Inhibition of Nuclear Protein Binding to the Human Ki-*ras* Promoter by Triplex-Forming Oligonucleotides†

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ABSTRACT: The human Ki-*ras* promoter contains a 22 base pair homopurine·homopyrimidine (pur·pyr) motif within a region that is nuclease-hypersensitive in both native chromatin and supercoiled plasmids. Gel mobility shift analysis and competition experiments show that this pur·pyr motif binds a nuclear protein(s) in a sequence-specific manner. Several observations suggest that the nuclear protein may be an important regulatory factor. Gel mobility shift analysis and DNase I footprinting demonstrate that oligonucleotides can be targeted to this motif forming sequence-specific purine*purine·pyrimidine (pur*pur·pyr) or mixed purine/pyrimidine*purine·pyrimidine (pur/pyr*pur·pyr) intermolecular triple helices through guanine (G) recognition of guanine·cytosine (G·C) base pairs and either adenine (A) or thymine (T) recognition of adenine·thymine (A·T) base pairs in the target sequence. Triple helices containing either T*A·T or A*A·T triplets are formed exclusively with oligonucleotides antiparallel to the homopurine target strand. The affinity of an oligonucleotide which forms T*A·T triplets is approximately equal to, or slightly greater than, the affinity of an oligonucleotide which forms A*A·T triplets. Oligonucleotide-directed triplex formation inhibits sequence-specific nuclear protein binding to the K-*ras* promoter. These observations suggest that triplex formation by the oligonucleotides described here may provide a means to specifically inhibit transcription of the K-*ras* oncogene.

There is a large body of evidence indicating that the *ras* oncogenes play a major role in the development of a wide variety of human malignancies (Barbacid, 1987; Bishop, 1987; Bos, 1989). The three functional *ras* genes in the human genome (c-Ha-*ras*1, c-Ki-*ras*2, and N-*ras*) are highly conserved and encode a structurally homologous 21-kDa signal transduction protein (p21). *Ras* genes which are activated by point mutation specify structurally altered forms of p21 that lead to malignant transformation. In the vast majority of human tumors, the transforming genes detected by gene transfer are members of the *ras* family. The Ki-*ras* gene has been found activated more often than any other single gene in human cancers. Activated Ki-*ras* has been found in 40% of human colon cancers, including premalignant adenomas, in 95% of pancreatic cancers and is commonly present in lung carcinomas (Bos et al., 1987; Forrester et al., 1987; Almoguera et al., 1988). In human colorectal and prostate cancers, overexpression of Ki-*ras* has also been reported (Gallick et al., 1985; Viola et al., 1986; Fujita et al., 1987). Increased expression of Ki-*ras* has been shown to augment its transforming potential (Pulciani et al., 1985; Winter & Perucho, 1986).

The Ki-*ras* promoter contains four GC box sequences but neither a TATA nor a CAAT box at their characteristic positions (McGrath et al., 1986). Previous studies on the chromatin structure of the human c-Ki-*ras* promoter revealed several sites which are hypersensitive to DNase I and micrococcal nuclease (Jordano & Perucho, 1986). One of these sites, located between approximately 250 and 400 bp upstream of the exon 0/intron 1 boundary, is also sensitive to endogenous nuclease and S1 nuclease in supercoiled plasmids. This region corresponds to the essential elements of the Ki-*ras* promoter (Jordano & Perucho, 1986) and contains a 22 base pair homopurine/homopyrimidine motif.

Purine- and pyrimidine-rich oligonucleotides targeted to purine·pyrimidine (pur·pyr)-rich sequences have been shown to form pur*pur·pyr and pyr*pur·pyr intermolecular triple helices (Moser & Dervan, 1987; Ferdorova et al., 1988; Lyamichev et al., 1988; Praseuth et al., 1988; Hanvey et al., 1989; Maher et al., 1989) and to block protein binding to DNA (Hanvey et al., 1989; Maher et al., 1989; Grigoriev et al., 1992; Gee et al., 1992). An oligonucleotide targeted to a site coincident with a PuF transcription factor binding site in the c-myc promoter has been shown to form triplex intracellularly and inhibit transcription in HeLa cells (Postel et al., 1991) while triplex-forming oligonucleotides targeted to a NFκB binding site in the interleukin-2 receptor α regulatory sequence have been shown to inhibit transcription of the native IL-2 R gene (Orsen et al., 1991) as well as reporter genes (Grigoriev et al., 1992) in human cells. In addition, oligonucleotides targeted to sequences coincident with Sp 1 binding sites in the HIV-1 LTR inhibit transcription in infected cells (McShan et al., 1992).

In an intermolecular triple helix, the oligonucleotide third strand occupies the major groove of the duplex, forming Hoogsteen hydrogen bonds with purine bases of the duplex (Moser & Dervan, 1987; Beal & Dervan, 1991). In the pyr*pur·pyr triplex, the pyrimidine-rich third strand is oriented parallel to the purine-rich strand of the duplex and consists of T*A·T and pH-dependent C^+*G·C triplets (Moser & Dervan, 1987). Pur*pur·pyr and mixed pur/pyr*pur·pyr triple helices have been formed at physiological pH with predominantly G*G·C triplets along with A*A·T or T*A·T triplets interspersed (Postel et al., 1991; Beal & Dervan, 1991; Durland et al., 1991). In these triplex types, both homopurine and

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mixed pur/pyr third strands have been shown to bind in an antiparallel orientation with respect to the purine-rich duplex strand (Beal & Dervan, 1991; Durland et al., 1991). However, mixed pur/pyr oligonucleotides oriented parallel to the purine-rich strands in IL-2 R (Orsen et al., 1991) and HIV-1 LTR (McShan et al., 1992) targets have also been reported to form mixed pur/pyr*pur·pyr-type triplex.

In an effort to develop specific transcriptional inhibitors of the human Ki-*ras* oncogene, we have designed oligonucleotides targeted to the 22 bp pur·pyr motif in the human Ki-*ras* promoter (−126 to −147). The relative ability of oligonucleotides to bind specifically to their target sequence in either a parallel or an antiparellel orientation and with either adenine or thymine to recognize seven A·T base pairs in an otherwise homo G·homo C sequence by forming A*A·T or T*A·T triplets, respectively, was evaluated. We have also determined the ability of the pur·pyr motif in the Ki-*ras* promoter to bind protein from a HeLa cell nuclear extract and the effect of triplex formation by the Ki-*ras*-targeted oligonucleotides on protein binding.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides were synthesized on a Milligen Cyclone DNA synthesizer using phosporamidite chemistry. All oligonucleotides were purified by preparative 16–20% polyacrylamide gel electrophoresis and their structural integrity and purity established by 5′-^{32}P-labeling using [γ-^{32}P]ATP and T_4 polynucleotide kinase followed by electrophoresis on polyacrylamide gels. Concentrations were determined from absorbance measurements at 260 nm using molar extinction coefficients.

Promoter Fragment Isolation. The −61 to −234 region of the human c-Ki-*ras*2 gene was PCR-amplified from HL-60 genomic DNA using Vent DNA polymerase. The 5′ PCR primer was designed with an *Eco*RI recognition sequence and the 3′ primer with a *Bam*HI recognition sequence. The PCR-amplified fragment was purified from a low-melting agarose gel, digested with *Eco*RI and *Bam*HI, and cloned into the pTZ 18R vector. The phagemid was digested with *Eco*RI and end-labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase. The fragment was then digested with *Bam*HI and the resulting 181 bp promoter fragment purified by preparative polyacrylamide gel electrophoresis. The sequence of the fragment was confirmed by Maxam–Gilbert sequencing (Maxam & Gilbert, 1980).

Gel Mobility Shift Analysis of Triplex Formation. For triplex shifts, the synthetic coding strand of the 22 bp c-Ki-*ras* triplex target sequence was 5′-^{32}P end-labeled with [γ-^{32}P]-ATP and T_4 polynucleotide kinase and annealed to its oligonucleotide complement. Potential triplex-forming oligonucleotides were heated at 65 °C for 10–15 min to reduce self-aggregation of the G-rich oligos and then quick-cooled on ice. They were then added to the labeled 22 bp target in 90 mM Tris–borate (pH 8.0)/10 mM $MgCl_2$ and incubated for 45 min at 25 °C. Samples were analyzed by electrophoresis on 16% native polyacrylamide gels at 100 V for approximately 15 h and then exposed at −70 °C for autoradiography. Both gel and running buffer contained 90 mM Tris–borate (pH 8.0)/10 mM $MgCl_2$.

DNase I Footprinting. After heating at 65 °C for 10–15 min and then quick-cooling on ice, olgonucleotides were incubated with ^{32}P-labeled promoter fragment in 20 mM Tris (pH 7.1) and 10 mM $MgCl_2$ for 45–60 min at 25 °C. Samples were precooled on ice and then digested with DNase I for 60 s on ice. Digestion was terminated by the addition of 20 mM EDTA in 90% formamide and then heating at 95 °C for 5 min to inactivate DNase I. Samples were quickly cooled on ice and then analyzed by electrophoresis on an 8 M urea/8% polyacrylamide sequencing gel at 42 W.

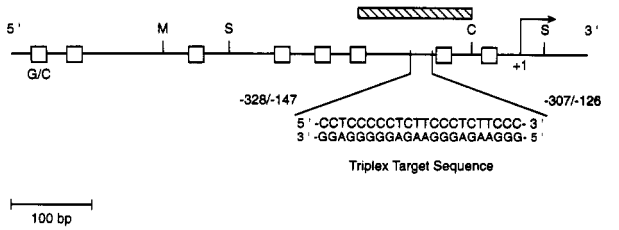

FIGURE 1: Map of the human KI-*ras* promoter showing the 22 bp purine·pyrimidine motif and triplex target sequence with the position relative to the transcription initiation site (−126 to −147) and exon 0/intron 1 boundary (−307 to −328). The previously identified nuclease-hypersensitive site is indicated by a slashed rectangle above the map.

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  -328/-147              -307/-126
                \       /
5′-CGCTGCCTCCCCCTCTTCCCTCTTCCCACACC-3′        Triplex Target
3′-GCGACGGAGGGGGAGAAGGGAGAAGGGTGTGG-5′          Sequence
     ||||||||||||||||||||||||||||||
     3′-GGTGGGGGTGTTGGGTGTTGGG-5′  KR22Tp
     5′-GGTGGGGGTGTTGGGTGTTGGG-3′  KR22Tap   Oligonucleotide
                                              Sequences
     3′-GGAGGGGGAGAAGGGAGAAGGG-5′  KR22Ap
     5′-GGAGGGGGAGAAGGGAGAAGGG-3′  KR22Aap
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FIGURE 2: Oligodeoxynucleotide sequences and their alignment with the target duplex. Oligodeoxynucleotides containing thymine (T) to recognize A·T base pairs in the target sequence are designated KR22Tp and KR22Tap while those containing adenine (A) to recognize A·T base pairs are designated KR22Ap and KR22Aap. The p and ap nomenclature indicate parallel or antiparallel orientation of the oligonucleotide relative to the homopurine target strand, respectively.

Protein Binding Assays. For protein shifts,^{32}P-labeled promoter fragment was incubated with a HeLa nuclear extract (Dignam, 1990) in protein binding buffer consisting of 20 mM Tris (pH 8.0)/100 mM KCl/1.5 mM $MgCl_2$/1 mM dithiothreitol/8% (v/v) glycerol for 30 min at 25 °C. For competition studies, unlabeled 22 bp Ki-*ras* promoter fragment or 22 bp nonspecific DNA was added to ^{32}P-labeled promoter fragment prior to adding extract. Samples were analyzed by electrophoresis on 5% native polyacrylamide gels at 150 V in 90 mM Tris–borate (pH 8.5)/2 mM EDTA followed by autoradiography. To determine the effect of triplex formation on protein binding, oligonucleotides were incubated with labeled 22 bp Ki-*ras* duplex in protein binding buffer consisting of 20 mM Tris (pH 8.0)/100 mM KCl/1.5 mM $MgCl_2$/1 mM dithiothreitol/8% (v/v) glycerol for 45–60 min at 25 °C. HeLa nuclear extract was added to the samples, and incubated for 30 min at 25 °C, and then analyzed by electrophoresis on 5% gels at 100 V. Both gel and running buffers contained 90 mM Tris–borate (pH 8.0) and 10 mM $MgCl_2$.

RESULTS

Oligonucleotide Design. The human Ki-*ras* promoter contains a 22 bp homopurine·homopyrimidine (pur·pyr) motif located at −307 to −328 with respect to the exon 0/intron 1 boundary and at −126 to −147 from the major transcription start site (Figure 1). Potential triplex-forming oligonucleotides targeted to the human Ki-*ras* pur·pyr motif were designed both parallel and antiparallel with respect to the homopurine duplex strand, containing guanine to recognize G·C bp (G*G·C triplets) and either adenine or thymine to recognize A·T bp (A*A·T or T*A·T triplets) (Figure 2). Since the *K-ras*-targeted oligonucleotides are highly asymmetrical, the parallel oligonucleotides can bind only in a parallel orientation, and

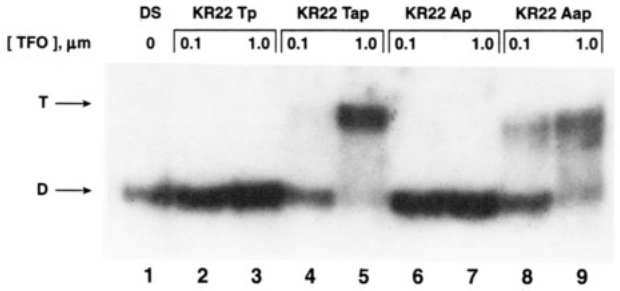

FIGURE 3: Gel mobility shift analysis of triplex formation by parallel and antiparallel K-*ras*-targeted oligonucleotides. The ^{32}P-labeled synthetic 22 bp target DNA (1.0 nM) was incubated in the absence or presence of oligonucleotide as described at the indicated concentration. The duplex control in the absence of oligonucleotide (lane 1) is indicated by DS. D = duplex DNA; T = triplex DNA.

the antiparallel oligonucleotides can bind only in an antiparallel orientation in order to form the defined triplets.

Triplex Formation and Third-Strand Orientation. The relative ability of each oligonucleotide to form triplex with its target sequence was determined by gel mobility shift analyses and DNase I footprint titrations. As shown in Figure 3, the addition of 1.0 μM oligonucleotide (1000-fold molar excess relative to duplex) designed to bind in an antiparallel orientation relative to the homopurine target strand with adenine to recognize A·T (KR22Aap) and with thymine to recognize A·T (KR22Tap) both result in the shift of duplex (D) to a distinct higher migrating band (T), indicating the formation of triplex DNA (T) (Durland et al., 1990, 1991). At the same concentration, oligonucleotides of identical sequence but in a parallel orientation relative to the homopurine strand do not give rise to any retarded bands resulting from triplex formation with either adenine (KR22Ap) or thymine (KR22Tp) opposite A·T base pairs. Thus, both pur*pur·pyr and mixed pur/pyr*pur·pyr triple helices by GA- and GT-containing oligonucleotides are formed exclusively with the third strand in an antiparallel orientation relative to the homopurine target strand.

The data from DNase I footprinting (Figure 4) are consistent with those obtained from gel mobility shifts, further demonstrating that triplex formation occurs with the oligonucleotide third strand oriented antiparallel relative to the homopurine target strand. Both the thymine- and adenine-substituted oligonucleotides give significant protection from DNase digestion of the target sequence in the antiparallel orientation at 5.0 μM (lanes 6 and 14). However, at the same concentration, neither of the oligonucleotides affords any protection in the parallel orientation (lanes 3 and 10).

*Relative Binding of Oligonucleotides Forming T*A·T or A*A·T Triplets.* Both KR22Aap and KR22Tap form little or no triplex with the target at 0.1 μM while both form triplex with essentially all of the target at 1.0 μM (1000-fold molar excess). At concentrations between 0.1 and 1.0 μM, gel mobility shift titrations (Figure 5) demonstrate that these oligonucleotides are still approximately equal in their ability to form triplex. At 0.5 μM, both KR22Tap (lane 4) and KR22Aap (lane 8) shift 50–75% of the target duplex to triple-helical DNA. The only distinction in triplex-forming ability between KR22Tap and KR22Aap is the apparent slight difference in the relative amount of duplex and triplex species in the presence of a 1.0 μM aliquot of each oligonucleotide. Comparison of lanes 5 and 9 in each of Figures 3 and 5 reveals perhaps a small increase in the percentage of triplex formation by KR22Tap compared to KR22Aap.

Comparison of the footprint titrations indicates that neither KR22Tap nor KR22Aap affords any DNase protection below 5.0 μM (Figure 4). At 5.0 μM, both KR22Tap (lane 6) and

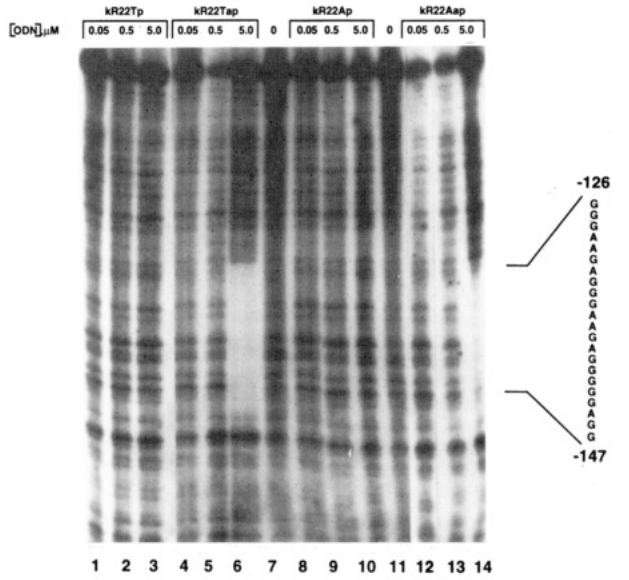

FIGURE 4: DNase I footprint titrations demonstrating sequence-specific binding by triplex-forming oligonucleotides. A 181 bp ^{32}P-labeled human Ki-*ras* promoter fragment (5.0 nM) was incubated in the absence or presence of oligonucleotide at the indicated concentation followed by DNase I digestion as described.

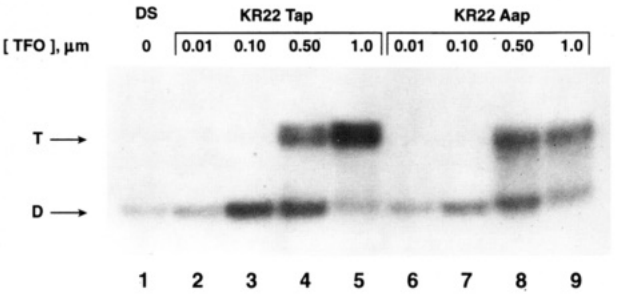

FIGURE 5: Gel mobility shift titrations comparing the relative binding of oligonucleotides which form T*A·T or A*A·T triplets. The ^{32}P-labeled synthetic 22 bp target DNA (1.0 nM) was incubated in the absence or presence of oligonucleotide as described at the indicated concentration. The duplex control in the absence of oligonucleotide (lane 1) is indicated by DS. D = duplex DNA; T = triplex DNA.

KR22Aap (lane 14) provide essentially complete protection of the entire target sequence. KR22Aap may provide slightly less protection than KR22Tap inasmuch as faint bands are present within the footprint of KR22Aap (lane 14). This conclusion would be consistent with the data from gel mobility shift titrations. Therefore, T*A·T triplets may stabilize triplex slightly more than A*A·T triplets within this particular sequence which otherwise consists of entirely G*G·C triplets. Nevertheless, any differene of this magnitude is probably within the margin of error involved in determining dissociation constants of these oligonucleotides. Data from gel mobility shifts are consistent with dissociation constants (K_d) of approximately 0.2–0.5 μM for both KR22Tap and KR22Aap on the basis of the equation $D/T = K_d \times 1/[\text{pur}]$ where D/T is the ratio of duplex (D) to triplex (T) and [pur] is the final concentration of the purine-rich oligonucleotide (Durland et al., 1991).

Sequence Specificity of Triplex Formation. The sequence specificity of triplex formation by the K-*ras*-targeted oligonucleotides is demonstrated by the DNase I footprinting. As shown in Figure 4, both KR22Tap and KR22Aap protect from DNase digestion at the triplex target site. The footprints obtained correspond to DNase protection of the entire 22 bp triplex target sequence as determined by alignment of Maxam–Gilbert sequencing data and DNase I footprints ran adjacent to each other.

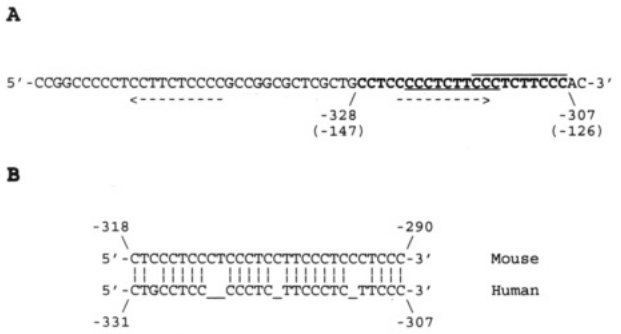

FIGURE 6: (A) Nucleotide sequence of the human Ki-*ras* promoter showing the 22 bp purine·pyrimidine motif and the relative position of 10 bp repeats. The 10 bp overlapping repeats are indicated by lines above and below the sequence while the 10 bp mirror repeats are indicated by arrows below the sequence to demonstrate relative polarity. The 22 bp purine·pyrimidine motif and triplex target sequence are indicated by boldface type. The position shown is with respect to the exon 0/intron 1 boundary and transcription initiation site (in parentheses). (B) Comparison of human and mouse purine·pyrimidine motifs showing sequence homology and relative positions with respect to the exon 0/intron 1 boundary.

The Ki-*ras* target sequence contains a 10 base pair overlapping repeat (Fiure 6A). We considered the possibility that this repeat could allow for alternative oligonucleotide alignments resulting in a seven base pair upstream or downstream shift in the binding of oligonucleotides. Such an alignment would involve the 5′ and/or 3′ ends of the oligonucleotide either binding with some mismatches or being left unbound. Other triplex-forming oligonucleotides have previously been shown by DNase I footprinting to bind in this manner based on the extent of protection of their target sequence (Durland et al., 1990, 1991). Different alignments of the K-*ras*-targeted oligonucleotides to either side of the target sequence would result in decreased occupancy of the seven base pair ends within the intended target relative to the central eight base pair region which would be occupied in all alignments. There appears to be no decrease in the extent of protection afforded over either seven base pair sequence relative to the common region of the target sequence (Figure 4, lanes 6 and 14). Misalignment would also be expected to yield triplex conformations of differing electrophoretic mobilities (Durland et al., 1991). However, gel shift analysis shows only one triple-helical complex, suggesting that KR22Tap and KR22Aap assume the correct alignment at the intended target sequence.

The Ki-*ras* promoter also contains a 10 base pair mirror repeat of a portion of the target sequence within a 16 base pair pur·pyr motif which is very similar to the triplex target sequence (Figure 6A). Because of the increased electrophoresis time (to enhance resolution of the target sequence) of the DNase I digests shown in Figure 4, the distal pur·pyr motif is not visible. DNase I protection and shorter electrophoresis (Figure 7) show both the target (upper brackets) and nontarget (lower brackets) pur·pyr motifs. Neither KR22Tp nor KR22Tap provides any DNase protection of the 16 bp distal pur·pyr motif (Figure 7, lower brackets). This observation is expected for KR22Tap since this repeat is opposite in orientation relative to that in the target sequence. The most favorable triplex alignment of KR22Tap would require parallel orientation with respect to the purine-rich target strand, and gel mobility shifts and DNase I footprinting with the parallel-designed KR22Tp show that triplex formation via this third strand orientation is not possible even with no mismatches (i.e., at the target site). However, at the distal pur·pyr motif, KR22Tp would allow an antiparallel alignment with the mirror repeat. Still, no interaction of the oligonucleotide at this sequence is

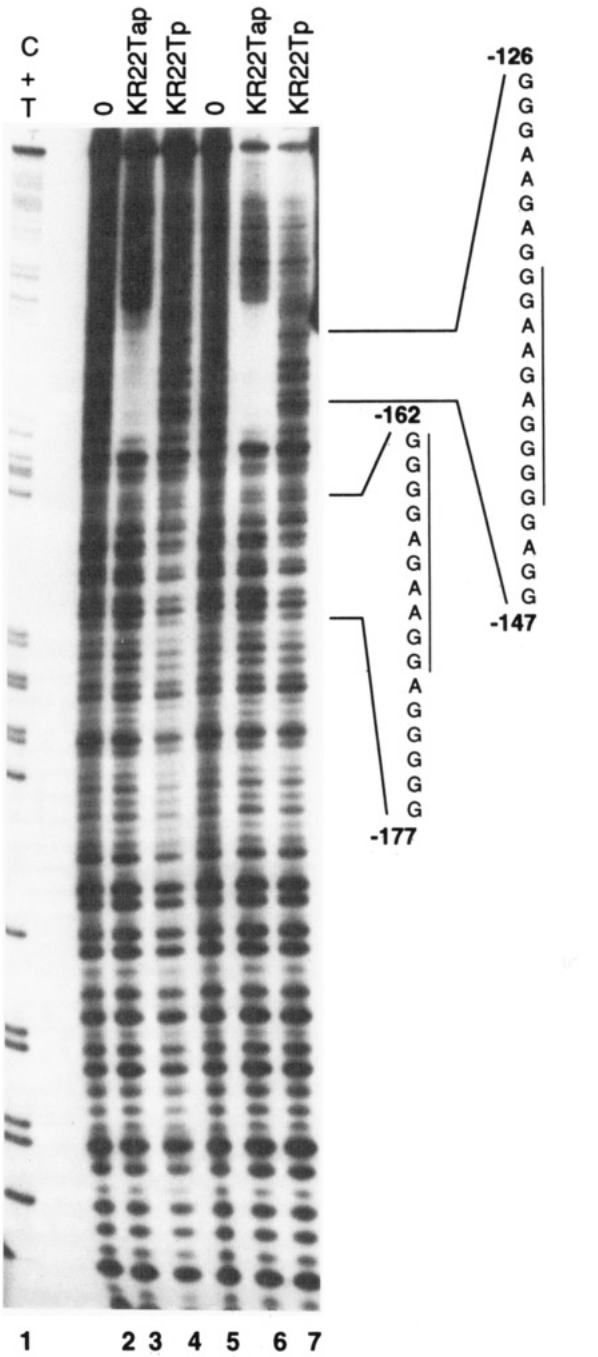

FIGURE 7: DNase I protection assay of oligonucleotide interaction at a distal nontarget pur·pyr motif. A 181 bp ^{32}P-labeled human Ki-*ras* promoter fragment was incubated in the absence or presence of KR22Tap or KR22Tp at the indicated concentration followed by DNase I digestion as described.

detected. These data suggest that triplex formation is not likely to occur when the oligonucleotide can form only a portion of the intended triplets and that the oligonucleotide may be able to discriminate between its target pur·pyr motif and commonly occurring nontarget pur·pyr motifs even though they match a portion of the target sequence.

Nuclear Protein Binding and Effect of Triplex Formation. Protein binding to the Ki-*ras* pur·pyr motif was documented by gel mobility shift experiments (Figure 8A). Incubation of the Ki-*ras* 22 bp pur·pyr motif with a HeLa nuclear extract results in the formation of major and minor highly retarded bands (B_2 and B_1, respectively), indicating the formation of protein–DNA complex (lane 2). The sequence-specificity of this protein interaction was examined by competition binding assay. As shown in Figure 8A, a 50-fold molar excess of

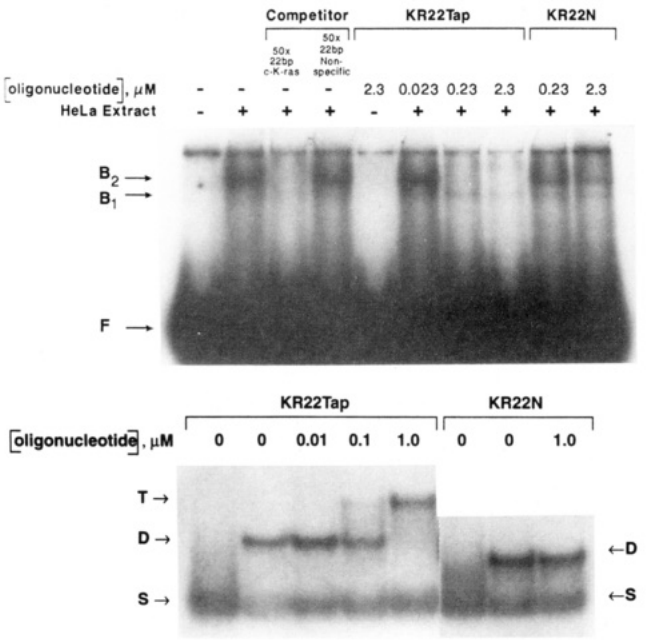

FIGURE 8: (A, top) Gel mobility shift analysis demonstrating sequence-specific nuclear protein binding to the purine·pyrimidine motif in the human Ki-*ras* promoter and its inhibition by triplex-forming oligonucleotides. ^{32}P-Labeled 22 bp human Ki-*ras* fragment was incubated with competitor duplexes or oligonucleotides as described and then HeLa nuclear extract added. Arrows indicate protein–DNA complexes or bound probe (B) and unbound DNA or free probe (F). (B, bottom) Gel mobility shift analysis demonstrating triplex formation with the 22 bp Ki-*ras* promoter sequence under protein binding conditions. The 22 bp target was incubated with oligonucleotide at the indicated concentrations under protein binding conditions identical to those used in (A).

unlabeled Ki-*ras* 22 bp pur·pyr motif effectively competes with the labeled sequence for protein binding (lane 3) while the same excess of an unlabeled 22 bp nonspecific DNA has no effect on protein binding to the target (lane 4). These data demonstrate that the nuclear factor binding to this Ki-*ras* promoter region involves a sequence-specific interaction.

The effect of triplex formation by KR22Tap on nuclear protein binding to the pur·pyr motif was also determined by gel mobility shift analysis (Figure 8A). Incubation of the 22 bp sequence with triplex-forming KR22Tap results in a concentration-dependent inhibition of nuclear protein binding (lanes 6–8). Protein binding is significantly inhibited at an oligonucleotide concentration of 0.23 μM and is completely abrogated by 2.3 μM. At the same concentration, a non-triplex-forming nonsense oligonucleotide (KR22N) has little or no effect on protein binding (lanes 9 and 10). Because of the low percentage (5%) gels used to analyze protein binding, migration of triplex DNA is indistinguishable from duplex (lanes 1 and 5) and is therefore not detectable in Figure 8A. However, gel mobility shift analysis in a 16% gel (Figure 8B) demonstrates that in identical protein binding conditions KR22Tap forms triplex with its target sequence (lanes 3–5) while the nonspecific oligonucleotide KR22N does not (lanes 6–8). KR22Tap is able to form triplex with approximately equal affinity as in standard triplex-forming conditions as evidenced by the similar amount of duplex target shifted under both conditions at 1.0 μM. These data suggest that inhibition of nuclear protein binding is a direct result of triplex formation by KR22Tap at the K-*ras* promoter target.

DISCUSSION

The human Ki-*ras* promoter contains a 22 bp pur·pyr motif −128 to −147 relative to the major transcription initiation site. We have demonstrated that oligonucleotides can be targeted to this pur·pyr motif, forming sequence-specific intermolecular triple helix. The oligonucleotides can form either pur*pur·pyr or mixed pur/pyr*pur·pyr triplex through formation of G*G·C and T*A·T or G*G·C and A*A·T triplets. Triple helices with either thymine- or adenine-containing oligonucleotides are formed exclusively with the oligonucleotide oriented antiparallel to the homopurine duplex strand. These results are consistent with previous studies with triple helix involving G*G·C as well as T*A·T and/or A*A·T triplets. Beal and Dervan (1991) have shown that triplex formation by 19mer oligonucleotide–EDTA*Fe analogs in which five T*A·T triplets are interspersed in an otherwise G*GC triplex or in which three of the T*A·T triplets are replaced with A*A·T triplets occurs in each case with the third-strand oligonucleotides binding antiparallel to the homopurine duplex strand. They also subsequently showed that shorter 15 mers derived from the same sequence that form four T*A·T triplets or in which a single T*A·T triplet was replaced with an A*A·T triplet also bind in the antiparallel orientation (Beal & Dervan, 1992).

The antiparallel orientation of binding by the adenine-containing oligonucleotide KR22Aap is also in agreement with models which show that A*A·T triplets adopt only the reverse-Hoogsteen hydrogen-bonding pattern (antiparallel orientation) (Giovannangeli et al., 1992). However, G*G·C and T*A·T triplets can adopt either the Hoogsteen or the reverse-Hoogsteen conformation, corresponding to a parallel or an antiparallel orientation of binding (Giovannangeli et al., 1992). On the basis of triplet models and energy minimization studies, it has been suggested that the antiparallel orientation is favored with oligonucleotides containing many GpT and TpG steps while a parallel orientation is favored when there are few GpT and TpG steps (Giovannangeli et al., 1992). The results obtained with the K-*ras*-targeted KR22Tap which contains 10 GpT and TpT steps seem to support this prediction experimentally. These findings are also consistent with studies with oligonucleotides which form a number of T*A·T triplets within mostly G*G·C triplexes in the promoters of the human c-myc, human EGF-R, and mouse insulin receptor promoters (Durland et al., 1991). However, oligonucleotides forming predominantly T*A·T and G*G·C triplexes designed parallel to purine-rich strands in the IL-2Rα gene promoter and the HIV-1 LTR have also been shown to form triplex (Orsen et al., 1991; McShan et al., 1992). It is likely that the preferred third-strand orientation is dependent on the individual sequence.

We have also compared the relative affinity of the K-*ras*-targeted triplex-forming oligonucleotide forming T*A·T triplets (KR22Tap) with that of an oligonucleotide forming A*A·T triplets (KR22Aap). The previous studies by Beal and Dervan also showed that both the thymine- and adenine-substituted 19mers provided efficient cleavage of the target DNA (Beal & Dervan, 1991). Quantitation of the data obtained with 15mers revealed that substitution of adenine (to form A*A·T triplet) for a single thymine decreases cleavage by less than a factor of 2 (Beal & Dervan, 1992). Similarly, the affinity of the K-*ras*-targeted oligonucleotide KR22Tap, containing thymine, is approximately equal to, or perhaps slightly greater than, the affinity of KR22Aap which contains seven adenines in place of thymines.

Oligonucleotide specificity was further explored by considering that overlapping repeats in the target sequence, and upstream mirror repeats of a segment of the target sequence could allow for partial binding of oligonucleotides. Oligonucleotides have been shown to bind to sequences in the c-myc

and insulin receptor genes with only a portion actually forming triplets with the duplex DNA (Durland et al., 1990, 1991). The Ki-*ras* oligonucleotides do not appear to interact to any extent with the partial target repeats in the Ki-*ras* promoter, suggesting that the triplex-forming oligonucleotides may be specific enough to discriminate between such sequences and its target *in vivo*.

We have shown that the 22 bp triplex-forming pur·pyr motif in the human Ki-*ras* promoter is also a binding site for a nuclear protein. The protein interacts with this motif in a sequence-specific manner. The identity of this nuclear protein and its significance are not known, but several observations suggest that it may be important in transcriptional regulation of the Ki-*ras* gene. The sequence is centered within the approximately 150 bp (−250 to −400 with respect to the exon 0/intron 1 boundary) shown to be DNase I and micrococcal nuclease hypersensitive in native chromatin and endogenous nuclease and S1 nuclease hypersensitive in supercoiled plasmids (Jordano & Perucho, 1986). This region represents the essential elements of the human Ki-*ras* promoter (Jordano & Perucho, 1986). The pur·pyr motif consists of 10 bp overlapping repeats of CCCTCTTCCC (or 7 bp tandem repeats of CCCTTCT) and another 10 bp mirror repeat of CCTTCTCCCC located immediately upstream (Figure 6A). Also, the promoter region of the human Ki-*ras* gene shares 82% nucleotide sequence homology with the mouse Ki-*ras* promoter (McGrath et al., 1983). The mouse Ki-*ras* promoter contains a 28 bp homopurine homopyrimidine motif at a very similar location (−290 to −318 from exon 0/intron 1) as the pur·pyr motif in the human promoter and exhibits considerable sequence homology with the human (Figure 6B). The mouse pur·pyr motif has been shown to be S1 nuclease sensitive in supercoiled plasmid, to bind at least one HeLa nuclear factor which also interacts with similar sequences in the promoter regions of the human EGF-R and I-R genes, and to result in a virtual complete loss of transcriptional activity upon deletion (Hoffman, 1990). Moreover, we have recently shown that the murine and human Ki-*ras* motifs compete for binding of the same HeLa nuclear factor and that the murine sequence also forms an intermolecular DNA triple helix. These observations are consistent with the human pur·pyr motif being an important positive regulatory protein binding site which may be essential for transcriptional activity of the human Ki-*ras* gene as with the mouse promoter.

We have demonstated that triplex formation at the target site in the Ki-*ras* promoter completely abrogates nuclear protein binding. Oligonucleotides are readily taken up by cells and can form triplex in intact cells (Postel et al., 1991). Moreover, triplex-forming oligonucleotides targeted to sequences coincident with protein binding sites in gene promoters have been shown to inhibit transcription of these genes in living cells (Postel et al., 1991; Orsen et al., 1991; McShan et al., 1992; Grigoriev et al., 1992). The sequence-specific oligonucleotide-directed triplex formation and inhibition of binding of a potential nuclear regulatory factor in the Ki-*ras* promoter represent important steps toward inhibiting expression of this biologically important oncogene at the transcriptional level *in vivo*.

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