# Unusual features of the urodele genome: do they have a role in evolution and development?

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**ABSTRACT Urodeles are amongst the organisms with the highest C-values. They provide a useful system for studies of genome organization at both the chromosomal and the molecular level. In this contribution we discuss the general features of "excess" DNA in urodeles and emphasize that the urodele genome is in a state of plasticity. That fluidity is due to various molecular mechanisms which are involved in its continuous turnover. The implications of this fluid, "excess DNA" for evolution and/or development are considered.**

KEY WORDS: *urodeles, genome, evolution, development, repeated DNA*

## Introduction

During the last two decades it has become increasingly apparent that the eukaryotic genome is not fully stable. For certain it is mendelially inherited after DNA replication and subject to change and rearrangement through the mechanisms of mutation and meiosis. In addition, the eukaryotic genome is, however, both dynamic and fluid. Several processes of genomic turnover (such as unequal crossing over, gene conversion, transposition, and slippage) are able to modify, scatter and amplify sequences by means of routine biochemical processes. Those processes supplement the classical, well known features of replication, mutation, and meiosis for generating change (reviewed by Dover, 1982, 1986, 1987, 1993).

Eukaryotes apparently have much more DNA than they need to code for all their expected gene expression functions (proteins and functional RNAs). This peculiar aspect of eukaryote genomes has been termed the *C-value paradox* (Thomas, 1971; Gall, 1981). Urodele amphibians provide the extreme example of such a paradox in that: (1) they have extremely high amounts of DNA (together with lungfishes and plants, they in fact have the highest DNA contents among eukaryotes); and (2) within urodeles there are cases of great variation in DNA amount even among closely related taxa.

Urodele amphibians usually also have large metaphase chromosomes and wonderful lampbrush chromosomes which can be easily studied and recognized under the microscope. In this sense they provide an ideal system for studying genome organization at both the chromosomal and molecular level.

In this contribution we will briefly review the general features of the genome of urodele amphibians. We will also discuss the possible significance of its organization and plasticity in an evolutionary and developmental perspective.

## Stability versus instability: karyotypic conservation and variation in DNA content

Among urodeles, the amount of DNA per diploid nucleus ranges from about 30 pg in some Plethodontids to nearly 200 pg in *Necturus* (Morescalchi, 1975; Olmo and Morescalchi, 1975; Olmo, 1991). Variation in the C-value can also be extreme in closely related taxa. Perhaps the best-known example of this is given by American salamanders of the genus *Plethodon*: different species of *Plethodon* that are very similar in external appearance have approximately multiple amounts of DNA. Yet they share the same number and general shape of chromosomes! Thus, it appears as though a balanced growth of their chromosomes occurred during evolution (Mizuno and Macgregor, 1974; Macgregor, 1982).

Because of their large chromosomes, karyotypes of urodele amphibians have been extensively studied by classical methods and by staining techniques such as C-banding, N-banding, etc. in order to gain insights into the phylogenetic relationships among different taxa (Mancino *et al.*, 1977; Schmid *et al.*, 1990). The results of these studies show that, in accordance with what has been observed in Apoda and Anura, there is a general evolutionary trend toward symmetrical karyotypes. The chromosome number decreases, and, in place of acrocentric elements and microchromosomes which can be found in species of the more generalized families, karyotypes of the higher families have fewer, almost exclusively metacentric, chromosomes (Morescalchi, 1975). On the whole there is evidence for a rather stable karyotype, in

*Abbreviations used in this paper:* IGS, intergenic spacer; rDNA, ribosomal DNA; NOR, nucleolus organizer region; pg, picograms; SINE, short interspersed repeated element; LINE, long interspersed repeated elements; LTR, long terminal repeat

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spite of great variation in the DNA amount. *But from where did all this excess DNA originate?*

## Repeated DNA is a source of excess urodele DNA

It has been estimated that as much as 80% of the total amount of urodele DNA can be made up of repeated sequences (Britten and Kohne, 1968; Strauss, 1971). Most of these repeats are noncoding sequences. The molecular organization and chromosomal distributions of the repeated sequences found in urodeles are not substantially different from those of other eukaryotes (Miklos, 1985; Barsacchi, 1991; Charlesworth *et al.,* 1994). Some of these repeated sequences are represented in very high copy numbers (highly repeated DNA) or are moderately redundant (middle repetitive DNA). They can be arranged either in "head-to-tail" tandem arrays or dispersed as single elements throughout the genome. In this latter case they are usually referred to as SINEs (short interspersed repeated elements; reviewed by Okada, 1991) or LINEs (long interspersed repeated elements; reviewed by Martin, 1991 and Finnegan, 1992), according to the length of their repetitive unit and independent of their degree of redundancy. This classification need not be viewed in too rigid a manner, since it is reasonable to assume that the whole spectrum of both copy number and molecular organization is represented in eukaryote genomes.

Among urodeles there are several examples of tandemly arrayed families of highly repeated sequences (often referred to as satellite DNA). They can exist as large blocks of repeats, usually associated with heterochromatin (Diaz *et al.,* 1981; Baldwin and Macgregor, 1985; Barsacchi-Pilone *et al.,* 1986; Cremisi *et al.,* 1988; Batistoni *et al.,* 1991), or as clusters dispersed throughout all the chromosomes with no preferential association with heterochromatin (Varley *et al.,* 1980b; Macgregor *et al.,* 1981; Batistoni *et al.,* 1986, 1995; Epstein *et al.,* 1986; Wu, 1987; Nagahashi *et al.,* 1991; Vignali *et al.,* 1991). In urodeles, the size of the basic repeat of these tandemly arrayed sequence families usually, but not always, falls in the range of a few dozen to a few hundred base pairs.

In contrast to satellite DNA, only a few elements of the SINE or LINE type have been characterized in urodeles. These sequences usually consist of singularly interspersed elements which are thought to derive from retrotransposition events. In urodeles, two cloned SINE-like families show a tandemly arrayed molecular organization; thus they are different from the typical SINEs of most eukaryotes (Nagahashi *et al.,* 1991; Batistoni *et al.,* 1995). Very little is known about LINE-like elements in urodeles, the only example being a family of Gypsy/Ty3-like elements found in the genome of the plethodontid *Hydromantes* (Nardi, 1991; Marracci *et al.,* 1996).

As we briefly mentioned in the Introduction, the eukaryote genome is subject to turnover mechanisms that are responsible for a state of plasticity of the genome. *In what way do urodele genomes, and specifically the above mentioned sequence families, provide examples for such a plasticity?*

## Plasticity of the urodele genome

In urodeles, several examples reveal that the genome is a fluidly evolving system. A first example, related to quantitative evolutionary variations in the total amount of nuclear DNA, was mentioned above and concerns the different C-values of closely related species of *Plethodon* (Mizuno and Macgregor, 1974; Macgregor, 1982). A second example, dealing instead with quantitative variations of a specific sequence, is given by TkS1, the centromeric satellite DNA of *Triturus karelinii* and related species. TkS1 is a family of tandemly repeated DNAs clustered in the centromeric heterochromatin of several newts. This family of sequences is highly conserved at the nucleotide level, at least in the five species most closely related to *Triturus karelinii*. In these same species, however, its abundance varies widely. An almost ten-fold variation has been detected between *Triturus karelinii*, which has the most, and *Triturus carnifex*, *Triturus cristatus* and *Triturus marmoratus*, all of which have comparably large amounts (Baldwin and Macgregor, 1985; Varley *et al.,* 1990). Thus, in spite of the similar C-values, it can be suggested that during evolution the TkS1 satellite has been differentially amplified in the various *Triturus* genomes. Correspondingly, these closely related newt species show different amounts of centromeric heterochromatin (although this does not imply that TkS1 satellite is the only component of centromeric heterochromatin: compare Cremisi *et al.*, 1988 with Varley *et al.,* 1990 and discussions therein).

All of the highly repeated DNA families cloned from urodeles are distributed on all or most chromosomes of the set, irrespective of their molecular organization (tandem arrays or single dispersed sequences) and regardless of whether they are preferentially associated with heterochromatin. It is difficult to imagine that they arose separately on each chromosome. Most likely they diffused from an original single locus by means of mechanisms such as transposition or non-homologous exchange of DNA sequences.

Newts provide cytological evidence in favor of exchanges occurring, not only between homologous chromosomes, but also between non-homologous elements of the set. Association of both homologous and non-homologous centromeres during meiosis and mitosis (Baldwin and Macgregor, 1985; Callan, 1991), and patterns of inheritance of centromere bars observed in lampbrush chromosomes of hybrids obtained by crossing different species of *Triturus* (Callan, 1982 discussed in Baldwin and Macgregor, 1985), strongly support this possibility. That evidence could explain the presence of specific clustered satellite sequences on all or most of the centromeres of the set (Diaz *et al.,* 1981; Baldwin and Macgregor, 1985; Cremisi *et al.,* 1988; Batistoni *et al.,* 1991). However, data from other eukaryotes reveal that classic exchange between chromosomes may not be the only mechanism. Other mechanisms may play a role in spreading sequences around chromosomes. For instance, transposition of minisatellite sequences has been reported in the midge *Chironomus* (Hankeln *et al.,* 1994); moreover, it has been observed that copies of the centromeric dodeca satellite of *Drosophila* may exist as circular extrachromosomal molecules, which could potentially mediate the transfer of sequences between chromosomes (Renault *et al.,* 1993) (see also below). *Could these same mechanisms be at work in diffusing dispersed families of sequences throughout the urodele genome?*

## Dispersion of repeated sequences throughout the urodele genome

The best example of a dynamic genome in urodeles is perhaps offered by some sequences which are present in the 18 S+28 S rDNA clusters. In all eukaryotes these clusters consist of tandemly arrayed repeats of several kilobases in length. A portion of the repeat codes for the precursor to the 18 S and 28 S ribosomal RNA

(coding region), while the other part represents the so called intergenic spacer (IGS). The genetic locus where the rDNA arrays are clustered is called the nucleolus organizer region (NOR), since it directs the formation of the nucleolus, the cytological site where transcription and processing of the 18 S and 28 S ribosomal RNA occurs. In newts of the genus *Triturus*, in addition to a constant locus bearing a NOR, several other additional rDNA sites, variable among individuals as to number and cytological position, may be present (Nardi *et al.*, 1977; Andronico *et al.*, 1985; De Lucchini *et al.*, 1993).

Although the existence of additional, non-constant ribosomal sites might be taken as another example of genome fluidity, it is but only part of the story. In fact, within the IGS of the rDNA unit some sequences have been identified that, although conserved in all the tested species of *Triturus*, in only one species — *Triturus vulgaris meridionalis* — have they formed "extra-ribosomal" clusters outside the ribosomal sites (De Lucchini *et al.*, 1988). These sequences contain a chi-like box partly homologous to the core region of the human hypervariable minisatellite, which is known to act as a recombinational hotspot (Jeffreys *et al.*, 1985; Wahls *et al.*, 1990). It is also homologous to the chi sequence of *Escherichia coli*, which promotes recombination in bacteria (Smith, 1983, 1991). The presence of chi-like sequences has been taken as a possible explanation for the existence, in *Triturus vulgaris meridionalis*, of additional rDNA arrays at various chromosomal loci (De Lucchini *et al.*, 1988). *Is it possible that this transfer of sequences was mediated through extrachromosomal circular DNA molecules?* Such molecules are known to occur for tandemly repeated sequences (Pont *et al.*, 1987; Degroote *et al.*, 1989; Renault *et al.*, 1993) and are a well known product of rDNA gene amplification in amphibian oocytes (Brown and Dawid, 1968).

## Possible retrotransposition events associated with the urodele genome

Finally we would like to mention a few more examples which argue in favor of retroposition events as mediators of gene dispersal. The first one is provided by the Nv2 satellite, a tandemly repeated sequence dispersed in the genome of the North American newt *Notophthalmus viridescens*. The Nv2 satellite is different from most other satellites in urodeles. It is transcribed in a tissue-specific manner into stable cytoplasmic transcripts (Epstein *et al.*, 1986; Epstein and Coats, 1991). Surprisingly, synthetic transcripts from dimeric cloned units of the Nv2 family of sequences are able to undergo an *in vitro* self-cleavage reaction directed by a catalytic core very similar to the one that some infectious agents of plants use for processing their RNAs during their replication cycle (Epstein and Gall, 1987a; Cremisi *et al.*, 1992). The similarity between these catalytic domains has suggested that a very ancient retrotransposition of an infectious agent took place during early salamandrid evolution (Epstein and Gall, 1987a,b).

More direct evidence of retroposition in urodeles comes from two other tandemly repeated DNA families, namely the PolIII/Tan family of *Cynops pyrrhogaster* (Endoh and Okada, 1986; Nagahashi *et al.*, 1991) and the HyPolIII family of the European *Hydromantes* (Batistoni *et al.*, 1995). Their repetition unit contains a tRNA-related segment, plus a tRNA-unrelated region. The whole repeat is flanked by short direct repeats, which are usually taken as an indication of a transposition event (reviewed by Finnegan, 1992). Moreover, the tRNA-related segments could not be originally derived by amplification of tRNA genes at the DNA level. In fact, eukaryote tRNA genes do not code for the CCA 3' terminus, which is added to tRNA molecules during their processing and which is instead found in both families of tRNA-related sequences (Nagahashi *et al.*, 1991; Batistoni *et al.*, 1995). This is a very strong indication that the tRNA-related parts of these DNA families were generated from tRNAs via cDNA intermediates. After integration of the cDNA intermediate these elements have been tandemly amplified along with some flanking sequences. The tandem organization of SINE-like elements in urodeles may reflect a strong tendency of their genomes to amplify any non-genetic sequences.

In conclusion, it is clear that urodeles provide evidence for a state of genome fluidity, where processes such as gene conversion (Whitehouse, 1982), transposition (Finnegan, 1992), unequal crossing-over (Smith, 1973, 1976), and slippage (Levinson and Gutman, 1987) play a crucial role in continuously shaping the genome from the inside (reviewed by Dover, 1982, 1986, 1987, 1993). The genome, through the phenotype it expresses, then copes with genetic drift and selection, which lead to changes in the genome influenced mainly by forces from the outside.

## Evolutionary and developmental implications of excess (?) DNA

As already remarked, a great deal of nuclear DNA in eukaryotes has been regarded as non-coding, excess DNA. Although the meaning of this excess DNA still remains rather elusive, several authors have proposed that it might have a variety of effects and/or functions. Thus, from time to time, this DNA has been considered important for gene regulation (Britten and Davidson, 1971), for determining nuclear and/or cell volume (Cavalier-Smith, 1985), for proper arrangement of chromosomes in the nucleus (Bennett, 1982), or for compacting DNA into chromosomes (Vogt, 1992; Pasero *et al.*, 1993). Others have instead made the point that some excess sequences might have no function at all (junk-DNA: Ohno, 1972). Yet others have proposed that they may serve merely for self-propagation/maintenance (selfish DNA), either because of autonomous replicative properties, or because of intrinsic biochemical properties that would confer to these sequences a better fitness in their genomic environment (Doolittle and Sapienza, 1980; Orgel and Crick, 1980; Doolittle, 1982). A full discussion on this topic would be beyond the intention of this contribution. However, new hints pointing towards a functional meaning of the excess DNA have recently been offered (Nowak, 1994). We would like, therefore, to keep an open mind into the possibility that absence of *known* function does not necessarily mean that this DNA is in reality functionless. Even in the event that it has no genic function (in the current sense of a gene coding for a protein or a functional RNA), this would not necessarily imply that it has no effect. Perhaps we should realize that the distinction between function and effect of a piece of DNA may be quite blurred when considered in an evolutionary perspective. In this sense DNA as a whole (both coding and noncoding) might be best regarded as "ignorant" (Dover, 1980), since it is simply subject to a series of biochemical processes which continuously scramble, disperse and modify its nucleotide sequence (Dover, 1987, 1993).

In principle, we should therefore consider that, at least in part, excess eucaryotic DNA might actually represent a by-product of

the above mentioned turnover mechanisms. That is, it might reflect evolution at work. In this continuous reshaping of genomes, however, pieces of DNA (and also of excess DNA) may be brought into new positions, where they could have new effects and/or acquire novel functions. We need not to assume that all such genetic novelties will become fixed in a population, although this has really happened in several instances. We can cite a few examples which involve transposable elements.

Transposable elements have long been regarded as something that is not directly related to gene function. Rather, they are often thought to represent extraneous, selfish parasites within the genome (Doolittle and Sapienza, 1980; Orgel and Crick, 1980; Doolittle, 1982; however, see Voytas and Boeke, 1993 for transposons as symbionts). Nonetheless, transposition may be evolutionarily important by generating allelic variants and changes in the fitness of individuals (Mackay, 1986; Pasyukova *et al.*, 1986; McDonald, 1993 and references therein). It might also generate new regulatory combinations that could be of developmental relevance. For instance, as part of their replicative strategy, transcription of retroelements is often sensitive to growth factors and hormones, due to responsive regulatory elements present in their LTRs (Cho *et al.*, 1990; Schiff *et al.*, 1991; Greene *et al.*, 1993). It has been shown that several genes have derived their regulatory sequences from LTRs of defective transposed elements (Banville and Boie, 1989; Stavenhagen and Robins, 1988; McDonald, 1993). In *Drosophila*, the *17.6* transposable element is transcribed in a regulated fashion during eye development, suggesting either a possible role of its transcripts in the development of the eye or an effect of its regulatory sequences in the innervation-dependent expression of nearby cellular genes (Mozer and Benzer, 1994). We have seen in the previous section that some repeated DNA families provide evidence that retrotransposition events take place in urodele genomes. Thus, it is possible that transposition may contribute to urodele genetic variability, genome turnover, and/or may affect the regulation of specific genes.

Genome turnover mechanisms can explain the concerted evolution of both coding and noncoding repeated sequences. That is, it explains why repeats of the same family of sequences tend to be homogeneous within a species (Arnheim *et al.*, 1980; Krystal *et al.*, 1981; Dover, 1982; Arnheim, 1983). The possible evolutionary consequences should be immediately clear if we think that this homogenization of families may happen differentially in separate populations; if the families are involved in the reproductive biology of the species, this may potentially lead to developmental incompatibilities between the separate populations and thus to speciation (Dover, 1982, 1986; Dover *et al.*, 1982).

Although most of the highly repeated DNA sequences known in urodeles are transcribed on lampbrush chromosomes (Varley *et al.*, 1980a,b; Diaz *et al.*, 1981; Macgregor, *et al.*, 1981; Baldwin and Macgregor, 1985; Barsacchi-Pilone *et al.*, 1986) they are not usually copied into stable RNAs. The only exception is that of the above mentioned Nv2 satellite of newts. The pattern of transcription of this satellite, its tissue specific transcription and/or processing, and the self-cleaving properties of its *in vitro* transcripts (Epstein *et al.*, 1986; Epstein and Gall, 1987a,b; Epstein and Coats, 1991; Cremisi *et al.*, 1992) strongly suggests it has a functional role. *Could it be possible that this example resides on the evolutionary border, or just beyond the borderline, between simple effect and direct function of a DNA sequence?*

## Additional roles for excess genomic sequences

In addition to these possible effects and/or functions of excess DNA which rest upon the specific characters of the sequences involved, it has been proposed that the genome has some "nucleotypic" effects which are not dependent on the quality of its nucleotide sequences, but rather on the mere quantity of DNA (Bennett, 1982; Cavalier-Smith, 1985). For instance, it has been remarked that there is a positive correlation between genome size and nuclear and cell size. Genome size is thought to serve to properly regulate the correct nuclear/cell volume (reviewed by Cavalier-Smith, 1985). Several reports have established this correlation as valid also for amphibians (Olmo and Morescalchi, 1975, 1978; Macgregor, 1982; Horner and Macgregor, 1983).

More significantly in our context, it has also been argued that DNA amount can exert an effect on development (reviewed by Cavalier-Smith, 1985) by affecting developmental rates. As a matter of fact, a negative correlation between C-value and developmental rates has been shown in amphibians: species with lesser amounts of nuclear DNA develop faster than those with high C-values (Goin *et al.*, 1968; Oeldorf *et al.*, 1978; Horner and Macgregor, 1983; Pagel and Johnstone, 1992). The relationship does not, however, hold true for all species, so that one can question to what extent developmental rates are set by nuclear DNA amounts ("nucleotypic effects"), rather than by the action of specific sets of genes. Although the relationship is grossly valid in general, it is difficult to establish a clear causal link between developmental rates and nuclear DNA amounts. In part this is difficult because it is not easy to envision suitable experimental tests. However, Sessions and Larson (1987) compared the rates of limb regeneration in several Plethodontid species. They monitored growth and differentiation rates in regenerating limbs. It was found that evolutionary changes in genome size are indeed correlated with changes in the rate of limb regeneration, in an inverse relationship. However, the inverse relationship is stronger for the differentiation rate rather than for the growth rate, suggesting that growth of the genome potentially may be one of the potential mechanisms leading to the uncoupling of growth rate and differentiation rate. Heterochronic changes which lead to neoteny are thereby favored. In this perspective it has been repeatedly emphasized that obligatory neotenes represent the urodele species with the highest DNA amounts (Goin *et al.*, 1968; Morescalchi, 1975; Morescalchi, 1992; Martin and Gordon, 1995).

## Genome content and neoteny

It seems more plausible that the evolutionary shift to neoteny did not, however, happen merely through an increase in genome size. Recent results showing that *Hoxd-13* gene disruption leads to neotenic limb development in the mouse (Dollé *et al.*, 1993) indicate that neoteny as a whole may instead result from coordinate and specific negative regulation of genes responsible for the development of the adult phenotype. It is thus reasonable to assume that the ontogenetic shift to neoteny normally occurs by shutting off these genes in response to specific external conditions.

Martin and Gordon (1995) have recently remarked that if conditions favorable to neoteny persist for many generations, there would not be any positive selection for wild-type adult genes which could be mutation-inactivated, thus compelling animals to obligate

neoteny. They proposed that a substantial part of the enormous genome of obligatory neotenes may be made up of unused, adult-stage somatic genes, which, during evolution, have been free to accumulate mutations and to duplicate in subsequent rounds of DNA replication, leading to the extremely high C-values of present day obligate neotenes. Their idea is also consistent with the possibility that the increase in genome size may have resulted from the genomic mechanisms discussed in the previous sections (for instance, by insertion of transposons and of repeated sequences into genes that are no-longer functional and subsequent successive rounds of tandem amplification). Moreover it cannot be excluded that the original key mutation(s) leading to obligate neoteny might also have arisen in a similar way. It has been reported that heterochromatization and gene silencing may be obtained by local tandem amplification of sequences (Dorer and Henikoff, 1994), that insertion of transposable elements often disrupts gene structure (McDonald, 1993), and that slippage mechanisms are responsible for genetic diseases (Dover, 1993; Kuhl and Caskey, 1993).

In conclusion, once obligate neoteny was genetically fixed, the genome would have become free to grow through the accumulation of extra-DNA in useless genes and/or through their duplication. It would be of great evolutionary significance to know whether genes controlling development of the adult phenotype are really silenced or disrupted by extra-DNA in the obligate neotenic species.

## Concluding remark

The observation that excess DNA has persisted during urodele evolution may imply that it contributes advantageous features to the development, growth, survival, and evolution of the species. The wide range of characteristics of the excess DNA has generated a variety of speculations about its function and/or effects. By increasing the database, in the future it should be possible to evaluate the validity of those speculations.

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