(A,G)-Oligonucleotides Form Extraordinary Stable Triple Helices with a Critical R·Y Sequence of the Murine c-Ki-*ras* Promoter and Inhibit Transcription in Transfected NIH 3T3 Cells†

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ABSTRACT: The promoter of the murine c-Ki-*ras* proto-oncogene contains a critical homopurine-homopyrimidine sequence which is recognized by a protein factor and is a potential site for triplex-forming oligonucleotides (TFOs). The TFOs designed to bind this critical c-Ki-*ras* target have either an AG or a GT sequence motif. Of the two types, the first is found to form triplexes with extraordinarily high stability. For instance, both d(AGGGAGGGAGGAAGGGAGGG) (20AG) and d(GGGAGGGAGG-GAAGGAGGGAGGGAGGGAGC) (30AG) are able to bind the c-Ki-*ras* target at 65 °C and to resist a polyacrylamide gel temperature of 55 °C. By contrast, the triplex formed by d(TGGGTGGGTGGT-TGGGTGGG) (20GT) is largely dissociated at a gel temperature of 55 °C. The affinity constants of the TFOs at 37 °C, 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$ (standard buffer) were determined through band-shift experiments and found to be respectively 1.0×10^6, 4.0×10^6, and 2.5×10^7 M^{-1} for 20GT, 30AG, and 20AG. The AG-triplexes exhibit in standard buffer monophasic melting profiles (T_m ∼ 75 °C) and circular dichroism spectra showing the typical negative ellipticity at 212 nm, which is a hallmark for triplex DNA. The rate at which the TFOs bind to the c-Ki-*ras* target at 37 °C was examined under pseudo-first-order conditions. When the TFOs are in excess over the target and in the micromolar concentration range, the kinetics of triplex formation are slow, characterized by association half-lives of about 1 h. The ability of the TFOs to act as artificial transcription repressors was examined in a cellular system employing transient transfection experiments. Cultured NIH 3T3 fibroblast cells were cotransfected with a DNA mixture composed by a TFO and plasmid pKRS-413 containing the chloramphenicol acetyltransferase (CAT) gene driven by the c-Ki-*ras* promoter. It was found that the CAT activity is specifically inhibited by the TFOs in a dose-dependent manner. As expected, stronger CAT repression is obtained with 20AG, the oligonucleotide which forms the more stable triplex. These data suggest that (A,G)-oligonucleotides may provide a valuable means for the selective repression of the c-Ki-*ras* gene expression.

DNA triple helices can be subdivided into three main classes according to the base composition of the Hoogsteen strand: CT-, GT-, and AG-triplexes. In the first class, a (C,T)-oligonucleotide binds to the major groove of a homopurine-homopyrimidine duplex (R·Y target), with a parallel orientation relative to the purine strand of the duplex, forming isomorphous T·AT and C^+·GC triads in the Hoogsteen configuration (Moser & Dervan, 1987; Françoise et al., 1987). These triple helices are particularly stable under slight acidic conditions and have been characterized by a variety of techniques including PAGE, UV-melting, circular dichroism (CD)[1] , microcalorimetry, IR, and NMR [reviewed by Sun and Hélène (1993); Frank-Kamenetskii and Mirkin (1995)]. In the second class of triplexes, the third strand is a (G,T)-oligonucleotide binding to the R·Y target with an antiparallel orientation in the reverse-Hoogsteen configuration (Cooney et al., 1988; Kohwi & Kohwi-Shigematsu, 1988; Beal & Dervan, 1991; Durland et al., 1991). These triplexes, whose triads are T·AT and G·GC, do not require base protonation and are thermodynamically stable under physiological conditions (Alunni-Fabbroni et al., 1996; Xodo, 1995b; Scaria et al., 1995; Durland et al., 1991; Olivas & Maher, 1995a,b; Mayfield et al., 1994; Gee et al., 1992, 1994). For these characteristics, (G,T)-oligonucleotides have been tested by several authors as transcription repressors for *in vitro* (Cooney et al., 1988; Alunni-Fabbroni et al., 1996; Maher, 1992; Mayfield et al., 1994) and *in vivo* experiments (Postel et al., 1991; McShan et al., 1992; Ing et al., 1993; Roy, 1993; Kovacs et al., 1996; Tu et al., 1995; Scaggiante et al., 1994; Orson et al., 1991; Hobbs & Yoon, 1994; Thomas et al., 1995; Grigoriev et al., 1993). The third class of triplexes (AG motif) is obtained by (A,G)-oligonucleotides and is apparently similar to the GT-triplex motif, except that T·AT base triplets are replaced by A·AT ones (Sun & Hélène, 1993). It should be noted that the triads in both GT- and AG-triplex motifs are not perfectly isomorphous as those in the CT-triplex motif (Sun & Hélène, 1993).

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[1] Abbreviations: bp, base pair(s); CAT, chloramphenicol acetyltransferase; CD, circular dichroism; TFO, triplex-forming oligonucleotide; 20AG, 5′-AGGGAGGGAGGAAGGGAGGG; 30AG, 5′-GGGAGGGAGGGAAGGAGGGAGGGAGGGAGC; 20GT, 5′-TGGGTGGGTGGTTGGGTGGG.

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Experimental evidence on the formation of a triplex containing A·AU base triplets was reported by Broitman et al. (1987), who demonstrated that under certain conditions two strands of poly(A) and one strand of poly(U) can form a poly(A)·poly(A)·poly(U) triple-helical structure. Later on, it was demonstrated by affinity cleavage that a 19-mer (A,G)-oligonucleotide was able to bind its target, but apparently not so tightly as the (G,T)-analogue (Beal and Dervan, 1991). The first thermodynamic data on the AG triplex motif pertain to the formation of a triplex from two molecules of d(GGGAAAAGGG) and one of d(CCCTTTTCCC) (Pilch et al., 1991).

It is by now clear that not all (G,T)- and (A,G)-oligonucleotides have the ability to form triplex structures with duplex DNA. This property strongly depends on both the sequence and the base composition of the target. However, studies on telomeric DNA have shown that G-rich sequences can self-associate into unusual structures such as dimers and/or tetraplexes (Williamson et al., 1989); thus, the failure by some (G,T)- and (A,G)-oligonucleotides to form triplexes is often due to TFO self-association (Olivas & Maher, 1995; Alunni Fabbroni et al., 1996; Cheng & Van Dyke, 1993; Noonberg et al., 1995; Milligan et al., 1993).

Recently, Svinarchuk et al. (1994) have discovered that some (A,G)-oligonucleotides directed against the promoter sequence 3′GGAGGGGGAGGGG of murine *c-pim-1* proto-oncogene formed triple helices with extraordinary stability (T_m up to 80 °C). These unexpected results suggested that our present understanding on DNA recognition by TFOs is still incomplete. What is surprising in this study is the unusual thermodynamic properties of AG- triplexes with respect to both GT and CT ones. For instance, AG-triplexes seem to denature with a single transition exhibiting a T_m equal to or higher than the T_m of the target (Svinarchuk et al., 1995a,b, 1996; Pilch et al., 1991). In light of these results, it is not clear whether the extraordinary stable triplexes made by the (A,G)-oligonucleotides directed against the *c-pim-1* promoter represent a specific rather than a general case. To gain insight into this matter, we have extended our study on the triple helices made by G-rich oligonucleotides targeted against a critical R·Y target contained in the murine c-Ki-*ras* promoter. In a previous work, we observed that at 25 °C and pH 7.4, d(AGGGAGGGAGGAAGGGAGGG) exhibited an affinity for this c-Ki-*ras* target which was weaker than that of the (G,T)-analogue (Alunni-Fabbroni et al., 1996). This was due to a stronger tendency of the first oligonucleotide to aggregate compared to the second one. In the present paper, we show that under conditions in which oligonucleotide aggregation is minimized ($T \geq 37$ °C), the two (A,G)-oligonucleotides we have designed for the c-Ki-*ras* R·Y sequence exhibit a high affinity for the target and form triplexes with $T_m > 70$ °C. As the (A,G)-triplex-forming oligonucleotides (TFOs) are found to form particularly stable triplexes at near-physiological conditions, we examined their ability to inhibit *in vivo* the activity of the murine c-Ki-*ras* promoter. For this purpose, we cotransfected NIH 3T3 cells with DNA mixtures composed by a TFO and plasmid pKRS-413 containing the chloramphenicol acetyltransferase (CAT) gene under the control of the murine c-Ki-*ras* promoter. The CAT activity measured in the presence of specific (triplex-forming) and nonspecific oligonucleotides allowed us to evaluate whether the TFOs examined in this study can act as specific artificial repressors for the c-Ki-*ras* promoter.

MATERIALS AND METHODS

Oligodeoxynucleotides. The oligodeoxynucleotides used in this study were synthesized on a DNA synthesizer (Applied Biosystem Model 380 B), using standard phosphoroamidite solid-phase chemistry. The deprotected oligonucleotides were purified by FPLC anion exchange chromatography, using a Mono Q column (Pharmacia), eluted with a linear gradient of ammonium bicarbonate. Sample purity was checked by 20% PAGE in the presence of 7 M urea. The samples were lyophilized and stored at −20 °C. Oligonucleotide concentrations of stock solutions in Milli Q water were determined by UV spectroscopy using as extinction coefficients at 260 nm for C,T, A, and G the values of 7500, 8500, 15 000, and 12 500 M^{-1} cm^{-1}, respectively.

Plasmids. Plasmid pKRS-413, used for the transient transfection experiments, was kindly supplied by D. George (University of Pennsylvania). This plasmid, whose structure has been previously described, is derived from the chloramphenicol acetyltransferase (CAT) vector pSVAOcat in which was cloned a 380-bp segment of the c-Ki-*ras* promoter (Hoffman et al., 1990). This construct is able to drive CAT transcription in host cells (Hoffman et al., 1990). In addition, plasmid pTKβgal, containing the β-galactosidase gene driven by the thymidine kinase promoter, was used as control (Park et al., 1994).

Electrophoresis Band-Shift Experiments. Band-shift experiments were performed using as target a 30-mer duplex with the sequence of the critical c-Ki-*ras* target. This duplex was prepared by annealing (30 min at 60 °C, 2 h at 37 °C, and overnight at room temperature) the pyrimidine strand, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, with the complementary purine strand. A fixed amount of labeled c-Ki-*ras* target was mixed with an excess of TFO in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5 mM $MgCl_2$ (standard buffer), and incubated overnight at given temperatures (20, 37, 55, and 65 °C). After incubation, the samples were immediately loaded in a 15% nondenaturing gel prepared in standard buffer and thermostated with a water-circulating Haake apparatus. Electrophoretic analyses were performed at different temperatures, from 20 up to 65 °C. As the temperature of the gel was significantly different from that of the Haake water-circulating bath, it was measured by placing in the middle of the gel a thermocouple collecting in continuum the temperature during a run at voltage and current conditions of 150 V and 50 mA. In this way, we found that bath temperatures of 20, 37, 55, and 65 °C corresponded to gel temperatures of respectively 20, 35, 46, and 55 °C. However, considering that the samples were incubated overnight before electrophoresis at 20, 37, 55, and 65 °C, and that the rate of triple formation under the experimental conditions is slow, the electrophoretic patterns obtained reflect the situation at the incubation temperature rather than at the gel temperature. After the running, the gel was dried under reduced pressure and exposed to autoradiography (Kodak film) for a couple of hours.

The kinetics of triple-helix formation by AG-triplex-forming oligonucleotides were examined at 37 °C, incubating a constant amount of labeled 30RY duplex (5 nM) with either 100- or 500-fold excess of cold TFO for increasing intervals varying from 0 to 40 h. Samples were removed from the reacting mixture at various times and freezed at −80 °C. They were then analyzed by electrophoresis in a thermostated

cell at a temperature near 0 °C, i.e., under conditions in which the kinetics of triplex formation were extremely slow on the electrophoretic time scale. The amount of triplex formed was evaluated by measuring the intensity of the autoradiographic bands by means of a scanner densitometer.

Transient Transfection Experiments. NIH 3T3 cells were cultured in Dulbecco's minimal essential medium containing 10% heat-inactivated fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) (Bio-Whittaker). Six hours before transfections, 4×10^5/mL cells were seeded in 60 mm diameter cultured plates. Cotransfections were carried out by the standard method of calcium phosphate precipitation, using 10 μg of plasmid pKRS-413 and up to 1 μg of TFO (Graham & van der Erb, 1973). The precipitates were allowed to stand for 45 min without agitation. They were added to the cells which were left to stand for 16 h. The medium was then substituted with fresh medium, and the cells were left to grow for another 24 h.

CAT Assay. Cells were harvested by scraping 40 h after transfection in 1 mL of PBS buffer. They were centrifuged for 10 min and resuspended in 150 μL of CAT buffer (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA). Cell extracts were obtained by five successive freezing−thawing cycles. Cell extracts were heated at 65 °C for 15 min, and the protein content was determined by the Bradford method. The CAT assay was performed by adding to the reaction tube 15 μg of extract, 20 μL of acetyl-coenzyme A (4 mM), 5 μL of [^{14}C]chloramphenicol (Dupont), and CAT buffer to a final volume of 150 μL. The reaction was allowed to proceed for 1.5 h at 37 °C (Gorman et al., 1982). The reaction was stopped by adding 1 mL of cold ethyl acetate, which was also used to extract the chloramphenicol. The organic layer was dried and resuspended in 20 μL of ethyl acetate, spotted on a silica gel thin-layer chromatography plate (Polygram silG, Macherey-Nagel), and run with chloroform−methanol (99:1 or 97:3) in an ascending way. After autoradiography, the separated bands were analyzed by a densitometer scanner.

Spectroscopic Measurements. Ultraviolet absorbance experiments were carried out on a Cary 4 spectrophotometer (Varian) equipped with a sample holder, thermostated at any temperature from 0 to 100 °C by a Peltier system. The temperature of the DNA solutions was determined by a thermocouple immersed in a quartz cuvette located in one of the six positions of the sample holder. The melting experiments were performed by heating and cooling the sample holder at a rate of 0.5 °C/min. In a typical melting experiment, the DNA solution was placed in a 0.5 cm path length cuvette, and the absorbance values as a function of temperature were collected every 0.2 °C. The data were transferred into a computer and analyzed by Sigma plot (Jandel Scientific).

Circular dichroism spectra were recorded on a Jasco 500A spectropolarimeter using a quartz cuvette with a 0.5 cm path length. The CD spectra shown are the average of four scans, from which the base line was subtracted. The CD spectra report the ellipticities in units of M^{-1} cm^{-1} (M is expressed in mol of strand/L).

RESULTS

c-Ki-ras Target for Triplex-Forming Oligonucleotides. The promoter of the murine c-Ki-*ras* gene contains a 29-bp polypurine-polypyrimidine sequence located at −290/−319 from the 3′ boundary of exon 0 (Figure 1). This DNA segment exhibits a number of properties: (a) it is CG-rich (73%); (b) it exhibits sensitivity to S1 nuclease; (c) it is recognized by at least one nuclear factor; (d) it is essential for the c-Ki-*ras* promoter activity, as its deletion yields a dramatic reduction of transcription (Hoffman et al., 1987, 1990; Pestov et al., 1991). Moreover, this sequence having a perfect R·Y motif is a potential site for TFOs. According to the known base-recognition code for triplex DNA, we designed three G-rich oligonucleotides, 20AG, 30AG, and 20GT, which can bind to the c-Ki-*ras* segment with the antiparallel orientation. In addition, we designed oligonucleotide 20AGr with a polarity opposite to that of 20AG, with the purpose of testing whether parallel triplexes can also be formed at the c-Ki-*ras* target. Finally, it should be noted that oligonucleotide 30AG, having the same sequence as the purine strand of the c-Ki-*ras* target, can only form an imperfect AG-triplex with one A·GC and one G·AT mismatched triads.

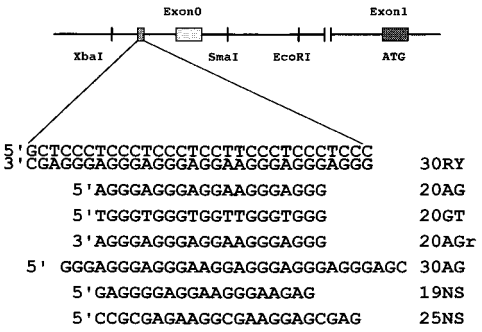

FIGURE 1: Structure of the c-Ki-*ras* promoter and sequence of the critical 29-bp R·Y target. For additional details, see Hoffman et al. (1987). The sequences of the triplex-forming oligonucleotides are also reported.

Electrophoresis: (A,G)-Oligonucleotides Form Very Stable Triplexes. The capacity of the designed TFOs to recognize and bind the c-Ki-*ras* target was investigated by band-shift experiments. Figure 2 shows the electrophoretic profiles at four different temperatures for the mixtures composed by target 30RY—a 30-bp duplex with the critical c-Ki-*ras* sequence formed by annealing the ^{32}P-labeled pyrimidine strand with an equimolar amount of purine strand—and by the triplex-forming oligonucleotides 20AG (lanes 2, 3), 30AG (lanes 4, 5), and 20GT (lane 6). Before electrophoresis in a thermostated cell, the mixtures were incubated overnight at the selected temperature. The results clearly show that triplex formation increases with temperature at both TFO/target molar ratios considered: 50 and 150. At 20 °C, the interaction between 20AG and 30RY produces a smeared band, indicating that at this temperature 20AG, although in excess over the target, is not able to push the 30RY + 20AG $\leftrightarrow$ T equilibrium completely to the right. Under the same conditions, the oligonucleotide 30AG shows practically no affinity for the target 30RY. By contrast, 20GT (TFO/target = 75) gives rise to a distinct and retarded band due to triplex formation. The affinity of the TFOs for c-Ki-*ras* increases significantly at 37 °C. At this temperature, 20AG pushes completely the duplex−triplex equilibrium to the right (lanes 2, 3), while 30AG is able to transform about half of the duplex molecules into triplex molecules (lanes 4, 5).

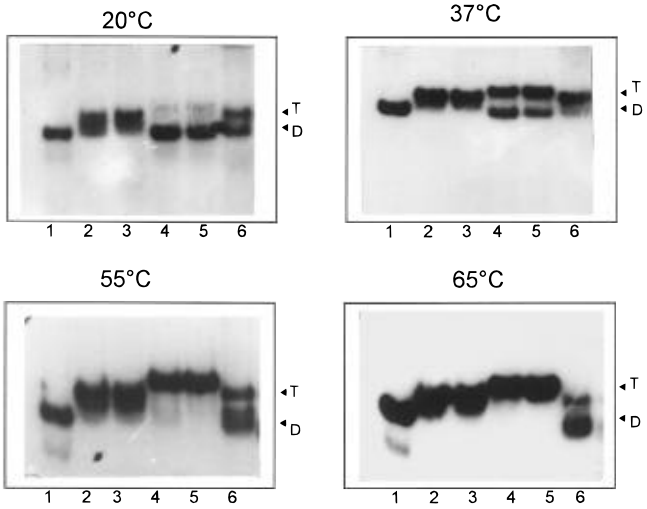

FIGURE 2: Triplex formation between 30RY (the R·Y target of c-Ki-*ras*) and the triplex-forming oligonucleotides 20AG (lanes 2, 3), 30AG (lanes 4, 5), and 20GT (lane 6) in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5 mM $MgCl_2$. Lane 1 shows the mobility of 30RY alone. Lanes 2−6 contain 28 nM 30RY plus a 50-fold (lanes 2, 4) or 150-fold (lanes 3, 5) excess of TFO. Oligonucleotide 20GT in 75-fold excess over 30RY is in lane 6. The target and the TFOs were combined, incubated, and analyzed at four different temperatures (20, 37, 55, and 65 °C).

Similarly, 20GT drives almost all the target duplex into triplex (lane 6). In order to examine the stability of these triplexes, we performed band-shift assays at 55 and 65 °C. These showed that, while the triplexes formed by 20AG and 30AG stand to these high temperatures, the triplex having 20GT as a third strand starts to dissociate at 55 °C and is largely disrupted at 65 °C. These experiments clearly demonstrate that the AG triple helices formed at the critical c-Ki-*ras* sequence can be very stable (T_m > 65 °C). Such a behavior is in keeping with the recent results obtained by Svinarchuck et al. (1994, 1995a,b).

As K^+ has been shown to inhibit triplex formation (Chen & van Dyke, 1993; Olivas & Maher, 1995a), the capacity of both 20GT and 20AG to bind to the c-Ki-*ras* target in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM $MgCl_2$ has been examined. We found that at 37 °C the K^+ ions weakly affected the affinity of 20AG for the c-Ki-*ras* target: for instance, at a 20AG/target ratio of 10, more than 50% of the target duplex is converted into triplex. By contrast, oligonucleotide 20GT exhibited a stronger dependence from K^+, requiring TFO/target ratios >10 for forming the triplex (not shown).

Affinity of Triplex-Forming Oligonucleotides for the c-Ki-ras Target. The affinity of the triplex-forming oligonucleotides 20AG, 30AG, and 20GT at 37 °C in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5 mM $MgCl_2$ was determined by gel electrophoresis. A constant amount of ^{32}P-labeled 30RY (28 nM) was incubated with increasing amounts of TFO to obtain TFO/target molar ratios varying from 0.5 to 200. The mixtures were incubated overnight at 37 °C and analyzed in a gel apparatus thermostated at 37 °C (Figure 3). The reaction taking place in each DNA mixture is

$$30RY + TFO \Leftrightarrow T \qquad (1)$$

The association constant, K_a, is given by

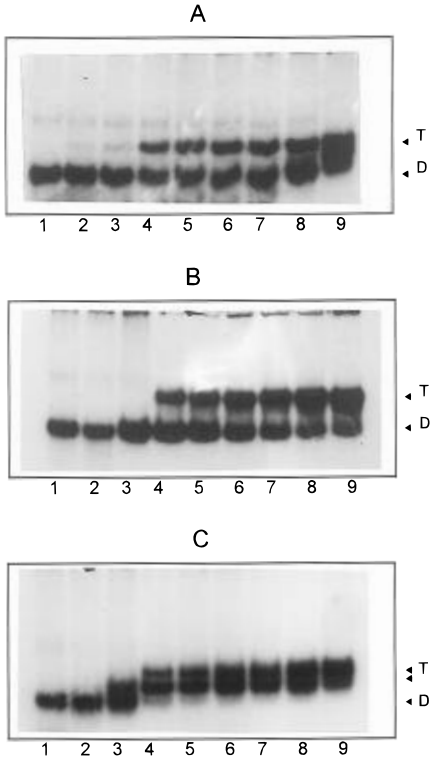

FIGURE 3: Determination of association constants at 37 °C, in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5 mM $MgCl_2$, for the binding of 30AG (A), 20GT (B), and 20AG (C) to the c-Ki-*ras* target. Increasing micromolar concentrations (lanes 2−9: 0.014, 0.028, 0.28, 0.56, 0.98, 1.54, 2.8, and 5.6 μM) of TFO were added to target 30RY (28 nM), and the mixtures were incubated overnight at 37 °C. The target 30RY was loaded in lane 1. The electrophoresis was carried out in a thermostated gel at 37 °C.

$$K_a = \frac{[T]}{[30RY]([TFO]_0 - [T])} \qquad (2)$$

where [T] and [30RY] (labeled target) are equilibrium concentrations and $[TFO]_0$ is the total concentration. The association constants of triplex formation (K_a) for 20AG, 30AG, and 20GT are found to be respectively 2.5×10^7, 4.0×10^6, and 1.0×10^6 M^{-1}. Thus, the free energy of triplex formation, determined from $\Delta G_{37} = -RT \ln K_a$ is, respectively, −10.5, −9.4, and −8.5 kcal/mol for 20AG, 30AG, and 20GT. These data are in substantial agreement with the results obtained from band shifts, and show that the thermodynamic stability of the triplexes follows the order 30RY·20AG >30RY·30AG > 30RY·20GT.

It is worth noting that the interaction between 30RY and 20AG produces, with respect to the mobility of the duplex, two retarded bands due to triplex structures. In order to explain why two bands are formed, we first checked out, through electrophoresis, whether 20AGr, having a polarity opposite to that of 20AG, is able to form a parallel triplex. Since we did not observe the formation of a parallel triplex (not shown), we ruled out the possibility that the two bands are due to 20AG binding to the target with both parallel and antiparallel orientations. It can be observed, however, that the slower of the retarded bands forms at 37 °C within hours, i.e., at a very slow rate which, as we observed, is pushed up at 45 °C and pushed down at 20 °C (not shown). This behavior suggests that a cooperative interaction may occur between 20AG and 30RY, in the sense that two molecules of 20AG bind one molecule of 30RY to form a complex

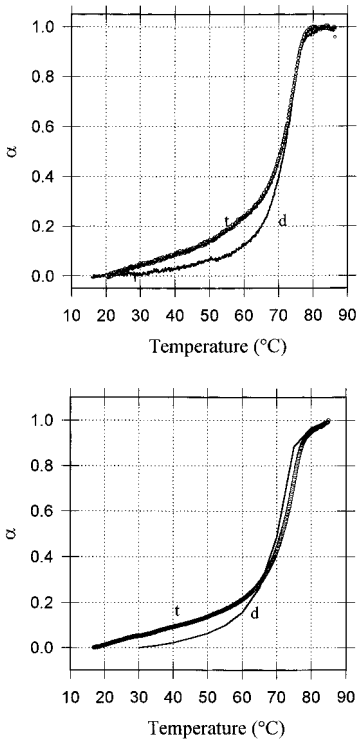

FIGURE 4: (Top panel) Absorbance *versus* temperature curves for the c-Ki-*ras* duplex 30RY (d) and the triplex formed by 20AG and 30RY (t), in 50 mM Hepes, pH 7, 50 mM NaCl, and 1 mM $MgCl_2$. (Bottom panel) Same melting curves but carried out in the Hepes buffer containing 5 mM $MgCl_2$. The melting curves have been obtained at a heating rate of 0.5 °C/min.

with the following primary structure (Colocci & Dervan, 1995):

5'GCTCCCTCCCTCCCTCCTTCCCTCCCTCCC

3'CGAGGGAGGGAGGGAGGAAGGGAGGGAGGG

5'AGGGAGGGAGGAAGGGAGGG**AGGGAGGGAGGAAGGGAGGG**3'

This complex, which contains 26 A·TA/G·GC canonical triads and 2 A·GC and 1 G·AT internal mismatched triads, should form a regular triple-stranded structure, characterized by no stacking discontinuity in the third strand at the R/R junction. Moreover, as two 20AG molecules have to align correctly in the major groove of duplex 30RY, it is not surprising to find that this cooperative complex forms with slow kinetics at 37 °C (hours). As expected, this complex is characterized by a lower mobility than that of triplex 30RY·20AG, as a result of its higher molecular weight. Colocci and Dervan (1995) have demostrated that when two triplex-forming oligonucleotides bind cooperatively to adjacent targets, the specific binding of each oligonucleotide is enhanced by a factor varying from 10 to 100. This phenomenon may explain the high affinity observed for 20AG with respect to 20GT.

Melting Experiments. Figure 4 (top panel) shows the melting curves in 50 mM Hepes, pH 7, 50 mM NaCl, and 5 mM $MgCl_2$ of both c-Ki-*ras* target and 30RY·20AG triplex. Both duplex and triplex structures melt with monophasic curves with T_m values of 72.8 and 73 °C, respectively. The different molecularity of the reactions of duplex and triplex formation (2 *versus* 3) results in a significant difference of the melting profiles in the pretransition regions: while the duplex melts with a symmetrical sigmoidal curve, the triplex melts with an asymmetrical curve. The melting profiles

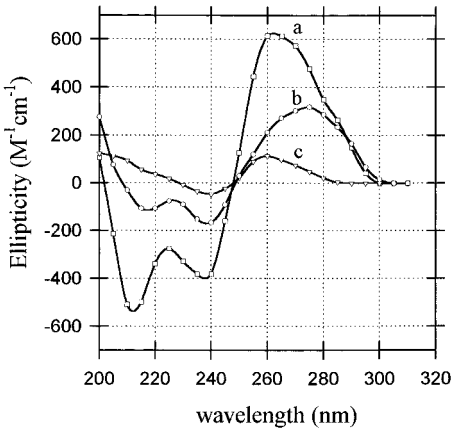

FIGURE 5: Circular dichroism spectra of (a) the triplex formed by 30RY and 20AG, (b) the duplex 30RY, and (c) the oligonucleotide 20AG, in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 10 mM $MgCl_2$.

appear rather reversible at a heating/cooling rate of 0.5 °C/min. When the $MgCl_2$ concentration of the buffer is lowered from 5 to 1 mM, the triplex T_m is 72.8 °C, while the duplex T_m is 70.5 °C (Figure 4, bottom panel). Instead, if in the buffer 10 mM $MgCl_2$ and 0.2 mM spermine are added, the triplex 30RY·20AG melts with a biphasic melting profile from which it is seen that the triplex T_m is 75 °C and duplex T_m is 80 °C (not shown). The triplex 30RY·30AG is found to melt in the 5 mM $MgCl_2$ buffer with a monophasic profile with a T_m of about 75 °C (not shown). Finally, when the oligonucleotides 20AG and 30AG are melted alone in the standard buffer, both show cooperative melting curves with $T_m = 40$ and 45 °C, respectively. This clearly indicates that the inability of these oligonucleotides to bind the c-Ki-*ras* target at 20 °C (see electrophoresis) is due to oligonucleotide self-association.

Circular Dichroism. A number of studies have shown that triplex formation is accompanied by a strong and negative ellipticity at 212 nm (Manzini et al., 1990; Xodo et al., 1990; Xodo, 1995b, Scaria et al., 1995; McShan et al., 1992). Figure 5 shows that the interaction between 30RY and 20AG produces this diagnostic band. The CD spectrum of the c-Ki-*ras* target (curve b) is typical for B-DNA showing one positive (275 nm) and two negative (220 and 240 nm) ellipticities. This spectrum is different from that of triplex 20RY·20AG (curve a). Curve c is the spectrum of 20AG. If one compares spectra a and b, a number of observations can be made: (1) the triplex structure is characterized by strong ellipticities at 260 nm ($\Delta\epsilon = 600$ M^{-1} cm^{-1}), 212 nm ($\Delta\epsilon = -550$ M^{-1} cm^{-1}), and 239 nm ($\Delta\epsilon = -400$ M^{-1} cm^{-1}); (2) the spectrum of the triplex differs from the sum of c-Ki-*ras* target and 20AG spectra, indicating triple-helix formation; (3) upon triplex formation, the ellipticity of the c-Ki-*ras* duplex at 275 nm is shifted to 260 nm and increased in intensity. This suggests that, in order to host a third strand in the major groove, the target duplex undergoes some structural changes which might be connected with the transformation from B-DNA to A-DNA (Ivanov & Kruglov, 1992). (4) The spectrum of the triplex shows a strong and negative ellipticity at 212 nm, which is a hallmark for triplex formation for both CT (Manzini et al., 1990; Xodo et al., 1990) and GT/GA (Xodo, 1995b; Scaria et al., 1995) triplexes.

The circular dichroism spectrum of the triplex formed by 30RY and 20GT is similar to that shown in Figure 5, except for a negative band at about 280 nm (Xodo, 1995b).

Kinetics of Triplex Formation by (A,G)-Oligonucleotides. The reaction of triplex formation is represented by eq 1. Under experimental conditions in which the TFO is in excess with respect to the target, the kinetics of triplex formation can be approximated to the pseudo-first-order:

$$F(t) = F_\infty[1 - \exp(-k_{app}t)] \quad (3)$$

where $F(t)$ and F_∞ are the fractions of duplex converted into triplex at time t and ∞, i.e., at equilibrium; k_{app} (h^{-1}) is a pseudo-first-order kinetic constant for triplex formation which depends on TFO concentration. The fraction of triplex formed as a function of time was estimated from band-shift experiments. The experimental data were best fitted with eq 3 in order to get an estimation of k_{app} (not shown). We found that at 37 °C the reactions of triplex formation between either 20GT or 20AG (2.5 μM) and labeled 30RY (5 nM), in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5 mM $MgCl_2$, occurred at rates characterized by k_{app} of respectively 0.44 and 1.03 h^{-1}. The half-lives of triplex formation, obtained from the relation $0.693/k_{app}$, were respectively 40 and 94 min for 20AG and 20GT. These findings show that the reaction of triplex formation by G-rich oligonucleotides is relatively slow, in accord with previous studies (Svinarchuk et al., 1994; Vasquez et al., 1995).

Inhibition of c-Ki-ras Promoter Activity by (A,G) Triple-Forming Oligonucleotides in Cultured Cells. We have tested the ability of the triplex-forming oligonucleotides 20AG, 30AG, and 20GT to inhibit the expression of the chloramphenicol acetyltransferase (CAT) gene driven by the c-Ki-*ras* promoter. To this purpose, NIH 3T3 cells were transiently cotransfected with a mixture of TFO and pKRS-413, a recombinant vector containing the CAT gene driven by the c-Ki-*ras* promoter, constructed by George and coworkers (Hoffman et al., 1990). Transfections were carried out by the calcium phosphate coprecipitation method (Graham & van der Erb, 1973), using DNA mixtures containing 10 μg of plasmid pKRS-413 and up to 1 μg of TFO. Before carrying out the transfections, the plasmid was combined with the TFO and the mixture incubated for 2 h at 37 °C, to allow saturation of the c-Ki-*ras* promoter sites by the TFO. The transfected mixtures were characterized by TFO/pKRS-413 molar ratios of 10, 25, and 50. Figure 6A shows typical CAT activities measured in the absence and in the presence of increasing amounts of 20AG. It appears clearly by autoradiography of the TLC plate that the CAT activity is inhibited in a dose-dependent manner by 20AG: when the TFO is 50-fold in excess over pKRS-413, a very low CAT activity is observed. The enzymatic assay was performed by using a constant dose of cellular extract (15 μg), which was quantified by the method of Bradford. To find out whether the observed CAT inhibition is specifically promoted by the triplex-forming oligonucleotide 20AG, we performed transfections in the presence of nonspecific G-rich oligonucleotides, 25NS and 19NS, which were experimentally shown to be unable to form a triplex with the c-Ki-*ras* promoter (not shown). Figure 6B shows the results of CAT assays which refer to transfections carried out at increasing 25NS/pKRS-413 molar ratios. It is noteworthy that 25NS promotes a nonspecific inhibitory effect on the CAT activity, which however is much less intense than that observed with 20AG. This nonspecific inhibitory effect is also observed with oligonucleotide 19NS. A direct comparison of specific and nonspecific effects is given by the histogram reported

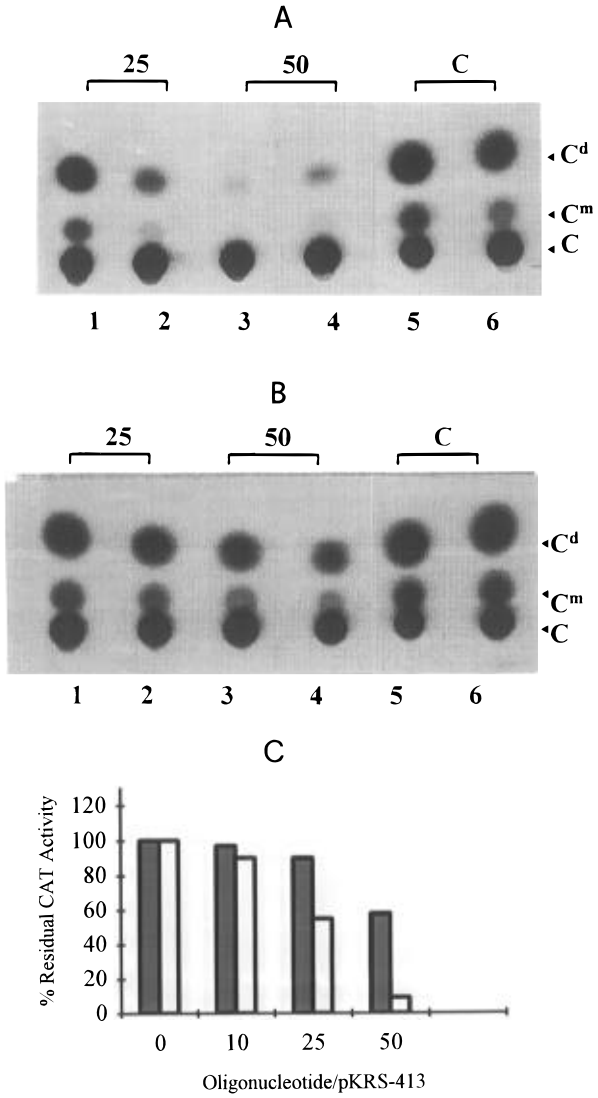

FIGURE 6: (A) Scanner copy of an autoradiogram showing the inhibition of chloramphenicol acetyltransferase (CAT) activity promoted by the triplex-forming oligonucleotide 20AG cotransfected with plasmid pKRS-413 by the calcium phosphate method. The transfections were carried out in duplicate, at oligo/plasmid molar ratios of 25 (lanes 1, 2) and 50 (lanes 3, 4). Lanes 5, 6 show the CAT activity obtained by transfecting the cells only with pKRS-413 (control). Before carrying out the transfection, the plasmid−TFO mixtures were incubated for 2 h in order to allow triplex formation at the c-Ki-*ras* promoter. The TLC spots represent the monoacetylated (C^m) and diacetylated (C^d) forms of chloramphenicol, migrating faster in the TLC plate than nonacetylated chloramphenicol. (B) Scanner copy of the autoradiogram showing the nonspecific inhibition of CAT activity promoted by the control oligonucleotide 25NS, which is not able to form a triplex with the c-Ki-*ras* target. Transfecting the NIH 3T3 cells with a mixture containing pKRS-413, pTKβgal, and 25NS, the nonspecific oligonucleotide, promoted a partial inhibition of roughly the same size on both CAT and b-galactosidase activities (not shown). (C) Autoradiograms as in panels A and B were quantitated by densitometry. The data are plotted as percent residual CAT activity defined as [100 × (areas of acetylated chloramphenicol in the presence of TFO)/(areas of acetylated chloramphenicol in the absence of TFO, control)]. Values obtained from three independent transfection experiments were averaged. Black bars refer to transfections carried out in the presence of the nonspecific oligonucleotide 25NS. White bars refer to transfections in the presence of c-Ki-*ras* promoter-specific 20AG. The percent residual CAT activity is shown for transfections carried out at oligo/pKRS413 ratios of 10, 25, and 50. At oligo/pKRS413 = 0, the transfection was carried out without the oligonucleotide (control). The difference between specific and nonspecific inhibition is attributed to triple-helix formation at the c-Ki-*ras* promoter.

in Figure 6C. Since the results can be affected by transfection efficiency, each experiment was carried out at least 3 times, and the results shown are the average values. In order to gain further insight into this nonspecific effect, we also cotransfected the NIH 3T3 cells with mixtures composed by oligonucleotide 25NS, pKRS-413, and a plasmid containing the β-galactosidase gene driven by the thymidine kinase promoter, pTKβgal. We observed that 25NS promoted inhibitory effects on both CAT and β-galactosidase activities of roughly the same size as those reported in the histogram of Figure 6C. As expected, when we transfected the cells with a mixture containing 20AG, pKRS-413, and pTKβgal, the TFO inhibited the expression of the CAT gene much more strongly than that of the β-galactosidase gene. Subtracting the nonspecific CAT inhibition from the specific 20AG-driven inhibition, one obtains a net effect which can be attributed to the effect of a triplex at location −290/−319 of the c-Ki-*ras* promoter: about 50% at TFO/pKRS-413 = 50, about 25% at TFO/pKRS-413 = 25. Figure 7 shows CAT assays obtained at TFO/pKRS-413 ratios of 10, 25, and 50 for 30AG (panel A), and 20GT and 20AG (panel B). The results are shown in the histogram of panel C. Similarly to 20AG, both 30AG and 20GT appear to promote in a dose-dependent manner the repression of the c-Ki-*ras* promoter activity.

Taken together, the results show that the TFOs tested *in vivo* in this assay appear to be effective in the specific inhibition of the c-Ki-*ras* promoter.

DISCUSSION

The murine c-Ki-*ras* promoter contains a 29-bp homopurine·homopyrimidine motif located at −290/−319 from the exon 0/intron 1 boundary, which serving as a binding site for at least one nuclear factor is essential for transcription activity (Hoffman et al., 1990). In this study, we have examined, through *in vitro* and *in vivo* experiments, the capacity of this critical c-Ki-*ras* promoter sequence to function as a target for TFOs. We show that two (A,G)-oligonucleotides, namely, 20AG and 30AG, form with this c-Ki-*ras* sequence triple helices of extraordinary stability (T_m > 70 °C) in comparison with the triplex formed by 20GT (T_m < 50 °C) (Xodo, 1995). This interesting property was not observed earlier, as (A,G)-oligonucleotides tend strongly to self-aggregate at a temperature below 37 °C (Alunni-Fabbroni et al., 1996; Noonberg et al., 1995; Olivas & Maher, 1995a,b; Cheng & van Dyke, 1993). Recently, Svinarchuk and co-workers have reported that a number of (A,G)-oligonucleotides targeted to R·Y promoter sequences located in the murine *c-pim-I* proto-oncogene, the bovine cytokeratin gene and the *vpx* gene of the SIV and HIV-2 viruses, form triple helices with unusual stability. Except for one case, these oligonucleotides have an invariable GGAGGGGAGG central core, containing a cluster of five successive guanines. It therefore seems plausible to suppose that this structural element is responsible for the very high stability of these triplexes. However, the behavior of oligonucleotides 20AG and 30AG suggests that clusters of only three guanines should be sufficient for a strong stabilization of the AG triplexes. This hypothesis is supported by the observation that when the target contains G clusters interrupted by one

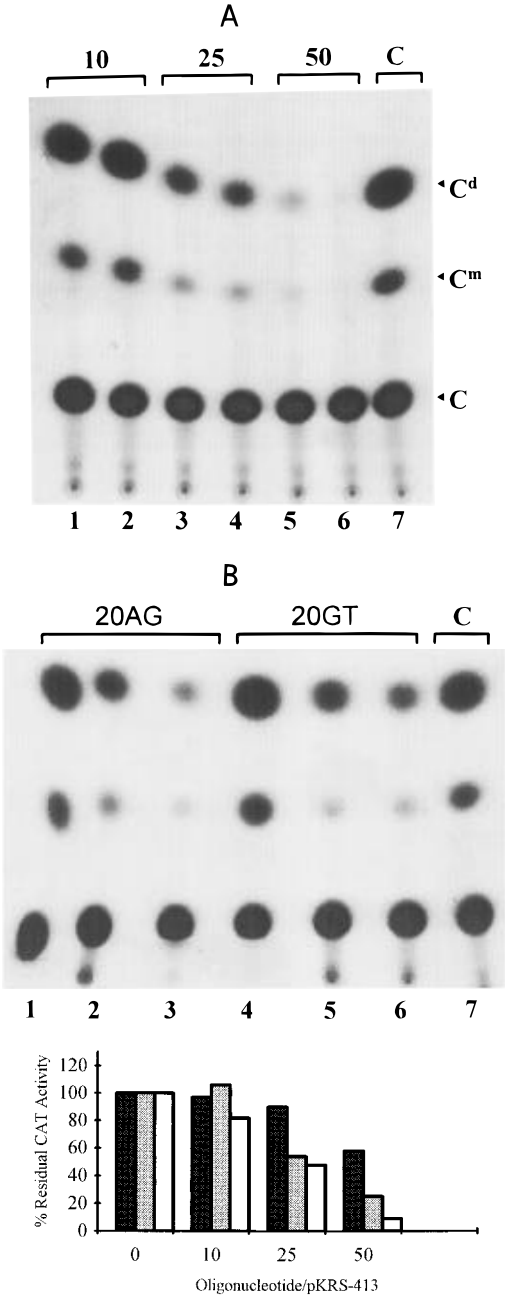

FIGURE 7: (A) Scanner copy of the autoradiogram showing the inhibition of CAT activity promoted by 30AG [30AG/pKRS-413 molar ratios of 10 (lanes 1, 2), 25 (lanes 3, 4), 50 (lanes 5, 6), and 0 (control, lanes 6, 7)]. (B) CAT inhibition promoted by 20AG (lanes 1−3) and 20GT (lanes 4−6) at TFO/pKRS-413 molar ratios of 10 (lanes 1, 4), 25 (lanes 2, 5), and 50 (lanes 3, 6). Lane 7 shows the control, i.e., CAT activity in the absence of TFO. (C) Histogram showing the quantitative results of experiments as in panels A and B. Black bars refer to the nonspecific oligonucleotide 25NS, gray bars refer to 20GT, and white bars refer to 20AG.

pyrimidine such as in

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GGCCCTCTTCCGCTTCCTCGCTC
CCGCGAGAAGGCGAAGGAGCGAG   target
   GAGAAGGGGAAGGAGGGAG    TFO
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the designed 19-mer oligonucleotide does not form a triplex (not shown). For the same reason, d(GG**C**GAGG**C**GGAGG) does not form a stable triplex with its target either (Svinarchuk et al., 1996).

In a previous work, we found that at room temperature 20AG exhibited a weaker affinity than 20GT for the c-Ki-

ras target because of its strong self-association property (Alunni-Fabbroni et al., 1996). In this study, we show that the capacity of the (A,G) oligonucleotides 20AG and 30AG to bind the c-Ki-*ras* target is surprisingly enhanced in the temperature range of 37−65 °C. Gel electrophoresis carried out as a function of temperature shows that the triple helices formed by 20AG and 30AG are stable even when they migrate in a gel thermostated at 55 °C. Instead, the triplex made by 20GT is not able to stand a gel temperature as high as 55 °C.

The affinity constants of 20AG, 20GT, and 30AG for the c-Ki-*ras* target were determined under near-physiological conditions by band-shift experiments and found to be on the order of 10^6-10^7 M^{-1}. These K_a values are in agreement with those found for the G-rich triplex-forming oligonucleotides designed to bind the human c-Ki-*ras* R·Y sequence (Mayfied et al., 1994). It is noteworthy that the human c-Ki-*ras* promoter, which shares about 80% nucleotide sequence homology with the murine c-Ki-*ras* promoter, also contains an R·Y sequence, located at −307/−328 from the exon 0/intron 1 boundary, which is essential for the promoter activity (Mc Grath et al., 1983; Jordano & Perucho, 1986). The ability of this site to form triple helices with TFOs has been studied by Mayfield et al. (1994). Through band-shift analyses carried out at 25 °C, these authors have shown that the affinity for the human c-Ki-*ras* promoter of 22-mer TFOs was approximately $(0.2-0.5) \times 10^6$ M^{-1}. Moreover, other K_a values determined under different experimental conditions (different T and $MgCl_2$ concentrations) have been reported in the literature for G-rich triplex-forming oligonucleotides, varying from 10^6 to 10^8 M^{-1} (Durland et al., 1991; McShan et al., 1992; Vasquez et al., 1995; Noonberg et al., 1995; Olivas & Maher, 1995a,b; Xodo, 1995; Alunni-Fabbroni et al., 1996).

The reaction of triplex formation investigated under pseudo-first-order conditions (2.5 μM TFO, 5 nM target) is found to occur at a slow rate, characterized by an association half-life of about 1 h. This behavior is similar to that found at 37 °C for the binding of d(GGGGAGGGGAGG) to the murine *c-pim-1* promoter ($k_{app} = 2.1 \times 10^{-2}$ min^{-1}, $t_{1/2} =$ 33 min) and for the binding of d(GGGGTTTGGGGGGTGGTGT) to the hamster adenine phosphoribosyl transferase gene ($k_{app} \sim 0.36-3.6$ h^{-1} and $t_{1/2} = 0.2-2$ h) (Svinarchuk et al., 1994; Vasquez et al., 1995). The same holds true for pyrimidine CT-triplexes, which also exhibit slow kinetics of triplex formation (Maher et al., 1990; Xodo, 1995a; Rougée et al., 1992; Shindo et al., 1993). There are two possible explanations for the slowness of triplex formation: (1) the TFOs self-aggregate in solution and have to disrupt their unusual structures before binding to the target; (2) the double-helix target has to switch from the B-DNA to A-DNA before hosting the TFO in its major groove. If this conformational transition is energetically unfavorable, only a small fraction of collisions between the target and TFOs might be able to generate a triplex. Triplex dissociation rates at 37 °C can be assessed through the relation $K_a = k_1/k_{-1}$. As 20AG shows an enhanced affinity for the c-Ki-*ras* promoter, probably due to the fact that it interacts in a cooperative manner with the R·Y target, the dissociation lifetime of the triplex is about 50 h, a property which makes 20AG a very promising TFO for *in vivo* applications.

We previously demonstrated that 20GT and to a lesser extent 20AG were able to inhibit *in vitro* the T7 RNA polymerase. This was observed by performing transcription assays at 25 °C and employing as template a linearized plasmid in which the critical c-Ki-*ras* R·Y site and also a reporter gene were cloned downstream from the T7 promoter (Alunni-Fabbroni et al., 1994, 1996).

In the present paper, we assayed *in vivo* the ability of the triplex-forming oligonucleotides 20AG, 30AG, and 20GT to repress the activity of the c-Ki-*ras* promoter. For this purpose, we performed a number of transient transfection experiments under different experimental conditions. The assay was simplified by directing the TFOs against a plasmid target rather than a genomic target, thus reducing the DNA complexity by about 6 orders of magnitude. It was found that both oligonucleotides 20AG and 30AG promote a stronger inhibition of CAT expression (about 50%) with respect to that observed with 20GT (about 25%). These results parallel those found by the band-shift experiments, which showed that at 37 °C both 20AG and 30AG form triple helices ($\Delta G = -10.5$ and -9.4 kcal/mol) more stable than that formed by 20GT ($\Delta G = -8.5$ kcal/mol).

An inhibitory effect similar in amplitude to the one described here has been obtained by cotransfecting adult rat cardiac fibroblasts with a 30-mer (A,G)-oligonucleotide and a plasmid containing the CAT gene driven by α1(I) collagen promoter (Kovacs et al., 1996). Instead, a smaller effect (20% inhibition) has been observed with a 13-mer (A,G)-triplex-forming oligonucleotide, cotransfecting cat fibroblast cells with a plasmid containing the *c-pim-1* promoter/luciferase construct (Svinarchuk et al., 1996).

As previously observed, synthetic oligonucleotides can be toxic to some cellular lines (Kovacs, 1996). In accordance with this observation, we found that nonspecific inhibitory effects are promoted by the control oligonucleotides 19NS and 25NS on both CAT and β-galactosidase activities, an effect which appears to depend on oligonucleotide concentration. At an oligonucleotide/plasmid molar ratio of 50 (which means less than 1 μg of TFO per 10 μg of plasmid for the transfections), the inhibition on CAT and β-galactosidase was approximately 40% (Figure 6C). The fact that the total protein content of cell extracts obtained after transfections was found to be rather constant suggests that the cell proliferation was not affected by the oligonucleotides and that the nonspecific effect on CAT and β-galactosidase might be due either to a systematic lower transfection efficiency caused by the presence of the oligonucleotides in the pKRS-413 solution or to an inhibition of the enzyme activity by the oligonucleotides. A similar problem has been pointed out by Kovacs et al. (1996) in cotransfecting cardiac fibroblasts with plasmids pColCAT410, containing the CAT gene driven by the rat *a1(I)* collagen promoter, pSV2gal, and an AG-triplex-forming oligonucleotide.

In conclusion, our data indicate that the critical 29-bp R·Y sequence, located in the murine c-Ki-*ras* promoter, can serve as target for G-rich triplex-forming oligonucleotides, in particular for those forming triplexes with A·AT and G·GC triads. Between the TFOs examined, 20AG seems to have interesting binding properties such as an enhanced affinity for the target (due to a cooperativity behavior) and a dissociation triplex lifetime of many hours, two features suggesting that this oligonucleotide can represent the basic sequence to find out an efficient transcriptional repressor specific for the c-Ki-*ras* gene.

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