Diploid gynogenesis in *Xenopus laevis* and the localization with respect to the centromere of the gene for periodic albinism \(a^p\)

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**SUMMARY**

Diploid gynogenetic *Xenopus laevis* were obtained by inseminating the eggs with u.v. irradiated spermatozoa, and treating them with hydrostatic pressure to inhibit the expulsion of the second polar body. A u.v. dose of 3000 ergs/mm\(^2\) genetically inactivates the spermatozoa without loss of their ability to activate egg development.

The use of a genetic marker on very large samples of eggs made it possible to verify the efficiency of the methods employed. The comparison of the development of diploid gynogenetic embryos with that of haploid gynogenetic, triploid and diploid controls makes it very probable that the relatively high mortality or abnormal development obtained with the pressurized eggs is not due to partial homozygosity but rather to physical damage to the egg structure by this treatment. Altogether about 2500 developing eggs were used.

In addition diploid gynogenetic reproduction from females heterozygous for a known mutation allows the mapping of the gene concerned relative to the centromere on the basis of the recombination rate. For this experiment we used the recessive mutation causing periodic albinism \((a^p)\) and found the position of this gene to be between 44 map units from the centromere and the end of the chromosome.

**INTRODUCTION**

Manipulation of the events accompanying fertilization, especially with physical perturbations such as irradiation (Beatty, 1964), hydrostatic pressure (Dasgupta, 1962), heat or cold shock (Fankhauser & Humphrey, 1942; Fankhauser & Watson, 1942; Fischberg, 1948; Swarup, 1956; Smith, 1958) has been used in a variety of combinations to produce cytogenetically modified amphibian embryos. For instance, diploid gynogenesis can be achieved through inhibition of the expulsion of the second polar body in eggs activated by irradiated spermatozoa, and yields viable diploid animals (Fischberg, 1947). These gynogenetic diploid animals can be used to determine which of the two sexes of a species is the heterogametic one, which is particularly useful for species without sex chromosome dimorphism as shown by unpublished results of our laboratory. They can also be used to detect recessive mutations in their mother (Krotoski & Tompkins, 1979; Droin & Colombelli, 1982) or to map mutant genes relative to
the centromere of the chromosome (Nace, Richards & Asher, 1970; Volpe, 1970; Colombelli, Thiebaud & Müller, 1984) but not to attribute these to a distinct chromosome. Finally, such animals can be used to establish inbred strains (Nace & Richards, 1969).

In the present paper we analyse the effects of the different treatments necessary to obtain diploid gynogenetic Xenopus laevis upon the viability of the offspring. We also describe the mapping of the periodic albinism \( (a^p) \) gene in Xenopus laevis by gynogenetic reproduction.

**MATERIALS AND METHODS**

**Animals**

All Xenopus laevis laevis used were laboratory reared animals. The periodic albinism mutants \( (a^p/a^p) \) X.I.I. (Hoperskaya, 1975) were originally provided by Dr O. A. Hoperskaya (Institute of Developmental Biology, Moscow, USSR) and given to us by the Hubrecht Laboratory, Utrecht.

**Artificial fertilization**

Ovulation was induced by two injections of 50 and 250 to 300 i.u. of human chorionic gonadotropin (Pregnyl, N.V. Organon, Oss, Holland) at 6 h intervals into the dorsal lymph sac of the females. A sperm suspension was obtained by macerating a pair of testes in 1 to 2 ml of amphibian Ringer and stored at room temperature (18–20 °C). This preparation could be successfully used for fertilization for up to 4 h. The sperm concentration was at least \( 3 \times 10^7 \) spermatozoa/ml.

Once the ovulating females started shedding their eggs spontaneously (2 to 6 h after the second injection) the eggs were stripped into a dry plastic Petri dish. About 100 eggs were collected at a time and inseminated with 0.05 ml of the sperm suspension diluted with 0.15 ml of aged tap water. Four minutes after insemination eggs were flooded with this water.

**Sperm irradiation**

Genetic inactivation of spermatozoa was carried out with u.v. irradiation. The quantity of sperm suspension used for one insemination was irradiated at 254 nm with an Universal u.v. lamp (Camag, Switzerland). We used a dose of 3000 ergs/mm². U.v. energy was measured by a Black-ray Ultraviolet Meter (Ultraviolet Products Inc., USA). A sperm suspension treated in this way should be used immediately, as its activity declines very rapidly (<10 min).

**Hydrostatic pressure**

Hydrostatic pressure is provided by a hydraulic press which acts on a pressure chamber in which the eggs to be treated were placed in a small plastic Petri dish (Müller, Thiebaud, Ricard & Fischberg, 1978). Hydrostatic pressure (400 at) was applied 5 min after insemination and maintained for 5 min.
Localization of the \( a^p \) gene in Xenopus

Controls

In order to check the efficiency of irradiation of the sperm we used spermatozoa of wild-type \( X. \) laevis to inseminate the albino eggs. Diploid gynogenetic reproduction of \( a^p/a^p \) females leads to an \( a^p/a^p \) offspring when the genetic inactivation of sperms was effective. If the spermatozoa are not sufficiently irradiated, triploid embryos \( a^p/a^p/+ \) are produced, which show development of melanophores and can therefore be recognized easily (Tompkins, 1978). The ploidy of embryos or tadpoles was determined by squashing whole individual embryos or tailtips of tadpoles and by counting the number of nucleoli (Müller et al., 1978).

Mapping of the gene \( a^p \)

The diploid gynogenetic progeny of heterozygous \( a^p/+ \) females should consist of 50% \( a^p/a^p \) and 50% \(+/+\) individuals, if no recombination between the \( a^p \) gene and the centromere occurs. Deviation from this segregation allows the localization of the \( a^p \) gene relative to the centromere.

The mapping function derived by Barratt, Newmeyer, Perkins & Garjobst (1954) for segregation of tetratypes in Neurospora, and applied in a closed form to facilitate computation in the work of Nace et al. (1970) for gene-centromere mapping in Rana pipiens has been used in this study. The proportion of heterozygous progeny (\( y \)) is related to the corrected map distance (\( x \)) through the following equations:

\[
y = \frac{2 \left( e^{3kt} - 1 \right) \left( 3^{2t} - 1 \right)}{3 e^{(2+k)t} \left( e^{2kt} - 1 \right)}
\]

\[
x = \frac{\left( 1 - e^{-2t} \right) kt}{\left( 1 - e^{-2kt} \right)}
\]

The parameter \( t \) is the uncorrected map distance. From the coefficient of coincidence (\( k \)) two values were chosen (\( k = 1.0 \) and \( k = 0.2 \)) which were proven to be reasonably predictive and thus delimit the most likely extremes (Barratt et al., 1954).

RESULTS

Viability after treatment

In order to analyse the effects of sperm irradiation and hydrostatic pressure on embryonic and larval development, the offspring of four different experimental situations have been analysed, namely: eggs of \( a^p/a^p \) females were submitted to pressure, or not, and fertilized artificially with either irradiated or untreated sperm of \(+/+\) males (Table 1).

The survival rates at different developmental stages of these different offspring, obtained in three independent experiments using \( a^p/a^p \) females, were
Table 1. Different types of offspring obtained by the use of u.v. irradiation and pressure

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>No UV irradiation</th>
<th>UV irradiated spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \varphi a^p/a^p )</td>
<td>( \sigma +/+ )</td>
<td>( a^p/+ )</td>
<td>( a^p )</td>
</tr>
<tr>
<td>No pressure</td>
<td>Diploid control</td>
<td>( a^p/a^p/+ )</td>
<td>Gynogenetic haploid</td>
</tr>
<tr>
<td>Pressurized eggs</td>
<td>Triploid</td>
<td>( a^p/a^p/a^p )</td>
<td>Gynogenetic diploid</td>
</tr>
</tbody>
</table>

analysed. As the survival rates were similar for the three females used (Table 2), the results were combined for each type of offspring (Fig. 1). Our results are based on a total of 2382 eggs which began with cleavage. Uncleaved eggs were discarded.

Two types of survival rates were obtained. By stage 30 (Nieuwkoop & Faber, 1975) the survival rate of the previously pressurized embryos (\( a^p/a^p/a^p \) and \( a^p/a^p \)) is drastically lower than that of the unpressurized ones (\( a^p/a^p \) and \( a^p \)). This indicates that the early mortality observed in a diploid gynogenetic progeny is due to physical damage to the eggs by pressure treatment.

Fig. 1. Survival rate curves for embryos and tadpoles of different genotypes. Curves 1 and 4 correspond to the different types of offspring according to Table 1. h: hours; d: days; stage according to Nieuwkoop & Faber (1975). Due to the large numbers the diploid and triploid tadpoles were discarded at 14 days. We know that no more than 5% of these animals should die between 14 days and metamorphosis if kept under good conditions.
Table 2. Survival of different types of offspring of three \( a^0/a^0 \) females

<table>
<thead>
<tr>
<th>Types of offspring (see Table 1)</th>
<th>Females used</th>
<th>No. of developing eggs</th>
<th>No. of embryos or tadpoles alive after</th>
<th>Homogeneity test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 days</td>
<td>3 days</td>
<td>4 days</td>
</tr>
<tr>
<td>diploid control</td>
<td>A</td>
<td>60</td>
<td>59</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>187</td>
<td>183</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>194</td>
<td>184</td>
<td>117</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>441</td>
<td>426</td>
<td>288</td>
</tr>
<tr>
<td>gynogenetic haploid</td>
<td>A</td>
<td>61</td>
<td>56</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>109</td>
<td>104</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>102</td>
<td>97</td>
<td>56</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>272</td>
<td>257</td>
<td>162</td>
</tr>
<tr>
<td>triploid</td>
<td>A</td>
<td>130</td>
<td>80</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>98</td>
<td>56</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>100</td>
<td>51</td>
<td>33</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>328</td>
<td>187</td>
<td>132</td>
</tr>
<tr>
<td>gynogenetic diploid</td>
<td>A</td>
<td>490</td>
<td>242</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>546</td>
<td>265</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>305</td>
<td>178</td>
<td>85</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>1341</td>
<td>685</td>
<td>329</td>
</tr>
</tbody>
</table>

The homogeneity of progeny mortality for each type of offspring was tested by contingency analysis. The chi-square obtained indicates that this mortality is uniform among the three females used. The data obtained can, therefore, be combined.
U.v. irradiation of the spermatozoa does not influence the early development of the embryos. Indeed, until stage 30, the survival rate of the diploid gynogenetic embryos (a'/a') is the same as that of the triploid embryos (a'/a' +) ($\chi^2 = 3.758; 0.05 < P < 0.1$), whereas the haploid embryos (a') have a similar mortality as the diploid controls (a' +) ($\chi^2 = 1.864; 0.1 < P < 0.2$). After stage 40, the retarded haploid embryos die selectively before they reach the feeding stage. This mortality of the haploid embryos was expected and they displayed the haploid syndrome (Hamilton, 1963).

Between stages 30 and 40, more triploid embryos (a'/a' +) survive than diploid gynogenetic ones (a'/a') and this difference is significant ($\chi^2 = 30.0; P < 0.001$). This increased early mortality might be due to the fact that diploid gynogenetic embryos are partially homozygous, enabling recessive lethal genes to be expressed. Triploid embryos, on the other hand, possess a third, paternal, genome which may counterbalance lethal genes.

After stage 40, no significant difference persists in the survival rate between triploid (a'/a' +) and diploid gynogenetic embryos (a'/a') which are from this stage on about as viable as the diploid controls (a' +). Thus, more than 18 %
Localization of the \( a^p \) gene in Xenopus (corresponding to 233 animals) of the gynogenetic diploid embryos reached sexual maturity.

Mapping of the \( a^p \) gene

In order to map the \( a^p \) gene relative to the centromere, two heterozygous \( a^p/+ \) females were used for gynogenetic reproduction. Again, the four types of offspring (cf. Table 1) were generated in order to control the effects of pressure and irradiation. The survival rates of the four different types of offspring in these experiments were similar to those obtained with the \( a^p/a^p \) animals (Fig. 1). Among the diploid gynogenetic animals, three genotypes were expected: \( a^p/a^p \), \( +/- \) and, in addition heterozygous \( a^p/+ \) (second-division segregants) resulting from recombination events. Before stage 33/34, all embryos were identical in their pigmentation pattern as all eggs of an \( a^p/+ \) female are pigmented regardless of their genotype. Beyond stage 37/38 two distinct phenotypes were observed: the albino and the pigmented larvae (Fig. 2). It is impossible to distinguish between the \( a^p/+ \) and the \( +/- \) genotype by morphological criteria, since the number of melanophores varies gradually also among \( +/- \) individuals.

Among the diploid gynogenetic offspring of the two \( a^p/+ \) females, 14.2\% were albino tadpoles. There was no significant difference in the results obtained from \( \varphi 1 \) and \( \varphi 2 \) (Table 3), as determined by contingency analysis.

In order to be sure that the low percentage (14.2\%) of albino tadpoles obtained was in fact due to recombination events and not to a sublethal condition of homozygous \( a^p/a^p \) individuals, a control mating between heterozygous \( a^p/+ \) animals was carried out by natural fertilization. Of the embryos 23.5\% (227 out of 965) were \( a^p/a^p \), which is statistically not different from the expected 25\% \( (\chi^2 = 1.122; 0.2 < P < 0.3) \); \( a^p/a^p \) animals are, therefore, not sublethal.

Since 14.2\% of the tadpoles obtained by gynogenetic reproduction were of the \( a^+/a^p \) genotype, another 14.2\% must be \( +/- \) genotypes. As we found a total of 85.8\% of pigmented tadpoles, 71.6\% of the animals should theoretically be \( a^p/+ \) heterozygous recombinants.

The map distance between the centromere and the \( a^p \) locus, calculated on the basis of this recombination rate and with a coefficient of coincidence \( k = 0.2 \), represents 44 map units. For \( k = 1.0 \) the gene \( a^p \) segregates independently of its

<table>
<thead>
<tr>
<th>( a^p/a^p )</th>
<th>( \varphi 1 )</th>
<th>( % )</th>
<th>( \varphi 2 )</th>
<th>( % )</th>
<th>Total</th>
<th>( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>13</td>
<td>16</td>
<td>15.8</td>
<td>35</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>( a^p/+ ) and ( +/- )</td>
<td>127</td>
<td>87</td>
<td>85</td>
<td>84.2</td>
<td>212</td>
<td>85.8</td>
</tr>
<tr>
<td>Total</td>
<td>146</td>
<td>100</td>
<td>101</td>
<td>100</td>
<td>247</td>
<td>100</td>
</tr>
</tbody>
</table>

Contingency analysis for progeny from these two females gave a \( \chi^2 = 0.39; P > 0.5 \).
centromere; the $a^p$ gene is thus located between 44 map units from the centromere and the end of the chromosome.

In order to check whether or not the $a^p$ gene and the sex-determining factor(s) are linked, we determined the sex of the $a^p/a^p$ animals obtained from the control mating between heterozygous $a^p/+ \times a^p/a^p$ parents. A sample of 100 $a^p/a^p$ tadpoles were raised of which 99 survived and reached sexual maturity. Of these 46 were males, 53 females. The sex-determining factors W and Z appear, therefore, not to be genetically linked with the $a^p$ gene.

**DISCUSSION**

Hydrostatic pressure inhibits the expulsion of the second polar body in 98% of the eggs that develop normally (Müller et al., 1978). However, fewer pressurized embryos survive until tailbud stage than unpressurized ones, probably due to mechanical damage of the egg's structure. The same early embryonic mortality had been observed by Jaylet & Ferrier (1978) with Pleurodeles. They blame recessive lethal genes for the mortality of the diploid gynogenetic embryos which are partially homozygous. Our results show that the early mortality is certainly due to the treatment with hydrostatic pressure (compare mortality of early haploid embryos with that of the triploid and gynogenetic diploid ones in Fig. 1). The mortality due to partial homozygosity of various recessive lethal factors in diploid gynogenetic embryos and tadpoles cannot be very high and must be similar to the difference in survival between the diploid gynogenetic individuals and the triploid ones at stage 39. The very low mortality from this stage until sexual maturity supports this interpretation, as there is no reason for different lethal factors to cause death at the same period.

Tompkins (1978) reports a low fertilization rate of the pressurized eggs, estimated on the basis of gastrulae obtained. However, from fertilization until gastrulation, the survival of pressure-treated fertilized embryos declines rapidly. This seemingly low fertilization rate of pressurized eggs might, therefore, be accounted for by postfertilization mortality.

U.v. irradiation inactivates the spermatozoa genetically by damaging DNA. Low doses, below 300 ergs/mm$^2$, induce aneuploid development which is lethal, whereas high doses, over 18,000 ergs/mm$^2$, completely inactivate the spermatozoa which then are unable to promote egg cleavage. A dose of 3000 ergs/mm$^2$ is satisfactory since both genetic inactivation of the spermatozoa and preservation of their capacity to activate the eggs is obtained. Kawahara (1978) also suggested 3000 ergs/mm$^2$ to be a reasonable dose for sperm irradiation. On the other hand, the u.v. dose indicated by Tompkins (1978) of 180 ergs/mm$^2$ induced only aneuploid embryos in our laboratory.

Since this study is based on a very large number of embryos, larvae and frogs, we determined the degree of ploidy by nucleolar counts of all individuals including the diploid controls. This method is frequently used when the ploidy of many
individuals has to be determined. It can of course only be applied to species which possess only one nucleolar organizer per chromosome set. It cannot be excluded that a few dying abnormal embryos are in fact aneuploid, but the method is quite satisfactory at least from stage 39 onwards.

The localization of the $a^p$ gene relative to the centromere by gynogenetic experiments represents a beginning of genetic mapping in *Xenopus*.

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