Fertility Investigation in F1 Hybrid and Backcross Progeny of Cattle (*Bos taurus*) and Yak (*B. gruniens*) in Mongolia. II. Little variation in gene products studied in male sterile and fertile animals.

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Summary

Crosses between cattle and yak produce and F1 hybrid, the khainag, which is fertile in the females but sterile in the males. Female khainag can be mated with either cattle or yak to produce a B1 backcross animal, the ortoom, which is also female fertile but male sterile. Further backcrossing of ortoom females to cattle or yak bulls will yield a B2 backcross animal, the usanguzee. Male usanguzees can be fertile, the male sterility in hybrids have been diluted by three consecutive backcrosses. We have shown that recovery of male fertility is associated with recovery of homologous pairing during meiosis (Tumennasan et al., 1997[1]). We have speculated that the loss of pairing and recombination or the related molecular events during meiotic prophase are responsible for lack of sperm in khainag and ortoom but not in the normal yak and cattle and usanguzee with recovered fertility. We have isolated several meiosis specific genes from lily meiotic cells (Kobayashi et al., 1994[2]) and the antibodies for such gene products have been prepared (Morohashi et al., 2000[3]). This paper reports the results obtained by using those to compare the meiosis specific gene expression in fertile and sterile animals. The expression of genes tested so far showed the absence of any noticeable difference between fertile and sterile animals but the plausible mechanism of recovery of fertility in consecutive backcrosses was discussed.

In Mongolian mountain areas, both yak and cattle are important economic animals and the cross products, khainag and ortoom, are also useful animals for farmers. However, F1 hybrid and F2 backcross males are sterile while females are fertile with yak and cattle (Fig.1). The karyotypes of the domestic cattle *Bos taurus* (2n=60) and yak *Bos grunniens* (2n=60) are identical in terms of both autosomes and X and Y sex chromosomes. Most of the interspecific hybrids are sterile in both sexes despite inheriting closely related or even identical chromosome sets from the two parents (reviewed by Hale et al. 1993[3]). In the hybrids structurally different chromosome sets exhibit major pairing abnormalities at meiotic prophase and this is associated with meiotic failure leading to sterility. In the case of cross between yak and cattle reported here only the male becomes sterile and the sterility starts to disappear after two sequential backcrosses. We sus-

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pected that the sterility may come from inactivation of some genes during meiotic prophase due to the one to one mixture of two sets of chromosomes in the male. Changing this mixing ratio the suppression may be reduced and the fertility increases. However, no meiosis specific product from gene(s) is known these animals and extremely difficult to obtain because of the cost. In the present report, we describe the use of antibodies raised against the selected gene products, LIM9, LIM10, LIM13, LIM14, LIM15, LIM16 and LIM18 which are all the products of meiosis specific gene, to detect the presence of such protein in testicular cells from male sterile and male fertile animals by means of Western blotting and immuno fluorescent microscopy (FISH method). Although these are the products of cDNAs isolated from lily meiotic cells, we applied for the fertile and sterile animals because of the well known facts that the presence of highly conserved nature of meiotic processes throughout eukaryotes (Hotta et al. 1997[4]).

The comparison of the expression of these genes in meiotic cells in testes from sterile and

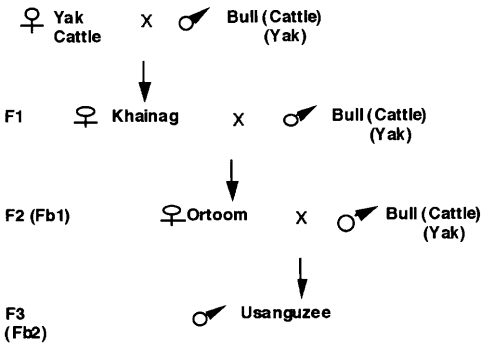


Fig. 1 Hybrid formation between yak and cattle and their backcross.

Mating either yak or cattle males with cow or female yak, repectively, produces male sterile and female fertile animals called khainag. Backcrossing of female khainag with male yak or ox produce F2 hybrids called ortoom which are also male sterile and female fertile. Backcrosseing of a female ortoom with male yak or ox will produces usanguzees which are both male and female fertile, although the male shows reduced fertility.

fertile animal were carried out by in situ hybridization and Northern hybridization but no significant differnce was found. The aim of these studies was to show the presence of phylogenic similarities in the meiosis specific gene-products and cellular distribution despite fertile or sterile and yak or cattle. Then, the possible mechanism of recovery of fertility in backcrossed male is discussed.

Materials and methods

Testicular samples were collected in the same area of Mongolia described in and earlier report[1], but the dates of harvest were delayed until the first week of June to obtain the meiocytes in a more advanced stage of spermiogenesis before castration. Animals aged 1.5 years and 5 years were castrated and small tissue pieces of testes were fixed in 4% paraformaldehyde solution for 30 minutes and stored in ice cold phosphate buffered saline (PBS) for cytological examination. Other pieces were ground with 5-10 volume of sample buffer (125mM Tris-HCl (pH6.8), 10% glycerol, β-merucaptoethanol) and the liquidified samples were brought back in cold. The remainder was frozen in liquid nitrogen for transport and eventual analysis.

For preparation of antibodies, cDNAs, Lim9, 10, 13, 16 and 18, were inserted into the plasmids, pQE30 or 31 or 33 (Qiagen Co.) and purified with QIAGEN plasmid kit (QIAGEN Co.). The cDNAs, Lim14 and 15, were incorporated into plasmid pET30b (Novagen Co.) followed by transformation of *E. coli.* In the case of LIM15, *E. coli* BL21(DE3) transformed with the expression plasmid, pFT-LIM15, were induced by the addition of 1mM IPTG (Isopropyl-thio-beta-D-galactoside) and harvested at the middle of the growth phase (optical density of 0.5-0.6) after 2 hours incubation. The pelleted cells were lysed in 6M urea-PBS buffer and the debris removed by centrifugation. The supernatant fraction was passed through a Nickel-agarose column (No-

vagen Co.) with and affinity for the 6xHis tag, and LIM15 recombinant protein was eluted by lowering the pH. The LIM15 recombinat protein was detected with an S-TagAPWestern kit (Novagen Co.) after electrophoretic separation and the final purification was carried out by Urea-SDS-polyacrylamide gel electrophoresis (1977[5]). The LIM15 recombinant protein was eluted from the gel and injected into rabbit. The obtained anti-LIM15 serum was purified by PVDP membrane methods described by Hammond (1990[6]).

For Western analysis, prarformaldehyde fixed and/or liquid nitrogen frozen samples were ground in 1-2 volumes of sample buffer (125mM Tris-HCl (pH6.8), 10% glycerol, 2.2% SDS (sodium dodecylsulfate), 1% β-mercaptoethanol) and boiled for 5 minutes. After centrifugation at 10,000 rpm for 30 minutes at room temperature using microfuge, the supernatant fraction was separated by acrylamide gel electrophoresis and transferred to PVDF membrane filter paper followed by the semi-dry-blotting system (Biocraft Co.) at 0.8mA/cm^2. The filters were treated with various antibodies raised against meiosis specific proteins (LIM 9, 10, 13, 14, 15,16 and 18). In place of LIM14, we used and anti-glycine-rich protein antiserum, because LIM14 protein is a glycine-rich protein. The filters were treated with alkaline phosphatase labeled primary antibody followed by incubation with anti-rabbit goat antibody, using commercially available detection kit (Biorad).

For histological and cytological examination, samples fixed with paraformaldehyde of after re-fixation with Bouin solution were embedded in paraffin-wax following standard dehydration and sectioned at 4μ followed by staining with hematoxylin-eosin (H+E stain) for observation.

For immuno-cytological examination, tissues fixed with paraformaldehyde were sectioned after wax embedding as above and attached to slides twice coated with poly-L-lysine. After removal of wax and hydration, the samples were washed three times with EGTA-phosphate buffer for 5 min. followed by a blocking step with 1% BSA (Bovine serum albumin) for 30 min. at room temperature. They were then treated with the primary antibody (500:1 dilution), either rabbit IgG (polyclonal) or mouse IgG (monoclonal), at 4℃ overnight. After washing three times with 0.05% Triton X-100 containing PBS, the samples were treated with the secondary antibody (100:1 dilution), either anti-rabbit IgG or anti-mouse IgG labeled with rhodamine or FITC at room temperature for 2 hours. Then, the samples were washed twice with PBS and stained with DAPI. After washing with PBS, the slides were mounted in DABCO (100mg/ml) containing 50% glycerine in PBS. Slides were examined under a fluorescent microscope.

For RNA isolation and gel blot analysis were carried out in similar fashion described previously (Mousavi et al. 1999[6]). Briefly, RNA was prepared from samles kept at -80℃ using aurintricarboxylic acid method (Kuhlemeier et al. 1988[7]). RNA samples were loaded onto 1% agarose-formaldehyde gels and blotted onto hybond N+ membrane according to the manufacture's protocol (Amersham). Hybridization of [32P]-labeled DNA probes to RNA blots was perormed in 50% formaldehyde, 5% SDS and 5XSSC (salt saline citrate). The filters were prehybridized for 2 hours and hybridized overnight at 42℃. The RNA blots were washed for 20 min. in 2xSSC, 1%SDS at 42 ℃, 50 ℃ and 60 ℃, briefly airdried and then autoradiographed with XAR-films (Fuji film).

Results

1. Absence of late meiotic cells in Khainag.

Histological examination of seminiferous tubules from 5 yea old and 1.5 year old yak (Fig. 2-a and b) and cattle (Fig.2-c and d) demo0nstrated active meiosis and production of round spermatids with no significant differece between the two species. However, in the tested of 1.5 year

old khainag (Fig.2-e) and 5 year old khainag (Fig.2-f) only stem cells and/or early meiotic cells at much reduced number could be seen. No late meiotic cells or spermaids were observed. This supports our previous observation (1) that meiosis probably terminates at early meiosis I.

In ortoom, one of two males had rather poor production o meiocytes (Fig.2-g) but other had more meiotic cells (Fig.2-h). However, neither showed completion of meiosis and production of spermatids, despite rather a rich abundance of spermatocytes in the seminiferous tubules. The suspected stage of meiotic breakdown was still early meiosis I.

In usanguzee, all the samples ontained from 1.5 year old animals showed many meiotic cells in the seminiferous tubules but some contained only small numbers of early meiotic cells (Fig.2-i and j), as onserved in Khainag and ortoom. Some of them apparently could reach the spermatid stage so that the animals may be fertile in spite of a greatly reduced spermatoigenic activity. However, we were unable to find any spermatids or sperm in the specimens that we examined.

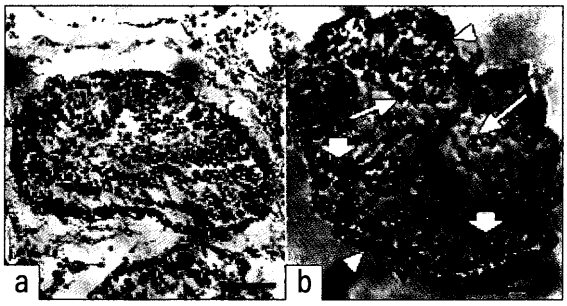


Fig. 2-a Fig. 2-b

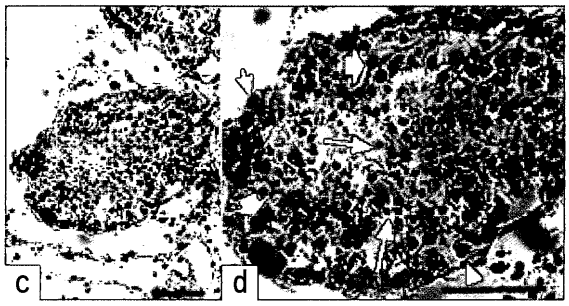


Fig. 2-c Fig. 2-d

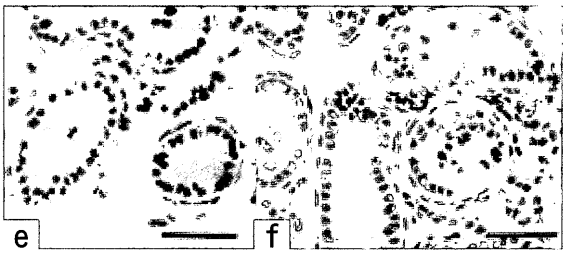


Fig. 2-e Fig. 2-f

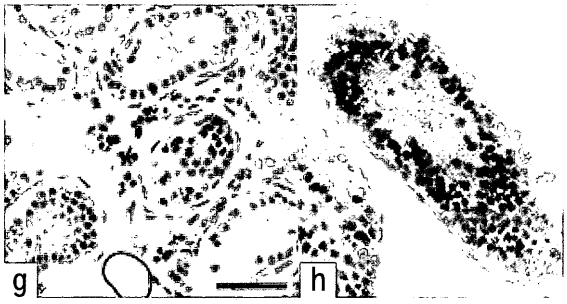


Fig. 2-g Fig. 2-h

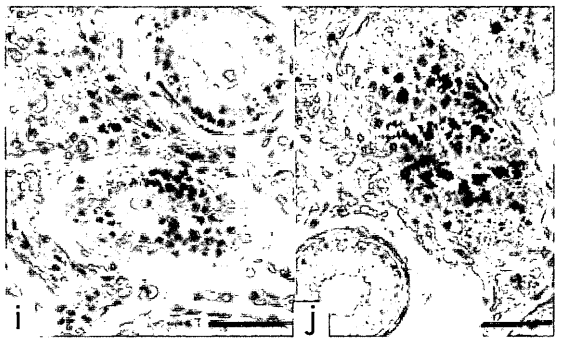


Fig. 2-i Fig. 2-j

Fig. 2 Histological sections of testes.

Yak 5 years old (a), yak 1.5 years old (b), ox 1.5 years old (c) and a higher magnification (d), indicating the normal development of spermetocytes in the seminiferous tubules. Arrowhead, arrow and openarrow in b and d indicate stem cells located at the bottom outer region of tubules, spermatids or sperms with a small nucleus and spermatocytes at meiosis I with a large nucleus, resoectively. Specimens from 1.5 year old khainag (e), 5 year old khainag(f), two 1.5 year old ortoom(gang h)and two 1.5 year old usanguzee (i and j)show increase of spermatocytes as back crosses proceeded. The bar indicates 50 μm.

Antibody used	Molecules found in cattle testis(kDa)	Molecules found in lily misrosporocyte(kDa)
LIM9	85	85
LIM10	27,23	18
LIM13	18,14	18,14
LIM14	35	15
LIM15	37	37
LIM16	41	55
LIM18	70	70

Table 1 Molecular weight of the proteins recognized on Western analysis.

2. Immunocytological observations.

The localization of several meiosis specific proteins was examined using the antibodies raised against each of the proteins produced in *E. coli* cells transformed by the corresponding cDNAs, namely, LIM 9, 10, 13, 154, 15, 16 and 18 (Table 1).

LIM15, considered as one of the key recombination proteins, homologous to RecA (8), was found in all of the nuclei of somatic cells, meiotic cells and even spermatids (Fig.3). A careful comparison of staining level in nuclei revealed that the most prominent staining was of nuclei inside the wall of the tubules, suggesting that more of thee protein or a protein(s) with high homology, is present in early meiotic nuclei. In the khainag seminiferous tubules, strong reactions were observed in a few meiotic cell nuclei in the tu-

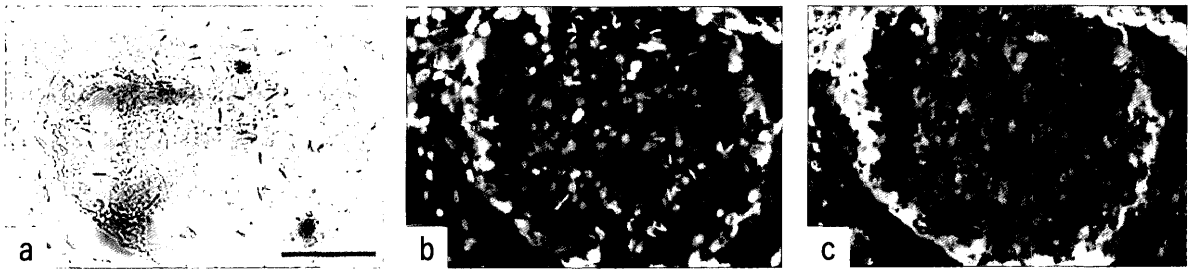


Fig. 3 Localization of LIM15-like protein(s) in yak testes.

Observation with phase contrast microscope(A), staining by DAPI(B), and staining by FITC-anti-LIM15 antibody. The bar is indicates 50μ.

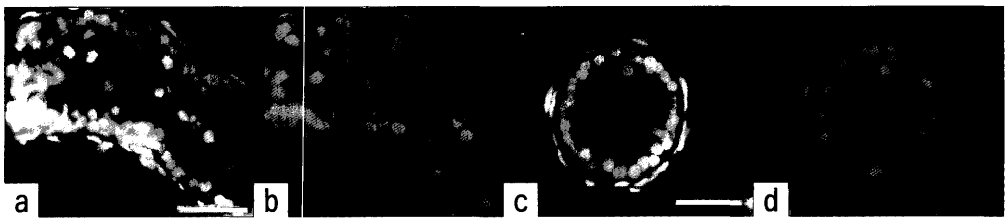


Fig. 4 Localization of LIM15 like protein(s) in seminiferous tubules from khainag testes.

(a) and (c) are DAPI atained tubules.(b) stained by Rhodamine-anti-LIM15 antibody.(d) stained by Rhodamine-anti-histone H1 antibody(purchased).

beules, although a weaker reaction was observed in surrounding somatic cells (Fig.4-a and -b). As a control, sections were stained with histone H1 antibody, which revealed localization restricted to nuclei, indicating good preservation of the tissue (Fig.4-c and d). Positive LIM15 staining was also found in the cytoplasm and somatic nuclei but the level of staining was muchi weaker than meiotic nuclei. LIM15 protein was initially identified in lily male meiocytes from late leptotene to pachytene and also found in the meiotic prphase cells of many other organisms, ranging from yeast to man(4). The presence of LIM15 in almost all nuclei of this study suggests that LIM15-like protein(s) and/or immunologically homologouse protein(s) are present at all times and in a variety of cells but the amount peaks at the prophase of msiosis (probably during pachytene).

LIM9 protein was detected with the anti-LIM9 antibody and a rhodamine labeled attached secondary antibody (Figs.5a and 5b), in the cytoplasm of all cells in the seminferous tubules, including the spermatogonia and spermatocytes of the khainag (Fig.5a left and right), and the ortoom (Fig.5b left and right). However, such staining was virtually absent in the somatic cells. Ortoom spermatocytes and spermatids exhibited cytoplasm binding of anti-LIM9 antibody (Fig.5b). A similar distribution of LIM9 was observed in samples from yak and cattle testes where many more spermatocytes were present (data not shown).

LIM10 was also distributed in the cytoplasm of the spermatocytes and probably spermatogonia of the khainag (Fig.5c) and ortoom (Fig.5d), as well as yak and cattle (data not shown).

The anti-LIM13 antibody reacted with the cytoplasm of connective tissue outside the seminiferous tubules in khainag (Fig.5e) but also reacted with the cytoplasm of spermatocytes (Fig.5f) and to some extend with spermatids in usanguzee, yak and cattle (data not shown).

The anti-LIM14 antibody reacted with both cytoplasm and nuclei of early spermatocytes in kkhainag (Fig.5g), ortoom (Fig.5h) and fertile animals. The LIM14 gene product has homology with cytokeratin in mammals and so a homologous protein having a glycine-rich structure would be expected in the tetes of both sterile and fertile animals.

The anti-LIM16 antibody sohowed the presenceo of LIM16 products, whose function is still unknown, in spermatogonia and spermatocyte nuclei of the khainag (Fig.5i), with eak staining also observed in their cytoplasm. Similar results were obtained for yak, cattle and ortoom (data not shown).

The anti-LIM18 antibody reacted mainly with khainag spermatocyte nuclei, but spermato goina and stem cells also showed a positive reaction in their nuclei (Fig.5j).

These immunocytological observations indicate that most of the antibodies raised against the meiosis specific proteins produced in *E. coli* cells react with spermatogonial and spermatocyte nuclei of yak, cattle and their hybrid animals, regardless of their fertility status. We could not examine the presence of reacting proteins during later stages of meiosis because of the absence of those cells in the seminiferous tubules.

3. Detection of LIM proteins in testicular cells by means of the western analysis.

Cytological studies alone are not sufficient because of the different nature of cell types present, which might cause false positive results, and quantitation requires immunochemical methods. Proteins extracted from cells can react with specific antibodies without disturbance. Western analysis of testicular tissue of cattle showed bands stained by anti-LIM antibodies as illustrated in Fig.6. A comparison of the sizes and numbers of proteins obtained from lily microsporocytes is given in Table 1. Anti-LIM9 gave four bands (85, 50, 45 and 28kDa) in cattle testes including the band detectable in lily microspores

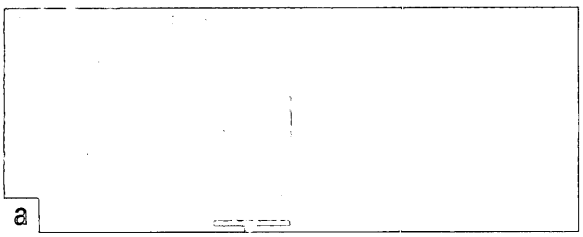
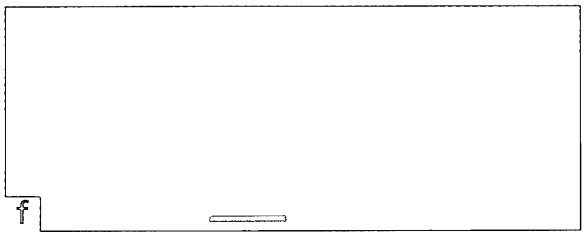


Fig. 5a



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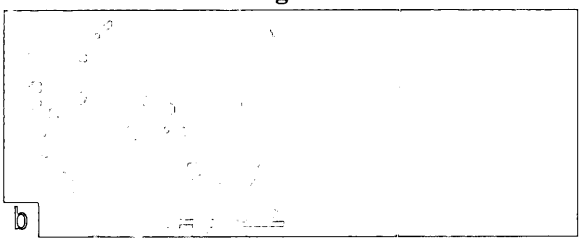

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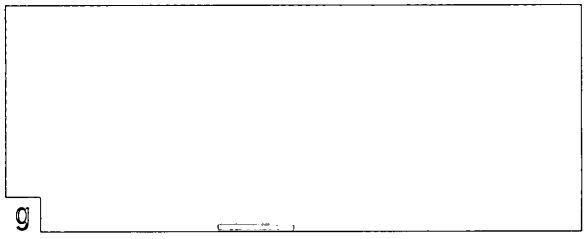
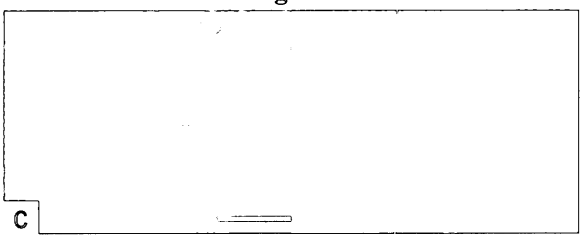


Fig. 5g



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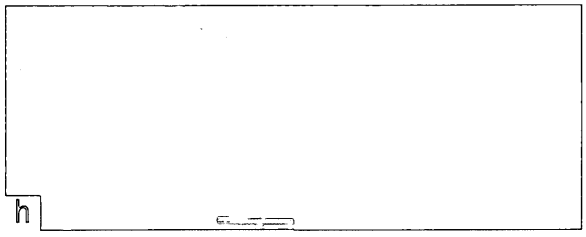

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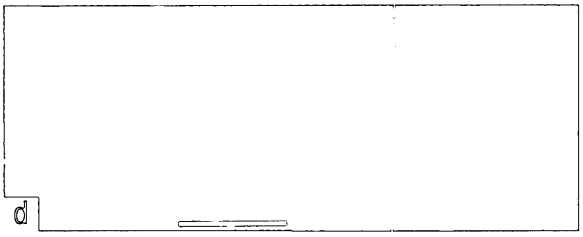

Fig. 5d

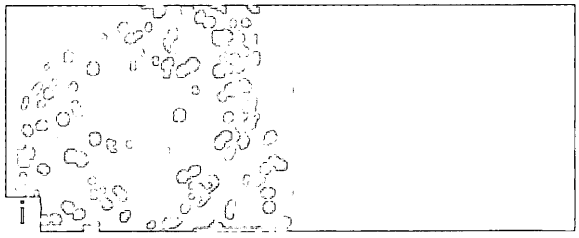

Fig. 5i

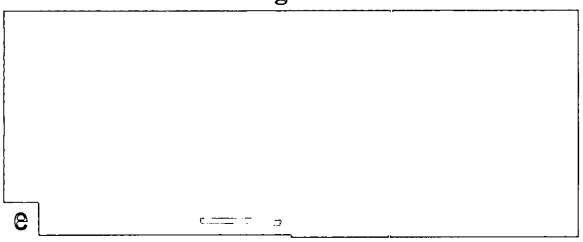

Fig. 5e

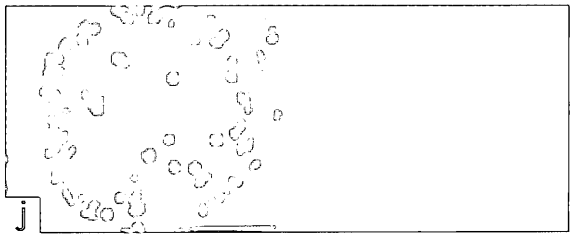

Fig. 5j

Fig. 5 Distribution of proteins reacting with the antibodies raised against LIM9,10,13,14,16 and 18, in seminiferous tubule of khainag and ortoom.

The left column pfotos from (a) to (j) are stained with DAPI, and the right column photos paired with the left are stained by antibodies. The photos in the right column of (b) and (d) are stained with FITC-labeled antibody and the other right column except (b) and (d) are atained with Rhodamine-labelled antibody. The photos (a) and (b) are from khainag and ortoom stained with anti-LIM9; the photos(c) and (d) are from khainag and ortoom stained with anti-LIM10, and photos (e) and (f) are from khainag and ortoom stained with anti-LIM13; the photos (g) and (g) are from khainag and ortoom stained with anti-LIM14, and the photos (i) and (j) are from khainag and ortoom stained with anti-LIM18.

(85kDa). Both LIM15 and LIM18 gave single band of the same moleculara size in both cattle and lily. At present all we can say is that similar or homologous protein(s) to each LIM protein are present in cattle testicular cells.

We further compared the amounts of testicular proteins reacting with anti-LIM-antibodies to determine whether this contribute to male sterility (Fig.7).

With anti-LIM9 shows very little variation was noted. In khainag the protein was only detectable up to the early spermatocyte stage, whereas it was present throughout meiosis in cattle and yak (Fig.5a). However, in early stage of meiosis, the 85kDa protein band was present at the same strength in all of the samples, regardless of the fertility (Fig.7-a).

The amount of protein reacting with anti-LIM10, also showed only slight variation, with some reduction in khainag and usanguzee (Fig.7-b).

Western analysis of LIM13 indicated two bands (18kDa and 14kDa) showing similar patterns in both sterile and fertile animals (Fig.7-c).

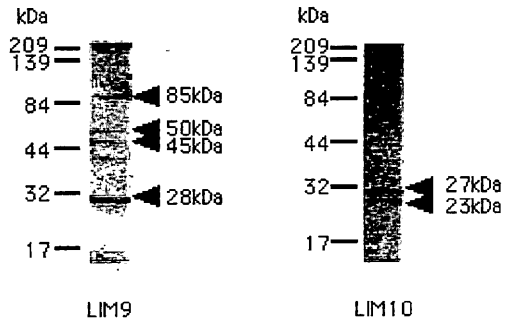

Fig. 6 Western analysis of testicular extract from cattle.

A piece of testicular tissue was ground in SDS-buffer and the supernatant fraction was separated by PAGE followd by transfer to a membrane filter. The filter was incubated with antibodies and/or serum prepared against each of the LIM proteins. The numbers on the left side of the colomns are size markers and the right side arrows and numbers indicating the proteins reacting with antibodies.

It should be noted that the meiosis specific protein ILM13 in lily microsporocytes is present in nonmeiotic cells of the male reproductive organ, despite the assumption that universarity of meiotic events occurs throughout phylogeny. Anti-LIM14 antibody reacted with 35kDa protein in the extracts of testes from both male sterile and fertile animals. A fuzzy band above 35kDa was also noted.

One band of 37lDa reacted with only anti-LIM15 antibody, LIM15 protein, was in the extracts of testes from both male sterile and fertile animals (Fig.6 and 7-e).

Anti-LIM16 antibody strongly reacted with a. 41lDa protein in testicular extracts of both sterile and fertile male, and also weakly woth 46kDa and 150kDa proteins.

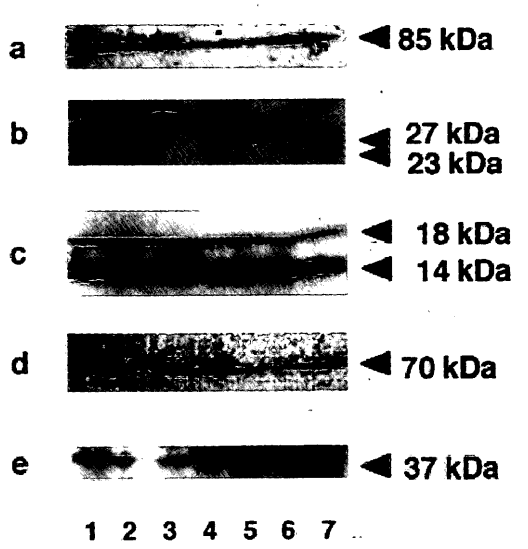

Fig. 7 Quantity of LIM-like proteins following the development of fertile male and sterile male testes.

Figures,(a), (b), (c), (d), and (e) demonstrate results of Western analysis with anti-LIM9, anti-LIM10, anti-LAM13, anti-LIM18 and anti-LOM15, respectively. The numbers at the bottom indicate the sources of samples, 1:1 year old cattle, 2:2 year old cattle, 3:1 year old yak, 4:2 year old khainag, 5:1 year old khainag,6:1.5 year old khainag and 7:1 year old usanguzee. The arrow heads at the right show the expected proteins. For fig.(e), 37kDa anti-serum was used instead of purified antibody.

Lim18 protein was detected in both male sterile and fertile testes at essentially the same level (Fig.7-d).

Western analysis thus indicted the presence of proteins reacting with all of the antibodies teste, with no relation to fertility. This, together with immuno-cytochemical analysis, may support the idea that there is a requirement for the proteins to functioning during meiosis which is common to many male reproductive tissues. However, the absence of LIM13 in the meiotic tissue, as indicated by immuno- cytochemical study (Figs.5 and 5b) suggests the ambiguity of its meiosis specific nature in testes.

Discussion

Many meiosis specific genes have been identified in yeast using mutants and their functions in both mitosis and meiosis have been subjected to extensive study(8,9,10).However, without known and experimentally usable mutants in higher organisms, it is difficult to investigate which genes governing meiosis and thus fertility. The failure of meiosis in male hybrids between yak and cattle, both with 60 nearly identical chromosome set with XY sex chromosomes, was confirmed in this study. But our speculation that loss of fertility (breakdown of meiosis) might arise through failure of expression of some meiosis specific genes did not prove correct. There have been suggestions that the meiotic processes are fairly conserved through phylogeny(11,12). We have isolated 18 cDNAs by differential hybridization from pollen mother cells of *Lilium longiflorum* at early prophase of meiosis I (2,13,14) Sequence analysis and a homology search revealed the presence of homologous genes in other organisms, and Northern hybridization showed that each of these cDNAs was expressed during meiosis. From these results, we could speculate their presumptive function in some of genes but the others remained to be analyzed.

Thus in the present study LIM proteins or homologous ones reacting to the anti-bodies were present in all the animal testes investigated, regardless of their fertility status. This suggests that other gene products which may be indispensable to meiosis need to be studied or we have to carry out more detailed and quantitative studies. Studies using isolated spermatogonia and spermatocytes may also be necessary to pin point the mechanisms of male sterility between yak and cattle. However this presents practical problems due to the lack of facilities where the animails are available. Further studies of this kind may become possible in future and more DNA clones which are not only specific to meiosis and active meiotically but also active somatically (3) should be isolated for future experiments.

The results of our immuno-cytochemisty demonstrated that proteins localized in the cytoplasm of lily microsporocytes, LIM9 and LIM10, are also present in the cytoplasm of cattle, yak and khainag spermatocytes, suggesting a similar function found in lily miocytes, namely, serine protease and heatshock protein, respectively (2,7). Comparable results were also obtained with LIM18 which is present in both cytoplasm and nucleus of meiocytes (7) are also present in both spermatocytes and spermatocytes. However, proteins localizing only in the nucle; or on the chaomosomes of microsporocytes, LIM13 and LIM(11) proved to be present in both nuclei and cytoplasm of spermatogonia and spermatocytes.

LIM14 has been observed in plastids of tapetal cells, microsporocytes, microspores and pollen (7) thus expected to be absent from testicular cells which do not have such subcellular particles. Our results from western analysis, however, clarely indicate the presence of LIM14 or at least in immunologically similar protein. Because the anti-LMI14 antibody can recognize LIM14 related glycine-serine rich protein in plants , it is possible that this antibody recoginized a glycine-serine rich cytokeratin-like protein(s) in testicular tissue. The cellular localization of LIM16 protein

has not been determined in spermatocytes and remains to be studied.

Meiotic breakdown in spermatocytes, in association with a failure of homologous pairing has been reported in Dmc-1 deficient mice(15). Lim 15 which is homologous to Dmc-1(11) is present in similar quantities in khainag, ortoom and fertile counterparts, despite the defferences regarding desynaptic breakdown at meiosis. This suggests the presence of facter(s) other than Dmc-1 causing meiotic breakdown in the hybrid condition. It may well be that meiotic failure in hybrids arides at a much earlier stage of germ cell development and synaptic failure is a secondary consequence, not the cause, of this breakdown.

In human today about 10 percent of adult couples are failimg to produce their offspring despite of their wishes. Among those, approximately half are due to male sterile and a significant portion presents meiotic failure. Even a healthy man, he produces various abnormal aperms at nearly 25 per cent, although he has sufficient fertility. These both serious and non-disterbing but unusable facts in mammals should be analyzed based on scientific and practical interest which no doubt make a cantribution to raise the quality of life.

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