DENATURED HELICAL SITES AND RIBONUCLEASE RESISTANT RNA ASSOCIATED WITH DNA FROM THE DORMANT SEEDS OF A HIGHER PLANT

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Summary

Denatured helical regions in the DNA of dormant cotton seeds were detected by means of ribonuclease interaction, methylated albumin kieselguhr chromatography and sedimentation analysis. Ribonuclease resistant RNA was also found associated with the dormant seed DNA. The implications of these two findings were discussed with regard to possible binding sites for RNA that stabilizes folded DNA.

Introduction

Recently, examination of genomic sized DNA in bacteria (1, 2, 3) has revealed that the DNA folds are stabilized by RNA molecules attached to the DNA at 2 or more different sites. Genomic sized DNA has also been isolated from some eukaryotic organisms including dormant seeds or seeds undergoing embryogenesis in higher plants (4, 5, 6, 7). It is probable that the DNA from these higher organisms is also folded and stabilized by RNA.

Through a procedural modification we were able to isolate DNA from cotton seeds with a higher sedimentation constant (30S) than that obtained (18-20S) by the original procedure (8). Although the 30S DNA molecules were not large enough to exhibit the stabilizing RNA linkages (1), the DNA isolates were still able to display the types of attachment sites either shown on postulated for the stabilizing RNA in procaryotic DNA (3). This communication therefore presents preliminary data on the nature of these attachment sites in the DNA from the dormant seeds of a higher plant, Gossypium hirsutum.

Methods and Materials

The DNA for this study was isolated from the dormant seeds of Gossypium hirsutum as described in detail previously (8). The only modifications of this procedure were made during the stage in which the defatted and saline-washed seed meal was combined with sodium dodecyl sulfate. Instead of continuously mixing this solution with a propeller stirrer for 3 hours, the mixture was allowed to stand overnight at 0-5° C with occasional gentle inversions of the containers. This modification resulted in a higher molecular weight preparation than that of the original method.

Before any of the RNA purification procedures were applied, the partially deproteinized DNA preparation from the described procedure was repeatedly extracted with a chloroform -1% octanol mixture until little to no denatured proteins were noted at the chloroform-aqueous-interphase after centrifugation.

The resulting DNA solution from the chloroform extraction procedure was incubated with 5X recrystallized pancreatic ribonuclease (Sigma) which has been pretreated by heating to 90° C for 10 minutes in a water bath to inactivate any possible contaminating deoxyribonuclease (9). The incubation conditions and final separation of the DNA from the ribonuclease enzyme and coprecipitating RNA were conducted as described previously (9).

In addition to the latter method three other commonly used procedures of DNA-RNA separation were utilized as controls for comparative purposes. The three additional methods were gel filtration (10), alkyl ammonium salt fractionation (11) and charcoal adsorption (12).

One to three micrograms of DNA were subjected to sedimentation analysis by means of a Beckman Model E analytical centrifuge (13).

One mg samples of DNA were applied to methylalbumin kieselguhr (MAK) columns and fractionated by discontinuous elution with buffered NaCl solutions (14).

The amount of ribonucleases resistant DNA associated RNA was determined by hydrolysis of ribonuclease treated DNA in 90% formic acid and subsequent chromatographic separation of the free purine and pyrimidine bases (8). The extinction coefficient for uracil was used to determine the concentration of this base eluted from the paper chromatograms and ultimately RNA in the DNA samples.

Results and Discussion

Although a recent publication (15) demonstrated that heat treated ribonuclease contained active DNA nucleases, we did not detect any degradation of our DNA samples with the particular brand of ribonucleases used in these experiments.

Both MAK chromatography and sedimentation analysis, which generally separates DNA according to molecular size or degree of denaturation (13, 14) were performed on the purified DNA preparations. Results of the chromatographic analysis are summarized in Fig. 1 as elution profiles for each of the DNA purification procedures. All treatments displayed identical profile patterns with the exception of the ribonuclease treated sample. The latter exhibited a noticeable shift towards a higher ionic elution step indicating either an increase in molecular weight or partial denaturation of the DNA sample. The sedimentation values resulting from the purification treatments are summarized in Table 1. The only significant increase in sedimentation velocity is that of the DNA purified by the ribonuclease method, and is in agreement with the chromatographic data.

The results of both the chromatographic and sedimentation studies could be attributed to a preferential binding of ribonuclease to localized denatured areas of the DNA molecule (10, 17). Unlike the latter reports, however, our DNA-ribonuclease mixture was exhaustively deproteinized with chloroform (9) before subjecting the purified DNA to sedimentation and chromatographic analysis. Apparently, (a) the denatured

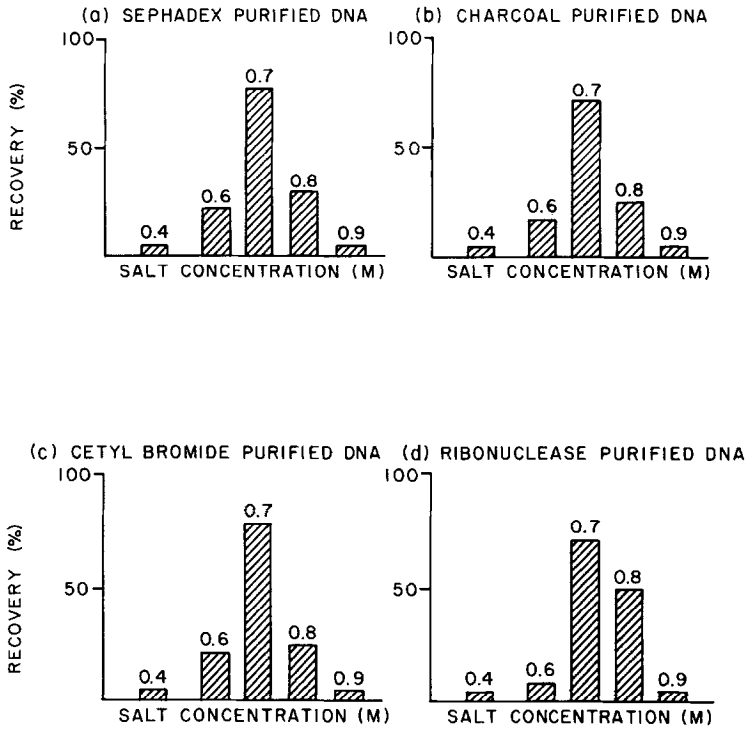

Figure 1

Distribution of DNA's, separated from light weight RNA by several purification procedures, on a methylated albumin column (MAK). One mg samples of each type of purified DNA in standard saline citrate solution were added to identically prepared columns of 10 ml MAK. Stepwise elution with increasing buffered salt concentrations was carried out. Each step resulted in two 5 ml and one 10 ml fractions. The number at the top of each histogram designates the salt concentration of the eluting buffer. The histogram at 0.4 M elution represents the scanty residual light weight RNA that was not completely separated from the DNA during the purification process.

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areas remained intact but more enlarged then that of the controls after elimination of the bound ribonuclease, or (b) some of the latter had resisted chloroform deproteinization and was still bound to the denatured areas. The second possibility was eliminated when the ribonuclease treated and chloroform deproteinized DNA was incubated with pronase (4). The resultant elution profile and sedimentation coefficient did not differ from that of the DNA samples which had only been purified with ribonuclease and then deproteinized with chloroform.

Table 1

Sedimentation coefficients of cotton seed DNA after four purification methods.[a]

DNA PURIFICATION	SEDIMENTATION COEFFICIENT Sw. 20
Sephadex C-100	29.3
Ribonuclease	34.0[b]
Cetyl Bromide	28.5
Charcoal	31.0

[a]Average mean of 2 separate determinations.

[b]Mean difference probability greater than 0.05 as determined by the t test.

As to the origin of the localized sites of denaturation, one of the postulated attachment points of stabilizing RNA is through an associated polymerase molecule at the site of RNA synthesis (3). It has been shown that the transcription of T7 phage and E. coli DNA by RNA polymerase produces localized "bubble like" denatured regions in the double helix (18, 19). Assuming that a similar process also takes place in the DNA of eukaryotic organisms, a rigorous deproteinization of our DNA samples with chloroform before application of ribonuclease or any of the other purification methods should tend to eliminate the polymerase enzymes associated with the denatured regions. The added ribonuclease would then interact and bind to those semi-stable denatured regions (16, 17). After the elimination of ribonuclease by chloroform, the ribonuclease treated DNA sample was often reduced to smaller sized molecules (18-23S) by rapid stirring or shaking. Apparently

the combination of ribonuclease interaction, binding and removal left the original denatured helical regions in the DNA sample slightly enlarged and unstable.

The other site or sites of DNA associated RNA binding are not clearly delineated. Attachment at these positions, however, may involve a direct RNA-DNA binding by a triple helix formation (3) which would be resistant to ribonuclease action (20). The following results from our experiments indicate a possibility of such a linkage. A portion of the DNA sample that had been treated with ribonuclease was subjected to an additional cetylbromide purification which in turn was followed by another ribonuclease treatment. Upon hydrolytic analysis, this DNA exhibited a 0.53 to 0.74% content of uracil. Assuming that the DNA associated RNA has approximately the same base composition as DNA (20, 21) the value of 32.5 mole percent for adenine of cotton DNA (8) was used to calculate the total amount of RNA associated with the exhaustively purified DNA. The percentage of RNA resistant to ribonuclease was then 1.6 to 2.3%. In view of our previous work (manuscript submitted), ribonuclease resistant DNA as a possible storage source of preformed m-DNA (22) can be ruled out. The storage form of m-RNA in dormant cotton embryos was found in a separate and purified isolate of heteronuclear RNA.

In conclusion, the semi-stable denatured helical regions in cotton seed DNA as detected by ribonuclease interaction and subsequent MAK chromatography or sedimentation velocity reflect one possible attachment site for stabilizing RNA. The small amount of ribonuclease resistant RNA associated with the cottonseed DNA would be indicative of still another type of binding site for the stabilizing RNA factor. Finally, the resistance of the 18 to 23S segments of DNA to further breakdown by repeated ribonuclease interaction and moderate shearing forces indicates that the binding sites of stabilizing RNA are prob-

ably located at specific points throughout the DNA molecules. Work is now in progress to demonstrate the presence and nature of the RNA stabilizing molecules in higher plants.

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