Detection of covalent triplex within human cells

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ABSTRACT

Triple helix-forming oligonucleotides covalently linked to psoralen can be specifically cross-linked to both strands of DNA at the triplex–duplex junction following UV irradiation. We have previously shown that a 15mer psoralen–oligonucleotide conjugate forming a triple helix on the promoter of the α subunit gene of the interleukin-2 receptor inhibits transcription of reporter plasmids transfected into living cells after irradiation. In the present work, we directly demonstrate covalent triple helix formation at the target site inside cells. A primer extension assay using Taq polymerase was developed to quantitate the DNA which had reacted with the psoralen of the triple helix-forming oligonucleotide. Photoaddition of the psoralen at the DNA target site was demonstrated, not only when the preformed triplex was electroporated inside cells, but also when the oligonucleotide was added to the culture medium after plasmid electroporation and before irradiation of the cells.

INTRODUCTION

Oligopyrimidine and oligopurine oligonucleotides have been shown to bind to the major groove of duplex DNA at polypurine·polypyrimidine sequences forming a triple helical structure (1–5). Intermolecular triplex-forming oligonucleotides have been shown to inhibit transcription in several systems at the level of either elongation (6–10) or initiation through inhibition of transcription factor binding (3,11,12). Several reports have described the effect of oligonucleotides in eukaryotic cells (9,12–17). However, in all these experiments there was no direct demonstration of triple helix formation inside cells.

Psoralen–oligonucleotide conjugates can be used to cross-link both strands of the target in a sequence-specific manner (18,19). Covalent triple helix can efficiently inhibit transcription of reporter plasmids transfected into living cells, as previously demonstrated on the promoter of the α subunit of the interleukin-2 receptor (IL-2Rα) (13). Inhibition was observed when UV-induced

cross-linking occurred both *in vitro* and *in vivo*. In these experiments a mutant of the target sequence was used to demonstrate that the inhibitory effect was due to triple helix formation (13,16).

In the present work, we have used a 15mer psoralen–oligonucleotide conjugate directed to the IL-2Rα promoter carried by a plasmid vector to directly detect triple helix formation inside human cells. An amplified primer extension assay has been developed to quantitate the site-specific psoralen photoadducts on DNA.

DNA polymerases are efficiently stopped at covalently modified bases in DNA (20,21). Recently it was shown that triple helix-forming oligonucleotide clamps coupled to a psoralen derivative induced, after irradiation, a stop of elongation at the photoaddition site (22). In the present work, the use of Taq polymerase which is stopped at psoralen-induced mono- and bi-adducts (20) allowed detection of photoproducts on an electroporated plasmid and to demonstrate triple helix formation inside cells.

MATERIALS AND METHODS

Plasmids and oligonucleotides

The plasmid constructs containing wild-type and mutant IL-2Rα promoter regions (positions –352 to +110) were described previously (1,13; see Fig. 1). The psoralen derivative of the 15mer oligonucleotide (Pso-15meC; see Fig. 1) was synthesized as previously described (18). Unmodified oligonucleotides were purchased from Eurogentec (Belgium). They were purified on denaturing polyacrylamide gels.

Photoinduced cross-linking experiments *in vitro*

Plasmids were incubated with various concentrations of Pso-15meC in the indicated buffer and irradiated using a Xenon lamp (150 W) in a Cunow housing system. The light was filtered through a Pyrex filter to remove radiation below 310 nm. A filter removing radiation below 390 nm (Type GG 395; Schott) was added to obtain a majority of mono-adducts. The conditions of

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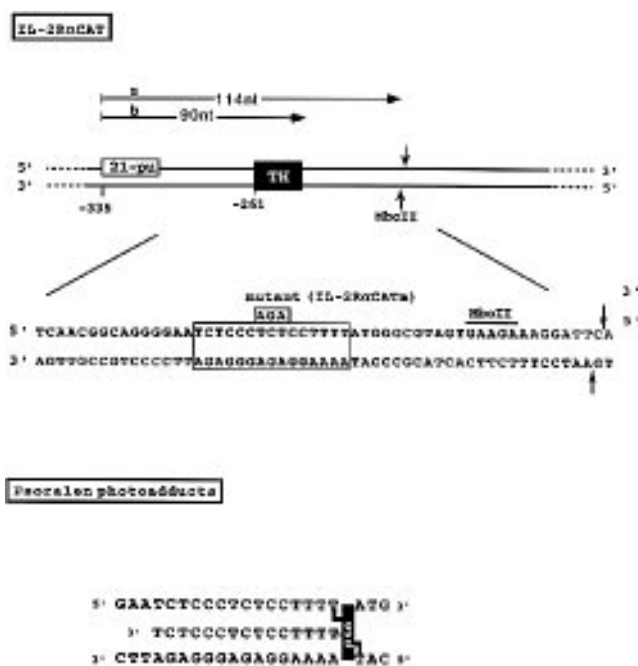+Present address: Genetics and Biochemistry Branch, NIKDD, NIH, Building 10, Room 9, Bethesda, MD 20892, USA

Figure 1. (**Top**) Plasmid construct containing the IL-2Rα promoter region (–352 to +110) (see 13). TH indicates the position of the oligopurine–oligopyrimidine 15 bp sequence. After cleavage with *Mbo*II, the primer 21pu (5′-ACTCAGCT-TATGAAGTGCTGG-3′) was used for replication experiments. The products of replication corresponding to the covalent reaction of psoralen on the template (90 nt, b) and unreacted template (114 nt, a) are represented at the top of the figure. (**Bottom**) Schematic representation of the psoralen bi-adducts obtained with Pso-15meC. The psoralen linked to the 5′-phosphate of the oligonucleotide preferencially reacts with the two thymines at the 5′-TpA-3′ site of the duplex.

DNA concentration, buffer incubation, temperature and time of irradiation are indicated in the figure legends.

Transfection experiments

Preformed triplex. IL-2Rα CAT plasmid (5 μg) was preincubated for 2 h at 4°C with 20 μM Pso-15-meC oligonucleotide in 10 μl buffer containing 10 mM KH_2PO_4, pH 6, 0.1 M NaCl. Samples of 10^7 cells (tumor T cell line C8166) were transfected by electroporation as previously described (12). After 15 min at 37°C, cells were irradiated at 4°C, washed three times with PBS and nuclei were isolated under mild lysis conditions (5 min on ice in a buffer containing 0.4% Nonidet P-40, 10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, centrifuged for 5 min at 500 *g* at 4°C and washed three times in PBS with 10 mM $MgCl_2$). This procedure was performed to eliminate extracellular plasmid (which could interfere in the extension reaction). Nuclei were lysed with 0.5% SDS. After proteinase K digestion (10 μg/ml, 2 h at 37°C), total DNA was extracted in phenol/chloroform saturated with 0.3 M sodium acetate, precipitated twice with 5 vol ethanol and digested with *Mbo*II endonuclease (0.2 U/μl).

Triplex formation in isolated nuclei. Samples of 10^7 cells were transfected with IL-2Rα CAT plasmid (5 μg). After 15 min at 37°C nuclei were isolated and incubated with 20 μM Pso-15meC at 37°C in PBS with 10 mM $MgCl_2$ in a final volume of 100 μl. After various incubation times nuclei were irradiated for 30 min

and DNA was extracted, precipitated and digested with *Mbo*II endonuclease as described.

Triplex formation in cells. Samples of 10^7 cells transfected with IL-2Rα CAT plasmid (5 μg) were incubated at 37°C. After 15 min, 20 μM Pso-15meC was added. Cells were irradiated after 1 h incubation. Nuclei were isolated before DNA extraction and cleavage with *Mbo*II endonuclease.

Linear amplified primer extension assay

The *Mbo*II endonuclease cleaves the template in the IL-2Rα promoter sequence 24 nt downstream of the 3′-end of the oligopyrimidine strand of the triple helix target sequence (see Fig. 1). A primer (21pu) corresponding to a sequence beginning 114 nt upstream of the *Mbo*II site and 90 nt upstream of the oligopurine tract 5′-end (Fig. 1) was used for extension reactions.This primer, 21pu (8 nM), was 5′-labeled with γ-^{32}P and mixed with DNA sample in 50 μl extension reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, 1.5 mM $MgCl_2$, 0.01% Tween 20], 1 mM each dNTP and 2.5 U Taq polymerase (Eurobio) were added and samples were overlaid with 50 μl mineral oil. The primer extension reactions were performed in a thermal cycling unit (Hybaid) under the following conditions: 95°C for 2 min, 55°C for 55 s, 72°C for 2 min; 25 cycles were performed. Samples were separated after ethanol precipitation by electrophoresis on an 8% polyacrylamide denaturing gel. The gel was dried and analyzed using a Molecular Dynamics PhosphorImager to quantitate the primer extension stops at the photoaddition sites.

Quantitation of the plasmid photoproducts

The plasmid IL-2Rα CAT (1 μg) was incubated with 10 μM Pso-15meC oligonucleotide, irradiated and digested at 65°C with *Tfi*I endonuclease liberating a 37 bp fragment containing the triple helix site (Fig. 3A). Samples were electrophoresed on a denaturing 20% polyacrylamide gel. After migration the gel was blotted on a nylon membrane (Hybond N^+; Amersham) in 10× SSC. Membrane was prehybridized for 2 h at 45°C in 5× SSC, 0.1% SDS, 200 μg/ml sonicated and denatured herring sperm DNA. Hybridization of 5′-^{32}P-labelled oligonucleotide 25pu (complementary to the purine-rich strand of the target) or 25py (complementary to the pyrimidine-rich strand of the target) (10 pmol) was performed in 5× SSC, 5% SDS, in the presence of 10 μg/ml denatured and sonicated herring sperm DNA, for 12 h at 47°C. The membrane was washed twice in 2× SCC, 0.1% SDS for 45 min at room temperature and analyzed using a Molecular Dynamics PhosphorImager. Hybridization of a control oligo-nucleotide complementary to another fragment of the cleaved plasmid allowed us to normalize the quantity of DNA.

Oligonucleotide stability and cellular uptake

For oligonucleotide uptake and stability studies, 5×10^6 C8166 cells were incubated at 37°C with 10 μM oligonucleotide Pso-15 meC in 75 μl RPMI 1640, 20 mM HEPES with 10% heat-inactivated fetal calf serum. After various times of incubation cells were centrifuged. The oligonucleotide was extracted from the culture medium with phenol/chloroform saturated with 0.3 M sodium acetate and precipitated with ethanol. The cell pellet was washed three times in PBS, lysed in 10 mM Tris–HCl, pH 7.2, 10 mM NaCl, 2 mM $MgCl_2$, 0.1% SDS

and treated with proteinase K (100 µg/ml) for 1 h at 37°C. Oligonucleotide was extracted with phenol/chloroform saturated with 0.3 M sodium acetate and precipitated with ethanol. Samples were electrophoresed in a denaturing 20% acrylamide gel. After migration the gel was blotted on a nylon membrane and hybridized with 5′-³²P-labelled 15mer AS (5′-AGAGGGA-GAGGAAAA-3′) complementary to Pso-15 meC.

RESULTS AND DISCUSSION

Detection of photoproducts on a plasmid template *in vitro*

A pyrimidine oligonucleotide–psoralen conjugate was previously shown to induce cross-linking of the two DNA strands after irradiation when it formed a triple helix, provided that there was a 5′-TpA-3′ step at the triplex–duplex junction (18). Analysis of cross-linked products showed that the 4′-5′ (furan) double bond mainly reacts with the thymine adjacent to the oligopurine target sequence. The resulting mono-adduct can absorb light at wavelengths near 365 nm and form a bi-adduct (Fig. 1). Mono-adducts involving the psoralen 3–4 (pyrone) double bond cannot be converted into bi-adducts.

The efficiency and specificity of triple helix formation with psoralen–15mer conjugates were studied on plasmid IL-2Rα CAT with a sensitive detection method using Taq DNA polymerase primer extension. After triple helix formation and irradiation, the plasmid was digested with the endonuclease *Mbo*II. Extension of the radiolabelled primer 21pu yielded a full-length product of 114 nt (Fig. 1). An arrest of Taq DNA polymerase by a psoralen adduct should give rise to a truncated product of 90 nt.

When irradiation was omitted, or in the absence of third strand, only a 114 nt product was obtained (Fig. 2, lanes 3 and 4). This fragment corresponded to termination of DNA synthesis at the *Mbo*II site. In the presence of the triplex-forming oligonucleotide and after irradiation (λ > 310 nm), a band appeared corresponding to termination of synthesis at the adenine preceeding the thymine which had reacted with the psoralen on the template strand. This result is in agreement with those obtained with triple helix-forming oligonucleotide clamps using modified T7 DNA polymerase (Sequenase) (22). A second minor band one base before the major stop appeared when irradiation time was increased (Fig. 2, lane 6). The same phenomenon has been previously observed by Sage and Moustacchi (21) using T4 DNA polymerase and DNA reacted with free psoralen. In experiments performed with the mutated plasmid IL-2Rα CATm, which cannot form a triple helix (see 1), no stop of polymerase was detected at the TpA site (Fig. 2, lane 2). This showed that both stops observed in the wild-type plasmid were specific and due to triple helix formation. Since two other TpA sites were present on the DNA fragment, the absence of stop at these sites for long irradiation times provided evidence for the specificity of psoralen reaction at the duplex–triplex junction directed by oligo-nucleotide binding to its target sequence.

The intensity of the truncated product reached a plateau after 6 min irradiation at λ > 310 nm. In subsequent experiments, an irradiation period of 10 min was adopted. With an excess of third strand oligonucleotide a plateau of 65% truncated product was reached. Polymerization conditions with Taq DNA polymerase (denaturation at 95°C and polymerization at 72°C) allowed dissociation of the three strands attached to the psoralen, but the cross-link between the two strands of DNA is expected to favour
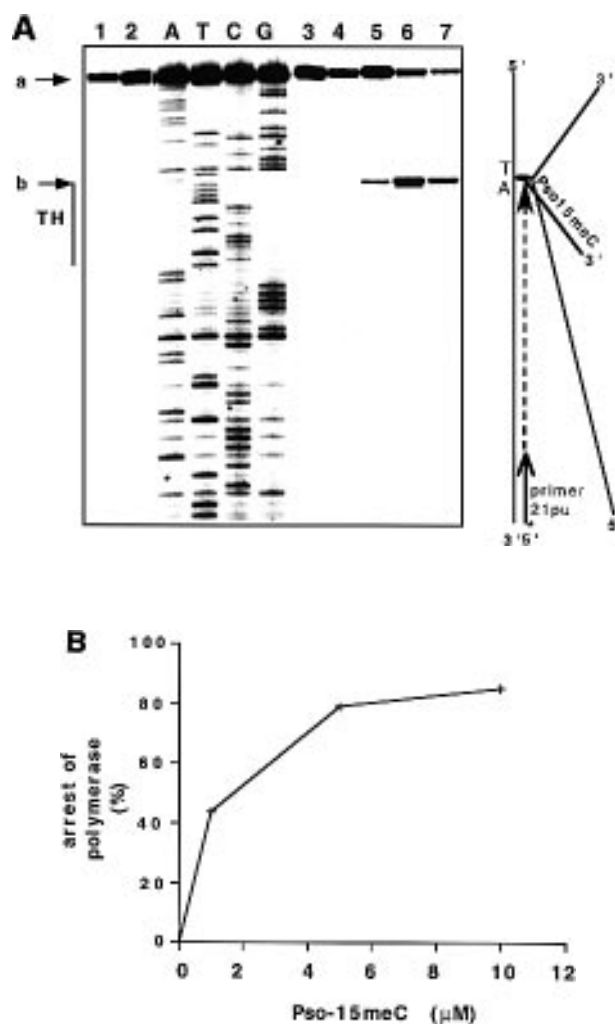

Figure 2. Detection of covalent psoralen adducts using a primer extension assay and the plasmid as a template. (**A**) Gel electrophoresis of the replication products. The purine strand of the plasmid digested with *Mbo*II was replicated using the primer 21pu (see Fig. 1). The plasmid IL-2Rα CAT (lanes 3–7) or IL-2Rα CATm (lanes 1 and 2) was incubated at 20°C for 2 h in 10 mM KH₂PO₄, pH 6.5, 0.1 M NaCl, in the presence of 10 µM Pso-15meC (lanes 1, 2 and 4–7). Samples were irradiated (λ > 310 nm) for 1 (lane 5), 5 (lane 6) or 10 min (lanes 1–3 and 7). Lane 4 is the non-irradiated control. Lanes A, T, C and G, Sanger sequence of the synthesized strand. The position of the arrest at the *Mbo*II digestion site is shown by arrow a, the arrest at the TpA photoaddition site is shown by arrow b. (**B**) Effect of increasing concentrations of Pso-15meC on the fraction of elongation product stopped at the photoaddition site (arrow b in A).

their renaturation during primer hybridization at 55°C. The template fraction which actually reacts with psoralen should therefore be underestimated by the primer extension method. To test this hypothesis, experiments were performed where mono-adducts were selectively produced by irradiation in the presence of a filter which removed radiations below 390 nm. Replication arrest at the photoaddition site was determined on the plasmid exposed for 10 min to irradiation in the presence of the filter (Fig. 2B). A plateau corresponding to 85% polymerase stop was reached. This value is in accordance with previous studies showing that 85% of furane side mono-adducts were formed on the oligopurine- containing strand of a comparable sequence (18).
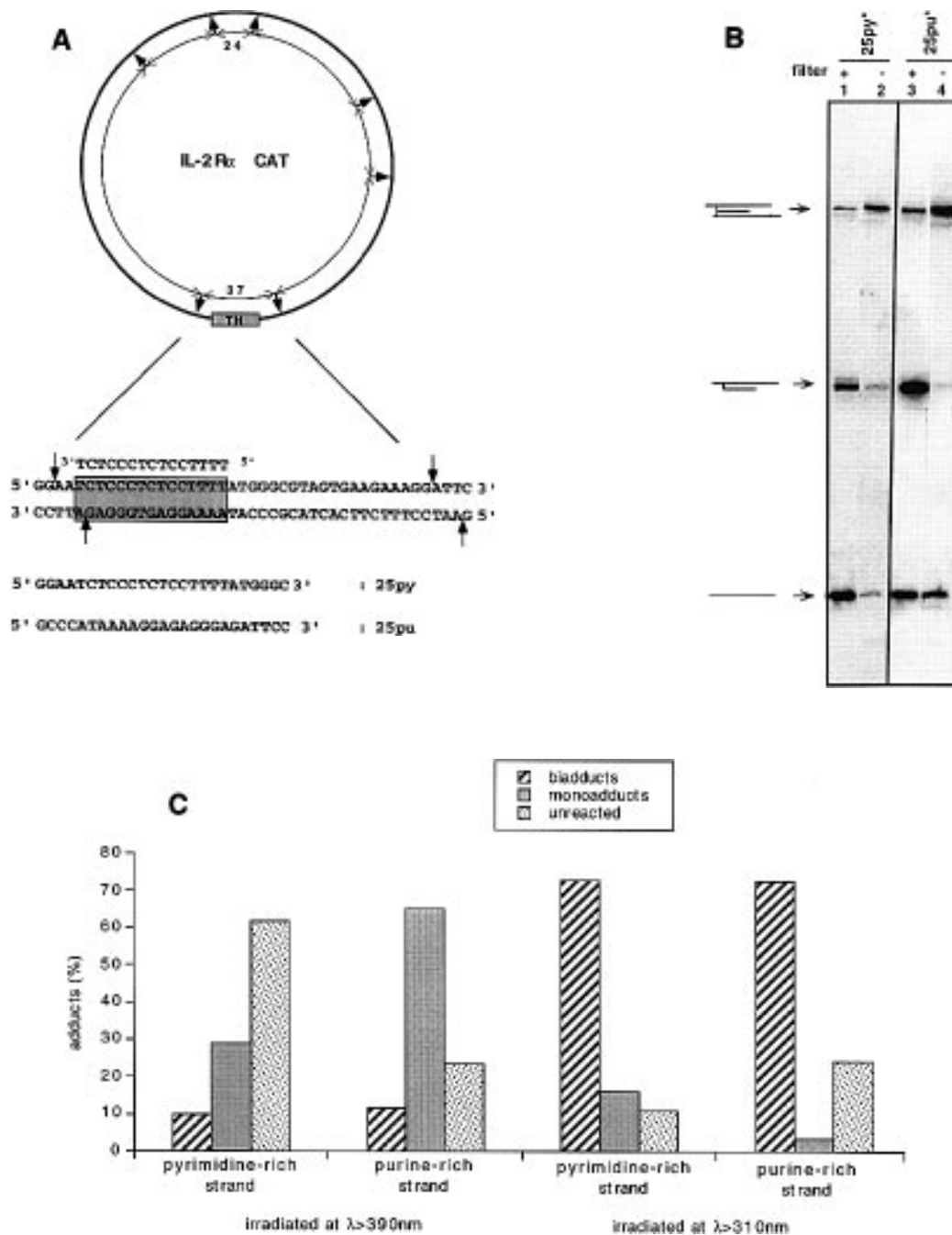
Figure 3. Detection of photoaddition products on plasmid IL-2Rα CAT. (**A**) Schematic representation of *Tfi*I cleavage sites on the plasmid (indicated by arrows). A 37 bp fragment, containing the oligopurine·oligopyrimidine sequence was obtained by cleavage at 65°C with *Tfi*I. The sequences of the oligonucleotides used as probes for hybridization experiments are indicated. (**B**) Southern blot of *Tfi*I-digested plasmid hybridized with oligonucleotide 25pu (lanes 1 and 2) or 25py (lanes 3 and 4). The plasmid incubated with 10 μM Pso-15meC was irradiated for 10 min in the presence of a filter removing radiation below 390 nm (lanes 1 and 3) or in the absence of the filter (λ > 310 nm) (lanes 2 and 4). A schematic representation of the photoaddition products is given on the left-hand side. (**C**) The percentage of each adduct was calculated from a quantitative analysis of the bands shown in (B).

To confirm that the primer-induced replication method allowed us to quantitate photoproducts, experiments were performed using another method of detection. After preincubation with the oligonucleotide and irradiation at λ > 310 nm or λ > 390 nm, the plasmid was cleaved at 65°C with the endonuclease *Tfi*I (Fig. 3A). Photoproducts were revealed on the purine and pyrimidine strands using radiolabelled 25mer complementary oligonucleotides to probe the different species after gel electrophoresis (Fig. 3B, lanes 1, 2 and 3, 4 respectively; see Materials and Methods). Quantitative analysis of photoproducts (Fig. 3C) showed that the presence of bi-adducts led to a slight underestimation of the psoralen reaction when Taq polymerase was used to detect photoproducts. When irradiation was performed without a filter (λ > 310 nm), the maximum of polymerase arrest at the
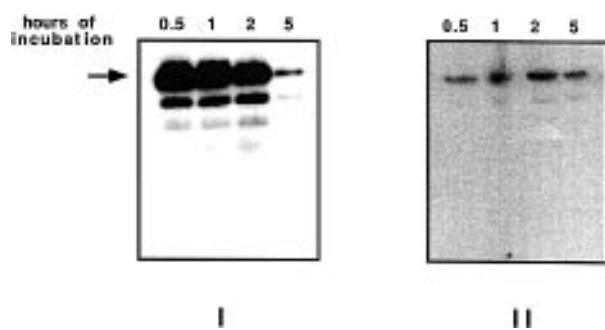
Figure 4. Oligonucleotide stability and cellular uptake. Pso-15meC unprotected at the 3′-end was incubated with C8166 cells and was extracted from the medium (I) or from cells (II) as described in Materials and Methods. After electrophoresis the gel was hybridized with the radiolabelled oligonucleotide 15mer AS, complementary to the Pso-15meC sequence (see sequence in Materials and Methods). The time of oligonucleotide incubation with the cells is indicated at the top of the gel in hours. The position of intact Pso-15meC is indicated by the arrows.

photoaddition site was 65%. Under the same conditions, 75% of photoproducts (mostly bi-adducts) were detected on the purine-rich strand by hybridization of radiolabelled 25py (Fig. 3B, lane 2). However, in the presence of the filter ($\lambda > 390$ nm) more than 80% of the purine-rich strand had reacted with the psoralen, forming mostly mono-adducts, in agreement with the plateau at 85% observed in the primer extension assay (see Fig. 2B). From these results, it was clear that the primer extension assay using a template irradiated with a filter ($\lambda > 390$ nm) was suitable for quantitating psoralen–DNA mono-adducts.

Oligonucleotide stability and cellular uptake

Oligonucleotide stability and uptake were studied by incubating cells or nuclei with the psoralen 15mer conjugate. After various times of incubation, oligonucleotide extracted from the cells was purified and analysed on a polyacrylamide gel (see Materials and Methods). The gel was subsequently blotted and hybridized to a ^{32}P-labelled oligonucleotide complementary to the 15mer sequence of Pso-15meC (15AS). This method avoided the problem of ^{32}P release due to phosphatase activity in the cell and allowed us to study cell uptake and degradation of 5′- and 3′-modified oligonucleotides.

The Pso-15meC degradation profile in the cell line C8166 is presented in Figure 4. After 2 h incubation, 50% of Pso-15meC remained intact in the medium. This oligonucleotide was 5′-protected by psoralen and was probably degraded by 3′-exonucleases and also by endonucleases. Degradation products (shorter than 10 nt) could not be detected by hybridization of the labelled complementary oligonucleotide. The oligonucleotide was extracted from cells at different times of incubation and analysed by gel electrophoresis (Fig. 4). The intact Pso-15meC was recovered from C8166 cells after 30 min incubation. The intracellular oligonucleotide concentration was maximal after 2 h incubation. This result, in accordance with several reports, showed that full-length oligonucleotide persisted for several hours within cells (14,23–25). When incubated with isolated nuclei, the 3′-unmodified Pso-15meC was quickly degraded in the medium (data not shown). After 1 h incubation, the full-length oligonucleotide was almost undetectable in the medium.

Triplex formation inside cells

The plasmid IL-2Rα CAT was transfected together with Pso-15meC into C8166 cells by electroporation. This cell line constitutively expresses the Tax protein, which transactivates IL-2Rα gene transcription (26–29).

Transfected cells were irradiated 15 min after electroporation. To avoid any contamination by external plasmid or plasmid associated with the cell membrane, plasmid DNA was extracted from nuclei and photoproducts were detected by primer extension (Fig. 5A). When cells were irradiated for 30 min (15 min after transfection), a significant fraction of the plasmid was detected as cross-linked to the oligonucleotide, as shown by the stop of polymerase at the photoaddition site (Fig. 5A). Of the purine-rich strand, 30% had reacted with the psoralen linked to the oligonucleotide after 30 min irradiation. To confirm that the signal was due to intracellular plasmid, the same experiment was performed after treatment of the cells with DNase I to degrade membrane-bound plasmids before isolating the nuclei. The fraction of the plasmid template that had reacted with psoralen and the signal intensity were similar to those obtained when cells were not treated with DNase I (data not shown). These experiments showed that the triple helical complex preformed *in vitro* was stable, at least to a certain extent, within the cells. However, the fraction of plasmid which was covalently modified by the psoralen was lower (50%) than that obtained *in vitro* (~85%). Several mechanisms can account for this difference. Thirty minutes irradiation might not be sufficient within cells to reach the plateau; the triplex may partially dissociate during electroporation or inside the cells. Previous studies have shown that CAT gene expression directed by the IL-2Rα promoter was inhibited after irradiation of HSB2 cells transfected with plasmid in the presence of Pso-15meC (13). The extent of inhibition was 70% when cells were irradiated 15 min after electroporation.

Experiments were then performed to demonstrate triplex formation inside cells. To eliminate the problem of oligonucleotide penetration into the cells, triple helix formation on the plasmid template was first demonstrated in isolated nuclei obtained from cells electroporated with plasmid in the absence of the oligonucleotide. The results are shown in Figure 5B. When nuclei of electroporated cells were incubated for 1 h with Pso-15meC, photoaddition products were detected. A significant fraction of the target reacted with the psoralen derivative, since 30% of the template was cross-linked to the oligonucleotide after 30 min irradiation. When nuclei were incubated for 2 h with the oligonucleotide before irradiation, the fraction of photoproducts was significantly reduced (Fig. 5B). This result is in accordance with the kinetics of degradation of the Pso-15meC oligonucleotide in the presence of nuclei. These experiments showed that the triple helix was formed inside nuclei, indicating that the oligonucleotide was able to find its target in the nuclear environment. As observed when triplex was preformed outside the cells, the fraction of template which reacted with the oligonucleotide did not exceed 30%. It has been previously shown that oligonucleotides are quickly incorporated in isolated nuclei (30), but an important fraction of the recovered oligonucleotide was associated with nuclear proteins (31,32).

After demonstrating triple helix formation in isolated nuclei, we performed experiments where cells were first electroporated with plasmid and then incubated with the oligonucleotide for 1 h. The cells were then irradiated and nuclei purified as described
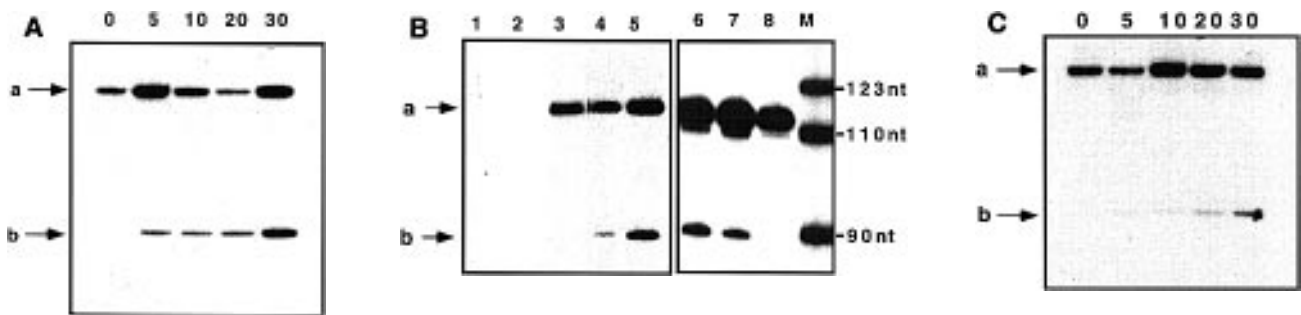
Figure 5. Detection of covalent triple helix on the plasmid inside cells. (**A**) Detection of photoproducts on the IL-2Rα CAT plasmid preincubated with Pso-15meC and electroporated inside C8166 cells as described in Materials and Methods. The times of irradiation of the cells are indicated at the top of the gel (in minutes). Polymerase arrest at the restriction site *Mbo*II and at the psoralen photoaddition site are indicated by arrows a and b respectively. (**B**) Detection of photoproducts formed in C8166 nuclei on the IL-2Rα CAT plasmid. (Left panel) Cells were electroporated with the plasmid, nuclei were isolated and incubated for 1 h with 20 μM Pso-15meC and irradiated for 10 (lane 4) or 30 min (lane 5). Lane 3 is the non-irradiated control. Non-electroporated cells were irradiated for 30 min (lane 1) or not irradiated (lane 2) in the presence of 20 μM Pso-15meC. (Right panel) Nuclei of cells previously electroporated with the plasmid were incubated for various periods of time with 20 μM Pso-15meC and irradiated for 30 min. Times of incubation were 30 (lane 6), 60 (lane 7) and 120 min (lane 8). M is a series of size markers obtained by digesting pBR322 with *Msp*I and radiolabelling the products. (**C**) Detection of photoproducts formed on the plasmid inside C8166 cells. Electroporated cells were incubated for 1 h with Pso-15meC and irradiated for various times (minutes) as indicated at the top of the gel.

above. The product of replication arrest at the photoaddition site was detected inside isolated nuclei, as shown in Figure 5C. When cells were irradiated for 30 min, 20% of the plasmid was detected as cross-linked to the oligonucleotide. This result indicated that the oligonucleotide Pso-15meC penetrates cells and binds to its target forming a local triple helix. As shown in Figure 5C, a plateau was not reached after 30 min irradiation. However, it was difficult to use longer irradiation times, due to the toxic effect on cell viability.

To make sure that covalent triplex was formed inside living cells and to eliminate dead cells we performed additional experiments. Viable cells were recovered by density gradient centrifugation on a sodium diatrizoate poly(sucrose) gradient (Histopaque-1077; Sigma) 24 h after electroporation. Cells were then incubated with the oligonucleotide, irradiated and the plasmid was extracted from nuclei. The fraction of cross-linked plasmid detected under these experimental conditions was the same as when cells were incubated with the oligonucleotide 15 min after electroporation (data not shown). This demonstrated that the photoproducts detected inside cells resulted from oligonucleotide penetration into viable cells and were not related to damaged cells resulting from the electroporation procedure. In all the experiments described above we did not detect any replication arrest at TpA sites other than the site targeted by the Pso-15meC oligonucleotide. Therefore the psoralen photoadducts detected by Taq polymerase are due to site-specific triple helix formation by the psoralen–15mer conjugate with its target site on the plasmid within cell nuclei.

CONCLUSION

Several reports have described specific inhibition of expression of endogenous genes inside cells, but in all these experiments direct evidence for triplex formation was not provided (10,14,15,17,33,34). Interpretation of the results can therefore be controversial. For example, Michelotti *et al.* (35) recently proposed that the previously reported inhibition of c-*myc* transcription (15) involved oligonucleotide interaction with *trans*-acting factors rather than triplex formation. The complexity of potential interactions of oligonucleotides with cellular compo-

nents other than the targeted nucleic acid sequence stresses the importance of correlating inhibition of gene expression to triple helix formation on the target DNA.

As shown previously in transient assays, the oligonucleotide Pso-15meC targeted to the IL-2Rα promoter leads after irradiation to a 70% decrease in reporter activity (13). In these experiments the plasmid and the oligonucleotide were co-transfected into the cells before irradiation. The results described above provide direct evidence that the oligonucleotide Pso-15meC does form a triple helix inside cells.

Recently, Svinarchuk *et al.* (36) studied triplex formation inside cells on a plasmid gene with an oligopurine third strand by *in vivo* DMS footprinting assay. They showed that a footprint was detectable only when the triple helix was preformed *in vitro* before electroporation. The differences between these data and our results may be explained by the use of different cell lines and because the methods of detection were not the same. In our experiments, we formed a covalent triple helix which is more favourable for detection than footprinting.

In the present study, we have shown that triple helix-directed cross-linking of the psoralen–oligonucleotide conjugate occurs not only when the plasmid and oligonucleotide are preincubated before electroporation followed by irradiation, but also when cells are incubated with the oligonucleotide after electroporation of the plasmid and then irradiated. Control experiments showed that the cross-linking reaction most likely occurred within the nucleus. Experiments with nuclei isolated from cells electroporated with the plasmid revealed that the target sequence was accessible to the oligonucleotide in the nuclear environment.

Several reports have described the repair of triple helix-induced photoproducts in cells (37–40). However, this mechanism seems to be inhibited under certain conditions (41,42). In our system we performed experiments indicating that the mono- and bi-adducts were not repaired either in HeLa nuclear extracts or in C8166 cells after 72 h incubation (manuscript in preparation).

Our data demonstrate that an oligonucleotide can form a specific triple helix inside cells on an exogenous target. The next step will be to demonstrate covalent triplex formation on the endogenous gene. For this purpose, modified oligonucleotides with increased stability in cell culture medium should be used.

The covalent attachment of a propylamino group at the 3′-terminus of Pso-15meC strongly reduced degradation in the medium, indicating that the 3′-unmodified oligonucleotide was mainly degraded by 3′-exonucleases (data not shown). Further work with this modified oligonucleotide will allow us to investigate triple helix formation in a quantitative way on the endogenous gene. Oligonucleotide analogues in which the phosphodiester linkages are replaced by N3′→P5′ phosphoramidate linkages form stable triple helices with targeted sequences (43). These analogues constitute an interesting tool to investigate the accessibility of DNA to triplex-forming oligonucleotides in cell nuclei.

ACKNOWLEDGEMENTS

REFERENCES

1 Le Doan,T., Perrouault,L., Praseuth,D., Habhoub,N., Decout,J.-L., Thuong,N.T., Lhomme,J. and Hélène,C. (1987) *Nucleic Acids Res.*, **15**, 7749–7760.
2 Moser,H.E. and Dervan,P.B. (1987) *Science*, **238**, 645–650.
3 Cooney,M., Czernuszewicz,G., Postel,E.H., Flint,S.J. and Hogan,M.E. (1988) *Science*, **241**, 456–459.
4 Beal,P.A. and Dervan,P.B. (1991) *Science*, **251**, 1360–1363.
5 Durland,R.H., Kessler,D.J., Gunnell,S., Duvic,M., Pettitt,B.M. and Hogan,M.E. (1991) *Biochemistry*, **30**, 9246–9255.
6 Young,S.L., Krawczik,S.H., Matteucci,M.D. and Toole,J.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10023–10026.
7 Duval-Valentin,G., Thuong,N.T. and Hélène,C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 504–508.
8 Maher,L.J. (1992) *Biochemistry*, **31**, 7587–7594.
9 McShan,W.M., Rossen,R.D., Laughter,A.H., Trial,J., Kessler,D.J., Zendegui,J.G., Hogan,M.E. and Orson,F.M. (1992) *J. Biol. Chem.*, **267**, 5712–5721.
10 Ross,C., Samuel,M. and Broitman,S.L. (1992) *Biochem. Biophys. Res. Commun.*, **189**, 1674–1680.
11 Maher,L.J., Wold,B. and Dervan,P.B. (1989) *Science*, **245**, 725–730.
12 Grigoriev,M., Praseuth,D., Robin,P., Hemar,A., Saison-Behmoaras,T., Dautry-Varsat,A., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1992) *J. Biol. Chem.*, **267**, 3389–3395.
13 Grigoriev,M., Praseuth,D., Guieysse,A.L., Robin,P., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3501–3505.
14 Orson,F.M., Thomas,D.W., McShan,W.M., Kessler,D.J. and Hogan,M.E. (1991) *Nucleic Acids Res.*, **19**, 3435–3441.
15 Postel,E.H., Flint,S.J., Kessler,D.J. and Hogan,M.E. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8227–8231.
16 Grigoriev,M., Praseuth,D., Guieysse,A.L., Robin,P., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1993) *C.R. Acad. Sci. Paris Life Sci.*, **316**, 492–495.
17 Ing,N.H., Beekman,J.M., Kessler,D.J., Murphy,M., Jayaraman,K., Zendegui,J.G., Hogan,M.E., O'Malley,B.W. and Tsai,M.J. (1993) *Nucleic Acids Res.*, **21**, 2789–2796.
18 Takasugi,M., Guendouz,A., Chassignol,M., Decout,J.L., Lhomme,J., Thuong,N.T. and Hélène,C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5602–5606.
19 Giovannangeli,C., Thuong,N.T. and Hélène,C. (1992) *Nucleic Acids Res.*, **20**, 4275–4281.
20 Hoepfner,R.W. and Sinden,R.R. (1993) *Biochemistry*, **32**, 7542–7548.
21 Sage,E. and Moustacchi,E. (1987) *Biochemistry*, **26**, 3307–3314.
22 Giovannangeli,C., Thuong,N.T. and Hélène,C. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 10013–10017.
23 Harel-Bellan,A., Ferris,D.K., Vinocour,M., Holt,J.T. and Farrar,W.L. (1988) *J. Immunol.*, **83**, 2431–2435.
24 Saison-Behmoaras,T., Tocqué,B., Rey,I., Chassignol,M., Thuong,N.T. and Hélène,C. (1991) *EMBO J.*, **10**, 1111–1118.
25 Yakubov,L.A., Deeva,E.A., Zarytova,V.F., Ivanova,E.M., Ryte,A.S., Yurchenko,L.V. and Vlassov,V.V. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6454–6458.
26 Hahn,B., Manzari,V., Colombini,S., Franchini,G., Galoo,R.C. and Wong-Staal,F. (1983) *Nature*, **303**, 253–256.
27 Inoue,J.I., Seiki,M., Taniguchi,T., Tsuru,S. and Yoshida,M. (1986) *EMBO J.*, **5**, 2883–2888.
28 Cross,S.L., Halden,N.F., Lenardo,M.J. and Leonard,W.J. (1989) *Science*, **244**, 466–468.
29 Maruyama,M., Shibuya,H., Harada,H., Hatakeyama,M., Seiki,M., Fujita,T., Inoue,J., Yoshida,M. and Taniguchi,T. (1987) *Cell*, **48**, 343–350.
30 Clarenc,J.P., Lebleu,B. and Léonetti,J.P. (1993) *J. Biol. Chem.*, **268**, 5600–5604.
31 Léonetti,J.P., Mechti,N., Degols,G., Gagnor,C. and Lebleu,B. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 2702–2706.
32 Geselowitz,D.A. and Neckers,L. (1992) *Antisense Res.*, **2**, 17–25.
33 Tu,G.C., Cao,Q.N. and Israel,Y. (1995) *J. Biol. Chem.*, **270**, 28402–28407.
34 Porumb,H., Gousset,H., Letellier,R., Salle,V., Briane,D., Vassy,J., Amor-Gueret,M., Israël,L. and Taillandier,E. (1996) *Cancer Res.*, **56**, 515–522.
35 Michelotti,E.F., Tomonaga,T., Krutzsch,H. and Levens,D. (1995) *J. Biol. Chem.*, **270**, 9494–9499.
36 Svinarchuk,F., Debin,A., Bertrand,J.R. and Malvy,C. (1996) *Nucleic Acids Res.*, **24**, 295–302.
37 Havre,P.A. and Glazer,P.M. (1993) *J. Virol.*, **67**, 7324–7331.
38 Sandor,Z. and Bredberg,A. (1994) *Nucleic Acids Res.*, **22**, 2051–2056.
39 Degols,G., Clarenc,J.P., Lebleu,B. and Léonetti,J.P. (1994) *J. Biol. Chem.*, **269**, 16933–16937.
40 Wang,G., Levy,D.D., Seidman,M.M. and Glazer,P.M. (1995) *Mol. Cell. Biol.*, **15**, 1759–1768.
41 Sandor,Z. and Bredberg,A. (1995) *FEBS Lett.*, **374**, 287–291.
42 Wang,G. and Glazer,P.M. (1995) *J. Biol. Chem.*, **270**, 22595–22601.
43 Escudé,C., Giovannangeli,C., Sun,J.-S., Lloyd,D.H., Chen,J.-K., Gryaznov,S.M., Garestier,T. and Hélène,C. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4365–4369.