Cloning of the glucose-6-phosphate dehydrogenase gene of *Drosophila melanogaster* using 17-base oligonucleotide mixtures as probes


Department of Zoology, Faculty of Science* and Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science**, Hokkaido University, Sapporo 060 and Oklahoma Medical Research Foundation, Oklahoma 73104***

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ABSTRACT

Mixtures of 17-base long oligonucleotides possibly encoding a hexapeptide of *Drosophila melanogaster* glucose-6-phosphate dehydrogenase were synthesized and used as probes for screening a genomic library of *D. melanogaster* constructed in Charon 4A vectors. A total of about 60,000 plaques were initially screened, and after two successive plaque purification three clones carrying the identical 13-kb EcoRI fragment were isolated.

That these clones contain the G6PD coding sequence was demonstrated by in situ hybridization of the cloned DNA fragments to salivary gland polytene chromosomes and in vitro translation of the hybrid-selected mRNA. As suggested by Torczynski et al. (1984), this method using short synthetic probes appears versatile for isolating low-copy genes from genomic libraries.

1. INTRODUCTION

In the course of studies on major genetic factors affecting the activity level of glucose-6-phosphate dehydrogenase (G6PD) in *Drosophila melanogaster*, we found an X-linked mutation which gives rise to constitutive expression of the G6PD gene (Tanda and Hori 1983a, b). This mutation as well as some other mutations of similar nature which were recently found in stocks artificially selected for high G6PD activity appear to be regulatory mutations probably resulted from insertion of a movable genetic element in the neighborhood of the G6PD coding region. The element mapped 0.02–0.04 units to the left of the G6PD locus (Hori *et al*. unpubl.) and its linkage to the X chromosome is stable as long as the mutants are sib-mated. However, loss of the high enzyme activity trait, presumably due to excision of the element, was frequently observed in flies of the outcross progeny, though in one direction only; i.e., when mutant females are mated with males of other strains, such as Muller-5, Canton S and Harwich, all the offspring exhibit a high level
of G6PD activity, while some of the progeny from the reciprocal crosses no longer show a high level of enzyme activity (Ito et al. 1984; Itoh and Hori 1985). Such a non-reciprocal effect is strikingly similar to hybrid dysgenesis, but we already have some evidence that it can not be explained by either P-M or I-R system. As an aid to future studies of this putative movable element of regulatory function an attempt was made in the present study to obtain a recombinant lambda phage carrying the Drosophila G6PD gene. A genomic DNA library of D. melanogaster cloned in phage lambda Charon 4A vectors was screened with 17-base oligonucleotides encoding the sequence for a hexapeptide of D. melanogaster G6PD, and three clones containing the coding sequence for G6PD were isolated.

2. MATERIALS AND METHODS

Purification of G6PD: G6PD was purified from D. melanogaster imagoes (strain 2512H) by the method of Hori and Tanda (1980) modified as follows: the eluate from a CM-Sephadex column was adjusted to pH 6.2 with 0.1M KH₂PO₄ and applied on a phosphocellulose column equilibrated with 20 mM phosphate buffer, pH 6.2. The column was washed with 0.1M phosphate buffer, pH 6.3, and the enzyme was eluted with 0.1M phosphate buffer, pH 6.7. The eluate was applied on a 2', 5'-ADP-Sepharose 4B column and the column was washed with 0.1M phosphate/0.1M KCl, pH 6.7 and then with 0.1M phosphate, pH 8.0. The enzyme was eluted with 30µM NADP⁺ in 0.1M phosphate, pH 8.0. The purified enzyme was electrophoretically homogeneous.

Sequence determination of G6PD: The purified enzyme (3.9mg) was digested with TPCK-treated trypsin at a substrate-enzyme ratio of 50:1 in 3 ml of 0.1M NH₄HCO₃, pH 7.8 at 37°C for 6 hr. The lyophilized digest was dissolved in 0.1% trifluoroacetic acid, pH 2.2, and fractionated on a reversed phase column Spherisorb 50DS with LC-18 packing using a Beckman HPLC as described previously (Takahashi and Tang 1983). The elution was carried out at a constant flow of 0.1% trifluoroacetic acid (1.2ml/min) with a linear gradient of acetonitrile (1.67%/min). Amino acid analyses were performed according to the method of Spackman et al. (1958) with a Durrum model D500 amino acid analyzer. Peptides were hydrolyzed in 0.2 ml of 5.7N HCl at 108°C for 20 hr. Sequence determinations were carried out with automated Edman degradation in a Beckman sequencer Model 890C. The PTH-amino acids were identified with a reversed phase 5 micron C-18 column as described (Takahashi and Tang 1983).

Synthesis of 17-base oligonucleotides and kination: Based on amino acid analyses of all peptide fractions from HPLC, fraction no. 10 (Fig. 1) was sub-
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jected to sequencing and found to contain two peptides: Tyr-Asp-Glu-Leu-Tyr-Lys (major peptide) and Ser-Asp-Gly-Ser-Arg (minor peptide). Possible cDNA sequences for the major peptide may be: 3' ATPu-CTPu-CTPy-GAX or AAPy-ATPu-TTPy 5'. Since deoxyadenosine is not suitable for the 3' end of oligonucleotides to be synthesized, the following two sets of 17-base long oligonucleotide mixtures were separately synthesized in a DNA synthesizer (Applied Biosystem Co., Model 380A): 3' T-Pu-C-T-Pu-C-T-Py-G-A-X-A-T-Pu-T-T-Py 5' (Probe A) and 3' T-Pu-C-T-Pu-C-T-Py-A-A-Py-A-T-Pu-T-T-Py 5' (Probe B). After deprotection with 80% acetic acid, the products were purified by a DEAE Toyopearl 650S column chromatography. The sequences of the synthesized oligonucleotides were confirmed by the method of Maxam and Gilbert (1980). The 5' ends of the oligonucleotides were phosphorylated with [γ-32P]ATP and T4 polynucleotide kinase (Maniatis et al. 1982) and used as hybridization probes (specific activity = 1 - 2 x 10^6 cpm/μg).

Construction of genomic library and plaque hybridization: A genomic library of D. melanogaster (strain 2512H) was constructed in phage Charon 4A using a λ in vitro packaging kit (Amersham) as described by Maniatis et al. (1982). The recombinant phages were amplified using E. coli strain DP50 supF. Plaque hybridization was performed as described by Benton and Davis (1977). Nitrocellulose filter plaque replicas were prehybridized in a solution contain-
ing 1.5M NaCl, 20mM tris-HCl, pH 8.0, 6mM EDTA, 0.1% SDS and 5 x Denhard't's solution at 37°C overnight. Hybridization was performed with the solution supplemented with 10% dextran sulfate and labeled probe at a concentration of 6 ng/ml at 20°C for 48 hr. Filters were washed 3 times (15 min per wash) at room temperature in 0.9M NaCl/90mM Na citrate, pH 7.0. Autoradiography was done for 3 days at -80°C.

Preparation of RNA: Flies were homogenized with 10mM vanadyl ribonucleoside complexes/25mM tris/0.25M each of NaCl, NH₄Cl and sucrose/50mM MgCl₂/5mM EDTA/0.5% 2-mercaptoethanol, pH 7.6. The homogenate was centrifuged at 12,000 x g for 5 min, made 0.1% with respect to Na N-lauroyl sarcosine, and extracted twice with phenol/CHCl₃ and once with CHCl₃. RNA was precipitated with 2.5 vols of ethanol and washed successively with 2M LiCl, 3M Na acetate and 70% ethanol. For separation of poly(A)+RNA, the total RNA fraction was subjected to oligo(dT) cellulose column chromatography as described (Aviv and Leder 1972).

Fig. 2. Plaque replicas of a genomic library of D. melanogaster hybridized with synthetic probe B.
Hybrid selection, Northern blot analysis and in situ hybridization: Hybrid selection of G6PD mRNA was performed as described by Ricciardi et al. (1979) with a 3.0-kb EcoRI-SacI fragment subcloned in M13mp18 and mp19 bacteriophages and poly(A)+RNA. Hybrid selected mRNA was translated in vitro in a rabbit reticulocyte lysate (Promega Biotec) with 3H-leucine as the labeled amino acid. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis before and after immunoprecipitation with rabbit anti-G6PD antibody. Northern blot analysis was performed as described by Thomas (1980) and in situ hybridization was as described by Langer-Safer et al. (1982) with biotin-labeled DNA fragments and a Vectastain ABC kit (Vector Lab.).

3. RESULTS AND COMMENTS

Pairs of duplicate plaque replicas of a genomic library of D. melanogaster were hybridized with 32P-labeled synthetic probes A and B. In an initial screening of about 6 X 10^4 plaques, 20 phage plaques appeared to hybridize to probe B, but none to probe A (Fig. 2). After two successive plaque purification, three clones, λDmG6PD 1, 3 and 14, containing the identical 13-kb EcoRI fragment were isolated. The restriction map of λDmG6PD 14 is shown in Fig. 3. Two lines of evidence indicate that this cloned fragment contains the coding region of G6PD: (1) the 5.8-kb and 3.9-kb EcoRI fragments, 3.0-kb EcoRI-SacI fragment and 7.4-kb SalI fragment were labeled with biotin and separately hybridized in situ to salivary gland chromosomes. All fragments tested hybridized only at the 18E region of the X chromosome in each nucleus (Fig. 4). This agrees with the cytological position of the G6PD locus (18D1-18F; Stewart and Merriam 1973). (2) Poly(A)+RNA was hybrid-selected with the 3.0-kb EcoRI-SacI fragments subcloned in M13mp18 and mp19, and translated in vitro. Electrophoretic analysis demonstrated that an RNA species

![Restriction map of λDmG6PD 14. Bars below the map represent the fragments used as probes. Black bars represent the probes hybridized to G6PD mRNA.](image-url)
selected with mp19 produced a polypeptide which migrated as fast as purified G6PD, and that this polypeptide was precipitated with rabbit anti-G6PD antibody (Fig. 5). Upon Northern blot analysis of total and poly(A)⁺RNAs with the 3.0-kb EcoRI-SacI fragment as probe, a single RNA band (2.2kb) was detected (Fig. 6). A similar result was obtained with a 2.7-kb BamHI fragment, but not with a 2.2-kb BamHI-EcoRI, a 0.9-kb EcoRI-BamHI, and a 3.4-kb EcoRI fragment. These data suggest that there are two exons in the cloned DNA sequence, one in the 2.7-kb BamHI fragment and the other in the 3.0-kb EcoRI-SacI fragment.

From the hybrid selection data that the non-coding DNA strand was in the M13mp19 vector, it was suggested that the direction of mRNA synthesis would be from the left to the right as shown in Fig. 3.

Preliminary experiments with the EcoRI-SacI fragment showed that the copy number of the G6PD gene per haploid genome was the same in wild flies and the high-G6PD activity mutants, thus the possibility being ruled out that the mutants contain multiple copies of the G6PD gene.
The method used for plaque hybridization in this study is almost the same as described by Torczynski et al. (1984) for the isolation of human interferon genes. As predicted by them, this method using short synthetic probes appears versatile for isolating low-copy genes from genomic libraries as well as from cDNA libraries.

Recently, molecular cloning of the D. melanogaster G6PD gene was reported by Ganguly et al. (1985), who used immunoselected poly(A)+RNA as probe. Comparison of their clone with zDmG6PD 14 showed that the two cloned fragments are the same as far as the restriction map is concerned.
Fig 6. Northern blots of total RNA and poly(A)+RNA. The blots were hybridized with a 3.0-kb EcoRI-Sacl fragment labeled with 32P by nick-translation. Lanes A and B represent 20μg and 40μg of total RNA, and lanes C and D, 0.5 and 1.0μg of poly (A)+RNA, respectively. HindIII fragments of lambda phage DNA were used as size standards.

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