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PCR based identification of short deletion/insertions and single nucleotide substitutions in genotyping of splotch (*Pax3*^{sp}) and truncate (*Noto*^{tc}) mouse mutants

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Abstract

Splotch (*Pax3*^{sp}) and truncate (*Noto*^{tc}) are spontaneously arisen mouse mutants with disturbed embryo development. Splotch carries a *Pax3* mutation and it is characterized by the neural tube defect. Corresponding mutation in human causes Waardenburg syndrome. Truncate is *Noto* mutant with disturbed development of the caudal notochord. In order to establish easy genotyping procedure of these mutations, it was tested whether simple PCRs with single primer pairs could be used for this purpose. As it was necessary to differentiate sequence variants on the scale of one to several nucleotides, the approach referred to as "3` variable primer ends" was applied. The method was based on the presence of discriminating nucleotides at the 3' end of the primer sequence. This approach was successfully applied in genotyping adult mice and embryos of splotch with a 6 bp deletion/insertion and truncate with a single nucleotide substitution. Described genotyping approach facilitates recognizing of these mutations and it could be in general used for detection of sequence differences in one to several nucleotides.

Key words: PCR, genotyping, splotch, truncate, mouse

1. Introduction

Single nucleotide substitutions (SNS; single-nucleotide polymorphisms (SNPs), point mutations) are single-base alterations in DNA sequence and represent the most common genetic variation [1] (nomenclature of sequence variants used according to recommendations of Human Genome Variation Society, [2]). In humans, SNSs are frequently related to disease and drug response. There are several main strategies for SNS detection, both in human and experimental animals. Mismatch analysis are based on chemical or enzymatic cleavage of mismatched DNA heteroduplexes, while the primer extension method is based on specific extension of the targeted sequence (reviewed in [3] and [4]). Modern approaches for SNS detection are fast and reliable, but as they are rather resource demanding, they are routinely used only for large scale SNS analysis or for specific diagnostic purposes.

Mouse mutants usually contain inserted sequences (e.g. transgenic animals) or exhibit larger insertions/deletions that can easily be distinguished by routine polymerase chain reaction (PCR) [5]. The need to recognize differences on the scale of one base pair when genotyping mutant mice is less frequent and involves the analysis of the spontaneously arisen mouse mutants and those produced from various mutagenesis screens. PCR, as a relatively fast and simple method, is in general only occasionally applied for SNS detection and specific modifications are usually recommended. For example, the amplification refractory mutation system used for detection of SNS is based on the introduction of additional mismatches in the corresponding primers, three to seven bases from the primers' 3' end, which adds to the primer specificity [6].

Another technique, tetra-primer allele specific amplification, uses two pairs of nested primers at two annealing temperatures. In the first round of amplification at higher temperature, a DNA fragment is amplified by the external primers because the temperature is too high for the annealing of SNS-specific internal primers. During the second round at lower temperature, the internal primers anneal and pair with the external primers, which results in the amplification products of unequal lengths, which could be subsequently distinguished by DNA electrophoresis [7].

The goal of this work was to test if careful adjustments of PCR parameters could yield a reliable protocol for human and mouse genotyping based on simple PCRs using single primer pairs. Two representative mouse lines were tested: splotch, *Pax3* mutant ($Pax3^{sp}$) which carried a short deletion/insertion of 6 bp (*Pax3*:c.456-7T_-2AdelinsCGTGTG) [8] and truncate, *Noto* mutant ($Noto^{tc}$), which carried a SNS (*Noto*:c.503T>G) [9].

Splotch homozygous embryos often die due to heart and neural tube defects. In humans, the corresponding *PAX3* mutation causes Waardenburg syndrome [10]. Adult heterozygotes can be recognized by their phenotype, which greatly facilitates the breeding of the line. However, the investigation of younger embryo stages requires a simple and a reliable method of genotyping.

Embryos of the truncate homozygotes lack the notochord in the caudal part [9, 11]. Adult homozygotes are viable and can mate, although the malformations of the tail and the lumbosacral region strongly influence their fertility. Establishing genotyping of truncate mice would facilitate the breeding, and in addition allow further analysis of the truncate developmental phenotype by comparing littermates of different genotypes.

The strategy applied was based on the use of primers, which contain the nucleotides specific for the sequence variants at their 3' end, a method referred to as 3' variable primer ends (3'VPE). The method was successfully applied for both splotch (deletion/insertion of 6 bp) and truncate (SNS bearing) animals.

2. Materials and Methods

2.1. Mouse strains

Two lines of mutant mice were used splotch (*Pax3*^{sp}), a *Pax3* mutant, characterized by short deletion/insertion of 6 bp (*Pax3*:c.456-7T_-2AdelinsCGTGTG) and truncate (*Noto*^{tc}), a *Noto* mutant characterized by a single nucleotide substitution (*Noto*:c.503T>G). In both lines the mutations arose spontaneously. Both lines were kept on the C57BL/6 background.

2.2. DNA isolation

Genomic DNA was isolated either from the adult mouse tails or from the embryonic forelimb buds using standard isopropanol-ethanol procedure [12]. Briefly, tissue was immersed in lysis buffer at 56°C overnight. (100 mM TrisCl, pH 8,0; 5 mM EDTA; 0,2 % SDS, 200 mM NaCl, 100 µg/ml proteinase K). DNA was precipitated by ispopropanol and ethanol, dried and dissolved in 100 µL of sterile water.

2.3. Polymerase chain reaction (PCR)

PCRs for the genotyping of both strains was performed in 25 µL of total reaction volume containing: approx. 500 ng of DNA sample, Taq polymerase (1 unit, Promega), deoxynucleotide mixture (200 µM), primers (400 nM), $MgCl_2$ (2 mM), 5x Green Buffer (5 µL, Promega) and sterile water. Annealing temperatures were 56ºC for splotch and 63ºC for truncate genotyping. Every sample of DNA was amplified in two separate reactions: one with primers corresponding to the wild type allele and the other with primers corresponding to the mutated allele. The unrelated pair of primers was used as a positive control in every reaction. The primers used are given in Table 1. Software used for primer design was "Primer Quest", freely available on http://www.idtdna.com/Scitools/Applications/PrimerQuest/. Primer analysis, including calculation of melting temperatures, was done using software "OligoAnalyzer 3.0", freely available on http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/.

3. Results

3.1. Selection of primers and PCR set-up

The primers expected to specifically amplify the sequences which differed in a single or in several nucleotides were designed according to the hypothesis that the ability of the 3` end of the primer to anneal to the target sequence would have the strongest influence

on the DNA amplification. If the 3` end of the prime failed to anneal to the target sequence, it was assumed that the Taq polymerase would not be able to further extend the newly synthesized DNA strand. Therefore, primers with different 3` ends were initially constructed for *Pax3*^{sp} deletion/insertion, which differed from its wild type allele in 6 bp. Subsequently, after this approach was shown efficient, the same was applied for SNS in the *Noto*^{tc} line (Fig. 1).

For both *Pax3*^{sp} and *Noto*^{tc} genotyping, sets consisting of 3 primers were designed: one primer in the 3` direction corresponded to the wild type sequence, another primer in the 3` direction corresponded to the equivalent but mutated sequence, and the common third primer was in the 5` direction. Two separate reactions were performed simultaneously, one with the wild type primer and the common primer, and another with the primer that corresponded to the mutated sequence and the common primer. In the case of the wild type animal, the amplification occurred only in the reaction including the primer that corresponded to the wild type sequence and in the case of the homozygote, only in the reaction including the primer that corresponded to the mutated sequence. In the case of the heterozygous animal, the amplification occurred in both reactions (Fig. 1A,B). To make sure that the lack of the amplified product was not a consequence of failed reaction, a control pair of unrelated primers, present in all samples, was used (within *Stam2* genomic sequence, at chromosome 2 in *Pax3*^{sp} genotyping, and within *Nol1* genomic sequence, at chromosome 6 in *Noto*^{tc} genotyping, Fig. 2 and 3).

Due to the difference in a single nucleotide, the PCR set-up for the truncate genotyping was carefully adjusted by testing different annealing temperatures. At temperatures higher than 62°C the primers which differed in only one base started to anneal

differentially. However, temperatures higher than 64°C yielded frequently no result especially using the primer specific for the wild type allele. This was in accordance with the calculations performed using the melting/annealing formula, which predicted that the primer corresponding to the wild type allele had a melting temperature almost 2°C lower than its mutant counterpart. In order to perform the PCR simultaneously, i.e. to have both wild type and mutated allele primer pairs at the same time in the PCR machine, temperature of 63°C was chosen as optimal for both reactions.

4. Discussion and conclusions

4.1. Reliability of splotch (*Pax3^{sp}*) and truncate (*Noto^{tc}*) strain genotyping

The usefulness and the reliability of the established PCR based genotyping for *Pax3^{sp}* and *Noto^{tc}* mice were carefully tested. Samples of DNA were taken from the adult *Pax3^{sp}* heterozygotes, which were recognized according to the discolorations of the fur, and from the control C57BL/6 mice. In all 10 samples the genotypes were correctly recognized. Furthermore, the method was tested on 30 *Pax3^{sp}* embryos 11.5 and 12.5 days old. All of the homozygous embryos recognized by the presence of the spina bifida were correctly genotyped (Fig. 2). Thus, the method of genotyping of *Pax3^{sp}* strain based on the 3` variable primer ends was absolutely reliable (total of 40 samples).

Testing of the reliability for the genotyping procedure for the *Noto^{tc}* mice was extensive and in total 95 mice obtained from the intercrosses of heterozygotes were tested. The adult *Noto^{tc}* homozygous mice (N=11), previously recognized according to the tail malformations were correctly genotyped as homozygotes. Nevertheless, there were 2

male and 3 female mice with normal tail morphology, which were also identified as homozygotes. Mating of these animals between themselves resulted in purely homozygotic litters. This confirmed that the *Noto*^{tc} adult homozygotes could reveal a normal tail phenotype (Fig. 3). From 95 genotyped mice, 53 were recognized as heterozygotes, 16 as homozygotes and 27 as wild type. Although 16 homozygotes instead of the 27 expected represented obvious decrease in the number of born homozygotes, the chi-square test did not show significant deviations from the expected ratio of 2:1:1 (χ^2=3,5625; 0.1<P<0.5). Decrease in the number of homozygotes born versus the number expected was already reported in similar studies. Moreover, detection of the homozygotes with normal tail morphology was in accordance with the previously reported variable severity of the *Noto*^{tc} phenotype [9, 11]. Theoretical possibility that the homozygote would be recognized as a heterozygote did not occur, as the mice without a tail were never recognized as heterozygotes. Moreover, heterozygote/wild type ratio did not change (1.96/1).

In conclusion, a simple, and reliable method was presented for the recognition of short deletion/insertions and single nucleotide substitutions. It is based on the PCR using 3` variable primer ends. As both mutants successfully genotyped by this approach are frequently used as animal models in the investigations of corresponding human diseases, successfully established genotyping method will further facilitate the use of these animals in future research. In addition, the applied approach can be in general extended to any similar application in experimental animals or humans, where detection of differences in one to several nucleotides is needed.

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REFERENCES

[1] Suh Y, Vijg J. SNP discovery in associating genetic variation with human disease phenotypes. Mutat Res 2005;573(1-2):41-53.

[2] den Dunnen JT, Antonarakis SE Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 2000;15(1):7-12.

[3] Sobrino B, Brion M, Carracedo A. SNPs in forensic genetics: a review on SNP typing methodologies. Forensic Sci Int. 2005;154(2-3):181-94.

[4] Ellis TP, Humphrey KE, Smith MJ, Cotton RGH. Chemical cleavage of mismatch: a new look at an established method. Hum Mutat 1998;11:345-353.

[5] Avraham KB. Positional-candidate cloning of genes from mouse mutants. Methods Mol Biol 2001;158:369-79.

[6] Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. 1989;17(7):2503-16.

[7] Ye S, Humphries S, Green F. Allele specific amplification by tetra-primer PCR. Nucleic Acids Res. 1992;20(5):1152.

[8] Goulding M, Sterrer S, Fleming J, Balling R, Nadeau J, Moore KJ, Brown SD, Steel KP, Gruss P. Analysis of the Pax-3 gene in the mouse mutant splotch. Genomics 1993;17(2): 355-63.

[9] Abdelkhalek HB, Beckers A, Schuster-Gossler K, Pavlova MN, Burkhardt H, Lickert H, Rossant J, Reinhardt R, Schalkwyk LC, Muller I, Herrmann BG, Ceolin M, Rivera-Pomar R, Gossler A. The mouse homeobox gene Not is required for caudal notochord development and affected by the truncate mutation. Genes Dev 2004;18:1725-36.

[10] Tachibana M, Kobayashi Y, Matsushima Y. Mouse models for four types of Waardenburg syndrome. Pigment Cell Res. 2003;16:448-54.

[11] Mitrečić D, Kostović-Knežević L, Gajović S. Morphological features of tail bud development in truncate mouse mutants. Cells Tissues Organs 2004;178:23-32.

[12] Hanley T, Merlie JP. Transgene detection in unpurified mouse tail DNA by polymerase chain reaction. Biotechniques 1991;10:56.

Table 1: Primers used in genotyping of splotch (*Pax3*^{sp}) and truncate (*Noto*^{tc}) mouse strains.

Primer pairs	**(5' → 3')**	
Pax3		
	Pax3wt3	Pax3wt5
D1Gaj1wt	gtgtgcgctcctcttttctcca	aattggacttagtattgttggaacg
	Pax3sp3	Pax3wt5
D1Gaj1sp	cgtgtgcgctcctctttcgtgtg	aattggacttagtattgttggaacg
Control primer pair (in *Stam2*)		
	Stam2wt3	Stam2wt5
D2Gaj1	gctttacagtggggatacat	ttatggcttttaggcaatct
Noto		
	Notowt3	Notowt5
D6Gaj12wt	gcaagagttggagaaggtgtt	ccacacacataaaaaggaggaagc
	Nototc3	Notowt5
D6Gaj12tc	gcaagagttggagaaggtgtg	ccacacacataaaaaggaggaagc
Control primer pair (in *Nol1*)		
	Nol1wt3	Nol1wt5
D6Gaj13	tctaccaggcccagtcagtc	actgtggggagacacaaagg

Figure 1: 3` variable ends approach in genotyping of splotch (*Pax3*^{sp}) and truncate (*Noto*^{tc}) strains.

3` variable primer ends method in genotyping of A) splotch (deletion/insertion of 6 pb) and B) truncate (SNS).

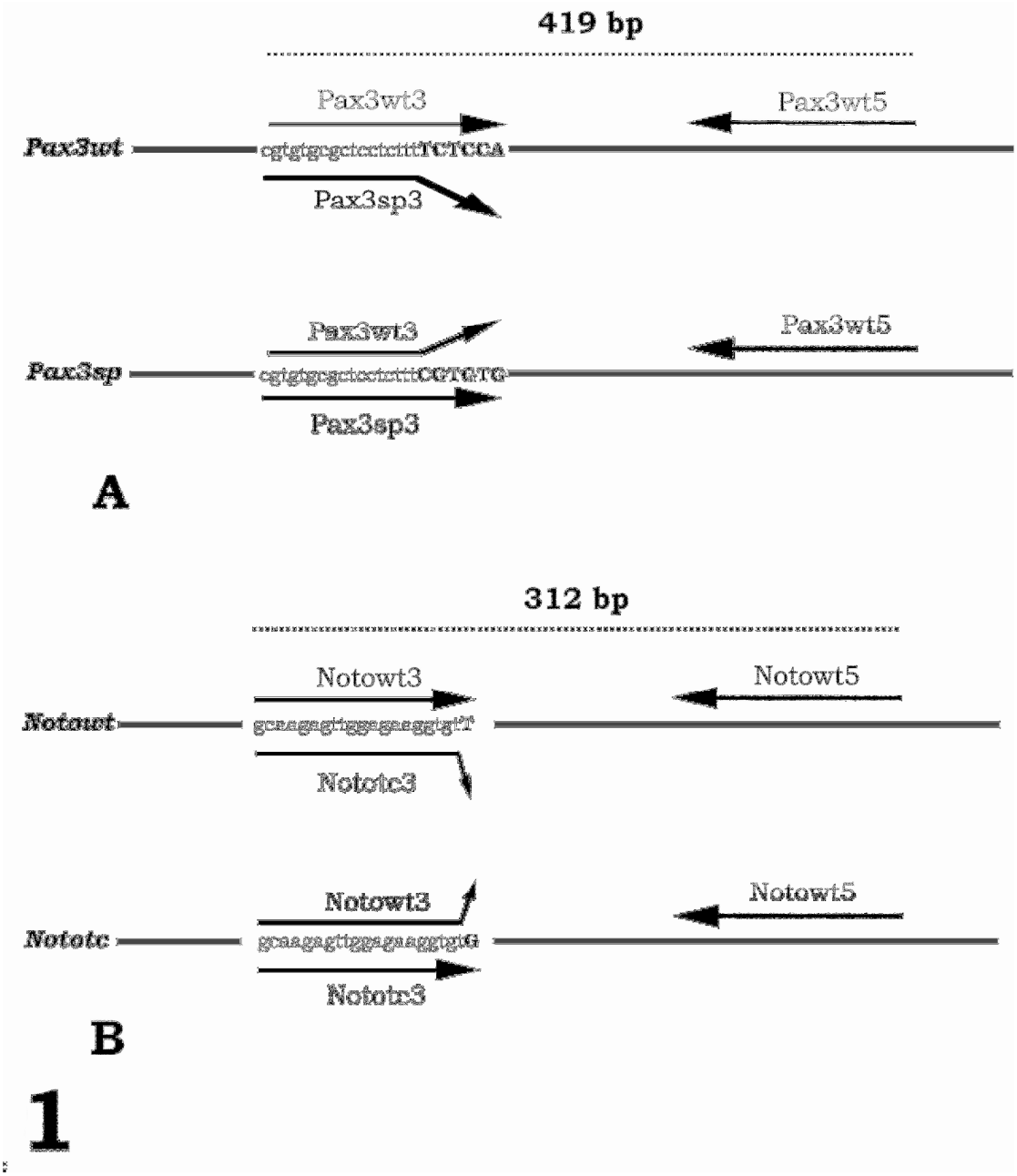
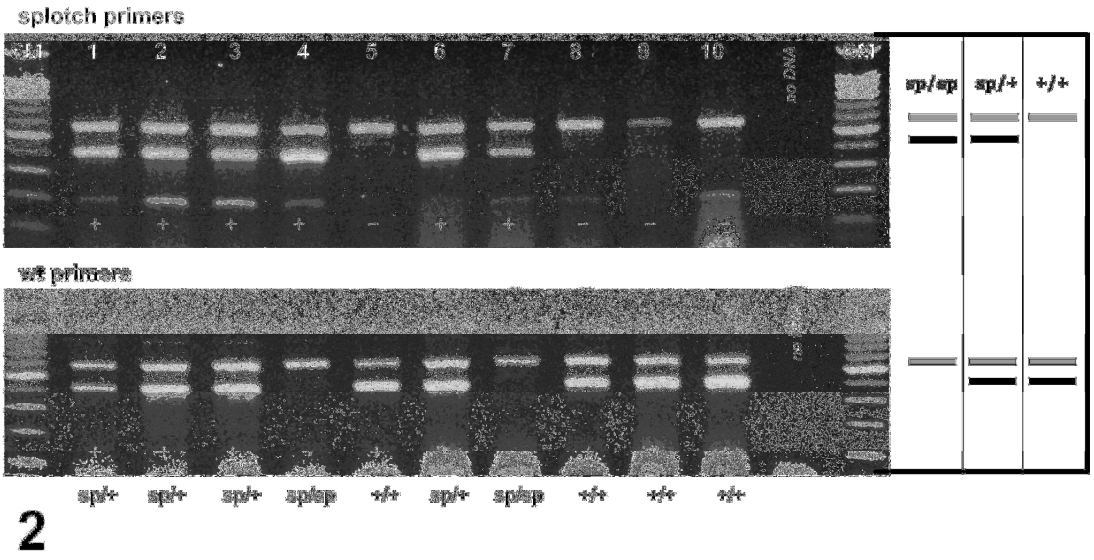


Figure 2: Gel electrophoresis of PCR products in genotyping of splotch (*Pax3*^{sp}) embryos.

As amplification with wild type *Pax3* and mutated (splotch) primers yields identical fragments (419 bp; black bands in the PCR diagram), two separated reactions were performed. Upper gel shows results of amplification with (*Pax3*^{sp}), and other gel, amplification of the same samples with wild type *Pax3* primers. To be sure that lack of amplified product is not a consequence of failed reaction, 600 bp control amplified product is present in all samples (*Stam2*, chromosome 2; grey bands in the PCR diagram). In the case of heterozygotes, both reactions yielded amplified product (lanes 1, 2, 3, and 6). Homozygotes were recognized in the lanes 4 and 7 because amplified fragment were present only with (*Pax3*^{sp}) primers. In the case of wild types embryos, only wild type primers yielded amplified products (lanes 5, 8, 9, and 10). SM – 100 bp DNA ladder as a size marker.



2

Figure 3: Gel electrophoresis of PCR products in genotyping of truncate (*Noto*^{tc}) embryos.

As amplification with wild type *Noto* and mutated (truncate) primers yields identical fragments (312 bp; black bands in the PCR diagram), two separated reactions were performed. Upper gel shows results of amplification with (*Noto*^{tc}), and other gel, amplification of the same samples with wild type *Noto* primers. To be sure that lack of amplified product is not consequence of failed reaction, 350 bp control amplified product is present in all samples (*Nol1*, chromosome 6; grey bands in the PCR diagram). In the case of heterozygotes, both reactions yielded amplified product (lanes 3, 6, 7, and 10). Homozygotes were recognized in the lanes 1, 5 and 8 because amplified fragment were present only with (*Noto*^{tc}) primers. In the case of wild types embryos, only wild type primers yielded amplified products (lanes 2, 4, and 9). SM – 100 bp DNA ladder as a size marker.