EXPRESSION OF THE KANDIYOHI GENE IN TRIPLOID FROGS PRODUCED BY NUCLEAR TRANSPLANTATION

ROBERT GILMORE MCKINNELL

Department of Zoology, Newcomb College of Tulane University, New Orleans, Louisiana

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A variant, known as mottled or kandiyohi, occurs in populations of the common leopard frog, *Rana pipiens*, in Minnesota and other north-central states (BRECKENRIDGE 1944; WRIGHT and WRIGHT 1949). The kandiyohi frog was described originally as a new species (WEED 1922), but genetic studies (VOLPE 1955) have revealed that it differs from the wild-type frog by a single dominant gene. The kandiyohi gene has been used as a genetic marker in nuclear transplantation studies. When a blastula nucleus containing the kandiyohi gene is transplanted to an enucleated egg obtained from a wild-type frog, it expresses itself in the appearance of the mutant character in the transformed frog (MCKINNELL 1960, 1962). Similar results are obtained in experiments involving the transplantation of nuclei obtained from the spotless (burnsi) mutant frog to enucleated eggs obtained from wild-type individuals (SIMPSON and MCKINNELL, 1964, in preparation).

Polyloid frogs may be produced with the nuclear transplantation procedure (SAMBUICHI 1959; SUBTELNY and BRADT 1960, 1963; KAWAMURA and NISHIOKA 1960). Triploid frogs occur among the individuals produced by transplanting diploid blastula nuclei to unfertilized eggs. The triploid condition has been interpreted as resulting from the fusion of a haploid maternal pronucleus with the transplanted diploid nucleus.

In the experiments to be described below, diploid blastula nuclei derived from wild-type frogs were transplanted to activated unfertilized eggs obtained from kandiyohi females. The reciprocal transplantation was also performed, *i.e.*, somatic nuclei heterozygous for the kandiyohi gene were transplanted to activated unfertilized eggs obtained from wild-type frogs. The objective of these experiments was to test the ability of the kandiyohi gene to express itself in the presence of two wild-type alleles, and to provide genetic evidence that fusion of the maternal gamete pronucleus with a transplanted diploid nucleus can occur. A preliminary report of this research was presented elsewhere (MCKINNELL 1963).

MATERIALS AND METHODS

Wild-type *R. pipiens* collected in the Lake Champlain area of northeastern United States were obtained in the fall of 1962 from the J. M. Hazen Company of Alburg, Vermont. *R. pipiens* (kandiyohi dominant mutant) were obtained from the J. R. Schettler Company of Stillwater, Minnesota during the same period.

TABLE 1

Types and extent of development resulting from the transplantation of diploid blastula nuclei to activated and unfertilized recipient eggs

	Transplantations attempted	Blastulae	Gastrulae	Embryos which hatched	Frogs carried to metamorphosis
<i>Kk or kk → k</i>	146	68	61	45	12
<i>kk → K or k</i>	176	103	85	53	13
Total numbers	322	171	146	98	25

The first line of the table shows the types of development obtained when diploid blastula nuclei (*Kk* or *kk*), obtained from crosses of wild-type frogs with kandiyohi frogs, are transplanted to unfertilized eggs from wild-type frogs (*k*). The second line shows the results of transplanting wild-type diploid blastula nuclei (*kk*) into the unfertilized eggs of kandiyohi females (*K*) or (*k*).

Ovulation was induced by the method of RUGH (1934). Freshly extruded eggs were fertilized with a sperm suspension made from testes minced in 10 percent Ringer's solution. Nuclear transplantation donor blastulae, Stage 8+ or 9 (SHUMWAY 1940), were dissociated in a modified Niu-Twitty solution (BRIGGS and KING 1960). Dissociated cells were transferred to a paraffin-lined Syracuse dish containing Niu-Twitty solution (FLICKINGER 1949). Eggs to be used as recipients of transplanted nuclei were activated with a clean glass needle while immersed in a 10 percent Ringer's solution. Outer jelly coats were removed with watchmakers forceps. The nuclear transplantation procedure used was that of BRIGGS and KING (1957).

Larvae were reared individually in one-quart squat bowls containing pond water. A diet of boiled lettuce was presented to the animals when the feeding stages were reached. The squat bowls were lined with filter paper and tilted at the time of forelimb emergence (Stage XX, TAYLOR and KOLLROS 1946) to provide both a dry surface as well as an aquatic environment for the transforming frogs.

Chromosomes were counted from aceto-orcein squash preparations (HUNGERFORD and DIBERARDINO 1958; DIBERARDINO, KING and MCKINNELL 1963) of tail-tips of pre-feeding stage embryos (Stage 22, SHUMWAY 1940).

RESULTS

Development and chromosome numbers: Table 1 summarizes the development of the 322 transplant embryos of these experiments. Frogs obtained from both kinds of transplantations were carried to metamorphosis. Diploid blastula nuclei, derived from the cross of kandiyohi and wild-type frogs, when transplanted to unfertilized eggs of wild-type females (line 1 of Table 1) resulted in embryos which were reared to metamorphosis. Similar results were obtained with the reciprocal transplantation, *i.e.*, eggs of kandiyohi frogs, to which wild-type diploid blastula nuclei had been transplanted, resulted in embryos which were also reared to metamorphosis (line 2 of Table 1).

An examination of chromosome squashes revealed that only diploid and triploid frogs were among the experimental animals that reached metamorphosis. Of the 25 transformed frogs, 16 were of triploid constitution. The remainder were diploid.

Table 2 summarizes the chromosome numbers of transplant embryos which cleaved on time (*i.e.*, those in which the two blastomere stage was reached about 3.5 hours after activation at 19° to 20°C) and of eggs which underwent a delay in cleavage of approximately one mitotic cleavage interval (*i.e.*, a delay of about 45 minutes).

TABLE 2

An analysis of the chromosome numbers of tadpoles resulting from the transplantation of diploid blastula nuclei to activated unfertilized eggs

	1n	2n	3n	4n	5n	6n
On time	4	18	25	.	.	.
Delayed	.	15	..	4	1	6

See text for explanation of terms "on time" and "delayed."

Haploid, diploid, and triploid embryos resulted from the eggs which cleaved on time. The haploid embryos are probably gynogenetic in origin, resulting from the failure of the introduced nucleus to fuse with the maternal haploid pronucleus. Diploidy is most likely due to development induced by the transplanted nucleus which failed to fuse with the maternal pronucleus. The triploid frogs are best explained as resulting from the fusion of the haploid maternal pronucleus with the transplanted diploid nucleus.

The phenotype of metamorphosed diploid frogs (all of which cleaved "on time") was consistent with the type of transplanted nucleus. Of 18 diploid embryos (Table 2), eight were reared to metamorphosis. One of the diploid frogs was of the kandiyohi phenotype. It was derived from a kandiyohi blastula nucleus transplanted to an egg derived from a wild-type frog. The seven other diploid frogs which reached transformation stages were of the wild-type phenotype and were derived from the reciprocal transplantation. These observations support an interpretation that diploidy among the experimental animals, which reached the two cell stage after a normal cleavage interval, is due to the transplanted nucleus and is not attributable to participation of the egg pronucleus.

Diploid, tetraploid, pentaploid, and hexaploid embryos were among the individuals which underwent a delay in cleavage. Diploidy among the delayed tadpoles may be ascribed to the reconstitution of the haploid maternal pronucleus to the diploid state. None of the delayed diploid embryos were reared to metamorphosis. They are believed to be comparable to the homozygous diploid embryos of SUBTELNY (1958). It is believed that the tetraploid condition resulted from the reconstitution of the introduced diploid nucleus to the tetraploid state after it failed to fuse with the maternal pronucleus. Hexaploidy may be due to the fusion of the maternal pronucleus with the transplanted nucleus to yield a triploid nucleus; this then reconstitutes to the hexaploid state after a delay in cleavage.

A pentaploid count was made of one embryo which had undergone a delay in cleavage. Reconstitution of the transplanted nucleus and its subsequent fusion with the egg pronucleus would account for the appearance of this single 5N embryo.

Triploid kandiyohi frogs: Twenty-five triploid embryos were produced in this study. Of this number, 16 were reared to metamorphosis. Nine metamorphosed triploid frogs were kandiyohi and seven were wild-type. Figure 1 is a photograph of a frog of *Kkk* genotypic constitution. It may be compared with a wild-type triploid frog, *kkk* (Figure 2).

FIGURE 1.—Triploid *Rana pipiens* (Kkk) showing mottled pattern characteristic of the kandiyohi variant. Frog derived from a diploid donor nucleus (kk) and a haploid (K) host nucleus. See text and Figure 3 for explanation.

FIGURE 2.—Triploid *R. pipiens* (kkk) showing typical wild-type spotting pattern. Frog derived from a diploid donor nucleus (kk) and a haploid (k) host nucleus. See text and Figure 3 for explanation.

Both spotted (wild-type) and mottled triploid frogs were expected in this study. All kandiyohi frogs thus far studied have been reported to be heterozygous (VOLPE 1955). Because of the heterozygous condition, only one half of the recipient eggs derived from kandiyohi females were expected to have the mutant gene (K). Diploid wild-type nuclei (kk) introduced into the eggs of kandiyohi females thus result in two types of triploid individuals, Kkk and kkk . Both types of triploid frogs also are recovered in the reciprocal transplantation, in which the donor nucleus is either Kk or kk and the recipient egg is k . Each type of transplantation is diagrammed in Figure 3.

There is no apparent dilution effect, *i.e.*, a triploid of genetic constitution Kkk does not appear to be any less mottled than a diploid mottled frog, Kk . A triploid mottled frog of this study (Figure 1) may be compared with kandiyohi frogs of other studies (Figure 3 of MCKINNELL 1962; Figure 1 of VOLPE 1960). However, it is difficult to evaluate objectively the extent of mottling in kandiyohi frogs. There is a wide spectrum of variation in diploid heterozygous kandiyohi frogs, and the genetic and nongenetic bases for the variable expression have not been ascertained. In studies of a related mutant frog (*burnsi*), VOLPE (1961) attributes the variation in spotting patterns principally to a complex of modifiers.

A triploid karyotype: Chromosome squash preparations were made of each experimental embryo which reached the tail-fin circulation stage (Stage 22, SHUMWAY 1940). Counts were made of metaphase plates incubated at 37°C in hypotonic saline solution followed by fixation and staining with aceto-orcein. Squashes prepared from embryos initially diagnosed as containing a triploid complement of chromosomes, which subsequently metamorphosed into kandiyohi

FIGURE 3.—Transplantation diagram illustrating methods of obtaining triploid frogs of genetic constitutions Kkk (kandiyohi) and kkk (wild-type). Eggs of mottled females may contain K or k genes. The triploid progeny of these eggs, into which kk nuclei have been introduced, would be expected to be either Kkk or kkk (left hand portion of diagram). Similar progeny are obtained when eggs of k constitution receive blastula nuclei of Kk or kk constitution (right hand portion of diagram).

frogs, were reexamined to confirm the early diagnosis of triploidy. Although the quality of the squash preparations varied, there were invariably one to four metaphase plates in each preparation in which 39 chromosomes ($3n=39$) were clearly discernible. In all preparations, there were many additional plates in which counts of "approximately 39" were made. It is unlikely that these additional metaphase plates contained an aneuploid complement of chromosomes. Unbalanced sets of chromosomes in amphibians typically result in reduced viability and abnormal appearance (FANKHAUSER 1945, 1955).

Figure 4 is a photograph of a metaphase plate chromosome squash derived from an embryo which metamorphosed into a kandiyohi frog. Although there were no loose chromosomes in the vicinity of this metaphase squash on the slide, one could postulate that the count of 39 chromosomes could be due to the addition of 13 stray chromosomes from an adjacent broken plate added to a normal diploid complement of chromosomes. If the 13 extra chromosomes were derived from another metaphase plate, one would predict that a random selection of 13 chromosomes would appear in a karyotype analysis of the squash. Figure 5, which is a karyotype of the squash illustrated in Figure 4, indicates that such is not the case. The 39 chromosomes can be arranged into 13 groups of three each. Six of the groups appear to have medial centromere (upper row of chromosomes of Figure 5) and seven of the groups of three have submedial centromeres (lower row of chromosomes in Figure 5). Three small chromosomes, each with a submedial centromere, possess a secondary constriction. The grouping of chromosomes in Figure 5 may be compared with the diploid karyotypes of DIBERARDINO (1962). It thus appears that the 39 chromosomes represent a true triploid complement and not a chance aneuploid grouping of 39 chromosomes.

Nuclear fusion: The appearance of the triploid kandiyohi frogs in these experiments provides genetic evidence that a transplanted diploid embryonic nucleus

FIGURE 4.—Metaphase plate chromosome squash prepared from the tail fin of a triploid nuclear transplant embryo. The embryo developed from an activated egg derived from a wild-type frog (k) into which was transplanted a diploid nucleus heterozygous for the kandiyohi gene (Kk). The embryo metamorphosed into a frog of the kandiyohi phenotype.

can fuse with a haploid gamete pronucleus. Five of the nine metamorphosed kandiyohi triploid frogs developed from kandiyohi eggs transplanted with diploid nuclei homozygous for the wild-type allele (kk). Triploidy could only have resulted from the fusion of the egg pronucleus with either the transplanted

FIGURE 5.—Triploid karyotype prepared from the chromosome squash illustrated in Figure 4 showing 13 groups of three chromosomes each ($3n = 39$).

nucleus or a retained first polar body. The latter possibility has been excluded by the reciprocal transplantation, which involved the transfer of a diploid embryonic nucleus heterozygous for the kandiyohi gene (*Kk*) into eggs of wild-type females. Four metamorphosed kandiyohi triploid frogs resulted from the latter experiments. There thus seems to be no satisfactory alternative explanation to account for the triploid condition and the kandiyohi phenotype among the frogs other than nuclear fusion of the transplanted embryonic diploid nucleus with the egg pronucleus.

DISCUSSION

Metamorphosed triploid frogs have been reported from a variety of anuran species (MOORE 1955). WICKBOM (1945) reported finding four adult triploid *Rana esculenta* from a natural population. *R. nigromaculata*, *R. japonica*, and hybrids of *R. japonica* with *R. temporaria ornativentris*, all having triploid constitution, have been reported by KAWAMURA (1951, 1952) and KAWAMURA and TOKUNAGA (1952). BRIGGS (1947) produced triploid *R. pipiens* by treating eggs with heat shortly after insemination. He reported that triploid tadpoles develop normally and reach metamorphosis at the same time as do diploid controls. Recently, DASGUPTA (1962) has reported a high incidence of triploid leopard frogs obtained by treatment of inseminated eggs with hydrostatic pressure.

The nuclear transplantation procedure has been used for the production of polyploid frogs. SAMBUICHI (1959) analyzed the chromosome constitution of 27 embryos produced from unfertilized eggs which had received a diploid nucleus. Nine of the 27 were triploid. SAMBUICHI provides two possible explanations for the triploid frogs. Either they developed because of nuclear fusion of the transplanted nucleus with the maternal nucleus, or as the result of the fusion of the maternal pronucleus with a suppressed first polar body. In similar experiments, SUBTELNY and BRADT (1960) record 48 triploid *R. pipiens* embryos among the individuals produced by transplanting diploid nuclei to uterine and body cavity eggs. Sixteen of the triploid embryos developed from eggs showing no suppression of the first and second polar bodies. These observations, together with the cytological studies of SUBTELNY and BRADT (1963) provide convincing evidence of nuclear fusion. The triploidy of the kandiyohi frogs, which developed as the result of nuclear fusion in the present study, supports the observations of SUBTELNY and BRADT.

The nonspotted variant, *R. pipiens* (burnsi dominant mutant) (MOORE 1942) has been the subject of recent studies of gene dosage effect on expressivity. DAVISON (1961) reported induced and spontaneous triploid frogs carrying the burnsi gene. He recorded that triploid burnsi frogs with one burnsi gene and two wild-type alleles (*Bbb*) have more dorsal spotting than diploid heterozygous burnsi frogs (*Bb*). A single burnsi tetraploid, *Bbbb*, showed an even greater spotting tendency in his study.

VOLPE and DASGUPTA (1962) have also produced diploid and triploid mutant burnsi frogs. They report, in contrast to DAVISON's findings, that the inhibitory effects of the burnsi gene prevail even in the presence of an increased dosage of

the wild-type allele. They maintain that any shift in the extent of spotting of the mutant phenotype toward the wild type is the result of dosage changes at the modifying loci, and not at the main spotting locus.

The decisive point established in the present paper is that the kandiyohi gene does express itself in a triploid frog containing two wild-type alleles.

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SUMMARY

Diploid blastula nuclei derived from wild-type parents were transplanted to activated unfertilized eggs obtained from kandiyohi frogs. The reciprocal transplantation was also performed, *i.e.*, blastula nuclei heterozygous for the kandiyohi gene were transplanted to eggs obtained from wild-type frogs.

Haploid, diploid, and triploid embryos developed from eggs which cleaved at approximately 3.5 hours after activation. Diploid, tetraploid, pentaploid, and hexaploid embryos were obtained from eggs which were delayed in cleavage.

Metaphase chromosomes of a triploid kandiyohi frog were analyzed and a karyotype presented which shows 13 groups of three chromosomes each.

Sixteen triploid frogs were reared to metamorphosis. The triploid frogs are best interpreted as resulting from the fusion of the maternal pronucleus with the transplanted diploid nucleus. Nine of the triploid frogs displayed the kandiyohi phenotype thus establishing that the kandiyohi gene can express itself in the presence of two wild-type alleles.

LITERATURE CITED

- BRECKENRIDGE, W. J., 1944 *Reptiles and Amphibians of Minnesota*. University of Minnesota Press, Minneapolis.
- BRIGGS, R., 1947 The experimental production and development of triploid frog embryos. *J. Exptl. Zool.* **106**: 237-266.
- BRIGGS, R., and T. J. KING, 1957 Changes in nuclei of differentiating endoderm cells as revealed by nuclear transplantation. *J. Morphol.* **100**: 269-312. — 1960 Nuclear transplantation studies on the early gastrula (*Rana pipiens*). I. Nuclei of presumptive endoderm. *Develop. Biol.* **2**: 252-270.
- DASGUPTA, S. 1962 Induction of triploidy by hydrostatic pressure in the leopard frog, *Rana pipiens*. *J. Exptl. Zool.* **151**: 105-122.
- DAVISON, J., 1961 A study of spotting patterns in the leopard frog. *J. Heredity* **52**: 301-304.
- DIBERARDINO, M. A., 1962 The karyotype of *Rana pipiens* and investigation of its stability during embryonic differentiation. *Develop. Biol.* **5**: 101-126.
- DIBERARDINO, M. A., T. J. KING, and R. G. MCKINNELL, 1963 Chromosome studies of a frog renal adenocarcinoma line carried by serial intraocular transplantation. *J. Natl. Cancer Inst.* **31**: 769-789.
- FANKHAUSER, G., 1945 The effects of changes in chromosome number on amphibian development. *Quart. Rev. Biol.* **20**: 20-78. — 1955 The role of nucleus and cytoplasm. pp.

- 126-150. *Analysis of Development*. Edited by B. H. WILLIER, P. A. WEISS, and V. HAMBURGER. Saunders, Philadelphia.
- FLICKINGER, R. A. 1949 A study of the metabolism of amphibian neural crest cells during their migration and pigmentation, *in vitro*. J. Exptl. Zool. **112**: 465-484.
- HUNGERFORD, D. A., and M. A. DIBERARDINO, 1958 Cytological effects of pre-fixation treatment. J. Biophys. Biochem. Cytol. **4**: 391-400.
- KAWAMURA, T., 1951 The offspring of triploid males of the frog, *Rana nigromaculata*. J. Sci. Hiroshima Univ., Ser. B. Div. 1 **12**: 11-20. — 1952 Triploid hybrids of *Rana japonica* Günther ♀ × *Rana temporaria ornativentris* Werner ♂. J. Sci. Hiroshima Univ., Ser. B, Div. 1 **13**: 129-138.
- KAWAMURA, T. and M. NISHIOKA, 1960 Amphidiploid frogs produced by artificial means. J. Sci. Hiroshima Univ., Ser. B, Div. 1 **18**: 195-220.
- KAWAMURA, T. and C. TOKUNAGA, 1952 The sex of triploid frogs, *Rana japonica* Günther. J. Sci. Hiroshima Univ., Ser. B, Div. 1 **13**: 121-128.
- McKINNELL, R. G., 1960 Transplantation of *Rana pipiens* (kandiyohi dominant mutant) nuclei to *R. pipiens* cytoplasm. Am. Naturalist **94**: 187-188. — 1962 Intraspecific nuclear transplantation in frogs. J. Heredity **53**: 198-207. — 1963 Expressivity of the kandiyohi gene in triploid frogs produced by nuclear transplantation. (Abstr.) Genetics **43**: 900.
- MOORE, J. A., 1942 An embryological and genetical study of *Rana burnsi* Weed. Genetics **27**: 406-416. — 1955 Abnormal combinations of nuclear and cytoplasmic systems in frogs and toads. Advan. Genet. **7**: 139-182.
- RUGH, R., 1934 Induced ovulation and artificial fertilization in the frog. Biol. Bull. **66**: 22-29.
- SAMBUICHI, H., 1959 Production of polyploids by means of transplantation of nuclei in frog eggs. J. Sci. Hiroshima Univ., Ser. B, Div. 1 **18**: 39-43.
- SHUMWAY, W., 1940 Stages in the normal development of *Rana pipiens*. I. External form. Anat. Record **78**: 139-147.
- SIMPSON, N. S., and R. G. McKINNELL, 1964 The burnsi gene as a nuclear marker for transplantation experiments. (In preparation.)
- SUBTELNY, S., 1958 The development of haploid and homozygous diploid frog embryos obtained from transplantations of haploid nuclei. J. Exptl. Zool. **139**: 263-306.
- SUBTELNY, S., and C. BRADT, 1960 Transplantations of blastula nuclei into activated eggs from the body cavity and from the uterus of *Rana pipiens*. Devel. Biol. **2**: 393-407. — 1963 Cytological observations on the early developmental stages of activated *Rana pipiens* eggs receiving a transplanted blastula nucleus. J. Morphol. **112**: 45-60.
- TAYLOR, A. C., and J. J. KOLLROS, 1946 Stages in the normal development of *Rana pipiens* larvae. Anat. Record **94**: 7-24.
- VOLPE, E. P., 1955 A taxo-genetic analysis of the status of *Rana kandiyohi* Weed. Syst. Zool. **4**: 75-82. — 1960 Interaction of mutant genes in the leopard frog. J. Heredity **51**: 151-155. — 1961 Variable expressivity of a mutant gene in leopard frog. Science **134**: 102-104.
- VOLPE, E. P., and S. DASGUPTA, 1962 Effects of different doses and combinations of spotting genes in the leopard frog, *Rana pipiens*. Develop. Biol. **5**: 264-295.
- WEED, A. C., 1922 New frogs from Minnesota. Proc. Biol. Soc. Wash. **35**: 107-110.
- WICKBOM, T., 1945 Cytological studies on dipnoi, urodela, anura, and emys. Hereditas **31**: 241-246.
- WRIGHT, A. H., and A. A. WRIGHT, 1949 *Handbook of Frogs and Toads of the United States and Canada*. Comstock, Ithaca, New York.