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Introduction of a human X-6 translocation chromosome into a mouse teratocarcinoma: Investigation of control of *HLA-A,B,C* expression

(human development/somatic cell genetics/β_2-microglobulin/*cis* control)

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ABSTRACT **We have developed an approach to human developmental biology which exploits somatic cell genetics. With this system we have examined the production of the HLA-A,B,C antigens. A human–mouse somatic cell hybrid was constructed which contained a human X-6 chromosome translocation carrying the *HLA* region; this hybrid was used as a donor of the X-6 translocation in the technique of microcell transfer. The X-6 chromosome recipient was the mouse embryonal carcinoma cell line PCC4. The microcell hybrid MCP-6 retained the embryonal carcinoma phenotype as judged by shape and absence of *H-2* expression. Nonetheless, the expression of the *HLA-A,B,C* genes was not extinguished. HLA-A,B,C antigen production at the cell surface, however, was not detected because this hybrid apparently could not make β_2-microglobulin.**

The investigation of human embryonic development is restricted, to a considerable extent, by ethical constraints. Cultured multipotent human teratocarcinoma cell lines, which would be of great use in this regard, have been difficult to obtain (1). We have adapted an alternative approach to study development based on somatic cell genetics.

The human major histocompatibility antigens HLA-A, -B, and -C are known to participate in cell–cell recognition phenomena (for review of *HLA* function, see refs. 2 and 3). The demonstration that mouse embryonal carcinoma cells do not contain the murine histocompatibility antigens H-2K and H-2D (4) and that human trophoblast membranes do not contain HLA-A and -B antigens (5) indicated that control of cell surface histocompatibility antigen expression might have important developmental implications. It was suggested, in the case of the human trophoblast, that the failure to produce HLA-A and -B antigens was associated with the lack of maternal rejection of the fetus and was explained by the target requirement of histocompatibility antigen expression for efficient T-cell killing (5, 6). Avoidance of T-cell killing was also suggested as a selection mechanism for the generation of tumors that lacked HLA-A,B, C antigens (7).

The HLA-A,B,C (and H-2K and -D antigens) are composed of two chains: a polymorphic 44,000-dalton glycoprotein coded for by a gene on chromosome 6, and β_2-microglobulin (β_2M) coded for by a gene on chromosome 15 (reviewed in ref. 8). The Burkitt lymphoma line Daudi, and some other human tumor cell lines, fail to express *HLA-A,B,C* normally on their cell surface because of a defect in β_2M synthesis (7, 9, 10). Genetic and biochemical investigation of Daudi has shown that, in the absence of β_2M, the 44,000-dalton chain is synthesized but apparently is not found at the cell surface (11–13). The reciprocal pattern is seen in term trophoblast and in some human tumors in which normal β_2M synthesis occurs in conjunction with apparent coordinate extinction of expression of the *HLA-A,B,C* genes (5, 7).

The failure of the murine embryonal carcinoma cells to produce H-2K and -D antigens has been studied by somatic cell genetics. Two groups have described somatic cell hybrids between murine embryonal carcinoma cells and thymocytes (14, 15); these hybrids fail to produce the H-2 antigens of the teratocarcinoma parent but continue to produce the thymocyte H-2 antigens, an example of *cis* control of expression. However, conflicting results have been reported by others who observed extinction of the thymocyte H-2D and -K antigens (16, 17).

We have analyzed the production of human histocompatibility antigens in interspecific somatic cell hybrids between mouse teratocarcinoma stem cells and human cells. The genetic analysis has been simplified by using the microcell technique (18, 19) to transfer a single human X-6 translocation chromosome to a mouse embryonal carcinoma cell recipient. The hybrid produced retains the teratocarcinoma phenotype and expresses several human genes. As well as studying control of HLA-A,B,C antigen production, we have used these hybrids to localize the *HLA* region on chromosome 6 and to demonstrate the reactivation of a human gene not expressed in the human parent. The introduction of specific human chromosomes into mouse embryonal carcinoma cells offers a general approach to studying the developmental regulation of the human genome.

METHODS

Cells and Culture Conditions. The Burkitt lymphoma line G3.32.2 was obtained from M. Steel (Medical Research Council Clinical Cytogenetics and Population Genetics Unit, Edinburgh). This cell line was isolated during *in vitro* mutagenesis studies on cell line F137 (20). Isoenzyme analysis suggests that F137 is identical to the Burkitt lymphoma line Jijoye (20). Steel found that the G3.32.2 subclone had acquired *de novo* an X-6 translocation chromosome. The mouse cells used were: PCC4 $8aza^R$ Ou^R, a clonal derivative of PCC4 $8aza^R$ (21) which is resistant to ouabain; PG19, a C57BL6 mouse melanoma (22) used as the positive control for H-2^b expression; 1R, an 8-azaguanine-resistant L cell (23). All the cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, penicillin, and streptomycin. Prior to fusion, PCC4 $8aza^R$ Ou^R and 1R were grown in 8-azaguanine at 30 μg/ml to remove any revertant cells; the

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Abbreviation: β_2M, β_2-microglobulin.

same medium was used to select the revertant mass culture G1R6RM.

Production of G1R6. G3.32.2 cells (5×10^6) were fused to 5×10^6 1R cells by using polyethylene glycol 6000 according to Pontecorvo (24). Hybrids were selected and grown in medium with added hypoxanthine (0.1 mM), methotrexate (10 μM), and (16 μM) thymidine (HAT medium). In addition the initial selection medium for G1R6 contained 10 μM ouabain (HAT/ouabain medium) to select against the human cells. The medium was also changed frequently to select for attached cells.

Microcell Production and Production of MCP-6. Subconfluent G1R6 was grown for 3 days in colchicine (0.2 μg/ml). Enucleation was achieved on discontinuous Ficoll gradients supplemented with cytochalasin B at 10 μg/ml (25). Microcell-containing fractions were pooled, diluted with medium, and pelleted by centrifugation. The microcells were separated by unit gravity sedimentation on a bovine serum albumin gradient (19). After 2 hr the upper two-thirds of the gradient was pooled, diluted in serum-free medium, and pelleted by centrifugation. The pellet was suspended in serum-free medium, and the 6×10^6 microcells were fused to 6×10^6 PCC4 $8aza^R$ Ou^R with inactivated Sendai virus by using the method of Kennett (26).

Indirect Radioimmunoassay and Antisera. The indirect radioimmunoassay was performed as described (27). The following monoclonal antibodies were used.

i. Monomorphic anti-HLA-A,B,C: W6/32 (28); PA2.6 (29); BB7.7 (30). The last antibody specifically recognizes the complex between human HLA-A,B,C chains and human β_2M and fails to recognize HLA-A,B,C chains complexed with mouse β_2M. W6/32 and PA2.6 can recognize HLA-A,B,C heavy chains complexed with mouse β_2M.

ii. Anti-human β_2M; BBM1 (31).

iii. Anti-H-$2D^b$; B-22-249 R1 (32). This antibody was a gift from G. Hammerling.

iv. Anti-H-$2K^k$; 11-4.1 (33). These antibody-producing cells were donated by V. Oi.

v. Anti-SSEA-1. (34). The antibody was a gift from B. Knowles and D. Solter.

vi. Control P3-X63.Ag8 immunoglobulin (35). This antibody was used as the control for nonspecific binding in the indirect radioimmunoassay.

W6/32, B-22-249, and SSEA-1 were obtained in ascites form; the other antibodies were concentrated tissue culture supernatants. All antibodies were used at concentrations that gave maximal binding to antigen-positive target cells. P3-X63.Ag8 antibody was used at a matched concentration.

Two conventional antisera were used in immunoprecipitation analysis: (*i*) goat anti-human β_2M (a gift from M. J. Crumpton) and (*ii*) 5996.3, a rabbit anti-human HLA-A,B,C heavy chain antiserum [a gift from N. Tanigaki (36)]. The specificity of the latter antibody for free HLA-A,B,C chains in the absence of β_2M has been demonstrated for *in vitro* cell-free translation (37) and for HLA-A,B,C chains produced by cells with a defect in β_2M synthesis (38).

Immunoprecipitation. About 5×10^6 cells were washed in methionine-free RPMI-1640 medium and resuspended in 1 ml of the same medium without fetal calf serum. After 30 min at 37°C, 1 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine (1000 Ci/mmol; The Radiochemical Centre, Amersham, England) was added, and incubation was continued for 3.5 hr. The cells were washed in complete medium, harvested, suspended in lysis buffer, and left on ice for 10 min. Nuclei were removed by centrifugation ($1000 \times g$ for 10 min) and the supernatants were passed through nitrocellulose filters (0.22 μm; Millipore). Aliquots containing 2×10^7 cpm from the samples prepared as above were made up to 1 ml in lysis buffer. The lysates were precleared by adding 10 μl of normal rabbit serum and incubating for 30 min at 4°C; then 50 μl of washed 10% formalin-fixed *Staphylococcus aureus* (Cowan strain 1) was added and incubation was continued for a further 30 min at 4°C, followed by centrifugation. Specific antisera were added to the cleared supernatants, and the immune complexes were precipitated and washed as described (13). The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction with 2-mercaptoethanol (38).

Karyotypic Analysis. The methods used have been described in detail (39). Human chromosomes were recognized by G11 staining (40) of chromosome spreads, and detailed analysis was performed by quinacrine banding (41) and trypsin-induced Giemsa banding (42).

RESULTS

The direct transfer of chromosomes from human cells to mouse recipient cells has proved to be difficult by using the colchicine method of inducing micronuclei. This difficulty can be circumvented by first producing human–mouse somatic cell hybrids and using these hybrids as the microcell donors of the human chromosomes. Because we wished to transfer the X-6 chromosome to the murine embryonal carcinoma cell PCC4, we first constructed a hybrid between G3.32.2 and another mouse cell line, the 8-azaguanine-resistant L cell 1R. The hybrid produced, G1R6, was used as the chromosome donor of the X-6 translocation to PCC4.

Production and Analysis of G1R6. G3.32.2 was fused with 1R by using polyethylene glycol, and the fusion mixture was plated in HAT/ouabain selective medium; the medium was changed frequently to select for attached cells. G3.32.2 grows in suspension and fails to grow in the presence of 10 μM ouabain; the attached 1R cells lack hypoxanthine phosphoribosyltransferase and cannot grow in HAT medium. The hybrids between G3.32.2 and 1R might be expected to retain the X-6 translocation if an active X were involved in the translocation. After 3 weeks, attached colonies were observed growing in the selective medium. Several clones were picked and one clone, G1R6, was expanded for further study.

G1R6 was shown to be a hybrid by the presence of a human–mouse heteropolymeric band for glucose-6-phosphate dehydrogenase (data not shown). In addition this hybrid produced several human cell surface antigens including HLA-A,B,C antigens as detected by the monoclonal antibodies W6/32 and BB7.7 in an indirect radioimmunoassay (Table 1). PA2.6, which is also a monomorphic anti-HLA-A,B,C, showed reactivity with the mouse parent 1R. This may be a specific crossreaction with the *H-2*^{k} haplotype because no reaction was observed with other non-*H-2*^{k} mouse cell lines. A similar apparently specific crossreaction was seen with W6/32 and the *H-2*^{b} haplotype (see below). Karyotypic analysis of G1R6 showed the presence of the X-6 translocation and absence of a normal chromosome 6. Further evidence that the X-6 translocation chromosome carried the *HLA-A,B,C* genes was obtained by back-selection in 8-azaguanine to remove the active human X chromosome contribution. The mass culture G1R6RM lost the active X chromosome as adjudged by the loss of human glucose-6-phosphate dehydrogenase. Concomitantly this mass culture also lost the ability to produce HLA-A,B,C antigens (Table 1). G1R6 and G1R6RM both produced human β_2M.

Production of MCP-6. The microcells from G1R6 were fused to PCC4 by using Sendai virus, and the fusion products were selected by plating in HAT medium. Several colonies that had a teratocarcinoma appearance were observed 21 days after the fusion. Three colonies, picked at random, all produced human

Table 1. Expression of HLA-A,B,C by G1R6

	Anti-HLA-A,B,C			Anti-human β_2M	Anti-H-2K^k	Control
	PA2.6	W6/32	BB7.7	BBM1	11.4.1	P3-X63.Ag8
G1R6	**9,604 ± 100**	**8,298 ± 900**	**7,212 ± 907**	**12,418 ± 994**	**13,494 ± 1258**	1369 ± 210
G1R6M	**5,585 ± 395**	3,010 ± 102	3,083 ± 85	**16,260 ± 2076**	**16,266 ± 790**	1573 ± 69
1R	**5,373 ± 726**	1,570 ± 267	2,159 ± 103	2,677 ± 135	**13,927 ± 670**	1448 ± 452
G3.32.2	**26,696 ± 2457**	**23,090 ± 1525**	**16,072 ± 217**	**20,838 ± 872**	1,583 ± 157	572 ± 29

The results are shown as mean ± SD of three determinations and represent cpm bound to 2.5×10^5 cells. Positive reactions (i.e., 3 times the P3-X63-Ag8 value) are in boldface.

glucose-6-phosphate dehydrogenase. One of these colonies, MCP-6, was chosen for further study. MCP-6 was indistinguishable from PCC4 morphologically. Typical of murine teratocarcinomas, it had a small cytoplasm-to-nucleus ratio, and one or two prominent nucleoli. The presence of the X-6 translocation and the absence of any other human chromosomes in the microcell hybrid was confirmed by detailed karyotypic analysis. Differential staining of human chromosomes by G11 banding demonstrated the presence of only one human chromosome (Fig. 1 *Left*). Further analysis by using trypsin-induced Giemsa banding indicated that the human chromosome involved a translocation between the long arm of the human X chromosome and the human chromosome 6 (Fig. 1 *Right*), confirming that the active X is in the X-6 translocation. The break points are Xq13 translocated to 6p21 (Fig. 2). The mouse chromosomes of MCP-6 are the same as PCC4, containing two marker metacentric chromosomes and a near diploid number.

Production of HLA-A,B,C Antigens by MCP-6. MCP-6 has an antigenic phenotype similar to that of PCC4 and other murine embryonal carcinoma cell cultures (34). It produced the antigen SSEA-1 (Table 2). The failure to produce H-2 antigens, as monitored by reaction with the H-2D^b-specific monoclonal antibody B-22-249, was also consistent with the embryonal carcinoma cell phenotype (4). PCC4 was derived from the *H-2^b* mouse strain 129 (21). The weak reaction of the monoclonal antibody W6/32 with the H-2D^b antigen-positive cell line PG19 apparently was due to a specific crossreaction with this haplotype of the mouse (unpublished results). The failure of MCP-6 to react with W6/32 indicated that this hybrid produced, at the cell surface, neither the X-6 chromosomally derived HLA-A,B,C antigens nor antigens of the *H-2D^b* haplotype. The failure to produce cell surface HLA-A,B,C antigens was further confirmed by the failure of MCP-6 to react with PA2.6, a monoclonal anti-HLA-A,B,C which does not crossreact with *H-2D^b*. As expected from the karyotypic analysis, MCP-6 did not express chromosome 15-controlled human β_2M.

The Burkitt lymphoma cell line Daudi fails to produce cell surface HLA-A,B,C antigens because of a defect in β_2M production (7, 11–13). The presence of uncomplexed intracellular HLA-A,B,C chains in Daudi can be demonstrated with the rabbit antiserum 5996.3; immunoprecipitation of [^{35}S]methionine-labeled cell extracts with this antibody precipitated 44,000-dalton polypeptide HLA-A,B,C chains (for example, see figure 1 in ref. 38). A similar 44,000-dalton polypeptide was precipitated by 5996.3 from [^{35}S]methionine-labeled extracts of MCP-6 (Fig. 3). Specific bands were not seen when the same extract was precipitated with W6/32, goat anti-human β_2M or a control normal rabbit serum; nor did 5996.3, W6/32 or the goat anti-mouse β_2M precipitate specific bands from PCC4. In contrast, all three specific antibodies precipitated a 44,000-dalton polypeptide (just below the nonspecific actin band seen with normal rabbit serum) from ^{35}S-labeled cell extracts of G3.32.2.

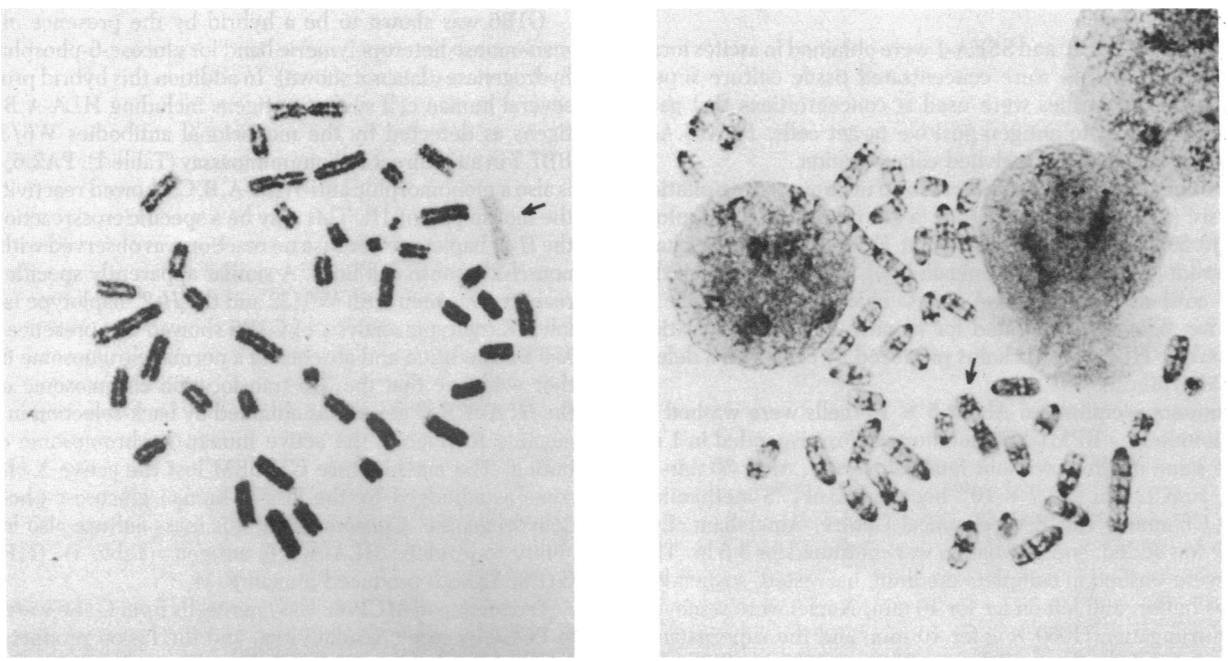

FIG. 1. Karyotypic analysis of MCP-6. (*Left*) Chromosome spread of MCP-6 stained by the G11 method. Arrow, lightly staining human X-6 translocation chromosome. (*Right*) Trypsin/Giemsa banded chromosome spread of MCP-6. Arrow, human X-6 translocation chromosome.

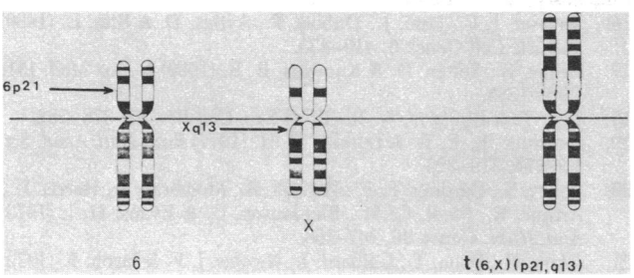

FIG. 2. A reconstruction of the break points in the human X-6 translocation chromosome.

DISCUSSION

The human–mouse hybrid MCP-6 retains a murine embryonal carcinoma cell appearance and antigenic phenotype despite the presence of an X-6 translocation chromosome derived from a human lymphoblastoid cell line. The maintenance of the stem cell phenotype has previously been seen in many intraspecific somatic cell hybrids (14–17, 43) and interspecific hybrids (44). MCP-6 can be used for investigation of developmental regulation of human chromosome X and 6 linked genes. In this communication we have described *HLA* expression.

The native cell surface HLA-A,B,C antigens are a complex between the chromosome 6 encoded heavy chains, which carry the polymorphic determinants, and β_2M. Genetic experiments with the β_2M-negative human cell line Daudi have shown that β_2M is necessary for normal cell surface HLA-A,B,C production (11). Karyotypic analysis (Figs. 1 and 2) and enzyme analysis [lack of human mannose phosphate isomerase (unpublished data)] demonstrate that MCP-6 lacks human chromosome 15, the chromosome that carries the structural gene for human β_2M (10). Although mouse β_2M is capable of supporting cell surface HLA-A,B,C antigen production in hybrids (45), murine embryonal carcinoma cells do not produce cell surface β_2M (46). The precipitation of a 44,000-dalton polypeptide chain from MCP-6 by antiserum 5996.3 suggests that the failure to produce cell surface HLA-A,B,C antigens is due, as in the case of Daudi, to the failure to synthesize either human or mouse β_2M. This conclusion is supported by recent experiments with conventional hybrids between G3.32.2 and PCC4 that have retained chromosome 15 as well as the X-6 translocation; these hybrids retain the embryonal carcinoma phenotype and produce HLA-A,B,C antigens and human β_2M at the cell surface although the murine H-2D^b antigens are not expressed (unpublished data).

The failure of the embryonal carcinoma cells to extinguish *HLA-A,B,C* gene expression is consistent with the results of several studies using intraspecific hybrids. Hybrids between teratocarcinoma stem cells and thymocytes retain the stem cell phenotype, cease expressing several thymocyte-specific antigens, and continue to express the thymocyte H-2 antigens but fail to switch on the dormant *H-2* genes of the teratocarcinoma parent (14, 15). A similar result has been reported in hybrids between embryonal carcinoma cells and myeloma cells (47).

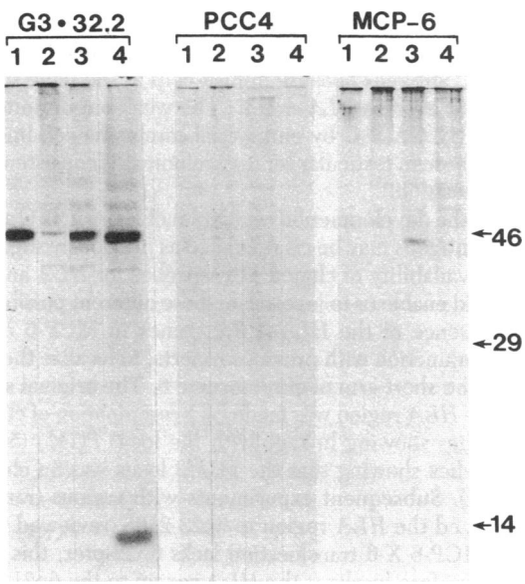

FIG. 3. Immunoprecipitation analysis of *HLA-A,B,C* expression by MCP-6. The immunoprecipitates were reduced and run on 12.5% acrylamide gels in the presence of $NaDodSO_4$. The following antibodies were used: lane 1, W6/32; lane 2, normal rabbit serum; lane 3, rabbit antiserum 5996.3; lane 4, goat anti-human β_2M. Molecular weights are shown $\times 10^{-3}$.

Apparently conflicting results were reported by two other groups (16, 17). The reason for this discrepancy is not clear but may be the precise antigens being studied (H-2K versus H-2D) or differences in the sensitivity of the assays used. Other possibilities include differences in the differentiated state of the hybrids produced by the different groups and segregation of the active β_2M genes.

The failure of the embryonal carcinoma cells to extinguish HLA-A,B,C synthesis could be due to one or more of several reasons. First, embryonal carcinoma cells may be incapable of regulating any differentiated functions. This seems unlikely in view of our experiments with thymocytes and those of others using embryonal carcinoma–Friend cell hybrids; hemoglobin synthesis and inducibility is clearly extinguished in hybrids that retain the teratocarcinoma phenotype (43).

Second, murine embryonal carcinoma cells may be incapable of regulating human genetic material. This is unlikely because we have previously demonstrated the "reactivation" of the gene controlling the human malic enzyme coded for by chromosome 6 but not expressed in G3.32.2, when the X-6 translocation is introduced into PCC4 either by microcell transfer or by normal direct fusion (48). Also, recent experiments on the expression of several monoclonal antibody-defined human X-linked antigens in MCP-6, and other similar hybrids, demonstrate regulation of these cell surface antigens by the teratocarcinoma phenotype (unpublished data). However, without more detailed analysis it is not clear if regulation is a transcriptional, transla-

Table 2. Expression of HLA-A,B,C by MCP-6

	Anti-HLA-A,B,C			Anti-human β_2M	Anti-SSEA 1	Anti-H-2D^b	Control
	PA2.6	W6/32	BB7.7	BBM1	480	B-22-249	P3-X63.Ag8
MCP-6	523 ± 76	544 ± 52	455 ± 57	709 ± 53	**3732 ± 342**	839 ± 123	507 ± 35
PCC4	655 ± 77	423 ± 56	347 ± 66	647 ± 57	**4271 ± 375**	437 ± 118	309 ± 5
PG19	605 ± 63	**3,502 ± 529**	427 ± 75	638 ± 31	292 ± 32	**6573 ± 607**	282 ± 64
G3.32.2	**16,608 ± 285**	**21,502 ± 850**	**10,504 ± 382**	**20,012 ± 958**	240 ± 40	262 ± 3	309 ± 5

See legend to Table 1.

tional, or post-translational phenomenon in these systems.

Third, embryonal carcinoma cells may be unable to regulate HLA-A,B,C antigens because human cells at the same stage of development express *HLA-A,B,C*. The weak but definite production of HLA-A,B,C by embryonal carcinoma cell lines derived from human testicular teratocarcinomas is consistent with this hypothesis (49).

Fourth, the developmental regulation of HLA-A,B,C and H-2K and D antigens may be *cis* regulated as previously suggested (15). The availability of cloned DNA probes for *HLA* and H-2 genes should enable us to investigate these different possibilities.

The presence of the *HLA-A,B,C* genes in MCP-6 can be used, in conjunction with previous reports, to localize the *HLA* region on the short arm of chromosome 6. The original assignment of the *HLA* region was made by a combination of classical family studies showing linkage with the locus *PGM3* (50) and further studies showing that the *PGM3* locus was on chromosome 6 (51). Subsequent experiments with various translocations localized the *HLA* region to 6p23-2105 (reviewed in ref. 52). The MCP-6 X-6 translocation lacks 6p22-pter; this translocation therefore localizes the *HLA* region to the 6p21 band. The microcell-hybrid approach we have described is a general approach which can be used to study developmental regulation of human genes. In addition, the hybrids can be used as immunogens and as source material for DNA cloning experiments to attempt identification of human embryonic material.

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