Analysis of the C-Value Paradox by Molecular Hybridization  
(complementary DNA/reiteration frequency/Amphibia)

MICHAIL ROSBASH*,†, PETER J. FORD‡, AND JOHN O. BISHOP*

* Institute of Animal Genetics, and † Department of Molecular Biology, King's Buildings, University of Edinburgh, Edinburgh EH9 3JN, Scotland

Communicated by Roy J. Britten, July 10, 1974

ABSTRACT Poly(A)-containing RNA was isolated from ovaries of Xenopus laevis laevis and Triturus cristatus carnifex and used as a template for the synthesis of radioactive complementary DNA with RNA-dependent DNA polymerase. When annealed with an excess of homologous DNA, the complementary DNA is rendered double-stranded with kinetics that suggest that the coding sequences are single-copy in both these organisms. In Triturus, these sequences are distinct from the majority of the genome, which consists of repeated sequences, and distinct from the ribosomal cistrons, which are present in proportion to the increase in C-value relative to the Xenopus genome. Moreover, the number of different poly(A)-containing molecules in the ovary (sequence complexity) is the same in Xenopus and in Triturus.

There exists an enormous variation in C-value (DNA content per haploid genome) among eukaryotic organisms (1). Even within a single class of organisms, large differences have been detected. These are most striking in the class Amphibia, in which C-values vary by two orders of magnitude (2). In particular, the Urodeles contain large amounts of DNA per haploid genome, e.g., Triturus cristatus carnifex has about 21 pg while Amphiuma and Necturus contain more than 100 pg of DNA per haploid genome (2). These values can be contrasted with Xenopus laevis laevis and most mammals, which have about 3 pg of DNA per haploid genome (3, 4).

On the basis of this range of DNA values and cytological evidence from various sources, Callan proposed that chromosomal DNA, and in particular genetically significant DNA, is arranged in tandemly repeated sequences (5). These repeated sequences consist of a "master" sequence and "slave" sequences; the "master" sequence would be subject to mutational and recombinational events while the "slave" sequences would be corrected once per life-cycle to reflect the sequence of the "master" sequence. The formation of extended lambrush loops during meiotic prophase was proposed as the stage at which this correction process takes place.

In order to test this proposal, and in order to understand the organization of the eukaryotic genome in Urodeles of high C-value, we have examined the reiteration frequency of DNA sequences complementary to poly(A)-containing RNA isolated from ovaries of Triturus cristatus carnifex. This fraction of the cellular RNA, assumed to be enriched for coding sequences, was transcribed with RNA-dependent DNA polymerase to make a radioactive DNA copy. This cDNA was annealed with an excess of unlabeled Triturus DNA in order to measure the reiteration frequency of the fraction of the genome corresponding to these sequences (6). The data, when compared to identical experiments performed on material from Xenopus laevis, strongly suggest that the coding sequences in Triturus occur once per haploid genome (single-copy), in contrast to the greater part of Triturus DNA. Moreover, kinetic experiments indicate that the number of different poly(A)-containing molecules present in the ovary (sequence complexity) is very similar, if not identical, in Triturus and Xenopus.

MATERIALS AND METHODS

Xenopus laevis laevis were obtained from the South African Snake Farm, Fish Hoek, South Africa, and Triturus cristatus carnifex from Haig and Co., U.K. Ovaries were removed and washed in Barth X medium. Nucleic acid was prepared by the method of Kirby, with the addition of 0.5% sodium lauryl sulfate to the aqueous phase, as described (7). High-molecular-weight RNA was prepared by homogenizing the pelleted nucleic acid in 3 M NaOAc (pH 6) to solubilize DNA, transfer RNA, and 5S RNA (8). The RNA precipitate was resuspended in "binding" buffer [0.4 M NaCl, 1 mM EDTA, 0.1% sodium lauryl sulfate, 10 mM Tris-HCl (pH 7.4), 10% glycerol] and passed over oligo(dT)-cellulose (9). The poly(A)-containing RNA was eluted in "elution" buffer [10 mM Tris-HCl pH 7.4, 0.1% sodium lauryl sulfate], and the RNA spectrum was measured in the Unicam SP800A spectrophotometer. The amount of poly(A) in this eluate was routinely assayed by hybridization with radioactive poly(U), as described (7). By comparing the amount of poly(A) with the total RNA bound by the column, it was estimated that the eluted RNA contained 20-50% ribosomal RNA. This was verified by sucrose gradient sedimentation of the RNA. RNA was precipitated and resuspended three times to remove sodium lauryl sulfate, and finally resuspended at a final concentration of 0.4 mg/ml in distilled H2O and stored at -20°C.

DNA was purified as described from Xenopus erythrocytes and from Triturus erythrocytes, liver, and sperm (10). The DNA was sonicated in 15 mM NaCl-1.5 mM sodium citrate or sheared in a Sorvall French Press at 50,000 lbs./inch² at 10-15°C and passed over a Sephadex SP-50-Chelax-100 column developed with 0.3 M NaCl-0.01 M NaOAc (pH 5). DNA prepared in this way was 450-550 nucleotides long if sonicated and 300-400 nucleotides long if sheared, as measured by the method of Studier in an analytical ultracentrifuge (11).

RESULTS

The reassociation of Triturus and Xenopus DNA was analyzed optically under standard conditions (Fig. 1). About 75% of

Abbreviations: cDNA, complementary DNA; phosphate buffer, equimolar Na2HPO4 and NaH2PO4; C40, product of DNA concentration and time in mol/sec/liter.

† Present address: Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Mass. 02154.
the *Xenopus* genome reassociates with a *C*\textsubscript{\textsc{ctg}}/\textsubscript{\textsc{i}} of between 500 and 1000 in 0.24 phosphate buffer M. Under identical conditions, single-copy *Triturus* DNA (with C-value seven times that of *Xenopus*) should reassociate at a rate seven times slower than *Xenopus*, or with a *C*\textsubscript{\textsc{ctg}}/\textsubscript{\textsc{i}} of between 3500 and 7000. These values are indicated by the arrows in Fig. 1. There is relatively little detectable single-copy DNA in the *Triturus* genome; most of the *Triturus* DNA is present as repeated DNA. Moreover, there is no discrete class of repeti-

**Fig. 1.** Optical renaturation of *Xenopus* and *Triturus* DNA. Denatured DNA was annealed in 0.30 M NaCl-0.030 M sodium citrate at 70\(^\circ\). At various *C*\textsubscript{\textsc{ctg}} values, aliquots were removed and quenched in ice-cold 15 mM NaCl-1.5 mM sodium citrate. The absorbance was monitored at 50\(^\circ\) and 90\(^\circ\) as described (12, 13). A complete gain of initial hypochromism was defined as 35\% (100\% duplex). All DNA samples were sheared. The arrows indicate the expected range of *C*\textsubscript{\textsc{ctg}}/\textsubscript{\textsc{i}} (3500-7000) of single-copy *Triturus* DNA. •, *Xenopus*; O, *Triturus*.

**Fig. 2.** DNA excess hybridisation with ribosomal RNA. 18S ribosomal [\textsuperscript{32}P]RNA was prepared by standard procedures (7). Briefly, *Xenopus laevis* kidney cells were incubated for 3 days in \(1/4\) normal concentration of phosphate, with 0.2 mCi/ml of [\textsuperscript{32}P] (carrier-free, Amersham). RNA was prepared and passed over oligo(dT)-cellulose to remove poly(A)-containing sequences. The 18S region from a sucrose gradient was pooled, precipitated, and repurified by acrylamide gel electrophoresis. The 18S peak was eluted, precipitated, and suspended in distilled H\textsubscript{2}O. The specific activity was \(0.5 \times 10^5\) cpm/\mu g. DNA excess hybridization was performed as described (12) with a DNA/RNA ratio in excess of \(3 \times 10^4\) (DNA > 3 mg/ml; RNA = 0.1 \mu g/ml). Aliquots were removed at the indicated *C*\textsubscript{\textsc{ctg}} values and assayed for the percentage of RNase-resistant [\textsuperscript{32}P] as described (12). The arrow indicates the *C*\textsubscript{\textsc{ctg}}/\textsubscript{\textsc{i}} = 6. •, *Xenopus*; O, *Triturus*.

tion frequencies. This is in agreement with the report of Straus (31), which presented similar data on DNA from various other Urodeles, as analyzed with hydroxyapatite.

In principle, a small amount of radioactive nucleic acid added to a vast excess of nonradioactive DNA can be used as a tracer in order to follow the reassociation of the portion of the genome complementary to the radioactive sequences (13, 14). The kinetics of this reassociation can be used to estimate the reiteration frequency of this subset of the genome. In Fig. 2, *Xenopus* ribosomal [\textsuperscript{32}P]RNA is hybridized in vast DNA excess to both *Xenopus* and *Triturus* DNA. Despite the 7-fold difference in C-value, the *C*\textsubscript{\textsc{ctg}}, and therefore the kinetics of hybridization, of the two reactions is very similar. Each *Triturus* genome, therefore, has about seven times as many ribosomal cistrons as *Xenopus*. This is in good agreement with a published report in which the number of ribosomal genes in these two organisms was measured by RNA excess filter hybridization (15).

DNA complementary to total ovary poly(A)-containing RNA was used in order to measure the reassociation kinetics of a fraction of the DNA that acts as a template for informational RNA transcription. Poly(A)-containing RNA was isolated from oocytes of both *Xenopus* and *Triturus*. In the presence of the primer (pT)\textsubscript{\textsc{ii}}, this RNA fraction is a good template for RNA-dependent DNA polymerase. The size of cDNA was analyzed on alkaline sucrose gradients; *Xenopus* cDNA and *Triturus* cDNA had S-values of 6.5 and 7.3 or weight-average sizes of about 500 nucleotides and 600 nucleotides, respectively (Fig. 3).
This cDNA was used as a radioactive tracer and annealed with an excess of homologous DNA (Fig. 4). As analyzed on hydroxyapatite (Fig. 4A), the Xenopus DNA complementary to this radioactive cDNA appears to reassociate with second-order kinetics. The C4t1/2 was about 200 to 400 under these conditions, faster than the single copy (final) transition of total Xenopus DNA (Fig. 1). This difference may be due to the difference between an optical assay and an assay with hydroxyapatite. Alternatively, it may be due to the presence of a small repetitive component or the lack of precise terminal values. There is relatively little (<15%) renaturation of Xenopus cDNA at low C4t. Therefore, it appears likely that most of the cDNA is complementary to single-copy DNA. Triturus cDNA, when renatured in the presence of a vast excess of Triturus DNA, also renatures with second-order kinetics but five to eight times slower than Xenopus DNA. In Triturus as in Xenopus, there is little highly repetitive DNA complementary to the cDNA (<10% at C4t 100).

The same reassociation experiments were analyzed with single-strand-specific nuclease S1 (Fig. 4B). The Xenopus cDNA reassociates at a rate two to three times slower than when assayed by hydroxyapatite. This difference is most likely a result of the fact that an assay with hydroxyapatite registers single-stranded regions adjacent to reassociated DNA as duplex, while an assay with nuclease S1 does not. Triturus cDNA begins to reassociate in such a way as to suggest a 10-fold slower rate than Xenopus cDNA. The kinetic data strongly suggest that the sequences complementary to the Triturus cDNA are present once, or at most twice, per genome.

The effect of base mismatching on the rate of renaturation is relatively small (19). Nevertheless, the Triturus cDNA-DNA duplexes considerably mismatched, this would lower the rate of reassociation and therefore lower the apparent reiteration frequency of these sequences. In order to test this possibility, the cDNA-DNA duplexes were melted (Fig. 5). The Tm values of the complexes from both organisms were the same (65.5 ± 0.5°C). The same Tm was obtained when the assay was performed with nuclease S1 instead of hydroxyapatite (data not shown). Triturus DNA has a Tm 1° greater than Xenopus DNA (unpublished results). The similarity of the Tm values of the two cDNA-DNA complexes suggests that mismatching of a magnitude sufficient to noticeably affect the rate of duplex formation is unlikely.

cDNA, transcribed from poly(A)-containing RNA, can be hybridized to its original template in RNA excess. If a large excess of RNA is used, the reaction is pseudo-first order and the rate is RNA-dependent. Consequently, the first-order rate constant (or constants) is interpretable in terms of the sequence complexity of the RNA in the reaction. To examine the relative sequence complexity of the two ovary poly(A)-containing RNA populations, each cDNA population was hybridized to its template, in RNA excess (Fig. 6). In both cases, the hybridization takes place with similar, and perhaps identical, kinetics. The data indicate that the sequence complexity of the poly(A)-containing RNA is very similar in the two cases.

The abscissa of Fig. 6 is proportional to R0t, where R0 is the initial concentration of RNA based on the measured concentration of poly(A). Since the cDNA is only 500–600 nucleotides long (on average) and its synthesis is completely dependent on priming by (pT)90, it is likely that it represents limited synthesis from the 3′-end of the poly(A)-containing RNA. The number of potentially reactive RNA molecules should, therefore, be proportional to the number of poly(A) termini and not necessarily to the absolute concentration of poly(A)-containing RNA. Provided that the size of the average poly(A)
segment is the same in the poly(A)-containing RNA of both *Xenopus* and *Triturus*, the concentration of RNA sequences complementary to cDNA will be proportional to the concentration of poly(A). The sizes of the poly(A) sequences were measured by acrylamide gel electrophoresis and found to be very similar in the RNA from the two organisms (Fig. 7). This justifies the use of poly(U) hybridization to measure "reactive" RNA concentration in the experiment shown in Fig. 6.

**DISCUSSION**

Although there is a general progression in C-value from primitive eukaryotes through nonchordate metazoa and chordates, there exists great variability within several phylogenetic groups. This is particularly true for fish, insects, and amphibia. The latter group includes related organisms that differ widely in their C-values; indeed, the family Pelobatidae (Order, Anura; Suborder, Anomoocoea) contains genera that differ by a factor of five in DNA content (2). It is unlikely that this difference is due to the high C-value organism being substantially more complex than the corresponding low C-value organism. There is also no obvious reason to expect *Triturus* to be phenotypically seven times more complex than *Xenopus*. Our results strongly suggest that the sequence complexity of ovary mRNA is the same for these two organisms. The results also suggest strongly that in each case mRNA is transcribed preferentially from the single-copy fraction of the DNA. This is in agreement with previous work from this and from other laboratories on mRNA sequences in various organisms (20-23). This result is particularly striking in *Triturus*, since the majority of the genome consists of repeated DNA. In contrast, the majority of *Xenopus* DNA renatures with a C4+1/2, consistent with the representation of these sequences once, or at most twice, per genome (Fig. 1). It should also be noted that the percentage of repeated DNA in the *Xenopus* genome is smaller as measured optically than as measured on hydroxypyhatite (about 25% as opposed to 45%) (Fig. 1 and ref. 25).

![Fig. 6. Hybridization between cDNA and its template RNA. All four experiments were plotted on an equivalent time axis corresponding to a poly(A) concentration of 5.45 μg/ml, which is equivalent to about 109 μg of poly(A)-containing RNA per ml. Samples were incubated at 70° in 0.24 M phosphate-EDTA. Poly(A)-containing RNA concentration was measured by hybridization with radioactive poly(U), as described in the text. cDNA was present at a concentration of 1.8 × 10-3 μg/ml. At the indicated (equivalent) times, aliquots were removed from the incubation and challenged with nuclease S1 in the presence of 50 μg/ml of denatured Escherichia coli DNA, as in the legend of Fig. 4. *Xenopus*: •, exp. 1; A, exp. 2. *Triturus*: O, exp. 1; Δ, exp. 2.

![Fig. 7. Size of poly(A). About 1 μg of poly(A)-containing RNA was degraded with pancreatic and T1 ribonuclease and subjected to electrophoresis on 15% acrylamide gels. The RNA was eluted and hybridized with radioactive poly(U) as described (7). The poly(U) was 5 × 10⁶ cpm/μg under the counting conditions used. The positions of the absorbance markers were determined on a parallel gel in which the dye migrated to an identical position. (A) *Xenopus*; (B) *Triturus*.

This is most likely due to interspersed single-copy sequences that are bound to hydroxyapatite at low Cct through the duplexed repetitive sequences to which they are attached and are, therefore, fortuitously scored as repeated DNA by this method (26).

Most mRNA species, with the exception of histone mRNA, contain a 3' sequence of poly(A) (23, 27-29). It has been shown by Perry that there exist substantial amounts of nuclear poly(A) that do not act as precursor to cytoplasmic messenger RNA (30). It is possible, therefore, that a substantial fraction of the cDNA is not complementary to mRNA sequences. Nevertheless, we chose to use the poly(A)-containing RNA from these two organisms as a probe for a fraction of the genome significantly enriched for protein-coding sequences. Previous work in this laboratory has demonstrated that *Xenopus* oocytes contain a large amount of poly(A)-containing RNA, which is likely to be the product of lambrush chromosome activity (7). A large proportion of this RNA is *bona fide* messenger RNA, as judged by its ability to stimulate amino-acid incorporation in various in vitro systems, and most likely represents masked messenger RNA inherited by the fertilized egg (C. M. Darnborough, P. J. Ford, and M. Rosbash, unpublished results). These earlier studies were performed with oocytes chemically stripped of follicle cells and divided into various size classes. *Triturus* oocytes are sensitive to this treatment and lyse in the presence of Pronase and EDTA. As a result, it was necessary to prepare the RNA from whole ovaries.

*Triturus* cDNA reassociates with an excess of *Triturus* DNA five to ten times slower than *Xenopus* cDNA with *Xenopus* DNA. Since *Triturus* has seven times as much DNA per cell as *Xenopus*, there appears to be no repetition in *Triturus* of the sequences that are transcribed into poly(A)-containing RNA. If there were extensive mismatching in either of these cases, this conclusion would be more tenuous. The Tm values of the cDNA·DNA duplexes (65.5 ± 0.5° in both cases) suggest that there is little mismatching in these duplex structures. If one applies the relationship \( T_m = 16.6 \log [\text{Na}^+] + 0.41 (G+C) + 81.5 \) (32), the cDNA·DNA duplexes would have a \( T_m \) value of 82 ± 0.5° in 0.12 M phosphate buffer. This is similar to the
value (82.5°) reported by Davidson and Hough (24) for the
Tm of reassociated nonrepetitive Xenopus DNA. Certainly,
there exists no mismatching of the extent necessary to affect
rates of reassociation and affect the conclusion drawn from
the experiment in Fig. 4 (19).

The data in Figs. 6 and 7 suggest that the ovaries of both
species contain the same sequence complexity of poly(A)-
containing RNA. In RNA excess, the cDNA provides an
effective tracer of the frequency distribution of the poly(A)-
containing RNA; the more complex an RNA population is,
the lower the concentration of the individual RNA sequence
and the slower the hybridization of the homologous cDNA.
The concentration of poly(A)-containing RNA was assayed
by measuring the poly(A) content by hybridization with
radioactive poly(U) (7). The advantage of this approach is
that it avoids three potential problems that are encountered
if measurements of this sort are made on the basis of RNA con-
centration. These are: (a) genuine differences in the mean size
of the poly(A)-containing RNA populations from the two
species, (b) limited cleavage of the RNA during preparation,
and (c) contamination of the poly(A)-containing RNA by
RNA that does not contain poly(A) (which is not transcribed
but contributes to the absorbance of the RNA).

The experiments presented in Figs. 3 and 6 strongly suggest
that the basic transcription patterns in Xenopus and Triturus
ovaries are very similar. In both cases, mRNA is transcribed
preferentially from the single-copy fraction of the genome, in
agreement with previous results on other systems (20–24).
Second, the number of different RNA species present in the
ovaries of the two organisms is similar. It is however, possible
that the first conclusion is valid only for transcription in the
ovary. Other cell types may synthesize larger percentages of
 messenger RNA from the repetitive portions of the genome.
Also, the first conclusion depends on the relative efficiency of
different mRNA molecules as templates for RNA-dependent
DNA polymerase. Were the mRNA molecules transcribed
from the repetitive fraction of the genome relatively poor tem-
plates for the enzyme, we would tend to underestimate the
contribution of repetitive DNA transcripts in the mRNA pop-
ulation. The second conclusion is subject to a similar criticism.
If mRNA molecules of a particular frequency class were poor
templates or unable to act as templates for RNA-dependent
DNA polymerase, this would change the relative contribution
of the various frequency classes to the total mRNA population.

Within these limits, the data strongly suggest that the Tri-
turus genome contains single-copy DNA that can be detected
by use of radioactive cDNA as a tracer. This single-copy DNA
is the preferential, if not exclusive, site for the synthesis of
informational RNA in the Triturus genome. The data argue
strongly against any serial repetition of gene sequences and,
therefore, argue strongly against the need for any correction
mechanism to maintain Mendelian inheritance of mutational
events incurred through evolution (5). The data also render
all the more puzzling the function, if any, the enormous quan-
tity of repetitive DNA in Triturus and perhaps in all organ-
isms with a high C-value.

We are grateful to Prof. H. G. Callan for helpful discussion.
We thank Fiona Gibson for excellent technical assistance. This
work was supported by a Helen Hay Whitney Fellow. We are indebted to Dr. J. W. Beard, Duke
University, Durham, N.C., for supplying us with avian myeloblas-
tosis virus.

1. Mirsky, A. E. & Ris, H. (1950) J. Gen. Physiol. 34, 451-
462.
2. Sexsmith, E. (1968) DNA Values and Karyotypes of Am-
204–207.
1408–1412.
Differentiation, eds. Monroy, A. & Tsanev, R. (Academic
15. Buongiorno-Nardelli, M., Antalid, F. & Lava-Sanchez, P. A.
774–786.
22. Goldberg, R. B., Galav, G. A., Britten, R. J. & Davidson,
23. Davidson, E. H. & Britten, R. J. (1973) Quart. Rev. Biol. 48,
565–613.
USA 63, 342–349.
491–506.
26. Davidson, E. H., Hough, B. R., Amenson, C. S. & Britten,
27. Greenberg, J. R. & Perry, R. P. (1972) J. Mol. Biol. 72,
91–98.
82, 315–331.
802.