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Short communication

GENE TRAPPING: AN ANTIBODY-DEPENDENT APPROACH FOR VERIFYING INTEGRATION IN YOUR FAVORITE GENE

ANNA GORELIK, TAMAR SAPIR and ORLY REINER*
Department of Molecular Genetics, The Weizmann Institute of Science,
76100 Rehovot, Israel

Abstract: Gene trapping is used to introduce genome-wide insertional mutations in embryonic stem cells. Determining the integration site is based on high-throughput PCR, which has inevitable possibilities for mistakes, thus necessitating clone verification prior to the generation of mutant mice. Here, we propose a rapid method to validate gene identity based on the fact that many high throughput gene-trapping integrations result in fusion proteins encompassing the N-terminal portion of the gene of interest and LacZ being expressed in embryonic stem cells. Our method utilizes an immunoprecipitation assay using a specific N-terminal-directed antibody to the protein product of the gene of interest followed by a color LacZ assay of the immunoprecipitate, strongly supporting the formation of a fusion protein when the color develops.

Key words: Gene-trap, LacZ fusion protein, Integration site

INTRODUCTION

Deciphering gene function is one of the main challenges of modern biology. The completion of the human and mouse genomes has just allowed us to realize the

* Author for correspondence. e-mail: orly.reiner@weizmann.ac.il

Abbreviations used: β-geo – fusion of β-galactosidase and neomycin transferase; DCLK2 – doublecortin-like-kinase 2; DCX – doublcortin; DsRed – *Discosoma sp*. reef coral red fluorescent protein; ES – embryonic stem; HEK293 – human embryonic kidney cell line; IGTC – International Gene Trap Consortium; IP – immunoprecipitation; LacZ – beta galactosidase reporter gene; PCR – polymerase chain reaction; 5'-RACE – Rapid amplification of 5' complementary DNA ends; X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside

daunting undertaking still ahead of us. Genome-wide analyses necessitate high throughput approaches. Gene trapping is a high throughput approach that is used to introduce insertional mutations across the genome in mouse embryonic stem (ES) cells (IGTC, International Gene trap Consortium, http://www.genetrap.org/) [1, 2]. The most commonly used gene trap vectors are based on the splice-acceptor-β-geo vectors first developed in the Soriano laboratory [3, 4]. These vectors contain a splice acceptor immediately upstream of a promoterless reporter. In many cases, gene trapping results in a fusion protein containing the N-terminus of the trapped gene product. According to the data on the IGTC web site (www.genetrap.org) dated 29/05/08, 132,966 cell lines were included, corresponding to 8868 gene loci; thus, the average calculated ratio is 15 cell lines per gene. Therefore, the existence of multiple integrations per gene is common, and each gene is likely to be represented by several clones. On receiving a gene trap clone from the repository, it is advisable to verify the identity of the clone and the site of integration. Usually only a 5'-RACE sequence is known, with the inference that the vector was inserted somewhere in the adjoining intron. Furthermore, since the original sequences are derived using high throughput protocols, mistakes are possible, and ideally, it is better to verify gene identity using a different assay from the original one. The generally recommended method is RT-PCR, which is very sensitive to any inaccuracy in the information about the integration site, since it requires the design of primers encompassing the site of integration. We propose an alternative procedure to verify the integration by recognizing the fusion protein expressed in the targeted ES cell line. Antibodies directed against the N-terminal of the protein are used for immunoprecipitation followed by a LacZ color assay of the immunoprecipitate. A blue color developed with the immunoprecipiation of the appropriate protein fused to LacZ, but did not develop using preimmune serum or the control cell lines. We propose this as a rapid and simple verification of the integration in the gene of interest.

MATERIALS AND METHODS

Cell lines

The ES cell lines were maintained according to the SIGTR protocols (http://www.sanger.ac.uk/genetrap/). HEK293 cells were grown in DMEM medium (Gibco, Auckland, New Zealand) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and transfected using calcium phosphate precipitation [5].

Antibodies

β-galactosidase rabbit antibodies were obtained from Cappel, MP Biomedicals (Solon, Ohio), and anti-myc mouse monoclonal antibodies from Sigma (Rehovot, Israel). The DCLK2 antibodies were generated in our laboratory. A six-histidine fusion protein of a portion of DCLK2 amplified by PCR with

5'-cactcgagtcaagagtaggacgatttcag-3' and 5'-gagatttcggcaacgggatcatcccc-3' was cloned in pRSET (Invitrogen, Carlsbad, CA). The fusion protein was purified and injected into rabbits to produce DCLK2 antibodies. The antibodies were tested for immunoprecipitation using HEK293 cells transfected with a myc-tagged DCLK2 expression plasmid (Fig. 1). As noted, the DCLK2 antibodies immunoprecipitated myc-tagged DCLK2, whereas the preimmune serum did not. No cross-reactive bands were noticed in untransfected cells.

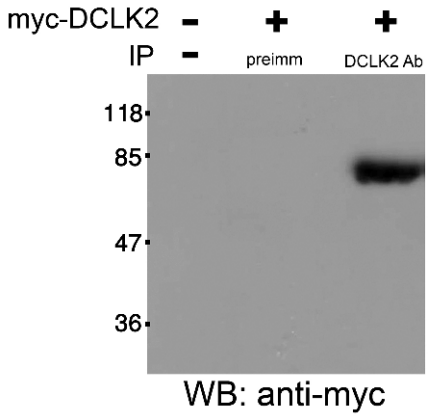

Fig. 1. DCLK2 antibodies immunoprecipitate myc-tagged DCLK2. HEK293 cells were transfected with myc-tagged DCLK2 and immunoprecipitated with preimmune serum or DCLK2 antibodies. The immunoblot was reacted with anti-myc antibody, which detected transfected DCLK2 only when the specific antibodies were used.

Immunoprecipitation

Protein lysates were prepared in IP buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% Triton X-100, 4 mM $MgCl_2$, 0.1 mM DTT) supplemented with protease inhibitors (Sigma, Rehovot, Israel). The lysates were incubated with polyclonal DCLK2 antibody or preimmune serum for 2 h at 4ºC. Following the incubation, 10 µl (bed volume) of protein A/G agarose (Santa Cruz, San Diego, CA), preblocked in IP buffer and supplemented with 10 mg/ml BSA (Sigma, Rehovot, Israel), was added to each of the samples for an additional 1 h. The immunoprecipitated proteins were pelleted by centrifugation and washed three times with IP buffer, and the remaining liquid was removed with a syringe.

X-gal staining of the immunoprecipitated proteins

The immunoprecipitated proteins were incubated in 50 µl of X-gal staining buffer (0.1 M phosphate buffer pH 7.3, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide) supplemented with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to a final concentration of 1 mg/ml.

Generating the Z/EG/DCX-DsRed ES cell line

A construct based on Z/EG [6] was created by removing the GFP cassette and inserting a DsRed-DCX/pA [7] following the loxP-PGKbgeo-tpA-loxP stop

sequence. This line constitutively expresses LacZ, whereas expression of the transgene (DCX-DsRed) is initiated following the removal of the stop sequence by Cre recombinase. The DNA was linearized and electroporated to R1 P10 ES cells (details at http://www.mshri.on.ca/nagy/r1.htm). The clones were screened for overall LacZ expression, and a single integration site was confirmed by Southern analysis using an internal DCX probe.

RESULTS AND DISCUSSION

Two embryonic stem (ES) cell lines (AC0704 and AZ0780) reported to contain β-geo (a fusion of β-galactosidase and neomycin transferase) insertions within the *Dclk2* gene sequence were purchased from the Sanger Institute Gene Trap Resource (SIGTR). Based on the deposited 5'-RACE sequence, a schematic presentation of the integration sites was drawn (Fig. 2). As a control line, we used the Z/EG DCX-DsRed ES line, generated in our laboratory. This line constitutively expresses β-geo (as detailed in the methods section). The ES cells were stained with X-gal, assaying for β-galactosidase activity. The three cell lines showed uniform and relative high levels of LacZ staining (Fig. 3).

Fig. 2. The schematic organization of *Dclk2*. The gene is composed of 16 exons (5' and 3' UTR are shown in black, and the translated exons are in grey). Based on the 5' race information, the position of the integration sites of the ES lines are shown by arrows. The antigen used to generate DCLK2 antibodies corresponds to exons 5 and 6 (underlined).

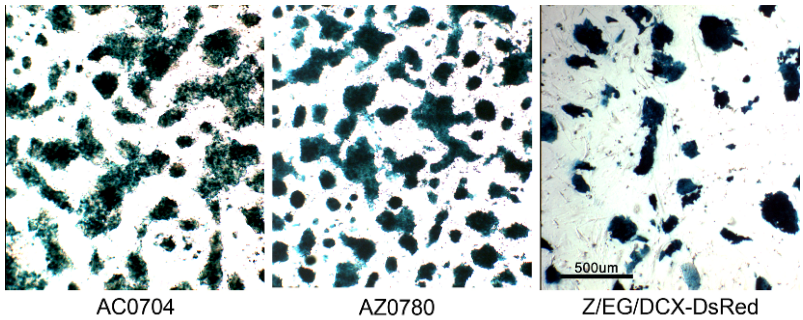

Fig. 3. LacZ staining of the ES cells used in this study. The three ES lines used in this study (AC0704, AZ0780 and Z/EG/DCX-DsRed) were grown on gelatin and stained as described in the methods section. The three lines showed positive and uniform staining. A scale bar is shown.

Immunoprecipitation was used to assay for the fusion of β-geo to DCLK2. Cell lysates were immunoprecipitated using either the DCLK2 antibody or the preimmune serum as a control. Immunoprecipitated proteins were stained with X-gal (Fig. 4). While the immunoprecipitate of AZ0780 turned blue after 2 h of incubation, no color change was noticed in the AC0704 or in Z/EG/DCX-DsRed, even following 24 h of incubation (Fig. 4, top row). Furthermore, no color developed when the preimmune serum was used (Fig. 4, bottom row).

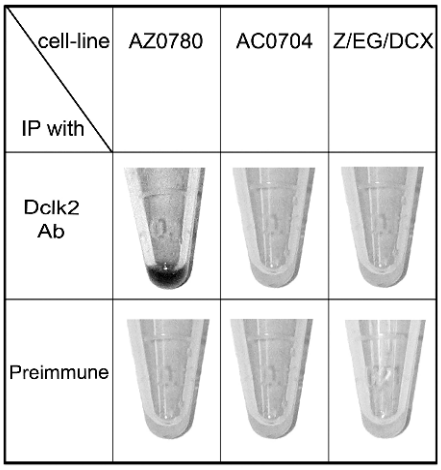

Fig. 4. The LacZ reaction of immunoprecipitated proteins. Cell lysates from AZ0780, AC0704 and Z/EG/DCX were immunoprecipitated with DCLK2 antibodies (top row) or preimmune serum (bottom row). Following washes, the precipitates were subjected to LacZ staining. Note the dark pellet corresponding to the blue color only in the case of AZ0780 immunoprecipitated with DCLK2 antibodies.

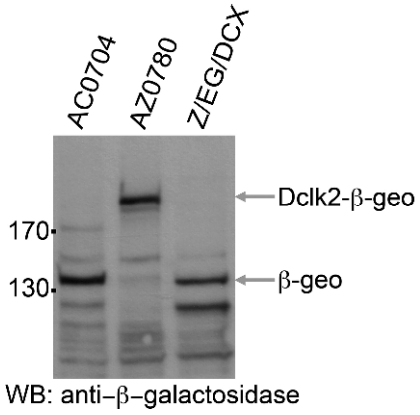

Fig. 5. Detection of β-geo using Western blot analysis. Cell lysates of AC0704, AZ0704 and Z/EG/DCX-DsRed were separated on SDS-PAGE and subjected to Western blot analysis using anti-β-galactosidase antibodies. Note the cross-reactive band in the size of β-geo in the AC0704 and Z/EG/DCX-DsRed lines, whereas in AZ0780, a higher molecular weight band is noted (marked as Dclk2-β-geo).

Our results indicate that only the AZ0780 cell line bore a DCLK2 β-geo fusion protein. The existence of a fusion protein was also suggested following Western blot analysis using anti-β-galatosidase antibodies. In the Z/EG/DCX-DsRed cell line, an immunoreactive band corresponding to the size of β-geo was noted, and a similar size was noted in the AC0704 cell line, although in AZ0780, a higher molecular-weight immunoreactive band was detected (Fig. 5). The absence of the desired fusion protein in AC0740 was demonstrated by inverse PCR, which indicated a β-geo insertion within the *Sarcc1* gene.

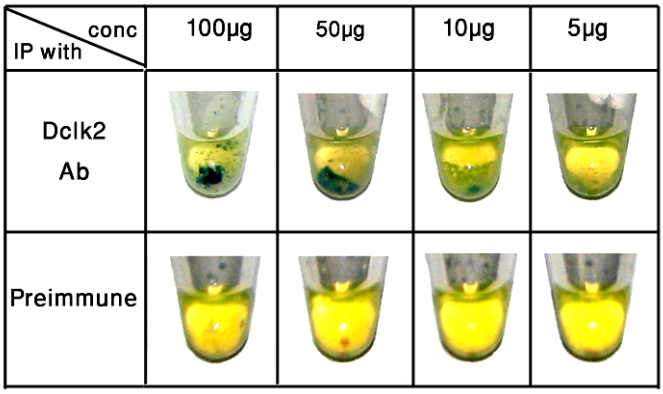

Fig. 6. The LacZ reaction of the immunoprecipitated proteins. The cell lysate from AZ0780 was diluted to different concentrations and immunoprecipitated with DCLK2 antibodies (top row) or preimmune serum (bottom row). Following washes, the precipitates were subject to LacZ staining. A concentration of 10 µg of cell lysate was sufficient to produce an observable LacZ staining.

In conclusion, we have shown that immunoprecipitation using an antibody directed to the N-terminal portion of a gene of interest followed by LacZ staining can assist in determining whether the gene trap insertion is located within the gene of interest. The technique is rather sensitive: gradual dilutions of the AZ0780 cell lysate showed that 10 µg of cell lysate were sufficient to obtain LacZ staining (Fig. 6). The two requirements of this technique are the expression of LacZ in the tested ES lines and the existence of an antibody directed to a portion of the protein residing upstream to the insertion. The technique is simple and provides results after a few hours. Although the exact integration site within the gene of interest is yet to be determined, our method allows a quick screen of multiple clones that potentially target a desirable locus. This could prove to save precious time and to markedly reduce costs.

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