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# Extraordinarily stable mini-hairpins: electrophoretical and thermal properties of the various sequence variants of d(GCGAAAGC) and their effect on DNA sequencing

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## ABSTRACT

**A small DNA fragment having a characteristic sequence d(GCGAAAGC) has been shown to form an extraordinarily stable mini-hairpin structure and to have an unusually rapid mobility in polyacrylamide gel electrophoresis, even when containing 7M urea. Here, we have studied the stability of the various sequence variants of d(GCGAAAGC) and the corresponding RNA fragments. Many such sequence variants form stable mini-hairpins in a similar manner to the d(GCGAAAGC) sequence. The RNA fragment, r(GCGAAAGC) also forms a mini-hairpin structure with less stability. The DNA mini-hairpins with GAAA or GAA loop are much more stable than DNA and RNA mini-hairpins with other loop sequence so far as has been examined. The stability difference between DNA and RNA mini-hairpins may be deduced to the stem structures formed by DNA (B form) and RNA (A form). The stable hairpins consisting of the GCGAAAGC sequence cause strong band compression on the sequencing gel. This phenomenon should be carefully considered in DNA sequencing.**

## INTRODUCTION

Recently it has been reported that some DNA and RNA sequences form stable hairpin structures (1–13). The DNA hairpin with a $(T)_4$ or $(T)_5$ loop possesses the most stable structure of those with other homonucleotide-containing loops (11–13). Stable RNA hairpins containing an UNCG or GNRA loop have been reported (2–10), to form the base-pair within the loops which is known to be a main stabilizing factor for the hairpin.

Previously, we found that a DNA fragment, d(GCGAAAGC), forms an extraordinarily stable hairpin structure consisting of a GAAA loop and only two G-C pairs at the terminus [we called this the mini-hairpin structure], whose melting temperature (Tm) is as high as 76.5°C in 0.1M NaCl solution (1). The DNA mini-hairpin structure formed by the d(GCGAAAGC) sequence is not denatured at room temperature, even in a 7M urea solution (1). Because of this stable compact structure, the mobility of the fragment exceeds even that of its own one-base-less fragment d(CGAAAGC), which lacks its 5′-terminal G of the d(GCGAAAGC) fragment, in 20% polyacrylamide gel electrophoresis containing 7M urea (see Fig. 1). This unusual mobility was found to cause band compression during the sequencing of fragments containing the d(GCGAAAGC) sequence, which was in fact observed (14) in the Maxam-Gilbert sequencing (15).

The mini-hairpin sequence GCGAAAGC is observed in some characteristic regions of natural DNAs, for example, in the replication origin of phage G4 (16) and in the rRNA genes (1,5,6 and Uhlenbeck personal communication).

There are very few reports (1,17) which refer to the formation of hairpin structures containing two G-C pairs, and it remains unknown why such hairpins are so stable. Here we clarify the characteristics of these hairpin structures by comparing the stability of various sequence variants of the d(GCGAAAGC) mini-hairpin, and demonstrate how these hairpin structures affect the sequencing of DNA by the dideoxynucleotide chain-termination method (18,19).

## MATERIALS AND METHODS

### Synthesis and gel-electrophoresis of DNA and RNA fragments

Each DNA fragment was synthesized using a DNA synthesizer (Applied Biosystems, 381A) and purified by high-performance liquid chromatography with a reverse-phase C-18 column (M&S Pack C18, 4.6 mm i.d.×15 cm). RNA fragments were synthesized using a DNA manual synthesizer (Nippon Zeon Co., Zeon Genet)(20,21) or by the phosphoramidite method with a DNA synthesizer (Applied Biosystems, 381A) using four nucleotide units (MilliGen/Biosearch)(22). Each oligomer was labeled at the 5′-terminus with [$\gamma$-$^{32}$P]ATP (Amersham) using T4 polynucleotide kinase (Boehringer)(23) and directly electrophoresed on 20% polyacrylamide gel containing 7M urea, 0.08M Tris-phosphate and 0.002M EDTA (pH 8.0).

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### Thermal denaturation analysis of the fragments

Purified oligonucleotides (1 $A_{260}$U/ml, approximately 0.01mM, each) were dissolved in 50mM sodium cacodylate and 0.1M NaCl (pH 7.0). The melting profiles were obtained at 260 nm using a Gilford spectrophotometer, Response II, at a heating rate of 0.5°C/min using 1 cm pathlength cuvettes. Tm values were calculated by the first derivatives of the melting curves, and then calculated automatically by the program supplied with the instrument.

### DNA sequencing

Synthetic oligomers (see Fig. 4a) were phosphorylated and ligated into *Eco*RI/*Hind*III-digested M13mp18 and M13mp19 vectors. Each phage ssDNA was sequenced by the dideoxynucleotide chain-termination method (18,19) using [$\alpha$-$^{35}$S]dCTP, T7 DNA polymerase (24) and substrates (dNTPs, 7-deaza-dGTP or dITP)(Sequenase, Toyobo). The reaction mixture was electrophoresed on 7% polyacrylamide containing 7M urea, 89mM Tris-borate and 2mM EDTA (pH 8.0) at room temperature.

### Sequencing-gel electrophoresis at high temperature

Sequencing was carried out using 7-deaza-dGTP, [$\alpha$-$^{35}$S]dCTP, T7 DNA polymerase, and M13mp19 vector DNA with synthetic fragments inserted. Electrophoresis was carried out at room temperature (approximately 20−40°C), and at 70°C using water-jacketed equipment (24×20×0.3 cm) attached on both sides of the sequencing-gel glass plates (25×40×0.03 cm) covered with aluminium plates (23×30×0.1 cm).

## RESULTS AND DISCUSSION

### Stability of mini-hairpin structures

As shown in Fig. 1, the d(GCGAAAGC) fragment displays abnormally rapid mobility in polyacrylamide gel electrophoresis with 7M urea, which is caused by the thermally very stable compact structure (mini-hairpin structure). We first examined the mobility of various sequence variants of the d(GCGAAA-GC) fragment in gel electrophoresis to see if any other variant could also possess the mini-hairpin structure. The mobility of each fragment was compared with that of its 5′ one-base-lacking fragment (Fig. 1).

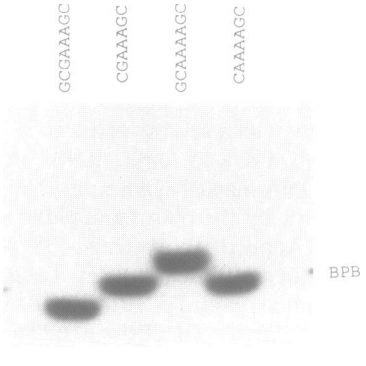


**Fig. 1.** Electrophoretic patterns of $^{32}$P-labeled d(GCGAAAGC), d(CGAAAGC), d(GCAAAAGC) and d(CAAAAGC) on a 20% polyacrylamide gel containing 7 M urea at room temperature, as described in 'Materials and Methods'.

Table 1 shows a comparison of the mobilities of various synthetic fragments on polyacrylamide gel containing 7M urea, and Table 2 presents the melting temperatures (Tm) of some of these fragments. There is a good correlation between the mobility in gel electrophoresis and the Tm of the fragments, indicating that a fragment showing an abnormal mobility on the gel containing 7 M urea always has a higher Tm (more than 60°C) than does a fragment with normal mobility (below 57°C; in some case no inflection point showing Tm was observed). Thus, a fragment forming the stable mini-hairpin structure could be identified, and many sequence variants were found to be capable of forming the stable mini-hairpin structure.

The stability of the mini-hairpin shown in Tables 1 and 2 can be characterized as follows.

1) The mini-hairpin structure requires more than two G-C base pairs at the stem region. The stability of the stem region correlates with the stability of the B-DNA double helix already elucidated as follows (25):

$$\begin{matrix}5'\\ d(GC)\\ \bullet\bullet\\ d(CG)\\ 3'\end{matrix}\begin{matrix}3'\\ \\ \\ \\ 5'\end{matrix} > \begin{matrix}d(CC)\\ \bullet\bullet\\ d(GG)\end{matrix} = \begin{matrix}d(GG)\\ \bullet\bullet\\ d(CC)\end{matrix} \geq \begin{matrix}d(CG)\\ \bullet\bullet\\ d(GC)\end{matrix} \quad \text{(Eq. 1)}$$

2) The mini-hairpin contains three or four nucleotides in the loop region, including at least one A. The hairpin with either a GAAA or GAA loop is the most stable of all these so far examined (Tables 1 and 2). These results are slightly different from those reported by Antao *et al.*(5,6), which may be due to differences in the stem sequences and lengths used; they studied with $^{5'}$GGAG---CUCC$^{3'}$ and $^{5'}$GGAC---GUCC$^{3'}$ sequences in the stem region (5).

3) The d(GCTTTCGC) fragment, complementary to the d(GCGAAAGC) fragment, does not form a mini-hairpin structure, although the sequence is similar to that of the already-

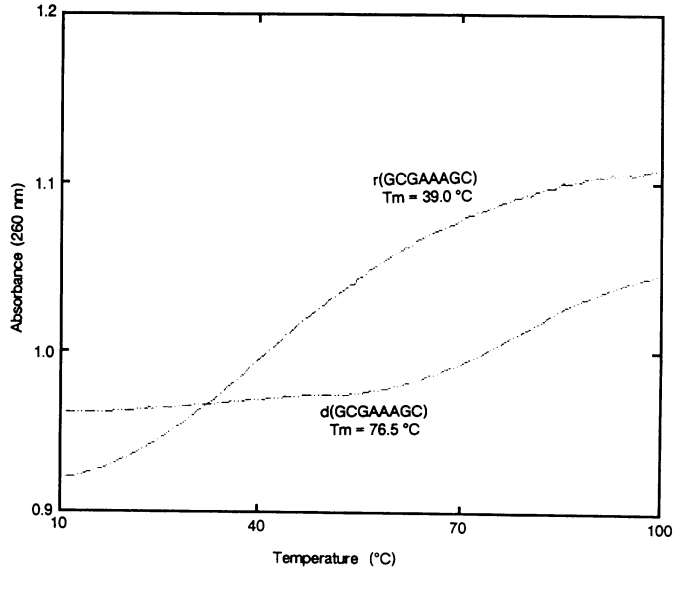


**Fig. 2.** Melting profiles of mini-hairpins d(GCGAAAGC) and r(GCGAAAGC) in 50mM sodium cacodylate (pH 7.0) and 0.1M NaCl.

known stable RNA hairpin with an UUUG loop (2,3). Antao et al. (5,6) reported that the corresponding DNA hairpin with a TTTG loop is unstable.

4) Westhole et al. (26), and Heus and Pardi (7) found that RNA hairpins containing a GNRA loop form the unusual G-A base pair within some rRNA loops. However, the third A in the GAAA loop of the d(GCGAAAGC) mini-hairpin is not essential in the DNA fragments described above (Tables 1 and 2).

In contrast to the extraordinarily stable d(GCGAAAGC) fragment (Tm = 76.5°C) in a 0.1M NaCl solution, the corresponding RNA hairpin, r(GCGAAAGC), is much less stable, the Tm value being 39°C as shown in Fig.2. The stability difference between the DNA and RNA mini-hairpins may be due to the conformational difference (DNA B form and RNA A form) in the stem structures (4−6), not the unusual G-A base pair in the loops.

To examine the effect of the stem region in the mini-hairpins, we synthesized eight kinds of DNA and RNA fragments, consisting of the same loop sequence but four different stem sequences; $^{5'}$GCGAAAGC$^{3'}$, CCGAAAGG, GGGAAACC, and CGGAAACG (20). The purity and chain length of each fragment were confirmed by electrophoresis for its $^{32}$P-labeled form on a 20% polyacrylamide−7M urea gel at 70°C, as shown in Fig.3a. In this case, no distinct mobility shift was observed among the eight different DNA and RNA fragments with the same chain lengths. Formation of the mini-hairpin structures for some of these fragments was confirmed only when electrophoresed on a native gel (20% polyacrylamide gel at room temperature), as shown in Fig. 3b. In the usual 20% polyacrylamide−7M urea gel, no mobility shift was observed in the RNA fragments (14), although both d(GCGAAAGC) and d(CCGAAAGG) ran more rapidly than d(GGGAAACC) and d(CGGAAACG), and as a whole, DNA fragments had higher mobilities than the corresponding RNA fragments.

As the GCGAAAGC and CCGAAAGG fragments in both DNA and RNA form showed higher mobilities than the GGGAAACC and the CGGAAACG fragments on the native gel (Fig. 3b), mini-hairpin structures must have been formed in the former two fragments. In these fragments, although d(CCGAAAGG) has considerably lower Tm (65°C) than d(GCGAAAGC) (76.5°C), both r(GCGAAAGC) and r(CCGAAAGG) have approximately the same Tm values (39°C and 41.5°C, respectively), which are much less stable than the corresponding DNA mini-hairpins (Fig. 3b). As in the case of the DNA mini-hairpin, the stability of the RNA mini-hairpins can be explained by the stability of the RNA helix in the stem regions, as shown by Eq. 2. This has been estimated by the free energy of each duplex (27−29):

$$\begin{matrix} 5' & & & & & & \\ & r(GC)^{3'} & & r(CC) & & r(GG) & & r(CG) \\ & \bullet\bullet & = & \bullet\bullet & = & \bullet\bullet & > & \bullet\bullet \\ 3' & r(CG)_{5'} & & r(GG) & & r(CC) & & r(GC) \end{matrix} \quad \text{(Eq. 2)}$$

However, the stability for RNA duplexes indicated by Eq. 2 is slightly different from that for DNA duplexes as shown by Eq. 1 above. One possible explanation could be the difference in the helical form between RNA (A form) and DNA (B form). There is a good correlation between the differences of the helical stability and the Tm values of mini-hairpin DNAs and RNAs. Therefor, the stem structures in the B form may be favored over those in the A form for the formation of stable mini-hairpins.

Another factor in stabilizing the hairpin structure may be the base pair adjacent to the loop region. As Antao *et al.* (5,6) and Tuerk *et al.*(2) have proposed, a C-G pair may be preferable to a G-C pair at the edge of the loop region. This idea explains well the observation that the GGGAAACC fragment in both the DNA and RNA forms does not form a mini-hairpin structure (Fig. 3). Thus, it seems that stability of mini-hairpins depends on the helical stability of the stem region, and on the loop-closing base pair, C-G.

The unusually high stability of the d(GCGAAAGC) mini-hairpin is still not explicable by these factors alone. The bendability (breaking ability) and the stacking ability in the loop region probably also contribute to the stability of the mini-hairpins. It is well known that poly (dA) forms by itself a single-stranded helical structure (30), because the stacking between two A residues is very strong (31,32).

**Table 1.** Comparison of mobility tendencies of various sequence variants of d(GCGAAAGC) in 20% polyacrylamide−7 M urea gel electrophoresis

| GCGAAAGC | | GCGAAAGC | | GCGAAAGC | | GCGAAAGC | |
|---|---|---|---|---|---|---|---|
| gcgNaagc | + | gcTTTTgc | − | CcgaaagG | + | gcgAgc | − |
| gcgaNagc | + | gcCCCCgc | − | CGgaaaCG | − | gcgAAgc | + |
| gcgaaNgc | + | gcAAAAgc | − | gAgaaaTc | − | gcgaaagc | + |
| gcgGGGgc | − | gcGGGGgc | − | gTgaaaAc | − | gcgaaaAgc | (+) |
| gcgTTTgc | − | | | | | gcgaaaAAgc | − |
| gcgCCCgc | − | | | | | | |
| gcgTTagc | + | GCGAAAGC | | GCGAAAGC | | others | |
| | | Acgaaagc | − | IcIaaaIc | − | GCTTTCGC | − |
| GCGAAAGC | | Tcgaaagc | − | CGCaaaCG | − | $CGAGTTTGACG^{17}$ | − |
| gcCaaagc | + | Ccgaaagc | − | | | | |
| gcAaaagc | − | Icgaaagc | (+) | | | | |
| gcTaaagc | − | gcgaaagA | − | | | | |

Abnormal mobility of each fragment was checked by comparison with the mobility of its 5'-one-base-lacking fragment as shown in Fig. 1. +, abnormal mobility; −, normal mobility; (+), the same mobility as its one-base-lacking fragment. The underlined upper-case letters in the sequence GCGAAAGC indicate that the bases are replaced by other bases shown by upper-case letters in the variants (N = A, G, C or T). The bases with no replacement in the variant are shown by lower-case letters.

**Table 2.** Tm (°C) of various sequence variants of d(GCGAAAGC) in 0.1M NaCl-50mM sodium cacodylate (pH 7.0)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| gcgaaagc | 76.5 | gcgaaTgc | 70.0 | gAgaaaTc | NO | gcgTTTgc | NO |
| gcgTaagc | 71.5 | gcgaaCgc | 67.0 | Icgaaagc | 59-62 | gcgCCCgc | 41-44 |
| gcgCaagc | 72.0 | gcgaaGgc | 70.5 | CcgaaagG | 65.0 | gcgaaagcT | >82 |
| gcgGaagc | 71.0 | gcgTTagc | 69.5 | gcgAAgc | 76.5 | | |
| gcgaTagc | 71.0 | gcCaaagc | 60-63 | gcTTTTgc | 33.0 | | |
| gcgaCagc | 72.0 | gcTaaagc | 55-58 | | | | |
| gcgaGagc | 71.5 | gcAaaagc | NO | | | | |

NO: No melting temperature was observed at 10–100°C. The bases are replaced by other bases shown by upper-case letters in the variants. Experimental errors of the Tm values are within ± 1°C, except for the values indicated by lower and upper limits.

$^1$H-NMR study of d(GCGAAAGC) leads to a structure in which each moiety of GCG and AAAGC form a reasonable helical structure (B form), and that is folded in double at the point between G3 and A4, based on the NOE experiment only within the above two moieties (1). However, the RNA hairpin with r(GCGAAAGC) has reported to fold in three in which A4 base stacks neither on G3 nor A5, and thus protrudes from the hairpin loop (7). Therefore, it is presumed that the base stacking in the DNA (B form) mini-hairpin is stronger than that in the RNA (A form) mini-hairpin.

In DNA mini-hairpins, the d(GCGAAAGC) fragment may be stabilized by both the stable helical structure of the AAA moiety and the bendability between the G3 and A4. As Table 1 shows that the hairpin-forming sequences with a GAAA or CAAA loop form mini-hairpin structures, either the GA or CA sequence may have high bendability. In contrast with the extra-stable d(GCGAAAGC) fragment, the d(GCAAAAGC) fragment does not form a mini-hairpin structure, as shown in Fig. 1 and Table 1. Although the stacking in the AAAA tract is very strong, the bendability of that region may be very low, with the consequence that the d(GCAAAAGC) fragment cannot make a break within itself so as to form the two terminal G-C pairs.

Thus, it seems that the stability of a mini-hairpin is determined by the balance between the bendability and the stacking stability in the loop, leading to a conclusion that the more base-stacks formed in a B form structure, the higher the stability of the mini-hairpin.

## Band compression in DNA sequencing in the regions containing the d(GCGAAAGC) sequence

As mentioned above, the extra-stable mini-hairpin shows rapid mobility even on polyacrylamide–7M urea gel, so it can be easily considered that such mini-hairpin-forming sequences in a DNA strand cause strong band compression in the sequencing of DNA.

To examine this possibility, we designed and synthesized model DNA fragments, including the three extraordinarily stable hairpin structures, on one strand [called a (+) strand], as shown in Fig. 4a (1). There are three regions that can be expected to form the hairpin structure, all of which comprise the sequence d(GCGAAAGC), but with different stem lengths. Thus, no alternative hairpin structure seems to be formed in the strand. This may be supported by the finding that band compressions could be observed in the regions corresponding to the GCGAAAGC-type mini-hairpin-forming sequences, as mentioned below, because it is known that compression is caused by any band migrating with anomalous rapidity on the sequencing gel, whenever the fragment length of the band is sufficient to form a stable hairpin structure (33).

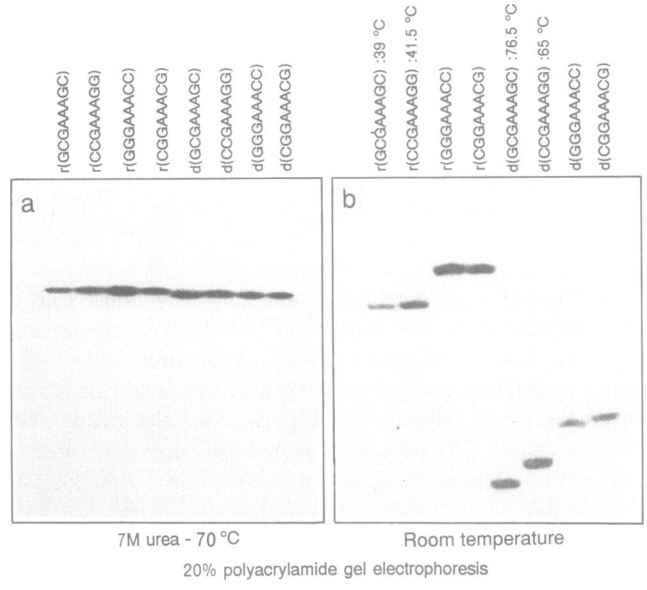


**Fig. 3.** Electrophoretic patterns of $^{32}$P-labeled DNA and RNA fragments on a 20% polyacrylamide gel containing 7M urea at 70°C (a), and on a 20% polyacrylamide gel without urea (native gel) at room temperature (b). The Tm's of the fragments forming the mini-hairpins are indicated in (b).

The synthetic fragments shown in Fig. 4a were inserted into the *Eco*RI-*Hind*III fragments of M13mp18 and M13mp19. These plasmids were sequenced by the dideoxy-termination method with T7 DNA polymerase using 2′-deoxynucleoside triphosphates (dNTPs) as substrates in three different ways: the normal case using four dNTPs, using 7-deaza-2′-deoxyguanosine triphosphate (7-deaza-dGTP) and/or using 2′-deoxyinosine triphosphate (dITP) instead of only d(GTP) (Fig. 4b) (33).

While sequencing the (+) strands of the plasmid using the (−) strand as the template, strong band compressions were observed (boxed in Fig. 4b) when dNTPs (left) and 7-deaza-dGTP (center) were used as the substrates. In using dITP (right), the sequencing ladders appeared to be aligned normally, but it actually contained another type of band compression caused by mismatching between dI residues in the synthesized strand and dN residues in the template strand (33), an example being shown by the arrow in Fig. 4b. This type of band compression induced by dI:dN mispairing is known to be caused by immobilization of DNA polymerase on the DNA template in the regions comprising a stable higher-ordered structure. The compression can be dissolved by using thermostable DNA polymerases such as Taq or Tth DNA polymerase at high temperature (34,35).

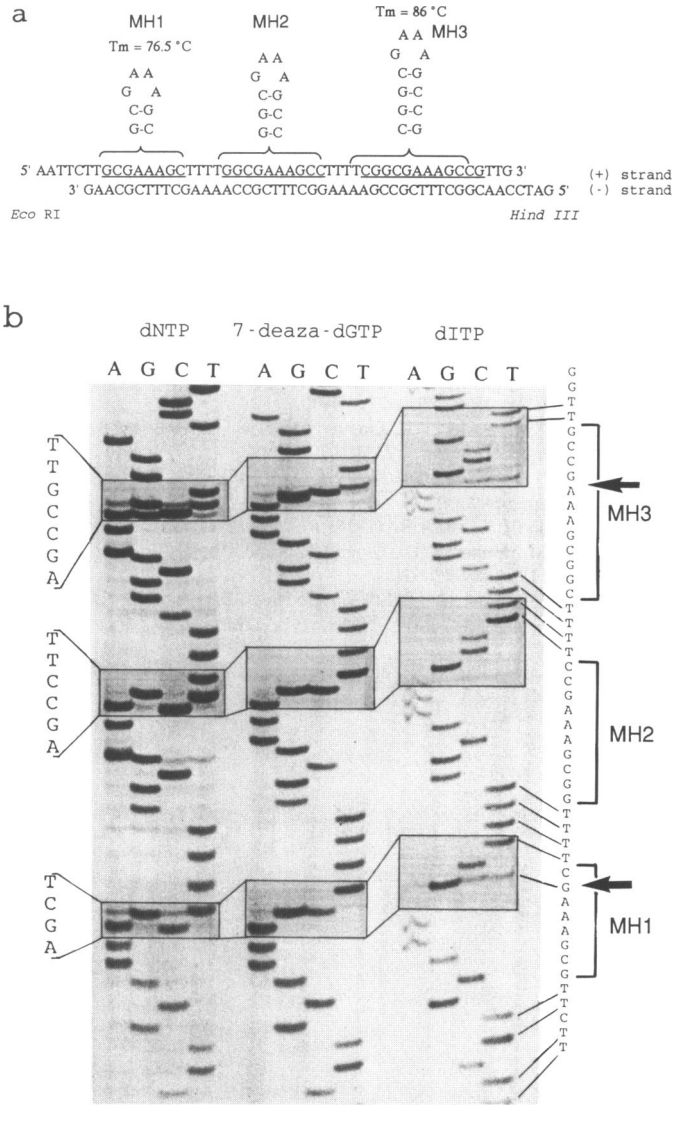


**Fig. 4.** (a) DNA duplex designed as a sequencing model. Three possible regions on the (+) strand expected to form hairpin structures are indicated by the secondary structures (named MH1−MH3); the Tm's of these fragments (MH1 and MH3) are also shown. (b) Electrophoretic patterns of sequencing the (−) strand template using dNTP (left), 7-deaza dGTP instead of dGTP (center), and dITP instead of dGTP (right) as substrates. The possible mini-hairpin-forming regions are indicate. The regions where compressions are observed are demonstrated by boxes and an arrow.

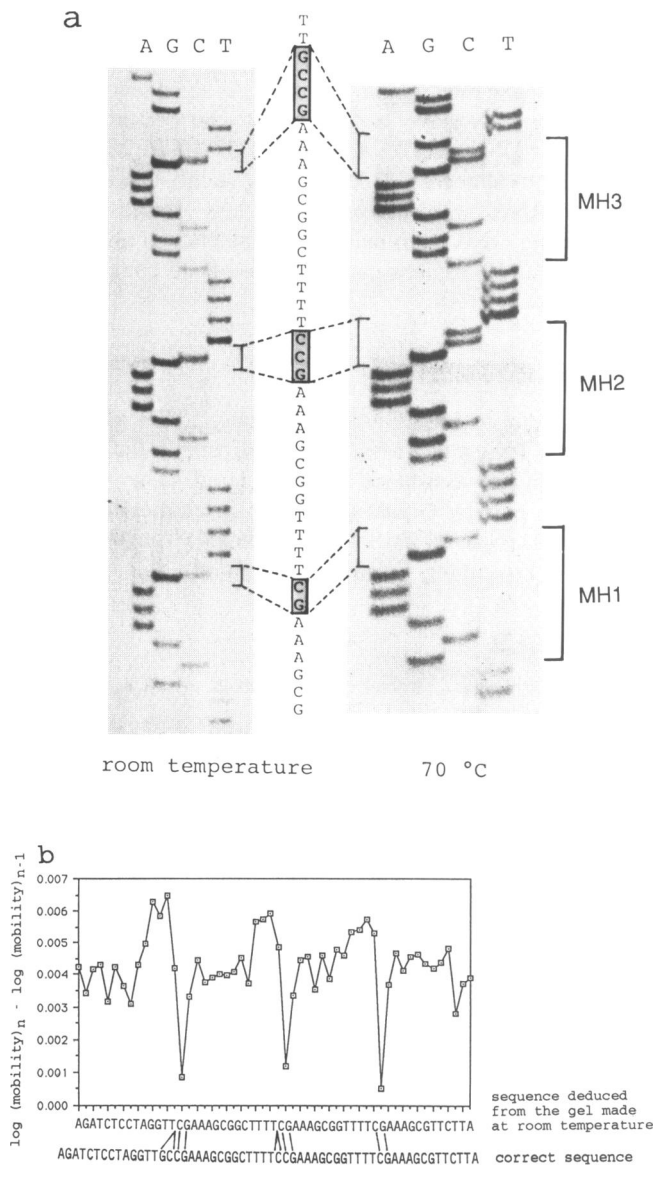


**Fig. 5.** (a) Electrophoretic patterns of sequencing the (−) strand template using 7-deaza dGTP instead of dGTP as substrates, at room temperature (left; this pattern is the same as that of Fig. 4b, center) and at 70°C (right). The regions where compressions are observed are indicated by boxes. (b) Correlation between the logarithm of the mobility ratio of the adjacent bands and the nucleotide sequence deduced from the ladder obtained at room temperature (Fig. 5a, left). The sequences are listed 3′ to 5′.

To dissolve the compression attributed to the extra-stable hairpin structure, we attempted to carry out gel-electrophoresis at high temperature (36−38). The region comprising the sequence d(CGGCGAAAGCCG) formed the most stable hairpin structure (Tm = 86°C in 0.1 M NaCl) in the (+) fragment synthesized from the (−) strand template. It is known that addition of urea to the solution to make a 7M concentration generally causes a decrease in the Tm of the fragments by 20−30°C (1). Electrophoresis was thus carried out under this condition at 70°C to denature any unusual higher-ordered structure in the fragment, with the result that band compressions were successfully dissolved using 7-deaza dGTP in the sequencing of the (−) strand template (Fig. 5a). The band compression was not completely dissolved by the gel-electrophoresis at up to 65°C (data not shown).

To examine the existence and the sites of band compression from only the sequencing ladder made at room temperature, we took the correlation between the logarithm of the mobility ratio of adjacent bands and the nucleotide sequence deduced from the ladder in the left part of Fig. 5a. As shown in Fig. 5b, three steep troughs appeared corresponding to the misread (compressed) regions. This plotting was found quite helpful for determining whether there would be any misreading in the deduced sequence as well as at the sites of compression. Another factor in band composition is known to be that the base composition and interactions between the 3′-terminal nucleotide and its neighboring 5′-nucleotide also affect the mobility of

oligomers in gel-electrophoresis (38,39); this also needs to be considered for more accurate sequence assessment.

Table 1 demonstrates that many sequences are capable of forming the stable mini-hairpin structure. As some sequences with abnormal mobility have been found to be present in some characteristic DNA and RNA region (1,5−10,16,26,40,41), it is necessary to take care in determining such DNA sequences. Although RNA mini-hairpins are less stable than the corresponding DNA mini-hairpins, as mentioned above, these RNA mini-hairpin regions can also cause band compression in RNA sequencing (40).

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