Molecular Biology of the Cell
Vol. 5, 1341–1352, December 1994

Splicing Components Are Excluded from the Transcriptionally Inactive XY Body in Male Meiotic Nuclei

Carmelit Richler,* Gil Ast,† Ruth Goitein,* Jacob Wahrman,* Ruth Sperling,* and Joseph Sperling†‡

*Department of Genetics, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; and
†Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Submitted May 17, 1994; Accepted November 2, 1994
Monitoring Editor: Elizabeth H. Blackburn

The study of the effect of programmed cessation of transcription in a large nuclear domain, on the distribution of elements of the pre-mRNA splicing machinery, is the main aim of this paper. To this end, we took advantage of the nuclear partitioning of mouse spermatocytes early in meiosis into *autosomal transcribing* and *XY nontranscribing* compartments. This system also allows to extend this study to stages in sperm differentiation that are accompanied by reduction and eventual cessation of transcription. We show by indirect immunofluorescence in spermatogenetic cells that 1) fluorescent signals of the pre-mRNA splicing factors SF53/4 and SC35, of the Sm antigens, and of RNA polymerase II, are largely absent from the nontranscribing, X-inactivated compartment, but are abundantly present in the transcribing autosomal compartment and 2) the presence, gradual reduction, and absence of transcriptive activity in nuclei undergoing the sperm formation sequence are positively correlated with the fluorescence patterns of the antibodies against SF53/4, SC35, and the Sm antigens. These data suggest that cessation of transcription during spermatogenesis is accompanied by exclusion of the splicing machinery from nontranscribing chromatin to its vicinity.

INTRODUCTION

The perfection of the nucleus leading to the evolution of the eukaryotic cell was achieved by structural adaptations to the requirements of DNA replication, transcription, RNA processing, and message transportation. This paper deals primarily with one aspect of nuclear functional organization, namely, the effect of cessation of transcription in a large nuclear region located within a transcribing environment on the distribution of pre-mRNA splicing components.

The maturation of most eukaryotic pre-mRNA involves posttranscriptional processing events that are required to convert the nuclear primary transcripts of RNA polymerase II (pol II) into functional mRNAs by splicing, capping, and polyadenylation. Numerous studies of splicing in vitro have established that it occurs only after the assembly of multicomponent ribonucleoprotein (RNP) complexes, termed spliceosomes, which sediment in density gradients as 40–60S particles. The assembly of functional spliceosomes involves an ordered binding to the pre-mRNA of five small nuclear RNP (snRNP) particles and of a number of non-snRNP proteins, among which several essential splicing factors have been identified (reviewed in Krainer and Maniatis, 1988; Steitz *et al.*, 1988; Green, 1991; Guthrie, 1991; Moore *et al.*, 1993). On the other hand, studies in vivo have shown that several specific nuclear pol II transcripts and their splicing products, as well as the general population of nuclear poly(A)$^+$ RNA, are packaged with all thus far known spliceosomal components and splicing factors in large nuclear RNP (lnRNP) particles of a uniform size that sediment at the 200S region in sucrose gradients (Sperling *et al.*, 1985, 1986; Spann *et al.*, 1989; Ast *et al.*, 1991). These studies indicate that nuclear pre-mRNA splicing in living cells occurs on structures more complex

‡ Corresponding author.

than individual spliceosomes. The lnRNP particles have thus been proposed to represent the nuclear RNA processing machinery (Sperling and Sperling, 1990).

The assembly of the splicing machinery in living cells is apparently coupled to transcription since the nascent pre-mRNA becomes covered with splicing factors as soon as it is transcribed. This positional relationship between sites of transcription and sites of pre-mRNA splicing is supported by a large body of observations at the light and electron microscope level (reviewed in Gall, 1992; Spector, 1993; Xing and Lawrence, 1993; Kramer *et al.*, 1994). In some cases, the pre-mRNAs that are found associated with splicing components in these nuclear sites are in a nascent form (Beyer and Osheim, 1988; Gall and Callan, 1989; Vazques-Nin *et al.*, 1990; Wu *et al.*, 1991; Tsvetkov *et al.*, 1992; Jiménez-Garcia and Spector, 1993; Wansink *et al.*, 1993; Xing and Lawrence, 1993; Zachar *et al.*, 1993). In other cases, the pre-mRNA is found at various stages of the RNA processing pathway (Fakan *et al.*, 1984; Carter *et al.*, 1991; Xing and Lawrence, 1993; Kramer *et al.*, 1994). In addition to this class of pre-mRNA containing sites, a second class of nuclear sites that contain splicing components but are devoid of pre-mRNA can be distinguished. Examples of such sites are the snurposomes of Xenopus oocytes (Gall and Callan, 1989; Wu *et al.*, 1991; Gall, 1992; Wu and Gall, 1993) and the coiled bodies of mammalian nuclei (Elicieri and Ryerse, 1984; Fakan *et al.*, 1984; Raska *et al.*, 1991; Spector *et al.*, 1991, 1992; Carmo-Fonseca *et al.*, 1992; Huang and Spector, 1992). Some of these organelles also lack certain splicing factors (e.g., SC35 [Wu *et al.*, 1991]). These findings, as well as experiments in yeast which have indicated that the amount of U1 snRNP is in excess of what is required for splicing efficiency and growth rate (Séraphin and Rosbash, 1989), suggest a role for these organelles as storage sites for splicing components (reviewed in Gall, 1992; Spector, 1993), although other roles are not ruled out. The relationship between the two classes of sites, those where pre-mRNAs and splicing components colocalize and those where splicing factors are present but pre-mRNAs are absent, is not well defined. A study comparing the distribution of splicing factors with that of pol II should provide insight into this issue—particularly when carried out in an unperturbed cellular system that normally develops large nuclear domains devoid of transcription, which exist alongside with transcriptionally active domains. Spermatogenesis of the mouse is suited for this purpose, as it comprises a sequence of complex cellular events exhibiting dramatic changes in the pattern of transcription, which is correlated with changes in chromatin density.

Three circumstances of spermatogenesis are particularly well suited for the study of the relationship between transcription and splicing phenomena. First, the nucleus of the early primary spermatocyte is divided into two *major* compartments of unequal size (Figure 1). In the larger, autosomal compartment many genes are transcribing, perhaps more than are functionally required (Davies and Willison, 1993), while in the smaller XY compartment the condensed chromatin is largely nontranscribing (e.g., Monesi, 1965; Tres and Kierszenbaum, 1981). This "XY body" occupies a well-defined portion of the nuclear volume. Second, the transition of postmeiotic, haploid spermatids into mature sperm cells is accompanied by a gradual chromatin condensation and silencing of gene activity (Davies and Willison, 1993). Third, elements of the RNA processing machinery and mRNA related to sperm differentiation accumulate outside the nucleus in the chromatoid body—an enigmatic transient structure unique to spermatogenesis (Biggiogera *et al.*, 1990; Saunders *et al.*, 1992).

Here we show by indirect immunofluorescence that the fluorescent signals of RNA polymerase II, of two different splicing factors and of the Sm antigens are almost completely absent from the largely nontranscribing XY body of early mouse spermatocytes, but are abundantly present in the autosomal compartment. We also show that in later haploid stages of spermatogenesis spermatid nuclei lose their splicing factors in correlation with their chromatin condensation and with the reduction and eventual cessation of transcription. Finally, the chromatoid body was found to harbor the tested splicing elements despite being empty of chromatin, hence of transcription.

MATERIALS AND METHODS

Cell Preparation

All experiments were performed on cells from testes of the laboratory mouse, *Mus musculus*, strain C57/Black. To release spermatogenetic cells, small pieces of testicular tubules were repeatedly pulled apart between two fine forceps. Microscopic slides were obtained in two ways. 1) Cells were suspended within drops of RNA-preserving buffer CSK [10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid (PIPES), pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$ (Fey *et al.*, 1986)] placed on silane coated slides, permeabilized on ice with Triton X-100 in CSK for 3 min, washed and fixed in 3.7% paraformaldehyde in CSK and then immediately immunostained. 2) Cells were microspread on clean slides in drops of 0.2 M sucrose, fixed in 4% paraformaldehyde in 0.1 M sucrose adjusted to pH 8.5, and washed in Photoflo (Fletcher, 1979). Slides were either stained with 50% silver nitrate for visualization of autosomal synaptonemal complexes and sex chromosome axes, or stored at −80°C until used. These two methods of cell preparation gave the same results. All photographs in this paper were obtained from cells prepared by the second method, which gave better cytological detail.

Antibodies

Monoclonal antibody (MAb) 53/4 (mouse) is directed against the essential pre-mRNA splicing factor SF53/4 (Ast *et al.*, 1991). Hybridoma cells producing MAb 53/4 were selected from a panel of MAbs prepared by injecting SJL/J mice with HeLa cell nuclear extract enriched by lnRNP particles that sediment at about 200S in sucrose gradients

(Spann *et al.*, 1989; Sperling *et al.*, 1985). MAb 53/4 from ascitic fluids was purified by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) as previously described (Ast *et al.*, 1991). MAb anti-SC35 (mouse), is directed against the essential splicing factor SC35 (Fu and Maniatis, 1990), a gift from Dr. J. G. Gall. MAb A52 (mouse) is an anti-DNA monoclonal autoantibody (Eilat *et al.*, 1984) kindly provided by Dr. D. Eilat. MAb 8WG16 (mouse) is directed against the highly conserved heptapeptide repeat of the largest subunit of RNA polymerase II (Thompson *et al.*, 1990) kindly provided by Dr. R.D. Kornberg. Anti-Sm human autoantibodies were from a patient with systemic lupus erythematosus (Arad-Dann *et al.*, 1987).

Immunostaining

Microscopic slides with spread cells were rinsed in 0.5% Triton X-100 in phosphate-buffered saline (PBS) at 4°C, for 3 min, washed twice in PBS containing 0.05% Tween-20 (PBS-Tween-20), and were incubated with the first antibody in PBS-Tween-20 for 1 h: MAb 53/4, 30 μg/ml; MAb anti-SC35, 5 μg/ml; MAb A52, undiluted culture supernatant; MAb 8WG16, 50 μg/ml; anti-Sm serum diluted 1:300. The slides were washed twice in PBS-Tween-20 for 10 min and incubated with the second antibody in the same buffer for 1 h. The secondary antibodies, diluted 1:50, were affinity-purified antibodies of the appropriate specificity, conjugated to either Texas red or fluorescin isothiocyanate (FITC) (all from Jackson, West Grove, PA). The slides were washed twice as before and once in PBS. For DNA staining, preparations already stained with second antibodies conjugated to Texas red or FITC were treated with 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/ml, or with propidium iodide at 1 μg/ml, respectively. In some experiments, anti-Sm antibodies were applied simultaneously with one of the mouse MAbs. Appropriate second antibodies, preadsorbed on immobilized serum proteins to remove cross-reactivity with antibodies of the other species, were used. The preparations were examined under a Nikon Microphot-SA photomicroscope with ×40 and ×100 Fluor objectives.

RESULTS

We have localized, by indirect immunofluorescence, two splicing factors, the Sm antigens, and RNA polymerase II in spread, XY/autosome compartmentalized meiotic nuclei of the mouse, and in haploid cells undergoing the progression towards mature sperm cells. The splicing antibodies were 1) a monoclonal antibody (MAb), one of a panel of MAbs raised against the lnRNP particle as a whole, that is directed against the essential splicing factor 53/4 (SF53/4) (Ast *et al.*, 1991); 2) a MAb directed against the non-snRNP splicing factor SC35 (Fu and Maniatis, 1990), which is a member of a family of serine/arginine (SR) rich essential protein splicing factors (Zahler *et al.*, 1992); and 3) an antiserum against the Sm antigens which are components of U1, U2, U4.6, and U5 snRNPs, known to be required for splicing, and of U7, U11, and U12 snRNPs, which are required for histone 3' end processing and nuclear RNA processing, respectively (reviewed in Baserga and Steitz, 1993). MAbs 53/4 and anti-SC35 enable the localization of splicing factors, whereas the anti-Sm serum enables the localization of proteins involved in pre-mRNA processing and not only those involved in splicing (Wu and Gall, 1993). DNA staining by DAPI and propidium iodide after the antibody staining proved reliable for cell type and stage identifications that were occasionally confirmed by phase microscopy.

The Deployment of Splicing Proteins Among Nuclear Compartments of Primary Spermatocytes

The two major compartments of primary spermatocytes at pachytene and part of diplotene, the autosomal and the XY compartment, appear strikingly different by several methods of visualization. For example, in silver-stained microspreads (Figure 1), the chromosomes of the peripherally located XY body are snugly coiled, with their telomeres in close proximity. The axes and chromatin of the sex chromosomes are deeply staining. These properties recall those of the somatic transcriptionally inactive Barr body in females of certain mammals (Walker *et al.*, 1991). The less dense autosomal bivalents, comprising much of the autosomal compartment, are relatively short, straight, well-defined bodies, readily separable from each other at the periphery of spread nuclei. Each bivalent has a central axis, the synaptonemal complex, and loops of chromatin in a configuration allowing the transcription of many genes (Davies and Willison, 1993).

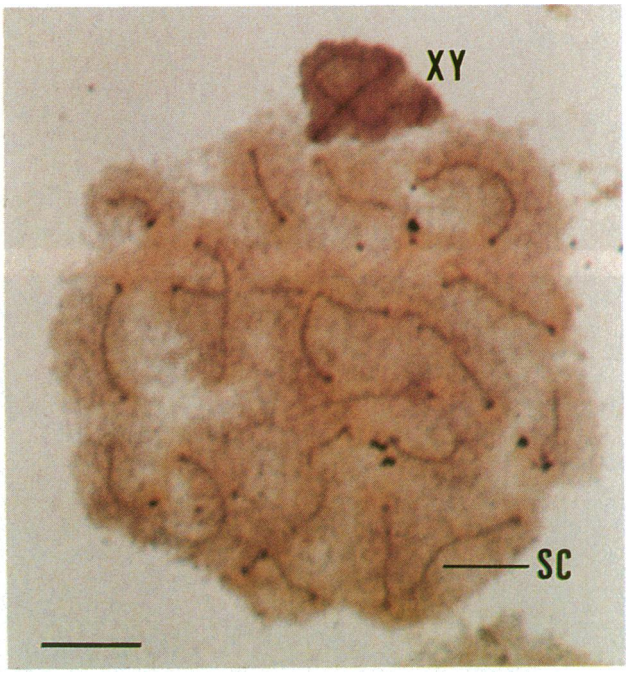

Figure 1. Partitioning of a meiotic nucleus at pachytene; silver staining. The nucleus consists of a large autosomal compartment, including 19 transcribing bivalents, and a smaller compartment of the nontranscribing X and Y (*XY*) chromosomes. The silver staining highlights the autosomal synaptonemal complexes (*SC*) and the axes of the sex chromosomes on the background of surrounding chromatin. The different chromatin staining of the two compartments is related to their state of condensation. All figures are from spread spermatogenetic cells of *Mus musculus;* scale bars, 10 μm.

Figure 2A shows that RNA polymerase II, which is present in the autosomal compartment, is almost totally absent from the XY body. To rule out the possibility that the lack of a fluorescent signal in the XY body was caused by hindrance to the penetration of antibodies to this compartment, we show that antibodies to DNA equally stained the autosomal and the XY compartments (Figure 2B1), while anti-Sm antibody, simultaneously applied to the same nucleus, stained only the autosomal compartment (Figure 2B2). This control experiment confirms that immunoglobulin molecules do penetrate the XY compartment.

Once the nontranscribing nature of the XY compartment was confirmed by the absence of pol II, we followed the distribution of splicing proteins in partitioned meiotic nuclei by using antibodies against the splicing factors SF53/4 and SC35 and against the Sm antigens. Typical results are shown in Figures 2–4, the outstanding feature of which is the almost total absence of SF53/4 (Figure 3C), SC35 (Figure 3, D and E), and the Sm antigens (Figures 2B, 3A, 3B, and 4) from the largely nontranscribing XY compartment. Accumulations of splicing components in-between the nontranscribing XY body and the transcribing autosomal chromatin (Figure 3, A2 and C2, triangles) are consistent with the view that the splicing machinery resides in domains defined by exclusion from areas of dense chromatin (Kramer *et al.*, 1994). Similarly,

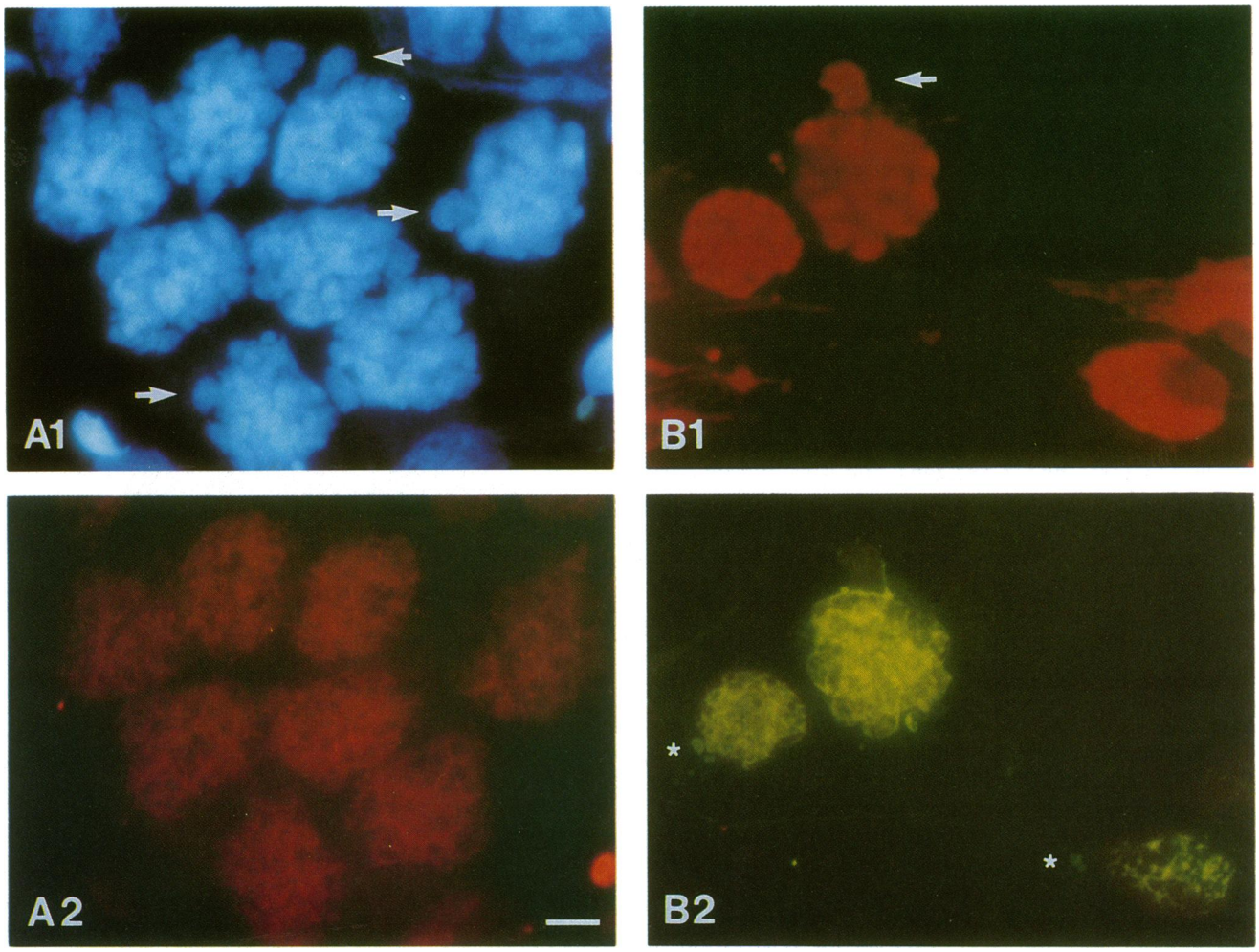

Figure 2. Meiosis, pachytene. (A1) DAPI staining; arrows, XY bodies. (A2) The same cells treated with anti-RNA polymerase II antibody (Texas red). The XY bodies are unstained, in line with the previously known suppression of transcription in the XY compartment. (B1 and B2) A test of the penetrability of the XY compartment to antibodies. The equal staining of the two compartments by anti-DNA antibody (B1, Texas red; arrow, XY body), while the autosomal compartment alone is stained by anti-Sm antibody (B2, FITC), indicate that antibodies penetrate into all parts of the meiotic nucleus. Note the round and elongating spermatids showing an anti-Sm antibody positive chromatoid body (B2, asterisks).

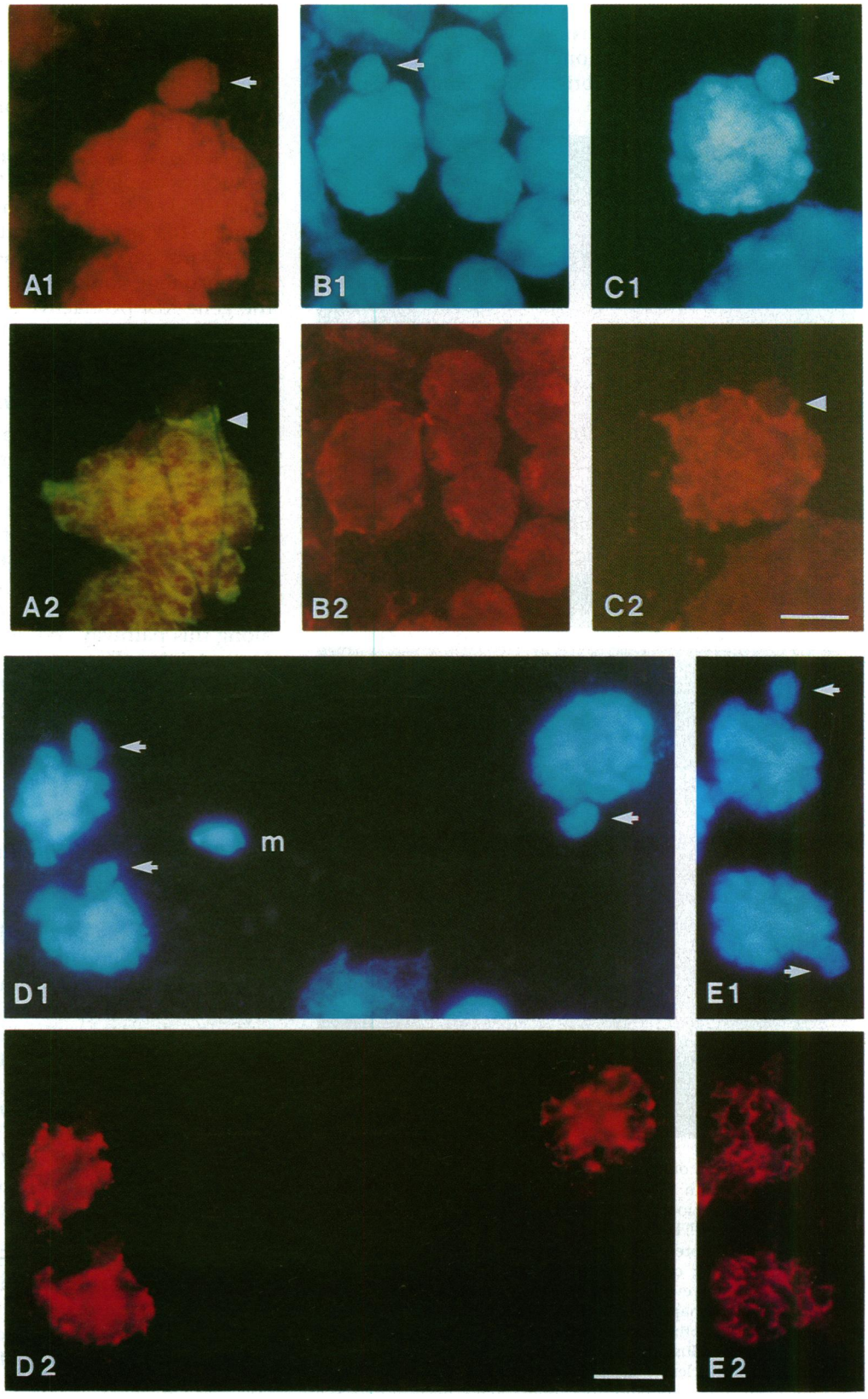

Figure 3. Splicing proteins are absent from the largely nontranscribing XY body at pachytene. (A1–E1) DNA staining. Propidium iodide (A1). DAPI (B1–E1). Arrows, XY compartments. (A2-E2) The same nuclei as in A1–E1 stained by anti-splicing protein antibodies. The XY compartments are not stained above background staining. The accumulation of presumably excluded splicing proteins in a chromatin-free region in-between the XY body and the autosomal compartment is especially well visible in A2 and C2 (triangles). (A2) Anti-Sm, FITC on propidium iodide background. (B2) Anti-Sm, Texas red. (C2) MAb 53/4, Texas red. (D2 and E2) Anti-SC35, Texas red. Note that D1 includes a mature sperm head (m), unstained by anti-SC35 antibodies (D2).

at the periphery of spread nuclei, apparent inlets free of chromatin (Figure 4, top, triangles) are filled with splicing components (Figure 4, bottom, triangles), an observation which may also be interpreted as an exclusion phenomenon. Figure 4 (bottom) also shows that splicing proteins are spread throughout the autosomal compartment in a network-like manner reminiscent of the interchromosomal channel network of somatic nuclei (Kramer *et al.*, 1994).

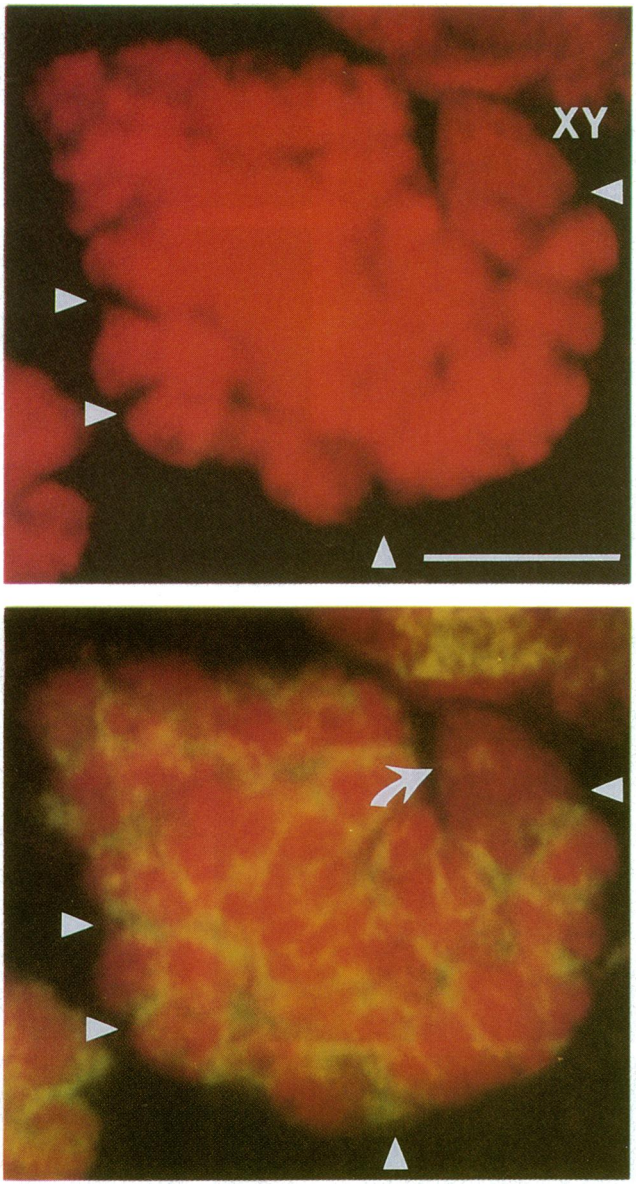

Figure 4. Exclusion of splicing components from the XY body and from dense chromatin. (Top) A pachytene nucleus stained for chromatin by propidium iodide; (Bottom) The same nucleus stained for the Sm antigens by FITC over a propidium iodide background. The absence of FITC fluorescence from the XY body (XY) indicates the exclusion of splicing components from this nontranscribing compartment. Note, however, the two fluorescent dots (bottom, curved arrow) resulting perhaps from transcription and splicing activity of the gene *Xist* in the otherwise inactive XY body (see text). Especially suggestive of the exclusion of splicing components are regions at the periphery of the nucleus and near the base of the XY body where apparent inlets into chromatin (top, triangles) are filled in by green signals arising from Sm antigens (bottom, triangles).

In some cases, the XY compartment of primary spermatocytes displays one or two fluorescent dots arising from SF53/4, SC35, and the Sm antigens (see Figure 4, bottom, for Sm). This limited, well-localized presence of splicing proteins in the generally nontranscribing compartment, could reflect the transcriptive activity of the gene *Xist* [X-inactive specific transcripts (Brown *et al.*, 1991)] in the testis (Richler *et al.*, 1992).

Splicing Proteins Distribution in Spermiogenesis

Along the pathway of sperm differentiation the shape of spermatid nuclei changes drastically. Starting from the ball-shaped nuclei of round spermatids, through the gradually elongating nuclei of elongated spermatids, the highly differentiated shape of the mature spermatozoon is reached. We observed that the intensity and extent of fluorescence of antibodies directed against SF53/4, SC35, and the Sm antigens, gradually decrease along this pathway. While round spermatids show intense staining (Figures 2B, 5, 6A, and 6B), in correlation with their transcriptive activity (Thomas *et al.*, 1989; Davies and Willison, 1993), elongated spermatids display only residual staining in patches (Figures 2B and 5), and mature spermatozoa show no staining at all (Figures 3D and 5).

The Presence of Splicing Proteins in the Chromatoid Body

Spermatocytes and spermatids possess a minute cytoplasmic body adjacent to the nuclear membrane. This body did not stain with the nuclear stains DAPI (Figure 5) and propidium iodide and was not detected by anti-DNA antibody (Figures 2B1 and 6B2), but it was strikingly visualized by antibodies against the splicing factors SF53/4 and SC35, and antibodies against the Sm antigens. Figure 5 (r) and Figure 6, A and B1, show spermatids following incubation with MAb 53/4 and anti-Sm antibody, respectively. The cells in Figures 2B2, 6A, and 6B1 display green FITC fluorescence of the extranuclear body while the nuclear signals, which appear over the red background of the Texas red anti-DNA antibody are yellow. This extranuclear body is, most likely, identical with the Sm-positive, DNA-negative chromatoid body earlier described (Biggiogera *et al.*, 1990, and literature cited therein). Anti-pol II antibody did not stain the chromatoid body. In a few spermatids, in which the nucleus was strongly fluorescent, the possibility of some staining of the chromatoid body could not be excluded, as the close proximity of the chro-

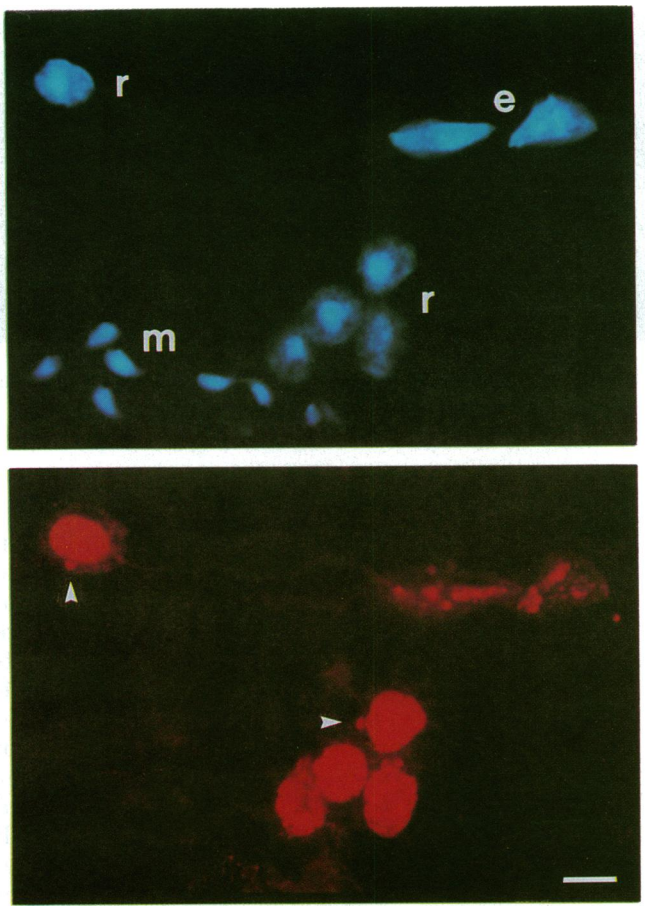

Figure 5. Two aspects of the same cells from the pathway of sperm maturation. The fluorescence signal of SF53/4 is gradually reduced as transcriptive activity diminishes along this pathway; *r*, round spermatids; *e*, elongated spermatids; m, almost mature spermatozoa. (Top) DAPI staining; (bottom) MAb 53/4 (Texas red). Note the unevenness of MAb 53/4 staining in the elongated spermatids, its total absence in the almost mature sperm, and the presence of chromatoid bodies (arrowheads) in the immunolabeled round spermatids and their nonstaining by DAPI (compare with Figures 2 and 6).

matoid body to the nuclear membrane made unambiguous determination of stained regions difficult.

DISCUSSION

Partitioning of Meiotic Nuclei

Colocalization of transcription and splicing has been demonstrated in numerous studies. A recent example, using viral transfection, even demonstrated a coordinated appearance of transcription and splicing components at sites of viral RNA transcription (Jiménez-Garcia and Spector, 1993). However, the question of whether cellular programmed cessation of transcription affects the nuclear organization of the splicing machinery has not yet been clarified. To address this question, we took advantage of the nuclear partitioning that occurs in spermatocytes early in meiosis.

In primary spermatocytes of groups with heterogametic males, sex chromosomes become transcriptionally inactive (e.g., Henderson, 1964; Monesi, 1965; Messthaler and Traut, 1975; Tres and Kierszenbaum, 1981), while the autosomes are intensively transcribing. The chromatin and axes of the sex chromosomes change appearance (Figure 1) in correlation with the cessation of transcription, forming an *XY body* (Solari, 1974). Two *major* compartments are thus defined in the meiotic nucleus: the *autosomal transcribing compartment* and the *XY nontranscribing compartment* (Richler *et al.*, 1989). This partitioning is a prominent feature of mid-late pachytene-, and of diplotene nuclei, despite the absence, in mammals, of membranes between the two compartments. The significance of the morphological and functional demarcation of the nontranscribing compartment is highlighted by observations made in exceptional chromosome situations. The existence of an XY body provides, e.g., a nuclear "shelter" for transcriptionally inactive B chromosomes in the rodent *Apodemus peninsulae* (Ishak *et al.*, 1991). Furthermore, autosomal segments ligated, through translocations, to sex chromosomes may become incorporated into XY bodies where they cease transcribing (Jaafar *et al.*, 1989). The functional aspect of this partitioning is shown by cases in which autosomal segments, although physically continuous with sex chromosomes, remain outside the sex body where they behave in accordance with their autosomal compartment location (Richler and Wahrman, unpublished observations on mouse X/autosome translocations). Strikingly, evolutionary-old, compound X chromosomes, like those of several species of the rodent genus *Gerbillus*, keep the origin of the various X chromosome segments in their cellular memory. During pachytene-diplotene, the original autosomal segments, now fused to the X chromosome, remain in the autosomal compartment, while the original-X chromosome segments form the sex chromosome body (see Figures 6, 11, 12, and 16 in Wahrman *et al.*, 1988).

Positional Aspects of Splicing Components in Partitioned Meiotic Nuclei

A large body of light and electron microscopy studies on the nuclear organization of the splicing machinery have demonstrated concentrations of splicing components in distinct subnuclear domains. While these studies have established the coupling between transcription and splicing, the physiological significance of the various patterns displayed by splicing components has not yet been clarified. These concentrations of splicing com-

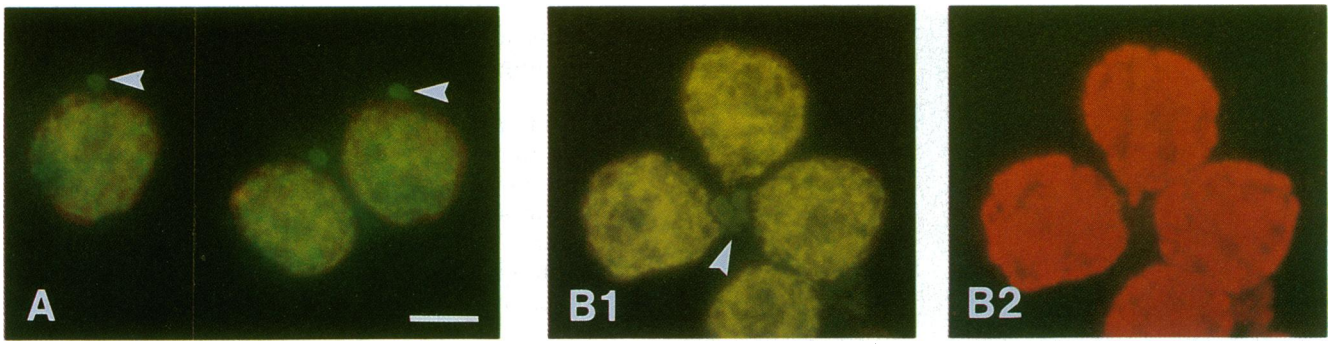

Figure 6. Round spermatids. Chromatoid bodies (arrowheads), extranuclear elements unique to spermatogenesis, are DNA-negative, but splicing protein-positive. (A and B1) Anti-Sm antibody, FITC. (B2) Anti-DNA antibody, Texas red.

ponents have been proposed to represent either sites of assembly of splicing components with nascent transcripts, or sites where splicing occurs, or storage compartments for splicing components where their catabolism may also occur (reviewed in Gall, 1992; Spector, 1993; Xing and Lawrence, 1993). Another intriguing controversial issue pertaining to the nuclear organization of the splicing machinery is the pathway and mechanism of transport of mRNAs from the transcribing gene to the cytoplasm (Rosbash and Singer, 1993). One model holds that the RNA processing events occur in an ordered fashion on defined tracks that lead from sites of transcription to nuclear pores (reviewed in Xing and Lawrence 1993). An alternative model argues that the intense nuclear signals represent concentrations of splicing components in association with nascent RNA and that the nuclear organization of these components, as well as their pathway out of the nucleus, is controlled by diffusion. The channels where nuclear pre-mRNAs are presumably processed and through which they are delivered to the nuclear pores are defined by exclusion from condensed nuclear structures (reviewed in Kramer *et al.*, 1994). Notwithstanding this controversy, it is clear that the nuclear sites where splicing components are concentrated belong to two classes. In sites belonging to the first class splicing components are associated with pre-mRNA transcripts, either nascent or partially processed (see INTRODUCTION). The second class comprises of sites that contain splicing components but no mRNA; these, presumably, play a role in storage of splicing components or as sites of their catabolism (for references see Gall, 1992). For brevity, we designate the first class as RNP sites and the second class as non-RNP sites.

Splicing may initiate at RNP sites containing nascent RNA, as splicing components assemble with pre-mRNA already during transcription (Gall, 1992; Spector, 1993). Indeed, specific examples of cotranscriptional splicing have been reported (Beyer and Osheim, 1988; Baurén and Wieslander, 1994). Is splicing completed within the nascent RNP sites, or does it continue in RNP sites that have been detached from sites of transcription and are on their way to the nuclear membrane? Evidently, different splicing patterns are expected for different individual transcripts, as the time point and kinetics of removal of a given intron depends on the strength of consensus sequences at its junctions with flanking exons, and on whether this particular splicing event is regulated (for references see Green, 1991; Moore *et al.*, 1993). It is therefore not surprising that incompletely spliced pre-mRNAs are found within RNP sites detached from their genes [e.g., the Balbiani ring 1 pre-mRNA of *Chironomus tentans* (Baurén and Wieslander, 1994), the *SgSLAC1* pre-mRNA in *Drosophila* (Zachar *et al.*, 1993) and CAD pre-mRNA in mammalian cells (Miriami *et al.*, 1994)]. Regarding the last example, it is interesting to note that the incompletely spliced CAD pre-mRNA is packaged in 200S lnRNP particles, which contain all known factors required for pre-mRNA splicing as integral components (Sperling *et al.*, 1985, 1986; Spann *et al.*, 1989; Sperling and Sperling, 1990; Ast *et al.*, 1991). Furthermore, almost the entire amount of nuclear CAD RNA, which is fully spliced or very close to maturation, is packaged in similar 200S lnRNP particles (Sperling *et al.*, 1985). These studies thus indicate that the structural integrity of the RNP particles that occupy the RNP sites is preserved throughout their way to the nuclear membrane.

How does cessation of transcription, like that of sex chromosomes, affect the nuclear distribution of splicing components? It might have been anticipated that cessation of transcription would cause the disappearance of RNP sites, which contain pre-mRNA. However, the fate of non-RNP sites, which are devoid of pre-mRNA, is less predictable. In the present paper we show that in adult fertile male mice the meiotic

sex chromosomes that are included in the essentially nontranscribing compartment are neither stained by anti-pol II antibody, nor by the antibodies to the splicing factors SF53/4 and SC35, and the Sm antigens. It follows that a large nuclear area where no transcription occurs does not contain components of the splicing machinery (see possible exception below)—whether or not associated with pre-mRNA. The splicing components appear excluded from the nontranscribing XY body and often are seen to accumulate in-between the XY body and the autosomal region (Figures 3, A and C, and 4).

How are splicing components distributed in the transcribing autosomal compartment? This compartment includes the autosomal bivalents, each possessing a central axis (the largely proteinaceous synaptonemal complex) from which chromatin loops spread out (Figure 1). The short, well-defined bivalents are surrounded by spaces occupied by splicing components forming a network-like pattern (Figure 4). It is possible that this pattern results from passive exclusion of RNA processing components and other clements from the axial and rather dense chromatin parts towards the periphery of the bivalents. Our observations on the XY body and the autosomes are consistent with previous findings in somatic interphase nuclei, suggesting that transcription and splicing components are localized in areas defined by exclusion from condensed nuclear structures (Zachar *et al.*, 1993; Zirbel *et al.*, 1993; Kramer *et al.*, 1994). The "exclusion principle" is thus extended to the meiotic system. Whether this exclusion forms the only basis for the intranuclear distribution of these components remains to be established.

An interesting gene in the context of this discussion is *Xist*, the only gene known to be transcribed exclusively from the inactive X chromosome (Brown *et al.*, 1991). The mRNA of this gene has been shown, in both man and mouse, to be spliced (Brown *et al.*, 1991; Brockdorff *et al.*, 1992). Since *XIST* (man) and *Xist* (mouse) are produced in the male by testicular tissue only (MacCarrey and Dilworth, 1992; Richler *et al.*, 1992; Salido *et al.*, 1992), the one or two fluorescent dots which are seen in some of the XY bodies (Figure 4, bottom) may reflect the splicing requirements of *Xist* during spermatogenesis. Further experiments are needed to show whether the observed sites displaying splicing proteins are indeed *Xist* related. Other possible candidates for activity in the XY body are genes escaping X inactivation in human female somatic cells, pseudoautosomal genes, and perhaps Y-linked genes required for spermiogenesis. Thus, the existence of splicing factors in a distinct small sub-domain located within the condensed XY body, from where the splicing machinery is generally excluded, should not be surprising, because the chromatin structure of a transcribing gene is expected to be decondensed and thereby accessible to splicing factors.

Positional Aspects of Splicing Components During Sperm Development

Haploid transcription at the early stages of spermiogenesis, especially in round spermatids, is intensive (Erickson, 1990), while transcription in succeeding elongated spermatids gradually decreases until it ceases in mature sperm (Davies and Willison, 1993). It was thus of interest to follow the distribution of splicing proteins along this sequence. Our observations show that while the strongest fluorescent signals of antisplicing factors 53/4, SC35, and anti-Sm were usually displayed over nuclei of round spermatids, the signals from later stages of spermiogenesis became less intensive and their distribution over nuclei became uneven until their eventual total disappearance (Figures 3D and 5). It thus seems that the positive correlation between the distribution of transcription activity and presence of splicing components generally holds also during haploid sperm differentiation. In this context it is pertinent to note that spermiogenesis displays a gradual condensation of chromatin (Davies and Willison, 1993). Therefore, the observed distribution of splicing components during spermiogenesis is also consistent with the notion that splicing components are excluded from condensed chromatin (Zachar *et al.*, 1993; Zirbel *et al.*, 1993; Kramer *et al.*, 1994).

Extranuclear Splicing Proteins

We have found in the present study splicing factors also at a DNA-free extranuclear location. These factors, SF53/4 and SC35, were detected in a well-defined cytoplasmic structure located near meiotic and spermatid nuclei (Figures 2B2, 5, and 6). The finding in this structure of splicing factors, as well as of Sm proteins (Figures 2B2 and 6; and Biggiogera *et al.*, 1990), establishes the extranuclear existence of proteins potentially involved in splicing in a spermatogenesis-specific organelle. As a functional designation for this organelle is not yet available, we use the last-century name *Chromatoid Body* (*chromatoider Körper*, Benda, 1891; quoted from Wilson, 1925), which has been applied in several recent papers to a minute organelle of fairly characteristic fine structure, located at the nuclear membrane. It includes the P1/P2 ribosomal proteins, hnRNP proteins, Sm proteins, and mRNA (e.g., Biggiogera *et al.*, 1990; Toppari *et al.*, 1991; Saunders *et al.*, 1992) and, as shown here, the splicing factors SF53/4 and SC35. The presence of hnRNP and splicing proteins in the chromatoid body is in line with early suggestions that it is extruded from the nucleus (for a summary of evidence for and against nuclear derivation; see Comings and Okada, 1972).

The list of known chromatoid body components together with its existence at a time interval in which transcription ceases, led to the suggestion that the chromatoid body is a storage device for mRNA species the products of which are required at the final stages of sperm formation, when transcription in the condensing nuclei ceases (Söderström and Parvinen, 1976). This hypothesis is supported by the finding in the chromatoid body of mRNA for transition protein 2 (TP2), an intermediate between histones and protamines required late in spermiogenesis (Saunders *et al.*, 1992). The possibility that the chromatoid body serves as an RNA processing organelle has also been raised, although no clear evidence is available on the presence of pre-mRNAs in it (Biggiogera *et al.*, 1990). Our finding of splicing factors in the chromatoid body extends the arsenal of potential RNA processing components that reside in this organelle. Further studies will show whether the chromatoid body is indeed a site of RNA processing, and perhaps translation, that has presumably evolved as an answer to the advent of nuclear quiescence and cytoplasm elimination—two important characteristics of late spermiogenesis. This concentration of nuclear splicing proteins outside the nucleus, away from a transcribing environment, is unique. Chromatoid bodies thus add their share to making the differentiation of sperm cells into a remarkable developmental pathway.

Concluding Remarks

The existence of splicing components at nuclear domains with transcriptional activity has been demonstrated in several instances (for recent reviews see Gall, 1992; Spector, 1993; Xing and Lawrence, 1993). These studies have established the coupling between transcription and RNA splicing in a transcribing environment. However, the presence of splicing components in large nontranscribing areas included in transcribing nuclei, was examined here for the first time. Primary spermatocytes present experimentally unperturbed cells, whose nuclei contain adjacent transcribing and nontranscribing areas with no barrier in-between. How is the silencing of transcription and RNA processing achieved in the nontranscribing compartment of such "transcription-chimeric" nuclei? In principle, silencing could have been achieved through inactivation of components involved in these activities, rather than by bringing about their absence. Our findings, nevertheless, indicate that the silencing of both transcription and splicing is correlated with chromatin condensation and is accompanied by the actual absence, presumably through exclusion, of RNA polymerase II and of splicing proteins from the silent areas. Such an extreme mechanism of silencing might have been critical for avoiding mistakes that could lead to unscheduled transcription in the sex chromosome compartment—the nuclear domain in which the expression of genes is generally not permitted.

ACKNOWLEDGMENTS

We thank Dr. J.G. Gall for valuable suggestions and comments on the manuscript.

REFERENCES

Arad-Dann, H., Isenberg, D.A., Shoenfeld, Y., Offen, D., Sperling, J., and Sperling, R. (1987). Autoantibodies against a specific nuclear RNP protein in sera of patients with autoimmune rheumatic diseases associated with myositis. J. Immunol. *138*, 2463–2468.

Ast, G., Goldblatt, D., Offen, D., Sperling, J., and Sperling, R. (1991). A novel splicing factor is an integral component of 200S large nuclear ribonucleoprotein (lnRNP) particles. EMBO J. *10*, 425–432.

Baserga, S.J., and Steitz, J.A. (1993). The diverse world of small ribonucleoproteins. In: The RNA World, eds. R.F. Gesteland and J.F. Atkins, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 359–381.

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

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

Biggiogera, M., Fakan, S., Leser, G., Martin, T.E., and Gordon, J. (1990). Immunoelectron microscopical visualization of ribonucleoproteins in the chromatoid body of mouse spermatids. Molec. Reprod. Develop. *26*, 150–158.

Brockdorff, N., Ashworth, A., Kay, G.F., MacCab, V.M., Norris, D.P., Cooper, P.J., Swift, S., and Rastan, S. (1992). The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell *7*, 515–526.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991). A gene from the region of the human X inactivation center is expressed exclusively from the inactive X chromosome. Nature *349*, 38–44.

Carmo-Fonseca, M., Pepperkok, R., Carvalho, M.T., and Lamond, A.I. (1992). Transcription-dependent colocalization of the U1, U2, U4/U6, and U5 snRNPs in coiled bodies. J. Cell Biol. *117*, 1–14.

Carter, K.C., Taneja, K.L., and Lawrence, J.B. (1991). Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. J. Cell Biol. *115*, 1191–1202.

Comings, D.E., and Okada, T.A. (1972). The chromatoid body in mouse spermatogenesis: evidence that it may be formed by the extrusion of nucleolar components. J. Ultrastruct. Res. *39*, 15–23.

Davies, P.O., and Willison, K.R. (1993). Molecular mechanisms of differentiation in mammalian spermatogenesis. Sem. Dev. Biol. *3*, 179–188.

Eilat, D., Hochberg, M., Pumphrey, J., and Rudikoff, S. (1984). Monoclonal antibodies to DNA and RNA from NZB/NZW F1 mice: antigenic specificities and NH2 terminal amino acid sequences. J. Immunol. *133*, 489–494.

Elicieri, G.L., and Ryerse, J.S. (1984). Detection of intranuclear clusters of Sm antigens with monoclonal anti-Sm antibodies by immunoelectron microscopy. J. Cell. Physiol. *121*, 449–451.

Erickson, R.P. (1990). Post-meiotic gene expression. Trends Genet. *2*, 264–269.

Fakan, S., Leser, G., and Martin, T.E. (1984). Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. J. Cell Biol. *98*, 358–363.

Fey, E.G., Krochmalnic, G., and Penman, S. (1986). The non-chromatin sub-structures of the nucleus: the ribonucleoprotein RNP-containing and RNP-depleted matrices analysed by sequential fractionation and resinless section electron microscopy. J. Cell Biol. *102*, 1654–1665.

Fletcher, J.M. (1979). Light microscope analysis of meiotic prophase chromosomes by silver staining. Chromosoma *72*, 241–248.

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

Gall, J.G. (1992). Organelle assembly and function in the amphibian germinal vesicle. Adv. Dev. Biochem. *1*, 1–29.

Gall, J.G., and Callan, H.G. (1989). The sphere organelle contains small nuclear ribonucleoproteins. Proc. Natl. Acad. Sci. USA *86*, 6635–6639.

Green, M.R. (1991). Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu. Rev. Cell Biol. *7*, 559–599.

Guthrie, C. (1991). Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science *253*, 157–163.

Henderson, S.A. (1964). RNA synthesis during male meiosis and spermiogenesis. Chromosoma *15*, 345–366.

Huang, S., and Spector, D.L. (1992). U1 and U2 small nuclear RNAs are present in nuclear speckles. Proc. Natl. Acad. Sci. USA *89*, 305–308.

Ishak, B., Jaafar, H., Maetz, J.L., and Rumpler, Y. (1991). Absence of transcription activity of the B-chromosome of *Apodemus peninsulae* during pachytene. Chromosoma *100*, 278–281.

Jaafar, H., Gabriel-Robez, O., and Rumpler, Y. (1989). Pattern of ribonucleic acid synthesis in vitro in primary spermatocytes from mouse testis carrying an X-autosome translocation. Chromosoma *98*, 330–334.

Jiménez-Garcia, L.F., and Spector, D.L. (1993). In vivo evidence that transcription and splicing are coordinated by a recruiting mechanism. Cell *73*, 47–59.

Krainer, A.R., and Maniatis, T. (1988). RNA splicing. In: Transcription and Splicing, eds. B.D. Hames and D.M. Glover, Oxford: IRL Press, 131–206.

Kramer, J., Zachar, Z., and Bingham, P.M. (1994). Nuclear pre-mRNA metabolism: channels and tracks. Trends Cell Biol. *4*, 35–37.

MacCarrey, J.R., and Dilworth, D.D. (1992). Expression of Xist in mouse germ cells correlates with X-chromosome inactivation. Nature Genetics *2*, 200–203.

Messthaler, H., and Traut, W. (1975). Phases of sex chromosome inactivation in *Oncopeltus fasciatus* and *Pyrrhocoris apterus* (Insecta, Heteroptera). Caryologia *28*, 501–510.

Miriami, E., Sperling, J., and Sperling, R. (1994). Mild heat shock treatment blocks the splicing of CAD pre-mRNA but not its packaging in lnRNP particles. Nucleic Acids Res. *22*, 3084–3091.

Monesi, V. (1965). Differential rate of ribonucleic acid synthesis in the autosomes and sex chromosomes during male meiosis in the mouse. Chromosoma *17*, 11–21.

Moore, J.M., Query, C.C., and Sharp, P.A. (1993). Splicing of precursors to mRNA by the spliceosome. In: The RNA World, eds. R.F. Gesteland and J.F. Atkins, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 303–358.

Raska, I., Andrade, L.E., Ochs, R.L., Chan, E.K., Chang, C.M., Roos, G., and Tan, E.M. (1991). Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. Exp. Cell Res. *195*, 27–37.

Richler, C., Soreq, H., and Wahrman, J. (1992). X inactivation in mammalian testis is correlated with inactive X-specific transcription. Nature Genetics *2*, 192–195.

Richler, C., Uliel, E., Rosenmann, A., and Wahrman, J. (1989). Chromosomally derived sterile mice have a 'fertile' active XY chromatin conformation but no XY body. Chromosoma *97*, 465–474.

Rosbash, M., and Singer, R.H. (1993). RNA travel: tracks from DNA to cytoplasm. Cell *75*, 399–401.

Salido, E.C., Yen, P.H., Mohandas, T.K., and Shapiro, L.J. (1992). Expression of the X-inactivation-associated gene *XIST* during spermatogenesis. Nature Genet. *2*, 196–199.

Saunders, P.T.K., Millar, M.R., Maguire, S.M., and Sharpe, R.M. (1992). Stage-specific expression of rat transition protein 2 mRNA and possible localization to the chromatoid body of step 7 spermatids by in situ hybridization using a nonradioactive riboprobe. Mol. Reprod. Dev. *33*, 385–391.

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

Söderström, K.-O., and Parvinen, M. (1976). Incorporation of [^{3}H]uridine by the chromatoid body during rat spermatogenesis. J. Cell Biol. *70*, 239–246.

Solari, A.J. (1974). The behavior of the XY pair in mammals. Int. Rev. Cytol. *38*, 273–317.

Spann, P., Feinerman, M., Sperling, J., and Sperling, R. (1989). Isolation and visualization of large compact ribonucleoprotein particles of specific nuclear RNAs. Proc. Natl. Acad. Sci. USA *86*, 466–470.

Spector, D.L. (1993). Macromolecular domains within the cell nucleus. Annu. Rev. Cell Biol. *9*, 265–315.

Spector, D.L., Fu, X.D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. EMBO J. *10*, 3467–3481.

Spector, D.L., Lark, G., and Huang, S. (1992). Differences in snRNP localization between transformed and nontransformed cells. Mol. Biol. Cell *3*, 555–569.

Sperling, R., Spann, P., Offen, D., and Sperling, J. (1986). U1, U2 and U6 small nuclear ribonucleoproteins (snRNPs) are associated with large nuclear RNP particles containing transcripts of an amplified gene in vivo. Proc. Natl. Acad. Sci. USA *83*, 6721–6725.

Sperling, R., and Sperling, J. (1990). Large nuclear ribonucleoprotein particles of specific RNA polymerase II transcripts. In: The Eukaryotic Nucleus, Molecular Biochemistry and Macromolecular Assemblies, eds. P.R. Strauss and S.H. Wilson, Caldwell, NJ: Telford Press *2*, 453–476.

Sperling, R., Sperling, J., Levine, A.D., Spann, P., Stark, G.R., and Kornberg, R.D. (1985). Abundant nuclear ribonucleoprotein form of CAD RNA. Mol. Cell. Biol. *5*, 569–575.

Steitz, J.A., Black, D.L., Gerke, V., Parker, K.A., Kramer, A., Frendewey, D., and Keller, W. (1988). Functions of the abundant U-snRNPs. In: Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles, ed. M.L. Birnstiel, Heidelberg: Springer-Verlag, 115–154.

Thomas, K.H., Wilkie, T.M., Tomashefsky, P., Bellvé, A.R., and Simon, M.I. (1989). Differential gene expression during mouse spermatogenesis. Biol. Reprod. *4*, 729–739.

Thompson, N.E., Aronson, D.B., and Burgess, R.R. (1990). Purification of eukaryotic RNA polymerase II by immunoaffinity chromatography. Elution of active enzyme with protein stabilizing agents from a polyol-responsive monoclonal antibody. J. Biol. Chem. *265*, 7069–7077.

Toppari, J., Kangasniemi, M., Kaipia, A., Mali, P., Huhtaniemi, I., and Parvinen, M. (1991). Stage-and cell-specific gene expression and hormone regulation of the seminiferous epithelium. J. Electr. Micros. Technol. *19*, 203–214.

Tres, L.L., and Kierszenbaum, A.L. (1981). Meiotic chromosomes of mouse spermatocytes: identification of bivalents, lampbrush organization, and transcription activities. In: Bioregulators of Reproduction, eds. G. Jagiello and H.J. Vogel, New York: Academic Press, 229–256.

Tsvetkov, A., Jantsch, M., Wu, Z., Murphy, C., and Gall, J.G. (1992). Transcription on lampbrush chromosome loops in the absence of U2 snRNA. Mol. Biol. Cell *3*, 249–261.

Vazques-Nin, G.H., Echeverria, O.M., Fakan, S., Leser, G., and Martin, T.E. (1990). Immunoelectron microscope localization of snRNPs in the polytene nucleus of *Chironomus thummi*. Chromosoma *99*, 44–51.

Wahrman, J., Richler, C., and Ritte, U. (1988). Chromosomal considerations in the evolution of the Gerbillinae of Israel and Sinai. In: The Zoogeography of Israel, eds. Y. Yom-Tov and E. Tchernov, Dordrecht: W. Junk, 439–485.

Walker, C.L., Cargile, C.B., Floy, K.M., Delannoy, M., and Migeon, B.R. (1991). The Barr body is a looped X chromosome formed by telomere association. Proc. Natl. Acad. Sci. USA *88*, 6191–9195.

Wansink, D.G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993). Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. J. Cell Biol. *122*, 283–293.

Wilson, E.B. (1925). The Cell in Development and Heredity. New York: Macmillan, 383.

Wu, C.-H.H., and Gall, J.G. (1993). U7 small nuclear RNA in C snurposomes of the *Xenopus* germinal vesicle. Proc. Natl. Acad. Sci. USA *90*, 6257–6259.

Wu, Z.A., Murphy, C., Callan, H.G., and Gall, J.G. (1991). Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle: loops, spheres, and snurposomes. J. Cell Biol. *113*, 465–483.

Xing, Y., and Lawrence, J.B. (1993). Nuclear RNA tracks: structural basis for transcription and splicing? Trends Cell Biol. *3*, 346–353.

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

Zahler, A.M., Lane, W.S., Stolk, J.A., and Roth, M.B. (1992). SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev. *6*, 837–847.

Zirbel, R.M., Mathieu, U.R., Kurz, A., Cremer, T., and Lichter, P. (1993). Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. Chromosome Res. *1*, 93–106.