Interspersion of repetitive with repetitive sequences in an amphibian, *Rana berlandieri*

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ABSTRACT

When conventional genome arrangement analyses performed on *R. berlandieri* DNA at a normal (60°C) and a high (75°C) reassociation temperature were compared, an additional interspersal pattern was detected which indicates that different classes of repetitive sequences are closely interspersed with each other. Our results further suggest that the genomic abundance of purified (or cloned) repetitive sequences can be accurately determined by solution hybridization with genomic DNA only when the reassociation is performed at a relatively high temperature ($T_m - 10^\circ\text{C}$).

INTRODUCTION

Because of its general occurrence, the interspersal of repetitive with single copy sequences in the DNA of many eukaryotes is presumed to be related to eukaryotic genome function¹. However, a more detailed knowledge of interspersal phenomena is required before the role of interspersal or the interspersing mechanism(s) can be ascertained.

High temperature reassociation was used in *Xenopus* to isolate a discrete class of repetitive sequences differing in thermal stability, frequency, sequence complexity, and genome arrangement from the remainder of the repetitive sequence². This paper describes the application of high temperature reassociation to the examination of genome arrangement in *Rana*, which led to a series of observations consistent with an interspersal of repetitive with repetitive sequences in this organism.

MATERIALS AND METHODS**DNA Isolation**

DNAs were isolated from whole *Xenopus laevis* (laboratory bred), *Rana berlandieri* (Mogul-Ed), *R. pipiens* (Mogul-Ed), or

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Ambystoma tigrinum (Lemberger Associates) as described³.

Nick Translation

DNAs were labeled by nick translation^{2,4} using ³H-labeled thymidine (40 Ci/mmol; ICN). Labeled fragments of increasing specific activity (10,000 cpm/ μ g to 250,000 cpm/ μ g) and decreasing length were prepared by using increasing amounts of DNase I (8×10^{-3} to 4×10^{-1} units/ μ g DNA; Calbiochem) to introduce nicks.

DNA Shearing

Long DNA fragments (lengths greater than 2400 nucleotides [NT]) were prepared by shearing native DNA preparations in 0.1 M sodium acetate, pH 6.0, at 12,000 rpm in an ice water bath in a VirTis 60 Homogenizer⁵. Driver DNAs for the interspersal curves were prepared by shearing native DNAs in 0.1 M sodium acetate, pH 6.0, and 67% glycerol at 30,000 rpm in an ice water bath. DNAs were collected by ethanol precipitation.

Agarose Chromatography

Chromatography on agarose A50m (BioRad) in 0.12 M NaPB (equimolar sodium phosphate buffer, pH 6.8) was performed as described² except that the agarose was poured over a matrix of 5-6 mm glass beads⁶ in a 1 cm diameter glass column. DNA sizes were determined by agarose chromatography, assuming $V_e/V_o=1.0$ to be equivalent to 1200 nucleotide pairs [NTP] (or 2400 NT) and $V_e/V_o=2.0$ to be equivalent to 135 NTP (or 270 NT)⁷, where V_e is the elution volume and V_o is the exclusion volume.

Reassociations

DNAs were dissociated in 5 mM NaCl by the addition of 2 M NaOH to a final concentration of 20 mM. After a short (5-15 min, depending on sample volume) incubation at the appropriate reassociation temperature, 2 M HCl was added to a final concentration of 20 mM. Unless otherwise stated, 0.1 volume of a stock buffer (3 M NaCl, 0.1 M PIPES [Sigma], pH 6.8) was added at the start of the reassociation to bring the solution to the appropriate salt concentration. Reassociations in PIPES buffer were maintained at 4°C above the desired temperature in order to compensate for the effect of additional sodium above 0.18 M.

Hydroxyapatite chromatography

Buffers contained 0.3 M NaCl in addition to 0.12 or 0.4 M NaPB. Points for the interspersal curves were analyzed by

collection of reassociated DNA on 1 ml hydroxyapatite columns (0.4 g; HTP; BioRad). Column fractions were counted in Aquasol (NEN) as described². Absorbances were determined using a Beckman model 25K spectrophotometer equipped with a digital printout.

S1 Nuclease Digestions

S1 nuclease digestions were performed as described² using S1 nuclease isolated through the DE-52 chromatography step⁸. S1 nuclease digestions were carried to completion using amounts of S1 nuclease (10 μ l/50 μ g DNA) and conditions that resulted in more than 95% digestion of single stranded DNA⁹. Unless otherwise stated, formaldehyde was added to 1% at the end of the reassociation. This concentration does not inhibit S1 nuclease digestion¹⁰. S1 nuclease resistant DNAs were separated from digestion products by chromatography on Sephadex G-100 in 0.12 M NaPB at room temperature or hydroxyapatite at 40°C as described above. In our hands there was no difference between these two methods of collection in the amount of DNA measured as S1 resistant or in the A50m chromatography profiles.

Melting Point Determinations

Melting point (T_m) determinations were made in 40 mM NaPB using a Beckman Model 25K recording spectrophotometer as described². T_m 's of a column series were normalized to the T_m value of the standard (horse DNA, 300 NTP) for a single melt. T_m 's of the same organism were all normalized against the same standard DNA's T_m .

RESULTS

Genome Arrangement Analyses at High and Normal Reassociation Temperatures

S1 digestion patterns⁹⁻¹¹ and interspersion curves¹²⁻¹³ performed at $T_m - 25^\circ\text{C}$ allow the determination of a variety of aspects of genome arrangement. For the purposes of this paper, two classes of repetitive sequence will be defined: interspersed repetitive represents short repetitive sequences interspersed with single copy DNA and clustered repetitive represents repetitive sequences of any length located adjacent to repetitive sequences (either the same or different) of any length.

An S1 nuclease digestion analysis of R. berlandieri DNA following 60°C reassociation (Figure 1) indicated that the DNA

Figure 1. Effect of a high temperature on the reassociation of clustered and interspersed repetitive sequences in *R. berlandieri*. Long DNA fragments (1.5 mg) were reassociated to *Ecot* 50 at either 60°C (—●—; 1.25 mg/ml) or 75°C (—○—; 0.14 mg/ml). Following reassociation, the DNA was digested with S1 nuclease and the resistant fragments were collected by hydroxyapatite chromatography and passed over agarose A50m.

was organized in a short period interspersion pattern; 17% of the total DNA being clustered repetitive sequences and 24% interspersed repetitive, the average size of the latter being 250 NTP. In *R. berlandieri*, more of the total repetitive sequences were present as clustered (41%) than in another frog, *Xenopus laevis*, whose clustered sequences make up only a quarter of the

repetitive DNA^{2,11}.

Since reassociation at a stringent criterion (75°C) can be used to induce repetitive sequences to form hybrids that are better paired than those that form at a normal criterion, increasing the stringency of criterion tends to alter the rates of reassociation of broadly related repetitive sequences^{2,14}. Too extensive a dissimilarity between the members of a repetitive sequence family would prevent formation of any hybrids at a low *Cot* under stringent conditions although they would eventually pair at more extended *Cots*. Sequences that can reassociate at a low *Cot* at 75°C are likely to be very similar in sequence and internal organization; these will be referred to as high temperature reassociating (HTR) sequences. In contrast, sequence families made up of members that are relatively dissimilar can not reassociate at 75°C. These are sequences that do not reassociate at high temperature (HTUR). In Xenopus, this was found to be a relatively discrete class of families².

At 75°C ($T_m - 10^\circ\text{C}$), there is only a small effect on the rate and extent of reassociation^{2,15} of single copy sequences, while there is a strong reduction in the amount of reassociation of dissimilar sequences². The selection of an *Ecot* which is in a relative plateau area (*Ecot* 50 for Rana and 10 for Xenopus) ensures that small changes in rate do not effect the interpretation of the results.

When Xenopus DNA was analyzed by S1 nuclease digestion after reassociation at 75°C, there was a substantial disappearance (40%) of interspersed repetitive sequences with relatively little loss (10%) of clustered repetitive sequences². In contrast to Xenopus, when R. berlandieri DNA was reassociated at 75°C and then digested with S1 nuclease, three-quarters of the clustered repetitive sequences were no longer present (Figure 1), compared to the disappearance of less than a third of the interspersed repetitive sequences. A very similar observation was made for a related ranid, R. pipiens (data not shown). From this observation, it appeared that much of the clustered repetitive sequence was being either partially or completely digested by S1 nuclease following 75°C reassociation.

The interspersion patterns of Rana DNA observed at 60°C and 75°C were consistent with the results from the S1 nuclease

digestion patterns. At 60°C, 36% of the DNA was repetitive at "zero length". Short period interspersion was observed, with about 30% of the DNA behaving as interspersed unreassociated DNA with an average length of 600-800 NT (Figure 2). Nearly the same interspersion pattern was observed at 75°C as at 60°C except that the amounts bound to hydroxyapatite were diminished, with 17% of the DNA scored as repetitive at "zero length". However, the amount of interspersed unreassociated DNA remained about the same, 29% of total DNA, and so did the average interspersed sequence length of approximately 600-800 NT.

At 75°C in *Xenopus*, there was little increase in the amount of tracer bound with increasing tracer length, indicating the disappearance of the short period interspersion pattern,

Figure 2. Effect of a high reassociation temperature on interspersion pattern measurement in *R. berlandieri*. Interspersion patterns at *Ecot* 50 were determined at either 75°C (—○—) or 60°C (—●—). Tracers (1000-3000 cpm) of increasing lengths were reassociated with 50 µg of total DNA (average size, 600 NT) in one ml of 0.12 M NaPB. The amount of DNA resistant to S1 nuclease digestion following reassociation of long fragments to *Ecot* 50 at each temperature is indicated by the squares at "zero length".

presumably as the result of the lack of reassociation of the interspersed repetitive sequences². In contrast, in Rana there was apparently the same amount of short period interspersion at either 60°C or 75°C, although there was a marked overall reduction in the amount of tracer bound to hydroxyapatite.

Thermal Stabilities of S1 Nuclease Digested Fractions

Diminished melting temperatures are an indication of mispairing at sites of base substitutions. Following 60°C reassociation, S1 digestion and agarose A50 chromatography, the thermal stabilities of the long, excluded fragments (clustered repetitive) and the short fragments (interspersed repetitive) can be used to determine the relative amounts of mispair in each class⁹⁻¹¹. In Xenopus, the thermal stability of interspersed repetitive sequences was more than 10°C lower than that of the clustered repetitive sequences¹¹ (Table 1).

If the apparent disappearance of clustered repetitive

Table 1. Thermal Stabilities of 60°C Reassociated, S1 Digested DNA Fragments.			
Organism	Long Rep. T_m , °C	Short Rep. T_m , °C	Difference °C
<u>X. laevis</u> ¹	82.0	71.1	10.9
<u>R. berlandieri</u> ²	85.5	72.6	12.9
<u>A. tigrinum</u> ³	83.2	74.4	8.8

Following S1 digestion, resistant DNAs were isolated by Sephadex G-100 chromatography and then passed over agarose A50m. Long resistant fragments were those excluded from the column and short fragments were those that chromatographed in the region of 300 NTP. Fractions were dialysed against 0.04 M NaPB prior to melting. Each melt was compared to the melting profile of a standard DNA in the same buffer. T_m 's were corrected for the effect of length using the formula $\Delta T = 650/L$, where ΔT is the temperature depression due to short fragment length and L is the length of the fragment in nucleotides⁴. The length correction was added to the observed T_m and the corrected value is presented above. The melting temperature correction for the long fragments was 0.54°C in each case.

¹ Reassociation was to Ecot 10. The short fragments had an average length of 325 NTP (length correction=2.0°C).

² Reassociation was to Ecot 50. The short fragments had an average length of 280 NTP (length correction=2.3°C).

³ Reassociation was to Ecot 1000. The short fragments had an average length of 300 NTP (length correction=2.2°C).

sequences in Rana had been due to high sequence divergence in clustered DNAs and relatively little divergence in interspersed DNA, then the long fragments would have been expected to have lower melting points than the short fragments. Instead, in both R. berlandieri and A. tigrinum, the tiger salamander, the reassociated short fragments had melting points approximately 10°C lower than the long (Table 1). From these data, it appeared that, as in other organisms, more base substitutions are present in the interspersed repetitive sequences of Rana and Ambystoma than in their clustered sequences.

When the melting points of DNAs reassociated at 75°C and then digested with S1 nuclease were compared (Table 2), the difference between the T_m 's of the long and short fragments was 5°C in Xenopus. The difference between the melting points of reassociated long and short sequences of Rana and Ambystoma was smaller (1-2°C). While the thermal stabilities of the interspersed repetitive sequences of Rana and Ambystoma would have been expected to increase following high temperature reassociation, the increase was greater than that of Xenopus.

From the 60°C data, it seemed that the interspersed repetitive sequences of all three amphibians had similar amounts of base substitutions; following 75°C reassociation and S1 digestion, it appeared that, in Rana and Ambystoma, sequences with higher intrinsic thermal stabilities (fewer base

Table 2. Thermal Stabilities of 75°C Reassociated, S1 Digested DNA Fragments.

Organism	Long Rep. T_m , °C	Short Rep. T_m , °C	Difference °C
<u>X. laevis</u> ¹	81.5	76.5	5.0
<u>R. berlandieri</u> ²	82.2	80.9	1.2
<u>A. tigrinum</u> ³	87.0	85.8	1.2

¹ Reassociation was to Ecot 10. The short fragments had an average length of 290 NTP (length correction=2.2°C).

² Reassociation was to Ecot 50 at a low DNA concentration (0.14 mg/ml). The short fragments had an average length of 250 NTP (length correction=2.6°C).

³ Reassociation was to Ecot 1000. The short repetitive fragments had an average length of 300 NTP (length correction=2.2°C).

substitutions) appeared in the putative interspersed repetitive sequence class.

Disappearance of Interspersed Repetitive Sequences at 75°C

The actual disappearance of interspersed repetitive sequences was difficult to assess in this situation. If clustered repetitive sequences were being partially digested by S1 nuclease following 75°C reassociation, then the presence of the resultant short precise sequences in the region of the chromatograph where interspersed repetitive sequences also occur would have tended to mask any diminution in the amount of interspersed repetitive sequences.

To get an estimate of the actual loss from the interspersed repetitive class after reassociation at 75°C, the following experiment was performed. Radioactively labeled interspersed repetitive sequences from a 60°C reassociation were isolated following S1 digestion and A50 chromatography. These sequences should be relatively, although not entirely, free of short lengths of clustered repetitive DNA. When re-reassociated with total DNA at 75°C to Ecot 50 and then S1 digested, half (48%) of these sequences were S1 resistant relative to the amount that was S1 resistant following re-reassociation with total DNA at 60°C and Ecot 50. It appears that, from the discrepancy between the estimated disappearance of repetitive sequences interspersed with single copy sequences (52%) and the observed disappearance of short S1 resistant fragments (27%), short S1 resistant fragments which are derived from the clustered repetitive class are being superimposed over the true interspersed repetitive class.

Although similar amounts of interspersed repetitive sequence were lost from Rana or Xenopus following 75°C reassociation, the shape of the short period interspersion pattern at 75°C stays the same for Rana (Figure 2) unlike the case for Xenopus². It seems likely that another interspersion pattern could exist within the clustered repetitive sequence class consisting of relatively short (about 300 NT) HTR repetitive sequences interspersed with longer (600-800 NT) HTUR repetitive sequences unable to reassociate at the stringent criterion.

DNA concentration and clustered repetitive sequence disappearance at 75°C

Following reassociation of R. berlandieri DNA to Ecot 50 at

75°C and at a low DNA concentration, there was a 73% reduction in the amount of clustered sequences in relation to those observed at 60°C (Table 3). However, when Rana DNA was reassociated to Ecot 50 at 75°C and at a high DNA concentration, there was only a 25% decrease in the amount of clustered repetitive DNA in relation to 60°C reassociation and a 176% increase compared to 75°C reassociation at a low DNA concentration (Figure 3; Table 3). When Rana sequences are reassociated at 60°C, there is little alteration in the S1 digestion pattern with changes in DNA concentration.

In comparison with 60°C reassociation, the amounts of short S1 resistant sequences were also reduced following 75°C reassociation, by 27% for the low concentration and by 31% for

Figure 3. Effect of DNA concentration during reassociation on the disappearance of clustered repetitive sequences at 75°C. Long R. berlandieri DNA fragments (1 mg) were reassociated to Ecot 50 at a concentration of 1.66 mg/ml (--- Δ ---) or 0.14 mg/ml (— $\circ$ —), S1 digested, isolated on Sephadex G-100, and passed over agarose A50.

Table 3. Quantitation of Results from R. berlandieri Experiments.

Parameter Varied	Reass. T, °C	Fig	DNA Conc. (mg/ml)	Ecot	Formaldehyde	Perc of Total Clus. (Ex)	Total Inters. (Short)
	60*	1	1.25	50	-	16.6	24.2
	60*	-	2.50	300	-	17.6	24.3
DNA concentration	75	3	1.65 ¹	50	+	12.4	16.7
	75	3	0.14 ²	50	+	4.5	17.6
Formaldehyde	75	4	0.14 ²	50	+	4.5	17.6
Addition	75	4	0.14	50	***	7.4	17.5
[low DNA conc]	75	4	0.11	50	-	10.1	15.3
Formaldehyde	75	-	1.65 ¹	50	+	12.4	16.7
Addition	75	-	0.99 ³	50	-	13.5	14.3
[high DNA conc]							
Ecot	75	5	0.99 ³	50	-	13.5	14.3
	75	5	0.84	300	-	4.8	25.7

*Included for comparison. At 60°C, similar values are obtained at high or low DNA concentrations, with or without formaldehyde.

**Formaldehyde was added after 5 min incubation at room temperature.

Superscripts indicate the same experiment.

the high concentration reassociation (Table 3).

Reassociation at 75°C and at DNA concentrations less than 100 µg/ml apparently resulted in pairings separated by regions susceptible to S1 nuclease digestion, while reassociation at DNA concentrations greater than 500 µg/ml resulted in the observation of longer S1 resistant fragments.

In addition to a changed concentration of DNA during reassociation, these experiments also differed in that the amount of S1 nuclease/ml was reduced in the low DNA concentration reassociation experiments relative to that of the high DNA concentration experiments, since in each experiment the S1 nuclease/DNA ratio was kept constant. However, when R. berlandieri DNA reassociated at a high concentration at 75°C was diluted to a low concentration [with PIPES at 75°C] before S1 digestion, the same amount of clustered repetitive sequences was seen as if it had not been diluted. Further, the A50 profile of

Xenopus DNA reassociated at 75°C and then S1 digested was the same whether or not it had been reassociated at a low or a high concentration. Therefore, the different observations of Figure 3 did not result from the difference in S1 nuclease concentration.

If HTR repetitive/HTUR repetitive interspersions were present, a reduction in reassociation criterion from $T_m - 10^\circ\text{C}$ would lead to immediate and extensive zippering since similar sequences would tend to be located close to their complements, unlike the situation in single copy/repetitive sequence interspersions. As would have been expected from this prediction, the degree of disappearance of clustered repetitive sequences in Rana was observed to be dependent on different experimental parameters. This variability was in strict contrast to the behavior of Xenopus DNA, for which the amount of clustered repetitive DNA observed following reassociation at 75°C remained similar despite change in experimental parameters such as DNA concentration and the presence or absence of formaldehyde.

Formaldehyde and Clustered Repetitive Sequence Disappearance at 75°C

The disappearance of clustered sequences following low DNA concentration reassociation was measured in relation to the addition of 1% formaldehyde to inhibit further reassociation¹⁶. Following immediate addition of formaldehyde, a smaller amount of clustered repetitive sequences was observed (Figure 4, curve a) than if five minutes elapsed at room temperature before its addition (Figure 4, curve b). Unless formaldehyde was added prior to S1 nuclease digestion, some zippering seemed to occur even during the digestion; that is, there was an increase in the amount measured as clustered (Figure 4, curve c). While the amount of short S1 resistant DNA decreased only slightly in these experiments (Table 3), the average size of the "short" S1 resistant sequences increased from 325 NTP with immediate digestion, to 400 NTP after five min at room temperature, to 540 NTP with no formaldehyde added. This was the result of an increase in the "valley" region of the A50 profile accompanied by a reduction in the amounts of the shortest S1 resistant sequences. Thus there seemed to be a transition in the average sizes of the partially digested sequences so that they approached the size of the longer, clustered sequences.

Figure 4. The effect of the addition of formaldehyde on the reassociation of clustered repetitive sequences at 75°C. Long *R. berlandieri* fragments (1 mg) were reassociated at low concentrations (0.11 mg/ml to 0.14 mg/ml) to Ecot 50. Formaldehyde was immediately added to 1% to one sample (—o—), added after a five minute incubation at room temperature to another (---Δ---), and not added to a third, which was immediately digested with S1 nuclease (---■---). The first two were digested with S1 nuclease as soon as the formaldehyde had been added. S1 resistant fragments were isolated on Sephadex G-100 and passed over agarose A50.

Although there were reductions in the amount of clustered sequences in the high concentration reassociations if formaldehyde was added, there seemed to be a continuum in its

effect related to increasing DNA concentration. For example, similar amounts of clustered DNA were observed (Table 3) following reassociations at 0.99 mg/ml, in the absence of formaldehyde (13.5%) or at 1.65 mg/ml, in the presence of formaldehyde (12.4%). That is, zippering seems to be enhanced by higher DNA concentrations.

The presence of formaldehyde during S1 digestion was not responsible for an loss of clustered sequences due to artifact. In Rana, the same results were observed at 60°C in the presence or absence of formaldehyde. In Xenopus, the same profiles were observed following 75°C reassociation whether or not formaldehyde was present. In addition, following high DNA concentration reassociation, the same amounts of clustered repetitive sequences were observed when S1 digestion was performed in either 1% or 5% formaldehyde.

These data are consistent with sequence arrangements that promote zippering when the stringent criterion is reduced. This zippering phenomenon also seems to be enhanced by increased DNA concentrations.

The Effect of Slowly Reassociating DNA on Clustered Repetitive Sequence Disappearance at 75°C

Reassociation to Ecot 300 at 75°C led to disappearance of clustered repetitive sequences (Figure 5), independent of changes in other parameters. There was a 64% disappearance of clustered repetitive sequences at Ecot 300 compared to an experiment at Ecot 50, 75°C, and a high DNA concentration. The clustered sequence disappearance at Ecot 300 is accompanied by an 80% increase in the amount of short S1 resistant DNA compared to the Ecot 50, 75°C, high DNA concentration results (Table 3). The larger total amount of S1 resistant DNA was due to the pairing of sequences that are able to reassociate at 75°C, but which do so slowly. In all likelihood, the increase in short S1 resistant fragments observed at 75°C and Ecot 300 was primarily due to partial digestion of clustered repetitive sequences, since there was no increase in these sequences as had been observed following Ecot 50 reassociation at high DNA concentrations at 75°C. Thus, these short S1 resistant sequences are likely to be derived from HTR sequences that reassociate between Ecot 50 and 300 and that are interspersed with rapidly reassociating HTR repetitive

Figure 5. Interrelationship between Ecot and the reassociation of clustered repetitive sequences at 75°C. Long *R. berlandieri* fragments were reassociated to either Ecot 50 (Δ ; 1.66 mg/ml; 1 mg total) or Ecot 300 ($\square$; 0.84 mg/ml; 2.5 mg total) and immediately digested with S1 nuclease; no formaldehyde was added in either case. The S1 resistant DNA was isolated on Sephadex G-100 and passed over agarose A50.

sequences, while unreassociated, S1 nuclease sensitive HTUR regions still exist between them.

Genome Size and the Loss of Clustered Repetitive DNA Reassociation at 75°C

It was possible that the phenomenon responsible for clustered repetitive sequence disappearance at 75°C might have been correlated with large genome size. The genome size of *R. pipiens* (7.5 pg¹⁷) is more than twice as large as that of *Xenopus*¹⁷. Presumably the genome size of *R. berlandieri* is similar to that of *R. pipiens*, as their single copy reassociation kinetics are similar (data not shown) and as they

are closely related species. We therefore examined the DNA of an amphibian possessing a much larger genome, the tiger salamander, Ambystoma tigrinum (43 pg¹⁸). Although short period interspersal was observed in the tiger salamander at 60°C (Figure 6), at 75°C a similar pattern of clustered sequence disappearance was observed as had been seen in Rana. This was a high concentration reassociation, but the results indicate that in the tiger salamander loss of the clustered sequences occurs

Figure 6. Effect of a high temperature on the reassociation of clustered and repetitive sequences in Ambystoma tigrinum. Long DNA fragments (1.9 mg/ml) were reassociated to Ecot 1000 at either 60°C (—●—) or 75°C (—○—). After reassociation and S1 nuclease digestion, resistant DNA was isolated by Sephadex G-100 chromatography and then passed over agarose A50.

under these conditions. This could indicate that a different sort of sequence arrangement of the clustered repetitive DNA exists in this organism. Alternatively, a more appropriate comparison could be between the Rana, Ecot 300 and the Ambystoma, Ecot 1000 data. It is possible that a relationship does exist between large genome sizes and types of sequence arrangement that inhibit the reassociation of clustered repetitive sequences at 75°C.

DISCUSSION

Sequence Arrangement within Clustered Repetitive DNA

Following reassociation at 75°C, certain characteristics of the clustered repetitive sequence class were observed:

- 1) Its extent of reassociation was concentration dependent (Figure 3),
- 2) It was reduced when zippering was prevented (Figure 4),
- 3) After reassociation to Ecots at which slowly reassociating sequences pair, its reassociation was consistently inhibited (Figure 5), and
- 4) The less clustered repetitive sequence reassociation, the more DNA was found in short S1 resistant fragments (Figures 3,5; Table 3).

At a low DNA concentration, if a long fragment containing different sequence elements has already undergone a pairing somewhere along its length, it is most likely to pair with a complementary sequence on the same fragment with which it originally paired. During reassociation at a high DNA concentration, a fragment with one pairing is more likely to pair with a complementary sequence on a different fragment.

The concentration dependence observations seem to imply that following reassociation at low DNA concentrations, these partially paired fragments have forms that hinder zippering, while at high DNA concentrations, zippering is promoted. It is possible that the alternation of different repetitive sequences with the same HTR family could lead to the formation (at low DNA concentrations) of "D-loops" that are not complementary to each other and which are sterically inhibited from pair formation.

Once the Ecot became large enough so that slowly reassociating sequences could pair, clustered repetitive sequence

reassociation disappeared, even at high DNA concentrations. This change could have resulted from the formation of structures at a higher *Ecot* that would strongly hinder zippering and that had S1 sensitive regions between the HTR and the slow HTR sequences.

Sequences that behaved as though extremely repetitive at 60°C could reassociate relatively slowly at 75°C. In *Xenopus* for example, a change from 3,000 copy to less than 10 copy was observed by increasing the reassociation criterion². Decreases in copy number were also observed in muskmelon repetitive DNA when increased reassociation temperatures were used¹⁴. The slowly reassociating HTR sequences could represent a family that reassociates relatively rapidly at 60°C, but has a much lower apparent copy number at 75°C. The average sequence of the slow HTR length is assumed to be about 300 NT, since there was an increase in resistant fragments of this size following *Ecot* 300, 75°C reassociation.

If HTR and HTUR repetitive sequences were interspersed, then after reassociation under conditions preventing HTUR sequence pairing, there would be sites in clustered repetitive DNA sensitive to S1 digestion. An HTR/HTUR repetitive interspersion pattern would explain the disappearance of some of the clustered sequences at 75°C, a similar (although diminished at 75°C) interspersion pattern at 60°C and 75°C, and also the increase in the interspersed ("short") repetitive sequence class following 75°C, *Ecot* 300 reassociation. Evidence from thermal stability studies suggests that a tandem arrangement of short repeat units may exist sea urchin clustered repetitive DNAs¹⁹. There is a molecular precedent for sequence interspersion in the repetitive/single copy interspersion pattern already present in the genome of *Rana*. A close relationship could exist between these repetitive/repetitive and repetitive/single copy interspersion mechanisms.

Application of High Reassociation Temperatures to Current Reassociation Questions

While cloning can yield information about individual interspersion events, at this time general interspersion phenomena can only be explored through the use of interspersion curves, S1 digestion patterns, or electron microscopy studies. From our high reassociation temperature analyses of sequence

arrangement in Rana and our speculation that there is an interspersion of repetitive with repetitive sequences, we may now ask by means of cloning if this hypothesis is valid. Before this work, there was little reason to expect the intermingling of such different repetitive sequence classes in clustered repetitive DNA.

Because repetitive sequences can vary within families as a result of base substitution or internal rearrangement, accurate interpretation of their reassociation kinetics has been difficult. While recombinant DNA technology and DNA sequencing methods have made it possible to compare the sequences of specific members of repetitive DNA sequence families, accurate values for the repetition frequency of the cloned DNA sequence in the genome can only be derived from reassociation experiments performed under high criterion conditions ($T_m - 10^\circ\text{C}$). A high reassociation temperature can be used in the extension of analyses of general sequence relatedness and/or repetitive sequence frequency, as we have reported in the isolation of a set of distinct sequences in Xenopus² and, in this paper, the observation of an unusual sequence arrangement in Rana.

The most sensitive probe we now have of repetitive sequence evolution is recombinant technology. But this probe could be effectively strengthened by using techniques, such as normal/high reassociation temperature comparisons, which allow a finer analysis at the reassociation level.

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