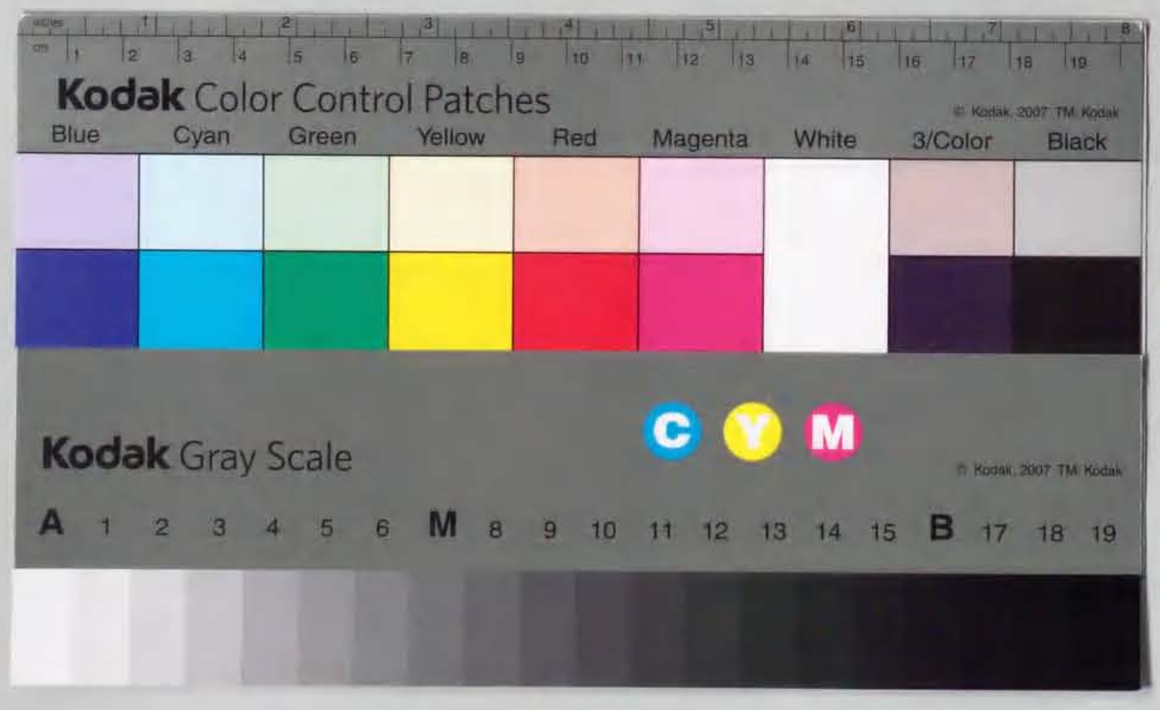
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GENETICAL STUDIES ON SEX DETERMINATION AND SEXUAL DIFFERENTIATION IN CHICKENS

(ニワトリにおける性決定および性分化の遺伝学的研究)

by

HIROSHI KAGAMI

GENETICAL STUDIES ON SEX DETERMINATION AND SEXUAL DIFFERENTIATION IN CHICKENS

（ニワトリにおける性決定および性分化の遺伝学的研究）

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Contents

Chapter I

General Introduction

Sex determination and sexual differentiation have been the most fundamental subject in biology to be elucidated. Until 1900, it was generally thought that the sex of a human embryo was decided by environmental factors like maternal nutrition. Since the rediscovery of Mendel's Laws, it has been shown that the sex of an individual is determined by the chromosomal constitution[1]. Though many scientific works have been performed to clarify the subject, many questions are still remained even today.

In higher animals, sexual reproduction involves the mixing of genomes from their parents to create offspring that are usually genetically different from each other. Since genetic variation is the key point of this system, sexual reproduction has tremendous advantages for the adaptation to various environments[2]. Development of the embryo follows the fertilization of an ovum with a spermatozoon. Some part of the embryo is destined to differentiate into a gonad and continues the differentiation into ovaries in the female or testes in the male depending on their genetic sex.

The first step of the elucidation of sex determination and sexual differentiation was the identification of the sex chromosomes in a genome. In chickens, cytogenetic methodologies have been attempted to describe the sex-determining mechanism correctly. It seemed that productive research in sex identification depended on a chromosome methodology which reveals the structure, activity or other functions on the chromosomes[3,4]. However it was very difficult to identify their sex chromosomes, especially identification of the W chromosome in female cells. Because the number of chromosomes in chickens is

numerous (2n=78) and the W chromosome is comparatively small. In addition the repeatability to obtain a clear metaphase preparation was low [5,6]. Karyotyping and also many other methods, such as immunological and biochemical methods, have also been tried to identify the genetic sex in chickens [7,8].

It is now clear that the chicken has 76 autosomes and ZZ sex chromosomes in male, and ZW sex chromosomes in female. Since the composition of sex chromosome in chickens is heterogametic in female and homogametic in male, the ovum which has completed the reproductive division, has a Z chromosome or a W chromosome in a fifty-fifty ratio. On the other hand, all spermatozoa have only the Z chromosome. If an ovum bearing a Z chromosome or W chromosome is fertilized with a spermatozoon a cock (ZZ) or a hen (ZW) is destined respectively. Thus sex determination in each bird depends on which ovum is fertilized. This is just opposite to the mammalian, where the sex chromosome is XX (female) and XY (male) and the sex determination is dependent on the spermatozoa. Therefore, it is important to clarify the mechanism for sex determination and sexual differentiation in chickens, as it compares to that of the mammals [1].

Recently, the knowledges about sex determination and sexual differentiation in mammals have been rapidly accumulated. For example, a gene locus that regulate the production of TDF (Testis Determining Factor), guiding the undifferentiated male gonad to the testes, has been nearly identified in a region of the short arm on the Y chromosome [9,10]. Furthermore, it was found that the sexual differentiation of the brain and the secondary

sex characteristics are induced by the testosterone secreted from the initially differentiated testes[11].

In contrast, the mechanism of the avian sex determination and sexual differentiation at molecular level has not yet been clarified. Since the composition of sex chromosome in avian is opposite to mammal, it has been considered that the major sex determining gene would be on the W chromosome.

In chickens, not only the sex chromosome composition but also the asymmetric growth of the gonad in female is making it difficult to analyze the mechanism for sex determination and sexual differentiation. In the hen, the ovaries and their duct systems develop asymmetrically between left and right sides of the body. During the gonadal sex differentiation, the left one alone differentiates into a functional ovary, while the right one ceases its growth and completely degenerates several weeks after hatching[12].

It was observed, however, that if the left gonad of female is degenerated due to disease or any other reason, the right one will make compensatory development in a rare case[13]. Along with such compensatory development, the secondary sex characteristics, such as comb, wattle and spur were developed similar to that of male. By surgical removing the left gonad of newly hatched female chicks, sex-reversal could be induced artificially[14]. The right gonad was caused to make compensatory development, and the androgenic steroid or the steroid secretory cell was confirmed. Histological structure of the right gonad widely varied from testis-like to ovary-like ones, and some one among those which were testis-like, a small number of spermatozoa were

observed[15,16]. It was considered to be important for the elucidation of the mechanism of sex determination and sexual differentiation in chickens to analyze the process of sex-reversal induced by the left gonadal removal.

However, the studies of artificial sex-reversal, referred to above, were done without confirmation of the genetic sex of the animals used. The males with retarded masculinization could be recovered with the treatment.

Recent progress of genetics enabled us to elucidate the riddle of creatures at the molecular level. This paper describes sex determination and sexual differentiation in chickens by use of modern genetical methods.

In chapter II, sexual differentiation of the gonad during embryonic life is described to illustrate the basic knowledge of sex determination and sexual differentiation in chickens. In chapter III, artificial sex-reversal from female to male by left ovariectomy in chickens whose genetic sex has been confirmed by the modern karyotyping techniques was performed. And interaction of gene and hormone on sex determination and sexual differentiation was considered. Also the mechanism of sex-reversal from hen to cock was discussed. To develop a new method of sex identification in chickens, the detection of female-specific repetitive DNA units was carried out in chapter IV. This method was applied for sexing of chicken embryos, too. For the purpose of molecular genetic analysis of the W chromosome, hybridization of the cloned W chromosome specific DNA probe to the initially XhoI digested DNA was

performed in chapter V. And the role of the W chromosome in sex determination and sexual differentiation in chickens was discussed. Based on the results obtained through these experiments, comprehensive discussion of the sex determination and sexual differentiation in chickens was described as general discussion in chapter VI.

Chapter II

Sexual Differentiation of the Gonad during Embryonic Life in Chickens

Introduction

The individual chicken embryo comes into existence as the result of the union of two gametes, the ovum and the spermatozoon. Chicken ovum is fertilized in the infundibulum, the anterior region of the oviduct. Several spermatozoa penetrate the inner perivitelline layer and enter the germinal disc, the small area of cytoplasm at the surface of the yolk mass where development is initiated [17].

The development of gonad is unique for its alternative differentiation. Most of the other organ rudiments can normally differentiate into only one type of organ. The gonad, however, normally has two options before sexual differentiation. During the undifferentiated stage, it has potential to develop into either an ovary or a testis. When the sexual differentiation is induced, the gonad is first led to develop into either an ovary or a testis, depending on their genetic sex [2].

During the sexual differentiation of the female chicken embryo, asymmetric development is observed between the left and right gonad. In the left gonad, cortical tissue differentiates secondary, and it develops into a functional ovary. On the other hand, the right gonad organized with medullary tissues develops relatively little during the incubation period. And a few weeks after hatching it degenerates completely [18].

In male, both left and right gonads develop equally and become functional testes.

However, it is not clearly determined when the asymmetric

development of gonads occurs in female.

In the present chapter, the initiation time of sexual differentiation of the gonad in the female embryo was determined with stereoscopic microscopical observations. And the mechanism of sexual differentiation of the gonad during embryonic stages was discussed.

Materials and Methods

Fertilized eggs from White Leghorn chickens were obtained. These eggs were incubated in a conventional egg incubator. Eggs were incubated in a vertical position with the large end set uppermost. Temperature was adjusted to 39°C constantly. Moisture in the incubator was kept at 80%. The eggs were turned automatically several times a day to prevent sticking of the embryo to the shell membrane. The incubation was stopped at 5, 7, 9, 11, 14, 21 days, respectively.

Egg shells of each fertilized egg were broken carefully without inflicting a wound to the embryos. Egg shell, albumin and yolk were removed from the embryo. Only the embryo was taken and was soaked with saline to remove the excess of yolk and blood. The embryo was floated in the saline for microscopical observation.

Whole body of the embryos were observed. Abdominal cavity of the embryo was surgically opened with a pair of forceps. The viscus of the embryo was removed carefully without injuring the gonads under the observation using a stereoscopic microscope.

The location of gonads at the left and right side was identified. Width and length of female gonad was measured. Photos of the whole body and the embryonic gonads were taken through the microscope.

Statistical analysis of the means of these gonadal measurements were applied with T-test. Significant differences between left and right gonads were calculated.

Results

The 5 day-old embryo of a chicken is presented in Fig. 2-1. At this stage the gonad developed as vestigial. And it was very difficult to identify the gonad accurately with microscopical observation. Consequently, length and width of the gonads could not be measured at the stage.

Gonad development of 7 day-old chick embryo is presented in Fig. 2-2. At 7 days of incubation the gonad development had progressed compared to 5 days. The gonads were easily identified. When the length and width between left and right were compared no significant differences could be detected(Table 2-1).

Fig. 2-1. The 5 day-old embryo of a chicken. In this stage of development, identification of the gonad with microscopical observation was very difficult.

At 9 days of incubation, secondary differentiation of the cortical tissue was observed in left gonad. In the right gonad the development had also progressed, but compared to the left gonad the development was not so distinguishable. When the statistical calculation was performed, significant differences were detected between left and right length, but could not be detected at width in female (Table 2-1).

As the time passes the differences in the gonad development has been increased between left and right.

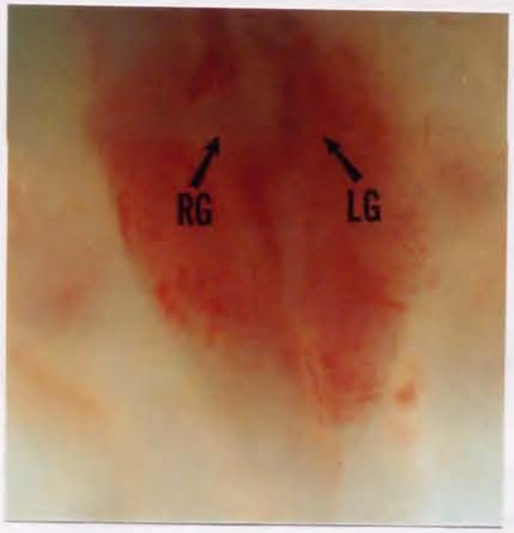


Fig. 2-2. Gonad development of a 7 day-old chick embryo. Significant differences between left and right gonad could not be observed (x 27). RG: Right gonad of the embryo. LG: Left gonad of the embryo.

At 11 days, significant differences were detected both in length and width between left and right gonads (Table 2-1).

At 14 days, development of the left gonad has been continued while the right gonad has nearly stopped its growth and reached its maximum size in female (Fig. 2-3 and Table 2-1).

In male, no asymmetric development was observed and both sides of the gonad continued to develop.

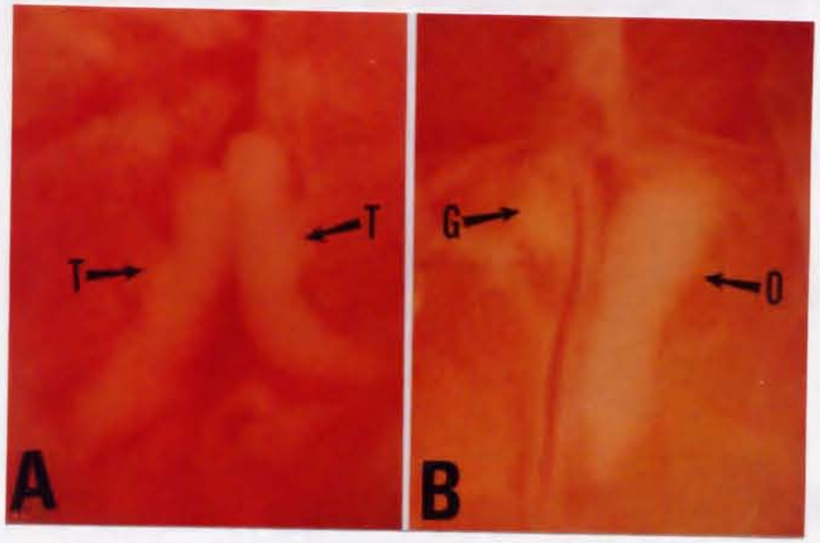


Fig. 2-3. Gonad development of a 14 day-old chick embryo. In male (A, x20), the left and right gonad developed equally. In female (B, x 20), asymmetric development was observed between left and right gonad. The left gonad developed into an ovarian gonad, while the right one degenerated. T: testicular gonad of a male embryo, O: left ovarian gonad of a female embryo, G: degenerating right gonad of a female embryo.

At 21 days the left gonad (Fig. 2-4) continued its development to a functional ovary and the right one has stopped of its development. The sign of degeneration was observed.

On the other hand, asymmetric development of the gonad could not be observed throughout all stages of incubation in male embryonic development.

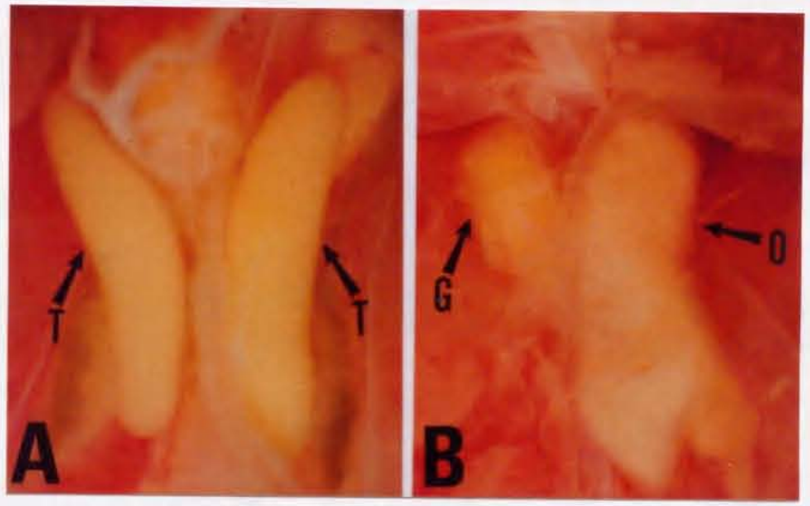


Fig. 2-4. Gonad development of a 21 day-old chick embryo. In male (A, x18), the left and right gonad developed equally. In female (B, x 18), asymmetric development between left and right gonad was distinguished. T: testicular gonad of a male embryo, O: left ovarian gonad of a female embryo, G: degenerated right gonad of a female embryo.

Table 2-1. Gonad development of female during embryonic life

days of incubation	no. of embryos		gonad(mm) a) left	gonad(mm) a) right
7 b)	10	length	1.87±0.21	1.88±0.25
		width	0.58±0.18	0.60±0.20
9	10	length	3.12±0.29 *	2.32±0.32
		width	0.92±0.19	0.72±0.34
11	10	length	4.45±0.41 *	2.64±0.30
		width	1.61±0.23 *	0.71±0.24
14	10	length	5.54±0.52 *	2.70±0.45
		width	1.92±0.22 *	0.70±0.21
21	10	length	6.50±0.59 *	2.68±0.51
		width	2.23±0.34 *	0.69±0.27

a) : Values were expressed as mean±S.D. in mm.

b) : In 7 days of incubation, morphological differences between left and right gonads could not be observed with microscopical observation. Therefore the sex of embryo examined could hardly be distinguished.

*: Significantly different ($p<0.05$).

Discussion

The genital system of chicken embryo comprises gonad and accessory genital organs. The primordial gonad is typically composed of a medulla and a cortex. The cortex is potentially ovarian tissue and the medulla is testicular. Germ cells are developed in gonad and conducted through accessory organ. During the sexual undifferentiated stage of gonad, there is no morphological difference between male and female [18].

The measurement of the width and length suggests the initiation time of the sexual differentiation of the gonad in chicken embryo. As the significant difference of the width and length between left and right gonads could not be observed, sexual differentiation was not yet be induced at 7 days. At 9 days significant difference was already observed. Therefore, the sexual differentiation of the left gonad in female was induced between 7 to 9 days, probably at about 8 days.

Since a pair of sex chromosomes is heterogametic (ZW) in female and homogametic (ZZ) in male, it was suggested that the induction of undifferentiated left gonad to ovary was regulated by the ovary determining gene on the W chromosome in chickens. Recently, the testis determining gene which induce the differentiation of the undifferentiated gonad to testes in male, was identified on the short arm of the y chromosome [10]. These findings also support the existence of ovary determining gene on the W chromosome.

But the most significant difference during the sexual differentiation in chicken embryo compared to mammals is the

asymmetric development of the gonad between left and right [18]. This phenomenon seemed to be induced by the difference in response to estrogen between left and right gonad. Estrogen target cells were searched for in the differentiation of chicken embryo. The cells were observed in the germinal epithelium of the left gonad but not in the right one. It was suggested that the absence of the target cells for estrogen in the germinal epithelium of the right gonad accounted for the lack of cortical differentiation to the right gonad [19].

In male, asymmetric development between left and right gonad could not be observed throughout all the stages of embryo development. It was suggested that no genetic regulation has been induced in the differentiation of undifferentiated gonad to testes. Consequently, it is suggested that the fundamental sex is not female but male in chicken.

Once the sexual differentiation of the gonad was induced, further development to ovary in female, or testes in male was regulated by sex hormones secreted from the initially differentiated gonad. Secondary sex characteristics such as comb, wattles, spurs and feather shape were also controlled by internal secretion of sex hormones. The male secondary sex characteristics is mainly induced by the regulation of the testosterone secreted from testes and that of female's is induced by estrogen secreted from ovary [20].

Summary

The initiation time of sexual differentiation of the gonad in female embryo was determined with stereoscopic microscopical observations.

At 7 days of incubation, the length and width between left and right gonads were not significantly different. At 9 days of incubation, secondary differentiation of the cortical tissue was observed at left gonad. And significant differences in the gonadal measurement were detected between left and right length in female. Consequently, the sexual differentiation of the left gonad in female has been initiated between 7 to 9 days probably at about 8 days.

On the other hand, asymmetric development of the gonad could not be observed throughout all the stages of incubation in male embryonic development.

Since a pair of sex chromosomes is heterogametic (ZW) in female and homogametic (ZZ) in male, it was suggested that the induction of the undifferentiated left gonad to ovary was regulated by the ovary determining gene on the W chromosome. It was suggested that the absence of the target cells for estrogen in the germinal epithelium of the right gonad is accounted for the lack of differentiation of the right gonad.

Chapter III

Artificial Sex-Reversal from Female to Male by Left Ovariectomy in Chickens

Introduction

It has been known for a long time that hens could be masculinized under rare circumstance[12,13,14]. An interesting case of natural sex reversal was reported. In one hen, a Buff Orpington, which had regularly laid eggs happened to be masculinized by chance. It was recognized by her secondary sex characteristics which changed completely to that of a male.

The experiment of artificial sex-reversal by left ovary removal was performed, and it was found that early left ovariectomy caused masculinization of the right gonad[14]. The presence of androgen and androgen-producing cells in the right gonad has been identified by some investigators[21,22,23]. In these sex reversed animals, their secondary sex characteristics such as comb growth, spur development, and plumage pattern appeared like that of a male.

The structure of the transformed right gonads varied according to the individual. In some cases the seminiferous tubule in the transformed right gonad was dispersed among fibrous strands, and/or lacked germ cells, and/or consisted of Sertoli cells alone. In some other cases fertile tubules with spermatogenesis proceeding to the spermatid or the spermatozoa stage were observed[12,24]. Only once a few spermatozoa from these transformed right gonads were isolated and inseminated into normal hens. But there was no evidence that these spermatozoa were fertile[16].

The works referred to above were done without confirmation of the genetic sex of the animals used. The males with retarded masculinization could be recovered with treatment. Recently, the development of karyotyping techniques in chickens enabled us to distinguish the genetic sex clearly and exactly. In the present chapter, artificial sex-reversal from female to male by left ovariectomy in chickens, whose genetic sex has been confirmed by modern karyotyping techniques, was described.

Materials and Methods

1. Animals

All birds used in this experiment were of the Single Comb White Leghorn strain. Immediately after hatching, the chicks were sexed by differences in the morphology and size of the genital process by a professional sex discriminator. Forty female chicks were ovariectomized. Nine of them died during the operation, and four of them died later of leg injuries. All the other ovariectomized chickens survived in good health. Five female and five male chicks were reared and used as controls.

2. Ovariectomy

Birds were first ovariectomized between one and eight days, and then two more successive operations between nine and fourteen days after hatching. The ovariectomy procedure was essentially the same as that for castration. The last intercostal space on the left side was cut open using a surgical blade. The portion of the peritoneum covering the ovary was removed, with the aid of a pair of forceps to grasp the tissues, the ovary was cut, and several pieces were removed one after another. Since the regeneration occurred rapidly, even if a small part of the ovary was left, the surgical removal was completed using an electric surgical blade.

3. Analysis of karyotype

Though all chicks were sexed by differences in morphology and size of the genital process, analysis of karyotypes was performed to determine their genetic sex.

At about three months of age their mitotic chromosome

preparations were examined using the leucocyte culture method [5,25,26]. Blood samples were collected from the wing vein and lightly centrifuged at 400 r.p.m. for 5 minutes. The plasma containing the leucocytes was transferred to sterile collection tubes containing a culture medium. The medium was composed of 5.0 ml of RPMI-1640, 0.2 ml of antibiotic solution (penicillin and streptomycin), and 0.2 ml of phytohemagglutinin (PHA). The collection tubes were incubated at 39.0 °C and 5% CO_2 in a water-jacket incubator for 72 hours. Colcemid was added to arrest cells at mitosis. The cell pellet was covered with 5 ml of 0.075 M KCl solution for hypotonic treatment. After the removal of the hypotonic solution the cell pellet was covered with a freshly-made solution of methanol and acetic acid at 3:1 for fixation. A solution of Giemsa stain diluted in distilled water was placed on the slide. After the slides were dried, chromosome spreads were examined with a light microscope for analysis of karyotypes.

4. Observation of the secondary sex characteristics

After the performance of ovariectomy, the differences of secondary sex characteristics between treated female chickens and control chickens (normal male and female) were recorded until maturity. Animals were sacrificed at about 9 months. For each animal the body weight, comb, wattles and feathers were weighed or measured.

5. Classification of sex-reversed chickens according to the secondary sex characteristics

Classification was based on their secondary sex characteristics for the degree of sex-reversal. The

classifications are listed as follows:

I, High degree of masculinity; II, Middle degree of masculinity; III, Low degree of masculinity.

6. Histological examinations

Autopsies were performed at about nine months of age, the testes of the male control and the transformed right gonads of the ovariectomized female were removed, then weighed and measured (length and width of the gonads) and they were cut into small pieces. These gonads (testes and right gonads) and the female controls' ovaries were fixed by Bouin's fluid, dehydrated in ethanol, cleared in xylene and embedded in paraplast. Serial sections were cut at 7 μm in thickness. Deparaplasted sections were stained with Hematoxylin and Eosin staining solution. These sections were then examined with a light microscope.

Results

1. Genetic sex

The metaphase chromosomes are presented in Fig. 3-1. The chromosomes were arranged into the 39 pairs according to decreasing size, and again divided into 9 pairs of macrochromosomes and 30 pairs of microchromosomes (Table 3-1). The macrochromosomes were identified according to the size and position of the centromere. The microchromosomes were too small to distinguish between them, but they might be acrocentric [25,27].

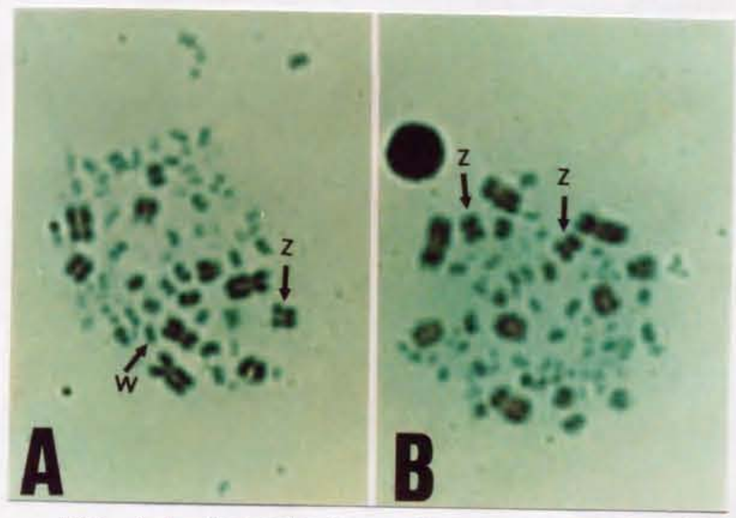


Fig. 3-1. The metaphase chromosomes produced by the leucocyte culture method (A: female, B: male). The Z and W chromosomes are marked by arrows. In some preparations the microchromosomes are difficult to identify, but the macrochromosomes are always distinct.

Table 3-1. Analysis of karyotype in the domestic fowl, based on chromosome size and centromere position

female		male	
size order	kinetochore	size order	kinetochore
1 and 2	metacentric	1 and 2	metacentric
3	acrocentric	3	acrocentric
4	submetacentric	4	submetacentric
Z or W	metacentric	Z	metacentric
6 and 7	acrocentric	6 and 7	acrocentric
8	metacentric	8	metacentric
9	submetacentric	9	submetacentric
10 - 39	acrocentric ?	10 - 39	acrocentric ?

Z: Z chromosome, W: W chromosome

?: Uncertain

It is known that the sex chromosomes of birds are a homomorphic pair (ZZ) in the male, and heteromorphic (ZW) in the female, and the Z chromosome (metacentric) is the fifth , the W chromosome (submetacentric) is the ninth according to decreasing size [25,27]. From this, the genetic sex of the chickens was determined, and it was confirmed that there had been no mistake in the initial sexing (Table 3-2).

Table 3-2. Comparison of sexing between morphological and genetic method*

morphological sex[1)]		genetic sex[2)]	
		male	female
male (control)	5	5	0
female (ovariectomized)	27	0	27
female (control)	5	0	5

* Values are expressed as number of the chickens examined.

1) Immediately after hatching chicks used for the present study were sexed by differences in morphology and size of the genital processes.

2) At about three months, their genetic sex was analyzed by their karyotypes.

2. Observation of the secondary sex characteristics

The secondary sex characteristics of ovariectomized chickens were very variable from that of normal female (Fig. 3-2; B, C, D, and E). As shown in Table 3-3, two of the ovariectomized females were classified as showing the high degree of masculinity (I), eleven of them to the middle degree (II) and fourteen of them to the low degree (III) according to observations of the secondary sex characteristics.

In highly masculinized chickens, the characteristics were very similar to that of normal males (Fig. 3-2; A and B).

(1) Head furnishing: In some cases (low degree) the combs, and wattles developed like the female controls. But in other cases (middle degree) the growth of the comb and wattles were significantly higher than that of normal females' ($p<0.05$). In the highly masculinized chickens their growth was very noticeable compared to the normal hens', but couldn't attain the size of the normal males'.

(2) Plumage: In almost all of ovariectomized chickens the sheen, shape and characteristics of the feathers were completely changed to those of a male. The saddle, neck and back feathers, remarkably changed. They were distinctly extended, and the tips were thinner and longer. The tail feathers also extended significantly longer ($p<0.05$) after the ovariectomy.

(3) Spurs: Normal hens rarely have spurs, but when the ovaries were removed, they began to develop. They were significantly longer than that of normal hens' ($p<0.05$) and not very different from males'.

Table 3-3. Comparison of secondary sex characteristics among male, female and ovariectomized female chickens*

		no. of chickens	body weight (kg)	wattles (g)	comb (g)	spurs (cm)
male	control	5	2.48±0.16[a]	12.77±1.11[a]	45.22±4.78[a]	1.59±0.17[a]
ovariectomized female[1)]	I	2	1.68±0.03[b]	8.91±1.10[b]	32.77±0.75[b]	1.59±0.24[a]
	II	11	1.68±0.09[b]	6.02±1.31[c]	19.13±5.58[c]	1.47±0.41[a]
	III	14	1.65±0.11[b]	1.50±0.57[d]	3.48±2.46[d]	1.27±0.33[a]
female	control	5	1.65±0.03[b]	1.52±0.32[d]	3.40±0.76[d]	0.31±0.05[b]

* Values are expressed as mean±S.D.

a,b,c,d Means within a column with different superscripts are significantly different ($p<0.05$).

1) I: High degree of masculinity, II: Middle degree of masculinity, III: Low degree of masculinity.

(continued in Table 3-3.)

tail feather		neck feather		back feather		saddle feather	
length (cm)	width	length (cm)	width	length (cm)	width	length (cm)	width
37.27±1.47[a]	3.43±0.30[a]	11.43±0.53[a]	3.03±0.14[a]	9.45±0.44[a]	2.46±0.35[a]	12.30±0.64[a]	2.59±0.41[a]
37.28±1.35[a]	3.24±0.29[a]	11.41±1.28[a]	3.04±0.10[a]	9.34±0.11[a]	2.44±0.41a	12.78±0.54[a]	2.77±0.42[a]
36.50±2.32[a]	3.21±0.32[a]	11.11±0.93[a]	2.90±0.28[a]	9.24±0.68[a]	2.38±0.29[a]	11.95±0.91[a]	2.73±0.25[a]
36.08±2.34[a]	3.23±0.24[a]	11.39±0.98[a]	3.00±0.35[a]	9.49±0.64[a]	2.47±0.26[a]	11.59±0.88[a]	2.54±0.26[a]
17.55±1.34[b]	3.44±0.35[b]	6.08±0.29[b]	1.47±0.35[b]	7.62±0.19[b]	2.59±0.30[a]	9.50±0.48[b]	2.70±0.29[a]

(4) Body size: The ovariectomized female chickens appeared almost like males on the outside. However, their body weights were significantly smaller compared to the male controls ($p<0.05$).

3. Histological structure of the transformed right gonads

Compared to the normal male testes, the size and weight of the transformed right gonads were significantly smaller ($p<0.05$), but varied from one to another (Table 3-4).

The right gonads which transformed in compensation after the ovariectomy had some variations in structures. In some cases the structure of the gonads were very similar to that of a normal hen's, in others it became testicular tissue showing various stages of spermatogenesis. And there was several variations of each types among these chickens. However, most of these tissues forming the gonads were composed of the medullary cord and cortical tissues. Consequently, the tissues constituting the right gonads may be classified as follows:

1) Testicular gonad, 2) Ovo-testis, 3) Ovary-like gonad

1) Testicular gonad: These tissues mainly originated from the medullary cord. They were consisted of several kinds of tissues. In some seminiferous tubules, many stages of the spermatogenesis were found. The development of the seminiferous tubules varied according to individual. In the most developed cases, the seminiferous tubules were regularly-shaped and enclosed a large lumen. In its lumen spermatogonia, spermatocytes and a few spermatid were observed. But when compared to the normal testes (Fig. 3-2; a) their spermatogenesis were not so active (Fig. 3-2; b).

Table 3-4. Comparison of gonad between male (testes) and treated female (right gonads) chickens*

		no. of chickens	weight (g)	length (cm)	width (cm)
male control		5	9.76 ± 0.68^{a}	4.30 ± 0.27^{a}	2.01 ± 0.06^{a}
ovariectomized female[1)]	I	2	0.51 ± 0.01^{b}	1.78 ± 0.04^{b}	0.94 ± 0.02^{b}
	II	11	0.49 ± 0.05^{b}	1.77 ± 0.12^{b}	0.87 ± 0.25^{b}
	III	14	0.47 ± 0.12^{b}	1.62 ± 0.22^{b}	0.80 ± 0.13^{b}

* Values are expressed as mean±S.D.

a,b Means within a column with different superscript are significantly different ($p<0.05$).

1) See Table 3-3 for detail.

2) Ovo-testis: These gonads were composed of both ovary-like and testicular tissues originated from cortical and medullary tissues, respectively.

A retarded seminiferous tubules contained a large number of spermatogonia and spermatocytes but no spermatid were found (Fig. 3-2; c and d). The cortical tissues were degenerated in some cases and they had very retarded ovarian follicles. Testicular tubules were found in the medullary tissues and their structures were varied from one to another.

In some of the most retarded seminiferous tubules, degeneration of tissues had taken place. In these tubules, most of the spermatocytes underwent regeneration and eventually disappeared, and a number of spermatogonia remained in the tubular wall.

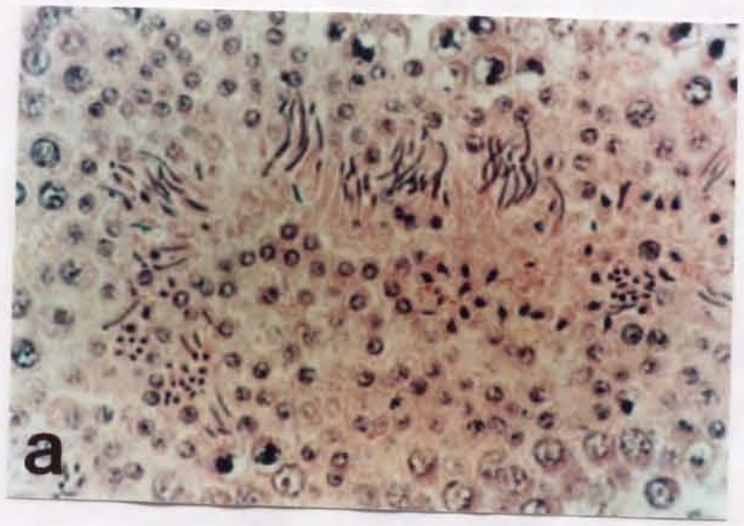
3) Ovary-like gonad: In some cases, the right genital gonads originated from cortical tissues (Fig. 3-2; e), and they were composed of many kinds of tissues.

The ovarian follicles were small in size, but follicles had basically the same structure as normal follicles (Fig. 3-2; f). Each of them had a wall formed by a layer of round follicle cells and ovum. In small follicles, the ovum was normal, and had no sign of degeneration.

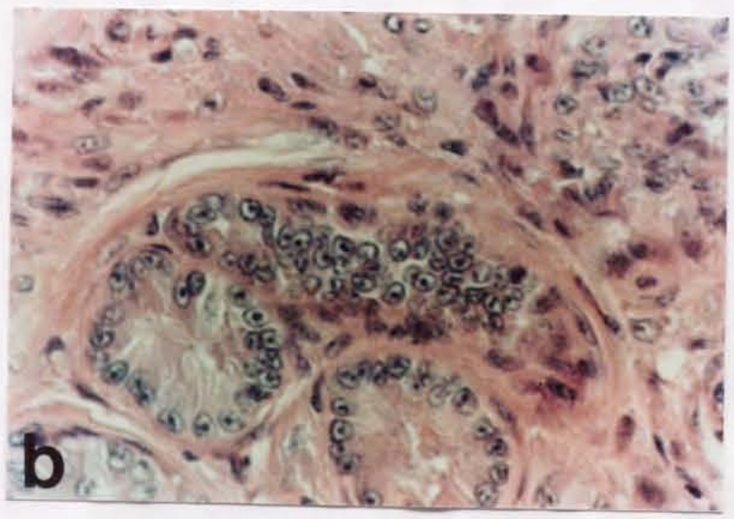
The degree of masculinity as determined from outward appearance, especially the comb growth, was almost commensurate with the masculinization of the transformed right gonad. Two of the gonads were classified to the testicular gonad, thirteen to ovo-testis and twelve to ovary-like gonad, respectively.

Fig. 3-2. The secondary sex characteristics of chickens used and histological observation of its gonad. Male control: Normal male appearance (A). It shows active spermatogenesis (a, x 600). High degree of masculinity: The secondary sex characteristics of this chicken have been completely changed to that of a normal male's (B). It shows various stages of spermatogenesis (b, x 600). Middle degree of masculinity: The secondary sex characteristics of this chicken have changed noticeably from that of a normal female's (C). It shows many spermatogonia and spermatocytes (c, x 600). Middle degree of masculinity: The secondary sex characteristics of this chicken have been very differed from that of a normal female's (D). It shows spermatogonia and spermatocytes (d, x 600). Low degree of masculinity: The secondary sex characteristics of this chicken have been very differed from that of a normal female's. But the development of wattles and comb is almost the same as that of a female's (E). It shows the complex of medullary and cortical tissues (e, x 600). Female control; Normal female appearance (F). Normal ovary (f, x 600).


A


a


B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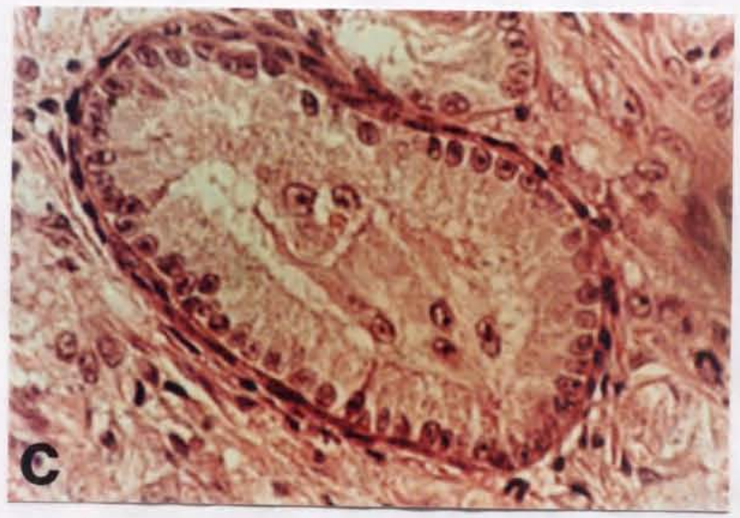
C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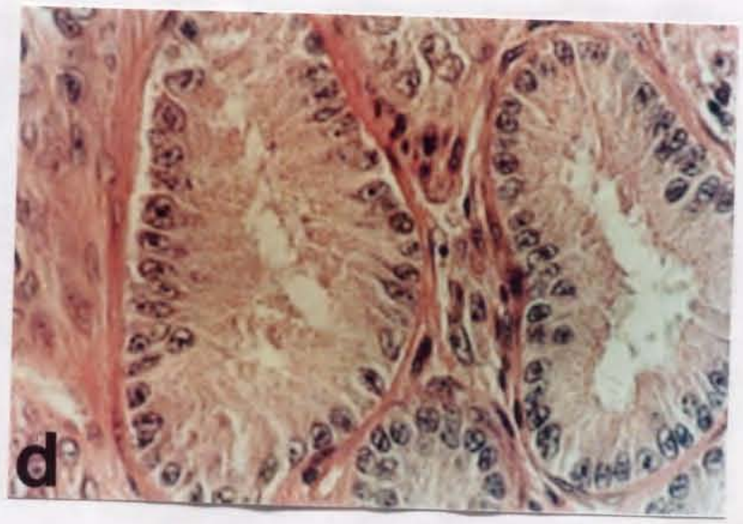
D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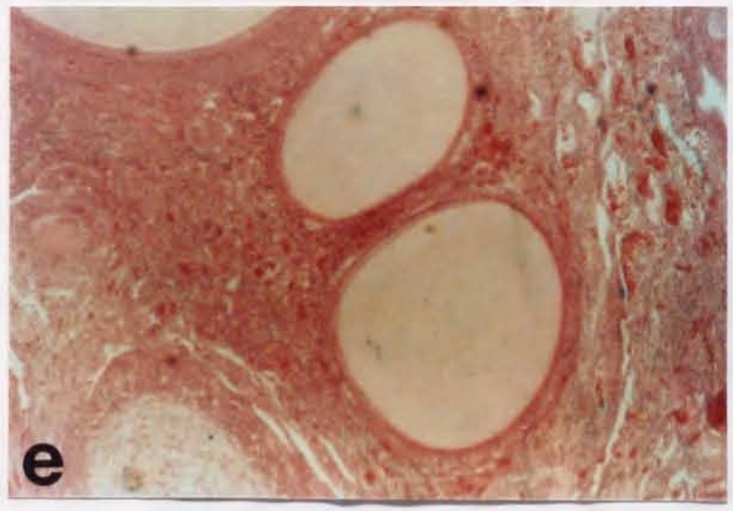
E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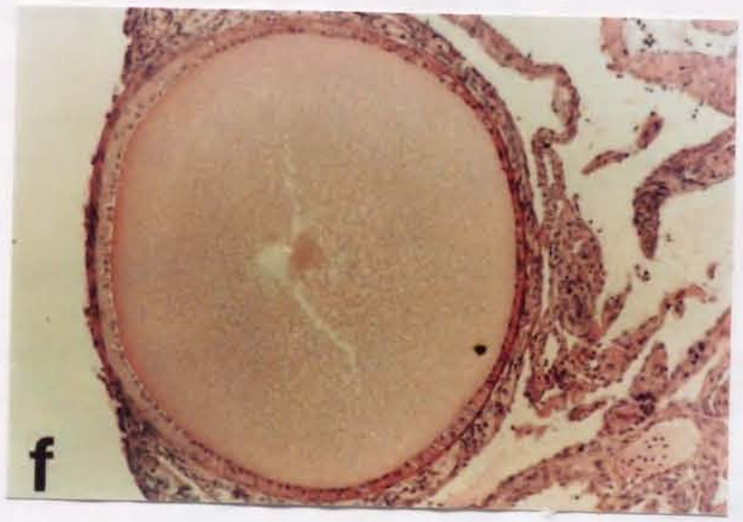
F


f

Discussion

The right gonad of the female chick normally degenerates after hatching. If an ovariectomy is performed while the medullary cord is still in the gonad, it will become a testicular structure, which can vary widely from an ovary to a testes[12,14,21]. It is natural to expect that the right gonad develops into an ovary, since it is also a female organ. Nevertheless, it can actually develop into a testicular gonad.

The mechanism for the transformation of the right gonad into a testicular gonad has not yet been confirmed. But the sex-reversal must depend on an increased release of gonadotrophins related to the stimulation of the hypophysis, since it doesn't occur in ovariectomized-hypophysectomized chickens[28]. It is well known in mammals that there is a functional link between the hypothalamus and the hypophysis. The hypothalamus secretes gonadotrophin-releasing factors and it regulates the level of gonadotrophin in the hypophysis, and thus the reproductive processes are influenced. In avian species, the hypothalamus and its link with the hypophysis are quite similar to those in mammals[29]. These may be important in controlling the release of the gonadotrophins such as LH and FSH.

Since the cortical tissue of the left gonad is a target organ for estrogen, ovariectomy involves removal of the target organ for estrogen. Consequently, negative feed back of the estrogen was induced. After the ovariectomy, changes are occurred in the anterior lobe of the hypophysis in a masculinized chicken.

The structure of the hypophysis becomes similar to those of castrated males[30]. The transformed hypophysis may cause an enhanced release of gonadotrophins, as it occurs for LH in the ovariectomized duck[31].

In the right rudimentary gonad, steroidogenic cells are reactivated by the gonadotrophins and some of them contribute to the formation of testicular tubules and to the Leydig cell population. Some other steroidogenic cells are also transformed into Sertoli cells[21].

Controlled by LH release from the hypophysis, testosterone may be secreted from the Leydig cell. Activation of the medullary tissue of the target organ for testosterone, was induced in the right gonad.

FSH might cause Sertoli cell hypertrophy and germ cell development which exist in the medullary cord of the right gonad at the time of ovariectomy. In both male and female of normal chicks, at about nine days after hatching, testosterone is secreted in small amounts from their gonads. Estrogen is mainly present in the female's left ovary. Then testosterone in the male and estrogen in the female become the predominant steroid at all stages until their maturity[32,33].

In the ovariectomized female it was shown that the right gonad, which had androgen-producing potential at nine days, produces more estrogen at two months. However, at five months the testicular gonad appears to produce twice as much testosterone as the two normal testes[22].

In the normal female's left gonad, the medullary tissue develops first and then the cortical tissue proliferates. The

medullary tissue gradually regresses, while the cortical tissue proliferates to form an ovary. In the male testis the medullary tissue gives rise to the development. The ovary begins to develop on the right side during the development of the chick embryo, but it soon stops developing and regresses. Just after hatching, the retarded medullary tissue still remains in the right gonad. When the ovary is removed the medullary is reactivated [14].

Tubular spermatogenesis is proceeded through some cell divisions. The spermatogonia divide spermatocytes and then the spermatocytes undergo a reduction division to yield spermatid, and finally spermatids differentiate into spermatozoa. These cell divisions may be promoted by the stimulation of testosterone which is secreted from Leydig cells.

The secretion of the testosterone from the initially transformed right gonad induced masculinization of the secondary sex characteristics, such as comb growth, spur development and plumage change. But the body weight of sex-reversed female could not attain the weight of male. It was suggested that the body weight is determined genetically.

In recent years, synthesis of steroid sex hormones has been demonstrated in young embryos during or even before sex differentiation. Enzymes that catalyze the synthesis of these hormones, such as hydroxysteroidodehydrogenase also have been discovered. Further research has been oriented toward the characterization of steroid hormones. Specific proteins are being isolated in the effectors. It is suggested that these

proteins work as receptors for steroid hormones. Nuclear receptors of estradiol have been discovered in the embryonic gonads and in the clocal wall at the time of sex differentiation. Thus a mechanism can be conceived in which protein and steroid hormones play mutual roles in the process of sex differentiation[23].

It has been expected that the reproductive cells of the transformed right gonad may contain the heterozygotic sex chromosome pattern (ZW), since the genetical female has heterozygotic chromosomes (ZW) in avian species.

A possible process of artificial sex-reversal from female to male is presented in Fig. 3-3.

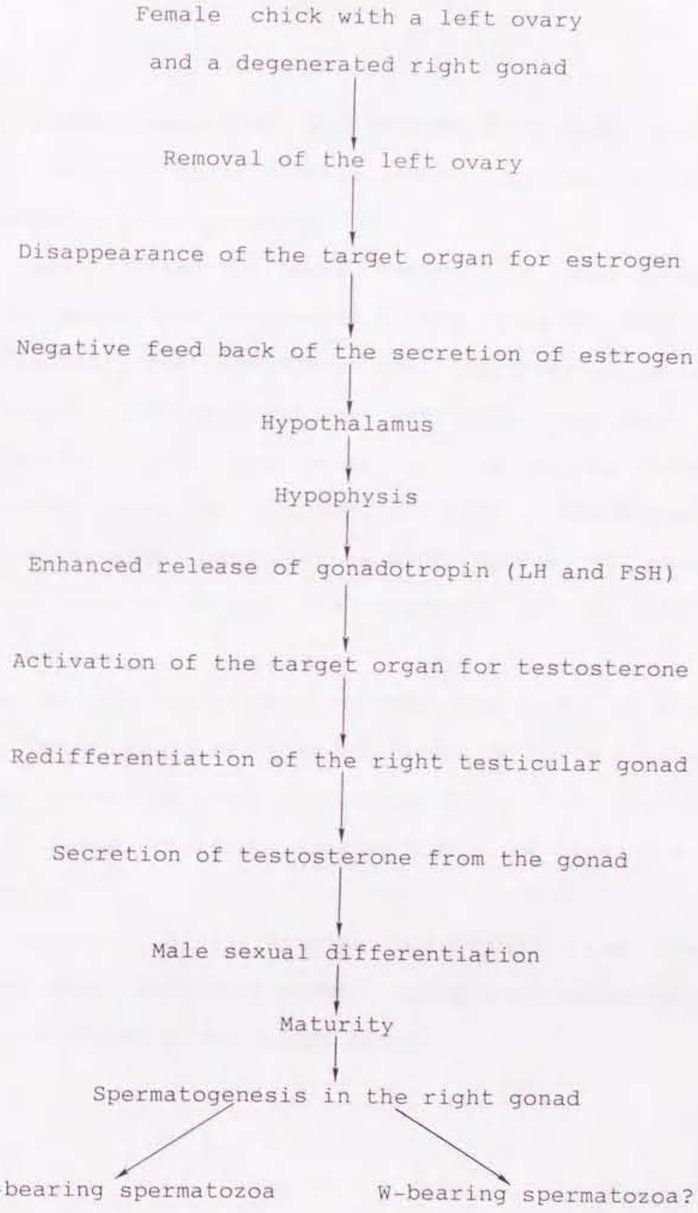


Fig. 3-3. A possible process of artificial sex-reversal of a female to a male chicken

Summary

The present experiment was conducted to cause sex-reversal in female chickens whose genetic sex has been confirmed by the modern karyotyping techniques.

The sex-reversed chickens induced by ovariectomy were classified into three categories on the basis of secondary sex characteristics. The secondary sex characteristics of these ovariectomized females were very different from that of the normal females, and, especially in the highly masculinized chickens, they were very similar to that of the normal males. Two chickens were classified as High degree of masculinity, eleven as Middle degree and fourteen as Low degree of masculinity.

When the left ovary was removed, the right gonad began to develop. Though there was gonadal variation in structure, they were also classified into three categories; two classified as testicular gonad, thirteen as ovo-testis and twelve as ovary-like gonad.

The degree of masculinity as determined from the outward appearance, especially comb growth was almost commensurate with the masculinization of the right gonad.

Chapter IV

Development of a New Method for Sex Identification in Chickens

Introduction

Sex identification of chicks at hatching by differences in morphology and genital process has been applied for commercial poultry breeding [34]. But this method is not available for identification of sex in chicken embryos needed for the studies in developmental biology. Therefore, genetic sex identification in chickens has been done by chromosome analysis [35,36]. A series of techniques for analyzing chicken karyotypes has been performed. Microculturing method for chick leukocytes has been reported. Since the W chromosome could be specifically stained, C-banding method was developed to identify the chromosome [37].

However it is very difficult to identify the sex, especially in females by means of chromosomal studies, because of the large number of chromosomes (2n=78), and the smallness of the W chromosome. Furthermore the repeatability of obtaining a clear metaphase preparation with culturing leukocytes was low [5].

Recently, it was found that the DNA fragments of about 0.7 kbp and 1.1 kbp could be separated specifically in female chickens by digestion of the genomic DNA with a restriction enzyme, XhoI [38,39].

For the purpose of developing a new method of sex identification in chickens, detection of the female-specific DNA fragments was applied. This method was also applied to early embryonic stages.

Materials and Methods

Preparation of the genomic DNA from whole blood.

Blood samples were taken from the wing vein of male and female chickens, with a heparin-wetted syringe. About 1 ml of blood was washed in 10 ml of Dulbecco's PBS(-) solution. After centrifuging at 1,500 r.p.m. for 5 minutes, red blood cells were resuspended in 10 ml of the buffer (50 mM Tris-HCl of pH 8.0, 25 mM EDTA and 100 mM NaCl). SDS and Proteinase K were added to the cell suspension at final concentration of 0.5% and 250 µg/ml respectively and incubated at 37°C for several hours. The samples were extracted three times with an equal volume of phenol that had been equilibrated with Tris buffer (pH 8.0). The nucleic acids were precipitated from the aqueous phase after adding two volumes of ethanol and was dissolved in a 1 X TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). The samples were further digested with RNase A and RNase T_1 (final concentration 50 µg/ml and 100 units/ml, respectively) at 37°C for 3 hours and purified by repeating extraction with phenol. The DNA was recovered by precipitation with ethanol and was finally dissolved in a 1 X TE buffer. The DNA concentration and the ratio of 260nm/280nm of the samples were measured with a spectrophotometer (Beckman's DS Series 60).

Digestion of genomic DNA with restriction endonuclease.

To determine the optimum reaction condition of XhoI for the digestion of genomic DNA, the dose of enzyme and reaction time were checked. Twenty five µg of male or female DNA was digested with 75 or 150 units of XhoI (Toyobo Co., Ltd.) at 37°C for 8

hours. The reaction was stopped at 8 hours by adding stopping solution (final concentration, 25mM EDTA, 0.2% SDS, 100μg/ml proteinase K and 100mM NaCl). After the digestion, DNA was precipitated with ethanol and dissolved in 1 X TE buffer. The digested DNA was loaded directory onto a conventional 1.2 % agarose gel and subjected to electrophoresis for 3 hours at 75 V. After the electrophoresis, the DNA in the agarose gel was stained with fluorescent dye, ethidium bromide, and photographed through a UV transilluminator at 254 nm.

The reaction time was also examined. Twenty five μg of male or female DNA was digested with 75 units of the enzyme at 37 °C. The reaction was stopped at 0.5, 4 and 8 hours by adding a stopping solution. The digested DNA was electrophoresised, stained and photographed by the same procedure as described above.

Application for sex identification of 7 day-old embryos.

After the determination of the applying condition, the procedure was applied to 7 day-old embryos. A small window was opened in the eggshell and eggshell membrane was removed carefully with a pair of forceps. Just beneath the membrane, a capillary vessel was observed. About 20 μl of blood was taken from the capillary vessel with a syringe for 1 ml having a needle (26G x 1/2, Terumo Co.,Ltd.). Using the same procedures described above, the whole blood samples were subjected for identification of sex.

Results

The original DNA of male and female chickens, extracted and electrophoresised was presented in Fig. 4-1. The DNA showed a high molecular weight.

The DNA samples of male and female were digested with XhoI. As shown in Fig. 4-2, pattern of digested DNA was obviously different between male and female. In the female sample, a 0.7 kbp DNA fragment was clearly detected as a dense band (Fig. 4-2; lane 6 and lane 7). On the other hand, it could not be detected in the male (Fig. 4-2; lane 3 and lane 4). This DNA fragment must be one of the W chromosome-specific DNA fragments. The 0.7 kbp fragment from the female DNA was useful to identify the sex of chickens, because it was fairly well separated from the other DNA fragments by the agarose gel electrophoresis. The optimum dose of XhoI for digestion of the chicken DNA was more than about 10 times needed for the digestion of the same quantity of the λ DNA.

When the reaction times of 0.5, 4 and 8 hours were compared, the 0.7 kbp fragment in the female was more clearly observed at 4 hours than 0.5 hour (Fig. 4-3; lane 2 and lane 3). There was little difference between 4 and 8 hours (Fig. 4-3; lane 3 and lane 4). Reaction time over 4 hours did not seem to improve the results.

When the procedure was applied to the 7 day-old embryo (Fig. 4-4), the individual could be identified for the sex (Fig. 4-5; lanes 2, 5, 7, 8, 9 and 10 were identified as females and the others in lanes 3, 4, and 6 were identified as males).

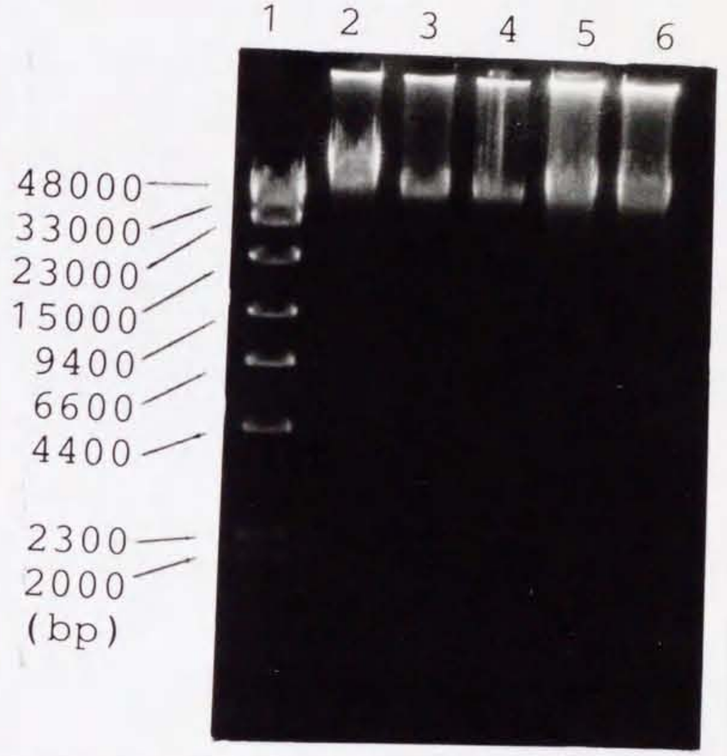


Fig. 4-1. Extraction of chicken genomic DNA from whole blood. The DNA size marker (λ-XhoI digest) in lane 1 and original DNA (25 μg) of female in lane 2, lane 3, lane 4 and male in lane 5, lane 6 were shown, respectively.

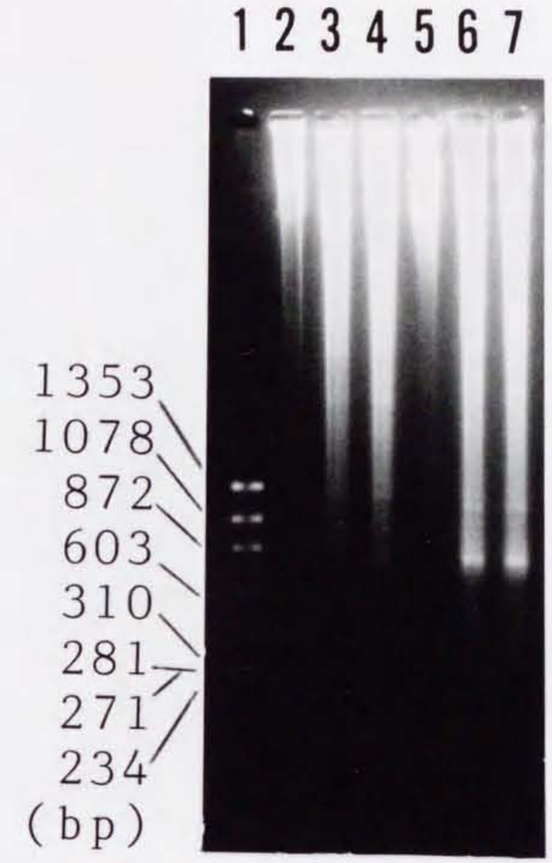


Fig. 4-2. Sex identification by detecting the female specific repetitive DNA fragments. The male DNA sample (25 μg) was digested with 75 (lane 3) and 150 units (lane 4) of XhoI. The female DNA sample (25 μg) was also digested with 75 (lane 6) and 150 units (lane 7) of XhoI. The DNA size marker (ϕx174-HaeIII digest) and the original DNA (25 μg) of the male and female are shown in lane 1, lane 2 and lane 5, respectively.

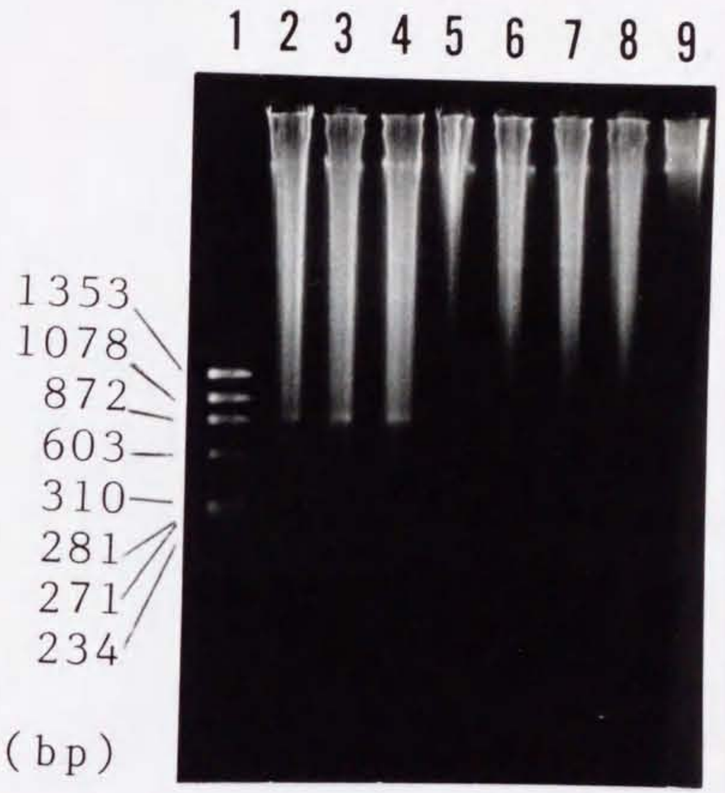


Fig. 4-3. Sex identification by detecting female specific repetitive DNA fragments. The female DNA sample (25 μg) was digested with 75 units of XhoI for 0.5 hour (lane 2), 4 hours (lane 3) and 8 hours (lane 4). The male DNA sample (25 μg) was also digested with 75 units of XhoI for 0.5 hour (lane 6), 4 hours (lane 7) and 8 hours (lane 8). The DNA size marker (ϕx174-HaeIII digest) and original DNA (25 μg) of female or male is shown in lane 1, lane 5 or lane 9, respectively.

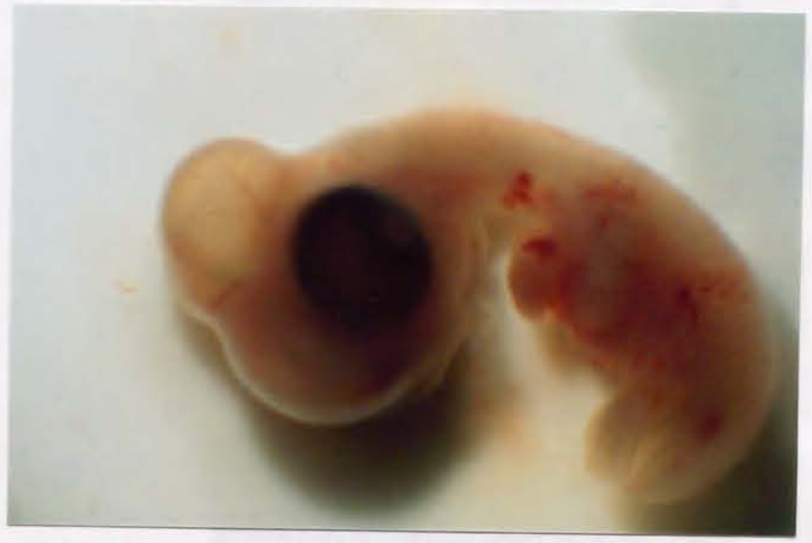


Fig. 4-4. The 7 day-old embryo of a chicken. In this stage, morphological differences in the gonads between male and female could not be observed. Also there was insufficient blood for karyotype analysis. Consequently, it was very difficult to identify the sex with morphological or karyotyping method.

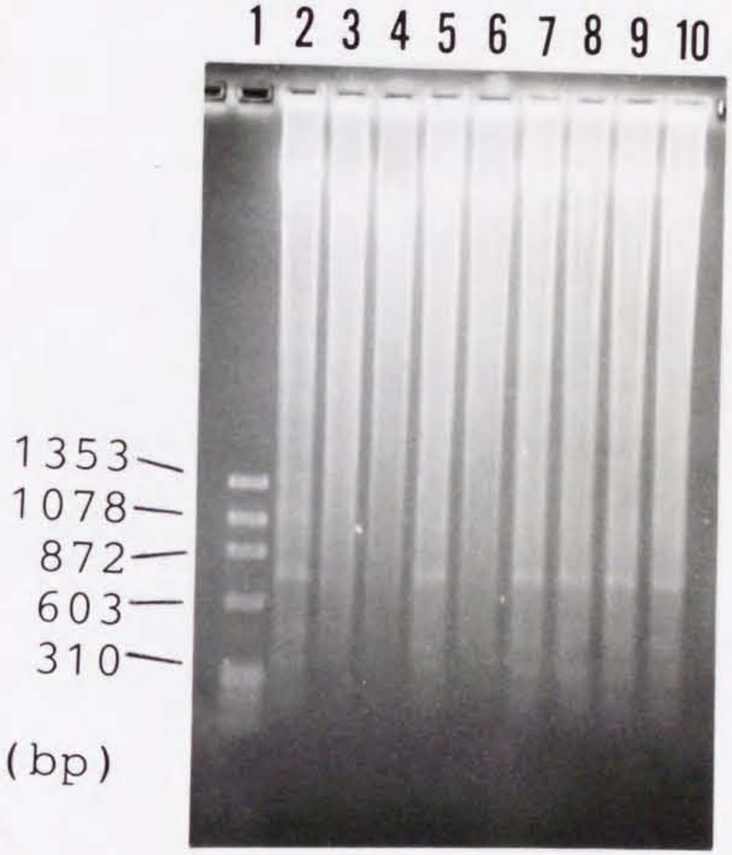


Fig. 4-5. Sex identification of 7 day-old embryos by detecting the female specific repetitive DNA fragments. The DNA samples were purified from whole blood and digested with 75 units of XhoI. The molecular size marker (ϕx174-HaeIII digest) was shown in lane 1. The individuals in lanes 2, 5, 7, 8, 9, and 10 were identified as females. And the others in lanes 3, 4, and 6 were identified as males.

Discussion

Sex identification in chickens has been tried with cytogenetic methodology which revealed the structural differences of the chromosomes[4]. It is now established that the chicken has sex chromosome of ZZ in males and ZW in females. However, karyological demonstration of the W chromosome in the female was very difficult. Because the chromosomes were so numerous (range = 52-92), and the microchromosomes often resembled each other[3].

When the chicken genomic DNA of female or male was digested by XhoI, a dense band was detected specifically in female at about 0.7 kbp but not in male. Since 0.7 kbp DNA sub-fragment is repeating about 14,000 times in the W chromosome of the chicken[40], it was easily detected as a clear and dense band. Consequently, it becomes a useful marker to identify the sex of chickens.

As the red blood cells of the chicken are nucleated, it is easy to obtain large quantities of DNA from a small volume of blood. Using the same method, sex identification could easily be achieved with only a small quantity of blood (less than 20 μl). When the procedure was applied to the 7 day-old embryo, which shows no morphological differences between male and female (see chapter II for detail), sex identification was also achieved. This technique could be expanded to identify sex at any stage in the life of the chickens.

Summary

In the present study, a simple and reliable method for sex identification in chickens is developed by detection of the W chromosome specific repetitive DNA units. The genomic DNA purified from blood cells was digested with restriction enzyme XhoI. A DNA fragment of 0.7 kbp was detected in female but not in male.

Since the red blood cells of the chicken are nucleated, it is possible to obtain enough DNA preparation from a small volume of whole blood. The detection of the female-specific repetitive DNA units is simple, reliable and efficient. By applying this method, sexing of chicken could be achieved even in the embryonic stages.

Chapter V

Molecular Genetic Analysis of the W Chromosome in Chickens

Introduction

Genetic sex is determined just after fertilization of an ovum (Z or W) with a spermatozoon (Z) in chickens. However, during the sexual indifferent stages, there is no morphological differences between male and female. Primary sex determination is initiated with gonadal differentiation into testes in male or ovary in female [10].

Since the W chromosome is female specific, it has been considered that the genes on the W chromosome plays important roles in primary sex determination.

Many scientific works have been performed to clarify the role of the W chromosome. In 1962 direct cytological evidence for the W chromosome, a female-specific sex chromosome, was presented as an unpaired acrocentric chromosome in the size class of No. 7-8 in female chicken cells [34]. It was reported that about 17% of chicken genome was composed of repetitive DNA. Two DNA subfractions were found, one at 1.715 and the other at 1.698 g/cm of CsCl centrifuge [37]. It was found that entire region of the W chromosome in metaphase chromosome are stained with C-banding method. These findings suggested that those chromosomal regions are heterochromatic [41].

Recently, analysis of the W chromosome at molecular level has been performed. It was found that about 50% of the DNA in the W chromosome consisted of a repetitive DNA family, in which fragments of about 0.7 kbp and 1.1 kbp. And the W chromosome-specific DNA fragments were cloned [38,39].

However, the role of the repetitive DNA in the W chromosome is still uncertain. And the knowledge about the W chromosome at molecular level is scanty compared to mammals [10].

In the present chapter, molecular analysis of the W chromosome was done by hybridization of the cloned W chromosome-specific DNA probe to the XhoI digested DNA. The roles of the DNA fragments for sex determination and sexual differentiation in chickens was discussed.

Southern transfer of the DNA from agarose gel to nylon membrane.

Blood samples of about 1 ml were taken from the wing vein of male and female chickens with a heparin-wetted syringe. Using the same procedure described in chapter IV, high molecular DNA was prepared and digested with XhoI. The DNA was subjected to electrophoresis in agarose gel, stained with ethidium bromide and photographed through a conventional UV transilluminator at 254 nm. The DNA was denatured by soaking the gel with 150 ml of alkaline buffer (0.2N NaOH and 600 mM NaCl) and neutralized with 150 ml of neutralization buffer (240 mM Tris-HCl (pH 7.5) and 600 mM NaCl). The gel was placed on a sheet of filter paper (Whatmann 3MM) saturated 10 x SSC buffer. A sheet of nylon membrane (Amersham, Hybond-N+), the same size to the gel, was placed on top of the gel without trapping air bubbles. Three sheets of filter paper and several sheets of paper towel were placed on the membrane. A glass plate was put on top and a weight about 700g was applied on the plate. Southern transfer of the DNA from the agarose gel to the membrane was continued for overnight at room temperature [42].

Preparation of the W chromosome-specific DNA probe.

One ng of plasmid DNA (pUGD06000) which contained the W chromosome-specific 0.7kbp DNA unit was added to 50μl of competent cells (Escherichia coli DH5α) on ice. One ml of LB medium was added and incubated for 1 hour at 37 °C. The transformed bacteria was streaked on LB plate containing

Ampicilin and incubated for overnight at 37°C. A single colony of the bacteria was transferred to 3.5ml of LB medium containing Ampicilin and incubated for overnight at 37°C. Lysis of the bacteria was done by adding 200μl of alkaline solution (0.2N NaOH and 1% SDS) and the plasmid DNA was harvested. The DNA was double digested with restriction enzymes BamHI and PstI. The digested DNA was subjected for electrophoresis in 0.8% agarose gel to obtain the W chromosome-specific 0.7kbp DNA unit. The DNA was labeled with horseradish peroxidase (Amersham) and used as a DNA probe.

Hybridization of the DNA probe to the digested genomic DNA.

The nylon membrane which contained the transferred DNA was set into the plastic bag and the hybridization buffer (Amersham) with 500 mM NaCl was poured. Pre-hybridization was done for 1 hour at 42°C. Initially labeled DNA probe of 100 μl was added to the bag and the hybridization was carried out at 42 °C for overnight. After washing the membrane with primary wash buffer (6M Urea, 0.4% SDS and 0.5xSSC) and secondary wash buffer (2xSSC), 0.0625ml/cm² membrane of the detection solution (Amersham) was added and incubated precisely 1 minute at room temperature. The membrane was placed to a film cassette with the DNA side up. A sheet of autoradiography film (Hyperfilm-ECL, Amersham) was put on the membrane and exposed for 1 hour. The stringency of the hybridization was chosen optionally changing the concentration of SSC in primary wash buffer and the temperature during washing the membrane.

Results

When the genomic DNA of the chickens were digested with XhoI and stained with ethidium bromide, the patterns of the DNA were easily distinguished between the male and female. In the female sample, a DNA fragment was clearly detected as a dense band at about 0.7 kbp (Fig. 5-1; lanes 6, 7 and 8). In the male the fragment could not be detected (Fig. 5-1; lanes 2, 3, 4 and 5).

Hybridization of the W chromosome-specific DNA probe to the initially digested and transferred genomic DNA showed clearly difference between male and female. In the high stringency which allowed less than 5% of base-pair mismatches, hybrid band was detected only at about 0.7 kbp in female (Fig. 5-2; lanes 5, 6 and 7). In the male any hybrid band could not be detected (Fig. 5-2; lanes 1, 2, 3 and 4).

In the more low stringency which allowed about 40% of base-pair mismatches, hybrid band was detected not only around 0.7kbp but also at about 0.6 and 0.3kbp specifically in female (Fig. 5-3; lanes 5, 6 and 7) but not in male (Fig. 5-3; lanes 1, 2, 3, and 4).

In another case (Fig. 5-4), hybridization was performed extremely low stringency which allowed about 60% of base-pair mismatches. Hybridization bands were detected at almost all region of the genome in female (Fig. 5-4; lanes 1and 2) but it could not be detected in male (Fig. 5-4; lanes 3 and 4).

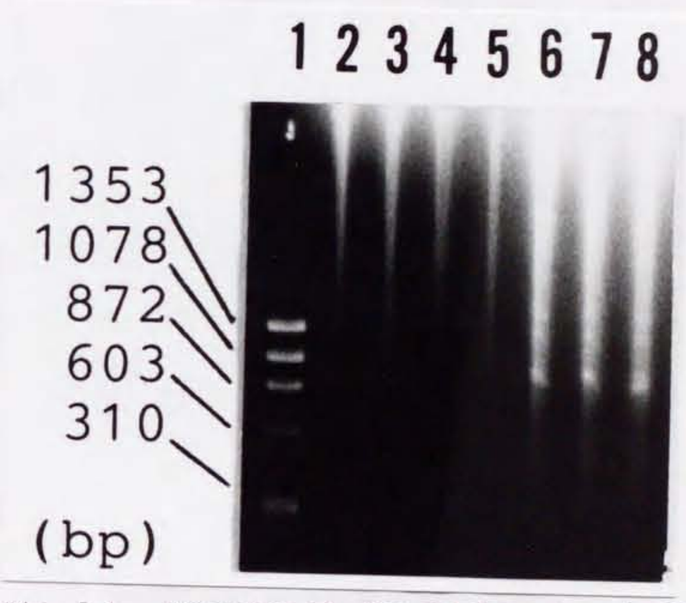


Fig. 5-1. Digestion of the chicken genomic DNA with XhoI and detection of the female specific DNA fragments. The male genomic DNA was digested with XhoI (lanes 2, 3, 4 and 5). The female genomic DNA was also digested with XhoI (lanes 6, 7 and 8). The DNA size marker (ϕx174-HaeIII digest) was shown in lane 1.

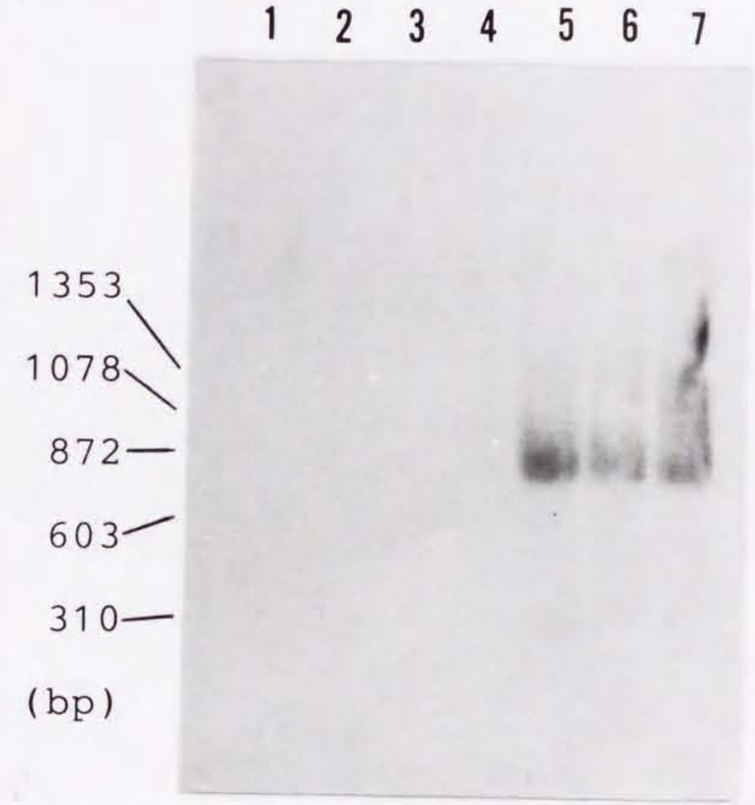


Fig. 5-2. Hybridization of the W chromosome-specific DNA probe to the initially digested chicken genomic DNA. The male genomic DNA was in the lanes 1, 2, 3 and 4. Any hybridization band could not be detected. The female genomic DNA was in the lanes 5, 6 and 7. A hybridization band with the probe was detected at about 0.7kbp. Hybridization was performed in the condition which allowed less than 5% of base-pair mismatches.

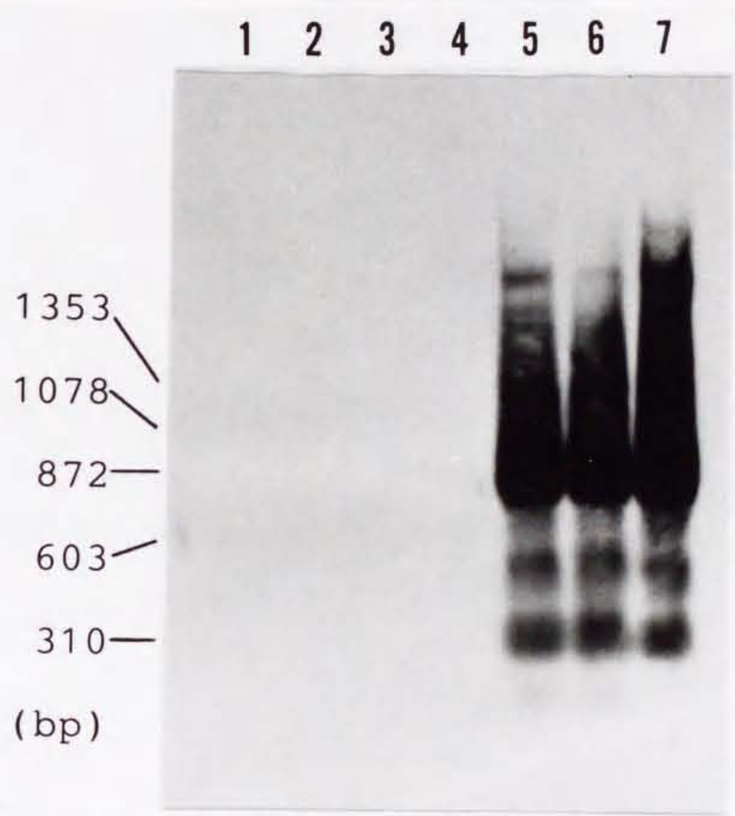


Fig.5-3. Hybridization of the W chromosome-specific DNA probe to the initially digested chicken genomic DNA. The male genomic DNA was in the lanes 1, 2, 3 and 4. Any hybridization band could not be detected. The female genomic DNA was in the lanes 5, 6 and 7. Hybridization bands with the probe were detected not only at 0.7kbp but also at about 0.6 and 0.3kbp. Hybridization was performed in the condition which allowed about 40% of base-pair mismatches.

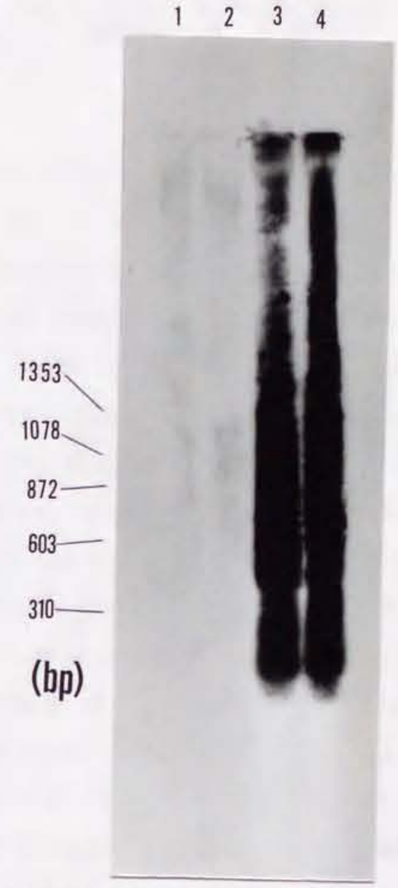


Fig. 5-4. Hybridization of the W chromosome-specific DNA probe to the initially digested chicken genomic DNA. The female genomic DNA was in the lanes 3 and 4. Hybrid was detected at almost all region of the genome. The male genomic DNA was in the lanes 1 and 2. Any hybridization could not be detected. Hybridization was performed in the condition which allowed about 60% of base-pair mismatches.

Discussion

Since the combination of sex chromosomes in chickens is heterogametic (ZW) in female and homogametic (ZZ) in male, it is suggested that the induction of the gonad to ovary was regulated by the ovary determining gene located on the W chromosome.

In chapter IV, digestion of the chicken genomic DNA with restriction enzyme XhoI and staining with ethidium led the detection of a DNA fragment at about 0.7 kbp in female. In the present chapter, hybridization of the W chromosome-specific DNA probe to the digested DNA in the high stringency also showed a hybrid band at about 0.7kbp in female. Consequently, it was identified that the initially detected band at about 0.7kbp correspond to the W chromosome-specific DNA fragment. As the stringency of the hybridization decreased, more hybrid bands were detected over wide regions in the genome of the female. These results suggested that the XhoI related sequences exist in the wide range of the female genome.

It was found that the cloned W chromosome-specific DNA fragment of about 0.7kbp contained open reading frames (ORFs) at each end of the unit[40]. Relatively long ORFs has been detected in mammals as long interspersed repetitive DNA sequences. Some of these sequences are transcribed and show significant homology with the sequence of reverse transcriptase[43,44]. In contrast, a number of tandem repeats of about 21bp exists within the repetitive DNA unit in chickens, suggesting few possibility of

the DNA regions to be transcribed to RNA. It is more likely that these ORFs were formed fortuitously as a consequence of the tandem duplication[45].

It was found that about 50% of the DNA in the W chromosome is shared by a repetitive XhoI family which fragments are constituting heterochromatin. XhoI family-related repetitive DNA sequences in the W chromosomes were found in the turkey and pheasant. The common structural features among 0.7 kbp XhoI (chicken), 0.4kbp PstI (turkey), and 0.5kbp TaqI (pheasant) repeating units are the appearance of Adenine and Thymine clusters[46]. Higher eukaryotes have highly repetitive DNA sequences. These repetitive DNA is generally existed around centromere. For example, in the human genome the majority of such sequences belong to a single family called the Alu family which is about 0.3kbp. However, these kinds of highly repetitive DNA sequences were not specific to male or female[47]. In the present studies, it was confirmed that the XhoI family of about 0.7 kbp is female specific. In this point, the character of the repetitive sequence in chickens are very different from that of the mammals.

In chickens, entire region of the W chromosome was stained with C banding method. Generally, this method is considered to stain constitutive heterochromatin which includes satellite or highly repetitive DNA[45]. Thus, it was considered that the detected female specific DNA fragments correspond to the heterochromatic region on the W chromosome. Heterochromatin was observed as highly compacted chromatin that remains visible in the light microscope during interphase. It has been suggested

that such region contain very few genes, giving rise to the idea that heterochromatic DNA doesn't have genetic activity to be transcribed into RNA[48].

Consequently, the ovary determining gene may be located on the rest of the region than the highly repetitive DNA fragments. The genes may be transcribed into RNA at the initiation of sexual differentiation and they may contribute to the induction of ovarian differentiation.

Summary

Hybridization of the W chromosome-specific DNA probe in the high stringency showed hybridization band at about 0.7kbp in female. Therefore, it was identified that the initially detected band described in the previous chapter at about 0.7kbp correspond to the W chromosome-specific DNA fragment. As the stringency of the hybridization decreased, more hybridization bands were detected over wide regions in the genome of the female. These results suggested that the XhoI-related sequences are existed in wide ranges of the female genome.

It was considered that the detected fragments constitute the heterochromatic regions of the W chromosome. Since such regions are considered inactive genetically, the ovary determining genes may be located on the rest of the regions than the highly repetitive DNA fragments.

Chapter VI

General Discussion

The individual chicken embryo comes into existence as the result of the union of two gametes, a ovum and a spermatozoon. After the fertilization, cleavage of the zygote is proceeded [18]. Cleavage is a series of extremely rapid mitotic divisions that the enormous volume of zygote cytoplasm is divided into numerous small cells.

During undifferentiated stages, there is no morphological differences between male and female embryos. Primary sex determination is characterized by the sexual differentiation of the gonad [9]. The development of gonad is unique for its differentiation. Most of the other organ rudiments are normally destined to differentiate into only one type of organ. The gonad, however, has normally two options before sexual differentiation. During the undifferentiated stage, it has potentials to develop to either an ovary or a testis. When the sexual differentiation is induced, the gonad is led to develop into either ovary or testis, depending on its genetic sex [20].

Since the W chromosome is female specific, it has been considered that the genes on the W chromosome plays important roles in primary sex determination. The process of sex determination that depend on morphogenesis and cellular differentiation are considered to be regulated by the activity of the genes in complex interacting networks or pathways. Within these pathways, specific genes may be rate-limiting or act as a switch [10]. In the present study, molecular genetic analysis of the W chromosome was performed by digestion of the chicken genomic DNA with restriction enzyme XhoI. This

led to the detection of the female chromosome-specific repetitive DNA fragments at about 0.7kbp. When the cloned W chromosome-specific DNA probe was hybridized to the initially digested genomic DNA, a DNA band was detected also at about 0.7kbp in female. Thus, it was identified that the initially detected fragment correspond to the W chromosome-specific DNA sequence. As the stringency of the hybridization decreased, more hybrid bands were detected over wide regions of the genome in female. Consequently, the existence of the XhoI related sequences was suggested.

It was found that the cloned W chromosome-specific DNA fragment of about 0.7kbp contains open reading frames (ORFs) at each end of the unit. But it is likely that these ORFs were formed fortuitously as a consequence of the tandem duplication[40]. The entire region of the W chromosome was stained with C banding method. Generally, this method is considered to stain constitutive heterochromatin which include satellite or highly repetitive DNA[45]. Thus, it was considered that the detected female-specific DNA fragments may correspond to the heterochromatic region on the W chromosome. It was reported that there are XhoI family-related repetitive DNA sequences in the W chromosomes of turkeys and pheasants, and that they share common structural features. The features among 0.7 kbp XhoI (chicken), 0.4kbp PstI (turkey), and 0.5kbp TaqI (pheasant) repeating units are the appearance of Adenine and Thymine clusters[46]. It seemed conceivable that these repetitive DNA sequences were particularly suitable for heterochromatization[38].

Heterochromatin was observed as highly compacted chromatin that remains visible in the light microscope during interphase. It has been suggested that such region contain very few genes, giving rise to the idea that heterochromatic DNA doesn't have a genetic activity to be transcribed into RNA[48]. Consequently, the ovary determining gene may be located on the rest of the region than highly repetitive DNA fragments. The DNA may be transcribed into RNA at the initiation time of sexual differentiation and it may contribute to the induction of ovarian differentiation.

Recently the testis determining gene, which induces the differentiation of the undifferentiated gonad to testes in male, was identified on the short arm of the male specific Y chromosome in mammals[10]. These findings in mammals also support the existence of the ovary determining gene on the female specific W chromosome in chickens. However, the most significant difference in sexual differentiation in chickens compared to mammals is the asymmetric development of the gonad. This phenomenon seemed to be dependent on the difference in response to estrogen between left and right gonad. Estrogen target cells were searched for in the differentiation of the chicken embryo. The cells were observed in the germinal epithelium of the left gonad but not in the right one. It was suggested that the absence of the target cells for estrogen in the germinal epithelium of the right gonad in female accounted for the lack of cortical differentiation of the right gonad[19].

In male, asymmetric development between left and right gonad was not be observed through out all the stages of embryonic development. Thus, it was considered that no genetic regulation

has been induced in the differentiation of undifferentiated gonads to testes. Consequently, it was suggested that the fundamental sex of chickens was male. Secondary sex characteristics such as comb, wattles, spurs and feather shape were controlled by internal secretion of sex hormones depending on its sex. The male secondary sex characteristics are mainly induced by the regulation of the testosterone secreted from testes and that of female's are induced by estrogen secreted from ovary [49,50,51].

When a female chick hatches, sexual differentiation has already begun and the right gonad also has begun degeneration. But it has been known for a long time that hens could be masculinized under some rare circumstance [12,13,14]. In the present studies artificial sex-reversal from female to male was conducted by left ovariectomy in female chicks. The secondary sex characteristics such as comb and wattles growth, plumage, and spurs of these ovariectomized chickens differed very much from that of control females'. The secondary sex characteristics of highly masculinized chickens were very similar to that of the normal males. It was considered that the secretion of testosterone from the initially transformed right gonad induced masculinization of these secondary sex characteristics. When ovariectomy was performed while medullary cord is still existed in the right gonad, it could retain a testicular structure, which varied widely from an ovary to a testes. Since the cortical tissue of the left gonad is the target organ

for estrogen, ovariectomy removed the target organ for estrogen. Activation of the medullary tissue, the target organ for testosterone, was induced in the right gonad[21].

These results of artificial sex-reversal also suggest that the fundamental sex is male and female is the estrogen induced sex in chickens.

The process of sex determination and sexual differentiation in chickens is summarized in Fig. 6-1.

On the developmental studies in chickens, sex identification of embryos used is very important. Therefore, genetic sex identification in chickens and the embryos has been done by chromosome analysis[25,35]. However it was very difficult to identify the sex, especially in females, by means of chromosomal studies, because of the large number of chromosomes (2n=78), and the smallness of the W chromosome. Furthermore repeatability of obtaining a clear metaphase preparation with culturing leukocyte was low[5].

When the chicken genomic DNA of female or male was digested with restriction enzyme XhoI, a dense band was detected specifically in female at about 0.7 kbp but not in male. Since 0.7 kbp DNA sub-fragment is repeating about 14,000 times in the W chromosome of the chicken[39,40], it was easily detected as a clear and dense band in the present condition. Consequently, it becomes a useful marker to identify the sex of chickens.

As the red blood cells of the chicken are nucleated, it is easy to obtain enough quantities of DNA from a small volume of whole blood. Using the same procedure, sex identification could easily be achieved with only a small quantity of blood (less

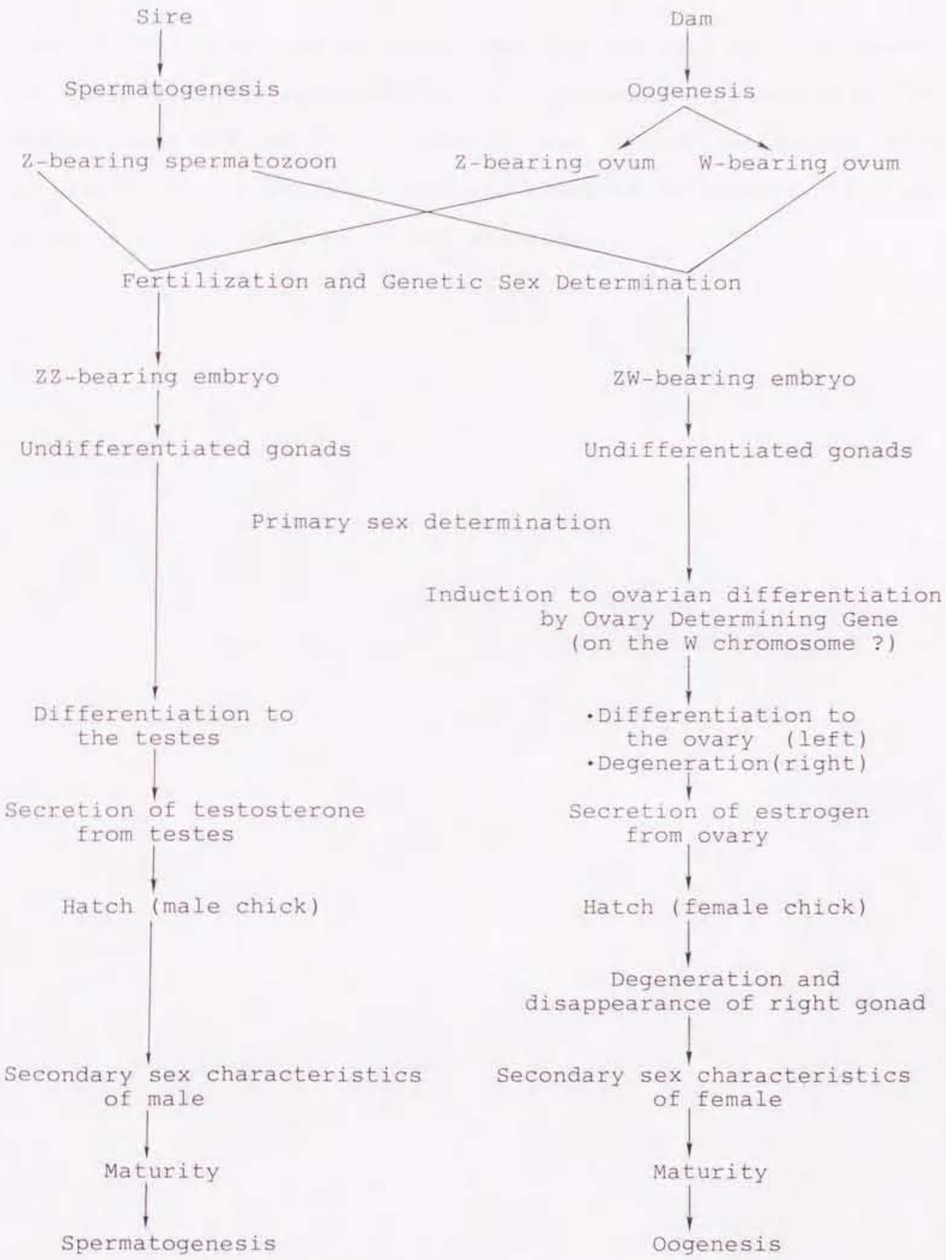


Fig. 6-1. Sex determination and sexual differentiation in chickens

than 20 µl). When the procedure was applied to 7 day-old chicken embryos, which has no morphological differences between male and female (see chapter II for detail), sex identification was also achieved. This technique could be expanded to identify the sex at any stage in the life of the chickens.

Chapter VII

General Summary

Sex determination and sexual differentiation in chickens were elucidated by use of modern molecular genetic methods. The results obtained through the experiments are summarized as follows:

1. When the development of gonad during embryonic stages was observed, asymmetric development was observed between left and right in females. It was considered that the sexual differentiation of the left gonad was initiated at about 8 days of incubation in females. On the other hand, asymmetric development of the gonad was not observed throughout all the stages of incubation in male embryonic development.

2. The secondary sex characteristics such as comb and wattles growth, plumage, and spurs, of ovariectomized chickens differed very much from that of control females'. The secondary sex characteristics of highly masculinized chickens was very similar to that of the normal males. But their body size didn't differ from that of control female chickens.

3. The sex-reversed chickens induced by ovariectomy were classified into three categories on the basis of their secondary sex characteristics. Two of them were classified to high degree of masculinity, eleven of them to middle degree and fourteen of them to low degree, respectively.

4. When the left ovary was removed, the right gonad began to develop compensatory. Though these gonads varied in structure, they were classified into three categories; twelve of them to have ovary-like gonads, thirteen to have ovo-testis and two to have testicular gonad.

5. Degree of masculinity as determined from the outward appearance, especially comb growth, was almost commensurate with the masculinization in structure of the transformed right gonad.

6. For the purpose of developing a new method of sex identification in chickens, the female-specific DNA fragments were detected at about 0.7 kbp in females with the XhoI digestion of genomic DNA purified from whole blood. It seemed that this method would be useful to identify the sex.

7. Since the detection of the female-specific repetitive DNA units is simple, reliable and efficient, by applying this technique sex identification could be easily achieved even at embryonic stages.

8. The cloned W chromosome-specific DNA probe was hybridized with the initially XhoI digested DNA. When the DNA probe was hybridized in high stringency which allowed about 5% of base-pair mismatches, a hybridization band was detected at about 0.7 kbp in females. But, the band could not be detected in males. Thus, it was identified that the initially detected female-specific DNA fragment corresponded to the cloned W-specific DNA.

9. It is considered that the detected female-specific DNA fragments constitute of the heterochromatic regions in the W chromosome. Since such regions generally contain very few genes and doesn't have genetic activity, the ovary determining gene may be located on the rest of the region of constituting highly repetitive DNA fragments.

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1) Kagami, H., and Tomita, T. Genetic and Morphological Studies on the Right Gonad of Ovariectomized Chickens.
Jpn. Poult. Sci., 27: 111-121. 1990.

2) Kagami, H., Nakamura, H., and Tomita, T. Sex Identification in Chickens by Means of the Presence of the W Chromosome-Specific Repetitive DNA Units.
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3) Kagami, H., and Tomita, T. Sexing of Chickens by Detection of the W Chromosome-Specific Highly Repetitive DNA Units.
Tokai J. Anim. Prod., 1: 43-47. 1990.
(in Japanese with English Summary)

4) Kagami, H., and Tomita, T. Identification of the Female-Specific DNA Fragments in Chickens.
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5) Kagami, H., and Tomita, T. Sexual Differentiation of the Gonad and Sex Identification in Chicken Embryos.
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