J Biomol NMR (2009) 45:9–16
DOI 10.1007/s10858-009-9350-y

COMMUNICATION

Preparation, resonance assignment, and preliminary dynamics characterization of residue specific $^{13}C/^{15}N$-labeled elongated DNA for the study of sequence-directed dynamics by NMR

Evgenia N. Nikolova · Hashim M. Al-Hashimi

Received: 13 June 2009 / Accepted: 19 June 2009 / Published online: 28 July 2009

Abstract DNA is a highly flexible molecule that undergoes functionally important structural transitions in response to external cellular stimuli. Atomic level spin relaxation NMR studies of DNA dynamics have been limited to short duplexes in which sensitivity to biologically relevant fluctuations occurring at nanosecond timescales is often inadequate. Here, we introduce a method for preparing residue-specific $^{13}C/^{15}N$-labeled elongated DNA along with a strategy for establishing resonance assignments and apply the approach to probe fast inter-helical bending motions induced by an adenine tract. Preliminary results suggest the presence of elevated A-tract independent end-fraying internal motions occurring at nanosecond timescales, which evade detection in short DNA constructs and that penetrate deep (7 bp) within the DNA helix and gradually fade away towards the helix interior.

Keywords DNA dynamics · Spin relaxation · A-tract · Sequence-specific flexibility · Domain elongation

Sequence-specific DNA flexibility plays essential roles in a variety of cellular processes that are key for gene packaging, expression and regulation (Travers 2004; Garcia et al. 2007; Gimenes et al. 2008). For example, intrinsic sequence-specific DNA flexibility is believed to play an important function in directing adaptive changes in DNA conformation that occur following protein and ligand recognition (Travers 2004; Shajani and Varani 2007; Fujii et al. 2007). Local deformability at the dinucleotide level and unusual elements such as adenine tracts have been implicated in nucleosome positioning and DNA accessibility by the transcriptional machinery, potentially providing a new layer of genetic regulation (Segal et al. 2006; Fernandez and Anderson 2007; Kaplan et al. 2009; Segal and Widom 2009). Sequence-specific modifications such as damage can also modulate DNA flexibility, which is believed to actively participate in the efficient recruitment of repair enzymes (Fuxreiter et al. 2002; Isaacs and Spielmann 2004; Stivers 2008).

Solution NMR techniques based on measurements of spin relaxation data (Palmer et al. 2001; Palmer and Massi 2006; Mittermaier and Kay 2006; Shajani and Varani 2007) and residual dipolar couplings (RDCs) (Prestegard et al. 2004; Bax and Grishaev 2005) provide a unique opportunity to probe DNA flexibility at the site-specific level over picosecond to millisecond timescales (Kojima et al. 1998; Spielmann 1998; Isaacs and Spielmann 2001; Ying et al. 2006). More recent examples of carbon relaxation studies have revealed multiscale dynamics in a methyltransferase enzyme DNA target sequence (Shajani and Varani 2005, 2008; Echodu et al. 2008) and unusual high-amplitude motions in the deoxyribose moieties of cytosine residues (Duchardt et al. 2008; Wu et al. 2003), while RDCs have allowed the detailed construction of conformational ensembles of the Dickerson dodecamer (Schwieters and Clore 2007; Wu et al. 2003). Despite advances in the application of spin relaxation and RDC techniques in studies of DNA dynamics illustrated above, methodological challenges abound. As shown for RNA, extended nucleic acids can undergo large-scale fluctuations

Electronic supplementary material The online version of this article (doi:10.1007/s10858-009-9350-y) contains supplementary material, which is available to authorized users.

E. N. Nikolova · H. M. Al-Hashimi (✉)
Department of Chemistry, Biophysics and Chemical Biology Program, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109-1055, USA
e-mail: hashimi@umich.edu

in global structure, such as bending, that can result in coupled changes in overall motions (Showalter et al. 2005; Zhang et al. 2003, 2006, 2007; Musselman et al. 2007; Hansen and Al-Hashimi 2007; Zhang and Al-Hashimi 2008). This in turn can lead to a breakdown in the "decoupling approximation", which is at the crux of formalisms currently used to quantitatively interpret NMR spin relaxation data and RDCs (Lipari and Szabo 1981; Tolman et al. 1997, 2001). In addition, motions occurring at nanosecond timescales remain difficult to address by solution NMR because they fall outside the detection limit of both spin relaxation and relaxation dispersion techniques. While RDCs are in principle sensitive to such fluctuations, their broad timescale sensitivity makes it complicated to tease out individual contributions from motions occurring at specific rates.

Our laboratory has developed an NMR-invisible domain elongation approach for decoupling internal from overall motions in RNA (Zhang et al. 2006; Hansen and Al-Hashimi 2007). The elongation not only serves to resolve internal from global motions, it also predefines the overall diffusion and alignment tensor to be axially symmetric with principal axis oriented along the elongated helix, thus further simplifying analysis of spin relaxation (Hansen and Al-Hashimi 2007; Zhang et al. 2006) and RDC data (Zhang et al. 2007). By reducing the rate of overall tumbling, the elongation extends the timescale sensitivity of relaxation data deeper within the nanosecond regime (Zhang et al. 2006). When combined with measurements of nitrogen and carbon spin relaxation, and RDCs, this approach allowed for the characterization of a network of local and collective motions in RNA spanning pico-to-millisecond timescales that play key roles in adaptive recognition (Zhang et al. 2006, 2007; Hansen and Al-Hashimi 2007; Zhang and Al-Hashimi 2008).

Here, we extend the elongation methodology and tailor it towards the atomic characterization of dynamical features in DNA. Specifically, we present a strategy for preparation and assignment of long DNA duplexes (E-DNA) that are $^{13}C/^{15}N$-labeled in a residue-selective manner to probe inter-helical bending motions induced by an A-tract sequence (Marini et al. 1982; Wu and Crothers 1984). A-tracts are defined as contiguous A_n or A_nT_m (n, $m \geq 3$) DNA elements that assume a distinctive B′-form conformation originally characterized by fiber diffraction and featuring minor groove compression, negative base inclination, and large propeller twist (Arnott and Selsing 1974; Alexeev et al., 1987a, b). When phased with the helical periodicity ($\sim$10.5 bp), short A-tracts separated by GC-rich stretches induce macroscopic curvature observed originally in anomalous gel retardation and circularization profiles of kinetoplast-derived DNA (Marini et al. 1982; Hagerman 1985; Koo et al. 1986; Ulanovsky et al. 1986). Despite decades of investigation, it remains unclear whether A-tracts induce static or dynamic bends. Although there are conflicting views, most studies suggest that A-tract sequences themselves are "straight" and locally stiff but that A-tract junctions with neighboring B-form DNA are flexible, and possibly, the source of deformation and inter-helical bending, reviewed in Beveridge et al. (2004) and Haran and Mohanty (2009). This highlights one of the biggest challenges in the field of DNA dynamics, which is to experimentally distinguish "static" versus "dynamic" deformations in a B-form duplex structure. Using the DNA elongation approach, we have probed rapid nanosecond bending motions in A-tract containing duplexes using ^{15}N and ^{13}C spin relaxation combined with qualitative intensity analysis. While we do not observe any concrete evidence for nanosecond bending dynamics, our results uncover deep penetrating end-fraying nanosecond motions, which seem to be independent of the A-tract at physiological temperatures.

In the new DNA elongation strategy, a sequence of interest, such as an A-tract, or a lesion-bearing construct, is flanked by short and long B-form helical domains, which act as reporters of any inter-helical motions occurring across the target sequence. The longer helix dominates overall diffusion and alignment whereas the short helix reports on local as well as collective dynamics occurring across the DNA target sequence. The sequence is devised in such a way that enables the site-specific incorporation of labeled nucleotides at particular positions in each helical domain (Fig. 1). The design minimizes resonance overlap, which is key for dynamics measurements, and yet provides adequate domain-specific information. The elongated DNAs provide a unique opportunity to resolve "static" versus "dynamic" bends using NMR spin relaxation measurements, as previously described for RNA (Zhang et al. 2006). For example, static directional bends are expected to reduce the angle between the nucleobase C–H (or N–H) bonds in the short helix and the slowly tumbling long axis of the DNA, thus resulting in an increase in the observed correlation times relative to perpendicular bonds in the elongated helix. Conversely, dynamic bends at timescales faster than overall tumbling are expected to reduce the observed correlation times for C–H (or N–H) bonds in the short relative to the elongated helix.

The elongated DNA (E-DNA) duplexes shown in Fig. 2 consist of 56 bps ($\sim$35 kDa) and are optimized to probe collective helical bending motions activated by an A-tract element. The E-DNA duplexes are prepared in a series of enzymatic reactions involving the target DNA, an unlabeled primer pair, a thermostable DNA polymerase and only one type of uniformly $^{13}C/^{15}N$-labeled nucleotides (G or T). Various efficient PCR and primer extension methods for the synthesis of large quantities of isotopically labeled

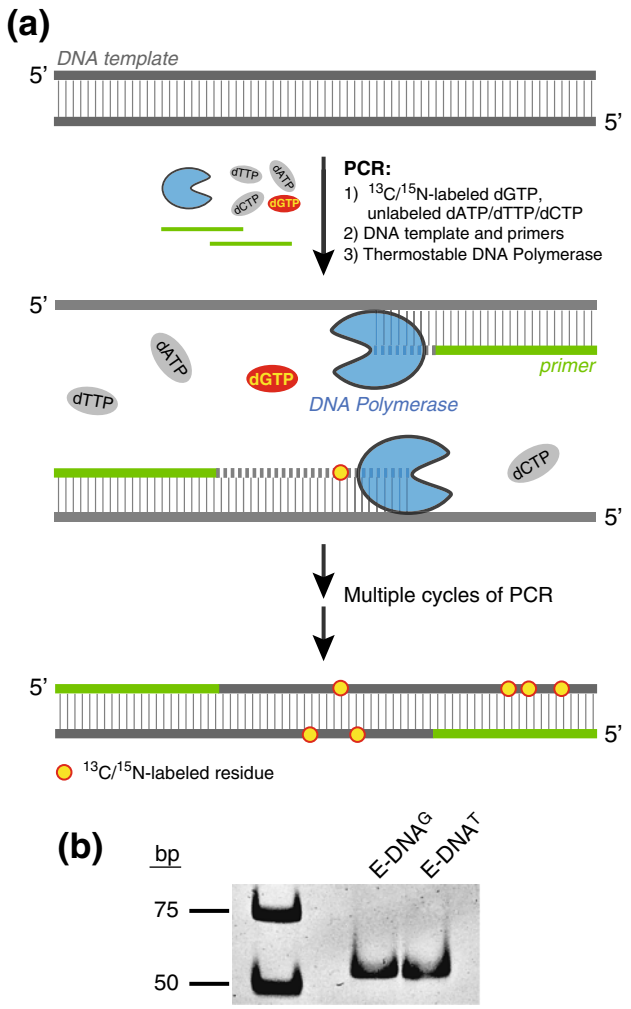

Fig. 1 PCR scheme for the synthesis of site-selective $^{13}C/^{15}N$-labeled E-DNA. **a** Shown is an example with isotopically labeled guanine (*G*) nucleotides, depicted as *yellow–red circles*. **b** 15% native polyacrylamide gel electrophoresis of 56 bp long E-DNA^{G} (*left*) and E-DNA^{T} (*right*) relative to a DNA standard

oligonucleotides with applications to NMR studies have been previously presented (Zimmer and Crothers 1995; Chen et al. 1998; Louis et al. 1998; Yan and Bushweller 2001; Masse et al. 1998) and are suitable for preparation of short or non-site-specifically labeled DNA. Here, a traditional PCR-based method combined with an appropriate sequence design and labeled nucleotide selection was implemented for the in vitro preparation of E-DNA (Fig. 1). Although the synthesis requires multiple reaction runs, current advances in large-scale PCR can facilitate the rapid production of E-DNA samples in NMR-friendly quantities. We prepared the three constructs shown in Fig. 2a, in which the identity of the labeled nucleotides and partially the sequence around them are altered. E-DNA^{G} and E-DNA^{T} contain G and T $^{13}C/^{15}N$-labeled residues, respectively, and share the same primer-defined regions, including the A-tract element and the short helical domain II. E-DNA^{GCtr} is used as a control and is identical to E-DNA^{G} except that the A_6-tract is replaced with an $(AC)_3$ element. The final DNA constructs contain three isotopically labeled nucleotides per domain placed in a unique sequence environment in order to reduce spectral crowding (Fig. 2a).

As shown in Fig. 2b (Fig. S1), the specific labeling strategy allowed us to collect well-resolved, excellent quality 2D spectra for the 56 bp E-DNA. The sparsely labeled, elongated DNA requires a non-traditional approach for resonance assignment given its large molecular size and mostly non-sequential positioning of the labeled nucleotides. To this end, we have implemented a "divide and conquer" approach for assigning resonances in E-DNA (Fig. 2). Here, assignments are first determined using conventional methods for unlabeled short duplexes (S-DNA) corresponding to the labeled fragments from the individual domains in E-DNA (Fig. 2a). The S-DNA constructs (Fig. S2) preserve the sequence identity of nearest neighbors and are expected to have chemical shifts comparable to those of labeled probes in E-DNA. The E-DNA resonances were then assigned by overlaying natural abundance 2D correlation maps (combined with 1D imino spectra) of S-DNA with those of E-DNA. As shown in Fig. 2 (Fig. S2), we observe excellent chemical shift correspondence between short and long DNA constructs allowing us to unambiguously assign the far majority of sugar and nucleobase resonances in the elongated duplexes. Since chemical shifts are sensitive reporters of nucleic acid structure, this observation supports local structural similarity between the short and long DNA molecules with the exception of the A-tract junction (Fig. S2). Decomposition of the E-DNA sequence into small fragments can also be employed in the reverse manner; inexpensive unlabeled DNA sequences can be used to predict optimal spectral quality and avoid potential overlap prior to the synthesis of large constructs.

Despite the agreement in chemical shift, 2D spectra of E-DNA^{G} and E-DNA^{T} recorded at 37°C featured marked differences in resonances intensities that were not apparent in corresponding short DNA duplexes. For example, simple examination of the spectra of Fig. 2 clearly shows that residue G53 in the short helix has elevated intensities compared to G83 in the elongated helix. Measurements of sugar backbone (C1′H1′) and base ($C7^{m}H7^{m}$, C8H8, N1H1, N3H3) resonance intensities revealed an interesting pattern in which values for the short helix are two to threefold higher than those belonging to the long helix (Fig. 3 and Fig. S3). This data suggest that residues in the short helix experience a greater degree of picosecond-to-nanosecond internal motions compared to residues in the elongated helix and/or that residues in the long helix experience a

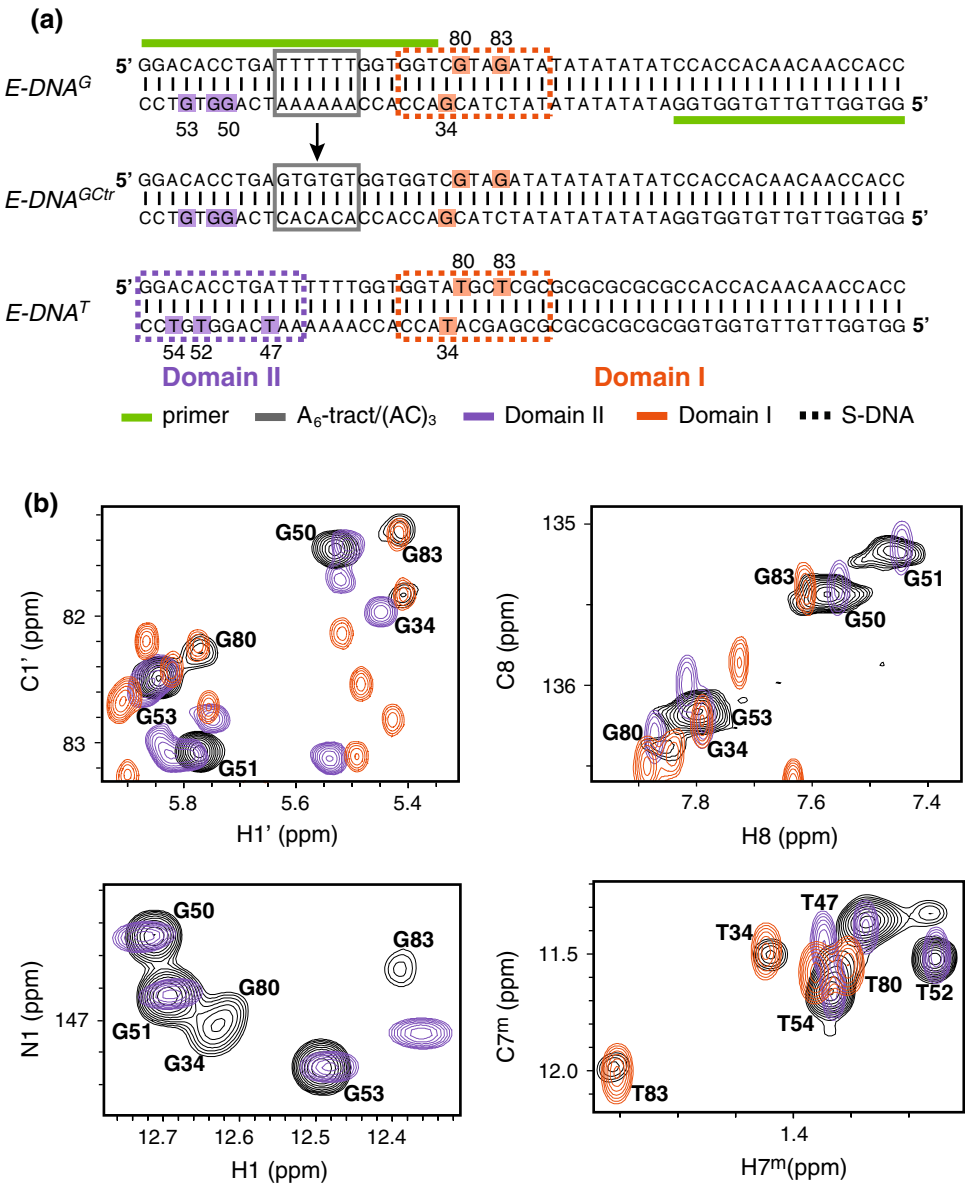

Fig. 2 Preparation and resonance assignment of E-DNA. **a** Sequence of the E-DNA constructs (56 bp) showing the location of the A_6-tract or $(AC)_3$ (*solid black*), the PCR primers (*solid green*), and the overlapping sequence with short S-DNA duplexes (*dashed purple/orange*) used for resonance assignments of E-DNAs. $^{13}C/^{15}N$-labeled residues are boxed and numbered. **b** 2D HSQC (S^3CT) or HMQC spectral overlays of E-DNA^G/E-DNA^T (*black*) with S-DNA (*purple*) and S-DNA^G/S-DNA^T (*orange*) for sugar C1'H1' (*top left*), base C8H8 (*top right*), imino N1H1 (*bottom left*), and methyl $C7^mH7^m$ (*bottom right*) showing excellent chemical shift correspondence

greater degree of linewidth broadening due to chemical exchange at the μs–ms timescale. A similar pattern of resonance intensities is observed in the E-DNA^{GCtr} control sample, indicating that the observed motional process is not induced by the A-tract (Fig. 3a).

To rule out the possibility of chemical exchange and gain further insights into the motions in E-DNA, we measured ^{15}N and ^{13}C transverse relaxation rate constants for the sugar C1′ and imino N1 nuclei in E-DNA^G and E-DNA^{GCtr} (Fig. 3b). Such quantitative measurements presented a challenge given the large size and relatively low concentration (0.1–0.2 mM) of the E-DNA samples. To overcome this problem, we used $R_{1\rho}$ experiments that employ selective Hartmann-Hahn polarization transfers to excite specific spins of interest and collect data in a 1D manner (see Supporting Information for details; Pelupessy et al. 1999; Korzhnev et al. 2005; Hansen et al. 2009). The 1D acquisition scheme affords shorter data collection times and thus improved sensitivity over corresponding 2D schemes. The experiment interrogates individual peaks in a single run and was thus perfectly suited for probing the small number of well-resolved resonances in E-DNA.

The on-resonance $R_{1\rho}$ relaxation data paint a picture analogous to that obtained from examination of resonance intensities. Similar R_2 values were measured in E-DNA^G and E-DNA^{GCtr} indicating that the A-tract does not induce fluctuations detectable by spin relaxation measurements under our experimental conditions (Fig. 3b). For both

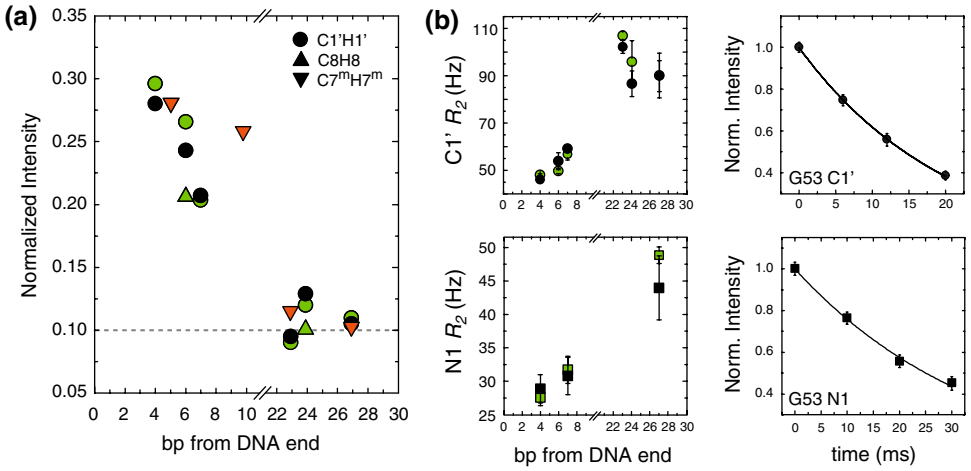

Fig. 3 Dynamics characterization of E-DNA. **a** Resonance intensities as a function of distance (in bp) from the DNA ends showing elevated motions in helical domain II. Data was derived from 2D correlation spectra of sugar C1'H1' (*circle*) and base C8H8 (*triangle*) and $C7^mH7^m$ (*inverted triangle*) recorded at 37°C (see Fig. 2a). Different constructs are color-coded with *green* ($E\text{-}DNA^G$), *black* ($E\text{-}DNA^{GCtr}$), and *red* ($E\text{-}DNA^T$) (see also Fig. S3). **b** ^{13}C (C1′, *top*) and ^{15}N (N1, *bottom*) R_2 relaxation rates (*left panels*) supporting fast dynamics in domain II. Data was collected using selective 1D $R_{1\rho}$ relaxation experiments with the spinlock carrier placed on-resonance and spinlock field strength of 3.5 kHz for ^{13}C and 1.0 kHz for ^{15}N (see Supplementary Information for details). Right panels show sample monoexponential decays for G53 in $E\text{-}DNA^G$ (Korzhnev et al. 2005; Hansen and Al-Hashimi 2007)

$E\text{-}DNA^G$ and $E\text{-}DNA^{GCtr}$, the R_2 data measured in the elongated domain is in excellent agreement with expectations based on a hydrodynamically estimated overall tumbling rate of $\sim$ 24 ns (37°C) and restricted internal motions ($S^2 \sim 0.85$; Fig. 3b). In contrast, for both $E\text{-}DNA^G$ and $E\text{-}DNA^{GCtr}$, the R_2 values measured for the shorter helix II were significantly smaller. The differences in R_2 measured in the long and short helices cannot be explained simply based on differences in the orientation of spin interactions relative to the elongated axis. First, as described in applications to E-RNA (Hansen and Al-Hashimi 2007; Zhang et al. 2006), for the nucleobases, the carbon and nitrogen interactions in the long helix are already positioned optimally perpendicular to the long axis and thus correspond to the lower limit of R_2 rates that can be measured in the absence of motions due to anisotropic tumbling. Second, hydrodynamic computations on an E-DNA model incorporating a bent A-tract verses a straight A-tract suggest that the curvature induces minor differences in R_2 in the opposite direction from what is observed in relaxation measurements (data not shown). Thus, the relaxation data exclude differential chemical exchange broadening as the source of large variations in resonance intensities and suggest the presence of internal motions at picosecond-to-nanosecond timescales.

Interestingly, the internal motions are not observed in short (13 bps) DNA constructs containing identical sequences. However, upon introduction of glycerol (18% w/v) to a short DNA duplex (14 bp, $\tau_m \approx 6$ ns) to increase solvent viscosity and decrease the rate of overall tumbling, we observed a relative increase in the intensities of resonances belonging to terminal as well as internal residues (Fig. S6). This suggests that the local fluctuations resolved in E-DNA occur at timescales comparable to overall tumbling of short DNAs (typically < 6 ns) and thus evade detection, as has been observed in a number of different RNA contexts (Zhang et al. 2006; Getz et al. 2007; Sun et al. 2007).

While the current preliminary analysis cannot allow for a detailed characterization of the nature of the motions, the resonance intensities and the relaxation data measured in the E-DNA indicate that the motions are gradually attenuated as a function of distance from the terminal end and are completely diminished at centrally positioned residues (Fig. 3b). It is possible that end-fraying motions occurring at nanosecond timescales reverberate deep (seven base-pairs) within the helix and gradually fade away as one penetrates further into the helix interior. This observation is supported by recent theoretical framework for spin-lattice relaxation of spin probes in weakly deformable DNA with internal flexibility (Smith et al. 2008). Moreover, the fast transitions could be accompanied by torsional or flexural backbone deformations potentially coupled to these internal modes near the DNA ends. Although the positional effect seems to dominate the trend, we cannot exclude contributions from sequence-specific local dynamics. Additional constructs, which contain domain-swapped residues, could be used in the future to resolve this ambiguity.

Finally, we note that our results do not rule out the presence of bending motions that occur at timescales much slower than can be probed by spin relaxation. Preliminary results on a short duplex containing the equivalent A_6-tract

provide evidence for microsecond exchange dynamics near the A-tract junctions, which could activate slower bending dynamics (manuscript in preparation). Measurements of additional relaxation data as well as RDCs in E-DNA should provide insights into the nature of both local and bending motions over a wider range (ps–ms) of timescales. A-tract induced bending has also been shown to be strongly dependent on temperature and presence/absence of divalent ions such as Mg^{2+} (Marini et al. 1984; Koo et al. 1986; Levene et al. 1986; Herrera and Chaires 1989; Chan et al. 1990, 1993; Jerkovic and Bolton 2001; Stellwagen et al. 2001; Hud and Feigon 2002; Movileanu et al. 2002; Tchernaenko et al. 2003, 2004). The high temperature and lack of Mg^{2+} ion conditions used in our experiments are believed to suppress A-tract induced bending. Thus, it will be important to also perform NMR dynamics studies at variable ambient conditions to comprehensively explore the landscape of A-tract induced dynamics.

In conclusion, we have implemented an approach for preparation, assignment, and analysis of dynamics in elongated DNA using NMR. Our results establish the ability to quantitatively study the dynamics of DNA, which are significantly larger and more anisotropic than customarily studied by NMR relaxation methods. We have detected rapid nanosecond motions that are manifested and enhanced when the residues approach the terminal ends and that likely can be explained due to a combination of internal position- and sequence-specific motions and possibly coupled backbone deformations. These effects are not observed in non-elongated DNA samples, indicating that the motions detected here occur at timescales greater than 6 ns. They comprise an interesting finding that could have implications, for instance, in the sensing and trapping of thermally driven transient fluctuations by DNA unwinding helicases at replication forks or other DNA end-interacting proteins (Delagoutte and von Hippel 2002). The observation of such gradual deep-penetrating end effects, which are normally thought to be localized at the first two base pairs, highlights the kinds of new dynamical insights that can be obtained from the new NMR domain elongation. In future experiments, we will resort to RDCs, variable length constructs and ambient conditions to probe these fraying effects and potentially slower bending motions with timescale sensitivity extending up to milliseconds.

Acknowledgments We thank Dr. Alexandar L. Hansen for stimulating discussions and for critically reading the manuscript and Dr. Alexander V. Kurochkin for maintenance of the NMR instruments. We gratefully acknowledge the Michigan Economic Development Cooperation and the Michigan Technology Tri-Corridor for support in the purchase of a 600 MHz spectrometer. This work was supported by a National Science Foundation CAREER award (MCB 0644278) received by H.M.A. E.N.N. was supported by a Rackham International Fellowship awarded by the University of Michigan.

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