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Inhibition of aromatase expression by a psoralen-linked triplex-forming oligonucleotide targeted to a coding sequence

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Abstract The cytochrome P450 enzyme aromatase (P450arom) is an important target in breast cancer treatment. We have designed a 20-base pyrimidine oligodeoxynucleotide (ODN) which forms a sequence-specific triple helix (triplex) with a purine-rich tract in the P450arom coding sequence. The psoralen-linked ODN (Pso20T) formed photo-induced cross-linked products with target double-stranded DNA. Cross-linked adducts formed in vitro between ODNs and P450arom expression constructs were used to transfect COS and human MCF-7 breast cancer cells. Levels of aromatase transcripts and enzyme activity were significantly lower in cultures transfected with Pso20T-treated cDNA relative to controls. Pso20T had a lesser inhibitory effect on aromatase expression from a mutant P450arom construct, consistent with predicted effects of the mutations on triplex formation. These results are compatible with triplex-mediated interruption of transcription within intact cells.

Key words: Triplex formation; Aromatase; Psoralen

1. Introduction

Triple helices (triplexes) form when a third DNA strand forms Hoogsteen-type hydrogen bonds with double-helical DNA. Recent interest has focused on induction of local triplex formation between double-stranded DNA and synthetic oligodeoxynucleotides (ODNs). This antigene approach is seen as a means of regulating gene expression, either as an investigative tool [1] or for potential therapy [2]. Triplex formation has several potential advantages over the antisense approach: there are fewer target molecules per cell and the regeneration rate of DNA is far lower than that of RNA. Thus low-dose administration could result in durable suppression of gene expression.

Triplex formation can occur by interaction of a pyrimidine third strand with a purine strand in a target duplex. In this Pyr·Pur·Pyr motif, thymine binds to an adenine in an AT base pair, and protonated cytosine binds to guanine in a GC pair [2]. The triplex approach has been used experimentally to modulate gene expression. However its use in biological systems is limited by sequence restrictions, a requirement for low pH in order for cytosine to be protonated, and the fact that the triplex interaction is of relatively low affinity. Various approaches have been used to overcome these problems, including the identification of alternative natural triplets [3], and the use of nucleoside analogues [2]. Binding affinity can be increased by covalently linking third strand ODNs to intercalating groups such as acridine [4] or psoralens [5,6]. The latter are photoactive intercalating groups which on irradiation at 320–400 nm form stable covalent adducts with pyrimidine bases, preferably thymine, at one or both ends of the psoralen molecule. Intercalation at a TpA step permits photo-induced cross-linking to both DNA strands, forming a stable bis-adduct.

The triplex approach has been used to target purine-rich sequences in upstream regulatory regions of genes such as c-*myc* [7,8], the IL2α receptor [6,9], and progesterone-responsive genes [10]. Because the transcribed region of a gene is generally much larger than the regulatory sequences, the ability to target coding sequences would increase the range of potential targets. Thus far this has been reported in a cell-free system [11], and in two studies in intact cells, targeting the β-galactosidase and *mdr*1 genes [12,13].

The cytochrome P450 enzyme aromatase (P450arom) catalyses the conversion of androgens to oestrogens and is an important target for the treatment of hormone-dependent breast cancer [14]. Here we report significant inhibition of P450arom expression within live cells, using a psoralen-linked third strand ODN which forms cross-linked adducts with a purine-rich region in the coding sequence of P450arom.

2. Materials and methods

2.1. Oligonucleotides

Unmodified ODNs were synthesised on an Applied Biosystems automated DNA synthesiser using standard phosphoramidite chemistry. Psoralen-C_5-phosphoramidite (Cambridge Research Biochemicals) was used to prepare ODNs linked at the 5′ end to 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; for experiments in cells these were functionalised at the 3′ end by a modified amine group using 3′ amine-on CPG columns (Cruachem Ltd). ODNs were purified by ion exchange HPLC and desalted using a Sephadex column. For psoralen-linked ODNs the presence of the psoralen was confirmed after purification by fluorescence spectroscopy. Fig. 1 shows the sequences of psoralen-linked third strand ODNs and the unmodified 30-mer ODNs used to reconstitute the double-stranded target.

2.2. Electrophoretic mobility shift assays

The ODN representing the coding strand of the target sequence was end-labelled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP, and purified by electrophoresis through a 20% polyacrylamide gel. It was annealed with a five-fold excess of the complementary strand by incubating in 1 × binding buffer (40 mM Tris-acetate, 1 mM EDTA (TEA), pH 5 or 6, or 40 mM Tris-acetate pH 7 with 10 mM $MgCl_2$) at 95°C for 5 min, 37°C for 15 min and room temperature for 15 min. Radiolabelled duplex (10,000 cpm, approxi-

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mately 10 fmol/reaction) was mixed with 80 fmol–8 nmol third strand ODN (final concentration 4 nM–400 μM) in 1 × binding buffer with 1 mM spermine (Sigma) and 10% sucrose. Reactions were incubated in siliconized Eppendorf vials for 16 h at 15–37°C. The samples were chilled on ice before irradiation at 365 nm. This was carried out for 10 to 30 min, 4–5 cm from the surface of a TL-33 transilluminator (UVP Ltd.). Reaction products were resolved by electrophoresis at room temperature on a denaturing 19% polyacrylamide/7 M urea gel in TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8.3) at 200 V for 4 h. The gels were wrapped in Saranwrap and visualised on a phosphorimager or exposed to autoradiographic film.

2.3. P450arom expression constructs

WT P450arom cDNA was cloned from human placental cDNA [16]. It was subcloned into expression vectors pCDM8 [17] (Invitrogen Co. San Diego) and pRc/CMV (Invitrogen) to create expression vectors p3610 and p3681 respectively [16].

2.4. Mutagenesis studies

Wild-type (WT) cDNA was subcloned into pBluescript (Stratagene) to prepare single-stranded DNA [15] as a template for mutagenesis. Mutant P450arom R265K, which lacks the *Hga*I site at base 837, was synthesised with ODN 5′-GCA·GAA·AAA·AGA·CGG·AAG··ATT·TCC·ACA·GAA··G-3′ in a mutagenesis reaction using the oligonucleotide-directed *In Vitro* mutagenesis system (Amersham) according to the manufacturer's instructions. Mutant progeny were screened by *Hga*I digestion and DNA sequencing, to confirm the presence of the desired mutation and the absence of other base changes. The mutant cDNA insert was subcloned into pCDM8 to create construct pR265K for transfection of COS cells.

2.5. Cell culture and transfection

COS cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) with 10% foetal calf serum (FCS), in a humidified atmosphere of 10% CO_2. Human MCF-7 breast cancer cells were originally obtained from the Michigan Cancer Foundation, Detroit, MI. They were cultured in RPMI-1640 tissue culture medium with 10% FCS. Cultures were negative for mycoplasma infection when stained with Hoechst 33258. Triplexes were formed in vitro by mixing 10 μg (2.5 pmol) expression vector p3610, pR265K or p3681 with 25 nmol ODN in 1 × TEA buffer pH 5. After one hour's incubation at room temperature the reactions were irradiated at 365 nm for 5 min on ice. Subconfluent cultures of COS and MCF-7 cells were transfected by electroporation in HEPES-buffered saline [16]. A β-galactosidase expression vector pCH110 (2 μg; Pharmacia) was cotransfected into COS cells for measurement of transfection efficiency. For each experiment, ODN-treated constructs were transfected into aliquots of the same cell culture within 5 min of one another, to minimise differences in transfection efficiency. After electroporation the cells were seeded into fresh culture medium with 10% FCS and incubated at 37°C. After 72 h parallel cultures were used for northern analysis, aromatase enzyme assay and β-galactosidase assay. In addition, experiments on MCF-7 cells were repeated over a longer time scale with G-418 selection. Cultures were transfected with ODN-treated p3681 as described above, and after 48 h 600 μg/ml G-418 (Gibco-BRL) was added to select for transfected cells. Fourteen days later aromatase assay was performed on neomycin-resistant cells.

2.6. Enzyme assays

Aromatase activity was measured in intact cells by the tritiated water release method, using as substrate 1 μCi of 250 nM [1β-^{3}H]androstenedione (specific activity 24 Ci/mmol, New England Nuclear) as previously described [16]. The activity was measured at three time points between 30 min and 4 h to confirm linearity of tritiated water release. Cell monolayers were then washed with 50 mM sodium phosphate buffer pH 7.4, dissolved in 0.5 M NaOH, and the protein content was determined [18]. These results were used to derive the mean ±S.E.M. specific aromatase activity expressed as pmol tritiated water released/mg protein/hour. For β-galactosidase assay, COS cell monolayers were washed once in phosphate-buffered saline (PBS) and scraped into 1 ml PBS. The cells were pelleted by centrifugation at 12,000 × *g* for 5 min at 4°C and resuspended in 200 ml fresh β-galactosidase solution (0.25 M Tris-Cl pH 7.5, 5 mM dithiothreitol, 15% glycerol). The cells were lysed by freeze-thawing and insoluble debris was pelleted at 12,000 × *g* for 5 min. The total protein content of the supernatant was assayed and duplicate aliquots of 10 μg were used for a β-galactosidase assay. The results were expressed as OD_{420} units/mg protein/min, and for comparative analysis the aromatase activity was corrected to a β-galactosidase value of 1 OD unit/mg/min.

2.7. Statistical analysis

Northern analysis was performed three times, and the images were analysed using ImageQuant software (Molecular Dynamics) or by densitometric scanning of autoradiographs. The results were expressed as the mean ±S.E.M. of three blots, expressed as a percent of the intensity in the 'no added ODN' control. Analysis of variance and Dunnett's test were used to assess the significance of differences in the results of Northern blots and enzyme assays.

3. Results

The target chosen was at bases 826–845 of the P450arom gene coding sequence [19], where there is a 20 bp purine-rich sequence interrupted by three CG pairs (Fig. 1). The third strand ODN was designed by placing a T for each AT base pair in the target sequence, and a C for every GC pair. At third strand positions corresponding to the three CG pairs in the target sequence, a thymidine was used, following a report that T·CG triplets could be tolerated [3].

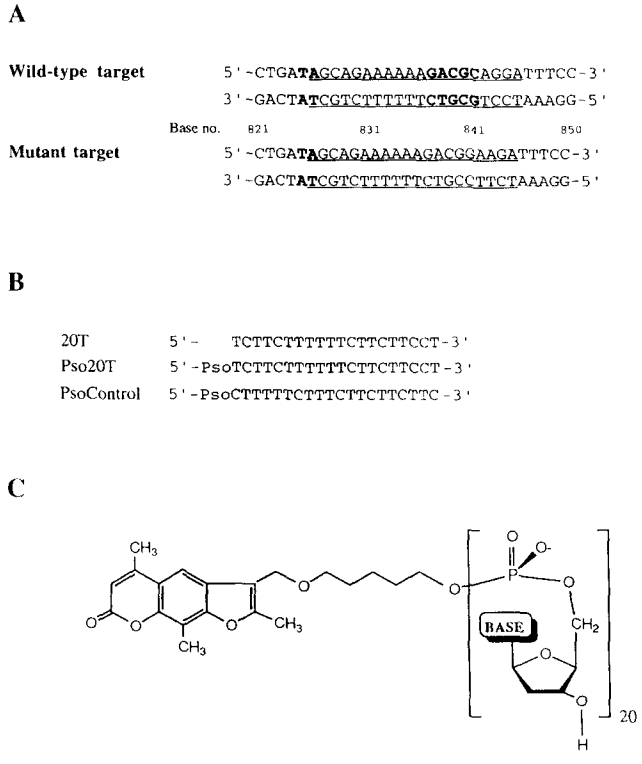

Fig. 1. (a) Target sequences within the P450arom gene coding sequence. For band shift assays the 30 bp WT and mutant sequences shown here were reconstituted using unmodified 30mer ODNs corresponding to the coding and noncoding strands. Within this sequence the 20 bp triplex target is underlined. The TpA step at the 5′ end of the target sequence and the *Hga*I site are shown in bold. Numbers indicate positions in P450arom cDNA. (b) Sequence of third strand ODNs used in this study. Pso indicates 4,5′,8-trimethylpsoralen. (c) Structure of 4,5′,8-trimethylpsoralen-linked ODNs. The psoralen derivative 4,5′,8-trimethylpsoralen was covalently linked via a pentamethylene linker to a cyanoethyl phosphoramidite. This was used to attach the psoralen to the 5′-hydroxyl of Pso20T and PsoControl ODNs (see section 2).

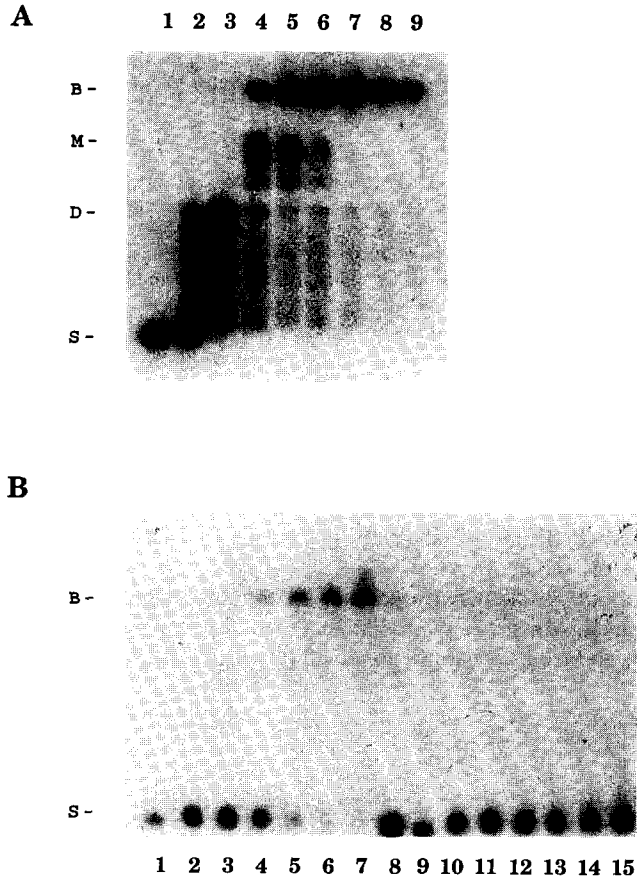

Fig. 2. Photo-induced cross-linking of Pso20T to 30bp target. (a) Time course of cross-linking. The ^{32}P-labelled duplex (approximately 10 fmol) was incubated with 800 pmol Pso20T (final concentration 40 μM) at pH 5, 15°C, irradiated at 365 nm for 10 s–30 min and electrophoresed through a denaturing 20% polyacrylamide gel. Lanes: 1, ^{32}P-labelled single strand alone; 2, ^{32}P-labelled duplex; 3–9, ^{32}P-labelled duplex plus Pso20T irradiated for: lane 3, 0 s; 4, 10 s; 5, 30 s; 6, 1 min; 7, 5 min; 8, 15 min; 9, 30 min. To the left are shown the positions of bis-adduct (B), monoadduct (M), duplex (D) and single-stranded DNA (S). (b) Sequence-specificity of cross-linking assessed using specific or control third strands. Radiolabelled duplex (10 fmol) was incubated at room temperature (23°C) with 80 fmol–8 nmol Ps20T or PsoControl (final concentration 40 nM–400 μM) in pH 7 binding buffer with 1 mM spermine (lanes 1–7 and 9–15) or without spermine (lane 8). After irradiation for 5 min at 365 nm the products were electrophoresed as above. Lanes: 1,9, ^{32}P-labelled single strand alone; 2,10, ^{32}P-labelled duplex; 3–7, duplex plus Pso20T, at 40 nM (lane 3), 400 nM (lane 4), 4 μM (lane 5), 40 μM (lane 6), 400 μM (lanes 7,8); 11–15, PsoControl, at 40 nM (lane 11), 400 nM (lane 12), 4 μM (lane 13), 40 μM (lane 14), 400 μM (lane 15). To the left are shown the positions of bis-adduct (B) and single strand (S).

3.1. Evidence for sequence-specific triplex formation in vitro

For electrophoretic mobility shift assays the target sequence was reconstituted using synthetic 30-mer ODNs. Preliminary assays were carried out under nondenaturing conditions, and confirmed that the unmodified 20T ODN, but not sequence-unrelated control 20-mer ODNs, could retard the mobility of ^{32}P-labelled duplex compatible with triplex formation. This effect was demonstrable at pH 5 and 15°C, but not at higher pH or temperature (not shown). The radiolabelled duplex was incubated with Pso20T third strand, irradiated at 365 nm and the products were resolved by denaturing gel electrophoresis. Initial incubations were performed at pH 5 and 15°C to assess the kinetics of photo-induced crosslinking. The results (Fig. 2a) showed that bis-adduct formation was detectable after only 10 s near-UV irradiation. Cross-linking was virtually complete after 5 min, when >95% radiolabelled target strand had been converted to bis-adduct. Two bands of intermediate mobility were attributed to dissociating duplex and mono-adduct formed after short (10 s–1 min) periods of irradiation [5]. Cross-linked products were also detectable after incubation of Pso20T and radiolabelled duplex at room temperature and pH7 (Fig. 2b). The effect was dependent on third strand concentration, with retarded mobility of the majority of labelled target in the presence of 4–400 μM Pso20T. This represented a 10^4- to 10^6-fold excess of third strand. At pH 5 there was little difference in the extent of bis-adduct formation at 4–37°C (not shown). At pH 7 formation of cross-linked products tended to fall with increased temperature, although there was no significant difference in the amount of bis-adduct formed at 22 and 37°C (not shown). To exclude nonspecific crosslinking, a control ODN was synthesised with a 5′-trimethylpsoralen group and the same number of thymidines and cytosines as Pso20T, but with an unrelated sequence (Fig. 1). This PsoControl ODN was incubated with radiolabelled target duplex, irradiated and subjected to denaturing gel electrophoresis in parallel with equivalent reactions using the specific Pso20T third strand. Under the conditions of this assay it was not possible to detect bis-adducts in the presence of PsoControl at concentrations up to 400 μM (Fig. 2b).

3.2. Effect of triplex formation in vitro on P450arom expression in COS cells

Triplexes were formed in vitro by incubating the P450arom expression vector p3610 with third strand ODNs at pH 5, using conditions previously shown to convert >95% duplex to triplex. After near-UV irradiation the cross-linked products were used to transfect COS cells, and P450arom expression was assessed after 72 h. Northern analysis (Fig. 3a,b) showed a reduction in the level of P450arom transcripts in cells transfected with psoralen-linked ODNs. The reduction was greater in cells transfected with Pso20T-treated p3610 (11% of the control level expressed in the absence of third strand, $P < 0.01$) than with PsoControl (40% control, $P < 0.05$). Reprobing for actin mRNA showed that this effect was not due to loading differences. However it could have been due, at least in part, to lower transfection efficiency in the presence of the psoralen-linked ODNs. Therefore the results of aromatase assay were corrected to a β-galactosidase activity of 1 OD unit/mg/min, to compensate for differences in transfection efficiency. The assay showed a time-dependent increase in tritiated water release in all cultures (not shown). In the cultures transfected with Pso20T-treated cDNA, aromatase activity was suppressed to 32% of the control level ($P < 0.05$; see Fig. 3c). The 20T and PsoControl ODNs had no effect on aromatase activity (105% and 112% control respectively), confirming that the differences in levels of P450arom transcripts on northern blots were due to variation in transfection efficiency.

COS cells were also transfected with the mutant P450arom construct R265K, following incubation and cross-linking in the absence and presence of Pso20T (Fig. 4). Both the WT and mutant cDNAs showed dose-dependent inhibition of aromatase enzyme activity in the presence of 1–100 μM Pso20T, with an IC_{50} of < 1 μM for the WT and between 1 and 10 μM for

the mutant enzyme. There was no difference in the magnitude of inhibition at the highest concentration of third strand. However at 1 μM Pso20T the activity of the WT enzyme was inhibited approximately twofold more than that of the R265K mutant (35% control compared with 65% for the mutant, $P < 0.01$), and there was a similar effect at 10 μM Pso20T (WT activity 13% control compared with 28% for the mutant, $P < 0.01$).

3.3. Effect of triplex formation on aromatase expression in MCF-7 cells

Third strand ODNs were incubated with mammalian P450arom expression vector p3681, irradiated and used to transfect human hormone-dependent MCF-7 breast cancer cells. Northern analysis was performed on RNA extracted after 72 h, and the results are shown in Fig. 5a,b. The unmodified 20T ODN had no effect on P450arom expression (130% control), and there was an apparent increase in P450arom transcripts in the presence of PsoControl (200%, $P < 0.01$). Cultures transfected with Pso20T-treated p3681 showed significant reduction in the level of full-length aromatase transcripts to 32% control levels ($P < 0.05$). This result was quantitatively similar to that obtained in COS cells. Northern blotting also suggested the presence of shorter transcripts, of approximately 0.8 kb, in MCF-7 cells transfected with the Pso20T-treated construct (Fig. 5a).

Enzyme assay of duplicate cultures showed aromatase activity which was linear with time (not shown), and as expected the level of activity with this expression system was much lower than in COS cells. The results (Fig. 5c) paralleled the northern blots, with a decrease in aromatase activity to 16% control in Pso20T-transfected cultures ($P < 0.01$). There was an apparent increase in activity in MCF-7 cells transfected with p3681 pretreated with 20T (180% control, $P < 0.01$) and PsoControl (140%, $P < 0.01$). To assess how far these results were attributable to differences in transfection efficiency, the experiments were repeated over a longer time-scale, using G418 to select for transfected cells. After 14 days G418-resistant colonies were analysed for aromatase activity. The release of tritiated water was linear with time in all cultures, and the specific activity was 5- to 10-fold higher than in cells which had not been subjected to selection (Fig. 6). The previously noted increase in aromatase activity in the 20T- and PsoControl-treated cultures was not seen here, suggesting that it may have been attributable to differences in transfection efficiency. However the relative reduction in aromatase activity in cells transfected with Pso20T-treated cDNA was very similar (2.0 pmol/mg/h, 14% control, $P < 0.01$) to that observed previously. This result was significantly lower than the activity measured in cultures transfected with 20T (2.0 vs. 10.5 pmol/mg/h, $P < 0.01$) and PsoControl (2.0 vs 14.9 pmol/mg/h, $P < 0.01$).

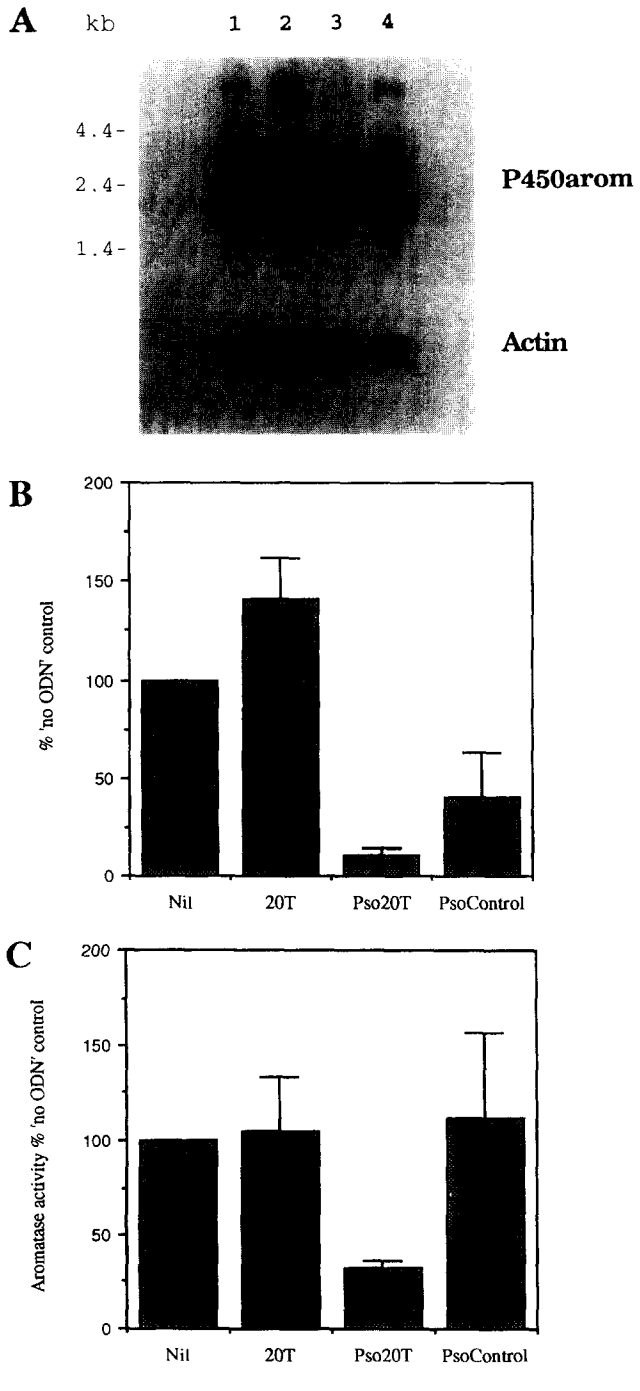

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Fig. 3. Effect of triplex formation on P450arom expression by COS cells. Triplexes were formed between third strand ODNs and COS cell expression vector p3610 as described in section 2. The samples were irradiated at 365 nm and used to transfect COS cells. After 72 h. total RNA was prepared by the method of Chomczynski and Sacchi [25], and 5–10μg was electrophoresed through a 1% agarose-formaldehyde gel, transferred to Hybond-N$^+$ (Amersham) and probed with a ^{32}P-labelled 1.5 kb insert made by digestion of p3681 with *Hin*dIII and *Not*I. Filters were stripped and reprobed for actin to correct for loading differences. Parallel cultures were assayed for aromatase and β-galactosidase activity. (a) Northern blot showing expression of P450arom transcripts after transfection with WT expression construct (p3610) irradiated in the absence of ODN or with 100 μM 20T, Pso20T or PsControl ODN. The filter was stripped and reprobed for actin to correct for loading differences. (b) Quantitative analysis of P450arom expression by COS cells. Bar chart shows the mean ± SEM of results from 3 separate experiments, corrected for the level of actin transcripts and expressed as % expression in the 'No ODN' control. (c) Aromatase enzyme activity expressed by transfected COS cells. The measured levels of aromatase activity were corrected to a β-galactosidase activity of 1 OD unit/mg/min to control for differences in transfection efficiency. Points represent the mean ±S.E.M. of the results of 3 separate experiments.

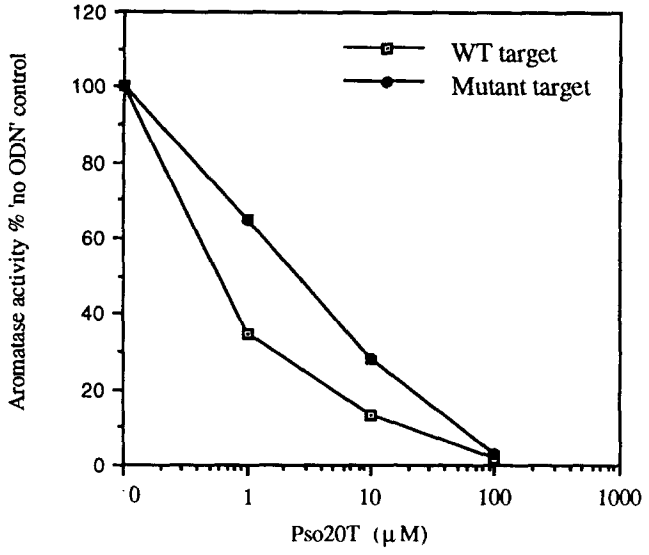

Fig. 4. Effect of Pso20T on aromatase activity expressed by COS cells transfected with WT or mutant P450arom cDNA. Triplexes were formed between Pso20T and COS cell expression vectors p3610 or pR265K. After cross-linking and addition of pCH110 the samples were used to transfect COS cells. The cultures were incubated for 72 h and were assayed for aromatase and β-galactosidase activity. Points represent the mean ± S.E.M. of 3 values, corrected for transfection efficiency.

4. Discussion

We have aimed to evaluate the potential of triplex formation to inhibit transcription elongation, and therefore chose a target within a coding exon of the P450arom gene. The selected 20bp sequence present within exon VII [19] included three CG pairs, and so did not represent a perfect triplex target. Using a modification of the established rules [3], it was possible to design a third strand ODN which demonstrated effects in electrophoretic mobility shift assays compatible with triplex formation. The use of psoralen-ODN conjugates has been shown to permit photo-induced cross-linking of the two target strands of DNA [5,6,20,21]. We adopted this strategy because the P450arom target sequence possesses a TpA step at the triplex–duplex junction, suggesting the potential for cross-linked bisadduct formation. We used 4,5′,8-trimethylpsoralen because this modification increases the dark binding affinity and quantum yield of photo-addition and is much more reactive than unsubstituted psoralen [22]. As predicted, the double-stranded target sequence formed sequence-specific bisadducts when cross-linked with the trimethylpsoralen-ODN conjugate, Pso20T. This effect was seen following incubations at physiological pH and temperature despite the absence of other modifications reported to reduce the requirement for low pH [11,23].

Having obtained results in cell-free systems compatible with triplex formation under physiological conditions, we evaluated the effect of triplexes formed *in vitro* on P450arom expression by intact cells. For this purpose we used psoralen-linked ODNs 3′-modified with an amine group. This modification has been shown to confer resistance to serum nucleases without impeding triplex formation ([9]; Macaulay et al., unpublished observations). In COS cells there was clear evidence of reduction in the level of P450arom transcripts in cells transfected with the WT expression vector previously cross-linked to Pso20T. In parallel with this we observed a reduction in aromatase activity as measured by the tritiated water release assay. These inhibitory effects on levels of P450arom transcripts and aromatase enzyme activity were not seen in the presence of the unmodified 20T or PsoControl ODNs.

As a further check on the specificity of the interaction with P450arom cDNA, we performed transfections using Pso20T cross-linked to WT or mutant expression constructs. Two

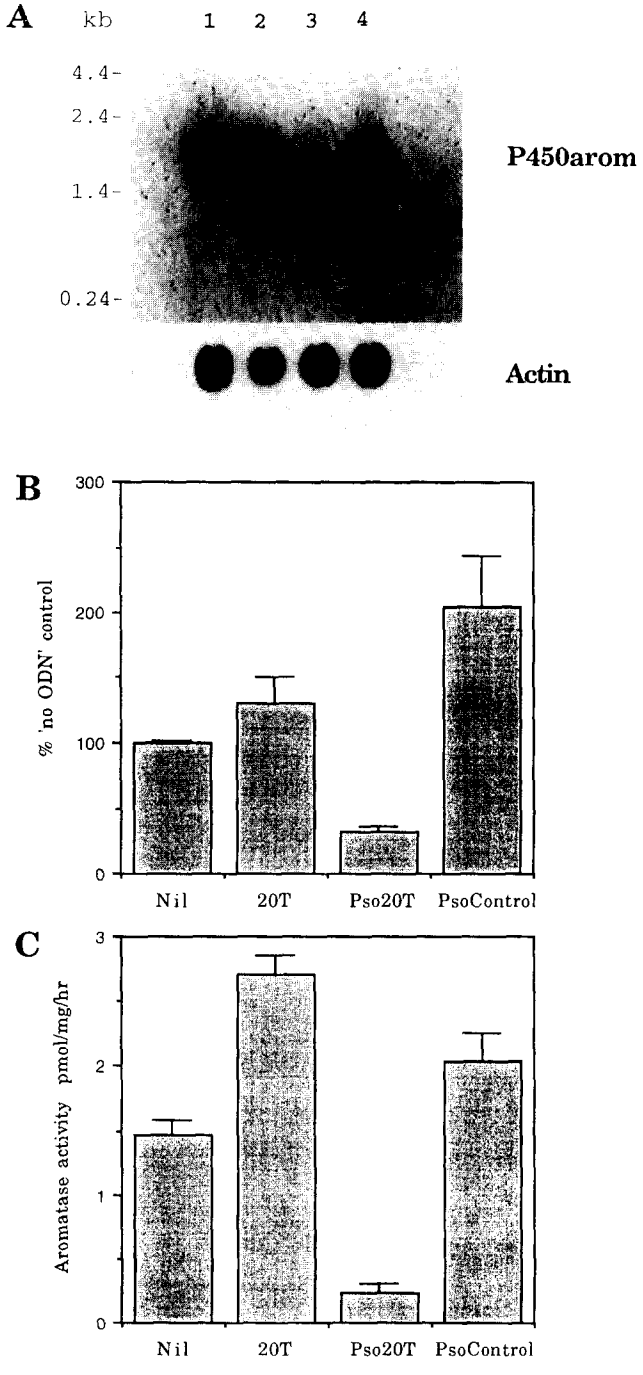

Fig. 5. Effect of triplex formation on P450arom expression by transfected MCF-7 breast cancer cells. (a) Northern blot showing expression of P450arom transcripts. (b) Quantitative analysis of P450arom expression by MCF-7 cells. (c) Aromatase enzyme activity expressed by transfected MCF-7 cells. Details as for legend to figure 3, except that pCH110 cotransfection was not used to correct the values shown in (c) for transfection efficiency.

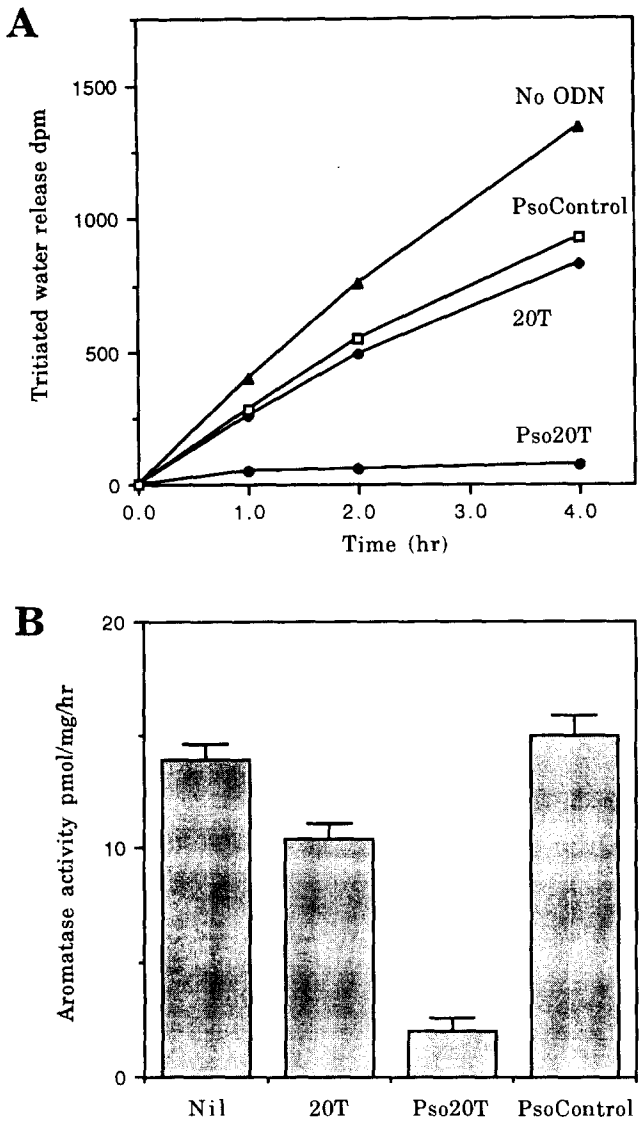

Fig. 6. Effect of triplex formation on expression of aromatase activity 14 days after G418 selection of transfected MCF-7 cells. (a) Tritiated water release increased with time in all cultures, and was suppressed in cultures transfected with P450arom expression vector cross-linked to 100 μM Pso20T. (b) Mean specific aromatase activity expressed by neomycin-resistant MCF-7 cells. There was no significant difference between results obtained for cultures transfected with P450arom expression vector irradiated in the absence of third strand ODN or with unmodified 20T or PsoControl. The aromatase activity detected in cultures transfected with expression vector cross-linked to 100 μM Pso20T was significantly lower ($P < 0.01$) than all other values.

changes were introduced into the WT sequence; a silent G to A mutation at base 843 which obliterated the *Hga*I site, and a C to G mutation at base 841. The effect of the latter is substitution of lysine for arginine at residue 265 of aromatase. Computer modelling suggests that this is a surface residue distant from the active site of human P450arom ([26]; C.A. Laughton, personal communication). The consequence for triplex formation is the replacement of stable triplets T·CG and C·CG by T·CG and C·AT, respectively. The latter is potentially destabilising according to previous reports [3,27]. COS cells transfected with pR265K expressed detectable aromatase activity which, like the WT activity, showed dose-dependent inhibition after cross-linking with Pso20T. The IC_{50} for the effect on the WT construct was, at <1 μM, approximately 10-fold lower than that for the R265K mutant. The individual values at 1 and 10 μM Pso20T suggested that the inhibition of WT activity was approximately twice as potent as the effect on the mutant activity. Thus the effect of the specific psoralen-linked ODN on aromatase activity was sensitive to individual base changes. The differential effect of Pso20T on suppression of aromatase activity expressed by WT and mutant aromatase activity was consistent with the predicted change in triplex binding affinity on the introduction of destabilising mutations [3,27].

We note that the specific 20T and Pso20T third strand sequences could form an imperfect heteroduplex, including a perfectly matched contiguous sequence of 10 bp, with P450arom mRNA transcribed from the target region of the P450arom gene:

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                                  ▽ ▽
WT mRNA 5'-CTGATAGCAGAAAAAAGACGCAGGATTTCC-3'
Pso20T    3'- TCCTTCTTCTTTTTTCTTCT-Pso-5'
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Thus it is possible that Pso20T-mediated inhibition of aromatase expression could be attributable to an antisense effect, similar to that reported by Praseuth and colleagues [28]. However of the bases mutated in pR265K (arrowed above), one involved an existing mismatch (underlined), and the other was outside the region of potential antisense interaction. Therefore these changes should have made no difference to an antisense effect. The observed differential effect of Pso20T on the WT and mutant cDNA targets is not consistent with this, but is consistent with the predicted effect on triplex formation

The COS cell expression system is based on run-away replication of the transfected plasmid [24], and it was possible that inhibition of DNA replication could cause the observed reduction in P450arom expression. Therefore these experiments were repeated in a non-replicative transfection system. WT MCF-7 cells express very low levels of aromatase activity detected only on prolonged (24 h) enzyme assay, and endogenous P450arom transcripts are not detectable by Northern blotting [16]. Transfected MCF-7 cultures expressed measurable P450arom transcripts and aromatase enzyme activity after 72 h. There was significant reduction in the level of full-length P450arom transcripts and enzyme activity in cultures transfected with P450arom cDNA cross-linked to Pso20T. The degree of inhibition was similar to that observed in COS cells. In addition, northern analysis suggested the presence of shorter transcripts which could indicate truncation of transcription, or degradation of full-length mRNA.

To exclude variation in transfection efficiency as a cause for differences in levels of full-length transcripts and enzyme activity, the experiments were repeated following G-418 selection for transfected cells. Assays performed after 14 days revealed significant reduction in aromatase activity, similar to that observed after 72 h. This confirmed that the fall in aromatase activity was not simply a transfection artefact, and that the effect on gene expression was durable. The continued expression of neomycin resistance from the same construct is consistent with a sequence-specific effect on P450arom transcription. The demonstration of reduction in enzyme activity after a relatively long time period argues against a significant antisense

effect: free ODN would be subject to degradation by cell and serum nucleases, and would be progressively diluted in dividing cells. The apparent durability of the effect we observed here is in contrast to studies in transfected HeLa cells, where triplex-mediated inhibition of reporter gene expression was reversed after 48–72 h due to repair of psoralen adducts [12]. It is possible that the inhibition we observed in MCF-7 cells after 14 days represented the residual effect of a greater degree of inhibition at earlier time points, or that MCF-7 cells have different repair mechanisms from HeLa cells.

In conclusion, these results are compatible with triplex-mediated stalling of RNA polymerase, suggesting that purine-rich tracts within coding sequences may be targeted by triplex forming oligonucleotides. The ability to demonstrate this in intact cells suggests the potential for use of the triplex approach to modulate gene expression in vivo.

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