Nucleic Acids Research, 1996, Vol. 24, No. 22 **4535–4532**

Chemical methods of DNA and RNA fluorescent labeling

Dmitri Proudnikov[2,+] and Andrei Mirzabekov[1,2,*,+]

Joint Human Genome Program: [1]Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA and [2]Engelhardt Institute of Molecular Biology, 32 Vavilov St., B-334, Moscow 117984, Russia

Received July 16, 1996; Revised and Accepted September 24, 1996

ABSTRACT

Several procedures have been described for fluorescent labeling of DNA and RNA. They are based on the introduction of aldehyde groups by partial depurination of DNA or oxidation of the 3′-terminal ribonucleoside in RNA by sodium periodate. Fluorescent labels with an attached hydrazine group are efficiently coupled with the aldehyde groups and the hydrazone bonds are stabilized by reduction with sodium cyanoborohydride. Alternatively, DNA can be quantitatively split at the depurinated sites with ethylenediamine. The aldimine bond between the aldehyde group in depurinated DNA or oxidized RNA and ethylenediamine is stabilized by reduction with sodium cyanoborohydride and the primary amine group introduced at these sites is used for attachment of isothiocyanate or succinimide derivatives of fluorescent dyes. The fluorescent DNA labeling can be carried out either in solution or on a reverse phase column. These procedures provide simple, inexpensive methods of multiple DNA labeling and of introducing one fluorescent dye molecule per RNA, as well as quantitative DNA fragmentation and incorporation of one label per fragment. These methods of fluorophore attachment were shown to be efficient for use in the hybridization of labeled RNA, DNA and DNA fragments with oligonucleotide microchips.

INTRODUCTION

Radiolabeling and fluorescent labeling have found wide application in DNA and RNA hybridization and sequence analysis. Although less sensitive, fluorescent dyes conjugated with nucleic acids offer some essential advantages over a radioactive label (1). Fluorescent dyes can be detected in real time with high resolution and several can be monitored in one experiment. Fluorescent dyes also lack radiation hazards and the consequent problems of waste handling and disposal.

Fluorescent labeling of nucleic acids is usually carried out by enzymatic reactions. Organic fluorophores are chemically introduced into primers or nucleoside triphosphates and are then incorporated either using PCR amplification or using DNA or RNA polymerases or terminal polynucleotide transferase (2–4).

The direct incorporation of fluorophores into nucleic acids by chemical means (1,5) has not found wide application. Instead, the introduction of active amino or thiol groups into synthesized oligonucleotides provides acceptors for subsequent chemical fluorescent labeling (1,6–8).

Sequencing analysis by hybridization to oligonucleotide microchips is being developed by different groups (8–15). The method can be applied to sequencing as well as to diagnostics of genetic diseases, gene polymorphism studies, quantitative analysis of gene expression, analysis of microorganisms in a sample, etc. (for review see 16). Fluorescent labeling allows one to carry out highly sensitive and rapid analysis of the hybridization of nucleic acids on microchips. A wide range of possible applications for hybridization with oligonucleotide microchips requires a procedure for fluorescent labeling that should be simple, efficient and inexpensive and also satisfy the following criteria: (i) it can be applied to both RNA and DNA, either isolated from cells or synthesized *in vitro*; (ii) it must be compatible with the fragmentation of nucleic acids (fragmentation is needed to decrease the formation of hairpin structures that interfere with nucleic acid hybridization to rather short microchip oligonucleotides); (iii) to carry out quantitative hybridization analysis, the amount of introduced label should not depend on length of the nucleic acid fragments; (iv) several labels may be incorporated for multiple coloring, based on the introduction of aldehyde groups into DNA by partial depurination and hybridization analysis.

Here we describe several procedures for fluorescent labeling developed with model oligonucleotides and applied to RNA and DNA. These procedures are regularly used by our group for fluorescent labeling of DNA and RNA, before or after their partial fragmentation. The labeled products have been used for hybridization with oligonucleotide microchips. These procedures are based on the introduction of aldehyde groups into DNA by partial depurination and into RNA by oxidation of its 3′-terminal ribonucleoside with sodium periodate, followed by the direct or indirect attachment of one fluorophore molecule to the aldehyde group of a fragment. The hybridization of RNA and DNA with oligonucleotide microchips demonstrated the efficiency of the labeling procedures described.

*To whom correspondence should be addressed at present addresss. Tel: +1 630 252 3161; Fax: +1 630 252 3387; Email: amir@everest.bim.anl.gov

+Present address: Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA

MATERIALS AND METHODS

The oligonucleotides 5′-d(T_6GT_8) and 5′-dA_8Ur were synthesized with a 394 DNA/RNA synthesizer (Applied Biosystems); poly(dA-dT)·poly(dA-dT) was purchased from Sigma.

Dimethyl sulfoxide (DMSO) was distilled *in vacuo* under calcium hydride. Pyridine and triethylamine were consecutively distilled under toluenesulfonyl chloride and calcium hydride. Acetonitrile was distilled under phosphorus pentoxide.

Reagents: tetramethylrhodamine (TMR) isothyocyanate, TMR succinimide ether, fluorescein isothiocyanate and fluorescein succinimide ether (Molecular Probes Inc.); T4 polynucleotide kinase (Promega); methanesulfonyl chloride, toluenesulfonyl chloride, hydrazine hydrate, *N*,*N*-dimethylacetamide and sodium cyanoborohydride (Merck). All other reagents were from Aldrich.

Chromatographic sorbents: RP-18 (Merck), DOWEX-50 (Dow). Chromatographic solvent systems for TLC on kieselgel plates (glass backed; Merck) were chloroform/ethanol 9:1 (A); acetonitrile/aqueous ammonium 8:2 (B); acetonitrile/water/chloroform/triethylamine 8:1:0.5:0.1 (C). Electrophoresis was carried out in 20% acrylamide:bisacrylamide (30:1) gels with 7 M urea in Tris–borate buffer, pH 7.8, at 300 V for 90 min. Autoradiograms were scanned with a 300A Computing Densitometer (Molecular Dynamics). UV spectra were measured with a Shimadzu UV-160A. The extent of fluorescent labeling was calculated from UV spectra at 260 nm, where the a_M (molar absorbency) value for adenosine is 15×10^3; for thymidine and 3-*N*-methyluridine, 10×10^3; for TMR, ~30×10^3; for fluorescein, 60×10^3; at 490 nm the a_M for fluorescein is 60×10^3; at 550 nm the a_M for TMR is 90×10^3 (17–18).

DNA depurination

In formic acid. DNA (10 µg or more) in 10 µl 80% formic acid was incubated at 20°C for 30 min and was then precipitated at –20°C for 15 min with 15 vol 2% lithium perchlorate in acetone. The precipitate was centrifuged, washed twice with 100 µl acetone and then with 100 µl diethyl ether and dried.

In HCl. DNA (1–10 µg) was suspended in 10 µl 0.2 N HCl, incubated at 37°C for 90 min, diluted with 40 µl 650 mM ethylenediamine hydrochloride, pH 7.6, and used for β-elimination without precipitation.

Complete depurination of d(T_6GT_8). Oligonucleotide (10 pmol radioactive or 1 nmol non-radioactive) was dissolved in 10 µl 80% formic acid and incubated at 60°C for 90 min. It was then precipitated at –20°C for 15 min with 15 vol 2% lithium perchlorate in acetone.

Synthesis of TMR hydrazine (7 in Fig. 1)

Triethylene glycol (1.5 g, 10 mmol) was dissolved in 50 ml dry pyridine and methanesulfonyl chloride (2.35 ml, 30 mmol) was added. The reaction was carried out at 0°C for 2 h and was monitored by TLC (solvent system A). The reaction was stopped by dilution of the mixture with 300 ml saturated sodium bicarbonate solution. The product was extracted using chloroform (three extractions, 50 ml each). The combined organic extracts were dried over sodium sulfate and evaporated to dryness. The residue (2.8 g) was dissolved in 30 ml *N*,*N*-dimethylacetamide and 4 ml (80 mmol) hydrazine hydrate was added. The reaction was carried out at room temperature for 3 h and was monitored by TLC (solvent systems A and B). The reaction mixture was diluted with 300 ml water and passed through a DOWEX-50 column (200 ml) in the H^+ form. The column was washed with 300 ml 15% aqueous isopropyl alcohol and then with 300 ml water and the reaction product was eluted with 500 ml 5% aqueous ammonium hydroxide. The solution was evaporated to dryness, diluted with 5 ml water and chromatographed on an RP-18 column (2.5×30 cm) eluted with water. The solution was evaporated to 50 ml and, after filtration, chromatographed on a DOWEX-50 column (200 ml) in the NH_4^+ form. The column was washed with 300 ml water and the product eluted with a linear gradient of 0–1% (0.5×0.5 l) aqueous ammonium hydroxide and then evaporated to dryness. Yield of bis-1,2-(hydrazoethoxy)ethane: 1.2 g (67%). Mass: 179.1 (M+1).

Tetramethylrhodamine isothiocyanate (1 mg, 2.25 µmol) was dissolved in 1 ml dry acetonitrile and a solution of bis-1,2-(hydrazoethoxy)ethane (2 mg, 11.2 µmol) in 20 µl dry acetonitrile and 3 µl triethylamine was added. After incubation at room temperature for 30 min (monitored by TLC, silvent system C), the reaction mixture was chromatographed on a TLC kieselgel plate (20×20 cm) eluted with solvent system C. The product-containing fraction was picked up from the plate, eluted from the sorbent with system C and lyophilized. Fluorescent dye was futher dissolved in 20% methanol to 4 mM concentration. Yield: 0.74 mg (53%). The solution of the chemical was stored in a freezer (–20°C, 3 months) without noticeable loss of activity.

Mass fragments found:
162.9 [$NH_2NH(CH_2CH_2O)_2CH_2CH_2$ NH +1];
386 [M– $NH_2NH(CH_2CH_2O)_2CH_2CH_2NHNHC(S)NH$ +1];
356.7 [$NH_2NH(CH_2CH_2O)_2CH_2CH_2NHNHC(S)NHC_6H_3$ (COO^-) +1];
325.7 [$(CH_2CH_2O)_2CH_2CH_2NHNHC(S)NHC_6H_3$ (COO^-) +1].

Fluorescent labeling of DNA with TMR hydrazine

Completely depurinated [^{32}P]d(T_6GT_8) (10 pmol) was dissolved in 10 µl 0.05 M sodium acetate buffer, pH 3.75–5.8, or sodium phosphate buffer, pH 6.5–7.0 (or 10 µg of depurinated DNA was dissolved in 10 µl sodium acetate buffer, pH 4.0) and 1 µl 4 mM TMR hydrazine (**9**) (>10-fold molar excess) in 20% methanol was added to the reaction mixture. The solution was incubated for 1 h at 37°C and then 1.5 µl 0.2 M $NaCNBH_3$ (or $NaBH_4$ or $PyBH_3$) in dry acetonitrile was added and the mixture was incubated at 20°C for 30 min. The mixture was diluted with 100 µl water and extracted five times with 100 µl *n*-butanol saturated with water. The DNA was precipitated with acetone and dried.

Fragmentation of depurinated DNA with ethylenediamine and its fluorescent labeling in solution or in a column

Depurinated DNA (up to 10 µg) or 10 pmol fully depurinated [^{32}P]d(T_6GT_8) was dissolved in 50 µl 0.5 M ethylenediamine hydrochloride, pH 7.4, and incubated at 37°C for 3 h. Four microliters of freshly prepared 0.1 M $NaBH_4$ was added at room temperature, followed by incubation at room temperature for 30 min. Then, 4 µl 20% ethylenediamine was added to the DNA solution and the DNA was precipitated with 1 ml 2% lithium perchlorate in acetone, washed twice with acetone and air dried. Alternatively, after the 30 min reduction, the DNA was diluted with 1.5 ml water, purified on a C18 Sep-Pak cartridge (Waters Corp.) eluted with 1 ml 50% methanol and lyophilized. However, ~50% of the DNA was usually lost on the column.

The oligonucleotide or DNA with attached ethylenediamine was dissolved in 10 µl absolute DMSO and then 1 µl freshly prepared 30 mM fluorescein (or tetramethylrhodamine) isothiocyanate (or succinimide ether) in dry DMSO and 0.2 µl triethylamine

were added. The reaction mixture was incubated at room temperature for 1 h and then 80 µl 0.1 M sodium acetate buffer, pH 4.0, was added. Unreacted fluorescein was extracted five times with 100 µl water-saturated *n*-butanol. The DNA was precipitated with acetone and dried.

For column labeling, 20 µg poly(dA-dT)·poly(dA-dT) was incubated in 200 µl 80% formic acid at 20°C for 30 min. The reaction mixture was diluted with 3 ml water, loaded onto the C-18 column (Alltech; 500 mg, high load) and the column washed with 5 ml water. All loading and washing procedures were performed with a syringe. The column was filled with 200 µl 500 mM ethylenediamine hydrochloride, pH 7.4, and incubated in a water bath at 37°C for 3 h. The column was washed with 2 ml water and then with 0.5 ml of a 20 mM solution of ethylenediamine hydrochloride in 100 mM sodium acetate buffer, pH 4.2. Nine microliters of 1 M sodium cyanoborohydride in dry acetonitrile was dissolved in 300 µl of a 20 mM solution of ethylenediamine hydrochloride in 100 mM sodium acetate buffer, pH 4.2, and loaded immediately onto the column. The column was incubated at room temperature for 0.5 h and washed with 10 ml water. Fresh saturated solution of fluorescein isothyocyanate (~50–100 µg) in 200 µl sodium carbonate/bicarbonate buffer, pH 9.1, was loaded, the column was washed with 1.5 ml water to distribute the dye throughout the whole column and then the column was washed with 1 ml sodium carbonate/bicarbonate buffer, pH 9.1. The column was incubated at room temperature for 5 h, washed with 10 ml water until the eluted solution became colorless, dried, washed with 2 ml acetone and dried again. The poly(dA-dT)·poly(dA-dT) was eluted with 50% acetonitrile. The fourth to the tenth drops were collected and labeled product was precipitated with 1.3 ml acetone. The poly(dA-dT)·poly(dA-dT) was dissolved in 100 µl water, extracted three times with 100 µl water-saturated *n*-butanol and precipitated with acetone.

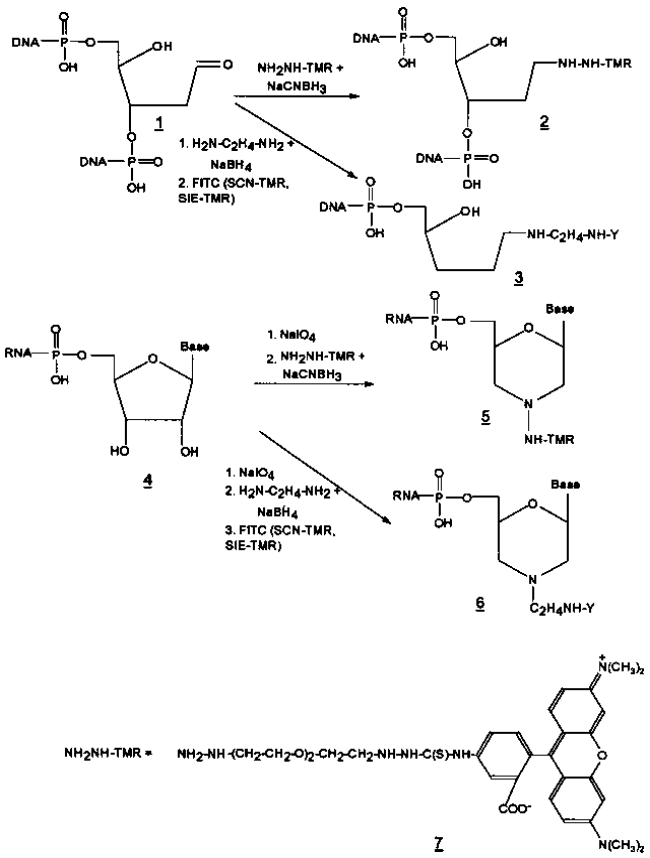

Figure 1. Scheme of fluorescent labeling of depurinated DNA and RNA. FITC, fluorescein isothiocyanate; SCN-TMR, tetramethylrhodamine isothiocyanate; SIE-TMR, tetramethylrhodamine succinimide ether; Y, fluorophore.

Fluorescent labeling of RNA with TMR hydrazine

Freshly prepared 0.1 M $NaIO_4$ (1 µl) was added to RNA (up to 20 µg) or dA_8Ur (10 pmol [^{32}P]dA_8Ur or up to 1 nmol dA_8Ur) in 5 µl water and the solution incubated at 20°C for 20 min. RNA or oligonucleotides were precipitated with 2% lithium perchlorate in acetone followed by two washings with acetone. Alternatively, the excess of $NaIO_4$ was reduced with 1 µl 0.2 M sodium hypophosphite for 20 min at room temperature. Then 8 µl 0.1 M sodium acetate, pH 4.0, and 4 mM TMR hydrazine in 20% methanol at 1 µl/0.5 nmol oligonucleotide or fragmented RNA were added. Coupling was carried out at 37°C for 1 h, then 1.5 µl 0.2 M $NaCNBH_3$ in dry acetonitrile were added and reduction was carried at room temperature for 30 min. The solution was diluted with 100 µl water, free fluorescent label was extracted with *n*-butanol and RNA was precipitated as described above for DNA.

To quantitatively separate fluorescently labeled oligonucleotides from unlabeled ones, the reaction solution was extracted four times with 50 µl phenol saturated with 1 M Tris–HCl, pH 8.5. Fluorescently labeled oligonucleotides were precipitated from the combined phenol solutions with a 10-fold excess of 2% $LiClO_4$ in acetone.

Fluorescent labeling of RNA through ethylenediamine attachment

$NaIO_4$-oxidized RNA or dA_8Ur (10 pmol [^{32}P]dA_8Ur or up to 1 nmol dA_8Ur) were dissolved in 10 µl 50 mM sodium acetate buffer, pH 4.0, and 3 µl 20 mM ethylenediamine hydrochloride, pH 7.2, were added. After incubation at 37°C for 1 h, 1.5 µl 200 mM $NaCNBH_3$ in dry acetonitrile was added at room temperature. Reduction was carried out for 30 min and then RNA (oligonucleotide) was precipitated with 200 µl of a 2% solution of $LiClO_4$ in acetone and dried. The precipitate was dissolved in 10 µl dry DMSO and then 1 µl of a 30 mM solution of fluorescein isothiocyanate in dry DMSO contaning 0.2 µl triethylamine was added. The mixture was incubated at room temperature for 60 min and then diluted with 80 µl 100 mM sodium acetate buffer, pH 4.0. The excess fluorescent label along with traces of fluorescently labeled ethylenediamine were extracted five times with water-saturated *n*-butanol. The RNA was precipitated with acetone and dried.

Hybridization of RNA and DNA with oligonucleotide microchips

Samples of 0.5 µg single-stranded DNA or 5 µg RNA, each ~200 nt long and complementary to the DQα region of the HLA gene, were prepared as described (15; Drobishev *et al.*, unpublished data). A microchip with immobilized 10-, 20- and 30mer was manufactured as described (15). RNA and DNA, fluorescently labeled as described above, were hybridized with the microchip in 10 µl 50 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl, 10 mM EDTA and 50% formamide at 4°C for 18 h. The microchip was washed once with the same hybridization solution at 4°C and fluorescence imaging of the hybridized microchip was

carried out with a multicolor fluorescence microscope equipped with a CCD camera and the necessary software (15).

RESULTS AND DISCUSSION

DNA and RNA fluorescent labeling (general description)

Highly reactive aldehyde groups can be easily formed in DNA after its partial depurination at acidic pH. DNA depurination has been well studied and has been used for DNA fragmentation (19–21), to detect DNA regions shielded by proteins or other ligands (21), in the Maxam–Gilbert sequencing method (22–24) and for DNA–protein crosslinking (25,26).

Figure 1 shows that the active aldehyde groups can be directly used for DNA labeling by reaction with fluorophores containing aldehyde-specific (e.g. hydrazine) groups. This reaction causes partial DNA fragmentation at depurinated sites. Alternatively, DNA can be quantitatively fragmented at depurinated sites through β-elimination reactions catalyzed by ethylenediamine (27). In the presence of reducing agents, ethylenediamine forms a stable secondary amine bond with the depurinated site. Then activated fluorescent dyes can be attached to the second primary amine group of the bound ethylenediamine. These procedures can be applied to any abasic DNA (Fig. 1).

Dialdehyde groups are easily incorporated into RNA by oxidation of the 3′-terminal ribonucleotide with $NaIO_4$ (Fig. 1). Such oxidized RNA has been used for fractionation (28,29) and for incorporation of a fluorescent label (30–33). These procedures were partly modified and applied for fluorescent labeling of RNA in the same way as described for DNA.

Though quantitative analysis of the reaction yields (Table 1) was carried out only on the model oligonucleotides, a wide range of RNAs, as well as single-stranded and double-stranded DNAs up to 1500 nt long, were labeled by the described methods and used for hybridization with oligonucleotide microchips.

DNA depurination

Purine glycosyl bonds in DNA are rather unstable and depurination is accelerated at low pH or at high temperature and neutral pH (34,35). Depurination can be facilitated by preliminary methylation of purine bases with dimethylsulfate (25). However, depurination under acidic conditions (pH 1–2) is more convenient, for it does not depend on the DNA secondary structure or the guanine and adenine composition and it is easy to perform (36–38). Depurination in 0.2 M HCl is preferable for small amounts of DNA, because the depurinated DNA can be directly used in the subsequent fragmentation procedures without purification. For large amounts, depurination is carried out in formic acid as a good solvent for DNA, followed by DNA precipitation before further use. Depurination at 20°C for 30 min in formic acid was shown to correspond to that in 0.2 M HCl at 37°C for 90 min. The optimal level for DNA depurination should be found experimentally by hybridization of the fluorescently labeled DNA samples. The complete depurination of a model oligonucleotide, 5′-d(T_6GT_8), was carried out at 65°C for 1.5 h in 80% formic acid.

Fluorescent labeling of partially depurinated DNA with TMR hydrazine. A scheme for fluorescent labeling of depurinated DNA is shown in Figure 1. Depurinated sites in DNA exist in an equilibrium of two forms: a cyclic hemiacetal and an open chain aldehyde; the latter constitutes only ~1% of the species, but is highly reactive (27). The aldehyde groups efficiently react with the hydrazine group of TMR hydrazine. The synthesis of TMR hydrazine consists of attachment of bis-hydrazine containing a polyether linker to the fluorophore. The basic alkylhydrazine group was shown to catalyze β- and δ-elimination reactions that cause DNA fragmentation (see below). The level of the fragmentation caused by β-elimination under the conditions we used varied from 20 to 30% (Table 1) and depended on the duration of the reactions. Since we have not observed a preference for β-elimination (relative to δ-elimination) in these reactions, it is not possible to obtain a high yield of fragmented, fluorescently labeled product. Double bonds between DNA and the dye can be stabilized by reduction with $NaBH_4$, $NaCNBH_3$ or $PyBH_3$, giving rise to fluorescently labeled fragmented or non-fragmented DNA.

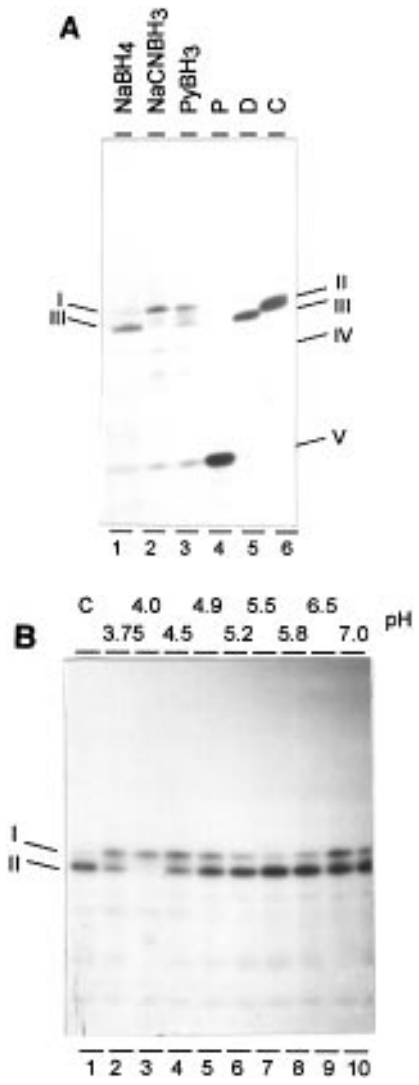

Figure 2. The effect of different reducing agents and pH on the condensation of depurinated [^{32}P]d(T_6GT_8) with TMR hydrazine. (**A**) Gel electrophoresis of depurinated oligonucleotide coupled with TMR hydrazine and reduced with $NaBH_4$ (lane 1), $NaCNBH_3$ (lane 2) and $PyBH_3$ (lane 3); [^{32}P]d(T_6GT_8) fully depurinated and treated with piperidine at 100°C for 1 h (lane 4); [^{32}P]d(T_6GT_8) fully depurinated (lane 5); [^{32}P]d(T_6GT_8) (lane 6). (**B**) Gel electrophoresis of fully depurinated [^{32}P]d(T_6GT_8) (lane 1) after coupling with TMR hydrazine in 50 mM sodium acetate or sodium phosphate buffers, pH 3.75–7.0, followed by reduction with $NaCNBH_3$.

Table 1. Yield of fluorescent labeling of RNA and DNA by different procedures

Oligonucleotide	Labeling procedure	Fragmentation (%)	Yield of fluorescently labeled oligonucleotide (%)
1. 5′-d(T_6GT_8)	TMR hydrazine[a]	20[c]	59[c]
2. 5′-d(T_6GT_8)	SCN fluorophore[b]	87[c]	55[d], 46[c]
3. 5′-dA_8Ur	TMR hydrazine[a]		65[d], 59[c]
4. 5′-dA_8 Ur	SCN fluorophore[b]		48[d]

[a]Coupling of TMR hydrazine to depurinated DNA or 3′-terminal ribonucleotide oxidized with $NaIO_4$.
[b]Coupling of SCN-fluorophore through an ethylenediamine bridge to depurinated DNA or 3′-terminal ribonucleoside oxidized with $NaIO_4$.
[c]Yield was measured by radioautograph scanning.
[d]Yield was measured by UV spectroscopy.

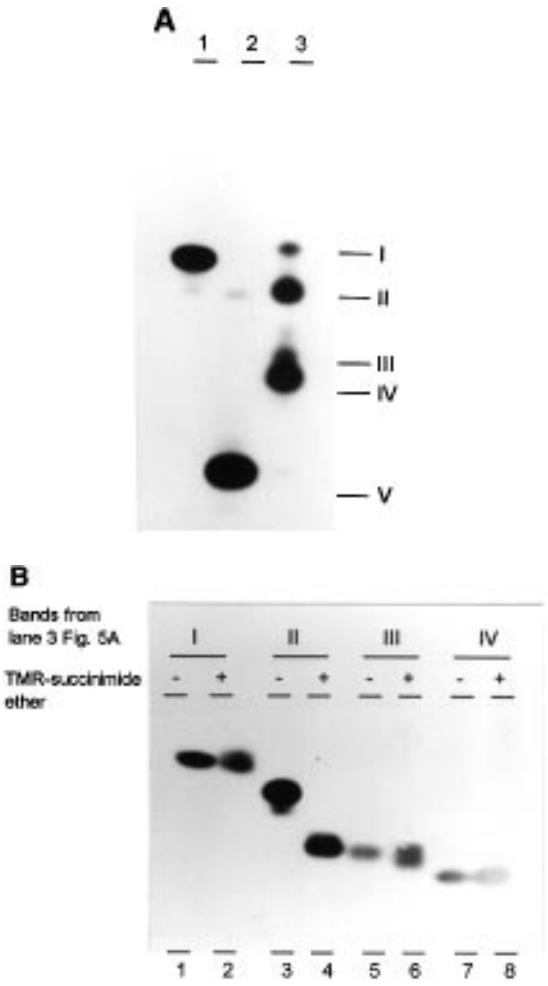

Figure 3. Fluorescent labeling of depurinated [^{32}P]d(T_6GT_8) after fragmentation at the depurinated site and ethylenediamine attachment. (**A**) Gel electrophoresis of the completely depurinated oligonucleotide [^{32}P]d(T_6GT_8) (lane 1), after treatment with piperidine at 100°C for 1 h (lane 2) or reaction with ethylenediamine followed by reduction with $NaBH_4$ (lane 3). (**B**) Gel electrophoresis of the compounds in bands I–IV (Fig. 5A, lane 3) treated with TMR succinimide ether.

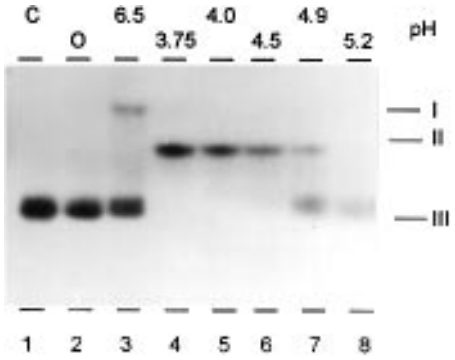

Figure 4. The effect of pH on the labeling of oxidized [^{32}P]dA_8Ur after reduction with $NaCNBH_3$. Lane 1, [^{32}P]dA_8Ur; lane 2, as lane 1 after oxidation with $NaIO_4$; lanes 3–8, oxidized [^{32}P]dA_8Ur, coupled with TMR hydrazine and reduced with $NaCNBH_3$ in 35 mM sodium acetate or sodium phosphate buffers, pH 3.75–6.5. Band I, unreduced product of TMR hydrazine condensation with initial oligonucleotide; band II, reduced fluorescently labeled product; band III, unlabeled initial oligonucleotide and this oligonucleotide after oxidation and reduction.

This method of fluorescent labeling was tested on the 15mer 5′-d(T_6GT_8), which was labeled at the 5′-terminus with [γ-^{32}P]ATP with polynucleotide kinase, was fully depurinated and was reacted with TMR hydrazine in the presence of different reducing agents.The products of these reactions were separated by gel electrophoresis (Fig. 2A). The initial 15mer (band II in lane 6), the fully depurinated 15mer (band III in lane 5) and [^{32}P]d(T_6)p (band V in lane 4, derived from the depurinated 15mer by treatment with piperidine) were electrophoresed as controls. Piperidine treatment causes complete fragmentation of DNA at depurinated residues (24). The binding of a bulky fluorochrome, TMR hydrazine, retards the electrophoretic mobility of the depurinated 15mer (band I in lanes 1–3) and 6mers (band IV). Scanning of the gel autoradiograph shows that the use of $NaCNBH_3$ as a reducing agent produced the highest yield (~60%) of non-fragmented fluorescently labeled 15mer (Table 1).

Figure 2B shows the effect of pH on the condensation of the depurinated 15mer (band II) with TMR hydrazine to produce fluorescently labeled DNA (band I). The reduction was carried out with $NaCNBH_3$. It appears that the most quantitative yield of the fluorescently labeled DNA is achieved at pH 4.0.

Fragmentation and fluorescent labeling of DNA. A scheme of fragmentation and fluorescent labeling of DNA at depurinated sites is also shown in Figure 1. The scheme is based on results of the studies of reductive attachment of amino compounds to depurinated DNA (39). Amines catalyze the scission of DNA at depurinated sites through a β-elimination reaction (27). Primary amines at the same time reversibly react with the aldehyde groups of depurinated sites to form aldimines (Schiff bases) (1). The

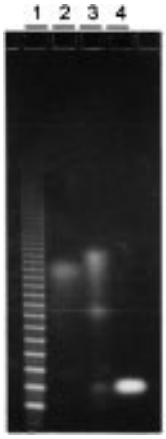

Figure 5. Gel electrophoresis of fluorescently labeled poly(dA-dT)·poly(dA-dT) through ethylenediamine attachment (see Fig. 6, path B). Lane 1, depurinated poly(dA-dT)·poly(dA-dT) fluorescently labeled through ethylenediamine attachment; lanes 2 and 3, fluorescein isothiocyanate and product of reaction of fluorescein isothiocyanate with ethylenediamine respectively; lane 4, bromphenol blue.

aldimines can be stabilized by reduction with various agents (1,40; reviewed in 41).

Ethylenediamine was shown to be particularly effective in the quantitative scission of depurinated DNA (25). The condensation of ethylenediamine with depurinated DNA sites also introduced a reactive primary amino group that can be used for fluorescent labeling by reaction with commercially available activated fluorophores, such as the isothiocyanates or succinimide ethers of fluorescein or TMR.

The product of the reaction of fully depurinated [^{32}P]d(T_6GT_8) with ethylenediamine followed by TMR succinimide ether treatment can be identified by gel electrophoresis. Figure 3A shows that [^{32}P]d(T_6NT_8) containing depurinated residue N (band I, lane 1) is converted into [^{32}P]d(T_6p) after piperidine treatment (band V, lane 2). The reaction of the same oligonucleotide, [^{32}P]d(T_6NT_8), with ethylenediamine followed by reduction with $NaBH_4$ results in the appearance of an additional three products (bands II–IV, lane 3). To identify some of these products, the substances from bands I–IV were eluted from the gel, reacted with TMR succinimide ether in dry DMSO and separated by electrophoresis. Figure 3B shows that only band II in lane 3 (Fig. 3A) changed its electrophoretic mobility after reaction with TMR succinimide ether. Piperidine treatment of band II did not produce any changes in its electrophoretic mobility (not shown), suggesting reduction with $NaBH_4$ of both the aldimine and the double C2′–C3′ bond in the terminal sugar moiety. A similar reduction of conjugated C=C and C=O bonds to aliphatic alcohols has also been described (42). Some side reactions led to unidentified products in Figure 3A and B. Thus, this procedure provides ~50% yield of ethylenediamine conjugation and ~90% efficiency of fragmentation at depurinated sites (Table 1).

The DNA labeling methods were carried out either in a solution through several rounds of precipitation or on a column by washing it with different reaction solutions (see Materials and Methods). The column procedure can be easily automated in order to perform many labeling reactions simultaneously, speeding up and simplifying fluorescent labeling.

Fluorescent labeling of RNA. The dialdehyde groups that are introduced into RNA by oxidation of the 2′,3′-terminal vicinal dihydroxy groups with $NaIO_4$ can react with TMR hydrazine (Fig. 1) with ~60% yield (Table 1). Reduction of the reaction product with $NaCNBH_3$ increases its stability. Figure 4 shows the effect of pH on fluorescent labeling of a model compound, [^{32}P]dA_8Ur, by reduction with $NaCNBH_3$. It appears that the optimal pH for the reaction is 3.75–4.5. If the RNA is phosphorylated at the 3′-terminal position (as the result of fragmentation induced by acids, alkalis or metals, for example), the phosphate groups can be easily removed by treatment with phosphatase before the labeling procedure.

Figure 1 also shows a method of fluorescent labeling through ethylenediamine attachment to the terminal 2′,3′-dialdehyde groups of oxidized RNA. The activated fluorescein dyes react with the primary amino group of ethylenediamine on the RNA as described above for DNA, with ~50% overall yield.

Purification of fluorescently labeled samples

A high excess of activated fluorescent label must be added in the condensation reaction so that we can ignore the traces of ethylenediamine, contaminated RNA and DNA after condensation. We have found that butanol extraction from acid aqueous solutions completely separates the labeled product from the excess of unreacted fluorophore without laborious procedures such us dialysis. Figure 5 shows that butanol extraction produces pure fluorescently labeled poly(dA-dT)·poly(dA-dT) with no admixture of other fluorescent products.

After labeling dA_8Ur with TMR hydrazine and butanol extraction of the unreacted fluorescent dye, the labeled product can be separated from non-labeled oligonucleotide by phenol extraction. This procedure transfers >90% of the labeled, but no unlabeled, 9mer into the phenol phase because of the hydrophobic nature of the fluorophore residue. The procedure works only for rather short oligonucleotides.

Hybridization of fluorescently labeled DNA and RNA with oligonucleotide microchip

Single-stranded DNA was first depurinated and then labeled with TMR hydrazine or was fragmented with ethylenediamine and labeled with activated fluorescein. RNA was enzymatically labeled or was oxidized and labeled with TMR hydrazine. Hybridization of the mixture of differently labeled DNAs and then RNA samples successively with the microchip (containing 10-, 20- and 30mers that are complementary to the RNA and DNA; Fig. 6A) is shown in Figure 6B. As expected, the fragmented DNA (6a) was hybridized more efficiently and produced stronger signals than the unfragmented DNA. The hybridization signals were higher with longer immobilized oligonucleotides. The unfragmented DNA (6b) and RNA (6c) was also preferentially hybridized on the periphery of the gel pads due to retarded diffusion of long nucleic acids into the gel and the possible formation of secondary structures in it. RNA labeled enzymatically (Fig. 6B, d) or after oxidation with either TMR hydrazine (Fig. 6B, c) or with activated fluorescein through a ethylenediamine linker (Gushin *et al.*, unpublished data) showed similar hybridization patterns. The hybridization shown in Figure 6B was carried out at 4°C. However, hybridization was also carried out at higher temperatures (up to 60°C) for up to

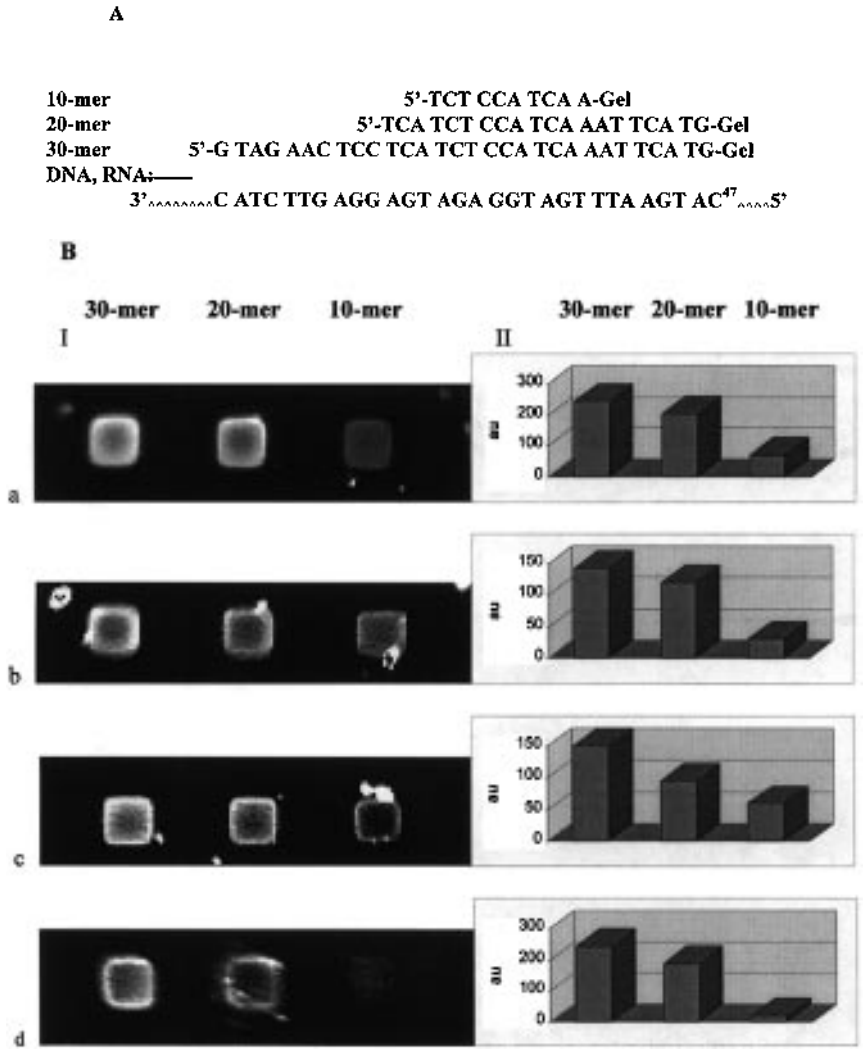

Figure 6. Hybridization of fluorescently labeled RNA and DNA with microchips. (**A**) DNA and RNA molecules ~200 nt long contain regions complementary to 10-, 20- and 30mer oligodeoxyribonucleotides. The oligonucleotides are immobilized on the microchip within 100× 100 × 20 µm polyacrylamide gel pads arranged 200 µm from each other on a glass slide (15). (**B**) Samples of DNA and RNA were each labeled using several labeling procedures and the samples were hybridized successively with the microchip; (a) partially depurinated DNA fragmented with ethylenediamine and labeled with activated fluorescein; (b) unfragmented, partly depurinated DNA labeled with TMR hydrazine; (c) RNA labeled with TMR hydrazine; (d) RNA labeled by incorporation of fluorescein-UTP during transcription with the Ambion MEGAshortscript kit according to the manual. Fragmented and unfragmented DNA labeled with fluorescein (a) and TMR (b) respectively were hybridized in a mixture with the microchips and their hybridization patterns were monitored separately with a two wavelength fluorescence microscope. I, fluorescence images of the microchip; II, quantitative measurements of the fluorescence intensities; au, an arbitrary unit of fluorescence intensity.

several hours without any sign of degradation of the fluorescence label (Drobishev *et al.*, Gushin *et al.*, unpublished data).

Depurination was carried out under conditions when ~1–2% of the bases were excised from DNA. The random removal of 2–4 bases within, for example, 200 base long DNA molecules did not appear to significantly affect the efficiency of hybridization. Depurination within the hybridization sequence has a low probability when hybridizing the labeled DNA with short oligonucleotides, although it significantly destabilizes the duplexes. Hybridization with longer oligonucleotides will increase the probability of depurination within the hybridized regions, but the destabilization effect of the excised base will be less strong.

Different fluorescent labels can be introduced into different samples of nucleic acids. The simultaneous hybridization of these samples in a mixture and differentiation of their hybridization pattern with a multicolor fluorescence microscope (shown in Fig. 6B, a and b) provides the possibility of more accurate quantitative comparison of the hybridization of these samples.

It appears that the described methods can provide efficient fluorescence labeling of DNA and RNA for their use in hybridization and for other procedures as well.

CONCLUSIONS

The procedures described for fluorescent labeling of DNA in solution or on a column and of RNA in solution are efficient, simple, inexpensive and based on commercially available reagents. They can be applied to samples extracted from cells or prepared by enzymatic or chemical methods. Fluorescent labeling of DNA can be carried out in parallel with quantitative or partial fragmentation. RNA can be labeled before or after standard procedures of fragmentation with acids, alkalis or metals and

followed by treatment with phosphatase to remove the 3′-terminal phosphate group. One fluorescent dye is introduced per RNA or DNA molecule after its fragmentation and this allows us to quantitatively analyze the labeling and hybridization procedures. These methods have been routinely used by our group at both ANL and EIMB for fluorescent labeling and hybridization of DNA and RNA with oligonucleotide microchips.

ACKNOWLEDGEMENTS

We are indebted to Prof. A.Krayevsky, Prof. V.Florentiev, Dr V.Shick and Dr P.Hemken for their support of this work. This work was supported by grant DE-FG02-93ER61538 of the US Department of Energy, by grants 558 and 562 of the Russian Human Genome Program and grant 96-04-49858 of the Russian Foundation of Fundamental Research.

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