LACK OF EXPRESSION OF HLA-B27 GENE IN TRANSGENIC MOUSE TROPHOBLAST

Conserved Genetic Pressures Underlying Extra-Embryonic Development

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Embryonic cells show developmentally regulated, differentiation-dependent expression of classical MHC class I genes (*HLA-A,B,C* in man; *H-2-K,D,L* in mouse) (1). Maturation of embryonic cells is generally accompanied by an increase in expression of MHC class I genes (1). In contrast, extra-embryonic trophoblastic cells show cell determination–dependent lack of expression of MHC class I genes: human chorionic villus trophoblasts do not express classical MHC class I antigens under any conditions, neither in vivo nor in vitro (2). Cell determination (trophectoderm determination) leads to a lack of expression of HLA-A,B (2).

Extravillous human trophoblasts, previously thought to express classical MHC class I heavy chains, have recently been demonstrated to express β_2-microglobulin-associated 40,000 dalton glycoproteins (3) which may be an equivalent of the murine MHC class I–linked (*Tl/Qa*-like) gene cluster (4). As for villous trophoblast, lack of expression for *HLA-A* and *-B* genes is retained in extravillous trophoblast (4).

Experimental data from murine embryonal carcinoma *F9* cells, which exhibit various properties of early embryos (5), suggest that developmental control of classical MHC class I genes in embryonal cells is controlled by a *cis*-acting regulatory DNA sequence located in the 5'-flanking region (5). This region called class I regulatory element (*CRE*) functions as a repressor or silencer of adjacent MHC class I promoter sequences in undifferentiated cells, but as an enhancer in differentiated cells. The mechanisms that control MHC class I gene expression in extra-embryonic trophoblast, in particular in extravillous trophoblast, are unknown at present. Exploration is complicated because purified cell suspensions of normal extravillous trophoblasts are difficult to obtain and to culture. The validity of carcinoma cell lines as substitutes remains to be established. Transgenic mice offer a better alternative for these and other types of genetic studies because, within certain limits, gene expression is subject to more physiological control. In this light, the recent establishment of transgenic mice with functional complementation of the genes encoding heavy (*HLA-B27*) and light (β_2-microglobulin) chains of HLA antigens (6) provide a convenient

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experimental model to study genetic regulation of MHC class I gene transcription in developing extra-embryonic cells, in particular in trophoblasts.

By applying in situ hybridization with biotinylated RNA probes complementary to HLA-B mRNA on tissue sections of embryonic and extra-embryonic tissues from transgenic mice (13.5 d after coitus), lack of expression of HLA-B genes was seen to be retained in transgenic trophoblast. In contrast, *HLA-B27* gene transcripts were readily detected in embryonic cells. This indicates that developmental control of MHC class I genes in extra-embryonic trophoblast is under conserved selective pressure that is retained across a species barrier. The regulatory mechanisms that control MHC expression in extra-embryonic trophectoderm are different from those operating in developing embryonic cells.

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Materials and Methods

Tissues. Female (*CBAxC57Bl/LiA*)F_1 mice carrying the human *HLA-B27* gene (4.3-kb Eco RI fragment of *HLA-B27K* genomic DNA) were mated with F_1 males harboring the human β_2-microglobulin gene (6). Embryonic and extra-embryonic tissues with adjacent decidua and myometrium were obtained at day 13.5 after coitus and fixed in 4% buffered formaldehyde for 18 h at 4°C, followed by conventional tissue processing and embedding in low melting point paraffin (T_m 52–54°C) (Paraplast).

Biotinylated RNA Probes. Eco RI–Hind III–digested 358-bp fragments of pHLA-1.1 (7) were obtained and purified as described (4). pHLA-1.1 contains 3′-untranslated sequences only and shows *HLA-B* locus specificity in Northern, Southern, and tissue hybridization under conditions of high stringency (0.1 × SSC, 68°C) (8). Fragments were ligated to Eco RI–Hind III sites of pGEM3 Blue vectors (Promega Biotec, Madison, WI) and used for transformation of competent *Escherichia coli* JM109. Plasmids were isolated from small (3 ml) overnight cultures of white colonies by a modified alkaline extraction procedure. Biotinylated RNA probes, both sense and antisense, were generated from linearized templates by promoter-mediated in vitro transcription according to Melton et al., (9) by using biotin-11-UTP (Enzo Biochem, New York, NY) and either SP6 or T7 RNA polymerase (Promega Biotec). Reaction mixtures contained 40 mM Tris, pH 7.5, 6 mM magnesium chloride, 2 mM spermidine, 10 mM DTT, 0.5 mM each of ATP, CTP and GTP, 1 mM biotin-11-UTP (Enzo Biochemicals), 200 U human placental ribonuclease inhibitor (RNasin) (Promega Biotec), 15 U of either SP6 of T7 RNA polymerase (Promega Biotec) and 1 μg linearized DNA template. Incubations were for 60 min at 37°C. Templates were removed by treatment with RNase-free RQ1 DNase (Promega Biotec) (1 U/μg DNA, 15 min, 37°C). Unreacted nucleotides were removed by chromatography through Sephadex G50 DNA grade columns (LKB Pharmacia, Bromma, Sweden). Elution was done with 10 mM Tris, pH 8.0, 1 mM EDTA, and 0.1% SDS. Labeling intensity was 32% (percentage of number of biotin molecules per total number of nucleotides) for antisense and 21% for sense probes. Both probes included 24 bases (6.7%) of vector sequences.

Tissue Hybridization. In situ hybridization was performed nonautoradiographically by using streptavidin-labeled colloidal gold (5 nm) with silver enhancement (Jansen Life Science Products, Beerse, Belgium) for detection and confocal laser scan microscopy (Zeiss, Oberkochen, FRG) with reflex contrast for visualization. This hybridization method is described in detail elsewhere (4). In brief, formalin-fixed secetions (3 μm) on poly-L-lysine (0.1%)-coated slides were dewaxed extensively, rehydrated, pretreated with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN), and postfixed with phosphate-buffered paraformaldehyde before hybridization. Hybridization was done for 2 h at 50°C in the presence of 50% (vol/vol) deionized formamide, 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate), 10% (wt/vol) dextran sulphate and Bacillus RNA (50 μg/ml). After hybridization, sections were washed in SSC and treated with RNase A. RNA-RNA hybrids were detected immunocytochemically by using streptavidin-labeled colloidal gold (5 nm) and silver enhancement. Conditions of stringency (final wash after hybridization in 0.1 × SSC for 60 min at 68°C) assures specific

hybridization of transgene transcripts with no crossreactions with endogenous murine *H-2-K,D* and *L* transcripts. Sections were counterstained with hematoxylin, dehydrated, and embedded in Depex.

Visualization. Images were visualized by using confocal laser scan microscopy (Zeiss) with an antiflex lens (×68) (reflex contrast). The advantages of confocal laser scan microscopy over conventional light microscopy have been described elsewhere (10). Increased light sensitivity and higher spatial resolution permit the detection of minor changes in a more specific, sensitive and accurate way (10). The confocal laser scan microscope used by us is a type II laser scan microscope with a synchronously scanned pinhole diaphragm in the detector beam path, a servo-controlled galvanometer scanner and a dual laser system (Ar, 488 nm; HeNe, 633 nm) (Zeiss). Resolution in lateral direction is about 0.2 μm. Depth of field is ~0.6–0.8 μm using high numerical aperture lenses. Digital image processing of scanned images was used to calculate experimental conditions optimal for hybridization and immunocytochemical efficiency and specificity. This procedure will be described in detail elsewhere (Oudejans, C. B. M., J. P. A. Baak, T. ten Kate, and C. J. L. M. Meijer, manuscript in preparation).

Results

Tissues from 13 transgenic (*HLA-B27*) mouse embryos (day 13.5 after coitus) with adjacent placenta, decidua and maternal uterus horn tissues were studied for the transcription of the *HLA-B27* gene by in situ hybridization. A schematic representation showing the organization of embryonic and extra-embryonic tissues in relation to each other and to the maternal surroundings is given in Fig. 1. The cell lineage relationships of embryonic and extra-embryonic tissues are given in Fig. 2. In situ hybridization was done with biotinylated antisense probes. Identically generated sense probes were used as negative controls. Probes were generated by promotor-mediated transcription of *HLA-B* locus-specific cDNA pHLA-1.1 templates (8). Transgenes segregated in a Mendelian fashion (6) and from 13 offspring (harvested as embryos); 6 obtained the *HLA-B27* transgene as evidenced by the presence of HLA transcripts

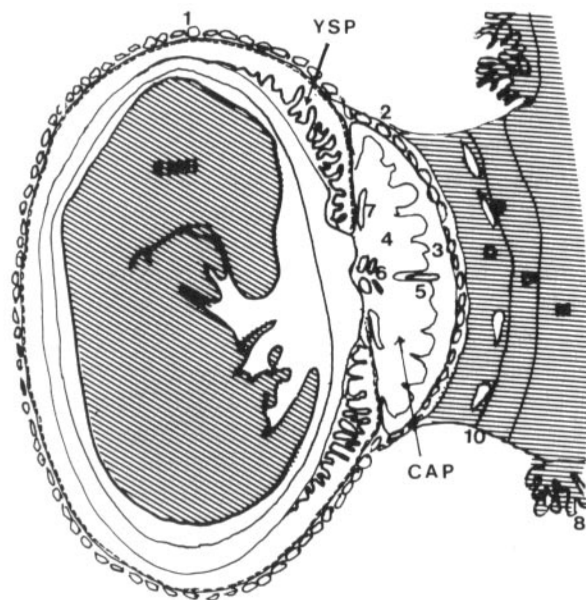

FIGURE 1. Longitudinal section through embryonic and extra-embryonic tissues of mouse embryo at 13.5 d of development. EMB, embryo(nic tissues); YSP, yolk sac placenta; CAP, chorio-allantoic placenta; D, decidua; M, uterus horn myometrium. *1*, Primary trophoblast giant cells; *2*, secondary trophoblast giant cells; *3*, spongiotrophoblast; *4*, labyrinthotrophoblast; *5*, maternal arterial channel; *6*, fetal umbilical vessels; *7*, endodermal sinuses; *8*, maternal uterus horn glands; *9*, maternal venous sinuses; *10*, compact decidua layer. See also Fig. 6 for cell lineage relationships.

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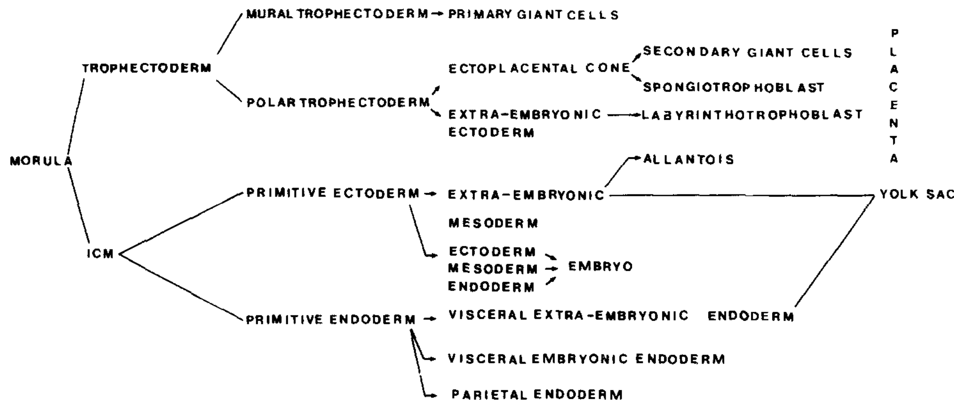

FIGURE 2. Cell lineage relationships of embryonic and extra-embryonic tissues during early mouse development (12).

in the latter but not in the remaining 7 mouse embryos. Maternal tissues were positive in all cases (Fig. 3). In these six positive embryos, organ-specific transcription levels were seen to vary. Quantitative assessment of these intrinsic variations will be described elsewhere (Oudejans, C. B. M, et al., manuscript in preparation). A representative experiment showing *HLA-B* mRNA in the cytoplasm of intestinal epithelial cells is shown in Fig. 4. In the placenta, however, trophoblastic cells, being either spongio-, labyrintho-, or trophoblast giant cells were consistently negative for *HLA-B27* gene transcription in all cases studied (Fig. 5). In contrast, placental stromal and endothelial cells did contain *HLA-B27* mRNA (Fig. 5). Moreover, visceral yolk sac endoderm was positive for *HLA-B27* transcripts (Fig. 6). Control experiments performed identically with sense-RNA probes confirmed the specificity of the reactions (Fig. 4c).

Discussion

Trophectoderm generation is likely to involve genetic mechanisms that modulate developmentally important control genes in a trophectoderm cell lineage-specific fashion. The genes and/or mechanisms that regulate their expression evolved for the sake of extra-embryonic development. Therefore, commitment of precursor cells to the development into trophoblast (trophectoderm determination) appears to be under selective genetic pressures, likely to be different from those operating in developing embryonic cells (11). For immunological reasons, the study of MHC expression in developing embryos has received considerable attention (1, 5). Here we show that in those extra-embryonic cells that are directly exposed to the maternal immune system, there is a complete lack of expression of classical MHC class I heavy chain gene products. The lack of expression of classical MHC class I genes in transgenic trophoblast across a species barrier is an example of the existence of a tissue/cell type-specific regulation. Trophoblastic cells did not transcribe the *HLA-B27* transgene despite the fact that embryonic cells of the same gestational age contained easily detectable levels of *HLA-B27* mRNA. Moreover, mother and positive embryos carry the same *HLA-B27* transgene and are isogenic with respect to MHC class I antigen

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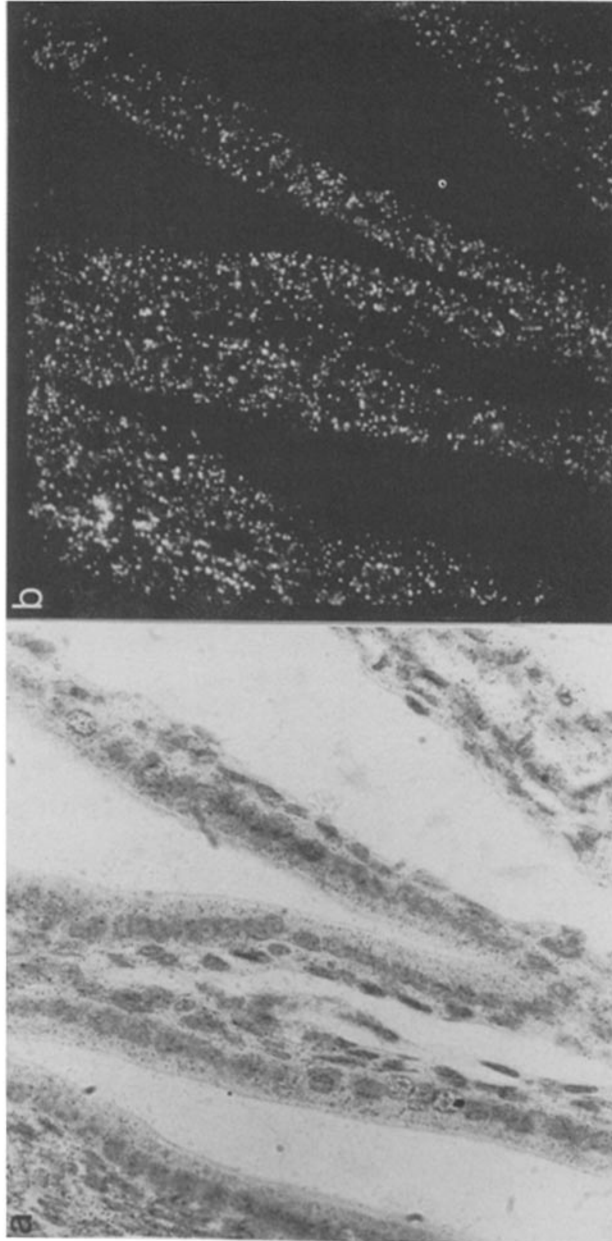

FIGURE 3. In situ hybridization of maternal uterus horn tissues from pregnant transgenic mice harboring the human *HLA-B27* gene. Hybridization was performed nonautoradiographically with biotinylated antisense RNA probes followed by immunocytochemical detection with streptavidin-gold/silver enhancement and visualization by confocal laser scan microscopy with reflex contrast. Transcripts of *HLA-B* are seen in the cytoplasm of endometrial gland epithelial cells. Counterstained with hematoxylin. (*a*) Conventional light microscopy. (*b*) Confocal laser scan microscopy with reflex contrast. ×650.

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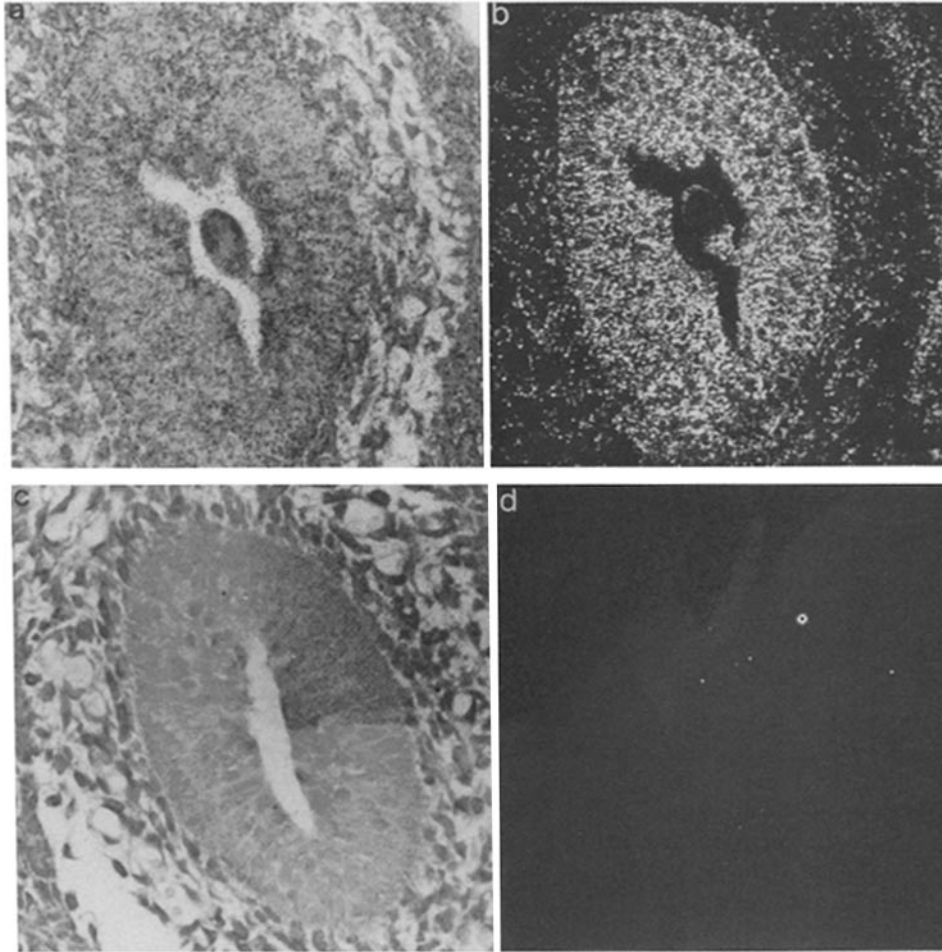

FIGURE 4. In situ hybridization of intestinal tissues of transgenic embryonic mice (day 13.5 after coitus) harboring *HLA-B27*. Procedures were as described in Fig. 3. Specific transcripts of *HLA-B* are seen clearly in the cytoplasm of epithelial cells when hybridization was performed with antisense probes (*a* and *b*). No signal was generated when sense-probes were used (*c* and *d*). (*a* and *c*) Conventional light microscopy. (*b* and *d*) Confocal laser scan microscopy with reflex contrast. ×650.

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expression. Despite this fact, silencing of the *HLA-B27* gene is retained in trophoblast. It must be noted that the visceral yolk sac endoderm cells that form the main component of the murine yolk sac placenta did contain *HLA-B27* mRNA (see Fig. 2 for cell lineage relationships). However, in contrast to trophoblasts that are derived from trophectoderm, the former cells are derived from the primitive endoderm (inner cell mass derivative). Therefore, lack of expression of *HLA-B* in extra-embryonic tissues of transgenic mice appears to be specific for trophectoderm. In this respect, it must be noted that the yolk sac placenta is rudimentary in man.

One possibility that could explain the differential expression pattern of *HLA-B* has not been addressed in the present study. Recent data show that murine (extra)em-

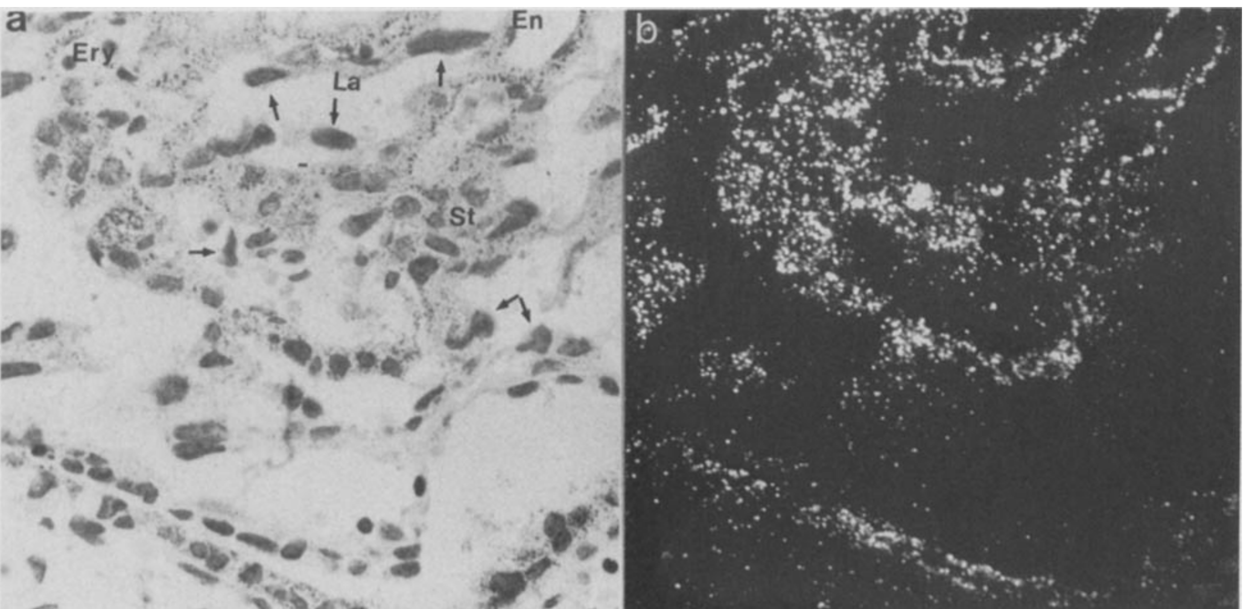

FIGURE 5. In situ hybridization of extra-embryonic tissues (chorio-allantoic placenta) of transgenic mice (day 13.5 after coitus) harboring *HLA-B27*. Procedures were as described in Fig. 3. In contrast to labyrinthal stromal cells and endothelial cells lining fetal placental vessels, no *HLA-B27* mRNA is detectable in the cytoplasm of trophoblastic cells bordering the maternal channels (*arrows*). La, labyrinthotrophoblast; En, endothelial cells; St, stromal cells (fibroblasts, monocytes/macrophages); Ery, fetal erythrocytes; MB, maternal peripheral blood cells.

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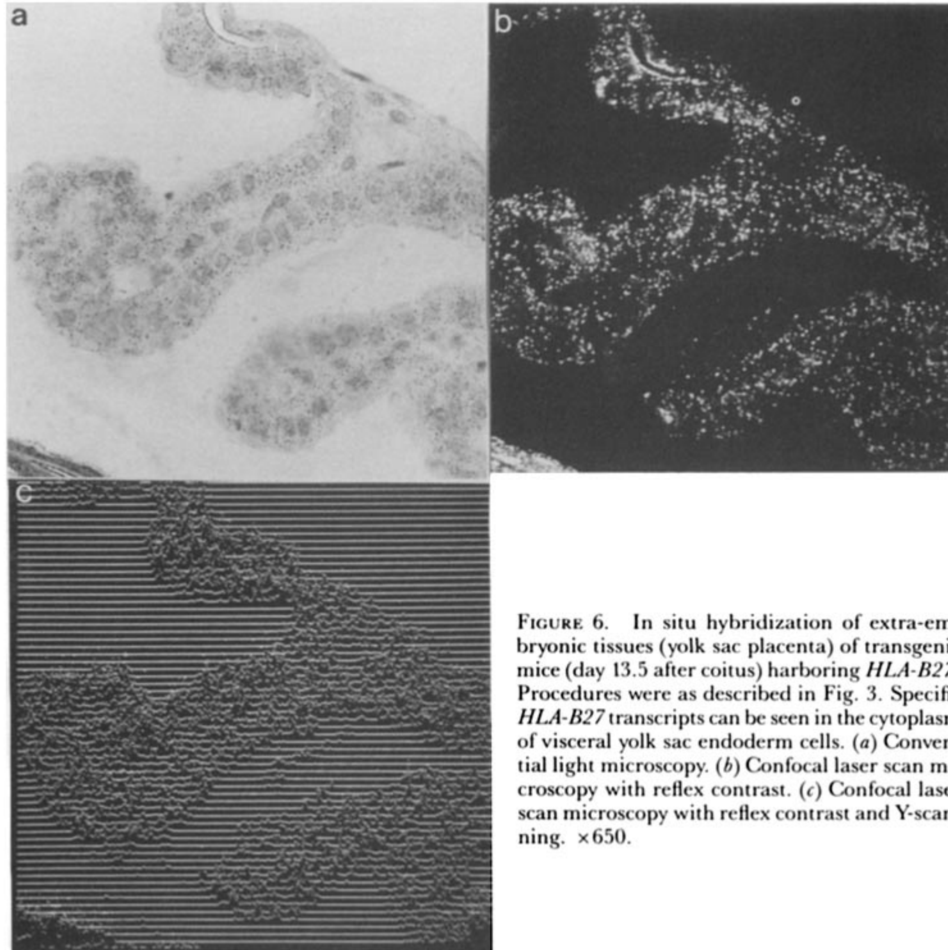

FIGURE 6. In situ hybridization of extra-embryonic tissues (yolk sac placenta) of transgenic mice (day 13.5 after coitus) harboring *HLA-B27*. Procedures were as described in Fig. 3. Specific *HLA-B27* transcripts can be seen in the cytoplasm of visceral yolk sac endoderm cells. (*a*) Convential light microscopy. (*b*) Confocal laser scan microscopy with reflex contrast. (*c*) Confocal laser scan microscopy with reflex contrast and Y-scanning. ×650.

bryonic development involves differential activity of homologous autosomal alleles according to parental origin. The maternal genome is essential for embryogenesis, the paternal genome is essential for development of the extra-embryonic tissues (11). To investigate this interesting possibility, future experiments should include studies on transgenic mice where the *HLA-B* gene segregates paternally.

At least two known genetic mechanisms could explain the lack of expression of *HLA-B27* in transgenic trophoblast. Negative *trans*-acting trophoblast-specific factors could remain bound to the 5'-flanking class I regulatory element (*CRE*) and repress *HLA-B* expression permanently in (transgenic) trophoblast. In embryonic cells, this repression was shown to be transient (5). Otherwise, trophoblast-specific modulations (e.g., structural modifications such as methylation, changes in chromatin structure of regulatory genomic DNA sequences flanking the MHC genes) could be involved that silence the genes. The fact that integrated copies of transgenes are usually found as long tandem repeats in other chromosomal locations than of their cognate loci

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implies that the regulatory sequences responsible for the lack of class I expression in trophectoderm are carried entirely by the genomic class I gene fragment used to generate the transgenes. In addition, the regulatory features are conserved as shown by their functioning across a species barrier and are tissue-specific as shown by their restriction to trophectoderm.

Summary

The mechanisms that regulate developmental control of the expression of MHC class I genes during generation of extra-embryonic tissues are largely unknown. In the present study, we studied the levels of transcripts of the human *HLA-B27* gene in extra-embryonic tissues of transgenic mice containing the *HLA-B27* (heavy chain) gene by in situ hybridization with biotinylated single-stranded RNA probes. In contrast to extra-embryonic stromal cells and embryonic tissues which contain (varying levels of) messenger RNA coding for *HLA-B27*, specific transcripts were not detected in labyrintho-, or spongiotrophoblast, nor in trophoblastic giant cells. These cells are devoid of HLA A and B locus class I transcripts in man. Regulation of expression of human MHC class I genes in extra-embryonic trophoblast in transgenic animals is thus under conserved selective pressure that is retained across a species barrier. Thus, in extra-embryonic tissues, regulation of expression of MHC class I genes is distinct from the mechanisms operating in developing embryonic cells.

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