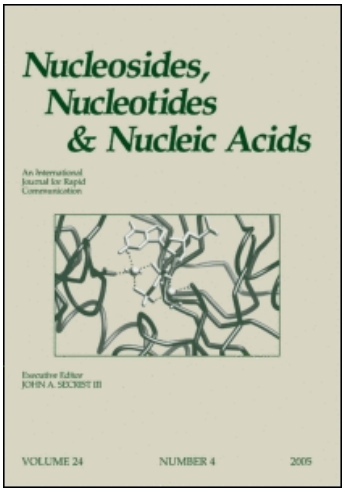

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Smith, C. L. , Simmonds, A. C. , Hamilton, A. L. , Martin, D. L. , Lashford, A. G. , Loakes, D. , Hill, F. and Brown, D. M.(1998) 'Use of 5-Nitroindole-2'-deoxyribose-5'-triphosphate for Labelling and Detection of Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 17: 1, 555 — 564
To link to this Article: DOI: 10.1080/07328319808005198
URL: http://dx.doi.org/10.1080/07328319808005198

NUCLEOSIDES & NUCLEOTIDES, 17(1-3), 555-564 (1998)

USE OF 5-NITROINDOLE-2'-DEOXYRIBOSE-5'-TRIPHOSPHATE FOR LABELLING AND DETECTION OF OLIGONUCLEOTIDES.†

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ABSTRACT: The 5'-triphosphate of 5-nitroindole-2'-deoxyriboside has been shown to be a good substrate for terminal deoxynucleotidyl transferase (TdT). An antibody has been prepared for the detection of 5-nitroindole and has been used for the detection of 5-nitroindole tailed DNA both in single-stranded form and after hybridisation to a template. This is therefore a new method for the detection of nucleic acid probes.

INTRODUCTION

Terminal deoxynucleotidyl transferase (TdT) is a mammalian enzyme which behaves like a DNA polymerase in synthesising DNA in the 5' to 3' direction using deoxynucleoside triphosphates, but without the need for a template. It has some structural similarity to β-polymerase which appears to have evolved independently of the non-mammalian DNA polymerases such as polymerase I from *E. coli*.[1]

TdT has been widely used for producing a 3'-tail on oligonucleotides and other single-stranded forms of DNA for labelling purposes. Tails of 150 nucleotides can typically be produced with the four natural nucleotides, including ^{32}P or ^{33}P labelled nucleotides, depending on the ratio of oligonucleotide to

† This paper is dedicated with great regard to the late Professor Tsujiaki Hata
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dNTP. The amount of divalent cations, such as magnesium or cobalt, is also important and will determine the relative incorporation of purines and pyrimidines.[2] When the nucleotide substrate is conjugated to reporter groups, such as biotin, fluorescein or digoxigenin, the tail size is, however, considerably reduced.[3,4]

It has been shown that the 5'-triphosphate nucleoside analogues of 5-nitroindole, 5-aminoindole and 3-nitropyrrole can be incorporated into DNA, albeit to a limited extent, by the use of Klenow fragment of DNA polymerase I or *Taq* polymerase.[5] We were therefore interested in answering two questions: Could these analogues behave as substrates for TdT and, if they could, would an antibody directed against an analogue recognise a tailed oligonucleotide hybridised to its target?

RESULTS AND DISCUSSION

Terminal deoxynucleotidyl transferase (TdT) tailing reactions.

The abilities of 5-nitroindole-, 5-aminoindole- and 3-nitropyrrole- 2'-deoxynucleoside 5'-triphosphates to act as substrates for terminal deoxynucleotidyl transferase (TdT) were compared with the natural dNTPs. The assay used a ^{33}P 5'-end labelled primer which was size-fractionated after tailing by polyacrylamide gel electrophoresis. Control incubation of the primer with enzyme alone resulted in the degradation of the primer and only a very small amount of the +1 product resultant from the incorporation of [γ-^{33}P] ATP from the 5'-end labelling reaction. As expected, incubation with any of the natural dNTPs gave 3'-tails in the region of 50-150 nucleotides. The longest tails were produced with dATP or TTP, followed by dCTP and then dGTP.

Surprisingly, we found that all three of the base analogue deoxynucleoside triphosphates are substrates for TdT (FIG. 1). For 5-nitroindole there was a smear of products indicating tails of 7-150 nucleotides in size. A lot of radioactivity failed to enter the gel probably because of the aggregation of the 3'-end labelled oligomers due to stacking interactions of the incorporated 5-nitroindole.[6] The 3'-tails produced by 5-aminoindole deoxynucleoside triphosphate are 7-35 nucleotides . Interestingly, there was significantly less material in the loading well when compared to 5-nitroindole. 3-Nitropyrrole-2'-deoxynucleoside-5'-triphosphate gave 3'-tails of a similar size to those produced by dCTP. Again a significant amount of material remained in the loading well which is presumed to be high molecular weight aggregates.

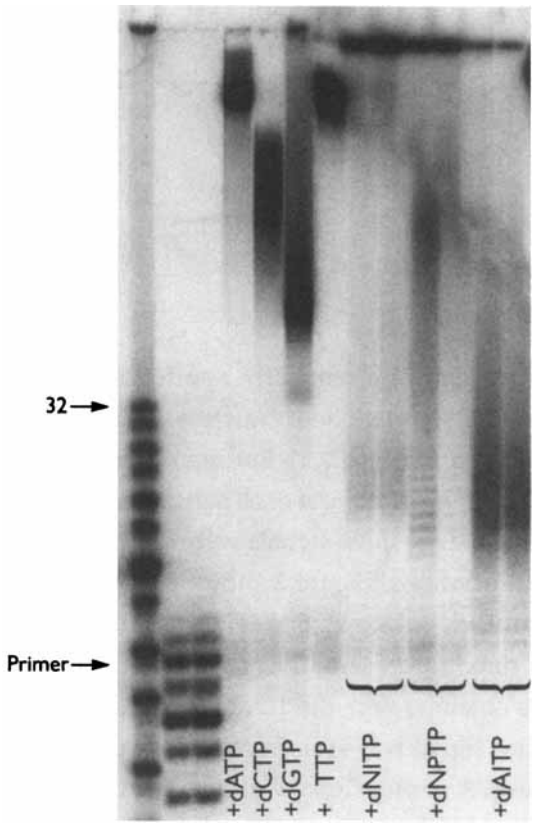
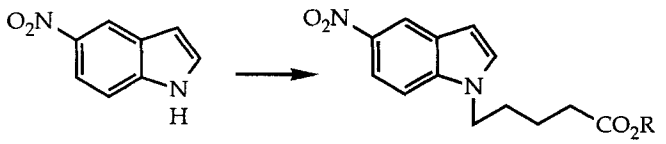


FIG. 1. Tailing reactions with terminal deoxynucleotidyl transferase using natural dNTPs and the triphosphate analogues of 5-nitroindole (dNITP), 3-nitropyrrole (dNPTP) and 5-aminoindole (dAITP). The first lane show oligonucleotide size markers.

Antibody detection.

Following the study of the tailing reactions we decided to see if 5-nitroindole-tailed oligonucleotides could be detected by means of an antibody directed against the nitroindole base (**1**). This could potentially form the basis of an oligonucleotide detection assay. 5-Nitroindole was coupled to methyl-5-bromovalerate *via* its sodium salt analogously to the glycosylation method of Revankar,[7] to give **2** (R=Me), which was then hydrolysed to the free acid (**2**, R=H) by refluxing in aqueous sodium hydroxide solution. **2** was then conjugated *via* its N-hydroxysuccinimide ester to keyhole limpet hemocyanin (KLH). The conjugate was then administered to three sheep. Serum samples were obtained at pre-immunisation, 6 and 10 weeks.



1 2

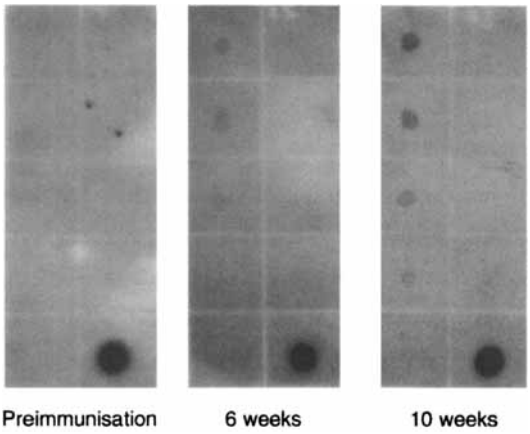
SCHEME 1

To test the antisera, a chemically synthesised 11-mer oligonucleotide incorporating three 5-nitroindole deoxynucleoside residues (n) at the 5'-end (5'-nnnTTCAGCGG-3') was fixed to a nylon membrane. After incubation with a 1:1000, 1:10 000 or 1:50 000 dilution of each serum sample, the presence of bound antibody was determined. Positive signals were seen from antisera obtained from all three sheep at all dilutions. Figure 2 shows an example of the response from one sheep with antisera taken at 6 weeks and 10 weeks after the primary immunisation. A maximum sensitivity of 0.1pmole 5-nitroindole-labelled oligonucleotide was obtained with the 1:1000 antiserum dilution. Preimmune sera were negative and no signal was seen from a control oligonucleotide lacking the 5-nitroindole residues. A strong signal was also seen on the blots from an aliquot of preimmune serum dotted out as a positive control.

Antibody detection of M13 following hybridisation with a 3'-end labelled oligonucleotide probe.

The -40 forward M13 sequencing primer was 3'-end labelled using TdT and 5-NITP. Dot blots were prepared on a nylon membrane using a range of M13 mp18 from 1 picomole down to 5 femtamoles. Unlabelled sequencing primer and herring sperm DNA dots were also added to the blots as controls as well as a 1:1000 dilution of the sheep pre-immune serum.

The labelled probe was hybridised to the dot blots, and after stringency washes the blots were treated in the same manner as the 5-nitroindole tailed oligomer dot blots. The sheep antiserum was used as a 1:10 000 dilution and the horseradish-peroxidase conjugated donkey anti-sheep IgG was used as a 1:25 000, 1:50 000 or 1:100 000 dilution. A strong signal was obtained from the whole range of M13 dots; the 5 femtamole dot was easily visible after a 2 minute film exposure (Figure 3). This is 20-fold greater sensitivity than that obtained with the synthetic 5-nitroindole containing oligomer dot blots, and indicates that the 3'-end 5-nitroindole tails must be significantly longer than 3 bases. The longer tails allow



10	0.05
5	0.01
1	
0.5	A
0.1	B

FIG. 2. Dot blots of an 11-mer oligonucleotide tailed with 5-nitroindole. Immunodetection was performed with serum obtained preimmunisation, after 6 and 10 weeks. Concentration of oligonucleotide is shown in the grid, and ranged from 10 - 0.01 pmoles. A: 15 pmoles of a non-tailed control 9-mer, B: 1:1000 dilution preimmune sera.

1	0.005
0.5	A
0.1	
0.05	B
0.01	C

FIG. 3. Dot blots of 5-nitroindole tailed M13 sequencing primer (tailed using TdT) hybridised to M13 mp18 DNA. Concentration range of bound M13 DNA as shown in the grid is 1 - 0.005 pmoles. A: Unmodified -40 M13 sequencing primer, B: 1µl of 0.5µg/µl herring sperm DNA, C: 1:1000 pre-immune serum.

increased antibody binding and therefore increased sensitivity of detection. The system has not been optimised and further improvements in sensitivity may therefore be achieved. The long 5-nitroindole tails do not affect the specificity of the probe as is clearly shown by the negative results obtained with the herring sperm DNA and the M13 -40 forward primer.

CONCLUSION.

The incorporation of the triphosphates of three indole nucleosides into 3' tails by the use of terminal deoxynucleotidyl transferase contrasts with the limited incorporation of these analogues by DNA polymerases as demonstrated in a previous study.[5] There it was shown that incorporation with Klenow or *Taq* polymerase is limited to one base with limited or no further extension. The reason for this is most likely the lack of any significant hydrogen bonding capability on the part of the indoles. This would endorse the currently held view that some base hydrogen-bonding is required for efficient chain extension catalysed by DNA polymerases. Although 5-aminoindole does have a primary amino group which theoretically could form a hydrogen bond, once in a duplex it is presumably incorrectly aligned to allow any significant bonding to its complementary partner in the duplex. The lack of hydrogen-bonding on the part of the indole nucleosides obviously does not limit their addition by TdT since TdT is not template-directed.

The production of specific antibodies to the 5-nitroindole base has led to the ability to recognise the 5-nitroindole moiety within a DNA oligomer both in the single-stranded form and after hybridisation to a template. With further optimisation to improve sensitivity, this method could therefore form the basis of an oligonucleotide detection or capture system.

MATERIALS AND METHODS

General methods

^{1}H-n.m.r. spectra were obtained on Bruker AM-360 in $CDCl_3$. Ultraviolet spectra were recorded on a Perkin Elmer Lambda 2 spectrometer in water unless otherwise stated. TLC was carried out on pre-coated F_{254} silica plates and column chromatography with Merck Kieselgel 60. The phosphoramidite monomer of 5-nitroindole was purchased from Glen research. Oligonucleotides were synthesised on a PE Applied Biosystems ABI 380B Synthesizer with the standard synthesis cycle. Purification was carried out by polyacrylamide gel electrophoresis using a 20% polyacrylamide gel, elution using 0.5M ammonium acetate, 1mM EDTA buffer followed by concentration and desalting using a

Sephadex G25 column (NAP-10, Pharmacia). The preparation of the analogue nucleoside triphosphates is described in the previous paper.[5]

Synthesis

1-(4-Methoxycarbonylbutyl)-5-nitroindole (2, R=Me). To a stirred solution of 5-nitroindole (0.32g, 2 mmol) in acetonitrile (10 ml) was added sodium hydride (57mg, 2.4mmol). After 45 minutes methyl-5-bromovalerate (0.43g, 2.2 mmol) in acetonitrile (1ml) was added and the solution stirred for a further 21 h. The reaction was poured into 1 M hydrochloric acid (20 ml) and extracted with ethyl acetate (2x20ml) and then washed with $NaHCO_3$ solution, dried and evaporated to a yellow oil. Yield 0.5g, 91%. Yellow crystals were formed from chloroform/hexane, mp 51-52°C. ^{1}H-n.m.r. ∂ (ppm) ($CDCl_3$) 1.65 (2H, m, CH_2), 1.90 (2H, m, CH_2), 2.34 (2H, t, J=7 Hz, CH_2), 3.66 (3H, s, OCH_3), 4.19 (2H, t, J=7 Hz, CH_2), 6.68 (1H, dd, J_1=3.3 Hz, J_2=0.6 Hz, H3), 7.25 (1H, d, J=3.3 Hz, H2), 7.35 (1H, d, J=9.1 Hz, H7), 8.12 (1H, dd, J_1=2.2 Hz, J_2=9.1 Hz, H6), 8.59 (1H, d, J=2.2 Hz, H4). IR (KBr) υ (cm^{-1}) 1718, 1517, 1336. m/z (CI) 294 $(M+NH_4)^+$, 277 $(MH)^+$.

1-(4-Carboxybutyl-5-nitroindole (2, R=H). Compound **2** (R=Me) (0.5g, 1.8mmol) was suspended in 1M sodium hydroxide solution (10 ml) and the solution heated at reflux for 90 minutes. The solution was cooled, diluted with water (10ml) and extracted with ethyl acetate (2x15ml). The aqueous layer was the acidified (1M HCl) and the resulting solution extracted with ethyl acetate (3x15ml), dried and evaporated to a yellow gum. The product was crystallised from chloroform/methanol; to give yellow needles. Yield 0.42g, 81%. M. pt. 141-2°C. ^{1}H-n.m.r. ∂ (ppm) ($CDCl_3$) 1.65 (2H, m, CH_2), 1.90 (2H, m, CH_2), 2.38 (2H, t, J=7 Hz, CH_2), 4.18 (2H, t, J=7 Hz, CH_2), 6.67 (1H, d, J=3.3 Hz, H3), 7.23 (1H, d, J=3.3 Hz, H2), 7.33 (1H, d, J=9.1 Hz, H7), 8.10 (1H, dd, J_1=9.1 Hz, J_2=2.2 Hz, H6), 8.57 (1H, d, J=2.2 Hz, H4). IR (KBr) υ (cm^{-1}) 1710, 1506, 1329. m/z (CI) 280 $(M+NH_4)^+$.

Preparation of 1-(4-carboxybutyl)-5-nitroindole KLH conjugate. To a solution of 1-(4-carboxybutyl)-5-nitroindole (19.5mg, 75μmol) in 0.5ml DMF was added N-hydroxysuccinimide (11.5mg, 0.1mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (29mg, 0.15mmol) and the solution stirred at room temperature under nitrogen for 90 minutes. TLC (toluene:methanol:acetic acid, 90:16:8) confirmed the formation of the active ester. Keyhole limpet hemocyanin (KLH) (45mg, 0.61ml) was dissolved in water (2.25ml) and pyridine (0.25ml). The active ester was added dropwise to the KLH solution over 5 minutes, and then stirring continued for 4 hours during which time a flocculent

yellow solid separated. The solution was dialysed against water for 2 days and then freeze-dried to give a yellow solid. Yield 60mg.

Antiserum Production. Three sheep were immunised with the 5-nitroindole-KLH conjugate (Polyclonal Antibodies Limited, Blaenwaun Farm, Ffostrasol, Llandysul, Ceredigion, SA44 5JT Wales). The primary immunisation was carried out with the conjugate formulated in Freund's complete adjuvant. Subsequent re-immunisations were carried out with the conjugate formulated in Freund's incomplete adjuvant at 4 weekly intervals. Blood samples were taken 2 weeks after each re-immunisation and serum prepared.

Testing of Antisera. Antisera were tested against dot blots of 5-nitroindole-tailed oligonucleotides on nylon membranes using a second antibody conjugated to horseradish peroxidase with ECL (Amersham International plc) substrate for detection. The response of pre-immune serum taken from each animal was compared with serum taken from the first and second bleeds.

Aliquots (1μl) containing 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 pmole of a chemically synthesised 5-nitroindole containing oligomer (sequence 5'-nnn TTC AGC GG, where n is 5-nitroindole) were dotted onto Hybond N+ membrane (Amersham International plc) as well as 1μl of a control oligonucleotide (5'-TGC TGG AGA). The DNA was then fixed by heating at 80°C for 90 minutes. After fixing, a 1μl aliquot of a 1:1000 dilution of pre-immune serum was dotted onto each blot and then incubated for 1 hour at room temperature with shaking in Liquid Block (Amersham International plc) diluted 1:10 with 10mM phosphate buffered saline (PBS). Individual blots were then incubated for 1 hour with shaking in 1:1000, 1:10 000 or 1:50 000 dilutions of each serum sample in 0.5% BSA solution in PBS. They were then washed three times for 10 minutes each in PBS containing 0.3% Tween 20, incubated for 1 hour in horseradish-peroxidase conjugated affinity-purified donkey anti-sheep IgG, H and L (Jackson ImmunoResearch Labs Inc) diluted 1:25 000 in 0.5% BSA in PBS, and again washed three times in PBS containing 0.3% Tween 20. They were then incubated in ECL detection reagent (Amersham International plc) for 1-2 minutes and then exposed to Biomax film for 2-5 minutes.

Positive signals were seen from antisera obtained after immunisation of all three sheep at all dilutions, but pre-immune sera gave a negative response. A maximum sensitivity of 0.1 pmole 5-nitroindole tailed oligonucleotide was obtained with the 1:1000 antiserum dilution. There was no signal from the control oligonucleotide.

Terminal Deoxynucleotidyl Transferase (TdT) Tailing. A 15-mer primer (5'-TGCATGTGCTGGAGA) was 5'-end labelled with [γ^{33}P] ATP and T4 polynucleotide kinase, and then heated at 100°C for 5 minutes to inactivate the enzyme. 4pmoles of the labelled primer, 25 units TdT and 32μM dNTP or analogue triphosphate were incubated in 25μl 100mM cacodylate buffer (pH 7.2), 2mM $CoCl_2$ and 0.2mM 2-mercaptoethanol at 37°C for 90 minutes. The reactions were terminated by addition of formamide/EDTA stop solution, and the products electrophoretically separated on a 19% polyacrylamide 7M urea gel. Gels were fixed, dried and exposed to Biomax autoradiography film.

Detection of M13 Dot Blots by Hybridisation with a 3'-End Labelled Probe. The -40 forward M13 sequencing primer (5'-GTTTTCCCAGTCACGACGTTGTA, 24μl, 48pmoles) was mixed with 40μl 5xTdT buffer, 120μl 400μM 5-nitroindole-2'-deoxynucleoside-5'-triphosphate and 200 units TdT, and the reaction incubated at 37°C for 90 minutes.

Aliquots containing 1, 0.5, 0.1, 0.05, 0.01 and 0.005 pmoles of M13 mp18 were dotted onto Hybond N+ membrane (Amersham International plc) with controls consisting of 1μl herring sperm DNA (0.5μg/μl) and 2μl (4pmoles) -40 forward M13 sequencing primer. The DNA was then fixed by heating at 80°C for 90 minutes. After fixing, a 1μl aliquot of a 1:1000 dilution of pre-immune serum was dotted onto each blot and allowed to dry at room temperature.

The dots were incubated for 30 minutes in hybridisation buffer (5% w/v dextran sulphate, 0.5% casein, 0.1% SDS and 5xSSC) at 42°C with shaking in a water bath. The 3'-end labelled probe was heated at 100°C for 5 minutes and then added to the hybridisation buffer solution. The blots were left at 42°C in the water bath for 60 minutes. They were then washed twice in 5xSSC, 0.1% SDS for 5 minutes at room temperature. This was followed by two further 15 minute washes with 1xSSC, 0.1% SDS (pre-heated) at 42°C. All subsequent steps were carried out at room temperature.

The blots were rinsed in 10mM PBS before transferring to a 1:10 dilution of Liquid Block (Amersham International plc) in PBS and incubated for 1 hour with shaking. Blots were then incubated for a further hour in 1:10000 dilution sheep anti-5-nitroindole serum in 0.5% w/v BSA in PBS. They were washed three times for 10 minutes each in PBS containing 0.3% Tween 20. Individual blots were then incubated for 1 hour with shaking in 1:25 000, 1:50 000 or 1:100 000 dilutions of horseradish-peroxidase conjugated donkey anti-sheep IgG (Jackson ImmunoResearch) in 0.5% w/v BSA in PBS. The blots were washed three times for 10 minutes each in PBS containing 0.3% Tween 20. Final incubation in ECL

detection reagent (Amersham International plc) for 1 minute before exposure to Hyperfilm MP (Amersham International plc) for 2-15 minutes.

ACKNOWLEDGEMENTS

We thank Richard Grenfell and Jan Fogg for oligonucleotide synthesis. We also thank Amersham International plc (to D.L.) and the Isaac Newton Trust (to F.H.) for financial support.

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