expected to produce a single very broad chevron and not the clearly separated double chevron. Squire's model, like that of Miller and Tregear[18] predicts that a large fraction of the myosin heads would be attached in rigor and would not be expected to explain the distribution of intensity in the equatorial X-ray diffraction pattern.

In vertebrate skeletal muscle the exact arrangement of neighbouring actin filaments is not known. If they are related by a 90° rotation[25], our construction shows that, as in insect flight muscle, the two heads of a myosin molecule may attach to neighbouring actin filaments. In this case, more distortion of the heads would be required to accommodate the varying interfilament distances produced at different sarcomere lengths. Squire and Luther have examined transverse sections through the ends of the A band of vertebrate skeletal muscle in rigor and have concluded that both single-filament and two-filament interactions occur (personal communication).

Conclusions

In biochemical experiments on suspensions of actin filaments the two heads of a myosin molecule probably interact, albeit with strain, with adjacent subunits in the same actin filament. But in the highly ordered filament lattice present in striated muscles considerations of geometry suggest that tighter binding could occur if the two heads bound to different actin filaments.

We have here been concerned with the rigor configuration, which is usually regarded as the termination of the drive-stroke of the cross-bridge cycle[2], but it would be reasonable to expect that the same two-filament interaction would occur during the earlier part of the stroke. In previous discussions of the mechanism of contraction in muscle, the two-headed nature of the myosin molecule has largely been ignored. Our ideas suggest a particular way in which the two heads are used; if correct, a new precision will have been given to the hitherto rather vague concept of the cross-bridge.

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X-chromosome inactivation during differentiation of female teratocarcinoma stem cells *in vitro*

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Evidence is presented that both X chromosomes are genetically active in clonal cultures of undifferentiated female mouse teratocarcinoma stem cells derived from a spontaneous ovarian tumour. As the cells differentiate in vitro *one of the X chromosomes becomes inactivated.*

EARLY in the development of the female mammalian embryo a process of X-chromosome differentiation, usually termed X-inactivation, occurs in individual embryonic cells[1,2]. Thereafter, the genes of only one of the two X chromosomes are expressed in that cell and its descendants. As a result dosage compensation occurs so that female cells which have two X chromosomes and male cells which have one produce the same amount of X-linked gene products. While there is ample documentation of this phenomenon, little is known about the mechanism by which it occurs, and there is controversy about the state of activity of the maternally and the paternally derived X chromosomes in female embryos during early post-fertilisation development[1-3]. The leading hypothesis is that both X chromosomes are active until some point in development when one or the other is inactivated and ceases to function as a template for messenger RNA (mRNA) synthesis. An alternative view is that the mechanism of X-chromosome differentiation involves not inactivation of an active X chromosome but activation of one of two inactive X chromosomes making the term X inactivation a misnomer.

One of the main obstacles to studies of X-chromosome differentiation is that it occurs very early in embryonic development and appropriate samples for biochemical analysis are consequently difficult to obtain. For example, in the mouse it has been estimated from cytogenetic analyses[4,5] and cell transfer experiments[6] that X-chromosome differentiation occurs at approximately the time of implantation (4 d of development) or shortly thereafter when there are relatively few cells in the embryo and it is particularly difficult to remove from the mother. A further difficulty is that male embryos cannot easily be distinguished from female embryos except by cytogenetic analysis, thereby complicating any experiemnt designed to determine whether one or two X chromosomes are biochemically active in the female embryos of a random population.

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Several experimental approaches have indicated that mouse teratocarcinoma stem cells (embryonal carcinoma cells) are very similar to the pluripotent cells of the normal early embryo[7-9]. It is particularly interesting that certain such cell lines behave like the pluripotent cells of the peri-implantation embryo. Their differentiation *in vitro* is apparently triggered when the cells form rounded aggregates; the first stage in their differentiation, like the first stage in the differentiation of the embryonic inner cell mass, is the formation of a layer of endodermal cells over the free surface of the cell clumps[10,11]. This suggested that female embryonal carcinoma cells might also be similar to normal embryonic cells with respect to X-chromosome activity and might undergo *in vitro* the changes typical of X-chromosome differentiation. If that were the case, sufficient embryonic material to study this phenomenon could be obtained by using clonal female embryonal carcinoma cells. We report here that a pluripotent female embryonal carcinoma cell line can serve as a model system for the study of X-chromosome differentiation *in vitro*.

Two active X chromosomes in undifferentiated female teratocarcinoma stem cells

We based our experiments on the hypothesis that initially both X chromosomes are genetically active in the embryo and that X-chromosome differentiation is an inactivation process. Therefore, if female teratocarcinoma stem cells are similar to the undifferentiated cells in the embryo at a time before X inactivation they should contain two genetically active X chromosomes. In that case, if both X chromosomes are producing gene products in a dosage dependent manner, the level of any X-linked gene product in female teratocarcinoma stem cells with two X chromosomes should be twice that in comparable male cells with one X chromosome. On the other hand, autosomal gene products should be present in the same amount in female and male cells, because both should contain the same number of copies of these genes.

To obtain appropriate female (XX) and male (XY) stem cell lines we used the methods of Martin and Evans[10,11] to isolate embryonal carcinoma cells which are maintained in the undifferentiated state by subculture every 3 d in the presence of a confluent feeder layer. These embryonal carcinoma cell lines were isolated from four different tumours: a spontaneous malignant ovarian teratocarcinoma of the LT mouse strain, a spontaneous testicular teratocarcinoma of the Large (Lg) mouse strain and two embryo-derived tumours of the AKR and 129 strains. As expected, the LT cells contained two X chromosomes (Fig. 1). The three other cell lines, however, each contained only one X chromosome but none had a Y chromosome even though one was derived from a spontaneous testicular tumour and was thus definitely male in origin. It should also be noted that none of the cell lines is euploid, although all have a diploid or near-diploid modal chromosome number (Fig. 1). These results are not unexpected for careful cytogenetic analysis usually shows that teratocarcinoma item cell lines are aneuploid

Table 1 Specific activities of enzymes in undifferentiated teratocarcinoma stem cells

a		LT–XX (clone 1)	PSA1–XO	LG–XO	AKR–XO	Ratio* LT (clone 1) PSA1
X-linked enzymes						
G6PD	Expt 1	148.0	73.0	72.0	75.0	
	2	52.0	27.0	32.0	25.0	1.93 ± 0.04
HGPRT	Expt 1	4.6	2.3	2.4	3.8	
	2	3.1	1.7	1.6	2.2	2.16 ± 0.07
α-gal	Expt 1	5.8	3.2	3.3	3.5	
	2	5.3	3.2	2.8	2.5	1.73 ± 0.06
Autosomal enzymes						
ICDH	Expt 1	20.0	21.0	22.0	20.0	
	2	46.0	44.0	43.0	40.0	0.94 ± 0.08
β-gal	Expt 1	2.5	2.7	2.0	3.2	
	2	2.8	3.5	2.8	2.5	0.95 ± 0.08
β-gluc	Expt 1	2.7	2.9	1.9	1.9	
	2	1.9	3.0	2.8	2.3	0.86 ± 0.11
β-NAC-gluc	Expt 1	75.0	72.0	73.0	58.0	
	2	68.0	68.0	66.0	70.0	0.99 ± 0.04

b		LT–XX (clone 1)	LT–XX (clone 2)	PSA1–XO	Ratio* LT (clone2) PSA1
X-linked enzymes					
G6PD	Expt 1	162.0	145.0	78.0	
	2	192.0	170.0	92.0	1.88 ± 0.02
HGPRT	Expt 1	4.0	4.2	2.0	
	2	5.8	4.7	2.9	1.94 ± 0.15
Autosomal enzymes					
APRT	Expt 1	4.8	3.9	4.6	
	2	3.5	3.2	4.5	0.77 ± 0.04
AK	Expt 1	1.4	1.5	1.7	
	2	1.6	1.6	1.9	0.93 ± 0.11
6PGD	Expt 1	95.0	108.0	107.0	
	2	133.0	140.0	123.0	1.03 ± 0.04

Undifferentiated cells (Fig. 3) were collected by exposure to trypsin at least 3 d after subculture, when in all approximately 5×10^7 cells were collected. Feeder cells represented no more than 10% of the cell population. All cells were washed three times with phosphate-buffered saline and centrifuged. Pellets were used immediately or frozen at −70 °C for up to 2 months. At the time of assay cell lysates were prepared by freezing and thawing followed by centrifugation. Specific activities of enzymes were determined as before: G6PD and 6PGD[30] ;HGPRT and APRT[31]; AK (unpublished results of L. Gudas, A. Cohen, B. Ullman and D. W. Martin); ICDH[19]; β-gal, β-gluc, β-NAC-gluc[32]. The assay for α-gal was similar to that for β-gal[32], with the appropriate form of the 4-methylumbelliferyl substrate. Each assay was linear with time and protein concentration. *a*, Specific activities of seven enzymes assayed in the cell lines derived from the tumours of all four mouse strains (Fig. 1). The data shown from two independent experiments represent the extreme variation between experiments. *b*, Comparison of specific activities of five enzymes in two independent clones derived from LT tumour 72484. The karyotype of clone 1 is shown in Fig. 1. Clone 2 differs as described in the text.

Specific activities are expressed as nmol product formed per min per mg of protein.

* Ratios given are based on the data from at least three independent experiments, including the two shown. They are reported with their standard errors.

and there is no teratocarcinoma stem cell line in which a Y chromosome has been demonstrated unequivocally[12]. In spite of chromosomal abnormalities, the XX–LT cell line was appropriate for our purposes because simple autosomal aneuploidy does not interfere with X-chromosome differentiation[2]. The three XO cell lines were also deemed appropriate because they each contained only one X chromosome and for our purposes could thus be considered 'males'.

To test our hypothesis that both X chromosomes in the LT cells are genetically active, we measured the levels of ten enzymes in the different cell lines. Three of these are known to be X-linked in the mouse[13,14]: glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), α-galactosidase (α-gal, EC 3.2.1.22), and hypoxanthine-guanine phosphoribosyl transferase (HGPRT, EC 2.4.2.8). Of the other seven, five are known to be autosomal in the mouse[14,15]: isocitrate dehydrogenase (ICDH, EC 1.1.1.41), β-galactosidase (β-gal, EC 3.2.1.23), β-glucuronidase (β-gluc, EC 3.2.1.31), adenine phosphoribosyl transferase (APRT, EC 2.4.2.7), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44); the remaining two are considered to be autosomal by analogy with man: adenosine kinase (AK, EC 2.7.1.20) and β-N-acetyl-glucosaminidase (β-NAC-gluc, EC 3.2.1.30).

The specific activities of these enzymes are shown in Table 1. Although the actual specific activities varied, the ratio of specific activities in the XX–LT cells compared with XO cells was constant for each enzyme from one experiment to the next (Fig. 2). For the six autosomal enzymes tested in all four cell lines, the levels were the same in the XX–LT cells as in the three XO lines. (APRT is not shown because it was not tested in the AKR and Lg cells.) In contrast, the levels of all three X-linked enzymes were twice as high in the XX–LT cells as in the XO cells.

It seems unlikely that these results are related to the aneuploid chromosome constitution of the different cell lines because similar results were obtained when LT cells were compared to three different XO cell lines, each with different chromosomal anomalies (Fig. 1). Moreover, similar results were obtained with a second LT cell line (clone 2, Table 1*B*) chromosomally different from the first. This cell line, isolated from the same

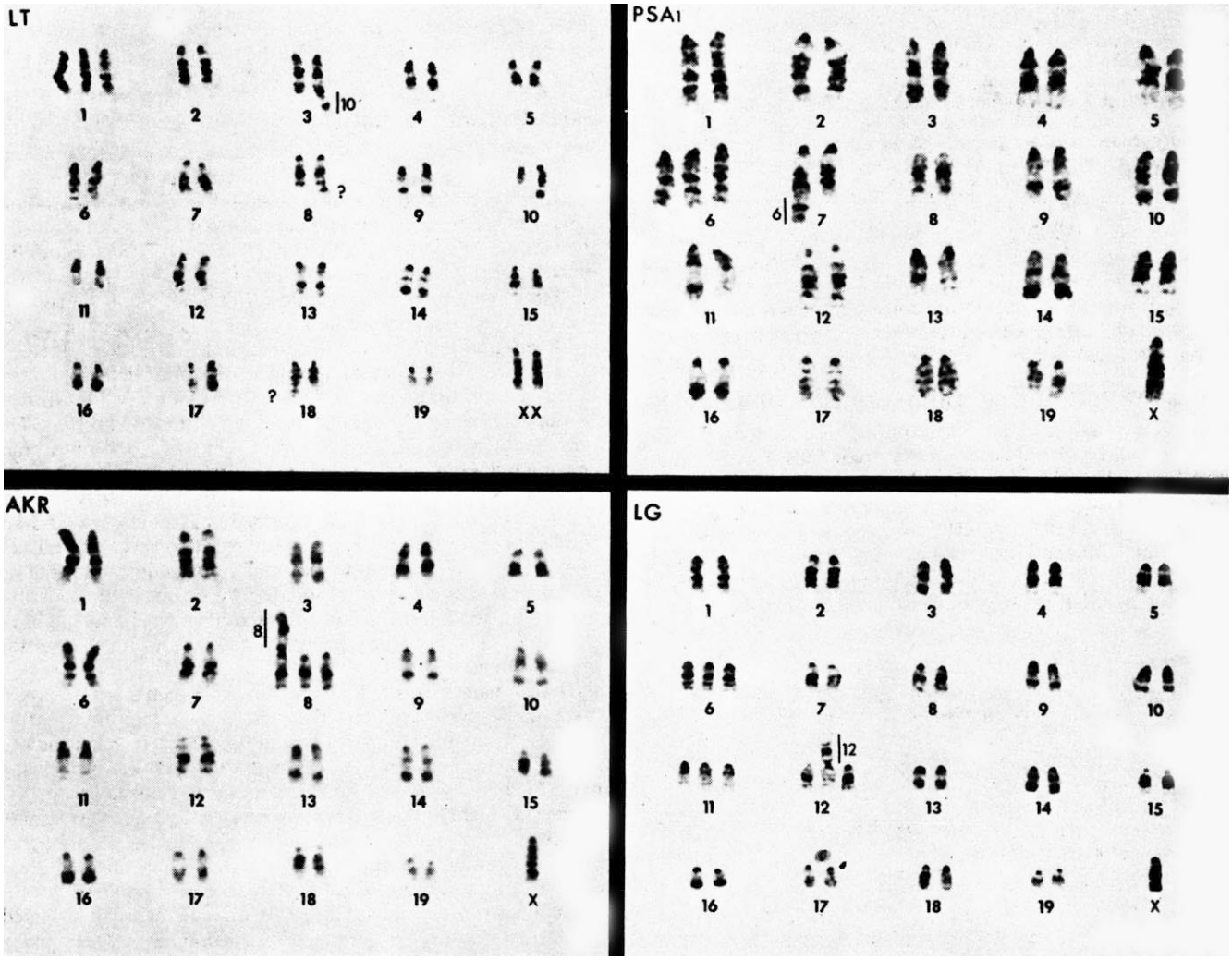

Fig. 1 Karyotypes of teratocarcinoma stem cell lines. Embryonal carcinoma cell lines were isolated as before[10,11] from the following teratocarcinomas: 34th transplant generation of LT spontaneous ovarian tumour 72482 and 16th transplant generation of Lg strain spontaneous testicular tumour 74218 (both provided by Dr L. C. Stevens, Bar Harbor); 9th transplant generation of AKR strain tumour FA-25-9-1, induced by implantation of a 6.5-d embryo under the kidney capsule of an adult mouse (provided by Dr D. Solter, Wistar Institute); 129 strain tumour OTT5568, induced by implantation of a 3-d embryo in an adult testis[27]. Each culture derived from these tumours is a homogeneous embryonal carcinoma cell line that can differentiate into various cell types *in vivo* and *in vitro*. Strain 129 (PSA1) cells have been described[28]. Cells were cultured as described in Fig. 3. Counts of chromosome spreads prepared as described below indicated the modal number of 41 chromosomes in 47/50 LT cells. Modal numbers for the other lines were 40 for PSA1 (50/50 cells examined), 43 for Lg (47/50) and 40 for AKR (48/50). While the banded karyotypes shown here are typical of most cells of each type, there was a significant proportion of the PSA1 cell population in which both number 7 chromosomes were normal. Giemsa-banded karyotypes were prepared as follows: 2–3 d after subculture undifferentiated cells were incubated for 1 h in medium containing 10^{-8}M vinblastine and collected by exposure to trypsin. Cells were suspended in 0.75 M KCl for 20 min and fixed in 1:3 acetic acid-ethanol overnight before being spread on slides. The slides were treated with 6M urea for 20 s at 37 °C and stained in 3% Gurr's Giemsa at *p*H 6.8 for 5 min. Chromosomes are numbered according to the system of Nesbitt and Francke[29].

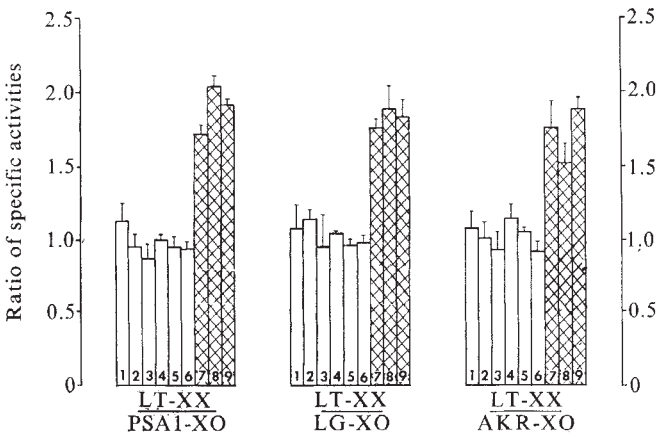

Fig. 2 Ratios of specific activities of autosomal (open columns) and X-linked (hatched columns) enzymes in undifferentiated XX compared with XO teratocarcinoma stem cells. Specific activities of nine enzymes were determined for all four cell lines (Fig. 1). The mean ratio (with s.e.m.) of the specific activity of each enzyme in the LT cells compared with each of the three XO cell lines is shown. Autosomal enzymes: 1, adenosine kinase; 2, β-galactosidase; 3, β-glucuronidase; 4, β-NAc-glucosaminidase; 5, isocitrate dehydrogenase; 6, 6-phosphogluconate dehydrogenase. X-linked enzymes: 7, α-galactosidase; 8, hypoxanthine-guanine phosphoribosyl transferase; 9, glucose-6-phosphate dehydrogenase.

tumour as the LT cells (clone 1) described above, has a modal chromosome number of 40 (trisomic for chromosome 11 and monosomic for chromosome 6, with a few small autosomal rearrangements). These results are consistent with the hypothesis that genes of both X chromosomes are expressed in the undifferentiated female teratocarcinoma stem cells.

X inactivation during differentiation of LT cells

If both X chromosomes are active in undifferentiated LT cells, it can be inferred that these tumour stem cells are similar to embryonic cells at a stage before X-chromosome inactivation. Because the teratocarcinoma stem cells can behave *in vitro* very much like inner cell mass cells during the period when X-chromosome differentiation is probably occurring *in vivo* it seemed likely that these cells would undergo changes in X-chromosome activity if they differentiated *in vitro*. To test this we cultured both the XX–LT cells and the XO–PSA1 (strain 129) cells in parallel. Separate samples of each were maintained in the undifferentiated state or in conditions conductive to differentiation (Fig. 3). At 2-3-d intervals all cells were assayed and the ratios of specific activities of two X-linked and two autosomal enzymes were determined in the XX compared with XO cells.

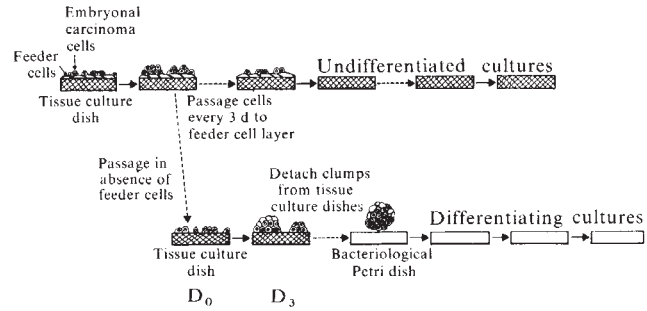

Fig. 3 Culture protocol for teratocarcinoma stem cells. The medium used in all experiments was Dulbecco's modified eagle's medium (containing glucose, 4.5 g l^{-1}) supplemented with 10% calf serum. To maintain embryonal carcinoma cells in the undifferentiated state cells were subcultured every 3 d and seeded at 5×10^6 per 9 cm^2 tissue culture dish containing a confluent layer of mitomycin C-treated STO cells[10,11]. To initiate differentiation cells were seeded at 10^7 per 9 cm^2 tissue culture dish without feeder cells. After 3 d of culture cell clumps formed and were detached by gentle pipetting, allowed to settle for several minutes, washed in fresh medium and seeded in bacteriological Petri dishes to which they did not adhere. The first differentiated cell type (endoderm) became apparent as a complete outer layer after the clumps were placed in suspension. In some cases differentiation proceeded in the sequence described in ref. 28, but not all the cell lines described here showed exactly that pattern of development.

The results of a typical experiment (Fig. 4) were those expected if X inactivation occurs during differentiation *in vitro*. A striking change occurred in the ratio of X-linked enzyme activity beginning approximately 9 d after the initiation of differentiation, with the ratio of first G6PD and then of HGPRT dropping from 2.0 to 1.0. As expected, the ratio of activity of autosomal enzymes remained 1.0 whether or not the cells differentiated.

Figure 5 shows that the change in X-linked enzyme activity was specific to the differentiative process. A sample of cells was allowed to differentiate while the parent cultures were maintained in the undifferentiated state. When G6PD activity in the former had decreased, indicating X-chromosome inactivation, a second sample of the undifferentiated parent culture was subcultured in conditions conducive to differentiation. In both cases the decrease in activity began approximately 9 d after the initiation of differentiation. No change in the karyotype of the LT cells occurred during differentiation.

Interpreting the data

The simplest explanation of these data is that both X chromosomes are active in the LT cells and that inactivation occurs when the cells are cultured in conditions conducive to differentiation. This conclusion depends on the validity of the assumption that the amount of gene product present in a cell reflects the number of active genes. More direct measures of X-chromosome activity such as the use of electrophoretic variants of X-linked products are not possible because teratocarcinomas heterozygous for X-linked markers are not yet available. However, there is increasing evidence that, in appropriately controlled conditions, quantification of enzyme activity can provide a valid estimate of the number of functional genes or chromosomes[16-18]. The only apparent anomaly in our results involves the autosomal enzyme ICDH, which is governed by the *Id-1* locus on chromosome 1. Recent experiments have shown that trisomy for chromosome 1 results in a proportional increase in ICDH activity in midgestation mouse embryos[19]. However, the LT cell line (clone 1) we used is also trisomic for chromosome 1, but no similar dosage effect was noted. There are many possible reasons for the failure to find a dosage effect when one might be expected, and we do not believe this exception invalidates the other findings.

The immediate precedent for our experiments is the assessment of X-chromosome activity in developing and mature oocytes by quantification of gene dosage[20-23]. In those experiments oocytes from female mice with two X chromosomes had twice the activity of X-linked enzymes as did oocytes from XO females (which had 39 chromosomes, with one X missing). Given that the conclusion, based on gene dosage studies, that both X-chromosomes function in oocytes was confirmed using electrophoretic variants of X-linked markers in human oocytes[24], and considering the consistency of our results with all X-linked and control autosomal enzymes except ICDH, it seems likely that our experiments represent a valid approach to the study of X-chromosome function in embryonal carcinoma cells.

In view of the close similarity between the teratocarcinoma stem cells and the pluripotent cells of the normal mouse embryo[7-9] these results, which provide the first clear evidence that X-chromosome differentiation is an inactivation process at the molecular level, support the hypothesis that the X-chromosome differentiation during embryonic development involves the biochemical inactivation of an X chromosome.

It is interesting that during differentiation the decrease in activity of G6PD always preceded that of the other X-linked enzyme, HGPRT, by 4 or 5 d. This could be interpreted as an indication that inactivation does not occur along the whole

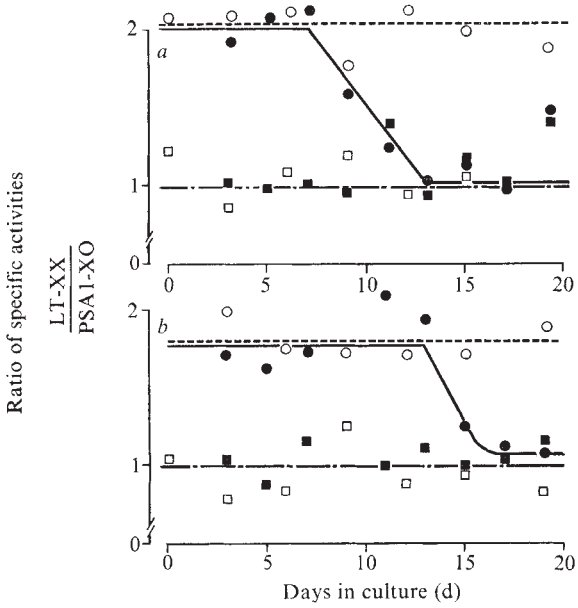

Fig. 4 Levels of X-linked gene products during differentiation of LT cells *in vitro*. Cultures of LT and PSA1 cells were maintained in the undifferentiated state or subcultured (at day 0) in the absence of feeder cells to begin differentiation. At various times during the subsequent 3 weeks, samples of XX–LT and XO–PSA1 cells were collected either by exposure to trypsin followed by centrifugation (for undifferentiated cells) or by centrifugation (for differentiating cultures), and the levels of G6PD, HGPRT, 6PGD and APRT were assayed as described in Table 1. The data shown are typical of the results obtained in two other such experiments. *a*: ○, G6PD in undifferentiated cells; ●, G6PD in differentiating cells; □, 6PGD in undifferentiated cells; ■, 6PGD in differentiating cells. *b*: ○, HGPRT in undifferentiated cells; ●, HGPRT in differentiating cells; □, APRT in undifferentiated cells; ■, APRT in differentiating cells.

length of the X chromosome at one time and that the G6PD locus is closer to an initiation site of X-inactivation than is the HGPRT locus. (The exact location of these genes on the mouse X chromosome is not known.) An alternative explanation is that the whole X chromosome undergoes inactivation at once, but that either the mRNA for HGPRT synthesis or the enzyme itself is considerably more stable than that for G6PD. Experiments to distinguish between these possibilities should lead to a better understanding of the molecular mechanism of X-chromosome inactivation.

Our results differ from those of McBurney and Adamson[25], who demonstrated by both biochemcial and cytogenetic tests that only one of the two X chromosomes appeared to be active in a female teratocarcinoma stem cell line. One possible explanation is that the cells they examined were isolated from a tumour obtained from a 6.5 d embryo and might have been derived from a later (post-inactivation) stage of development than the LT cells described here. A second possibility is that their cell selection and culture procedures, which resulted in feeder-independent stem cells, led to changes in the state of activity of the X chromosomes.

Whatever the reason for this difference, the availability of the LT cells described here makes possible several new approaches to the study of X-chromosome activity. For example, these cells provide a new means of mapping X-linked markers, because the levels of enzymes or even structural proteins suspected of being X-linked can be assayed in the XX–LT and XO–PSA1 cells as described here. LT cells can also be used to study the temporal relationship between biochemical inactivation and the onset of late replication (heterochromatinisation) which is a cytological marker of the inactive X chromosome[26]. We have determined that in undifferentiated LT cells both X chromosomes replicate at the same time (data not shown). We are investigating whether the changes in enzyme activity found in the LT cells after differentiation are accompanied by changes in the time of replication of one of the two X chromosomes in these cells. The LT cells should also be useful for studies of molecular aspects of X-differentiation, which may provide clues to understanding the way in which genes are regulated during embryonic development.

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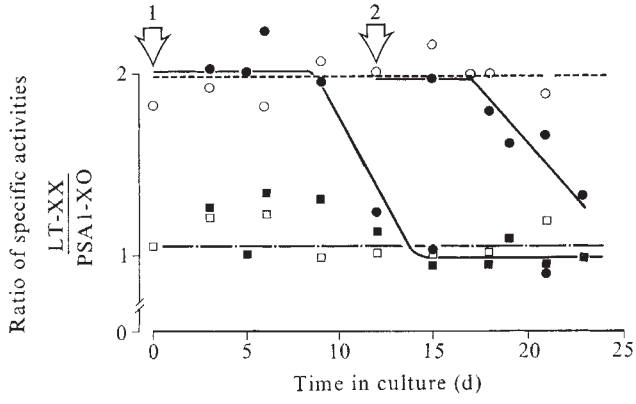

Fig. 5 Relationship between levels of X-linked gene products and the initiation of differentiation. Cultures of LT and PSA1 cells were maintained in the undifferentiated state. At day 0 (arrow 1) one sample of LT–XX cells and one of PSA1–XO cells were subcultured in the absence of feeder cells to initiate differentiation. At day 12 (arrow 2) a second sample each of undifferentiated LT and of PSA1 cells was subcultured in the absence of feeder cells to begin differentiation. Portions of each cell population were collected at various times and the specific activities of G6PD and 6PGD were determined. ○, G6PD in undifferentiated cells; ●, G6PD in differentiating cells; □, 6PGD in undifferentiated cells; ■, 6PGD in differentiating cells.

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