Amplification of the rudimentary gene in a PALA-resistant *Drosophila* cell line

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In *Drosophila melanogaster* the rudimentary locus encodes for a multifunctional protein catalyzing the first three enzymatic activities of pyrimidine biosynthesis. Cell lines were selected which were resistant to PALA (*N*-(phosphonoacetyl)-L-aspartate), a specific inhibitor of aspartate transcarbamylase, the second enzyme of this pathway. In a cell line where the enzyme production is increased 5 times, Southern blot analyses show that the rudimentary gene and surrounding regions are amplified about 5 times. In this case gene amplification could therefore account for the observed enzyme overproduction.

Aspartate transcarbamylase N-*PALA resistance* Drosophila melanogaster *Rudimentary gene*
Enzyme overproduction *Gene amplification*

1. INTRODUCTION

Inhibitors of enzymes catalyzing a reaction essential for the growth of cells have allowed the selection of resistant clones in mammalian cells in culture. Many such resistant lines overproduce the target enzyme. The molecular basis for this overproduction is the amplification of the gene coding for this enzyme in at least 3 cases [1–3].

In mammalian cells one multienzymic polypeptide, CAD, encoded by one gene has the 3 enzymatic activities, carbamyl phosphate synthetase (CPSase, EC 2.7.2.9), aspartate transcarbamylase (ATCase, EC 2.1.3.2) and dihydroorotase (DHOase, EC 3.5.2.3) which catalyze the 3 first steps of the de novo pyrimidine biosynthesis [4].

Using the transition state analog PALA [5] a potent and specific inhibitor of ATCase as the selective agent, authors in [6] obtained by stepwise selection many mutants of simian virus 40-transformed Syrian hamster cells resistant to high levels of PALA (25 mM). In some mutants resistant to 25 mM PALA the specific activity of ATCase is increased more than 100-fold as compared to wild-type cells. The specific activities of CPSase and DHOase are increased coordinately with that of ATCase in PALA-resistant mutants. Elevated levels of CAD protein result from an increase of the corresponding mRNA due to an amplification of the corresponding gene [2,7]. The mammalian system is however very complex [2] and we sought for a simpler model of CAD gene amplification in *D. melanogaster*. Clones of *D. melanogaster* cells resistant to PALA which display a concomitant increase in the 3 enzymatic activities CPSase, ATCase and DHOase (submitted) have been isolated in our laboratory. These activities are encoded by the rudimentary gene (*r*: 1–55.3) localized at the locus 15 A1 on chromosome X [8]. Authors in [9] have selected from a genomic library of *D. melanogaster* a clone which hybridized to the hamster CAD gene. Starting from this clone BS 313 (fig.1) they 'walked' along the chromosome and isolated a 90 kb region containing the whole rudimentary gene (about 26 kb long). Here, we used the probes kindly provided by B.P. Jarry and C. Louis to analyze at the

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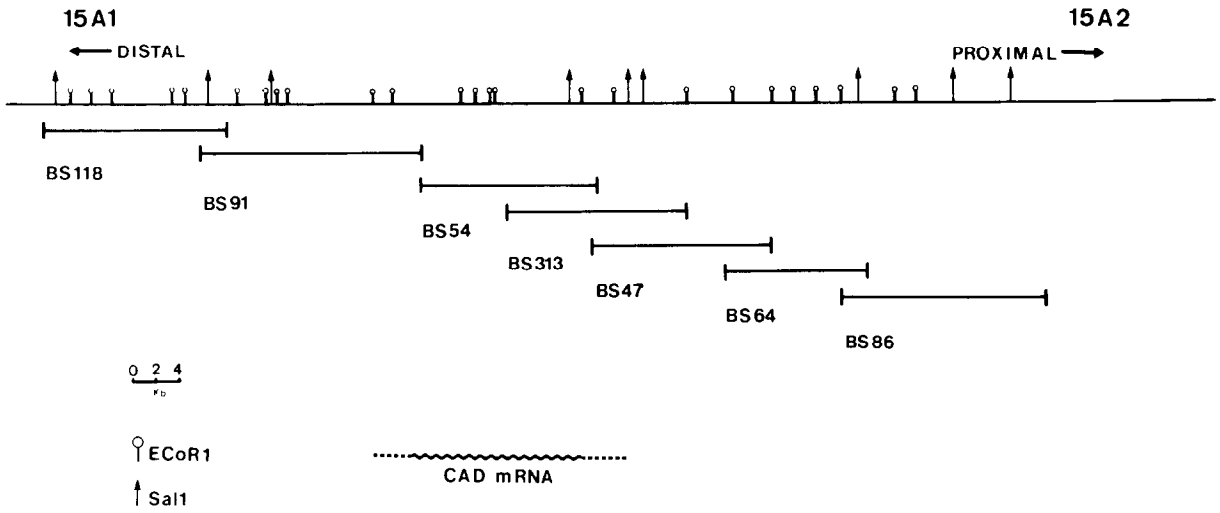

Fig.1. Restriction map of the recombinant phages derived from the rudimentary locus on the X chromosome [9]. Each probe is composed of a *Drosophila* DNA fragment inserted in Charon 4A phage by *Eco*RI linkers [13]. The proximal side is situated near the centromere and the distal side is far from the centromere.

DNA level the molecular basis for the overproduction of CAD protein in a PALA-resistant (PALA-r) *Drosophila* cell clone.

2. MATERIALS AND METHODS

2.1. *Cell culture conditions*

A *D. melanogaster* cell line (77 OM_3) established from the Oregon stock in the Laboratoire de génétique des virus (Gif sur Yvette), was obtained from Dr A. Ohanessian and used for selection of PALA-resistant cells (submitted). The cells were grown either on plastic plates or in suspension when large amounts were needed in a 1/1 mixture of D 22 medium [10] and the medium in [11], supplemented with 5% (v/v) decomplemented fetal calf serum.

The clone used here is resistant to 10 mM PALA and cultured in the absence of PALA for one year. These cells have an increased activity of the 3 enzymes CPSase, ATCase, DHOase of 5–6-fold (submitted).

2.2. *Preparation of cellular DNA*

High-M_r DNA was isolated as in [12]. Cells were washed twice in an isotonic buffer (0.14 M NaCl, 25 mM HEPES, pH 7.2), then resuspended in 10 ml/g cells of 10 mM Tris–HCl (pH 7.9), 5 mM EDTA, 1% SDS and incubated overnight in the presence of 100 μg/ml proteinase K. The hydrolysate was extracted twice at room temperaure for 2 h with an equal volume of phenol–chloroform–isoamyl alcohol (50:48:2). The aqueous phase was dialysed 18 h, against TEN buffer (10 mM Tris, pH 7.9, 2 mM EDTA, 1 M NaCl), then dialysed several days against TE buffer (10 mM Tris, pH 7.9, 2 mM EDTA). The dialysate was incubated with pancreatic RNase (20 μg/ml) for 1 h at 37°C, and extracted as above with a phenol–chloroform–isoamyl alcohol mixture. After exhaustive dialysis, the DNA was ethanol-precipitated and stored in solution in TE buffer at 4°C.

2.3. *Genomic probes*

2.3.1. Phage DNA preparation

Recombinant phages corresponding to the rudimentary region [13,9] (fig.1) were propagated on *Escherichia coli* strain KH802 plated on LAM medium (Luria Broth with 10 mM $MgSO_4$). After an overnight incubation at 37°C, 10 ml PS buffer (10 mM Tris, pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, 0.05% gelatin and chloroform) were layered on the plates and the incubation was resumed for 24 h at 4°C. The resulting lysate was then collected and stored at 4°C. Phage DNA was isolated from the lysate as in [14].

2.3.2. Probe for the tubulin 3 gene

The plasmid pDmT$_{3\alpha}$ [15] used in control experiments was kindly provided by P.C. Wensink. For hybridization studies DNA was nick-translated with [^{32}P]dXTPs as in [16].

2.4. *Electrophoresis of DNA and transfer*

After complete *Eco*RI digestion (1 unit/μg DNA for 4 h at 37°C) the cellular DNA fragments were electrophoresed on a 1% agarose gel in Tris–borate buffer (0.089 M Tris base, 0.089 M boric acid, 2 mM EDTA, pH 8) for 18 h at 1 V/cm and transferred to nitrocellulose filters as in [17]. The filters were prehybridized for more than 4 h at 68°C in 6 × SSC, 2 × Denhardt's solution [17,18] and hybridized in 6 × SSC, 2 × Denhardt's solution, 0.1% SDS containing 50 μg/ml yeast tRNA in the presence of 10^6 cpm of the nick-translated probe (specific radioactivity 10^7–10^8 cpm/μg). The filters were washed 3 × 1 h in 2 × SSC, 0.1% SDS at 68°C, then 3 × 1 h in 0.2 × SSC, 0.1% SDS at room temperature, dried and exposed to Kodak X-Omat R films with intensifying screens at −80°C for 1–3 days.

3. RESULTS

3.1. *Amplification of the rudimentary locus*

As illustrated in fig.1 3 recombinant phages, BS 91, BS 54, BS 313 (covering 40 kb) include the whole rudimentary gene [9]. The intensity of hybridization of these 3 probes to *Eco*RI digests of equal amounts of genomic DNA from PALA-r and wild type OM cells has been compared (fig.2B–D). PALA-r cells show a 5-times more intense signal than wild-type cells indicating a 5-fold amplification of the rudimentary region.

3.2. *Amplification of sequences flanking the rudimentary locus*

Clone BS 118 contains a 15 kb sequence flanking the rudimentary locus on the distal side [9]. Clones BS 47, BS 64 and BS 86 cover a 38 kb region on the proximal side of the locus [9]. The

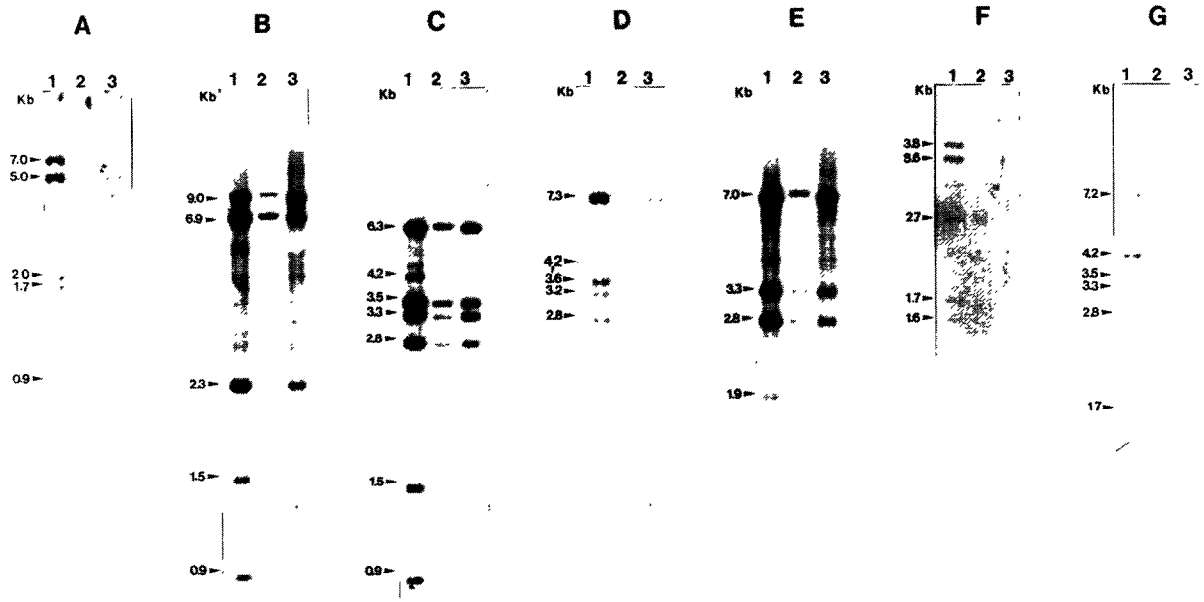

Fig.2. Southern hybridization pattern of probes from rudimentary region to PALA-r and wild-type OM cell DNAs. DNAs digested with *Eco*RI were electrophoresed in a horizontal 1% agarose gel (1 V/cm, 18 h), transferred to a nitrocellulose filter (Schleicher and Schüell BA 85) and hybridized to the various probes (10^7–10^8 cpm/μg; 10^6 cpm/blot). The filters were washed under stringent conditions. Fragment sizes were estimated from *Hin*dIII and *Eco*RI digests of bacteriophage λ DNA run in a parallel track (not shown). A = BS118; B = BS91; C = BS54; D = BS313; E = BS47; G = BS86. Track 1: 10 μg PALA-r DNA. Track 2: 1 μg PALA-r DNA. Track 3: 10 μg OM DNA. F = BS64. Track 1: 5 μg PALA-r DNA. Track 2: 1 μg PALA-r DNA. Track 3: 5 μg OM DNA.

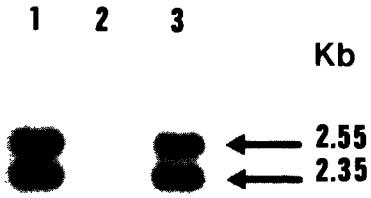

Fig.3. Southern hybridization pattern of a tubulin probe to PALA-r and wild-type OM cell DNAs. The experimental conditions were the same as those in fig.2 except that the probe was pDmT3α. As a matter of control, *Eco*RI fragments of PALA-r and OM DNAs were electrophoresed in lanes adjacent to those shown in fig.2C,E. After transfer, the nitrocellulose filter was cut, and the control lanes hybridized with the pDmT3α (tubulin) probe. Track 1: 10 μg PALA-r DNA. Track 2: 1 μg PALA-r DNA. Track 3: 10 μg OM DNA.

comparison of the signals obtained after hybridization of these 4 probes to *Eco*RI DNA digests of PALA-r and wild-type cells led to the conclusion that the regions flanking the rudimentary gene, about 20 kb on the distal side and 40 kb on the proximal side, are also amplified (fig.2A,E–G). Consequently, there is no evidence that attenuation of amplification is detectable on either side of the rudimentary gene. A possible error in the estimation of DNA used for blot analysis was ruled out since identical signals were obtained for an equal amount of *Eco*RI-digested DNA of PALA-r and wild-type cells hybridized to a probe for the tubulin 3 gene (localized on chromosome 3 at 84D4-8 ([16], fig.3).

4. DISCUSSION

By growing *D. melanogaster* cells in the presence of increased concentration of PALA, a competitive inhibitor for ATCase, it is possible to select clones resistant to up to 10 mM PALA. This resistance is due to the elevated activity of the multifunctional protein catalyzing the 3 enzymatic activities CPSase, ATCase, DHOase (submitted) as is the case for PALA-r Syrian hamster cells selected in [6]. An analysis of metaphase spreads from the parental and PALA-r cells (submitted) revealed no difference in their respective karyotypes (2 n, with 20% 4 n). Our objective was to investigate the molecular basis of this overproduction at the DNA level by assaying for a possible amplification of the rudimentary gene in PALA-r cells. The CAD protein coding sequence was quantitated both in PALA-r and wild-type cells and was found to be amplified about 5-fold in the mutant cells. The increase in the 3 enzymatic activities (5–6-times) observed in these cells could thus be accounted for by the amplification of the rudimentary gene. Scanning of autoradiograms indicated the same degree of amplification throughout the 100 kb studied region including the rudimentary locus; no reduction was detectable at the borders of this domain. This situation differs from that observed for the chorion genes of *Drosophila*. Single-copy chorion genes are amplified by the follicle cells during oogenesis prior to their expression [19]; maximal amplification has been demonstrated in the center of the domain where the chorion genes are located as well as decreasing amplification of flanking sequences to both sides [20]. In our case the exact limits of the amplified unit have therefore to be determined by 'walking' along the chromosome on both sides of the 100 kb region surrounding the rudimentary locus.

Another characteristic of the amplification of the rudimentary gene is the absence of concomitant modification of the DNA structure in this region. As in the case of PALA-r hamster cells [2,6,7] the amplification of the rudimentary gene appears not to be an extrachromosomal phenomenon, since after one year of culture in the absence of the selective inhibitor the PALA-r *Drosophila* cells used here retain their characteristic increase in the CAD protein and rudimentary gene.

The phenomenon of amplification has been shown to occur in different situations in Biology throughout evolution to mediate either resistance of prokaryotes [21] or eukaryotes [1–3] to antiproliferative drugs or high-level synthesis of specific proteins in developmental time [19]. One of the molecular mechanisms leading to gene amplification could be a series of unequal crossing-overs occurring during the successive selection steps as discussed in [22].

The investigation of the amplification process may be easier in this *D. melanogaster*-resistant cell clone than in mammalian systems for the following reasons:

(i) The genome is of lower complexity;
(ii) Artefacts caused by genomic rearrangements are minimized by the low level of gene amplification;
(iii) Repeated sequences whose frequency hampers amplification studies in mammalian systems (O. Brison, personal communication) are not found in the region of the *Drosophila* genome studied here.

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