Improved Technique for Detection of Enhanced Green Fluorescent Protein in Transgenic Mice

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ABSTRACT

One of the most exciting recent advances in cell biology is the possibility to use the green fluorescent protein and its various mutated forms as reporter proteins in studies carried out in vitro and in vivo. In the present study, several detection techniques for the enhanced green fluorescent protein (EGFP) were compared in transgenic mice, using fluorescence and confocal microscopy. In addition, different tissue preparation techniques (squash preparations, vibratome sections, frozen sections) were evaluated. As a model we used transgenic mice expressing EGFP under the control of a 5.0-kb fragment of the glutathione peroxidase isoenzyme 5 protein promoter (GPX5-EGFP) or under a 3.8-kb fragment of the cysteine rich protein-1 promoter (CRISP1-EGFP). In the GPX5-EGFP mice, expression of EGFP was observed in the distal part of the caput epididymis, while the CRISP1 promoter directed EGFP expression in the tubular compartment of the testis. Among the various tissue preparation procedures tested, the best morphological and histological preservation, and reproducibility in EGFP detection, were obtained using frozen sections after a slow tissue-freezing protocol developed in the present study. After slow tissue freezing, specimens of testis and epididymis could be stored at -70°C for at least six weeks without any affect on EGFP fluorescence. Hence, the method developed offers the possibility to analyze EGFP fluorescence in tissues several weeks after specimen collection. The sensitivity achieved was equal to that found in immunohistochemistry, applying biotin-streptavidin-FITC detection. Confocal microscopy is known to have the advantage that fluorescence can be detected from cells in different layers. This was found to be important as regards detecting EGFP fluorescence because the fluorescence was destroyed at the cut surfaces of sections produced by either vibratome or cryomicrotome.

INTRODUCTION

The green fluorescent protein (GFP) can be used as a detection tool for cell sorting, detection of gene expression, analyzing transfection efficiency, and measuring cell growth. In addition, GFP and its various mutated forms have become popular reporter proteins in transgenic animals, for evaluating promoter activity in vivo in different tissues and cell types (1–7). Site-directed mutagenesis of the primary sequence of GFP has offered researchers the possibility to choose among an expanded number of variants of GFPs with different spectral properties. We have recently developed transgenic mouse models, using the enhanced green fluorescent protein (EGFP) as a marker, for evaluation of tissue-specific expression of promoters of interest in vivo (3). EGFP was chosen because of its brighter fluorescence and higher signal-to-noise ratio compared with GFP. In the transgenic mice used in the present study, EGFP expression was driven by a 5.0-kb-long 5′-flanking region of the glutathione peroxidase 5 protein (GPX5) promoter, which is especially expressed in the caput epididymis (3), or by a 3.8-kb-long 5′-flanking region of the cysteine rich protein-1 (CRISP-1) promoter, which directs transgene expression in the testis. For detection of EGFP, we used conventional fluorescence and confocal microscopy and compared different specimen preparation techniques with the aim of evaluating and optimizing the system.

MATERIALS AND METHODS

Production of Transgenic Mice

Production of the transgenic mice used in the present study has been described elsewhere (3). In these mice, a 5.0-kb-long 5′-fragment of the mouse GPX5 promoter corresponding to nucleotides -5012 to +24 was used to drive EGFP reporter gene expression in the caput epididymis. Similarly, a 3.8-kb-long 5′-fragment of the mouse CRISP-1 promoter corresponding to nucleotides -3714 to +138 was used to drive EGFP expression in the testis. The University of Turku Ethics Committee on Use and Care of Animals approved all the procedures involving mice. The mice were specific pathogen-free and were fed with complete pelleted chow and tap water *ad libitum* in a room with controlled light (12 h light, 12 h darkness) and temperature (21°C ± 1°C).

Tissue Handling

Mice were sacrificed by CO_2 asphyxia, and the tissues were removed to a Petri dish containing PBS. Caput epididymides were dissected out, separated from the other parts of the epididymides, and fat was removed. Testes were dissected out, punctured with a needle, and the tissues were then fixed for 2 h in 4% paraformaldehyde (PFA) in PBS at room temperature.

Frozen Sections

Rapid freezing. Fixed tissues were washed once in PBS and embedded in Tissue-Tek® O.T.C. (Sakura Finetek, Torrance, CA, USA). The embedded tissues were then quickly frozen by immersing the specimens in isopentane at -70°C and kept at -70°C until sectioned.

Slow freezing. Fixed tissues were washed once in PBS and embedded in Tissue-Tek at room temperature. The embedded tissues were then kept in the dark at 4°C for 24 h and then slowly frozen at -70°C in a box covered with cotton wool. The tissues were stored at -70°C for one, two, and six weeks before sectioning.

Squash Preparations

The fixed tissues were washed once in PBS and placed under a stereomicroscope. Tubules were separated from each other with microsurgery scissors and forceps. Squash preparations were mounted in PBS or fluorescent mounting medium (FMM; Dako, Carpinteria, CA, USA) and kept in the dark at 4°C until analyzed.

Sectioning Techniques

Vibratome sections. Fixed tissues were washed once in PBS (pH 7.4) and dried quickly. Before sectioning, the tissues were embedded in 1% low melting point agarose in 0.9% NaCl (BMA,

Rockland, ME, USA) at 40°C and cooled to room temperature. Alternatively, the fixed tissues were supported in agarose and glued onto the vibratome stage. The stage was put into a chamber filled with PBS, 70-µm-thick sections were cut with a vibratome (model 752HA; Campden Instruments, Leicestershire, UK), and the sections were removed carefully from the bath with cover slips. The sections were mounted on microscope slides in PBS or FMM. When PBS was used as mounting medium, the cover slips were sealed with rubber cement.

Cryosections. Tissues were processed using either the rapid or slow specimen freezing protocol. Before sectioning, the tissues were kept for 30 min at -20°C. Then, 10- to 50-µm-thick sections were cut with a cryomicrotome (model CM 3050; Leica, Heidelberg, Germany) and collected on poly-L-lysine (1%)-treated microscope slides. During sectioning, the material was kept at room temperature to avoid several freezing and thawing cycles. Finally, the sections were rinsed in PBS and mounted in PBS or FMM as described above and kept in the dark at 4°C until analyzed.

Immunohistochemistry

Tissues were fixed for 2 h in 4% PFA, followed by 1–2 h in 4% PFA - 5% sucrose, and finally incubated overnight in PBS - 20% sucrose. Frozen sections (10 µm) were prepared using the rapid freezing technique and immunostained by means of rabbit anti-GFP antibodies (Living Colors™ Peptide Antibody; Clontech Laboratories, Palo Alto, CA, USA), using 1:500 to 1:5000 dilutions in PBS supplemented with 1% normal goat serum (Vector Laboratories, Burlingame, CA, USA). The antigen-antibody complexes were visualized by using biotinylated anti-rabbit antibody (Vector Laboratories) combined with streptavidin-FITC (Dako, Glostrup, Denmark) complex.

Microscopy

The fluorescent light emitted by EGFP was evaluated using fluorescein isothiocyanate (FITC) optics (Leica) and a research microscope (Model DM RBE; Leica), and by using a confocal laser microscope setup (model TCS SP scanner and DMRE microscope; Leica). In the research microscope, the sections were exposed to UV light through a 450–490-nm bandpass filter, via a 510 nm DM (dichroic mirror), and the emission was captured through a 520-nm longpass filter with a digital camera (Model DC 100; Leica). In the confocal laser microscope, the sections were exposed to a 488-nm excitation wavelength, and emission was obtained at 500–520 nm. Ten images with 2-µm intervals in the z-axis were collected with a confocal scanner (model TCS SP), equipped with an Argon-Krypton-ion laser system (Omnichrome, Chino, CA, USA) coupled with a LeicaSCANware™ 4.2a program (Leica).

RESULTS AND DISCUSSION

Tissues obtained from transgenic mice expressing the EGFP under the control of GPX5-EGFP and CRISP1-EGFP appeared to be valuable resources for evaluating different specimen preparation and EGFP detection techniques. In the GPX5-EGFP mice, EGFP expression was present in the distal region of the caput epididymis, while, in the CRISP1-EGFP mice, the protein was present in the tubular compartment of the testis. All the specimens prepared by different techniques were first analyzed using conventional fluorescence microscopy, and then confocal microscopy images were captured.

The disadvantage in specimens prepared as squashes was that EGFP expression could not be localized to a specific region of the caput epididymis (data not shown). Furthermore, the images captured from these specimens by conventional fluorescence microscopy were not of satisfactory quality because of high nonspecific background fluorescence. This high nonspecific background fluorescence was partially caused by the thickness of these specimens and the 520-nm longpass emission filter used. Another disadvantage of the squash specimens was difficulty in sealing the specimens properly. This led to quick drying and loss of EGFP fluorescence.

In vibratome sections and frozen

sections prepared by the slow freezing protocol, EGFP fluorescence could be detected and was seen to be strictly localized to the cells expressing the protein (Figure 1, A–E and H). In GPX5-EGFP mice, the fluorescence was found in segment IV of the distal caput epididymis (Figure 1, A–E), while, in the CRISP1-EGFP mice, the fluorescence was localized in the developing sperm (Figure 1H). However, when the tissues were processed using the rapid freezing protocol, all of the EGFP fluorescence was lost (Figure 1G). Therefore, it is apparent that the slow freezing protocol preserves the structure of the EGFP protein, thus emitting fluorescence also after freezing. We suggest that rapid freezing possibly changes the conformation of the protein, leading to a structure that does not emit fluorescence properly. The presence of EGFP in the sections after the rapid freezing protocol was confirmed by immunohistochemistry (Figure 1F). It is also possible that when keeping the specimens in Tissue-Tek at 4°C for 24 h, the compound soaks into the tissue and preserves the fluorescent properties of EGFP by an unknown mechanism. As the tissues were fixed with a cross-linking fixative, it is unlikely that membrane damage would lead to leakage of the protein and to less bright fluorescence in the sections prepared by the rapid freezing procedure. The data indicate that the sensitivity of EGFP detection is clearly dependent on the specimen preparation technique used, and when using frozen sections, the type of tissue freezing protocol is of key importance.

With the vibratome, 70-µm-thick sections could be prepared, but the sections were not always intact. As expected, 10–50-µm-thick sections could routinely be prepared with a cryomicrotome. The ease of preparing frozen sections is an additional advantage of analyzing EGFP fluorescence by applying the slow freezing protocol. Furthermore, with the slow freezing technique, EGFP fluorescence was detectable at the same intensity even after storing the specimens at -70°C for six weeks (Figure 1, A–C). The high sensitivity achieved with the slow freezing protocol developed was equal to that found using a routine immunohistochemical method (rapid freezing protocol) involving the biotin-streptavidin-FITC technique (Figure 1F). Furthermore, when using confocal microscopy, it was found that in both vibratome and cryomicrotome sections the amount of fluorescent cells increased rapidly when the fluorescence was analyzed at about 5 µm below the cutting surface. The

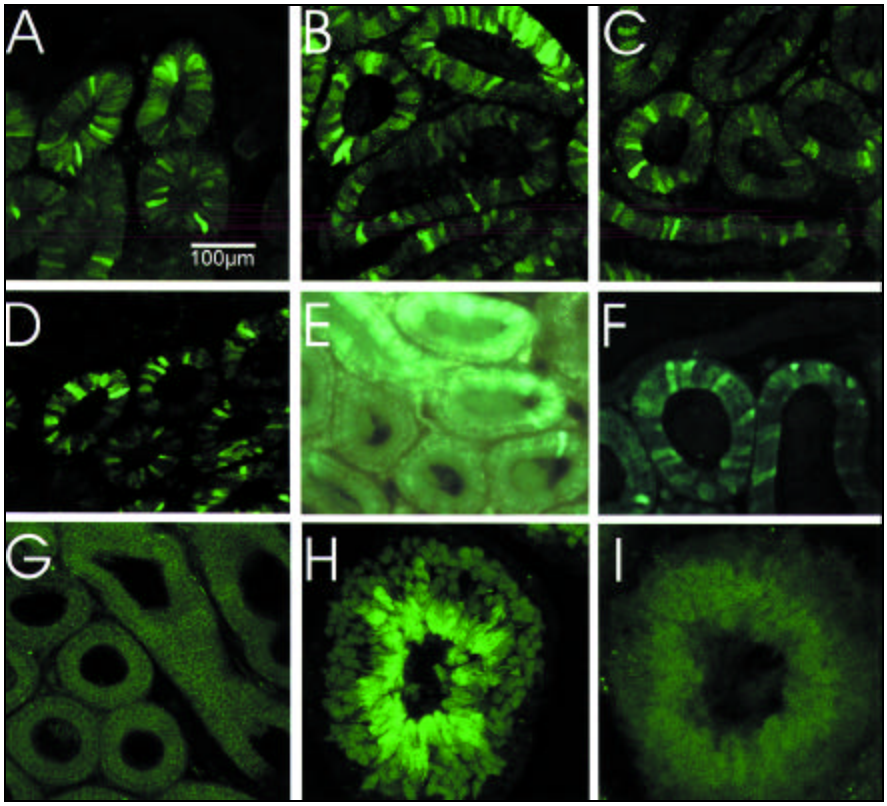

Figure 1. Localization of EGFP fluorescence in caput epididymis of transgenic mice expressing EGFP under control of a 5.0-kb-long 5′-flanking region of GPX5 promoter (A–G) and in the tubular compartment of the testis of CRISP1-EGFP mice (H and I) utilizing the 3.8-kb-long 5′-flanking region of CRISP-1 promoter. (A–C) Confocal images taken from sections prepared by the slow tissue freezing protocol. The epididymides were kept at -70°C for one, two, and six weeks before sectioning and EGFP detection, respectively. (D) Confocal image of a 70-µm-thick vibratome section. (E) Conventional fluorescence microscopy image taken from a 50-µm-thick section prepared by the slow tissue freezing protocol. (F) Immunohistochemical localization of GPX5-EGFP transgene in the epididymis, using the biotin-streptavidin-FITC technique. (G) Confocal image taken from an epididymis section prepared by the rapid tissue freezing protocol. (H) Confocal image representing EGFP fluorescence in developing sperm after the slow freezing protocol. (I) After rapid freezing, no specific EGFP fluorescence was detected in CRISP1-EGFP mouse testis. The magnification is the same in all images shown.

reason for this is not known at the moment. In line with this observation, we noticed that only a few fluorescent cells could be detected in 10-µm-thick frozen sections prepared by the slow freezing protocol (data not shown).

The fluorescence of EGFP obtained in PBS- and FMM-mounted specimens was equally strong. However, it was evident that the intensity of EGFP fluorescence was reduced one week after preparation, even when the specimens were stored in darkness at 4°C. This phenomenon is probably due to the degradation of paraformaldehyde fixation in the specimens. Among the various preparation procedures used, the best morphological and histological preservation and reproducibility were obtained using frozen sections after the slow tissue freezing protocol. In images taken by confocal microscopy, EGFP fluorescence was often detected in places where no fluorescent cells could be found by conventional fluorescence microscopy. This was due to the fact that the nonspecific background fluorescence in the 50–70-µm-thick specimens was high. This problem will be at least partially solved with specific narrow band emission filters. Another problem that would still persist is the fact that for EGFP detection 50–70-µm-thick specimens had to be used, as we found only few fluorescent cells in 10-µm-thick section. With conventional fluorescence microscopy, it is not possible to have all the fluorescent cells in the different layers (50–70-µm-thick specimens) in focus at the same time: therefore, one will not notice EGFP in cells that are not in focus and poses low fluorescence. By using confocal microscopy (model TCS SP), one can also abolish the nonspecific background fluorescence by means of appropriate settings of the emission wavelength. At the same time, one can collect images in z-axis from different layers of the specimen. Hence, with confocal imaging techniques, it is currently possible to detect EGFP fluorescence in cells that do not show fluorescence in conventional fluorescence microscopy. In conclusion, with the technology currently available, the highest sensitivity for detection of EGFP was obtained by applying confocal microscopy in frozen sections prepared according to the slow freezing protocol described in the present report.

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