Genet Sel Evol (1996) 28, 129-139

Original article

Induction of chromosomal fragile sites in goats: a preliminary study

NL López-Corrales, MV Arruga *

Departamento de Producción Animal y Ciencias de los Alimentos, Facultad de Veterinaria de la Universidad de Zaragoza, c/ Miguel Servet 177, 50013 Zaragoza, Spain

(Received 9 November 1995; accepted 16 January 1996)

Summary – The current study describes the results obtained from different methods of detection of folate-sensitive fragile sites in goat chromosomes. Two different types of expression of chromosomal fragility have been observed: telomeric non-staining gaps, in 20 out of 21 animals studied, and chromatidic breaks in ten animals. The non-staining gaps have been identified mainly in chromosome 5, and their frequency of occurrence ranged from 30 to 66% of the cells. The chromatidic break occurrence ranged from 2 to 5% of the cells among the break carriers. From the methods used and the observed frequency of expression in cultures, the gaps were classified as common folate-sensitive fragile sites. Significant differences between the induction methods used were obtained.

goat / fragile site / folate deficiency

Résumé – Induction de sites chromosomiques fragiles chez les chèvres : étude préliminaire. *Cette étude décrit les résultats de différentes méthodes de mise en évidence de sites chromosomiques fragiles sensibles au folate chez la chèvre. Deux types différents d'expression de la fragilité chromosomique ont été observés : des espaces télomériques ne prenant pas la coloration, sur 20 des 21 animaux étudiés, et des cassures chromatidiques sur 10 animaux. Les absences de coloration ont été localisées principalement sur le chromosome 5 et leur fréquence d'apparition allait de 30 à 66 % des cellules. Chez les porteurs de cassures, la fréquence de ces dernières allait de 2 à 5 % des cellules. D'après les méthodes utilisées et les fréquences observées dans les cultures, les zones chromosomiques non colorées peuvent être considérées comme appartenant à la catégorie commune des sites fragiles sensibles au folate. Des différences significatives entre les méthodes d'induction ont également été observées.*

chèvre / site fragile / déficience en folate

* Correspondence and reprints: Laboratorio Citogenetica.

INTRODUCTION

About one hundred chromosomal fragile sites have been detected in humans since the first description was made by Dekaban in 1965 (Sutherland, 1991). Human fragile sites have been successively related to different pathologies and one of the most well known is the association between the mental retardation syndrome and the fra.Xq27.3 (Sutherland and Baker, 1990; Vogel et al, 1990; Oberlé et al, 1991; Craig, 1991). Furthermore, implications of chromosomal fragility in different processes like Bloom syndrome (Fundia et al, 1992), chromosomal viral integration points (Caporossi et al, 1991), chromosomal evolution (Miró et al, 1987; Popescu et al, 1990) and the relationship between fragile sites, oncogenesis and tumoral events (Yunis, 1983; Yunis and Soreng, 1984; DeBraeckeler, 1987; Dal Cin et al, 1991; Austin et al, 1991) have been well documented in human cytogenetics. In animals only a few chromosomal fragile sites have been reported, mainly in domestic species. In pigs, Riggs and Chrisman (1989, 1991) have described aphidicolin and folate-sensitive fragile sites like the ones detected by Yan and Long (1993). More recently, folate, 5-BrdU and aphidicoline fragiles sites have been found in equine, rabbit, bovine, mole rat, dog and sheep karyotypes by Rønne (1992), Poulsen and Rønne (1991), Uchida et al (1986), Gripemberg (1991), Stone et al (1991a) and Matejka et al (1990). Although no close relationship with any pathology has yet been observed, Tewari et al (1987) have indicated a possible effect on the fertility of female rats and Stone et al (1991b) have suggested the implication of some fragile sites in tumoral chromosomal rearrangements in the mammary glands of dogs. These features and some of the published results, indicate that the fragile sites may be distributed in the majority of domestic species in a similar way as for humans. This highlights the importance of knowing the distribution and morphological characteristics of animal fragile sites, as a first step to finding the possible relationships between any defined pathology or syndrome and the presence of chromosomal fragility.

There is no knowledge of the induction methodologies or chromosomal fragility expression forms in goats and the aim of this work has been the adaptation of induction methodologies to begin studies of the detection and identification of folate-sensitive fragile sites in this karyotype.

MATERIAL AND METHODS

Twenty-one adult goats were used, including Saanen, Toggenburg and cross-bred animals.

An adaptation of published protocols (Sutherland et al (1985); Howard-Peebles (1991); Fisch et al (1991); Jacky et al (1991)) was used to induce the expression of fragile sites in lymphocyte cultures.

Whole blood (1 mL) was cultivated in 10 mL of low folate M-199 medium (Flow) supplemented with 5% SFB (Gibco), 1% penicillin-streptomycin (Gibco), 5 IU of PHA (phytohaemaglutinine) (Wellcome) and 5 IU of Pokeweed (Gibco)-like mitogens. The culture pH was adjusted to 7.6–7.8 by the addition of bicarbonate. Three modifications to this basic culture were used: protocol 1: 5 μM of fluorodeoxyuridine FdU (Sigma, F 0530) and 30 mg/mL of thymidine (Sigma, T 5018)

were added during the last 24 h of cell culture; protocol 2: 5 μM of fluorodeoxyuridine (Sigma, F 5030), 30 mg/mL of thymidine (Sigma, T 5018) and 10 μg/mL 5-bromo-2′-deoxyuridine BrdU (Sigma, B 5002) were added during the last 24 h of cell culture; protocol 3: 5 μM of fluorodeoxyuridine (Sigma, F 5030), 30 mg/mL of thymidine (Sigma, T 5018), and 10^{-5}M of amethopterin and methotrexate (Sigma A 6770) were added during the last 24 h of cell culture.

The cultures were harvested and fixed according to a standard technique (Moorhead et al, 1960). Two cultures for each treatment were made and 50 cells from each one were observed. Control cultures were used for each protocol according to the standard methodology: 1 mL of whole blood in 10 mL of RPMI 1640 (Gibco), supplemented with 20% SFB (bovine calf serum) (Gibco) and 1% penicilline-streptomycin (Gibco) and 1% L-glutamine (Sigma). The cultures were incubated at 37 °C in the absence of CO_2 and harvested after 72 h of growth.

The identification of chromosome pairs was accomplished by an adaptation of the original Seabright's G banding method (Seabright, 1971). An ANOVA test was used to establish the differences between treatments (Stat View, Macintosh).

RESULTS

Two different types of chromosomal alterations were observed. The numbers given below refer to protocol 3.

Non-staining gaps at the telomeric region

The results are shown in figure 1a and 1b. The minimum expression value considered was 4% of the total observed cells, and only one animal presented an expression percentage below this. Among the remaining 20, the gaps were present at a frequency of 30–66% of the cells, with a mean value of $48.3 \pm 2.1\%$. Cells with more than one gap occurred at a frequency ranging from 0–38% of the total, with a mean value of $10.5 \pm 2.3\%$ (table I).

After destaining and subsequent G-banding the autosome pair number 5 could be identified as the main carrier of gaps (fig 2a, 2b and 2c). In 18 animals (90% of the 20), gaps on homologous chromosome 5 could be observed on this pair (fig 2a and 2b).

Chromatidic breaks

The occurrence of chromatidic breaks ranged from 0% (breaks were detected only in ten animals) to 4%, with a mean value of $1.5 \pm 0.4\%$ (table I). Unlike the gaps, the break locations were detected in different regions and chromosome pairs, and these ruptures were observed in only one chromatid in all the analyzed cases (fig 3a and 3b).

Methodologies used

The three variants described were useful for detecting gaps and break induction. Considering the ANOVA test performed, treatment 3 showed significant differences ($p < 0.05$) from treatments 1 and 2, taking into account only gap expression (fig 4).

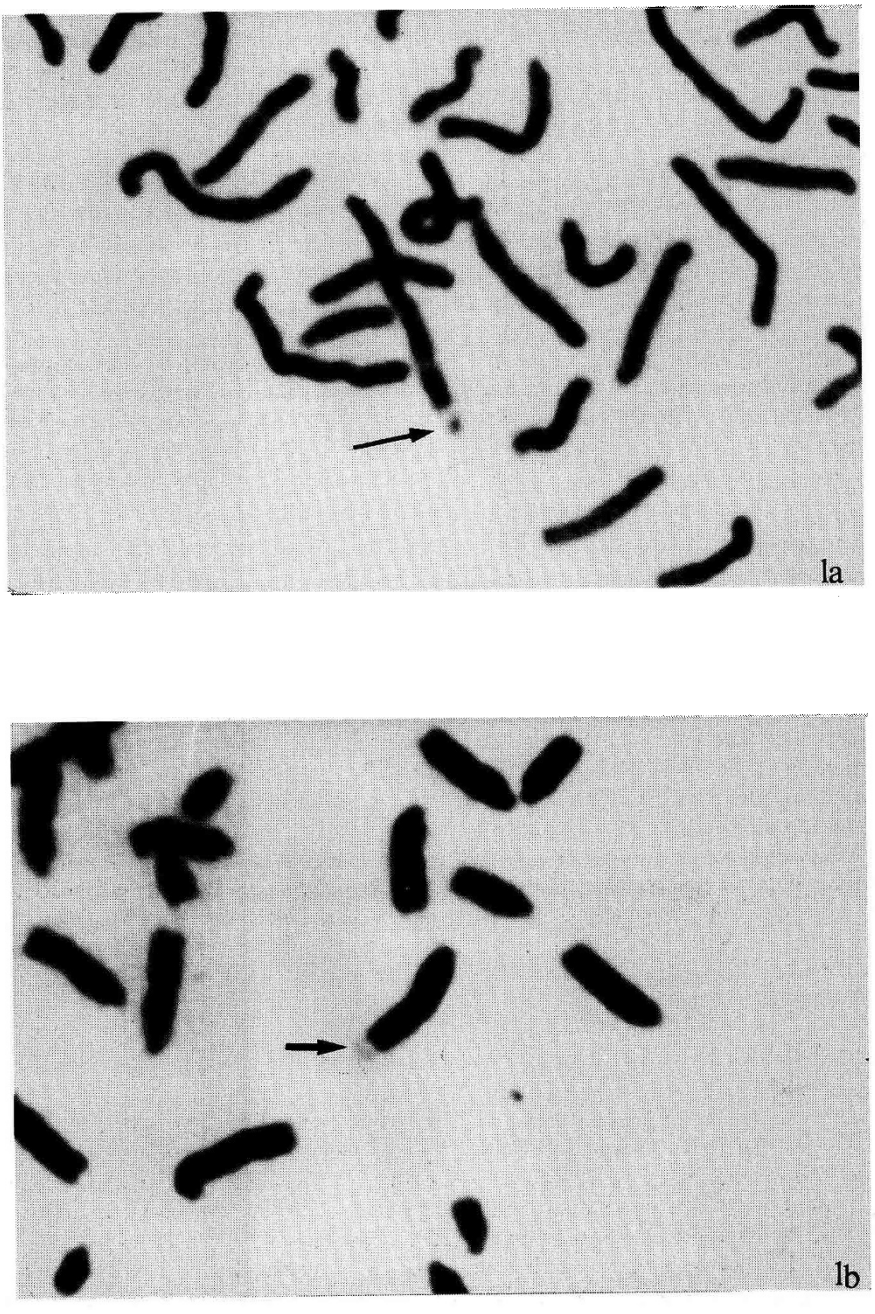

Fig 1. Chromosomal gaps located at the telomeric position.

Table I. Gap and break expression detected per individual.

Animal	*No of cells observed*	*Gap expression*[b] *(%)*	*Cells with more than one gap (%)*	*Cells with breaks (%)*
H	104	48	17.3	0
Mp	100	50	6	0
Mg	100	36	24	0
HP	100	52	4	0
Hg	100	60	4	2
989	100	46	6	0
39	100	66	8	0
16	100	60	8	4
38	100	42	18	0
108	100	44	12	2
612	100	58	20	4
616	100	44	28	2
755	100	52	38	0
104	50[a]	52	0	4
195	100	46	2	2
121	100	56	2	0
771	100	44	0	0
102	60[a]	30	67	3.3
129	100	34	6	4
130	80[a]	45	0	5
Total : 20				
Mean		483	10.5	1.5

[a] Cell cultures with low mitotic index; [b] percentage of cells with at least one gap.

The proliferation cell rate was lower when protocol 2 was employed. For this reason, another comparison test between protocols 1 and 3 was carried out using 18 animals (fig 5). The ANOVA test did not show significant differences, but a slight increase in gap expression in treatment 3 could be detected in both comparison tests (fig 4 and 5).

DISCUSSION

From a morphological point of view, the two types of lesions found can be considered as different chromosomal fragility expression forms in the goat karyotype. The high frequency of gaps and the constancy of their location are two features in agreement with the descriptions of other species whose gaps have been the most frequent expression of chromosome fragility. Matejka et al (1990) reported a mean value of 40% expression for a BrdU sensitive fragile site located at the eighth pair in sheep, and Gripenberg et al (1991) detected up to 86% expression in a fra.X. of deer. In humans the fra.12q24.2 and fra.10q25 are both fragile sites with a high level of expression in cultures (Voiculescou et al, 1991; Tommerup et al, 1981; Gollin

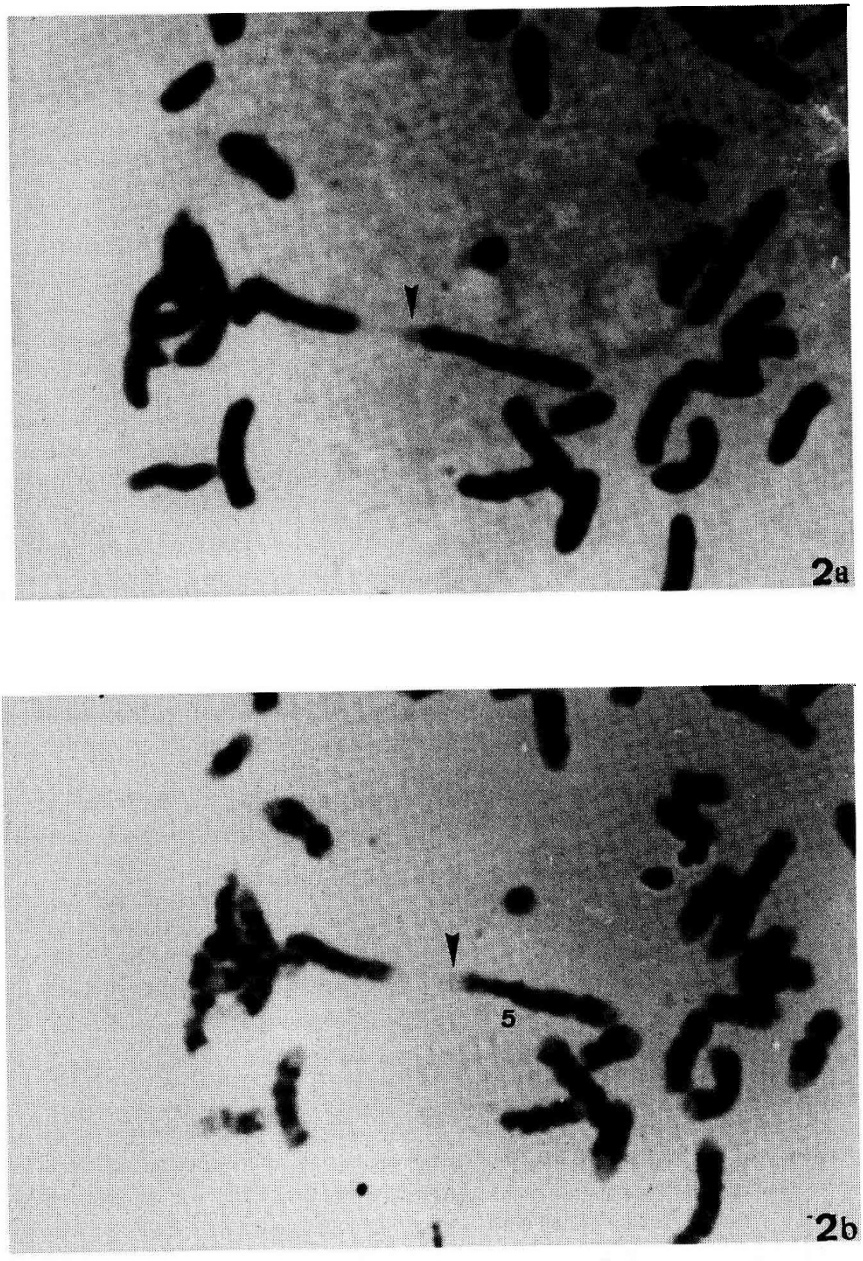

Fig 2.a. Giemsa staining and sequential G-banding of a gap in a chromosome pair carrier. **b.** The banding pattern reveals the pair number 5.

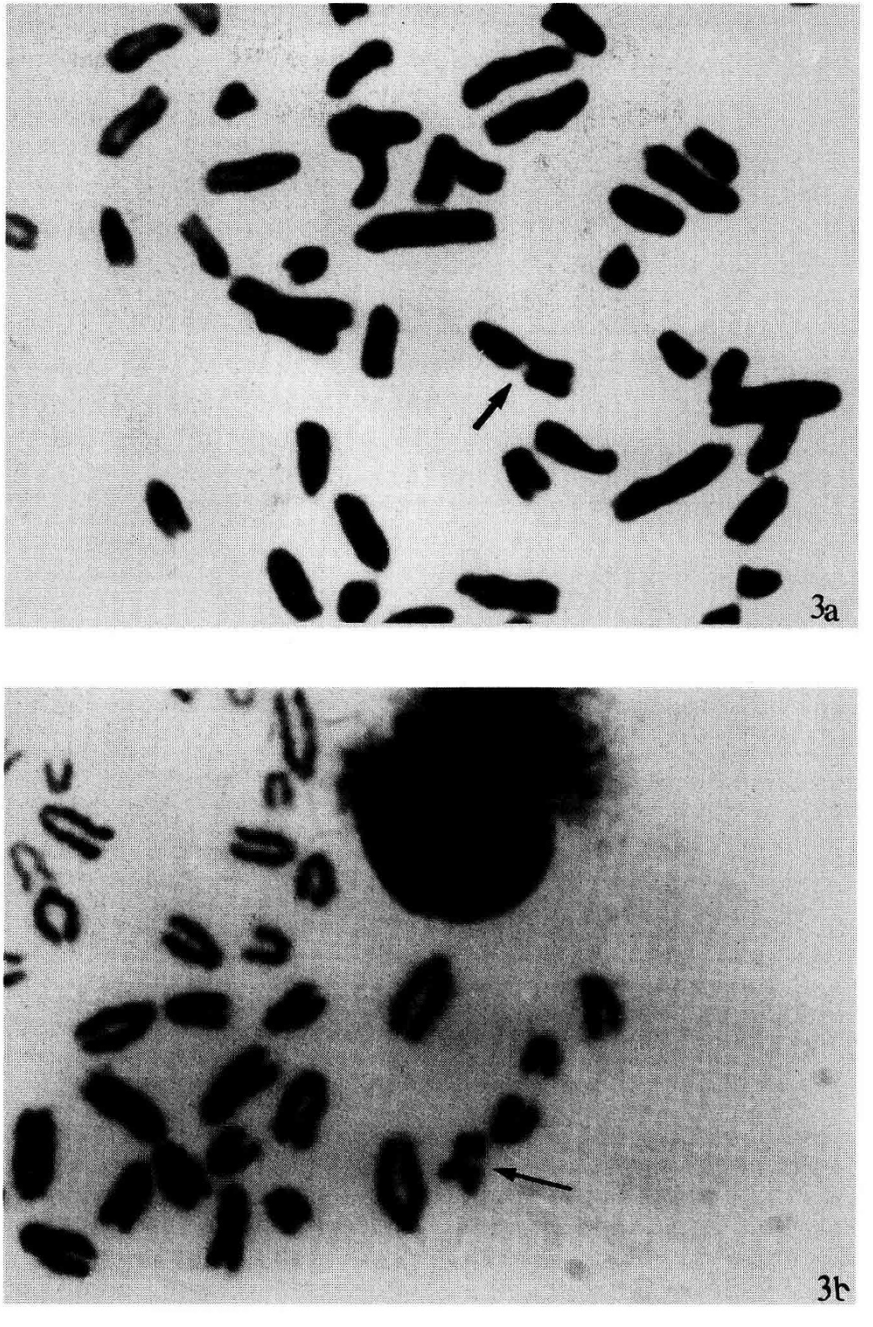

Fig 3. Chromatidic breaks located on different chromosome pairs.

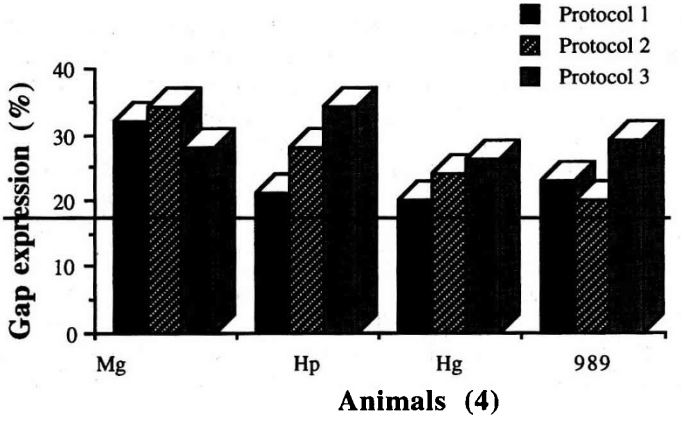

Fig 4. Gap expression on pair number 5 (comparison between protocols and 3).

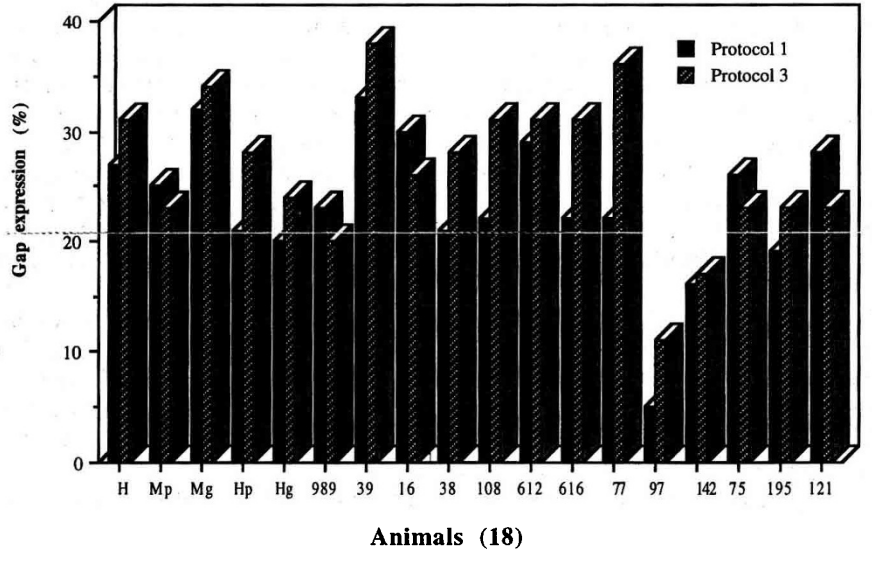

Fig 5. Gap expression on pair number 5 (comparison between protocols 1 and 3).

et al, 1985). These high expression values are in agreement with the mean expression observed by us, and reinforce the fact that our results may represent a common folate-sensitive fragile site, which shows a chromosome gap as its form of expression in cell culture.

Furthermore, the three protocol modifications used were useful for gap induction. Even though some of the drugs used might have enhanced the expression, it can be pointed out that the absence of folic acid in the culture medium was the main factor in provoking the fragile expression. A slight positive effect on gap expression was

detected only when methotrexate was added 24 h before harvesting (a difference was observed mainly when a comparison was made between treatments 1 and 3). If the effect produced by amethopterin on the condensation and chromosome elongation is considered (Laird et al, 1987), an increase of the degree of stretching at the fragile region, and consequently a clearer observation on optical microscopy, could be a logical explanation for the observed high level of expression when this drug is added.

Even though in some animals (three), different chromosome pairs were detected to be telomeric gap carriers, their expression levels were <4% of the cells and no chromosome repetitive location was confirmed; only in the pair 5 were the gap location and frequency expression values good enough to be considered a possible expression of chromosomal fragility. One important fact is that the gaps overlap the telomeric nucleolar organization region (NOR) in this pair. Consequently it is necessary to assume that any stretching of the NOR region could provoke a variable percentage of false-positive fragile detection. In this work no triple staining (Giemsa-G-Banding-NORs) was employed, and it is difficult to estimate what percentage of gaps might be false-positive due to active NOR regions being detected as fragile sites, or indeed if all the fragile sites described here are only NOR stretchings. There is some evidence that the latter is not the case. First, telomeric gaps were detected only in one (pair 5) of the five chromosome carriers of NORs in goat (according to Di Meo et al, 1991) pairs 2, 3, 4, 5 and 28 are NORs carriers in goats. Secondly, the gaps were not limited to the telomere only, the gap region involving all the telomeric positive R or negative G bands of the carrier chromosome. The gaps are bigger than the NOR region detected with normal AG-NOR (silver staining NOR) staining. These features indicate that it is not valid to assume that all the gaps are extensions of the NORs, though a percentage of false-positives due to this cannot be excluded.

Telomeric Structural Changes (TSC) have been indicated as another source of error when human telomeric fragile sites are analyzed (Butler et al, 1990). These TSC are chromosomal lesions which can be detected as fragile sites and the authors mentioned that about 10% of positive detection in human fra.X is due to this kind of alteration. According to this it would be necessary to take into account another variable percentage of error in the results observed.

The expression rate and the different positions of the breaks are subjects for discussion. The breaks were not found in the control cultures and they presented morphological features similar to some of the fragile sites described in humans (isochromatidic breaks as reported by Sutherland et al, 1985, and Sutherland, 1991). On the other hand, in no case did the break frequency raise the minimum level of expression estimated in this work (4%) and only eleven animals expressed these breaks with no repetition established in the position (a variation was noted in the location within each chromosome and between chromosome pairs).

Taking into account that the main source of variation for the fragile expression is related to the induction methodology (as described by Fisch et al, 1991 for the fra.X syndrome in humans), the possibility that the methods used here caused a higher rate of break or, in some cases gap, inductions cannot be excluded.

CONCLUSIONS

Although this is a preliminary study, some conclusions may be drawn. The expression of chromosomal fragility and its induction in goat karyotype are similar to observations in related and unrelated species. Furthermore, gaps seem to be the main form of expression. Even though a combination of FdU and a low folate medium seems to be the only condition necessary for induction, some drugs like amethopterin and thymidine are important for improving fragility expression in this karyotype.

A telomeric gap could be detected successfully only in pair 5; according to its expression frequency and culture conditions it could be classified as a virtual folate-sensitive fragile site. Further study is needed of the sources of error which can lead to a significant percentage of false-positive results (a combination of Telomeric Structural Changes and NORs). A study of its genetic inheritance is also required to reach a definitive conclusion.

It is not possible to draw a conclusion about the chromatidic breaks detected. The low frequency of expression and the variation in location on different chromosome pairs indicate that different induction methods are necessary for a better understanding of the nature of their expression.

REFERENCES

Austin MJF (1991) Expression of common fragile sites on the X chromosome corresponds with active gene regions. *Cancer Genet Cytogenet* 54, 71-76

Butler MG, Allen GA, Haynes JL, Clark S (1990) Chromosome lesions which could be interpreted as 'fragile sites' on the distal en of Xq. *Am J Med Genet* 37, 250-253

Craig I (1991) Human genetic–methylation and the fragile X. *Nature* 349, 742-743

Caporossi D, Bacchetti S, Nicoletti B (1991) Synergism between aphidicolin and adenoviruses in the induction of breaks at fragile sites on human chromosomes. *Cancer Genet Cytogenet* 54, 39-53

Dal Cin P. De Wever I, Moerman P, Van Den Berghe H (1991) Translocation X;12 in mesothelioma. *Cancer Genet Cytogenet* 55, 115-118

De Braekeler M (1987) Fragile sites and chromosomal structural rearreangements in human leukemia and cancer. *Anticancer Res* 7, 141-142

Di Meo GP, Iannuzzi L, Ferrara L, Rubino R (1991) Identification of nucleolus organizer chromosomes in goat (*Capra hircus*). *Caryologia* 44, 309-316

Fisch GS, Silverman W, Jenkins EC (1991) Genetic and other factors that contribute to variability in cytogenetic expression in fragile X males. *Am J Med Genet* 38, 404-407

Fundia AF, Gorla NB, Bonduel MM, Azpelicueta O, Lejarraga H, Sackman F, Larripa I (1992) Increased expression of 5q31 fragile site in a Bloom syndrome family. *Human Genet* 89, 569-572

Gollin SM, Holmquist GP, Ledbetter DH (1985) Fra (10)(q25): the BrdU effect is substitution-dependent. *Am J Hum Genet* 37, 208-214

Gripenberg U, Huuhtanen S, Wessman M, Nieminen M (1991) A fragile site in the X chromosome of the reindeer (*Rangifer tarandus* L). *Genet Sel Evol* 23, 135s

Howard-Peebles PN (1991) Fragile X expression: use of a double induction system. *Am J Med Genet* 38, 445-446

Jacky PB (1991) Guidelines for the preparation and analysis of the fragile X chromosomes in lymphocytes. *Am J Med Genet* 38, 400-403

Laird CD (1987) Proposed mechanism of inheritance and expression of the human fragile-X-syndrome of mental retardation. *Genetics* 117, 587-599

Matejka M, Cribiu EP, Ricordeau G, Eychenne Durand V, Lajous D, Darre R (1990) Evidence for rare heritable fragile site in Romanof sheep chromosome 8. *Reprod Domest Anim* 25, 227-234

Miró S, Clemente IC, Fuster C, Egozcue J (1987) Fragile sites, chromosome evolution and human neoplasia. *Hum Genet* 73, 345-349

Moorhead PS, Nowell PC, Mellman NJ, Battips J, Hungerford M (1960) Chromosome preparation of leukocytes cultured from human peripheral blood. *Exp Cell Res* 20, 613-616

Oberlé I, Rousseau F, Heitz D, Kretz D, Devys D, Hanauer A, Boué J, Bertheas MF, Mandel JL (1991) Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* 252, 1097-1102

Popescu NC, Zimonjic D, Di Paolo J (1990) Viral integration, fragile sites and proto-oncogenes in human neoplasias. *Human Genetics* 84, 383-386

Poulsen BS, Rønne M. (1991) High-resolution banding and localization of fragile sites in *Oryctolagus cuniculus*. *Genet Sel Evol* 23, 183s

Riggs PK, Chrisman CL (1989) Preliminary analysis of aphidicolin-induced fragile sites in goat chromosomes. *Sixth North American Colloqium on Cytogenetics on Domestic Animals*, West Lafayette, IN, abstr 4

Riggs PK, Chrisman CL (1991) Identification of aphidicolin-induced fragile sites in domestic pig chromosomes. *Genet Sel Evol* 23, 187s

Rønne M (1992) Putative fragile sites in horse karyotype. *Hereditas* 117, 127-136

Seabright M (1971) A rapid banding technique for human chromosomes. *Lancet II*, 971-972

Shuterland GR, Baker E, Fratini A (1985) Excess thymidine induces folate sensitive fragile sites. *Am J Med Genet* 22, 433-443

Stone DM, Jacky PB, Hancock DD, Prieur D (1991a) Chromosomal fragile site expression in dogs: I. Breed-specific differences. *Am J Med Genet* 40, 214-222

Stone DM, Jacky PB, Hancock DD, Prieur D (1991b) Chromosomal fragile site expression in dogs: II. Expression in boxer dogs with mast cell tumors. *Am J Med Genet* 40, 223-229

Sutherland GR (1991) Chromosomal fragile sites. Review. In: *Genetic Analysis Techniques and Applications* 8, 161-166

Sutherland GR, Baker E (1990) The common fragile site in band q27 of the human X chromosome is not coincident with the fragile X. *Clin Genet* 37, 167-172

Tewari R, Juyal RC, Thelma BK, Das BC, Rao SRV (1987) Folate-sensitive fragile sites on the X-chromosome heterochromatin of the Indian mole rat *Nesokia indica*. *Cytogenetic Cell Genet* 44, 11-17

Tommerup N, Poulsen H, Nielsen KB (1981) 5-Fluoro-2-deoxyuridine induction of the fragile site on Xq28 associated with X-linked mental retardation. *J Med Genet* 18, 373-376

Uchida IA, Freeman VCP, Basrur PK (1986) The fragile X in cattle. *Am J Med Genet* 23, 557-562

Vogel F, Crusio WE, Kovacs C, Fryns JP, Freund M (1990) Selective advantage of fra(X) Heterozygotes. *Hum Genet* 86, 25-32

Voiculescou I, Back E, Schempp W (1991) Homozygous condition for a BrdU-requiring fragile site on chromosome 12. *Hum Genet* 86, 416-417

Yang MY, Long SE (1993) Folate sensitive fragile sites in chromosomes of the domestic pig *(Sus scrofa)*. *Res Vet Sci* 55, 231-235

Yunis J (1983) The chromosomal basis of human neoplasia. *Science* 221, 227-236

Yunis J, Soreng AL (1984) Constitutive fragile sites and cancer. *Science* 26, 1199-1204