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Production of transgenic birds

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Abstract. The avian embryo presents a tremendous challenge for those interested in accessing and manipulating the avian germ line. By far the most successful method of gene transfer is by retrovirus vector. The efficacy of retrovirus vectors has been demonstrated by germ line insertion of replication-competent retroviruses as well as the insertion of replication-defective retrovirus vectors carrying bacterial marker genes. Retroviral vectors have also been shown to be useful for the transfer and expression of genes in somatic cells. Further, germ line transgenesis has been reported in both the chicken and the Japanese quail. In addition, several alternative gene transfer methods are under development. These include transfection of avian sperm, development of germ line chimeras using primordial germ cells and blastodermal cells, and the development of embryonic stem cell lines. Potentially, basic research and the poultry industry will derive substantial benefit from this revolutionary technology.
Key words. Gene transfer; transgenic birds; retrovirus vectors.

Introduction

The ability to transfer genes into the germ line of birds has created new and revolutionary opportunities for both basic research and the poultry industry. Currently, there is much interest in using gene transfer technology to examine gene expression, evaluate genetic control elements, and to correlate these findings to developmental and physiological processes in birds. Equally exciting is the prospect for creating new and more efficient strains of poultry. Many investigators are transferring genes that may provide commercial birds with resistance to disease and improved feed efficiency and growth rates.
The development of gene transfer technology for avian species, however, has not progressed as rapidly as mammalian gene transfer technology. Progress in producing transgenic birds has been hampered, in part, by the avian reproductive and embryonic developmental system. The newly fertilized avian ovum is very large, fragile and yolk-filled. Embryonic development begins in the oviduct during egg formation. At the time the egg is laid, the blastoderm is composed of approximately 60,000 cells organized into a 1–2 layer disc[12, 22]. This has rendered the use of DNA microinjection into isolated early embryos, which has been used so successfully in mammalian gene transfer, ineffective. In fact, to date there have been no reports of transgenic birds produced by this method.
Thus, by necessity, avian gene transfer methodology has focused on several alternative approaches, most of which are in the early stages of development. As discussed in previous reviews[5, 8, 13, 14, 39, 43], successful gene transfer involves the identification, isolation and characterization of genes of interest, appropriate modification, and (if possible) preliminary testing in cultured cells. This paper will review the progress that has been made in producing transgenic birds including potential gene transfer methods that are currently under investigation. Applications of this technology to basic research and commercial poultry improvement will also be discussed.

Gene transfer methods

The ultimate goal for avian transgenics is to develop several methods for producing transgenic birds which will meet the needs of specific gene transfer applications. For example, retroviral vectors may be favored in situations where small genes are to be transferred and where regulated expression is not critical. Transfection (or injec-

tion) of DNA will be necessary for larger genes and when expression of the transferred gene must be regulated in a tissue-specific or developmentally defined manner. The use of embryonic stem cells (or similar totipotent cells) is envisioned for targeted gene insertion using homologous recombination. This will allow gene replacement and/or targeting to a specific chromosome.

To date, there are reports of three methods that have been used successfully to transfer genes into the germ line of birds. By far the most common approach is to infect multi-celled embryos with retrovirus vectors[1, 25, 35, 36, 45]. Recently, however, a new approach has been introduced which uses transfected sperm cells as vectors for the generation of transgenics[17]. The third method involves the use of irradiated spermatozoa to introduce new genes[6, 27].

Retroviruses as gene delivery systems

Although transfer of genes into the germ line using retrovirus vectors was first reported in the mouse[48], birds are particularly well suited for retroviral vector-mediated gene transfer. Retroviruses are able to overcome the difficulty in accessing germ line cells in multi-celled embryos, the most convenient developmental stage for avian manipulation. To produce transgenic birds by retrovirus infection of multi-celled embryos, a window is made in the shell of a freshly laid egg and the retrovirus stock is microinjected near the blastoderm[1, 25, 35, 36]. The shell is then sealed and the egg is incubated until hatching. Retroviral vectors circumvent the difficult task of having to manipulate the embryo at an earlier developmental stage while the egg is in the oviduct. Retroviruses are also appealing as gene transfer vectors from the standpoint that through the course of evolution, they have naturally entered the germ line and are genetically transmitted as endogenous viruses in the chicken[47].

Retroviruses are successful gene transfer vectors because of their unique life cycle. The first phase of the cycle begins with attachment of the virus particle to the host cell[52 - 54]. This adsorption process is mediated via cell surface receptors that recognize specifically the viral envelope glycoprotein located on the surface of the virus particle. The viral RNA genome is released upon entering the host cell cytoplasm and the viral enzyme, reverse transcriptase, directs the synthesis of a DNA intermediate copy of the viral genome. This DNA is then randomly integrated into the host genome. Once it has integrated, it is called a provirus and becomes a stable part of the host's chromosomes. The second phase of the infection cycle involves the replication of new virus particles[10, 11]. The provirus contains the coding information for viral proteins and uses the transcriptional and translational system of the host cell to produce viral RNA and proteins. The viral components are assembled into new virus particles which are released from the cell to begin a new round of infection.

Thus, the strategy for using retroviruses to transfer genes is to take advantage of the virus infection cycle. The gene to be transferred is inserted into the retrovirus genome so that the virus particle can carry the gene into the host cell and chromosome directly. Based on modifications to the viral sequences, two types of retroviral vectors are recognized: replication-competent and replication-defective vectors. In addition to the transferred gene, replication-competent vectors contain all the viral sequences needed to complete both phases of the life cycle and produce virus particles. These viral sequences include the *trans*-acting regions that encode the viral proteins as well as the *cis*-acting regions that contain other information required for replication. There are three main *trans*-acting viral regions. The *gag* region which encodes the structural and processing proteins of the virus core, the *pol* region which encodes the reverse transcriptase and integrase enzyme activities, and the *env* region which encodes the envelope glycoproteins. Among the important *cis*-acting regions are the genetic regulatory sequences including the promoter and enhancer that control the expression of the virus, the termination signal, the primer binding site that initiates DNA synthesis, the encapsidation or packaging signal that directs packaging of the viral RNA into the virus particle, and the terminal sequences that are required for integration of the viral DNA into the host's chromosome[53, 54].

Replication-competent vectors are useful for transferring genes to cultured cells or to embryos when the presence of the replicating virus does not interfere with development or present a hazard. These vectors have a distinct advantage in that they can replicate and spread from cell to cell producing a high rate of infection that is not dependent on the titer of the virus stock produced. The main disadvantage is the possibility of pathogenesis from the vector and decreased immune response to retrovirus infection as well as the risk of forming new pathogenic virus strains in transgenic birds.

Replication-defective retrovirus vectors are modified such that they contain the gene to be transferred but are missing either *trans*- or *cis*- and *trans*-acting regions of the virus. These vectors are unable to complete more than one round of the life cycle and cannot produce infectious virus particles. In its simplest form, a replication-defective vector replaces all or part of the viral protein coding region with the gene to be transferred (see fig.). More complex forms of these vectors include deletions in the 3′ viral promoter and enhancer region of the vector construct[58]. Called self-inactivating or suicide vectors, these vector viruses lose their regulatory sequences upon integration into the host genome. Without the viral promoter, the production of vector RNA in host cells is precluded leaving only the transferred gene with its regulatory sequence active.

Replication-defective vector virus stocks are produced in specially constructed cells called helper cells[56]. These cells contain the viral protein coding sequences designed

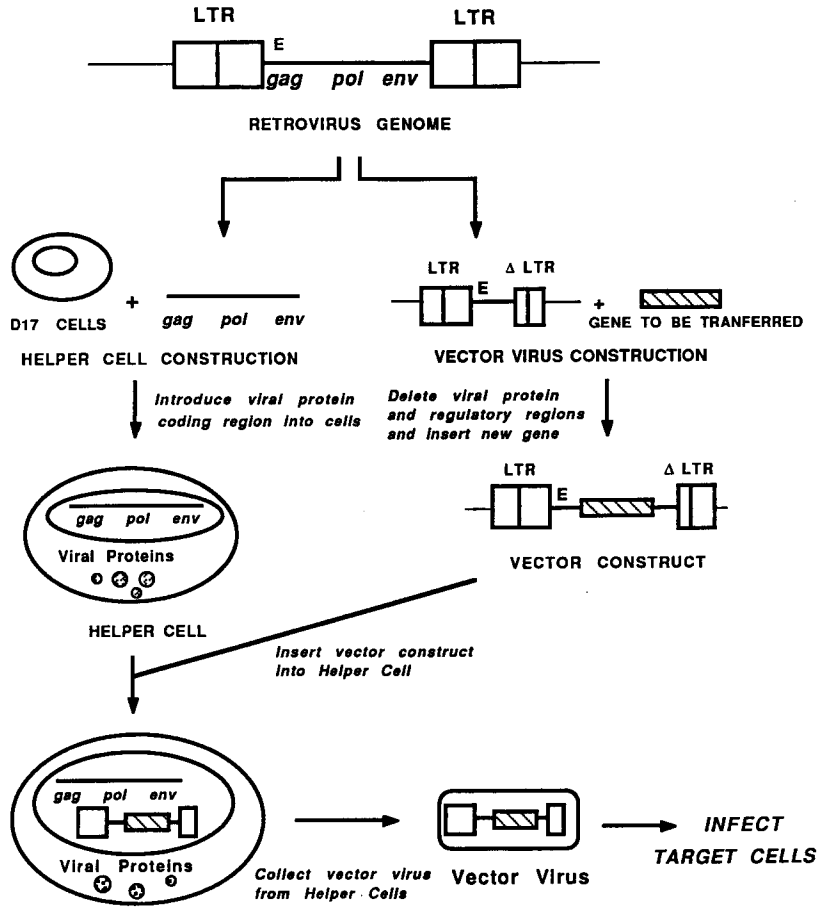

Diagram of the production of replication-defective retrovirus vector stock in helper cells. Construction begins with a proviral clone of the retrovirus genome. Helper cells are produced by inserting the protein coding region (*gag*, *pol* and *env*) of the virus into a suitable cell line such as the transformed canine cell line D17 which supports the growth of the avian reticuloendotheliosis viruses. The vector is produced by replacing the protein coding region and, for self-inactivating vectors, the portion of the 3′ long terminal repeat (LTR) that contains the viral genetic regulatory elements, with the gene to be transferred (hatched box). The vector also retains the encapsidation signal (E). The vector containing the inserted gene is then introduced into the helper cells. The helper cells are able to produce vector virus stock that is free of helper virus which may be used to infect target cells such as early embryos.

to supply the *trans*-acting proteins from the *gag*, *pol* and *env* regions of the virus. To reduce the possibility of producing helper virus (replication-competent virus), the encapsidation site and other *cis*-acting sequences are frequently removed. Helper cells have been shown to be capable of producing vector virus stock that is helper virus-free (Shuman, unpublished results).

In most gene transfer applications, replication-defective vectors are preferred over replication-competent vectors for transferring genes to the germ line, especially for commercial applications. Replication-defective vectors that are free of helper virus are viewed as being safer than replication-competent vectors due to their inability to replicate in host cells. Use of self-inactivating vectors further improves safety by reducing the possibility of expressing the vector sequences in the host cell. This reduces the chance that the vector will recombine with related viruses that may be encountered in the field. Also, deletion of the viral regulatory elements helps to ensure that the virus sequences do not influence the expression of the transferred gene. The primary disadvantage of using defective vectors compared to competent vectors is that successful gene insertion depends greatly on the titer of the virus stock produced. Unfortunately, virus titer is usually relatively low, especially in self-inactivating vectors, which results in fewer infected germ line cells when embryos are inoculated with vector virus stocks and consequently fewer transgenics are produced.

Advantages and limitations of using retrovirus vectors

Especially for avian species, retrovirus vectors have the advantage of providing an efficient and nontraumatic method of gene delivery. In addition, typically only one provirus is found per integration site with 1–2 integration sites per cell (depending on the titer of the virus stock and whether the vector is replication-competent). Thus, the number of transferred genes inserted is similar to that found normally for unique host genes. Although retro-

virus vectors have been the most successful method for avian gene transfer, this approach has several limitations. Foremost is the problem of germ line mosaicism. The number of germ line transgenics ultimately produced from a vector virus-infected embryo is relatively low. Typically, hundreds to thousands of eggs must be inoculated and a similar number of progeny screened to identify one transgenic bird. There are also limitations with the vector. Each vector construct must be tested to determine that the inserted gene does not interfere with production of vector virus so that high-titer virus stocks can be produced. Also, although theoretical predictions suggest that viral vectors can transfer 10 kb of genetic material[50], the titer of the virus stock is inversely proportional to the size of the transferred sequence making the practical limitation much smaller.

Insertion of retroviruses into the germ line of birds

Retrovirus vectors were first used to transfer genes to cultured cells. Replication-defective vectors were constructed from the avian retrovirus reticuloendotheliosis virus (REV; for review see ref. 50) and more recently from avian leukosis virus (ALV)[26] to transfer bacterial marker genes[38]. Retroviral vector technology improved with the advent of helper cell lines which made possible the production of vector virus stock free of helper virus[56]. Replication-competent vectors have been constructed from Rous sarcoma virus[20]. In these vectors, the *src* oncogene has been replaced with a synthetic linker to allow insertion of the gene to be transferred. Replication-competent vectors have been used to transfer bacterial marker genes[16] as well as to study genetic regulatory sequences[21].

The first germ line transgenic birds reported in the literature were produced by infection of embryos with replication-competent avian leukosis viruses[35, 36]. In this study, embryos from Line 0 chickens (a White Leghorn strain free from endogenous viral genes and exogenous infection) were inoculated with two recombinant ALVs and an exogenous ALV to determine if these viruses had the potential to enter germ line cells. The recombinant viruses contained the long terminal repeats (LTR) of the endogenous ALV, RAV-0, and the subgroup A envelope gene of exogenous ALV. Rous-associated virus type 1 (RAV-1), also a subgroup A type virus, was the exogenous virus used in this study. Twenty-one transgenic males identified by Southern blot analysis in the first generation (G-1) were found to transmit 23 proviral inserts that were stably inherited for at least two generations (summarized in the table). Twenty-one of these inserts encoded complete ALV genomes and were found to produce virus particles giving evidence of expression. Interestingly, in the second generation (G-2) and beyond, progeny were identified that had additional proviral inserts that were not carried by their parents[9]. Apparently, these new inserts arose through reinfection of the germ line cells in transgenic birds expressing infectious virus. These results showed clearly that replication-competent ALV can infect germ line cells resulting in stably inherited proviruses.

In this same study, two out of the 23 inserts were not found to produce infectious virus particles when tested for virus production[34]. These fortuitous events provided the opportunity to test the idea that expression of the viral envelope gene can produce resistance to viral infection. One insert, called *alv*11, was found to produce the *gag* and *env* viral proteins as well as virus particles as revealed by electron microscopy; however, restriction enzyme analysis revealed a 0.5 kb deletion in the *pol* gene. The lack of infectivity was, therefore, presumed to be due to a loss of reverse transcriptase activity. The other insert, called *alv*6, was found to lack p27 (a *gag* product) and typical ALV virus particles but did express the ALV envelope glycoprotein. Cultured cells and birds containing *alv*6 and *alv*11 were extensively examined for resistance to infection and oncogenesis by ALV. Previous

Summary of the production of transgenic birds by retrovirus infection

Species	Gene transferred	Vector virus	Type of vector	No. G-0 tested[a]	No. G-0 transmitting	No. G-1 transgenic	No. G-1 negative	No. expressing transgene	Reference
Chicken, Line 0 White Leghorn	Not attempted	RAV-0 recombinant[b]	Rep.-competent	14	4	21	794	15	34
"	Not attempted	RAV-0 recombinant[b]	Rep.-competent	9	4	5	532	4	34
"	Not attempted	RAV-1	Rep.-competent	14	1	2	551	1	34
"	Not attempted	CSV	Rep.-competent	8	0–5%	3	Unreported	Unreported	36
Chicken, commercial broiler	*neo* and *tk*	SNV	Rep.-defective	4[c]	4	34	720	yes	1, 4
Chicken, commercial Leghorn	None	RSV	Rep.-competent	Unreported	0–40%	Unreported	Unreported	yes	6
Japanese quail	*cat*	SNV	Rep.-defective	29	1	1	1594	1	25
	None	SNV	Helper virus	29	1	2	1593	1	25

[a] The cells of G-0 birds are mosaic; germ line infection is verified by analyzing G-1 progeny for evidence of the transgene.
[b] RAV-0 recombinant contains RAV-0 long terminal repeats plus the subgroup A envelope glycoprotein.
[c] 33 birds were identified that had vector sequences in blood and semen samples. Only 4 of these birds were mated to test germ line transmission.

studies of endogenous viruses that express envelope genes showed that cell cultures and chickens were very resistant to infection with subgroup E ALVs (the endogenous virus subgroup type) but not to other subgroups. The objective was to determine whether *alv*6 and *alv*11 similarly produced resistance to subgroup A viruses. They found that *alv*6 and to a lesser extent *alv*11 cultured cells and birds showed resistance to infection by subgroup A viruses but not to subgroup B viruses as predicted. The *alv*6 line, therefore, represents a model system for introducing genes from the pathogen into the host to induce host resistance to the pathogen.

It has also been shown that a modified replication-competent Schmidt-Ruppin A strain of Rous sarcoma virus (RSV) can enter germ line cells following infection of early embryos [7]. In this study, a nontransforming vector containing the bovine growth hormone gene in a downstream position from the viral genes was used to infect early commercial Leghorn embryos. Resulting viremic hens were used to produce progeny with the strategy that embryos would be exposed to virus in the oviduct of the hen. Male offspring were then tested for germ line transmission of the RSV vector. The males were found to transmit RSV proviral inserts with a frequency that varied from 0–40% (table), however, none of the birds tested were found to have retained the bovine growth hormone gene. Transgenic birds were also tested to determine whether the proviral inserts would protect the bird against challenge with transforming RSV. Protection was found when transgenic birds were challenged with subgroup A RSV while no protection was found with subgroup B RSV. These results are in agreement with the findings of Salter and Crittenden [34] for birds containing *alv*6 and it is presumed that protection is derived from the expression of the subgroup A envelope gene.

Reticuloendotheliosis virus has also been shown to be artificially inserted into the germ line via infection. Salter et al. [36] infected embryos and hatched chicks and showed that chick syncytial virus (CSV), a type of REV, could be transmitted through the germ line of chickens. Interestingly, all transmission came from chicks infected at 1 day after hatch with the frequency of transmission ranging from 0 to 5%. Three G-1 chicks were identified with proviral inserts that appeared to arise through germ cell infection, however, all chicks died at an early age. In addition, Lee and Shuman [25] have found that infection of Japanese quail embryos with spleen necrosis virus (SNV), a type of REV, resulted in two birds carrying SNV proviral inserts. One of these birds expressed the proviral insert and transmitted the provirus to her progeny. All of her progeny, however, showed signs of viremia and died within a few weeks of hatching. An attempt has also been made to produce transgenic quail with ALV inserts, however, the quail were shown to be resistant to infection and germ line transmission with replication-competent ALVs [33] (and Shuman, unpublished results).

Using retrovirus vectors to insert foreign genes into the germ line

In addition to germ line insertion of replication-competent retroviruses, it has also been shown that it is possible to transfer replication-defective retrovirus vectors containing bacterial marker genes into the germ line of birds [1, 25]. Bosselman et al. [1] have used a replication-defective SNV vector to transfer two bacterial marker genes (in a single vector) to germ line cells of a commercial broiler strain of chickens. The bacterial marker genes contained in the vector included the neomycin resistance gene driven by the SNV promoter and the herpes simplex virus type 1 thymidine kinase gene expressed from its own promoter. As in the studies using replication-competent virus, freshly laid eggs were inoculated with vector virus stock and resulting birds were screened for evidence of infection. 23% of the birds that hatched showed evidence of vector sequences in DNA extracted from blood cells (table). Four males that were found to have vector sequences in their blood as well as their semen were tested for germ line transmission. All four birds produced transgenic progeny which totaled 34 different insertion sites. Expression analysis of birds containing transgenes showed that they were highly expressed [4].

Germ line transmission of a retroviral vector containing a bacterial marker gene has also been shown in Japanese quail. Lee and Shuman [25] used a replication-defective SNV vector containing the bacterial marker gene chloramphenicol acetyltransferase (*cat*) to infect early quail embryos. In this study, 81% of the birds that hatched showed evidence of vector sequences in DNA extracted from blood cells (table). 16 males and 13 females that were found to have vector sequences in their blood were tested for germ line transmission. One male transmitted the *cat* vector provirus to one of his progeny. This insert has been stably transmitted over 5 generations. Expression of the *cat* gene as detected by enzyme analysis has been shown in a wide variety of tissues; highest enzyme activities were found in the intestine, thymus, skin, and muscle while lower activities were found in the spleen, gonad, and kidney. Transgenic birds were also examined for evidence of the presence of replication-competent SNV by culture of whole blood samples with chicken embryos fibroblasts and were found to be negative.

Using retrovirus vectors to insert genes into somatic cells

The first reported use of a retrovirus vector to transfer a foreign gene in vivo in birds was by Souza et al. [49] in the chicken. A replication-competent RSV vector virus containing the chicken growth hormone gene was inoculated into embryos at nine days of incubation. It was determined that growth hormone levels were elevated in the serum of some of the infected birds following hatching. However, growth rate was not significantly different from control birds.

More recently, Bosselman et al. [2] has used a replication-defective SNV vector to transfer the chicken growth hor-

mone gene to early embryos. At 7 days of incubation, DNA was extracted from inoculated embryos and screened for the presence of vector and SNV sequences. 52% of the embryos tested hybridized to the vector probe while none of the embryos showed evidence of hybridization to virus-specific sequences not contained in the vector. This suggested a relatively high rate of infection in the absence of helper virus. Circulating growth hormone levels as measured by radioimmunoassay in 15-day-old embryos were found to be 10-fold higher than uninfected controls. DNA extracted and screened from adult birds also showed evidence of the presence of the vector containing the growth hormone gene. In a similar study, Chen et al.[7] transferred the bovine growth hormone gene to early chicken embryos using a replication-competent ALV vector. Bovine growth hormone was measured in growing and adult birds and was found to be expressed into adulthood.

Retrovirus vectors have also been used to transfer bacterial marker genes to early embryos. Shuman and coworkers[23-25] have transferred the *cat* gene using a replication-defective SNV vector to both chicken and quail embryos. Expression of the *cat* gene was detected in 60–95% of the infected embryos during the early, middle and late embryonic period and in hatched chicks examined at 1–3 weeks of age and as adults. In addition, *cat* activity was detected in a wide variety of organs examined including the brain, bursa, heart, intestine, liver, skeletal, muscle, spleen, and thymus. Bosselman et al.[2] has transferred the neomycin resistance and thymidine kinase genes using a SNV vector to early chicken embryos. DNA analysis showed that the bacterial genes and vector could be detected in embryos as well as adult birds.

Sperm-mediated gene transfer

Spermatozoa are appealing targets for gene transfer studies because they are easy to obtain and offer a direct access to the germ line. It has recently been reported that chicken sperm can be used to deliver exogenous genes to the ovum[17]. In this experiment, sperm cells were incubated in a buffer containing the bacterial *lac*Z or *cat* genes and then used to inseminate hens. The resulting embryos or chickens were analyzed for the presence and expression of the transferred genes. Polymerase chain reaction and Southern blot analysis revealed that 30–60% of the transfected birds contained the genes. Enzyme assays for CAT and β-galactosidase activity confirmed, however, that these G-0 birds were mosaics. Mating of G-0 birds has shown that the transferred genes were transmitted through the germ line to the G-1 generation. If shown to be repeatable and stable, this method could offer a promising alternative to retroviral gene transfer.

Previous attempts, however, to use sperm as delivery vehicles have not met with much success[13] (and Salter and Crittenden, personal communication). In these studies, the strategy was to coat the sperm with DNA prior to insemination. Although it was found that DNA readily attaches to the sperm, successful integration of transferred DNA by this method has not been reported.

Another reported sperm-mediated approach to transfer genes is by irradiating sperm. In these studies, hens were initially inseminated with irradiated semen obtained from donor males carrying marker genes. 24 h later the hens were reinseminated with nonirradiated semen obtained from males that were the same strain as the hen. Using this method, Pandy and Patchell[27] and Tomita et al.[51] showed that 3–5% of the resulting progeny had feather and egg color characteristics of the marker male. This approach was also used by Bumstead et al.[6] who found that 0.4% of the resulting progeny carried the major histocompatibility haplotype marker of the irradiated sperm in addition to the haplotypes of the dam and nonirradiated sire. In contrast, Shoffner et al.[40] have reported that they were unable to find evidence of marker genes in progeny following sperm irradiation. Nevertheless, this approach may have utility for transferring genes between different breeds of chickens or other birds.

Transfection of early embryos

Another method recently employed to introduce DNA into early embryos is chemically-mediated transfection. Han et al.[19] examined the use of DNA complexed with either calcium-phosphate or liposomes. An RSV/*lac*Z construct was complexed and then injected into freshly laid embryos and embryos incubated for 24 h. Embryos screened at 36–60 h of incubation were found to have β-galactosidase activity. In a similar experiment, Rosenblum and Chen[32] used liposomes to transfer a construct containing the firefly luciferase gene expressed from a RSV promoter. Luciferase was detected at high levels in 3-day-old embryos and had detectable activity in 8-day-old embryos. These methods may be suitable for examining gene activity in early embryos.

Gene transfer methods under development

There are several promising strategies under development that may lead to alternative and/or improved methods for gene transfer in birds. One such method is the in vitro culture of embryos from shortly after fertilization until hatching[28]. In this technique, eggs were isolated from the magnum of the oviduct and incubated through a series of artificial shells until hatching. Unfortunately, the hatch rate was relatively low and few chicks can be produced at one time. This technique has been used, however, to examine the fate of DNA injected into the fertilized ovum prior to the first cleavage division[29,37]. Using an RSV*cat* construct, it was found that the DNA was lost after the first 24 h of embryonic development. Betagalactosidase activity was examined following injection of the *lac*Z gene under control of the cytomegalovirus early promoter. Expression was first observed at 12 h of development and more than 90% of the embryos contained stained cells at 24 h. After 24 h the

number of stained cells decreased dramatically and after 7 days only 7% of the surviving embryos had stained cells in embryonic tissues. This suggests that stable integration of injected DNA is a rare event in this system but this may provide a means to study gene expression at very early embryonic stages.

Another approach of great interest for gene transfer technology is the production of germ line chimeras. There are several strategies for producing chimeras including the transfer of primordial germ cells, blastodermal cells, and embryonic stem cells. Primordial germ cells (PGCs) may be isolated from the germinal crescent region of early embryos and transferred to host embryos where they have been found to colonize the host gonad [18, 31, 41, 44] and are passed through the germ line [57]. In addition, there is a recent report that it is possible to infect PGCs with retrovirus vectors. Simkiss et al. [46] have isolated chicken PGCs which were exposed to a replication-defective vector containing the *lac*Z gene. PCR analysis of DNA extracted from embryonic tissues including the gonad showed that the *lac*Z gene was present.

It has also been shown by Petitte et al. [30] that it is possible to isolate chicken blastodermal cells from one embryo and transfer them to another embryo where they participate in the development of a variety of tissues and organs including the germ cells. In this study, progeny were produced that carried the marker traits of the donor embryo. Further, it has also been shown that it is possible to introduce DNA constructs containing the *lac*Z gene into blastodermal cells using lipofection and then to transfer these cells to host embryos [3, 55]. At 48–65 h of incubation, the embryos were found to contain a small number of expressing cells suggesting the possibility of using this technique for gene transfer studies.

There are several groups currently attempting to produce embryonic stem cell lines for the chicken. Embryonic stem cells would have the advantage that donor cells could be selected prior to transfer and they could also be used for homologous recombination to allow targeted insertion. Cells lines of this type would have tremendous value for gene transfer studies. To date, however, there are no reports of bird embryonic stem cell lines. The main limitation of using embryonic stem cells, or chimeras in general, is the low number of progeny produced from the transferred cells. In the future, it may be possible to alleviate this problem by devising strategies to reduce host germ cell populations.

Applications of gene transfer technology in birds

Gene transfer and genetic engineering potentially may be applied as a tool for basic research, as a method for improving the genetic characteristics of commercial poultry, and as a new method to produce pharmaceuticals and other biologically important proteins. As a new tool for basic research, this technology permits the study of cloned genes in the intact bird. This will undoubtedly aid in the understanding of complex processes such as growth and reproduction. The study of genetic material by this method will likely be an important step in the process of identifying and testing genes that will be used for improving poultry production via new animal products or by producing improved breeding stock.

One of the main objectives of gene transfer technology has been the genetic improvement of commercial poultry. Research to date has focused on possible growth enhancement by transferring growth hormone genes by retrovirus vector to early embryos [2, 7, 49], and on disease resistance by transferring viral genes that provide resistance to infection. These include the ALV envelope gene transferred to the chicken germ line via replication-competent vectors [35, 36] and to cultured cells via replication-defective vector [42] and, also, the murine Mx1 protein gene, a mediator of resistance to influenza virus, transferred by replication-competent vector to cultured cells [15]. Other areas that appeal to poultry breeders include the transfer of genes that will provide a general enhancement of the immune and reproductive systems, improving feed efficiency, reducing fat and cholesterol in meat and eggs, controlling sex determination, identifying the sex at hatch (or before), and generally improving meat and egg quality.

Gene transfer may also make it possible to use the chicken as a bioreactor for the production of pharmaceuticals and other proteins. At least two possibilities are envisioned both involving the hen's remarkable reproductive system. One idea is to express the pharmaceutical gene in either the oviduct of the hen so that the protein product is incorporated into the albumen of the egg [43]. Alternatively, the gene could be expressed in the liver and manipulated in such a way that it will be incorporated into the egg yolk (Gibbins, personal communication). The ultimate goal would be to provide a new manufacturing system that could produce bioproteins at costs which are lower than mammalian or bacterial cell culture while providing a correctly processed, biologically active product. Because of the high reproductive rate and relatively short generation interval of chickens and high protein ratio in eggs, chickens may have an advantage over mammals, including rabbits, mice, goats, sheep, and cattle that are currently being tested as possible bioprotein production systems (see Wilmut et al., this review).

Conclusion

The use of retroviral vectors has made germ line gene transfer a reality for researchers in avian science. Conceivably, this methodology could be used to produce the first genetically engineered commercial poultry as well as providing a valuable tool for the study of genes in avian species. For the future, it is anticipated that avian gene transfer methodology will expand to include techniques that will improve the overall efficiency of producing transgenic birds as well as provide methods to refine and

make more predictable the outcome of the gene transfer process. As with any new technology, research, development and application will require a stepwise approach that includes considerable testing and evaluation. However, this appears to be a technology that holds great promise, one that is capable of revolutionizing basic avian research as well as the poultry industry.

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Production of pharmaceutical proteins in milk

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Abstract. There is every reason to expect that it will be possible within the next few years to begin to use farm animals to produce large quantities of some of the human proteins that are needed for the treatment of disease. Revolutionary new opportunities for the production of novel proteins in milk have been created by the development of methods for gene transfer. Exploitation of these opportunities depends upon selection and cloning of milk protein genes and identification of the sequences that govern tissue specific hormonally induced expression in the mammary gland. Studies with three genes, ovine β-lactoglobulin, rat β-casein and whey acidic protein of rat and mouse, suggest that they may all meet this requirement. Fragments of the ovine β-lactoglobulin, murine whey acidic protein and rabbit β-casein genes have directed production of novel proteins in the milk of transgenic mice, sheep, rabbits and pigs. The proteins were biologically active and usually co-migrated with authentic proteins. In early experiments, protein concentration was low, but our recent observations suggest that fusion genes containing genomic clones direct production of concentrations of protein that are suitable for commercial exploitation.

In the longer term, two approaches may offer the potential of more reliable expression. Control elements capable of directing expression that is independent of site of insertion of the gene, but dependent on the number of copies of the gene, have been identified for a small number of genes. The availability of such elements for the milk protein genes would increase the reliability of gene expression considerably. Alternatively, targeted mutation of genes may allow the insertion of coding sequences within an existing gene so avoiding position effects.

Key words. Gene transfer; gene modification; gene expression; livestock; transgenic animal; pharmaceutical proteins; milk composition.

Introduction

The ability to transfer genes into the germline of livestock creates revolutionary new opportunities for the modification of animal production traits, including the composition of milk. The mammary gland of a dairy animal can be envisaged as an efficient vat for the production of specific proteins, sugars and fats. In this role it has long been exploited by Man as a source of food, particularly protein. Changes may be envisaged either to proteins that are normally produced in milk[48] or to direct production of novel proteins to the mammary gland[26].