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Limitation of Differential Expression of HLA-A,B,C Antigens on Choriocarcinoma Cell Lines by Messenger RNA for HLA Heavy Chain but not by β_2-Microglobulin

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ABSTRACT

The relative amounts of HLA-A,B,C antigens, β_2-microglobulin (β_2m), and trophoblast antigens (Trop-1 and Trop-2) were determined on nine choriocarcinoma cell lines including seven lines of gestational origin and two lines of nongestational origin (from ovary and stomach) by quantitative immunofluorescence analysis using a fluorescence-activated cell sorter. Most of these lines expressed surface HLA to variable extents, but one had none detectable. However, all lines secreted readily measurable amounts of β_2m.

We analyzed total RNA extracted from these lines using northern blot molecular hybridization with HLA-A,B,C- and β_2m-specific complementary DNA probes. We found no messenger RNA species which hybridized with the HLA probe in cells with no detectable HLA surface antigen and only small amounts of HLA-specific RNA in cells with low levels of HLA membrane antigen. Cells exhibiting surface HLA levels greater than about 30% of that on lymphocytes had much higher amounts of HLA-specific RNA than did choriocarcinoma cells with no or low HLA antigen expression. In contrast, RNA hybridizing with β_2m-specific probes was present at the 20% level or higher (relative to lymphocytes) in all the cell lines tested. Thus, the expression of HLA-A,B,C is apparently limited in choriocarcinoma cells by the level of HLA heavy-chain RNA and not by the level of β_2m RNA. We discuss these findings in relation to the normal trophoblastic or other origins of this tumor type and with respect to the regulation and function of HLA in trophoblasts.

INTRODUCTION

CCs[4] are believed to arise from normal trophoblasts or hydatidiform moles (4). These tumors are unique because of their semiallogeneic or allogeneic origin. Their potential to express allogeneic antigens may contribute to the rejection of these tumors as allografts. Cell lines have been established from such tumors and from CC of nongestational origin (16, 18, 24, 28, 29, 32, 37, 39). Normal trophoblasts and some CC cell lines lack HLA antigens (11, 12, 14, 41); nevertheless, patients with CC often develop anti-HLA antibodies (20, 21, 45). These considerations and observations led us to reexamine the question of the presence of HLA-A,B,C antigens on cells of a number of CC cell lines of gestational and nongestational origin.

How HLA-A,B,C antigens are regulated is of considerable interest because they are present on all normal nucleated cells with the notable exception of trophoblasts (1, 11, 12). Furthermore, some tumors, e.g., Daudi (a Burkitt lymphoma), have been found to lack HLA-A,B,C antigens (2). Examination has shown this lack in Daudi cells to be due to a single base mutation resulting in an untranslatable β_2m mRNA (30). In contrast, we have shown that normal, placental-derived cytotrophoblasts, which have at most traces of HLA, have only traces of heavy-chain mRNA but moderate levels of β_2m mRNA. We concluded that cytotrophoblast HLA production is limited by HLA mRNA, in contrast to the Daudi situation.[5]

In the work to be presented, we found that CC lines vary from having no detectable HLA expression to having levels equal to those of lymphoid cells. The levels of surface HLA correlate approximately with amounts of HLA heavy-chain mRNA but not with amounts of β_2m mRNA or rates of β_2m protein secretion.

MATERIALS AND METHODS

Cells. Seven gestational choriocarcinoma cell lines derived from 6 patients were used: JAR (29), BeWo (28), JEG (18) [received from Dr. H. Sussman, Pathology Department, Stanford University; the JEG line is a clone derived from BeWo], HCCM-5 (24), NUC-1 (37), GCH-1 (39), and ENAMI-1 (39); and 2 nongestational choriocarcinoma cell lines [IMa (of ovarian origin) (32) and SCH (of gastric origin) (16)]. Other cell lines included LCL-721 (lymphoblastoid) (17), provided by Dr. P. Kavathas; Daudi (Burkitt lymphoma) (2), provided by Dr. N. Holmes (Department of Structural Biology, Stanford University); and F9 (mouse undifferentiated teratocarcinoma) (8). Trypsin (0.05%)-EDTA (0.02%) solution was used to release cells for subculture. PBL were isolated from freshly drawn venous blood by flotation on a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Inc., Piscataway, NJ).

Medium. RPMI 1640 or Waymouth's (Irvine Scientific, Santa Ana, CA) with 10% newborn calf serum was used.

Monoclonal Antibodies. MB40.5 (6) and W6/32 (26), which identify a framework component of HLA-A,B,C heavy chain, and BBM.1 (5) (anti-human β_2m) were kindly provided by Dr. P. Parham (Department of Structural Biology, Stanford Unviersity). Monoclonal antibodies against human β_2m (L368) (19) and HLA-DR (L243) (19) were provided by Becton-Dickinson Monoclonal Center, Inc. (Mountain View, CA). Anti-Trop-1 and anti-Trop-2 are trophoblast antigen-specific monoclonal antibodies, previously produced in our laboratory (22). As a control for nonspecific binding, we used isotype-matched monoclonal antibodies against the dansyl hapten (9), provided by Dr. J. Reidler of our laboratory.

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[4] The abbreviations used are: CC, choriocarcinoma; β_2m, β_2-microglobulin; FACS, fluorescence-activated cell sorter; PBL, peripheral blood lymphocytes; cDNA, complementary DNA; SSC, 0.15 M NaCl-0.015 M sodium citrate; SDS, sodium dodecyl sulfate.

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All of the antibodies were titrated on known positive target cells, and saturating concentrations were used in the assays. The same concentrations of anti-dansyl served as unstained controls. Regents were stored at 4° with 0.1% sodium azide and deaggregated by centrifugation immediately before use.

Immunofluorescence Staining and FACS Analysis. Cells were detached from flasks using 0.05% trypsin-EDTA solution and stained by the direct or indirect immunofluorescence technique described previously (13). Photomultiplier voltages were converted by logarithmic amplification for display on a multichannel analyzer. The relative intensity of specific immunofluorescence is given as the difference in mean fluorescence intensity of antibody-stained cells minus that of cells with irrelevant antibody control. Relative antigen density values were calculated by dividing the mean specific fluorescence intensity for each cell line by its cell surface area, which was obtained from impedance volumes measured on a FACS Research Analyzer (B-D FACS Division, Sunnyvale, CA).

Secretion of β_2m. Cells were harvested from optimally growing stock cultures, washed, and incubated (in duplicate) at a density of 5×10^5 viable cells/ml for 65 hr at 37° as described previously (25). At the time of harvest, the culture supernatant was centrifuged at $400 \times g$ for 5 min, quick-frozen, and stored at −20° until measurement. β_2m was quantitated by a solid-phase radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden).

cDNA Probes. An HLA-B7 cDNA clone which cross-hybridizes with HLA-A,B,C genes and mRNA and contains a 1400-base pair insert (33) was generously provided by Dr. S. Weissman (Yale University). A human β_2m cDNA clone containing 328 base pairs of protein-coding sequence and 217 base pairs of 3′-untranslated region (34) was the kind gift of Dr. K. Itakura. For both clones, the largest *Cfo*I fragment (containing primarily cDNA insert plus 337 base pairs of pBR322) was isolated and labeled with ^{32}P by nick translation to a specific activity of 2×10^8 cpm/μg.

RNA Gels and Hybridization. RNA was extracted from frozen cell pellets by homogenization in the presence of 4 M guanidine thiocyanate, centrifugation through a cushion of 5.7 M cesium chloride, and ethanol precipitation as described by Chirgwin *et al.* (7). The amount of RNA was determined by measuring the A_{260}. Twelve μg of each RNA sample in 50% formamide, 2.2 M formaldehyde, and 1 × 3-(*N*-morpholino)-propanesulfonic acid buffer were denatured by heating for 5 min at 70°, quick-chilled, and electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde and ethidium bromide (6.25 μg/ml) in 1 × 3-(*N*-morpholino)propanesulfonic acid buffer. RNA separated by electrophoresis was subsequently transferred to nitrocellulose filters according to the procedure of Thomas (40). Blots were hybridized to ^{32}P-labeled HLA-A,B,C or β_2m probes for 18 hr at 42° in 40% formamide, 4 × SSC, 1 × Denhardt's solution, 20 mM Tris (pH 7.6), 0.1% SDS, denatured herring sperm DNA (100 μg/ml), and 50% dextran sulfate. The filters were then washed at room temperture with 4 changes of 2 × SSC and 0.1% SDS for 20 min each, followed by 3 washes at 55° for 1 hr each with 0.1 × SSC and 0.1% SDS. The filters were then dried and exposed to XAR-5 film (Kodak) at −70° overnight. The intensities of the hybridizing HLA-A,B,C and β_2m mRNA bands after autoradiography were quantitated by densitometry. The positions of the 18S and 28S rRNA markers were determined by the ethidium bromide staining pattern of the gel prior to blotting.

RESULTS

HLA-A,B,C Antigen Density. Nine CC cell lines and several controls were analyzed using immunofluorescence staining and FACS analysis. Log fluorescence distributions were homogeneous with all lines. Representative patterns for JAR, BeWo, ENAMI-1, IMa, and SCH are shown in Chart 1. Mean fluorescence intensities, calculated by computer, closely approximate the model values. We stained all these cell lines separately with either of 2 monoclonal antibodies, W6/32 or MB40.5, directed against different determinants of the HLA heavy chain, or with either of 2 monoclonal antibodies, L368 or BBM.1, directed against β_2m determinants found, respectively, on HLA heavy chain-associated β_2m or on all β_2m polypeptides. The relative fluorescence intensities per cell or per unit surface area are similar with all of these antibodies (Tables 1 and 2). Table 1 also shows the wide variation in antigen density from one CC line to another, ranging from undetectable HLA antigen on JAR to approximately the same as that of lymphocytes on SCH. It is worth noting that all surface β_2m appears to be associated with HLA-A,B,C molecules. If there were any detectable amount of β_2m associated with polypeptides other than HLA-A,B,C heavy chain, we would have seen higher fluorescence intensities with BBM.1 than with L368. The CC cells are large, with cell volumes some 14 to 17 times and surface areas 6 to 7 times those of lymphocytes. Nevertheless, there is no correlation between cell size and amount of HLA antigens per cell, either among cells

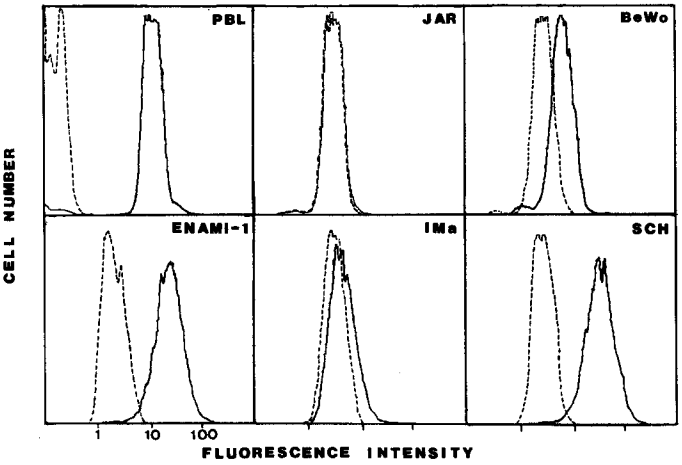

Chart 1. Immunofluorescence staining of choriocarcinoma cell lines and normal lymphocytes with fluorescein-conjugated monoclonal antibody against HLA-A,B,C framework determinant (W6/32). ——, stained histograms; – – –, stained with medium alone.

Table 1
Surface antigens on CC cell lines

Choriocarcinoma cell line	Relative antigen density with monoclonal antibodies: HLA-A,B,C antigen[a] W6/32 (HLA)	MB40.5 (HLA)	BBM.1 (β_2m)	L368 (β_2m)	Trophoblast antigens[b] Trop-1	Trop-2
Gestational						
JAR	<0.1	<0.1	0.2	1	273	2
HCCM-5	3	3	7	9	79	118
BeWo	5	3	4	7	238	53
NUC-1	12	7	7	13	83	0
JEG	13	9	8	12	116	35
GCH-1	36	29	32	38	215	87
ENAMI-1	39	44	45	33	195	89
Nongestational						
IMa	1	1	4	2	119	11
SCH	70	93	102	85	207	108

[a] Relative antigen density on choriocarcinoma cell lines compared with that on PBL:

$$\frac{\text{Mean of fluorescence intensity of choriocarcinoma (− autofluorescence)}}{\text{Difference in surface area} \times \text{mean of fluorescence intensity of PBL (− autofluorescence)}} \times 100$$

[b] $\frac{\text{Mean of fluorescence intensity of choriocarcinoma (− autofluorescence)}}{10}$

within a line or between lines.

Secretion of β_2m was assayed in culture supernatants by radioimmunoassay. All the CC cells secreted readily measurable amounts of β_2m (10 to 80 ng/ml/65 hr) which did not correlate with either the amount of HLA or the β_2m determinants on the membranes of these cells.

We did not detect HLA-DR on any CC lines.

Two monoclonal antibodies specific for trophoblast and CC antigens were also tested quantitatively for reaction with all the cell lines. Anti-Trop-1 reacted with all CC cells, and anti-Trop-2 reacted with most. Anti-Trop-2 did not react with either JAR [also found previously (22)] or NUC-1.

Analysis of HLA and β_2m RNA. We chose 5 independently derived CC cell lines of gestational and nongestational origin for RNA analysis. These cell lines (JAR, BeWo, ENAMI-1, IMa, and SCH) represented the range of differences in membrane HLA antigen levels that we observed among CC cell lines.

Fig. 1 shows an autoradiograph of a northern blot of RNA from these CC cell lines hybridized to an HLA-A,B,C-specific cDNA probe. For comparison, we have included RNAs from a lymphoid cell line (LCL-721), normal PBL, and Daudi cells. The major hybridizing band which comigrates with 18S rRNA represents the mature HLA-A,B,C mRNA species. The larger bands probably represent unprocessed and/or partially spliced intermediates. Quantitative measurements of the mature mRNA species were done by densitometry (see Table 3). The relative amounts of HLA-A,B,C mRNA in the CC cell lines correlated with the relative antigen densities on the surfaces of the respective cells (Fig. 1, *Lanes a* to *e*; Table 3). The control cells (LCL-721, PBL, and Daudi) all had higher and relatively equal amounts of HLA-A,B,C mRNA (Fig. 1, *Lanes f* to *h*; Table 3).

Fig. 2 shows the results of hybridization of a similar northern blot to a β_2m probe. In sharp contrast to the variations in HLA-A,B,C mRNA, the levels of β_2m mRNA varied only 3-fold among the CC cell lines (Table 3) despite much greater variation in the level of β_2m cell surface expression (Table 1). In particular, extremely low levels of β_2m were detectable with FACS on the surface of JAR, BeWo, and IMa cell lines; nevertheless, these cell lines contain readily detectable levels of β_2m mRNA. In addition, there is no meaningful relationship between the level of β_2m mRNAs in the CC lines and the amount of β_2m either on the cell surface or secreted into the culture supernatant. As shown in Fig. 2, *Lane h*, Daudi cells contain significant amounts of β_2m mRNA, which was recently proven to be abnormal (nontranslatable mRNA) by Rosa *et al.* (31) and dePreval and Mach (10).

The relative ratio of HLA-A,B,C heavy-chain mRNA to β_2m mRNA in LCL-721 cells (measured by densitometry) is similar to that in normal PBL. In contrast, the ratios of these mRNA levels in JAR, BeWo, and IMa cells are quite low, strongly suggesting that the very low amounts of HLA-A,B,C antigen expression on the cell surface are regulated at the level of HLA-A,B,C heavy chain and not β_2m transcription. In ENAMI-1 and SCH cell lines, the mRNA ratios are similar to that in normal PBL, but the presence of free, secreted β_2m in the culture supernatants suggests that, in these cells as well, HLA-A,B,C antigen expression is limited by the level of HLA-A,B,C heavy-chain mRNA and not by a low level of biosynthesis of β_2m molecules. On the basis of these findings, different modes of regulatory mechanisms are obviously used for each chain of HLA-A,B,C antigens, which are finally expressed as heterodimeric molecules on the cell surface.

Table 2

HLA-A,B,C antigens and β_2m of control cells

	Relative antigen density		β_2m culture supernatant (ng/ml/65 hr)
	HLA-A,B,C antigen[a]	β_2m cell surface antigen[a]	
LCL-721	130	122	384
PBL	100	100	ND[b]
Daudi	0	0	0
F9	0	0	0

[a] Relative antigen density on cell lines compared with that on PBL as described in Table 1, Footnote *a*.
[b] ND, not done.

Table 3

Relative amounts of HLA-A,B,C and β_2m mRNAs

Cells	HLA-A,B,C mRNA[a]	β_2m mRNA[a]
JAR	0.0	29.0
BeWo	13.1	27.0
ENAMI	78.9	45.7
IMa	4.8	16.2
SCH	38.3	33.8
LCL-721	105.3	57.1
PBL	100.0	100.0
Daudi	90.9	19.0

[a] Relative mRNA level (measured with a densitometer) compared with that in PBL (= 100) (we must compare these relative mRNA amounts with those in PBL, not those in LCL-721, because we compared HLA antigen density on CC cells with that on PBL).

DISCUSSION

We have shown that CC cell lines have levels of HLA membrane antigen varying from none detectable to levels approximating those of lymphocytes. Examination of mRNA levels in these lines showed that the 3 cell lines with no or low HLA antigen had either no detectable or low levels of HLA mRNA but higher levels of β_2m mRNA. We have previously found that normal cytotrophoblasts isolated from placenta had no detectable HLA on their membranes and (at most) traces of HLA heavy-chain mRNA, but moderate levels of β_2m mRNA.[5] The other 2 CC cells examined for mRNA had membrane HLA levels averaging 40 and 87% the levels of lymphocytes and both HLA and β_2m mRNA levels in the same relative range. All the CC cell lines secreted β_2m polypeptide. Thus, in both a presumptive normal cell precursor of CC (normal cytotrophoblasts) and the transformed CC cells themselves, membrane HLA glycoprotein seems to be limited by the amount of HLA heavy-chain-specific mRNA.

This contrasts with the basis of regulation in the well-known cell line, Daudi, derived from a Burkitt lymphoma (of B-lymphocyte derivation). Daudi makes no membrane HLA but has moderate levels of both HLA mRNA and β_2m mRNA (10). It, however, has a mutation resulting in a single base change in β_2m RNA preventing translation of β_2m polypeptide (10, 30, 31). In addition, Parnes and Seidman (27) recently reported that a mouse thymoma mutant cell line fails to express cell surface β_2m, H-2, or TL antigens resulting from defects in both copies of the β_2m gene in this cell line. These data led us to conclude that β_2m is critically required for cell surface expression of HLA-A,B,C (or H-2) antigens. Clearly, the regulation of surface HLA in Daudi and these other β_2m mutants is mediated by quite different mechanisms than that used by the trophoblast-like cells studied here. This conclusion has also been made by Trowsdale *et al.* (41).

Because β_2m polypeptide is made in the CC cells and since β_2m is known to be associated with Major Histocompatibility

Antigen Class I molecules other than HLA-A,B,C or H-2 (23, 38), we determined whether these CC cells have any surface β_2m that is not associated with HLA. The monoclonal antibody BBM.1 reacts with both free β_2m and HLA-associated β_2m. The staining intensities with BBM.1 were similar to those found with the antibody L368, which reacts with a conformational determinant requiring β_2m to be associated with HLA. This evidence strongly indicates that there are no other Class I molecules on CC cells (or normal trophoblast).[5]

It is intriguing to consider the possible reasons for the extensive and characteristic differences in amounts of HLA on the various CC cell lines studied here. The cytotrophoblast in placentas and chorion membranes of middle or late pregnancy is essentially devoid of HLA (11, 36)[5] and (gestational) CC are believed to be of trophoblast origin. *A priori,* one would expect CC to be HLA negative, as has in fact been reported (14). We propose 4 possibilities: (*a*) CCs arise from some other, unknown cell type (which probably closely resembles trophoblast); (*b*) CCs arise from trophoblasts at different stages of differentiation, with different levels of HLA expression; (*c*) some gestational CCs are of androgenic origin (complete type hydatidiform moles) (15, 43), while others are of fetal origin (placental trophoblasts), and HLA levels could be (systematically) different on these 2 types of CC; and (*d*) HLA levels in the trophoblast progenitor cells are changed after neoplastic transformation to a new and characteristic level.

The conflicting and incomplete data in the literature on CC origins and the presence of HLA antigens on CC tumors (3, 4, 42) make it difficult to discuss possibilities *a*, *b*, and *c*. Comparison of restriction fragment length polymorphisms (44) of mother, father, and CC will make it possible to readily determine the genetic origins of CC. Immunohistological staining of tissue with monoclonal antibodies as well as cell dissociation studies followed by FACS analysis, as described,[5] should also help clarify the cell type origins of CC.

A recent study concludes that early, nonvillous, trophoblast displays HLA-A,B,C antigens (35). If these cells are precursors of later (villous) trophoblasts, which we and others have shown are HLA-A,B,C negative (11, 12),[5] then the HLA heavy chain genes must be turned off in the further differentiation of trophoblasts after the early stage in which they are turned on. The molecular events responsible for the first "turn-on" of these genes may not be precisely reversed by the later "turn-off," and it may be more probable for these genes to be reactivated in the process of neoplastic transformation which results in a CC. This could account for the fact that some CCs produce HLA antigens. Experiments could be designed to determine if the chromatin structure in the region of HLA genes and the methylation patterns of these genes and their DNA flanking sequences change in the transition from early to late trophoblast or if these characteristics differ among CC lines and between these and cytotrophoblasts. Studies of HLA gene regulation in CC cell lines may help in understanding regulation of HLA expression in the development of trophoblast. The function of these cells in the fetal part of the fetal-maternal junction during gestation may thereby become better understood.

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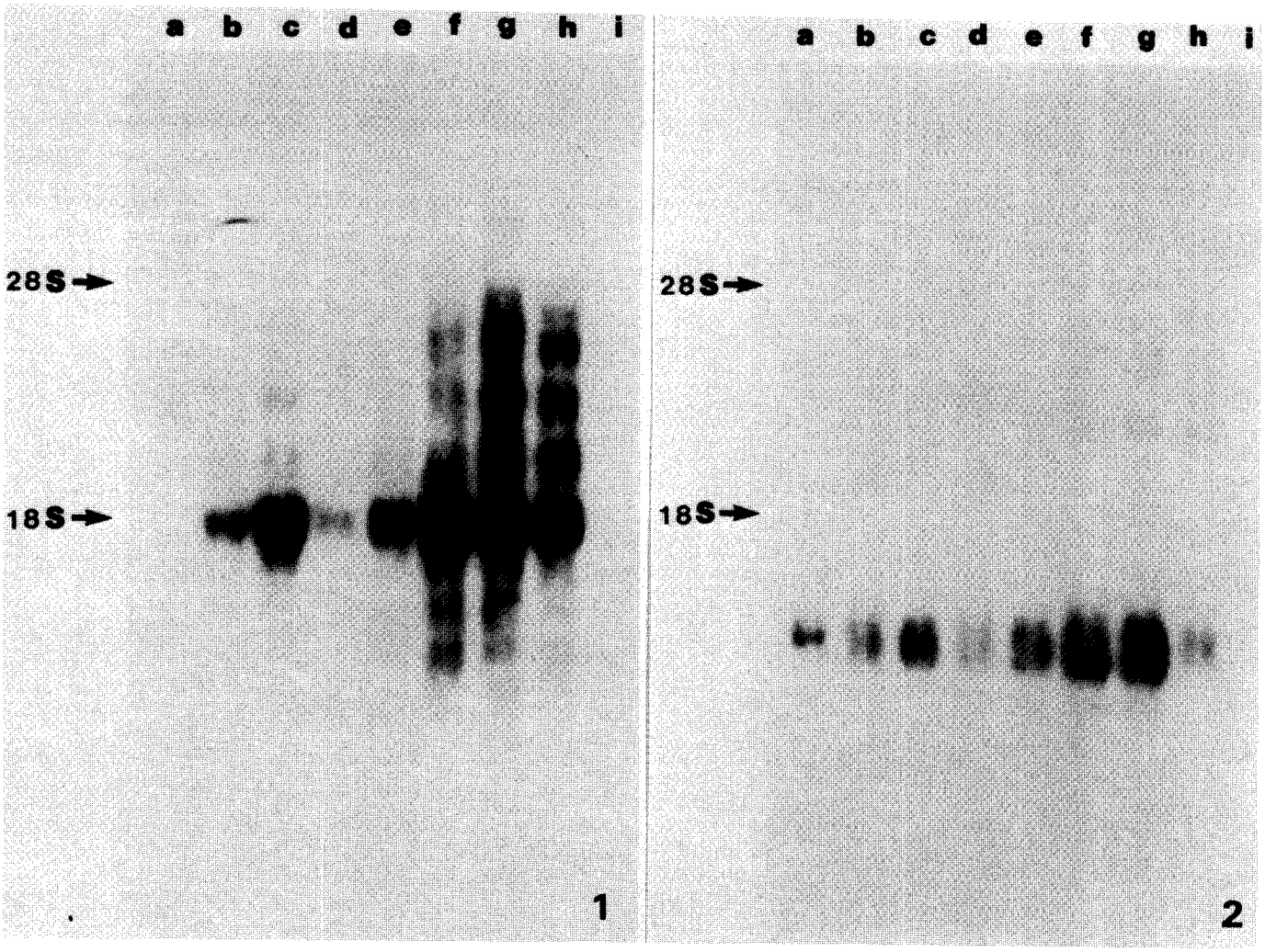

Fig. 1. Autoradiography of northern blot hybridization of choriocarcinoma cell lines with a HLA-A,B,C heavy-chain cDNA probe. The same amount (12 μg) of RNA isolated from cell lines was subjected to agarose electrophoresis, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled HLA-A,B,C cDNA probe. The positions of 28S and 18S RNAs are indicated. *Lane a,* JAR; *Lane b*, BeWo; *Lane c*, ENAMI-1; *Lane d*, IMa; *Lane e*, SCH; *Lane f*, LCL-721; *Lane g*, normal PBL; *Lane h*, Daudi; *Lane i*, F9.

Fig. 2. Autoradiography of northern blot hybridization of choriocarcinoma cell lines with a human β_2m cDNA probe. *Lane a,* JAR; *Lane b*, BeWo; *Lane c*, ENAMI-1; *Lane d*, IMa; *Lane e*, SCH; *Lane f*, LCL-721; *Lane g*, normal PBL; *lane h*, Daudi; *Lane i*, F9.