Synthesis of 8-(2-4 dinitrophenyl 2-6 aminohexyl) amino-adenosine 5' triphosphate: biological properties and potential uses

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ABSTRACT
We have synthesized 8-(2-4 dinitrophenyl 2-6 aminohexyl) amino-adenosine 5' triphosphate (in short: rATP-DNP), a derivative of ATP which carries a dinitrophenyl group. We show that rATP-DNP is a substrate for calf thymus deoxyribonucleotidyl terminal transferase (EC 2.7.7.31) and E. coli DNA polymerase I (Kornberg polymerase EC 2.7.7.7.). It can therefore be incorporated into DNA molecules by elongation from 3' ends or by nick translation. The incorporated dinitrophenyl group can be recognized by specific antibodies which can then be detected by anti-antibodies coupled to an enzyme. DNP groups could also be introduced into DNA after enzymatic incorporation of 8-aminohexyl adenosine 5' triphosphate and reaction with 1-fluoro-2-4-dinitrobenzene. Thus, DNA molecules carrying DNP groups can ultimately be revealed by enzymatic coloured reactions. Potential uses of this enzymatic labelling as a substitute to the radioactive detection of nucleic acids, are discussed.

INTRODUCTION
Nucleotide analogs are of widespread use in biology, particularly as mutagens and inhibitors of enzymatic reactions. They have often been the source of important methodological advances. A recent example is the use of deoxynucleotides in DNA sequencing (1-3).

We and others (4-6) have become interested in introducing chemical modifications into nucleic acids which could be specifically recognized by enzyme-linked molecules. In this way, nucleic acids could be detected by coloured enzymatic reactions rather than by radioactive labels. Manning et al. (7) described a way of linking biotin to RNA; they showed that biotinylated ribosomal RNA, hybridized to DNA, could be visualized under the electron microscope by use of avidin (an egg white protein with very high affinity for biotin) coupled to polymethacrylate spheres. Following a similar procedure, we found that DNA-RNA hybrids formed in liquid could be detected by avidin coupled to β-galactosidase (Cam, B., Guesdon, J.L., Avrameas, S., Kourilsky, P., 1978, unpublished results). We, however, failed to develop a convenient filter hybridization method and followed other approaches (described below). In the
meanwhile, Lauger et al. (8) have synthetized a biotin-substituted deoxyuridine 5' triphosphate. This nucleotide analog is incorporated into DNA by DNA-polymerase I and can be detected by avidin or antibiotin antibodies coupled to peroxidase (9).

In seeking alternatives to radioactive labelling, we looked for enzyme labelling substitutes to both internal and end labelling of nucleic acids. Internal labelling is particularly important for the preparation of probes for hybridization, whereas end labelling is useful in a variety of analytical procedures, especially DNA sequencing. An internal labelling procedure will be described elsewhere (Tchen et al., in preparation). We report here on a procedure devised for the 3' end labelling of DNA. It is known that calf thymus deoxynucleotidyld terminal transferase (EC 2.7.7.31) is capable of adding a few ribonucleotides (1 to 4) at the 3' end of DNA molecules (10). After alcali treatment, which breaks the ribose-ribose, but not the ribose-deoxyribose bonds, only one nucleotide remains attached. This yields a homogeneous population of DNA molecules with one added residue. If the latter was detectable by an enzyme-antibody system, analysis of 3' terminal enzyme labelled DNA molecules would become conceivable.

Following this rationale, we have synthetized a new derivative of ATP, namely 8-(2-4 dinitrophenyl-6-aminohexyl) amino-adenosine 5' triphosphate (in short: rATP-DNP). We describe the synthesis of this compound and show that it is suitable for 3' end labelling by calf thymus deoxynucleotidyld terminal transferase. In addition, we found that it can also be used by E. coli DNA polymerase I in the nick translation reaction (11). We also show that the commercial precursor of rATP-DNP, 8-aminohexyl adenosine 5' triphosphate, can be incorporated into DNA by these two enzymes, and that the DNP group can be subsequently added. Both procedures yield DNA labelled with DNP groups, which can be detected by enzyme-linked antibodies. Potential uses are discussed.

MATERIAL AND METHODS

Materials: Unlabelled deoxynucleotides, rATP and E. coli DNA polymerase I were purchased from Boehringer Mannheim and 8-aminohexyl adenosine 5' triphosphate from Sigma. Radiolabelled nucleotides were from Amersham. Deoxynucleotidyld terminal transferase was from P.L. Biochemicals; rabbit anti-dinitrophenyl antibodies from Miles Laboratories; goat IgG-antirabbit coupled to peroxidase from Institut Pasteur Production; 1-fluor-2-4-dinitrobenzene was purchased from Sigma and H_2O_2 110 vol from Merck; plasmid pKCl is a derivative
of pBR322 carrying the 2 kb BamHI fragment of the human β globin gene inserted in its BamHI site (12); phage lambda DNA was purchased from Biolabs.

Synthesis of 8-(2-4-dinitrophenyl-6-aminohexyl)-amino-adenosine 5'-triphosphate: 8-aminohexyl adenosine 5'-triphosphate (in short: 8-aminohexyl ATP) (10 mg, 16 μmoles) in 500 μl of 100 mM MgCl₂, 200 mM NaHCO₃, pH 8.8, was treated with 40 μl of 10% (w/v) solution of 1-fluoro-2-4-dinitrobenzene (25 μmoles). The solution was heated at 40°C in the dark for 90 minutes, then extracted four times with equal volumes of ethylether. The reaction mixture was applied to a 10 ml column of DE 52 DEAE cellulose (Whatman). After washing with 50 ml of 0.05 M LiCl, pH 5, the products were eluted by using a 1-liter linear gradient of 0.05 M LiCl, pH 5, to 0.35 M LiCl, pH 2. A major peak absorbing at 278 nm and 360 nm contained the desired product. After desalting through a G10 Sephadex (Pharmacia), the absorbing fractions were lyophilized and analysed by thin layer chromatography on cellulose (isobutyric acid - NH₄OH EDTA saturated 4-1 v/v) and the U.V. spectrum of the obtained compound was determined in a Beckman model 25 spectrophotometer.

Enzymatic incorporation of rATP and of its derivatives by deoxynucleotidyl terminal transferase: A 35 bp HindIII-DdeI fragment from the globin sequence in pCK1, carrying a single 5' terminal ³²P label at the HindIII end, was prepared as for conventional DNA sequencing. This fragment (8 pmoles) was incubated with 3 units of deoxynucleotidyl terminal transferase and 5 μM of rATP, rATP-DNP or 8-aminohexyl ATP in a final volume of 20 μl in 100 mM potassium cacodylate buffer, pH 7.5, 10 mM dithiotreitol, 1 mM CoCl₂ and 50 μg/ml bovine serum albumin. Incubations was at 37°C for the indicated times.

Enzymatic incorporation of rATP-DNP inside plasmid pKCl by E. coli DNA polymerase I: Reaction mixtures (50 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 50 μg/ml bovine serum albumin, 8 μM dTTP, dGTP and dCTP, 3.3 mM α-²³²P rATP (400 Ci/m mole) (Amersham France), 5 mM MnCl₂, 0.5 μg plasmid pKCl. For substitution of rATP by 3.3 μM rATP-DNP, we used 3.3 μM α-²³²P dCTP (400 Ci/m mole). The reactions were run with 12 units of DNA polymerase I at 14°C. Aliquots (5 μl) were removed at the indicated times, acid precipitated with trichloracetic acid and filtered through nitrocellulose paper. The samples were counted in a liquid scintillation spectrometer (Intertechnique SL 32).

Chemical dinitrophenylation of 8-aminohexyl ATP incorporated into DNA: A PstI-EcoRI 750 bp DNA fragment from pKCl was elongated with 8-aminohexyl ATP and deoxynucleotidyl terminal transferase as above. Phage lambda DNA was nick-translated with 8-aminohexyl ATP as previously described. These DNAs (3 μg of
the PstI-EcoRI fragment of pKCl, or 10 μg of λDNA) were pretreated with 2 μl
of 1-fluoro-2-4-dinitrobenzene (10 % in ethanol) in 200 μl of 200 mM sodium bi-
carbonate buffer pH 8.8, at 37°C for 2 hours and then at 4°C overnight. Excess
reagent was removed by repeated extraction (4 times) with 500 μl of diethyle-
ther and DNA was purified by chromatography through a G75 Sephadex column.
Part of the DNP labelled 750 bp fragment was digested with Ddel to yield a
420 bp DNA fragment free of DNP labelled 3' OH extremities which was purified
from a 5 % acrylamide gel.

Visual recognition of DNA-DNP: 1 μl aliquotes of DNP-labelled DNA dissolved
in 20 mM Tris, 1 mM EDTA, pH 8, containing from 15 ng to 10 pg of DNP-labelled
DNA per μl, were spotted on nitrocellulose filters. Filters were baked at 80°C
for 1 hour, then saturated in proteins by soaking in a solution of albumin
(3 % bovine serum albumin, 1 SSC) for 1 hour at room temperature. Filters were
incubated overnight at 4°C with a rabbit antiserum to DNP (Miles Laboratories,
code No. 61-006-1) diluted 1/100 with 3 % albumin, 2 SSC, 1 % NP 40. Filters
were rinsed in 2 SSC, 1 % NP 40 for a total of 8 hours with 3 changes and in-
cubated with a horseradish peroxydase conjugated goat antiserum to rabbit IgG
(Miles Laboratories, code No. 61-202-3) diluted 1/100 with 3 % albumin, 2 SSC,
1 % NP 40. Filters were then rinsed as previously. Peroxydase activity was
detected by the purple insoluble precipitate formed in a 30 minutes incubation
with 2 mg of 3-amino-9-ethyl-carbazol dissolved in 0.5 ml of N-N' dimethyl
formamide, 9.5 ml of 0.05 M sodium acetate buffer, pH 5, and 10 μl of 30 %
H2O2.

RESULTS
1) Synthesis of 8-(2-4-dinitrophenyl-6-aminohexyl)-amino-adenosine 5' triphos-
phate.

Synthesis of rATP-DNP (see formula in figure 1) was carried out in one step
from two commercially available reagents as outlined in Materials and Methods.
In brief, 8-aminohexyl ATP was reacted with 1-fluoro-2-4-dinitrobenzene. It
was necessary to add MgCl2 to the reaction mixture to prevent the addition of
dinitrophenyl groups to the phosphates. After purification of the product, the
overall yield of the reaction was 48 %. In thin layer chromatography on cellu-
lose plates, the purified compound was homogeneous showing only one spot, the
migration of which indicate an Rf of 0.85 (as compared to an Rf of 0.70 for
8-aminohexyl-ATP). The U.V. spectrum is compatible with the addition of a sin-
gle DNP group since the ε 280 nm/ε 360 nm observed is 4.2 with regard to an
expected theoretical value of 4.0 (figure 2).

2) Substrate activity of rATP-DNP with DNA polymerases

rATP-DNP was tested for its ability to function as a substrate for two DNA polymerases: calf thymus deoxynucleotidyl terminal transferase and E. coli DNA polymerase I.

(i) It is known that deoxynucleotidyl terminal transferase is able to incorporate a small number of ribonucleotides at the 3' OH end of DNA molecules (10). We analysed the addition of rATP or rATP-DNP to a purified DNA segment of 35 bp labelled with $^32$P at one of its 5' ends and the products were run on a 8 % acrylamide sequencing gel (Figure 3). Incubation with enzyme and rATP or rATP-DNP results in the progressive addition of one or a few residues as seen from the upper bands observed after 2 and 4 hours (lanes c and d). Quantitation was difficult because deoxynucleotidyl terminal transferase contained a contaminating exonuclease activity. The latter was responsible for the appearance of the lower bands of 34 and 33 nucleotides seen after all times of incubation with (lanes a, b, c, d) or without (data not shown) nucleotide substrates. Mi-

Figure 1: Dinitrophenylation of 8-aminohexyl adenosine 5' triphosphate.

Figure 2: U.V. spectrum of 8-(2-4-dinitrophenyl-6-aminohexyl) amino-adenosine 5' triphosphate in sodium phosphate buffer 100 mM, pH 8.
The migration of the rATP and rATP-DNP elongated molecules are slightly different. This may be due to hydrophobic interactions between the DNP groups and the macromolecular matrix of the gel. Further evidence for the incorporation of rATP-DNP is the enzymatic detection reported below.

The kinetics of the elongation reaction is slow, but similar for rATP and rATP-DNP. In our conditions, it took about 24 hours to elongate more than 95% of the DNA molecules, as judged from the addition of $^{32}$P labelled rATP in a control reaction. Using the same conditions, we could prepare DNA quantitatively elongated by rATP-DNP.

(ii) As previously reported (13), ribonucleotide triphosphates may serve as substrates for DNA polymerase I provided that MgCl₂ is replaced by MnCl₂. We initially used 5 mM MnCl₂ in the reaction, as described (10). Only 0.1% of the adenine bases were substituted by rAMP (in contrast, 20% of the cytosine bases were substituted by dCMP as a control with 5 mM MgCl₂ in the nick-translation protocol of Rigby et al. (11) (Figure 4A). In order to increase this low yield we determined the optimal salt conditions. We found that a 100 fold more rAMP (10%) was substituted in the presence of 0.5 mM MnCl₂ (Figure 4B). Similar yields were obtained with rATP-DNP instead of rATP. Since radiolabelled DNP-ATP was difficult to prepare, we measured incorporation of the unlabelled analog by following incorporation of $\alpha^{-32}$P dCMP into DNA. DNP labelled DNA was thus prepared from 0.5 µg of pKCl, 3.3 µM $\alpha^{-32}$P dCTP (400 Ci/m mole), 8 µM rATP-DNP, 0.5 mM MnCl₂ and other constituents as described in Material and Methods.
purified on Sephadex G50, precipitated with alcohol and analysed in an agarose gel. Degradation was observed, as usual in nick translation reactions, but there was less degradation in 0.5 mM MnCl₂ and 1 mM MgCl₂.

3) Incorporation of 8-aminohexyl adenosine 5' triphosphate into DNA followed by in situ addition of the dinitrophenyl group.

Since rATP-DNP was synthesized from 8-aminohexyl ATP reacted with 1-fluoro-2-4-dinitrobenzene, we looked for incorporation of 8-aminohexyl ATP into DNA. Using the same approaches as above, we found that ATP, rATP-DNP and 8-aminohexyl ATP are incorporated equally well by deoxynucleotidyl terminal transferase and DNA polymerase I (data not shown). Reaction with 1-fluoro-2-4-dinitrobenzene (as described in Materials and Methods) should then yield the same DNA products as those obtained by incorporation of rATP-DNP. Chemical analysis of the low amounts of modified DNA was difficult. To assess the specificity of addition of DNP groups to the aminohexyl arms, we prepared plasmid DNA with a 3' extension of 8-aminohexyl ATP residues. DNP was added. Modified DNA was cleaved by a restriction enzyme cutting close to the 3' end and fractionated by electrophoresis. DNP groups were revealed by the enzymatic procedures described below. There was no trace of internal labelling, a strong indication that addition reactions proceeded as predicted (see below, Figure 5, lanes 4 and 5).
Figure 5: Visual recognition of DNP-labelled DNA.

Lane 1: DNP-labelled lambda DNA obtained by nick-translation with 8-aminohexyl adenosine 5' triphosphate followed by chemical dinitrophenylation;
Lane 2: Lambda DNA;
Lane 3: Lambda DNA + 1-fluoro-2-4-dinitrobenzene.
Spots a) 15 ng; b) 5 ng; c) 500 pg; d) 50 pg.
Lane 4: 3' OH DNP labelled DNA fragment (750 bp);
Lane 5: Same fragment cut with Ddel.
Spots a) 10 ng; b) 1 ng; c) 100 pg; d) 10 pg.

4) Accessibility of the DNP group to antibody

To check whether the DNP group is accessible to antibody, we spotted various amounts of DNA carrying DNP groups onto nitrocellulose filters. DNA was internally labelled by DNA polymerase I or end labelled by deoxynucleotidyl terminal transferase, using either rATP-DNP, or 8-aminohexyl ATP, followed by reaction with 1-fluoro-2-4-dinitrobenzene (direct or indirect DNP addition). Filters with spotted DNA were exposed to a rabbit antiserum to DNP and then to a goat antirabbit antiserum coupled to peroxydase. After washing, peroxydase bound to filter was allowed to react with a solution of 3-amino-9-ethyl-carbazol to yield a red coloured precipitate, making coloured spots on the nitrocellulose filters, as in the histochemical reactions. Results are shown in figure 5.

Over a low background, 500 pg of phage lambda DNA labelled with DNP by nick-translation to the extent of about 2 DNP groups per 100 nucleotides can be detected (lane 1). In the case of 3' end labelling, quantities in the order of 100 pg of a 750 bp fragment could be detected (lane 4). Molecules with only one terminal rATP-DNP residue obtained after alcali treatment, ethanol precipitation and dialysis also react with the antibody. Quantities in the order of
DISCUSSION

In this publication, we describe a new nucleotide analog carrying a dinitrophenyl group which can be recognized by specific antibodies. Synthesis of the analog is carried out in one step with high yield by a very simple procedure, from two commercially available products. The analog, rATP-DNP is a substrate for both calf thymus deoxynucleotidyl terminal transferase and E. coli DNA polymerase. Other DNA polymerases have not been tried. 8-aminohexyl ATP, the precursor of rATP-DNP which carries a highly reactive aliphatic amine at the end of the hexane arm, is also incorporated into DNA by the same enzymes. Dinitrophenyl groups can be chemically added afterwards, and the reaction displays the expected specificity for the aliphatic amine. This alternative procedure is very versatile, since it does not require the preparation of rATP-DNP while DNA directly reacted with 8-aminohexyl ATP and DNP is very easily and rapidly purified (see Materials and Methods).

DNP groups in DNA can be detected by specific antibodies (monoclonal antibodies against DNP are available but have not been tried). The latter can be revealed by anti-antibodies coupled to an enzyme such as peroxidase. This does not imply that all DNP groups are accessible to antibody. Detection of DNA internally labelled with DNP is less sensitive than expected, when compared to another method of internal labelling (Tchen et al., in preparation). This suggests that, in spite of the hexane arm, internal DNP groups interact with bases in the DNA. This low sensitivity precludes, at this stage, the use of this method for the internal labelling of DNA probes for hybridization.

The end labelling method based on the incorporation of rATP-DNP by terminal deoxynucleotidyl transferase, easily detects $10^{-3}$ pmole of DNA. This is about 10 fold less sensitive than routinely obtained after $^{32}P$ end labelling of DNA. Optimization of detection by antibodies and enzymes, and/or amplification of enzymatic signals might fill this moderate gap in sensitivity. As it stands, the method is usable for 3' end labelling of DNA when high sensitivity is not required. In the future, such procedures may become significant in automation of DNA sequencing techniques. It may also be noted that rATP-DNP per se could be useful to follow a number of enzymatic reactions which utilize ATP.

It is likely that DNA-polymerases would accept as substrates other analogs of the same type as rATP-DNP. The method described here can easily be adapted to synthesize many ribo- and deoxyribonucleotides carrying various antigenic
groups. This could be helpful both in the understanding of DNA-polymerase reactions and in increasing the versatility of enzyme labelling techniques.

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REFERENCES