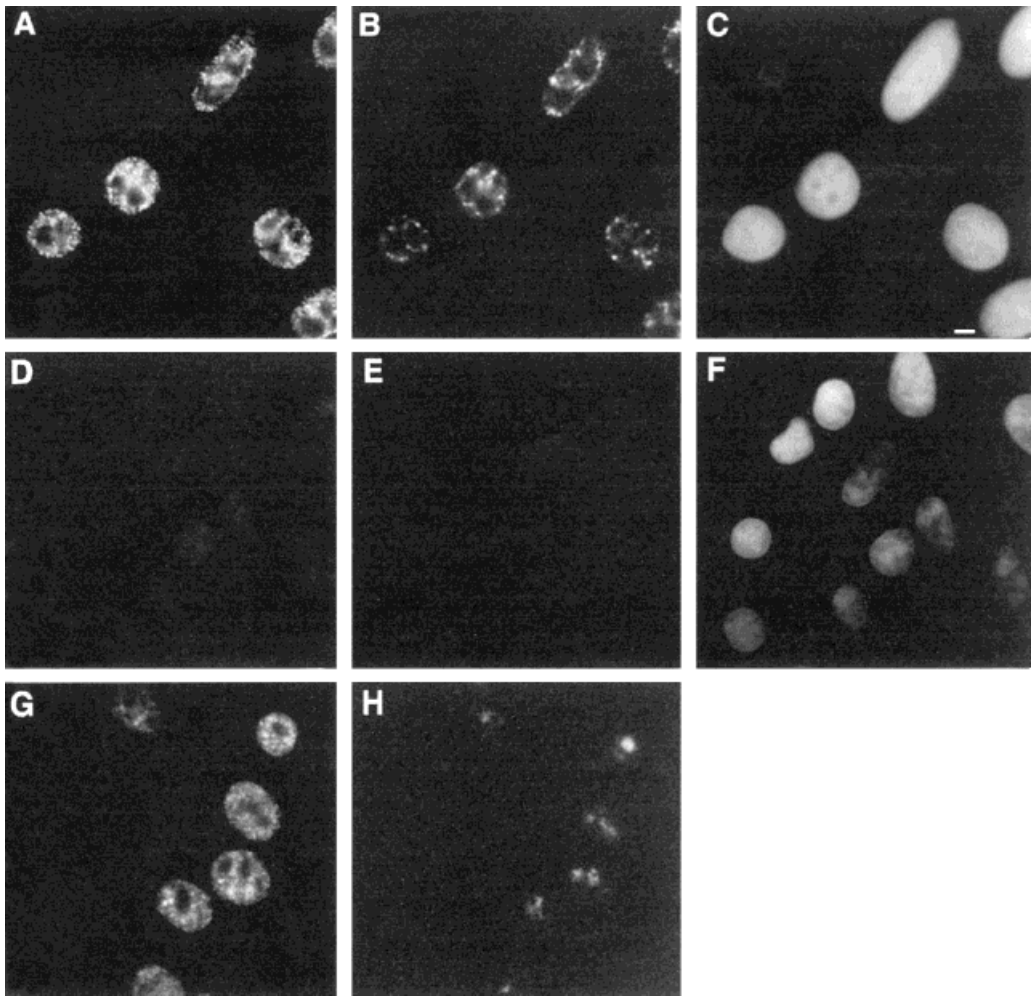

Fig. 3. Association of PSF with RNA. HeLa cells were grown on coverslips and triple labeled for immunofluorescence with antibodies to **A:** PSF (B92 MAb), **B:** snRNPs (anti-Sm), **C:** DNA Hoechst counterstain. The cells underwent RNase A digestion and stained for: **D:** PSF, **E:** snRNPs, **F:** DNA counterstain, or underwent DNase I digestion and stained for: **G:** PSF, **H:** DNA counterstain.

Raska et al., 1991]. They vary in size and in number in different cells. Several publications indicate that Cajal body numbers are high in differentiating cells and low in undifferentiated cells [Santama et al., 1996; Boudonck et al., 1998; Dahm et al., 1998]. In the mouse hemopoietic system, anti-p80 coilin antibody did not stain differentiated myeloid cells (Fig. 8A,C) whereas other cell types usually contained 1–3 Cajal bodies (Fig. 8A,C, arrowheads). In addition, in most HL-60 nuclei stained for PSF, two or three foci appeared overpronounced, in addition to the regular nucleoplasmic granular focus pattern (Fig. 9A,C,D, arrowheads) and were sometimes seen in nucleoli (Fig. 9C). Such staining resembled snRNP-rich foci that were shown to colocalize with Cajal bodies [Carmo-Fonseca et al., 1992]. Immunostaining of HL-60 cells with anti-p80 coilin showed in most cases no colocalization of PSF and p80 coilin (compare Fig. 9C,D–E), although in several cases PSF-rich foci colocalized with a faint Cajal body (Fig. 9C,E).

PSF Nuclear Distribution in Stem Cell Systems

Having observed differentiation-dependent modifications in the nuclear distribution of PSF and other splicing factors in the hemopoietic system, we turned to examine PSF in other stem cell systems. In spermatogenesis, changes in nuclear structure of spermatocytes occur as differentiation and silencing of the transcriptional machinery proceeds. Immunofluorescent staining of frozen sections of mouse testis with the B92 antibody showed that mature spermatozoa with condensed nuclei did not stain (Fig.

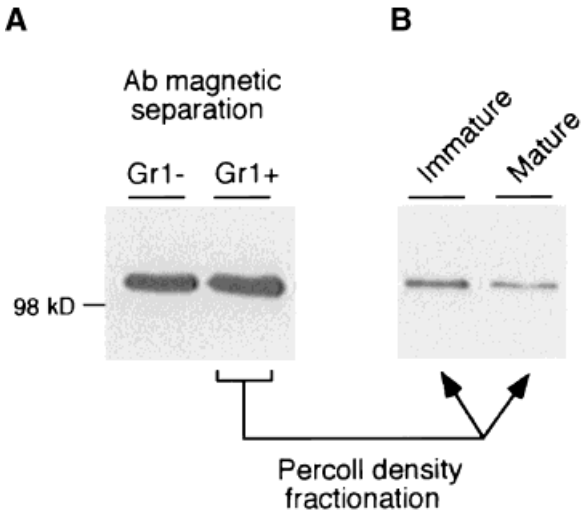

Fig. 4. PSF protein levels in immature and mature granulocytes. Bone marrow granulocytes were separated from non-granulocytic cells on a magnetic column using the anti-Gr-1 Ab. Granulocytic cells were further separated to immature and mature fraction on a Percoll density gradient. Protein extracts were made from each fraction and Western blots were probed with the B92 Mab. **A:** separation of granulocytic cells, **B:** separation of immature and mature granulocytes.

10B, arrowheads), whereas undifferentiated spermatogonium, spermatocytes and spermatids all stained to a certain degree (Fig. 10A,B, asterisks).

In the skin, stem cells are found in the basal cell layer of the epidermis [Watt, 1989]. As they mature they give rise to progenitors, termed transit amplifying cells, that move away from the stem cells clusters [Jensen et al., 1999]. They then are able to migrate out, toward the keratinized squames and undergo terminal differentiation. Immunofluorescence of PSF showed differential staining in cells of the basal layer of the murine skin layers. Some nuclei stained very strongly for PSF (Fig. 10C,D, arrowheads) while other nuclei did not (Fig. 10C,D). In addition, less PSF staining was seen in the outer skin layers. Cells growing in the hair follicle move distally to the follicle, mature and finally die. In tissue sections proximal to the base of the hair follicle, nuclei have regular shape and stain positively for PSF (Fig. 10E,F, left hand follicle). However, in sections distal to the base of the hair follicle, where cells are more mature and their nuclei are more condensed and of irregular shape, PSF immunofluorescence became less pronounced until it finally disappeared (Fig. 10G,H—same hair follicles, section distal from the base of the follicle).

DISCUSSION

Re-Distribution of Splicing Factor-Rich Foci During Myeloid Differentiation

The functional status of cells manifests in the localization and degree of condensation of chromatin. Changes in chromatin condensation were shown to be accompanied by alterations in interchromatin granule distribution [Krzyzowska-Gruca et al., 1983]. Using antibodies to different splicing factors (PSF, snRNPs, $U2AF^{65}$), we observed changes in the redistribution of splicing factor-rich foci in mature bone marrow cells of the granulocytic lineage. The nucleus of terminally differentiated human granulocytes consists of heterochromatin tightly packed in three or four lobes joined by thin DNA-containing filaments. In mouse granulocytes, lobulation is less pronounced and connecting filaments are not seen. Nuclear morphogenesis of the myeloid lineage starts with the myelocyte, and is dramatic in the next, metamyelocyte stage, in which the nucleus becomes doughnut shaped. The metamyelocyte loses proliferative capability and can only differentiate and give rise to mature granulocytes with segmented nuclei. The latter spend 7–10 days in the peripheral blood and can translocate through blood vessels to areas of infection. This movement, called diapedesis, is attributed to the unique, flexible, structure of the granulocyte nucleus. It has been suggested that nuclear restructuring may result from changes that reduce the interactions of chromosomes and transcription factors [Carter and Lawrence, 1991]. We found in relatively more mature granulocytes, that condensed nuclei exhibited a thread-like PSF staining pattern through the interior of the doughnut-shaped nucleus while in immature cells, which have less condensed chromatin, a regular nuclear pattern of splicing factor-rich foci was observed. Western blotting of PSF from mature and immature granulocytes showed that there is no reduction in PSF levels in mature cells. This thread-like pattern is unique to mature granulocytes and has not been observed in other cell types.

The changes in the distribution of the splicing factor rich foci in mature myeloid cells can be correlated to the transcriptional status and changes in chromatin structure during differentiation. Several studies have demonstrated active chromatin in the nuclear periphery. These studies also showed that transcription-

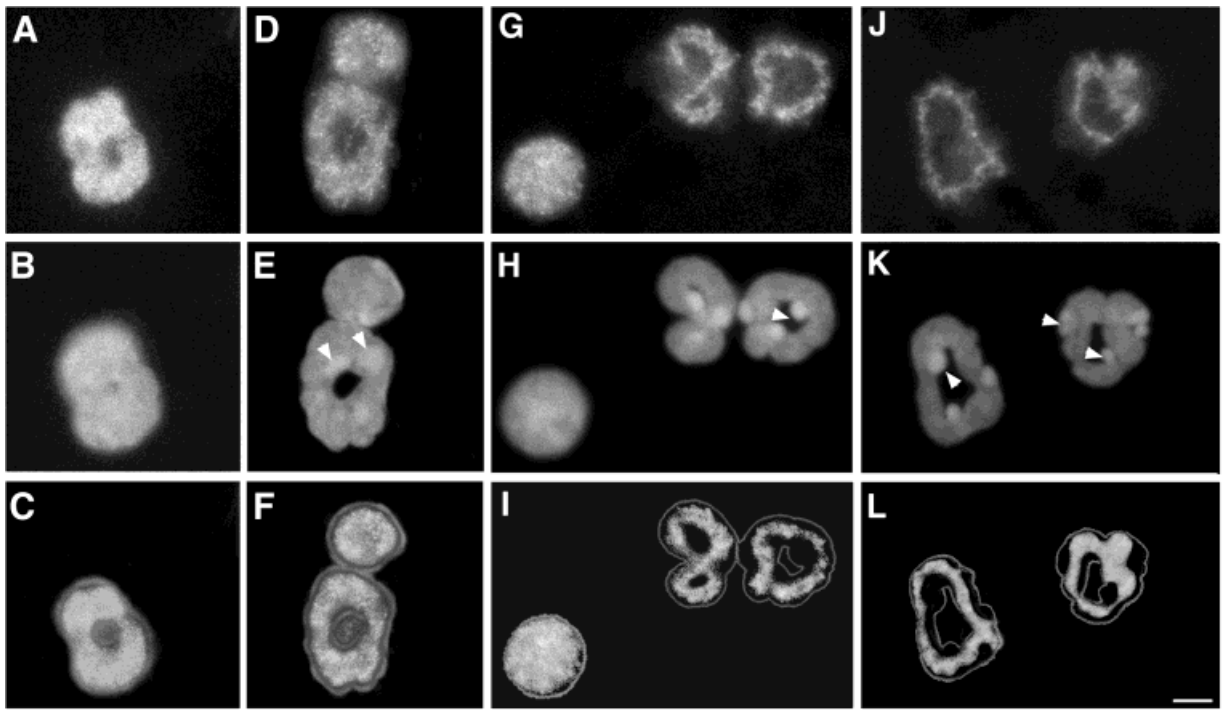

Fig. 5. Reorganization of PSF during myeloid differentiation of mouse bone marrow cells. **A, D:** PSF immunostaining (B92 MAb) is found in whole nuclear volume in immature granulocytes with uncondensed nuclei. **G, J:** in mature cells with condensed chromatin PSF staining is compact. **B, E, H, K:** DNA Hoechst counterstain, respectively. **C, F, I, L:** Computerized overlay of PSF threads (yellow) and nuclear boundaries (blue). Arrowheads point to areas of highly condensed chromatin. [Color figure can be viewed in the online issue, which is avaiable at www.interscience.wiley.com.]

ally active genes reside in close proximity to the nuclear membrane and thus allow efficient nuclear export of mRNA [for review, see Haaf and Schmid, 1991]. However, other studies show heterochromatin localization in the nuclear periphery, mRNA localization in the nuclear interior and active genes in the inner nuclear volume of lymphocytes [for review, see Carter and Lawrence, 1991]. Topographically, mRNA transcripts and the splicing machinery are excluded from the interior regions of individual chromosomes and are believed to reside in an interchromatin channel network [Zirbel et al., 1993; Kramer et al., 1994]. We found in differentiated myeloid cells that the splicing machinery is compactly localized to the interior of the nucleus and is surrounded by condensed chromatin. The nuclear periphery is devoid of splicing factor-rich foci. This suggests that chromatin condensation in these cells occurs from the periphery inward, in a constrictive manner, thus compacting the interchromatin granules to the center of the nucleus. Although this might cause difficulty in the movement of mRNAs out of the nucleus, the ring-shaped structure of the nucleus allows for most of the nuclear volume to be in contact with the cytoplasm and, therefore, the relative distance from the nuclear interior to periphery is rather small.

Distribution of Splicing Factor-Rich Foci Versus Differentiation State of Hemopoietic Cells

During granulocytic differentiation most of the DNA condenses into heterochromatin, nucleoli disappear, and there is a reduction in endoplasmic reticulum and ribosomes. As such, mature granulocytes are considered cells with low levels of transcription and protein synthesis. It is also known that these cells are capable of selective de novo synthesis of molecules important for their motility, including membranal and cytoskeletal proteins. Granulocytes further require mediators of phagocytic activities such as interleukins and colony-stimulating factors, especially after stimulation [Jack and Fearon, 1988; Beaulieu et al., 1992]. The reduction in the spatial distribution of splicing factor-rich foci that we observed in maturing granulocytes, correlates well with the reduced transcriptional activity attributed to these cells.

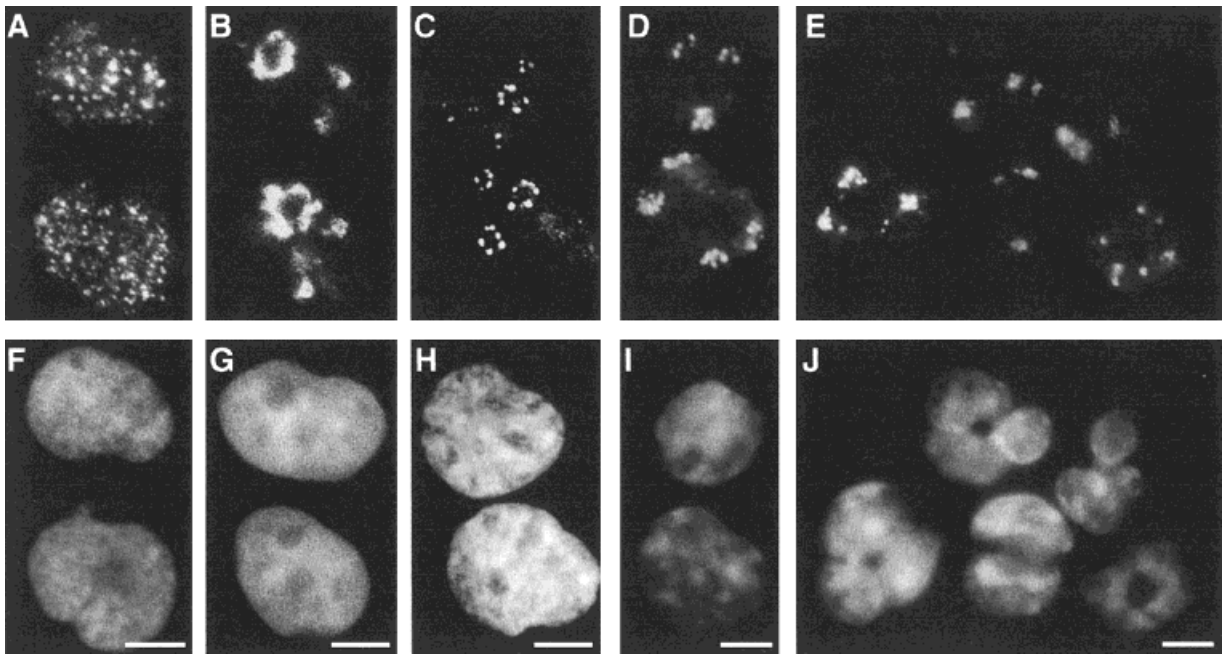

Fig. 6. Nuclear reorganizaiton of PSF during transcriptional inhibition in myeloid cells. Granulocytic cells were immunostained with the anti-PSF B92 MAb. Immunofluorescent staining of nuclear foci was observed in **A:** untreated cells, in comparison to: **B:** petal-like structures in cells treated with DRB, or **C:** actinomycin D for 2 h. Transcriptionally inhibited mouse bone marrow **D:** immature and **E:** maturing granulocytes treated with the transcriptional inhibitor DRB. **F–J:** DNA Hoechst counterstain.

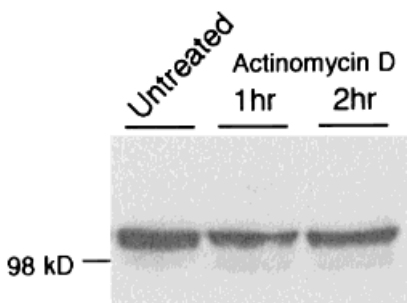

Fig. 7. PSF protein levels in transcriptionally inhibited cells. HL-60 cells were treated with actinomycin D for 2 h. Protein extracts were made from untreated and treated cells after 1 and 2 h and immunoblotted with the B92 MAb.

We previously showed that FACS sorted early bone marrow stem cells had very delicate granular staining while the relatively more mature progenitors had pronounced and abundant PSF-rich foci [Lee et al., 1996]. We now show that this strong pattern changes and becomes compact as the cells differentiate. Our data concerning PSF-rich foci numbers in cells with different proliferative and transcriptional indexes, i.e., stem cells (low proliferative), progenitors (highly proliferative) and mature granulocytes (unproliferative) are in agreement with experiments done with lymphocytes; Mitogenic stimuli in lymphocytes, which enhance RNA and protein synthesis and cause changes in nuclear morphology, are accompanied by high levels of interchromatin granules as compared to their numbers in resting lymphocytes [Krzyzowska-Gruca et al., 1988] and by repositioning of genes [Brown et al., 1999]. Stein et al. [1996] concluded that the structural modifications associated with proliferation and gene expression during differentiation are accommodated by the constituents of the nuclear matrix. Many of these proteins, such as certain hnRNPs and lamins, have recently been shown by high-resolution 2D gel electrophoresis to be absent in mature granulocytes and present in other hemopoietic cells [Gerner and Sauermann, 1999]. Thus, we conclude that the distribution of PSF-rich foci during different points of hemopoietic differentiation from the stem cell level, through progenitors and intermediately differentiated cells, up to fully differentiated specialized cells is dynamic and correlates with the transcriptional state of all these stages. Differentiation-dependent relocalization of nuclear components occurs in a variety of tissues, however, the pattern of redistribution seems to

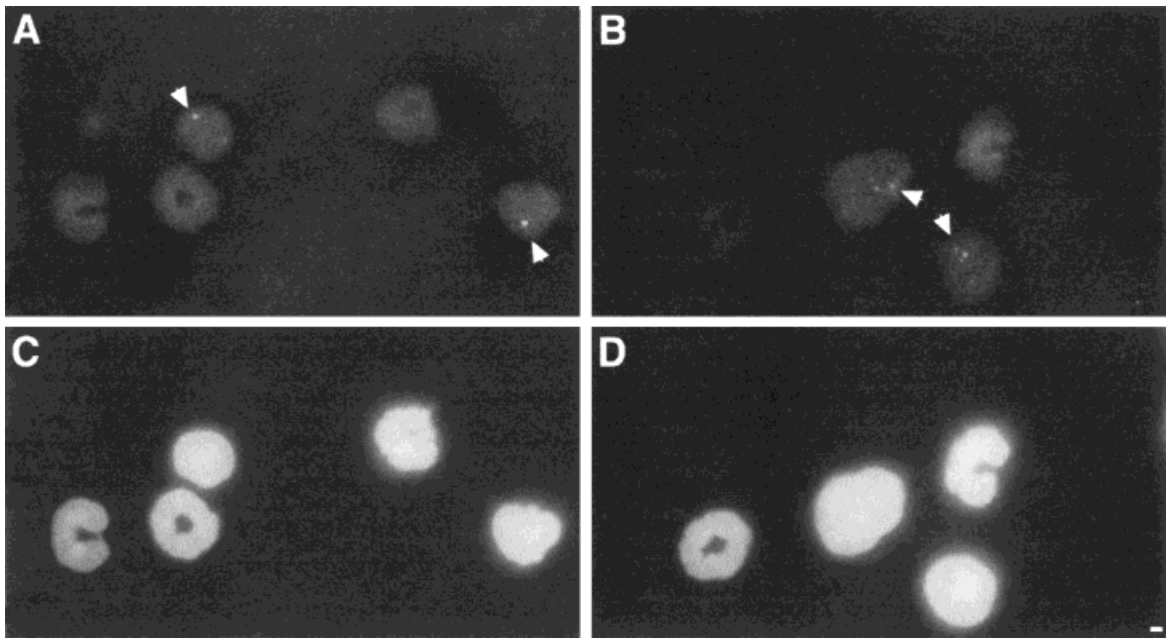

Fig. 8. Cajal bodies in mouse myeloid cells. p80 coilin immunolabeling is found in **A, B:** immature nuclei (arrowheads), but not in mature cells (doughnut shaped nuclei). **C, D:** DNA Hoechst counterstaining.

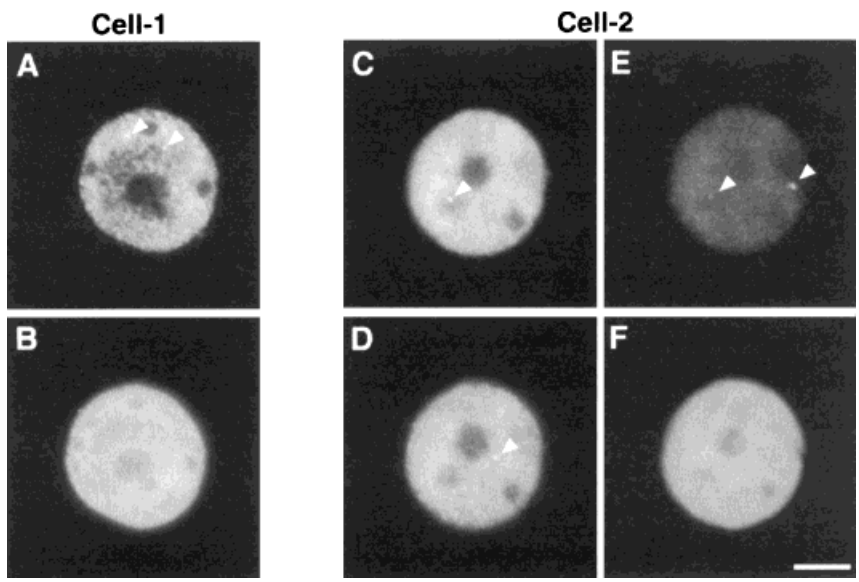

Fig. 9. Double staining of PSF and Cajal bodies. HL-60 immature myeloid leukemia cells immunostained with **A, C, D:** anti-PSF B92 MAb (C and D are the same cell seen from two different planes). PSF rich foci (C, D arrowheads) partially colocalize with **E:** p80 coilin (arrowheads). **B:** is the DNA counterstain for A. **F:** is the DNA counterstain for C, D, E.

be tissue type specific; Lelievre et al. [1998] showed that human mammary epithelial cell morphogenesis in vitro entails relocalization of the splicing factor SRm160 and follows a pattern distinct from that reported here for PSF in granulocytes. Whether these varied changes have a determining role in differentiation, remains to be seen.

Cajal Bodies, PSF and Transcription Inhibition in Differentiating Cells

Cajal bodies (as recently coined by Gall, 1999), first described by Ramon y Cajal [1903] are round nuclear structures consisting of snRNPs, nucleolar and cell-cycle components, transcription factors and the Cajal body (CB) marker—

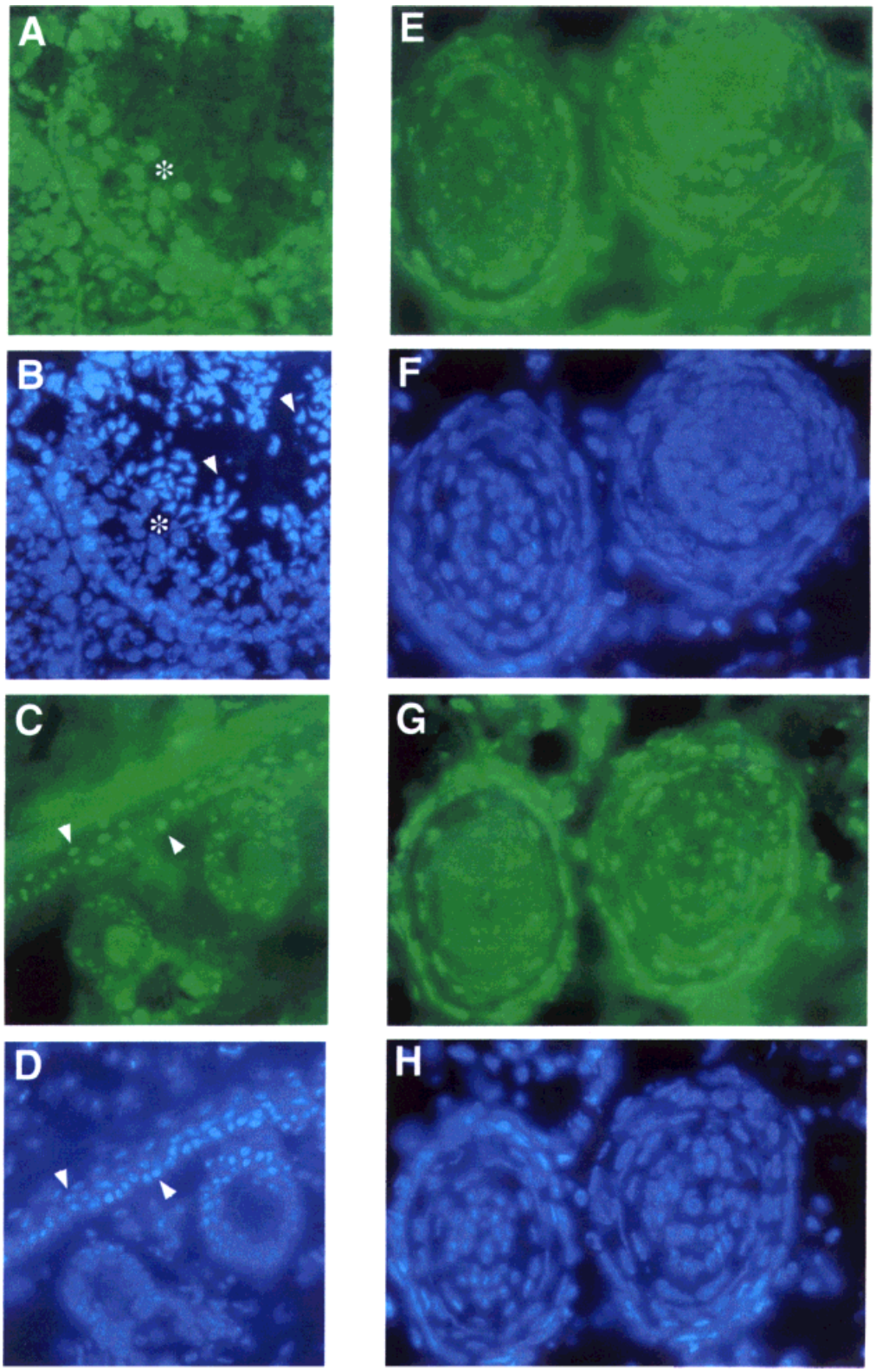

Fig. 10. PSF immunostaining on mouse tissue sections. Anti-PSF B92 MAb was used for immunofluorescence of: **A, B:** Testis, **C, D:** skin (arrowheads point to nuclei that stain for PSF), **E:** hair follicles (proximal section, and **G** distal section of the same hair follicles). Left follicle is less mature than the right one. B, D, F, H: DNA Hoechst counterstaining. [Color figure can be viewed in the online issue, which is avaiable at www.interscience.wiley.com.]

p80 coilin [for review, see Matera, 1999]. The numbers of CBs vary from cell to cell [Raska et al., 1991], they may be found in nucleoli [Malatesta et al., 1994]. We found that mature myeloid cells did not contain CBs, whereas immature cells had 1–3 CBs. In addition, in the HL-60 immature myeloid leukemia cell line CBs in most cases did not localize with PSF-rich foci which were sometimes seen within the nucleolus. Since there is heterogeneity in the composition of the CB population in individual cells [Alliegro and Alliegro, 1998] and some CBs do not contain coilin [Bauer and Gall, 1997] it remains to be examined whether these structures might belong to the family of CB related structures [for review, see Sleeman and Lamond, 1999a]. Changes in the organization of CBs have been seen during differentiation [Santama et al., 1996; Dahm et al., 1998]. Most interestingly, in the *Arabidopsis* root epidermis, quiescent cells had one CB, rapidly dividing meristematic cells had up to 20 CBs, elongating nondividing cells had between 3 and 6, and differentiating cells had one [Boudonck et al., 1998]. The function of CBs is unclear, although recently it has been suggested that CBs are a station in the maturation pathway of snRNPs [Sleeman and Lamond, 1999b] and sites of RNA polymerase I, II and III complex assembly [Gall et al., 1999]. It is then plausible that differentiated granulocytes which have low levels of transcription, and thus pre-mRNA splicing, make do with the existing snRNPs and RNA polymerases.

In HL-60 cells, PSF-rich foci transformed during complete transcriptional inhibition into compact petal-like peri-nucleolar forms. This was not due to changes in PSF protein levels in transcriptionally inhibited cells. This petal-like organization has been observed for the U1-70K and U2AF proteins [Carmo-Fonseca et al., 1991a,b]. Interestingly, both U2AF and PSF are polypyrimidine-tract binding proteins and it has been suggested that PSF can displace $U2AF^{65}$ on the polypyrimidine tract during the second step of splicing [Gozani et al., 1994]. In addition, it has been shown that PTB is normally found in the perinucleolar compartment [Ghetti et al., 1992; Matera et al., 1995] which is functionally active in RNA metabolism [Huang et al., 1998]. Recently, it has been shown that most of the PSF is not in complex with PTB [Meissner et al., 2000; Shav-Tal et al., 2000] and it is possible, therefore, that such conditions of transcriptional inhibition allow preferable binding of PSF to PTB, thus targeting PSF to the perinucleolar region. In mature myeloid cells that do not have nucleoli, complete transcriptional inhibition caused concentration of PSF in compact foci in the different lobes, once again indicating a specific interaction of PSF with a nuclear component, which might be PTB or nucleolar remnants.

We found that PSF disappears completely in mature sperm cells as is the case for other splicing factors as judged by immunofluorescent detection methods. The sperm nucleus has the most highly condensed eukaryotic DNA known, is transcriptionally inactive and in no need of splicing machinery. In skin layers we observed cells that differentially stained for PSF, perhaps indicating different levels of maturation. In addition, in hair follicles PSF becomes undetectable by immunofluorescence as hair cells differentiated. We have found that, although PSF is undetectable by our antibody in certain cells or cell states, the PSF protein remains intact [Shav-Tal et al., unpublished observations]. This "disappearance" of PSF antigenicity seems to be related to the cell state and to modifications occurring on the protein and might be relevant to the mechanism of splicing factor dynamics within the nucleus.

Nuclear structure affects cell growth and differentiation [Georgatos, 1994]. Our study points to such a connection in the hemopoietic stem cell system, in which major nuclear restructuring events occur. It seems that Sheldon Penman [1991] was correct a decade ago in asking "If genes just make proteins and our proteins are the same, then why are we so different?", and in concluding that the answer lies in the study of biological architecture.

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