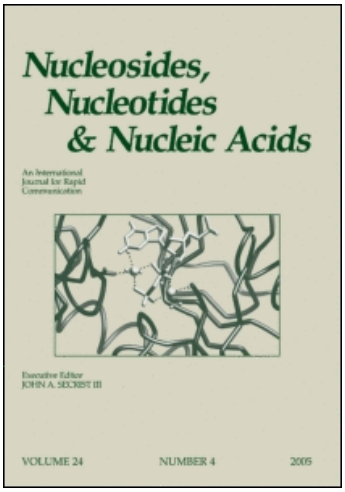

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5-BROMOURIDINYLATED OLIGONUCLEOTIDE FOR HYBRIDIZATION ANALYSIS OF DNA AND RNA ON MEMBRANES AND IN SITU.

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Abstract. Oligodeoxynucleotide probes complementary to oxytocin m-RNA and to human N-ras gene were labeled with 5-bromo-2′-deoxyuridine (5-BrdU) and used in in situ hybridization and dot blot analysis. The hybridizations were detected with monoclonal antibody to 5-BrdU.

INTRODUCTION

Nucleic acid hybridization with synthetic oligonucleotides has found indispensable application in genetic research, biomedical research and clinical diagnostics.

There are two basic concepts for reporter groups detection, either direct by means of attaching radioisotopes (1) or flurochromes (2) or indirect detection after the attachment of enzymes (3), after attaching biotin as the reporter group (4) and after chemical modification, eg. sulphonation of the probes (5).

We have developed a new method, based on the enzymatic incorporation of 5-bromo-2′-deoxyuridinylic moieties to the 3 -end of an oligodeoxynucleotide with terminal deoxynucleotidyltransferase. A monoclonal antibody directed against 5-bromodeoxyuridine is used for detection of the hybrids.

MATERIALS AND METHODS

Tailing with 5-BrdU

To 5pmol oligonucleotide, for reaction control previously labelled with $\gamma^{32}P$ at the 5'-end, in a reaction volume of 20 µl were added: 100mM potassium cacodylate (pH 7.0), 1mM $CoCl_2$, 1mM β-merca ptoethanol, 100µg/ml bovine serum albumine (nuclease free, Boehringer, FRG), 12 units of terminal deoxynucleotidyltransferase (Pharmacia, Sweden) and 400µM 5-bromo,2'deoxyuridinetriphosphate (Sigma, USA). The reaction was incubated at 37 °C for 45 min, being stopped by boiling for 2 min.

Hybridization

In situ hybridization was performed as previously described (6)

For dot blot hybridization , DNA was extracted from human leucocytes by the method described in (7). Amplification of target sequences was done essentially as described (8). RNA was extracted from Wistar rat hypothalmus by the single-step method (9).

The purified nucleic acid was spotted into a Biodyne membrane, 1.2µm (PALL, UK) at different concentration (see figure legends) and treated in accordance with the suppliers instruction.

Pre-hybridization was performed in a solution containing: 5x SSPE, 7% SDS, 120µg/ml t-RNA, 50% formamide. and 5x Denhardt solution.

Filters were pre-hybridized for two hours, and hybridized in the same solution after the addition of the labelled probe to a final concentration of 1 pmol/ml. Hybridization was carried out at 37°C for two hours.

After hybridization the filters were washed twice with 2x SSPE + 0.1% SDS at room temperature for 15 min, and the stringent washing was done in 5x SSPE + 0.1% SDS at 60°C for 5 min.

Immunodetection

In situ hybridization

Histological sections were prepa red as previously described (6).

After hybridization the sections were incubated with monoclonal antibodies raised in mouse against 5-BrdU (Progen, FRG) diluted 1:1000 in PBS (0.1M phosphate buffer pH 7.2, 0.9% NaCl) for 2h in a moisture chamber at room temperature. Thereafter the sections were incubated for 30 min at room temperature with rabbit anti-mouse antibodies. diluted 1:100 in PBS, followed by a 30 min incubation with rabbit peroxidase- antiperoxidase complex, diluted 1:50 in PBS. Immuno- precipitates were visualized with 3,3′-diaminobenzidine and H_2O_2.

Membrane hybridization

The hybridized membranes were incubated for 2h in a protein saturation solution (5% casein hydrolysate in PBS). The membranes were then incubated for 1h with the same monoclonal antibody diluted in PBS (30µg/ml). After incubation with biotinylated anti mouse antibody diluted 1:100 in PBS for 30 min at room temperature precipitates were visualised by streptavidin gold complex (Jansen, FRG) and silver enhancement (Jansen, FRG).

RESULTS

In this work two oligonucleotides were utilized (TAB.1): N59wt, a 20-base fragment complementary to human N-ras gene; and Ox1, a 20-mer inversely complementary to oxytocin m-RNA (10). Both fragments were radiolabelled at their 5′-end with γ-^{32}P ATP and subsequently tailed with 5-BrdU as described in methods, with aliquots being taken every 15 min.

From the addition pattern presented in figure 1, it can be concluded that 5-BrdU is added very efficiently and the difference in the addition mode between the two primers confirms the observation that the reaction is primer

TABLE 1. The structure of the oligonucleotides used.

Oligonucleotide	Sequence (5'-3')	Length	Td (°C)
0x1	GAACTGCCCCCTGGGCGGCA	20	70
N59wt	TGGATACAGCTGGACAAGAA	20	58

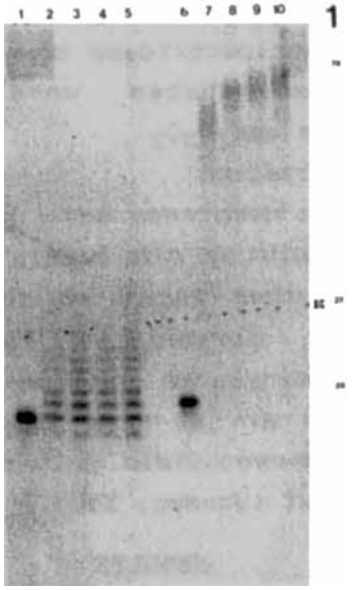

Figure 1. 20% PAGE-7M urea gel showing the addition pattern obtained during the tailing of 0x1 (20:mer) and N59wt (20:mer) with 5-BrdU by the enzyme terminal deoxynucleotidyltransferase. Lanes 1 to 5 are aliquots of the reaction with 0x1 withdrawn successively at times 0, 15, 30, 45, and 60 min. Lanes 6 to 10 are N59wt at times 0, 15, 30, 45, and 60 min.

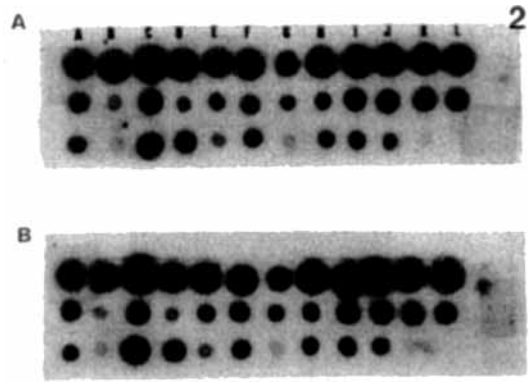

Figure 2. The hybridization profile of human DNA hybridized against oligonucleotide N59wt labeled solely at its 5'-end with γ-^{32}p ATP (Panel A) and the same oligonucleotide labeled at the 5'-end with γ-^{32}p ATP and tailed with 5-BrdU (Panel B) are indistinguishable. Lanes A-L: different clones amplified with the same amplimers. First row is hybridization control.

dependent (11). Thus while fragment N59wt gives a monomodal reaction, with the product corresponding to 70 additions of bromodeoxyuridylate, the pattern from the reaction with Oxi is more complex, giving bands corresponding to less than ten additions and a band corresponding to 70 additions. This mode of reacting is consistent with the literature, and was attributed to the fact that with increased length the primer is more accessible for the enzyme (12).

To determine whether the presence of a 5-BrdU tail on an oligonucleotide probe alters the stability of the duplex formed upon hybridization, the oligonucleotide N59wt (1pmol) tailed with 5-BrdU as above, was compared in a dot blot hybridization procedure with the same oligonucleotide labelled only at its 5'-end with γ-^{32}P ATP.

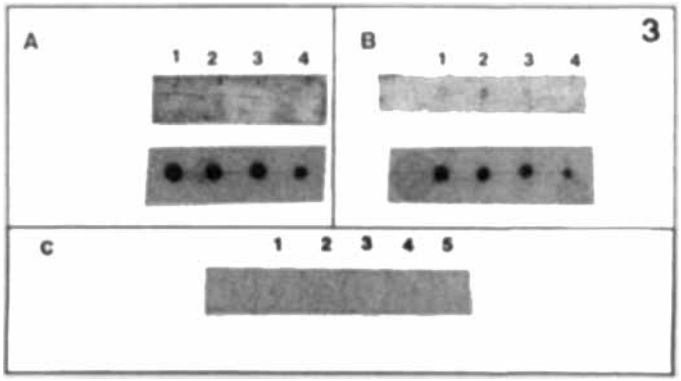

Figure 3. Panel A: Dot blot analysis of total human DNA amplified for N-ras (spots 1 to 3 contain 1µ, spot 4 contains 0,5µg) and hybridized to N59wt (1pmol/ml) labeled with γ^{-32}p at the 5'-end and tailed with 5-BrdU at the 3,-end. The stripe was exposed to a X-ray film over night (beneath) and immunostained with anti-BrdU antibodies (above) as described in the text. Panel B is the same experiment as above with the difference that the DNA spotted was total cellular DNA unamplified (spotted at 2µg, 1µg, 0.5µg, and 0.1µg). Panel C is total cellular RNA extracted from rat hypothalamus and spotted successively at 2µg, 1µg, 0.5µg, 0.1µg, and 0.05µg, and hybidized to Ox1 tailed with 5-BrdU and stained with anti-BrdU antibody.

Both oligonucleotides were hybridized against cellular DNA amplified for human N-ras oncogene. The results are shown in figures 2A and 2B. There was no detectable difference in the hybridization profile of both probes.

To assess if monoclonal antibodies raised in mouse against 5-BrdU were able to bind to the BrdU-tailed oligonucleotide after hybridization, both oligonucleotides N59wt and Ox1 were tailed with 5-BrdU and hybridized to different amounts of target sequence in dot-blot experiments. It can be seen from figure 3, that although it was possible to detect low amounts of target sequence, the procedure was not optimal with respect to the membrane used, since it binds antibodies unspecifically.

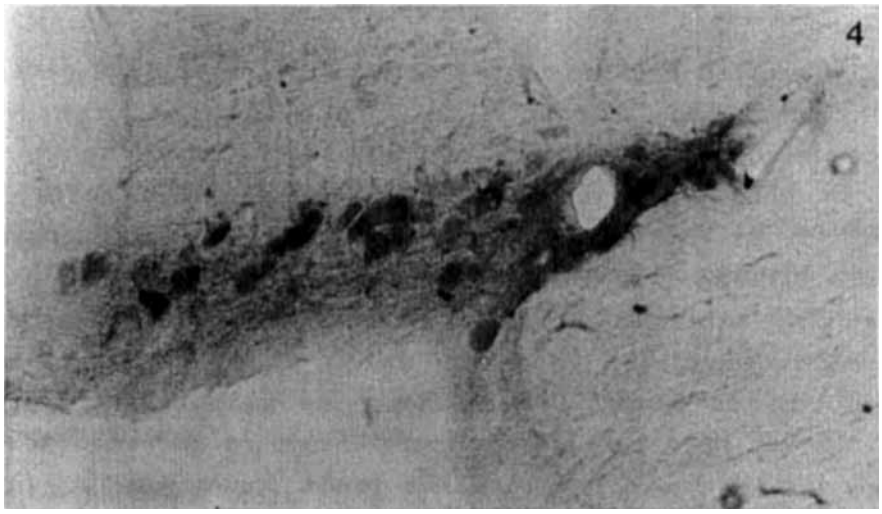

Figure 4. Immunodetection of in situ hybridization with a 5-BrdU tailed oligonucleotide probe complementary to oxytocin m-RNA. Note the single cells resolution of the method!

It is know that commercial menbranes differ widely in their affinity for proteins and the optimization of the detection makes necessary a comparative study between the different membranes commercially avaible.

For in situ hybridization, 5-BrdU tailed Ox1 was hybridized as describrd in methods section. The results obtained after incubation with the antibodies and enhancing with a secondary antibody peroxidase-antiperoxidase conjugated are shown in figure 4.

Sections pre-treated with RNA:se (20µg/ml) for 20 min. were devoid of signal, confirming the specificity of the method.

It is clear that resolution is greatly increased. making possible the visualisation of subcellular structure not revealed by other labelling techniques.

CONCLUSION

Enzymatic tailing with 5-bromouridinylate is a powerful tool for non radioactive labelling of synthetic oligonucleotide probes for dot blot experiments as well as for in situ hybridization to cellular nucleic acids, the stability of the hybrids formed in vitro, is not changed by 5-BrdU tailing. For in situ hybridization the best level of detection reported sofar was achieved with ^{3}H-labelled genomic probes, and is in the range of $10^{-18}g$ of specific nucleotide sequence (13). Although an exact quantification has not yet been carried out, comparison of our results with those published for ^{3}H-labelled probe shows similar levels of detection with higher resolution.

The method here described is routinely used in our laboratory for in situ hybridization experiments. It can obviously be a good alternative to methods employing biotin, specially in tissues with high levels of endogenous biotin.

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