DNA denatures upon drying after ethanol precipitation

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ABSTRACT

We have observed that ethanol precipitation and subsequent drying of small (<400 bp) radiolabelled DNA fragments is able to induce a transition to a form that migrates aberrantly on acrylamide gels. This unusual form has increased sensitivity to S1 nuclease, decreased sensitivity to restriction enzymes, and a concentration dependence for the reversion to the duplex form. Apparently, DNA denatures upon dehydration so that redissolving at low dilution will allow the collapse of DNA fragments into single-stranded hairpin structures. These structures are stable enough at low dilution to prevent complete reannealing of single stranded species. These single stranded species show strong binding to unidentified proteins present in nuclear extracts. This may give rise to misleading interpretations of mobility shift assays, especially if the single-stranded conformers have a similar mobility to the duplex fragment, which can occur in fragments that are 50-100 bp long. Evidence is presented that DNA, in general, denatures upon dehydration, but that hindrances to rotation in the solid state may prevent long fragments from dissociating.

INTRODUCTION

The mobility shift assay is a powerful tool for detecting very small amounts of protein that will bind specifically to a radiolabelled DNA sequence. A small amount of radiolabelled DNA and crude nuclear extracts are used in this assay to detect specific DNA binding proteins, to define conditions required for binding, to locate the binding region more precisely, and to assay purification of DNA binding proteins during chromatography procedures. The inherent sensitivity of the technique requires that conditions be adjusted to enhance specific protein binding while minimizing binding of non-specific proteins that are present in nuclear extracts. A critical assumption when mobility shifts are used is that the DNA used for binding is homogeneous in conformation. Although the validity of this assumption is usually not questioned, we have observed that misleading gel shifts can be obtained when the radiolabelled DNA fragment is inadvertently induced to undergo a structural transition to a conformer that has some single-stranded character.

METHODS AND MATERIALS

Isolation of Nuclear Extracts

Nuclear extracts from HTC cells were obtained using a method described by Sealey and Chalkley (1). Isolated nuclei from 5×10^8 cells were washed sequentially with 0.1, 0.3, and 0.5 M NaCl buffers. The 0.1 to 0.3 M NaCl fraction was precipitated with 70% ammonium sulfate and the pellet was redissolved in 2 ml of 10 mM Hepes pH 8.0, 200 mM NaCl, 7 mM BME, and 50% glycerol.

Polyacrylamide Gel Electrophoresis

The mobility shift gel in Figure 1 was 4% polyacrylamide gel run in 25 mM Tris pH 7.4, 190 mM glycine, 1 mM EDTA at 150 volts. Subsequent gels were run in 90mM Tris pH 7.4, 90 mM Borate, 1 mM EDTA (TBE). In both cases, gel and running buffer have the same composition.

Radiolabelling of DNA fragments

Plasmid DNA was digested with the appropriate restriction enzyme (using manufacturer's conditions) in a 20 μ l volume which was then adjusted to 0.5 mM dCTP, dGTP, and dTTP. 5 μ l of 10 μ Ci/ μ l α - 32 P-dATP and 5 units of DNA polymerase I were added to the mixture cooled on ice. After 30 minutes, the reaction was terminated by adjusting to 0.2% SDS, 10 mM EDTA, and 2 μ g proteinase K. The mixture was analyzed by electrophoresis on a 6% TBE acrylamide gel which was stained with ethidium bromide. The fragment of interest was excised from the gel and electroeluted in 1/10 TBE.

Phenol extraction/ethanol precipitation/dehydration

The aqueous DNA solution was extracted once with an equal volume of phenol/chloroform (1:1). A second extraction was performed with an equal volume of chloroform/isoamyl alcohol (24:1). A 1/10 volume of 3 M sodium acetate and 2 volumes of cold 100% ethanol were added. The material was mixed and placed in a freezer at -20°C for at least 15 minutes. Samples were centrifuged 15 minutes in eppendorf centrifuge and supernatant was discarded. The residual ethanol (~10 μ l) was removed by at least 20 minutes of lyophilization. Samples were redissolved in either 10 mM Tris pH 7.4, 1 mM EDTA (TE) or 10 mM Tris, 0.1mM EDTA (T1/10E) to a concentration of approximately 1 ng/ μ l.

S1 nuclease digestions

S1 nuclease was incubated with DNA fragments in a buffer containing 10 mM sodium acetate pH 4.5, 200 mM NaCl, 1mM MgCl₂, 0.1mM ZnCl₂ at 37° C. S1 nuclease was used at 5 units S1 nuclease/100 μ l volume. The products were run on an 8% acrylamide, 7.7 M urea, 13% formamide sequencing gel in 1X TBE. Purine cleavages were performed as described elsewhere (2).

Computer structure programs

An RNA secondary structure program was used as a convenient, albeit approximate, device to explore possible conformations of single stranded DNA (3).

Micrococcal Nuclease Digestions

Nuclei from 1×10^8 cells were washed and resuspended in 8 ml of 0.25 M sucrose, 10 mM Tris pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM $CaCl_2$. 20 units/ml of micrococcal nuclease were added and the mixture was incubated for 30 minutes at 37° C. The reaction was terminated by adjusting to 20 mM EDTA and placing at 0° C. The samples were centrifuged at low speed to pellet nuclei and an aliquot of the supernatant was run on an agarose gel. The supernatant was found to contain a discrete monomer band (150-160 bp) with no detectable multiples of this size (data not shown). The supernatant was precipitated with ethanol (but not dried) and redissolved in TE.

Trichloroacetic Acid Precipitations

100 µg of salmon sperm DNA was added to the sample before addition of 5 ml of ice-cold 10% trichloroacetic acid (TCA). The mixture was placed on ice for 15 minutes and then filtered through a Whatman GF/A glass fiber filter. The filter was washed four times with 5 ml of cold 10% TCA and once with 5 ml of cold 100% ethanol. The filter was allowed to dry before adding scintillation fluid and counting.

RESULTS**Appearance of an anomalous form of DNA with unusual protein binding capacity.**

We first detected an anomalously migrating form of fragment DNA while searching for proteins that might bind to the 93 base pair repeats found in rat satellite DNA. There are four related (but not identical) 93 bp sequences which are repeated, strictly in order, in long tandem arrays in the rat genome (4,5). All four types of these repeats, which are all bounded by Eco RI sites, have been individually cloned into pBR322, so that an Eco RI digest of one of these plasmids yields a 93 bp band which can easily be separated on a gel from vector DNA (Figure 1, lane A). The 93 bp fragment was combined with nuclear extracts from rat hepatoma tissue culture (HTC) cells in a standard mobility shift assay with a large excess of poly dI:dC as non-specific competitor (Figure 1, lanes D,E). The band near the top of the gel that appears at the higher protein concentration in lane D is almost certainly due to a small amount of histones present in higher salt extracts (>0.3 M) of nuclei. This band can be removed if the extracts are passed through sepharose which selectively and irreversibly binds histones (R. Chalkley, unpublished observations). Since we were not able to see any specific binding under these conditions, the fragment was extracted with phenol to remove any impurities that might inhibit binding.

After phenol extraction and ethanol precipitation of the DNA, addition of nuclear extract gives rise to shifts which are efficiently competed for by the unlabelled fragment but not by similarly sized fragments obtained from Hha I

Figure 1. Preferential binding of some nuclear proteins to an aberrant form of DNA.

A) Eco RI digested p93-2 (digest from which 93 bp fragment was obtained)
 B) 93, 186, 279 bp standards
 C) electroeluted 93 bp fragment
 D) 93 bp fragment + 1µg poly (dI-dC) + 500 ng protein of 0.3 M NaCl nuclear extract
 E) same as previous lane except ten fold less nuclear extract was added
 F) phenol extracted, EtOH ppt'd, 93 bp fragment
 G) lane F material + 1 µg poly (dI-dC) + 500 ng nuclear extract
 H-K) competitions with 1X, 2X, 5X, 10X molar excess of unlabelled 93 bp fragment (using 50 ng protein, the amount used in lane E)
 L-O) competitions with 1X, 2X, 5X, 10X unlabelled 100 bp fragment from Hha I digested vector pTZ18R
 Arrow on right shows position of unusual form of DNA fragment.

digested pTZ18R (lanes H-O). However, as can be seen in the lane with 93 bp DNA alone (lane F), there is an additional form that is present in the radiolabelled probe (see arrow) that has a slightly slower mobility than the 93 bp DNA, but is clearly not a dimer. Furthermore, when a large amount of protein is used in the binding assay (lane G), it seems that this unusual form is preferentially shifted. Competition with unlabelled 93 bp DNA is able to restore the amount of this material to its original level (lanes H-K). Furthermore, assays employing fragments that lack the additional form do not show these same shifts (lanes C-E).

Figure 2. Dehydration induces the aberrant form which has different sensitivity to nucleases than the parental form.

Electrophoresis was done on a 6% acrylamide gel at 150 volts for three hours.

- A) electroeluted 93 bp DNA (starting material for lanes B-M)
- B) ethanol precipitated, not dried
- C) precipitated with ethanol containing 10% phenol, not dried
- D) ethanol precipitated, washed with 100% ethanol, not dried
- E-H) ethanol precipitated, lyophilized for 1,2,5,20 minutes
- I) ethanol precipitated, lyophilized for 20 minutes with flask cooled in dry ice bath (flask was warmed slightly in a water/ice bath during the last five minutes to ensure complete removal of the ethanol)
- J) 93 bp fragment in 10 μ l TE, lyophilized 20 minutes
- K) same as previous lane, except lyophilization was for 16 hours
- L) 93 bp fragment incubated at 68° C 20 minutes
- M) 93 bp fragment in 90% formamide
- N) EtOH ppt'd, dried 93 bp DNA
- O) 93 bp fragment in S1 nuclease buffer 20 minutes 37°C
- P) same as lane O, with 5 units of S1 nuclease added to 50 μ l rxn mixture
- Q) EtOH ppt'd, dried fragment in restriction enzyme buffer 20 minutes 37°C
- R) same as lane Q, digested with 5 units Hae III for 20 minutes
- S) same as lane Q, digested with 5 units Hinf I for 20 minutes
- T) 186 bp standard

It appears that a substantial amount of protein in these extracts is capable of binding the anomalous form because the amount of nuclear extract necessary to obtain efficient binding is 80 fold less than that normally used in such assays for enhancer-binding proteins (data not shown). We note that most of the anomalous form of DNA is driven into complex formation even under these dilute conditions. All members of the cloned 93 bp families are capable of developing this anomalous form which binds protein with great avidity in gel retardation

assays. The appearance of this material was surprising since the DNA had been obtained by excision of a single band from an acrylamide gel. Even more puzzling was the observation that the digest from which this material was obtained (lane A) did not contain the additional band. Furthermore, lane C shows that electroelution of the fragment from the gel slice has not produced this form. Therefore, we conclude that the appearance of the aberrant form is correlated in some way with the procedure that was used to concentrate and purify the labelled fragments as described in the Methods section.

Drying of ethanol-precipitated DNA is critical in anomalous band formation.

We indicated above that fragments that were not further purified after electroelution from an acrylamide gel do not show anomalously migrating material, nor do we obtain the mobility shifts which are generated when the anomalous form is present. In our search for the conditions that induce the formation of this band, we checked all the steps involved in the phenol extraction/ethanol precipitation procedure that was used to purify our fragments. Since ethanol and other organic solvents have been reported to reduce the T_m of DNA (6,7) and to reduce helical twist (8), we suspected that exposure to these agents might induce some conformational change. However, we found that exposure to phenol and/or ethanol is not sufficient to induce formation of the protein-binding conformer. As can be seen in Figure 2, fragments that are precipitated but not dehydrated are able to maintain their normal structure (lane B). However, as shown in lanes E-H, if the precipitate is dried in a lyophilizer we now detect the anomalous band. Such drying of the pellet of ethanol precipitation is routinely done to remove the last traces of ethanol. In addition, the aberrant form can be produced simply by dehydrating an aqueous solution of the DNA fragment under vacuum at room temperature (lanes J,K). Although exposure to ethanol or ethanol/phenol does not induce the aberrant form unless drying occurs (lanes B-D), nonetheless, the presence of organic solvents does facilitate the transition, quite possibly by subsequently allowing the DNA to dehydrate more rapidly. Finally, the transition occurs during lyophilization at room temperature. In order to see whether the transition was temperature dependent in any way, we dried an ethanol precipitation with the lyophilization flask in a dry ice bath. As shown in the lane I, the transition is much reduced if the lyophilization occurs at lower temperature.

The Nature of the Anomalously Migrating Material.

The aberrant form appears to have single-stranded regions by two criteria: (1) the aberrant form is resistant to restriction enzymes which attack the parental duplex fragment (Figure 2, lanes Q-S). These restriction enzymes seem to attack the aberrant form as well but at a much slower rate. Hae III and Hinf

I probably slowly attack single stranded DNA at duplex sites that form transiently between single strands in solution (9,10). (2) the anomalous form is sensitive to S1 nuclease (as shown by a decrease in the amount of aberrant form in lanes O,P--the S1 nuclease degradation products have migrated off the gel). Three possible structures for the anomalous form suggest themselves: (1) a largely random coil single stranded molecule with little intrastrand pairing, (2) a single stranded hairpin structure (i.e. a single strand containing fairly stable duplex regions) or (3) a double stranded molecule that is partly denatured but still retains elements of the original duplex organization (i.e. a cruciform type of structure). Analyses of the 93 bp satellite DNA sequences reveals that there is substantial capacity for intrastrand pairing. The random coil model, therefore, seems unlikely because the temperature and ionic strength (TE) used to dissolve the precipitated DNA would seem to be adequate to maintain even somewhat imperfect duplex regions--whether they be inter- or intra- strand. Furthermore, the hairpin model is supported by observations that denaturation by heat or 90% formamide produces a band of the same mobility as the anomalous form (Figure 2, lanes L,M), though this does not exclude the cruciform model.

A decisive experiment to determine the correct model involves finding the concentration dependence of reversion of the aberrant form to the parent band under appropriate conditions. If the transition to the aberrant form is due to an intramolecular rearrangement--without strand separation--then the half-life for reversion back to the parent form will be independent of concentration. On the other hand, reannealing of single strands to the duplex form would be a bimolecular reaction and its half life must be concentration dependent. We have found that we cannot induce significant transition to the aberrant form using concentrations of DNA that can be seen by ethidium staining, suggesting that we do indeed have single-stranded material. We have also observed a greater amount of anomalous band formation when the dried pellet is dissolved in a larger volume (at greater dilution). However, if the anomalous form is incubated at room temperature for an extended period we can detect no significant shift to the parental form, even after several days at the concentration of DNA we employ (1 ng/ μ l). This indicates that if the material were indeed a single strand, it must contain a substantial degree of stable secondary structure. We reasoned that the stability of some hairpin-like conformation may be able to prevent the aberrant form from reannealing. Accordingly, we tried a range of elevated temperatures and ionic strengths in order to determine if we could selectively destabilize any imperfect intrastrand pairing. At 65°C and 200 mM NaCl, we can follow reversion to the duplex form at different DNA concentrations (Figure 3). The results indicate that the half-life of reversion is indeed concentration dependent. This rules out any unimolecular rearrangement that would be involved

Figure 3. DNA concentration dependence of reversion of aberrant form to parental form.

Electrophoresis was performed on a 5% acrylamide gel at 150 volts for two hours. Ethanol ppt'd, dried 93 bp DNA were incubated at 65°C, 200mM NaCl+TE at DNA concentrations shown. Timepoints represent aliquots removed from incubation mixture and put at 0°C until gel was run.

in cruciform reversion to a stable duplex. Furthermore, the elevated temperature required for reversion indicates that the aberrant form that we see on our gels is indeed stabilized at room temperature in some sort of intrastrand hairpin.

To verify this, we decided to run the products of an S1 nuclease cleavage reaction on a sequencing gel. In this case, we employ a larger (280 bp) fragment that contains two 93 bp repeats. After ethanol precipitation and dehydration, S1 digestion clearly shows a regional hypersensitivity and does not attack all points in the molecule equivalently (Figure 4). Clearly then, the anomalously migrating material has substantial elements of single strandedness, quite possibly interspersed with some duplex regions. Nucleic acid structure computer programs have been used to derive several possible structures for one 93 bp repeat sequence. Because of technical reasons, we were not able to perform S1 nuclease mapping on the 93 bp aberrant form. However, extension of the aberrant form by the Klenow fragment of DNA polymerase I shows one major band that is 20 bp longer than the fragment (data not shown). We surmise that elongation is initiated through an intrastrand primer present in a hairpin conformation. In accord with this data, a likely structure of the denatured form of the 93 bp fragment is given in Figure 4. For reference, the S1 cleavages of the 93 bp sequence within the 280 bp aberrant form are shown on this structure.

Effect of Fragment Length on Drying Induced Anomalies.

We have conducted cross-hybridization experiments in which a variety of short, labelled fragments are co-precipitated and dried with longer unlabelled fragments which include the complete sequence of the shorter. The details of

Figure 4. A possible structure for aberrant form of 93 bp repeat DNA.

The sequencing gel shows the products of an S1 digestion of the aberrant form of a fragment containing repeat DNA. The fragment was digested for 5 (lane 2) and 10 minutes (lane 3) using conditions outlined in the Methods section. The fragment used is a 280 bp fragment containing two 93 bp repeats (clones 2A and 2B) which are flanked by 30 bp (Hind III-Eco RI) and 70 bp (Eco RI-Dde I) of pBR322. Lane 1 is a purine cleavage of this fragment which was labelled at the Hind III site by addition of ^{32}P -dATP and non-radioactive dTTP, dGTP, and dCTP. Unlabelled dATP was added to fill out the ends. The S1 digestion pattern is shown for the portion of this fragment containing one 93 bp repeat and 70 bp pBR322.

The secondary structure below (for clone 2B) is based upon results from Klenow extensions of the aberrant form of the 93 bp fragment alone (see Discussion). The positions of S1 hypersensitivity within the longer fragment are shown by the numbered arrows.

mixing of different fragments are indicated in Figure 5. In each case, some of the labelled fragment, upon drying of the co-precipitate, is shifted up to the position of the longer unlabelled fragment. (Just as a denatured fragment runs more slowly than the duplex, it appears that the hybrid between strands of two different sizes migrates more slowly than a duplex of the longer size.) Obviously then, both the labelled fragments and the longer unlabelled fragments (186 and 279 bp) must denature sufficiently to allow hybridization between these fragments. The amount of cross-hybridization obtained by dehydration is comparable to that found when the mixture of fragments is completely denatured by heat (compare lanes 6 and 7, 12 and 13, 14 and 15, 18 and 19). In addition, it appears that the 59 bp fragment, when denatured, has an aberrant form that actually migrates faster than the duplex form (lane 17-19). Certain DNA fragments between 50 and 100 bp may have denatured conformers that have the same

Figure 5. Cross-hybridization of fragment mixtures denatured by heat or dehydration.

Electrophoresis was performed on a 5% acrylamide gel at 150 volts for two hours. Numbers give the fragment size in base pairs for the labelled and unlabelled fragments. In the lanes which have both labelled and unlabelled DNA fragments, there is at least a five fold molar excess of unlabelled fragment relative to the labelled DNA. Equal volumes of these mixtures were either ethanol precipitated and lyophilized 30 minutes (E) or were boiled for 3 minutes in TE and then allowed to cool at room temperature for one hour (Δ). A shorter exposure is given in panel B in order to resolve the parent bands. The 59 and 73 bp fragments were prepared by digestion of the 93 bp fragment with Hind III and Hae III, respectively. The 186 and 279 bp fragments are multimers of the 93 bp repeat clones.

mobility as the parent duplex in a given gel system. For one system of fragments, the cross-over point where single and double stranded molecules have the same mobility is 68 nucleotides (11).

Although the above experiment shows that even 279 bp fragments can denature to a certain extent upon dehydration, we wanted to ascertain how length of fragment influences the ability of repeat DNA to develop an altered conformation upon drying after ethanol precipitation. A 180 bp fragment containing 150 bp of repeat DNA (Figure 6, lanes 7,8) is capable of dehydration-induced changes, although the proportion is much less than for the 93 bp fragment (lanes 5,6).

Figure 6. Length and sequence dependence of transition to aberrant form. Electrophoresis was performed on a 5% acrylamide gel at 150 volts for two hours. Lanes 1-12 show ethanol precipitations of repeat DNA within increasing fragment lengths from 59 to 372 bp. Lanes 13-20 show some fragments that are not repeat DNA but are also capable of dehydration-induced aberrant forms.

- 1) 60 bp Eco RI-Hind III fragment of 93 bp repeat clone 10B
- 2) EtOH ppt. of lane 1 material
- 3) 70 bp Eco RI-Hae III fragment from 93 bp repeat clone 2A
- 4) EtOH ppt. of lane 3 material
- 5) 93 bp repeat clone 2A
- 6) EtOH ppt. of lane 5 material
- 7) Hind III p93-10 (contains 150 bp repeat DNA and 30 bp pBR322)
- 8) EtOH ppt. of lane 7 material
- 9) 280 bp Dde I-Hind III p93-2 fragment (186 bp repeat DNA flanked by pBR322 sequences)
- 10) EtOH ppt. of lane 9 material
- 11) 372 bp fragment of repeat DNA Hind III p93-50
- 12) EtOH ppt. of lane 11 material
- 13) 51 bp fragment Eco RI-Hind III pTZ18R
- 14) EtOH ppt. of lane 13 material
- 15) 95 bp fragment Hind III-Pvu II pTZ18R
- 16) EtOH ppt. of lane 15 material
- 17) 121 bp human serine tRNA gene
- 18) EtOH ppt. of lane 17 material
- 19) Xho I-PvuI 225 bp fragment from gag gene of Rous Sarcoma Virus
- 20) EtOH ppt. of lane 19 material

Longer exposure also reveals a small amount of anomalous band formation for the 280 and 372 bp fragments containing repeat DNA (lanes 9-12). In general, as the length of the fragment increases, the proportion of fragment that becomes single stranded after drying decreases. We have also observed that dehydration

generates aggregates that cannot leave the wells in larger (> 200 bp) fragments (lanes 10,12,20). Although the nature of these aggregates is not known, we speculate that dehydration-induced denaturation might allow formation of interstrand networks.

The ability to form anomalous structures is not limited to repeat DNA.

In order to determine whether denaturation induced by dehydration is unique for the 93 bp repeats or if it is a general phenomenon, we have also assayed whether other small DNA fragments can undergo such anomalous transitions. 51 and 95 bp fragments from the pTZ18R vector (U.S. Biochemicals) were precipitated with ethanol and dried as described before. The 51 bp fragment has an anomalous band that migrates slightly faster than the duplex (Figure 6, lanes 13,14). Although, the 95 bp fragment does not show an anomalous band, it may have a denatured form that cannot be resolved from the duplex on this gel. This is in contrast to the 93 bp fragment of repeat DNA which has an aberrant form that is easily resolved on acrylamide gels. We also used a 121 bp tRNA gene fragment (provided by P.A. Weil) which can form extensive hairpin structures in single stranded form as well as a 225 bp fragment from the gag gene of the Rous sarcoma virus (provided by L. Karnitz). In both cases, we observe formation of an anomalously migrating form of DNA following drying of an ethanol precipitation (Figure 6, lanes 17-20).

Although we have shown that other sequences besides repeat DNA are capable of a dehydration-induced conformational change, the sequences chosen probably have rather stable single stranded conformers. Indeed, they were selected on

Table I. Dehydration renders a complex mixture of DNA fragments more sensitive to S1 nuclease.

Treatment of EtOH ppt.	Control	+ S1 nuclease	Heat denatured + S1 nuclease
Not dried	6529	5533	1955
Dried	5644	3207	2119

The numbers are TCA-precipitable counts that remain after incubation with S1 nuclease. ³H-DNA fragments of 150-160 bp were prepared from the rat genome by incubating HTC nuclei with micrococcal nuclease. Two samples of 3 µg of this DNA (labelled to specific activity of 15,000 cpm/µg) were precipitated with ethanol. After the supernatants were poured off, one of the samples was dried in vacuo for two hours. The samples were redissolved in 200 µl of 10 mM Tris, 0.1 mM EDTA with vigorous vortexing. Both samples were divided into four aliquots of 50 µl each. A control aliquot was placed in S1 buffer, incubated at 37° C for 30 minutes, and precipitated with 10% TCA. Another aliquot was treated the same as the control, except that 10 units of S1 nuclease were added to the 100 µl reaction volume. The last column in the table refers to an aliquot which was boiled for three minutes and then cooled immediately to room temperature. This sample was then placed in S1 buffer and digested with S1 nuclease in the same manner as above.

this basis. Therefore, we wanted to know if DNA in general could be denatured in the same way. To this end, we decided to examine the effect of dehydration on unique sequence DNA in the rat genome. Rat hepatoma tissue culture (HTC) cells were labelled with ^3H -thymidine so that we could easily follow the DNA through subsequent manipulations. Nuclei were isolated and incubated with micrococcal nuclease to produce 150–160 bp fragments representing all classes of DNA. These molecules cover a wide range of possible sequences and the unique sequences are present at sufficiently low concentration that if they are denatured, the C_0t attained during experimental manipulation is insufficient to permit significant reannealing of complementary strands. Single stranded DNA can then be quantitated by digesting with S1 nuclease. We precipitated some of this DNA with ethanol and then tested to see if its sensitivity to S1 nuclease was dependent upon whether or not the ethanol precipitate was dried. Such precipitates were dissolved to 15 $\mu\text{g}/\text{ml}$ in 10 mM Tris (T1/10E) with vigorous mixing. Aliquots of these solutions were adjusted to optimum conditions for S1 nuclease and the amount of S1 sensitive material generated by dehydration was assayed. The results from this experiment are shown in Table I. We see that heat denaturation allows S1 nuclease to digest 60–70% of the DNA. In contrast, treatment of DNA, which was not dried, with S1 nuclease leads to the digestion of only 15% (presumably reflecting some single-stranded material produced during the micrococcal nuclease digestion which was used to obtain uniformly sized DNA from the rat genome). If, on the other hand, the same DNA is dehydrated, 45% of the DNA is now rendered sensitive to S1 nuclease, clearly indicating that a substantial fraction of the total unique sequence DNA was denatured during drying. Nonetheless, as expected, the denaturation induced by dehydration is not as complete as that induced by heat. We believe that this is due to complementary strands remaining in close proximity to each other as the DNA is dehydrated. Even though, base pairing may not be maintained in the solid state, complementary strands stay in close proximity because dehydration prevents diffusion. In support of this, we have found that sensitivity to S1 nuclease of precipitated, dried DNA is dependent upon the vigor of mixing during dissolving.

DISCUSSION

We have found that anomalously migrating species of DNA molecules appear following drying of the pellet of an ethanol precipitation. The anomalous forms of DNA are, in general, identified by a slower rate of migration in polyacrylamide gels than the parental fragments from which they were obtained. They are characterized by regions of single stranded DNA and by their great stability against reversion to the duplex form, even upon prolonged incubations at 25°C, 10 mM Tris. The concentration dependence of reversion at 65° clearly

shows that the aberrant form is single stranded. Thus, we surmise that the anomalous forms are single stranded molecules with substantial duplex content (hairpin structures). Nucleic acid structure programs reveal many possible structures for the single-stranded 93 bp DNA and the S1 nuclease results confirm the existence of secondary structure by showing that only parts of the denatured fragment is susceptible to S1 cleavage (Figure 4). The tendency to form such single strand hairpins is not just a quirk of repeat DNA, but appears to reflect a general tendency for short DNA molecules to denature upon extensive drying.

The observation that DNA denatures upon drying at room temperature was surprising. Presumably, the loss of water reduces the entropic contribution to the stabilization of the duplex normally seen in aqueous environments. Although ethanol itself is capable of reducing the T_m of DNA, we have established that exposure to the organic solvent per se does not cause DNA denaturation during an ethanol precipitation. On the other hand, the presence of ethanol does increase the efficiency of the subsequent dehydration and denaturation, presumably by reducing the amount of water bound to the DNA. It has been shown that phenol extraction/ethanol precipitation permits cruciform extrusion to occur at lower supercoil densities than normal (12,13). The authors surmised that exposure to phenol was the critical factor in reducing the activation energy, but dehydration-induced denaturation would also be expected to facilitate this process. The surprising aspect of this transition is that the DNA molecules are sufficiently free in the solid state to permit the rotation necessary for complete denaturation. As the denatured fragments are redissolved (in TE in our case), we expect that two competing events take place: the development of stable intrastrand structure, and reannealing of complementary strands to form normal duplex. The final proportion that remains single stranded will depend upon the rate of duplex formation, the rate of folding of the single strand, and the stability of intrastrand hairpins. These variables, in turn, are dependent on DNA sequence, ionic strength, temperature, and DNA concentration such that adjustment of these conditions will influence the proportion of DNA that becomes single stranded when the pellet is dissolved. It is not clear if total denaturation occurs for very large DNA fragments. Presumably, local areas of denaturation occur in larger DNA fragments, but hindrances to rotation may be able to maintain the strands in register.

The identity of the abundant proteins that preferentially bind to the aberrant conformer is not clear at this point. It seems unlikely that these are HMG proteins because they are found in a 70% ammonium sulfate pellet. The most obvious possibility is that we have a very sensitive assay for replication-associated single stranded DNA binding proteins. However, other proteins such as lactate dehydrogenase (14), and proteolyzed heterogeneous nuclear RNA binding

proteins (hnRNP proteins) are also capable of binding ssDNA (15).

It is advantageous to use as small a fragment as possible in mobility shift assays in order to minimize binding of non-specific proteins to the DNA flanking specific binding sites. However, the use of smaller fragments may give rise to misleading shifts that actually represent binding of non-specific proteins to single-stranded conformations that can be induced by manipulation procedures. Furthermore, an aberrant conformation may not always have a slower mobility in the mobility shift gel system and may, in principle, have even the same mobility as the duplex (11). The appearance of aberrant conformers is probably only a minor concern if DNA concentration is kept high (> 0.5 mg/ml). Nonetheless, it would be prudent to avoid drying of ethanol precipitates used in preparing fragments for techniques that require low concentrations of duplex DNA (mobility shift assays, cloning, etc.). If it is imperative to concentrate small quantities of DNA by ethanol precipitation, we have found that denaturation can be minimized if the precipitate is cooled in a dry ice bath while the ethanol is evaporated.

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