

# Protein hnRNP A1 and its derivative Up1 unfold quadruplex DNA in the human *KRAS* promoter: implications for transcription

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## ABSTRACT

The promoter of the human *KRAS* proto-oncogene contains a structurally polymorphic nuclease hypersensitive element (NHE) whose purine strand forms a parallel G-quadruplex structure (called 32R). In a previous work we reported that quadruplex 32R is recognized by three nuclear proteins: PARP-1, Ku70 and hnRNP A1. In this study we describe the interaction of recombinant hnRNP A1 (A1) and its derivative Up1 with the *KRAS* G-quadruplex. Mobility-shift experiments show that A1/Up1 binds specifically, and also with a high affinity, to quadruplex 32R, while CD demonstrates that the proteins strongly reduce the intensity of the 260 nm-ellipticity—the hallmark for parallel G4-DNA—and unfold the G-quadruplex. Fluorescence resonance energy transfer melting experiments reveal that A1/Up1 completely abrogates the cooperative quadruplex-to-ssDNA transition that characterizes the *KRAS* quadruplex and facilitates the association between quadruplex 32R and its complementary polypyrimidine strand. When quadruplex 32R is stabilized by TMPyP4, A1/Up1 brings about only a partial destabilization of the G4-DNA structure. The possible role played by hnRNP A1 in the mechanism of *KRAS* transcription is discussed.

## INTRODUCTION

The mammalian *KRAS* gene encodes for a guanine nucleotide-binding protein of 21 kDa that activates several cellular pathways controlling important events such as proliferation, differentiation and signalling (1). The Ras proteins behave as a molecular switch cycling between

inactive GDP-bound and active GTP-bound states. The state of nucleotide occupancy is regulated by specific proteins named guanine nucleotides exchange factors (GEFs) and GTPases activating proteins (GAPs) (1,2). The *RAS* genes are frequently mutated in solid and haematological neoplasias with single point mutations at exons 12, 13 and 61 (3,4). The most common mutated *RAS* gene in solid tumours is *KRAS*, with a 90% incidence in pancreatic adenocarcinomas (5,6). As the mutated Kras protein has a defective GTPase activity, it is not inactivated by GAPs (7). It remains locked into the GTP-bound active state which continuously transmits to the nucleus mitotic signals that contribute to the neoplastic phenotypes in cancer cells (8–10). As pancreatic adenocarcinomas are refractory to conventional treatments, the discovery of new drugs capable to sensitize tumour cells to chemotherapy is being pursued in many laboratories. In our laboratory, we focused on *KRAS* and in order to design anti-*KRAS* drugs we investigated how the transcription of this proto-oncogene is controlled. Previous studies have shown that a nuclease hypersensitive element (NHE), located in the *KRAS* promoter upstream of the transcription start between –327 and –296, is responsible for most of the transcription activity (11). Earlier we reported that the purine strand of NHE is structurally polymorphic, as its tract of sequence recognized by nuclear proteins is able to fold into stable G-quadruplex structures (12,13). Using the purine strand of NHE (called 32R) in quadruplex conformation as a bait, we pulled down from a pancreatic nuclear extract three proteins with affinity for the *KRAS* quadruplex. By SDS-PAGE and mass spectrometry, we identified these proteins as poly[ADP-ribose] polymerase 1 (PARP-1), ATP-dependent DNA helicase 2, subunit 1 (Ku70) and heterogeneous ribonucleoprotein A1 (hnRNP A1) (13). Protein hnRNP A1 (from now on A1) is a member of the heterogeneous ribonucleoprotein family, which is highly abundant in the nucleus of actively

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growing mammalian cells (14,15). All members of the hnRNP family are characterized by two highly conserved RNA recognition motifs (RRMs) at the *N*-terminus and by a glycine-rich domain at the *C*-terminus (16,17). Although a recent structure of a co-crystal of Up1 (a proteolytic portion of A1 retaining binding activity) bound to the telomeric repeat (TTAGGG)<sub>2</sub> suggests that both RRM1s interact with DNA (18), it has been reported that only one motif (RRM1) is sufficient for strong and specific binding to single-stranded telomeric DNA (19) and that its sub-element RNP11 mediates destabilization of quadruplex (CGG)<sub>n</sub> (20). Proteins hnRNP play various roles in mRNA metabolism (14,15) and in the biogenesis of telomeres (21). As protein A1 (and its derivative Up1) was reported to have a telomere-lengthening effect in erythroleukemia cells (21,22), it is suspected to function as an auxiliary factor of the telomerase holoenzyme (23). Considering that the 3' G-rich repeats of the telomeres are folded in stable G-quadruplex structures, it has been hypothesized that A1 stimulates telomere elongation by disrupting high-order structures formed by the telomere repeats. Indeed, Up1 was reported to destabilize the bimolecular quadruplex formed by human telomere repeats d(TTAGGGTTAGGG), d(TTAGGG)<sub>4</sub> and the intramolecular quadruplex of d(GGCAG)<sub>5</sub> (23–25).

Since we discovered that A1 is associated to the *KRAS* promoter, in this study we have investigated the interaction between recombinant A1/Up1 and the *KRAS* G-quadruplex. Electrophoretic mobility shift assay (EMSA) showed that A1/Up1 binds to the *KRAS* quadruplex with high affinity and specificity, while CD and fluorescence resonance energy transfer (FRET) experiments revealed that A1/Up1 destabilizes this non B-DNA structure of the *KRAS* promoter. The results of our study support a transcription mechanism in which A1 should function as a G-quadruplex destabilizing protein, as it seems to occur in the G-rich 3' overhang strand of the telomeres (23). In conclusion, this study sheds some light on the mechanism of *KRAS* transcription regulation and may be useful for the rationale design of anticancer drugs specific for oncogenic *KRAS*.

## MATERIALS AND METHODS

### DNA and proteins hnRNP A1/Up1

The oligonucleotides used in this study (Table 1) were obtained from MWG (Germany) and Microsynth (Switzerland). They have been purified by 20% PAGE (acrylamide: bisacrylamide, 19:1) in TBE, under denaturing conditions (7 M urea, 55°C). The bands were excised from the gel and eluted in water. The DNA solutions were filtered (Ultrafree-DA, Millipore) and precipitated. DNA concentration was determined from the absorbance at 260 nm of the oligonucleotides diluted in milli Q water, using as extinction coefficients 7500, 8500, 15000 and 12500 M<sup>-1</sup> cm<sup>-1</sup> for C, T, A and G, respectively. Dual-labelled F-32R-T (5' end with FAM, 3' end with TAMRA) were HPLC-purified.

Recombinant proteins Up1 and A1 tagged to GST were expressed in *Escherichia coli* BL21 using plasmids

pGEX-Up1 and pGEX-hnRNP A1. After transformation, the bacteria were grown for 2 h at 37°C with 50 µg/ml ampicillin to an A<sub>600</sub> of 0.5–2.0 prior to induction with IPTG (100 µM final concentration). Cells were allowed to grow for 7 h before harvesting. The cells were centrifuged at 5000 r.p.m., 4°C. After centrifugation the supernatant was removed carefully and the cells washed twice with PBS. The pellet was re-suspended in a solution of PBS with PMSF 100 mM and DTT 1 M. The bacteria were lysed by sonication, added with Triton X-100 (1% final concentration) and incubated for 30 min on a shaker at room temperature. The lysate was then centrifuged for 10 min at 4°C at 10000 r.p.m. Glutathione Sepharose 4B (GE Healthcare) (50% slurry in PBS) was added to the supernatant and incubated for 30 min at 4°C on a shaker. The mix was centrifuged for 5 min at 500g and the pellet was washed 5 times in PBS and eluted with elution buffer containing 20 mM NaCl, 20 mM reduced glutathione, 200 mM Tris-HCl, pH 9.5 for A1 elution and pH 7.5 for Up1 elution. Alternatively, to remove the GST tag, the mix was centrifuged for 5 min at 500g, washed with PreScission Cleavage buffer (GE Healthcare) and centrifuged 5 min at 500g. The pellet was incubated for 4 h at 4°C with PreScission protease to cleave the GST tag from the purified proteins. After PreScission cleavage, the A1 or Up1 moieties were detached from GST which remained bound to the Glutathione Sephadex beads. The reaction mixtures were centrifuged for 5 min at 500g, 4°C, and the untagged proteins collected from the supernatant. Finally, the purification of tagged and untagged Up1 and A1 proteins were checked by SDS-PAGE.

### CD and fluorescence experiments

CD spectra have been obtained with a JASCO J-600 spectropolarimeter equipped with a thermostatted cell holder. CD experiments were carried out with oligonucleotides (3 µM) in 50 mM Tris-HCl, 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) and are reported as ellipticity (mdeg) versus wavelength (nm). Each spectrum was recorded three times, smoothed and subtracted to the baseline.

Fluorescence measurements were carried out with a Microplate Spectrofluorometer System (Molecular Devices) using a 96-well black plate, in which each well contained 50 µl of 200 nM dual-labelled F-32R-T in 50 mM Tris-HCl, pH 7.4 and KCl as specified in the figure captions. Before adding the protein, the samples were incubated for 24 h at room temperature in the specified buffer. The protein (Up1, A1 or BSA) was added 30 min before fluorescence analysis. The emission spectra were obtained by setting the excitation wavelength at 475 nm, the cut-off at 515 nm and recording the emission from 500 to 650 nm. Upon addition of KCl, F-32R-T assumes a folded quadruplex conformation and FRET is expected between the 5' and 3' fluorophores. The emission

intensity of the donor (FAM) decreases while the intensity of the acceptor increases, correspondingly, as  $K^+$  is added to the sample solution. The energy transfer from the donor to the acceptor and vice versa can be empirically represented by the parameter  $P$ :

$$P = \frac{I_D}{(I_D + I_A)}$$

where  $I_D$  and  $I_A$  are the intensities of the donor and acceptor (26,27). Fluorescence melting experiments were performed on a real-time PCR machine (iQ5, BioRad), using a 96-well plate filled with 50  $\mu$ l solutions of dual-labelled F-32R-T. The protocol used for the melting experiments is the following: (i) equilibration step of 5 min at low temperature (15°C); (ii) stepwise increase of the temperature of 1°C per min for 76 cycles to reach 95°C. All samples in the wells were melted in 76 min.

Kinetic experiments were carried out using the iQ5 real-time machine. Oligonucleotide F-32R-T (200 nM) in 100 mM KCl, i.e. in the quadruplex conformation, was mixed with the complementary 32Y strand and the increase at 525 nm of the fluorescence was measured as a function of time. The experiment was also performed adding to F-32R-T a mixture containing 32Y (8-fold) and Up1 (400 nM). The increase of fluorescence  $\Delta F = F - F_0$ , where  $F_0$  and  $F$  is the fluorescence at 525 nm (FAM) at  $t = 0$  and at any time  $t$ , was best-fitted to a single or double-exponential curve. The half-life of the reaction is given by  $t_{1/2} = 0.693/k$ .

## EMSA

Oligonucleotides 32R, *HRAS-1*, *HRAS-2*, *CMYC*, *CKIT*, *VEGF*, 32Y, Gmut1 and Gmut2 were end-labelled with [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase. Duplex dsNHE was prepared annealing (10 min at 95°C, overnight at room temperature) a mixture containing equimolar amounts of radiolabelled 32R and complementary 32Y in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. Before EMSA, the quadruplex-forming oligonucleotides were allowed to form their structure in 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 37°C (overnight incubation). Radiolabelled oligonucleotides (35 nM) were treated for 30 min at room temperature with different amounts of A1/Up1, ( $r$  ([protein]/[oligonucleotide]) ratios are specified in Figure 3) in 20 mM Tris-HCl, pH 8, 30 mM KCl, 1.5 mM  $MgCl_2$ , 1 mM DTT, 8% glycerol, 1% Phosphatase Inhibitor Cocktail I (Sigma, Milan, Italy), 5 mM NaF, 1 mM  $Na_3VO_4$ , 2.5 ng/ml poly [dI-dC]. After incubation, the reaction mixtures were loaded in 8% TBE (1 $\times$ ) polyacrylamide gel, thermostatted at 16°C. After running the gel was dried and exposed to autoradiography (G E Healthcare, Milan) for 24–36 h at –80°C.

## Polymerase-stop assay

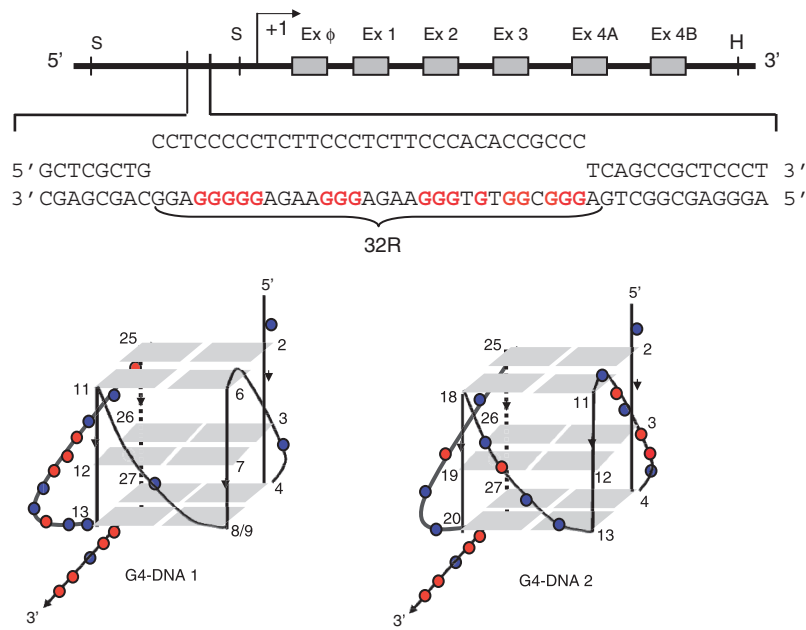
A linear DNA fragment of 87 nt, containing the G-rich element of NHE, was used as a template for Taq polymerase primer-extension reactions. This DNA sequence was purified by PAGE. The template (100 nM) was mixed with the labelled primer (50 nM) in 100 mM KCl, Taq buffer

1 $\times$  and overnight incubated at 50°C. The primer extension reactions were carried out for 1h, by adding 10 mM DTT, 100  $\mu$ M dATP, dGTP, dTTP, dCTP and 3.75 U of Taq polymerase (Euro Taq, Euroclone, Milan). The reactions were stopped by adding an equal volume of stop buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol, 0.1% bromophenol blue). The products were separated on a 15% polyacrylamide sequencing gel prepared in TBE, 8 M urea. The gel was dried and exposed to autoradiography. Standard dideoxy sequencing reactions were performed to detect the points in which DNA polymerase I was arrested.

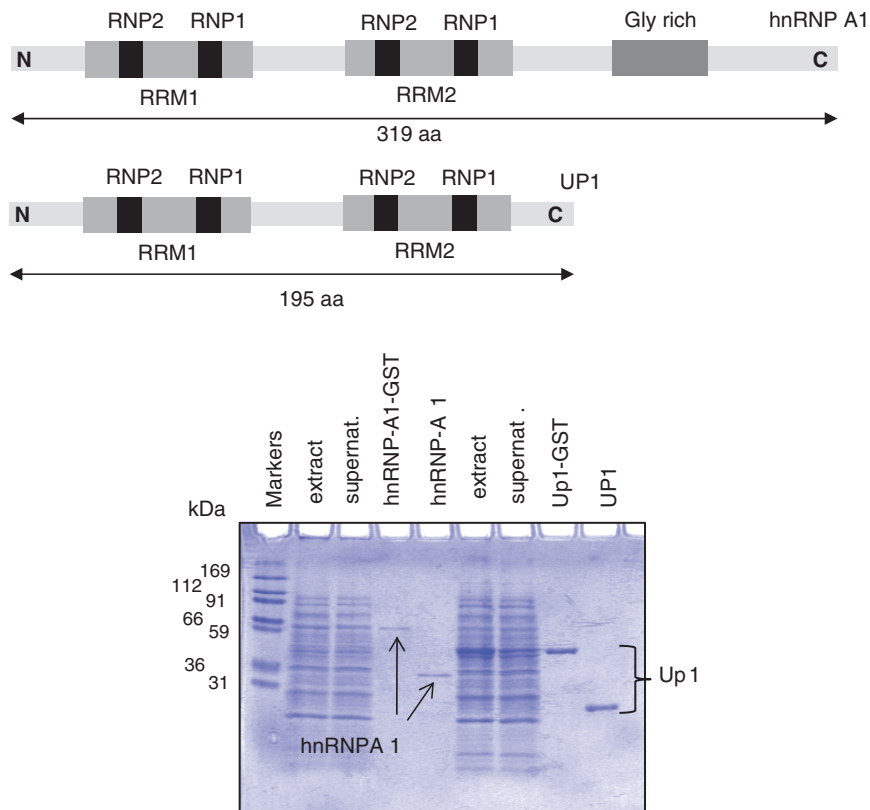
## RESULTS

We previously demonstrated that the G-rich strand of NHE can form G-quadruplex structures (13,28). By means of CD and DMS-footprinting experiments we found that the G-tract called 32R forms a parallel G-quadruplex characterized by three G-tetrads ( $T_m$  of 70°C in 100 mM KCl) (Figure 1). Pull-down assays with a pancreatic nuclear extract combined to mass spectrometry showed that quadruplex 32R binds to three proteins: PARP-1 (116 kDa), Ku70 (72 kDa) and A1 (34 kDa) (13). Since A1 is involved in the biogenesis of the telomeres as a G4-DNA destabilizing protein (23) and is able to disrupt the secondary structures of the hypervariable minisatellite sequence d(GGCAG)<sub>5</sub> (24), we asked whether A1/Up1 can have a similar functional role in the human *KRAS* promoter. To address this question, recombinant A1 and its derivative Up1 were expressed in *Escherichia coli* as proteins fused to GST and purified by affinity chromatography with glutathione sepharose 4B. The GST moiety was removed with a pre-scission protease and recombinant tagged and untagged proteins were obtained with a high purity level (Figure 2). Up1 is a proteolytic fragment (195 aa) of A1 (319 aa) that retains the two RNA-recognition motifs (RRMs) responsible for binding to nucleic acids (18,22).

The interaction between A1/Up1 and a variety of DNA substrates, some of which were structured in G4-DNA and some not, was analysed by EMSA.  $^{32}$ P-labelled 32R (35 nM) was first incubated for 24 h in 100 mM KCl to allow quadruplex formation, then incubated for 30 min with increasing amounts of Up1 or A1:  $r$  ([protein]/[32R]) = 0, 0.5, 1, 2, 5, 10, 20, 50, 100. As preliminary experiments showed that GST-tagged and untagged proteins behave in the same way, we performed EMSA with the tagged proteins. Figure 3a and b shows that quadruplex 32R forms with A1/Up1 a DNA-protein complex that, being detected even at  $r = 0.5$ , should have a 1:1 stoichiometry. In addition, for  $r > 20$ , another slow-migrating DNA-protein complex appears in the gel, most likely due to a 1:2 complex. When  $r$  was increased to 200 and the samples run in a longer gel, 32R migrated essentially as 1:2 complex (Figure 3c). The formation of two DNA-protein complexes by A1/Up1 is in keeping with the results of Zhang *et al.* (23) and the crystal structure of d(TTAGGG)<sub>2</sub> bound to Up1 (18). Since a tract of 12 nt functions as a minimum binding unit, 32R has

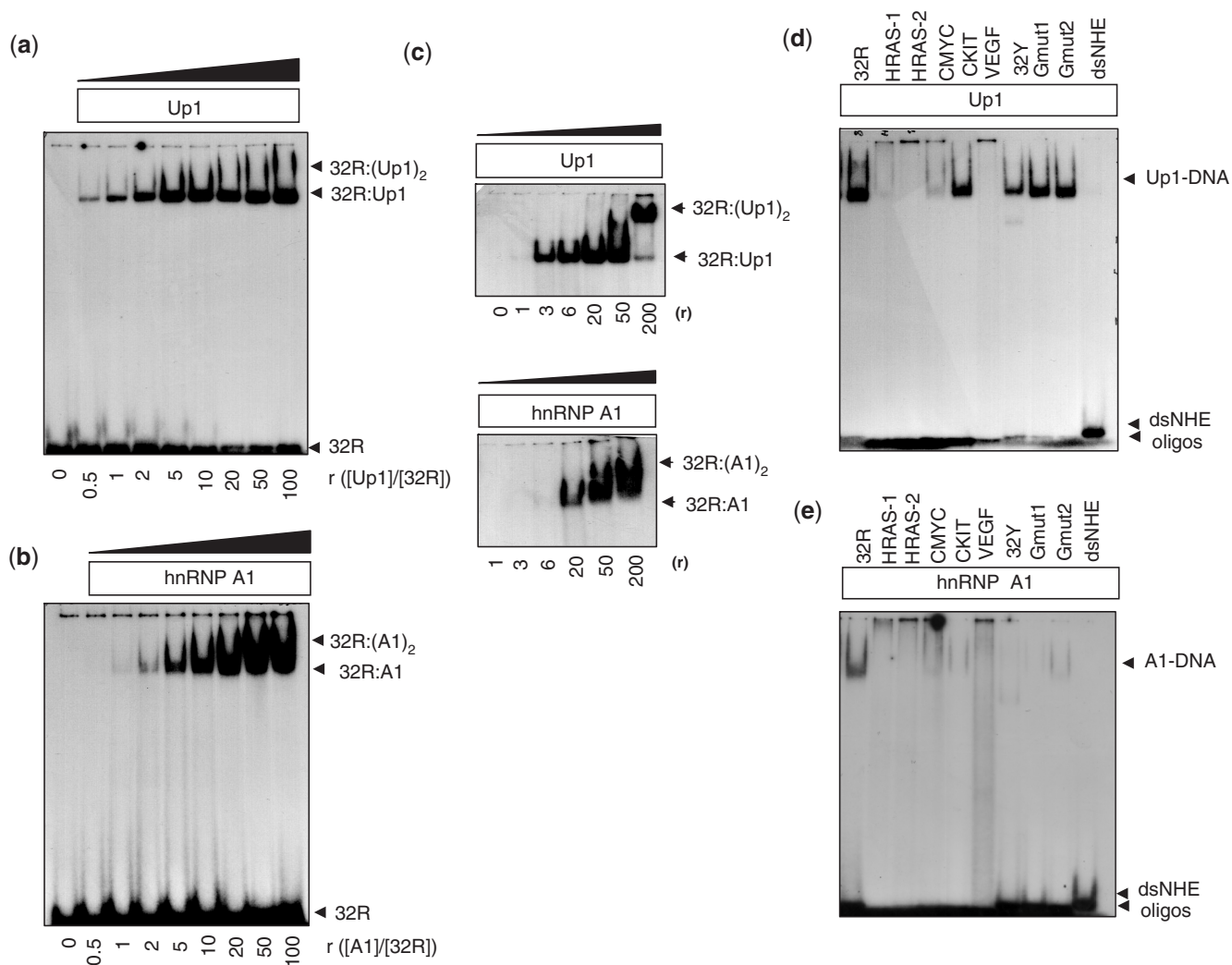


**Figure 1.** Sequence of the nuclease hypersensitive element (NHE) in the human *KRAS* promoter. The G-rich sequence 32R forms a G-quadruplex whose putative structure, consistent with CD and dimethyl sulfate footprinting, is G4-DNA1, which is characterized either by a flipped-out thymidine connecting G7 to G9 or a GGGT triad (13). The expected G4-DNA2 structure is not supported by dimethyl sulfate footprinting. The nucleotides of 32R (Table 1) are numbered from the 5'-end.



**Figure 2.** Schematic representations of proteins hnRNP A1 and Up1. The two RNA-recognition motifs (RRMs), that mediate ssDNA binding, contain each two conserved RNP2 and RNP1 submotifs. Up1 encompasses the amino-terminal two-third of the hnRNPA1 sequence. SDS-PAGE of GST-tagged and untagged hnRNP A1 and Up1, after glutathione sepharose 4B purification. Lane 1, protein markers; lane 2, total extract (hnRNP A1); lane 3, supernatant; lane 4, purified GST-tagged hnRNP A1; lane 5, purified untagged hnRNP A1; lane 6, total extract (Up1); lane 7, supernatant; lane 8, purified GST-tagged Up1; lane 9, purified untagged Up1.





**Figure 3.** (a, b) EMSA of 35 nM <sup>33</sup>P-labelled quadruplex 32R after 30 min incubation with increasing amounts of Up1 or A1 at the specified *r* values, in 20 mM Tris-HCl, pH 8, 30 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 8% glycerol, 1% Phosphatase Inhibitor Cocktail I (Sigma), 5 nM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2.5 ng/μl poly dI-dC, for 25°C. The analyses were carried out in 8% polyacrylamide gel (29:1) in TBE (1×) at 16°C. Before the EMSA, 32R was incubated overnight in 100 mM KCl to get it in the G-quadruplex conformation; (c) EMSA as in (a, b) but with *r* values up to 200; (d, e) EMSA of A1/Up1 mixed to various DNA substrates [G-quadruplexes 32R, *HRAS*-1, *HRAS*-2, *CMYC*, *CKIT*, *VEGF*, dsNHE (32R:32Y) and unstructured oligonucleotides Gmut1, Gmut2, 32Y]. PAGE carried out in 8% polyacrylamide gel (29:1) in TBE (1×) at 16°C.

potentially two binding sites, which can in principle form two DNA-protein complexes by binding one or two protein molecules. By quantifying the intensity of the electrophoretic bands, we roughly estimated that the dissociation constant  $K_d$  of the 1:1 complex is about 50 nM for Up1 and 200 nM for A1. We also tested the binding specificity of A1/Up1 for a variety of well known G-quadruplex structures obtained from *CMYC*, *CKIT*, *VEGF* and *HRAS* promoter sequences (29–32) (for *HRAS* quadruplexes, see Supplementary Data S1) (Figure 3d and e). The various DNA substrates have been <sup>33</sup>P-labelled and treated with an excess of protein ( $r = 50$ ). It can be seen that A1 shows good specificity for the *KRAS* quadruplex, as it does not bind to the other quadruplex-forming sequences, unstructured oligonucleotides Gmut1, Gmut2, 32Y (the complementary polypyrimidinic NHE strand) and dsNHE (32R:32Y) (Table 1). Instead, protein Up1, besides quadruplex 32R,

shows affinity also for the *CKIT* quadruplex and unstructured oligonucleotides.

To analyse the effect of A1/Up1 on the *KRAS* G-quadruplex, we could not employ electrophoresis because the mobility between an *intramolecular* quadruplex and its unfolded form is not very different. Therefore, we used spectroscopic techniques such as circular dichroism (CD) and FRET. Figure 4 shows that in 100 mM KCl, 32R is characterized by a CD signature typical of a parallel G-quadruplex: a strong and positive ellipticity at 260 nm and a weak and negative ellipticity at 240 nm (33). When quadruplex 32R is denatured by increasing the temperature, the positive 260 nm band is dramatically reduced and its spectrum becomes similar to that of unstructured oligonucleotides (data not shown). Thus, the structural transition from quadruplex-to-ssDNA is accompanied by a strong reduction of the 260 nm ellipticity. A similar transition was obtained by adding to

quadruplex 32R increasing amounts of A1/Up1 ( $r = 1, 2, 4, 6$ ). It can be seen that the protein causes a progressive reduction of the 260 nm ellipticity, indicating that the G4-DNA structure is unfolded by the protein. As a control, we treated quadruplex 32R with an unrelated protein, the trypsinogen inhibitor, and found that the 260 nm ellipticity was not affected and remained constant at all protein concentrations used. The CD spectra of Up1 at increasing concentrations show that the protein between 240 and 320 nm does not have any negative band, but below 240 nm it shows a negative band typical of the polypeptide

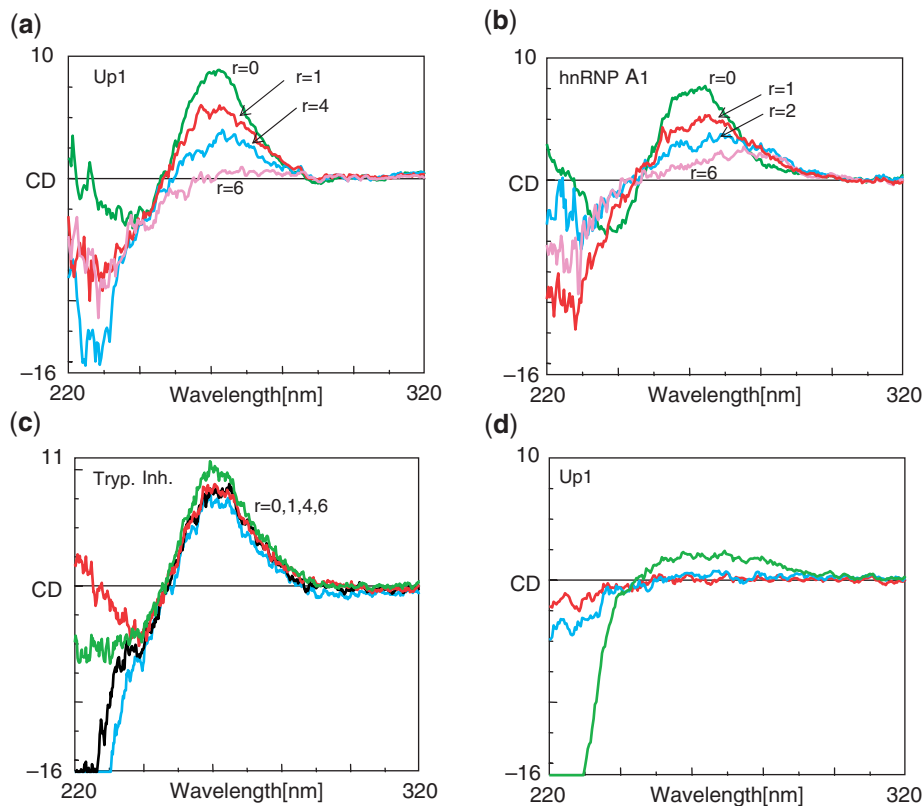
**Table 1.** Oligonucleotides (5' > 3') used in this study

AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG	32R
F-AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG-T	F-32R-T
AGGGAGGGCGCTGGGAGGAGGG	CKIT
GGGCGGGCCGGGGCGGGTCCCAGCGGGG	VEGF
TGGGAGGGTGGGAGGGTGGGGAAGG	CMYC
TCGGTTGCGGGCGCAGGGCACGGGCG	HRAS-1
CGGGCGGGGCGGGGCGGGGGCG	HRAS-2
GCGGTGTGTGAAGAGTGAAGAGTGGGATGCAG	Gmut1
GCATTCTGATTACAGTATTACCTTCACTCCA	Gmut2
CCTCCCCTCTTCCCTCTTCCCACACCGCCCT	32Y
GTACTACACTTGATA	primer
ACCTTGATGAATCCAGGGCGGTGTGGGAAGAG	template
GGAAGAGGGGGAGGAATCGCTACCGTTAAGCA	
TCGATCATATCAAGTGATAGTAC	

F: FAM; T: TAMRA.

backbone. The CD data showing G-quadruplex unfolding are in keeping with those previously obtained with the telomeric TTAGGG repeat (24,25) and the hypervariable minisatellite sequence d(GGCAG)<sub>5</sub> treated with Up1 (24).

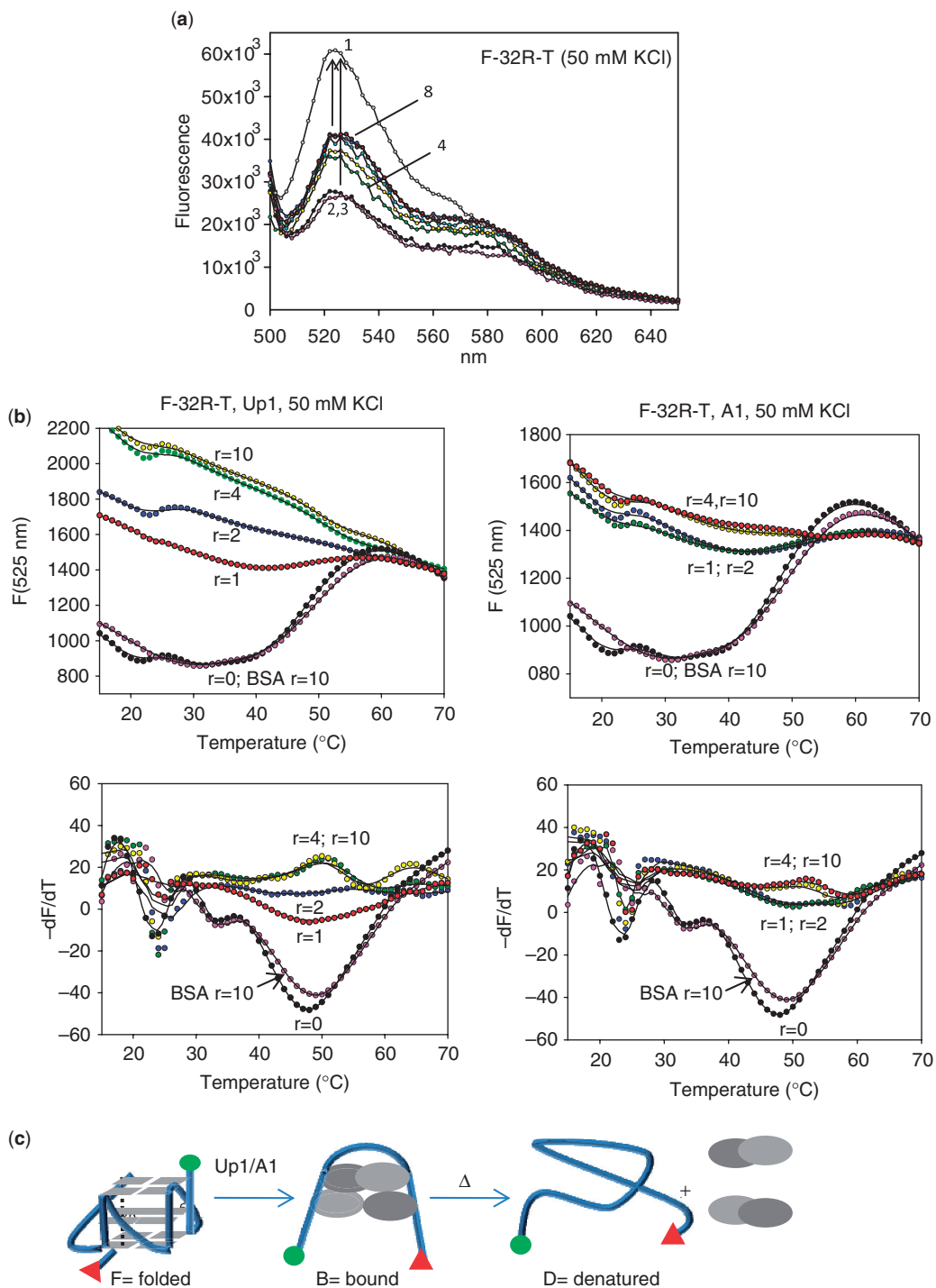
The unfolding of the human *KRAS* quadruplex by A1/Up1 was also investigated by FRET, using the quadruplex-forming sequences tagged at the 5' and 3' ends with FAM (donor) and TAMRA (acceptor) (34). By exciting F-32R-T at 475 nm, the emission intensity of the donor at 525 nm decreases while the emission intensity of the acceptor at 580 nm increases, as the KCl concentration is increased from 0 to 140 mM (Supplementary Data S2). F-32R-T folded in the G-quadruplex conformation ( $T_m = 75^\circ\text{C}$  in 140 mM KCl) is characterized by a  $P$ -value of 0.52 (see 'Materials and methods' section). This  $P$ -value is higher than that observed for the quadruplex formed by the human telomeric repeat d(GGGTTAGGGTTAGGGT) (26), because F-32R-T forms a parallel quadruplex where the two fluorophores are at opposite ends of the structure (13). When the G-quadruplex is destabilized by scaling down the KCl concentration to zero or by adding the complementary 32Y strand, that transforms the G-quadruplex into a B-DNA duplex where the donor and acceptor are separated by about 115 Å, the donor fluorescence significantly increases (for instance, from spectrum 2 to spectrum 1, Figure 5a) and the  $P$ -value becomes 0.75. This means that the unfolding of quadruplex F-32R-T is accompanied



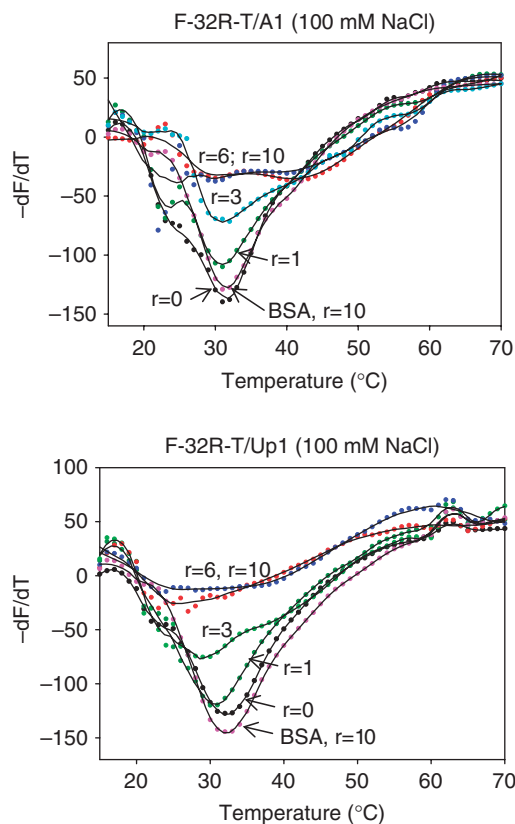
**Figure 4.** CD of 32R (2  $\mu\text{M}$ ) in 50 mM Tris pH 7.4, 100 mM KCl in the presence of increasing amounts of Up1 ( $r = 0, 1, 4, 6$ ) ( $r = [\text{protein}]/[\text{DNA}]$ ) (a); hnRNP A1  $r = 0, 1, 4, 6$ , (b); trypsinogen inhibitor (TI) ( $r = 0, 1, 4, 6$ ) (c). The CD of Up1 at three concentrations is reported (2, 4 and 8  $\mu\text{M}$ ) (d). Spectra have been recorded at room temperature with a path length cuvette of 0.5 cm. Ordinate reports ellipticity values in mdeg.

by a  $\Delta P = 0.75 - 0.52 = 0.23$ . We then asked if quadruplex F-32R-T is unfolded by A1/Up1. To choose at which ionic strength the FRET experiments in the presence of A1/Up1 should be performed, we measured the  $T_m$  of

quadruplex F-32R-T in KCl and NaCl solutions (in 50, 100 and 140 mM KCl,  $T_m$  is 48, 70 and 75°C, respectively; in 100 mM NaCl, the  $T_m$  is 32°C). Figure 5a shows the effect on quadruplex F-32R-T in 50 mM KCl ( $T_m = 48^\circ\text{C}$ ,



**Figure 5.** (a) Fluorescence spectra of 200 nM F-32R-T in water (spectrum 1) or 50 mM Tris-HCl, pH 7.4, 50 mM KCl in the absence (spectrum 2) or presence of BSA ( $r = 10$ , spectrum 3) or Up1 ( $r = 0.5, 1, 3, 6, 10$ , spectra 4–8); (b) row FRET-melting curves ( $F_{525}$  versus  $T$ ) obtained with the iQ5 real-time PCR machine of quadruplex F-32R-T treated with A1/Up1 at various [protein]/[DNA] ratios, in 50 mM Tris pH 7.4, 50 mM KCl. As reference a melting curve of F-32R-T in the presence of BSA ( $r = 10$ ) is reported. Bottom panels show the corresponding first derivative curves,  $-dF_{525}/dT$  versus  $T$ . The G-quadruplex was incubated with the protein for 30 min prior to melting; (c) schematic representation of the U-shape structure of the DNA-protein complex between F-32R-T and Up1.



**Figure 6.**  $-dF_{525}/dT$  versus  $T$  curves in 50 mM Tris pH 7.4, 100 mM NaCl of F-32R-T in the absence or presence of A1 (a), Up1 (b), BSA at various [protein]/[DNA] ratios.

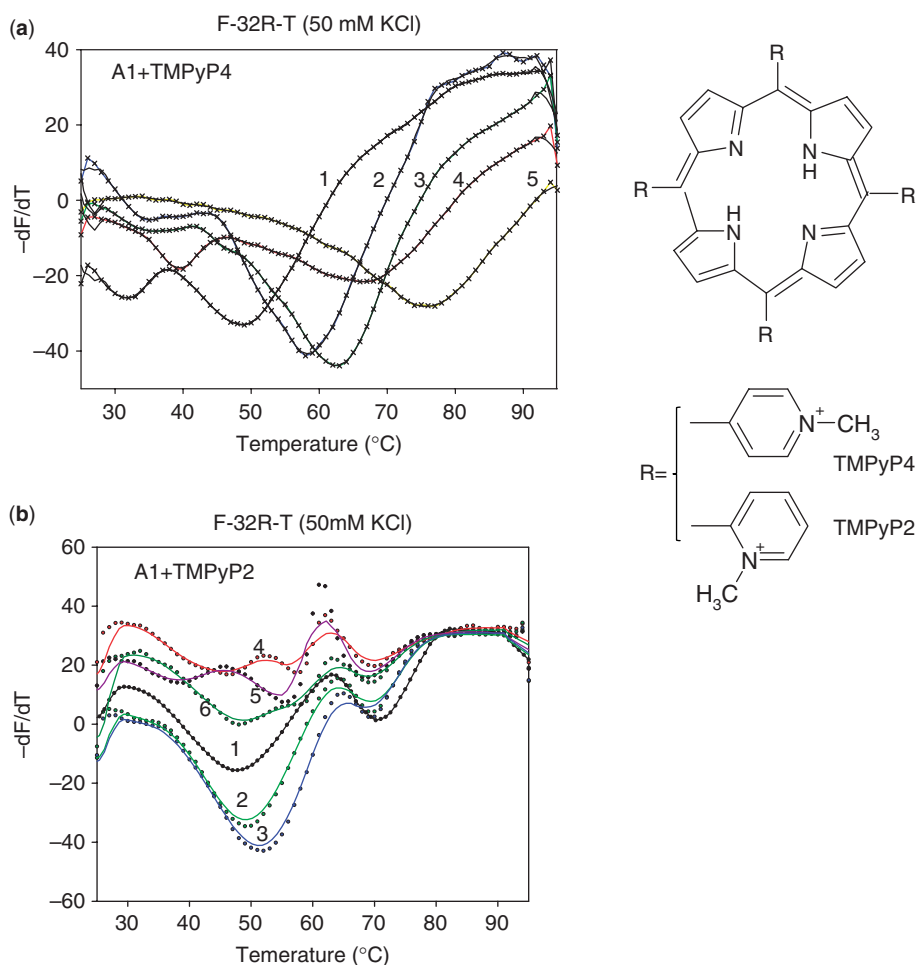
$P = 0.62$ ) of Up1 and A1 at  $r = 1, 2, 3, 6, 10$ . It can be seen that, compared to complementary 32Y, Up1 does not promote a significant increase of the donor emission, a behaviour that might suggest that Up1 has little effect on the quadruplex conformation [for instance the  $P$ -value is 0.62 at  $r = 0$  (spectrum 2), 0.67 at  $r = 10$  (spectrum 8),  $\Delta P = 0.05$ ]. If we assume that  $\Delta P = 0.23$  reflects total opening of the G-quadruplex,  $\Delta P = 0.05$  suggests that F-32R-T bound to Up1 is partially opened (20%). Alternatively, it is possible that F-32R-T in the DNA-protein complex is completely opened but with the 5' and 3' ends brought close to one another so that FRET takes place. To gain insight into this possibility we performed melting experiments. We reasoned that in case the quadruplex is partially unfolded, its  $T_m$  would be lowered, whereas in case it is completely opened by A1/Up1, the quadruplex-to-ssDNA transition should be abrogated. Figure 5b shows typical melting curves for quadruplex F-32R-T in 50 mM KCl, obtained with a real-time PCR machine, after the DNA was incubated for 30 min with A1/Up1 ( $r = 1, 2, 4, 10$ ) or BSA ( $r = 10$ ) just before melting. It can be seen that an excess of BSA does not change the  $T_m$  of the G-quadruplex, as one expects with an unspecific protein which does not interact with DNA. In contrast, when quadruplex F-32R-T is incubated with A1/Up1, a strong change of the melting curves is observed. The cooperative transition relative to the denaturation of

the G-quadruplex ( $T_m$  of 48°C) is completely abrogated and replaced with a broad and non-cooperative curve, which reflects the disruption of the DNA-protein complex. The abrogation of the quadruplex-to-ssDNA transition is clearly observed with both  $F$  versus  $T$  and  $-dF/dT$  versus  $T$  curves. A similar behaviour has been reported for the UV-melting of the virus type 1 nucleocapsid protein bound to the quadruplex formed by d(GGGTTGGTGTG GTTGG) (35). In 100 mM NaCl, where quadruplex F-32R-T shows a cooperative transition with a  $T_m$  of 32°C, we also observed the abrogation of the cooperative transition by A1/Up1 (Figure 6). These data suggest that when F-32R-T is bound to A1/Up1, its secondary structure is completely disrupted and F-32R-T in the DNA-protein complex is in the single-stranded form. The fact that the opening of the quadruplex by Up1 is accompanied by a  $\Delta P$  which is 20% of that observed with 32Y (0.05 against 0.23) can be rationalized on the basis of the crystal structure between Up1 and the telomeric repeat (T TAGGG)<sub>2</sub> (18). In the crystal, the two RRM elements within a Up1 molecule bind to two separate 12mer oligonucleotides, which are antiparallel and separated by an *interstrand* distance of 25–50 Å. Thus, we expect that F-32R-T bound to A1/Up1 adopts a U-shape with the two fluorophores close enough to promote energy transfer (18) (Figures 5c).

It is well known that the cationic porphyrin TMPyP4 stabilizes quadruplex DNA by stacking externally to the G-tetrads and interacting with the loop nucleotides (36). We therefore tested whether TMPyP4 reduces the quadruplex destabilizing action of A1/Up1. Quadruplex F-32R-T (200 nM) was incubated for 12 h in 50 mM KCl, in the presence of 200 and 600 nM TMPyP4. Figure 7a shows that TMPyP4 enhances the  $T_m$  of quadruplex F-32R-T from 48°C (curve 1) to 68 (curve 4) and 76°C (curve 5). The mixtures were treated for 30 min with 1 μM A1 ( $r = 5$ ) and then melted. While A1 at  $r = 5$  is able to completely disrupt the *KRAS* quadruplex in 50 mM KCl (see Figure 5b), in the presence of the porphyrin it promotes only a partial destabilization of the G-quadruplex: the  $T_m$  is reduced from 68°C to 58°C (in the presence of 200 nM TMPyP4, curve 2, Figure 7a) or from 76°C to 63°C (600 nM TMPyP4, curve 3, Figure 7a). So, the stabilizing effect of the porphyrin partially inhibits the capacity of the protein to unfold the G-quadruplex. To exclude the possibility that TMPyP4 directly interacts with and inhibits A1, we performed a control experiment with TMPyP2, the positional isomer of TMPyP4 showing little affinity for quadruplex DNA (Figure 7b). As expected, TMPyP2 neither stabilizes appreciably quadruplex 32R, nor impairs the unfolding of the quadruplex structure by A1. These experiments provide a possible molecular mechanism that explains how TMPyP4 is found to repress the activity of the *KRAS* promoter (12,13).

Krainer and co-workers (23) showed that A1/Up1 binds to the single-stranded and structured human telomeric repeat (TTAGGG)<sub>*n* = 2, 4</sub>. They suggest that A1 is likely to function as an auxiliary factor of the telomerase holoenzyme and propose that the protein stimulates telomerase elongation through unwinding of the G-quadruplex structures formed during the



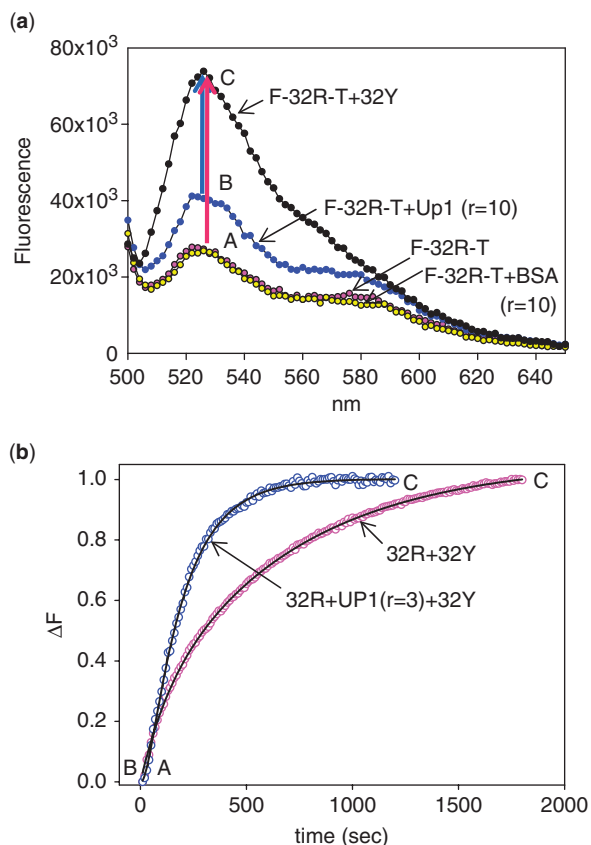


**Figure 7.** (a)  $-dF_{525}/dT$  versus  $T$  melting curves of 200 nM F-32R-T in 50 mM Tris pH 7.4, 50 mM KCl (curve 1), in the presence of 200 nM (curve 4) or 600 nM (curve 5) porphyrin TMPyP4. Curves 2 and 3 show the melting curves obtained by F-32R-T treated with 200 nM TMPyP4 + A1 ( $r = 5$ ) or 600 nM TMPyP4 + A1 ( $r = 5$ ), respectively; (b)  $-dF_{525}/dT$  versus  $T$  melting curves of 200 nM F-32R-T in 50 mM Tris pH 7.4, 50 mM KCl (curve 1), in the presence of 200 nM (curve 2) or 600 nM (curve 3) porphyrin TMPyP2. Curves 4, 5 and 6 show the melting curves obtained by F-32R-T treated with A1 ( $r = 5$ ); 200 nM TMPyP2 + A1 ( $r = 5$ ); 600 nM TMPyP2 + A1 ( $r = 5$ ), respectively. Exc 475 nm, Em 525 nm; (c) Structures of TMPyP2 and TMPyP4.

translocation steps. Our study suggests that protein A1, being a component of a multiprotein complex formed within NHE (13), may have a similar function for the *KRAS* promoter: i.e. to resolve the folded quadruplex conformations. The destabilizing activity of A1 should facilitate a quadruplex-to-duplex transformation, that seems to be necessary to activate transcription (12,13). To test this hypothesis, we investigated whether the kinetic of hybridization between quadruplex F-32R-T and the complementary 32Y strand becomes faster in the presence of Up1. When quadruplex F-32R-T in 100 mM KCl ( $T_m = 70^\circ\text{C}$ ) is mixed at  $25^\circ\text{C}$  with the 32Y strand the quadruplex sequence is transformed into the more stable duplex ( $T_m = 78^\circ\text{C}$ ) and the fluorescence of the donor increase as in the duplex it is separated from the acceptor (Figure 8a, from A to C). This assembly process can be monitored by measuring the increase of donor (FAM) fluorescence,  $\Delta F$ , as a function of time ( $\Delta F = F - F_0$ , where  $F_0$  is the FAM fluorescence at 525 nm at  $t = 0$  and  $F$  the fluorescence at time  $t$ ). The  $\Delta F$  versus  $t$  curve

shows an exponential shape that was best-fitted to a double-exponential equation (37). For the slow phase a constant  $k_{\text{slow}}$  of  $1.56 \times 10^{-3} \pm 6 \times 10^{-5} \text{ s}^{-1}$  was obtained (Figure 8b). The hybridization performed in the presence of Up1 occurs with a faster kinetic which was nicely best-fitted to a single-exponential equation:  $k = 5.2 \times 10^{-3} \text{ s}^{-1}$ . In this case the assembly occurs between F-32R-T bound to Up1 and 32Y, the fluorescence increases from B to C (Figure 8b). The half-life  $t_{1/2}$  for the hybridization of 32R to 32Y in the presence of Up1 is 133 s, while in the absence of Up1 is 444 s, i.e. more than 3 times higher. This demonstrates that Up1 is indeed a G4-DNA destabilizing protein that facilitates the quadruplex-to-duplex transformation within NHE.

Finally, by a primer extension assay using a template containing the *KRAS* G-rich element we tested whether A1/Up1 is able to remove the block to Taq polymerase caused by quadruplex formation (12,13,24). Figure 9 shows that when the template is incubated in 100 mM KCl prior to primer extension, Taq polymerase is arrested



**Figure 8.** (a) FRET spectra of 200 nM F-32R-T in 50 mM KCl, in the presence of BSA ( $r = 10$ ), Up1 ( $r = 10$ ) and 6-fold complementary 32Y strand. (b) Increase of fluorescence  $\Delta F$  as a function of time following the addition to 200 nM F-32R-T of 6-fold complementary 32Y in 50 mM Tris pH 7.4, 100 mM KCl. Exc 475 nm; Em 525 nm. The experiment has been conducted in the absence and presence of Up1 ( $r = 3$ ). The solid lines are the best-fits of the experimental points with an exponential equation (SigmaPlot 11, Systat Software Inc).

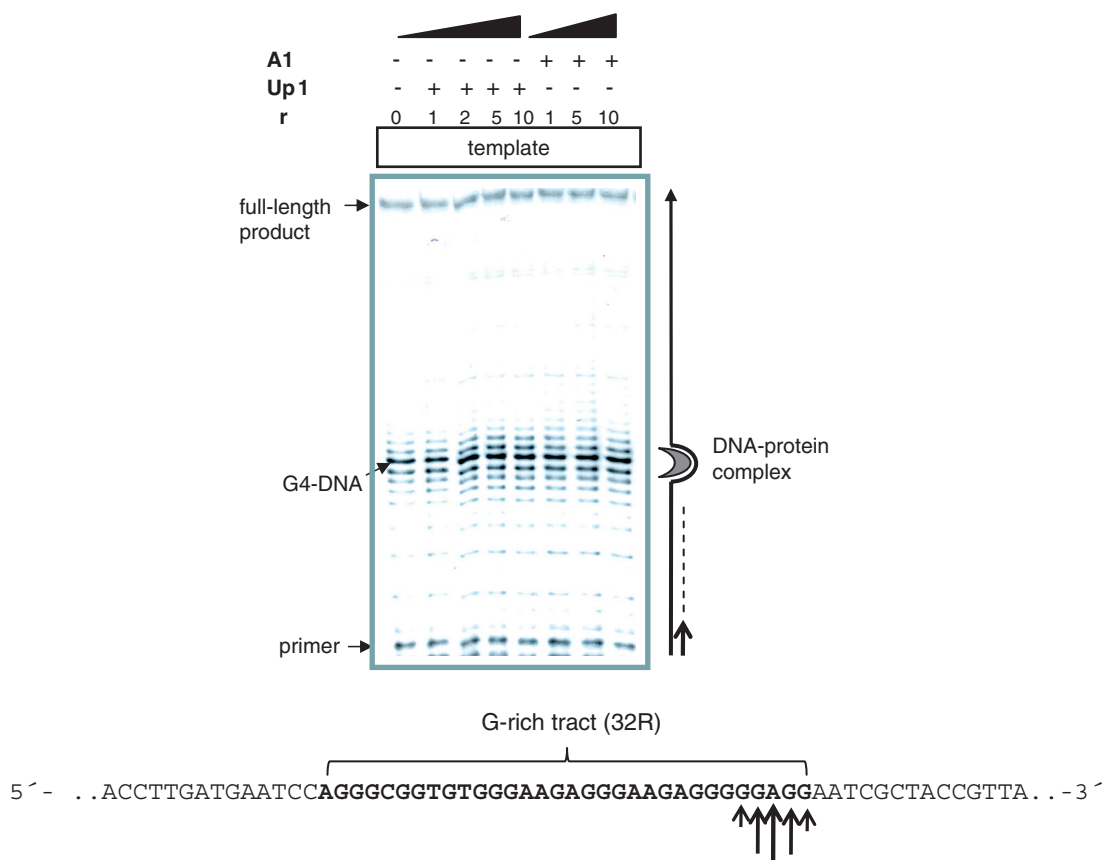
at the 3' end of the G-rich tract, as this element folds into a G-quadruplex structure. Contrarily to what we expected, the addition of increasing amounts of protein A1/Up1 strengthened the pause of Taq polymerase. The precise points at which Taq polymerase was arrested were determined by Sanger sequencing reactions and are indicated with arrows in the template sequence. This suggests that A1/Up1 forms with the DNA template a complex which is sufficiently stable to arrest the processivity of Taq polymerase. That's why A1/Up1 enhances the block of Taq polymerase at the G-rich element. However, to corroborate this hypothesis DNA footprinting experiments should be done to demonstrate direct binding of A1/Up1 to the site of arrest. The complex between A1/Up1 and 32R is destabilized when the G-rich strand hybridizes to its complementary sequence to afford a B-DNA duplex for which A1/Up1 has no affinity (see EMSA). Finally, in keeping with the results in Figure 3, the primer-extension assay shows that A1/Up1 binds to the G-rich tract of NHE with a high selectivity, since significant arrests of polymerase at other points of the template are not observed.

## DISCUSSION

This work describes the ability of A1, and its derivative Up1, to destabilize the quadruplexes of the *KRAS* promoter and to facilitate their hybridization to the complementary polypyrimidine strand. In accord with pull-down experiments (13), EMSA confirmed that recombinant Up1 and A1 bind to the *KRAS* quadruplex with a high affinity and sequence-specificity, as the binding to other G-quadruplex structures such as *HRAS1*, *HRAS2*, *CMYC*, *VEGF* appeared either weak or inconsistent. Only the quadruplex from the *CKIT* sequence (Table 1) is recognised by Up1. The association of A1 to the *KRAS* promoter is restricted to the polypurine strand, as EMSA shows that A1 does not bind to the complementary polypyrimidine strand, nor to NHE in duplex conformation. Considering that the minimum length for strong binding to Up1 is a stretch of 12 nucleotides (18), 32R, being composed by 32 nucleotides, has potentially two binding sites. In fact, EMSA shows that 32R forms two DNA-protein complexes that are expected to have a stoichiometry of 1:1 and 1:2 (DNA:Up1). This is in accord with the results of Zhang *et al.* (23) showing that Up1 forms with the telomeric repeats (TTAGGG)<sub>4</sub> two DNA-protein complexes.

In accord with previous observations (24,25), A1 and Up1 promote a significant reduction of the 260 nm ellipticity, typical of G4-DNA in the parallel conformation. This demonstrates that both proteins are able to unfold the quadruplex structures of the *KRAS* promoter. This conclusion is further supported by FRET-melting experiments showing that the quadruplex formed by F-32R-T is completely disrupted by A1 or Up1. When the *KRAS* G-quadruplex is incubated for 30 min with A1/Up1 before melting, the cooperative transition of the G-quadruplex is replaced by a non-cooperative transition. This suggests that when the *KRAS* sequence is bound to A1/Up1, it is open and in a single-stranded conformation, as shown by the crystal of Up1 with the telomeric repeat (18). In contrast, when a 10-fold excess BSA is added to the G-quadruplex, no change in the quadruplex transition is observed. We interestingly found that protein A1/Up1 facilitates the assembly into a duplex of the two complementary NHE strands. In fact, the half-life of renaturation is reduced from 444 to 133 s in the presence of Up1, 100 mM KCl. This is in accord with earlier studies reporting that A1 promotes a rapid renaturation of nucleic-acid strands, probably by melting the secondary structures that are formed transiently during the annealing process (38). The finding that A1 resolves the *KRAS* quadruplexes has an important biological significance because previous studies supported the notion that the *KRAS* G4-DNA might behave as a transcription suppressor (12,13,28).

The role of A1 *in vivo* has been investigated in the context of the telomere biogenesis (21–23). One possible function of the protein would be to disrupt the G4-DNA structures of the telomere G-repeats, allowing proper elongation by the telomerase (23). The data of our study suggest that A1 could have a similar function in the transcription of *KRAS*. This is in keeping with the fact that A1: (i) binds to the folded G4-DNA conformations of



**Figure 9.** Primer extension assay showing that Taq polymerase pauses at the G-rich element of *KRAS* where the template forms G-quadruplex structures in the presence of KCl. The 87-mer DNA template (100 nM) was mixed with  $^{33}\text{P}$ -labelled primer (50 nM) (Table 1) and incubated for 24 h in 140 mM KCl to allow quadruplex formation by the G-rich element. The mixtures were added with increasing amounts of Up1 (lanes 2–5) or A1 (lanes 6–8),  $r = [\text{protein}]/[\text{DNA}]$  as specified, and incubated for 30 min prior to primer extension. Taq polymerase is arrested at the G-rich element due to quadruplex formation. In the presence of Up1 or A1 the polymerase arrest is stronger. The points in which Taq polymerase is arrested, have been identified by standard Sanger sequencing reactions. Primer extension reaction performed at 37°C for 1 h. Reaction products separated in a 12% Urea-TBE denaturing gel.

NHE but not to the complementary pyrimidinic strand or duplex NHE; (ii) disrupts G4-DNA and (iii) facilitates the assembly of the NHE strands into a duplex. A possible model for transcription regulation of *KRAS* is the following. NHE should exist in equilibrium between a folded (quadruplex) and a double-stranded conformation. In the folded form the promoter is locked into a form that might inhibit transcription (12,13). To activate transcription, the folded form of NHE should hybridize to the complementary strand in order to restore the duplex. As the quadruplex-to-duplex transformation is likely to be kinetically slow, the functions of A1 would be of destabilizing the quadruplex and allow the G-rich strand to hybridize to its complementary within a time compatible with a response of the cell to molecular stimuli. There are a number of genes with C+G-rich elements in the region surrounding the transcription start site that seems to be characterized by a transcription regulation mechanism involving G-quadruplex structures (12,13,39–45).

Several proteins from different organisms that interact with quadruplex DNA have been reported (46). They can be classified by function into five major groups: (i) proteins that increase the stability of DNA quadruplexes;

(ii) proteins that destabilize quadruplex DNA in a non catalytic way; (iii) proteins that unwind catalytically quadruplex DNA in an ATP-dependent fashion; (iv) proteins that promote the formation of quadruplex DNA; (v) Nucleases that specifically cleave DNA at or adjacently to a quadruplex domain. Like other members of the hnRNP family such as hnRNP A2 (20) and CBF-A (20,47) that destabilise the G-quadruplex formed by the d(CGG)n fragile X expanded sequence, protein A1 acts on DNA in a non-catalytic way, i.e. remaining bound to the DNA substrate. Another protein with a similar property is POT-1 which binds to the telomere G-rich DNA overhangs and disrupts G4-DNA structures (48,49). However, contrarily to A1/Up1, POT-1 causes a significant increase of the *P*-value of the quadruplex from the human telomeric repeat, because in the DNA-protein complex the telomeric repeat assumes an extended conformation in which the donor-acceptor are separated by a distance that is too long for FRET (26). Similarly, A1 disrupts the G4-DNA structures assumed by NHE and its remaining bound to the G-rich sequence prevents the DNA from assuming again the folded conformation. We were indeed surprised to observe by primer extension experiments that

at 37°C, A1/Up1 did not remove the block to Taq polymerase and the protein even enhanced the polymerase arrest. This clearly indicates that after interaction the protein remains bound to the template, and the resulting DNA–protein complex is sufficiently strong to arrest the processivity of the polymerase.

Finally, the proposed transcription regulation model suggests two strategies to downregulate the *KRAS* oncogene and sensitize pancreatic cancer cells, which are refractory to conventional treatment, to chemotherapy. First, use of G4-ligands that lock the promoter in the non-transcriptable form by stabilizing the G-quadruplexes; second, use of decoy molecules specific for the proteins that recognize the G4-DNA structure of NHE (28). Work is in progress in our laboratory along this direction.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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