Structural polymorphism within a regulatory element of the human KRAS promoter: formation of G4-DNA recognized by nuclear proteins

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ABSTRACT

The human KRAS proto-oncogene contains a critical nuclease hypersensitive element (NHE) upstream of the major transcription initiation site. In this article, we demonstrate by primer-extension experiments, PAGE, chemical footprinting, CD, UV and FRET experiments that the G-rich strand of NHE (32R) folds into intra-molecular G-quadruplex structures. Fluorescence data show that 32R in 100 mM KCl melts with a biphasic profile, showing the formation of two distinct G-quadruplexes with \( T_m \) of \( \approx 55^\circ C \) (Q₁) and \( \approx 72^\circ C \) (Q₂). DMS-footprinting and CD suggest that Q₁ can be a parallel and Q₂ a mixed parallel/antiparallel G-quadruplex. When dsNHE (32R hybridized to its complementary) is incubated with a nuclear extract from Panc-1 cells, three DNA–protein complexes are observed by EMSA. The complex of slower mobility is competed by quadruplex 32R, but not by mutant oligonucleotides, which cannot form a quadruplex structure. Using paramagnetic beads coupled with 32R, we pulled down from the Panc-1 extract proteins with affinity for quadruplex 32R. One of these is the heterogeneous nuclear ribonucleoprotein A1, which was previously reported to unfold quadruplex DNA. Our study suggests a role of quadruplex DNA in KRAS transcription and provides the basis for the rationale design of molecular strategies to inhibit the expression of KRAS.

INTRODUCTION

KRAS is one of the most frequently mutated oncogenes detected in human cancer. It encodes for a guanine nucleotide (GTP/GDP)-binding protein acting as a signal transducer (1). The normal (wild-type) RAS protein is transiently activated by signals from cell receptors that promote the exchange of bound GDP with GTP. The protein returns to its inactivated state through the hydrolysis of GTP to GDP. It has been shown that the KRAS gene may harbour a number of point mutations resulting in aminoacid substitutions (in particular, at codons 12, 13 and less frequently at codons 59, 61 and 63) that yield a protein with a reduced capacity to hydrolyze GTP (2-4). As a result, the mutated RAS protein remains locked in the activated state, transmitting constitutively signals for cell proliferation to the nucleus, via the MAP-kinase cascade (5). Activating KRAS mutations are most prevalent in pancreatic (60%), biliary tract (33%), colorectal (32%) and lung (19%) carcinomas (6). Due to its role in the pathogenesis of cancer, KRAS is an ideal target for anticancer drugs. Several anti-KRAS molecular strategies have been proposed: (i) inhibition of KRAS expression by antisense, ribozyme and antigen oligonucleotides (7-11); (ii) blockade of RAS protein to anchor to the plasma membrane by farnesyl transferase inhibitors (12,13); (iii) inhibition of downstream effectors of the RAS proteins (14,15). The human KRAS gene is located in chromosome 12, at the 12p12.1 locus, and its promoter contains a polypurine nuclease hypersensitive element (NHE) that plays an essential role in transcription (16-18). Nuclease-hypersensitive sites rich in guanines are commonly found in the regulatory regions of eukaryotic genes and have been associated with non-B-DNA conformations (19). The most investigated nuclease-hypersensitive sequence is the one located in CMYC, upstream of the P1 promoter that controls 80–90% of transcription (20–22). Hurley and co-workers have demonstrated that the CMYC hypersensitive site adopts stable G-quadruplex (or G4-DNA) structures that seem to behave as a transcription repressor (21). These data strongly support the notion that certain nuclease hypsersensitive sites can assume two possible conformations: a double-stranded, transcription-active form or a folded,
transcription-inactive form. A similar picture was recently proposed for the murine KRAS gene that also contains a G-rich NHE in its promoter (23). Other promoter/enhancer sequences composed by multiple runs of guanines are structurally polymorphic. For instance, three overlapping G-rich sequences within the promoter of BCL-2 form distinct G-quadruplex structures, each characterized by a different loop topology (24); a polypurine tract of the hypoxia-inducible factor 1α promoter forms a parallel quadruplex (25); G-rich regions forming G4-DNA are present in promoter/enhancer sequences of VEGF (26); the PDGF-A gene is regulated by multiple promoter and silencer elements that are CG-rich and exhibit a single-stranded folded character (27); CKIT (28) and muscle-specific human sarcomeric mitochondrial creatine kinase (sMtCK) (29) contain sequences that form quadruplex structures. This leads to the suggestion that G4-DNA in the control region of the genes may contribute to the regulation of gene expression (30,31).

In this work, we demonstrate that the G-rich strand of NHE, located in the human KRAS promoter, can fold into a dynamic intra-molecular G-quadruplex that can assume two distinct conformations for which we propose, on the basis of circular dichroism (CD) and DMS-footprinting data, folding topologies with three and four loops. Pull-down assays were used to isolate from a pancreatic adenocarcinoma nuclear extract proteins that have affinity for the G-quadruplex of 32R. LC-MS/MS of in-gel tryptic digests of these proteins allowed the identification of poly[ADP-ribose] polymerase I (PARP-1), ATP-dependent DNA helicase 2 subunit 1 (Ku70) and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1). PARP-1 and the dimer Ku70–Ku83 bind also duplex NHE (dsNHE). The proteins of the hnRNP family are of particular interest, as some of them show unfolding properties against G4-DNA (for a comprehensive review see ref. (32)). The observation that KRAS transcription in Panc-1 cells is reduced by TMPyP4 (but not by TMPyP2), a cationic G4-DNA stabilizing ligand, confirm the notion that folded G-quadruplex structures within critical promoter region behaves, indeed, as a transcription repressor. The implications of these findings in the regulation of KRAS transcription as well as in the development of new molecular approaches to specifically inhibit oncogenic KRAS are discussed.

MATERIALS AND METHODS
Oligonucleotides and porphyrins
The oligonucleotides (sequences in Table 1) were obtained from Sigma-Proligo (Paris, France). They were purified by electrophoresis using a denaturing 20% polyacrylamide gel (acrylamide: bisacrylamide, 19 : 1) in TBE, 7 M urea, 55°C. The bands were excised from the gel and eluted in water. The DNA solutions were filtered (Ultrafree-DA, Millipore, Billerica, MA) and precipitated. The concentration of each DNA was determined from the absorbance at 260 nm in milli Q water, using as extinction coefficients 7500, 8500, 15 000 and 12 500 M⁻¹cm⁻¹ for C, T, A and G, respectively. Dual-labelled 32R [5’ end with fluorescein (FAM), 3’ end with tetramethylrhodamine (TAMRA)] was HPLC purified. Porphyrins TMPyP2, TMPyP3 were purchased from Porphyrin Systems (Lübeck, Germany), while TMPyP4 from Sigma (Milan, Italy).

Polymerase stop assay
A DNA fragment of 80 bp, containing the NHE sequence (access number L00044 bases 228–306), was cloned in plasmid pBSKS+ at SmaI, in the orientation that the polyuridine strand of NHE was the template for T3-primer extension reactions. Vector pBSKS-NHE (15 nm) was denatured at 95°C in the presence of NaOH (100 mM) then neutralized with an equivalent amount of HCl. Following this treatment, the vector was added with T3 primer for DNA polymerase I (7.5 nm), 100 mM KCl,
Circular dichroism, ultraviolet and fluorescence experiments

CD spectra were obtained using a JASCO J-600 spectropolarimeter equipped with a thermostatted cell holder. CD experiments were carried out with oligonucleotides (1 or 3 μM) in Tris 50 mM, pH 7.4, 100 mM KCl. Spectra were recorded in 0.5 cm quartz cuvette. A thermometer inserted in the cuvette holder allowed a precise measurement of the sample temperature. The spectra were calculated with J-700 Standard Analysis software (Japan Spectroscopic Co., Ltd, Tokyo, Japan) and are reported as ellipticity (millidegrees) versus wavelength (nanometres). Each spectrum was recorded three times, smoothed and subtracted to the baseline. UV measurements were obtained with a (Perkin–Elmer Spectrophotometer, Waltham, MA) equipped with a thermostatted cell holder. Spectra were recorded in 0.5 cm quartz cuvette containing 3 μM DNA solution.

Fluorescence measurements were carried out with a Microplate Spectrofluorometer System (Molecular Devices, Concorde, Canada) using a 96-well optical-bottom plate, in which each well contained 50 μl of 0.30 nm dual-labelled 32R in 50 mM Tris–HCl, pH 7.4, and lithium or potassium chloride as specified in the figure captions. The samples were overnight incubated at 37°C before measurements. The emission spectra were obtained setting the excitation wavelength at 475 nm, the cut-off at 515 nm and recording the emission from 500 to 650 nm. Fluorescence melting experiments were performed on a real-time PCR apparatus (iQ5, BioRad, Hercules, CA), using a 96-well plate filled with 50 μl solutions of dual-labelled 32R, prepared as described above. The protocol used for the melting experiments is the following: (i) equilibration step of 5 min at low temperature (20°C); (ii) stepwise increase of the temperature of 1°C/min for 76 cycles to reach 95°C. All the samples in the wells were melted in 76 min. After melting the samples were re-annealed, melted again to give the same melting curves.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides 32R, 32Y and 32deg were end-labelled with [γ-32P]ATP and T4 polynucleotide kinase (30 pmol) and purified by denaturing polyacrylamide gel electrophoresis (PAGE). Duplex dsNHE was prepared annealing (10 min at 95°C and overnight at room temperature) a mixture containing equimolar amounts of radiolabelled 32R and complementary cold 32Y in 50 mM Tris–HCl, pH 7.4, 100 mM NaCl. To observe quadruplex formation, 32R was incubated overnight at 37°C in 50 mM Tris–HCl, pH 7.4, 140 (or 50) mM KCl, 10 mM MgCl2. The samples were also incubated with and without TMPyP4, as specified in Figure 2. After incubation, the samples were loaded in a 15% native polyacrylamide gel in TB, containing 50 mM KCl in the gel and running buffer. After running, the gel was dried and exposed to autoradiography (Hyperfilm, GE Healthcare, Milan, Italy) for few hours.

DMS-footprinting and TMPyP4-photo cleavage experiments

PAGE-purified oligonucleotide 32R (25 nm), end-labelled with [γ-32P] ATP (GE Healthcare, Europe, GmbH) and polynucleotide kinase (New England Biolabs, MA, USA), was incubated overnight at 37°C, in 50 mM sodium cacodylate, pH 8, 1 mM EDTA, 0, 50 or 100 mM KCl, 1 μg sonicated salmon sperm DNA. Some DNA solutions were added with 50 or 100 nm TMPyP4. Dimethyl sulphate (DMS) dissolved in ethanol (DMS : ethanol, 1 : 10, vol/vol) was added to the DNA solution (1 μl to a total volume of 20 μl) and left to react for 5 min at room temperature. For photo cleavage experiments, the DNA solutions containing 100 nm TMPyP4 were incubated in cacodylate buffer containing 100 mM KCl, and treated for 5, 10 or 15 min with white light from a Solaris 2 (J615) projector (150 W) at a fluence rate of 64 mW/cm². Both DMS and photo cleavage reactions were stopped by the addition of 1:4 volume of Stop solution (1.5 M sodium acetate pH 7, 1 M β-mercaptoethanol, 1 μg/μl tRNA). DNA was precipitated with 4 volumes of ethanol and resuspended in piperidine 1 M. After cleavage at 90°C for 30 min, reactions were stopped by chilling in ice followed by precipitation with sodium acetate 3 M pH 5.2 and 3 volumes of ethanol. The DNA samples were resuspended in 90% formamide and 50 mM EDTA, denatured at 90°C and run for 2 h on a denaturing 20% polyacrylamide gel, prepared in TBE and 8 M urea, pre-equilibrated at 55°C in a Sequi-Gen GT Nucleic Acids Electrophoresis Apparatus (BioRad, CA, USA), which was equipped with a thermostouple that allows a precise temperature control. After running, the gel was fixed in a solution containing 10% acetic acid and 10% methanol, dried at 80°C and exposed to Hyperfilm MP (GE Healthcare) for autoradiography.

Preparation of nuclear protein extract and EMSA

Nuclear extracts were obtained as described by Abmayr and Workman (33). Briefly, 10 plates of 10-cm diameter of confluent Panc-1 cells were collected and resuspended in hypotonic buffer (10 mM HEPS, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 5 mM NaF, 1 mM Na3VO4). Swollen cells were homogenized with a Dounce homogenizer and the nuclei, pelleted by centrifugation, were resuspended in low-salt buffer (20 mM HEPS, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). Release of nuclear proteins was obtained by the addition of a high-salt buffer (as low-salt buffer, but with 1.2 M KCl). Protein concentration was measured according to the
Bradford method. Protein–DNA interactions were analysed by electrophoresis mobility shift assays (EMSAs). End-labelled duplex 5nM dsNHE or 32R-quadruplex were incubated in 20 µl solution containing 20 mM Tris–HCl, pH 8, KCl 30 mM, MgCl₂ 1.5 mM, DTT 1 mM, glycerol 8%, Phosphatase Inhibitor Cocktail I (Sigma, Milan, Italy) 1%, NaF 5 mM, Na₃VO₄ 1 mM, poly [dI-dC] 2.5 ng/µl, nuclear extract 0.25 µg/µl, for 2 h at 37°C. The analyses were carried out in 5% polyacrylamide gels in TBE at 20°C.

Pull-down assays
Ten milligrams of nuclear protein extracts (0.71 mg/ml) were incubated for 2 h with 85 nm biotinylated 32R or duplex NHE in a solution containing Tris–HCl pH 8 20 mM, glycerol 8%, KCl 150 mM, MgCl₂ 1.5 mM, poly [dI-dC] 2.5 ng/µl (about two times more concentrated than biotinylated DNA), Na₃VO₄ 1 mM, NaF 5 mM, DTT 1 mM, PMSF 0.2 mM. One milligram of Streptavidin MagneSphere Paramagnetic Particles (Promega, Milan, Italy) was added and incubated for 30 min at room temperature. Particles were captured with a magnet and the proteins were eluted with the buffer containing increasing amounts of NaCl (0, 0.1, 0.25, 0.5, 0.75 and 1 M). For further analyses the proteins eluted were concentrated and desalted with Microcon YM-3 filters (Millipore, Billerica, MA).

SDS–PAGE protein analysis
Silver staining. Proteins were separated in 8% SDS–PAGE, the gel was fixed overnight with three changes of 5% acetic acid, 30% ethanol, sensitized in 0.02% Na₂S₂O₃ solution of 1.29 M (NH₄)₂SO₄, 0.1% Coomassie G250, methanol, 1% acetic acid, then stained overnight with a Colloidal Coomassie blue staining. Proteins were run on 8% SDS–PAGE, the gel was fixed for 1 h with 45% methanol, 1% acetic acid, then stained overnight with a solution of 1.29 M (NH₄)₂SO₄, 0.1% Coomassie G250, 0.5% acetic acid and 34% methanol. Gel was destained with water for a good visualization of the bands that were excised for LC-MS/MS analysis.

Southwestern blots. Twenty-five micrograms of nuclear protein extract were loaded in each lane, separated in 8% PAGE–SDS gel and electroblotted in 25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS on a nitrocellulose transfer membrane. Denaturation was performed with 6 M guanidine–HCl for 30 min at 4°C, followed by renaturation with increasing dilutions with HEPES binding buffer (25 mM HEPES pH 7.9, 4 mM KCl, 3 mM MgCl₂), 1:1, 1:2, 1:4, each wash was carried out at 4°C for 15 min. The blotted membrane was treated for 1 h with 5% non-fat milk in HEPES-binding buffer. Hybridizations with radiolabelled 32R or mutant sequence 32R-4C were carried out in 0.5% non-fat dried milk, 1% NP40, 10 µg/ml poly [dI-dC], in HEPES-binding buffer, 1000 c.p.m./ml of radiolabelled probe, which was preincubated in Tris–HCl 50 mM pH 7.4, KCl 100 mM for 2 h at 37°C. Washes were performed with HEPES-binding buffer.

Protein identification by mass spectrometry
After the separation of proteins by SDS–PAGE, Coomassie-stained protein bands were excised and processed to obtain a tryptic digest as previously described (34). LC-MS/MS analyses were performed on a Micromass CapLC unit interfaced to a Micromass Q-Tof Micro mass spectrometer (Waters, Milan, Italy) equipped with a nanospray source. For each gel spot, 6.4 µl of tryptic digest were injected at a flow rate of 20 µl/min onto an Atlantis dC18 Trap Column. After valve switching, the sample was separated on an Atlantis dC18 NanoEase column (150 × 0.075 mm², 3 µm particle size) at a flow rate of 4.0 µl/min using a gradient from 10% B to 55% B in 27 min (solvent A: 95% H₂O, 5% acetonitrile, 0.1% formic acid; solvent B: 5% H₂O, 95% acetonitrile, 0.1% formic acid). Instrument control, data acquisition and processing were achieved with MassLynx V4.1 software. The MS/MS data were analysed by the online MASCOT software (Matrix Science, http://www.matrixscience.com) against the human section of the MSDB database (release 31 August 2006) (35). The following parameters were used in the MASCOT search: trypsin specificity; maximum number of missed cleavages: 1; fixed modification: carbamidomethyl (Cys); variable modifications: oxidation (Met) and acetyl (N-term); peptide mass tolerance: ±1.0 Da; fragment mass tolerance: ±0.5 Da; protein mass: unrestricted; mass values: monoisotopic. All the identifications were considered reliable if at least two different peptides matched to the protein with significant scores. Only in the case of sample p34, one tryptic peptide was considered enough for the identification because it was associated with the MS/MS spectrum with a high score.

RNA extraction, RT and real-time PCR
Total RNA was extracted from differently treated cells (Panc-1 cells untreated or treated with 50 µM TMPyP2, TMPyP3 or TMPyP4) using Trizol reagent (Invitrogen, Carlsbad, CA), following manufacturer instruction. cDNA synthesis: 5 µl of RNA in diethyl pyrocarbonate (DEPC) treated water (extracted from about 12 500 Panc-1 cells) was heated at 70°C and placed in ice. The solution was added to 7.5 µl of mix, containing (final concentrations) 1× buffer; 0.01 M DTT (Invitrogen); 1.6 µM primer dT [MWG Biotech, Ebersberg, Germany; d(T)ₐ₋₁]; and 1.6 µM Random primers (Promega); 0.4 mM dNTPs solution containing equimolar amounts of dATP, dCTP, dGTP and dTTP (Euroclone, Pavia, Italy); 0.8 U/µl RNase OUT; 8 U/µl of M-MLV reverse transcriptase (Invitrogen). The reactions were incubated for 1 h at 37°C and stopped with heating at 95°C for 5 min. As a negative control the reverse transcription reaction was performed with 5 µl of DEPC water. Real-time PCR reactions were performed with 1× iQ™ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA), 300 nm of each primer, 1/10 of RT reaction. The sequences of the primers used for KRAS and GAPDH amplifications are reported in
Table 1. The PCR cycle was: 3 min at 95°C, 30 cycles 10 s at 95°C, 30 s at 60°C with an iQ5 real-time PCR controlled by an Optical System software version 2.0.

Cell culture
The human cell line used is from pancreatic adenocarcinoma (Panc-1). The cells were maintained in exponential growth in Dulbecco’s modified eagle’s medium (DMEM) medium containing 100 U/ml penicillin, 100 mg/ml streptomycin, 20 mM L-glutamine and 10% foetal bovine serum (Euroclone, Pavia, Italy). Cell viability was measured by MTT assays following standard procedures.

RESULTS
The nuclease hypersensitive element of KRAS forms quadruplex structures
The promoter of the human KRAS gene contains a NHE from −327 to −296, positions relative to the exon q/intron 1 boundary (16–18) (accession no. L00044). Evidence that this critical promoter element is structurally polymorphic was obtained by primer extension assays, using as template a vector containing NHE. Figure 1 illustrates the results of a typical primer-extension experiment where DNA polymerase is arrested at the 3'end of the G-rich strand of NHE. Considering that this segment contains four runs of guanines and that the arrest of DNA polymerase is potassium-dependent, it is likely that the G-rich strand assumes a G-quadruplex structure. In the presence of NaCl, the pause of DNA polymerase I is weak (lane 5) and a full-length product is observed in the well (data not shown). In contrast, with KCl or KCl+TMPyP4, a cationic porphyrin-stabilizing G4-DNA (36,37), the arrest of DNA polymerase appears stronger, after 1 or 3 h of reaction (lanes 6–9) and a low amount of full-length product is observed in the wells (data not shown). In light of this experiment, we focused on a sequence of 32 bases (32R), comprising fours runs of guanines and located immediately downstream from the DNA polymerase arrests (Figure 1).

To see if 32R assumes a secondary structure we first performed spectroscopic experiments. In 100 mM KCl, but not in 100 mM LiCl, 32R shows a thermal differential spectrum (TDS) typical of quadruplex DNA, with negative bands at 295 and 225 nm, positive bands at 250, 260 and 275 nm (38) (Figure 2a). For comparison we also report the TDS of the CMYC sequence (Table 1), which is known to form a parallel G-quadruplex (39). The CD spectrum of 32R is typical of DNA in the parallel quadruplex conformation (40), as it is characterized by a strong and positive ellipticity at 260 nm, which upon denaturing is strongly reduced (from 23 to 8 mdeg) (Figure 2b).

In order to determine the molecularity of the G-quadruplex formed by 32R, we performed native electrophoresis, taking advantage of the fact that oligonucleotides folded into compact G-quadruplex structures migrate faster than oligonucleotides of the same length that are unstructured or self-associated into inter-molecular structures. Figure 2c shows that, in a non-denaturing gel containing 50 mM KCl, 32R migrates as an intra-molecular G-quadruplex. The addition of five equivalents of TMPyP4 to the samples containing 32R in 50 or 140 mM KCl, gives rise to a new fast-moving species (band Q2). This suggests that the porphyrin stabilizes a new G-quadruplex conformation that migrates faster than Q1. The transformation of Q1 into Q2 is likely to require an intra-molecular rearrangement. That’s why Q2 is observed after a pre-incubation of 12 h at 37°C, but not at 4°C. As expected, when the PAGE experiment was carried out in 50 or 140 mM LiCl, with and without TMPyP4,
32R migrates as 32Y and 32deg. This indicates that 32R does not form G4-DNA structures in the lithium buffer (Figure 2d).

Fluorescence melting experiments show that two quadruplexes are formed by the G-rich strand of NHE

Tandem repeats of guanines can adopt a variety of intramolecular G-quadruplex structures, each characterized by a definite folding topology. To investigate the folding of the G-rich strand of NHE we used fluorescence spectroscopy and labelled the 5' and 3' ends of 32R with FAM and TAMRA, respectively. In LiCl (up to 100 mM), upon excitation at the FAM absorption band (475 nm), a fluorescence emission band is observed at 525 nm due to FAM. As fluorescence emission is not observed at 585 nm (TAMRA), there is no fluorescence resonance energy transfer (FRET) from FAM to TAMRA, in keeping with the fact that in lithium 32R does not form any structure (Figures 2 and 3a). When 32R is in the random-coiled conformation, the two fluorophores are expected to be at a...
distance of about 130 Å, which is higher than the Förster radius ($R_0 = 45–60$ Å) for efficient FRET (41). The quenching effect on FAM observed upon addition of lithium is probably due to the guanines nearby to the fluorophores (42). Replacing lithium with potassium, the fluorescence emission spectrum of the dual-labelled 32R sequence shows bands at both 525 and 585 nm (Figure 3b). This indicates that potassium induces the folding of 32R into a G-quadruplex structure in which the two dyes come within the Förster distance. Two observations can be made.

First, the fluorescence intensity of FAM decreases when potassium is increased, because of the energy transfer from FAM to TAMRA, due to quadruplex formation. Second, TAMRA emission increases with potassium. This is evident if one measures the intensity ratio $F_{585}/F_{525}$; it increases from 0.24 to 0.58 in 0–100 mM KCl, while it remains constant at ~0.24 in 0–100 mM LiCl. It has been suggested that the modest increase of TAMRA, which is generally observed, might be due to a decrease in molar absorptivity of the molecule and/or to a static quenching caused by interactions with the FAM moiety (43). Excitation spectra monitored at wavelength of TAMRA emission (585 nm) showed two bands that corresponded to TAMRA and FAM absorption maxima, supporting the presence of FRET when 32R is exposed to potassium (data not shown).

To determine the stability of the G-quadruplex we performed fluorescence melting experiments as previously described (44,45). Basically, upon fluorescein excitation at 475 nm one observes an increase in the emission at 525 nm between the low-temperature (folded/quenched) and high-temperature (unfolded/bright fluorescent) form. Figure 3c shows the first derivative of the fluorescence (dF/dT) versus T for dual-labelled 32R, after incubation in the buffer added with increasing amounts of KCl. Two clear transitions are observed in the KCl concentration range of 20–100 mM (at 10 mM KCl only one transition is observed). The $T_m$ of these transitions are reported in Table 2. The first transition occurs in the temperature range of 44–55 °C, while the second occurs in the range of 67–72 °C. As these transitions are only observed in the presence of potassium ions, they are due to the melting of two distinct G-quadruplex structures. On the other hand, the existence of two-folded conformations for 32R is not only suggested by the melting experiments but also by gel electrophoresis. Figure 2c showed that in the presence of five equivalents of TMPyP4, 32R migrates, after incubation at 37 °C, with two-folded fast-running compact conformations (Q1 and Q2). As TMPyP4 promotes the transformation of Q1 into Q2, we investigated the melting of 32R in the presence of TMPyP4. Figure 4a shows the melting curves of 32R in Tris-buffer containing 100 mM KCl and different amounts of TMPyP4 (TMPyP4/32R ratios of 0, 1, 3 and 5). In 100 mM KCl, 32R shows the typical biphasic profile due to the melting of quadruplexes Q1 and Q2, as previously observed. The addition of TMPyP4 induces the transformation of Q1 into Q2. At the TMPyP4/32R ratios of 3 or 5, 32R shows a monophasic

![Figure 3](image_url)

**Figure 3.** Fluorescence emission spectra (500–650 nm) of 300 nm dual-labelled 32R incubated at 37°C in 50 mM Tris–HCl, pH 7.4, at different LiCl (a) or KCl (b) concentrations (10, 20, 40, 60 and 100 mM, the arrow indicates the KCl(LiCl)-increment direction). Excitation wavelength was 475 nm; (c) First derivative fluorescence melting curves (dF/dT versus T) of 300 nm dual-labelled 32R in 50 mM Tris–HCl, pH 7.4, at different KCl concentrations (10, 20, 40, 60, 100 mM). Before measurements, the samples were incubated overnight in the appropriate buffer.

<table>
<thead>
<tr>
<th>KCl (mM)</th>
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$^a$Value uncertainty is ±1°C.
denaturing curve with a $T_m \sim 72^\circ C$ (Q$_2$). In conclusion, FRET data shows that the polypurine strand of NHE can potentially adopt one of the two possible G-quadruplex conformations, which are inter-convertible by increasing the temperature and/or adding TMPyP4.

It is interesting to observe that the CD of 32R in 100 mM KCl changes upon addition of five equivalents of TMPyP4, the intensity of the 260 nm ellipticity is reduced and a new positive ellipticity at 295 nm is generated (Figure 4b). This might suggest that the folding topology of quadruplex 32R changes from a parallel (46) to a mixed parallel/anti-parallel quadruplex (47). This finding is in accordance with previous data showing that, upon binding, TMPyP4 and Se2SAP promote the conversion of the G-quadruplex of CMYC from the parallel to the mixed parallel/anti-parallel conformation (48,49).

G-quadruplex structure of 32R: DMS footprinting and CD studies

To gain an insight in the folding of 32R we used chemical footprinting with DMS. After overnight incubation in 100 mM LiCl, 37°C, 32R does not show any footprinting (lane 2 and Figure 5a), suggesting that the sequence does not form any secondary structure, in accordance with
FRET and PAGE data. However, in 100 mM KCl, 37°C, 32R shows a clear and reproducible footprinting where 11 guanines out of 21 appear protected from DMS/piperidine cleavage (G2 is not seen in the gel). Although 32R contains four runs of guanines that could allow a straightforward fold into a parallel quadruplex with two external loops of 4 bases and one of 6 bases (Figure 5b, Q0), the footprinting data indicate that 32R folds into a G-quadruplex with a different folding topology. This is indicated by the fact that the central run of guanines G18–G20 is cleaved and the couple of guanines G6–G7 is protected from cleavage under a variety of conditions: 37°C or 50°C, 50 or 140 mM KCl, with or without 5 eq. of TMPyP4 (Figure 5a and lanes 3–8). Since 32R does not fold into a hairpin (this structure should have also been observed in Li-buffer), its DMS-footprintung, which is highly reproducible, is consistent with the formation of a parallel G-quadruplex with external loops of 1, 2 and 11 bases (Figure 5b, quadruplex Q1). Quadruplex Q1 is likely to have thymidine T8 directly involved in the formation of the G-tetrad core. On the other hand, mixed guanine/thymine tetrads have been previously observed by NMR and the structure of the putative GGGT tetrad is derived from that reported for GTGT (50) (Figure 6a). It is worth noting that thymidine has been reported to also form TTTT (51) and ATAT (52) tetrads. Compared to a canonical G-tetrad that is stabilized by eight hydrogen bonds, GGGT has one less H-bond. Alternatively, one cannot exclude that guanine G9 is involved in the formation of the G-tetrad. In this case, the run of guanines G6–G9 should contain T8 as a flipped-out base and the resulting G-quadruplex should have two one-base double-chain reversal loops. This possibility, however, is not supported by the fact that G9 is reactive to DMS. One could argue that T8, being an intervening base between G7 and G9, might cause a backbone distortion in the neighbourhood of G9, which weakens the hydrogen bond involving N7 of G9. Another aspect that is worth noting is that a close inspection of the autoradiographies showed that G25 and G28 appear always partially protected (lane 10). This may result from the fact that two loop isomers of Q1 may exist in solution: one involving the run

(a) Structures of the putative GGGT and GGGG tetrads; (b) CD spectra of 32R mutant sequences in 50 mM Tris-HCl, pH 7.4, 100 mM KCl. Oligonucleotide concentration was 3 μM, pathlength cell 0.5 cm, ellipticity expressed in millidegrees.
of guanines G25-G26-G27, the other the run G26-G27-G28. The preference of forming Q1 with 1/1/11 or 1/2/11 loops instead of Q0 with 4/4/6 loops can be rationalized by the finding that single-nucleotide loops stabilize quadruplex DNA more than larger loops (53).

To obtain further evidence that 32R in 100 mM KCl folds into Q1, we analysed the CD of a number of 32R mutant sequences (Figure 6b). It can be seen that: (i) when the run of guanines G18–G20, which is supposed to be part of the 11-base loop, is replaced with T's (mutant 32R-3L), the CD does not change; (ii) when G9, G16 and G23, which should be located in the external loops, are replaced with T's (32R-3T), the CD does not change either; (iii) when G28 and G29, located in the penta-guanine stretch near the 3' end of the sequence, are replaced with T's (32R-2T), the CD does not appreciably change; (iv) when G2, G12, G19 and G26, bases located in the runs of guanines (32R-4T), are replaced with T's, the 260-nm ellipticity is dramatically reduced, indicating that the mutant does not assume any secondary structure. Together, these CD spectra support the structure assigned to Q1.

As shown by fluorescence-melting and PAGE, 32R in the presence of TMPyP4 adopts a more stable quadruplex conformation with $T_m = 728$ C (Q2). The CD of 32R obtained in the presence of TMPyP4 suggests that Q2 should have a structure with both parallel (260 nm ellipticity) and anti-parallel (295 nm ellipticity) characters. We propose for Q2 a mixed parallel/anti-parallel topology with one-base double-chain reversal loop and two lateral loops of 2 and 11 bases (Figure 5b). Since TMPyP4 is a porphyrin that upon irradiation generates reactive oxygen species that cleave DNA in the neighbourhood of the binding site, we performed photocleavage experiments with 32R. We observed that the bases that are strongly photocleaved are located in the lateral 11-base (G18, G19 and G23) and 2-base (G9) loops (Figure 5a, lanes 11–13). This is consistent with the finding that the porphyrin preferentially binds to the loops of intramolecular quadruplexes (54). But, the fact that G25 and slightly also G11 and G6 are photocleaved supports the notion that TMPyP4 binds also to the external G-tetrads (36,37). This photocleavage pattern is substantially in accordance with the folding proposed for quadruplex Q2.

The binding of nuclear proteins to duplex NHE is competed by 32R in the G-quadruplex form

NHE is a regulatory element of the KRAS promoter that is essential for transcription (16–18). When dsNHE is incubated with a pancreatic adenocarcinoma (Panc-1) nuclear extract, three DNA–protein complexes, namely C1, C2 and C3, are observed by EMSA (Figure 7a,

![Figure 7. Competition binding experiments.](image-url)
In order to see if the proteins that bind to dsNHE also recognize the G-rich strand (32R), folded in its G-quadruplex conformation, we performed competition experiments. We found that 32R competed efficiently the DNA–protein complex C1. The addition of 50-fold excess of 32R inhibits the formation of complex C1 by >50%, 100-fold excess practically abrogates complex C1. In contrast, mutant sequences 32R-4C, 32R-8C and 32R-12C (Table 1), which do not fold into a quadruplex structure (according to CD), did not affect the formation of C1 at all, suggesting that 32R competes with dsNHE for protein binding through its secondary structure. It is interesting to note that the competition of C1 results in an increase of C3, suggesting that C3 is probably a partially assembled form of the DNA–protein complex C1. We also observed that complex C1 is competed, though less efficiently than 32R, by the CMYC quadruplex-forming sequence (Table 1), which is known to form a parallel quadruplex (39), but it is not competed by the pyrimidine strand of NHE (32Y)(Supplementary Data S1). Direct evidence that the G-quadruplex formed by 32R binds to proteins from Panc-1 nuclear extract was obtained by EMSA (Figure 7b). Radiolabelled 32R, pre-equilibrated in 100 mM KCl and successively incubated with the nuclear extract for 30 min, formed three distinct DNA–protein complexes (B1, B2 and B3). As complex B1 is partially competed by mutants 32R-4T, 32R-8C, 32R-12C (Table 1) and dsNHE, it is due to a protein binding nonspecifically to DNA. Complexes B2 and B3 are not competed by the mutant sequences, but are strongly competed by dsNHE. This suggests that B1 and B2 contain proteins that recognize both quadruplex and double-stranded conformations of NHE.

Identification of nuclear proteins binding to the G-rich strand of NHE

We used streptavidin–biotin paramagnetic beads to pull down from Panc-1 nuclear extract the proteins binding to dsNHE under physiological conditions: in 20 mM Tris–HCl pH 8, 1.5 mM MgCl2, 2.5 ng/μl poly [dI-dC], 150 mM KCl, 37°C. After binding, the proteins were eluted from the beads in three steps with Tris-buffers containing increasing concentrations of NaCl (0.25, 0.5 and 0.75 M). The fractions obtained were desalted and analysed by SDS–PAGE/silver staining. Three polypeptides co-eluted in the high salt fraction: p114, p86 and p70 (Figure 8a, lanes 5–7). In parallel, the nuclear extract was applied to magnetic beads coupled to 32R in the G-quadruplex conformation. Again, the bound proteins were eluted in three steps and the fractions analysed by SDS–PAGE (lanes 2–4). In this case, the polypeptides co-eluting in the high salt fraction are p114, p70 and p34. When the pulled down experiment was performed with the beads coupled with a scramble oligonucleotide (B-scram, Table 1), these specific bands in SDS–PAGE were not obtained (data not shown). The bands were excised from the gel and the proteins identified by mass spectrometry analysis; (b, left) Southwestern blot showing that radiolabelled 32R-quadruplex, but not mutant 32R-4C, recognizes 34 and 70 kDa proteins (hnRNP A1 and Ku70) present in the extract; (b, right) Southwestern blot showing that radiolabelled 32R recognizes bands at 114 and 70 kDa (PARP-1 and Ku70) present in the 0.75 M NaCl pull-down fraction (lane 4); as a control it is shown that 32R recognizes recombinant PARP-1 (rPARP-1).

![Figure 8.](image-url)
two proteins associated with NHE of KRAS (PARP-1 and Ku70) have affinity for both the folded and duplex conformation of regulatory NHE and may provide a rationale for the competition experiments illustrated in Figure 8. It is worth noting that the three proteins binding to quadruplex 32R have been previously found to recognize unusual DNA structures (see Discussion section). The protein that is likely to have an important function in the regulation of KRAS transcription is hnRNPA1, as it possesses unfolding capacity against G4-DNA (55). The binding of these proteins to quadruplex 32R was also observed by Southwestern blots. The nuclear extract was run on SDS–PAGE, electroblotted onto nitrocellulose and the proteins renatured in HEPES buffer (see Materials and methods section). The membrane was treated with radiolabelled 32R or 32R-4C (separate experiments). As expected, 32R but not mutant 32R-4C recognized bands at ~34 and 70 kDa (hnRNPA1, Ku70). As for PARP-1, its binding to 32R was difficult to detect, probably because this large protein did not completely renature after SDS–PAGE. However, when the 0.75 M NaCl fraction of the pull-down was electroblotted (lane 4 of Figure 8a) a clear band around 113 kDa was obtained, as expected. As a control, we used recombinant PARP-1 (rPARP-1).

**Effect of cationic porphyrins (TMPyP2, TMPyP3 and TMPyP4) on KRAS transcription**

Considering that the G-rich sequence of NHE can form stable G-quadruplex structures, we asked if these unusual DNA structures have any effect on transcription of genomic KRAS in pancreatic adenocarcinoma cells. To this purpose we treated Panc-1 cells with cationic porphyrins that are known to stabilize G4-DNA: TMPyP3 (more specific for parallel inter-molecular quadruplexes) and TMPyP4 (more specific for folded quadruplexes) (56). As a control molecule of interest, we used TMPyP2, which isomers TMPyP2, TMPyP3 and TMPyP4, in which the on the pyridyl ring is either in the ortho or meta or para positions, relative to its connection to the porphine core, are shown.

![Figure 9. Real-time PCR determination of the level of endogenous KRAS mRNA in Panc-1 cells untreated or treated for 12 and 24 h with 50 μM of TMPyP2, TMPyP3 and TMPyP4. The ordinate reports the percent residual KRAS mRNA T/C100, where T is (KRAS mRNA/GAPDH mRNA) in treated cells and C is (KRAS mRNA/GAPDH mRNA t untreated cells). The structures of the three positional isomers TMPyP2, TMPyP3 and TMPyP4, in which the N-methyl group on the pyridyl ring is either in the ortho or meta or para positions, relative to its connection to the porphine core, are shown.

TMPyP4 on human KRAS transcription is evaluated at genomic level; the repression reported is lower probably because the porphyrins do not localize efficiently into the nucleus of the cells (57). Finally, the fact that TMPyP2, the porphyrin that cannot stabilize G4-DNA, does not reduce KRAS mRNA, suggests that the bioactivity of TMPyP4 is due to specific binding to the G-quadruplex extruded by the regulatory element and not to a non-specific strand breakage.

**DISCUSSION**

In this study, we demonstrate that a nuclease hypersensitive element of the KRAS promoter, located upstream of the major transcription initiation site, is structurally polymorphic. Primer-extension experiments showed that potassium and TMPyP4 induce the arrest of DNA polymerase I
factor 1-

At 37

G-quadruplex structures, namely Q 1 and Q 2. When

a double-to-single strand transition (64). In vitro data indicate that Ku70 binds to and stabilize the tetrahelical form of the Fragile X syndrome d(CGG) n expanded sequence (65). However, the protein associated with

binding to NHE, which is particularly interesting in the context of

Kras transcription is hnRNP A1, as its N-terminus proteolitic portion (residues 1–195), referred to UP1, shows the ability to resolve G4-DNA. Recombinant UP1 has been demonstrated to have G-tetrad destabilizing activity against intra-molecular G-quadruplexes formed by the

minisatellite (MN)Pc-1 tandem repeat d(GGCAG) n (55) and by the telomeric d(TAGGGT) q repeat (66). It is not yet known how hnRNP A1 and UP1 resolve G4 DNA, but like the proteins active at the telomeres, UP1 should trap single-stranded DNA and shift the single-stranded ⇐ quadruplex equilibrium away from the quadruplex (54). As hnRNP A1 and PARP-1 have been reported to interact with each other, it could be that PARP-1 rybosylates hnRNP A1 and modifies the functional properties of the protein in the context of gene regulation (67).

In the light of these data, we asked how a folded secondary structure formed within NHE might be involved in transcription regulation. The Kras promoter at NHE may undergo, in the presence of superhelical stress, a duplex-to-quadruplex equilibrium. When NHE is in the folded G-quadruplex form, transcription should be inhibited, in accordance with the fact that the stabilization of this structure by TMPyP4 results in a transcription
repression (21, 23, 27). It is possible that the G-quadruplex of NHE is stabilized by Ku70 (65). The complementary unpaired pyrimidine strand of NHE should be bound by single-stranded binding protein hnRNP K (68). To activate transcription, the G-quadruplex of NHE should be unfolded and this could involve hnRNP A1, because of its capacity to resolve intra-molecular G4-DNA (55, 66). Once the promoter assumes a double-stranded conformation, a transcriptional active DNA–protein complex is rapidly assembled. In this scenario, G-quadruplex DNA behaves as a transcription repressor. The model proposed for the control of KRAS transcription suggests several strategies to down-regulate KRAS in tumour tissues: (i) use of planar macrocycles capable to stabilize G4-DNA and lock the promoter into the folded non-active form (69); (ii) use of triplex-forming oligonucleotides to block the formation of DNA–protein complexes at NHE (70); (iii) use of decoy oligonucleotides against proteins binding to NHE (hnRNP A1/Ku70/PARP-1 and others).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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