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Control of H-2 antigen and β_2-microglobulin gene expression in mouse trophoblast cell clones

(histocompatibility antigen/flow microfluorometry/teratocarcinoma/endodermal cells)

KENICHI TANAKA*, KEIKO OZATO†, GILBERT JAY‡, JANE R. PARNES§¶, LATA RAMANATHAN*, J. G. SEIDMAN§‖, KENNETH S. S. CHANG*, AND ETTORE APPELLA*

Laboratories of *Cell Biology and ‡Molecular Virology, National Cancer Institute; and †Pregnancy Research Branch and §Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20205

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ABSTRACT **We have investigated the expression of H-2 antigen and β_2-microglobulin in recently established mouse trophoblast cell clones. These clones, derived from C57BL/6 ($H\text{-}2^b$) or BALB/c ($H\text{-}2^d$) strains, synthesized extra-embryonic endoderm- and trophectoderm-specific cytoskeletal proteins, termed "Endo A" and "Endo B," and no detectable SSEA-1 embryonic antigen. Flow microfluorometry indicated that H-2 antigen expression on the cell surface of trophoblast cells was very low, corresponding to 2–5% of the amounts found on differentiated teratocarcinoma cells (12-1a) and BALB/c 3T3 cells, respectively. Expression of β_2-microglobulin was reduced to ≈14% of the amounts found on 12-1a cells. Immunoprecipitation and polyacrylamide gel electrophoretic analysis indicated that the synthesis of H-2 antigen and β_2-microglobulin in the trophoblast cells was lower than that found in normal spleen cells. In addition low, but unequal, levels of mRNA specific for H-2 antigen and β_2-microglobulin were found in trophoblast cells by blot hybridization with cDNA probes. The low mRNA levels may be due to transcriptional control of the genes encoding H-2 antigen and β_2-microglobulin.**

In murine embryogenesis, an early blastocyst begins to be formed at about the 32-cell stage, when the first morphologically detectable differentiation occurs. At this time, the wall of the blastocyst forms a single layer called the trophectoderm. This layer subsequently proliferates and differentiates, giving rise to a number of trophoblast cell populations. Cell lines designated as trophoblast have been established from mouse cultured mid-term placentas and have been shown to produce transplantable tumors (1). In subsequent studies, we isolated two clones from these cells, which originated from BALB/c and C57BL/6 strains. They are fibroblastoid in morphology, adhere to glass, divide every 20–24 hr, and can be subcultured with or without trypsinization. Their karyotype is diploid. These cells have some properties of undifferentiated cells—i.e., they are restrictive for growth and expression of polyoma virus, simian virus 40, and murine retrovirus and are not susceptible to the antiviral action of murine interferon (1, 2).

The expression of the major histocompatibility complex, particularly of H-2 antigens, on the trophoblast has long been of interest because of an apparent immunological paradox in pregnancy in which the semi-allogeneic fetus fails to elicit immunological rejection in the pregnant host (3–7). Another interesting observation is the finding that, when the mouse placenta was separated into its two major components, spongiotrophoblasts and labyrinthine trophoblasts, H-2 antigens were detected only on the spongiotrophoblasts (8). Trophoblast cells offered us the advantage of studying the homogeneous population and they served as a unique model system for undifferentiated cells. Since β_2-microglobulin is always associated with H-2 antigen molecules (9), we also investigated the expression of β_2-microglobulin in parallel.

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MATERIALS AND METHODS

Cell Cultures. The trophoblast cells were cloned in soft agar as described by Laskov and Scharff (10) and all cells were maintained in RPMI 1640 medium (GIBCO)/10% fetal bovine serum (GIBCO) containing gentamycin (M. A. Bioproducts, Walkersville, MD) at 50 μg/ml at 37°C in humidified 5% CO_2/95% air. F9 embryonal carcinoma stem cells, F9AcC1 9 endoderm cells, and 12-1a, a differentiated clone derived from the embryonal carcinoma stem cells (11, 12), provided by C. Croce (Wistar Institute), were also maintained in the above condition.

Antisera. Anti-H-2^b serum was prepared by immunizing BALB/c mice with spleen cells from BALB·B mice and was a gift from Michael Rogers (National Cancer Institute).

Monoclonal anti-H-$2K^d$ and/or -D^d (clones 34-1-2, 31-3-4, 34-2-12), anti-H-$2K^b$ and/or -D^b (clones 20-8-4, 34-4-20, 34-7-23), and H-$2K^k$ (clone 16-3-22) antibodies were prepared as described (13, 14). Each test antibody used for fluorescence staining was a mixture of equal amounts of culture supernatants of the three hybridoma clones indicated in the parentheses. Monoclonal anti-H-$2K^k$ (clone 16-3-22) was used as a negative control. Fluorescein-conjugated affinity-purified goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Cappel Laboratories (Cochranville, PA).

Rabbit antiserum against β_2-microglobulin was prepared as described (15). Rabbit antisera directed against the extra-embryonic endoderm- and trophectoderm-specific cytoskeletal proteins termed "Endo A" and "Endo B" proteins and against anti-SSEA-1 antigen mouse monoclonal antibody were gifts from R. Oshima and B. Knowles, respectively (16–18).

Fluorescein Staining of Cells and Flow Microfluorometry Assay. SSEA-1 antigen was stained as described (16). For flow microfluorometry assay, single cell suspensions were obtained from monolayers of cells and were stained as described (13, 14). Cells were analyzed with a fluorescence-activated cell sorter (FACS II; Becton Dickinson FACS Systems) interfaced with a PDP 11 computer.

Preparation of [^{35}S]Methionine-Labeled Cell Extracts. The two trophoblast cell clones (BALB/c and C57BL/6), BALB/c

Abbreviations: 1× SSC, 0.15 M NaCl/0.015 M Na citrate, pH 7; pfu, plaque-forming unit(s).

¶ Present address: Div. of Immunology, Stanford Univ. Medical Center, Stanford, CA 94305.
‖ Present address: Dept. of Genetics, Harvard Medical School, Boston, MA 02115.

3T3 cells, F9 cells, and normal spleen cells of C57BL/6 strain (3×10^7 cells) were labeled with [^{35}S]methionine (Amersham) at 50 μCi/ml (1 Ci = 37 GBq) at 37°C in humidified 5% CO_2/95% air. The cells were then washed three times with phosphate-buffered saline and lysed with 0.5% Triton X-100 for 30 min at 4°C, and the extract was clarified by centrifugation at 10,000 × *g* for 60 min. The cellular supernatants routinely had a specific activity of about 1×10^8 dpm/mg of protein.

Immunoprecipitation. The labeled extracts (1×10^6 dpm) were suspended in 100 μl of immunoprecipitation buffer (10 mM Tris·HCl, pH 8.0/0.02% $NaDodSO_4$/0.15 M NaCl) containing 0.1% aprotinin/0.1% soybean trypsin inhibitor. The extracts were cleared by mixing them with 100 μl of a 10% (wt/vol) suspension of heat-killed formalin-fixed *Staphylococcus aureus* (Cowan I strain) and incubating the mixture for 30 min at room temperature. The supernatant obtained after centrifugation was immunoprecipitated overnight at 4°C with 10-μl samples of the different test antisera, and the immunocomplexes were bound to *Staphylococcus aureus* (Cowan I strain) as above. These complexes were pelleted by centrifugation, suspended in Laemmli buffer, analyzed on 12% $NaDodSO_4$/polyacrylamide gels (19), and processed for radioautography (20). Immunoprecipitation analysis for the presence of two cytoskeletal proteins, Endo A and Endo B, was carried out as described (17, 18).

Electrophoresis of RNA. For H-2 mRNA detection, total RNAs were extracted from cells by the guanidine hydrochloride method (21). For β_2-microglobulin mRNA, total RNAs were isolated from 2×10^8 cells by using the guanidine thiocyanate procedure of Chirgwin *et al.* (22). Samples (10 μg) of each RNA were heated to 70°C for 5 min in 50% deionized formamide/2.2 M formaldehyde/1× running buffer (20 mM 4-morpholinepropanesulfonic acid/5 mM sodium acetate/1 mM EDTA) and then cooled on ice. The RNA samples were electrophoresed overnight at 100 V on a 2% agarose gel containing 2.2 M formaldehyde and 1× running buffer. The RNA was transferred to nitrocellulose filters as described by Thomas (23).

Hybridization of RNA Blots. Nitrocellulose filters were baked at 80°C for 3–4 hr, maintained at 45°C for 4–6 hr in 50% formamide/5× SSC (1× SSC = 0.15 M NaCl/0.015 M Na citrate, pH 7)/0.1% polyvinylpyrrolidone/Ficoll/bovine serum albumin/0.1 M sodium phosphate, pH 7.5 containing denatured herring sperm DNA at 250 μg/ml. Hybridization was carried out in the same buffer for 16–48 hr at 45°C. After hybridization, filters were washed in 3× SSC at 68°C with several changes of buffer and then with 0.5× SSC at 68°C. Blots were dried and exposed to Kodak XAR-5 film at −70°C. The β_2-microglobulin probe was a nick-translated 600-base-pair *Sst* I/*Kpn* I fragment of the β_2-microglobulin gene as described (24). Two H-2 antigen probes were used, one from the highly conserved third external domain including amino acids 151–236 and the other from the 3′ noncoding region unique to one subclass of H-2 transcripts (25).

Titration of Virus Infectivity. Each cell culture was infected with mutant polyoma virus (PyTr-39) at a multiplicity at 1.0 plaque-forming unit (pfu) per cell (26). The virus yield was measured by plaque-forming assay in secondary mouse embryo fibroblasts at day 0 and day 5 at 37°C in each cell line.

RESULTS

Characterization of Markers on the Trophoblast Cell Clones. To define the nature of our cell clones, we tested for the presence of markers distinguishing these cells from undifferentiated stem cells, endodermal cells, and adult cells. As shown in Table 1, the clones lack the stage-specific embryonal antigen SSEA-1, which is expressed in cells at the eight-stage through the morula, in the inner cell mass and all embryonal carcinoma cells (16). The clones also contain Endo A and Endo B, two cytoskeletal proteins present on both trophoblast and extra-embryonic endoderm cells (17, 18). In addition, a polyoma virus mutant (PyTr-39) grew well in trophoblast, 12-1a, and 3T3 cells but not in F9 and F9AcC1 9 cells (ref. 26; Table 1). High amounts of estradiol are detected only in culture supernatants of trophoblast cells (Table 1). These results indicate that our cell clones have certain unique characteristics indicating that they are trophoblastic in nature.

Cell Surface Expression of H-2 Antigen and β_2-Microglobulin. To examine the surface expression of H-2 antigen and β_2-microglobulin on these trophoblast cells, sensitive flow microfluorometry analyses were carried out. Monoclonal antibodies reacting against H-2K and H-2D antigens of $H\text{-}2^b$ or $H\text{-}2^d$ haplotype were pooled for the binding assay. As shown in Fig. 1, pools of anti-$H\text{-}2^d$ and of anti-$H\text{-}2^b$ monoclonal antibodies reacted strongly with BALB/c 3T3 and 12-1a cells, respectively. Virtually all the cells were positive for H-2 antigens as evidenced by a single fluorescent cell peak. In contrast, trophoblast cells of either the BALB/c or the C57BL/6 strain showed only marginal staining with these antibodies. Extremely low levels of H-2 antigen were, however, detected by the pooled antibodies; i.e., the mean fluorescence values obtained with the test antibodies were always about 10% higher than those of controls obtained by staining with anti-$H\text{-}2K^k$ antibody or staining without antibodies (with fluorescein alone). The fluorescence profile indicated that very low levels of H-2 antigen are present uniformly on most of the trophoblast cells.

Table 1. Markers that distinguish trophoblast cloned cells from teratocarcinoma stem cells (F9), parietal endoderm cells (F9AcC1 9), and 12-1a and BALB/3T3 cells

			Trophoblast cells			
	F9	F9AcC1 9	BALB/c	C57BL/6	12-1a	BALB/3T3
% cells SSEA-1-antigen positive*	>95	<1	<1	<1	<1	<1
Growth yield of polyoma virus mutant†	0.67	0.50	42	58	120	150
Synthesis of estradiol‡	12.9	12.0	132.8	183.8	ND	12.7
Synthesis of Endo A and Endo B§	−	+	+	+	−	−

* Determined by indirect immunofluorescence assay using mouse anti-SSEA-1 antibodies.
† Determined as pfu/ml in infected culture on day 5/pfu/ml in infected culture on day 0.
‡ Results represent (pg/ml)/48 hr per 10^6 cells.
§ Bands at 55 (Endo A) and 50 (Endo B) kilodaltons were immunoprecipitated from trophoblast cells and F9AcC1 9 cells with anti-Endo A and anti-Endo B serum. −, Absent; +, present.

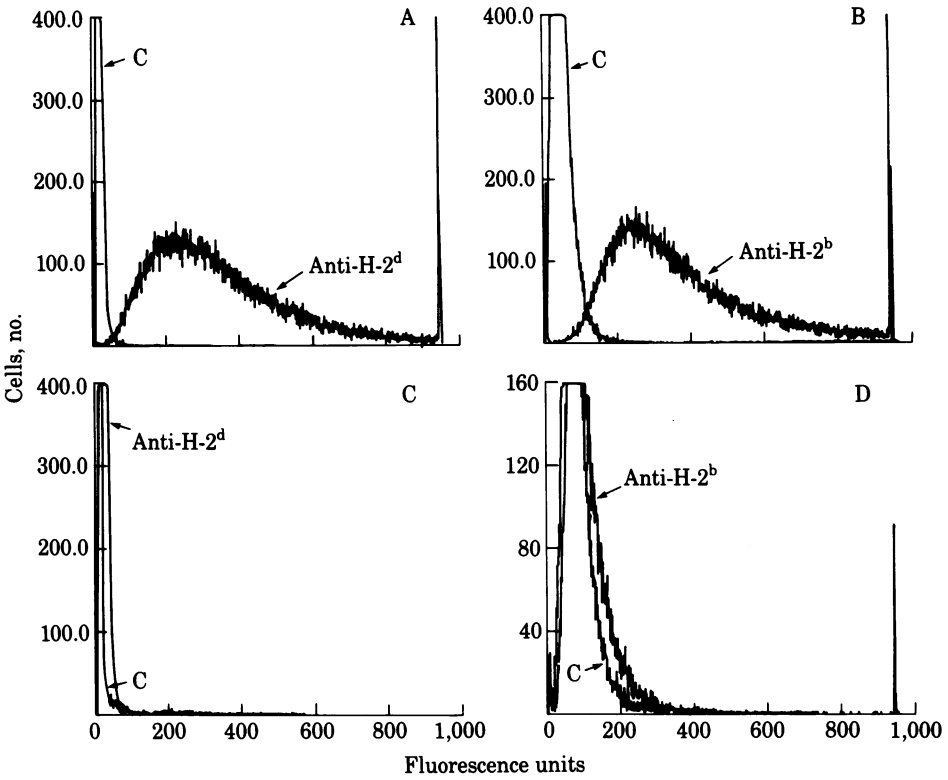

FIG. 1. Surface expression of H-2 antigens assayed by flow microfluorometry. (*A* and *C*) Binding of anti-H-2^d antibodies (mixture of equal volumes of clones 34-1-2, 31-3-4, and 34-2-12) on BALB/c 3T3 and BALB/c trophoblast cells, respectively. (*B* and *D*) Binding of anti-H-2^b antibodies (mixture of equal volumes of clones 34-4-20, 34-17-23, and 20-8-4) on 12-1a and C57BL/6 trophoblast cells, respectively. The negative control (*C*) was anti-H-2^k antibody (clone 16-3-22). Cells were mixed with 50 μl of monoclonal antibodies in culture supernatants and mixtures were incubated with excess fluorescein-labeled goat anti-mouse F(ab')$_2$.

To quantitate the relative amount of H-2 antigen expressed on trophoblast cells, the binding of the anti-H-2 antibodies at various dilutions was examined for 3T3, 12-1a, trophoblast, and normal spleen cells. Binding to 3T3, 12-1a, and normal spleen cells reached saturation when antibody dilution was less than 0.1. The anti-H-2^d antibody binding at the level of saturation for BALB/c trophoblast cells was about 2% of that for BALB/c 3T3 cells. Similarly, the level of H-2^b antigen present on the surface of C57BL/6 trophoblast cells was 5% of that on 12-1a cells. By comparing the maximum fluorescein binding to spleen cells (which express 10^4–10^5 H-2 molecules per cell; unpublished data) with the binding to trophoblast cells, one may estimate that approximately 10^3 H-2 molecules are expressed on the surface of a trophoblast cell.

The binding of rabbit anti-β_2-microglobulin antibody to trophoblast cells of C57BL/6 origin and to 12-1a cells was significantly higher than the background whereas the binding to F9 cells was not (Fig. 2). However, the amount of β_2-microglobulin present on the cell surface was quantitated by maximum fluorescein binding and binding to C57BL/6 trophoblast cells was about 14% of that on 12-1a cells. Similar results were obtained with BALB/c trophoblast cells.

Synthesis of H-2 Antigen and β_2-Microglobulin. To investigate whether the low level of expression of H-2 antigen and β_2-microglobulin on the surface of trophoblast cells was due to impaired synthesis, increased turnover, or deficiency in glycosylation of the H-2 antigens, we examined the synthesis of these antigens. The level of synthesis of the H-2 antigen and β_2-microglobulin in C57BL/6 trophoblast cells is compared with that of C57BL/6 spleen cells in Fig. 3. A strong doublet corresponding to the H-$2K^b$ and H-$2D^b$ antigens, which probably have different carbohydrate contents (27), was seen in spleen cells (lane 1) while only a faint band in the H-2 region was detected in trophoblast cells (lane 6). A band corresponding to β_2-microglobulin at 1.2 kilodaltons which was immunoprecipitated with either alloantiserum or rabbit anti-β_2-microglobulin serum was substantially less dense with trophoblast cells than with spleen cells (lanes 3 and 8).

Approximately equal amounts of H-2 antigen and β_2-microglobulin were immunoprecipitated in the case of spleen cells (lane 3), while only free β_2-microglobulin was immunoprecipitated in the case of trophoblast cells (lane 8). These results suggested that there is more free β_2-microglobulin than H-2 heavy chain available for complex formation in trophoblast cells.

The low levels of immunoprecipitable H-2 antigens and β_2-microglobulin found in these trophoblast cells were not due to accelerated degradation because extracts obtained after a shorter (0–90 min) labeling period also did not yield any immunoprecipitable antigens from the trophoblast cells.

The Level of mRNA of H-2 Antigens and β_2-Microglobulin. Because H-2 antigen and β_2-microglobulin were detected at low levels in the trophoblast cells, we attempted to determine the levels of accumulation of their mRNAs by hybridization of total RNAs with H-2 and β_2-microglobulin cDNA probes. Two H-2-specific cDNA probes were used, one derived from the highly conserved third external domain including amino acids 151–236 (Fig. 4A, lanes 1–4) and the other, from the 3′ noncoding region unique to one subclass of H-2 transcripts (lanes 5–8). Hybridization with the cDNA probe to total liver RNA from BALB/c (lanes 1 and 5) and SWR (lanes 2 and 6) mouse strains detected a transcript of $\approx$1,800 bases (25). The level of expression of these H-2-specific transcripts in BALB/c trophoblast cells (lanes 4 and 8), however, is greatly reduced (<2%) and is somewhat comparable with that seen in F9 teratocar-

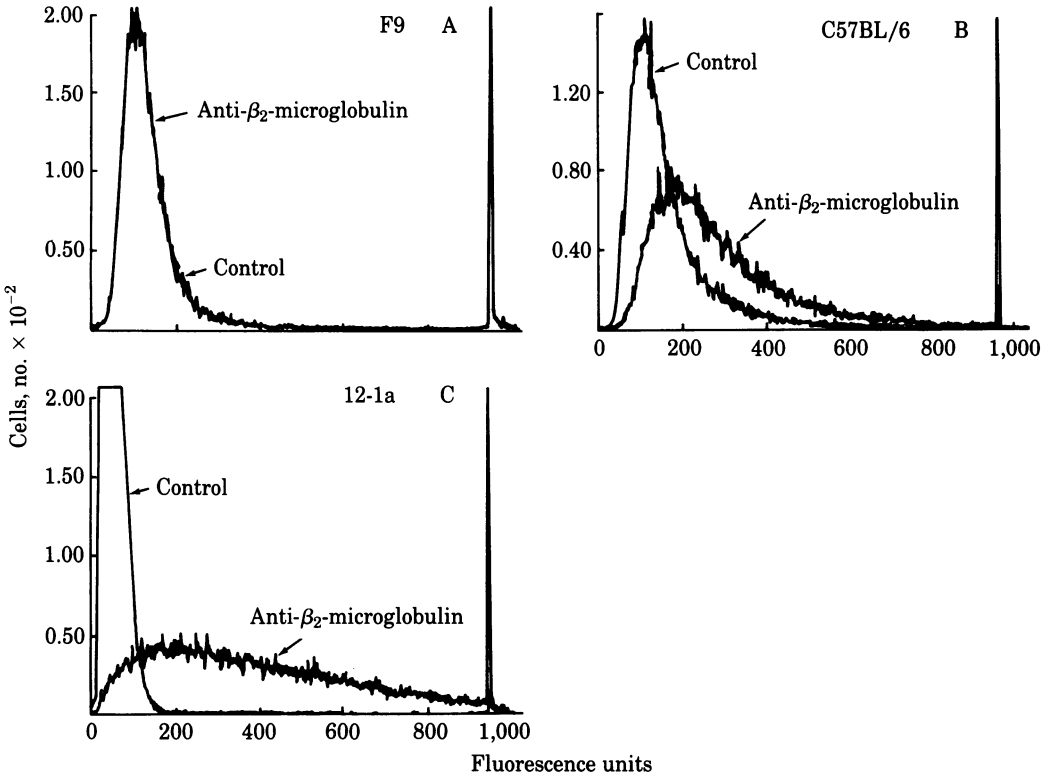

FIG. 2. Surface expression of β_2-microglobulin assayed by flow microfluorometry. F9 cells (*A*), C57BL/6 trophoblast cells (*B*), and 12-1a cells (*C*) were treated with 50 μl of a 1:20 dilution of antiserum and then incubated with excess fluorescein-labeled goat anti-rabbit F(ab')$_2$ fragment. The negative control was normal rabbit serum.

cinoma cells (lanes 3 and 7). Hybridization with a ^{32}P-labeled cloned β_2-microglobulin cDNA probe (24) detected major transcripts of ≈1,250 and ≈1,050 bases (Fig. 4*B*); the levels of expression, again, are significantly less (<18%) in the trophoblast cells (lanes 2 and 3) than in BALB/c 3T3 cells (lane 1).

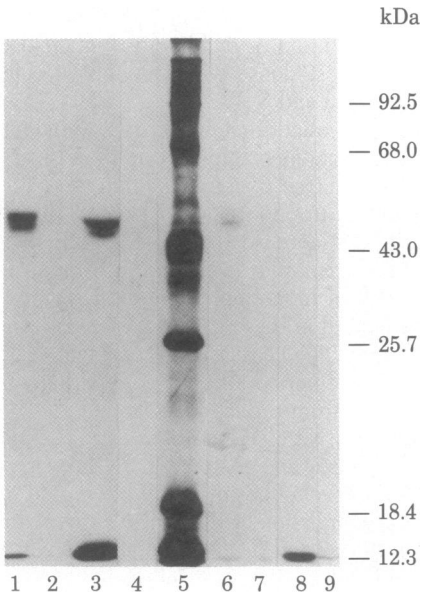

FIG. 3. Synthesis of H-2 antigen and β_2-microglobulin by C57BL/6 spleen cells and C57BL/6 trophoblast cells. ^{35}S-Labeled whole cell lysates (1 × 10^6 dpm) of C57BL/6 spleen cells (lanes 1–4) and C57BL/6 trophoblast cells (lanes 6–9) were analyzed by immunoprecipitation with anti-H-2^b alloantiserum (lanes 1 and 6), normal mouse serum (lanes 2 and 7), rabbit anti-β_2-microglobulin serum (lanes 3 and 8), and normal rabbit serum (lanes 4 and 9) and electrophoresis on a 12% Na-DodSO$_4$/polyacrylamide gel. Lane 5: marker standards. kDa, Kilodaltons.

DISCUSSION

In this paper, we have reported that the expression of surface H-2 antigens and β_2-microglobulin is reduced on trophoblast cells in accordance with a low level of synthesis of these proteins. Further, these results correlate with low levels of mRNAs for H-2 antigen and β_2-microglobulin. Thus, the reduced H-2 antigen and β_2-microglobulin expression is not due to inaccessible surface antigens, aberrant protein synthesis, glycosylation, or membrane insertion. Rather, it appears that it is due to transcriptional control of the H-2 antigen and β_2-microglobulin genes, although rapid turnover of the mRNAs could also explain the results.

Recently, it has been reported that in undifferentiated teratocarcinoma cells the expression of H-2 antigens, and possibly β_2-microglobulin, detectable on the cell surface is under transcriptional control (28, 29). It is interesting to note that our cell clones differ from those teratocarcinoma cells in several important aspects. First, the trophoblast cells express extra-embryonic endoderm- and trophectoderm-specific cytoskeletal proteins, as reported here, while the teratocarcinoma cells do not (17, 18). Second, although embryonal carcinoma cells have been shown to express a stage-specific embryonal antigen (SSEA-1), this antigen has not been detected in the trophoblast cell clones by indirect immunofluorescence using anti-SSEA-1 monoclonal antibody.

Trophoblast cells and endoderm cells share several biochemical and immunological properties (17, 18, 30). However, unlike retinoic acid-induced parietal endoderm cells, which show a characteristic change in morphology when treated with dibutyryl cAMP (31), trophoblast cells do not express any changes

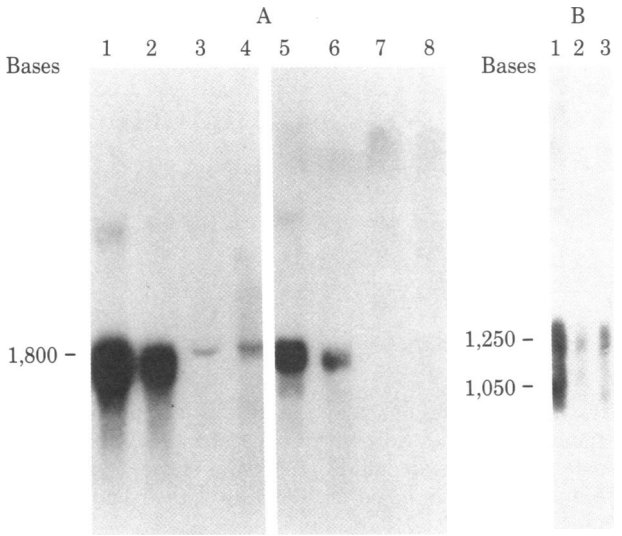

FIG. 4. Hybridization of H-2- and β_2-microglobulin-specific RNA in liver cells from BALB/c and SWR mice, F9 cells, BALB/c 3T3 cells, BALB/c trophoblast cells, and C57BL/6 trophoblast cells. (*A*) Total cellular RNAs were hybridized to ^{32}P-labeled H-2 DNA. One probe (including amino acids 151–236) was from the highly conserved third external domain (lanes 1–4) and the other was from the 3′ noncoding region (lanes 5–8). Lanes: 1 and 5, liver cells from BALB/c; 2 and 6, liver cells from SWR; 3 and 7, BALB/c trophoblast cells; 4 and 8, F9 cells. (*B*) Total cellular RNAs were hybridized to ^{32}P-labeled β_2-microglobulin DNA. The probe was a nick-translated 600-base-pair *Sst* I/*Kpn* I fragment of β_2-microglobulin gene. Lanes: 1, BALB/c 3T3 cells; 2, BALB/c trophoblast cells; 3, C57BL/6 trophoblast cells. The origin is at the top of the figure.

on treatment with cAMP or retinoic acid (data not shown). In addition, trophoblast cells are nonpermissive for gene expression of simian virus 40 (2), which is able to express the simian virus 40 large tumor antigen in parietal endoderm cells as in adult cells (32). By evaluating the growth of a polyoma mutant in a series of murine cell lines, we have observed that this mutant selected for growth on trophoblast cells grew well in differentiated cells and trophoblast cells but did not grow in undifferentiated cells (ref. 26; Table 1). These observations suggest that the mechanism regulating gene expression of polyoma virus mutant in these cells may reflect cellular regulatory processes specific to stage of differentiation. Trophoblast cells thus represent a different stage of differentiation from that of murine embryonal carcinoma cells and endodermal cells, even though they have similar properties in the regulation of H-2 antigen and β_2-microglobulin genes. These cells should be a useful source for studying various processes in mouse embryonic cells because they are a homogeneous population with defined differentiation capacity. These clones constitute a valuable addition to the frequently used F9 teratocarcinoma cells.

Studies of the expression of the H-2 antigen and β_2-microglobulin in oocytes and preimplantation embryos add another facet to the question of gene regulation. Recently, Sawicki *et al.* (ref. 33; J. Sawicki, personal communication) suggested that synthesis of β_2-microglobulin and H-2 antigen may not be coordinated in early mouse embryos. Similar observations had been made in human spermatozoa and gonocytes in which HLA expression is not detectable while β_2-microglobulin expression is readily demonstrable (34, 35). Our results show a higher level of β_2-microglobulin than of H-2 antigen expression in trophoblast cell clones. It appears, therefore, that the transcription of H-2 antigen and β_2-microglobulin is controlled independently in these systems.

Our finding of very low H-2 antigen expression on trophoblast cell surfaces may contribute to the resolution of the immunological paradox of pregnancy alluded to in the *Introduction*. With few H-2 antigens on the surface of most placenta cells, there might be less chance of triggering an allogeneic immune response in the pregnant female.

We thank Drs. Carlo Croce, Davor Solter, Robert Oshima, and Michael Rogers for providing us with F9 stem cells, F9AcC1 9 cells, 12-1a cells, anti-SSEA-1 monoclonal antibodies, anti-Endo A and anti-Endo B antibodies, and H-2^b alloantiserum. We also thank D. Stephany and S. Sharrow for assistance with the cell sorter analysis and Sarah Butler for expert aid in the preparation of the manuscript.

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