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In Vitro Stability of Polymerase Chain Reaction-generated DNA Fragments in Serum and Cell Extracts

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ABSTRACT. The potential use of polymerase chain reaction (PCR)-generated DNA fragments (PCR-DNAs) as pharmaceutical agents has previously been suggested, with the demonstration of the *in vitro* cellular internalization and biologic activity of PCR-DNA decoy molecules targeted to human estrogen receptor gene. In order to provide information on the stability of these double-stranded DNA molecules, the nuclease resistance of PCR-DNAs of different sizes was studied in different conditions and experiments. Simulating *in vitro* and *in vivo* transfection protocol, we demonstrated that PCR-DNAs exhibited good stability toward fetal bovine serum (FBS) and adult human serum nuclease digestion. In addition, when the protective activity of liposome-based formulations toward nuclease digestion was tested, it was shown that the stability of PCR-DNAs could be further increased (up to 7 days) when a liposome-mediated delivery system was employed. BIOCHEM PHARMACOL **56**;6: 703–708, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. PCR-generated DNA fragments; DNA stability; human serum; cell extracts; liposome

Our laboratory has recently focused upon usage of PCR-DNAs§ as decoy molecules suitable for modulating gene transcription [1]. PCR-DNAs belonging to hER gene promoter, without chemical modifications and without the use of carrier systems, were transfected into MCF7 breast cancer cells, producing a modulation of hER gene expression. In spite of this well-documented activity [1], the stability of these PCR products under cell culture conditions or in human serum is still an open question. This knowledge represents an essential prerequisite for their use as pharmaceutical agents. It is well known that nucleic acid molecules such as antisense and double-stranded oligonucleotides, triple helix and ribozymes used as novel therapeutic agents for specific modulation of gene expression are scarcely stable *in vivo* [2]. Different methods aimed at overcoming the limitations appearing during their practical use have been extensively analyzed.

In order to study the *in vitro* and *in vivo* stability of different DNA molecules, it should be considered that: a) nuclease activities are present both in serum and in different intracellular compartments [3, 4]; and b) an understanding of the interactions between DNAs and nucleases is generally critical in determining how the transfected DNAs act within a biologic system. The objective of this paper was to analyze the stability of PCR-DNAs in various environments: a) in the presence of FBS diluted in water or in cell culture medium; b) in conditioned media from different cell lines, simulating the extracellular environment present during *in vitro* transfection; c) in nuclear and cytoplasmic fractions, simulating intracellular environment; and d) in whole human serum, simulating *in vivo* conditions.

All the stability experiments were carried out using agarose gel electrophoresis as analytical method to quantitate the DNA. In addition, the stability of PCR products delivered by using cationic liposomes as carrier system was assessed.

MATERIALS AND METHODS

Nucleic Acids

PCR-DNA fragments belonging to hER gene promoter [5, 6] were used. PCR-DNAs were obtained by PCR amplification using pGHER1 [5] or pBLCAT8ERCAT1 [6] recombinant plasmids as templates and specific primers localized at -3258/-3156, -2753/-2539, and -128/-108 [5, 6] to obtain PCR-102, PCR-214, and PCR-120, respectively. PCR amplifications were carried out using 2.5 U of Taq DNA

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§ *Abbreviations:* CD, cationic detergent dioctadecyl-dimethyl-amonium bromide; CH, cholesterol; ER, estrogen receptor; FBS, fetal bovine serum; MEM, minimum essential medium; PC, phosphatidylcholine; PCR, polymerase chain reaction; and REV, reverse-phase evaporation.

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polymerase (Perkin Elmer Cetus) and by performing 30 cycles on a Violet Thermal Cycler. The cycles were programed as follows: 30 sec at 94° (denaturation), 60 sec at 55° (annealing) and 60 sec at 72° (extension). All PCR products were purified and concentrated using Microcon-30 (Amicon) ultrafiltration devices, as previously described [1]. PCR products were then analyzed by 2% agarose gel.

Preparation of Nuclear Extracts and Cytoplasmic Fractions

MCF7 breast cancer, RD/18 rhabdomyosarcoma and K562 erythroleukemic cell lines were utilized. Cells were maintained in α minimum essential medium (MEM, Life Technologies) supplemented with 10% FBS (Boehringer Mannheim) at 37° in 5% CO_2 as described [7]. Nuclear extracts were prepared essentially according to the method of Dignam *et al.* [8]. Aliquots containing 1 μg of proteins were incubated at 37° for appropriate times with PCR products. After incubation, all samples were phenol/chloroform extracted to analyze the physical state of DNA and exclude the possibility of DNA fragments remaining complexed with lipids or proteins in the complex.

The cytoplasmic fractions were obtained from 1×10^6 cells harvested and treated with a solution containing 150 mM NaCl, 10 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$ and 0.5% NP40. After centrifugation at 10,000 *g* for 10 min, a supernatant representing crude cytoplasmic fractions was obtained. This includes cytosol with different kinds of membranes and components originating from dissolution of cellular vesicles such as endosomes, lysosomes, and small size organelles. Aliquots corresponding to 1/10 of the cytoplasmic fractions were utilized for incubation with PCR products.

Human Sera

Human blood samples obtained from healthy volunteers were allowed to clot for 3 hr and the cells were removed by centrifugation.

Liposome Preparation

Cationic liposomes were prepared by the REV method as previously reported [9]. The aqueous phase consisted of 1 mL of water, and the organic phase was a solution of egg PC, CH and the cationic detergent CD in 4 mL of diethyl ether. The molar ratio of liposome constituents was PC: CH:CD 8:2:1, mol/mol/mol.

The biphasic system was vortexed and sonicated at 0° for 5 min in a bath-type sonicator. The ether present in the obtained stable emulsion was removed by rotary evaporation under reduced pressure at room temperature, resulting in a turbid, white liposome dispersion. In order to obtain homogeneously sized vesicles, the REV liposomes were then extruded through polycarbonate filters with different pore sizes. The formation of the liposome-PCR-DNA complex was carried out by mixing 2.5 μL of unilamellar vesicles composed of PC, CH and CD (10 mg/mL of total lipid) with an equal volume solution containing 40 ng of PCR-DNA.

DNA Stability and Quantification Analysis

PCR-DNAs were incubated at 37° in a) 10% FBS diluted in water; b) 10% FBS diluted in α MEM; c) 10–90% human serum; and d) cell-conditioned medium obtained after harvesting cells grown at confluence. Samples were removed after different lengths of times and analyzed by electrophoresis in 2% agarose gel to determine PCR-DNA content. Liposome-associated PCR-DNAs were incubated at 37° in 10–90% human serum diluted in water. All sera used were not heat inactivated.

Before electrophoresis analysis, PCR-DNAs incubated in the presence of human serum and liposomes were phenol-extracted in order to avoid the formation of DNA-protein or DNA-liposome complexes, resulting in nonmigrating bands in the gel. Traces of phenol in the aqueous fraction were eliminated by three extractions with a chloroform/isoamyl alcohol solution (24:1, v/v). This treatment also led to the solubilization and extraction of the liposomal material. Then, samples were Microcon-purified [1], concentrated, and loaded on a 2% agarose gel.

The quantification of DNA on agarose gel was performed by densitometry. To determine the accuracy of measurements, serial 1:2 dilution of known amounts (nanograms) of PCR products corresponding to time 0 undegraded band were separated on 2% agarose gel and visualized by ethidium bromide staining. An Imaging Densitometer (Bio-Rad Model GS 700, Bio-Rad) was used to analyze DNA levels. The ODs of the examined band were plotted against the nanograms of DNA loaded on the gel to create a standard curve. A linear regression was observed.

Where indicated, DNA levels corresponding to the amount of PCR product resistant to nuclease activity were deduced from the standard curve, and the percentage of DNA degradation was consequently calculated.

RESULTS

In order to assess the stability of PCR-DNAs, we defined incubation conditions performing a preliminary investigation with double-stranded DNA in the form of a 4.3 Kb pBR322 plasmid restricted with *Hae*III and a 48.5 Kb lambda phage DNA (λDNA). As a source for nucleases, we used non-heat-inactivated FBS, because it is routinely employed in cell culture experiments, at a final concentration of 10% in water (Fig. 1, panel A) or in α MEM (Fig. 1, panel B), and 250 ng of plasmid and λ DNAs were incubated in the presence of FBS at 37° from 2 to 180 min, then analyzed by electrophoresis.

When incubated in the presence of FBS diluted in water, plasmid and λ DNA was rapidly degraded as demonstrated by the presence of low mol. wt DNAs in the lanes b, c and d of the panel A of Fig. 1. When incubated in the presence of FBS diluted in medium, the DNAs remained substan-

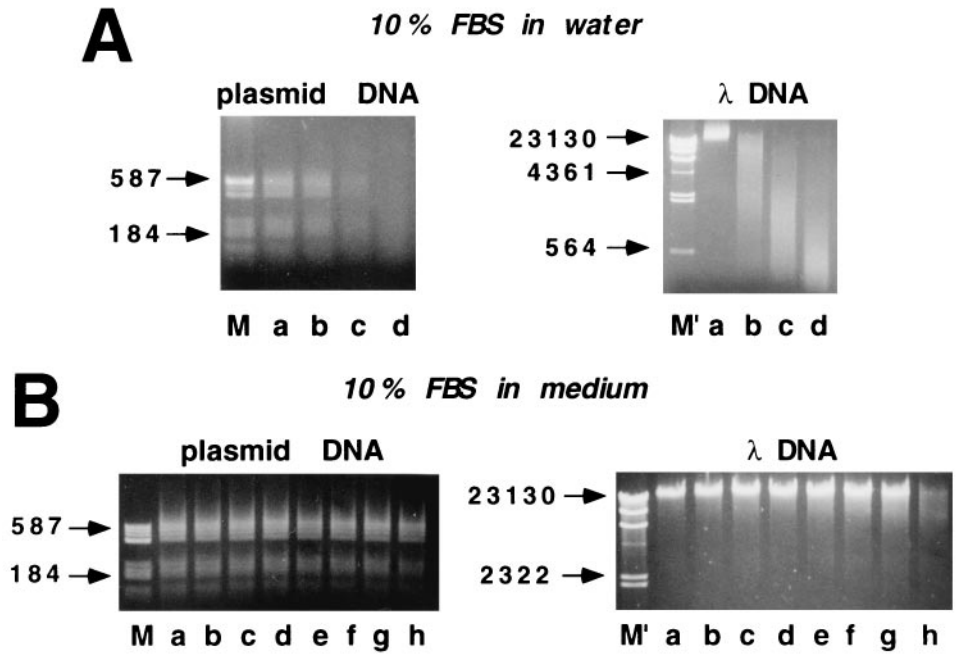

FIG. 1. Effect of FBS nucleases on *Hae*III restricted pBR322 plasmid DNA and on λ DNA. 250 ng of plasmid and λ DNA were incubated in the presence of 10% non-heat-inactivated FBS in water (panel A) and in α MEM (panel B) at 37° for 2 (lane a), 15 (lane b), 30 (lane c), 60 (lane d), 90 (lane e), 120 (lane f), 150 (lane g), and 180 (lane h) min. After incubation, samples were electrophoresed on 1.5% agarose gel. The mol. wt markers were: M = *Hae*III restricted pBR322 DNA; M′ = *Hind*III restricted λ DNA.

tially undegraded for up 120 min, as indicated by the presence of strong bands still evident in the lanes f of the panel B. The results of these experiments indicated that: a) the nucleases present in the FBS rapidly degraded these DNA molecules; and b) the degradation kinetic was faster when FBS was diluted in water.

When purified PCR products, 102-, 120- and 214-bp fragments in size, (namely PCR-102, PCR-120 and PCR-214), were incubated in the presence of 10% FBS in water, a different pattern of degradation was observed. Representative electrophoretic gels of PCR-DNAs incubated under these conditions are shown in panel A of Fig. 2. Increasing degradation was observed in the order: 102 < 120 < 214, indicating that differences in stability appeared to be related to the DNA mol. wt. Results of densitometric measurement of the electrophoretic bands visualized by ethidium bromide staining are presented in the table below panel A as a percentage of degradation compared to time 0 undegraded band. After 90 min (lane e), PCR-102 appeared substantially undegraded, while PCR-120 and PCR-214 were degraded at 40% and 68%, respectively. After 120 min (lane f), both PCR-120 and PCR-214 were completely degraded, whereas PCR-102 was degraded only at 5%. The experiments were repeated five times giving the same percentage of degradation.

According to the data obtained for plasmid and λ DNA, all PCR products showed a higher nucleases resistance when incubated in 10% FBS in α MEM, remaining substantially undegraded for 48 hr. Figure 2B shows the electrophoretic migration pattern relative to the experiment conducted with PCR-214, confirming the decreased activity displayed by FBS nucleases when diluted with medium. Stability was also determined by incubating PCR-DNAs for 48 hr in conditioned media obtained from MCF7 breast cancer (lane d), RD/18 rhabdomyosarcoma (lane e), and K562 erythroleukemic (lane f) human cell lines. In all cases, little or no degradation occurred under the conditions examined, as indicated by the substantially unaltered PCR-DNA bands on agarose gel.

In order to verify whether PCR-DNAs were stable in different intracellular environments and under varying stressing conditions, 100 ng each of the three PCR-DNAs (PCR-102, PCR-120 and PCR-214) were incubated up to 48 hr at 37° with nuclear extracts and crude cytosolic fractions obtained from MCF7, K562 and RD/18 cells as described in the Materials and Methods section. The results of the experiments are depicted in panel C of Fig. 2. At the shorter times, there were no significant differences between the different DNAs. All PCR-DNAs were stable up to 4 hr in the presence of both nuclear extracts and cytoplasmic fractions, regardless of the cell line (data not shown). After 24 hr, high levels of degradation were detectable for all PCR-DNAs incubated in RD/18 cytoplasmic fraction. On the contrary, PCR-214 and PCR-120 incubated in cytoplasmic fractions from MCF7 and K562 cells did not show any detectable degradation, while PCR-102 showed a low level of degradation. After 48 hr, a comparable degree of DNA degradation was observed for all the cytoplasmic fractions, whereas PCR products incubated in nuclear extracts were still present. Considering these results, further stability tests in non-heat-inactivated FBS and adult human serum were carried out using PCR-214 as a model. As

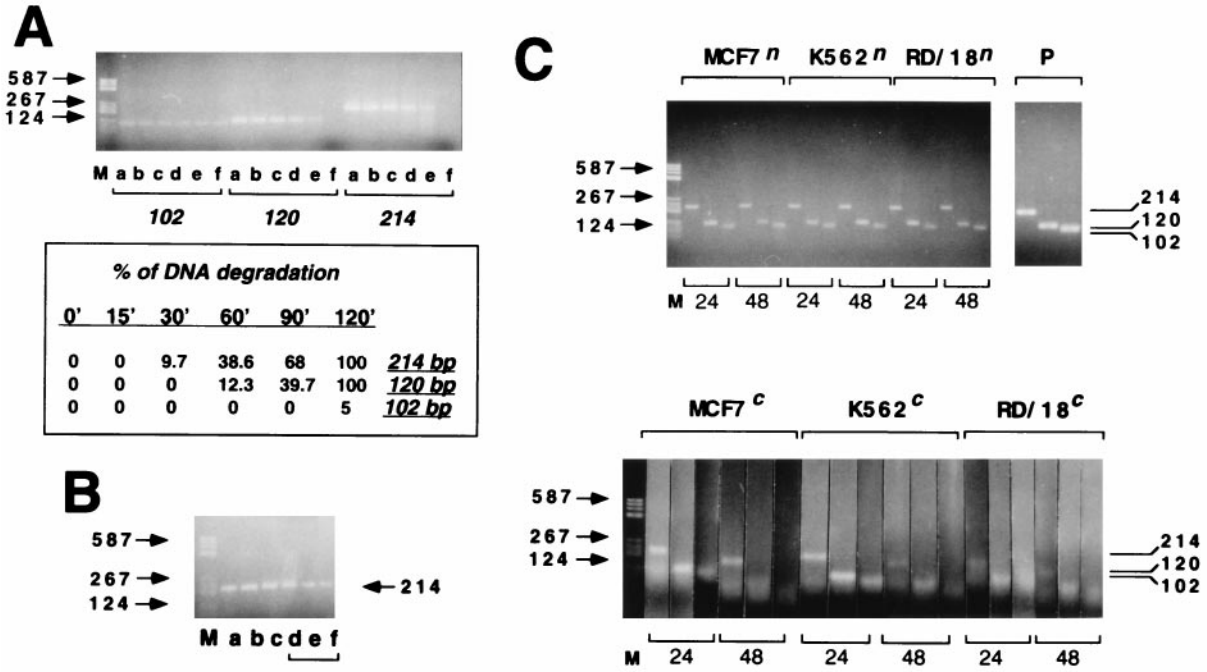

FIG. 2. Panel A: effect of FBS nucleases on PCR products. Agarose gel electrophoresis patterns of PCR-102, PCR-120, and PCR-214 incubated in the presence of 10% non-heat-inactivated FBS diluted in water at 37° for 0 (lane a), 15 (lane b), 30 (lane c), 60 (lane d), 90 (lane e), and 120 (lane f) min. The amount of PCR product remaining intact was determined by densitometric measurements of the fluorescence signals in agarose gel after ethidium bromide staining. The percentage of DNA degradation of the three different PCR products, in comparison with the PCR products that were not incubated, is indicated in the inserted table. Panel B: protective effect of α MEM on FBS nuclease digestion of PCR-214. Agarose gel electrophoresis patterns of PCR-214 incubated at 37° for 48 hr in the presence of water alone (lane a), α MEM (lane b), 10% FBS diluted in α MEM (lane c), and conditioned medium from MCF7 (lane d), RD/18 (lane e) and K562 (lane f) cells, respectively. Panel C: effect of cellular nucleases on PCR products. PCR-214, PCR-120, and PCR-102 were incubated at 37° for 24 and 48 hr in the presence of MCF7^n, K562^n, and RD/18^n nuclear extracts and MCF7^c, K562^c, and RD/18^c cytoplasmic fractions. PCR products that were not incubated are also indicated (P). In each panel, M = mol. wt marker (*Hae*III restricted pBR322 DNA).

shown in Fig. 3, PCR-214 was degraded more rapidly in aqueous 10% FBS than in aqueous 10% human serum. After 30 min (lane d), DNA was completely degraded in FBS, whereas even after 120 min (lane f) the same PCR product was stable in human serum.

In a second set of experiments, the stability of PCR-214 was determined in aqueous 10% and 90% human serum from volunteers of both sexes. The degradation kinetic was determined by sampling after different lengths of time, from 2 min to 48 hr. Before agarose gel electrophoresis, samples were phenol-extracted and Microcon-concentrated in order to remove the serum proteins. This purification step was inserted in order to prevent aspecific interactions occurring between nucleic acids and proteins, which would result in high mol. wt unmigrating bands. As shown in panel A of Fig. 4, during the first 8–12 hr of incubation a low degradation level was observed, suggesting that PCR products may be considered quite stable DNA molecules in adult human serum. On the contrary, after 48 hr no DNA bands were detectable, indicating that PCR-214 was, at that time, completely degraded. Therefore, the degradation of PCR product mediated by human serum nucleases was found, as expected, to be time-dependent. Instead, both 10% and 90% FBS exhibited a similar degradation rate pattern. In addition, no differences were observed between DNA incubations with male and female FBS samples (data not shown).

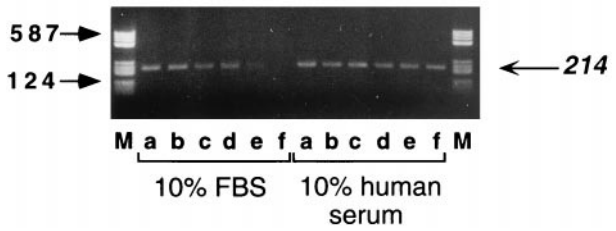

FIG. 3. Comparison of FBS and human serum nuclease activity on PCR-214. Agarose gel electrophoresis pattern of PCR-214 incubated in the presence of 10% FBS or 10% human serum in water at 37° for 0 (lane a), 2 (lane b), 15 (lane c), 30 (lane d), 60 (lane e), and 120 (lanes f) min. M = mol. wt marker (*Hae*III restricted pBR322 DNA).

In a further set of experiments, the possible protective effect of liposome was tested for PCR-DNAs incubated in human serum. In contrast with conventional liposomes, cationic liposomes do not entrap DNA molecules within their interior. The nucleic acid molecules are bound by ionic interactions on the surface of preformed cationic liposomes. The protection of cationic liposomes, constituted by PC:CH:CD 8:2:1 mol/mol/mol, was studied on degradation of PCR-214 catalyzed by human serum nucle-

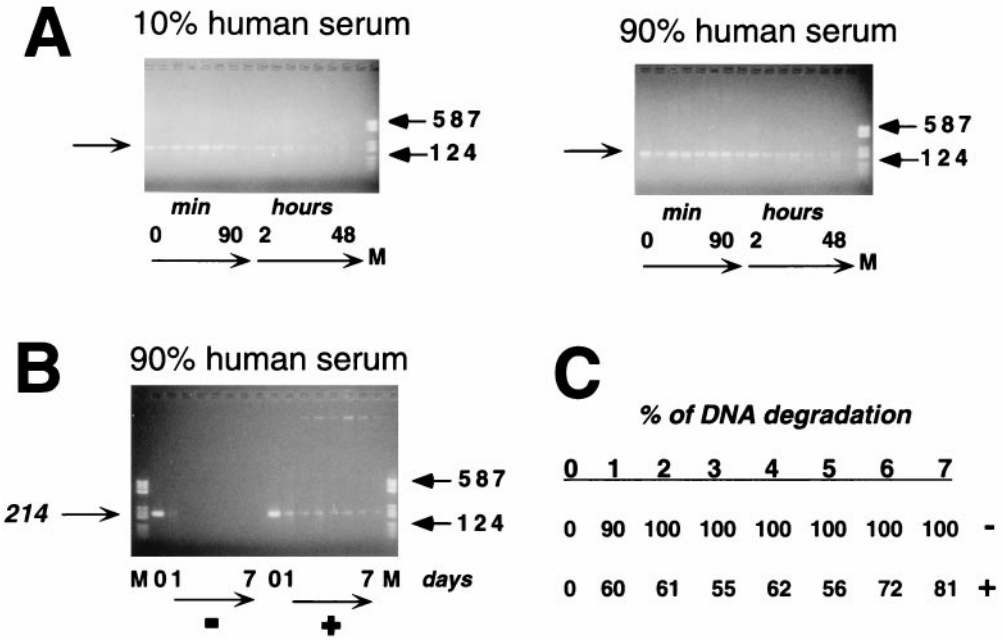

FIG. 4. Panel A: effect of human serum nucleases on PCR-214. Agarose gel electrophoresis patterns of PCR-214 incubated at 37° in the presence of 10% and 90% human serum. Samples were incubated for 0, 2, 15, 30, 45, 60, and 90 min (lanes 0 → 90) and 2, 4, 6, 8, 10, 12, 18, 24 and 48 hr (lanes 2 → 48), then phenol-extracted and loaded on agarose gel. The figure is representative of triplicate sets of experiments. Panel B: long-term protective effect of liposome on degradation of PCR-214 catalyzed by human serum nucleases. PCR-214 was incubated in the presence of 90% human serum with (+) or without (−) liposome. Samples were then phenol-extracted and loaded on agarose gel. The retardation of the DNA band in the gel resulted from DNA complexed with liposome not completely phenol-extracted. M = mol. wt marker (*Hae*III restricted pBR322 DNA). Panel C: densitometric analysis of the electrophoretic signals visualized by ethidium bromide staining in panel B of the figure. The percentage of DNA degradation of PCR-214 incubated in the presence of 90% human serum with (+) or without (−) liposome, in comparison with the PCR product that was not incubated, is reported. To calculate the percentage of degradation, the sum of densitometric scanning of the retarded and the expected normal band was considered. All liposome-DNA incubations were repeated a minimum of four times giving comparable results.

ases. Liposome/PCR-214 complexes were incubated at 37° from 0 to 7 days in the presence of 90% human serum diluted in water, treated as described in the Materials and Methods section and electrophoresed. As shown in panel B of Fig. 4, liposome incubation prolonged stability of PCR-214 in the presence of human serum up to at least 7 days, resulting in a protective effect toward nuclease digestion. Results of densitometric analysis of the electrophoretic bands visualized by ethidium bromide staining are presented in panel C, as a percentage of degradation compared to time 0 undegraded band. It is noteworthy that after 7 days of incubation PCR-214 were still detectable at *ca.* 20%. The same liposome protection experiment performed with 10% FBS diluted in water gave comparable results (data not shown).

DISCUSSION

Synthetic DNA molecules provide a potential therapeutic tool in the control of diseases and pathological processes at the genomic level. One of the major problems that has to be solved to increase the biologic effects of synthetic DNA molecules, such as oligonucleotides which are to be transfected into the cells, is to reduce their sensitivity to nucleases. The aim of this study was to elucidate the extracellular and intracellular fate of short double-stranded DNA molecules produced by PCR amplification (PCR-DNAs) by assessing their stability under conditions they would encounter outside and inside cellular compartments. The absence of chemical modifications in such molecules, proposed as competitors for transcription factors in a decoy approach, presents the advantage that the targeted proteins may have more opportunities to correctly bind the specific DNA sequence. Therefore, the possibility of PCR-DNAs to exert their biologic effect in term of control of gene expression rely on their ability to be resistant to nuclease activity of FBS or human serum and lysosomal enzymes.

The experiments reported here indicate that unmodified PCR-DNAs are quite stable molecules in stressing conditions favoring DNA degradation. Differences on stability appear to be related to size of DNA fragments, type of serum dilution and cell cytoplasmic fraction. The observation that DNA molecules are more resistant to serum nuclease activity when incubated in 10% FBS diluted in culture medium than when incubated in 10% FBS diluted in water, demonstrates a protective effect of the medium. This means that medium components (such as salts or nutrients) or possibly the medium pH could in some way inhibit the degradation of DNA fragments. It is noteworthy that in incubation with cell conditioned media representing the culture extracellular environment all the PCR-DNAs examined are stable for at least 48 hr, which corresponds to

the incubation time needed for transient cellular transfection experiments.

Interestingly, the experiments performed in the presence of adult human serum reveals a kinetic of PCR-DNAs degradation slower than that observed in the presence of FBS; this may suggests that human serum could contain lower amounts of endo- and exonucleases or less active ones.

The stability of PCR-DNAs inside the cellular compartments appears to be different in crude cytoplasmic fraction with respect to nuclear fraction. In fact, PCR-DNAs are quite stable in the presence of nuclear extracts regardless of the cell line. Differently, the degradation pattern up to 24 hr observed in presence of crude cytoplasmic fractions may vary according to cell type. This suggests that the nuclease activity of cytoplasmic fractions from RD/18 rhabdomyosarcoma cells is more effective toward DNA degradation than cytoplasmic fractions from K562 erythroleukemic and MCF7 breast cancer cells. It is not surprising that cytoplasmic degradation occurs in a more rapid fashion, since crude cytosolic extracts are well known to contain lysosomal enzymes, including a battery of hydrolases which may be responsible for nucleic acid degradation [10]. Nevertheless, it is interesting that the whole degradation of unmodified PCR product occurs only after 48 hr, and that a same PCR product degrades in MCF7 more slowly than in RD/18 cells. This finding suggests that: a) different cells may have different levels and types of nucleases; and b) in the range of 4–24 hr these PCR products are relatively stable toward these enzymes.

When complexed with cationic liposomes, as a delivery system, PCR-DNAs prolong their stability up to 7 days and, consequently, their potential biologic activity, providing a promising new class of therapeutic compounds potentially suitable to control disease-related genes.

It is important to note that, up to now, the nuclease activity present in FBS (or FCS) and adult human serum has been determined only toward ss- or ds-oligonucleotides with different chemical modifications [11–16], to obtain information concerning the ideal modified oligonucleotide to be used as antisense or decoy molecule. Moreover, stability experiments have been generally performed for shorter incubation times than those employed here. On the basis of our observations, the ds DNA molecule in the form of PCR-generated DNA fragment, thanks to its stability, may be considered an interesting transcriptional decoy molecule suitable for gene modulation approaches as suggested in our preliminary experiments [1]. Moreover, we have demonstrated that, by using a cationic liposome delivery system, the nuclease resistance of PCR products can be greatly increased, thus confirming that liposome represents a good system to administer *in vivo* PCR-DNAs, protecting them against breakdown by serum nucleases.

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