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Deletion of specific sequences or modification of centromeric chromatin are responsible for Y chromosome centromere inactivation

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Summary. Stable dicentric chromosomes behave as monocentrics because one of the centromeres is inactive. The cause of centromere inactivation is unknown; changes in centromere chromatin conformation and loss of centromeric DNA elements have been proposed as possible mechanisms. We studied the phenomenon of inactivation in two Y centromeres, having as a control genetically identical active Y centromeres. The two cases have the following karyotypes: 45,X/46,X,i(Y)(q12) and 46,XY/47,XY,+t(X;Y)(p22.3;p11.3). The analysis of the behaviour of the active and inactive Y chromosome centromeres after Da-Dapi staining, CREST immunofluorescence, and in situ hybridization with centromeric probes leads us to conclude that, in the case of the isochromosome, a true deletion of centromeric chromatin is responsible for its stability, whereas in the second case, stability of the dicentric (X;Y) is the result of centromere chromatin modification.

Introduction

Dicentric chromosomes are stable and self-perpetuating when one of the two centromeres does not function. Such chromosomes are referred to as pseudodicentric, the non-functional centromeres being termed inactive or suppressed by Therman et al. (1974) and latent by Hsu et al. (1975). It appears likely that inactivation is the result of the incapacity of the kinetochore to be joined by the spindle fibres, but it is not clear how a centromere becomes inactive. In principle, either the deletion of specific DNA sequences or the modification of the relationship between proteins and the kinetochore might mediate centromere inactivation. However, even the use of techniques such as indirect immunofluorescence using CREST autoantibodies (Merry et al. 1985; Peretti et al. 1986; Earnshaw et al. 1989) or in situ hybridization with specific centromeric DNA sequences (Jabs et al. 1984; Jabs and Persico 1987; Worsham et al. 1989) have not clarified this point.

We selected for this study two cases of dicentric chromosomes in which we could compare the behaviour of the inactive and active Y centromeres, which are both, by definition, derived from the same Y chromosome and therefore are genetically identical.

Materials and methods

We studied the chromosome constitution of a girl with short and asymmetric limbs (case 1) and of a male with slight mental retardation and moderate hypogonadism (case 2) by G-, Q-, Da-Dapi- and Cd-staining. Centromeric antigens have been studied using autoantibodies from sera of four CREST patients and immunofluorescence detection (Haaf and Schmid 1989). Since CREST sera contain mixtures of centromere antibodies directed against three centromere proteins, we used sera from different patients on different slides. In situ hybridization was performed with two Y-linked probes (pTDF-1 and pFr35-II) and with two alphoid probes namely pYα-1 and p82H (Table 3). These alphoid probes, at high stringency conditions, hybridize respectively to the Y chromosome centromere and the chromosome 14 centromere, but at medium-low stringency conditions hybridize to all the centromeres. Hybridization with p82H was performed assuming that its corresponding genomic sequences represented an homogeneous class of conserved alpha DNA (Alexaindre et al. 1987), but according to Waye et al. (1988) p82H recognizes an alphoid domain of chromosome 14. The probes were labelled either with radioactive or with non-radioactive deoxynucleotides. Nick translation of the entire probe-containing plasmid was carried out using two ^{3}H-labelled deoxynucleotides to a specific activity of 10^7–10^8 cpm/μg. Hybridization was carried out at 37°C in 50% formamide overnight using probe concentrations of 25 ng/slide in case 1 and 12.5 ng/slide in case 2 for p82H, of 17 ng/slide in both cases for pYα-1, of 11 ng/slide for pFr35-II, and of 1.25 ng/slide for pTDF-1. Slides were washed in 40% formamide/2 × SSC at 37°C, when alphoid probes were used and in 50% formamide/2 × SSC at 37–39°C for pTDF-1 and pFr35-II. After autoradiography, the chromosomes were Q-banded and scored for grain distribution. Labelling of probe pYα-1 was carried out also by the random primed labelling technique using digoxigenin-11-dUTP (Boehringer, Mannheim, FRG). Hybridization was carried out at 37°C in 50% formamide overnight using decreasing concentrations of the labelled probe-containing plasmid

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Table 1. Details of cases studied. *n*, Number of metaphases

Case	Gender/ year of birth	Karyotypes		Findings
		Lympocytes *(n)*	Fibroblasts *(n)*	
1	F/1976	45,X[31]/46,X,i(Y) (pter→q12::q12→pter)[19]	45,X[15]/46,X,i(Y) (pter→q12::q12→pter)[6]	Short, asymmetric limbs Gonadal streaks
2	M/1961	46,XY[15]/47,XY, +dic(X;Y)(p22.3;p11.3)[75]	47,XY,+dic(X;Y) (p22.3;p11.3)[59]	Moderate hypogonadism Normal male phenotype

for different slides (25, 12, 2.5 ng/slide) in competition with the non-labelled one to reach the final concentration of 25 ng/slide. Slides were washed in 40% formamide/2 × SSC at 40°C. Labelling grains were detected according to the manufacturers' recommendations. Since Q-banding patterns were not easily recognizable after the colour reaction, metaphases were stained and photographed after Giemsa staining. The Q-banded metaphases were then rephotographed after the elimination of the labelling grains by immersion of the slides in a methyl-acetic (3:1) mixture.

Results

Phenotypic and clinical data of the two cases and their chromosome constitution in lymphocytes and cultured fibroblasts are provided in Table 1. The parents of the two patients all have normal karyotypes.

Chromosome banding

Case 1. The morphology of the normal Y chromosome was that of a D chromosome with a single primary constriction and a central block of bright Q-fluorescence that indicated that the chromosome could be an isochromosome (Fig. 1a). After Da-Dapi-, C- and Cd-banding, only the site of the primary constriction showed the characteristic centromeric staining, whereas no band was visible at the presumptive site of the inactive centromere (Fig. 1b, c, Table 2).

Case 2. The morphology of the abnormal X;Y chromosome was that of a chromosome no. 2 with a single primary constriction at the site of the X centromere (Fig. 1a). After Da-Dapi- and Cd-banding, the site of the inactive Y centromere and the primary contriction of the normal Y chromosome were both positive (Fig. 1b, c, Table 2).

Anticentromere immunofluorescence

In neither case was positive fluorescence present at the site of the inactive centromeres, whereas the active centromeres of the Y chromosome showed the characteristic fluorescent dots (Fig. 1d, Table 2).

In situ hybridization

The results of radioactive in situ hybridization are given in Table 4.

Case 1. Hybridization with pTDF–1 and pFr35–II indicated that both probes hybridize at the short arm and at the distal long arm of the abnormal Y chromosome and thus that it is indeed an isochromosome. No difference in the autoradiographic labelling was detected at the distal portion of the isochromosome with respect either to the proportion of labelled metaphases at the active or inactive Y centromeres, or to the distribution of informative grains. Similarly, with probe p82H, no labelling differences were found at the active or the inactive centromeres of the Y chromosome.

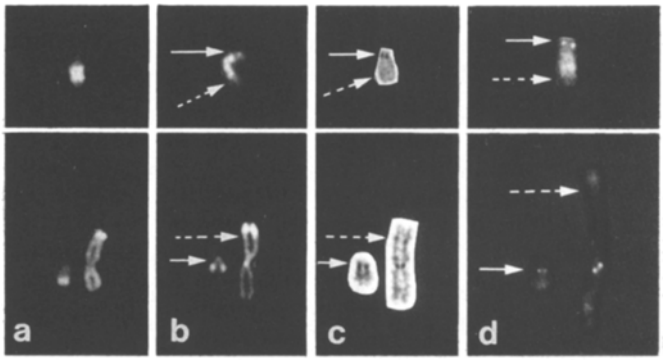


Fig. 1a–d. *Upper part:* the isochromosome (Y) (case 1) and *lower part:* the dic(X;Y) with the normal Y chromosome (case 2) after Q- (**a**), Da-Dapi- (**b**), and Cd-staining (**c**) and anticentromere immunofluorescence with CREST sera (**d**). *Continuous arrows* point to the active Y centromere; *dotted arrows*, to the inactive one

Table 2. Behaviour of the suppressed centromere after different staining

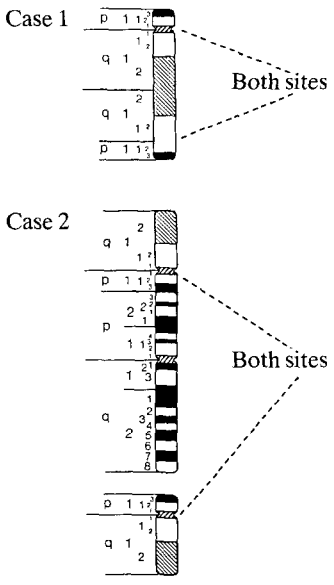
Staining	Case 1 iso(Y)	Case 2 t(X;Y)
Da-Dapi	–	+
Cd	–	+
CREST antigens	–	–

Table 3. Molecular probes used

Probe	References	Y localization (or locus)
pTDF-1	Scherer et al. (1989)	ZFY
pFr35-II	Waibel et al. (1987)	DYS95
pYα-1	Tyler-Smith and Brown (1987)	cen
p82H	Waye et al. (1988)	cen

Table 4. Results of radioactive in situ hybridization. Number of mitoses with at least one grain at either centromeric region of the Y chromosomes

Probes Y chromosome localization		pYα1 Centromere	p82H Centromere	pTDF-1 Short arm	Fr35-II Short arm
Case 1		171	151	15	115
	Both sites	30	41	1	70
		108	144	16	105
Case 2		52	7		
	Both sites	32	12		
		57	17		



After radioactive hybridization with pYα–1, the active centromere appears more densely labelled than the inactive one regarding either the proportion of informative metaphases (Z score, $P < 0.001$) or the distribution of grains (square root transformation, paired t-test, $P < 0.002$). On analysis of the nonradioactive competition hybridization, each centromere was scored as absence of spots, presence of spots, or presence of very large multiple spots. Data analysed by the sign test (Siegel 1956) revealed a significantly higher degree of hybridization at active centromere with 25 ng/slide of labelled probe ($P = 0.002$). Even better results in discriminating hybridization at active vs inactive centromere was achieved by 12 ng/slide of labelled probe in competition with 13 ng/slide of unlabelled probe ($P < 0.001$). Experiments with 2.5 ng/slide of labelled probe in competition with 22.5 ng/slide of unlabelled probe resulted in very few labelled mitoses. We recovered only 7 labelled mitoses, and this number is too small to allow a reliable statistical test.

Case 2. No difference in the labelling of alphoid DNAs pYα–1 and p82H was found at the site of the Y inactive centromere with respect to that of the normal one.

Discussion

Two mechanisms of centromere inactivation have been proposed: a functional modification or a deletion of critical DNA sequences. In favour of the first hypothesis is the persistence of the following characteristics at the active centromere: C-banding (references in Earnshaw et al. 1989), CREST-immunofluorescence, although weakened (Merry et al. 1985; Peretti et al. 1986; Wandall 1989), and alphoid DNA (Jabs et al. 1984; Jabs and Persico 1987). On the other hand, the deletion hypothesis is supported by the finding, in an apparently pseudodicentric chromosome, of an actual deletion of one of the two centromeres, the deleted one still being present as a separate entity (Vianna-Morgante and Rosenberg 1986). Our results indicate that, in pseudodicentric chromosomes, both mechanisms, i.e. modification of the protein/DNA interaction and deletion of DNA sequences, may be responsible for the chromosome stability.

We conclude that, in the case 1, stability of the isochromosome is the result of a deletion of some centromere sequences. This is demonstrated by the following findings: (1) The absence of centromeric C- and Da-Dapi-banding from the presumptive site of the suppressed centromere. (2) Probe pYα–1 gave, at the Y inactive centromere, a signal that is significantly weaker than that at the normal one. With non-radioactive hybridization, differential labelling in active or inactive centromeres is mainly qualitative using the labelled probe but allows clear-cut discrimination using a 50% labelled/unlabelled probe mixture. We wish to stress that the centromere polymorphism detected by alpha satellite DNA probes (Willard and Waye 1987; Jabs et al. 1989) cannot be responsible for the difference of the signal intensities between the two Y centromeres since they originate from the same Y chromosome. After hybridization with p82H no difference in the frequency and amount of labelling was detected between the active and the inactive Y centromeres. This is likely to be due to the probe concentration (25 ng/slide), which saturated all the hybridizable sites. The cytogenetic data and the results of the hybridization indicate that a deletion has occurred, viz. a deletion that involves part of the alphoid DNA and the DNA responsible for the Da-Dapi staining.

Since alphoid DNA is as rich in A + T as the region sensitive to Da-Dapi staining, the two regions could be coincident. If so, the residual alphoid DNA sequences at the inactive centromere would not be sufficient to produce a Da-Dapi band. In case 2, there is no evidence for the occurrence of a deletion event. Indeed, the Da-Dapi staining and the labelling after hybridization with p82H and pYα–1 have the same intensity at both Y centromeres. Thus, we conclude that a modification of the centromeric structures could be responsible for the stability of this (X;Y) dicentric.

The results obtained with Cd staining and CREST immunofluorescence are in line with our conclusions. Cd staining, considered by some authors specific for active centromeres since is not observed in inactive centromeres (Daniel 1979; Maraschio et al. 1980; Wandall 1989), was found to be present at the suppressed centromere of two abnormal Y chromosomes by Magenis et al. (1985). The lack of Cd staining at the inactive centromere of our case 1 and its presence at that of our case 2 supports our hypothesis of the two different inactivation mechanisms, irrespective of the significance of Cd staining.

Regarding CREST immunofluorescence, previous studies on inactive centromeres have demonstrated that

it is still present, although weakened, in autosomal suppressed centromeres (Merry et al. 1985; Peretti et al. 1986; Wandall 1989) but it is absent in Y suppressed centromeres (Peretti et al. 1986). CREST sera contain mixtures of three classes of centromere antibodies (Earnshaw and Rothfield 1985). Among them, only the so-called anti-CENP-B seem to be present in all sera, plus either anti-CENP-C or anti-CENP-A (Earnshaw et al. 1986). Earnshaw et al. (1989) demonstrated that in a pseudodicentric chromosome 13, CENP-C but not CENP-B was absent from its inactive centromere. Working with whole sera, we suggest that absence of immunofluorescence at the site of both our inactive centromeres is the results of the absence (case 1) or the steric modification (case 2) not only of CENP-C or CENP-A but also of CENP-B. It is possible that, in case 2, the immunofluorescent behaviour of the inactive centromere is caused by the late replication of the dic (X;Y). Indeed, similar results have been described in some late-replicating (X;X) dicentric chromosomes (Earnshaw and Migeon 1985; Peretti et al. 1986).

Assuming that the deletion and modification of the centromeric chromatin are both involved, but separately, in centromere inactivation, what is their relative frequency? The findings that inactive centromeres usually maintain their Da-Dapi staining (personal observation) and that CREST immunofluorescence is present at autosomal suppressed centromeres indicate that the centromere inactivation is most frequently achieved by a modification of the protein/DNA kinetocore relationship.

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