A Method to Demonstrate Transformation in *Ephestia* 1,2

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Larvae of *Ephestia* of the genotype *ml ml* were injected on the 8th day after the last larval molt with purified, high molecular DNA from + animals. After the animals hatched, the wings were scored for the occurrence of black pattern scales of type IV. Ten type IV scales were found on 606 wings of treated animals, leading to an estimate of \(10^{-4} - 10^{-5}\) for aberrant scales. Injection into animals in the process of wing regeneration led to the appearance of clusters of 2—3 aberrant scales. While it is not yet certain that the aberrant cells constitute transformants, the method appears to be sufficiently specific and sensitive to solve this problem.

Transformation consists in the uptake of DNA from one cell into another and its incorporation into the host's genome. If the donor DNA and that of the host differ in one pair of alleles, transformation implies the replacement of the donor's allele and, if it were to occur in a multicellular organism, would be expected to give rise to a somatic mutation.

Transformation has been observed and studied in a number of bacterial species, though it has not been possible to observe it in all bacterial species investigated. The literature on bacterial transformation has been thoroughly reviewed by Ravin 4. In the cells of higher organisms, several attempts have been made to demonstrate transformation, using particularly pigment cells in birds 5 and biochemical characters in human cell lines 6 as experimental materials.

In order to achieve transformation, a system must be developed in which the phenomenon can be observed whenever it occurs. It cannot be assumed that even if these conditions are met transformation will be observed, since in well-investigated bacterial species such as *E. coli* and *S. typhimurium*, transformation has not yet been found. But the conditions outlined below are certainly necessary for successful transformation to occur.

It is necessary to develop a method to extract pure, high molecular DNA. While methods for microorganisms and mammalian tissues are available and fairly routine, their application to insect tissues has been accomplished only rarely. Furthermore it is necessary to investigate the possibility of protecting the injected DNA from DNAases which may be present in the system.

More difficult is the establishment of a mutational system in which transformation can be observed. In the original experiments with bacteria, transformation was a very rare event, and only with improvement of the technique, particular clarification of the conditions of competence, could a higher yield of transformants be obtained. The methods used in bacteria and in human cell lines rely, therefore, on the creation of selective conditions under which only the transformants can grow. This method does not appear to be applicable to somatic transformation in vivo.

**Materials and Methods**

It appears that the wing scales of *Ephestia* constitute a favorable system, since a large population of characteristically differentiated cells can be observed. The scales of the hind wing have been used successfully for the study of induced mutations 7 and are able

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1 This paper is dedicated to Professor Dr. Alfred Kühn on the occasion of his 80th birthday, as a token of my gratitude to him as an inspiring teacher and of my admiration for his research work. E. C.

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to demonstrate events which occur with a frequency of $10^{-5}$ or less. Unfortunately, for the study of transformation, no mutations are known which affect the scales of the hind wing. The fore wing has the disadvantage that the scales are not homogeneous but that the color pattern is formed by scales of different shape and pigmentation.

It was decided, therefore, to work with the mutant ml described by Kühn; ml is a "recessive" gene which abolishes the pattern on the fore wing, substituting two light colored types of pattern scales (types VII and VIII of Kühn and Henke) for the several types which make up the normal color pattern. Not all of the types of scales found on a wild-type Ephestia wing can be easily distinguished from the ml scale type; but the black pattern scale (type IV) which occurs in most of the wild-type pattern elements is sufficiently different from the ml scale to be easily recognizable whenever it occurs. Not only does it differ by its black pigmentation but it is also more slender and narrow in shape, and can therefore be observed by studying the wing under a dissecting binocular at low magnification.

Two disadvantages of the system should be mentioned here. The ml ml males are inefficient at copulation. The strain is therefore kept routinely by mating ml ml females to +/ml males, resulting in segregating progeny. Since no means exist to distinguish ml from ml or ml animals in the larval stage, injections were made into both types of larvae, resulting in a waste of some of the DNA. Furthermore, it turned out that the mutant gene ml is not completely recessive. Heterozygotes show the wild-type pattern, but it is less distinct and looks with the naked eye as if it were covered with a white powder. Microscopic examination shows that in heterozygotes isolated scales of type VIII are interspersed with the pattern scales. It was possible in a segregating F2 to distinguish wild-type homozygotes from heterozygotes, and to confirm their genotype by outcrossing to ml ml animals.

DNA was prepared from Ephestia larvae, pupae and imagos. The method of preparation and the properties of the DNA preparations will be described elsewhere. It should only be mentioned that two methods of extraction were used, a modification of the phenol extraction method of Kühn, and the Duponol method of Marmur. The resulting preparations were high molecular, double-stranded DNA contaminated with not more than 2 per cent protein.

The following procedure was used: DNA solutions protected by spermine, gelatine, or both were injected into larvae from crosses $\varnothing ml ml \times ml/+ at the eighth day after the last larval molt. Since at this time many mitoses are found in the wing disc, it was assumed that the cells might be in a competent state. The injected larvae were permitted to develop into adults. Their wings were severed, spread out on Caedax, and scored under a dissection binocular for the occurrence of black pattern scales.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Method of DNA extraction</th>
<th>DNA concentration [μg/ml]</th>
<th>Amount injected [μl]</th>
<th>Additions</th>
<th>Survivors</th>
<th>No. of mutants</th>
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<td>I</td>
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<td>300</td>
<td></td>
<td>Sp. + G.</td>
<td>19</td>
<td>5</td>
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<td>4</td>
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<td>8</td>
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<td>0</td>
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<tr>
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<td>1</td>
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<td></td>
<td>Sp. + G.</td>
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<td>1</td>
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<td>Controls</td>
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<td></td>
<td>G</td>
<td>56</td>
<td>2</td>
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Table 1. Results of transformation experiments with Ephestia. (I = larval DNA, p = pupal DNA, a = adult DNA; Sp. = spermine, G = gelatine).


10 A. Kühn, Biol. Zbl. 61, 109 [1941].
Results

The total material of survivors from injections consists of 392 ml/+ heterozygotes and 334 ml ml animals, indicating a slightly lower survival of the homozygote mutant. The results are summarized in Table 1. Fig. 1 * shows the appearance of a black pattern scale of type IV on an ml ml wing; the dark areas below the aberrant scale are shadows. Excluding the animals injected with a low concentration of 33 µg/ml DNA, 10 mutant scales were found in 303 animals, i.e. on 606 wings. On 300 ml ml wings from untreated animals, no black pattern scales were found.

The data in Table 1 show that dark pattern scales may obtained if larvae are injected with 2 – 3 µg DNA per larva. Larval, pupal and adult DNA are all effective. Gelatine and gelatine + spermine protect the DNA sufficiently to permit the occurrence of type IV scales. Digestion of DNA with DNAase results in toxicity: none of 62 larvae injected with DNA-digest, 2 µg per larva, survived the treatment (Table 1, Exp. II last line).

In all 10 cases, isolated single dark scales were found. If the appearance of these scales is due to a genetic change, it should be transmitted to the offspring of the transformed cell, and spots of more than one dark scale would be expected to appear. For this reason, injections were carried out in larvae of different ages. As Table 2 shows, this experiment was not feasible since the survival of larvae injected at earlier ages was poor.

<table>
<thead>
<tr>
<th>Age of host (days after last molt)</th>
<th>No. of animals treated</th>
<th>Survivors ml/+ ml ml</th>
<th>No. of mutants</th>
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<tr>
<td>6</td>
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<td>70</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>10 (prepupae)</td>
<td>23</td>
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<td>0</td>
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</tbody>
</table>

Table 2. Results of transformation experiments, injecting + Ephestia DNA into host larvae of different ages. (DNA from + – larvae extracted with Duponol. 2.6 micrograms injected into host larvae, with addition of spermine and gelatine.)

Another method was attempted by using regenerating wings 13. The left wing bud was extirpated in larvae on the 8th day after the last larval instar. Two days after the operation, they were injected with 8 µl DNA extracted with Duponol, 300 µg/ml, with the addition of spermine + gelatine. Of 26 controls which only underwent extirpation, 13 survived, while after injection of extirpated animals with 0.9% saline, 13 out of 35 animals survived. Of the 26 surviving control animals, 12 were ml ml none of which showed black pattern scales. Of 162 larvae injected with DNA, 64 survived; survival was therefore not reduced as compared to the controls. Of the survivors, 29 were ml ml and, on two of their wings, spots consisting of 2 and 3 identical pattern scales were found. One of the spots occurred in the region of the marginal spots, while the other was located in the region of the central spots. One of them was found, as expected, on the regenerated wing, while the other spot appeared on the non-operated control wing.

Discussion

Previous attempts to demonstrate transformation in multicellular organisms have not been completely convincing. Fahmy and Fahmy 14 treated Drosophila sperm with DNA from Drosophila and other sources, and observed the appearance of a large number of Minutes, indicating the non-specific induction of chromosome breaks. Benoit et al. 5 induced changes in ducks’ pigmentation and morphology by injection of DNA, which changes could be transmitted through several generations. Since the genetic basis of the characters investigated is not clear, it cannot be decided whether actual transformation has occurred. Szybal'ska and Szybalski 6, working with cells of human origin in tissue culture, obtained transformation for a gene locus controlling the enzyme IMP-pyrophosphatase, but could not induce transformation at the 8-azahypoxanthine resistance locus.

In all but the last case, the methods employed are not sufficiently sensitive and specific to demonstrate rare mutational events. In the Ephestia system, the specificity of mutation in ml/ml to scales of type IV is high; the sensitivity cannot be exactly established, since direct counting of the visible pattern scales proved to be unreliable. An estimate can, however, be attempted on the basis of known facts. The total number of scales on the upper side of the hind wing

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is about 6,250. The fore wing has slightly more than half the area of the hind wing, and the scales are more densely arranged; the factor appears to be 2.2 fold that of the hind wing, so that it may be assumed that the upper side of the fore wing carries about 6,000–7,000 scales. According to Kühn, the pattern scales constitute about 20% of the total number of scales, so that an estimate of 1,200 pattern scales on the fore wing appears reasonable. The number of dark scales induced described in Table 1 corresponds to 1 on 60 wings, or in 72,000 an estimated frequency of somewhat above $10^{-5}$. This number may be an underestimate of the actual number of mutational events, since black pattern scales of type IV constitute between 15 and 20 percent of all the scales on a normal wing. At all events, a frequency of $10^{-4}–10^{-5}$ for induced dark scales of type IV appears to be reasonably accurate.

It seems to be fairly certain that the type IV scales observed on ml ml wings have been induced by the treatment; but it cannot yet be confidently stated that it is due to incorporation of + DNA into the affected cells. But it appears that the scale system of *Ephesia* may be sufficiently sensitive and specific to permit a decision on this question.

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Zur Frage des Zeitpunktes der Entscheidung zwischen abortivem oder rekombinativem Verhalten eines transduzierten Chromosomenfragmentes in der Empfängerzelle

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Herrn Prof. Dr. A. Kühn zum 80. Geburtstag gewidmet


In wild-type transduction of auxotrophic strain *E. coli* B/r/thr-leu-ara colonies auxotrophic for leucine or threonine do not all arise at the same time after plating. In such crosses 48 hrs. after plating from about 20% of minute colonies grown from single abortively transduced cells there can be isolated cells capable to form genetically stable colonies prototrophic for leucine or threonine. Turbidity-measurements on cell populations derived from isolated minute colonies prove that such leu'-cells arise on the plate up to at least 96 hrs. after transduction. Linkage-data of the sites leu'-1 or thr'-1 with ara-12 for these cells disprove the occurrence of the thr' or leu'-state by back-mutation. Transduction with *E. coli* B/r/ara as donor with selection for arabinose-fermentation demonstrates the failure of delayed arising leu' or thr'-cells in crosses yielding no minute colonies caused by abortive transduction. The experiments are discussed as evidence for the occurrence of recombination between the acceptor-chromosome and the abortively transduced chromosomal fragment of a donor cell within a minute colony many cell generations after injection of this fragment.

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Fig. 1.