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PAAn-1b and PAAn-E: Two Phosphorothioate Antisense Oligodeoxynucleotides Inhibit Human Aromatase Gene Expression

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Estrogen-dependent diseases, especially breast cancers, are frequently treated with aromatase inhibitors. Another more recent strategy is the antisense technology. In this study, after predicting aromatase mRNA secondary structure, we describe the design, the efficiency, and the toxicity of two antisense phosphorothioate oligodeoxynucleotides (PAAn-1b and PAAn-E) directed toward aromatase mRNA. Indeed, 2 μM PAAn-1b and PAAn-E encapsulated with 54 μM polyethylenimine inhibit aromatase activity by 71 and 79%, respectively, in transfected 293 cells, with IC_{50} values of 0.2 and 0.6 μM. The mechanism of inhibition appears to be specific after using sense and scramble oligodeoxynucleotides as controls and largely decreases aromatase mRNA and protein amounts. Moreover, PAAn-1b and PAAn-E are not cytotoxic for 293 cells. This study finally provides a new strategy for aromatase inhibition. It offers new tools for studying aromatase gene expression and its role in cancer for instance, and this could be of help for the therapy of estrogen-dependent diseases.

The study of the role of estrogens and the regulation of aromatase gene expression, for instance in male reproduction, remains a challenge [1]. Moreover, estrogen-dependent diseases, such as breast cancer, may be amplified by an overproduction of these hormones by the breast tumor itself or its adjacent tissues [2–8]. This synthesis is due to an enzymatic complex formed by the specific cytochrome P450 aromatase (aromatase herein) and the ubiquitous cytochrome P450 NADPH reductase. In 1973, Griffiths *et al.* [9] suggested developing aromatase inhibition as a treatment for estrogen-dependent cancers. At least three strategies can be carried out today to achieve this goal. The first one is to screen, *in vitro,* new molecules designed from the known structure of substrates and inhibitors of this enzyme, and to evaluate their inhibition potency [10–15]. The second strategy is to design new inhibitors according to information concerning the aromatase active site provided by molecular modeling and site-directed mutagenesis studies [16–23]. The third and more recent strategy is to inhibit specific gene expression by annealing of an antisense oligonucleotide (AS-ODN) to its complementary genomic DNA or mRNA sequence [24–27]. Such a study was previously undertaken by forming a triple helix between a psoralen-linked 20-mer pyrimidine oligodeoxynucleotide and the genomic aromatase coding sequence [28]. Although the "triplex formation" offers advantages compared to the "antisense method" (mRNA target) such as fewer target DNA molecules per cell, this latter approach was however more flexible and efficient enough to be applied in the fields of cardiovascular medicine, virology and oncology (see [24] for review). Thus, Ackermann *et al.* [29] developed an AS-ODN complementary to the translation start region of human aromatase transcripts able to inhibit the activity by 60–70% but only at a relatively high concentration of 100 μg/ml. In our study, since the phosphorothioate group significantly increased the stability of the AS-ODN as previously described [25], we designed two phosphorothioate antisense oligodeoxynucleotides (PAAn-1b and PAAn-E) specifically directed against aromatase mRNA. This strategy allowed us to strongly inhibit the aromatase gene expression in cell culture. We will further discuss the design, the efficiency and the action mechanism of these antisense oligodeoxynucleotides.

MATERIAL AND METHODS

Chemicals. All chemical products were obtained from Sigma (St. Quentin Fallavier, France) or GibcoBRL (Cergy Pontoise, France). The [1β,2β-^{3}H]-androstenedione was from Dupont NEN (Les Ulis, France), solvents from Carlo Erba (Val de Reuil, France) and from sds (Peypin, France), alkaline phosphatase substrate kit from Bio-Rad (Ivry sur Seine, France), culture media from BioWhittaker

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(Gagny, France), Thermo Sequenase Kit from Amersham (Les Ulis, France), pCMV plasmid and SNAP Total RNA Isolation kit from Invitrogen (NV Leek, The Netherlands), M-MLV Reverse Transcriptase from Promega (Charbonnières, France) and Qiagen Plasmid Maxi Kit from Qiagen (Courtaboeuf, France). Human aromatase cDNA was kindly provided by E. R. Simpson (USA). The oligodeoxynucleotides were synthesized from our design by Cybergene (Saint-Malo, France).

pCMV-human aromatase cDNA construction. The plasmid used in this study was previously described [15, 23]. Briefly, the human aromatase cDNA (2920 pb, [30]) was cloned into pCMV (*Eco*RI site at position 753). The length, the concentration and the purity of the plasmid–cDNA construction were checked by sequencing and by 1% agarose electrophoresis and ethidium bromide staining.

293 cells culture and transfections. Human embryonal kidney 293 cells (ECACC number: 85120602) were grown in EMEM medium red phenol free supplemented with 2 mM glutamine, 10% new-born calf serum (supreme serum) and 1% nonessential amino acid at 37°C in an atmosphere of 5% CO_2 and 95% air. Fifty thousand cells were grown up to 50% confluence on 24-well cell culture plates 18 h before transfection, and then washed with serum-free cell culture medium, supplemented with 500 μl serum-free medium and transiently transfected with 2 μg pCMV-human aromatase cDNA and 54 nmol of a 10 mM polyethylenimine aqueous solution (pH 7.0) using a modification of the method of Boussif *et al.* [31]. After 2h incubation at 37°C, cells were transfected with oligodeoxynucleotides (0.4–2 μM) and PEI as described above. A control with PEI alone was performed. Cells were further incubated 2 h at 37°C and then supplemented with 500 μl medium containing 10% supreme serum. After a further 18 h incubation, cells were washed with serum-free medium and the aromatase activity was measured "in cell."

Aromatase activity and inhibition "in cell." In this study, we evaluated aromatase activity "in cell" according to Zhou *et al.*[32] by measuring ^3H_2O released from 200 nM [1β,2β-^{3}H]androstenedione (a 2 μM substrate solution was prepared by adding 3.5 nmol of tritiated androstenedione, specific activity 1554 GBq/mmol, to 76.5 nmol nonradioactive androstenedione in 40 ml final volume of ethanol). Control incubation was realized by transfecting in the same conditions the pCMV plasmid alone instead of the pCMV-cDNA plasmid. The results are the mean of triplicate experiments ± SD and expressed as percentage of control.

Enzyme-linked immunosorbent assay. According to the previously described method [23], cells were scraped, resuspended in water, sonicated 4-fold 10 s and the expressed aromatase was evaluated by an ELISA method adapted to our model. Briefly, 200 μl lysate and 800 μl anti-equine aromatase polyclonal antibodies (Ac. I) (1/10,000^e) were preincubated 2 hours and then added (100 μl per well) into plates, previously coated at 37°C with 50 ng/well purified equine aromatase and washed. The fixation of the Ac. I was then evaluated by incubating 1 hour with anti rabbit IgG antibodies coupled with alkaline phosphatase (1/6000^e), washing and incubating 1 hour with the substrate *p*-Nitrophenylphosphate as described by the manufacturer. The absorbance was finally read on a Bio-Tek EL800 apparatus at 405 nm. The results were the mean of triplicate experiments ± SD and were expressed as ng aromatase/well. The anti equine aromatase antibodies were prepared in our laboratory and are known to specifically cross-react with the human enzyme by western blotting.

RNA secondary structure prediction and determination of the antisense oligodeoxynucleotides. This experiment was performed with the automatic software MacDNASIS demo version 3.7 (Hitachi Software). DNAsis used the RNA energy values in the calculations. The maximal bulge and interior loop were fixed to 30 nucleotides. The AS-ODN sequences chosen were PAAn-1b (^{s}GAT^{s}GCCT^{s}TTC-T^{s}CAT^{s}G), PAAn-E (^{s}TCGA^{s}GTC^{s}TGTG^{s}C), Sense-1b (^{s}CAT^{s}GAG-A^{s}AAGG^{s}CAT^{s}C), Sense-E (^{s}GCAC^{s}AGA^{s}CTCG^{s}A), Scramble-1b (^{s}CTG^{s}TCTA^{s}GTTA^{s}CGC^{s}T) and Scramble-E (^{s}GATC^{s}GTT^{s}GC-CG^{s}T). The symbols (s) indicate the phosphorothioate positions. The human aromatase specificity of these oligodeoxynucleotides was evaluated by Gap-Blast search on GenBank, EMBL, DDBJ and PDB protein database according to Altschul *et al.* [33]. This step will be further discussed in results. The integrity of both AS-ODN after transfection and incubation was verified by a denaturing electrophoresis (8% acrylamide, 3.9 M urea) and staining was realized by the Silver Nitrate method according to Tunon and Johansson [34].

RT-PCR. Aromatase and actin mRNA were reverse-transcribed from total RNA, which was extracted from scraped cells with the SNAP Total RNA Isolation kit. The RT reaction (20 μl) was performed from 150 ng total RNA during 1h at 37°C with 100 U M-MLV Reverse Transcriptase, 0.8 mM dNTPs, 10 U RNAguard Ribonuclease Inhibitor and 0.5 μM 3′ primer. The primers were (^{1140}TTGGT-GATCCACATCTGCTG^{1121}) and (^{404}GTGCAGCCCAAGTG-TGCTG-CCGAA-TC^{379}) for actin and aromatase respectively. The PCR was realized on a Robocycler (Stratagene, France) in a total volume of 25 μl containing 10 μl of the previous reaction, 2 μM of each primer, 0.8 mM dNTPs and 2.5 U of *Taq* DNA polymerase. It was performed with 1 cycle: 5 min at 94°C, 3 min at Tm (58°C for actin and 75°C for aromatase), 3 min at 72°C, 30 cycles: 1 min at 94°C, 1 min at *T*m, 2 min at 72°C and 1 extracycle of 10 min at 72°C. The 3′ primers are described above and the 5′ primers were (^{609}GACTACCTCATGAA-GATCCT^{628}) and (^{276}TATGGAGAATTCGTGCGAGTCTGGA-TC^{302}) for actin and aromatase respectively. The expected lengths of the amplicons were respectively 531 bp and 128 bp. The RT-PCR products were then analyzed with a 2% agarose electrophoresis and ethidium bromide stained. The products were finally quantified with the NIH Image computer software and expressed as arbitrary units ± SD.

Cytotoxicity study. The cytotoxicity of ODN on 293 cells was performed according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [35]. The wavelengths were determined on our model and were 565 nm and 750 nm for the test and the reference wavelengths respectively.

Statistical study. A *t* test with unpaired values was performed with the Statview demo software version 4.5.1 (Abacus Concepts).

RESULTS

Design of the antisense oligodeoxynucleotides. We have defined, from the aromatase mRNA secondary structure prediction, seven exposed domains (A-F and 1b, Fig. 1) within the coding sequence (Fig. 2). The A loop was in the coding region of the aromatase cDNA from position 431 to 445, the 1b loop from 417 to 431, the B loop from 1070 to 1097 (this domain was divided into two oligodeoxynucleotides), the C loop from 1517 to 1530, the D loop from 259 to 270, the E loop from 1238 to 1249 and the F loop from 874 to 885. To increase the antisense specificity, we tested the nucleotidic sequences of these seven loops against other mammalian mRNAs by a Gap-Blast search (July 1998). This study allowed us to define the two more specific phosphorothioate AS-ODN: PAAn-1b and PAAn-E, complementary to loops 1b and E respectively. Their optimal size (12–16 pb) was chosen according to Schu and Ramalho Ortigao (not published). Modified phosphorothioate oligonucleotides (30% of nucleotides) were chosen because they retain the property of aqueous solubility and Watson-Crick base pair hybridization, but are also nuclease-resistant. How-

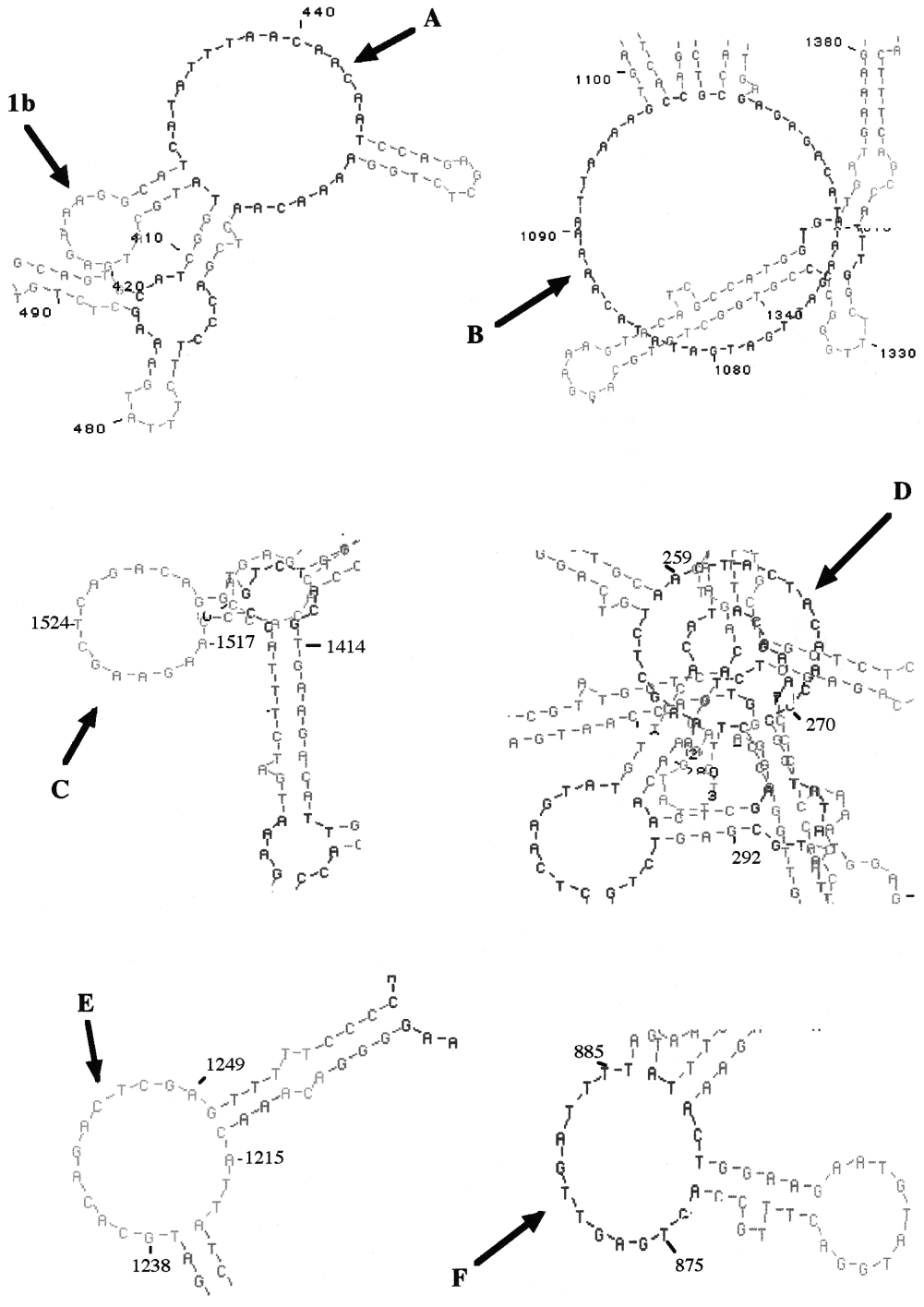

FIG. 1. Aromatase mRNA secondary structure predictions. These predictions were realized with MacDNASIS demo version 3.7 (Hitachi Software). DNAsis used the RNA energy values in the calculations. The maximal bulge and interior loop were fixed to 30 nucleotides. Seven domains (A–F and 1b) are indicated as a possible target for an AS-ODN.

ever, although PAAn-1b was totally complementary only with the aromatase mRNA, PAAn-E showed a partial hybridization (8 bp) with a loop of the pOO71 mRNA and an almost complete one (11 bp) with a loop of the cadherin-6 mRNA, but this should not decrease the interest of PAAn-E, as will be further discussed.

Inhibition of the human aromatase. When 2 μM AS-ODN was transfected into 293 cells previously transfected with the human aromatase, the aromatase activity significantly decreased with both AS-ODN (from 100 to 29.4 $\pm$ 9.3% and to 21.3 $\pm$ 9.5% for PAAn-1b and PAAn-E respectively, Table 1). More-

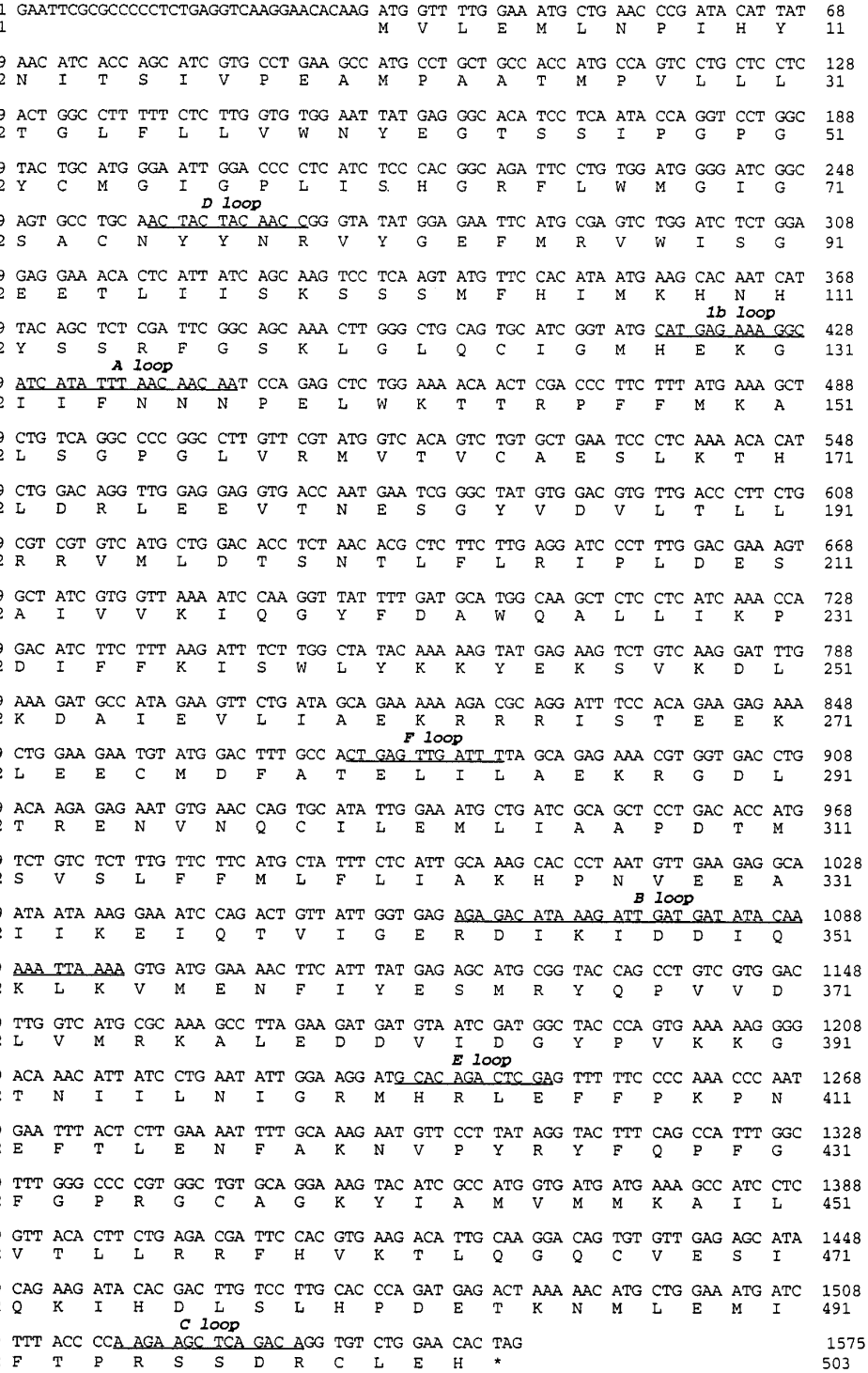

FIG. 2. cDNA and protein sequences of the human aromatase. The human cDNA was from Corbin *et al.* [30]. Seven domains (A–F and 1b) are indicated as a possible target for an AS-ODN.

over, the protein quantity largely decreased with these AS-ODN. Use of PEI *per se* did not induce a significant modification of aromatase activity and protein quantity (Table 1). It should be noted that activity and protein amount obtained with sense and scramble oligodeoxynucleotides were significantly different from corresponding AS-ODN but not from WT. Moreover, Fig. 3, which shows an AS-ODN concentration-dependent inhibition, evidences IC_{50} values of 0.2 μM and 0.6 μM for PAAn-1b and PAAn-E respectively.

TABLE 1

Effects of the Antisense Oligodeoxynucleotides PAAn-1b and PAAn-E (2 μM) Transfected with 54 μM PEI on the Human Aromatase in 293 Cells at 24 h

	Activity (%)	Protein (ng)
WT	100 ± 17.7	1.1 ± 0.2
WT + PEI	94.6 ± 15.2	0.66 ± 0.0
PAAn-1b	29.4 ± 9.3[a,b]	0.4 ± 0.4[a,b]
PAAn-E	21.3 ± 9.5[a,b]	0.3 ± 0.3[a,b]
Sense-1b	67.7 ± 21.4	1.5 ± 0.2
Sense-E	92.0 ± 27.7	1.9 ± 0.7
Scramble-1b	133.4 ± 43.5	1.5 ± 0.3
Scramble-E	69.5 ± 0.0	1.3 ± 0.2

Note. 50,000 cells in 24-wells plates were transfected with 2 μg pCMV-human aromatase cDNA with 54 μM polyethylenimine as transfecting agent [31]. "Control" was realized with pCMV alone. The ODNs were transfected with 54 μM PEI 2 h after the aromatase cDNA transfection and incubated 24 h. "Control + PEI" was realized with 54 μM PEI but without ODN. Aromatase activity was evaluated by measuring ^3H_2O released from 200 nM [1β,2β-^{3}H]-androstenedione incubated in culture medium at 37°C–5% CO_2 atmosphere during 45 min. Results are expressed as % ± SD to a standard control which was incubated under the same conditions and are the means of three experiments. The aromatase protein quantity was evaluated by the ELISA method as described under Material and Methods. Results are the means ± SD of two experiments with triplicate values.

[a] $P < 0.05$ comparing to WT.

[b] $P < 0.05$ comparing to corresponding sense and scramble ODN.

Cytotoxicity studies. To detect mammalian cell survival and proliferation, Mosmann [36] developed a quantitative colorimetric assay based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). In our study, the treated cells were cultured during 24, 72, and 96 h. The MTT assay showed that both PAAn-1b and PAAn-E did not appear to be cytotoxic for 293 cells (Fig. 4). Indeed, cellular growth was intact with both AS-ODN since absorbance, corresponding to living cells, increased during 24, 72, and 96 h. Moreover, absorbance was in general about 74.6% of the control WT but was not significantly different, except for PAAn-E at 72 h (63.7 ± 14.5%) but not at 96 h. This difference appears to be due to a kinetic variability of cellular growth, as evidenced by Fig. 4. Sense and scramble controls were not cytotoxic (data not shown). Moreover, cell morphology was unchanged during treatment with AS-ODN (Figs. 5C and 5D). In the case of PAAn-E, that did not evidence any significant cytotoxicity (Fig. 4), the nucleus/cytoplasm ratio seemed to be slightly increased by this treatment (Fig. 5C). The absence of toxicity was not due to the degradation of AS-ODN which was visualized on a denaturing electrophoresis after 24 h of treatment (data not shown).

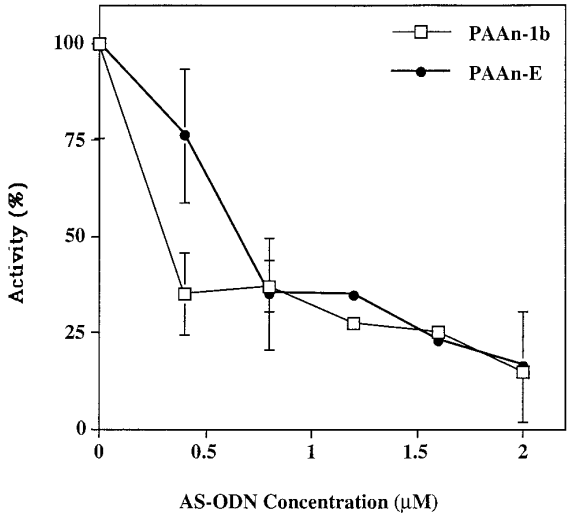

FIG. 3. Inhibition of the human aromatase activity by 2 μM antisense oligodeoxynucleotides PAAn-1b and PAAn-E after different times of incubation. For the transfection with 54 μM PEI and aromatase activity methods, see Table 1. The concentrations of each AS-ODN ranged from 0.4 to 2 μM.

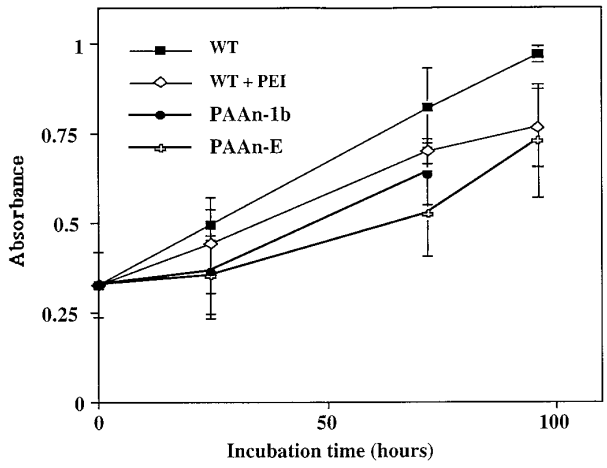

FIG. 4. Effects of 2 μM antisense oligodeoxynucleotides PAAn-1b and PAAn-E transfected with 54 μM PEI on the viability of 293 cells. Cytotoxicity was evaluated by measuring the mitochondrial succinate-dehydrogenase substrate (MTT) transformation in blue formazan product. The absorbance at reference wavelength (750 nm) was subtracted from the test absorbance (565 nm) and results are the mean of triplicate values ± SD. The incubation times of the AS-ODN with 293 cells were 24, 72, and 96 h.

Action mechanism of the antisense oligodeoxynucleotides PAAn-1b and PAAn-E. Total RNAs were extracted from 293 cells after 24 h of treatment with both PAAn-1b and PAAn-E. The aromatase and actin mRNAs were reverse-transcribed and amplified by PCR. Products, analyzed on a 2% agarose gel (Fig. 6A), were at the expected length. This figure shows that aromatase mRNA is less abundant in treated cells whereas the actin mRNA rate is constant. This observation is reinforced by Fig. 6B which evidences

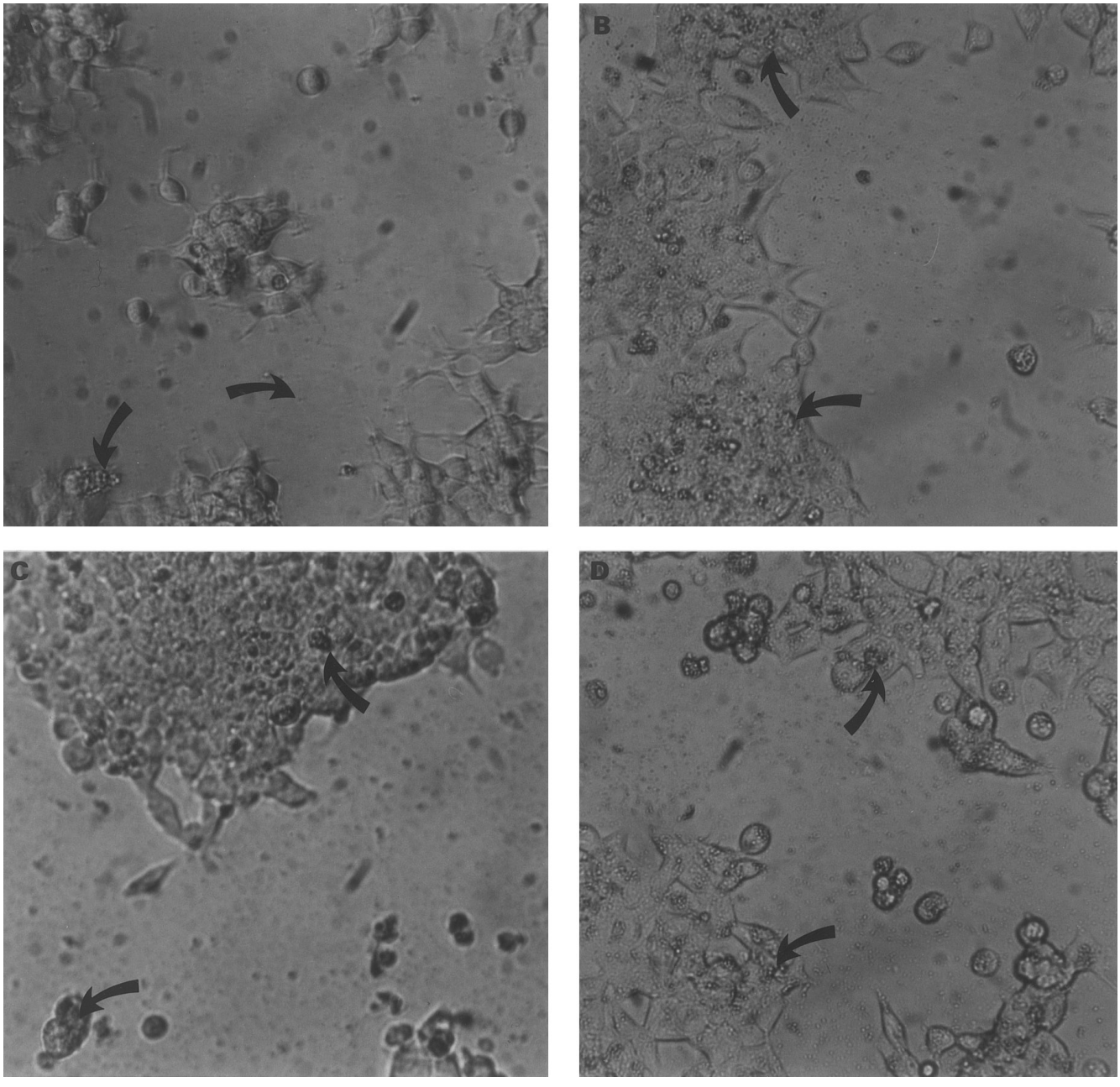

FIG. 5. Cytotoxicity study of both PAAn-1b and PAAn-E antisense oligodeoxynucleotides (×160). 50,000 cells in 24-wells plates were transfected with 2 μg pCMV-human aromatase cDNA with 54 μM polyethylenimine as transfecting agent and incubated 2 h for control (A). No difference was observed with nontransfected cells. The transfected cells then received after 2 h incubation either 54 μM PEI alone (B), 2 μM PAAn-E (C) or PAAn-1b (D) both with PEI. Pictures were taken 24 h later for B–D. The arrows indicate the PEI/DNA complexes.

aromatase/actin mRNAs ratios considerably lower in treated cells (0.39 ± 0.03 and 0.62 ± 0.03 with PAAn-E and PAAn-1b, respectively) than in the control (1.05 ± 0.04). These small values were not due to the PEI since the control PEI one was 0.91 ± 0.05. Lack of genomic DNA amplification was checked with the reverse transcriptase negative control (data not shown with actin primers). Sense and scramble ODN were without effect on aromatase amounts.

DISCUSSION

As described in results and Figs. 1 and 2, we designed seven domains in the aromatase mRNA corresponding to specific loops. Indeed, a good antisense oligodeoxynucleotide has to be not only specific for an mRNA sequence, but its target has to be free of secondary structure [24]. Thus, we designed PAAn-1b and PAAn-E which were the more specific ones for loop

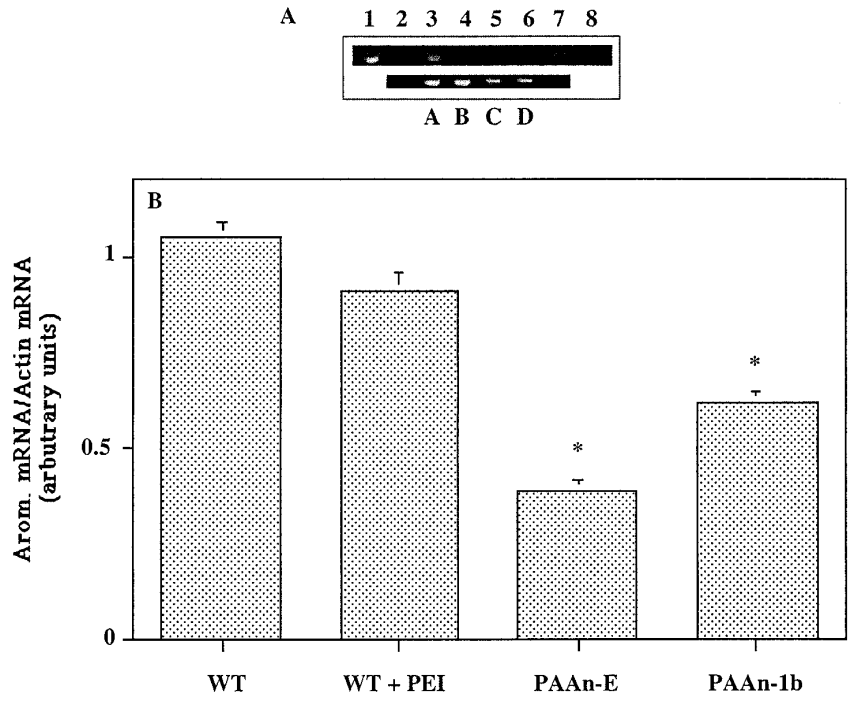

FIG. 6. Effect of both antisense oligodeoxynucleotides PAAn-1b and PAAn-E on mRNA levels. (A) RT-PCR products from aromatase (1–8) and actin (A–D) mRNA after 24h of treatment with both PAAn-1b and PAAn-E oligodeoxynucleotides (2 μM). Lanes 1, 3, 5, and 7 were WT, WT + 54 μM PEI, PAAn-E and PAAn-1b respectively with aromatase primers. Lanes A, B, C, and D were the equivalent samples with actin primers. Lanes 2, 4, 6, and 8 were the corresponding control with aromatase primers without M-MLV Reverse Transcriptase. (B) Aromatase/actin mRNA ratios expressed in arbitrary units. (*$P < 0.05$). P-450 and actin mRNA were reverse-transcribed from total RNA which was extracted from scraped cells. The RT reaction was performed from 150 ng total RNA. The PCR was realized with 10 μl of the previous reaction. The expected lengths were respectively 531 and 128 bp for aromatase and actin respectively. The RT-PCR products were then analyzed by electrophoresis on a 2% agarose gel.

target in aromatase mRNA. Our protein data bank consulting evidenced that PAAn-E could hybridize with two mRNA sequences coding for the p0071 and cadherin-6 proteins. The ubiquitous p0071 protein is an armadillo family member associated with the junctional plaque [36]. However, the p0071 mRNA secondary structure prediction evidenced that the target loop seemed to be weakly accessible to the PAAn-E and that this AS-ODN would accordingly not be able to inhibit the translation of this protein. Cadherin-6 is a cell adhesion molecule preferably expressed in tumoral tissues [37, 38], and Paul *et al.* [38] reported that the overexpression of cadherin-6 could be associated with progression of renal cell carcinoma. If the antisense oligodeoxynucleotide PAAn-E could inhibit the translation of the cadherin-6, it could thus have a beneficial effect on cellular proliferation in tumoral tissues.

We then evaluated the effects of both PAAn-1b and PAAn-E on human aromatase activity in transfected 293 cells. Although nude AS-ODN could be injected, several authors showed that efficiency was improved by adding a transfecting agent such as cationic lipids [39–41] or polyethylenimine [42]. As previously described [15, 23], we used polyethylenimine as a transfecting agent and evidenced that the best ratio in our model was 9 nmol of PEI for 1 nmol of phosphate. This ratio was applied to the antisense strategy and 250 nmol of PEI (250 μM in the reactional volume) were thus necessary for transfection of 10 μg AS-ODN (30 nmol of phosphate). However, this quantity (with or without AS-ODN) was too high and strongly decreased the cellular viability (about 50%) of the treated cells without decreasing the protein quantity (data not shown). This fact was previously reported by Lambert *et al.* [42] since these authors evidenced that 190, 240, or 360 μM PEI decreased cell survival by 37, 56, or 75% respectively. They finally proposed a PEI concentration of less than 180 μM in their model. Since 54 nmol PEI was the concentration used for human aromatase cDNA transfection in our model, we chose a ratio of 1.8 (54 nmol PEI and 30 nmol of phosphate for AS-ODN) which finally appeared to be efficient (Table 1). Consequently, a concentration-dependent decrease of aromatase activity was evidenced by the antisense oligodeoxynucleotides PAAn-1b and PAAn-E (Table 1 and Fig. 3), with a considerable decrease of protein quantity. We evidenced IC_{50} values (0.2 and 0.6 μM for PAAn-1b and PAAn-E, respectively) much lower than the results previously obtained by Macaulay *et al.* [28] who observed an IC_{50} of 1 μM with a Pso20T directed

towards the genomic DNA. The antisense strategy thus seemed to be more efficient *in vitro*. Moreover, we observed that PAAn-1b seemed to be 3-fold more potent than PAAn-E. These results are thus very interesting since this antisense oligodeoxynucleotide was more specific for the aromatase mRNA. Moreover, a concentration of 2 μM significantly decreased the aromatase activity by 70–80% which is a better effect than that previously described by Ackermann *et al.* [29] especially as these authors used much higher AS-ODN concentrations (100 μg/ml) than ourselves (about 10 μg/ml). On the other hand, as described in Figs. 4 and 5, no cytotoxic effect was observed during the treatment of 293 cells with 2 μM PAAn-1b or PAAn-E, and these AS-ODN were not degraded by the cellular DNAses.

To understand the action mechanism and to check the specificity of both antisense oligodeoxynucleotides, PAAn-1b and PAAn-E, we evaluated the aromatase mRNA level with a semiquantitative RT-PCR (Fig. 6). After evidencing the integrity of the total RNA (data not shown), we observed that both AS-ODN strongly decreased the P450arom mRNA level without modifying the actin one. These results were in agreement with the decreasing protein quantity previously evidenced (Table 1). Our results showed that the effect of both antisense oligodeoxynucleotides, PAAn-1b and PAAn-E, seemed to be specific for the aromatase mRNA; this could lead to an mRNA degradation by the RNAse H and a lower translation, as previously described (see [24] for review).

Recently, Branch [43] specified that an efficient AS-ODN is specific only if it can comply with three conditions: (1) there is no gross loss of cell viability, (2) the mRNA target decreases significantly and (3) the protein quantity is lower than the control. In this study, we easily met these three criteria. Moreover, low concentrations of AS-ODN and PEI were specific and active. These results could be confirmed on non-transfected aromatase positive cells and a differential display RT-PCR could verify if these AS-ODN extinguish other unexpected gene expressions. This study finally led to the development of a new strategy for aromatase inhibition which could offer new tools for studying the aromatase role and gene expression. This could in turn provide further help for the therapy of estrogen-dependent diseases.

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