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Association of Chromosomal RNA with Repetitive DNA

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ABSTRACT **Chromosomal RNA, which is a component of the chromosomes of higher organisms, and whose participation is required for sequence-specific interaction of chromosomal proteins with chromosomal DNA, occurs in chromosomes bound to DNA. We have found that the DNA sequences to which chromosomal RNA binds are repetitive ones.**

It has been shown earlier that the participation of a species of chromosomally associated RNA, chromosomal RNA, is essential to sequence-specific interaction of chromosomal proteins with DNA. This is true for pea plant chromatin (1), and for chromatin of chick embryo (2). In this function chromosomal RNA interacts with and binds to DNA (3). The DNA of higher organisms is composed of sequences each repeated a single time in the genome (the unique sequences) and of sequences repeated a few to many times per genome (the repetitive sequences) (4). We now ask the question, with which of these classes of DNA does chromosomal RNA interact? We show below that chromosomal RNA interacts with the repetitive sequences. Since chromosomal RNA is established as a control element of the chromosomes of higher organisms, it follows that the repetitive sequences of the DNA are likewise, in part at least, also control elements.

MATERIALS AND METHODS

Preparation of chromosomal RNA

Chromosomal RNA from the buds of pea seedlings was prepared according to the method of Bonner and Widholm (5). The apical 1-cm portions of pea seedlings grown in the dark at 25°C for 6 days were removed, cooled, and homogenized in a Waring Blendor for 60 sec in grinding medium consisting of 0.25 M sucrose, 0.001 M $MgCl_2$, and 0.05 M Tris buffer, pH 8. The homogenate was filtered through cheesecloth and Miracloth and the crude chromatin was pelleted by centrifugation at 4000 × *g* for 30 min. The crude chromatin was resuspended and washed by centrifugation five times in 0.01 M Tris, pH 8. The final crude chromatin was dissolved, with homogenization in CsCl to a final concentration of 4 M, and centrifuged for 20 hr at 36,000 rpm in the Spinco 40 rotor. The pellicle of chromosomal RNA and protein was removed after centrifugation, washed five times by centrifugation in 70% ethanol, and then incubated with pronase (2 mg/ml in 0.01 M Tris, pH 8, predigested for 90 min at 37°C). After incubation for 6 hr, any remaining and denatured protein was removed by phenol extraction and the chromosomal RNA (made 0.2 N in KOAc) was precipitated from the supernate by the addition of 2 volumes of 95% ethanol. The precipitated chromosomal RNA was dissolved in 0.2 M NaCl–7 M urea–0.01 M Tris, pH 8, and chromatographed on an A-25 DEAE-Sephadex column, eluted with a linear gradient of NaCl, 0.2–1.0 M. The chromosomal RNA, which is eluted at 0.55 M NaCl, was collected, dialyzed against 0.01 M Tris, lyophilized, redissolved in 0.01 M Tris, and again precipitated from 0.2 M KOAc with ethanol.

The chromosomal RNA separated thus was labeled *in vitro* with tritium-labeled dimethylsulfate as described by Smith, Armstrong, and McCarthy (6). To 0.5 ml of chromosomal RNA in 0.1 M phosphate buffer, small aliquots of dimethylsulfate were added for 6 successive 2-hr periods. The reaction was conducted at room temperature. It yielded RNA of specific activity approximately 13,200 cpm/A_{260} unit.

Preparation of pea bud DNA

Pea bud DNA was prepared by standard procedures as outlined in ref. 7.

Reannealing of DNA

Pea DNA sheared by sonication to an average length of about 500 base pairs was dissolved in 50% (v/v) formamide made up to 5× SSC (SSC is 0.15 M NaCl–0.01 Na citrate) and melted by heating to 100°C. It was then cooled and introduced into two cuvettes. One, of 1-mm path length, contained 20 A_{260} units of DNA; the other, of 1-cm path length, contained 2 A_{260} units of DNA. Two identical cuvettes, containing solvent only, served as blanks for each. The stoppered cuvettes, sealed with paraffin, were incubated at 37°C and their absorbances were measured intermittently in a Gilford multiple-channel absorbance spectrometer equipped with a thermostatically-controlled cuvette chamber and maintained at 37°C. Rate of reannealing was monitored by the decrease in absorption at 270 nm. These reannealing conditions minimize base-pair mismatching (8). The use of formamide permits the use of a low temperature, which minimizes DNA degradation during long reannealing times (9). Rate of reannealing in the ionic strength of the solvent used is corrected to that of 0.12 M phosphate buffer by the data of Britten (10).

Hybridization

The three separated fractions of DNA were denatured (100°C, 10 min in 0.01 SSC) and applied to filters in 6× SSC as described by Gillespie and Spiegelman (11). Hybridization was carried out in 50% formamide, 5× SSC at a temperature of 37°C for a period of 17 hr. After incubation, filters were washed in 2× SSC, treated with previously heated (80°C for 15 min) RNase (20 μg/ml, 1 hr), again washed with 2S× SSC, dried, and counted in a Beckman 200-B liquid scintillation system. Retention of DNA by the filters, monitored

Abbreviation: SSC, 0.15 M NaCl–0.015 M Na citrate.

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TABLE 1. *Composition of pea genome with respect to components of various degrees of sequence redundancy**

Category of DNA	Amount per genome (g)	$Cot_{1/2}$	Genome size relative to *E. coli*	$Cot_{1/2}$ expected if all sequences unique	Redundancy ($Cot_{1/2}$ found/ $Cot_{1/2}$ expected)
Rapidly reannealing	1.5×10^{-12}	6×10^{-2}	3.8×10^{2}	5×10^{2}	1×10^{4}
Less-rapidly reannealing	1.5×10^{-12}	2.1	3.8×10^{2}	5×10^{2}	2.4×10^{2}
Slowly reannealing	2.0×10^{-12}	8×10^{2}	5×10^{2}	7×10^{2}	1.1

* Calculated from data of Fig. 1 and based on the further facts that (1) the haploid pea genome consists of 5×10^{-12} g [Birnstiel, Chipchase, and Flamm (14)], and (2) that the $Cot_{1/2}$ for reannealing of *E. coli* DNA under the present conditions is 1.3.

by perchloric acid hydrolysis of the filter-bound DNA, was complete.

RESULTS

Pea DNA contains repetitive segments, as would be expected of the DNA of a higher organism, and as is shown by the reannealing profile of Fig. 1. The data of Table 1, derived from Fig. 1, show that about 40% of the pea genome (5×10^{-12} g per haploid complement) consists of unique sequences. A further 30% consists of sequences of various degrees of repetitiveness, but of average redundancy 1×10^{4}. The final 30% consists of sequences of widely varying repetitiveness, but of average degree of multiplicity 240.

To separate the pea DNA segments of various degrees of repetitiveness, whole genomal DNA was sheared to an average length (as observed by electron microscopy) of about 500 base pairs. It was then denatured by heating to 100°C for 5 min, and reannealed at 66°C to a Cot (moles of bases per liter × seconds) value of 0.4. Inspection of Fig. 1 will reveal that this is sufficient to reanneal the bulk of the most repetitive fraction. The reannealed material was passed through a hydroxyapatite column and single-stranded material was eluted with 0.12 M phosphate buffer at 66°C as described by Britten and Kohne (4). This was followed by elution of double-stranded DNA with 0.4 M phosphate buffer, again at a temperature of 66°C. The double-stranded material was remelted and again separated into single- and double-stranded material. This procedure results in two-cycle purified, most repetitive, DNA. The single-stranded material from the first column separation was again annealed at 66°C to a Cot of 100 (sufficient to reanneal essentially all of the intermediately repetitive DNA) and again subjected to separation of double-stranded from single-stranded material by hydroxyapatite column chromatography. Each of the resulting fractions was subjected to a second round of melting, reannealing, and hydroxyapatite purification. These procedures provided three fractions of pea DNA, namely the most repetitive, the middle repetitive, and the unique sequences, each purified by two cycles of melting, reannealing, and hydroxyapatite chromatography.

The extent of hydridization at saturation of pea chromosomal RNA to each fraction is shown by the data of Fig. 2. It is clear that under the conditions of hybridization the two repetitive fractions of DNA hybridize with chromosomal RNA extensively, while the unique DNA hybridizes but little. The extent of hybridization of chromosomal RNA with whole pea DNA based on the data of Fig. 2 is somewhat more than 5%, in agreement with earlier data based on the hybridization of chromosomal RNA to whole pea DNA (5).

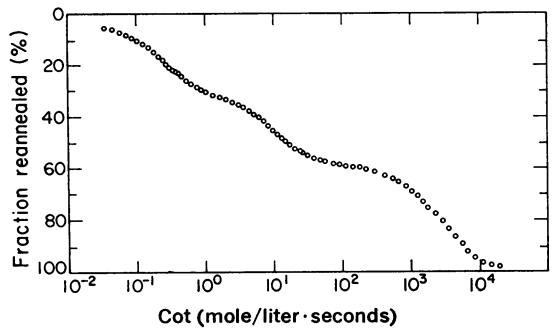

FIG. 1. Reannealing profile for denatured pea DNA. Data obtained by measurement of decrease in absorbance of denatured DNA (hyperchromicity on melting = 36%). A solution of 2 A_{260} units in a cell of 1-cm path length was used for the upper portion of the curve, one of 20 A_{260} units in a cell of 1-mm path length for the lower portion of the curve.

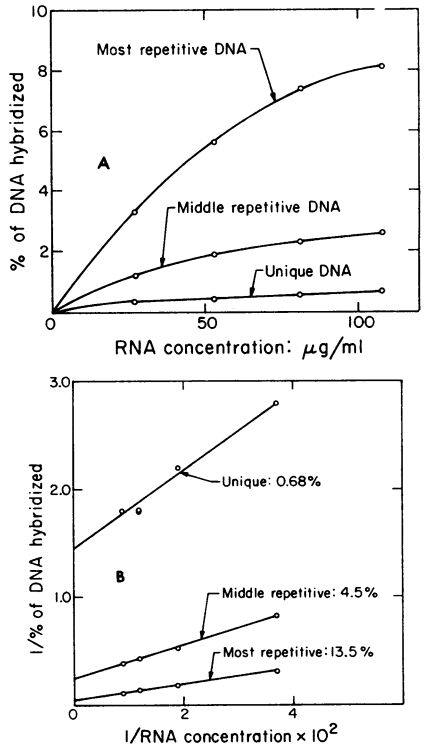

FIG. 2. Hybridization saturation curves for chromosomal RNA and the three differently repetitive classes of pea DNA. *A*, percentage of DNA hybridized as a function of input RNA concentration. *B*, double reciprocal plot of the data of *A*. From the intercepts, the amount of DNA hybridized at saturation may be obtained.

It is clear, then, that chromosomal RNA is complementary to, and hybridizes with, repetitive DNA. The remaining question is, does it hybridize to any extent whatsoever with the unique DNA of the pea genome? The answer to this question is obscured by the fact that the "unique sequence" fraction of pea DNA may not be completely free of repetitive sequences. This is supported by the fact that chromosomal RNA hybridizes to our unique sequence DNA fraction at Cot values characteristic of repetitive sequences. That the unique sequence fraction should contain some repetitive sequences would be expected on the basis of the model of Britten and Davidson (12), according to which the repetitive sequences are interspersed among the unique ones.

DISCUSSION

In one sense it is already known that chromosomal RNA is complementary to and interacts with the repetitive sequences of the genome. Thus, in the studies of Bonner and Widholm (5), hybridization to saturation of pea chromosomal RNA with denatured pea DNA was obtained at a Cot value of about 10. Significant hybridization of RNA to the unique DNA sequences does not occur over this concentration and time range. Values similar to those found by Bonner and Widholm have been reported by Dahmus and McConnell (13) and Huang and Huang (2) for hybridization of chromosomal RNAs of rat ascites tumor and of chick embryos to their respective DNAs. The findings of the present study underscore the earlier ones, and, by a different experimental approach, emphasize the interaction of chromosomal RNA with the repetitive sequences of the genome. Chromosomal RNA is, as is already known for the pea and chick chromatin systems, a control element responsible for sequence-specific interaction of chromosomal proteins with chromosomal DNA. The interaction of chromosomal RNA with the repetitive sequences of the genome implies that the latter also are control elements of the genetic material of higher organisms.

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