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Expression of HLA Class I Genes in Meiotic and Post-Meiotic Human Spermatogenic Cells[1]

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ABSTRACT

In human spermatogenic cells, in contrast to somatic cells, expression of major histocompatibility complex (MHC) class I molecules is undetectable. This lack of expression may contribute to the absence of female immune reaction against spermatozoa and may be necessary for gamete fusion. Among the molecular repressor mechanisms that may be used at the DNA level, we investigated 5′ CpG methylation of the different class Ia and class Ib loci in meiotic pachytene spermatocytes and postmeiotic round spermatids, which had been purified from human testes by centrifugal elutriation. These results were compared with those obtained with mature spermatozoa and peripheral blood mononuclear cells. Using methylation-sensitive restriction enzymes and DNA locus-specific probes, we found that HLA-A, HLA-B/C, and HLA-E loci were similarly unmethylated in the germ and somatic cells tested, whereas HLA-F and HLA-G were even less methylated in the former cells. Together with the observation that spermatozoon DNA contains class I genes that are transfectable and able to direct transcription and protein synthesis in murine L cells, these data suggest that HLA class I genes are in an active conformation in male germ cells. We indeed found that both spermatocytes and spermatids contained low levels of class Ia and class Ib mRNA. Using reverse transcriptase-polymerase chain reaction, followed by DNA sequencing, we also detected three HLA-G transcriptional isoforms, resulting from alternative splicings, which suggested that this class Ib gene may have a potential function in these germ cells. Although intracellular expression of β_2-microglobulin (the light chain that associates with HLA class I heavy chains) was found in spermatocytes but not in round spermatids, no membrane-bound nor intracellular translated HLA class I heavy chain was detected in either germ cell type, when monomorphic anti-HLA class I monoclonal antibodies were used. Thus, lack of expression of HLA class I proteins in the male germ line is likely to involve post-transcriptional mechanisms of regulation.

INTRODUCTION

In the human species, three major histocompatibility complex (MHC) class I genes, designated *HLA-A*, *HLA-B*, and *HLA-C* and referred to as classical or class Ia genes, encode extremely polymorphic proteins that are expressed in most somatic tissues and whose major function is well established: they bind intracellularly derived peptides and present them to cytotoxic T cells. They contribute to the elimination by the immune system of autologous virally infected cells, cells that have undergone oncogenic transformation, or allogenic grafted cells [1]. The remaining MHC class I genes, designated *HLA-E*, *HLA-F*, and *HLA-G* and referred to as nonclassical or class Ib, encode less polymorphic molecules; their tissue distribution is more restricted, and their function is still unclear (reviewed in [2]). Of particular interest are the HLA-G products, which have been reproducibly detected in a subpopulation of trophoblast cells, suggesting that, among other mechanisms, they may play a role in maintaining immunological tolerance between mother and fetus [2]. Furthermore, there is increasing evidence to support the hypothesis that they may present antigenic peptides [3].

Human male germ cells do not express detectable amounts of class Ia molecules at their cell surface [4]. It is generally thought that this lack of expression may contribute to the relative immunological privilege observed in the testis [5]. Such an absence of HLA class I expression at the moment of fertilization may be necessary for gamete fusion and may prevent potential female alloimmune reaction against spermatozoa [4]. Little is known with respect to the expression of class Ib genes in human spermatogenic cells. Transcription of *HLA-G* has been detected in human testis [6] and in the testis of HLA-G transgenic mice [7]. A recent paper also mentioned the detection by reverse transcriptase-polymerase chain reaction (RT-PCR) of HLA-G mRNA in mature human spermatozoa [8]. The molecular regulatory mechanisms that control the absence of expression of class Ia molecules at the cell surface of male human germ cells are unknown. They may operate either at the transcriptional and/or post-transcriptional levels [2]. Among the various transcriptional mechanisms that may be used, we investigated the DNA methylation status of each MHC class I locus. All human class Ia and class Ib genes sequenced to date harbor CpG islands in their 5′ region and thus are rich in potential cleavage sites for methylation-sensitive, rare-cutter restriction enzymes [2]. We have demonstrated an inverse relationship between methylation status and transcriptional expression of the different class I genes in the human trophoblast-derived cell line JAR: all class I loci except *HLA-E* are methylated and repressed [9]. In this report, we first investigated the possible involvement of such DNA methylation in the absence of detectable ex-

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pression of class I genes in enriched populations of pachytene spermatocytes, round spermatids, and spermatozoa. Second, in view of the unmethylated status that class I genes exhibited in these spermatogenic cells, we then examined whether class I genes might be transcribed and eventually translated in male germ cells.

MATERIALS AND METHODS

Cell Lines

The human choriocarcinoma cell lines JAR and JEG-3 were obtained from the American Type Culture Collection (Rockville, MD). HHK is a human lymphoblastoid cell line [9]. These cell lines were grown in RPMI 1640 (Life Technologies, Inc., Cergy-Pontoise, France), supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HLA-A-expressing murine L transfectants are LMTK$^-$ cells that have been transfected with total genomic human sperm DNA and selected for HLA class I cell surface expression by fluorescent-activated cell sorting, using monomorphic anti-HLA class I W6/32 monoclonal antibody (mAb) [10]; they are referred to here as "L-transfectants." These transfectants were cultured in Dulbecco's Modified Eagle medium supplemented with hypoxanthine/aminopterin/thymidine, 10% FCS, and antibiotics. These transfectants express an HLA-A2.2 allelic variant, as determined by one-dimensional isoelectric focusing gel electrophoresis (M.G. Guttridge, unpublished data). HLA-G-expressing murine L transfectants are LMTK$^-$ cells that have been cotransfected with an HLA-G 6.0-kb *Hin*dIII genomic fragment (gift of Dr. H.T. Orr, Minneapolis, MN) and the pSV2-neo plasmid that contains the neomycin-resistant gene, using the calcium phosphate method [11]. Stable transfectants were selected by growth in 500 μg/ml of G418 (Life Technologies, Inc.).

Spermatogenic Cell Populations

Pachytene spermatocytes and round spermatids were collected from human testes obtained from patients undergoing therapeutic orchidectomy for metastatic carcinoma of the prostate. The patients had received no hormonal treatment before castration. Cell suspensions were prepared according to the method of Meistrich et al. [12] with the following modifications: decapsulated testes were rinsed in PBS, chopped into 1–2-mm^3 pieces with scalpels, and washed three times to remove the remaining blood. Pieces of one testis were incubated for 30 min at 32°C in PBS containing 0.1% trypsin and 20 μg/ml DNAse I. This enzymatic treatment was stopped by incubation with 20 μg/ml of soybean trypsin inhibitor (Sigma Chimie, Saint Quentin Fallavier, France). The tissue was again mechanically dissociated and incubated with agitation for 15 min at 32°C in PBS containing 0.05% collagenase type I and 20 μg/ml DNAse I. The cell suspension was filtered through nylon gauze (100-μm pore size) and then nylon wool to remove spermatozoa, and was centrifuged at 75 × *g* for 10 min. The cellular pellet was resuspended in PBS containing calcium and magnesium, and filtered through nylon gauze (20-μm pore size).

Germ cells were separated with an elutriator rotor (JE5 Beckman Instruments, Inc., Fullerton, CA) as previously described [12]. Elutriation conditions are outlined in Table 1. At the end of the elutriation procedure, phase contrast microscopy was used to select two cellular fractions corresponding to round spermatids and one fraction corresponding to pachytene spermatocytes (Table 1). DNA flow cytometry [13] was used to determine the relative DNA content of these germ cell fractions and therefore their respective enrichment (see *Results*, Fig. 1). After fixation in 70% (v/v) ethanol, cells were washed in PBS and incubated in 0.4% pepsin for 15 min at 37°C and in a mixture of 10 mg/ml of ribonuclease A (Sigma type 1-A), ethidium bromide (10 mg/ml), and 0.3% Nonidet P-40 for 30 min at room temperature. The suspensions were filtered through a nylon filter (70-μm pore size) and processed through a Cytofluorograph FACS-Scan (Becton-Dickinson, Immunocytometry Systems, San Jose, CA).

TABLE 1. Conditions for separation of spermatogenic cell fractions from human testis by centrifugal elutriation.

Rotor speed (RPM)	Flow rate (ml/min)	Collected volume (ml)	Cell fractions	Fraction numbers
3000	13.5	180[a]	Discarded	
3000	17.9	150	**Round spermatids**	I
3000	31.3	150	**Round spermatids**	II
2000	23.2	150	Discarded	
2000	30.0	150	Discarded	
2000	40.0	150	**Pachytene spermatocytes**	III

[a] 100 ml of the starting cell suspension was loaded in the chamber.

Human spermatozoa obtained from healthy, fertile donors were purified from contaminating cells as previously described [14]. From the same donors, fresh blood samples containing mononuclear cells, referred to here as peripheral blood leukocytes (PBL), were collected and treated as previously described [14].

Purification of Leydig Cells

Purification of Leydig cells from normal human adult testes was performed as described [15].

Flow Cytometry

Indirect immunofluorescence was performed as described [11]. The following primary murine mAbs were used: W6/32, monomorphic anti-HLA class I heavy chains associated with β_2-microglobulin (β_2m); B1.23.2, monomorphic anti-HLA-B,-C class I heavy chains associated with β_2m; B8.12 monomorphic anti-HLA class II; B2.62.2 and B1G6 anti-human β_2m [16]; HC10 monomorphic anti-denatured HLA-B,-C class I heavy chains [17], a gift from Pr. Hidde Ploegh, Cambridge, MA; CD45 anti-leukocyte common antigen (Immunotech, Marseille, France), and 10.3.6 anti-murine MHC class II IA^k [16], used as a negative control. After washings, cells were incubated with an F(ab')$_2$ goat anti-mouse IgG secondary antibody conjugated with fluorescein isothiocyanate (Immunotech). Dead cells, identified by uptake of propidium iodide, were excluded. Fluorescence was read with a Coulter EPICS-Elite flow cytometer (Margency, France).

Immunocytochemical Studies

Purified pachytene spermatocytes, round spermatids, or control HHK lymphoblastoid cells were cytocentrifuged, air-dried, and fixed with cold acetone for 5 min. Cytospun cells were incubated for 30 min at room temperature either with W6/32 or B1G6 mAbs or with a mouse IgG$_1$ negative control (Immunotech), washed three times in Tris-buffered

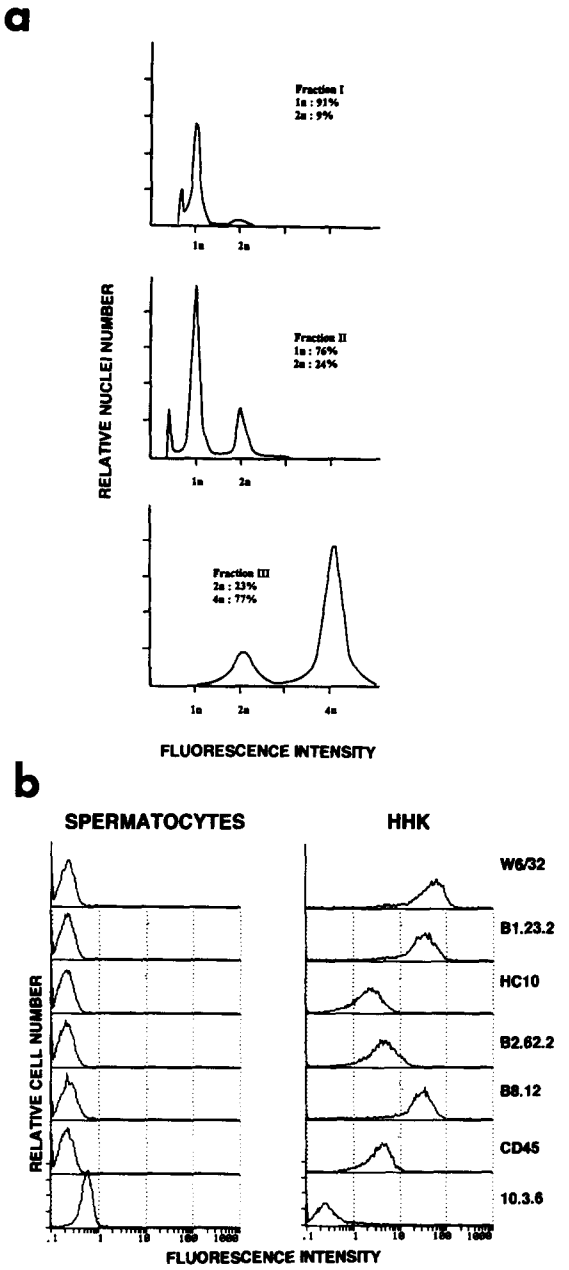


FIG. 1. a) Flow cytometry analysis of ethidium bromide-stained nuclei from human testicular spermatogenic cell fractions after centrifugal elutriation. b) Flow cytometry analysis of purified fraction III containing pachytene spermatocytes (left) and HHK lymphoblastoid cell line (right), stained with the following mAbs (from top to bottom): W6/32 (monomorphic anti-HLA class I), B1.23.2 (monomorphic anti-HLA-B, anti-HLA-C class I), HC10 (monomorphic anti-denatured HLA class I heavy chains), B2.62.2 (anti-human β_2m), B8.12 (monomorphic anti-HLA class II), CD45 (anti-leukocyte common antigen), and 10.3.6 (anti-murine MHC class II IA^k). Trypsin and collagenase treatments performed on the HHK cell line under the same conditions as those described in *Materials and Methods* for preparation of germ cells did not change the level of W6/32 binding (data not shown).

saline (TBS), incubated with a biotinylated F(ab')$_2$ goat anti-mouse IgG antibody (Immunotech) for 30 min, and washed twice in TBS. After incubation with a streptavidine alkaline phosphatase complex for 30 min at room temperature and two washes in TBS, positive reactions were visualized with a chromogen substrate. Slides were counterstained with the Mayer hematoxylin reagent.

Preparation of Genomic DNA, Restriction Enzyme Digestion, Southern Hybridization, and DNA Probes

Genomic DNA was prepared from cell lines, germ cells, or PBL, contained in agarose plugs (1×10^6 haploid cells, 5×10^5 diploid cells, or 2.5×10^5 tetraploid cells/plug), as described [11]. DNA was first digested with *Hin*dIII and then with one of the following methylation-sensitive restriction enzymes: *Bss*HII (recognition sequence, GCGCGC), *Eag* I (CGGCCG), *Mlu* I (ACGCGT), *Nar* I (GGCGCC), *Sma* I (CCCGGG), or *Sac* II (CCGCGG). Digestions, electrophoresis, blotting, and hybridizations were performed as previously described [11].

The following probes were used: a 1.2-kb HLA-A-specific probe derived from the 3′ untranslated region (3′UT) of the *HLA-A3* gene [11]; a 3′UT 0.55-kb HLA-B/C-specific probe derived from the *HLA-B7* gene [11]; a 33-bp antisense oligomer oLG2-C1 (aa 143 to 153) specific for *HLA-E* [18]; a 3′UT O.51-kb HLA-F-specific probe [14]; a 3′UT 0.45-kb HLA-G-specific probe derived from the *HLA-G/6.0* gene [7]; a 2.2-kb mouse β_2m-specific probe encompassing exons 2 and 3 and known to cross-react with the human β_2m [19]; and a 1.26-kb glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) rat cDNA probe [11]. DNA probes were labeled by the random hexamer priming method [11] with [α-^{32}P]dCTP (3000 Ci/mmol; DuPont-NEN, Les Ulis, France). Oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase (Boehringer Mannheim, Meylan, France) and [γ-^{32}P]ATP.

Northern Blot Analysis

Total RNA extraction, electrophoresis, blotting, and hybridizations were performed as described [11].

RT-PCR Amplification, cDNA Cloning, and Sequencing

Complementary DNA was synthesized from 2.5 μg of total RNA, extracted as above, with oligo(dT) primers and 200 U of reverse transcriptase, using a Superscript II preamp kit (Life Technologies Inc.). For HLA-G amplification, the PCR reaction was carried out as described [20], with use of the following HLA-G primers: Gc1.2 (5′-CCCAAGCTTCCCTGACCCTGACCGAGACC-3′, from the leader sequence) and Gc.2 (5′-CCGGAATTCG-GCTGGTCTCTGCAC-3′, from the 3′UT). Human β_2m primers were 5′β_2 (5′-CATTCCTGAAGCTGACAGCA-3′) and 3′β_2 (5′-CATGGTTGTGGTTAATCTGG-3′) from the exon 1 and the 3′UT of the human β_2m, respectively [21]. Amplification protocol for the β_2m cDNA synthesis was as follows: 94°C for 1 min, 55°C for 90 sec, and 72°C for 90 sec for 35 cycles. Reaction products were separated by electrophoresis in a 1.2% agarose gel and were stained with ethidium bromide after the run. The specificity of the PCR products was confirmed by alkaline blotting of the fragments onto an Appligene (Illkirch, France) positive membrane and hybridization with ^{32}P-labeled HLA-G-specific or mouse β_2m-specific probes.

Amplified cDNA products were purified on a Quiaquick-spin Promega column (Promega France, Charbonnières, France). The DNA fragments were ligated with the PGEM-T vector system I kit (Promega France) and sequenced with the femtomole DNA sequencing system (Promega France) with use of [γ-^{32}P]dATP.

RESULTS

Pachytene Spermatocytes and Round Spermatids, Purified from Human Testes, Do Not Express HLA Class I Molecules at Their Cell Surface

After centrifugal elutriation, cell DNA content of three spermatogenic fractions highly enriched in either tetraploid pachytene spermatocytes or haploid round spermatids were analyzed by flow cytometry (Fig. 1a). The enrichment of round spermatids (1n) was around 90% (fraction I) or 80% (fraction II), and the enrichment of pachytene spermatocytes (4n) was around 80% (fraction III). Fractions I and III were selected for further studies. Such degrees of purity allow a direct analysis of DNA methylation within individual genes in germ cells [22]. Absence of significant non-germ cell contamination in these fractions was further assessed by the lack of somatic cell surface markers (Fig. 1b): cells contained in fractions III (Fig. 1b, left profiles) and I (data not shown) did not bind any of the anti-HLA class I, HLA class II, human β_2m, and CD45 mAbs. In contrast, a human lymphoblastoid cell line reacted with all of these mAbs (Fig. 1b, right profiles). Furthermore, cells present in these two fractions did not bind CD4 (helper/inducer T-cell and monocyte marker) or CD8 (cytotoxic/suppressor T cell marker) mAbs (data not shown). Thus, it was shown that the two selected spermatogenic fractions were not contaminated by somatic cells, according to the three criteria of DNA cell content (Fig. 1a), absence of somatic cell surface markers (Fig. 1b), and morphology observed by phase contrast microscopy (data not shown). It was also established that HLA class I heavy and light chains are undetectable at the cell surface of both meiotic and postmeiotic germ cells.

The Unmethylated Status of HLA-A, -B/C, and -E Class I Genes Remains Unchanged during Spermatogenesis and Does Not Differ in Spermatogenic and Somatic Cells

To investigate whether DNA methylation may play a role in the absence of HLA class I expression in spermatogenic cells, we studied the methylation status of some selected CpG sites, present in the 5′ part of HLA class I loci, including the first three exons, in the genomic DNA of different germ cell populations and in spermatozoa. We first studied the methylation status of *HLA-A* in round spermatids (Fig. 2a). Genomic DNA was first digested with the *Hin*dIII enzyme and then with a methylation-sensitive restriction enzyme. After DNA fractionation by agarose gel electrophoresis and transfer, the blot was hybridized with an *HLA-A* locus-specific probe. After a single digestion with *Hin*dIII, the expected 5.1-kb *Hin*dIII fragment containing the *HLA-A* genes was detected. After a second digestion with either *Eag* I, *Nar* I, *Sma* I, or *Sac* II, the 5.1-kb *Hin*dIII fragments disappeared and gave rise to smaller bands, indicating that these CpG sites were cleavable and unmethylated. *Bss*HII and *Mlu* I sites do not occur within *HLA-A3* and the other HLA-A loci sequenced to date and, accordingly, after incubation of genomic DNA with these restriction enzymes, the 5.1-kb *Hin*dIII fragments were not modified. The methylation pattern of *HLA-A* in pachytene spermatocytes was identical (data not shown).

We then investigated the methylation status of the HLA-A2-expressing murine L cells that were transfected with total human sperm DNA and selected for HLA class I cell surface expression [10]. Filters containing either single- or double-digested genomic DNA were similarly hybridized with the HLA-A probe (Fig. 2b). A single digestion with *Hin*dIII or a double digestion with *Hin*dIII and either *Bss*HII or *Mlu* I revealed similar 5.1-kb *Hin*dIII fragments. After a double digestion with *Hin*dIII and either *Eag* I, *Nar* I, *Sma* I, or *Sac* II, the *Hin*dIII fragments disappeared and were replaced by smaller bands, indicating that these CpG sites were cleavable and thus were unmethylated in this sperm-derived *HLA-A2* gene.

We then analyzed the methylation status of *HLA-B/C* loci in spermatocytes and spermatids (Fig. 2, c and d). After a single digestion with *Hin*dIII, the expected 21-kb *Hin*dIII fragments containing the *HLA-B* genes were detected in both germ cell types. An upper band of 23 kb, corresponding to the *HLA-C* locus [9], was also observed in spermatids. This band is generally present in spermatocytes but, because of a shorter migration in this particular experiment, was almost unseparated from the 21.0-kb fragment. These *Hin*dIII fragments containing *HLA-B* and *HLA-C* disappeared after a second digestion with either *Bss*HII, *Eag* I, *Nar* I, *Sma* I, or *Sac* II, demonstrating that none of these sites were methylated in these class Ia loci. *Mlu* I digestion was partial in *HLA-B* but total in *HLA-C*, suggesting either that the single *Mlu* I site present in most of the *HLA-B* loci was partially methylated, most likely on one chromosome, or alternatively that some *HLA-B* loci not yet sequenced lack such a CpG site.

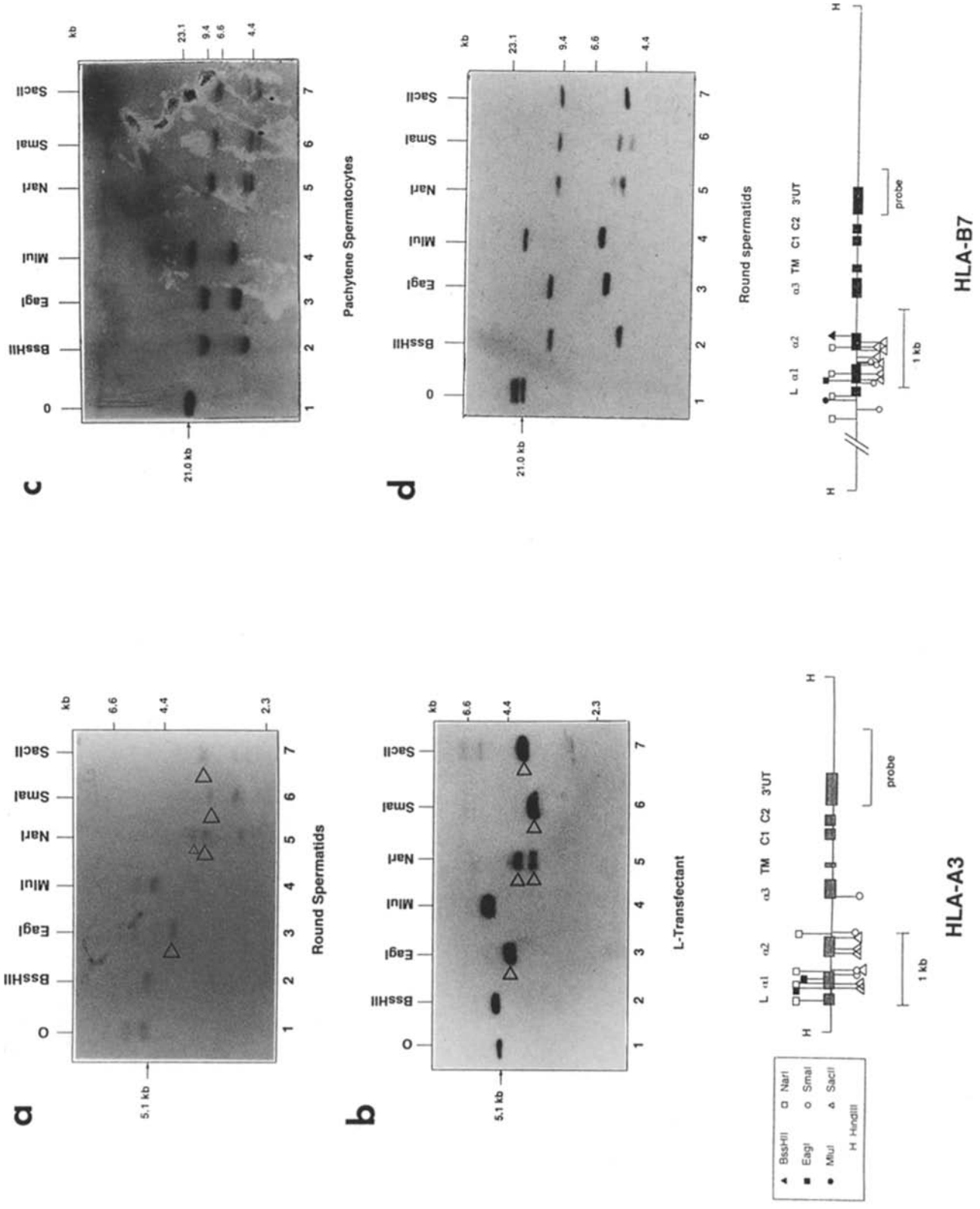
Finally, we investigated the methylation status of the *HLA-E* class Ib locus. After a single digestion with *Hin*dIII, the expected 6.2-kb *Hin*dIII fragment containing the *HLA-E* gene was obtained in both germ cell populations (data not shown). All the CpG sites tested that are present in the 5′ part of this locus (*Eag* I, *Nar* I, *Sac* II, and *Hpa* II/*Msp* I) were cleavable and thus unmethylated (data not shown).

These data showed that the unmethylated status of *HLA-A*, *HLA-B/C*, and *HLA-E* class I genes remain unchanged during the meiotic and postmeiotic stages and are similar to those observed in spermatozoa and in the somatic PBL [14].

Differences in the Methylation Status of HLA-F and HLA-G Class Ib Genes Are Detected Between Germ Cells and Somatic Cells

We have shown previously that several CpG sites in the 5′ part of *HLA-G* and *HLA-F* were unmethylated in human spermatozoa, but methylated in somatic cells from the same individuals [14]. We investigated whether the same CpG sites also appeared unmethylated in spermatocytes and

FIG. 2. Methylation patterns of *HLA-A* in round spermatids (**a**) and sperm L transfectant expressing HLA-A2.2 (**b**) and of *HLA-B/C* in pachytene spermatocytes (**c**) and round spermatids (**d**). Genomic DNA was digested by *Hin*dIII alone (lane 1) or double-digested by *Hin*dIII and one of the following methylation-sensitive rare-cutter enzymes present in the 5′ part of the HLA-A and/or HLA-B/C loci: *Bss*HII (lane 2), *Eag* I (lane 3), *Mlu* I (lane 4), *Nar* I (lane 5), *Sma* I (lane 6), and *Sac* II (lane 7). Enzymes used in the second digestion are given above each lane; Lane 1 ("0") shows the absence of second digestion. After electrophoresis and Southern blotting, filters were hybridized with HLA-A or HLA-B/C locus-specific probes derived from *HLA-A3* or *HLA-B7* genes, respectively [11]. The sizes of the *Hin*dIII fragments containing *HLA-A* or *HLA-B* loci are indicated on the left (arrows). DNA size markers (in kb) are shown on the right. Arrowheads on autoradiographs **a** and **b** point at the smaller fragments of *HLA-A* generated after double digestion. Restriction maps of the *HLA-A3* and *HLA-B7* genes with the *Hin*dIII (H) and the rare-cutter enzyme sites used in this study, as well as location of probes, are shown at the bottom. The black boxes indicate exons: 5′ exon 1 (L), left; 3′ exon 8 (3′UT), right.



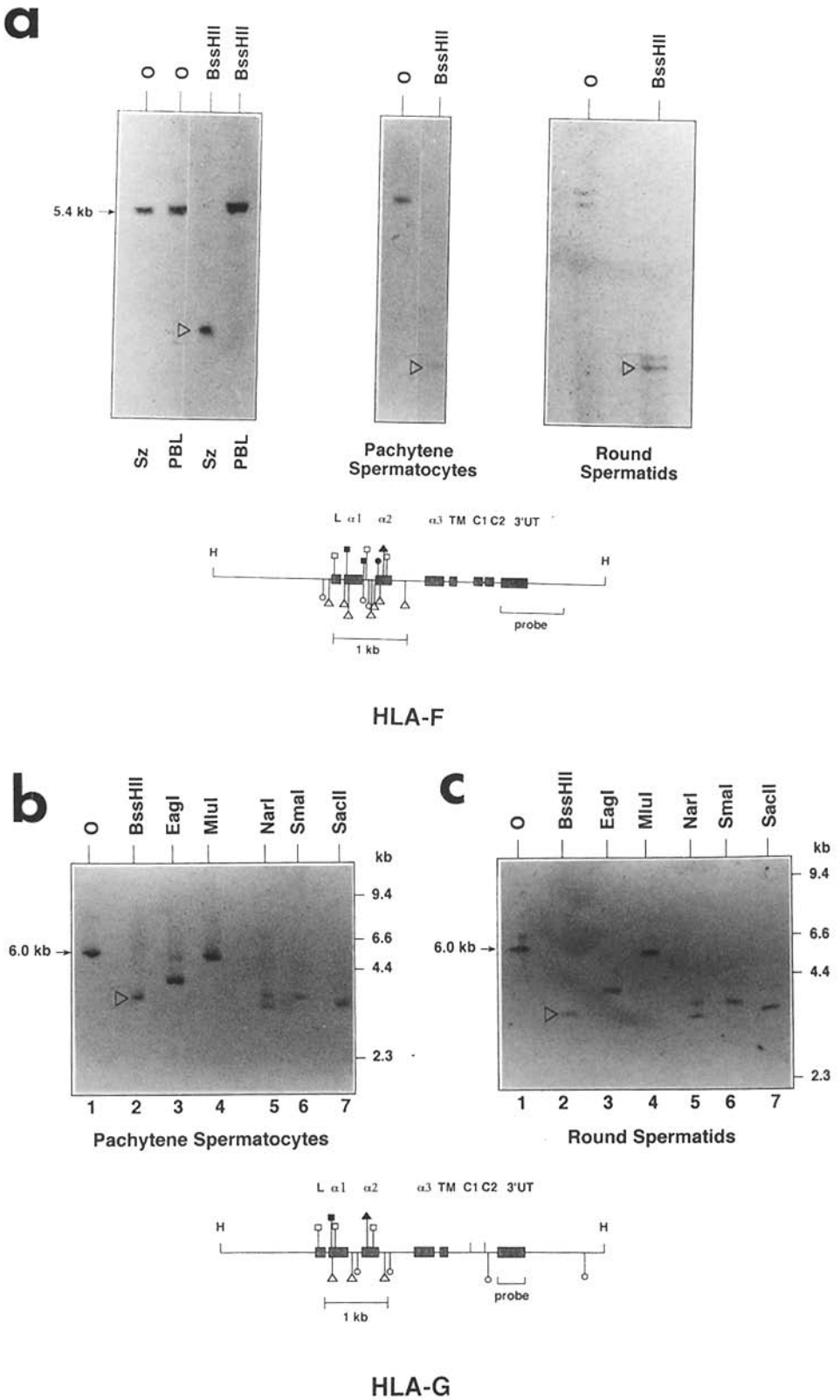


FIG. 3. **a**) Methylation status of *HLA-F* at the *Bss*HII restriction enzyme site in spermatozoa (Sz), PBL, pachytene spermatocytes, and round spermatids. Genomic DNA was digested by *Hin*dIII alone (lane O), or double-digested by *Hin*dIII and *Bss*HII (lane *Bss*HII). After electrophoresis and Southern blotting, filters were hybridized with the *HLA-F* locus-specific probe [14]. The size of the *Hin*dIII fragments containing the *HLA-F* gene is indicated on the left. The arrowheads point at unmethylated and cleavable *Bss*HII sites in germ cells. A restriction map of the *HLA-F* gene shows the *Hin*dIII (H) and rare-cutter enzyme sites used in this study (see Fig. 2 for restriction enzyme site legends), as well as the location of the HLA-F locus-specific probe from which it is derived. **b, c**) Methylation patterns of *HLA-G* in pachytene spermatocytes (**b**) and round spermatids (**c**). The same procedure as described in the Figure 2 legend was followed, except that hybridization was performed with an HLA-G locus-specific probe [7]. The size of the *Hin*dIII fragments containing the *HLA-G* gene is indicated on the left. The arrowheads point at unmethylated, cleavable *Bss*HII sites in spermatogenic cells. A restriction map of the *HLA-G* gene shows the *Hin*dIII (H) and rare-cutter enzyme sites, as well as the location of the HLA-G locus-specific probe from which it is derived [7].

spermatids (Fig. 3, a–c). After a single digestion of DNA with *Hin*dIII and hybridization with an HLA-F locus-specific probe, a 5.4-kb *Hin*dIII fragment was obtained in spermatozoa, pachytene spermatocytes, round spermatids, and PBL (Fig. 3a, lane O). A double digestion with *Hin*dIII and *Bss*HII revealed clear differences between spermatogenic cells and PBL: the *Bss*HII site present in *HLA-F* was cleavable (i.e., unmethylated) in spermatocytes, spermatids, and spermatozoa but resistant to cleavage (i.e., methylated) in PBL (Fig. 3a, lane *Bss*HII). Several cleavable *Nar* I and *Sac* II sites detected in the spermatogenic cells remained partially cleavable in PBL, whereas no difference was observed after digestion either with *Eag* I or *Sma* I (data not shown). Similar observations were made on the *HLA-G* lo-

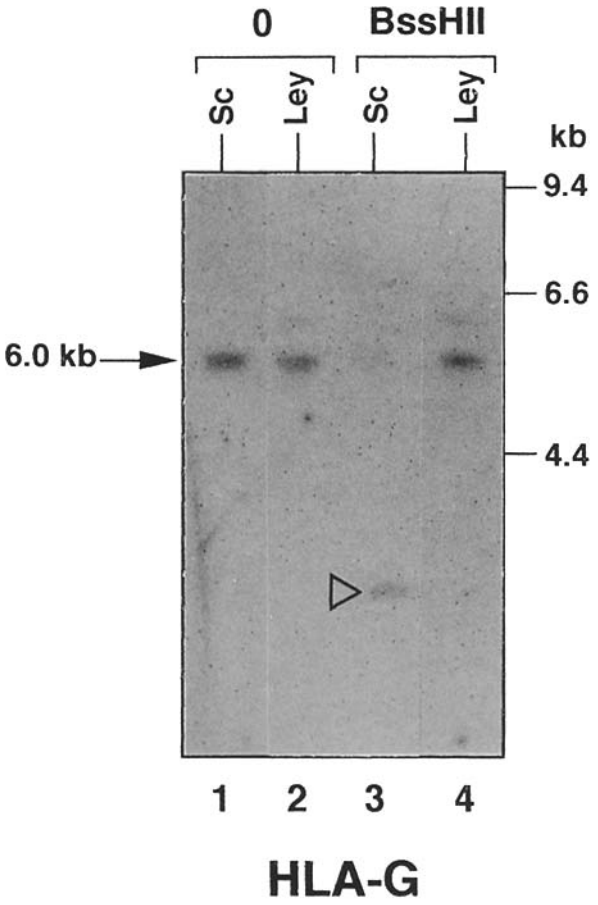


FIG. 4. Methylation status of *HLA-G* at the *Bss*HII site: comparison between pachytene spermatocytes (Sc) and Leydig cells (Ley). The same procedure was followed as that described in the Figure 2 legend, except that hybridization was performed with an HLA-G locus-specific probe [7]. DNA was digested either with *Hin*dIII alone (lanes 1 and 2) or double-digested with *Hin*dIII and *Bss*HII (lanes 3 and 4). The arrowhead points at unmethylated, cleavable *Bss*HII site in spermatocytes. The size of the *Hin*dIII fragments containing the *HLA-G* gene is indicated on the left.

cus. After a single digestion with *Hin*dIII and hybridization with an HLA-G locus-specific probe, a 6.0-kb *Hin*dIII fragment was obtained in spermatocytes and spermatids (Fig. 3, b and c, lane 1). After a second digestion with *Bss*HII, the single *Bss*HII site present in *HLA-G* was cleavable and thus unmethylated in pachytene spermatocytes and round spermatids, the 6.0-kb *Hin*dIII fragment being replaced by a shorter one (Fig. 3, b and c, lane 2); in contrast, we have already shown that the *Hin*dIII fragments were resistant to *Bss*HII cleavage and thus methylated in PBL [14]). After *Nar* I digestion, two unmethylated sites were detected in spermatogenic cells (Fig. 3, b and c, lane 5), whereas only one appeared in PBL [14]. No detectable difference was observed between these cells after *Eag* I, *Sma* I, or *Sac* II digestions (Fig. 3, b and c, lanes 3, 6, and 7). The *Mlu* I site is absent in HLA-G, and therefore the 6.0-kb *Hin*dIII fragment was never affected after incubation with this enzyme (Fig. 3, b and c, lane 4).

To determine whether the unmethylated status of specific CpG sites in the *HLA-F* and *HLA-G* class I genes was testis-specific rather than germ cell-specific, we performed a similar analysis on the DNA of Leydig cells, purified from human testis [15], as compared with the DNA of pachytene spermatocytes. Results obtained after hybridization with an HLA-G locus-specific probe are shown in Figure 4. After a single digestion with *Hin*dIII, the expected 6.0-kb fragment was found in both cell types (Fig. 4, lanes 1 and 2). After a double digestion with *Hin*dIII and *Bss*HII, the 6.0-kb HLA-G *Hin*dIII fragment was cleavable in spermatocytes (Fig. 4, lane 3), whereas it remained unmodified in Leydig cells (Fig. 4, lane 4). Similar results were obtained after hybridization with an HLA-F locus-specific probe (data not shown). Therefore, the *Bss*HII sites present in the *HLA-F* and *HLA-G* class I loci are similarly methylated in Leydig cells and PBL. Despite their testicular localization, Leydig cells maintain a "somatic methylation pattern" in these class Ib loci.

Class Ia, Class Ib, and β_2m Genes Are Transcribed in Pachytene Spermatocytes and Round Spermatids

The undermethylated status of all class Ia and class Ib loci in meiotic and postmeiotic spermatogenic cells prompted us to examine whether these genes could be transcribed in these germ cells, using Northern blot analysis. We first examined *HLA-A* and *HLA-B/C* transcription (Fig. 5, a and b). HLA-A and HLA-B/C transcripts were present in pachytene spermatocytes and round spermatids, although at low levels, and in the HHK control lymphoblastoid cell line, but at much higher levels. In contrast, no class Ia transcript was detected in the negative control JAR cell line.

Two HLA-E mRNA species of 1.8 and 2.6 kb were detected in each of the germ cell types and in all of the somatic cells studied, including JAR (data not shown). In contrast, using this method, we failed to detect any HLA-G transcript in spermatocytes and spermatids (data not shown). To investigate whether *HLA-G* could nevertheless be transcribed in germ cells at levels that are undetectable by Northern analysis, we performed an RT-PCR analysis, using HLA-G-specific primers. Complementary DNA amplified products from pachytene spermatocytes and round spermatids, as well as control JEG-3 and JAR cell lines, were analyzed by agarose gel electrophoresis, followed by ultraviolet visualization and Southern blot hybridization, using an HLA-G locus-specific probe (Fig. 5c). Three predominant bands of approximately 1200, 900, and 600 bp were detected in JEG-3 and in both types of germ cells by ethidium bromide staining (Fig. 5c, upper panel). It should be noted that a low intensity of the HLA-G2 band was always observed. These bands were hybridized with a radiolabeled HLA-G locus-specific probe (Fig. 5c, lower panel). In contrast, no HLA-G-specific cDNA could be amplified in JAR. Therefore, it is concluded that three HLA-G isoforms are transcribed in human testis germ cells.

We also analyzed β_2m transcription by RT-PCR, using primers from exon 1 and 3′UT regions (Fig. 5d). Among the several fragments obtained in the stained agarose gel (Fig. 5d, upper panel), a single band of 945 bp was specifically hybridized with a radiolabeled human β_2m-specific probe in germ cells and HHK but not in the mouse L-G cells (Fig. 5d, lower panel).

We then investigated whether the three HLA-G mRNA species detected in germ cells corresponded to the alternatively spliced isoforms previously described in several human somatic tissues [2, 6, 11, 20]. The HLA-G cDNA-amplified products from germ cells were purified, ligated with PGEM-T vector, and sequenced. Relevant portions of autoradiograms are presented in Figure 5, e and f. The full-length HLA-G1 isoform present in pachytene spermatocytes (Fig. 5e) and round spermatids (data not shown) was

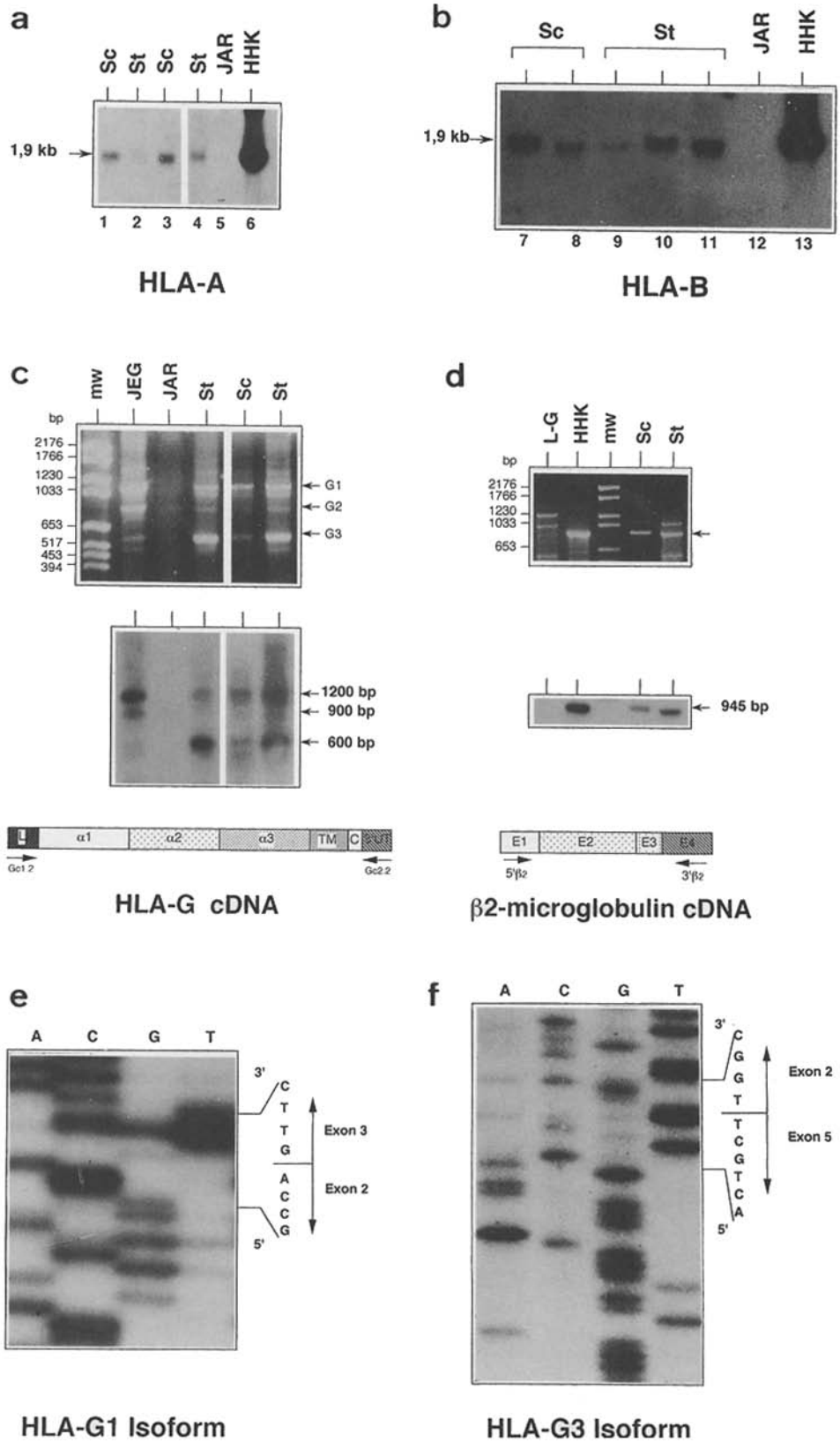


FIG. 5. **a, b**) Detection of classical HLA-A and HLA-B/C class I mRNA by Northern blot analysis. Total RNA (10 μg) from human pachytene spermatocytes (Sc) or round spermatids (St), purified from different human testes (lanes 1, 2, 7, 9; lanes 3, 4, 8, 10; and lane 11 correspond to germ cells purified from first, second, and third testes, respectively) as well as from JAR and HHK human control cell lines, was separated on a formaldehyde agarose gel, transferred onto GeneScreen membranes, and hybridized with ^{32}P-labeled class Ia probes: (**a**) HLA-A probe (lanes 1–6); (**b**) HLA-B/C probe (lanes 7–13). The sizes of mRNAs were calibrated with RNA markers. Ethidium bromide staining indicated that all lanes received a similar loading of RNA. **c**) RT-PCR amplification of HLA-G cDNA from human pachytene spermatocytes (lane Sc), round spermatids purified from two different testes (lane St), and from JAR and JEG-3 cell lines. Upper panel: ethidium bromide-stained gel. Size markers (lane mw) are indicated in bp. G1, G2, and G3 bands correspond to the three HLA-G isoforms described in the text. Middle panel: Southern blot hybridization with a radiolabeled HLA-G locus-specific probe derived from 3'UT of the *HLA-G 6.0* gene [7]. The arrows point to the HLA-G1 (1200 bp), HLA-G2 (900 bp), and HLA-G3 (600 bp) species. Bottom panel: The Gc1.2 and Gc2.2 primers used for cDNA amplification [20] are indicated in the HLA-G cDNA sequence from which they are derived. L (left), leader; α1, α2, α3, external domains; TM, transmembrane domain; C, cytoplasmic domain; 3'UT (right). **d**) RT-PCR amplification of β_2m cDNA from human pachytene spermatocytes (Sc), round spermatids (St), murine HLA-G-expressing L cells (L-G), and HHK human lymphoblastoid cell line. Upper panel: ethidium bromide-stained gel. Size markers (lane mw) are indicated in bp. The arrow points to the β_2m-specific band. Middle panel: Southern blot hybridization with a radiolabeled β_2m-specific probe. Bottom panel: 5'β_2m and 3'β_2m primers used for cDNA amplification are indicated below the β_2m cDNA sequence from which they are derived [21]. E1, E2, E3, and E4: exons 1, 2, 3, and 4. **e, f**) Sequence analysis of PCR-amplified cDNAs derived from pachytene spermatocyte mRNA. The autoradiograms show the respective cDNA sequences where alternative splicing has occurred to form the G1 (**e**) and G3 (**f**) mRNAs. The HLA-G1 isoform has been sequenced with the 5' primer and HLA-G3 with the 3' primer (complementary sequence: exon2–5'-GCCA/AGCAGT-3'-exon5).

similar to the previously described sequence [20]. The 900-bp isoform corresponded to that spliced out of exon 3 (data not shown). The 600-bp HLA-G3 isoform was also found to be alternatively spliced, leaving exon 2 in contact with exon 5, in pachytene spermatocytes (Fig. 5f) and round spermatids (data not shown). These data indicate that it is unlikely that the three HLA-G mRNA species herein detected are germ cell-specific HLA-G isoforms.

The results of both transcriptional expression and methylated status of the different HLA class Ia and class Ib

TABLE 2. Summary of the methylation status and transcriptional activity of HLA class I genes in pachytene spermatocytes (Sc), round spermatids (St), PBL and trophoblast-derived JAR cell line.*

Gene	Sources	*Bss*HII	*Eag* I	*Mlu* I	*Nar* I	*Sma* I	*Sac* II	Transcription
HLA-A	Sc	–	□	–	□	□	□	+
	St	–	□	–	□	□	□	+
	PBL[a]	–	□	–	□	□	□	+++
	JAR[a]	–	■	–	■	■	■	–
HLA-B/C	Sc	□	□	◪	□	□	□	+
	St	□	□	◪	□	□	□	+
	PBL[a]	□	□	◪	□	□	□	+++
	JAR[a]	■	■	■	■	■	■	–
HLA-E	Sc	–	□	–	□	–	□	+
	St	–	□	–	□	–	□	+
	PBL[a]	–	□	–	□	–	□	+
	JAR[a]	–	□	–	□	–	□	+
HLA-F	Sc	□	□	□	□	□	□	ND
	St	□	□	□	□	□	□	ND
	PBL[a]	■	□	◪	◪	□	◪	ND
	JAR[a]	■	■	■	■	■	■	ND
HLA-G	Sc	□	□	–	□	□	□	+[b]
	St	□	□	–	□	□	□	+[b]
	PBL[a]	■	□	–	◪	□	□	+[b]
	JAR[a]	■	■	–	■	■	■	–

[a] From Boucraut et al. [9] and Guillaudeux et al. [11, 14].
[b] Transcription only detectable by RT-PCR.
* Relative extents of methylation of the individual cutter sites most proximal to the 3′ probe are designated as follows: ■ = complete methylation; ◪ = partial methylation; □ = unmethylated; – = sites absent; ND = not done. Relative transcription levels are designated as follows: + = low transcription; +++ = high transcription.

genes in male germ cells as compared with PBL and the trophoblast-derived JAR cell line [9, 11] are summarized in Table 2.

Class Ia and Class Ib Transcripts Are Not Translated in Human Germ Cells, Whereas Intracellular β_2m Proteins Are Detectable in Spermatocytes

As shown above, using a panel of monomorphic anti-HLA class I and β_2m mAbs, we were unable to detect, by flow cytometry analysis, HLA class I and β_2m molecules at the cell surface of meiotic and post-meiotic spermatogenic cells (Fig. 1b). We thus wanted to know whether the class I transcripts detected by Northern blot (Fig. 5, a and b) or by RT-PCR analysis (Fig. 5c) could nevertheless be translated in intracellular proteins. We thus performed immunocytochemical analysis on cytocentrifuged, fixed pachytene spermatocytes and round spermatids, as compared with a control lymphoblastoid cell line (Fig. 6). After incubation with the negative control mouse immunoglobulin G1 (IgG_1), no staining was observed on either cell type (Fig. 6, a, d, and g). After incubation with the anti-HLA class I W6/32 mAb, HHK cells were clearly stained (Fig. 6b), whereas both pachytene spermatocytes (Fig. 6e) and round spermatids (Fig. 6h) remained unstained, demonstrating that HLA class I heavy chains were not translated in these spermatogenic cell subpopulations. After incubation with an anti-human β_2m mAb, HHK (Fig. 6c), but also pachytene spermatocytes (Fig. 6f), were clearly stained, whereas round spermatids appeared negative (Fig. 6i).

DISCUSSION

In view of the data presented in this paper, the following three major findings can be drawn and will be discussed: meiotic and post-meiotic spermatogenic cells 1) do contain HLA class I genes in an active conformation; 2) exhibit apparently functional class Ia and class Ib messages; and 3) do not produce detectable HLA class I heavy chain proteins although pachytene spermatocytes translate β_2m.

Spermatogenic Cells Do Contain HLA Class I Genes in an Active Conformation

Three pieces of evidence argue in favor of this possibility: 1) *HLA-A*, *HLA-B/C*, and *HLA-E* class I loci are similarly unmethylated in spermatogenic and somatic cells. Although our data gave information on a limited number of CpG sites, reproducible results were found among different individuals, correlating with a transcriptional activity. 2) *HLA-F* and *HLA-G* class I loci are even more demethylated in spermatogenic than in somatic cells. The absence of methylation at some CpG sites of these class Ib loci in spermatogenic cells and the presence of methylated cytosines at the same sites in somatic cells, including testicular Leydig cells, confirm that de novo methylation events must take place after fertilization during embryonal development [22, 23]. Moreover, unmethylated CpG sites were also detected in several parts of the HLA class I chromosomal region, outside the known loci, in the DNA of spermatozoa [14]. The rather unmethylated status of this chromosomal region in spermatogenic cells is surprising, as, up to the present work, it was thought that the DNA of spermatozoa was highly methylated, more than oocyte DNA, both in terms of overall methylation and of methylation of specific genes [24, 25]. Since we know that transcriptional expression of a particular gene is generally inversely correlated to the level of methylation of its CpG island [2], our observations suggested that some coding sequences present in this chromosomal region, including HLA class I genes, were transcriptionally active, and perhaps functionally important, during spermatogenesis. 3) HLA class I genes derived from DNA of spermatozoa are transfectable in mouse L cells and indeed can direct HLA class I transcription and protein synthesis [10], although this does not imply that these genes can do so in the testicular germ cells. Thus, it appears that HLA class I genes were in an active conformation throughout the spermatogenetic process, suggesting that neither early prophase meiosis, nor the beginning of spermiogenesis, exerts an influence at this DNA level.

Spermatogenic Cells Contain Apparently Functional Class Ia, Class Ib, and β_2m Messages

As indicated earlier, the undermethylated status of CpG islands is generally correlated with the transcriptional activity of associated coding sequences [2]. Although there is no definitive proof, the unmethylated status of class I loci in male germ cells might reflect at least a basal transcription. In this study, we first presented direct evidence that a transfected *HLA-A2.2* gene, derived from human spermatozoa and able to direct transcription and protein synthesis [10], exhibited an unmethylated status. We then demonstrated the presence of both class Ia and class Ib as well as β_2m transcripts in pachytene spermatocytes and round spermatids. These mRNAs are unlikely to derive from contaminating somatic cells since none of the different somatic cell surface markers tested, including CD4, CD8, CD45, β_2m, and MHC class I and class II antigens were ever detected. These data are in accordance with the previous report of Janitz et al. [26], who showed by in situ hybridization performed on human testis the presence of HLA class

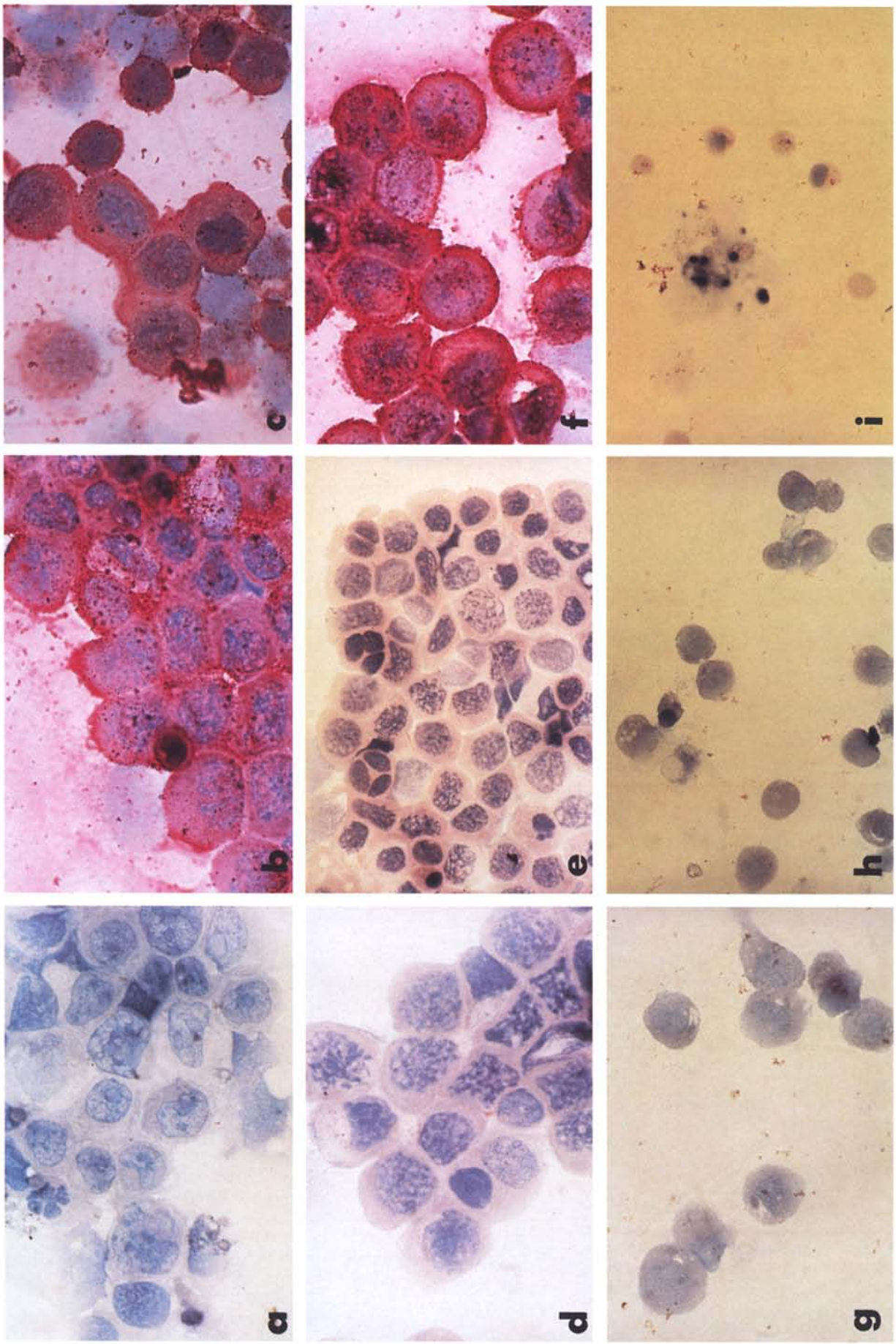


FIG. 6. Immunocytochemical analysis. Human lymphoblastoid cell line HHK (**a–c**), purified pachytene spermatocytes (**d–f**), and round spermatids (**g–i**) were acetone-fixed and cytospun. Cells were incubated with mouse IgG, negative control (**a, d, g**), or with W6/32 anti-HLA class I (**b, e, h**) or B1G6 anti-human β_2m (**c, f, i**) mAbs, followed by biotinylated goat anti-mouse-IgG. All samples were then incubated with alkaline phosphatase conjugate and substrate, and poststained with hematoxylin. Magnifications: (**a–d, f–g**) ×700; (**e, h**) ×450; (**i**) ×200.

I messages in the first layer of gametogenic cells in the peribasal compartment of the seminiferous tubules. Few other reports mentioned the presence of class Ia transcripts in human spermatogenic cells (reviewed in [4]). Thus, it appears that transcription of class Ia genes already occurs at the meiotic stage of spermatogenesis and is not modified thereafter. Whether or not transcription of class Ia genes also occurs in germ cells earlier than spermatocytes (i.e., spermatogonia) remains to be established. The presence of HLA-E and HLA-G class Ib transcripts in pachytene spermatocytes and in round spermatids was also shown, and a recent report has produced evidence of HLA-G mRNA in mature human spermatozoa [8]. Our data suggest that these transcripts can be stored from the early phase of the spermatogenetic process and still be present in spermatozoa.

Spermatogenic Cells Do Not Produce Detectable HLA Class I Proteins, Although Pachytene Spermatocytes Do Translate β_2m

Although we found class Ia and class Ib transcripts in pachytene spermatocytes and round spermatids, no cell surface or intracellular translated product was detectable in male germ cells by flow cytometry or immunocytochemical analysis, respectively, with use of monomorphic anti-HLA class I mAbs. However, the possibility that at least some HLA-G protein isoforms that could not be detected by such antibodies were present in these germ cells cannot be excluded. The use of anti-HLA-G-specific mAbs, when available, will help to answer this question. We also cannot exclude the possibility that such HLA class I proteins are present at low levels in spermatogonia or early germ cells, as has been reported in the rat testis [27] and in transgenic mice carrying an allogenic MHC class I transgene under the control of the c-*fos* promoter [28]. However, the regulation of class I gene expression in human spermatocytes and spermatids is likely to occur at the translational level, which would not be surprising, given the knowledge that as much as 60–75% of the total RNA from mouse testis may not be associated with polysomes [29]. Many spermatogenic cell genes are indeed subject to translational regulation [30]. This is the case for cytochrome c_T, a mitochondrial electron transport protein of spermatogenetic cells. Cytochrome c_T transcripts in mouse pachytene spermatocytes are associated with polysomes and are translated, whereas transcripts in round spermatids are present predominantly in nonpolysomal fractions [31]. A post-transcriptional blockage in synthesis, assembly, or transport of class I proteins may be responsible for their deficient expression. The molecular regulatory mechanisms involved remain to be elucidated.

We have shown that both pachytene spermatocytes and round spermatids transcribe β_2m but that the corresponding protein product was detectable only in the former cells. A relationship has been established between the concentration of β_2m in seminal plasma and sperm number [32]. The origin and function of this protein in the genital tract remains to be determined.

Biological Significance of HLA Class I Messages in Spermatogenic Cells

Although no translated product has been detected in human spermatogenic cells, we have shown that class Ia and class Ib transcripts are present during spermatogenesis. Such a reservoir of MHC class I messages in male germ cells is likely to have biological significance. One report mentioned a stable presence of class I proteins on spermatogenic cells obtained from infertile individuals [33]. The presence of class I transcripts in germ cells should make possible a translation following viral infection or tumor appearance in the testes or after disruption of the spermatogenetic process. Expression of HLA class I molecules on virally infected or tumoral cells is vital for CD8+ T cell recognition of viral or tumoral antigenic peptides and subsequent lysis of target cells [2]. It has been shown that lymphocytes could appear in large numbers in the testis of HIV-1 seropositive men [34]. Although MHC class I molecules were not detected at the cell surface of human preimplantation embryos at the 2- to 8-cell stage and no MHC class I transcript was detected in unfertilized murine oocytes [4], one can hypothesize that the male-derived HLA-G class I transcripts may, after implantation, direct an early protein synthesis in restricted cell populations, such as the extravillous invasive cytotrophoblast, in which translated HLA-G products have been reproducibly detected [2]. This would contradict an accepted belief that early protein synthesis is directed solely by maternally derived mRNA, but it has been shown that the paternal genome contributes preferentially to the normal development of extraembryonic tissues [35].

Taken together, the data presented in this study confirm the powerful regulatory mechanisms that control both transcriptional and translational expressions of each HLA class Ia and class Ib gene during the spermatogenetic process.

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