Microsatellite analysis of mouse TNF and Cypla2 loci for polymorphism: detection and evaluation of genetic contamination

by Abdolhossein Rezaeian1,2, Mohammad hossein Alimohammadian3 & Sirous Zeinali1
1Biotechnology Research Center Pasteur Institute of Iran, Institute Pasteur of Iran, Pasteur St., Tehran, Iran
2Research and Clinical Center of Infertility, Yazd, Iran
3Department of Immunology, Institute Pasteur of Iran, Pasteur St., Tehran, Iran

Summary
The mouse TNF locus comprises tandemly arranged genes coding for Tnf α (cachectin) and Tnf β (lymphotoxin). The mouse TNF and Cypla2 loci were selected to detect simple sequence length polymorphism on genomic DNAs of four laboratory mouse strains. These polymorphisms might be useful in the search for tumor suppressor genes involved in specific cancers. The primary aim of this work was to analyse the use of these polymorphic microsatellite markers, which could differentiate between four typical laboratory mouse strains (i.e. BALB/c, C57BL/6, CBA as three inbred mouse strains and Syrian Hamster as an outbred strain) and the second was to define possible genetic contamination of a BALB/c mouse colony. In this study, we investigated the applicability of DNA fingerprinting using short tandem repeats (STRs) by the use of PAGE and silver staining. We found that all microsatellite DNA sequences varied in size in the TNF locus in the four strains tested, but for the Cypla2 locus no polymorphism was found within these strains. To our knowledge this is the first report of lack of polymorphism in the Cypla2 locus within these strains. The other techniques for inbred mouse identification were skin grafting and a coat colour test. We found no signs of rejection during skin grafting within the BALB/c mouse strain and also as expected all strains showed normal phenotypes. These experiments suggested lack of genetic heterogeneity in Cypla2 locus and no evidence of genetic contamination was also found in the BALB/c mouse.

Introduction
The tumor necrosis factor (TNF) locus comprises the closely linked genes coding for Tnfα (cachectin) and Tnfβ (lymphotoxin). The TNF locus is contained within the MHC, at the boundary of the class 2 and class 3 region both in mouse and man. (Jongeneel et al. 1990, Benavides et al. 1998). TNFα has been shown to play an important role in inflammation, immunoregulation, proliferative and antiviral responses, and is also important during infection with various protozoan parasites (Iraqi & Teale 1998). The development of DNA-based markers has had a revolutionary impact on gene mapping and more generally, in animal and plant genetics. The construction of high resolution maps from mouse and human genomes with markers at every 1 centi-Morgan (cM) requires the identification of a large number of polymorphic DNA markers (Love et al. 1990). Studies of population dynamics, social structure and mating strategies in wildlife have been broadened since the development of DNA fingerprinting technology in 1985 (Lambert et al. 1997). DNA fingerprinting has since been used in a diverse range of applications, including individual identification, analyzing heterozygosity, determination of paternity and population genetics (David & Christian, 1999). Initially, man was the emphasis of DNA fingerprinting studies (Lambert et al. 1997), but subsequently the focus has widened.
to include domestic animals, and more recently, wild animals and free range populations (Jackson & Abbott, 2000). Most DNA polymorphisms or variations have been detected using restriction enzymes, which detect sequence variations at restriction enzyme sites. These, by their nature, are rare and their detection can require the use of 20-30 different enzymes. In addition, blotting and hybridization protocols are time consuming, as tools for genetic mapping and markers with more than two alleles are considerably more useful than standard, diallelic restriction fragment length polymorphisms (RFLP) (Love et al. 1990). Variable numbers of tandemly repeated (VNTR) DNA polymorphisms or minisatellites are extremely polymorphic and are widely distributed throughout human and mouse genomes. They can be analyzed by the use of methods similar to conventional RFLP analysis and also by using the polymerase chain reaction (PCR). The repeated unit can be up to 50 bp and because of the large number of repeats, the utility of PCR as a way for analyzing VNTR length may be limited (Love et al. 1990). Then simple sequence length polymorphisms (SSLPs) will provide an alternative methodology to the conventional analysis of RFLPs in studies of loss of heterozygosity (LOH) in tumors (Wiseman et al. 1994), as well as for genetic mapping in the mouse (Dietrich et al. 1992).

Eukaryotic genomes contain many short tandem repeats (STR) of very simple motifs (usually dinucleotides), among which, (CA)n seems to be the most abundant. This so-called microsatellite is more or less distributed throughout the genome, and is commonly found within the non-coding parts of the genes. The length of a given microsatellite is variable in the population, but is inherited as a relatively stable allele (Jongeneel et al. 1990).

There are now more than 3000 different genetically defined inbred, congenic mutant and transgenic strains of mice, which are used for biomedical research (Jackson & Abbott, 2000). The lack of authenticity or contamination of inbred strains has become a serious concern as the use of contaminated stocks may result in considerable financial loss and a waste of time (Jackson & Abbott, 2000). Since investigations concerning genetic purity had never yet been performed in Iran, we decided to analyze a set of three mouse inbred strains (BALB/c; C57BL/6 and CBA). BALB/c mouse strain is mainly used in cancer and immunological research. This strain is the most widely requested strain from the animal facilities at the Pasteur Institute of Iran.

Therefore, the aim of this work was to describe a set of polymorphic microsatellite markers, which can help discriminate between the classical laboratory inbred strains BALB/c, C57BL/6, CBA. As a comparison, an outbred strain of Syrian Hamster was added to our study. Other conventional typing of mouse strains by using skin grafting and coat color test were also used for inbred mouse identification.

**Materials and Methods**

**Experimental animals**

36 adult (i.e. 4-8 weeks old) female BALB/c mice obtained from the breeding farm of Pasteur Institute of Iran were used in this assay. Eighteen of the mice were used both in the reciprocal abdominal skin transplantation method and in the extraction of DNA from their tails tips. Another 18 BALB/c female mice were used as a parent in the test cross with C57BL/6 for a coat colour test. All animals used in this study (i.e. C57BL/6, BALB/c, CBA and Syrian Hamster) were bred under controlled environmental conditions of temperature (20-22°C), humidity (50%) and light (12/12 h = light/dark). Commercial pelleted food and water were available ad libitum (Jackson & Abbott, 2000).

**DNA samples**

High-molecular weight genomic DNA samples were prepared from tail tips of mice collected from C57BL/6, BALB/c, CBA and Syrian Hamster strains. DNA was extracted in the absence of phenol by modification of a salting out procedure (Sambrook & Russell, 2001, Glover & Hames, 1995). Briefly, one-half inch of mouse-tails were ground then digested overnight at 55°C with proteinase K (0.5
mg/ml). Proteins were then precipitated with a saturated NaCl solution followed by centrifugation at 13,000 rpm for 10 min. DNA was precipitated by adding two volumes of absolute ethanol and the DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.5).

**Skin grafting**
The reciprocal abdominal skin grafting method was used. Three cycles of six animals each were performed. The observation period was extended to 120 days. Auto graft, allograft and syngenic were carried out as controls.

**Microsatellite markers**
We selected three loci containing 1) Tnf α; simple direct repeats of two nucleotides, 2) Tnf ß; complex nucleotide repeats, and 3) Cypla2; tetramere repeats. TNF locus containing the tandemly arranged genes coding for Tnfα and Tnfß (Tab. 1).

**PCR amplification**
PCR amplifications were performed in aliquots of 25 µl volumes containing 50 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl, PH 8.3, 2 mM MgCl₂, 0.001% spermidine) 200 mM each dATP, dCTP, dGTP, and dTTP (Euobio, France), 4 ng of each primer (Gensetoligos, France), and 1 unit of Taq DNA polymerase (CinnaGen, Iran). PCR reactions were carried out in Mastercycler gradient (Eppendorf) as follows: an initial incubation at 95°C for 5 min; followed by 30 cycles, each consisting of 1 min at 94°C, 1 min at 61°C, 3 min at 72°C; a final extension at 72°C for 10 min; and then incubated at 4°C. PCR products were subjected to electrophoresis on agarose gel. Allele sizes were measured relative to a standard size marker (100 bp ladder, Gibco BRL-UK) (Fig. 1) (Glover & Hames 1995, McPherson & Muller 2000).

Fig. 1
PCR amplified fragments generated from a panel of inbred mouse strains using the primers depicted in Table 1, and were determined on 3% agarose gel.

Table 1.
Simple repeat-containing DNA sequences or microsatellites in the mouse and the chromosomal locations and distance from the centromere in cM as taken from the April 1990 edition of the ‘Locus Map of the Mouse ‘from the Jackson Laboratory.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr name</th>
<th>Sequence</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Repeat unite</th>
<th>map location</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>17 MMU17</td>
<td>5’ GGACAGAGAAGAAATGGGTTTC 3’</td>
<td>(CA)₃₀</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ TCGAATCTGGGGCCAATCAGGAGGG 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFß</td>
<td>17 MMU17</td>
<td>5’ TTCCTGTGGCGGCCTTATCAG 3’</td>
<td>(TC)₆C₄(TC)₆TT(CT)₅</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ AGACAATGGGTAACAGAGGCA 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cypla2</td>
<td>9 MMCYO3</td>
<td>5’ AGTTTTAGGCTAGTATAGGTT 3’</td>
<td>(CAAG)₁₂</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’ ACTGGAACCTTAGAAGCATGAG 3’</td>
<td></td>
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Microsatellite typing
PCR products were resolved by electrophoresis through a vertical non-denaturing 12% polyacrylamide gel electrophoresis (PAGE). 15 microliters of each PCR product were mixed with 5 µl of loading buffer, loaded on the gel, and subjected to electrophoresis for 5 h at a constant current of 22 mA. After electrophoresis, the gel was stained with ethidium bromide or silver by standard protocols (David & Christian 1999).

Results
A coat colour test was used for the BALB/c strain inbred mouse assay. All CXBF1 animals obtained from the test crossing were observed once the F1 progeny had reached the age of full expression of coat colour. All hybrid F1 mice had the same expected black-agouti phenotype.

The second test for inbred mouse assay was skin grafting. Skin grafts were inspected daily from day 10 for 3 weeks, and once a week up to day 120 post grafting. No signs of rejection were observed during this time in these animals, suggesting no genetic heterogeneity.

Microsatellite analysis by PCR was the third method used for the above purpose. Comparative analysis using microsatellite markers revealed that TNFa and TNFB microsatellites varied in size among the four mouse strains tested. The C57BL/6 had the shortest microsatellite (CA) repeat at 130 base pair (bp), followed by BALB/c and CBA with 110, 120 bp respectively. Syrian Hamster had the middle size for the Tnfa locus (Fig. 2 A and also Fig. 2 B, samples on the left of size markers). The size of PCR bands for TNFβ were; 160, 140, 150, 140-160 bp for C57BL/6, BALB/c, CBA and Syrian Hamster respectively (Fig 4, samples on the left of size marker). All microsatellite DNA sequences varied in size among the four strains tested, except for Cypla2 locus in which no polymorphism was observed (Fig 2, 3 & 4; samples on the right of size markers). It should be noted that polymorphism for this site had already been reported in other studies. (Love et al. 1990).

We also observed co-segregation phenomena between inbred lines and RI line (Syrian Hamster) which is an indication of genetic linkage between these polymorphisms (Fig. 2 A; samples 7, 8 & Fig. 2 B; sample 1 and Fig 4; sample 5).

![Figure 2a](image1)
Polymorphism assay using Tnf α STR stained with ethidium bromide (A) or silver (B). A: Size marker (100 bp ladder) (1); C57BL/6 (2,10); BALB/c (3,4); CBA (5,6); Syrian Hamster (7,8); negative control (9).

![Figure 2b](image2)
B: Syrian Hamster (1); CBA (2); BALB/c (3); C57BL/6 (4); Size Marker (100 bp ladder) (5); Cypla2 locus for Syrian Hamster (6), CBA (7), BALB/c (8), C57BL/6 (9), positive control (10).
Figure 3 AB
PCR product sizes for murine strain BALB/c A: 1-4 for TNF β locus, 7-9 for Cypla2 locus, negative control (5), size marker (100 base pair ladder) (6); B: 2-7 for TNFα locus, negative control (1), size marker (100 base pair ladder) (8). Microsatellite markers were determined by Silver stained (A) and ethidium bromide (B).

Figure 4
The results of PCR amplification for polymorphism assay using Tnf β (1-5) and Cypla2 (7-10) STRs stained with silver. Negative control (1), C57BL/6 (2), BALB/c (3), CBA (4), Syrian Hamster (5), size marker (100bp base pair ladder) (6), C57BL/6 (7), BALB/c (8), CBA (9), Syrian Hamster (10).
Discussion
This study showed the presence of polymorphism in Tnf α and Tnf β genes of the different mouse strains studied. These polymorphisms (in Tnf α and Tnf β genes) may contribute to a more detailed genetic mapping with these loci, and may be useful in the search for tumor suppressor genes involved in some specific cancers, such as thymic lymphomas. Other studies may clarify this possibility. The mouse strains BALB/c, C57BL/6 and CBA represent the type of strains used for the susceptibility and the resistance phenotype respectively (Glover & Hames 1995), specially in studies with Leishmania major (Kropf et al. 1998). F1 hybrids of these strains may be typed for lymphoma susceptibility using simple sequence length polymorphisms (SSLPs) as markers. Therefore, studies on genetic mapping of these strains an mice would be an interesting field.

Our results for genetic contamination assay for the two loci showed a match with control strain DNA and perfect correlation with the expected BALB/c alleles: i.e. Tnf α (110 bp) and Tnf β (140 bp). No difference in DNA band was observed in these lines, therefore it seems that there is no difference in gene expression in these mouse strains. We can conclude that BALB/c strains used in our study are perfectly inbred with no genetic contamination as shown for loci studied.

DNA fingerprinting is a valuable addition to the various methods that already exists for the authentication of inbred strains of animals (Jackson & Abbett, 2000). In our knowledge, this experiment is the first genetic monitoring in experimental animals comprising different methods including coat color, immunological and molecular genetic markers which is carried out in an inbred mouse colony. This study was carried out in BALB/c strains as a quality control measure, because BALB/c mice are, by far, the most requested strain from our institute animal facilities. Fortunately in our investigation no evidence of genetic contamination for these sites was found for this strain. Because of the importance of genetic homogeneity within an inbred strain, we suggest that these lines be refreshed again.

Microsatellite loci are associated with high levels of tandem repeat number variability, reflecting high rates of spontaneous mutations to new length alleles. However it seems that no major mutation has been happened in the studied colony. These results suggest that the polymorphisms between different strains are due to an inheritance influence but not due to Mendelian distribution as reported by other investigations (Tom & Read 1999). At the most variable loci, the mutation rate could be directly measured by pedigree analysis. As breeding analysis was not performed, the pattern of allelic inheritance is unknown. Of course, heterogeneity may be due to incomplete extention by the polymerase and/or terminal transferase activity of the enzyme and/or polymerase slippage during copying of the short tandem repeats or to out-of-frame priming within the repeats (David & Christian 1999, Tom & Read 1999) in pattern of PCR as demonstrated in our study (Fig 2, B; 8 and Fig 4 for Tnf β locus). Of interest, no obvious heterogeneity was found for Cypla 2 locus in these selected mice strains. Our data show inconsistency with report of other authors (Love et al 1990). The reason for this contrary result is not clear, but may be attributed to mutation has accured in the strains studied. To our knowledge, this is the first report on the lack of polymorphism for Cypla2 locus between selective three mouse strains studied.

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