ARTIFICIAL PARTHENOGENESIS AND CLONING

Artificial parthenogenesis

Biotechnology aspires to modify biological processes so that they effectively work for humans at all levels of biological organization: molecular, cellular, ontogenetic, population and global. From the genetic point of view among biological processes we highlight, first, transfer of hereditary attributes from generation to generation during processes of reproduction and, secondly, realization of the genetic information during individual development. The point between any two consecutive generations passes through the activated...
egg, which gives rise to a new organism. We consider activation as the exit of an egg from a blocked condition which is the final result of oogenesis and maturation, the deblockage being caused by various factors. If there is no activation, there is no egg development.

Activation is not always caused by penetration of a sperm inside the egg. In the case of natural parthenogenesis insemination is absent, and the activation is caused by other factors, for example, aerobic oxygen as in the stick insect Carausius morosus (Pijnacker and Ferverda, 1976). In other cases (wasps) the sperm penetration inside the egg is not followed by activation; the activating function is performed by the female egg laying apparatus, which strongly deforms an egg and causes activation through extension of the egg membrane. The male nuclear material then fuses with the female pronucleus (Went, 1982). In the case of natural gynogenesis (e.g., some fishes), on the contrary, the sperm carries out only the activating function and the fusion of male and female nuclei does not take place (Schultz, 1977; White, 1973). Thus, the mechanism of egg activation can be set up by various factors and in nature the sperm penetration inside the egg is not always the cause of its activation.

Attempts to understand the activating impact of the sperm by replacement of insemination with various physico-chemical agents resulted in the discovery of artificial parthenogenesis. It was in the silkworm that the Russian zoologist (Tikhomiroff, 1886) obtained activation of unfertilized eggs with various acids, alkali, electrical stimuli, and hot water (45°C). In these experiments development frequently reached the stage of egg pigmentation but hatching was never recorded because of abnormal embryogenesis. Sato in Japan observed complete artificial parthenogenesis (up to larval hatching) for the first time 40 years later using hydrochloric acid treatments. He found that, as a result of such processing, both maturation divisions take place in unfertilized eggs and, as in the normal case, the female pronucleus forms (Sato, 1925, 1931). According to modern classification this type of artificial parthenogenesis as well as one caused by freezing at -11°C for 30 min (Terskaya and Strunnikov, 1974) in addition to spontaneous parthenogenesis are referred to as “meiotic”. In all of these cases parthenogenetic progeny were mostly male.

In the years 1936-1940 in the Soviet Union, Astaurov carried out extensive and thorough research on parthenogenesis caused by aqueous heat shock at different temperatures and protocols. He established his “sacred” formula for thermic parthenogenesis: 46°C, 18 min. In this case parthenogenetic progeny were practically all female, the maternal genotype being repeated or cloned (Astaurov, 1940).

**The mechanism of thermic parthenogenesis and cloning**

On the basis of his genetic analysis, Astaurov proposed a cytological mechanism for this new type of artificial parthenogenesis. However, even he did not use his completely correct scheme because of its discordance with some cytological data found by the well-known cytologist Frolova. In 1978, after more careful cytological analysis, Astaurov's original explanation for ameiotic thermic parthenogenesis was completely confirmed and reinstated (Klimenko and Spiridonova, 1979; Klimenko, 1990). The suppression of the first (reductional) maturation division is the main feature of this type of artificial reproduction. It is owing to the absence of crossing over in the silkworm female and retention of the single equational division in meiosis that the parthenogenetic progeny inherits the genotype and idioype of the mother moth and becomes its clone. Thus, artificial cloning in sericulture was realized more than 60 years ago (Fig. 1).

**Fig. 1.** The fates of elimination chromatin (ec) in the different schemes of thermoparthenogenesis. After normal insemination (norm-right) at early anaphase I the homologues detach from the bands of modified synaptonemal complexes. These bands are destroyed after heat shock at 46°C (right, 2). The sister chromatids are pulled to the opposite poles by the retained (equational) fibers of the spindle, and the next division (3) in the newly formed diploid nuclei (comprised of the female pronucleus and the single polar body) is only a mitotic one (Klimenko and Spiridonova, 1979).
According to Frolova the suppression of the reductional meiotic division by heating at 46°C appears to be so strong that homologues remain paired forever, the elimination chromatin remaining built-in between homologous chromosomes (see Astaurov, 1968).

In the new scheme the elimination chromatin collapses after heat shock and so, presumably, do spindle strings engaged in the reductional meiotic division. During the only equational meiotic division the sister chromatids of each of the 56 chromosomes are carried to the opposite poles by retained “equational” spindle strings. The single polar body and the diploid female pronucleus form, the latter being the first cleavage nucleus as well.

Some factors for successful artificial parthenogenesis

In analyzing and carrying out artificial parthenogenesis for reproduction we should take into account some general factors that substantially, if not completely, determine success in applying any experimental stimulation to the egg.

Egg status at the moment of stimulator application: The age of an egg is usually timed after the termination of egg maturation processes. Conditionally we accept the imago’s emergence from a cocoon as a benchmark in egg aging. Eggs can also be activated successfully, however, before that moment if taken from the pupa. The conditions of moth maintenance, the imago female’s age at the moment of egg excision from the ovarioles, as well as temperature, humidity during drying of the excised eggs and duration of the latter fundamentally influence the efficacy of both egg activation and complete parthenogenesis.

Long experience shows that conditions during rearing and the pupal period, through the processes of oogenesis, can significantly influence the oocyte’s properties, and hence its capability for artificial parthenogenesis.

Setting up activation processes: The efficiency of artificial egg stimulation depends on the method of activation, more precisely, the nature of the activator and how it is applied. It is necessary to take into account the changes in intensity of the activation treatment during the whole period of egg processing. The study of artificial parthenogenesis in different species has revealed qualitative and quantitative distinctions, depending on both the activator and mode of its application, between the pattern of induced activation reactions and the pattern of normal (sperm) activation processes in the egg. The former approximate the latter to a different degree (Epel, 1978). Is the closest approx-

imimation the best approach in searching for effective techniques of artificial parthenogenesis?

Cleavage nucleus ploidy level compatible with development: In the case of meiotic parthenogenesis at the diploid level segregation of the haploid female pronucleus can be up to 100% after treatment with some activators; however, haploid development in the silkworm is abnormal and does not reach hatching. The high success rate in meiotic artificial parthenogenesis induced by cryoactivation (-11°C, 30 min) is due to the fact that the freezing favors in some way the restoration of diploidy at the first division of the haploid female pronucleus (Vereyskaya and Terskaya, 1986). The success of thermic activation (46°C, 18 min) is mainly the success of diploidization as the activating heat shock simultaneously suppresses reductional meiotic division and the diploid female pronucleus is inevitably segregated after the single equational division.

Resistance of the parthenogenon to ooplasmic damage caused by the activator: Last but not at least, even with the proper nucleus the cell differentiation processes do not always result in the formation of a normal larva because following artificial parthenogenesis development proceeds on the basis of ooplasm modified (“imprinted”) by the artificial activator. It is obvious that these ooplasmic changes most likely do not optimize but complicate nuclear-cytoplasmic and cellular interactions in differentiation processes compared with normal development. Therefore we can search for resistance or tolerance of parthenogenetic development to such ooplasmic modifications and for the genetic basis of this phenomenon. In the silkworm the resistance in question positively correlates with the level of individual heterozygosity of the female moth (Fig. 2) used for testing its capability for thermic parthenogenesis or cloning (Altukhov and Klimentko, 1978).

Cloning in the silkworm

The silkworm appears to have been the first artificially cloned animal thanks to its unique capability for thermic parthenogenesis and absence of crossing over in females. This uniqueness as to thermoactivation requires explanation in terms of egg activation mechanisms and heat shock egg resistance in different species and is a big problem for future comparative studies on artificial parthenogenesis.

As based on ameiotic parthenogenesis, cloning in the silkworm is inevitably female sex limited. The problem of cloning a male genotype in the silkworm can be solved through androgenesis (a different type of artificial parthenogenesis) only in the case of absolute homozygosity of the male. Such clones were for the
first time obtained in the silkworm in the early 1980’s (Strunnikov, 1983). A more general approach for cloning any genotype in the silkworm is proposed later in this article.

In view of the previously mentioned strong and positive correlation between the ability to undergo complete thermoparthenogenesis and the level of individual heterozygosity, the attempts to clone female genotypes of different silkworm stocks and inbred strains have been definitely frustrated, especially concerning genotypes of top quality thread cocoons.

Cloning of female inbred genotypes was achieved in our experiments on ovary transplantation between inbred lines and their maternal parthenocline. The ability of the inbred line eggs to undergo complete thermoparthenogenesis was 30-50% higher after ovary transplantation in the third/fourth instar from the inbred line into maternal clone larvae. The inbred line eggs were larger after such transplantation, their protein spectrum approaching that of the recipient. The larvae hatched from “transplanted” eggs of a single donor are genetically identical and can be used for further cloning (Klimenko, 1988).

**Intraclonal variability in the silkworm**

Genetic variability in parthenocloning: A peculiar feature of artificial reproduction by thermic parthenogenesis is the indispensable presence of tetraploid eggs in the layings of diploid parthenoclines. The proportion depends on the genotype of a clone and upon conditions of reproduction. It is supposed that during cleavage there is, as a consequence of thermoactivation, a certain probability for diploid nuclei to fuse and give rise to tetraploid “patches” in some tissues and organs that can later result in the appearance of tetraploid oocytes (Astaurov, 1940).

Tetraploid eggs can be activated with the Astaurov technique as effectively as diploid ones but hatching and viability of hatched larvae is close to zero. This explains why we cannot find tetraploid individuals in diploid clones but only a few diplo-tetraploid chimeras. It is not clear how simple doubling of a genotype can so considerably and negatively influence viability, although the ability to undergo thermoactivation (judged by pigmentation) remains unchanged.

Admixed tetraploid eggs have quite a different fate following spontaneous parthenogenesis and upon cryoactivation (-11°C, 30 min), both being of the meiotic type. In these cases, as was shown using genetically marked material, there arise diploid whole chromosome recombinants (crossing over is absent), mostly female (Fig. 2). We explain the prevalence of females in diploid recombinant progeny by the preferential conjugation of ZZ - WW in tetraploid oocytes.

The occurrence of mosaics after reversion to the diploid level is attributable to the participation of the polar body in development. From one such mosaic female we obtained two complementary diploid clones (Klimenko and Spiridonova, 1978; Klimenko, 1988).

It is still possible that admixed tetraploid eggs may start development spontaneously, hence meiotically, before thermoactivation. Some “contamination” of a diploid clone with diploid recombinants depends then only upon the thermo resistance of the embryo stage to the scheduled heat shock (46°C, 18 min). In practice such cases of “contamination” are found very seldom. This type of variability within a clone was named transpolyploidal (Klimenko and Spiridonova, 1978).

Especially rare are the cases of surviving absolutely homozygous male embryos, which might also arise spontaneously from diploid eggs before the sublethal heating of thermoactivation.

**Artificial populations based on a cloned genotype:**
Applying meiotic parthenogenesis to a clone we can generate an artificial population on the basis of the clonal genotype. Indeed, homozygous and recombinant parthenogenetic sons resulting from cryoactivation of clonal diploid and tetraploid eggs can be crossed with maternal clone and recombinant females. At this specific “self-fertilization” the maternal chromosome of any zygotic linkage group comes unchanged from the maternal clone set (due to the absence of crossing over). The homologous “paternal” chromosome is in fact also a maternal one, which comes from the parthenogenetic son and can also remain genetically unchanged if the corresponding linkage group in the mated son is completely homozygous. This is always the case when the sons are completely homozygous males (Fig. 3). The paternal autosomes that come from the non-homozygous paternal linkage groups (in parthenogenetic sons obtained from tetraploid eggs) are also maternal in origin but may be recombinant as the result of crossing over.

Given genetic markers in all autosomal linkage groups we can in the first zygotic generation after “self-fertilization” directly select the parent pairs or founder female moths which, in the next generation, produce bisexual strains or, respectively, partheno-clones absolutely homozygous for the desired linkage groups, Z-chromosomes being identical in all such strains. Of particular interest would be an “absolutely” homozygous bisexual strain, in which the only possible difference between any two individuals would be sex (the presence or absence of a W-chromosome). Such strains would be very favorable for genetic analysis of many hereditary traits and biological problems.

For a long time after the discovery of ameiotic parthenocloning by Astaurov, researchers paid attention only to the phenotypic similarity of individuals within a clone, differences being attributed to some anomalies of embryonic development that occur more frequently at lower levels of complete parthenogenesis. When selected clones had reached practically 100% of complete parthenogenesis (Astaurov, 1973) it became an unspoken rule that at sufficient synchronization of development and under identical conditions the distinctions between individuals of a clone would disappear.

The situation changed when traits with incomplete penetrance were introduced into parthenoclines (Fig. 4). Now it was shown that such characters as well as quantitative ones vary rather widely in clones and sometimes more than in inbred lines, penetrance and expression depending on the genotype of a clone and conditions of development, for example, temperature.

**Fig. 3.** Analysis of female progeny obtained through “self-fertilization” in clone 4a heterozygous for loci w₂, ch and lem. An absolutely homozygous parthenogenetic son (w₂, ch, lem) was crossed with the maternal clone. The marked daughters that had been produced were grouped according to level (12, 13, 14, 15, 27) and composition (1, 2, 3) of their heterozygosity and their ability to undergo Astaurov thermoparthenogenesis was estimated (percentage given). The diagrams present normalized means for six other traits (a - cocoon weight, b - cocoon shell weight, c - a/b, d - anterior wing length, e - posterior wing length, f - number of eggs in the abdomen). There is positive correlation between the trait complex (polygon areas), estimates of heterozygosity and ability to undergo thermoparthenogenesis.

**Fig. 4.** Control of trait penetrance (additional pigment spots on the body segments) by incubation temperature (°C). “Clean”, spotted or mixed larvae can be taken for parthenogenetic reproduction, the effect of temperature during egg incubation on the character penetrance being almost the same.
during egg incubation (Klimenko et al., 1980; Klimenko, 1988).

It is worth noticing that many quantitative characters of the clone founder female are never realized in its parthenogenetic progeny. We must conclude that artificial parthenogenesis is a form of somewhat oppressed development realized only because of the extremely high heterozygosity (ameiotic thermoparthenogenesis) or due to absence of lethal genes in a genotype (meiotic parthenogenesis).

**SPONTANEOUS PARTHENOGENESIS**

We can consider the ability to undergo spontaneous parthenogenetic development, i.e., without apparent cause, as a quantitative character with very wide variation. Artificially produced parthenogenetic clones are ideal material for studying such characters. This opportunity gave rise to some new data in the area of spontaneous parthenogenesis almost ignored by earlier researchers.

**Genetic variation of the ability to undergo spontaneous parthenogenesis (ASP)**

During selection and breeding of parthenoclones we can easily observe spontaneous parthenogenesis because all females in such experimentation are carefully isolated from males and some time after emergence start laying unfertilized eggs. The relative amount of pigmented eggs is an old and reliable estimation of the ability to undergo spontaneous parthenogenesis (ASP). Long-term observations show that the specific level of ASP in a clone depends both on rearing conditions and on maintenance conditions for cocoons and moths in good correspondence with all old observations on different silkworm stocks. Different clones have different ASPs.

It is interesting that ASP has not appeared to be highest in record parthenoclones reproduced by the Astaurov method, although some positive correlation between the two types of parthenogenesis is not excluded. For example, unique parthenoclones, found in 1985-1990, with almost 100% pigmentation of laid unfertilized eggs (up to 5% of complete parthenogenesis) were not the best ones for thermoparthenogenesis. In one of these clones spontaneous parthenogenesis exceeded the record parameters of hatching for artificial meiotic parthenogenesis registered in clone P29 after cryoactivation (Terskaya and Strunnikov, 1974), which made us more attentively consider this phenomenon.

A meiotic mechanism was evident from obtaining absolutely homozygous males and transpolyploid females in these experiments. The former were used in crosses with the maternal clone for the further analysis of ASP in the first zygotic progeny females. We found that, unlike thermoparthenogenesis, inbreeding as a consequence of such “self-fertilization” can increase ASP up to a level of 20% and more in some daughters of such crosses. Thus we recorded the highest parameters of ASP in the study of this phenomenon (Klimenko, 1988).

The data obtained allowed us to assume essential distinctions in the genetic systems associated with these two types of parthenogenesis: thermic ameiotic and spontaneous meiotic. In the latter case we can assume the presence of some genes in *Bombyx mori* L., which in homozygous condition increase ASP (Fig. 5). This suggests that selection of lines with high ASP is possible; however, a value of 50% ASP cannot be exceeded as the constitution WW is not viable.

In the light of the data obtained we cannot answer in the complete negatively as to the question of the existence of parthenogenetic reproduction in nature or, in principle, among the ancestors of the domesticated silkworm. Some data show the possibility of the appearance of parthenogenetic daughters and restitution of the maternal genotype (Sato, 1931; Kawaguchi, 1934) in meiotic parthenogenesis; in addition, tetraploidization as a factor in normal reproduction cannot be excluded. Thus, even in the case of complete isolation of one female, bisexual parthenogenetic progeny might allow sexual reproduction of the species and further evolution of parthenogenesis genes proved to be useful for survival. In the absence of the necessary ecologo-genetical data such an assumption is only speculative.
Non genetic variation of ability to undergo spontaneous parthenogenesis

The parthenogenesis in question is referred to as spontaneous only because its causal factors are not yet established. Some external factors affecting an egg that has left the oviduct are considered responsible somehow to weaken or remove the blocking mechanism of the mature egg and to start its development without insemination. The variety of external factors usually taken into account is not very wide: temperature, humidity, illumination, basic components and pollution of the air and air exchange. As a rule, we admit that each of these factors affects all of the laid eggs to the same extent if the experiments are carried out under constant laboratory conditions. Variability of ASP among individual females was investigated in clone p29 (the best one for thermoparthenogenesis) and is presented on the histogram in Fig. 5.

It was A.A. Tikhomiroff who first began to use washed eggs taken from ovarioles in experiments on artificial parthenogenesis. In this way he was able to prepare sufficient numbers of synchronized eggs, exclude the influence of factors accompanying egg laying and observe only the efficiency of the stimulator under investigation because eggs prepared in this way and left without any treatment did not develop.

The factors that Tikhomiroff eliminated are, in my opinion, of major interest for disclosing the nature of spontaneous (natural) parthenogenesis. Since that time researchers have not reported an exception from the simple rule: eggs “gently” excised from ovarioles show practically no signs of development but when laid they always proceed to some extent through parthenogenetic development. When clones with 100% laid pigmented unfertilized eggs were selected this rule was maintained: eggs taken out of ovarioles and washed gently, or left in extirpated ovarioles, or artificially retained in the moths show extremely rare and poor pigmentation attributable to the influence of the experimental manipulations.

These facts incline us to assume that parthenogenesis in laid eggs is caused by the action of intrinsic maternal factors upon the egg during its advance from ovarioles onto the substrate. The glue that sticks eggs to the substrate is not a necessary causal factor of egg activation. The record ASP clones laid loose eggs. If we could identify these factors we could already talk about natural instead of spontaneous parthenogenesis. On the other hand, these prospective factors, be they physical or chemical in nature, remain external in relation to the egg, similar to any stimulator of artificial parthenogenesis. Thus, in future studies of egg activation we will probably find that there is no basic difference between artificial parthenogenesis and natural parthenogenesis.

There is one more important issue. I have come gradually to the belief that in the silkworm and, probably, many other insects and arthropods, sperm impregnation of the egg by the female sexual apparatus is not a necessary and/or the sole factor for setting up egg activation. Rather, as in wasps and bees with haplo-diploidy as a sex determination mechanism, insemination is necessary for the formation of a diploid cleavage nucleus, parthenogenetic haploid development being abortive unless diploidization occurs during or soon after the first division of the female pronucleus.

ARTIFICIAL PARTHENOGENESIS AND EGG ACTIVATION

Managing the process of activation

Temperature dosage in thermoactivation: The outwardly simple method of thermoactivation poses many riddles. First of all is the obscure mechanism itself for thermic stimulation of unfertilized eggs to develop. Cytogenetic analysis allows only the conclusion that in meiosis of thermoactivated eggs the reductional maturation division is suppressed, but there is no inference as to the trigger for the activation processes in the heat shocked egg. Astaurov believed, from the most general considerations, that the basis of the initiation mechanism of thermoactivation would involve reversible conformational changes in some proteins caused by sublethal heat shock (46°C, 18 min) (Astaurov, 1940). Our studies on thermic egg stimulation in the silkworm confirmed Astaurov’s hypothesis.

To begin with, the dependence of thermoactivation (egg pigmentation) on the temperature dosage (treatment at a certain temperature) is obvious, the temperature dose being timed by fast cooling of the treated eggs in room temperature water upon termination of the chosen treatment (Fig. 6). If unfertilized eggs heated for optimum treatment cool down in air instead of cooling in water, the percentage of activated (pigmented) eggs falls sharply, and compensation for the inevitable excess of optimum dosage by reducing the heat treatment does not improve poor results (Astaurov, 1940).

Deactivation and reactivation, reversibility of activation processes: For a long time this finding of Astaurov remained without any explanation until it was shown in our experiments that it is the abrupt cooling after heat shock that starts activation and parthenogenetic development using the Astaurov method. On the contrary, sufficiently gradual cooling
after heating returns the egg to its initial, nonactivated status, in the sense that it can then be reactivated in both (meiotic and ameiotic) ways. This implies that not only are reversible changes actually connected to egg activation, but also changes resulting in suppression of the reductional meiotic division (Klimenko, 1980a, b).

As the cooling from 46°C at a rate not higher than 0.5°C/min produces a deactivating effect on the activated egg (Fig. 7) the procedure was named temperature-gradient deactivation (TGD).

Cooling the treated eggs in water at room temperature (16-23°C for 10 min) and placing them at 16°C for further development terminates the water heat shock in the method of thermoactivation. During about 10 min (at 16°C) from the end of heating TGD appears quite effective but later the deactivating effect quickly falls and disappears, signaling that parthenogenetic development has advanced so that it can no longer be reversed. Hence, we can assume that the proteins and/or other macromolecules involved in the mechanism of Astaurov thermoactivation have properties of reversibility. The abrupt cooling in the Astaurov technique blocks this reversibility so that development can start (Klimenko, 1982).

**Activation by “nonactivating” temperatures:** It is known that 0°C and 40°C are the upper and lower limits of effective temperature for cryoactivation and, respectively, thermoactivation of unfertilized eggs (Astaurov, 1940). This means that at these temperatures activation of the unfertilized egg fails to start even after long treatments. We assumed that the absence of activation in this case is actually the result of deactivation, i.e., the reversibility of activating changes was not block-

![Fig. 6. Dependence of activating heat shock dosage at 46°C upon the rate of cooling. 1, cooling according to the Astaurov technique confirms the optimum treatment for 18 min. 2, cooling at 0.5°C/min for the same treatments (deactivated eggs obtained in interval 0 - 30 min can be reactivated). 3 - spontaneous parthenogenesis.](image)

![Fig. 7. Egg activation by gradual cooling terminated by the usual abrupt placement of the eggs in 16°C water. For 15 min (up to 40°C) this change does not affect the result (more than 90% pigmentation achieved). Deactivation processes start after this point.](image)
It has been shown that eggs deactivated in this way can be fertilized and can develop up to an imago practically the same as normal eggs, the sex ratio being normal (Klymenko, unpublished). However, this does not mean that the stresses gained by this treatment have no effect. On the contrary, our earlier experiments have shown that the temperature stresses at the egg stage have far-reaching effects on different processes including such genetic ones as crossing over frequencies (Palii and Klymenko, 1995, 1996). Nevertheless, the reversibility of activational changes with temperature-gradient deactivation seems quite complete as the fertilization appears normal and development can proceed to hatching and the imago stage, although the in-body-activated (nondeactivated) and then inseminated eggs are mostly unfertile.

Reversibility of egg activation at normal insemination: With TGD it is possible to deactivate eggs that have been activated without using heat shock, for example, by freezing (-11°C, 30 min) or through normal insemination. Deactivation of freshly inseminated eggs creates an interesting situation: sperm are inside the egg but their presence is not sufficient for renewal of meiosis and for the transformation of the spermatozoids into nuclei. The eggs perish, having within them experienced everything that is usually necessary for development except egg activation. In fact, we make thereby use of insemination just for introducing the sperm inside unfertilized eggs. If such eggs are reactivated, for example by freezing at -11°C for 30 min, development resumes and fertilization occurs. Reactivation by the Astaurov method is also effective, the diploid (not reduced) female pronucleus fusing with a haploid sperm pronucleus and forming a triplod cleavage nucleus with sex chromosome constitution ZZW (Klimenko and Spiridonova, 1982; Klimenko, 1982, 1988).

On the basis of these experiments we can predict that microinjection of DNA, sperm, nuclei and cells into mature unfertilized eggs followed by effective artificial activation can be purposefully used to obtain cleavage nuclei needed for creating new biological forms. As a special case triploids were obtained simply through Astaurov thermoactivation of freshly inseminated eggs, the deactivation step being skipped (Klimenko, 1982).

PARTHENOGENETIC ENGINEERING

Transgenesis has been recently put into practice in the silkworm. This provides an opportunity for introducing into the egg not only foreign DNA but also, in the same way, any organic and non-organic agents, cellular components, somatic or germ line nuclei, or cells at different stages of embryogenesis and/or differentiation taken from the same or different species. In this connection, we consider as intriguing the future prospects of several research directions.

Expanding opportunities for cloning in the silkworm

Nothing prevents us at present from introducing into the unfertilized egg nuclei and cells of somatic or germ line origin and then activation of this virtually parthenogenetic system with an effective activator, the egg’s own nucleus being inactivated or removed in some way. With this system all the basic problems of transnuclear cloning in mammals could be studied using Bombyx mori L., with any silkworm genotype (not only female as in parthenocloning), in principle, being cloned. Using record parthenoclones as the best source of eggs to support artificial parthenogenetic development we, at last, could clone any unique genotype even if the latter would be highly inbred (homozygous). That is why the best parthenoclones could play an important role in future selection as well.

New opportunities for artificial insemination

Microinjection of mature spermatozoa or spermatocytes (at different stages of differentiation) into unfertilized eggs with a subsequent egg activation of the meiotic type (for example, -11°C for 30 min) could be used for direct artificial insemination in insects and also to approach some problems of insemination and fertilization in general. For example, the importance of egg cytoplasm for studying the penetrance and expression of a gene (mutation) would find a convenient experimental system for its deeper analysis.
Expansion of hybridization

1. To expand the opportunities of hybridization in insects, microinjection of spermatozoa of one species into the unfertilized eggs of another species followed by artificial egg activation could be exploited to overcome the inability to perform interspecific crosses. The approach could be of value in preserving endangered species, or to learn more directly about evolutionary relationships between genes/chromosomes, etc. in closely related species.

2. Transferring nuclei of unfertilized eggs blocked in metaphase I (spindles) into the unfertilized egg of *Bombyx mori* L. is of special interest. In this case Astaurov thermoactivation might result in fusion of recipient and donor diploid female pronuclei and formation of a tetraploid (the donor is *B. mori*) or an amphidiploid (if the donor is a different species) cleavage nucleus. Polyploids of increasing degree could be obtained with this technique.

3. Having the recipient egg nucleus inactivated we can expect androgenetic development in case 1 and cloning in case 2, with the donor sperm possibly belonging to another species (nuclear-cytoplasmic hybridization).

Transgenesis

The parthenogenetic experimental system (reactor-like) described here can be a means for testing different ways of introducing (attaching) DNA in (to) sperm, different nuclei, and cells before microinjecting them into unfertilized silkworm eggs and triggering off the cytogenetic reaction. This artificial activation can be one of the two types: meiotic or ameiotic. The inactivation of the recipient nucleus may be purposeful in some experiments.

CONCLUSION

Experimental deactivation of freshly inseminated eggs and their artificial reactivation clearly indicate prospects for what I would call “parthenogenetic engineering” (Fig. 9). The further study and use of artificial activation in combination with microsurgical delivery of different genetic, nuclear and cellular material inside the egg will allow us to make the mature oocyte a cytogenetic reactor for creating and cloning a wide spectrum of genotypes, transnuclear cloning being only a special case.

It would be a mistake and possibly just wasting time to try developing some of these prospects without using the advantages offered by the Astaurov thermoparthenogenesis. Already selected record parthenoclines, as shown by rich experimental practice, provide the best arsenal of experimental material: oocytes that are genetically identical, ready reproducible for many years, capable of undergoing different types of parthenogenesis, and giving viable, hybrid, vigorous progeny in a wide spectrum of crosses. In this case female genotypes produced by parthenogenetic engineering would gain the capability for thermic parthenogenesis and give rise to new parthenoclines that could be then reproduced with the Astaurov technique and/or modified as described in this article.

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