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Mitotic Separation of Two Human X-Linked Genes in Man–Mouse Somatic Cell Hybrids

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ABSTRACT Six interspecific somatic hybrid cell lines were derived from a mouse line deficient in hypoxanthine: guanine phosphoribosyltransferase (HGPRT) and human diploid cells with normal enzyme activity. Human HGPRT was present in all six hybrids and the clones derived from them. However, in two of the six, and in some clones from another two, human glucose-6-phosphate dehydrogenase (G6PD) was absent. Since the structural loci for both these enzymes are X-linked in man, these findings suggest that these two loci have separated quite frequently through chromosome breakage and that they must be rather far apart on the X chromosome.

The potential importance of man–mouse somatic cell hybrids for the genetic analysis of man became apparent after the discovery by Weiss and Green (1) that this type of interspecific hybrid undergoes a rapid loss of human chromosomes. They attempted to determine the chromosomal location of the human structural gene for thymidine kinase (TK) by obtaining cell hybrids between normal human diploid cells and mouse cells that were deficient in TK and therefore unable to grow in HAT selective medium (2). The appearance of hybrid clones that grew in HAT medium suggested that human *tk* locus was being retained and expressed in the hybrid cells. Presumably, the human chromosome carrying the *tk* locus was selectively retained. Further studies (3, 4) led to the conclusion that the chromosome in question belongs to the human E-group, since a small submetacentric chromosome was present in all of the hybrid cells grown in HAT medium, even though other human chromosomes were lost. This conclusion was strengthened by the results of a back selection experiment: when these cells were grown in 5-bromodeoxyuridine (which selects for TK deficiency) this chromosome was lost. Recently, Migeon *et al.* have shown (5) that the electrophoretic mobility of the TK produced by the hybrid cells grown in HAT medium resembles that of human enzyme. An alternative hypothesis—of a reverse mutation at the mouse *tk* locus—was therefore unlikely.

Abbreviations: HGPRT, hypoxanthine: guanine phosphoribosyltransferase (EC 2.4.2.8); Hgprt, phenotype denoting ability or otherwise to synthesize this enzyme; *hgprt*, gene directing this synthesis; G6PD, G6pd, and *g6pd*, similarly for glucose-6-phosphate dehydrogenase (EC 1.1.1.49); TK, thymidine kinase (EC 2.7.1.21); HAT, hypoxanthine–aminopterin–thymidine selective growth medium.

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Using similar methods, Nabholz *et al.* (6) showed that the hybrid clones derived from a fusion between normal human lymphocytes and a mouse line deficient in hypoxanthine: guanine phosphoribosyl transferase (HGPRT) always retained a genetic marker of the human X-chromosome, glucose-6-phosphate dehydrogenase (G6PD), and that G6PD was always lost by hybrid cells that survived back-selection (for (HGPRT) deficiency) in 8-azaguanine. Since the X-linkage of the human loci for *hgprt* and *g6pd* has been established beyond doubt by pedigree analysis (7, 8) and other studies (9, 10), these findings provide indirect support for the hypothesis that the survival of the hybrid cells in HAT selective medium depends on the retention and expression of at least part of the human X-chromosome. Direct support for this conclusion has been provided by Shin *et al.* (11), who showed that the HGPRT produced by these hybrids is electrophoretically indistinguishable from the human HGPRT, but clearly different from the mouse enzyme.

The purpose of this paper is to report the first evidence of mitotic separation of linked loci in somatic cell hybrids. A preliminary account of some of this work has been given elsewhere (12, 13).

METHODS

Human diploid cells were obtained from three sources: blood of an erythroblastotic newborn male infant (RBC), with red-cell precursors making up 50% of the nucleated cells; peripheral blood lymphocytes from three adults (two female, JW and ADC, and one male, CF) grown for 3 days in the presence of phytohemagglutinin; and fibroblasts from an embryonic female (HF_2) and an adult with the sex chromosomal complement of XXXXY (4XY). The mouse cells were of the A_9 line, which is deficient in HGPRT, and the B_{82} line, deficient in TK. Both lines were originally derived from the L-cell line by Littlefield (14, 15). Neither cell line will grow in HAT medium (2, 14). Murine and human cells were fused by the use of UV-inactivated Sendai virus (16). Six viable hybrid lines free of the parental A_9 cells and one free of the parental B_{82} cells were obtained by growth of cultures containing the fused cells in HAT medium. Colonies of hybrid cells appeared in 2–5 weeks. Mass cultures were obtained by transferring individual colonies to glass culture vessels and passing them in HAT medium at approximately weekly intervals.

The chromosome complement and enzyme content of each hybrid cell line were determined at intervals. G6PD activity was determined by the method of Motulsky *et al.* (17), and

TABLE 1. *Presence and activity of enzymes in mouse–human hybrids*

Cell type	Identification number*	No. of metaphase cells analyzed	Modal no. chromosomes	Enzyme studies					
				Human		Mouse		Activity	
				HGPRT	G6PD	HGPRT	G6PD	HGPRT†	G6PD‡
A_9	...	21	56,57	0	0	0	+	0.02	0.156
L_{929}	3	...	...	0	0	+	+	101±20§	0.154
A_9–RBC2	5	36	60,61	+	+	0	+	15–39	0.176
A_9–CF	6	28	61	+	+	0	+	18–27	0.220
A_9–ADC	7	24	56	+	+	0	+	13–38	0.257
A_9–4XY	8	20	57,58	+	+	0	+	13–40	0.257
A_9–RBC1	9	45	57	+	0	0	+	34–50	0.149
A_9–JW	10	31	55	+	0	0	+	4–96	0.126
B_{82}–HF2	11	23	64	0	0	+	+	118	0.134

* Corresponds to channel number in Figs. 1 and 2.

† Nanomoles of IMP formed per hr per mg protein. The values are not directly comparable as they increased with time after hybridization.

‡ International units per mg of soluble protein. Values usually increased slightly with time after hybridization.

§ SD, $n = 7$.

expressed in international units per mg of soluble proteins. The type of G6PD, whether human or mouse, was determined electrophoretically by the method of Rattazzi *et al.* (18). The activity of HGPRT was determined by the method of Harris and Cook (19), and the enzyme was characterized electrophoretically as described by Shin *et al.* (11). Control enzyme preparations were obtained from normal human lymphocytes, normal diploid fibroblastic cells, or mouse L-cells. Usually, sonicates of 1–3 × 10_6 cells were required for each set of quantitative and qualitative enzyme assays. Growth of L-cells in medium 199 + 10% fetal calf serum or Dulbecco-modified Eagle's medium + 10% fetal calf serum with or without HAT did not affect the electrophoretic mobility of either enzyme.

Hybrid cell lines were cloned in HAT or 8-azaguanine in microtest tissue-culture plates (Falcon). All the hybrid lines and the clones derived from them have been stored at −90°C for future use.

RESULTS

Chromosome studies

The A_9 mouse cells used in these experiments had modal numbers of 56 and 57 chromosomes, including several biarmed chromosomes indistinguishable from the human X. The mouse–man hybrid cells had modal numbers of 55–61 chromosomes. Because several mouse and human chromosomes were morphologically similar, the number of mouse or human chromosomes in any hybrid could not be determined, although most of them were clearly of mouse origin.

Enzyme studies: HGPRT

The A_9 mouse cell had no detectable HGPRT activity, but considerable enzyme activity was present in the other parental cell types and in every hybrid cell line (Table 1). The electrophoretic mobility of mouse HGPRT is clearly different from that of human HGPRT (Fig. 1). The HGPRT activity in all of the A_9–human hybrid cell lines has the same mobility as the human enzyme, while the B_{82}–human hybrid cells have HGPRT with the mobility of the mouse enzyme.

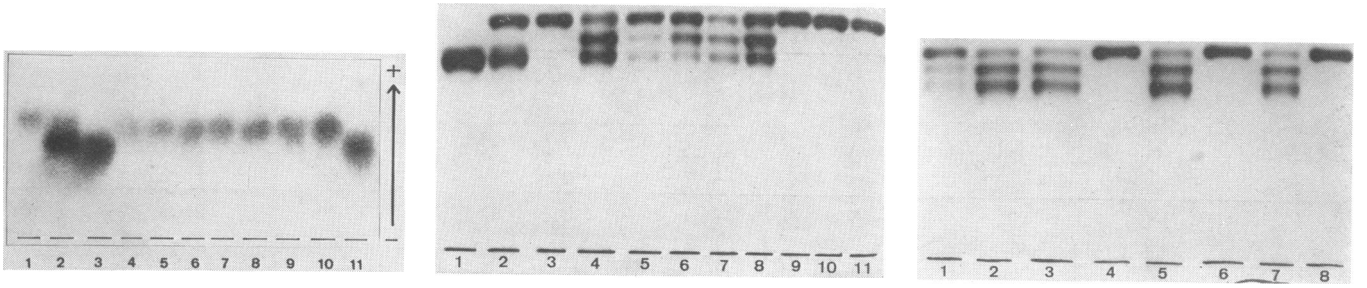
These results suggest that every A_9–human hybrid of the present series owes its survival in HAT medium to the production of HGPRT of human type. The B_{82}–human hybrid cells, for which a condition for survival in HAT is the retention of the human *tk* locus and not necessarily of human HGPRT, produce mouse HGPRT but not human HGPRT, probably because they have lost the human X-chromosome. This conclusion is supported by the absence of human G6PD in these cells.

Enzyme studies: G6PD

All the parental cell lines used for the hybridization experiments and all hybrid lines had G6PD activity (Table 1). However, electrophoretic studies performed on cell sonicates of the hybrid lines and on controls showed differences in the G6PD patterns (see Fig. 2). Human G6PD and mouse G6PD migrate as single bands (channels 1 and 3). A mixture of the two does not lead to the formation of additional bands (channel 2). Four of the six A_9–human hybrids (channels 5–8) showed an enzyme band with intermediate mobility in addition to both human and mouse G6PD. This probably represents heteropolymeric G6PD molecules made up of murine and human G6PD subunits (6). The other two A_9–human hybrids (channels 9 and 10), as well as the B_{82}–human hybrid (channel 11), contained only murine G6PD. The relative intensities of the murine, heteropolymeric, and human G6PD bands in the four A_9–human hybrids (channels 5–8) were different for each hybrid; in two instances (channels 5 and 6) the murine G6PD was clearly in excess of the human G6PD (M > H); in two other hybrids (channels 7 and 8) the amount of human G6PD was equal to, if not greater than, the amount of murine G6PD (M < H). Under our assay conditions, the banding pattern of G6PD of similar man–mouse hybrid clones, which were kindly provided by Drs. Ruddle (20) and Bodmer (6), was always found to be of the type M < H (see channel 4).

Cloning studies

The variable banding pattern of G6PD in our series of A_9–human hybrids might reflect mixed populations of cells with



(*Left*) FIG. 1. Electrophoretic mobility of murine and human HGPRT in man–mouse somatic cell hybrids and controls.

Channel *1*, human fibroblasts; *2*, mixture of human and mouse cells; *3*, mouse cells (line L-929); *4*, man–mouse hybrid line (3W4) from Nabholz *et al.* (6) derived from a HGPRT-deficient mouse line and human lymphocytes; *5–10*, the man–mouse hybrid lines of the present series, derived from the A_9 mouse line (HGPRT-deficient) and human diploid cells of different types; *11*, the man–mouse hybrid line derived from the B_{82} mouse line (TK-deficient) with human diploid fibroblasts.

The murine HGPRT moves more slowly than the human enzyme. All the man–mouse hybrids isolated in the HAT medium and derived from a HGPRT-deficient mouse line produce a "human-like" HGPRT (channels 4–10). The man–mouse hybrid derived from the TK-deficient mouse line (channel 11) produced a "murine-like" HGPRT.

(*Center*) FIG. 2. Electrophoretic mobility and intensity of murine (fast), human (slow), and heteropolymeric (intermediate) G6PD in hybrids and controls. Channels are the same as for Fig. 1.

The normal banding pattern is the one of channel 4 (the man–mouse hybrid of Nabholz *et al.*), in which the intensity of the human G6PD is greater than that of the murine G6PD.

The hybrids of channels 9 and 10 possess only murine G6PD (though they still possess human HGPRT, Fig. 1). The hybrid of channel 11 has lost human G6PD and human HGPRT (Fig. 1). The variable banding pattern of the hybrids of channels 5–8 is due to mixture of cells having the "normal" banding pattern (as in channel 4) with cells having only murine G6PD.

(*Right*) FIG. 3. G6PD patterns in a mass culture (channel 1, A_9–RBC2) and in representative clones derived from it (channels 3, 4, 5, 6, 7, and 8). Channel 2 shows the control hybrid (3W4) provided by Nabholz *et al.* Three of the man–mouse hybrid clones have only murine G6PD. The banding pattern of G6PD in the clones of channels 3, 5, and 7 is now identical to the normal banding pattern of channel 2, where the human G6PD is in excess of the murine G6PD.

the intensity pattern of cloned hybrids (M < H) (channel 4) together with hybrids possessing only murine G6PD. In order to test this possibility, we cloned the six A_9–human hybrid lines and the B_{82}–human hybrid line in HAT medium and studied each clone for its HGPRT and G6PD enzyme patterns. Table 2 summarizes the results for a total of 121 such clones. Studies of HGPRT showed that all the 105 hybrid clones derived from the six A_9–human mass cultures possessed human HGPRT, while the 16 hybrid clones derived from the B_{82}–human cell line possessed murine HGPRT. Of these first 105 hybrid clones, which were derived from six independent fusion experiments between A_9 and human cells, only 58 possessed human G6PD and a triple-banded pattern. In 50 of these, the pattern of murine, heteropolymeric and human G6PD was of the type M < H. The remaining 8 "clones" still had a triple-banded pattern of the type M > H, which suggests that they still may be mixtures of cells, some of which possess only murine G6PD. Of the two A_9–human hybrids that contained a mouse G6PD band of greater intensity than the human band (M > H), one (A_9–RBC2, see Fig. 3, channel 1) yielded clones of two types—some clones possessing the triple-banded pattern M < H (Fig. 3, channels 3, 5, and 7) like that of clones of the hybrids produced by Nabholz *et al.* (Fig. 3, channel 2), and other clones possessing only murine G6PD (Fig. 3, channels 4, 6, and 8). The 16 clones derived from the mass culture of B_{82}–human hybrid had only murine G6PD.

DISCUSSION

In somatic cell hybrids derived from normal human diploid cells and HGPRT-deficient mouse cells, survival in HAT medium depends on the retention and expression of the human structural locus for HGPRT. Since the loci for both HGPRT and G6PD are located on the X-chromosome, failure to find human G6PD in 47 out of 105 clones derived from four of the six hybrid lines studied is unexpected.

The simplest explanation of these findings is that the X-chromosome has broken between the loci for *hgprt* and *g6pd*, with obligatory retention of the *hgprt* locus and occasional loss of the *g6pd* locus. Such a loss of loci may provide a mechanism for "mitotic segregation" in somatic hybrid cells. The two human X-linked loci in question must necessarily be far apart from one another for breakage to have occurred so frequently between them.

Alternative explanations for the absence of human G6PD in the A_9–human hybrid lines that possess human HGPRT include (*i*) use of human cell donors who have a G6PD-negative allele and therefore are deficient in G6PD activity, (*ii*) fresh somatic mutation to deficiency, (*iii*) repression of the human *g6pd* locus, and (*iv*) back mutation or virus-dependent enzyme induction at the mouse *hgprt* locus.

The first possibility has been ruled out by the direct examination of the donors. All were found to be of normal phenotype, generally called Gd(+);B (21).

Fresh somatic mutation cannot be excluded, but it is known to be a very rare event. It is unlikely that fresh mutation could account for the high frequencies of exceptions observed (47 in 105), expecially in the absence of a selective pressure for cells lacking human G6PD.

Repression of the synthesis or inhibition of the activity of human G6PD also seems unlikely in view of the normal production of mouse G6PD. In addition, after cloning, the cells never underwent further phenotypic changes even after numerous cell divisions, and those that had been derived from the mass cultures with murine G6PD alone never produced human G6PD. Back mutation or virus-dependent enzyme

TABLE 2. *Characteristics (human or murine) of enzymes in hybrids and clones*

Hybrid	No.*	Mass cultures		Clones			
					G6PD†		
		HGPRT	G6PD	HGPRT	MHyH M > H	MHyH M < H	M
A_9–RBC2	5	H	MHyH (M>H)	H	1	18	3
A_9–CF	6	H	MHyH (M>H)	H	5	12	
A_9–ADC	7	H	MHyH (M≤H)	H	2	6	5
A_9–4XY	8	H	MHyH (M≤H)	H		14	
A_9–RBC1	9	H	M	H			20
A_9–JW	10	H	M	H			19
B_{82}–HF2	11	M	M	M			16

H, human; Hy, hybrid; M, murine. M > H means more murine than human enzyme present.
* Corresponds to channel number in Figs. 1 and 2.
† Numbers refer to the number of clones in each category.

induction at the mouse *hgprt* locus, resulting in the production of HGPRT with an electrophoretic mobility indistinguishable from that of human HGPRT, can be excluded with reasonable confidence because the reversion to Hgprt$^+$ was never observed in the control fusions within the A_9 mouse parental line. Furthermore, the HGPRT produced by all the A_9–human hybrids, with or without the human G6PD, is always of the same type and is electrophoretically indistinguishable from the human HGPRT. Even if the A_9 cell could revert to Hgprt+, the possibility that the new HGPRT activity would be always "human-like" is rather small.

Loss of the structural locus is therefore the most likely explanation for the absence of the human G6PD. This could be the result of a chromosomal breakage followed by the loss or suppression of that part of the human X-chromosome carrying the *g6pd* locus.

On the basis of the frequent separation of the two loci observed in our series of A_9–human hybrids, it can be concluded that the genes for HGPRT and G6PD are not closely linked. Pedigree analysis of the only family so far reported as segregating at both these loci (22) suggests that they are indeed "loosely linked". This is in accord with our conclusion.

In contrast to our results, Nabholz *et al.* (6) did not observe loss of human G6PD in 42 clones derived from five man–mouse somatic cell hybrids of the same type. Ruddle (personal communication), however, found cytological evidence of X-chromosome breakage in one of the hybrid clones derived from his series of man–mouse hybrids, and Migeon and Miller (4) suggested the occurrence of similar chromosome instability in hybrids derived from human diploid cells and mouse cells deficient in TK. They noted a steady increase with time in the number of cells able to grow in HAT medium despite the absence of detectable human E-group chromosomes and suggested that the human *tk* locus may have been translocated to a mouse chromosome. It seems probable, therefore, that we have observed the separation of linked loci at such high frequencies because our hybrids have all been grown in the HAT selective medium for an extended period of time (over a year).

Live Sendai virus can produce chromosome breaks in cultured cells (23). Chromosome breakage and rejoining have also been observed in mammalian interspecific somatic cell hybrids produced by fusion with inactivated virus (24). Saksela *et al.* (25) have shown that chromosome breakage may even be an unavoidable consequence of virus-induced fusion. This could be a serious source of error when one is determining new linkage groups by means of the man–mouse somatic cell hybrid technique. On the other hand, it is an ideal tool, if not a necessary requirement, for estimating the genetic distance between linked loci, as demonstrated in the present series of experiments with the two X-linked markers.

The analysis of interspecific somatic cell hybrids may also be utilized for mapping the chromosomes of other animals, since preferential loss of the chromosomes of one of the species in interspecific hybrids is not limited to man–mouse hybrids (24, 26). Linkage groups can best be determined under conditions of maximum chromosome stability, while map distances and gene order can perhaps be determined most efficiently by exposure of the test cells to a chromosome-breaking agent under standard conditions prior to hybridization.

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