# Nitrosamine-Induced Cancer: $O^4$-Alkylthymine Produces Sites of DNA Hyperflexibility†

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ABSTRACT: The carcinogenic properties of *N*-nitroso compounds are associated with their ability to alkylate DNA, in particular to form $O^6$-alkylguanine and $O^4$-alkylthymine. DNA duplexes containing either $O^6$-alkylguanine or $O^4$-alkylthymine were synthesized, and each duplex was ligated to form a set of DNAs of increasing length with the alkylated base out of phase (16 base-pairs apart) or in phase (21 base-pairs apart) with the helical repeat of the DNA. The DNA contained the sequence 5′ CAA 3′, which is the 61st codon of the K-*ras* gene, because this codon is a preferred site of mutation for a number of carcinogens including the methylating carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). $O^4$-Methylthymine or $O^4$-ethylthymine replaced thymine in either of the two A·T base-pairs of this codon (normally CAA), and $O^6$-methylguanine replaced the guanine in the G·C pair. All the sequences containing $O^4$-alkylthymine exhibited anomalous, slow, gel migration and ligated to form circles of unusually small diameter. In general, the effect was seen when the alkylated base-pair was out of phase with the helical repeat as well as when it was in phase, suggesting that the alkylated base-pair confers flexibility which is largely isotropic, i.e., has no preferred direction, rather than anisotropic flexibility or bending. However, at pH 8.3 the 21-base-pair set containing $O^4$-alkylT·A had significantly greater anomalous migration than the 16-base-pair set, suggesting that the flexibility produced by this base-pair has a significant anisotropic component and thus resembles true bending. This anisotropic component was eliminated when the pH of the gel was decreased from 8.3 to 6.5. NMR studies have previously suggested that the adenine in this base-pair is protonated at pH 5.5 and that this proton forms a hydrogen bond between $N^1$ of the purine and $O^2$ of the pyrimidine. Protonation of adenine at pH 6.5 (but not at 8.3) and the consequent formation of a second hydrogen bond in the base-pair may explain the difference in electrophoretic behavior at the different pHs. DNA duplexes containing $O^4$-alkylT·A base-pairs were more retarded and had lower thermal melting points ($T_m$) than DNA duplexes containing $O^4$-alkylT·G base-pairs. The effect of $O^4$-methylthymine was much greater than that of $O^6$-methylguanine, and the effect of $O^4$-ethylthymine was slightly greater than that of $O^4$-methylthymine.

DNA molecules become curved when certain sequences are repeated in phase with the periodicity (10.5 base-pairs) of B-DNA [for recent reviews see Crothers et al. (1990) and Hagerman (1990)]. Most of the DNA loci of biological significance now considered to be curved, among them promoters (Struhl, 1985; Ryder et al., 1986; Plaskon & Wartell, 1987; Kawamoto et al., 1989) and origins of replication (Zahn & Blattner, 1985; Anderson, 1986; Dean et al., 1987; Williams et al., 1988; Eckdahl & Anderson, 1990), share the same motif of dA·dT tracts half a helical turn long separated by an integral number of helical turns (Wu & Crothers, 1984). The question of whether curvature per se is of functional importance, or whether the dA·dT tract is important with curvature being fortuitous and insignificant, is yet to be answered. There are, however, a number of reports suggesting that a correlation exists between the degree of DNA curvature and biological function (Deb et al., 1986; Bracco et al., 1989; Collis et al., 1989) and between curvature and DNA recognition by proteins (Zahn & Blattner, 1987). The postulate (Husain et al., 1988) that some DNA repair enzymes recognize loci of DNA curvature rather than the DNA sequence itself is particularly attractive because a damaged base has to be recognized irrespective of the surrounding DNA sequence.

*N*-Nitroso compounds are potent carcinogens in most species. Their carcinogenic effect results from the alkylation of DNA, in particular alkylation of the $O^6$ position of guanine (Loveless, 1969) and the $O^4$ position of thymine (Lawley et al., 1973). Both of these modified bases are pro-mutagenic: the presence of $O^6$-alkylguanine leading to the incorporation of thymine during DNA replication (Abbott & Saffhill, 1979), and that of $O^4$-alkylthymine leading to the incorporation of guanine (Singer et al., 1978; Saffhill, 1985). These mutations are believed to initiate carcinogenesis (Zarbl et al., 1985; Kumar et al., 1990), but *N*-nitroso compounds are complete carcinogens and can bring about all the multistage processes of carcinogenesis, presumably including the subtle changes in gene expression associated with promoters such as the phorbol esters. The mechanism by which nitroso compounds carry out these changes is not known, but it is possible that the conformational changes in DNA following alkylation play a role. A further reason for studying the conformation of alkylated DNA is to understand how $O^6$-alkylguanine-DNA-alkyltransferase, which removes the alkyl groups from $O^6$-alkylguanine and $O^4$-alkylthymine in DNA and is the most important protective response to these carcinogens [reviewed by Lindahl and Sedgwick (1988); Pegg, 1990], recognizes the alkylated base. For these reasons we have measured the extent of DNA bending and other conformational changes produced by the presence of $O^4$-alkylthymine and $O^6$-alkylguanine. This was assessed by nondenaturing gel electrophoresis. Curved

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DNA migrates significantly slower than normal DNA during electrophoresis on a nondenaturing polyacrylamide gel. This property, first observed in a study of kinetoplast DNA (Marini et al., 1982, 1984), led to the discovery of DNA curvature and has been used in almost every subsequent investigation of DNA curvature. The melting point ($T_m$) of the alkylated DNA was also measured, providing a measure of the destabilizing effect of the altered base-pair.

Within the oligomer used was the sequence 5′ GT CAA GAG 3′ corresponding to that around codon 61 (CAA) of the mouse K-*ras* gene. *ras* genes are activated by mutation of codons 12, 13, or 61 in a large number of animal tumors induced by chemical carcinogens [reviewed by Balmain and Brown (1988)]. This particular sequence was chosen because mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), an important carcinogen in tobacco-induced cancer, had the K-*ras* oncogene activated via point mutations in codon 61 as well as in codon 12 (Belinsky et al., 1989, 1990). The T·A to C·G mutations occurred in the second position of this codon and could be explained by miscoding following the formation of $O^4$-alkylthymine.

While this work was in progress Voigt and Topal (1990) reported that $O^6$-methylguanine produces a small destortion of DNA. We have shown that $O^4$-methylthymine and $O^4$-ethylthymine produce much greater conformational distortion than $O^6$-methylguanine. The conformational change produced by $O^4$-alkylthymine was primarily an increase in the flexibility of the DNA and was produced by both $O^4$-alkylT·A and $O^4$-alkylT·G base-pairs. Only in the case of $O^4$-alkylT·A at pH 8.3 did this flexibility have an anisotropic component suggesting a curved structure; but at pH 6.5 both $O^4$-alkylT·A and $O^4$-alkylT·G base-pairs appeared to produce an isotropic increase in flexibility.

## Materials and Methods

*Chemicals and Enzymes.* Acrylamide/methylenebisacrylamide (19:1 w/w) was from Sigma, and all other general chemicals were from Aldrich or BDH. [$\gamma$-$^{32}$P]ATP (sp radioact. 3000 Ci/mmol) was from the NEN Division of Du Pont; T4 DNA ligase and polynucleotide kinase were from New England Biolabs; alkaline phosphatase and snake (*Crotalus durrissus*) venom phosphodiesterase were from Sigma.

*Oligonucleotide Synthesis.* Oligonucleotides were made with a Cruachem automatic machine (Cruachem Ltd., Glasgow, G20 0UA, Scotland). The oligonucleotide sequences are shown in Figure 1. The modified bases were $O^4$-methyl- and $O^4$-ethylthymine at either positions 1 or 2 and $O^6$-methylguanine at position 3. The oligomers containing $O^6$-methylguanine were prepared as before (Smith et al., 1990). Those containing $O^4$-methyl- and $O^4$-ethylthymine were prepared by a new technique in which a chemically reactive pyrimidine derivative is incorporated into the oligonucleotide and then converted into the $O^4$-alkylthymine after synthesis (Xu & Swann, 1991). This technique has the advantage that the product of a single oligonucleotide synthesis can be subdivided and a number of 4-substituted pyrimidines can be made from it. After cleavage from the solid support and removal of the base and phosphorus-protecting groups, the oligomers with the 5′-terminal (4,4′-dimethoxytriphenyl)methyl (DMT) protection still on were partially purified, and the DMT group was removed using NENsorb Prep cartridges (Du Pont) according to the maker's instructions. Then they were purified by ion-exchange chromatography. The presence of the correct modified base and the completeness of the deprotection was confirmed by base analysis (Graves et al., 1989).

*$^{32}$P Labeling and Formation of Ligation Ladders.* The oligomers containing the modified base, i.e., the top strand in Figure 1, was 5′-phosphorylated using 5 $\mu$L of 10 mM ATP and 10 units of T4 kinase in 40 $\mu$L of 50 mM Tris-HCl/10 mM $MgCl_2$/7 mM dithiothreitol (DTT). After 30 min of incubation at 37 °C, the kinase was inactivated by heating at 65 °C for 10 min. The complementary DNA strand, i.e., the bottom strand in Figure 1, was 5′-$^{32}$P-labeled with 50 $\mu$Ci of [$\gamma$-$^{32}$P]ATP under the conditions described above except that after the initial 30 min of incubation, 5 $\mu$L of 10 mM ATP was added and the incubation was continued for 30 min to ensure complete 5′-phosphorylation. Forty picomoles of the top strand in Figure 1 and 20 pmol of the $^{32}$P-labeled complementary strand were annealed in 67 $\mu$L of 66 mM Tris-HCl, pH 7.6/6.6 mM $MgCl_2$/1 mM ATP by heating to 80 °C and slow cooling to room temperature. Subsequently, 7.5 $\mu$L of 100 mM DTT and T4 DNA ligase (0.1 Weiss unit) were added, and the ligation reaction was carried out at room temperature. At 5, 10, and 15 min, 25 $\mu$L was transferred into a single tube containing 25 $\mu$L of 2% SDS and 1 $\mu$L of 500 mM EDTA. Mixing aliquots of different incubation times was found to give more even size distribution of oligomers. Samples were extracted with 100 $\mu$L of phenol/chloroform (1:1); DNA was precipitated by the addition of 300 $\mu$L of ethanol and resuspended in 30 $\mu$L of Tris–EDTA, pH 8, containing 5% glycerol, bromophenol blue, and xylene cyanol.

*Gel Electrophoresis.* The ligation products were electrophoresed on a nondenaturing 4% polyacrylamide gel at room temperature until the bromophenol blue had migrated 25 cm. The buffer for electrophoresis at pH 8.3 was 89 mM Tris–borate/10 mM EDTA, and that for electrophoresis at pH 6.5 was 89 mM Tris–phosphate/10 mM EDTA. The voltage applied at pH 8.3 was 10 V/cm, but the gel formed in the pH 6.5 buffer had a much lower resistance and this voltage drop would have produced significant heating of the gel. Any heating of the gel is undesirable in these experiments since it has been shown that DNA duplexes undergo conformational changes in temperatures below the $T_m$ (Diekmann, 1987). Thus, in the acidic gels a voltage drop of 5 V/cm was applied which gave the same power input (4–5 W) as at pH 8.3. Following electrophoresis the gels were dried and autoradiographed. The autoradiographs were scanned by a Joyce-Loebl scanning densitometer (Chromoscan 3; Joyce-Loebl, Marquisway, Tyne and Wear, England). The relative mobilities were calculated by dividing the distance migrated by each alkylated oligomer by the distance of migration of the control oligomer with the same number of base-pairs. The curved molecules migrate more slowly as though they were larger than the control DNA. The *K*-factor (Diekmann, 1987) is the ratio of apparent length to actual chain length and was obtained using as molecular weight markers the $(dN_{16})_x$ polymers. A graph of the logarithm of the molecular weight vs the mobility of the control $(dN_{16})_x$ oligomers gave the linear equation from which the apparent lengths of the other oligomers were calculated. *K*-factors were then plotted vs the number of repeats of each polymer.

*DNA Melting Curves.* Oligonucleotides 21 bases long were synthesized having sequence complementary to that of the top strand of Figure 1 so that blunt-ended 21-mer duplexes could be formed. For the $T_m$ determination, 1 nmol of each complementary strand was annealed in a volume of 0.6 mL and in a solution containing 1 mM Tris-HCl, pH 8.3, 0.1 mM EDTA, 1 mM $MgCl_2$, and 25 mM NaCl. The temperature-dependent change in absorbance at 260 nm was followed using a CARY 3 spectrophotometer connected to a Cary temperature controller (Varian Techtron Pty Ltd, Australia).

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A) 21-MER

positions                    123
              GGACC.TCC.CTC.TTG.ACC.TGC.T
                 G.AGG.GAG.AAC.TGG.ACG.ACCTG


B) 16-MER

positions                 123
              GGACC.CTC.TTG.ACGC.T
                 G.GAG.AAC.TGCG.ACCTG
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FIGURE 1: Sequences used for the formation of ligated polymers containing base-pair substitutions (A) in phase and (B) out of phase with the helical repeat. The numbers above each duplex refer to the positions where base-pair substitutions were introduced.

The rate of temperature increase was 0.5 deg/min. The $T_m$ values were determined as the maximum values of the first-derivative graph of the absorbance vs temperature.

## Results

Oligonucleotides containing $O^4$-methylthymine, $O^4$-ethylthymine, and $O^6$-methylguanine were synthesized, 16 or 21 bases in length, and annealed with the complementary strands forming 12- and 17-base pair duplexes with 4-residue single-strand overhangs (Figure 1). In the duplexes $O^4$-alkylthymine was paired with either adenine or guanine, and $O^6$-methylguanine was paired with cytosine. To see whether the neighboring bases have an effect on the structural change in DNA caused by $O^4$-alkylthymine, the modified base was placed in two of the positions normally occupied by thymidine. $O^6$-Methylguanine replaced the guanine in the G·C following the T·A base-pairs which in other oligomers were replaced with $O^4$-alkylthymine. Analogous sequences containing the normal T·A base-pair and sequences containing T·G and C·A mismatches were also included for comparison.

Assuming that there are 10.5 base-pairs per helical turn of B-DNA, ligation of the 21-mers containing one modified base would give a series of oligomers $(dN_{21})_x$ with the lesion always on the same side of the DNA helix (in phase). Any asymmetric distortion of the DNA produced by these modified bases would be additive, producing a plane curved structure. A curved DNA molecule has less electrophoretic mobility than normal DNA. However, bending is not the only cause of abnormal migration (Hagerman, 1990). Changes in mobility not connected with bending were evaluated by electrophoresis of ligated oligomers with 16-base-pair sequence repeats $(dN_{16})_x$. These would have the alkylated bases positioned every one and a half helical turns, and the asymmetric effect of one alkylated base would be counteracted by the next alkylated base because it would lie on the other side of the helix. Electrophoresis was carried out at the normal alkaline pH 8.3 and also at pH 6.5.

A typical autoradiograph of the gel after electrophoresis of ligated 16-mers at pH 8.3 is shown in Figure 2. From the autoradiograph, relative mobilities were obtained as described under Materials and Methods and plotted versus the number of 16-base-pair repeats in the polymer (Figure 4A). The DNA containing a T·G mismatch at every 16th base-pair migrated at the same rate as the control sequence. All oligomers containing $O^4$-alkylthymine migrated more slowly than the corresponding control sequences. In summary: (1) sequences containing alkylT·A migrated considerably more slowly than the corresponding sequences containing alkylT·G; (2) sequences containing ethylT paired either to A or to G migrated more slowly than the corresponding sequences containing methylT; (3) the effect of alkylT was greater when it was in position 1 than in position 2 (Figure 4A). The rank order of *reduced* mobility for the $(dN_{16})_x$ oligomers was

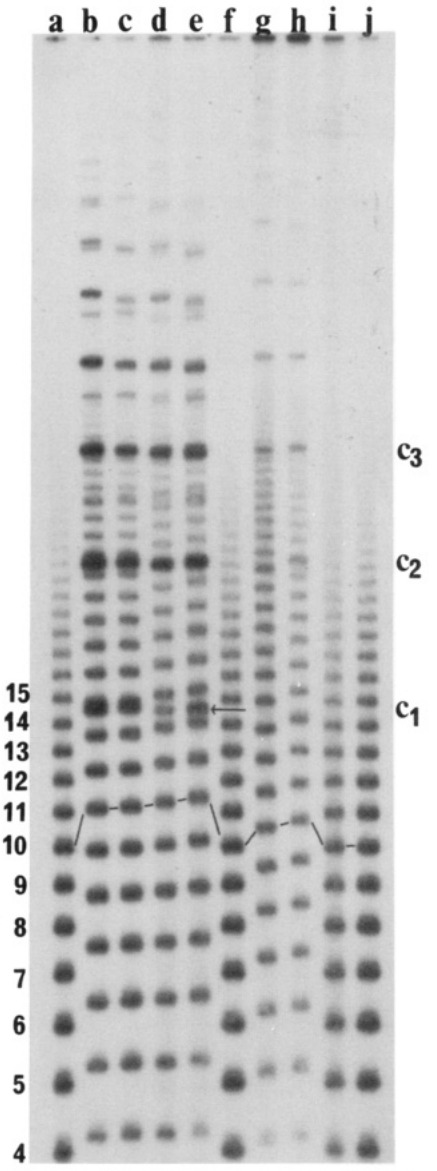


FIGURE 2: Nondenaturing gel electrophoresis of ligated 16-mer oligomers on a 4% polyacrylamide gel at pH 8.3. Oligomers were ligated and electrophoresed as described under Materials and Methods. The number of 16-base-pair monomers in each band is indicated along the left side of the autoradiograph. The cyclic products are indicated along the right side of the autoradiograph. Bands corresponding to multimers of the same length (160 base-pairs) are indicated. Control ligation ladder: lanes a, f, and j. At position 2, the oligomers contain meT·A (lane b), etT·A (lane c), meT·G (lane g), etT·G (lane h), or T·G (lane i). At position 1, the oligomers contain meT·A (lane d) or etT·A (lane e).

etT·A position 1 > meT·A position 1 >
etT·A position 2 ≥ meT·A position 2 ≫ etT·G position 2
> meT·G position 2 > T·G position 2 = T·A

These $(dN_{16})_x$ ligation ladders showed two families of bands (Figure 2). The first comprised the linear DNA molecules while the other consisted of circular molecules. The rate at which T4 DNA ligase converts linear DNA fragments into circles reflects the flexibility of the DNA molecule (Shore et al., 1981; Ulanovsky et al., 1986). There was a correlation between the formation of circles and the degree of retardation of the linear molecules containing $O^4$-alkylthymine. The most

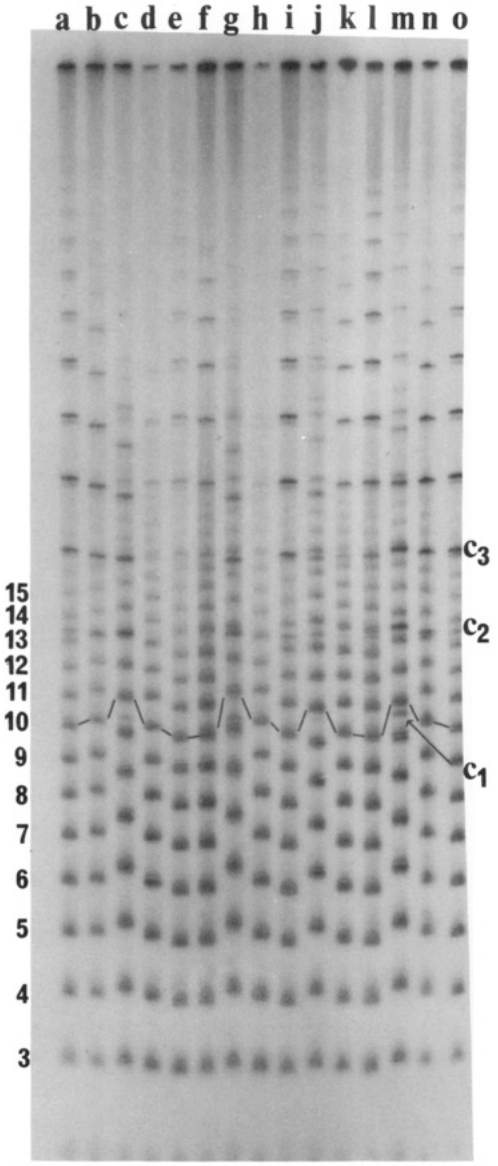


FIGURE 3: Nondenaturing gel electrophoresis of ligated 21-mers on a 4% polyacrylamide gel at pH 8.3. The number of 21-base-pair monomers ligated in each band are indicated along the left side of the autoradiograph. The cyclic products are indicated along the right side of the autoradiograph. Bands corresponding to multimers of the same length (210 base-pairs) are indicated. Control ligation ladder: lanes a, i, and o. At position 2, the oligomers contain C·A (lane b), meT·A (lane c), meT·G (lane d), T·G (lane e), etT·A (lane g), or etT·G (lane h). At position 1, the oligomers contain meT·A (lane j), meT·G (lane k), T·G (lane l), etT·A (lane m) or etT·G (lane n). At position 3, the oligomers contain meG·C (lane f).

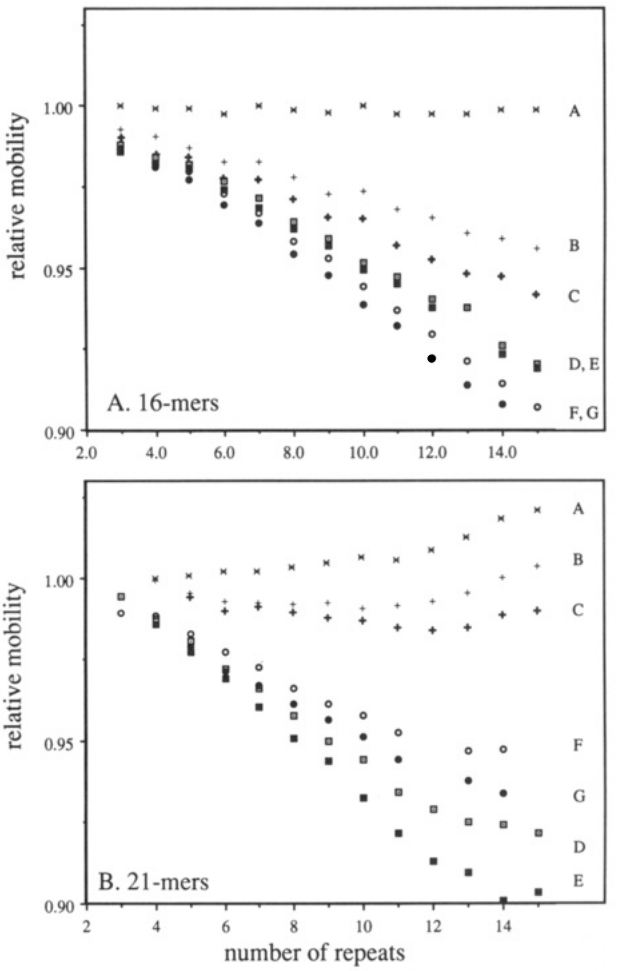


FIGURE 4: Changes in relative electrophoretic mobilities of (A) multimers with 16-base-pair repeats and (B) 21-base-pair repeats as a function of the number of repeats. The relative mobilities were calculated from the polyacrylamide gels shown in Figures 2 and 3 as described under Materials and Methods. The figure shows that the presence of an alkylT reduces the mobility of the DNA and that alkylT·A pairs have a greater effect than alkylT·G pairs. Values for polymers with base-pair substitutions at position 2: ⊡ (D) meT·A; ■ (E) etT·A; + (B) meT·G; + (C) etT·G; ⋇ (A) T·G. Substitutions at position 1: ○ (F) meT·A; ● (G) etT·A.

slowly migrating sequences, i.e., those containing alkylT·A, produced smaller circles and a greater proportion of circles than the alkylT·G-containing sequences (Figure 2). The control $(dN_{16})_x$ ligation mixture and the sequences containing T·G mismatches, which had normal mobility, did not produce small-diameter circles.

Nondenaturing electrophoresis of the 21-base-pair multimers gave the autoradiograph shown in Figure 3. In summary: (1) the sequences containing alkylT·A base-pairs migrated most slowly; (2) sequences containing ethylT base-paired to either A or G migrated more slowly than the analogous sequences containing methylT (Figure 4B); (3) the most slowly moving linear sequences formed the smallest circles; (4) in contrast to the considerable retardation caused by the presence of alkylT, the presence of meG·C caused only a small reduction in mobility which was even less than that produced by the C·A mismatch. The rank order of reduced mobility for the $(dN_{21})_x$ polymers was

etT·A position 2 > meT·A position 2 > etT·A position 1 > meT·A position 1 ≫ C·A position 2 > etT·G position 2 > etT·G position 1 > meT·G position 2 > meT·G position 1 > meG·C position 3 > T·A > T·G position 1 ≥ T·G position 2

The exact comparison between the migration of the 16-mers and 21-mers is discussed more fully below, but in general terms there was a parallel between the electrophoretic behavior of the 21-base-pair multimers $(dN_{21})_x$ (Figure 3) and that of the 16-base-pair multimers $(dN_{16})_x$ (Figure 2).

One aspect in which the results with these $(dN_{21})_x$ oligomers did not parallel those from the $(dN_{16})_x$ oligomers was in the effect of the position of the alkylT·A base-pair on the mobility. An alkylthymine in position 2 in the $(dN_{21})_x$ oligomers had a greater effect than an alkylthymine in position 1. This was the opposite of what was observed for the ligated 16-mers.

While this work was being carried out, an unexpected observation was made. The ligation product of the control 21-mer contained relatively small circles. These small-size circles would not be expected to form in normal DNA (Shore & Baldwin, 1983), and they were not seen in the ligation ladder

of the control 16-mer sequence. Electrophoresis in parallel of the control $(dN_{21})_x$ and $(dN_{16})_x$ ligation mixtures suggests that the sequence used as a control has sequence-dependent curvature which could explain the formation of circles. The 16-mer sequence had three fewer bases at the 5′-end and two fewer bases at the 3′-end than the 21-mer, but the relative base composition was almost identical to that of the 21-mer sequence. Thus, the 21-mer and the 16-mer should have very similar relative electrophoretic mobilities. However, the $(dN_{21})_x$ oligomers migrated more slowly than expected (Figure 6). Interestingly, when the adenine in position 1 or 2 was replaced by a guanine, forming a T·G mismatch, the resultant $(dN_{21})_x$ ligation ladder migrated faster than the control 21-mer ligation ladder (Figure 4B).

*pH Effect.* In addition to the normal pH 8.3 gels, electrophoresis was also carried out at pH 6.5 because the structure of alkylT·A pairs in solution is known at acidic pH but is not as yet known at alkaline pH (Kalnik et al., 1988a,b). At pH 6.5 the rank order of *reduced* mobility for the $(dN_{21})_x$ polymers was

etT·A position 1 > etT·A position 2 ≥ meT·A position 1 > meT·A position 2 ≫ etT·G position 2 > meT·G position 2 ≥ T·A > C·A position 2 ≥ T·G position 2

while the order for the $(dN_{16})_x$ oligomers was

etT·A position 1 ≥ etT·A position 2 > meT·A position 1 > meT·A position 2 ≫ etT·G position 2 > meT·G position 2 > T·G position 2 = T·A

All the oligomers containing base-pair substitutions, whether in the $(dN_{21})_x$ or the $(dN_{16})_x$ series, had less relative retardation at pH 6.5 than at pH 8.3. However, the pH effect was not of the same magnitude for all the sequences. There was an extraordinary difference between relative mobilities of $(dN_{21})_x$ multimers containing alkylT·A or C·A mismatches at the two pHs (Figure 5B), but there was only a moderate change in relative mobilities of $(dN_{21})_x$ multimers containing meG·C, T·G, or alkylT·G. The relative mobilities of all the $(dN_{16})_x$ oligomers were changed only slightly by the change in the pH and the extent was similar for all the sequences (Figure 5A).

Because of the differences in electrophoretic behavior of the 16-mer and 21-mer multimers used as controls, one cannot distinguish a phase-dependent effect on mobility over and above the phase-independent effect from the data as shown in Figure 4. However, the studies at the two different pHs allowed the phase-dependent effect to be distinguished from the phase-independent component of retardation of the $(dN_{21})_x$ multimers. The $(dN_{16})_x$ and $(dN_{21})_x$ ligation ladders were run in parallel at both pHs, and the *K*-factor (Diekmann, 1987) of the oligomers was obtained (see Materials and Methods) using as molecular weight markers the $(dN_{16})_x$ multimers. Higher *K*-factors for the $(dN_{21})_x$ than those of the respective $(dN_{16})_x$ multimers is an indication of anisotropic flexibility or bending of DNA. In order to avoid the possibility that the differences observed in retardation at the two pH conditions was an artifact caused by the difference in the voltage drop previously used for the acidic and basic gels (see Materials and Methods), the same voltage drop of 5 V/cm was used for both pH.

The *K*-factor increase as a function of the number of repeats for the $(dN_{21})_x$ control sequences was similar at both pH's (Figure 6). However, the $(dN_{21})_x$ polymers containing alkylT·A base-pairs had significantly higher *K*-factors than the respective $(dN_{16})_x$ polymers, at pH 8.3 but not at pH 6.5 (Figure 7A,B). The *K*-factor difference between 16- and

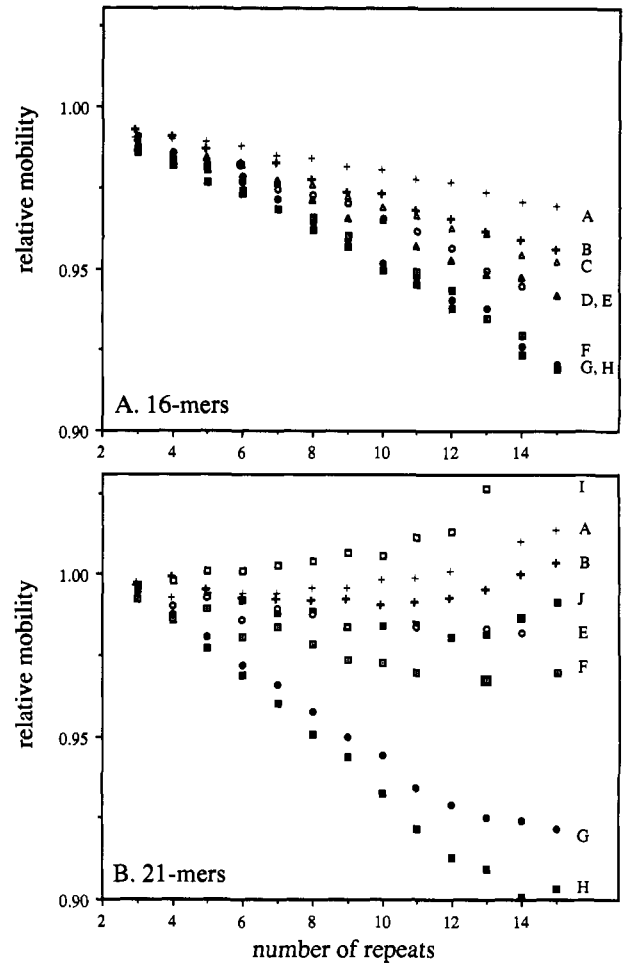


FIGURE 5: The effect of pH on the relative mobility of (A) 16-base-pair and (B) 21-base-pair multimers containing $O^4$-alkylthymine and 21-base-pair multimers containing C·A mismatches. The figure shows results from sequences in which the alkylthymine was in position 2. Very similar results were obtained with sequences where the substitution was in position 1. The closed or bold symbols correspond to the values at pH 8.3 and the open symbols to the values at pH 6.5. ●, ○ (G, E) meT·A; ⊞, ⊡ (H, F) etT·A; ■, □ (J, I) C·A; +, + (B, A) meT·G; ▲, △ (D, C) etT·G.

21-base-pair multimers was greater when the alkylT·A base-pair was in position 2 than when it was in position 1.

*Stability of 21-mer Duplexes.* All base-pair substitutions caused a decrease in the melting point ($T_m$) (Table I). The smallest decrease in $T_m$ was caused by the T·G base-pair at position 2 while the greatest decrease was caused by an meG·C base-pair at position 3. In all cases examined, the duplex containing the alkylT·G base-pair was more stable than the duplex containing the alkylT·A base-pair. All base-pair substitutions were more destabilizing when present at position 1 than at position 2. The size of the alkyl group (methyl or ethyl) did not have any significant effect on the $T_m$ values.

## Discussion

The control sequence used in the present study was unexpectedly found to be curved. The degree of gel retardation (e.g., the $(dN_{21})_{15}$ multimer had a *K*-factor of 1.1) is considerable for a low percentage acrylamide gel (Ulanovsky et al., 1986) and suggests a significant curvature. The curvature was lost by substitution of one of the A's in the TTG/CAA by a G. The curvature would not have been predicted from the DNA sequence; for although Trifonov and Sussman (1980) suggested a model for DNA curvature based on the difference between the wedge angle of the dinucleotide ApA and that

Table I: $T_m$ Values for 21-mer Duplexes with Base-Pair Substitutions at Positions 1, 2, and 3

| | position 1 | | position 2 | | position 3 | |
|---|---|---|---|---|---|---|
| base-pair | $T_m$ (°C)[a] | $\delta T_m$ (°C)[b] | $T_m$ (°C) | $\delta T_m$ (°C) | $T_m$ (°C) | $\delta T_m$ (°C) |
| control | 70.9 | – | – | – | – | – |
| C·A | ND | ND | 64.2 | 6.7 | | |
| T·G | ND | ND | 67.7 | 3.2 | | |
| meG·C | | | | | 63.1 | 7.8 |
| meT·A | 63.6 | 7.3 | 64.9 | 6.0 | | |
| etT·A | 63.3 | 7.6 | 64.9 | 6.0 | | |
| meT·G | 64.8 | 6.1 | 65.7 | 5.2 | | |
| etT·G | 64.8 | 6.1 | 65.6 | 5.3 | | |

[a] $T_m$ values were calculated as described under Materials and Methods. [b] $\delta T_m$ values were calculated by substracting the $T_m$ value of the control oligomer from each observed value. ND indicates no data available.

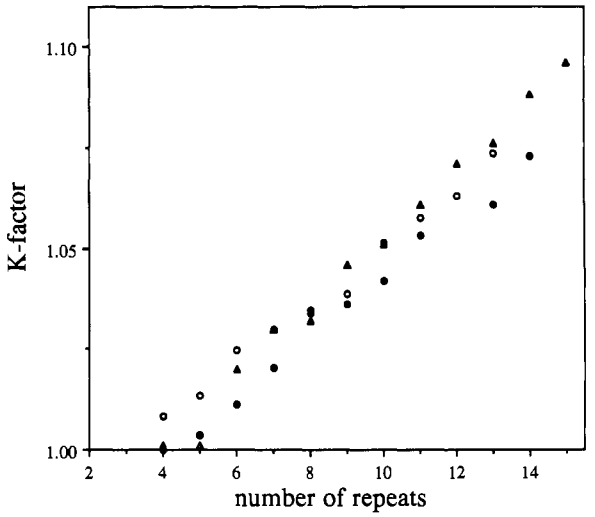


FIGURE 6: The effect of pH and voltage gradient on the *K*-factor of the control 21-base-pair multimers as function of the number of repeats. The *K*-factor determination was based on the migration of the control 16-base-pair multimers (see Materials and Methods). The figure shows that at both pH 6.5 and 8.3 the DNA migrated more slowly than expected and that this slow migration was not affected by pH or the voltage gradient in the gel: electrophoresis at 10 V/cm and pH 8.3 (△); electrophoresis at 5 V/cm at pH 8.3 (○) and at pH 6.5 (●).

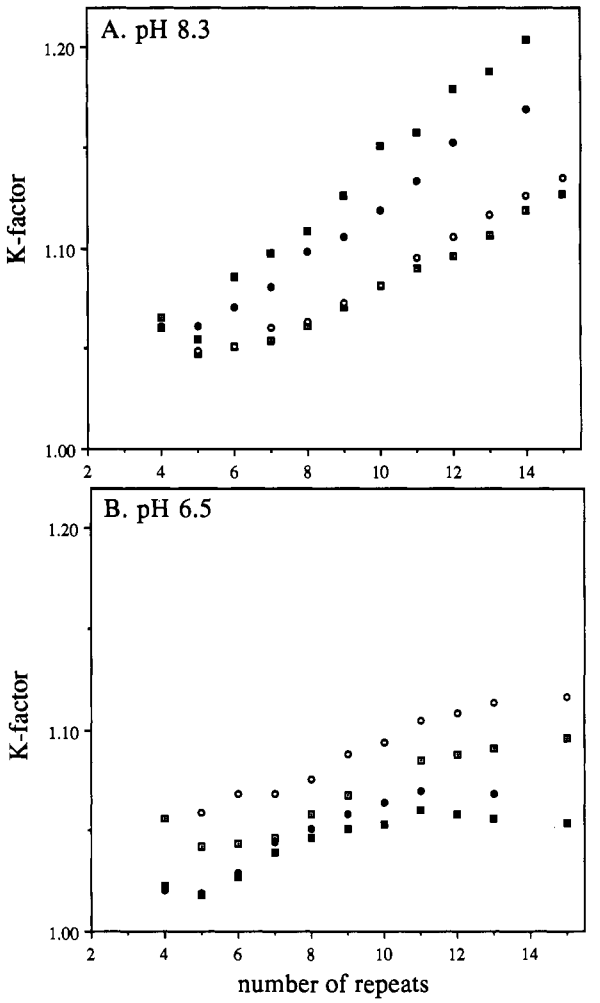


FIGURE 7: Evidence for a degree of anisotropic flexibility, or bending, of sequences containing methylT·A base pairs at pH 8.3 (A) but not at pH 6.5 (B). The *K*-factors were calculated as under Materials and Methods using the 16-base-pair multimers as control: 16-mers with meT·A at position 2 (⊡) or meT·A at position 1 (○); 21-mers with meT·A at position 2 (■) or meT·A at position 1 (●).

of any other dinucleotide, previous results indicate that an A tract must have at least three consecutive adenines in order to induce sufficient curvature to be detected on a nondenaturing gel (Diekmann, 1986). When this curvature was first observed, it seemed to suggest that the K-*ras* might have curvature centered on the 61st codon. However, the sequence used is only partially similar to that of the K-*ras*, and subsequently it was found that the sequence 5′ AC ACA GCA GGT CAA GAG GAG T, which corresponds exact to codons 58–63 of the mouse K-*ras* gene, has no significant electrophoretic abnormality.

While much evidence exists for the direction (Zinkel & Crothers, 1987; Koo & Crothers, 1988) and the magnitude (Koo et al., 1990) of DNA curvature induced by oligo(dA·dT) tracts, little is known about the distortions of the DNA helix caused by modified bases. Electrophoretic mobility studies have been performed for 2-(acetylamino)fluorene–guanine adducts (Schwartz et al., 1989), thymine dimers (Husain et al., 1988), and $O^6$-methylguanine (Voigt & Topal, 1990). The presence of all these modified bases retarded the migration of the oligomers through the gel. The 2-AAF–guanine-induced retardation was not phase-dependent (Schwartz et al., 1989), but the thymine dimer (Husain et al., 1988) and $O^6$-methylguanine (Voigt & Topal, 1990) induced retardation was much greater when the lesions were repeated in phase with the 10.5-base-pair helical turn of the DNA. Therefore, it was suggested that 2-AAF–G produces isotropic flexibility of DNA while thymine dimers and $O^6$-methylguanine induce largely asymmetric (anisotropic) flexibility or bending.

$O^4$-Alkylthymine produced a considerable reduction of the electrophoretic mobilities of oligomers when it is repeated either in phase or out of phase with the helical repeat. This effect was much greater when the modified base was paired to adenine than when paired to guanine, and the magnitude of the effect depended on the size of the alkyl group. $O^4$-Ethylthymine always caused greater retardation than $O^4$-methylthymine.

One possible reason for this retardation is curvature. One

characteristic of curved DNA is that gel retardation is observed only if the sequence responsible for the bending occurs in phase with the helical repeat(=10.5 base-pairs)(Koo et al., 1986). There are a number of other reasons for gel retardation which do not have this phasing dependence and in which the degree of retardation is proportional to the number of lesions but independent of their alignment in relationship to the helical repeat. These include cruciforms, localized single strandedness or bulges in the DNA sequence, chemical or physical interaction with the gel matrix, and isotropic flexibility. Isotropic in this context implies an increased flexibility which is symmetrical around the helical axis, while anisotropic flexibility, discussed further below, implies an asymmetrical increase in flexibility.

Oligomers used in this study lack any self-complementarity that could induce hairpin or cruciform formation. Furthermore, any physical or chemical interaction of the modified thymidine with the gel matrix is unlikely to be the cause of the retardation because in that case one would expect the same effect when the alkylated thymine is paired either with guanine or adenine. Voigt and Topal (1990) suggested that the gel retardation they observed in $O^6$-methylguanine-containing oligomers was due in part to a localized bubble formation. This does not seem to be the case for the $O^4$-alkylthymine-containing oligomers because, although no extensive study of the distribution of small circles was performed, there seemed to be a correlation between retardation and the formation of small circles. The observation of these circles could only lead to the conclusion that oligomers containing $O^4$-alkylT are more flexible than the control oligomers because the more flexible a molecule, the higher the probability of the two ends coming together and being ligated to form a circle (Zahn & Blattner, 1985; Ulanovsky et al., 1986). Localized single strandedness or bulges could reduce the mean circle distribution only if these regions of single strandedness were regions of increased flexibility. The flexibility we observed could be either isotropic, i.e., bending in all directions is equally possible, or anisotropic, i.e., bending in some directions is thermodynamically favored. In the latter case the DNA molecule would spend more time bent toward the preferred directions, and a multimer of 21-base-pair repeats, where the lesion is always on the same side of the DNA helix, would migrate more slowly than DNA of the same length containing 16-base-pair repeats.

To test the issue of phasing, the $(dN_{16})_x$ and $(dN_{21})_x$ ladders were run in parallel. The control $(dN_{16})_x$ ligation ladder was used as a reference. The increase in the $K$-factor of the oligomers containing the alkylT·A base-pair was far greater when the modified base was repeated every 21 base-pairs, i.e., in phase with the helical repeat, rather than every 16 base-pairs, i.e., with the alkylated base alternately on one side of the helix then on the other (Figure 7A). The phasing factor was greater at position 2 than at position 1 of the sequence. Evidence that the phase dependence of the retardation was caused by the alkylT·A base-pair, and not by inherent DNA curvature, came from the studies at pH 6.5. The $K$-factor of the control $(dN_{21})_x$ sequence was virtually unaffected by the decrease of pH (Figure 6), but the $K$-factor of the alkylT·A-containing $(dN_{21})_x$ multimers was reduced (Figure 7B), so that at pH 6.5 the retardation of the $(dN_{21})_x$ multimers, in which the alkylT·A pair was in phase, was similar to the retardation of the $(dN_{16})_x$ oligomers in which the alkylT·A pair was out of phase.

These observations lead to the conclusion that there is a significant difference in the structure of the oligomer containing alkylT·A pairs at pH 8.3 and pH 6.5. At pH 8.3 the conformational change and flexibility produced by an alkylT·A pair is asymmetric (anisotropic). A greater anisotropic factor for the alkylT·A at position 2 could explain the difference in the ranking order of relative mobilities of the $(dN_{16})_x$ and $(dN_{21})_x$ polymers. At pH 6.5 similar retardations and ranking orders were observed, suggesting that the conformational change and flexibility produced by the alkylT·A base-pair is isotropic (Koo et al., 1986). The anomalous migration caused by the alkylT·G base-pair at pH 8.3 did not have a phasing characteristic, and no significant difference was observed in the decrease of retardation of the alkylT·G-containing $(dN_{21})_x$ and $(dN_{16})_x$ oligomers when the pH was reduced to 6.5. In this case, an isotropic flexibility model would be more plausible.

However, a pH effect, similar to the one observed for the oligomers containing an alkylT·A base-pair, was observed for the oligomers containing a C·A base-pair, which suggests that this pH effect could be a reflection of similarities in the structures for these mismatches. NMR studies have shown that methylT·A base-pairs are more stable at acidic than at neutral pH and that at the lower pH there is a strong similarity between the structure of the alkylT·A and C·A base-pairs (Kalnik et al., 1988a,b; Swann, 1990). These NMR studies and X-ray crystallography of DNA duplexes containing C·A mismatches (Hunter et al., 1986) suggest that the C·A and methylT·A pairs have one hydrogen bond between the $NH_2$ of A and $N^3$ of the pyrimidine and possibly a second hydrogen bond between $N^1$ of A and the $O^2$ of the pyrimidine, with the hydrogen on $N^1$ resulting from protonation of the adenine. Thus, the protonation of adenine at pH 6.5 (but not at pH 8.3) with the formation of a second hydrogen bond may be the explanation for the difference in the structure and flexibility of DNA at pH 6.5 and pH 8.3.

The effect of the position of the alkylT-containing base-pair on the mobility of the $(dN_{16})_x$ oligomers at pH 8.3, and of both the $(dN_{16})_x$ and $(dN_{21})_x$ oligomers at pH 6.5, could be explained by the difference in stacking interactions. The alkylT at position 2 is in the sequence 5′ T alkylT G 3′, and in position 1 it is in the sequence 5′ C alkylT T 3′ (Figure 1). The theoretical expectation that the pyrimidine–purine combination of nearest neighbors at position 2 should be more effective at stabilizing the $O^4$-alkylthymine in an intrahelical stacked conformation than the pyrimidine–pyrimidine of position 1 (Saenger, 1984) is supported by the melting point ($T_m$) measurements of 21-mer duplexes (Table I). These results show that the sequences with the alkylT in position 2 have a higher melting point than those with the alkylT at position 1.

Another interesting aspect of this study is the difference in migration anomaly caused by $O^4$-alkylT·A and $O^4$-alkylT·G base-pairs. The retardation caused by $O^4$-alkylT·A was dependent upon phasing of the alkylated base-pairs with the helical turn, while that caused by $O^4$-alkylT·G base-pair was not. In addition, the anomalous migration caused by the $O^4$-alkylT·A base-pair was greater than that caused by the $O^4$-alkylT·G base-pair. A difference would have been predicted because previous NMR studies suggest that the $O^4$-alkylT·G retains the Watson–Crick alignment while the $O^4$-alkylT·A base-pair adopts a wobble conformation (Kalnik et al., 1988a,b). Furthermore, this difference correlates with the relative decrease in the melting point ($T_m$) of the two base-pairs (Table I). Although previous studies on a self-complementary dodecamer in our laboratory suggested that $O^4$-alkylT·A is more stable than the $O^4$-alkylT·G base-pair (Li et al., 1987), measurements on non-self-complementary 21-mers (Table I) show that the opposite is in fact the case.

One striking aspect of these results is the discovery that $O^4$-alkylthymine causes a much greater effect on the flexibility

of DNA than $O^6$-methylguanine [Figure 3 and Voigt and Topal (1990)]. These differences may be relevant to the differences in the recognition of these modified bases by repair enzymes and even suggest a different role for them in the process of carcinogenesis. Carcinogenesis is a multistage process, and the historic division into two stages, initiation and promotion, plainly covers a complex process. *N*-Nitroso compounds can produce all these changes associated with promotion as well as with initiation. If alkylation of DNA does produce epigenetic changes then one would predict that alkylT in DNA would have a greater role than alkylG because of the greater change in DNA conformation that it produces.

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