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Inhibition of gene transcription by purine rich triplex forming oligodeoxyribonucleotides

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ABSTRACT

Several oligodeoxynucleotides (ODNs) were designed in order to interact with the purine rich element of the IRE (Interferon Responsive Element) of the 6-16 gene by triplex formation. An ODN of 21 bases, the sequence being identical to that of the purine strand of the IRE (48% G), but in reverse orientation, was able to interact with the IRE (K_D: 20 nM). The binding was Mg^{2+} dependent. The two purine strands of the triplex were oriented antiparallel as confirmed by DNAase I and copper-phenanthroline footprinting experiments. An ODN in which A were replaced by T, also interacted with the same target, but with a lower affinity. Exonuclease III action indicated that the two IRE repeats of the 6-16 promoter interacted with each other through Hoogsteen base pairing, the third strand being parallel to the paired Watson – Crick strand. This led to a potential H-DNA structure which could be destabilized by adding ODNs able to form a triplex structure. 6-16 IRE driven-reporter gene constructs lost their interferon stimulability when co-transfected with triplex forming ODNs. The range of effective ODN concentrations was compatible with the affinity determined when measuring their direct interactions with the DNA.

INTRODUCTION

Elements of gene sequences having a high purine (pur) content have been the first to be considered as potential targets for triple helix formation (1–3). The rules defining the pairing of the third strand to the duplex are those defined by Hoogsteen (4). The third strand, composed of pyr, lies in the major groove of the duplex DNA. More recently, a number of different studies were designed to interfere with gene expression by targeting a critical region of the promoter of a given gene through triple helix formation (5–10). This led to the demonstration of new possibilities for pairing single stranded DNA to double stranded DNA. Other studies, examining specific properties of some genes, came to the conclusions that within the genome, H-DNA conformations might exist (11–14). Such structures also involved triplex formation. Now, a number of situations where a single strand DNA had been shown to interact with a duplex DNA, had been described. Depending on the model under study, the third strand had to be oriented parallel (6,15–17) or antiparallel (5,8,10, 18, 19) to the pur strand of the duplex DNA with which it is supposed to interact. Some of the models studied needed an acidic medium to allow the protonation of the cytosine. In a few cases, the addition of polyamines was necessary (20). In other cases, a triple helix could be formed at neutral or slightly alkaline pH, in the presence or absence of added magnesium (8, 21).

Interferons (IFNs) are regulators of many cellular functions. They have immunomodulatory actions, antiproliferative effects and antiviral activities (22–24). A consensus sequence could be noted on the basis of the comparison of the 5′-flanking regions of a number of IFN-inducible genes (25–26). It can confer IFN responsiveness to a reporter gene (27,29). This IFN-Responsive Element (IRE) can be considered as a purine (pur) rich element. Considering the possibility of interfering with IFN-induced gene expression within the cell, conditions for triple helix formation which will allow a physiological pH are a necessary prerequisite. In addition, since the nuclei are known to contain a high Mg^{2+} content, it can be expected that the triple helix structures requiring Mg^{2+} may find conditions for their formation at their site of interaction. These requirements mainly concern the possibility of forming pur*pur-pyr triplex. (13, 21).

Thus, the case of the IFN-inducible genes appeared favorable. Among these genes, the 6-16 gene is of interest. Its IRE lies within a 39 bp sequence. Two copies of this sequence, one of which contains a dinucleotide insert, are in tandem upstream to the transcription initiation site (28). Except for one base, the length of the purine tract was 21 bases. Each purine tract was separated by 20 bases. Therefore, besides testing the interference of triple helix formation at the level of the purine tracts on IFN action, the possibility that each of the purine tract could interact with the other through Hoogsteen base pairing was investigated. The data reported here show that it was possible to interfere with the IFN-induced expression of a transfected reporter gene under the control of the 6-16 IRE by using ODNs shown to form triple helix with the IRE. Footprinting experiments indicated that the two tandem purine tracts may interact with each other, forming a H-DNA structure as suggested in a preceeding paper (30).

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MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides (ODNs) were purified by gel electrophoresis under denaturating conditions and gel-eluted ODNs concentrated using Sep-Pak cartridges (Waters). Alternatively, they were purified after synthesis using Oligo-Pak columns (Waters). ODNs were labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP (ICN).

Construction of IRE-containing plasmids

The ODNs 5′ IRE and 3′IRE shown in Table I were cloned individually or after appropriate ligation to each other in the BamHI or Sma I sites of the pT3T718U vector (Pharmacia). For transfection experiments, these constructions containing the IRE(s) were cut with Eco RI and then filled with the Klenow fragment. The second cut was at the Xba I site. In order to minimize false positive, the ampicillin resistance site was destroyed by cutting with Sca I. The insert of interest was separated from other fragments by gel agarose electrophoresis and cloned in the pBLCAT2 vector (31 modified according to ref. 32) previously cut with XbaI and Hind III, the latter site being filled with the Klenow fragment. Screening of the colonies was performed using one of the ODN constituting the IRE and an ODN which sequence was complementary to that of thymidine kinase (TK) promoter. The latter ODN served as a primer for sequencing the insert. All the plasmids were used after purification on Qiagen columns.

Triplex gel shift experiments

Triplex formation was allowed to proceed in 50 mM tris−HCl, pH 7.4, 10 mM Mg^{2+} and 15% sucrose. Samples were heated to 65°C and then allowed to cool to 37°C for at least one hour. Studies involving only ODNs were performed using non denaturating polyacrylamide gel electrophoresis (acrylamide/bis acrylamide, 19/1; 8%, w/v acrylamide), those involving plasmids or restriction fragments, agarose (0.6%) gel electrophoresis. Electrophoresis were performed at 4°C using as electrophoresis buffer 1×TBE where EDTA had been replaced by 10 mM Mg^{2+}. At the end of agarose gel electrophoresis, the gels were transferred on Zeta probe membrane (Bio-Rad) using 20×SSC as transfer buffer. The membrane was then autoradiographed and when necessary each band was cut and counted by Cerenkov radiation. At the end of polyacrylamide gel electrophoresis, the gel were dried and autoradiographed.

Triplex mediated footprints

The conditions for copper-(1,10)-phenantroline (OP-Cu), DNAase I and exonuclease III footprinting experiments are given in the legend of the Figs. Probes were labeled with T4 polynucleotide kinase after cutting with Eco RI (or Hind III) and alkaline phosphatase treatment. After a second digestion with HindIII (or Eco RI), the probes were purified on polyacrylamide gel electrophoresis and eluted from the gel. Sequencing was performed according to Maxam and Gilbert (33).

Transfection procedure

For transfection experiments, in order to minimize the degradation of ODNs added to the cell culture medium, the fetal calf serum was heat-treated to reduce endogenous nuclease activities (34, 35). Confluent HeLa cells were splitted at a ratio of one to four in 6-well trays. 24 h later the medium was changed omitting serum. DNA (10 μg), mixed together with 10 μl lipofectin (BRL) in 1 ml of serum free medium, was added and left for 4 h on the cells at 37°C. IFN addition, when appropriate, was 5 h later this last medium change. Cells were harvested after 40 h growth. The chloramphenicol acetyl transferase (CAT) assay was performed as described in ref. 36 using butyryl-CoA and performing the extraction of the butyrylated product with xylenes before liquid scintillation counting. Amounts of cell extract were adjusted so that the extent of substrate conversion was kept below 20%. Results were normalized to the protein content of the samples measured according to Bradford (37). Each experiment was performed at least twice.

RESULTS

Rationale and design of the third strand

The ODNs used in this study are listed in Table I. The ODNs constituting the 5′IRE and the 3′IRE were synthetized for cloning either of the repeat or both in different vectors (see Methods). IRE corresponded to an ODN already used in other studies in which its interactions with proteins were studied upon IFN addition (27,30). IRE-7 and IRE-14 were sequences of the IRE deleted respectively by 7 or 14 bases. In the case of IRE-14, 2 bases supposed to be involved in triplex formation (underlined sequence), are missing when compared to IRE or to IRE-7. 'Pur anti//' has the same sequence as the strand with which it is supposed to pair, but in reverse orientation. It should be able to lead to the formation of the G*GC and A*AT triplets (pur*pur-pyr triplex)(5, 8, 10, 18, 19). 'GT anti//' should hybridize in the same way, the A being replaced by T (8). 'Pur//' (6) and 'pyr//' (38) represent respectively the upper and the lower strands of the IRE. 'Pur//' might lead to the possibility of G*GC and A*AT triplets, the two Hoogsteen paired strands being parallel (6). 'Pyr anti//' (same sequence as the pyr strand of the IRE, but reverse orientation) could potentially lead to T*AT and C*GC pairings (pyr*pur-pyr), the ODN being oriented parallel to the pur strand (1, 15−17). Finally, 'GC//' according to ref.15, would

Table I. ODNs used in this study

5′IRE	5′GATCGCGGAGCTGGGAGAGAGGGGAAAATGAAACTCTGCACAGTGCAG 3′CGCCTCGACCCTCTCTCCCCTTTTACTTTGAGACGTGTCACGTC
3′IRE	5′GAGCTGGGAGAGAGGGGAAAATGAAACTGCAGAGTGCAGAAA 3′CTCGACCCTCTCTCCCCTTTTACTTTGACGTCTCACGTCTTTCTAG
IRE	5′GAGCTGGGAGAGAGGGGAAAATGAAACTGCAGAGTGCAG 3′CTCGACCCTCTCTCCCCTTTTACTTTGACGTCTCACGTC
IRE-7	5′GAGCTGGGAGAGAGGGGAAAATGAAACTGCAG 3′CTCGACCCTCTCTCCCCTTTTACTTTGACGTC
IRE-14	5′GAGAGAGGGGAAAATGAAACTGCAG 3′CTCTCTCCCCTTTTACTTTGACGTC
pyr anti//	5′CCCTCTCTCCCCTTTTGCTTT
pur anti//	5′AAAGTAAAAGGGGAGAGAGGG
GC//	5′GGGGTGGGGCCCCGCGCGCCC
GT anti//	5′TTTGTTTTTGGGGTGTGTGGG

5′ IRE and 3′IRE correspond to the two repeats of the 6-16 gene, in this order among the transcription initiation site. The differences between the repeats are indicated by the 4 bases in small capitals. The non-paired bases correspond to the Bam HI cloning site added. The underlined sequence indicates the targeted region for forming triplex. The bold large T and A are the points of mismatch for defining triplex formation.

lead to G*TA and C*CG pairing and had to be parallel to the pyr strand of the duplex.

IRE is a target for triplex formation

A number of ODNs were tested for their ability to form a complex with IRE using a band shift assay under non denaturating polyacrylamide gel electrophoresis conditions (Fig. 1). 'Pur anti//' and 'GT anti//' elicited a retardation in the migration of the IRE. None of the strands constituting the IRE demonstrated any ability to interact with the duplex. These data were obtained with Mg^{2+} during the initial stage of formation of the complex and during the electrophoresis. If Mg^{2+} was omitted during the incubation period, the amount of retarded IRE was decreased. The same two ODNs were able to induce a band shift. It is likely that before entering the gel (which contained Mg^{2+}), some triplex formation had time to occur. This suggested that the rate of interaction should be rapid. Heating the samples before loading the gel still allowed the demonstration of retarded bands with the same ODNs. Such an observation confirmed in another way the rapid association of the ODNs with the duplex. Experiment using the same incubation conditions, but without Mg^{2+} during the electrophoresis step, could not allow the demonstration of triple helix formation (not shown).

The working hypothesis defined precisely the region of the IRE involved in triplex formation and was supported by the data presented in Fig. 2. Using IRE-14, no retardation of the probe was apparent with the ODNs shown to be able to promote such an effect. In this case, 2 bases of the targeted motif were deleted. In addition, the truncated motif was on one of the extremity of the duplex, which should not favor the stability of the base pairing due to gaping (in addition, at the other end of the motif, there should be a weaker association of strands, due to the mismatch according to the pairing rules)(see legend to Table I and Results below). With the IRE-7 probe a clear retardation pattern was observable with 'pur anti//'. With 'GT anti//' only a smear, starting from the most retarded band, was observed. In no instance 'GC//' interacted with any of the duplex used. Identical

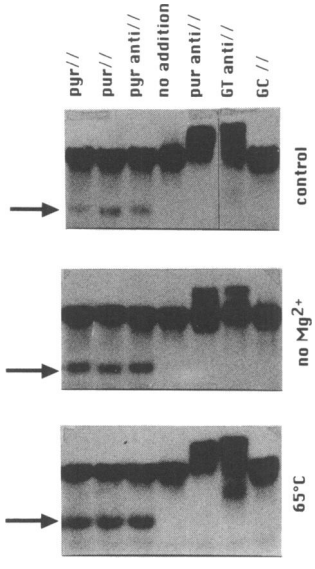

Figure 1. Specificity of triplex formation probed by band shift. Tracer amounts of IRE (50 fmoles), labeled on both strands, were incubated with 1 nmole of the indicated ODNs under 20 μl in the medium described under the 'Methods' section. In the case of the middle panel, Mg^{2+} was omitted. All samples were heated to 65°C and then allowed to cool to 37°C. For the lower panel, samples were heated to 65°C and put immediately in ice before loading on the gel. In all conditions, both the gel and the running buffer contained Mg^{2+}. The band indicated by an arrow corresponded to one of the strand of the IRE which was probably in small excess over the other (upper panel) or to strands which did not have time to reassociate before loading on the gel.

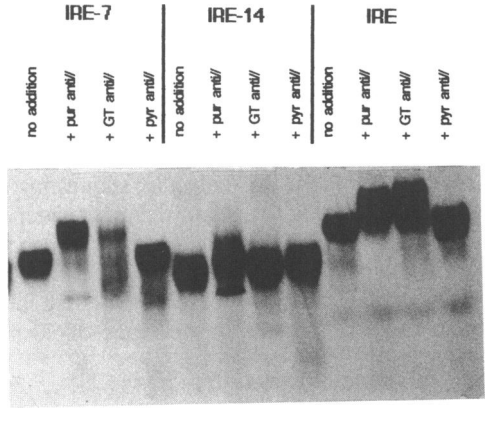

Figure 2. Ability of shortened IRE to be retarded by triplex forming ODNs. The double stranded ODNs were labeled and mixed with the indicated single stranded unlabeled ODNs using the same concentrations as that described for Fig. 1.

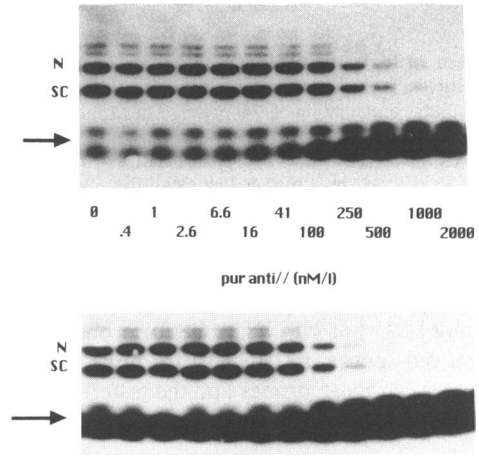

Figure 3. Binding of triplex forming ODN on IRE-containing plasmids. Two amounts of plasmids were used: 1 μg (upper panel) and 100 ng (lower panel). To a tracer amount of 'pur anti//', increasing amounts of unlabeled ODN were added as indicated on the abscissa. For the lowest plasmid concentration, the specific activity of the probe was increased 5 times. The incubation volume was 30 μl of which 20 were loaded on horizontal agarose gel. Considering the presence of two IRE repeats in the plasmid, the total IRE concentrations during the incubation were respectively 30 and 3 nM when using 1 μg or 100 ng plasmid. The analysis of the data using Scatchard analysis gave K_D values of 13.5, 12.2 and 12.4 nM and B_{max} values of 5.3, 12.7 and 17.7 nM for respectively the concatenated, the relaxed and the supercoiled forms of the plasmid when 1 μg of the later was used. Using 10 times lower amounts of the plasmid, its affinity for the ODN, as far as the concatenated forms were concerned could not be determined. For the nicked (N) and supercoiled (SC) forms, the K_D values were respectively 20.8 and 13.8, the B_{max} values, 1.1 and 1.7 nM. The migration of the free probe is indicated by an arrow. The reason for the band just ahead of the free probe was not explained. It was present when probe alone was loaded on the gel..

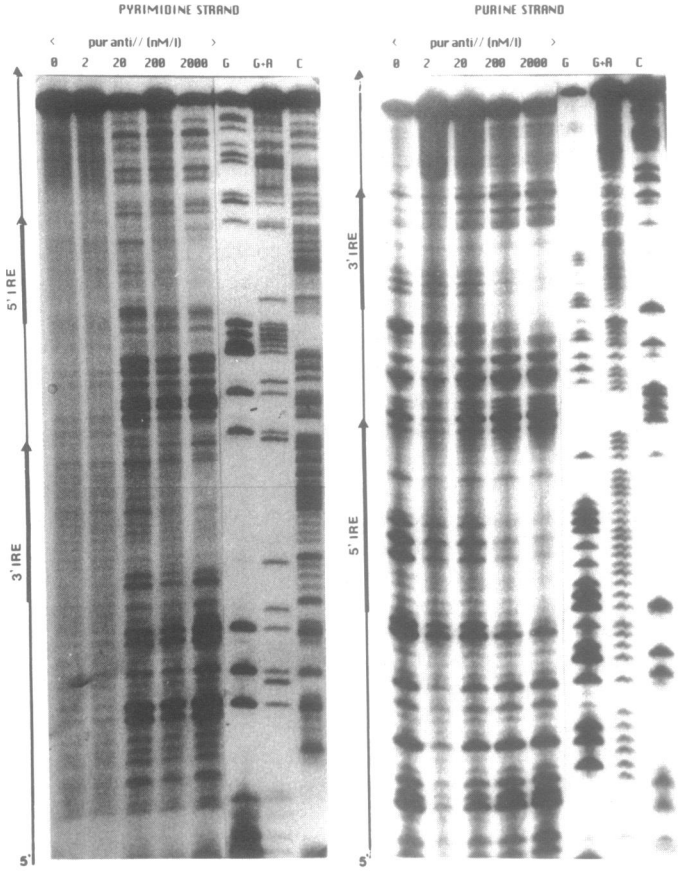

Figure 4. Effects of triplex formation on the pattern of DNAase I digestion. Conditions of triplex formation were those defined in 'Methods'. DNAse I was then added at a final concentration of 400 pg/ml. After 1 min at 37°C, its action was stopped by phenol extraction. Samples were ethanol precipitated and analyzed by denaturating polyacrylamide (8%) gel electrophoresis. The positions of both 5′ and 3′ IRE is indicated by a thicker line along the autoradiogram.

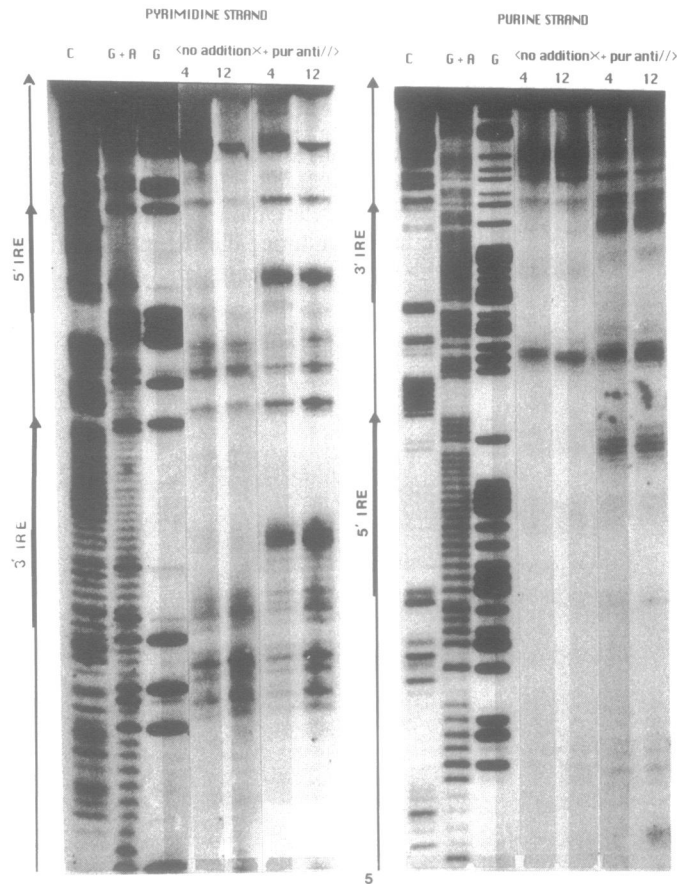

Figure 5. Triplex formation enhanced cleavages due to OP-Cu. Triplex formation was allowed to proceed under 30 μl using or not 'pur anti//' concentration equal to 1 μM. 1.4 μl of solution A (4 mM of (1−10) phenanthroline and 1 mM $CuSO_4$) and 1.4 μl of β-mercaptopropionic acid were added to each samples. Reaction was stopped either 4 or 12 min later with 4 μl of 2−9 dimethyl (1−10) phenanthroline. After dilution and ethanol precipitation, samples were analyzed as in Fig. 4.

results were obtained, except for a greater mobility shift, when the ODNs were labeled instead of the duplexes (results not shown). Using PRD-I or NF-κB probes or probes containing the IREs of various genes (HLA-A3, IFI-56K, HLA-DR), no interaction between these probes and the tested ODNs could be demonstrated (results not shown).

Triplex formation within plasmids

The basic features of the model being verified, it had to be validated on a system of higher complexity such as a plasmid. Either form of a plasmid (nicked, supercoiled or concatenated) was able to retard 'pur anti//' (Fig. 3). The vector containing the two repeats as in the 6-16 gene, interacted with 'pur anti//' in a dose dependent manner. This process was saturable within the range of concentration which had been observed with the synthetic IRE. The 39 bp IRE was an effective competitor for the binding of 'pur anti//' to the cloned sequence. The specificity of the interaction was identical to that described above with the duplex IRE under conditions where the IRE (39 bp) represented approximately 1% the number of bp of the vector. The vector without insert was not able to elicit such an effect even when increasing the amount of ODN (not shown).

The determination of the affinity of 'pur anti//' for each of the form of the plasmid containing one or two IRE motifs was done by quantitating each of the retarded bands after agarose electrophoresis. Whatever the form of the IRE-containing plasmid (nicked, supercoiled or concatenated), the affinity of 'pur anti//' for the IRE was the same (Fig.3). Its affinity was independent on the number of IRE repeats. The total number of binding sites determined from the Scatchard analysis of the data was in a good agreement with the estimate deduced from the DNA concentration in the medium and the number of IRE motifs in the DNA (see legend to Fig. 3). There was no indication of an interaction of the IRE repeats within the DNA molecule, suggesting therefore that 'pur anti//' interacted with each of the motif independently. All the data being consistent with the Scatchard analysis of the data, it could be conclude that the formation of the triple helix structure followed the characteristics of a reversible interaction. The K_D for this interaction was in the range of 20 nM.

Footprinting of triplex formation

DNAase I footprinting. The data presented in Fig. 4 showed that at high 'pur anti//' concentration, two regions of the probe were less sensitive to DNAase I action. These protected regions corresponded to the supposed site involved in triplex formation for either strand of the probes. This agreed with the DNAase

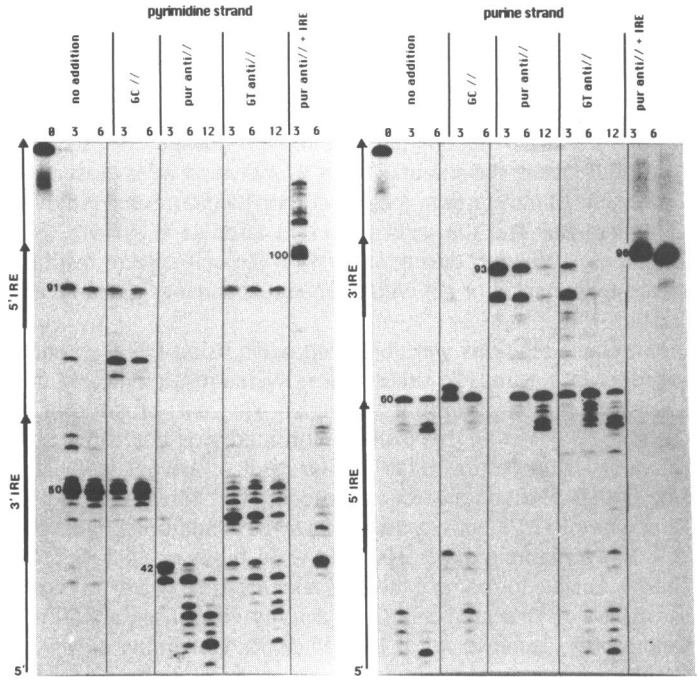

Figure 6. H-DNA structure suggested by exonuclease III action: effects of triplex forming ODNs. Incubation conditions were those previously defined except for the presence of 1 mM dithiothreitol and 1 μg of *E.coli* DNA. The volume was 40 μl to which were added 4 μl of exonuclease III (20 U). From zero time, 7 μl aliquotes were taken at 3, 6 and 12 min and reaction quenched with NaOH-containing gel loading buffer. Samples were analyzed on denaturating gel electrophoresis along with degradation products of the probe according to Maxam and Gilbert (not shown here due to the difference in exposure times of the autoradiogram). The ODN concentration was 5 μM, that of IRE, 1μM.

I properties which action could be blocked by a triple helical structure on one hand, and which had a poor activity in digesting single strand DNA on the other. The domain of 'pur anti//' concentration which promoted a significant protection of the probes was in a good agreement with the affinity determined from the Scatchard analysis, albeit one order of magnitude higher. A 'pur anti//' concentration equal to the K_D (20 nM) would lead to 50% complexation of the probe in the triple helix complex. Using such a technique, a protection by the ODN could be difficult to detect. However using a 10 fold higher concentration of ODN, i.e. 10 K_D (200 nM), 90% of the probe will engaged in the triplex, which then would allow to detect a significant protection by the ODN against the DNAase I action.

'GT anti//' altered the cleavage pattern due to DNAase I but only at the highest concentrations (not shown), which was consistent with its lower ability to promote triplex formation (Fig. 2). ODNs whose ability to promote a band shift had not been demonstrated, were unable to promote any protection of the probes (not shown).

Interestingly, preliminary experiments (not shown) showed that about 10 times more DNAase I was necessary to get a significant digestion of the probe when it was not involved in triplex formation. The poor pattern of digestion shown in lanes 0 and 2 (left panel, Fig. 4) reflected such a situation. Data presented below suggested the possibility for the probe to adopt an H-DNA conformation, this structure being destroyed upon triplex formation. Such a structure which leads to a folding of the DNA on itself may well be responsible for a low sensitivity of the probe to DNAase I action due to a limited access. Obviously, high enough DNAase I can introduce breaks in the structure and then further allows enzyme action (note that in the presence of Mg^{2+}, DNAase I cleaves each strand of the DNA independently). It can also be noted that the pyr strand of the probe appeared to be more resistant to DNAase I than the pur one (Fig. 4). This was also consistent with the H-DNA model proposed below in which part of the pyr strand should not be paired and therefore be a poor substrate for DNAase I.

1,10-phenanthroline-copper (OP-Cu) footprinting. In the absence of any ODN added some preferential cleavages were observed (Fig. 5). This might due to the rate of the cleavage reactions in relation to local sequence (39, 40) as well as to secondary structure within the DNA molecule (the oxidative species generated by OP-Cu reacts with the sugar portion of the DNA). The pur and pyr rich regions of the IRE were the less sensitive to OP-Cu action. The addition of 'pur anti//' increased the rate of cleavage within the region where it was supposed to interact on either strand of the probe. Looking more precisely to the region of the DNA for which an increase cleavage was observed, it turned out that it concerned the bases centered on the point for which a mismatch was pointed out as far as the rules of triple helix formation were concerned (see Table I). The triple stranded structure seemed reasonnably stable since increasing the length of Cu-OP action did not modify the cleavage pattern observed. Omitting Mg^{2+} led to identical cleavage patterns whether or not 'pur anti//' was added (not shown).

The formation of the triple stranded structure protected the pur rich strand at the site of interaction from DMS action as expected for Hoogsteen interactions (not shown). The G at the 3′ end of the motif was equally sensitive to DMS action in the presence or absence of the third strand which validated the model predicting that the presence of a T should generate a mismatch. No other effects of 'pur anti//' on the sensitivity to DMS could be detected outside of the targeted motif, nor on the other strand of the duplex.

Protection of exonuclease III action by triplex formation

Considering the purine rich strand, in the absence of triplex forming ODN, one major stop of the exonuclease action was observed at the position 60 (Fig. 6). It corresponded to both the end of one of the IRE repeat and also to the middle of the probe. When 'pur anti//' was added a transient protection of the probe was observed up to position 93, which corresponded to approximately the middle of the first repeat encountered by the exonuclease III. Increasing the incubation time or the amount of enzyme led to the same pattern as observed in the absence of added 'pur anti//'. With the 'GT anti//' the protection of the probe was less efficient with the incubation time, consistent with a lower affinity of this ODN for the triplex formation (see above). On the pyr rich strand, two stops (positions 50 and 91) for exonuclease III action were observable: they corresponded to the middle of each of the IRE repeat.

These data were consistent with the possibility for the probe to form an H-DNA stucture. Such a structure can be achieved through parallel pairing of the 3′ IRE (or 5′ IRE) pur strand with the 5′ IRE (or 3′ IRE) double stranded motif. This will lead to single stranded pyr regions. Exonuclease III being unable to digest single stranded DNA, stops for its action will then occur within the 3′ IRE (position 91) or 5′ IRE (position 50) of the pyr strand. Upon the addition of 'pur anti//', the exonuclease III action will

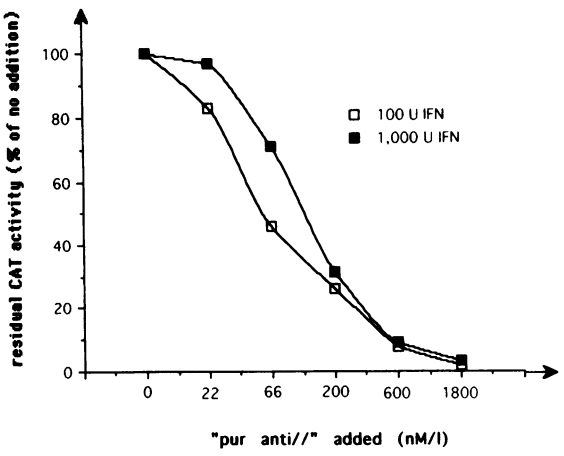

Figure 7. Inhibition of IFN-stimulation of IRE-controled CAT gene by triplex forming ODN. For transfection conditions, see 'Methods'. Time of IFN treatment was 40 h. The 'pur anti//' concentrations indicated were those used at the time of transfection. The CAT activities measured in the absence, or with either 100 or 1 000 U $\alpha\beta$ IFN, were respectively equal to 400, 12497 and 40900 dpm/h/μg protein.

be stopped at the first IRE repeat encountered (position 93 on the pur strand). IRE addition led to the same result albeit the extent of protection was somewhat higher. When adding IRE, the DNA did not have to fold back on itself, the pairing occurring between the synthetic IRE and the IRE-containing DNA. Thus constraints on the DNA molecule were minimized (these constraints due to the folding of the DNA may lead to gaping of the extremities of the pairing regions, favoring the exonuclease III action). Due to the pairing of the double stranded IRE with the pur region of any IRE motif of the probe, the pyr region of the probe will be single stranded and therefore not be sensitive to exonuclease III action (stop at position 100). The absence of protection observed when 'pur anti//' was added (stop at position 42) was compatible with the possibility for exonuclease III to digest the pyr strand in a pur*pur-pyr complex, the pyr strand being paired by means of Watson–Crick bonds to the pur one and not engaged in interactions with the third strand.

The DMS footprinting agreed with this interpretation since when triplex formation occurred in a parallel orientation between the pairing strands, the N-7 of the guanine of the duplex is not involved in the association with the third strand and thus reactive. Furthermore, such an analysis fitted the observations presented above concerning the sensitivity of the probe to DNAase I action.

Triplex formation inhibited gene transcription

Several attempts were made in order to get inhibition of the IFN-induced CAT activity by adding triple helix forming ODNs in the cell culture medium. Concentrations of ODNs up to 5 μM were added in the cell culture medium, before IFN addition and during the IFN treatment. At most a 40–60% inhibition of CAT activity could be observed (not shown). Therefore, it was decided to co-transfect both the reporter gene and the triple helix forming ODN. Under these conditions, a dose-dependent inhibition of the measured CAT activity was observable as a function of the 'pur anti//' concentration (Fig. 7). The maximum inhibition, leaving only% or less of the original activity was observed with micromolar concentration of 'pur anti//'. Half-maximum inhibition occurred at approximately 50–100 nM of ODN. This value had to be compared to those determined from the Scatchard analysis of the binding data described above and from the DNAase I footprinting experiments. They fell within the same range especially if one takes into account the transfection procedure used: the amounts of ODN indicated were those used at the time of transfection, the transfection medium being removed 3 hours later and the cells harvested after 44 h growth (See Methods). Whether during these 44 h the cell culture medium was supplemented or not with ODN did not change the observed results.

Similar inhibitions were obtained when using DNA amounts of reporter plasmids 25 times lower. Such a result indicated that the amount of plasmid transfected was not proportionally decreased as the ODN concentration increased due to limiting amounts of lipofectin. In fact similar results were obtained using the DEAE-dextran transfection procedure but with lower absolute amounts of CAT activity (not shown). As additional controls, ODNs (containing either 100% GA, 15 bases or 45% GA, 21 bases), unable to promote the formation of triple helix, promoted at most a 17% decrease in CAT activity when used at 150 nM and a 50% decrease at 1.5 μM. Plasmids containing either the SV40 enhancer and promoter or no enhancer upstream of the TK promoter, were inhibited respectively by 20 and 17% at 150 nM of 'pur anti//' and by 40 and 34% at 1.5 μM. The levels of these inhibition could in no way be compared to those described above (not shown).

Using an inframaximum dose of IFN, an inhibition of the response was observable for lower doses of added 'pur anti//'. Such an observation might be taken as an indication for a competition of transcription factors known to bind to the IRE with the IRE-directed triple forming helix ODN (Fig. 7).

DISCUSSION

The data presented in this paper showed that a triple helix structure of the pur*pur.pyr type, the third strand being antiparallel to the pairing strand of the duplex, could be formed in vitro. The extrapolation of the data suggested that the same could occur within the cell, albeit not demonstrated. Anyhow, the results of gene expression inhibition supported this conclusion. A number of studies demonstrated the possibility of targeting specific DNA sequence by forming triplex. In most cases no functional consequence had been looked at. ODNs were shown to block the action of restriction enzyme when pairing to its site of recognition (41, 42) or to prevent the interaction of DNA-binding proteins with the DNA (17). Other works reported the specific strand cleavage of duplex DNA once paired with a third strand with one of its extremity bearing a cleavage reagent (1, 16, 43–47).

In comparison, fewer examples of interference with gene expression by triple helix formation had been reported. Some data were obtained with eukaryotic cell-free transcription system (6, 7). An inhibitory effect on the transcription was observed when using ODN forming triple helix structure supposed to bind at the Sp1 binding site, i.e. upstream the initiation site (9). Other studies showed that triplex formation just dowstream the *E.coli* RNA polymerase binding site of the bla gene blocked the transcription (48). ODNs were also designed to bind to the site of interaction of transcription factors, such as NF $\varkappa$B (49). In the case of the c-myc promoter, the c-myc mRNA level could

be reduced by 50% when using ODN concentrations outside of the cells equal to 25 μM (50). A 2-fold decrease in the nascent mRNA transcription had been described for the IL2Rα gene in lymphocytes by promoter region colinear triplex formation using ODN concentration equal to 15 μM (8). We observed similar inhibitions of reporter gene expression when adding similar amounts of third strand in the cell growth medium.

By using a cotransfection procedure, the amounts of ODN needed to get significant inhibition of gene expression were lowered. At the highest concentrations used (μM range) an almost complete inhibition was obtained. Such a procedure made compatible the values determined in vitro for the affinity of the triplex formation and those determined for the biological effect. At this point several points have to be raised. The transfection procedure used was not compatible with the transfection of a preformed triplex (mainly because of the low Mg^{2+} content of the cell transfection medium). At most, both the reporter gene and the ODN were enable to enter simultaneously in the cells. The observed effects strongly suggested that the conditions for forming the triplex were indeed found in the cell. It is of interest to note that the time at which the samples were prepared for CAT assay was rather long after the entry of both ODN and reporter gene in the cell. One might have expected a significant degradation of the ODN, which would have lower its apparent efficiency in inhibiting gene expression. In fact if samples were prepared for CAT assay at shorter times after transfection, the relative potency of each concentration of ODN was not changed (the only difference was in the amount of CAT activity measured, due to the steady state of CAT expression not being reached; results not shown).

The possibility that the ODN might be a competitor for the reporter gene due to limiting amounts of lipofectin could not account for the data. Using amounts of reporter DNA differing by 25 fold, the curves of the dose dependent inhibition of CAT activity observed were superimposable once normalized to the activity measured in the absence of added ODN. In addition, the control experiments performed with either other plasmids or ODNs unable each to promote triplex formation, did not yield similar inhibitions (see results). Therefore, it might be suggested that triplex formation could enhance the intracellular stability of the ODN. Alternatively, the route by which it was delivered in the cell may localize the ODN, still accessible to its target, in a compartment where degradation was reduced. A number of studies indicated that the amount of ODN taken by cells could lead to intracellular concentrations at least equal to those in the cell culture medium (8, 51, 52). If this was indeed the case in our study, the low effects observed mainly rised the problem of intracellular targeting of the ODNs (besides that of intracellular delivery).

The data presented in this paper led to the conclusion that the IREs when present in tandem could form H-DNA structure. There are no evidence that such a structure might exist in the cell and/or it might have a functional role. It could be possible that the H form of the promoter controled the basal rate of transcription and that upon IFN addition, it is destroyed upon ISGFs binding. The possibility of forming H-DNA structures had been raised in the case of the promoter of IFN-stimulated genes (30) and demonstrated in the case of homopur-homopyr sequences (11–14). In theses cases, depending on the presence or not of Mg^{2+}, the triplex pur*pur-pyr or pyr*pur-pyr were respectively formed.

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