

X-chromosome deletions in embryo-derived (EK) cell lines associated with lack of X-chromosome inactivation

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SUMMARY

The predictions of a model for the initiation of X-chromosome inactivation based on a single inactivation centre were tested in a cytogenetic study using six different embryo-derived (EK) stem cell lines, each with a different-sized deletion of the distal part of one of the X-chromosomes. Metaphase chromosomes were prepared by the Kanda method from each cell line in the undifferentiated state and after induction of differentiation, and cytogenetic evidence sought for a dark-staining inactive X-chromosome. The results confirm the predictions of the model in that when the inactivation centre is deleted from one of the X-chromosomes neither X present in a diploid cell can be inactivated, and in addition considerably further localize the position of the inactivation centre on the X-chromosome.

INTRODUCTION

In somatic cells of female mammals one of the two X-chromosomes is genetically inactivated resulting in dosage compensation for X-linked genes. The onset of X-chromosome inactivation in embryogenesis is linked to cellular differentiation, occurring first in the trophectoderm, the first tissue to differentiate, then in the primary endoderm, and finally in cells of the pluripotential epiblast, at around 6 days of development (Epstein, Smith, Travis & Tucker, 1978; Kratzer & Gartler, 1978; Monk & Harper, 1979; Takagi, Sugawara & Sasaki, 1982). This concept is supported by the presence of two active X-chromosomes in at least some embryonal carcinoma (EC) stem cell lines, with X-inactivation occurring as the cells differentiate (Martin, 1978; Takagi & Martin, 1984), although there have been some conflicting results with other EC stem cell lines (McBurney & Adamson, 1976; McBurney & Strutt, 1980).

The mechanism of X-inactivation is unknown, but is believed to involve an inactivation centre on the X. Models have differed over the position or number of

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such centres (e.g. Russell & Cacheiro, 1978; Distèche, Eicher & Latt, 1981), but evidence from mice carrying structural alterations of the X-chromosome (Russell & Montgomery, 1965, 1970; Russell & Cacheiro, 1978; Takagi, 1980; Rastan, 1983) points to the presence of a single inactivation centre on the X-chromosome located somewhere between the breakpoints in Searle's translocation, T(X;16)16H (Rastan, 1983) and the Oak Ridge translocation R(X;7)6R1 (Russell & Cacheiro, 1978). The X-chromosome controlling element (*Xce*) locus maps between these two breakpoints and is a strong candidate for the inactivation centre (Cattanach & Papworth, 1981; Johnston & Cattanach, 1981; Rastan, 1982; Rastan & Cattanach, 1983).

It is known that the mechanism of X-chromosome inactivation is such that in a diploid cell a single X remains active; in individuals with supernumerary X-chromosomes all X-chromosomes except one become inactivated. The activity of an X-chromosome is thought to depend on the state of its inactivation centre (Russell & Cacheiro, 1978). Rastan's (1983) model proposed that only one centre in any cell may be 'blocked' and the X with a blocked inactivation centre remains active. Any other inactivation centres present in the same cell are free to receive a signal which leads to inactivation. If an X is divided into two segments by an X-autosome translocation only a segment including the centre can undergo inactivation. It has been shown that the segment without the inactivation centre remains active at all times (Russell & Cacheiro, 1978; Russell & Montgomery, 1965, 1970). The predictions of the model are further supported by two cytogenetic studies (Takagi, 1980; Rastan, 1983); in embryos carrying Searle's translocation, T(X;16)16H, in both balanced and unbalanced form, the X¹⁶ chromosome (i.e. the translocation product with the centromeric part of the X-chromosome) was never inactivated, even when inactivation of this chromosome would have restored genetic balance. Furthermore, a second type of rare unbalanced embryo was found containing two complete X-chromosomes and the 16^X chromosome (i.e. the translocation product with the centromeric part of chromosome 16), in which two chromosomes were inactivated in every cell (Rastan, 1983). These results position the proposed inactivation centre distal to the breakpoint on the X-chromosome in T16H.

In the present study the model has been tested using various embryo-derived pluripotential stem cell lines (EK lines – Evans & Kaufman, 1981) derived from both parthenogenetic embryos and fertilized embryos (Kaufman, Robertson, Handyside & Evans, 1983) in which one of the two X-chromosomes shows a deletion in the distal region (Robertson, Kaufman, Bradley & Evans, 1983a; Robertson, Evans & Kaufman, 1983b). The extent of the deletion varies between different lines but the position of the breakpoint appears to be constant for a given line. This is probably the result of clonal inheritance and fixation by cell selection of a particular karyotype. In this study the Kanda method was used (Kanda, 1973; Rastan, Kaufman, Handyside & Lyon, 1980) to determine cytogenetically whether X-inactivation had occurred in differentiated and undifferentiated cells from various embryo-derived (EK) stem cell lines carrying different-

sized deletions of one of the X-chromosomes. The model predicts that in cell lines where the inactivation centre has been deleted from the X-chromosome, neither the deleted X-chromosome nor the intact X-chromosome can be inactivated.

MATERIALS AND METHODS

Cell lines

The cell lines used, HD1, HD2, HD3, HD6, CP4-2 and A13, were embryo-derived (EK) stem cell lines isolated directly from mouse embryos (Evans & Kaufman, 1981). Details and methods of isolation are given in Kaufman *et al.* (1983).

Haploid-derived lines HD1 and HD2 were isolated from parthenogenetically derived embryos from 129/SvEv mice and lines HD3 and HD6 from parthenogenetically derived embryos from (C57BL×CBA)F1 mice. Lines CP4-2 and A13 were both derived from normal fertilized embryos from 129/SvEv mice (Robertson *et al.* 1983a).

Detailed karyotype analysis has shown that the EK cell lines retain a normal diploid autosomal component but frequently show a deletion of the distal region of one of the two X-chromosomes, or sometimes even loss of one X-chromosome (Robertson *et al.* 1983b). This appears to be a feature of EK lines irrespective of whether they are derived from haploid parthenogenetic embryos or normal fertilized embryos. The X-chromosome constitution of the cell lines used in this study is shown in Fig. 1.

Cell culture

All cell lines were routinely maintained in the undifferentiated state by culture in DMEM supplemented with 10% foetal calf serum and 10% newborn calf serum on feeder layers of inactivated fibroblasts (Evans & Kaufman, 1981). In order to minimize spontaneous differentiation the cells were maintained in exponential growth by plating at high densities ($3 \times 10^6/6$ cm petri dish) and subculturing at 2- to 3-day intervals. At the time of the experiment the cell lines had undergone between 10 to 15 passages, and we estimate that there are about four generations per passage. The stem cells were induced to differentiate, when required, by suspension culture of cellular aggregates (Martin & Evans, 1975). The cells were plated onto gelatinized dishes at an initial density of $10^6/6$ cm dish to remove the feeder cells. Following 2 days' culture the cells were briefly trypsinized in order to dislodge small clusters of cells. These cellular aggregates were collected in DMEM plus 10% newborn calf serum and transferred into bacteriological petri dishes to which they cannot adhere. This suspension culture procedure induces the differentiation of stem cells resulting in the formation of an outer layer of endodermal cells to

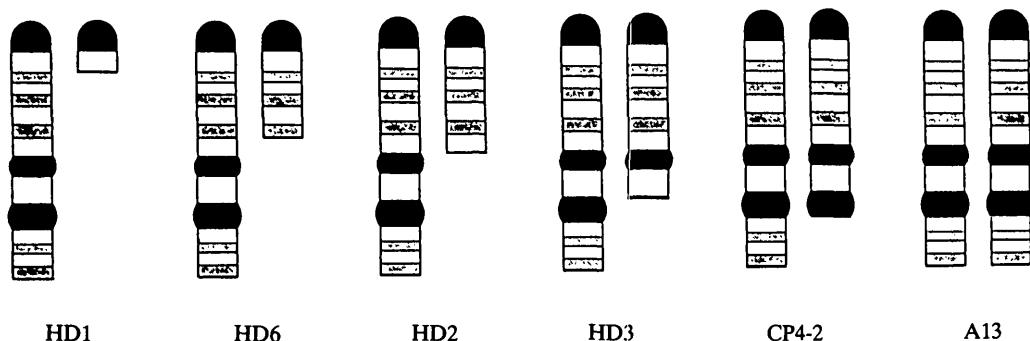


Fig. 1. Diagram of the X-chromosome constitution of the embryo-derived (EK) stem cell lines used, based on G-banded karyotypes.

form structures termed embryoid bodies (Martin & Evans, 1975). The differentiated endoderm cells represent 30–40 % of cells and the remainder appear to be undifferentiated after 4 days.

Preparation of metaphase spreads by the Kanda method

(i) Undifferentiated cells

Exponentially growing cultures were exposed to colcemid at a final concentration of $0.05 \mu\text{g ml}^{-1}$ for 1 h. The cells were then collected by trypsinization, pelleted by centrifugation at 500 r.p.m. for 5 min and resuspended in 0.56 % (w/v) potassium chloride solution. The cell suspension was then incubated in a waterbath at 50°C for 15 min (Kanda, 1973), pelleted by centrifugation at 500 r.p.m. for 5 min and fixed in 3:1 methanol:glacial acetic acid. The fixative was changed a further two times and air-dried preparations made. Slides were stained in 2 % Giemsa (buffered at pH 6.8) for 20 minutes.

(ii) Embryoid bodies

Metaphase spreads were collected from embryoid bodies after a period of 4 days' suspension culture, using the modified Kanda method described above. After this treatment any inactive X-chromosomal material present stains darker than any of the other chromosomes (Kanda, 1973; Rastan *et al.* 1980; Rastan, 1983).

Analysis

All slides were coded and scored blind. Metaphase cells were scored for the presence or absence of a dark-staining (inactive) X-chromosome. Only unbroken metaphase cells with 40 chromosomes were counted.

RESULTS

Table 1 shows the results for each cell in the undifferentiated and differentiated state. Cell lines HD3, CP4-2 and A13 in the undifferentiated state showed a very low proportion of metaphase spreads (6 %, 5 % and 22 % respectively) with a clearly visible dark-staining inactive X-chromosome. However, when these cell lines were allowed to differentiate the proportion of metaphase spreads with a dark-staining X-chromosome (Fig. 2) increased substantially to 57 %, 37 % and 40 % respectively (Table 1). In line A13 two intact X-chromosomes are present and in line CP4-2 it is not easy to distinguish between the deleted and intact X in metaphase spreads treated by the Kanda method. However, in line HD3, where it is possible to distinguish the two X-chromosomes on the basis of size, either the intact X (Fig. 2C) or the deleted X (Fig. 2D) could be seen to be inactivated. Of the 99 metaphase spreads from line HD3 in the differentiated state which showed a clear dark-staining X-chromosome, 48 metaphases had the intact X dark staining and 51 metaphases had the deleted X dark staining.

Cell lines HD1, HD2 and HD6 on the other hand showed no unequivocal dark-staining X-chromosome in either the undifferentiated or differentiated state (Fig. 3). As all cell lines were maintained in the undifferentiated state in exactly the same conditions and induced to form embryoid bodies with an outer layer of differentiated endodermal cells in exactly the same way, it appears that in lines HD1, HD2 and HD6 X-chromosome inactivation does not occur. These results

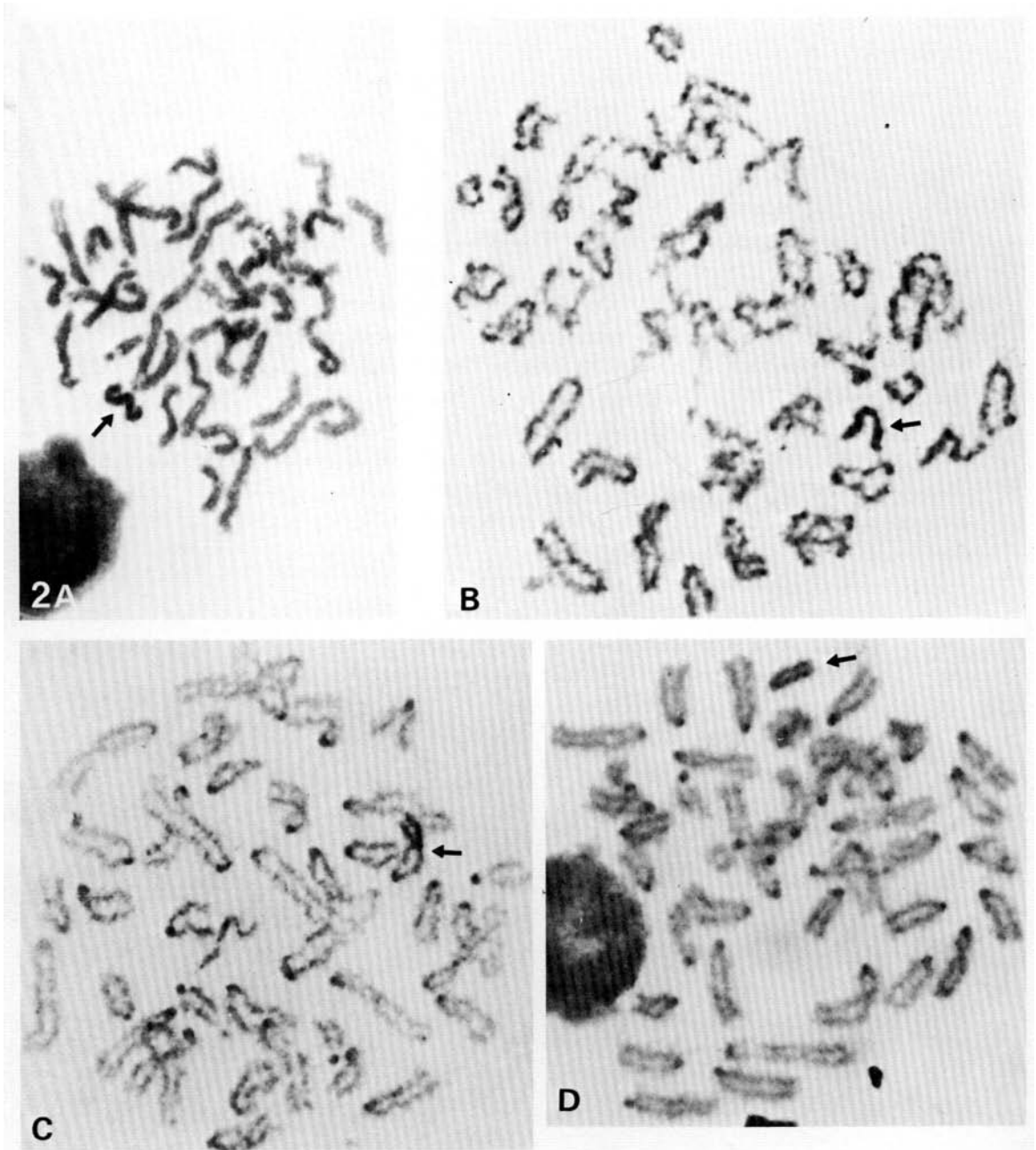


Fig. 2. Metaphase spreads from embryo-derived (EK) stem cell lines treated by the Kanda method after induction of differentiation to show dark-staining inactive X-chromosomes. (A) metaphase from line A13; (B) metaphase from line CP4-2; (C) metaphase from line HD3 with intact X inactive; (D) metaphase from line HD3 with deleted X inactive (inactive X-chromosomes arrowed).

support the hypothesis that an inactivation centre on the deleted X-chromosome in these cell lines has been removed.

DISCUSSION

The bulk of the cytogenetic and biochemical evidence on female embryonal carcinoma (EC) stem cells suggests that prior to differentiation both X-chromosomes are in the active state and that X-inactivation occurs following the initiation of differentiation *in vitro* (Takagi & Martin, 1984). The results presented here suggest that, like EC cells, embryo-derived (EK) stem cells tend to have a very low proportion of cells which show cytogenetic manifestation of an inactive X-chromosome when maintained in the undifferentiated state and that this proportion increases considerably when the cells are induced to differentiate to form embryoid bodies. The fact that in cell lines HD3, CP4-2 and A13D a differentially dark-staining inactive X-chromosome was detected in some cells even when the cells were being maintained in the undifferentiated state need not be taken as evidence against the stem cell model for the onset of X-chromosome differentiation (Monk, 1981) as one can only minimize but not totally eliminate spontaneous differentiation by culture conditions.

The model for the initiation of X-chromosome inactivation proposed by Rastan (1983) to explain inactivation of only one translocation product in reciprocal X-autosome translocations and the situation found in embryos carrying T16H in unbalanced forms, located the inactivation centre somewhere distal to the break-point in T16H and in addition predicts that when the inactivation centre of one of

Table 1. *Proportion of metaphases in undifferentiated and differentiated states*

Cell line		Total metaphases scored	Metaphases with dark X (%)	Metaphases with no dark X (%)
HD1	U	42	0	42 (100 %)
	D	10	0	10 (100 %)
HD2	U	27	0	27 (100 %)
	D	69	0	69 (100 %)
HD3	U	18	1 (6 %)	17 (94 %)
	D	175	99 (57 %)	76 (43 %)
HD6	U	79	0	79 (100 %)
	D	23	0	23 (100 %)
CP4-2	U	44	2 (5 %)	42 (95 %)
	D	174	64 (37 %)	110 (63 %)
A13D	U	45	10 (22 %)	35 (78 %)
	D	198	79 (40 %)	119 (60 %)

U = Undifferentiated; D = Differentiated.

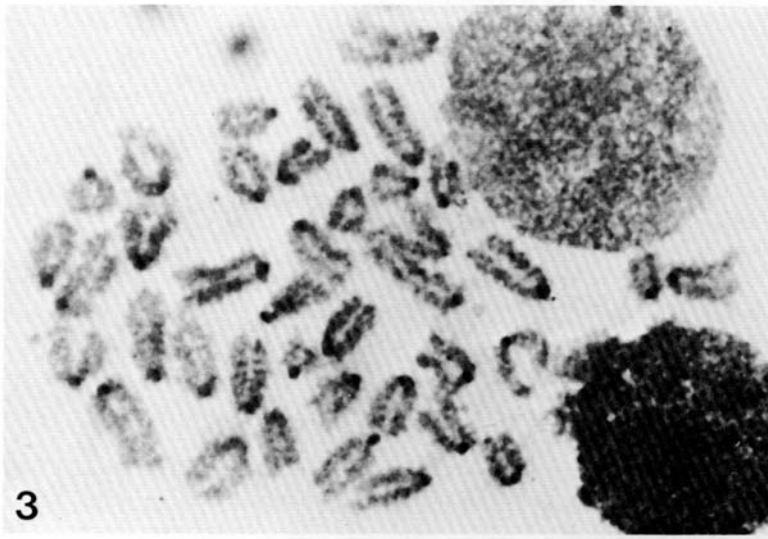


Fig. 3. Metaphase spread from line HD2 treated by the Kanda method after induction of differentiation showing no dark-staining inactive chromosome.

the two X-chromosomes in a diploid cell has been deleted neither the deleted X nor the intact X will be capable of being inactivated. The predictions of the model have been confirmed in the present study; for cell lines HD1, HD2 and HD6, in which a substantial portion of the distal part of one of the X-chromosomes has been deleted, no differentially dark-staining inactive X-chromosome could be detected by the Kanda method (Table 1). In lines A13D (with two intact X-chromosomes), and CP4-2 and HD3 (with smaller portions of the distal end of the second X-chromosome deleted), on the other hand, a dark-staining inactive X-chromosome was detectable in a considerable proportion of cells (Table 1). The most likely interpretation is therefore that in lines A13D, CP4-2 and HD3 the inactivation centre is present on both the intact X-chromosome and the deleted X chromosome and so random X-inactivation can occur normally, whereas in lines HD1, HD2 and HD6 the inactivation centre is no longer present on the deleted X-chromosome. According to Rastan's model, the single inactivation centre present on the intact X in the latter cell lines thus always receives the blocking factor and so is protected from inactivation, and the deleted X cannot be inactivated as it does not possess an inactivation centre. Thus there is no X-inactivation in these cell lines even when they are induced in differentiate.

For line HD3, where the deleted X can be easily distinguished from the intact X on the basis of size in metaphase spreads treated by the Kanda method, our results show that random inactivation of either the intact X or the deleted X is seen in 4-day embryoid bodies. *A priori* consideration of gene dosage and cell selection arguments might lead one to expect to see inactivation of the deleted X-chromosome in the majority of cells, as inactivation of the intact X-chromosome would result in a cell which is functionally nullisomic for genes on the distal part of

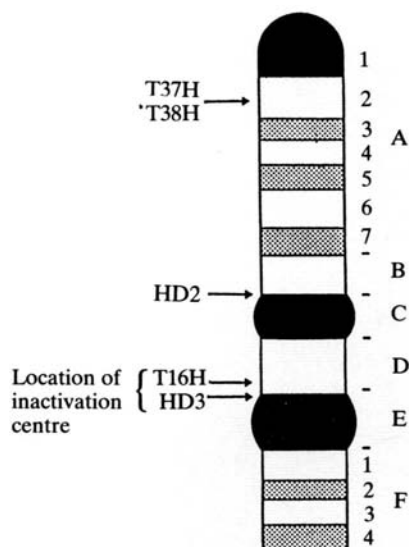


Fig. 4. Diagram of the G-band pattern of the X-chromosome to show location of the inactivation centre with respect to the breakpoints of deletions of the X-chromosome and X-autosome translocations.

the X-chromosome. However, it is possible either that embryo-derived stem cells growing and differentiating *in vitro* are freed from the stringent selective constraints operating in the developing embryo *in vitro*, or that in 4-day embryoid bodies there has not yet been time for cell selection against cells with the intact X inactive to have occurred. An examination of X-inactivation in later embryoid bodies would test the latter possibility.

Although cell lines HD1, HD6 and HD2, which show no X-inactivation, are derived from parthenogenetic embryos and cell lines CP42 and A13, which exhibit X-inactivation, are derived from fertilized embryos, the fact that line HD3, which also exhibits X-inactivation, is derived from a parthenogenetic embryo shows that the results are a function of the size of the deletion and do not depend on the origin of the cell lines.

The results presented in this paper therefore support the location of a controlling centre for X-inactivation on the X-chromosome between the breakpoints in the deletions in lines HD2 and HD3. However, it is known from previous work (Takagi, 1980; Rastan, 1983) that the inactivation centre is located distal to the breakpoint in T16H, so in effect the inactivation centre is considerably further localized by this study to between the breakpoints in T16H and HD3 (Fig. 4). Whereas future work may localize the inactivation centre even further (perhaps even to a single locus) at present it appears that the breakpoints in T16H and HD3 define a critical region of the X-chromosome that must be present on more than one X-chromosome in order for X-inactivation to occur at all.

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