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Application of the nonradioactive in situ hybridization for the localization of acetylcholinesterase mRNA in the central nervous system of the rat; comparison to the radioactive technique

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Abstract. In this preliminary report nonradioactive digoxigenine - based and radioactive *in situ* hybridization procedures for the localization of acetylcholinesterase mRNA were tested and compared in rat brain. General patterns of *Ache* mRNA localization observed by both techniques did not differ significantly and were practically the same as reported in previous *in situ* studies on the mammalian brain. Shorter procedure time and avoidance of precautions necessary at work with radioactive materials are major advantages of nonradioactive technique. Under- and over- staining can be prevented by direct examination of coloring reaction. Faint staining in the control experiment with heterologous DNA suggests that proper stringency is essential for the specificity of staining.

Key words: Acetylcholinesterase - Acetylcholinesterase mRNA - *In situ* hybridization - Rat brain - Digoxigenine

Introduction

In situ hybridization is the only technique enabling localization of nucleic acids in various tissues. Classical *in situ* procedures were based on the hybridization of target nucleic acid with radioactively labeled complementary polynucleotide detected subsequently by the autoradiography. However, offering several advantages, nonradioactively labeled probes started recently to replace radioactive ones.

In case of localization of mRNA encoding enzyme acetylcholinesterase (AChE) nonradioactive *in situ* hybridization technique has been successfully applied in studies on the *in vitro* innervated human skeletal muscle [2]. However, it has not been used yet for the localization of AChE transcripts (*Ache* mRNA) in the mammalian brain. In this study nonradioactive, digoxigenine labeled double stranded DNA probe has been applied for the localization of *Ache* mRNA in the rat brain. Results were compared to the *Ache* mRNA localization obtained by the radioactive method using (^{35}S) end-labeled oligonucleotides.

Materials and methods

Nonradioactive technique

Experimental conditions were basically the same as described before [2]. Digoxigenine labeled double stranded DNA corresponding to the 339 bp upstream from the Ser 200 active site region was used as a probe. Dry ice frozen brains were cut to 10μm coronal sections, mounted, fixed in 4% phosphate-buffered paraformaldehyde for 5 min, washed in phosphate buffered saline, dehydrated in 70% ethanol and stored in 95% ethanol at +4 °C until processed. Sections were prehybridized for 4 hours at 46°C in solution containing 5 x SSC, 5 x Denhardt's, 50% deionized formamide, 250 μg/ml yeast tRNA, 250 μg/ml freshly denatured herring sperm DNA and 4 mM EDTA. After prehybridization, sections were dehydrated in series of ethanol and air dried briefly. Hybridization was carried out for 15h at 46°C in the same solution as used at prehybridization except that herring sperm DNA was replaced by freshly denaturated AChE DNA probe (concentration was approximately 5 ng per 10 μl) labeled with digoxigenine according to the instructions supplied with DIG DNA Labeling and Detection Kit (Boehringer, Mannheim). Sections were rinsed two times for 15 min each at 46 °C with 2 x SSC buffer, one time for 15 min with 0..2 x SSC and 3 h in several changes of 0.1 x SSC. Hybridized probe was detected with the alkaline phosphatase-coupled anti-digoxigenine antibody, according to the DIG DNA Labeling and Detection Kit protocol. In control experiment probe was replaced by digoxigenine labeled BamHI, Bgl I and Hinf I digests of pBR328 plasmid (containing 16 fragments with lengths varying between 4907 and 154 bp). Two other controls included detection without the application of the anti-DIG antibodies and the probe and detection without the application of the probe.

Radioactive technique

For the preparation and fixation of tissue see above. Experimental conditions were basically the same as described before [5]. Synthetic antisense 3' -end-labeled (^{35}S) oligonucleotides (62 bp corresponding to bases 1090-1152 of the rat AChE gene) were used as a probe. The specific activities of the labeled probes ranged from 0.8 to 1.2 x 10^9 cpm/μg. The labeled probes were diluted at a concentration of 3x 10^3 cpm/μl and 100 μl of hybridization buffer with labeled probe (i.e. 3 x 10^5 cpm) were applied to each slide, covered with a strip of parafilm and incubated overnight (16h) at 42°C. Sections were then washed for 1 h at 55°C in 1 x SSC and thereafter for one hour at the room temperature. Sections were then dehydrated through series of alcohol , dried and exposed to x-ray film (Amersham Hyperfilm βmax). The autoradiograms were exposed for 28 days at room temperature.
No staining could be observed in control sections hybridized in the presence of 50 fold excess of nonlabeled probe.

Results

Distribution of *Ache* mRNA in rat brain observed after nonradioactive procedure (Fig. 1A) did not differ significantly from the distribution obtained after radioactive technique (Fig. 1B). Pattern of *Ache* mRNA localization was practically the same as reported in previous *in situ* studies on the mammalian brain [1,3,4]. Higher magnification of the striatum region revealed two types of *Ache* mRNA positive cells: larger, intensively stained but sparsely distributed cells and more numerous smaller cells with lower intensity of staining (Fig. 1C,D). The number of larger cells corresponded approximately to the number of cells detected in the same brain area by the radioactive technique, while smaller *Ache* mRNA positive cells could be observed only after nonradioactive procedure. No staining could be observed in the control experiments carried out without antibodies and/or without the probe. However, if heterologous DNA was applied instead of the probe some nonspecific staining was observed predominantly in the cerebellum area (Fig. 2).

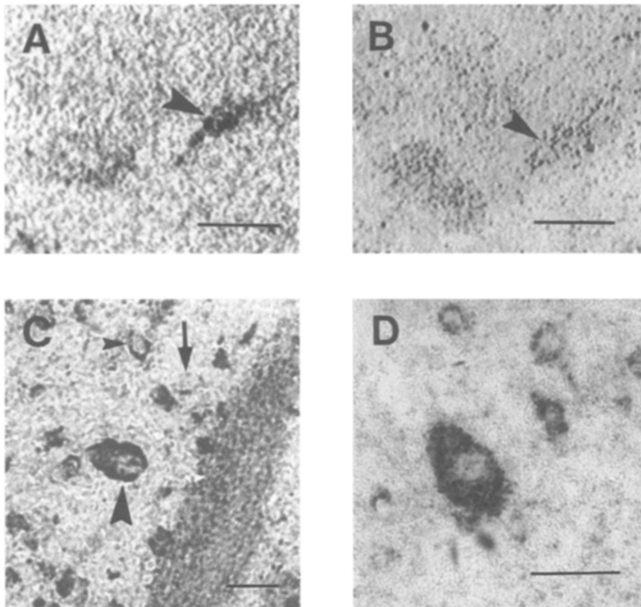

Fig. 1. Localization of *Ache* mRNA in the substantia nigra - pars compacta (arrowhead) in rat brain detected radioactively (A) and nonradioactively (B,C,D). Slightly different patterns of staining are due to the inherent differences between autoradiography and enzymatically catalyzed coloring reactions. Three types of cells could be detected in the striatum area: large, intensively stained (C- large arrowhead) cells, smaller faintly stained cells (C - small arrowhead) and cells, which did not stain above the control level (arrow). Another grupation of cells from the striatal area is shown at higher magnification in D. (Scale bars = 500 μm (A,B) ; 25 μm (C,D)).

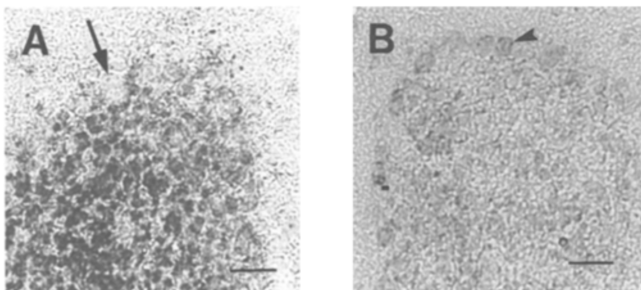

Fig. 2. Localization of *Ache* mRNA in the cerebellum : (A). Faint staining was observed in the control experiment, where heterologous plasmid DNA was applied instead of the probe (see Methods): (B). Note also differences in staining distribution in A and B: Purkinye cells were stained by heterologous DNA (B - arrowhead) but not by the probe (A - arrow). Cerebellum had the highest intensity of nonspecific staining among all brain areas. (Scale bars = 50 μm).

Discussion

According to these preliminary results nonradioactive, digoxigenine based technique is a good alternative to the classical radioactive approach used in other reports on *Ache* mRNA localization in mammalian brain [1,3,4]. Procedure time (two days) was much shorter than in case of radioactive technique (at lest ten days), mostly due to the relatively short detection phase. Avoiding all special precautions necessary at work with radioactive materials and better environment protection are also important advantages of the nonradioactive procedure. Nonradioactive technique also enabled direct examination of slices during the detection phase so that coloring reaction could be stopped at the appropriate moment preventing in this way under-and over- staining.
Nonspecific staining observed in the control experiment with heterologous DNA was most prominent in the cerebellum. This observation is in accord with previous reports that this tissue is notorious for nonspecific binding of DNA probes [3]. However, at present we have no explanations for the staining of white substance occasionally observed in our experiments. In our future work we will attempt to minimize the nonspecific staining observed in present experiments.

Acknowledgments. The excellent technical assistances of Zvonka Frelih, Marjan Kadunc and Helena Kupšek is gratefully acknowledged. This research was supported by the Ministry of Science of Slovenia.

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