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Isolation of discrete repetitive sequence classes from Xenopus DNA by high temperature reassociation

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ABSTRACT

Sequences that did or did not reassociate at 75°C (stable and unstable, respectively) were isolated from total repetitive Xenopus laevis DNA. Sequence complexities or frequencies were determined by self (minicot) or DNA excess (slave minicot) reassociations at 60°C. Stable sequences were five times shorter and four times more frequent than unstable sequences. Reassociations at 75°C or at 50°C were used to establish apparent sequence frequencies at these criteria. Interspersion curves at either 60°C or 75°C and low Cot reassociation of long fragments of total X. laevis DNA at either 60°C or 75°C, followed by S1 digestion and agarose chromatography, were used to determine genome arrangement of the stable and unstable classes of sequence. Reassociation at high temperature was found to permit the fractionation of repetitive sequences into two populations of differing characteristics.

INTRODUCTION

Most repetitive sequence families display a continuum of relatedness so that even a self reassociation yields a continuum of mispair.[3] When repetitive sequences from different organisms are reassociated, the superimposition of two sets of mispaired sequences weakens the interpretation of sequence evolution. Because of this, attempts to determine rates of evolution of repetitive sequences have not been effective.

If it were it possible to reduce the sequence families to disparate sets, rather than continua, relationships between heterologous sequences could be examined more rigorously. Unlike single copy sequences, repetitive sequence families whose members have varying degrees of relatedness can be defined by their reassociation temperatures since their reassociation characteristics (rate, extent, and thermal stability) should be disproportionately affected by changed reassociation temperatures compared to those of single copy sequences. Thus, lowering

reassociation temperature can lead to an apparent increase in the number of members of repetitive sequence families. For example, in most studies T_m - 25°C is used as the standard reassociation criterion.[3,4] Yet in the analysis of the evolution of mouse satellite DNA in sibling species,[5,6] a lower reassociation temperature was used to reduce specificity to allow recognition of putative ancestrally related sequences.

In contrast, by increasing reassociation temperatures, and thus increasing the sequence specificity required for reassociation,[7,8] one can obtain a minimum estimate of repetitive sequence copy number in broadly related families and can isolate two relatively different classes of repetitive sequences by high criterion reassociation. We conclude that the use of a more stringent criterion presents an additional method for fractionating populations of repetitive sequences and is useful in the analysis of repetitive sequence arrangement.

MATERIALS AND METHODS

DNA Isolation

X. laevis were obtained from the South African Reptile Farm or were laboratory bred. DNA was isolated from whole frogs as previously described.[9] DNA was isolated similarly from horse testes, the kind gift of C.D. Heinze, D.V.M. B. subtilis DNA was purchased from Calbiochem.

Nick Translation of DNA

A modified nick translation procedure[10,11] was used to label DNA in vitro. The DNA was nicked by DNase in .1 ml of solution containing: 10 mM $MgCl_2$, 70 mM potassium phosphate buffer, pH 7.4, .263 units of DNase I (Calbiochem, 798 units/mg) and one μg of DNA. After incubation at 37°C for 15 min, the solution was incubated at 70°C for 10 min to inactivate the DNase. The nicked DNA was transferred to a tube in which had been evaporated .4 μg each of unlabeled dATP, dGTP, and dCTP, .1 to .4 μg of TTP, radioactively labeled (details are given in figure legends), and in some cases, .2 μg of unlabeled TTP. β-mercaptoethanol was added to a concentration of 10 mM and the mixture was preincubated for 15 min at 15-18°C. DNA polymerase I (1.8 units; Boehringer Mannheim; Grade I; or Miles Labs) was added and the solution was incubated for 2-5 hr at 15-18°C. The reaction was terminated by a ten min incubation at 70°C. Labeled DNA was separated from precursor nucleotide triphosphates by passage through Sephadex G-100. The average single stranded size of the resulting labeled DNA was 300 NT. Specific activities ranged from 6×10^5 to 4×10^6 cpm/μg. To produce labeled DNAs of

longer lengths, the same procedure was followed except that the amount of DNase added was reduced or the DNase step was omitted. Under these conditions, DNAs with lower specific activities but with lengths >3000 NT were obtained.

DNA Shearing and Fragment Length Determinations

Unlabeled DNA was sheared in a VirTis 60.[12] To obtain 300 nucleotide (NT) long fragments, DNA in 66% glycerol and .1 M sodium acetate, pH 6.0, was sheared for 15 min at 30,000 rpm in an ice water bath. To obtain fragments about 3000 NT long, DNA in .1 M sodium acetate, pH 6.0, was sheared for 15 min at 12,000 rpm in an ice water bath. The speed of the shaft was measured directly with an electronic stroboscope (Strobotac) or with a digital readout from a photocell that monitored shaft speed. The single-stranded length of the DNA was determined by chromatography of dissociated DNA on agarose A-50m (Biorad, 100-200 mesh) in .12 M NaPB (NaPB is equimolar Na_2HPO_4 and NaH_2PO_4) and .6% formaldehyde. DNA was denatured by heating to 97°C in low salt (5 mM NaCl). When the solution reached 97°C, 37% formaldehyde was added to a final concentration of .6%; the solution was rapidly chilled and loaded onto the column. Prior to each measurement, the column was calibrated using native DNA (exclusion marker) and uridine (inclusion marker). A Kav of .5 was assumed to be equivalent to a single stranded size of 300 NT.[13] Unless otherwise indicated, all DNA sizes are single-stranded.

Reassociation Kinetics

Tracer and driver DNAs were routinely dialysed against 5 mM EDTA and then against 5 mM NaCl prior to reassociation. The DNAs were dissociated by heating to 97°C and were brought to the appropriate salt concentrations by the addition of 1 M NaPB and 5 M NaCl. They were then incubated at the designated temperature to the desired Ecot [Ecot = $OD_{260}/2$ x t (hr) x S (salt factor)].[12] The final salt concentration usually was .12 M NaPB (.18 M Na^+). Incubations in higher salt concentrations (.3 M Na^+ and .6 M Na^+) were maintained at temperatures 4°C and 8°C higher to compensate for the effect of salt on T_m. Reassociation mixtures in small volumes were brought to 4 ml with .12 M NaPB, preheated to either 60°C or 75°C, and loaded on hydroxyapatite columns ("filtrators")[12] maintained at either 60°C or 75°C. In most of the reassociation reactions, 50 μg of DNA was loaded onto a 1 ml hydroxyapatite column (.4 g hydroxyapatite powder; HTP; DNA grade, Biorad). Where there was less than 50 μg DNA, the column was preloaded with horse DNA to adjust the final amount of DNA loaded to 50 μg. Under these conditions, single-stranded DNA

is not adsorbed and is washed from the column with 10 column volumes of .12 M NaPB. Duplex DNA structures were eluted with 8 column volumes of .4 M NaPB. In some experiments, .3 M NaCl was present in both buffers to improve discrimination between single- and double-stranded DNA. Total radioactivity in each fractthe ion was monitored by adjusting ionic strength of all fractions to ~.2 M Na^+ and adding two volumes of Aquasol (New England Nuclear). The hydroxyapatite and its supporting triacetate filter (metricel GA-8; Gelman) were counted by adding 3 ml of water and 8 ml of Aquasol. Counts still associated with the hydroxyapatite were corrected for the small amount of quench and calculated as reassociated DNA.

S1 Nuclease Digestion

S1 nuclease was isolated from crude α-amylase (Aspergillus oryzae; Sigma) as described by Vogt[14] through the DE-52 cellulose (Whatman) chromatography step. DNAs to be digested by S1 were reassociated in .3 M NaCl, .01 M PIPES (Sigma), pH 6.7. At the end of the reassociation formaldehyde was added to .6% to prevent any further reassociation. Case and Baker[15] and our unpublished observations have shown that formaldehyde in low concentrations does not affect S1 digestion. We obtain more reproducible results in the presence of formaldehyde, particularly for "zero time" reassociation points. Following reassociation, an equal volume of a stock acetate solution (50 mM sodium acetate, pH 4.3, .2 mM $ZnSO_4$) and 50 μg/ml .5 M β-mercaptoethanol were added, as was 10 μl od S1 nuclease/50 μg of DNA. The reaction mixture was incubated at 37°C for 45 min. (With the enzyme preparation used, this is equivalent to a digestion condition of 4500 $\mu l\text{-}min\text{-}mg^{-1}$).[13] To terminate the digestion, 1 M NaPB was added to a final concentration of .12 M. Samples were fractionated on hydroxyapatite at 60°C or 75°C into enzyme sensitive (unbound) or enzyme resistant (bound) structures, which were subsequently eluted with .4 M NaPB.

Thermal Stability Measurements

Optical determinations of T_m were made using a Beckman Model 25K recording spectrophotometer. Jacketed cuvettes were heated using a programmed Lauda circulating waterbath and temperatures were monitored automatically, using a Beckman temperature readout device modified at Purdue University. The DNAs were melted in .04 M NaPB.

For thermal chromatography, 8,000-10,000 cpm of reassociated DNA was loaded onto a 1 ml jacketed hydroxyapatite column (maintained at the reassociation temperature), preloaded with 250 μg horse DNA. To ensure the accurate determination of T_m of the

native standard DNA, short (100 NT long) fragments were used so that more than half would be eluted by 95°C; fragments 300 NT long are not. After the column was washed with 10-12 column volumes of .12 M NaPB, the temperature was increased approximately 5°C. The column was then washed with 8 column volumes of .12 M NaPB to elute DNA that had become single-stranded and this temperature was increased again. The process was continued up to 95°C. The column was then washed with .12 M NaPB as before and finally with 10 column volumes of .4 M NaPB to elute any double-stranded DNA.

Electron Microscopy

Fragment lengths were determined for DNAs prepared by a formamide method[16] slightly modified by Tim Leathers (personal communication), using a Philips EM300 electron microscope and an HP digitizer. S13 RF molecules, the gift of Ethel Tessman, were used as the standard.

RESULTS AND DISCUSSION

High Temperature Reassociation

In nucleic acid reassociation experiments, the choice of appropriate reassociation temperature can be crucial. In studies using prokaryote DNAs, it was observed that at higher temperatures the reassociation rate falls, while specificity increases.[7-9] At lower reassociation temperatures the rate also falls but specificity decreases as well. In general, between T_m - 20°C and T_m - 30°C, there exists a broad plateau in optimum reassociation rate.[4-17]

The effect of increased reassociation temperature on DNA reassociation was determined for Bacillus subtilis DNA, which is virtually entirely single copy and which has a G+C content (40%)[18] similar to that of X. laevis (39%).[19]

In a preliminary optical reassociation study, several elevated reassociation temperatures were investigated. At 70°C (T_m - 18°C) there was relatively little change in the rate or extent of reassociation of either bacterial (single copy) or repetitive X. laevis sequences, while at 80°C (T_m - 8°C) the rate of reassociation of even the bacterial DNA was markedly reduced. This is consistent with observations that DNAs do not reassociate well at temperatures close to their melting points.[4-17] A reassociation temperature of 75°C (T_m - 13°C) was chosen for further experimentation since there was a slight depression of the reassociation rate of the bacterial DNA and a strong depression of the extent of reassociation of the repetitive DNA.

The reassociation kinetics of B. subtilis DNA were

determined using a hydroxyapatite assay at 60°C and 75°C. Compared to the standard criterion (60°C), the extent of reassociation at the stringent criterion (75°C) was diminished by 17% and the reassociation rate was .744 that at 60°C (Figure 1). Wetmur and Davidson[4] observed that reassociation rates of bacterial and viral DNAs at T_m - 15°C and T_m - 14°C were .836 and .854 those at T_m - 25°C. Since relatively short fragments of DNA have significantly lower T_m's,[21] the slightly lower extent of reassociation at 75°C is probably due to the inability of any very short DNA fragments to form stable duplexes at an elevated temperature.

High Temperature Reassociation of Repetitive Sequences

Families consisting of very similar sequences should show little change in copy number or complexity with increasing reassociation temperature, analogous to the effect of increased temperature on the rate of reassociation of single copy DNA. Families consisting of broadly related members should display increased complexity and decreased frequency if reassociated under a more rigorous criterion. The behavior of repetitive sequences under a more stringent criterion (75°C) was contrasted with that at a standard criterion (60°C) by the reassociation of isolated repetitive Xenopus laevis DNA with itself.

By Ecot 10, two repetitive components have reassociated in Xenopus DNA. One is a "fast" fraction, about 5% of the total DNA. The major component is 30% of the total DNA, and is of an "intermediate" frequency class.[22] When isolated repetitive DNA was self-reassociated at 75°C, the extent of reassociation was markedly diminished at low Ecots compared to its reassociation at 60°C (Figure 2). Some of the repeated sequences apparently no longer behave as if they were repetitive. When DNA that had not reassociated at 75°C (Ecot 12) was reassociated with an excess of X. laevis DNA at 60°C (Ecot 16), it reassociated more than 90%. Therefore, extensive thermal degradation was not the cause for a substantial fraction of the drop in extent of reassociation. Some of the loss is presumably due to the inability of the smaller fragments to reassociate at higher criterion. The diminution of extent of reassociation was comparatively small for the bacterial DNA (17%) and is probably about the same for the repeated DNA.

When the reassociation rate at 75°C was corrected for the effect of temperature and the decreased extent of reaction, it was four times faster than the 60°C rate (4.75 $lm^{-1}sec^{-1}$ at 60°C compared to 17.5 $lm^{-1}sec^{-1}$ at 75°C). Thus the DNA that reassociated at 75°C behaved as though it were less complex. The

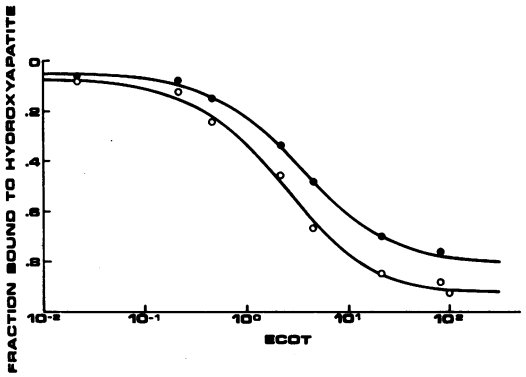

Figure 1. Kinetics of Reassociation of Single Copy DNA under stringent criterion. B. subtilis DNA was reassociated at either 60°C (—o—) or 75°C (—●—). Labeled tracer was prepared using 4 μg of ^{14}C-dATP (510 mC/mmol; Amersham-Searle), and 5 μg of DNA. The 75.3% of the DNA that was not bound following a "zero time" strip was used as tracer. It had a specific activity of 40,000 cpm/μg and a length of 350 nucleotides. It was mixed with 700 μg of unlabeled B. subtilis fragments (450 nucleotides long) and incubated in 0.4 M NaPB to an Ecot of 115 at 64°C. After reassociation, the 96.7% of the tracer which bound to hydroxyapatite was used for this experiment. The data were fit using a computer program[20] in this and the other kinetic curves assuming second order kinetics. The rates determined were .64 $lm^{-1}sec^{-1}$ for 97% reassociation (60°C) and .391 $lm^{-1}sec^{-1}$ for 80% reassociation (75°C).

rate of reassociation of the repetitive DNA at 75°C could have been: 1) slower, if all the reassociating repetitive sequences were present in greater apparent complexity; 2) .744 the rate at 60°C if there were no effect of reassociation temperature, other than on extent, on the reassociation of the stable repetitive DNA; or 3) faster if there were families of sequences with very similar and less complex members than the repetitive DNA average. These sequences would have little mispairing and would pair almost as well at 75°C as at 60°C. Apparently, there might be cryptic repetitive classes undetected in the reassociation kinetics of total X. laevis repetitive DNA.

Sequence Complexities of Unstable and Stable DNAs

If reassociation at 75°C had allowed only the pairing of relatively similar sequences, only the most similar members of the families would have been isolated. In that case the stable class would not represent a set of sequences different from the unstable ones. On the other hand, if there were "discontinuous" families with some having relatively similar members and others

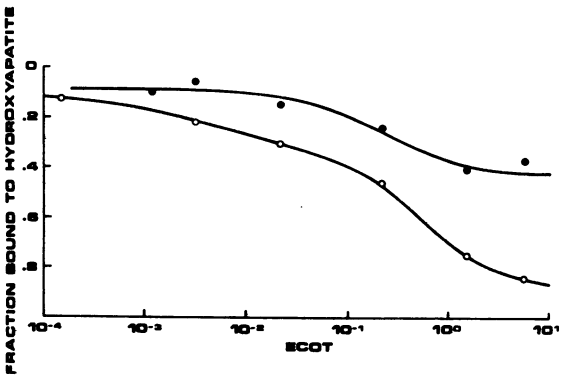

Figure 2. Kinetics of Reassociation of Repeated DNA under Stringent Criterion. X. laevis repetitive DNA was reassociated at 60°C (—o—) or 75°C (—●—). To prepare the tracer, 10 μg of total X. laevis DNA and 4 μg of ^{3}H-dTTP were used; the specific activity was about 580,000 cpm/μg. The 84.9% of the DNA that was not bound after a "zero time" strip was dialysed and then mixed with 10 mg of X. laevis DNA fragments (300 nucleotides long). After dissociation by heating to 97°C and incubation at 60°C to an Ecot of 20, 42.8% of the DNA was bound to hydroxyapatite. After dialysis, the DNA was dissociated again and then reassociated to an Ecot of 5 at 60°C. The 68.4% of the DNA that bound to hydroxyapatite (28.5% of the total DNA) was designated repetitive DNA. The rates determined were 3.28 for 94% reassociation (60°C) and 4.44 for 45% reassociation (75°C).

having internally rather dissimilar members, then it should be possible to separate these types of families by high temperature reassociation. To test if the reassociation at 75°C had actually fractionated the repetitive sequences into discrete families, stable sequences were isolated by reassociation to Ecot 1 at 75°C. The unbound DNA was then reassociated at 60°C to Ecot 10 to isolate repetitive sequences incapable of reassociation at 75°C. The DNA that reassociated at 75°C constituted the stable class while that which did not constituted the unstable class.

These two classes of sequence represent populations enriched for different characteristics. The unstable sequences could contain sequences that would have reassociated at 75°C, had they been slightly longer.

The average sequence complexities of these DNAs were determined by self-reassociation (Figure 3; Table 1). The stable class, with a k(pure) of 21.7 $lm^{-1}sec^{-1}$, was five times less complex than the unstable class, which had a rate of 4.21 $lm^{-1}sec^{-1}$.

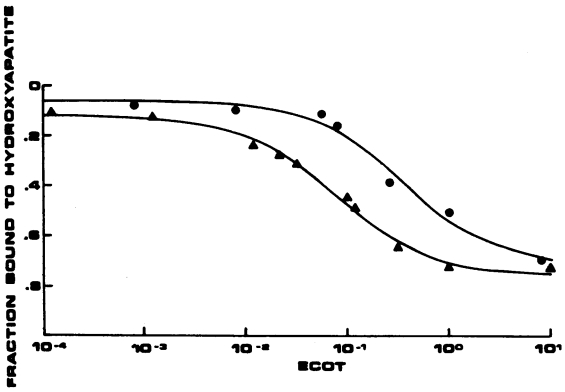

Figure 3. Self Reassociation of Unstable and Stable repetitive sequences. "Zero time" stripped, radioactively labeled and unlabeled total X. laevis DNA were reassociated at 75°C to Ecot 1.3 and fractionated on hydroxyapatite. The bound sequences, 21.3% of the total DNA, represent the stable class (—▲—). The unbound DNA was self reassociated at 60°C to Ecot 9. The unstable class (—●—) was that set of sequences that reassociated (26.3% of the total DNA). Each of these fractions was self reassociated at 60°C.

Frequencies were determined by reassociating stable and unstable DNAs with an excess of total DNA. Stable DNA was four times more frequent, with a k(pure) of 4.44 $lm^{-1}sec^{-1}$, than unstable DNA, with a k(pure) of 1.12 $lm^{-1}sec^{-1}$ (Figure 4, Table 1).

Thermal Stabilities of Stable and Unstable Sequences

If the stable repetitive sequence class were capable of reassociation at high temperature due to a high G+C content, its reassociated T_m would be expected to be similar to or greater than that of native DNA. However, the presence of a G+C rich class of sequences seemed unlikely. Native X. laevis DNA is characterized by a relatively homogeneous melt with a narrow transition, indicative of a similarity of G+C content throughout the DNA. Only very small G+C rich components have been observed in somatic cell DNA of Xenopus by CsCl analytical centrifugation.[19] Furthermore, the stable repetitive sequence class is relatively large so that a G+C rich component of this magnitude should have been observed. To explore this point, unlabeled stable and unstable sequences were isolated and their melting points determined optically (Table 2). Total DNA was reassociated to Ecot 10 at 60°C, and the reassociated DNA was

Table 1. Summary of Rate Data and Fits

DNA	k fixed	RMS	F ± S.D.[1]	C ± S.D.[1]	k ± S.D.[1]
Self Reassociations[2]					
S(60)	free --	.0209	.250±.018	.621±.022	13.5±1.89
	2.74 U(60)	.0871	.129±.075	.634±.095	2.74 --
U(60)	free --	.0307	.282±.044	.650±.047	2.74±.78
	13.50 S(60)	.0954	.443±.069	.570±.119	13.5 --
Driven Reassociations[3]					
S(60)	free --	.0176	.165±.02	.675±.025	3.00±.046
	.59 U(60)	.0816	.232±.088	.699±.113	.59 --
	1.81 S(75)	.0319	.124±.028	.674±.04	1.81 --
S(75)	free --	.0283	.282±.052	.604±.06	1.81±.69
	3.00 S(60)	.0403	.33±.04	.593±.07	3.00 --
U(60)	free --	.0184	.342±.015	.529±.02	.592±.125
	1.54 U(50)	.0416	.379±.024	.539±.04	1.54 --
	3.00 S(60)	.0672	.405±.038	.549±.072	3.00 --
U(50)	free --	.0187	.189±.019	.676±.026	1.54±.25
	.59 U(60)	.0540	.116±.045	.672±.067	.59 --

[1]F indicates the fraction of the DNA that did not reassociate; C the component of the DNA that reassociated; and k, the rate fit. Computer fits were made either without fixing the rate (free fit) or, in the indicated cases, by fixing the rates to those of the curves shown.
[2]Taken from Figure 3.
[3]Taken from Figures 5 and 6.

collected on hydroxyapatite, dissociated, and then re-reassociated to Ecot 1 at 75°C. Following hydroxyapatite fractionation, the DNA that was not bound was reassociated to Ecot 10 at 60°C and collected using hydroxyapatite. These DNAs, stable and unstable, were then melted optically, using native X. laevis DNA 300 NTP long as a standard. From the T_m's, it is clear that sequences that reassociate at 75°C are more stable thermally than those sequences that can not reassociate at 75°C. In addition, the T_m of the stable class was 6.7°C less than that of native DNA of a similar fragment size, an observation consistent with the assumption that some phenomenon other than

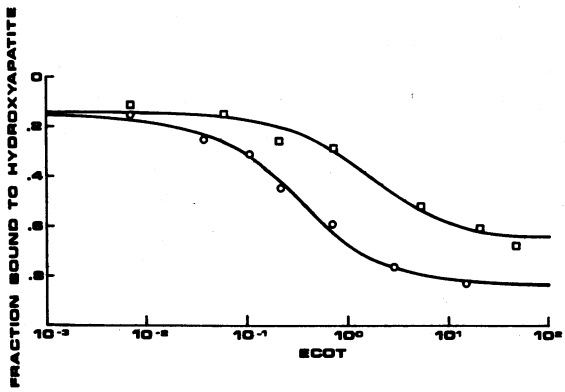

Figure 4. Reassociation Kinetics of Unstable and Stable DNAs Driven with Excess DNA. Stable (—o—) and unstable (—¤—) DNAs (Figure 3) were reassociated at 60°C in the presence of a 90-100 fold excess of total X. laevis DNA.

Table 2. Comparison of Thermal Stabilities of Stable and Unstable Sequences

DNA	T_m, °C	ΔT_m, °C	Trans[3]
Native[1]	81.2	---	4.0
Stable[2]	75.1	6.7	9.5
Unstable[2]	64.5	17.3	13.0

[1]Native X. laevis DNA, sheared to a length of 300 NT.
[2]Stable and unstable DNAs were isolated by reassociating 5 mg of X. laevis DNA as described in the text. DNAs were melted in 0.04 M NaPB as described in Materials and Methods.
[3]Trans is the degrees increase between 25 and 75% of the hyperchromicity change.

that of G+C content was responsible for its ability to reassociate at an elevated temperature.

If there were a G+C rich component comprised of a set of very dissimilar sequences, then this approach would not be conclusive. Due to the homogeneity of G+C content of Xenopus DNA, a component of this type would be relatively small, if it does exist.

Frequencies Determined at Different Criteria

A family of repetitious sequences whose members have varying degrees of relatedness should show significant changes in reassociation rate and thermal stability at differing reassociation temperatures. In addition, at lower temperatures more members of the family would reassociate at low Cots while fewer would reassociate at higher temperatures. The thermal stabilities of hybrids from such DNAs generally show strong dependence on the temperature of reassociation. Discrete, closely related families of repeated sequences should show little change in their thermal stabilities or reassociation rates with change in reassociation criterion.

To explore further the sequence relatedness within these families, the DNAs were reassociated at different temperatures in the presence of excess total X. laevis driver DNA. We used the thermal stabilities of the hybrids and the formula suggested by Bonner and coworkers[17] to account for the effect of mispair and to estimate the reassociation rates more accurately. Single copy DNAs reassociate at their optimum rates within a broad range of temperatures (T_m - 30°C to T_m - 20°C) and show diminished rates outside these limits. Mispair affects the rate of reassociation of heterologous and chemically modified single copy DNAs.[7,18,19] The optimum reassociation temperatures for such reassociations have been empirically determined to lie between T_p - 30°C and T_p - 20°C, where T_p is $(T_n + T_m)/2$, T_n is the melting temperature of well-paired DNA and T_m is the melting temperature of reassociated heterologous DNA.[17]

Unstable sequences reassociated at 75°C show little or no repetitive sequence reassociation (9.9% reassociation at Ecot 1). This amount is the same or less than that observed at lower temperatures at the lowest Ecots used. When unstable sequences were incubated at 75°C to Ecot 240, 29.4% reassociated. By Ecot 4090, 46.1% had reassociated. By extrapolating from the relative decrease in extent of reassociation at 60°C from that at 50°C, we predict that only 60% of this DNA would be reactable at 75°C. Eventually the unstable DNA should reassociate at 75°C, unless it has been thermally degraded. While this DNA does reassociate at 75°C, it behaves as though present in one or a few copies. When unstable DNA was reassociated at 60°C, the normalized rate of reassociation was 1.12 $lm^{-1}sec^{-1}$; the same DNA reassociated at 50°C had a normalized k of 2.28 $lm^{-1}sec^{-1}$ (Figure 5; Table 1). When the reassociation rates of the unstable sequences at 50°C and 60°C were corrected using the Bonner et al.[17] formulation, they were 2.56 and 1.2 $lm^{-1}sec^{-1}$, respectively (Table 3),

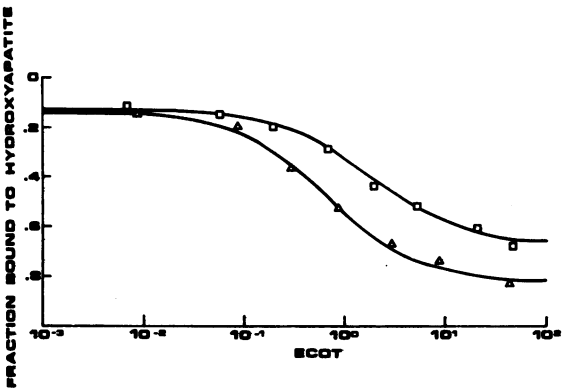

Figure 5. Effect of Lowered Criterion (50°C) on the Reassociation of Unstable DNA. Unstable DNA and excesses of driver DNA used were the same as in Figure 4. Unstable DNA was reassociated at either 60°C (—¤—) or at 50°C (—Δ—) and then assayed on hydroxyapatite at either 60°C or 50°C.

Table 3. Effect of Changed Criterion on Reassociation Rates of Repetitive Fractions.

DNA[1]	T_m[2]	T_p[3]	$T_i - T_p$	Rate Relative[4] to Optimum Rate	Obs.[5] k	Adj.[6] k
U(50)	70	79.5	-29.5	.89	2.28	2.56
U(60)	72	80.5	-20.5	.93	1.12	1.20
S(60)	81.7	85.3	-25.3	.96	4.44	4.62
S(75)	82.1	85.5	-10.5	.6	3.00	5.00

[1]U is unstable; S is stable; number in parenthesis is T_i, the incubation temperature.
[2]T_m of the reassociated DNA.
[3]T_p is $(T_n + T_m)/2$. T_n is the melting temperature of well-matched DNA, 88.9°C for X. laevis DNA 300 NT long on hydroxyapatite.
[4]Taken from Figure 8 of Bonner, et al.[17]
[5]Taken from the data in Figures 5 and 6. Rates are normalized to 100% reassociation.
[6]Calculated from: (observed rate)/(rate relative to optimum rate).

equivalent to 7100 and 3333 copies (determined by comparison of the rates with a single copy rate of .00036 $lm^{-1}sec^{-1}$).[22] These families of sequences appear to be broadly related, since the members change in relatedness from few copies (75°C) to 3333 copies (60°C) to 7100 copies (50°C).

In contrast, using the Bonner et al. formulation,[17] the stable sequences have very similar reassociation rates at both 60°C and 75°C (5.0 $lm^{-1}sec^{-1}$ at 75°C compared to 4.62 lm^{-1} sec^{-1} at 60°C; Figure 6; Table 3). Thus changing criterion has little effect on the reassociation of this family of sequences, the members of which remain at approximately 13,000 copies (12,800 at 60°C and 13,900 at 75°C).

The same preparations of stable and unstable DNAs were used to develop several kinetic curves, with relatively few points used for each curve. The fits and standard deviations for these curves are given in Table 1. In addition, the root mean square errors (RMSs) are given for fits forced to the rates for companion curves. In every case but one, the RMSs were more than twice as poor for the forced fits, indicating that the data did not fit these rates. The single exception is the forcing of fits between the S(60) and S(75) curves, in which case they almost agreed. The latter observation is consistent with the rate of reassociation of stable DNA being little affected by change in criterion.

Since smaller fragments have reduced T_m's and reduced ability to reassociate at higher temperatures, there could have been a selection for a small fragment size class (unstable) and a larger fragment size class (stable) using fractionation by high temperature reassociation. Smaller fragments from the stable class that do not reassociate at 75°C due to their length will be found in the unstable class. This would tend to reduce the average length of the unstable sequences. The difference in reassociation rates for self reassociation (Figure 3; Table 1) was a factor of five. The ratio of the square roots of fragment lengths determines the effect of length on rate for self reassociations.[4] Assuming the stable DNA to be at least 300 NT long, in order to account for the factor of five difference in self reassociation rate, the unstable DNA would have to be 12 NT long. The difference in rates for driven reassociation (Figure 4; Table 1) was a factor of four. The effect of fragment length on reassociation is either dependent on the ratio of tracer fragment lengths, assuming a constant driver length,[23] or on the ratio of [.0077][tracer length][(tracer length)$^{-.55}$ + (driver length)$^{-.55}$].[24] In driven reassociations, if the ratio of length

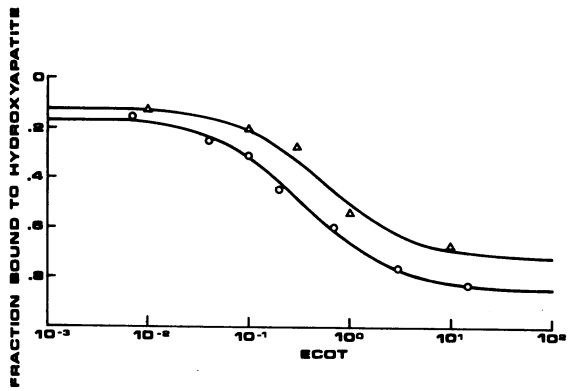

Figure 6. Effect of Increased Criterion (75°C) on the Reassociation of Stable DNA. Stable DNA and excesses were the same as in Figure 4. Stable DNA was reassociated at either 60°C (—o—) or at 75°C (—Δ—) and then assayed at either 60°C or 75°C, respectively.

determines the rate difference and assuming stable DNA to be 300 NT long, the unstable DNA would have to be 75 NT long to account for a difference of four in rate. If one uses Hinnebusch and coworkers' formula,[24] which accounts for the effect of driver length, the unstable fragments would have to be smaller than 50 NT. The hydroxyapatite assay can not detect efficiently duplexes smaller than 50 nucleotide pairs (NTP) at 60°C.[25] The results of reassociation of fragments even 100 NT long would be markedly impaired extent of reassociation and greater scatter, since the average duplex size would be half the fragment length (50 NT) during most of the reassociation.

Size measurements by electron microscopy showed that the stable class (n=91; 589 ± 196 NT) was only 22% larger than the unstable class (n=72; 461 ± 148 NT).

Implications for the Analysis of Repeated Sequences

A conclusion from the present data is that repeated sequence reassociation is more criterion dependent than that of single copy DNA. By choosing Ecots and reassociation temperatures carefully, one may be able to analyze or fractionate eukaryotic DNAs into sequence classes with characteristics different enough to allow the examination of subclasses of repetitive sequence populations (Table 4).

Genome Arrangement of Stable and Unstable Sequences

In Xenopus, reassociated interspersed repetitive sequences are thermally much less stable than noninterspersed repetitive

Table 4. Characteristics of Stable and Unstable Sequences

CHARACTERISTIC	STABLE	UNSTABLE
Percent of Repeated DNA	50.9	49.1
Percent of Total DNA	14.4	13.9
Self Reassociation k(pure)[1]	21.7	4.21
Sequence Complexity (NTP) (from Self Reassociation)	74,000	460,000
Driven Reassociation k(pure)[1] (from Driven Reassociation)	4.44	1.12
Copy Number	12,000	3,000
Percent of Total as Interspersed	10.9	7.2[2]
Percent of Total as Noninterspersed	6.8	1.0[2]
Percent of S1 Resistant as Interspersed	37.6	87.8
Percent of S1 Resistant as Noninterspersed	62.4	12.3

[1]Rate normalized to 100% reassociation.
[2]By difference.

sequences.[26] In the context of this paper, interspersed repetitive sequences are defined as repetitive sequences of relatively short length, interspersed with single copy sequences.[22,27,28] Noninterspersed repetitive sequences are defined as long repetitive sequences or as blocks of repetitive sequences. We wished to determine which type of sequences, interspersed or noninterspersed repetitious, were lost on reassociation at 75°C. The expectation would be that interspersed repetitive sequences would be primarily lost, but it was possible that sequences forming poor pairs at 60°C made better pairs at 75°C. Therefore, sequences could be lost specifically from the noninterspersed or interspersed classes, or randomly from both classes. In addition, the long fragment lengths used would allow comparison of the relative proportions of stable and unstable repetitive DNA, without losses during reassociation due to an initially rather short (300 NT) fragment length. A mixture of >3000 nucleotide long X. laevis tracer and >3000 nucleotide long X. laevis driver DNA was dissociated and reassociated to Ecot 10 at either 60°C or 75°C to permit repetitive sequence reassociation. At the end of the

reassociation period, any single-stranded DNA was digested with S1 nuclease. The enzyme resistant structures were collected on hydroxyapatite, at either 60°C or 75°C and their relative sizes determined by A50m agarose chromatography (Figure 7). Noninterspersed repetitive structures are presumed to be DNA 2000 NTP or larger, while interspersed repetitive structures are assumed to be smaller DNA structures, in the range of 300 NTP.[18] Following 60°C reassociation, 7.8% and 18.1% of the total DNA were in the large molecular weight (noninterspersed repetitive) and small molecular weight (interspersed repetitive) peaks, respectively. These values are consistent with other determinations on Xenopus DNA.[26] However, at 75°C, 6.8% and 10.9% of the total DNA were noninterspersed (13% loss) and interspersed (40% loss). These losses at 75°C were very reproducible. Thus it appears that at least 80% of the unstable sequence is interspersed repetitive DNA. In addition, the average size of the interspersed repetitive sequences present at 75°C was somewhat larger, 420 NTP compared to 370 NTP at 60°C. Under the relatively severe digestion conditions used, it was possible that reassociated noninterspersed repetitive DNA was sufficiently digested to be moved into the shorter DNA peak. However, digestion using five times less enzyme showed little difference.

Sequences no longer capable of reassociation at 75°C seem to be derived primarily from interspersed sequences and seem so reduced in apparent copy number at 75°C that they do not find a complement during a relatively short incubation. Of the DNA capable of reassociation at 60°C, 67% was still able to reassociate at 75°C, when the initial fragment size was much larger than that used for the previous fractionations and kinetic studies. This estimate is probably more accurate in defining the proportions of stable and unstable sequences (65/35) than that obtained from reassociation with small fragments (50/50). Thus the rate differences observed were probably underestimates.

By reassociating tracers of varying lengths with excess unlabeled DNA of constant length, it is possible to determine whether there is short period interspersion, and if so, the amount of DNA involved and the length of the short period interspersed single copy sequences.[27] In our hands, at 60°C, Xenopus DNA showed the presence of short period interspersion with a short period interspersed single copy length of approximately 700 NT, consistent with previous observations.[22] At 75°C, however, little or no short period interspersion was apparent. Rather long period interspersion was observed (Figure 8). The data do not exclude the presence of a small amount of

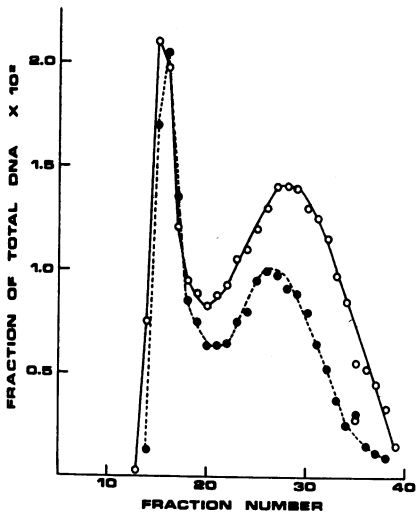

Figure 7. Fraction of Total DNA as Noninterspersed or Interspersed Repetitious Sequences under Normal and Stringent Criteria. Long ^{3}H tracer (170,000 cpm/μg) was prepared omitting the DNase digestion and the "zero time" strip. More than two-thirds of the preparation was 3000 nucleotides long or longer. X. laevis driver (75μg; more than 3000 nucleotides long) and ^{3}H-labelled tracer (50,000 cpm) were reassociated to an Ecot of 10 at 64°C. After reassociation, the DNA was digested with S1 nuclease. The enzyme resistant DNA (37.3%) was collected on hydroxyapatite at 60°C and then chromatographed on an A50m agarose column (100-200 mesh) (—o—) in the presence of exclusion (native horse DNA) and inclusion (uridine) markers. The same driver (100μg) and tracer (100,000 cpm) were reassociated at 79°C and digested as described and collected on hydroxyapatite at 75°C. The enzyme resistant DNA (28.5%) was chromatographed on the same agarose column (--•--), using the same markers. To account for "zero time" reassociation, 50μg of driver and 150,000 cpm of tracer were dissociated, rapidly cooled to 70°C and then digested with S1 nuclease. The enzyme resistant DNA (10.5%) was collected on hydroxyapatite at 60°C and chromatographed on the same agarose column in the presence of the same markers. To plot the data, the proportion of enzyme resistant DNA in each fraction was multiplied by the amount of total DNA that was enzyme resistant in that experiment. The values derived from the "zero time" experiment were subtracted, fraction by fraction, from the values derived for each of the 60°C and 75°C fractions. The values plotted are the proportion of the total DNA in each fraction, less the "zero time" value.

DNA in short period interspersion. In fact, if the amount of DNA that is S1 resistant at 75°C (18%) is set as the y-ordinate intercept, a small amount of DNA is observed as short period interspersed (25%). This fraction appears to have a slightly longer interspersed single copy sequence length (900 NT, compared to 700 nucleotides; Figure 8; broken line). This might indicate that the shorter interspersed sequence elements are also those primarily involved with short period interspersed single copy DNA and that the longer interspersed repetitive sequences observed after reassociation at 75°C (Figure 7) may be associated with long period interspersed single copy DNA.

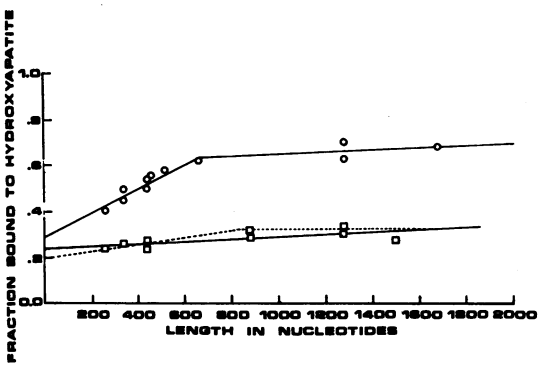

Figure 8. Interspersion Patterns Determined at 60°C or 75°C. X. laevis total DNA was nicked with units of DNase, dissociated, "zero time" stripped, and fractionated on A50m in 0.05 M Na_3PO_4, pH 11.9. Fragment sizes were determined by analytical agarose chromatography in 0.6% formaldehyde. Tracers were reassociated with 50 μg Xenopus DNA 300 nucleotides long to an Ecot of 10 at either 60°C (—o—) or 75°C (—¤—) in 0.12 M NaPB and then fractionated on hydroxyapatite at either 60°C or 75°C. The dotted line indicates the fit to the 75°C data, assuming the amount of long tracer resistant to S1 nuclease digestion (18%) to be equivalent to the y-intercept value.

Implications for Genome Evolution

It seems likely that sequences produced recently by saltatory replication[4,29] would be present as blocks of repetitious sequences and that older repetitive sequences would tend to be interspersed throughout the genome. The idea that less mispair is equated with more recent origin is consistent with the observation that reassociated nonrepetitive DNA has a greater thermal stability than does reassociated interspersed repeated DNA.[26,30] In addition to being noninterspersed, multiple copies that had been produced recently would be likely to be more similar to each other, and in greater copy number and smaller complexity, as were those of the stable class. However, the higher thermal stability of the noninterspersed sequences could be due to sequence conservation. Conversely, dimininished thermal stability of the interspersed repetitious sequences could be a relict of an increased rate of base substitution due to either the process of becoming interspersed, or, once interspersed, to an increased susceptibility to change. The observation that longer interspersed repetitive DNA may be interspersed with long period interspersed single copy DNA would be consistent with the

hypothesis that repetitious sequences would become increasingly more interspersed during evolutionary time. In turn, this would imply that more divergent or older interspersed sequences would tend to be shorter in length and be associated with shorter interspersed single copy DNA.

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