Transgenic chickens as bioreactors for protein-based drugs

Simon G. Lillico, Michael J. McGrew, Adrian Sherman and Helen M. Sang

The potential of using transgenic animals for the synthesis of therapeutic proteins was suggested over twenty years ago. Considerable progress has been made in developing methods for the production of transgenic animals and specifically in the expression of therapeutic proteins in the mammary glands of cows, sheep and goats. Development of transgenic hens for protein production in eggs has lagged behind these systems. The positive features associated with the use of the chicken in terms of cost, speed of development of a production flock and potentially appropriate glycosylation of target proteins have led to significant advances in transgenic chicken models in the past few years.

The possibility of using transgenic animals for the production of therapeutic proteins was raised shortly after the development of the first method for the genetic modification of mice. Significant progress has been made towards this goal based on targeting expression of pharmaceutical proteins to secretory organs of animals, particularly the mammary gland of livestock mammals [1–4]. The predicted advantages of such production systems compared with synthesis in microbial cells or mammalian tissue culture cells include the ability to produce large quantities of posttranslationally modified and complex proteins and the possibility of providing a cheaper alternative to the use of large-scale fermentors. The cost of production of glycosylated proteins from large-scale cell cultures is considerable, and it is predicted that transgenic animal production will be more cost effective [5]. A key requirement for the production of human proteins for therapeutic purposes is that the production method should result in a protein that incorporates the posttranslational modifications of the naturally expressed protein [6]. The posttranslational modification of many proteins is essential for protein function, and if a protein is not appropriately modified it could have a short half-life in the patient and poor therapeutic efficacy. Absence of specific glycosylations can result in a protein that is immunogenic or unmask peptide epitopes that might be antigenic. The requirement for appropriate glycosylation of each therapeutic protein for overall efficacy must be evaluated for each target protein.

The basic strategy for targeting expression of a foreign protein to, for example, the mammary gland, has been to identify the regulatory sequences of milk protein genes required for high-level, tissue-specific expression and subsequently link these to the coding sequence of a therapeutic protein. Next, various methods can be used to introduce the resultant transgene into the genome of the host species [2,3]. Over the past decade, various recombinant human proteins, for example, proteins that are typically isolated from human blood products and monoclonal antibodies [7], have been successfully produced in the milk of transgenic mammals such as cows, goats, sheep and rabbits [5]. With the promise of cheaper production cost and ease of purification, it was expected that biopharmaceutical products produced in transgenic animals would quickly pass from the barnyard to the market place. Although progress has been slower than predicted, significant

Simon G. Lillico
Michael J. McGrew
Adrian Sherman
Helen M. Sang*
Roslin Institute,
Roslin,
Midlothian,
UK, EH25 9PS
*e-mail:
helen.sang@bbsrc.ac.uk

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TABLE 1

Composition of an egg produced by a hen

Constituent	Proportion of egg by weight (%)	Components of individual constituents (%)	
Albumen	60	Water	88
		Protein	11
Yolk	30	Water	50
		Lipid	30
		Protein	20
Shell	10		–

Major albumen proteins in a 60 g egg[a]

Constituent	Weight (g)	Fraction of total protein (%)
Ovalbumin	2.20	54.0
Ovotransferrin	0.50	12.0
Ovomucoid	0.50	12.0
Lysozyme	0.15	3.4

[a]Figures expressed as percentages of the total albumen proteins.
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advances are now being made and several products have reached various stages of clinical trials. Recombinant C1 inhibitor (Pharming Group NV) purified from the milk of transgenic rabbits has recently entered Phase III clinical trials. If successful, market launch for this product is expected in 2005. In addition, recombinant human antithrombin III protein produced in the milk of transgenic goats (GTC Biotherapeutics) has completed Phase III clinical trials. This product is currently under review for market authorization in Europe, the successful completion of which promises to galvanize the field of animal biopharming.

More recently, the development of genetically modified plants for production of pharmaceuticals, a potentially competitive approach to that of using animal bioreactors, has made significant progress, with many companies currently in start-up phase [8]. These methods entail the production of recombinant human proteins in the leaves or seeds of transgenic plants. The advantages of this system include low production costs and absence of mammalian-derived viral sequences and pathogens. Several recombinant proteins produced in plants have entered clinical trials and lipase from transgenic maize has been granted orphan drug status (www.meristem-therapeutics.com). However, there are two key issues that need to be addressed before plant-derived biopharmaceuticals will reach the market place. First, the glycan groups that are added to proteins are not the same in animals and plants. This problem could be circumvented by additional manipulation of the production plant species to 'humanize' the glycosylation patterns of the proteins produced. Second, and more importantly, the real or perceived issue of environmental biosafety, which involves the risk of food crop contamination by the horizontal spread of the introduced transgene to the wild-type population, must be resolved.

Why develop chickens as bioreactors?

Although there are an increasing number of options for production systems for therapeutic proteins, it is recognized that the resources of commercial production create a bottleneck. Pharmaceutical proteins produced in eggs might have significant advantages for specific target drugs, including appropriate glycosylation, lower costs than either cell culture or transgenic mammalian systems and faster scale-up.

Production of proteins in eggs

Modern layer hens produce eggs in a 20–24 h cycle, with each ovulated yolk initially acquiring layers of egg white, followed by shell membranes and eventually a shell during its passage through the 50–70 cm length of the mature oviduct. At lay, a typical egg weighs around 55–60 g, with the yolk constituting ~30%, the white 60% and the shell 10% of the total weight (Table 1) [9]. Albumen (protein) is the main constituent of dried egg white, and is biochemically relatively simple. Nine different proteins account for 87% of the total protein mass, with ovalbumin, ovotransferrin and ovomucoid being the most abundant (54%, 12% and 12%, respectively). The relatively low complexity of egg white should facilitate purification of recombinant proteins from albumen. There is considerable commercial expertise available in processing of eggs and purification of some components, for example, lysozyme.

The genes encoding egg white proteins are translated in the secretory cells of the magnum of the oviduct of the laying hen (Figure 1a,c). Secretion is stimulated as the yolk passes down the oviduct (Figure 1b). Transgenic expression of therapeutic proteins in egg white is likely to be achieved by using the regulatory sequences of the genes encoding egg white proteins to drive expression of a sequence that encodes a therapeutic protein and that has been modified to promote secretion. The regulatory sequences of two egg white protein genes (ovalbumin and lysozyme) have been characterized in detail at the molecular level and are therefore the most obvious candidate genes to modify to target expression to the oviduct. Ovalbumin has been less well-characterized because the only system in which the regulatory elements can be studied is through transient transfection of cells isolated from the oviduct of female chicks that have been stimulated by hormone injections to develop prematurely. Significant regulatory elements have been identified, including steroid response elements and a negative regulatory region that inhibits expression of ovalbumin in tissues other than the oviduct [10,11]. There is also evidence that sequences within the transcribed region of the ovalbumin locus contribute to the stability of the ovalbumin mRNA, and therefore the total amount of ovalbumin protein synthesized. It could be important to include such sequences in an

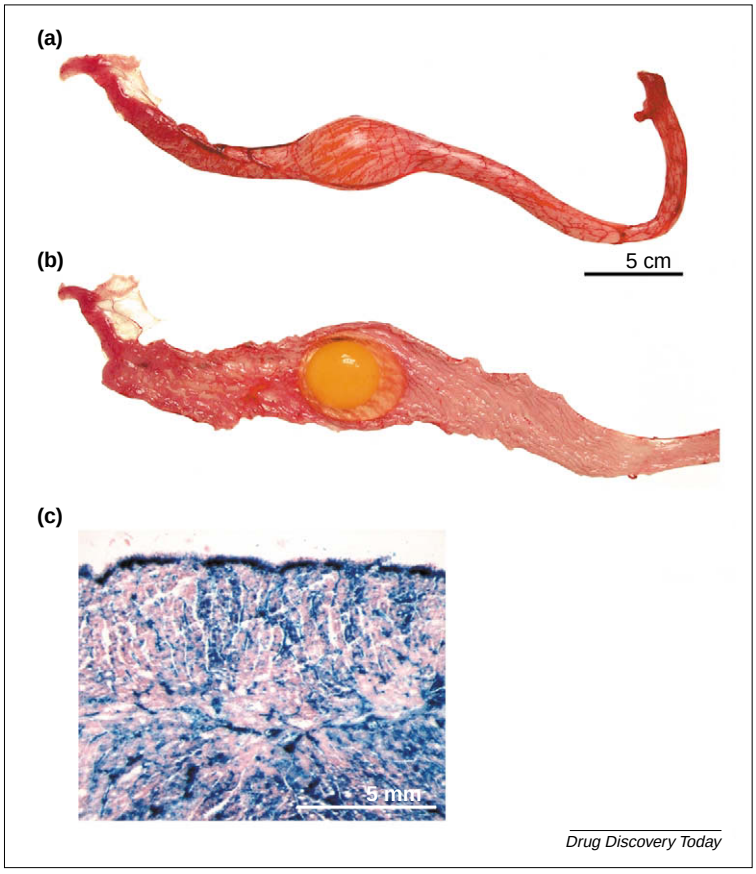

FIGURE 1

The oviduct of a laying hen. (a) An oviduct from a hen isolated during passage of a yolk through the magnum. **(b)** The oviduct opened to show the yolk and the accumulating egg white. **(c)** A section through the magnum of a transgenic hen that has been stained to detect expression of the *lacZ* reporter gene [34].

ovalbumin-derived transgene, if high levels of therapeutic protein expression are to be achieved. Functional elements of the lysozyme gene have been investigated in more detail than those of ovalbumin because the chick lysozyme gene can be introduced into transgenic mice, where it is expressed in the macrophages. Analysis of the elements involved and the effects of deletion of specific sequences indicated that the entire lysozyme region of ~20 kb is required for tissue-specific, copy number-dependent expression [12,13].

In contrast to egg white, which contains modest concentrations of lipid, a complex mixture of lipids form the bulk of dried egg yolk [9], the components of which can include triglycerides, sterols (mainly cholesterol), phospholipids and glycolipids. The protein fraction of the yolk comprises a variety of products, including vitellogenins and low-density- and high-density-lipoproteins. Vitellogenins, and some other yolk proteins, are synthesized in the liver and accumulate in the yolk via a receptor-mediated process during the days preceding ovulation. If recombinant protein sequestration in the yolk is required, internal recognition sequences involved in this process would need to be incorporated in a recombinant protein. The yolk also sequesters up to 400 mg of IgY, a form of IgG. Uptake of a chimeric human IgG has been demonstrated [14] and the sequences important for receptor-mediated uptake into egg yolk identified [15]. Synthesis of recombinant antibodies in transgenic hens and recovery from yolk could be exploited specifically for therapeutic antibody production. Recombinant antibodies that are designed to be sequestered in yolk could be purified using a process already established for the isolation of polyclonal antibodies raised in laying hens.

Glycosylation of proteins

A potential advantage of using chickens as bioreactors is that some of the oligosaccharide moieties added to nascent polypeptides in the chicken have greater similarities with the sugars used by humans than those of other mammals, although the data available on glycosylation in birds are limited. A study of the glycosylation of IgGs in different species highlighted the species-specific variation in the sialylation of *N*- and *O*-linked glycans, a major mode of posttranslational glycosylation [16]. IgGs from some mammals, including cows, sheep and goats, comprise oligosaccharides with *N*-glycolylneuraminic acid (NGNA), whereas other species, including the rabbit, contain NGNA and *N*-acetylneuraminic acid (NANA). Of the species investigated, it was observed that the IgG oligosaccharides of humans and chickens only incorporate NANA. Furthermore, humans produce Hanganutziu–Deicher antibodies that recognize NGNA, resulting in potential rejection of proteins carrying this epitope.

The absence of some glycosylations of proteins in humans is unusual in comparison with most other mammals. For example, α1–3-galactose (α1,3-Gal) epitopes are found on tissues and secreted glycoproteins of mammals, with the exception of humans, apes and Old World monkeys [17]. In humans, the gene for the enzyme α1,3-galactosyltransferase is inactive, and 1% of circulating B-lymphocytes produce anti-α-Gal antibodies in response to enteric bacteria, which contributes to the rejection of tissues in transplants (e.g. pig xenografts) and could prove problematic for proteins produced in the milk or other body fluids of livestock species. By contrast, chickens do not produce α1,3-Gal [18], reducing the potential risk of an adverse immune response to pharmaceutical proteins produced in eggs.

Additional potential advantages of chicken bioreactors

Production of human proteins in hens could be the method of choice for some proteins that are toxic to mammals. For example, although expression of human erythropoietin in the mammary gland of rabbits had a deleterious effect [19], it is unlikely that human erythropoietin will be active in chickens [20].

The egg is an attractive vehicle for the recovery of therapeutic proteins because the contents of the egg are sterile and proteins in egg white are stable, suggesting that therapeutic proteins could have a long half-life in egg white [21]. Vaccines for human use have been produced in the eggs of hens for many decades, and thus established

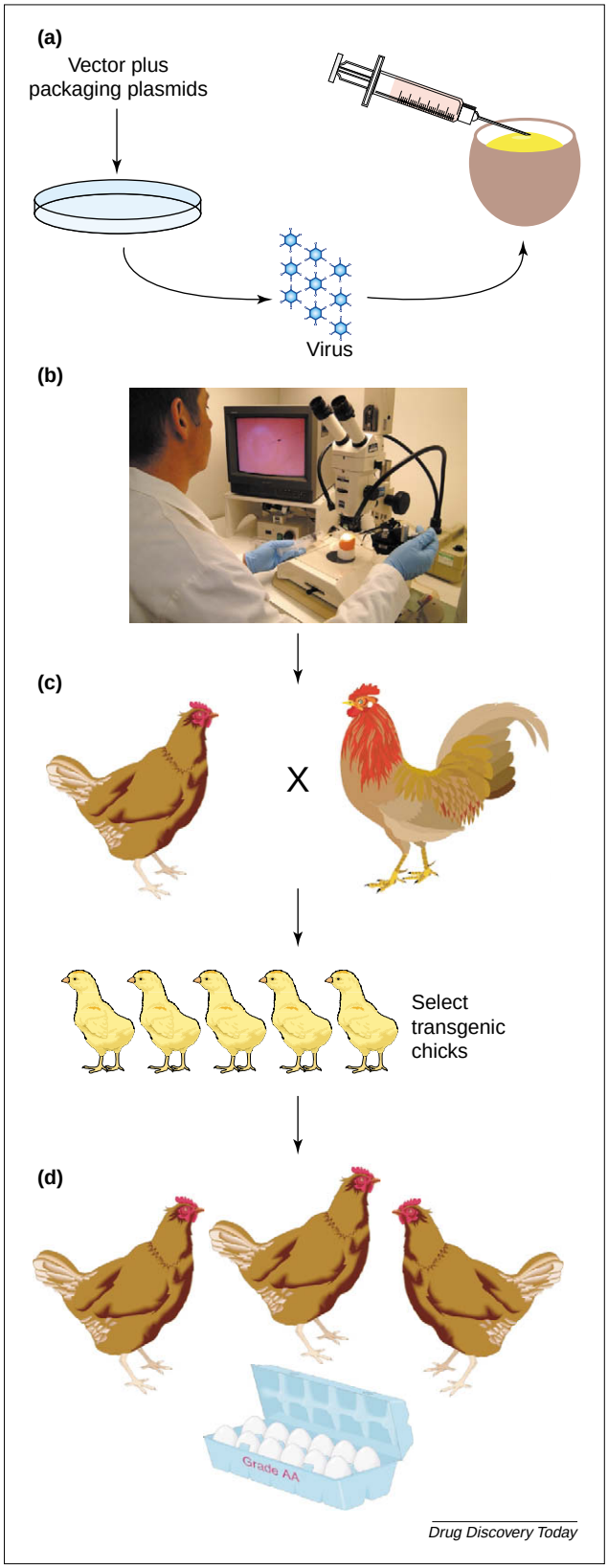

FIGURE 2

Production of transgenic birds using a lentiviral vector. (a) After cotransfection of the viral vector–transgene construct into tissue culture cells, the virus particles are harvested, concentrated and approximately 2 μl is subsequently injected below the embryonic disc of a newly laid egg. **(b)** Injection of an egg with a virus preparation. The eggs are then cultured to hatch (three weeks incubation). **(c)** Hatched chicks are raised to sexual maturity (approximately 20 weeks) and males are screened to detect the viral transgene in sperm. They are crossed to stock hens and hatched chicks are screened to identify hemizygous transgenic birds. **(d)** The transgenic birds are raised to maturity and can be bred to generate a transgenic flock or analysed for transgene expression and accumulation of therapeutic protein in the eggs of the transgenic hens.

regulations are likely to facilitate the development of regulatory procedures for therapeutic protein production in eggs.

A significant advantage of the use of hens as bioreactors, in comparison with the utilization of cattle, sheep or goats, is the short incubation time of three weeks and the relatively short generation time of ~20 weeks. A transgenic production flock can be built up within a comparatively short period of time (Figure 2). Cockerels can be mated to ten hens every day or so and each hen will then lay ~10 fertile eggs. Once a transgenic cockerel has been identified, he can be used to breed over 100,000 transgenic offspring in a year. The costs of developing a transgenic production flock for a specific therapeutic protein have been estimated; however, because the companies developing this approach are still at an early stage no definite costs are currently available. The production of founder transgenic birds, from which a flock can be developed, could potentially cost a few thousand dollars, if the recent advances in transgenic technology prove to be applicable for this purpose, which is considerably less than the cost of producing transgenic cattle. Regulations have yet to be established for the conditions for rearing transgenic birds for protein production: a convention for rearing birds in specific pathogen free environments has been established but conforming to these rules would increase the cost of production. The speed at which a production flock could be generated and the high rate of production (300 eggs $year^{-1}$ hen^{-1}) suggest that protein production in hens could prove extremely useful for target proteins that are required in large amounts. For example, if each transgenic hen deposited 100 mg of therapeutic protein in an egg, then a single hen could produce 30 g of protein each year.

Progress towards development of chicken bioreactors

Methods for production of transgenic chickens

There are significant differences between the reproductive physiology of birds and mammals: avian embryos develop from a large yolky egg that is enveloped in a hard shell after fertilization and the embryo then develops in the incubated egg. By the time a fertile egg is laid, the chick embryo has already developed on the yolk to a stage at which it consists of ~60,000 cells. Most methods for genetic modification of mammals involve manipulation of oocytes, fertilized eggs or early embryos recovered from female donors, with transfer to recipient females after manipulation to introduce transgenes. The large size and fragility of the egg produced by a hen (i.e. the yolk) and problems in recovering eggs shortly after fertilization make

manipulation of the egg for injection of DNA transgenes or nuclear transfer at this early stage difficult. By contrast, it is easy to obtain fertile newly laid eggs but, as the embryo develops, attempts to modify embryos genetically at this stage face different challenges [22].

Despite the technical challenges involved, several different approaches have been taken to developing a method for the efficient genetic modification of chickens. These have been directed at manipulation of the avian embryo at three key stages of development: (i) the newly fertilized egg; (ii) the embryo in newly laid eggs; and (iii) embryos that have reached ~2 days of incubation, when the primordial germ cells (PGCs), the precursors of the gametes, can be accessed. The different approaches and their relative successes have been discussed extensively [23–27], with the most successful methods described thus far being based on the use of viruses as gene transfer vectors.

Retroviral vector gene transfer

The first genetically modified birds were produced using vectors derived from avian retroviruses, which were obvious candidates for gene transfer vectors because they integrate into the chromosomes of their host as an obligatory step in their normal life cycle. Modification of vectors derived from avian leucosis virus (ALV) and reticuloendotheliosis virus resulted in replication-defective vectors that only undergo a single cycle of integration within the host [22,23]. A replication-defective vector comprises a copy of the viral genome from which the genes encoding viral proteins have been removed and the transgene of interest has been inserted; this modified copy of the viral genome retains the sequences required for viral DNA integration and viral genome packaging. Copies of the viral protein genes, including a gene encoding viral coat protein, are maintained as separate plasmids. The vector and viral protein genes are cotransfected into tissue culture cells to enable the synthesis of complete viral particles. The viral particles are collected and, for introduction into a chick embryo, injected into the embryo in a newly laid egg. Because the chick embryo at this stage of development is multicellular, any transgenic birds that hatch will be chimeras with respect to integration of the viral vector. An ALV vector was used to generate transgenic birds, but the frequency of transgenic cockerels produced was low (10%) and only one, from a total of 56 cockerels screened, produced transgenic offspring. The frequency of production of transgenic offspring from this male was also low – ~0.7% [21]. Although this method is inefficient, it has been used to generate transgenic birds that express low levels of a therapeutic protein. An increased germline transduction frequency was obtained using a vector derived from the avian spleen necrosis virus, with one of 15 males demonstrating a germline transmission frequency of ~0.9% [28].

These vectors are derived from oncogenic retroviruses and the results from their use in birds, and from studies using murine retroviral vectors in mice and tissue culture cells, suggest that they have low efficiencies in the production of transgenic animals and that expression of transgenes introduced using these vectors is likely to be susceptible to silencing. A novel group of vectors has recently been derived from the lentivirus class of retroviruses [29]. These vectors, which were developed principally for gene therapy applications, have potential advantages over oncoretroviral vectors for production of transgenic chickens. Moreover, they have been used to generate transgenic mice, pigs [30,31] and cattle [32] at high efficiency and, in mice, reliable tissue-specific expression that was maintained after germline transmission has been demonstrated [33,34].

The efficiency with which lentiviral vectors, which are derived from equine infectious anaemia virus, could be used to transduce the chicken germline was investigated [35]. Vectors carrying a reporter transgene, which would facilitate analysis of expression in transgenic birds, were packaged using an envelope coat protein from vesicular stomatitis virus. The virus was concentrated to give high titres and injected into newly laid eggs that were subsequently cultured to hatch. Twelve cockerels were generated and all identified as chimeric transgenic animals. Ten of these birds were mated and transgenic chicks detected in offspring from all the cockerels tested at frequencies from 4% to 45%. Analysis of the reporter transgenes in several independent transgenic lines showed that the expression pattern was conserved. Moreover, the expression pattern was maintained after germline transmission to the next generation with no detectable silencing of transgene expression. The key limitation of lentiviral vectors for production of transgenic animals is that the size of the transgene is restricted to ~8 kb. The high efficiency of this system will facilitate the testing of many different transgene constructs, specifically those designed to express therapeutic proteins for incorporation in the egg white or yolk of transgenic hens.

Expression of proteins in transgenic chickens

To date, there is limited information in the literature describing the deposition of foreign proteins in eggs. Analysis of eggs from transgenic birds, which were generated using an ALV vector containing a CMV promoter linked to the coding sequence for human interferon α-2b, detected up to 200 μg of human interferon in the white of a laid egg [36]. Although the CMV promoter is often considered ubiquitous, considerable variation in expression levels of the reporter gene *lacZ*, which is driven by the CMV promoter, were detected between tissues in transgenic hens, with expression in the oviduct among the lowest (Figure 1c) [35]. The *O*-linked glycans of recombinant human interferon α-2b expressed in transgenic hens were analysed and an estimated 38% were correctly glycosylated with respect to the naturally occurring human protein [36].

Conclusions

The recent improvements of transgenic technology for chickens and preliminary results on protein expression in birds are encouraging. It is probable that the promise of the development of transgenic hens for the production of therapeutic proteins will be tested in the next few years, which will be numerous years after this potential application was initially suggested. Several biotechnology companies are actively pursuing this goal [4]. Many questions remain to be answered in terms of levels of protein production, posttranslational modification of proteins and protein purification to a standard that meets regulatory approval. The data available describing posttranslational modifications in the chicken are intriguing. However, the level and nature of glycosylation of foreign proteins that are synthesized at high levels in the oviduct necessitate the successful production of transgenic birds expressing these high levels of protein to enable the analysis of the resultant material. Glycosylation of chicken IgGs is similar to that of human IgGs, but native chicken IgGs also contain high mannose structures that are not found in human IgGs [16].

The effects on integrity of the eggs produced by transgenic hens expressing additional proteins also require investigation, and are likely to depend on the nature of the specific proteins expressed. The possibility of production of human antibodies is particularly exciting because these are unlikely to have adverse effects on the birds and would meet the growing requirement for large-scale production of therapeutic antibodies. Once these have been established, the relative value of production of proteins in hens' eggs, compared with other protein production systems such as plants and cell culture that are also undergoing continuous improvement, can be assessed. An avian system will need to be competitive in terms of the time taken to generate the protein product, reliability of production, cost-effectiveness and quality of product. If these criteria are met, the avian system could potentially become the method of choice for the production of specific proteins.

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