A short purine oligonucleotide forms a highly stable triple helix with the promoter of the murine c-pim-1 proto-oncogene

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ABSTRACT
A homopurine–homopyrimidine region of murine c-pim-1 proto-oncogene was chosen as a target for triple-helix-forming oligonucleotide. Oligonucleotide 5'-GGG-GAGGGGGAGG-3' was shown to bind to its target sequence in the presence of 50 mM Na+ or K+, 10 mM MgCl2 and 20 mM Tris–acetate, pH 7.5. This oligonucleotide is bound in an antiparallel orientation with respect to the homopurine sequence. As was shown by co-migration assay the triplex is stable up to 65°C. At 37°C it was practically irreversible: after 24 hours of co-migration assay there was no traces of triplex dissociation. The rate of triplex formation was highly accelerated with increase of temperature and Mg2+ concentration. This rate was higher for superhelical DNA when compared to the linear and circular ones and the preference was dependent on temperature and Mg2+ concentration. The precision of this interaction is extremely sequence dependant in the c-pim-1 promoter region. Indeed only one difference when compared to the target gave negligible triplex formation under investigated conditions. These data suppose that natural triplex structures could play an important role in eukaryotic gene regulation and/or chromatin structure formation.

INTRODUCTION
Homopurine–homopyrimidine regions in DNA have attracted a great deal of attention in connection with their possible role in gene regulation in eukaryotes [1,2]. On the other side these regions raise the possibility of manipulating gene expression through artificial triple helix formation [3,4]. Displacement of DNA-bound regulatory proteins from their recognition sites by triplex forming oligonucleotides might provide a general strategy for the alteration of sequence-specific function in eukaryotes [5]. While the kinetic parameters of formation of an oligodeoxyribo-nucleotide-directed triple helix as well as its stability were carefully investigated for the pyrimidine–purine–pyrimidine structural motif [6–9] a few is known about purine–purine–pyrimidine triple helical structural motif [10,11] that can be also utilized for sequence-specific DNA recognition. In the present work we have investigated the triplex complex formation with a polypurine oligonucleotide 5'-GGGGAGGGGGAGG-3' targeted to the promoter region of c-pim-1 gene. This proto-oncogene encodes a highly conserved serine/threonine phosphokinase which is predominantly expressed in hematopoietic organs and gonads in mammals [12,13]. Its overexpression predisposes to lymphomagenesis in mice [14]. The oligonucleotide 5'-GGGGAGGGGGAGG-3' was shown to bind to its target sequence in the presence of 50 mM Na+ or K+, 10 mM MgCl2 and 20 mM Tris–acetate, pH 7.5. This oligonucleotide is bound in an antiparallel orientation with respect to the homopurine sequence. As was shown by co-migration assay the triplex is stable up to 65°C. At 37°C it was practically irreversible: after 24 hours of co-migration assay there was no traces of triplex dissociation. The rate of triplex formation was highly accelerated when the temperature and Mg2+ concentration were increased. This rate was higher for superhelical DNA when compared to the linear and circular one and the preference was dependent on temperature and Mg2+ concentration. The precision of this interaction is extremely sequence dependant in the c-pim-1 promoter region. Indeed only one difference when compared to the target gave negligible triplex formation under investigated conditions. These data suppose that triplex could play an important role in eukaryotic gene regulation and/or chromatin structure formation.

MATERIALS AND METHODS
Oligonucleotide preparation
Oligonucleotides were synthesized using the Applied Biosystems 391A DNA synthesizer and purified by electrophoresis in 20% polyacrylamide denaturing gel. The 5' end of the oligonucleotides were radiolabelled with [γ-32P] ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs) as per the manufacturer's instructions. Oligonucleotides were labelled at a specific activity of 2000 Ci/mmole.

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Plasmids

The c-pim-1 promoter region was obtained by the amplification of genomic DNA of NIH 3T3 and IW35 [15] cell lines with two sets of primers: 1) lower 5'-GAGGCAGGCGAAGGCGAG-AGGC-3', upper 5'-TTCTCTGCTTCTCCGGCGCTC-3'; 2) lower 5'-CCCTAGAGCTTCTGCTGCGG-3', upper 5'-GCCGGCCACACAAAGGCTC-3'. Primers were chosen on the basis of published mouse c-pim-1 promoter sequence [12].

The amplification conditions were as follow: 95°C for 80 s, 62°C for 80 s and 74°C for 180 s; Tag polymerase was from Appligene. The first set of primers gave rise to the fragment of 1138 bp long (positions -1018 to +220 concerning the mRNA start and after cutting with KpnI and PstI it was cloned in the plasmid pBluescript II SK by the same sites. The final length of the DNA corresponding to the c-pim-1 promoter is 891 bp (positions -905 to -14). We named these plasmids pPimNl and pPimWl correspondently to the DNA from NIH 3T3 and IW35 cell lines. The second set of primers give rise to the fragment of 616 bp (positions -597 to +19) (in this case DNA only from IW35 cell was used) and after cutting with PstI it was cloned in PstI-EcoRV sites of pBluescript II SK vector. The final length of DNA, corresponding to c-pim-1 promoter is 583 bp (positions -597 to -14) (plasmid pPimW2). All plasmids were grown in bacterial strain XL Blue 1 (Stratagene). Circular DNA of plasmid pPimW2 was obtained by HindUl digestion of superhelical DNA in the presence of 16 µM ethidium bromide at 15°C.

DNA sequencing

The region of interest was sequenced by the Sequenase Version 2.0 protocol (United States Biochemical) with the primer 5'-CG-ACCCCTCTCCAAACCCCAATTCC-3' in the plasmids pPimN1, pPimW1 and pPimW2. For the pPimW2, G and A+G reactions were also performed according to Maxam and Gilbert protocol [16].

Purine oligonucleotide-directed triple-helix formation

In general, less than 0.015 pM of [5'-32P] labelled purine oligonucleotide was added to 0.9 µg (approximately 0.4 pM) plasmid DNA in 10 µl buffered solution containing 10 mM MgCl2, 50 mM NaCl and 20 mM Tris- acetate, pH 7.5. To prevent unspecific adsorption of the oligonucleotide to the tubes wall, 5 µM of oligonucleotide 5'-GGGCGGCGAG-AGGC-3' was added to the mixture. After incubation of the mixture at temperature and time specified in the figure legends the triple helix formation was monitored by agarose or polyacrylamide gel electrophoresis.

Co-migration assay

After triple helix formation 2 µl of 50% saccharose solution was added and samples were loaded on the starts of 1% agarose gel. Electrophoresis was performed at room temperature for 4 hours in the presence of 20 mM Tris- acetate, pH 7.5, 50 mM Na acetate and 10 mM MgCl2. DNA was visualized by brief staining in ethidium bromide solution (0.1 µg/ml) and bands corresponding to the linear and superhelical DNA were cut. The quantity of the radioactivity, bound to the bands was evaluated by Chenenkov with 1900 TR counter (Packard). Gel was prepared for autoradiography by drying in flow of air on the glass surface at 60°C. For co-migration assay at 65°C and 70°C electrophoresis was performed in 5% polyacrylamide gel with the same buffer as for the agarose gel.

Probing with dimethyl sulfate (DMS)

Linear DNA. To prepare a DNA fragment for modification by DMS the pPimW2 plasmid was cut with ClaI restriction enzyme, 3' labelled with Klenow fragment of DNA polymerase I, and digested with BamH1 restriction enzyme. A smaller labelled fragment Bam H1—ClaI was used for further experiments. The prepared fragment (about 0.3 pM) was dissolved in 20 µl of the buffer: 50 mM MOPS, pH 7.2, 50 mM NaCl and 10 mM MgCl2. Then 20 pM of the oligonucleotide 5'-GGGCGGAGGGAGGAG-3' was added. The mixture was incubated overnight at room temperature. Then 2 µl of 5% DMS was added and the reaction was performed for 4 min at 24°C. The reaction was stopped by the addition of a 5 µl solution containing 10% mercaptoethanol, 1 mM EDTA and 0.1 M Na acetate. After double precipitation in ethanol the samples were treated with 50 µl of 10% piperidine at 95°C for 20 min and the products of cleavage were separated in 6% polyacrylamide denaturing gel.

Superhelical DNA. The superhelical DNA was treated at first with DMS in the same conditions as the linear one. The same radiolabelled fragment was then prepared and treated with piperidine.

RESULTS AND DISCUSSION

The target sequence in murine c-pim-1 promoter is shown in Figure 1. It should be noted that the chosen sequence is highly conserved: there are only 4 differences between mouse and man in the region going from 198 to 269. In human cell lines part of this conserved sequence interacts with the transcription factor PPF-348 [17]. To avoid mistakes which could be due to variations in genomic sequences and to amplification procedures we have sequenced the region from the base 100 to 250 (Fig. 1) in plasmids pPimN1, pPimW1 and pPimW2. We have found in this region 4 differences with published sequences [17]. Since in all three plasmids the sequences were the same, we have concluded that we cloned the natural murine c-pim-1 promoter region. It is interesting to note, that all four differences with published results have improved the homology with human c-pim-1 promoter region.

Analysis of specificity of triple helix formation

A co-migration assay was performed with linear, circular and superhelical plasmid pPimW2 either intact or after digestion with restriction enzymes HindIII—XbaI, and with parent plasmid pBluescript II SK (Figs 1 and 2). These experiments clearly indicated that under experimental condition the interaction with the oligonucleotide occurs only with DNA containing the promoter sequence. Moreover, in this region there are two other sequences which differ in one position from the target (Fig. 1B). After digestion of the plasmid pPimW2 with the restriction enzyme BstUl, these three sequences are located in three different fragments. One fragment of 267 bp (positions 623 in parent plasmid pBSBluescript SK to 191 in c-pim-1 promoter) contain the targeted sequence with a mismatch in position 173; the fragment of 96 bp (positions 194 to 292) contain the targeted sequence and the fragment of 66 bp (positions 392 to 458) contain the targeted sequence with a mismatch in position 419 (Fig. 1). There was no trace of triplex formation after co-migration assay at 25°C with the fragment of 267 and 66 bp long (Fig. 3). When all procedures were performed at 8°C there was a weak band
of radioactivity corresponding to the length of 267 bp (Fig. 3). We did not find any radioactivity in the position corresponding to the length 60–70 bp (Fig. 3). To further clarify the nature of interaction, we have used DMS footprinting.

DMS modifies the N7 position of guanines leading to chain scissions after treatment with piperidine. This chemical will not react with the purine—pyrimidine—purine triplex because the N7 position of purines is protected by Hoogsteen base pairing [18].

The procedure was performed for the linear and superhelical plasmids as described in Materials and Methods. As shown in Fig. 4, the guanines located within the target site for the purine oligonucleotide binding are less reactive with DMS than guanines external to the third-strand binding site indicating triplex formation under experimental conditions. Moreover, this picture clearly shows that the site of triplex formation are the same for linear and superhelical DNA. In both cases it is most probable that the oligonucleotide binds the purine tract of the double helical DNA.

Figure 1. Scheme of the pPimW2 plasmid (A) and nucleotide sequence of murine c-pim-1 promoter region (B) [12]. (A) The plasmid pPimW2 was obtained as described in Materials and Methods. Restriction sites which are involved in analysis of triplex structure are indicated. Numbers in brackets indicate positions of the restriction sites in the plasmid pBluescript II SK. (B) Fragment from nucleotide 100 to 250 was also sequenced by us. Numbers above the sequence designate the differences with published sequence (1, 3 are A and G instead of G and A; 2, 4 are insertions of G and C correspondently) [12]. The oligonucleotide 5′-GGGGAGGGGGAGG-3′ is positioned under the targeted sequences. The small italic in the oligonucleotide indicate ‘mismatch’ for triplex formation. (1) Recognition sites for BstUI restriction enzyme. (**) Putative site for PPF-348 transcription factor [17].

Figure 2. Co-migration assay with circular (A-1), superhelical (A-2), linear and superhelical (C-1) forms of the plasmid pPimW2. The same with the plasmid after digestion by HindIII and XhoI restriction enzymes (A-3) and with the parent plasmid pBluescript II SK (C-2). DNA was incubated with oligonucleotide 5′-GGGGAGGGGGAGG-3′ at 24°C during 4 hours at pH 7.5 under the condition described in Material and Methods. Running took place through agarose gel. B and D are the same gel as A and C after staining with ethidium bromide. O, S, L, indicate correspondently the positions for circular, superhelical and linear forms of the plasmid pPimW2. F and R correspond to the positions of the HindIII-XhoI restriction fragment containing the c-pim-1 promoter region (due to the lower concentration we can not see it after ethidium bromide staining) and the rest of the plasmid. P indicate the superhelical form of the parent plasmid pBluescript II SK.

Figure 3. Co-migration assay after BstUI digestion of the plasmid pPimW2. 1: electrophoresis at 25°C; 2: all procedures were performed at 8°C. M: markers [X174 RF DNA—HaeIII Digest (New England Biolabs)]. Arrows indicate the targeted fragments 270 bp (one mismatch) and 96 bp (no mismatch).
DNA in the major groove antiparallel to the Watson—Crick purine strand.

Stability of the triplex

We have evaluated the stability of triplex by performing a co-migration assay up to 70°C. First the assay was done for superhelical and linear DNA under 50°C in agarose gel. There was no traces of triplex disruption during two hours of migration (data not shown). The same experiment was done with HindIII—XbaI cut plasmid pPimW2 in polyacrylamide gel at 65°C during 2 hours. After autoradiography there was a negligible smear of radioactivity under HindIII—XbaI restriction fragment (Fig. 5). Complex dissociation started when the temperature was increase up to 70°C (Fig. 5). It is remarkable that the complex was very specific: no binding of the oligonucleotide to the initial plasmid occured (Fig. 5). We carefully checked the stability of the complex at 37 °C. For this purpose we performed a co-migration assay with superhelical DNA during 24 hours in 5% polyacrylamide gel. After 24 hours of run, the band corresponding to the superhelical DNA was cut and the DNA associated radioactivity was counted. Within 10% accuracy there was no sign of triplex dissociation. To confirm the high stability of the triplex we have performed another experiment. In this case after triplex formation in equimolar mixture of the plasmid and the [5'- 32P] labelled oligonucleotide we have added 100 times molar excess of the same non-labelled oligonucleotide. After incubation in 10 µl buffered solution containing 10 mM MgCl2, 50 mM Na+ or K+ acetate and 20 mM Tris—acetate, pH 7.5 during 24 hours at 37°C the amount of radioactivity which remains bound to the plasmid was evaluated by a co-migration assay. In the conditions of this experiment more than 90% of the [5'- 32P] labelled oligonucleotide remains bound to the plasmid. To our knowledge it the first report of such a stable triplex with a relatively short (13mer) nonmodified oligonucleotide. It is interesting to mention on one hand, that the melting temperature of a triplex with spermin-conjugated oligonucleotide is 42°C [19]. On the other hand, the highest melting temperature of triplexes was achieved i) for specially constructed hydrophobic derivatives of oligonucleotides, where stabilization was due to inter-cholesteryl interaction: 68°C [20] and ii) for a 15mer oligonucleotide with methylated cytosines and covalently attached to acridine in the presence of 4 mM spermine: 42°C [5]. When associated with double helical DNA a bound pyrimidine 21mer with methylated cytosines had a half life of approximately 9 hours (37°C, pH 6.8) [21].

Oligonucleotide association kinetics

Due to the high stability of the triplex it was possible to estimate some kinetic parameters of triplex formation. In the case of a triplex-forming reaction between a duplex and a oligopurine third strand the equation rate may be symbolized by:

\[ \frac{d[D]}{dt} = \frac{d[M]}{dt} = -\frac{d[Tr]}{dt} = -k_{in}[D][M] + k_{out}[Tr] \] (1)

where D, M and Tr are the duplex, the third strand and the triplex respectively, and the brackets indicate concentrations. Since the plasmid DNA concentration in our experiments is at least 20 times fold the concentration of the oligonucleotide and \( k_{out} \) substantially higher than \( k_{in} \) we can transform this second order reaction to a pseudo first order one:

\[ \frac{d[M]}{dt} = -K[M] \] where \( K = k_{in}[D] \) (2)

Or in the integrated form:

\[ \ln[M]/[M_0] = Kt \] (3)

Where \([M_0]\) is the initial concentration of the oligonucleotide which is able to form triplex. In the co-migration assay we can

Figure 4. Autoradiogram of a 6% polyacrylamide sequencing gel showing the results of DMS footprinting experiments carried out with linear (A) and superhelical (B) DNA. Experimental conditions are given in Material and Methods. (1) DMS treatment in the presence of the oligonucleotide 5'-GGGGAGGGGAGG-3'; (2) the same without oligonucleotide. (A line 3) for linear DNA A+G reaction were also performed according to [16].

Figure 5. Autoradiogram of a nondenaturating polyacrylamide gel showing the results of the co-migration assay at 65°C (1) and 70°C (2). (3) the same gel as (2) after staining with ethidium bromide.
estimate the quantity of the oligonucleotide coupled with plasmid DNA to be [Tr]. Practically we used the following expression for determination of K:

\[ K = \frac{\ln([M_0] - [Tr])/[M_0])}{t} \quad (4) \]

From the rate of reaction it is easy to evaluate the half-life (t) of the oligonucleotide (the time required for the one half of the oligonucleotide to be associated in triplex) under our experimental conditions:

\[ t = \frac{0.693}{K} \quad (5) \]

The experimental values of K and t for linear DNA at different temperatures are presented in Table 1.

Preliminary experiments have shown that the rate of triplex formation in the presence of K$^+$ instead of Na$^+$ does not vary a lot. This is in contrast with the data reported by [22]. We have observed a great influence of K$^+$ on the oligonucleotide adsorption to the propylene tubes wall: in the presence of K$^+$ the adsorption was 5-7 times higher than with Na$^+$. Up to half of the oligonucleotide can be adsorbed even in the presence of 0.5-1.0 μM of another competing purine rich oligonucleotide (5'-GAGGCGGCAGGGCGAGAGGC-3'). The authors of the previously mentioned paper have used restriction endonuclease protection assay for triplex determination and cannot measure the adsorption phenomenon. Another reason for this discrepancy could be related to a sequence-specific influence of potassium on the adsorption phenomenom. Another reason for this discrepancy is under torsional stress like in superhelical DNA [23,24] the preferential formation of triple helix structure in superhelical DNA may have biological significance. Further physico-chemical work is needed for explanation of this effect.

### Influence of superhelicity on the rate of triplex formation

To compare the rate of triplex formation between linear and superhelical DNA the following experimental conditions were used: linear and superhelical DNA (final DNA concentration 0.09 μg/ml) were mixed at an equimolar concentration in the presence of K$^+$. The authors of the previously mentioned paper have used restriction endonuclease protection assay for triplex determination and cannot measure the adsorption phenomenon. Another reason for this discrepancy could be related to a sequence-specific influence of potassium on the rate of triplex formation.

It has to be emphasized that the rate of triplex formation at 37°C looks very promising regarding the possibility of targeted gene manipulation.

### Table 1. Kinetic parameters of triplex formation

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>K (×10^4) (min⁻¹)</th>
<th>Association half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.6 ± 0.2</td>
<td>2600</td>
</tr>
<tr>
<td>25</td>
<td>28.7 ± 3</td>
<td>240</td>
</tr>
<tr>
<td>37</td>
<td>210 ± 20</td>
<td>33</td>
</tr>
<tr>
<td>55</td>
<td>1560 ± 100</td>
<td>4.4</td>
</tr>
<tr>
<td>65</td>
<td>3200 ± 300</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Data are based on the analysis of association rate curve (not shown). For each temperature the association curve is based on 5 to 7 incubation times, each performed in duplicate.

### Table 2. Influence of temperature on the relative rate of triplex formation of the purine oligonucleotide with superhelical and linear targeted DNA

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>k₀/k₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>25</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>37</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>55</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
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REFERENCES