BIOLOGY OF REPRODUCTION **60,** 1273–1278 (1999)

A High Proportion of Bovine Blastocysts Produced In Vitro Are Mixoploid[1]

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ABSTRACT

Fluorescence in situ hybridization with chromosome 6- and chromosome 7-specific probes was used to assess the extent of chromosome abnormalities in developing bovine blastocysts at 7–8 days after insemination in vivo or in vitro. Interphase nuclei (N = 10 946) were analyzed from 151 blastocysts produced in vitro and from 28 blastocysts recovered from superovulated animals. This revealed that 72% (109 of 151) of the in vitro-produced blastocysts were mixoploid, i.e., were a mixture of normal, diploid, and polyploid cells. However, only a small fraction of the total number of cells were chromosomally abnormal. Of the mixoploid blastocysts, 83% (91 of 109) contained less than 10% polyploid cells, 13% (14 of 109) contained 11–25% polyploid cells, and only 4% (4 of 109) of the blastocysts had more than 25% polyploid cells per blastocyst. In contrast, a significantly lower proportion (25%) of mixoploidy was found in 28 bovine blastocysts developed in vivo ($p < 0.0001$). All of the mixoploid blastocysts that had developed in vivo contained less than 10% polyploid cells. No entirely aneuploid blastocysts, i.e., blastocysts in which all cells had the same type of chromosome abnormality, were found in either of the groups. Taken together, the most common chromosome abnormalities observed were diploid-triploid mixoploidies and diploid-tetraploid mixoploidies. Thus, our results confirm earlier reports that morphologically normal bovine blastocysts developed in vivo are often mixoploids. We further show that in vitro-produced bovine blastocysts have a high rate of mixoploidy. Although the difference in mixoploidy rate detected in this study may not be general, it is an interesting phenomenon for further studies.

INTRODUCTION

Chromosome analysis of developing mammalian embryos has provided evidence that a considerable proportion of morphologically normal embryos are chromosomally abnormal [1, 2]. The biological significance of the elimination of chromosomally abnormal embryos has been illustrated in humans, where approximately half of spontaneous abortions in the first and second trimester are chromosomally abnormal [3]. Chromosome analyses have, however, also revealed that a rather high proportion of mammalian embryos are mixoploid, i.e., contain a mixture of normal cells, diploid cells, and cells carrying more or less than two sets of chromosomes. The biological significance of this phenomenon has been addressed in a number of experiments in mice that have taken advantage of the presence of embryonic stem cells and of tetraploid cells produced by electrofusion. Results from diploid-tetraploid chimeras have confirmed that even high proportions of tetraploid cells are tolerated, but the ability to trace the tetraploid cells has also revealed that they are eliminated in the developing embryo and are preferentially allocated to the extraembryonic membranes [4–6]. Although there is no detectable increase in embryonic loss in the mouse diploid-tetraploid chimeras, James et al. [6] reported that chimeras had retarded development at Day 7.5 and heavier placentas than normal controls at Day 12.5.

Embryonic mixoploidy is also a common phenomenon in domestic animal species. An early and extensive study of bovine embryos developed in vivo revealed that 41.5% of morphologically normal blastocysts were diploid-tetraploid mosaics [7]. This was surprising, since earlier studies had revealed only few diploid-tetraploid mosaics in pig, cattle, sheep, rabbit, and mouse embryos. In fact, only one study [8] had reported a high proportion of polyploidy similar to that found by Hare et al. [7]; specifically, 219 of 361 (66.6%) pig embryos contained polyploid cells. Although studies of embryos from domestic animals have been reported, the true frequency of mixoploidies in normally developing embryos is still uncertain, and estimates of the number of chromosomally abnormal embryos vary considerably. However, in a compilation of results of chromosome abnormalities found in different stages (Day 2–32) of embryos from cattle, sheep, and pigs [1], a majority of embryos (52.4%) fall in the mixoploid category. So far, studies of bovine embryos produced in vitro [9] provide no evidence that the abnormality rate is different from that found in embryos produced in vivo.

A caveat of cytogenetic analysis for determining the extent of mixoploid embryos is that only a minor fraction of the total number of cells can be studied. If only a minor proportion of embryonic cells are chromosomally abnormal, they may easily remain undetected. However, chromosome analysis based on fluorescence in situ hybridization (FISH) with chromosome-specific probes makes it possible to estimate the rate of numeric chromosome abnormalities in interphase cells in addition to the mitotic cells that give rise to metaphase spreads. In the experiments reported here, we used FISH, with DNA probes specific for bovine chromosomes 6 and 7, to estimate the rate of polyploidy in morphologically normal bovine blastocysts developed in vivo, i.e., recovered from superovulated cattle, and produced in vitro.

Accepted December 30, 1998.
Received September 8, 1998.
[1]Supported by the Danish Agricultural and Veterinary Research Council. The UK Ministry of Agriculture Fisheries and Food is acknowledged for a research grant to J.L.W.
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MATERIALS AND METHODS

Metaphase Spreads and Embryo Production

Metaphase spreads of male bovine cells were prepared from lymphocyte cultures using standard cytogenetic methods [10]. The lymphocyte cultures were supplemented with 200 μg/ml bromo-deoxyuridine 6–7 h prior to harvesting in order to induce R-banding.

In vivo-developed blastocysts were recovered from 12 superovulated heifers with multiple injections of FSH (Folltropin-V; Vetrepharm, London, ON, Canada) initiated between Days 9 and 11 of the estrous cycle. At 48 h after the first FSH injection, 500 μg cloprostenol (Estrumat vet; Schering-Plough, Farum, Denmark) was injected to induce luteolysis. Estrus occurred 48 h after cloprostenol application, and the donors were inseminated twice at an interval of 12 h. On Day 7 after insemination, embryos were recovered by nonsurgical flushing of the uterine horns with 300 ml PBS plus 1% steer serum (Danish Veterinary Laboratory, Copenhagen, Denmark) using established procedures. Morphologically excellent and good-quality embryos were cultured for up to 6 h in vitro in SOFaa medium [11] until the blastocyst or expanded blastocyst stage, at which stage the embryos were vitrified and stored in liquid nitrogen until warming [12] immediately before fixation for FISH.

In vitro blastocysts were produced as described earlier [13, 14] except that the maturation medium contained 1 mg/ml polyvinylalcohol (Sigma Chemical Co., St. Louis, MO) instead of serum. Expanded or hatched blastocysts that were evaluated as being morphologically excellent and of good quality were obtained at Day 7 or 8 after insemination.

All blastocysts were spread using the method described previously for whole human embryos [15]. Briefly, individual blastocysts were washed in PBS and transferred to a small drop of spreading solution (0.01 N HCl, 0.1% Tween 20). The blastocyst was constantly observed using an inverted phase-contrast microscope. As the zona pellucida and the cytoplasm gradually dissolved, the nuclei were removed using a finely drawn Pasteur pipette and transferred to a clean glass slide. Nuclei were fixed by careful addition of drops of 3:1 methanol:glacial acetic acid. Specimens were subsequently fixed in 3:1 methanol:glacial acetic acid at 4°C for at least 24 h. Slides were then air dried and aged at 60°C overnight before the FISH procedure was initiated. Slides that were not immediately hybridized were stored at −20°C.

FISH

DNA probes were the plasmid 33E39 (p33E39) that produces a strong signal at the subcentromeric region of cattle chromosome 6 [16] and the cosmid clone JAB8 (cJAB8) that produces a strong signal at the subcentromeric region of cattle chromosome 7. In addition, a microsatellite isolated from this cosmid clone maps to cattle chromosome 15 [17]. DNA from p33E39 and from cJAB8 was isolated using the Qiagen DNA purification system (Diagen GmbH, Hilden, Germany). The DNA from cJAB8 was labeled with digoxigenin-11-dUTP, and DNA from p33E39 was labeled with biotin-14-dUTP by a standard nick-translation reaction [18]. FISH was performed essentially as described by Thomsen et al. [19] except that slides containing blastocyst nuclei were predigested for 15 min in a solution of 100 μg pepsin per milliliter of 0.01 N HCl at 37°C in order to provide access for the probe to the hybridization target. Briefly, slides were then treated with 100 μg/ml RNase A (Sigma), washed in double-strength SSC (single-strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), fixed in 1% phosphate-buffered paraformaldehyde for 2 min, washed in double-strength SSC, and air dried after being dehydrated in an ascending ethanol series. Chromosomal DNA was denatured by immersing slides in 70% formamide, double-strength SSC (pH 7) for 2 min at 65–68°C, and the slides were then immediately dehydrated in an ice-cold ascending ethanol series and air dried. The biotinylated p33E39 DNA and the digoxigenated cJAB8 DNA were added to the hybridization solution (50% deionized formamide, 10% dextran sulfate, double-strength SSC, 10 μg salmon sperm DNA, 12 μg genomic DNA) at a final concentration of 15–30 ng/μl, denatured by incubation at 70°C for 5 min, and quenched on ice. Aliquots (15 μl) of this solution were placed on each slide, coverslipped, sealed, and incubated overnight at 42°C. After hybridization, slides were washed twice in 45% formamide, double-strength SSC for 3 min and three times in double-strength SSC for 3 min, all at 42°C. After washing, slides were incubated at 37°C for 10 min in 4-strength SSC, 0.1% Tween 20 containing 5% skim milk powder to reduce nonspecific antibody binding. Hybridization sites of biotinylated probes were visualized using fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories, Burlingame, CA) after two rounds of amplification using biotinylated goat anti-avidin antibodies (Vector). Hybridization sites of digoxigenated probes were visualized using the fluorescent antibody enhancer set (Boehringer Mannheim, Mannheim, Germany) except that the third layer was replaced by anti-mouse rhodamine (Boehringer Mannheim). Nuclear DNA was counterstained with either diamidino-phenyl-indole (DAPI, 1 μg/ml) or propidium iodide (PI, 400 ng/ml) in Dabco antifade solution (Sigma). Cattle chromosomes 6 and 7 were identified by R-banding using 20–40 μg PI per milliliter alkaline (pH 11) mounting medium [20]. The slides were examined using epifluorescence microscopy, and images of FITC, rhodamine, and DAPI fluorescence were recorded separately using a Quantix CCD camera (Photometrix, Tucson, AZ) and subsequently merged using IPLab Spectrum software (Signal Analytics, Vienna, VA).

Analytical Criteria

Blastocyst nuclei were scored only if they were intact and nonoverlapping. The specific signals detected in a given blastomere were considered to reflect a true chromosome constitution if the signals were of similar size, shape, and intensity and were more than the diameter of a single signal apart. A nucleus was considered diploid if it was possible to count either 2+2 (Fig. 1A), 2+1, or 2+0 signals; triploid if 3+3 (Fig. 1B), 3+2, 3+1, or 3+0 signals were found; and tetraploid if 4+4 (Fig. 1C), 4+3, 4+2, 4+1, or 4+0 signals were observed. Nuclei with higher ploidy were classified accordingly (Fig. 1D). Thus, nuclei with monosomy of either chromosome 6 or 7 were disregarded in this study. In addition, nuclei lacking signals such as 0+0, 0+1, 1+1, 1+diffuse, or 0+diffuse were considered as false negative, and only blastocysts having less than 20% false-negative nuclei were included in the analysis. Furthermore, only embryos in which 30 or more nuclei could be analyzed were included. Embryos that had nuclei with more than one type of chromosome complement were considered mixoploid.

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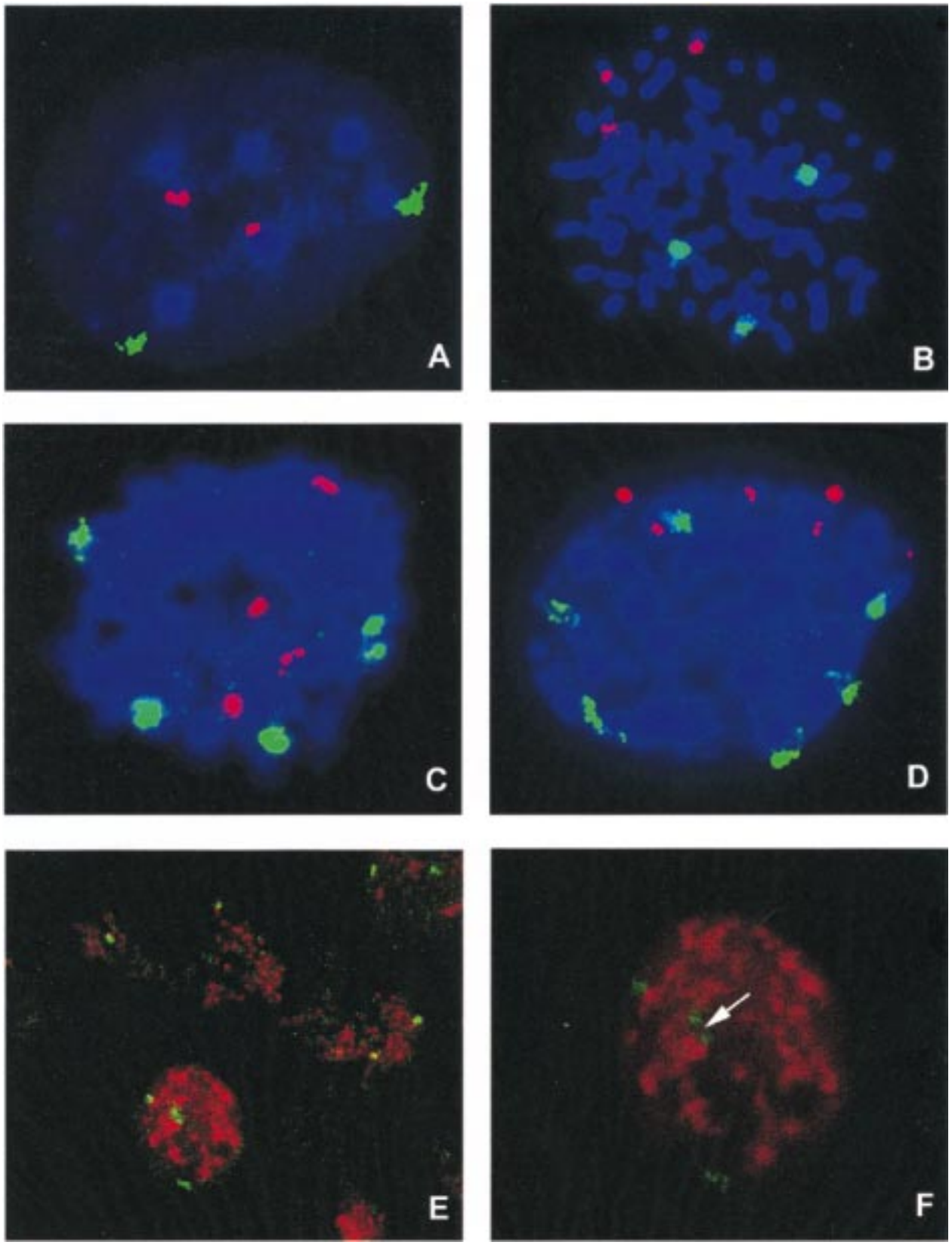

FIG. 1. FISH with chromosome 6 (green)- and chromosome 7 (red)-specific DNA probes on extracted interphase nuclei (**A, C, D, F**) and on metaphase spreads (**B, E**) from bovine blastocysts. The nuclei were counterstained with DAPI (blue) or PI (red). **A**) A normal diploid blastomere with two signals from each chromosome. **B**) A metaphase from a triploid blastomere showing 3 chromosomes with a green signal and 3 chromosomes with a red signal. **C**) A tetraploid blastomere with 4 signals from each chromosome. **D**) A hexaploid blastomere with 6 signals from each chromosome. **E, F**) A spread from a bovine blastocyst hybridized with the chromosome 6 probe. The metaphase spread (upper half) shows 4 chromosomes at the center. Arrow shows a large signal that seems to be split on two domains.

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TABLE 1. Distribution of chromosome abnormalities in bovine blastocysts produced in vitro and in vivo.

Chromosomal abnormalities %	In vitro blastocysts		In vivo blastocysts	
	%	(N)	%	(N)
0	27.8	42	75	21
0–5	43	65	21.4	6
6–10	17.2	26	3.6	1
11–15	3.3	5	0	0
16–20	3.3	5	0	0
21–25	2.6	4	0	0
26–30	0.7	1	0	0
31–35	0.7	1	0	0
36–40	0	0	0	0
41–45	0	0	0	0
46–50	0.7	1	0	0
>50	0.7	1	0	0
Total	100	151	100	28

Statistical Analysis

The relative frequency distribution of chromosome abnormalities in in vitro- and in vivo-produced bovine embryos was compared using Pearson chi-square analysis in a 2×2 frequency table.

RESULTS

Two-color FISH with the chromosome 6-specific probe (p33E39) and the chromosome 7-specific probe (cJAB8) produced different signals: p33E39 produced a large and rather diffuse signal containing a number of small spots, whereas cJAB8 showed small and more well-defined signals.

In Vitro-Produced Bovine Embryos

In 151 blastocysts, a total of 9294 nuclei were scored, ranging from the minimum of 30 to a maximum of 167 nuclei per blastocyst. An average of 6.7% false-negative nuclei were encountered. As illustrated in Table 1, we found mixoploidy in 72% (109 of 151) of the blastocysts. Most mixoploid blastocysts contained less than 25% polyploid cells: there were 83% (91 of 109) that contained 10% or fewer polyploid cells and 13% (14 of 109) that contained 11–25% polyploid cells, and only 4% (4 of 109) of the blastocysts had more than 25% polyploid cells. However, more than one type of chromosome abnormality in mixoploid blastocysts was often observed: the 40 blastocysts containing only one abnormality were 24 diploid-triploid mosaics and 16 diploid-tetraploid mosaics. Two, three, or more than three types of ploidy abnormalities were found in 28% (31), 24% (26), and 11% (12) of the 109 mixoploid blastocysts, respectively (Table 2).

In Vivo-Produced Bovine Embryos

A total of 1652 nuclei were analyzed from 28 blastocysts, the number of nuclei ranging from 30 to 144 per blastocyst. An average of 8.7% of nuclei were false negative. As illustrated in Table 1, mixoploidy was detected in 25% (7 of 28) of the blastocysts analyzed—significantly less than in the in vitro group ($p < 0.0001$). Six of these blastocysts contained less than 5% polyploid cells. Of the 4 blastocysts presenting only one abnormality, there were 3 triploid-diploid mosaics and 1 tetraploid-diploid mosaic. Two and three different abnormalities were observed in 2 and 1 blastocyst, respectively (Table 2).

TABLE 2. Abnormalities in bovine blastocysts produced in vitro and in vivo.

No. of abnormalities/ blastocyst	In vitro blastocysts		In vivo blastocysts	
	%	(N)	%	(N)
1	36.7	40	57.1	4
2	28.4	31	28.7	2
3	23.8	26	14.3	1
4	8.3	9	0	0
5	2.8	3	0	0
Total	100	109	100	7

DISCUSSION

FISH with bovine chromosome 6- and 7-specific probes reported here has for the first time been used to provide estimates of the extent and types of polyploidy within bovine blastocysts produced in vitro and developed in vivo. An average of approximately 60 nuclei were analyzed per blastocyst, and the results revealed a mixoploidy rate of 25% for blastocysts developed in vivo and of 72% for blastocysts produced in vitro. It is assumed that the embryos recovered from the superovulated animals are representative for normal in vivo embryos [21]. Although the difference in mixoploidy rates in the two populations is statistically significant, the findings may be valid only for the particular in vitro production system used and cannot be generalized at this point. It is also clear that the mixoploidy rate in the in vivo-developed blastocysts was not estimated as accurately as the mixoploidy rate of blastocysts produced in vitro. Still, the sampling of 28 morphologically normal blastocysts from 12 heifers is sufficient to allow detection of a statistical difference.

Previous studies on chromosomal abnormalities in bovine embryos have all been carried out by karyotyping, in which the whole embryo is incubated overnight with colchicine to arrest dividing blastomeres at metaphase, followed by attempts to obtain metaphase spreads by fixing and air drying the intact embryo. This method does allow identification of supernumerary or missing chromosomes and detection of polyploidy. In a cytological study of 7-day-old bovine in vivo embryos, no abnormalities were observed in 23 morphologically normal embryos together with more than 100 normal embryos in unrelated studies [22]. In that study, chromosome abnormalities were found only in morphologically abnormal embryos. The fact that the investigators did not find any chromosome abnormalities as compared to our observed rate of mixoploidy (25%) can be explained by the different techniques and thus the number of blastomeres available for analysis. We analyzed from 30 to 144 nuclei per in vivo embryo, whereas King et al. [22] analyzed from 1 to 7 metaphases. Thus, it is more likely that abnormal nuclei would have been detected in our study. Hare et al. [7] analyzed more than 150 in vivo-fertilized bovine Day 12 blastocysts and found 41.5% mixoploid blastocysts. The most likely explanation for the discrepancy between that study and ours is the difference in the age of the embryos analyzed: we analyzed blastocysts at Day 7–8 whereas Hare et al. [7] analyzed blastocysts at Day 12–15. An increasing mixoploidy rate throughout blastocyst development is conceivable, as data from the mouse have shown a rise of ploidy through gestation [23].

The in vivo-developed blastocysts were subjected to vitrification before chromosome analysis. One might speculate whether vitrification could influence the frequency of detectable polyploid cells. It has been reported that vitrifica-

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tion produces ultrastructural changes that are lethal to some cells [24]. We also realize that a higher proportion of nuclei in the vitrified, in vivo-developed blastocysts displayed a lack of hybridizing signals in comparison to in vitro-produced blastocysts. This phenomenon is most likely related to degenerative processes induced by the vitrification procedure. However, only if the vitrification procedure is more harmful to polyploid cells than to normal diploid cells can there be a decrease of mixoploidy frequency in the vitrified embryos. Although we find this unlikely, the possibility cannot be excluded.

Among the difficulties of cytogenetic investigations of embryonic metaphase spreads is identification of cattle chromosomes, a fact well illustrated by the ongoing debate in the scientific literature of the standard cattle karyotype [25]. The identification of individual chromosomes is even more difficult in metaphase spreads from embryos, mainly because of chromosome condensation and overlapping chromosomes. Because of these difficulties, combined with the fact that not all blastomeres produce metaphase spreads, most studies are based on only few metaphase spreads per embryo. Thus, embryos that contain a low percentage of polyploid cells and are classified as polyploid, may in fact be mixoploid. For example, if most embryos contain 10% polyploid cells, the chance of finding a polyploid embryo by analyzing only one cell is between 5% and 10%. Actually, the frequencies of tetraploidy and triploidy estimated from cytogenetic investigations of cattle embryos are 4% and 8%, respectively [1]. We consider it likely that these embryos have been mixoploid, as in the present FISH-based study we detected no true triploid or tetraploid embryos in a total of 179 embryos examined.

Compared to cytogenetic methods, the FISH-based analysis has provided a sensitive new tool for estimating the proportion of mixoploid embryos. However, FISH using two chromosome-specific DNA probes will not reveal all chromosome aberrations in embryos, for example trisomies, except those affecting chromosome 6 or 7, and all monosomies will not have been detected in this study. Furthermore, this study has demonstrated that the genomic organization of the repetitive elements present in the chromosome-specific probes introduced some limitation to their use. Using p33E39 we found a large diffuse signal that made it difficult to distinguish two distinct signals in some nuclei. This is in agreement with the report of Solinas-Toldo et al. [16] describing a ''long punctuated signal'' on bovine interphase nuclei for cosmid IOBT33 and for the 4.3-kilobase *Eco*RI fragment subcloned in plasmid 33E39. The authors pointed out that a similar hybridization signal was observed with repeat elements to chromosomes 8 and the X in mice [26, 27] and that analysis of the target sequences on these mouse chromosomes revealed a complex pattern of repeat units encompassing a relatively large genomic distance of about 1 megabase [28]. Thus, a repeat probe hybridizing to a relative small chromosome region is preferred for this type of study.

In the total material, false negatives were found in 7.5% of the nuclei and may be attributed to DNA degradation in apoptotic cells, loss of DNA during denaturation, poor penetration of the probes, insufficient binding of detection reagents, and in some cases overlapping signals. In order to improve the hybridization efficiency, we used HCl/Tween 20 to dissolve the zona pellucida and the cytoplasma of the embryos, resulting in clean nuclei with good morphology. Furthermore, pepsin digestion prior to the FISH procedure removed remnants of cytoplasm and made the DNA accessible for hybridization to the probes. Occasionally nuclei that remained covered with cytoplasm failed to hybridize with either probe, thus demonstrating the importance of having clean preparations for FISH.

It is surprising that a majority of the mixoploid embryos are diploid-triploid. In the study of Hare et al. [7] there were 66 (96%) diploid-tetraploid mosaics and only 1 diploid-triploid mosaic in the 69 embryos that contained polyploid cells. An explanation for the high diploid-triploid proportion in our study could be that some of the nuclei with three signals were actually tetraploid but that the chromosome homologues of the extra sets did not separate properly. Some support for this explanation was seen for an embryo that was hybridized with the p33E39 probe alone and revealed several interphase nuclei plus a metaphase spread. In this embryo, we consistently observed three signals in the interphase nuclei, but the metaphase spread was tetraploid (Fig. 1, E and F).

Mixoploid embryos constitute a sizable proportion of blastocysts produced in vitro. It could be argued that all embryos, chromosomally abnormal or not, have the potential to develop to the blastocyst but may die at a later stage. This is likely to be true, as bovine parthenogenetic embryos reached the blastocyst stage in percentages similar to that for normal bovine embryos, although all the parthenotes presented chromosome aberrations, with a tetraploid chromosome complement being the predominant common type [29]. Results from human as well as from mouse work further demonstrate that tetraploid embryos can develop beyond the blastocyst stage and begin implantation, although they exhibited a high degree of abnormalities [30, 31]. We are confident, however, that the majority of the mixoploid blastocysts found in our study are to a high degree developmentally competent, as bovine blastocysts produced by the same in vitro procedure as used in this study have resulted in a pregnancy rate of 64% in our laboratory [32]. This rate does not deviate significantly from results obtained from transfer of in vivo-developed embryos [33], and therefore a high frequency of mixoploidy does not seem to influence the developmental potential of the embryo. It is also noteworthy that as reported by Kawarsky et al. [34], mixoploid embryos exhibited a rate of development similar to that of the diploid embryos on at least Day 5 postfertilization, and that as concluded by Hare et al. [7], a level of 25% tetraploid trophoblast cells was compatible with pregnancy. It is conceivable that the polyploid cells in bovine blastocysts are preferentially allocated to the extra-embryonic membranes, as has been shown in mice [6]; but this remains to be studied in detail.

There could, however, be serious consequences of highly mixoploid blastocysts. Assuming that the difference of mixoploidy rate between in vitro-produced and in vivo-developed blastocysts detected by us is a general phenomenon, this question could be rephrased to ''What differences have been found between in vitro-produced calves and those resulting from in vivo development?'' A number of reports describe increased frequencies of calving abnormalities including large calf size, placental abnormalities, and reduced viability of in vitro-produced calves [32, 35–38]. The crucial question is whether these differences are caused by the difference in mixoploidy rate that we have detected for blastocysts produced in vitro. It is impossible to answer that question, but a hypothetical link between a high proportion of embryos with polyploid cells and the so-called ''large calf syndrome'' could simply be the result of an overactivity of the placenta due to an increased number

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of polyploid trophoblast cells. Studies to elucidate this hypothesis could be an interesting extension of the present investigation, since James et al. [6] found heavier placentas at Day 12.5 in diploid-tetraploid mouse chimeras than in normal controls.

ACKNOWLEDGMENTS

We are grateful to Ms. Nazia Wahla, Mrs. Inger Heinze, and Mrs. Zaida R. Rasmussen for excellent technical assistance and to Dr. Knud Christensen, Dept. of Animal Science and Animal Health, for lymphocyte cultures.

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