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The identification of nuclear proteins that bind the homopyrimidine strand of d(GA·TC)n DNA sequences, but not the homopurine strand

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ABSTRACT

Alternating d(GA·TC)$_n$ DNA sequences, which are abundant in eukaryotic genomes, can form altered DNA structures. Depending on the environmental conditions, the formation of (GA·GA) hairpins or [C$^+$T(GA·TC)] and [GA(GA·TC)] intramolecular triplexes was observed *in vitro*. *In vivo*, the formation of these non-B-DNA structures would likely require the contribution of specific stabilizing factors. Here, we show that Friend's nuclear extracts are rich in proteins which bind the pyrimidine d(TC)$_n$ strand but not the purine d(GA)$_n$ strand (NOGA proteins). Upon chromatographic fractionation, four major proteins were detected (NOGA1–4) that have been purified and characterized. Purified NOGAs bind single-stranded d(TC)$_n$ with high affinity and specificity, showing no significant affinity for either d(GA)$_n$ or d(GA·TC)$_n$ DNA sequences. We also show that NOGA1, -2 and -3, which constitute the three most abundant and specific NOGA proteins, correspond to the single-stranded nucleic acid binding proteins hnRNP-L, -K and -I, respectively. These results are discussed in the context of the possible contribution of the NOGA proteins to the stabilization of the (GA·GA) and [GA(GA·TC)] conformers of the d(GA·TC)$_n$ DNA sequences.

INTRODUCTION

DNA is structurally polymorphic. *In vitro*, DNA structure depends both on the nucleotide sequence and the environmental conditions. Alternating d(GA·TC)$_n$ DNA sequences constitute a case of highly polymorphic DNA sequences that, depending on the experimental conditions, can form [C$^+$T(GA·TC)] intramolecular triplexes at acidic pH, or [GA(GA·TC)] intramolecular triplexes, in the presence of some transition metal ions (Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}) (1–3). At high metal-ion concentration, the formation of (GA·GA) intramolecular hairpins is observed (4). The formation of bitriplex structures, in which [C$^+$T(GA·TC)] and [GA(GA·TC)] intramolecular triplexes coexist, has also been reported (5). Alternating d(GA·TC)$_n$ sequences are abundant in eukaryotic genomes, accounting for ~0.4–0.5% of the total mammalian genome (6,7). These sequences are often found at gene regulatory regions (8,9), suggesting a role in transcription regulation as demonstrated in several cases (10–13). d(GA·TC)$_n$ sequences are also frequent at 'hot-spots' for genetic recombination (8,9) and they have been shown to arrest DNA replication both *in vitro* and *in vivo* (14–18). Though the extent to which the structural properties of the d(GA·TC)$_n$ sequences influence their biological function(s) is still a matter of debate, their potential to form altered DNA structures was found to correlate with their effects on DNA replication and recombination (15,16,18,19). For any double-stranded DNA fragment, the regular right-handed B-DNA conformation is thermodynamically favored. Therefore, the formation of altered DNA structures would require the contribution of stabilizing factors. Negative supercoiling is known to facilitate the formation of triple-stranded DNA at d(GA·TC)$_n$ sequences *in vitro* (1–3). Local increases in negative supercoiling, occurring during DNA replication or transcription, could also stabilize these altered DNA structures *in vivo*, at least transiently. Specific nuclear proteins, by themselves or in conjunction with negative supercoiling, can also play an important stabilizing role. In this respect, it was recently shown that the GAGA factor of *Drosophila* binds d(GA·TC)$_n$ sequences both as double- and triple-stranded DNA (20) and the identification of a few other triple-stranded DNA binding proteins has also been reported (21,22). Single-stranded DNA binding proteins which preferentially bind to either strand of the d(GA·TC)$_n$ sequences could also play a stabilizing role. Here, we show that Friend's nuclear extracts are rich in proteins binding the pyrimidine d(TC)$_n$ strand and four major binding activities are detected. On the other hand, no strong binding to the purine d(GA)$_n$ strand is observed. The polypeptides responsible for the four major d(TC)$_n$ binding activities have been purified and characterized. These proteins, called NOGA, show no significant affinity for the complementary d(GA)$_n$ strand and,

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therefore, could be instrumental in the stabilization of the [GA(GA·TC)] and (GA·GA) intramolecular forms of the $d(GA·TC)_n$ sequences.

MATERIALS AND METHODS

Preparation of nuclear extracts

Friend's cells were grown to a final density of 2×10^6 cells/ml in Dulbecco's modified Eagle's medium containing 10% heat inactivated fetal bovine serum. Extracts were obtained from a total of 5×10^{10} cells according to the method of Dignam *et al*. (23) and dialyzed against buffer C (20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 20 mM HEPES pH 7.9, 0.1 mM PMSF, 0.5 mM DTT). After dialysis, nuclear extracts were spun for 20 min at 20 000 r.p.m. in a SS34 rotor, rapidly frozen and stored at –80°C.

Fractionation of extracts and purification of NOGA proteins

For the purification of the NOGA proteins, crude nuclear extracts were subjected to chromatographic fractionation. All the solutions used were prepared in buffer C and all fractions were monitored for the presence of NOGAs by EMSA as described below. For the first purification step, the crude nuclear extract was applied to a 5 ml HiTrap-Blue column (Pharmacia Biotech) equilibrated in buffer C. The column was washed with 0.1 M KCl, eluted with a 40 ml 0.1–2.0 M KCl linear gradient and then washed again at 2.0 M KCl. All the steps were carried out at a flow rate of 3.5 ml/min in an FPLC System (Pharmacia). Fractions containing the bulk of the $d(TC)_{20}$-binding activity, that eluted from this column as a broad peak between 1.2 and 2.0 M KCl, were pooled and dialyzed against buffer C. Then, the solution was incubated in 0.03% NP-40–buffer C for 20 min at 4°C, with an excess of biotinylated $d(TC)_{20}$ oligonucleotide in the presence of a similar excess of heat denatured single-stranded *Escherichia coli* DNA added as non-specific competitor. After incubation, the mixture was loaded onto a streptavidin–agarose column (Sigma) equilibrated in buffer C. Elution was carried out at a flow rate of ~0.5 ml/min in three steps with 10 vol of 0.1 M KCl, 10 vol of 0.6 M KCl and 25 vol of 2.0 M KCl, respectively. Though some activity was detected in the 0.6 M KCl elution, the bulk of the $d(TC)_{20}$-binding activity eluted at 2.0 M KCl. These high salt fractions were pooled and loaded onto a Phenyl-Superose HR 5/5 (Pharmacia) column equilibrated in 2.0 M KCl–buffer C. The column was washed at 2.0 M KCl, eluted with a 20 ml 2.0–0.1 M KCl linear gradient and washed again at 0.1 M KCl. All steps were carried out at a flow rate of 0.35 ml/min in an FPLC System (Pharmacia). The elution profile of this column showed four major $d(TC)_{20}$-binding activities that were named NOGA1–4 (Fig. 1B).

NOGA1 eluted from the PhenylSuperose column at 1.8–1.3 M KCl as a single polypeptide.

Fractions containing NOGA2, eluting at 1.0–0.8 M KCl from the Phenyl Superose column, were pooled, desalted in a HiTrap Desalting (Pharmacia Biotech) column to 50 mM KCl, loaded onto a ResourceQ (Pharmacia) column equilibrated in 50 mM KCl–buffer C and, after a wash at 50 mM KCl, eluted with a 20 ml 0.05–1.0 M KCl linear gradient followed by a wash at 2.0 M KCl. All steps were carried out at a flow rate of 1 ml/min in an FPLC system (Pharmacia). From this column NOGA2 eluted at 0.3–0.4 M KCl as a single polypeptide. The same polypeptide is also present in the 0.6 M KCl elution of the $d(TC)_{20}$–streptavidin–agarose column from where it can also be purified to homogeneity in a ResourceQ column.

NOGA3 eluted from the PhenylSuperose column at 0.1 M KCl as a doublet of ~62–64 kDa. The very last fractions of the 2.0 M KCl elution of the $d(TC)_{20}$–streptavidin–agarose column are enriched in the NOGA3 doublet that can also be purified by chromatography through the PhenylSuperose column eluted with a 5 ml 0.25–0.1 M KCl linear gradient.

Fractions containing NOGA4 eluted at 2.0 M KCl from the PhenylSuperose column. They were pooled, desalted to 50 mM KCl, loaded on a ResourceQ (Pharmacia) column and eluted with a 10 ml 0.05–0.2 M KCl gradient. From this column NOGA4 eluted at 0.13 M KCl as a single polypeptide.

At each step of purification, the protein content of the relevant fractions was determined by SDS–polyacrylamide gel electrophoresis (PAGE).

EMSA experiments

Synthetic $d(TC)_{20}$ and $d(GA)_{20}$ oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Double-stranded $d(GA·TC)_{20}$ oligonucleotide was prepared by mixing equimolar amounts of labeled $d(TC)_{20}$ and $d(GA)_{20}$. Both oligonucleotides were annealed by heating at 90°C for 5 min, 58°C for 2 h and then left to cool at room temperature overnight.

All binding reactions were carried out with ~2 ng radiolabeled $d(TC)_{20}$ and $d(GA)_{20}$ or 4 ng duplex $d(GA·TC)_{20}$ in a 25 µl final volume consisting of 4% glycerol, 0.2 M KCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10 mM β-mercaptoethanol and 0.03% NP-40. Samples were incubated for 20 min at 4°C in the presence of a 125-fold excess (w/w) of heat denatured single- or double-stranded *E.coli* DNA as non-specific competitors. When the binding of purified proteins was analyzed, no competitor DNA was added to the reaction except for the competition experiments that were performed in the presence of increasing amounts of various competitor DNAs, as indicated in each case. Competition experiments were performed at the protein concentration that, in the absence of any added competitor, corresponds to ~50% binding of radiolabeled $d(TC)_{20}$. The DNA–protein complexes were resolved on 5% polyacrylamide–0.5× TBE (0.45 mM Tris-base, 0.45 mM boric acid, 1 mM EDTA, pH 8.3) gels run at 4°C and 10 V/cm. After electrophoresis, gels were dried and autoradiographs were recorded in Hyperfilm (Amersham) at –80°C. For quantitative analysis of the results, autoradiographs were scanned with a MolecularDynamics laser densitometer using the internal integration to determine peak areas.

Western blot assays

For western blot analysis, protein samples were subjected to 12% SDS–PAGE and transferred to nitrocellulose membranes. Rabbit αhnRNP-K and αPTB antibodies, generous gifts from Drs Bomsztyk and Zakin, and monoclonal αhnRNP-L (Ab4D11), a gift from Dr Dreyfuss, were used at the appropriate dilution. After incubation with the specific antibodies, the membranes were washed, incubated with anti-rabbit (1:5000 dilution) or anti-mouse (1:10 000 dilution) horseradish peroxidase-conjugated secondary antibody and developed by chemiluminescense following the ECL protocol (Amersham).

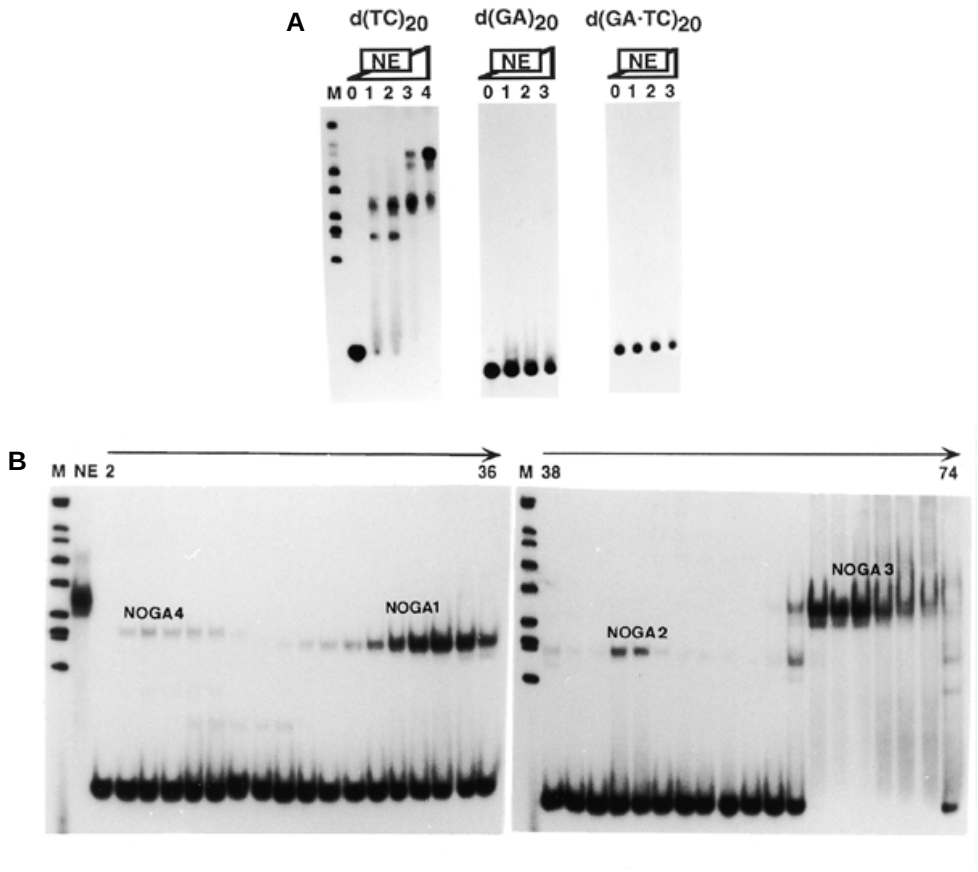

Figure 1. (**A**) The binding of nuclear extracts (NE) to single-stranded $d(TC)_{20}$, $d(GA)_{20}$ or double-stranded $d(GA{\cdot}TC)_{20}$ is analyzed by EMSA as a function of increasing amounts of NE. Panel $d(TC)_{20}$: 0 µl (lane 0), 0.25 µl (lane 1), 0.5 µl (lane 2), 1 µl (lane 3) and 2 µl (lane 4). Panels $d(GA)_{20}$ and $d(GA{\cdot}TC)_{20}$: 0 µl (lane 0), 2 µl (lane 1), 4 µl (lane 2) and 8 µl (lane 3). (**B**) The elution profile corresponding to the PhenylSuperose chromatography (see Materials and Methods for details). Aliquots of 2 µl of every other fraction were assayed for binding to $d(TC)_{20}$. Numbers on top indicate the corresponding fraction number. Lanes M correspond to DNA molecular weight marker VI (Boehringer Mannheim).

Peptide sequencing

For peptide sequencing proteins were immobilized onto PVDF membranes (Hyperbond, Beckman) by either vacuum transfer (NOGA1) or electrotransfer (NOGA2, -3 and -4). Internal NOGA2 peptides were obtained through digestion of purified protein with thrombin in 50 mM KCl, 2.5 mM $CaCl_2$, 20 mM Tris–HCl, pH 8.0 at 20°C overnight. Peptides were resolved in 12–18% SDS–PAGE gels. NOGA3 and NOGA4 were resolved in 8 and 12% SDS–PAGE gels, respectively. Before electrophoresis, NOGA3 was precipitated with 25% TCA. Sequencing was performed by automated Edman degradation on a Beckman LF3000 Protein Sequencer.

RESULTS

Four major NOGA activities are detected in crude Friend's nuclear extracts

Figure 1A shows EMSA experiments in which crude nuclear extracts obtained from Friend's cells were assayed for binding to either a homopurine $d(GA)_{20}$ or a homopyrimidine $d(TC)_{20}$ oligonucleotide. Upon the addition of increasing amount of nuclear extract, a number of intense activities binding the $d(TC)_{20}$ oligonucleotide are detected [Fig. 1A, panel $d(TC)_{20}$]. The formation of several protein–DNA complexes can be observed indicating that, most likely, several different nuclear proteins are capable of binding to the single-stranded $d(TC)_{20}$ oligonucleotide. On the other hand, in the case of the $d(GA)_{20}$ oligonucleotide, only weak binding activities are detected [Fig. 1A, panel $d(GA)_{20}$] even in the presence of large amounts of nuclear extract [Fig. 1A, panel $d(GA)_{20}$, lane 3]. Contrary to the $d(TC)_n$ DNA sequences, which remain single-stranded under most experimental conditions, homopurine $d(GA)_n$ sequences can self-associate giving rise to the formation of both intramolecular and intermolecular homoduplexes (24,25). In the EMSA experiments described in Figure 1A, protein binding was performed in the presence of 200 mM KCl where the $d(GA)_{20}$ oligonucleotide forms stable homoduplexes. However, no significant binding was detected when the incubation was performed at low ionic strength, under conditions where the $d(GA)_{20}$ oligonucleotide is only partially structured (not shown). Similarly, binding of the nuclear extract to a $d(GA{\cdot}TC)_{20}$ duplex was also found to be very weak [Fig. 1A, panel $d(GA{\cdot}TC)_{20}$]. These results show that Friend's nuclear extracts contain a number of proteins which bind strongly to single-stranded $d(TC)_n$ sequences, but which are poor in their

Table 1. Amino acid sequences obtained from purified NOGA proteins

NOGA	SEQUENCE	Homologue	Reference
NOGA1	AAAGGGGGGGRYYGGG**N**EGGRAPKR AAAGGGGGGGRYYGGG**S**EGGRAPKR 5 29	hnRNP L (human)	26
NOGA2	GGDLMAYDRRGRPGDRYDGMVGF GGDLMAYDRRGRPGDRYDGMVGF 317 339	hnRNP K (human)	28
NOGA3a	RGSDELFST–VSNGPFIM RGSDELFST**C**VSNGPFIM 14 31	hnRNP I (mouse)	27
NOGA4*	SDAAEEQPMETT SDAAEEQPMETT 2 13	CBF-A(mouse)	30

The N-terminal sequences of the purified NOGA1, NOGA3a and NOGA4* are presented. The sequence of NOGA4* corresponds to that of the fast migrating polypeptide of the triplet containing NOGA4 (see text for details). In the case of NOGA2, the N-terminal sequence of an internal peptide prepared by digestion with thrombin is presented. The differences in amino acid sequence are indicated in underlined bold face.

ability to bind $d(GA)_n$ sequences, either single- or double-stranded, or $d(GA{\cdot}TC)_n$ duplexes.

To characterize the different factors capable of binding the $d(TC)_{20}$ oligonucleotide, the nuclear extract was subjected to chromatographic fractionation as described in Materials and Methods. Briefly, the crude nuclear extract was first loaded onto a HiTrap Blue-Sepharose column where the bulk of the $d(TC)_{20}$ binding activity elutes at high salt concentration (1.2–2.0 M KCl). Positive fractions were then subjected to a $d(TC)_{20}$-affinity column, and elution was performed in three steps of increasing salt concentration: 0.1, 0.6 and 2.0 M KCl. Though some $d(TC)_{20}$ binding was detected in the 0.6 M KCl elution, most of the binding was found in the 2.0 M KCl elution. These high salt fractions were then loaded onto a hydrophobic PhenylSuperose column that was eluted with a 2.0–0.1 M KCl gradient. The elution profile of this column showed four major $d(TC)_{20}$ binding activities, none of which binds significantly to a $d(GA)_{20}$ fragment (not shown). The polypeptides responsible for these activities were named NOGA1–4 (Fig. 1B). NOGA1 and NOGA3 are the two major activities. NOGA3 interacts strongly with the hydrophobic matrix, eluting at 0.1 M KCl. On the other hand, NOGA1 elutes at relatively high salt concentration: 1.8–1.3 M KCl. The two other activities, NOGA4 and NOGA2, elute at 2.0 and 1.0–0.8 M KCl, respectively.

Purification and characterization of NOGA1

The PhenylSuperose fractions corresponding to the NOGA1 activity are highly enriched in a polypeptide of about ~68 kDa in molecular mass (Fig. 2A, lane 4); therefore, no further purification was required in this case. As judged by EMSA, NOGA1 shows a strong affinity for single-stranded $d(TC)_{20}$ [Fig. 2C, panel $d(TC)_{20}$]. On the other hand, purified NOGA1 does not show any significant binding to either single-stranded $d(GA)_{20}$ or double-stranded $d(GA{\cdot}TC)_{20}$ DNA fragments [Fig. 2C, panels $d(GA)_{20}$ and $d(GA{\cdot}TC)_{20}$]. The specificity of the interaction of NOGA1 with single-stranded $d(TC)_{20}$ was analyzed through competition experiments using single-stranded $d(TC)_{20}$ as specific competitor, and heat denatured single-stranded *E.coli* DNA (ss*E.coli*DNA) or single-stranded M13 DNA (ssM13DNA) as non-specific competitors. As shown in Figure 3D, single-stranded $d(TC)_{20}$ is a much more efficient competitor than either ss*E.coli*DNA or ssM13DNA. One-fold excess of $d(TC)_{20}$ results in 50% competition while, in the cases of the non-specific competitors, a higher excess of ~20-fold is required to reach 50% competition. The low affinity of NOGA1 for double-stranded DNA was confirmed when double-stranded *E.coli* DNA (ds*E.coli*DNA) was used as non-specific competitor (Fig. 2D, panel ds*E.coli*). In this case, 50% competition is obtained only after the addition of ~150-fold excess of competitor.

Table 1 shows the N-terminal amino acid sequence corresponding to purified NOGA1. This sequence shows a very high identity to human hnRNP-L (residues 5–29) (26) with only one amino acid change (S21→N21), strongly suggesting that NOGA1 corresponds to the murine hnRNP-L. In agreement with this interpretation, NOGA1 is recognized by specific monoclonal antibodies raised against human hnRNP-L (Fig. 2B). The difference in amino acid sequence mentioned above was confirmed when the cDNA corresponding to the mouse protein was cloned (not shown).

Purification and characterization of NOGA3

Fractions eluting from the PhenylSuperose column at low KCl concentration, corresponding to the NOGA3 activity, are highly enriched in a doublet of ~62–64 kDa (Fig. 3A, lane 4). When these fractions are analyzed by EMSA two retarded bands are observed (Fig. 1B), strongly suggesting that the two polypeptides are capable of binding the single-stranded $d(TC)_{20}$ probe. The very last fractions of the $d(TC)_{20}$-affinity column, corresponding to the last high salt 2.0 M KCl elution, are enriched in the doublet mentioned above (Fig. 3A, lane 3), constituting a better substrate for the purification of the NOGA3 polypeptides than the high salt fractions containing the bulk of the $d(TC)_{20}$ binding activities (Fig. 2A, lane 3).

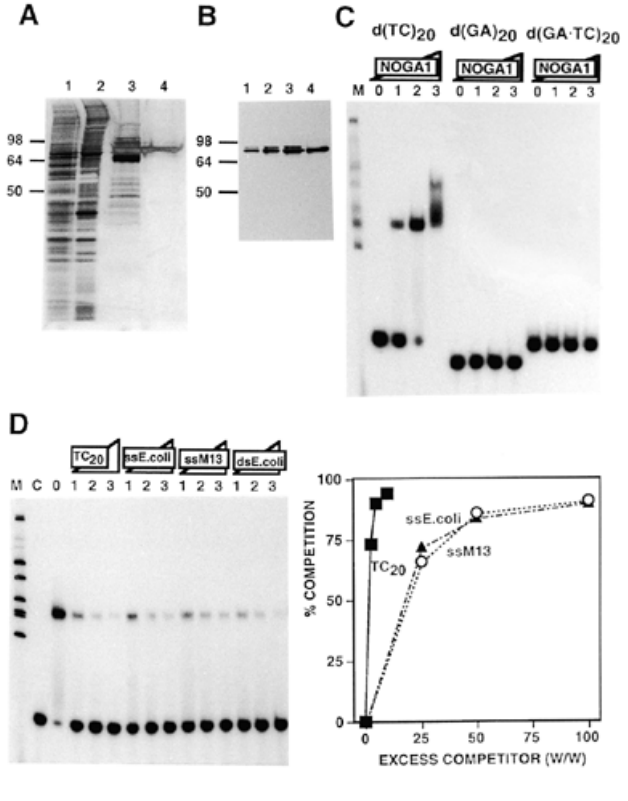

Figure 2. Purification and characterization of NOGA1. (**A**) 12% SDS–PAGE of: the crude nuclear extract (lane 1), the pool of the fractions containing the bulk of the $d(TC)_{20}$ binding activity which elute at 1.2–2.0 M KCl from the HiTrap-Blue column (lane 2) and at 2.0 M KCl from the $d(TC)_{20}$-affinity column (lane 3). Lane 4 corresponds to the fractions of the PhenylSuperose column containing the NOGA1 activity. The positions of markers of known molecular weight (in kDa) are indicated. (**B**) Western blot analysis of the same samples as used in (A) using an αhnRNP-L-specific antibody. (**C**) The binding of purified NOGA1 to single-stranded $d(TC)_{20}$, $d(GA)_{20}$ and double-stranded $d(GA{\cdot}TC)_{20}$ is analyzed by EMSA as a function of increasing purified protein. Panel $d(TC)_{20}$: 0 µl (lane 0), 0.1 µl (lane 1), 0.3 µl (lane 2) and 0.9 µl (lane 3). Panels $d(GA)_{20}$ and $d(GA{\cdot}TC)_{20}$: 0 µl (lane 0), 0.3 µl (lane 1), 0.9 µl (lane 2) and 2.7 µl (lane 3). Lane M corresponds to DNA fragments of known molecular weight used as markers as in Figure 1. (**D**) The efficiency of competition of single-stranded $d(TC)_{20}$ (TC_{20}), single-stranded *E.coli* DNA (ssE.coli), single-stranded M13 DNA (ssM13) and double-stranded *E.coli* DNA (dsE.coli) is shown as a function of increasing excess of competitor DNA (w/w). Panel TC_{20}: 0 (lane 0), 2.5 (lane 1), 5 (lane 2) and 10 (lane 3). Panels ssE.coli and ssM13: 25 (lane 1), 50 (lane 2) and 100 (lane 3). Panel dsE.coli: 250 (lane 1), 500 (lane 2) and 1000 (lane 3). Quantitative analyses of the results are shown on the right.

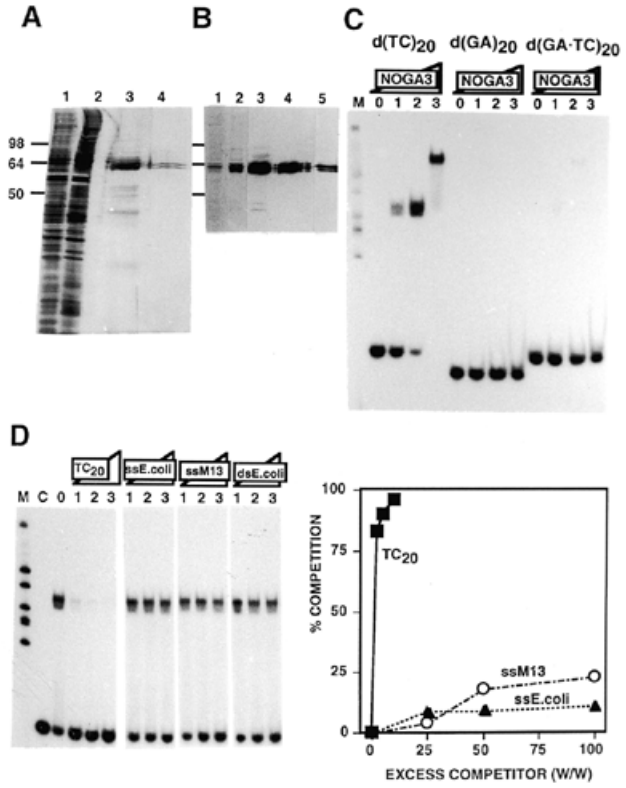

Figure 3. Purification and characterization of NOGA3. (**A**) 12% SDS–PAGE of: the crude nuclear extract (lane 1); the pool of the fractions containing the bulk of the $d(TC)_{20}$ binding activity which elute at 1.2–2.0 M KCl from the HiTrap-Blue column (lane 2); the pool of the very last 2.0 M KCl fractions of the $d(TC)_{20}$-affinity column (lane 3) and the pool of the fractions of the Phenyl-Superose column containing the NOGA3 activity (lane 4). The positions of markers of known molecular weight (in kDa) are indicated. (**B**) Western blot analysis using an αhnRNP-I-specific antibody of: the crude nuclear extract (lane 1); the pool of HiTrap positive fractions (lane 2); the pool (lane 3) or the very last (lane 4) of the fractions eluting from the $d(TC)_{20}$-affinity column at 2.0 M KCl and the pool of the PhenylSuperose fractions containing NOGA3 activity (lane 5). (**C**) The binding of purified NOGA3 to single-stranded $d(TC)_{20}$, $d(GA)_{20}$ and double-stranded $d(GA{\cdot}TC)_{20}$ is analyzed by EMSA as a function of increasing purified protein. Panel $d(TC)_{20}$: 0 µl (lane 0), 2 µl (lane 1), 6 µl (lane 2) and 18 µl (lane 3). Panels $d(GA)_{20}$ and $d(GA{\cdot}TC)_{20}$: 0 µl (lane 0), 6 µl (lane 1), 18 µl (lane 2) and 54 µl (lane 3). Lane M corresponds to DNA fragments of known molecular weight used as markers as in Figure 1. (**D**) The efficiency of competition of single-stranded $d(TC)_{20}$ (TC_{20}), single-stranded *E.coli* DNA (ssE.coli), single-stranded M13 DNA (ssM13) and double-stranded *E.coli* DNA (dsE.coli) is shown as a function of increasing excess of competitor DNA (w/w). Panel TC_{20}: 0 (lane 0), 2.5 (lane 1), 5 (lane 2) and 10 (lane 3). Panels ssE.coli and ssM13: 25 (lane 1), 50 (lane 2) and 100 (lane 3). Panel dsE.coli: 250 (lane 1), 500 (lane 2) and 1000 (lane 3). Quantitative analyses of the results are shown on the right.

Purification of the NOGA3 polypeptides from these fractions is also achieved by fractionation through the PhenylSuperose column. As shown in Figure 3C, purified NOGA3 shows strong binding to single-stranded $d(TC)_{20}$ and the formation of two protein–DNA complexes of slightly different electrophoretic migration is detected at intermediate protein concentration [Fig. 3C, panel $d(TC)_{20}$, lanes 1 and 2], which is consistent with the two NOGA3 polypeptides being capable of binding single-stranded $d(TC)_{20}$. Purified NOGA3 does not show any significant binding to single-stranded $d(GA)_{20}$ or double-stranded $d(GA{\cdot}TC)_{20}$ [Fig. 3C, panels $d(GA)_{20}$ and $d(GA{\cdot}TC)_{20}$]. As judged from the competition experiments shown in Figure 3D, the interaction of NOGA3 with single-stranded $d(TC)_{20}$ is of high specificity, higher than that of NOGA1. No significant competition is observed upon the addition of up to a 100-fold excess of ss*E.coli*DNA or ssM13DNA. On the other hand, a 5-fold excess of $d(TC)_{20}$ completely abolishes the binding and 50% competition is obtained in the presence of just 1-fold excess of competitor (Fig. 3D). The affinity of NOGA3 for double-stranded DNAs

is very low since a very significant binding to $d(TC)_{20}$ is detected even in the presence of 1000-fold excess of ds*E.coli*DNA (Fig. 3D, panel dsE.coli, lane 3).

The N-terminal amino acid sequence of the NOGA3 polypeptide of higher electrophoretic migration (NOGA3a) was shown to correspond to murine hnRNP-I or polypyrimidine tract binding protein (PTB; residues 14–31) (Table 1) (27). Consistent with these results, NOGA3a is recognized by specific αhnRNP-I antibodies (Fig. 3B, lane 5). No sequence information could be obtained from the direct sequencing of the polypeptide of slow electrophoretic migration (NOGA3b). However, NOGA3b is also recognized by αhnRNP-I-specific antibodies, indicating that it corresponds to a closely related protein. Actually, purified hnRNP-I frequently appears as a triplet (27) and a third polypeptide of similar electrophoretic mobility is also recognized by the antibodies in crude nuclear extracts or partially purified fractions (Fig. 3B, lanes 1–4). This third polypeptide elutes from the PhenylSuperose column at lower ionic strength than the NOGA3 fractions and, though it does not bind single-stranded $d(TC)_{20}$ in the presence of NOGA3, it does show some weak binding once purified (not shown). The western blot analysis shown in Figure 3B corroborates the presence of the two NOGA3 polypeptides in the last high salt 2.0 M KCl elution of the $d(TC)_{20}$-affinity column (Fig. 3B, lane 4).

Purification and characterization of NOGA2 and NOGA4

NOGA2 elutes from the PhenylSuperose column at ~1.0–0.8 M KCl (Fig. 1B) and the corresponding fractions show a prominent polypeptide of ~66 kDa in addition to several other polypeptides (Fig. 4A, lane 4). Further purification of this activity was obtained by fractionation through a ResourceQ (Pharmacia) column that was loaded at 50 mM KCl and eluted with a 0.05–1 M KCl linear gradient. From this column, NOGA2 elutes at ~0.3–0.4 M KCl and, as shown by PAGE, corresponds to the 66 kDa polypeptide mentioned above (Fig. 4A, lane 5). The same binding activity is also present in the fraction corresponding to the 0.6 M KCl step elution of the $d(TC)_{20}$-affinity column. NOGA2, which is actually more abundant in the 0.6 M KCl fractions, can also be purified from these fractions through the same chromatographic steps. Purified NOGA2 binds single-stranded $d(TC)_{20}$ strongly (Fig. 4C) with a specificity similar to that shown by NOGA1 (compare Fig. 4D with Fig. 2D). NOGA2 binds single-stranded $d(GA)_n$ or double-stranded DNA poorly (Fig. 4C and D). No N-terminal sequence information was obtained from the direct analysis of the purified protein. In this case, an internal peptide was prepared by the proteolytic digestion of purified NOGA2 with thrombin and its N-terminal amino acid sequence showed full identity to that corresponding to an internal region of human hnRNP-K (residues 317–339) (Table 1) (28). In good agreement, purified NOGA2 was recognized by αhnRNP-K-specific antibodies (Fig. 4B, lane 6). The western blot analysis shown in Figure 4B corroborates the presence of NOGA2 in both the 2.0 and 0.6 M KCl step elutions of the $d(TC)_{20}$-affinity column (Fig. 4B, lanes 3 and 4).

NOGA4 is the least abundant of the $d(TC)_{20}$ binding activities we have detected. It elutes from the PhenylSuperose column at high ionic strength (Fig. 1B) and the corresponding fractions are not particularly enriched in any specific polypeptide (Fig. 5B, lane 4). Further purification of the fraction corresponding to NOGA4 activity was reached by fractionation

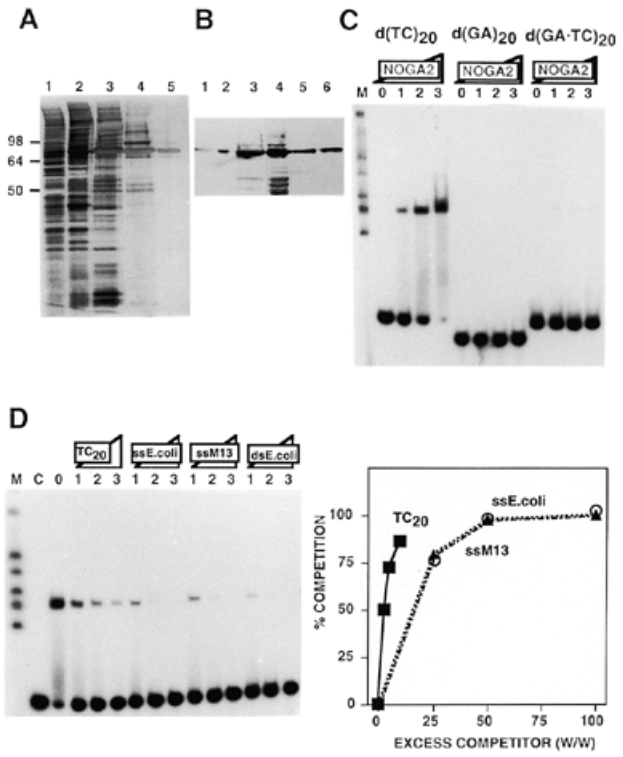

Figure 4. Purification and characterization of NOGA2. (**A**) 12% SDS–PAGE of: the crude nuclear extract (lane 1); the pool of the fractions containing the bulk of the $d(TC)_{20}$ binding activity which elute at 1.2–2.0 M KCl from the HiTrap-Blue column (lane 2); the pool of the fractions eluting from the $d(TC)_{20}$-affinity column at 0.6 M KCl (lane 3); the pool of the PhenylSuperose (lane 4) or ResourceQ (lane 5) fractions corresponding to the NOGA2 activity. The positions of markers of known molecular weight (in kDa) are indicated. (**B**) Western blot analysis using an αhnRNP-K-specific antibody of: the crude nuclear extract (lane 1); the pool of HiTrap positive fractions (lane 2); the pool of the fractions eluting from the $d(TC)_{20}$-affinity column at 2.0 M KCl (lane 3) or 0.6 M KCl (lane 4); the pool of the PhenylSuperose (lane 5) or ResourceQ (lane 6) fractions corresponding to the NOGA2 activity. (**C**) The binding of purified NOGA2 to single-stranded $d(TC)_{20}$, $d(GA)_{20}$ and double-stranded $d(GA{\cdot}TC)_{20}$ is analyzed by EMSA as a function of increasing purified protein. Panel $d(TC)_{20}$: 0 µl (lane 0), 0.1 µl (lane 1), 0.3 µl (lane 2) and 0.9 µl (lane 3). Panels $d(GA)_{20}$ and $d(GA{\cdot}TC)_{20}$: 0 µl (lane 0), 0.3 µl (lane 1), 0.9 µl (lane 2) and 2.7 µl (lane 3). Lane M corresponds to DNA fragments of known molecular weight used as markers as in Figure 1. (**D**) The efficiency of competition of single-stranded $d(TC)_{20}$ (TC_{20}), single-stranded *E.coli* DNA (ssE.coli), single-stranded M13 DNA (ssM13) and double-stranded *E.coli* DNA (dsE.coli) is shown as a function of increasing excess of competitor DNA (w/w). Panel TC_{20}: 0 (lane 0), 2.5 (lane 1), 5 (lane 2) and 10 (lane 3). Panels ssE.coli and ssM13: 25 (lane 1), 50 (lane 2) and 100 (lane 3). Panel dsE.coli: 250 (lane 1), 500 (lane 2) and 1000 (lane 3). Quantitative analyses of the results are shown on the right.

through a ResourceQ (Pharmacia) column loaded at 50 mM KCl and eluted with a 0.05–1 M KCl linear gradient from which it elutes at ~0.13 M KCl (Fig. 5A). The positive fractions are enriched in a polypeptide of ~50–52 kDa (Fig. 5A, lane 5). In this case, no sequence information was obtained from the direct sequencing of the purified protein. As in the case of NOGA2, the NOGA4 polypeptide is also found in the 0.6 M KCl step elution of the $d(TC)_{20}$-affinity column, from where it can be purified following the same chromatographic steps. The

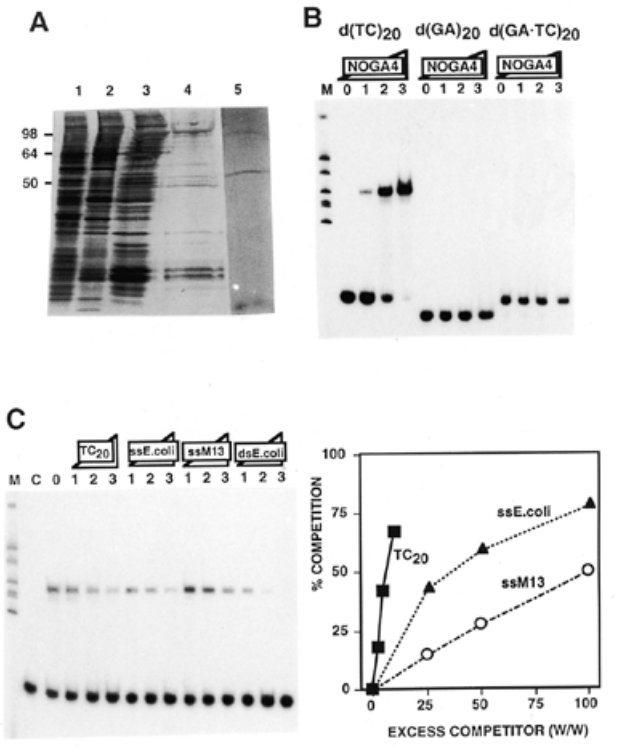

Figure 5. Purification and characterization of NOGA4. (**A**) 12% SDS–PAGE of: the crude nuclear extract (lane 1); the pool of the fractions containing the bulk of the $d(TC)_{20}$ binding activity which elute at 1.2–2.0 M KCl from the HiTrap-Blue column (lane 2); the pool of the fractions eluting from the $d(TC)_{20}$-affinity column at 0.6 M KCl (lane 3); the pool of the PhenylSuperose (lane 4) or ResourceQ (lane 5) fractions corresponding to the NOGA4 activity. The positions of markers of known molecular weights (in kDa) are indicated. (**B**) The binding of purified NOGA4 to single-stranded $d(TC)_{20}$, $d(GA)_{20}$ and double-stranded $d(GA·TC)_{20}$ is analyzed by EMSA as a function of increasing purified protein. Panel $d(TC)_{20}$: 0 µl (lane 0), 0.7 µl (lane 1), 2 µl (lane 2) and 6 µl (lane 3). Panels $d(GA)_{20}$ and $d(GA·TC)_{20}$: 0 µl (lane 0), 2 µl (lane 1), 6 µl (lane 2) and 18 µl (lane 3). Lane M corresponds to DNA fragments of known molecular weight used as markers as in Figure 1. (**C**) The efficiency of competition of single-stranded $d(TC)_{20}$ (TC_{20}), single-stranded *E.coli* DNA (ssE.coli), single-stranded M13 DNA (ssM13) and double-stranded *E.coli* DNA (dsE.coli) is shown as a function of increasing excess of competitor DNA (w/w). Panel TC_{20}: 0 (lane 0), 2.5 (lane 1), 5 (lane 2) and 10 (lane 3). Panels ssE.coli and ssM13: 25 (lane 1), 50 (lane 2) and 100 (lane 3). Panel dsE.coli: 250 (lane 1), 500 (lane 2) and 1000 (lane 3). Quantitative analysis of the results is shown on the right.

affinity of NOGA4 for single-stranded $d(TC)_{20}$ is high (Fig. 5B) but the specificity of this interaction is lower than in the cases of NOGA1–3, a significantly higher excess of $d(TC)_{20}$, ~6-fold, being required to reach 50% competition (Fig. 5C). The affinity of NOGA4 for single-stranded $d(GA)_{20}$ or double-stranded $d(GA·TC)_{20}$ is also low (Fig. 5B).

DISCUSSION

It is known that alternating $d(GA·TC)_n$ sequences can form different types of altered DNA structures *in vitro* (Fig. 6), that have been proposed to play a role(s) in a number of different biological processes (1–3). However, in the absence of specific stabilizing factors, as for any double-stranded DNA fragment, the regular B-DNA conformation is favored over any of the other non-B-DNA conformations. In the case of the $d(GA·TC)_n$ DNA sequences, the non-B-DNA conformers contain regions of single-stranded DNA that would have a high tendancy to re-bind to the complementary strand, destabilizing the altered DNA conformation and restoring the regular double-stranded character of the sequence. Nuclear proteins binding to these single-stranded regions, such as those described in this paper, could help to maintain them unpaired. Additional factors interacting with the structured elements of the conformers (triplex DNA, hairpins) could also play a crucial role in the stabilization of these altered DNA structures. Actually, several proteins have been shown to be capable of binding triplex DNA as well as homopurine DNA fragments *in vitro* (20–22,29). The four NOGA polypeptides described in this paper bind single-stranded $d(TC)_{20}$ fragments very strongly. Though each NOGA protein differs in the degree of specificity of its interaction with $d(TC)_{20}$, all four NOGAs show no significant affinity for either single-stranded $d(GA)_{20}$ or double-stranded $d(GA·TC)_{20}$ DNA fragments, a binding behavior that would be expected for proteins capable of stabilizing the [GA(GA·TC)] and/or the (GA·GA) intramolecular forms of the $d(GA·TC)_n$ sequences (Fig. 6).

NOGA1, -2 and -3 correspond to the single-stranded nucleic acid binding proteins hnRNP-L, -K and -I, respectively. In the case of NOGA4, we were not able to identify the corresponding polypeptide. However, NOGA4 corresponds to the slow migrating band of a triplet of polypeptides of similar molecular weight (Fig. 5A, lane 4) that, all through the chromatographic fractionation, show a similar behavior suggesting that they might correspond to related proteins. The fast migrating band, which is the most intense of the triplet, elutes from the ResourceQ column at ~0.15 M KCl as a single polypeptide that also binds $d(TC)_{20}$, though with a significantly lower affinity than NOGA4 itself (not shown). The N-terminal sequence of this polypeptide was shown to correspond to the mouse CArG box binding factor CBF-A, residues 2–13 (Table 1) (30), that also contains several single-stranded nucleic acid binding motifs.

Several indications strongly suggest that the NOGA proteins described here might also interact with DNA *in vivo*. Most of the proteins known to bind RNA, including the hnRNP proteins, are also capable of binding single-stranded DNA with similar, or even higher, affinity and/or specificity (31,32). Most frequently, ssDNA recognition takes place through the same structural domains involved in RNA binding. Secondly, several hnRNP proteins were shown to remain attached to RNase-treated lampbrush chromosomes being capable of binding ssDNA (33). Moreover, in several cases, RNA-binding proteins have been shown also to act at the DNA level *in vivo*. Actually, one of the best documented examples corresponds to hnRNP-K/NOGA2 that has been shown to interact, both *in vitro* and *in vivo*, with pyrimidine-rich sequences of the κB enhancer, the c-*myc* promoter, the regulatory region of the neuronal nicotinic acetylcholine receptor gene and the SV40 control region (34–38), influencing their expression levels. Interestingly, depending on the promoter and the activator, hnRNP-K/NOGA2 can either potentiate transcription or act as a repressor (38–43), suggesting that its effect on transcription regulation is indirect, perhaps through changes in DNA

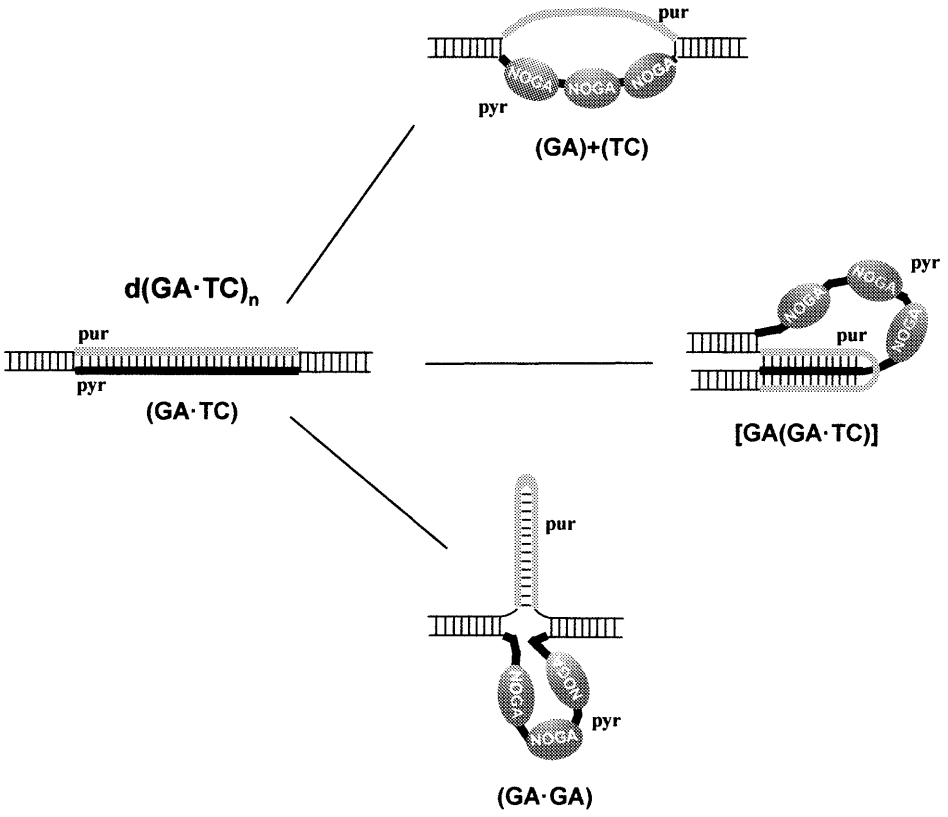

Figure 6. Non-B-DNA conformers of the $d(GA{\cdot}TC)_n$ DNA sequences and the potential contribution of NOGA proteins to their stabilization. The formation of $[C^+T(GA{\cdot}TC)]$ intramolecular triplexes is observed at acidic pH. At neutral pH, in the presence of some specific transition metal ions, the formation of [GA(GA·TC)] intramolecular triplexes and (GA·GA) hairpins is detected. The NOGA proteins described in this paper could be instrumental in the stabilization of the [GA(GA·TC)] intramolecular triplexes and/or (GA·GA) hairpins.

conformation. On the other hand, the role played by hnRNP-K/NOGA2 in RNA metabolism remains obscure though it appears to regulate translation of specific transcripts (44,45).

HnRNP-I/NOGA3 corresponds to PTB that was proposed to be involved in different aspects of RNA metabolism, particularly in RNA splicing (31,46). However, in addition to its functions in RNA processing, hnRNP-I/NOGA3 has also been shown to interact with pyrimidine-rich sequences of the rat tyrosine aminotransferase liver enhancer, the human transferrin promoter and the adenovirus major late promoter (47–49). HnRNP-I/NOGA3 shows a heterogeneous nuclear distribution, being concentrated in a few more intense spots, and contains four single-stranded nucleic acid binding domains which are only distantly related to the canonical RNP consensus sequence (31). HnRNP-L/NOGA1 appears to be closely related to hnRNP-I/NOGA3, sharing a similar molecular organization (four highly degenerated RNP motifs) and nuclear distribution (31,50). Interestingly, a positive interaction between these two proteins was demonstrated in yeast (50). HnRNP-L/NOGA1 contains an N-terminal glycine-rich domain, which is absent in hnRNP-I/NOGA3. Similar glycine-rich domains contained in related RNA-binding proteins, such as the *Drosophila* sex-lethal, have been shown to act as transcription activation domains in yeast (51). The role(s) of hnRNP-L/NOGA1 at the RNA level is still unclear, though it was proposed to be involved in the processing of intronless pre-mRNAs (52).

In the case of the CArG box-binding protein CBF-A, no specific RNA binding has yet been reported, although the protein contains canonical RNP motifs. In contrast, CBF-A was shown to bind CArG boxes located at the regulatory regions of various genes as well as telomeric DNA sequences both as single-stranded and tetra-stranded forms (30,53,54).

The four NOGA proteins studied here account for the majority of the single-stranded $d(TC)_n$ binding activities detected in crude nuclear extracts obtained from Friend's cells that, as shown in Figure 1A, give rise to three different types of complexes of increasing electrophoretic mobility. From the electrophoretic mobilities of the complexes obtained with the purified NOGA proteins, we conclude that NOGA1 and NOGA2 contribute to the complexes of higher electrophoretic mobility detected in crude extracts, while NOGA3 is responsible for the complexes of intermediate mobility. The slow migrating complexes detected in crude extracts are also likely to arise from the binding of NOGA3 since they are detected in the presence of increasing amounts of the purified protein (Fig. 3C). The complexes obtained with purified NOGA4 are not detected in crude extracts corroborating both the low abundance of the protein and its weak affinity and specificity for single-stranded $d(TC)_{20}$. These results indicate that murine

cells contain only a limited number of $d(TC)_n$ binding proteins that, as summarized in Figure 6, could participate in the *in vivo* stabilization of the non-B-DNA conformers of the $d(GA{\cdot}TC)_n$ sequences, setting up an appropriate framework from which to address the question of the functional consequences of their formation. As a consequence of its high flexibility, higher than duplex DNA, the formation of single-stranded DNA might facilitate the direct interaction of proteins bound at non-adjacent *cis* elements *in vivo*, as was recently proposed in the case of hnRNP-K/NOGA2 (55). Interestingly, the ability of hnRNP-K/NOGA2 to bind the pyrimidine-rich element of the c-*myc* promoter *in vivo* was demonstrated through a mammalian one-hybrid assay in which a fusion protein, carrying the VP16 transactivation domain, was found to activate transcription of a reporter gene when the corresponding promoter contains the pyrimidine-rich sequence mentioned above (41). Similar experiments, performed either in yeast or mammalian cells, would also be very informative in the evaluation of the ability of the rest of NOGA proteins to bind CT sequences *in vivo*.

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