Nucleic Acids Research, 1999, Vol. 27, No. 11 **2299–2303**

Benzimidazolium triflate-activated synthesis of (6–4) photoproduct-containing oligonucleotides and its application

Shigenori Iwai*, Toshimi Mizukoshi, Yoshie Fujiwara, Chikahide Masutani[1], Fumio Hanaoka[1] and Yoshihiro Hayakawa[2]

Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan, [1]Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan and [2]Graduate School of Human Informatics, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Received February 22, 1999; Revised and Accepted April 12, 1999

ABSTRACT

In the solid-phase synthesis of oligonucleotides containing the pyrimidine(6–4)pyrimidone photoproduct using a dinucleotide building block, considerable amounts of by-products were found as the chain length increased. The by-products were the major product when a 49mer was synthesized on a 40 nmol scale. It was assumed that these by-products were formed by the coupling of phosphoramidites with the N3 imino function of the 5′ component of the (6–4) photoproduct. We examined imidazolium triflate and benzimidazolium triflate to find an alternative activator for DNA synthesis. Imidazolium triflate prevented by-product formation to some extent, but the coupling yields were low. Benzimidazolium triflate was comparable to tetrazole in coupling efficiency and reduced by-product formation to a great extent, without modification of the synthesizer program. The obtained 49mer was used to detect proteins that recognize UV-damaged DNA in HeLa cell extracts. Two major protein–DNA complexes were found when a 49mer duplex was used as probe, while a 30mer duplex failed to detect one of them. This application showed the usefulness of long chain 'damaged' oligonucleotides in biochemical studies.

INTRODUCTION

Ultraviolet (UV) light, as well as ionizing radiation and various chemicals, causes chemical changes of the base moieties in DNA, which result in genetic mutations. One of the most important forms of DNA damage induced by UV light is the pyrimidine(6–4)pyrimidone photoproduct [abbreviated as (6–4) photoproduct] formed between two adjacent pyrimidine bases, which induces mutations at a very high frequency (1–3). For applications to biological and biochemical studies in the fields of mutagenesis and DNA repair, we previously developed a method of synthesizing oligonucleotides containing the (6–4) photoproduct at a specific site in defined sequences, using a dinucleotide building block (4,5). Oligonucleotides synthesized by this method have been used in studies of the molecular biology of mutation (3,6) and repair (7,8).

In our previous study (4), (6–4) photoproduct-containing oligonucleotides with chain lengths of up to 30 nt were synthesized using the building block on a DNA synthesizer. In the 30mer case, considerable amounts of by-products were found on HPLC analysis of the crude mixture after chain assembly and deprotection. It was assumed that these by-products were formed by coupling of the phosphoramidites with the N3 imino function of the 5′ component of the (6–4) photoproduct. Since formation of the (6–4) photoproduct destabilizes the duplex (6,9), the preparation of long chain oligonucleotides by ligation of short fragments (10) is not practical. Therefore, the question remains as to whether long chain oligonucleotides, which would be more useful than shorter ones in biochemical experiments, can be synthesized in good yield. A decrease in the yield of the desired product, due to by-product formation, is also a serious problem in large-scale synthesis for structural biology and parallel synthesis of different sequences may be unsuccessful, since the prolonged coupling time for the (6–4) building block can affect by-product formation.

In this study, we tested several activators other than tetrazole and found that benzimidazolium triflate was an efficient activator in the synthesis of (6–4) photoproduct-containing oligonucleotides that reduced by-product formation to a great extent. A 49mer, which could not be synthesized successfully by the ordinary tetrazole activation method, was obtained in satisfactory yield. We also describe an application of this 49mer to show the effectiveness of long chain oligomers in biochemical experiments.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligonucleotides were synthesized on a Perkin-Elmer Applied Biosystems model 394 DNA/RNA synthesizer and SNAP

*To whom correspondence should be addressed. Tel: +81 6 6872 8208; Fax: +81 6 6872 8219; Email: iwai@bioorg.beri.co.jp

polystyrene columns (Perkin-Elmer) were used for the 40 nmol synthesis. The building block of the (6–4) photoproduct was prepared as described (4), except that tetrahydrofuran was used as the solvent for the phosphitylation (5). Imidazolium triflate and benzimidazolium triflate were prepared as described (11,12) and were dried over phosphorus pentoxide *in vacuo* before use. For the normal bases, nucleoside phosphoramidites bearing the (4-*tert*-butylphenoxy)acetyl group for protection of the exocyclic amino functions (13), purchased from PerSeptive Biosystems, were used, in combination with (4-*tert*-butylphenoxy)acetic anhydride as a capping reagent, because the (6–4) photoproduct is labile under alkaline conditions for base deprotection. As shown in Table 1, the synthesizer program was changed only to extend the coupling reaction of the (6–4) photoproduct building block to 20 min (with tetrazole or benzimidazolium triflate) or to 30 min (with imidazolium triflate) and that of the other phosphoramidites was changed to 1 min when imidazolium triflate was used as activator. After chain assembly, the oligonucleotides were cleaved from the support and were deprotected simultaneously by treatment with 30% ammonia water at room temperature for 2 h. The ammonia was removed by evaporation and, after the aqueous solutions were washed with ethyl acetate, aliquots were analyzed by HPLC on a Gilson gradient-type analytical system equipped with a Waters 996 photodiode array detector, using a Waters μBondasphere 5μ C18 300 Å column (3.9 × 150 mm) at a flow rate of 1.0 ml/min with a linear gradient of acetonitrile (7–15% during 20 min) in 0.1 M triethylammonium acetate (pH 7.0). Purification of the 49mer was carried out on the same HPLC column using a 9–13% acetonitrile gradient. For anion exchange HPLC, a TSK-GEL DEAE-2SW column (4.6 × 250 mm) was used with a linear gradient of ammonium formate (0.4–1.4 M during 20 min) in 20% acetonitrile.

Table 1. The 40 nmol synthesis cycle used in this study

Step	Reagents[a]	Time[b]
Detritylation	0.29 M CCl_3COOH in CH_2Cl_2	39 s
Coupling	0.10 M nucleoside phosphoramidite or 0.13 M (6–4) photoproduct phosphoramidite + (in CH_3CN)	
	0.53 M tetrazole	33 s (or 20 min[c])
	0.10 M imidazolium triflate	75 s (or 30 min[c])
	or 0.20 M benzimidazolium triflate	33 s (or 20 min[c])
Capping	0.13 M (4-*tert*-butylphenoxy)acetic anhydride in THF + 2.1 M 1-methylimidazole in THF	19 s
Oxidation	20 mM iodine in THF/pyridine/H_2O (7:2:1 v/v/v)	32 s

[a]The concentrations of the reservoir solutions are shown.
[b]The time for solution delivery is included.
[c]The reaction time for the (6–4) photoproduct building block was extended to 20 or 30 min.

Characterization of the 49mer

An aliquot (0.08 A_{260} units) of the purified 49mer was incubated with nuclease P1 (12 μg) in 30 mM ammonium acetate (pH 5.3, 10 μl) at 37°C for 15 h. The mixture was diluted with water (58 μl) and 0.5 M Tris–HCl (pH 9.0, 10 μl) and alkaline phosphatase (from calf intestine, 2 μl, 2 U) was added. After an incubation at 37°C for 2 h, ethanol (600 μl) was added and the mixture was kept at –20°C for 2 h. The proteins were pelleted by centrifugation and the supernatant was concentrated *in vacuo*. The residue was dissolved in water and an aliquot was analyzed by reversed phase HPLC under conditions similar to those described above, except that the acetonitrile gradient was from 0 to 10%.

Detection of protein–DNA complexes by electrophoretic mobility shift assays

The 49mer and a 30mer containing the (6–4) photoproduct (10 pmol) were labeled using [γ-^{32}P]ATP (560 kBq, ~3 pmol) and T4 polynucleotide kinase (6 U) and each was annealed to its complementary strand. HeLa cell extracts were prepared as described previously (14). The mixtures for binding (10 μl) included 5 nM ^{32}P-labeled duplex, 0.1 mg/ml poly(dI)·poly(dC) and the cell extract in 13.6 mM HEPES–KOH (pH 7.9), 0.14 mM EDTA, 3.4 mM $MgCl_2$, 0.14 mg/ml BSA, 10 mM DTT and 3% glycerol. The estimated amounts of whole proteins, used in Figure 6, were 2, 5 and 10 and 2, 8 and 20 μg for the nuclear and cytoplasmic extracts, respectively. After an incubation at room temperature for 15 min, a dye solution (1 μl) containing xylene cyanol and bromophenol blue in 50% glycerol was added and the mixtures were loaded onto a non-denaturing 5% polyacrylamide gel (acrylamide:*N*,*N*′-methylenebisacrylamide 19:1). The gel size was 15 × 15 cm and the buffer contained 50 mM trizma base, 0.4 M glycine and 2 mM EDTA. Electrophoresis was performed at 20 mA and the bands were detected on a BAS-2500 bioimaging analyzer and by autoradiography. The specificity of binding was determined by adding unlabeled duplexes with and without the photoproduct as competitors, as shown in Figure 7.

RESULTS AND DISCUSSION

Synthesis of a 49mer by the tetrazole activation method

To examine by-product formation in the synthesis of long chain oligonucleotides, a 49mer, d(AGCTACCATGCCTGCACGAAT(6–4)TAAGCAATTCGTAATCATGGTCATAGCT), in which T(6–4)T represents the (6–4) photoproduct at thymidylyl (3′–5′)thymidine, was synthesized by the ordinary method using tetrazole as activator, starting from 0.2 μmol of the 3′-terminal nucleoside on controlled pore glass (Fig. 1). This sequence was originally prepared by ligating a UV-irradiated hexamer with two oligonucleotides (10). After detritylation on the solid support, the oligonucleotide was cleaved from the support and was deprotected simultaneously with ammonia. A reversed phase HPLC analysis of the crude mixture is shown in Figure 2. The highest peak was assumed to be the desired 49mer, because it co-eluted with the undamaged 49mer in an anion exchange HPLC analysis, and after purification by reversed phase HPLC, this 49mer was characterized with (6–4) photolyase (7). The amount of the by-products, with retention times longer than that of the highest peak, was much larger as compared with that of the previous 30mer synthesis (4).

We assumed that the by-products in question were formed by the coupling of the phosphoramidites with the N3 imino function of the 5′ component of the (6–4) photoproduct. The reasons for this assumption are as follows. (i) The amount of the by-products

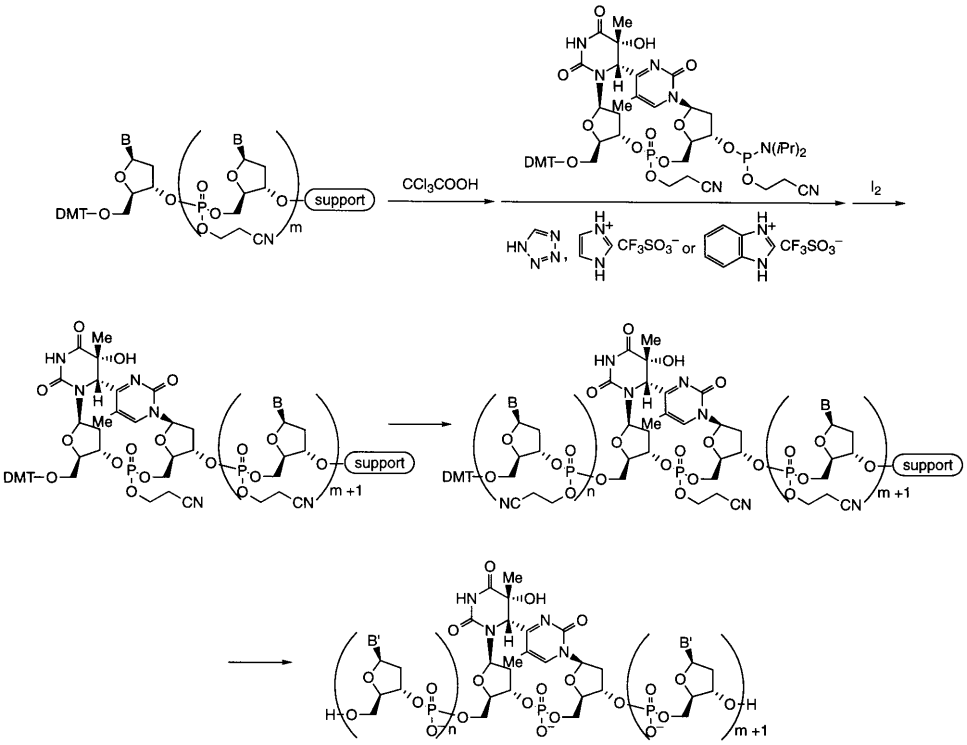

Figure 1. Scheme for the synthesis of oligonucleotides containing the (6–4) photoproduct. Tetrazole, imidazolium triflate or benzimidazolium triflate was used as activator.

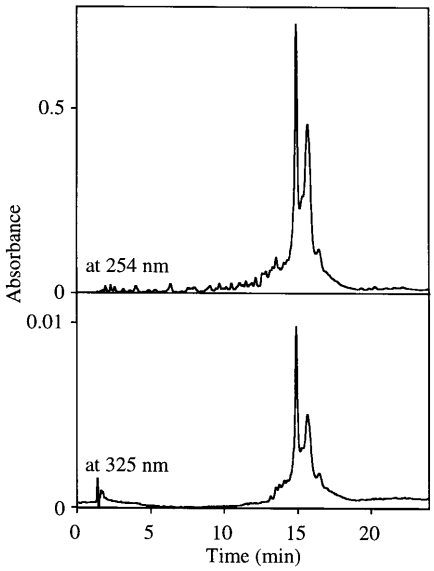

Figure 2. Reversed phase HPLC analysis of the crude mixture after the synthesis of a 49mer on a 0.2 µmol scale. The elution profile monitored at 325 nm, which is characteristic of the (6–4) photoproduct, shows the accurate molar ratio of the by-products to the desired 49mer, because the ε_{325} value is independent of chain length.

depended on chain length. (ii) The retention times of the by-products were longer than that of the desired product in both reversed phase and anion exchange HPLC analyses. (iii) The $\varepsilon_{260}/\varepsilon_{325}$ ratio of the by-product was larger than that of the desired product. These observations strongly suggested that the by-products were oligonucleotides branching at the (6–4) photoproduct. The result of acylation at the N3 position in our previous study (4) and the formation of a similar by-product in the TC (6–4) case (5) suggested that the branch point was N3.

Another result supporting our assumption was obtained when the same 49mer was synthesized on a 40 nmol scale. As shown in Figure 3A, the peak of the desired 49mer (indicated by an arrow) was very small and the by-products were the major product, probably because the phosphoramidites were used in a much larger excess over the elongating oligonucleotide. To obtain long chain oligonucleotides in good yield, we tried to protect the N3 position, but it was too difficult to find a protecting group that was compatible with the other protecting groups and with the labile (6–4) photoproduct. As an alternative method, activators other than tetrazole were examined using the extreme conditions of the 40 nmol synthesis.

Synthesis using imidazolium triflate and benzimidazolium triflate as activators

First, imidazolium triflate was used as activator. Since this compound was used as a highly O-selective activator in oligonucleotide synthesis without base protection (11), it might prevent coupling with the imino function. The 49mer was synthesized by replacing the tetrazole solution with a 0.1 M solution of imidazolium triflate in anhydrous acetonitrile. The reversed phase HPLC elution profile of the deprotected mixture is shown in Figure 3B. Although the reaction time for coupling of the dA, dG, dC and T phosphoramidites was extended to 1 min, according to the literature (11), shorter oligonucleotides, due to low coupling yields, were found before elution of the desired

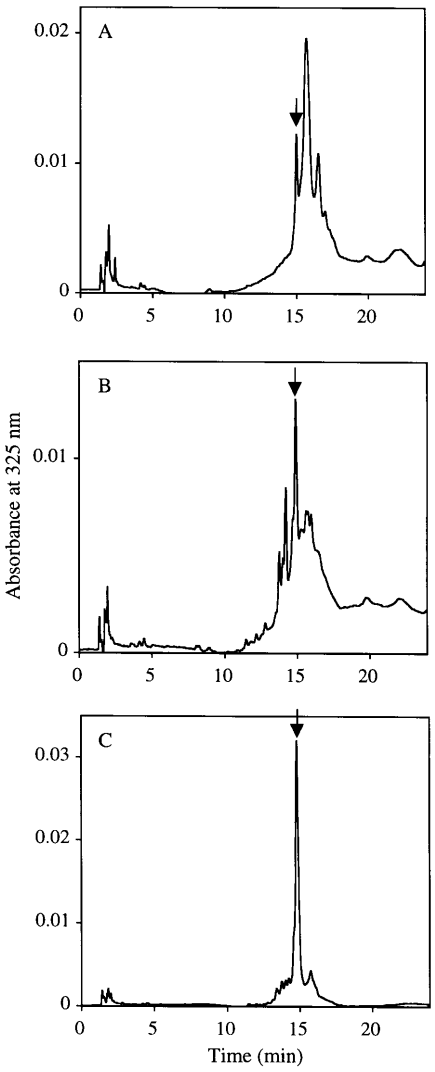

Figure 3. Reversed phase HPLC elution profiles of the crude mixtures of the 49mers synthesized on a 40 nmol scale using tetrazole (**A**), imidazolium triflate (**B**) and benzimidazolium triflate (**C**). The peak of the desired 49mer in each analysis is indicated by an arrow.

49mer. Formation of the by-products was prevented to some extent, but the result was not satisfactory.

Next, benzimidazolium triflate, which has a lower pK_a value than imidazolium triflate (12), was tested. In this case, a 0.2 M solution in acetonitrile was used and the procedure was exactly the same as that for the tetrazole-activated synthesis. Reversed phase HPLC analysis of the deprotected mixture (Fig. 3C) revealed that this activator was comparable to tetrazole in coupling efficiency and reduced by-product formation to a great extent. This (6–4) photoproduct-containing 49mer was purified to give a single peak in both reversed phase and anion exchange HPLC analyses, as shown in Figure 4. Its overall yield from the first nucleoside was 23%. Degradation of this 49mer with nuclease P1 and alkaline phosphatase gave an HPLC elution profile indistinguishable from that of the degradation products from the tetrazole-activated 49mer (Fig. 5) and the nucleoside composition was dA:dG:dC:T = 14.8:8.6:12.0:11.6 (the theoretical value is 15:9:12:11).

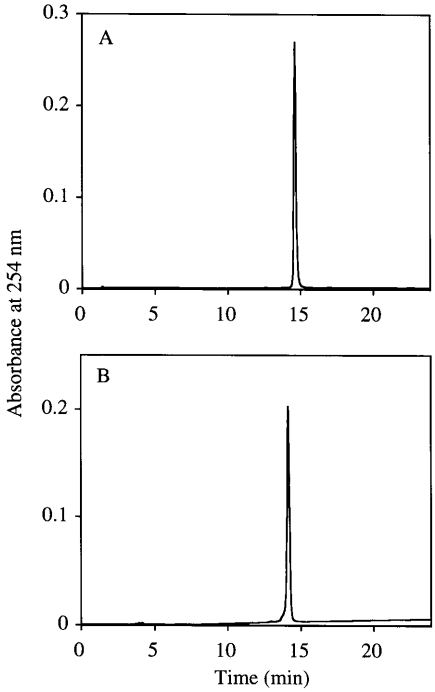

Figure 4. HPLC analyses of the purified 49mer on reversed phase (**A**) and anion exchange (**B**) columns. In a co-injection experiment on the anion exchange column, this 49mer was not separated from an undamaged 49mer with the same sequence.

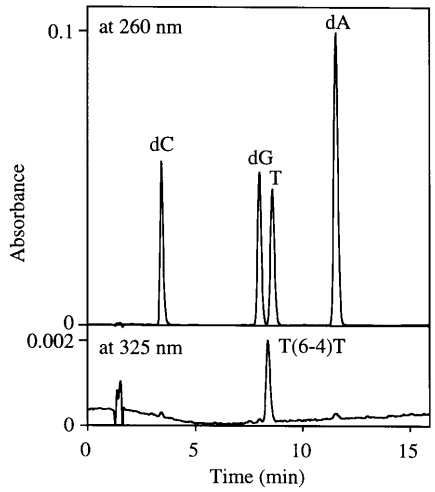

Figure 5. HPLC analysis of the nucleoside components of the 49mer produced by degradation with nuclease P1 and alkaline phosphatase. The (6–4) photoproduct was detected at 325 nm and its retention time was identical to that of the authentic dinucleoside monophosphate of the (6–4) photoproduct prepared by UV irradiation of d(TpT).

Detection of proteins that bind the (6–4) photoproduct-containing DNA

The 49mer was used to detect proteins that recognize DNA containing this photoproduct. A previously synthesized 30mer, d(CTCGTCAGCATCT(6–4)TCATCATACAGTCAGTG) (4), was used for comparison. These oligonucleotides were labeled

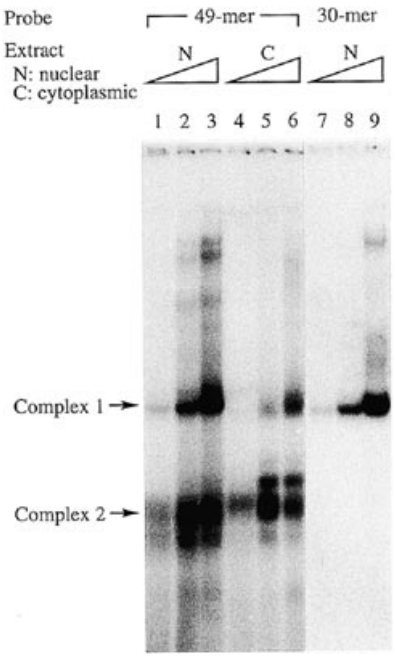

Figure 6. Detection of proteins that bind the (6–4) photoproduct-containing DNA by electrophoretic mobility shift assays. Nuclear (lanes 1–3 and 7–9) and cytoplasmic (lanes 4–6) extracts from HeLa cells were mixed with the (6–4) 49mer (lanes 1–6) or (6–4) 30mer (lanes 7–9) probe and the mixtures were subjected to electrophoresis on a non-denaturing 5% polyacrylamide gel. The free probes were run off in this experiment.

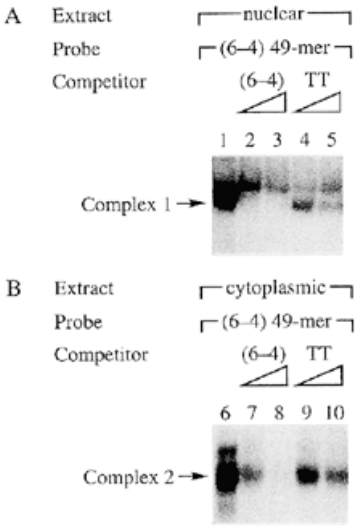

Figure 7. Analysis of photoproduct specificity by competition experiments. Nuclear and cytoplasmic extracts were used to determine the specificity in complexes 1 (**A**) and 2 (**B**), respectively. The ^{32}P-labeled (6–4) 49mer duplex was used as probe and an unlabeled (6–4) photoproduct-containing (lanes 2, 3, 7 and 8) or undamaged (lanes 4, 5, 9 and 10) duplex was added as competitor. The competitor/probe ratios were 0 (lanes 1 and 6), 200 (lanes 2, 4, 7 and 9) and 800 (lanes 3, 5, 8 and 10).

using [γ-^{32}P]ATP and T4 polynucleotide kinase and were annealed to each complementary strand. Using these oligonucleotide duplexes as probes, electrophoretic mobility shift assays of nuclear and cytoplasmic extracts from HeLa cells were carried out. As shown in Figure 6, several bands of ^{32}P-labeled DNA–protein complexes were obtained. When the (6–4) 49mer was used as a probe, two major bands (complexes 1 and 2) were detected in both nuclear and cytoplasmic extracts, but in the 30mer case, only a single band was detected for the nuclear extract, with a mobility corresponding to that of complex 1 in the 49mer experiment. These results were highly reproducible and no clear complex formation was observed when the cytoplasmic extract was assayed using the 30mer probe (data not shown). As a result, the difference between the 49mer and 30mer probes showed the effectiveness of long chain oligonucleotides in biochemical experiments.

To determine whether complex formation was specific to the (6–4) photoproduct, competition experiments were carried out using unlabeled duplexes with and without the photoproduct as competitors, as shown in Figure 7. In consideration of the clarity of each band, nuclear and cytoplasmic extracts were used to test complexes 1 and 2, respectively, and the results demonstrated that these bands were obtained by formation of complexes specific to the (6–4) photoproduct in DNA. Purification and characterization of the proteins detected in this study are in progress.

CONCLUSION

In this study, benzimidazolium triflate was found to be an efficient activator in the synthesis of (6–4) photoproduct-containing oligonucleotides and to improve the yield and the purity of the desired product. Although short and medium length oligonucleotides containing this photoproduct have been used in studies of the molecular biology of mutation and DNA repair, long chain oligomers, which this activator enabled us to synthesize, will make a greater contribution to various fields in biology.

REFERENCES

1 LeClerc,J.E., Borden,A. and Lawrence,C.W. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9685–9689.
2 Smith,C.A., Wang,M., Jiang,N., Che,L., Zhao,X. and Taylor,J.-S. (1996) *Biochemistry*, **35**, 4146–4154.
3 Kamiya,H., Iwai,S. and Kasai,H. (1998) *Nucleic Acids Res.*, **26**, 2611–2617.
4 Iwai,S., Shimizu,M., Kamiya,H. and Ohtsuka,E. (1996) *J. Am. Chem. Soc.*, **118**, 7642–7643.
5 Mizukoshi,T., Hitomi,K., Todo,T. and Iwai,S. (1998) *J. Am. Chem. Soc.*, **120**, 10634–10642.
6 Fujiwara,Y. and Iwai,S. (1997) *Biochemistry*, **36**, 1544–1550.
7 Hitomi,K., Kim,S.-T., Iwai,S., Harima,N., Otoshi,E., Ikenaga,M. and Todo,T. (1997) *J. Biol. Chem.*, **272**, 32591–32598.
8 Sugasawa,K., Ng,J.M.Y., Masutani,C., Iwai,S., van der Spek,P.J., Eker,A.P.M., Hanaoka,F., Bootsma,D. and Hoeijmakers,J.H.J. (1998) *Mol. Cell*, **2**, 223–232.
9 Jing,Y., Kao,J.F.-L. and Taylor,J.-S. (1998) *Nucleic Acids Res.*, **26**, 3845–3853.
10 Smith,C.A. and Taylor,J.-S. (1993) *J. Biol. Chem.*, **268**, 11143–11151.
11 Hayakawa,Y. and Kataoka,M. (1998) *J. Am. Chem. Soc.*, **120**, 12395–12401.
12 Hayakawa,Y., Kataoka,M. and Noyori,R. (1996) *J. Org. Chem.*, **61**, 7996–7997.
13 Sinha,N.D., Davis,P., Usman,N., Pérez,J., Hodge,R., Kremsky,J. and Casale,R. (1993) *Biochimie*, **75**, 13–23.
14 Masutani,C., Sugasawa,K., Yanagisawa,J., Sonoyama,T., Ui,M., Enomoto,T., Takio,K., Tanaka,K., van der Spek,P.J., Bootsma,D., Hoeijmakers,J.H.J. and Hanaoka,F. (1994) *EMBO J.*, **13**, 1831–1843.