

## GENE AMPLIFICATION IN *RHYNCHOSCIARA* (1955-1987)

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*A review is made of the evidence indicating the existence of gene amplification in Rhynchosciara, from the early cytological work to the more recent studies using cloned sequences from the DNA puffs. Mention is made of work still in progress which indicates that the transcription unit of a DNA puff is surprisingly complex.*

### Cytological evidence

Gene amplification in somatic cells was detected by Breuer & Pavan (1955) in the course of their morphological studies on the salivary gland chromosomes of larvae of *Rhynchosciara americana*. They found that certain bands of these chromosomes swelled greatly and that in these bands either simultaneously or after this phenomenon there was a disproportionate increase in DNA content as compared to that found in neighbouring bands. To distinguish these formations from the RNA puffs which were known to be found in many polytene chromosomes, including those of *Rhynchosciara*, these formations have been called "DNA puffs" by several authors. Breuer & Pavan concluded that the DNA puffs constituted an exception to the rule of DNA constancy. Fick & Pavan (1957) working with *Rhynchosciara* demonstrated that the incorporation of <sup>3</sup>H-thymidine in a DNA puff site in chromosome C was much greater than that found in the other sites of the same chromosome. This was consistent with the idea that at the DNA puff site there was taking place an increase in gene number, although such an increase could hardly be measured at the time.

The first quantitative measurements of gene amplification in *Rhynchosciara* were carried out by Rudkin & Corlette (1957) using microspectrophotometry and adequate digestion of the preparations with RNase and DNase in order to identify what they were measuring. They studied the tip of chromosome B, where one of the major DNA puffs of Breuer & Pavan was located. Their results indicate that at the site of this puff there was a 2.5 increase in integrated absorbancy, corresponding to DNA, as compared to the increase found in a neighbouring region where no puff was formed.

Due to its peculiar mechanism of sex determination, and to other unknown causes, it is

very difficult to raise *Rhynchosciara* under laboratory conditions. Thus, the search for gene amplification proceeded in larvae of related flies, and the most readily available was *Sciarcoprophila*, the American fungus gnat, which has already been extensively studied by Metz et al. (1938). Working with this organism, Gabrusewycz-Garcia (1964) was able to demonstrate the faster rate of <sup>3</sup>H-thymidine incorporation in certain chromosomal bands as compared to others in the same chromosome, in accord with the data of Fick & Pavan (1957). Also a very careful microspectrophotometric measurements of regions of the salivary glands in the course of development was carried out by Crouse & Keyl (1968). The results obtained indicated rather conclusively that at the DNA puff site studied there occurred two extra DNA replication cycles in relation to another measured site in which no puff developed. These measurements constituted until recently the most conclusive evidence for the existence of gene amplification in the salivary gland chromosomes of *Sciara* and gave credibility to the results obtained with *Rhynchosciara*. Since then, the occurrence of DNA puffs was found in several other species belonging to the Sciaridae family, such as *Bradysia hygida* (Laicine et al., 1984) and *Trichosia pubescens* (Amabis & Amabis, 1984). In these and other cases studied the evidence for gene amplification consisted in microscopic observations and in the finding of differential rates of thymidine incorporation at the presumed DNA puffs sites.

### Biochemical and molecular studies

These studies are better carried out in *Rhynchosciara americana* one of the largest Sciaridae, because of the peculiarities of the biology of this organism. After fertilization the female of this species lays about a 1.000 eggs, in a short time, and the larvae which hatch from them keep body contact from the time they hatch

until pupation, having a synchronized development (Pavan, 1958). This in turn facilitates collection of sufficient quantities of specialized tissues, such as the salivary glands, for the biochemical analysis. A larva has two salivary glands and one of these, at the developmental period corresponding to maximal development of the DNA puffs, contains about 0.9  $\mu\text{g}$  DNA.

The DNA puffs occur in late 4th instar. About 10 days before pupation the larvae stop feeding and enclose themselves into a loose common cocoon made from a silk-like secretion produced by the salivary glands, which are in more like silk glands. This loose cocoon thickens as more secretion is produced and eventually each larvae is found enclosed in an individual cell, within the common cocoon, which takes the overall aspect of a honeycomb. The chronological age of a group, even under controlled conditions might differ from its physiological age, which has to be determined in every case. This is easily done by examination of the chromosomal morphology, and from the general aspect of the group (Stocker et al., 1984).

The initial studies were centered in determining the biological function of the DNA puffs. Experimental approaches to this problem were facilitated by the fact that the B-2 puff develops fully only in the proximal section of the gland in period IV, and that here the C-3 (de Moraes & Miranda, 1970) DNA puff is not fully developed. This enables work with cells expressing only one type of puff. Biochemical events taking place at the proximal segment of the gland at this period were compared with those taking place in period III (no DNA puffs).

The first significant progress was made in the search for changes in the poly(A)<sup>+</sup> RNA profile in the proximal gland cells at these two key periods. It was found that there is a correlation between the appearance of a 16S poly(A)<sup>+</sup> RNA species in these cells and the degree of expansion of the B-2 DNA puff (Okretic et al., 1977). Subsequently it was found that this RNA could be isolated from microdissected B-2 puffs and that it hybridizes "in situ" to DNA at the B-2 puff site (Bonardo et al., 1979). More recently a correlation between an 18S poly A RNA and the C-3 DNA puff was also found (de Toledo & Lara, 1978).

In accord with data mentioned above it was found that the transition from period III to periods IV-V is accompanied by dramatic changes in the pattern of protein synthesis of the gland. Winter et al. (1980) detected five

new peptides which are synthesized in the gland only at period IV-V when the DNA puffs are formed. They also found that the appearance of one of these peptides could be correlated with the appearance of puff B-2 and another to that of puff C-3. Both the 16S and the 18S poly(A)<sup>+</sup> RNA's have been transcribed "in vitro"; the main translation product in each case corresponds to the peptide which had previously correlated with the appearance for the B-2 and C-3 puffs, respectively (de Toledo & Lara, 1978). Taken together the evidence just presented strongly indicates that the "DNA puffs" are involved in messenger RNA production.

The formation of the DNA puffs and its biochemical consequences which take place in the course of normal development, can also be brought about experimentally by injection of the insect molting hormone, ecdysterone (Amabis & Amabis, 1984; Stocker & Pavan, 1974; Stocker et al., 1984; Berendes & Lara, 1975).

It is interesting to note that the first reaction to either normal or artificially raised titer of ecdysterone in the hemolymph of the larvae is the formation of a series of "early" RNA puffs (Berendes & Lara, 1975). Under experimental conditions DNA amplification can be detected at the DNA puff sites after 20 hours after ecdysterone injection (Berendes & Lara, 1975). There is indication that these early RNA puffs are involved in the synthesis of special "activator RNA", which are able to induce the formation of DNA puffs (Graesman et al., 1974). Work carried out by Amabis & Amabis (1984) in a related organism, *Trichosia pubescens*, corroborate these observations, and indicate that the "activator RNA" must be translated to become effective. There is a great need to repeat these experiments and achieve a better definition of these "activator RNA's", and of their activations mechanism.

#### *Molecular biology of the DNA puffs*

There is transcription in many sites in the salivary glands chromosomes of period V larvae in late 4th instar. At this period of development many DNA puffs are very active and transcripts made from them are sufficiently abundant in the RNA population so that they can be separated either by electrophoresis or sucrose gradient sedimentation. Advantage was taken of this fact to prepare a library of cloned DNA constructed from poly(A)<sup>+</sup> RNA obtained from whole glands of animals in period V (Glover et al., 1982). This library was then screened for

colonies that would hybridize strongly with  $^{32}\text{P}$ -DNA complementary to poly(A)<sup>+</sup> RNA from period V glands, but that would not hybridize to poly(A)<sup>+</sup> RNA from period III glands (when DNA puffs are not active). Among 600 colonies so screened 80 were detected which had these properties and thus represented developmentally regulated sequences. From these we selected for further study three clones. One of these pRa 3.65 corresponding to a 1.25 Kb RNA hybridized "in situ" to the C-3 region. The other pRa 3.81 hybridized "in situ" to the C-8 region and the hybridization site of the other pRa 3.46 was not determined as yet, but we know that its sequence is not amplified.

Genomic clones were isolated by Millar et al. (1985) using the c-DNA clones to probe genomic banks, made using  $\lambda$  vectors. These clones or their subclones were also used to quantitate the amplification. The results obtained confirm that amplification results in 3 extra replication cycles at both C-3 and C-8. The same degree of amplification was also found in regions neighbouring the mapped C-3 and C-8 exons. Roberto V. Santelli (unpublished results) has been able to further probe the genomic banks, and has found a series of overlapping phages which, together with the ones previously identified, cover an extension of 40 Kb for the C-8 puff and 22 Kb for the C-3 puff. He is attempting to measure the degree of amplification along these chromosomal extensions in order to define the model according to which amplification is achieved at the DNA puffs. His measurements are however not completed and thus we have no conclusion regarding this important point.

It is crucial for an elucidation of the model of operating in *Rhynchosciara* gene amplification to determine how the DNA puff genes are controlled. Thus I have started an attempt to define the transcription unit of the C-8 puff. This was necessary in order to carry out a collaborative research with Allan Spradling, which aims to insert a transcription unit of a DNA puff in *Drosophila* by P-mediated transformation, in order to study the amplification control in *Rhynchosciara*. For this work, I have used the available C-8 cloned material. Surprisingly, I found that over the 34.4 Kb walk covered by Santelli for this puff, there is only a 550 bp exon present. Since the messenger coded by this puff has a length of 1.9 Kb and the protein coded by this messenger has a molecular mass of 35.000 Da (Navarro et al., 1983) the coding region should be around 1 Kb. Thus one or more

exons may still be found if longer chromosome segments are probed. Otherwise, if the exon found is the only one, the discrepancy between its length and that of its mRNA must still have an unknown explanation.

In conclusion, it is evident that much needs to be done to obtain an understanding of gene amplification in *Rhynchosciara*. Research here offers interesting new perspectives and the system itself is amenable to many experimental approaches, particularly with respect to elucidation of the amplification mechanisms. Effort along these lines might prove fruitful, and gene amplification in *Rhynchosciara* might help to understand what happens in other cases of gene amplification.

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