Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Antibody manufacture in transgenic animals and comparisons with other systems

Louis-Marie Houdebine

Various forms of recombinant monoclonal antibodies are being used increasingly, mainly for therapeutic purposes. The isolation and engineering of the corresponding genes is becoming less of a bottleneck in the process; however, the production of recombinant antibodies is itself a limiting factor and a shortage is expected in the coming years. Milk from transgenic animals appears to be one of the most attractive sources of recombinant antibodies. None of the production systems presently implemented (CHO cells, insect cells infected by baculovirus, or transgenic animals and plants) has yet been optimized. This review describes the advantages of using milk for antibody production in comparison with the other systems.

Addresses
Biologie du Développement et Biotechnologies, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France; e-mail: houdebine@diamant.jouy.inra.fr

Current Opinion in Biotechnology 2002, 13:625–629

0958-1669/02/$ – see front matter

Abbreviations
ADCC antibody-dependent cellular cytotoxicity
CHO Chinese hamster ovary
GlcNac *N*-acetylglucosamine
NANA *N*-acetylneuraminic acid
NGNA *N*-glycosylneuraminic acid

Introduction

Animals are the natural producers of the antibodies they use for protection against diseases. Vaccination and passive immunization exploit this property extensively. The great variety of antibodies, as well as their high specificity and affinity for antigens, makes it possible to use these molecules for purposes other than passive protection against diseases. Indeed, antibodies are one of the favourite tools of biologists and are extensively used for various *in vivo* and *in vitro* diagnostics. Antibodies may also have natural enzymatic activities, which can be optimized by mutations [1]. These antibodies, known as abzymes, might become a new source of enzymes. Furthermore, antibodies could be used to manage environmental pollution by neutralizing toxic substances [2].

The major uses of monoclonal antibodies are expected to be in the medical domain. For example, the administration of recombinant antibodies can be used to protect patients against respiratory syncytial virus infection [3]. Such an approach is particularly attractive when no vaccines or antibiotics are available or when the pathogens have become resistant to antibiotics [4]. Interestingly, not only IgG but complete recombinant chimaeric IgA can also be used to inactivate pathogens in patients [5]. Antibodies can also mediate protection against intracellular pathogens [6]. In this respect, they could provide a major line of defence against biological attack by terrorists [4], particularly as they can be kept as a powder and easily self-injected in case of attack.

Antibodies neutralize pathogens via different mechanisms. A simple binding of the antibody to a key molecule of the pathogen may be sufficient to provide protection. In other cases, antibody-dependent cellular cytotoxicity (ADCC) is needed [7••]. Recent studies have shown that the simultaneous use of monoclonal antibodies directed against several different conformational epitopes of the human immunodeficiency virus envelope, which binds CD4 in human lymphocytes, prevented infection by various mutants of the virus [8]. Antibodies can specifically block the action of natural factors *in vivo*, and are used to inhibit some rejection mechanisms after organ transplantation.

Antibodies can also be used as vehicles to target active molecules to specific cells. For example, radioactive ions can be bound to antibodies that specifically recognize tumour cells. This approach proved to destroy Hodgkin's [9] and non-Hodgkin's lymphoma [10]. Toxins bound to antibodies can have similar effects [11].

The versatility of antibodies is further demonstrated by their use in transfection studies. Plasmids can be non-covalently bound to antibodies and these complexes allow for efficient and specific *in vivo* transfection. This method, called antifection [12], was used to destroy human tumours grafted to severe combined immunodeficiency disease (SCID) mice: genes that induce cell death by apoptosis were used for this purpose. Other 'killer' genes can also be used (F Hirsch *et al.*, personal communication). The same approach may be extended to tumour cell genes coding for enzymes capable of locally transforming a prodrug into an active antitumour molecule. This versatile method could also provide cells with genes coding for growth factors, leading to tissue regeneration.

As can be seen, antibodies have a vast range of uses both *in vivo* and *in vitro*, all of which require different forms of these molecules. Methods for efficient antibody production are therefore of significant interest to biotechnologists. In this review we consider some of the advantages and disadvantages of the available antibody expression systems and discuss future considerations.

Antibody gene isolation and engineering

Complete antibodies or monovalent or bivalent fragments can be synthesized and used for many of the applications

described above. Antibodies can be murine, chimaeric (i.e. largely murine but containing the human constant region) or humanized. In the case of humanized antibodies, only the regions that recognize the antigen are not of human origin [13]. Humanization minimizes as much as possible the immune reactions of patients against the injected antibodies. Antibodies produced in this way may be IgGs, which contain light and heavy chains, or IgAs which have, in addition to heavy and light chains, a junction chain and a secretory component. Ideally, the production system should provide antibodies requiring no modifications before use.

The vast majority of monoclonal antibodies used to date are of murine origin. Rabbits, which have a broad antibody repertoire, are a possible alternative, but both of these approaches require antibody humanization [14].

The most attractive strategy is to prepare human monoclonal antibodies; however, the cloning of the corresponding genes from human cells has proved to be difficult. Phage display [15] or polysome display [13] allow the systematic cloning of genes or gene fragments coding for heavy or light chains, which are capable of forming functional antibodies after their random association. These approaches may require laborious antibody mutations in order to obtain the appropriate specificity and affinity.

Pioneering work showed that recombinant antibodies could be obtained from the blood of transgenic animals [16,17]. However, these antibodies were hybrids containing host chains. Nevertheless, antibodies obtained using this approach were able to protect fish against haemorrhagic septicaemia virus [18] and mice against prion disease [19].

The simplest way to obtain human antibody genes is to use transgenic mice. These animals harbour human Ig loci and their own Ig loci have been eliminated by homologous recombination [20–22]. These 'immunized' mice can potentially provide most of the human antibodies required for human therapy (see also Update).

Antibody expression systems

Several studies have concluded that bacteria and yeast are only suitable for the synthesis of antibody fragments. By contrast, insect cells and Chinese hamster ovary (CHO) cells can be the source of intact antibodies fully capable of recognizing antigens [23,24•,25].

Cultured cells, even when optimized, are expected to have limited capacity to produce large amounts of antibodies [23,24•,25,26••]. Transgenic animals and plants appear to be the only tools enabling high production levels [26••,27–29,30••].

Animal and plant cells have a similar capacity to assemble antibody subunits and active IgAs were prepared from plants [27] as well as from CHO cells [25]. However, these systems are not equivalent as far as the post-translational modifications of antibodies are concerned. The different systems vary in their capacity to glycosylate antibodies. This point is essential, as glycosylation is required to obtain antibodies that are stable *in vivo* and capable of inducing complement and ADCC [31].

Antibodies extracted from plants (so-called plantibodies) have *N*-glycans that are very different from those secreted by mammalian cells [32,33]. The *N*-glycans of plantibodies are not only unable to provide them with some biological properties, but might also induce various undesirable side-effects in patients. Preliminary data indicate that plantibodies in mice do not provoke a significant immune response [33], or at least not after a limited number of injections, but additional studies will be required before this problem can be considered to be negligible.

Murine IgGs prepared from tobacco were able to prevent tooth infection by *Streptococcus mutans* in mice without causing any side-effects [34]. Interestingly, human anti-Rhesus D IgG1 antibody produced in *Arabidopsis* inactivated Rhesus-D antigen even though natural killer (NK)-mediated ADCC did not occur. Thus, unexpectedly, this inactivating effect seems to be mediated by a mechanism different from ADCC. This suggests that plantibodies might have a broader pattern of therapeutic activity than anticipated [35].

Native proteins are often heterogeneously glycosylated. This phenomenon occurs on a greater scale in recombinant proteins secreted from CHO cells or mammary glands [36]. This seems to be due to a saturation of the glycosylation machinery, as recombinant proteins are less completely glycosylated when their concentration in milk is higher. The under-glycosylated antibodies may be less stable *in vivo* and might not have all the expected biological properties.

Plantibodies are not sialylated. Antibodies found in CHO culture medium and in milk are only partially sialylated. Sialic acid exists in two forms: *N*-glycosylneuraminic acid (NGNA) and *N*-acetylneuraminic acid (NANA). Human proteins contain the NANA form of sialic acid and ruminant proteins the NGNA form, whereas rabbit proteins have both forms and chicken proteins only the NANA form [37]. It is expected that antibodies with the NGNA form of sialic acid could provoke some undesirable side-effects including immune response in patients.

NK-mediated ADCC is known to be induced by antibodies only if the *N*-glycans grafted to Asp297 in the human constant region of the antibody is properly glycosylated. The presence of *N*-acetylglucosamine (GlcNac) in the triantennary *N*-glycan is also thought to be required for inducing ADCC. It is known that plant cells do not add GlcNac and that this is also the case for several types of animal cell. The presence of terminal GlcNac in recombinant antibodies extracted from milk has not been documented so far.

Glycosylation of antibodies can be improved in plants and animals by transferring the genes encoding enzymes capable of adding GlcNac, sialic acid, fucose and galactose to the *N*-glycans. This has been achieved in CHO cells [38•,39•] and is under study in plants [32] and animals.

The production of a few monoclonal antibodies in milk has been documented [30••]. Mouse monoclonal antibodies, both humanized and non-humanized, that are capable of neutralizing coronavirus have been produced in mouse milk at a concentration of up to several grams per litre [40,41].

Anti-CD6 [42], anti-CD19 [43] present on the cell surface, and antitransferrin receptor–RNase fusion protein [44] have been prepared in mouse milk. Several antibodies from goat milk are also available [30••].

Protein purification is known to be a key stage in the preparation of biopharmaceuticals. The culture medium of CHO cells may contain cell debris, lipids, DNA, cellular proteins, viruses and other pathogens and, potentially, serum albumin, transferrin and serum. Immunoglobulin purification from milk does not raise particular problems: lipids are eliminated in an early step by centrifugation and caseins and lactose can be separated from immunoglobulin by membrane filtration. Affinity chromatography exchangers can provide antibodies with 99.9% purity with a yield of 65% [30••]. The presence of host animal IgG and IgA in milk may complicate the purification protocol in some cases. Available chromatographic systems have given satisfactory results so far.

Antibody purification from plants can be achieved with existing methods. The problems that are encountered are different when antibodies are stored in seeds rather than in leaves. It is acknowledged that it may be more difficult to separate antibodies from seed proteins than from leaf cellular proteins; however, products extracted from leaves are more likely to contain contaminants that will have undesirable side-effects when injected into patients.

Conclusions

The available data leave little doubt as to the capacity of CHO cells and transgenic plants and animals for the large-scale preparation of diverse recombinant antibodies. Different experts have their own view on the advantages and limitations of these different systems, but the data are still too scarce to allow precise conclusions.

It is claimed that up to 10 kg of recombinant antibodies per acre can be obtained from transgenic plants [27]. Grams of antibodies per litre of milk have been repeatedly obtained. Optimization of vectors and, particularly, the use of gene insulators will allow increased and more predictable production of antibodies in milk [45]. Recent work has shown that loci are bordered by DNA regions insulating their genes from those of the neighbouring loci. The known insulators contain silencers preventing cross-talk between genes of neighbouring loci, chromatin openers to give free access to the transcription machinery, and enhancers [46]. One insulator from the chicken β-globin locus allowed most if not all transgenic lines to express foreign genes under the control of a ubiquitous promoter [47] and a milk protein gene promoter (S Rival-Gervier, unpublished results). Interestingly, long genomic DNA fragments containing two independent milk protein genes (α-lactalbumin and whey acidic protein) allowed the highly efficient expression of these genes in transgenic mice [48–50]. These data strongly suggest that the long DNA fragments contained insulators, which can be associated to gene constructs to optimize their expression in milk. Although CHO cells have a more limited production capacity, they could still provide a cheaper production system than goat milk for quantities ranging from 10 to 50 kg per year [51].

Transgenic plants are expected to provide up to 1 kg of plantibodies after 36 months [28]. The same levels could be obtained from rabbit milk, but not from goat milk.

One advantage of transgenic plants and animals over CHO cells is their flexibility. Building a 100 000 L fermentor requires four years and costs $400 million [7••]. Scaling up production in CHO cells is therefore much more difficult than simply using more plants or animals.

An argument commonly used as a reason to favour the use of transgenic plants rather than animals to produce recombinant antibodies is that plants are devoid of human pathogens. The reality is more subtle: it is possible to breed animals in conditions where they are not subjected to infections [52••]. Animals can be bred that are not contaminated by prions and the presence of prions in purified proteins can be detected. In the future, animals with an inactive prion protein gene could be available. One species, the rabbit, is known to transmit only rare and minor diseases to humans. Moreover, this animal is not susceptible to prion diseases and may prove useful for antibody production in the future.

In view of concerns about infection, the US Food and Drug Administration and the European Medicines Evaluation Agency have laid down points to consider when preparing recombinant proteins from milk. These guidelines do not appear to be a bottleneck when using animals for the preparation of recombinant antibodies.

One of the major advantages of transgenic plants and animals over cultured cells is their robustness. Domestic transgenic plants and animals can be maintained in already defined standard conditions and their levels of production are very stable. Milk secretion is essentially constant for weeks or months (depending on the species) and the same is true for the secretion of recombinant proteins in milk. In cell culture, protein glycosylation is known to be dependent on CHO metabolism, which is variable according to

culture conditions [24•,31]. By contrast, the metabolism of the mammary cell is much more stable and the glycosylation of the recombinant protein secreted in milk is constant for weeks or months. Lines of transgenic plants and animals can be preserved using well-known techniques. Seeds and milk can be easily stored until it is time to purify the antibodies without any loss of activity.

Transgenic plants producing antibodies may raise environmental concerns. Indeed, plants cultured in fields could release recombinant proteins that have an effect in humans. Furthermore, transgenic plants are acknowledged to disseminate their genes in neighbouring fields in an uncontrolled manner. This phenomenon is expected to have a negligible impact for agriculture in most cases. The situation is quite different for pharmaceutical-producing plants. These environmental problems are expected to be solved using systems to control plant reproduction. Such problems are unlikely to be encountered with farm animals, which are kept in enclosed areas. Biological fluids, other than milk, from transgenic animals can theoretically be the source of pharmaceutical proteins [53]. Among these systems, egg white from transgenic chickens appears the most attractive [54] (see also Update). At present, the production of antibodies in milk is more technically mature than other systems using transgenic animals, including chickens [54] or plants [27].

At this time, 11 recombinant antibodies have been approved by the Food and Drug Administration. About 400 have been prepared in different ways and are currently under testing. The present total worldwide capacity for the production of recombinant proteins in cultured cells is estimated to be 400 000 L, but about five to six times this capacity will be needed before the end of the present decade to fulfil our manufacturing needs [7••,26••]. The imminent shortage of cell culture capacity suggests that the use of both transgenic plants and animals will be required to reach this goal.

Update

The production of recombinant antibodies is now being extended to cow. A single human artificial chromosome harbouring the unrearranged human heavy (H) and lambda (λ) chain loci has been introduced into the cow genome using micro cell- mediated chromosome transfer and cloning techniques. Mature and functional human immunoglobulins were found in the blood of the transchromosomic calf [55,56]. A recent review by Dove [57] gives additional data supporting the idea that transgenic animals are an inevitable tool to produce the needed recombinant antibodies in the coming years.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Fletcher MC, Kuderova A, Cygler M, Lee JS: Creation of a ribonuclease abzyme through site-directed mutagenesis. *Nat Biotechnol* 1998, 16:1065-1067.
2. Harris B: Exploiting antibody-based technologies to manage environmental pollution. *Trends Biotechnol* 1999, 17:290-295.
3. Simoes EA: Respiratory syncytial virus infection. *Lancet* 1999, 354:847-852.
4. Casadevall A: Antibodies for defense against biological attack. *Nat Biotechnol* 2002, 20:114.
5. Corthesy B: Recombinant immunoglobulin A: powerful tools for fundamental and applied research. *Trends Biotechnol* 2002, 20:65-71.
6. Casadevall A: Antibody-mediated protection against intracellular pathogens. *Trends Microbiol* 1998, 6:102-107.
7. •• Gura T: Therapeutic antibodies: magic bullets hit the target. *Nature* 2002, 417:584-586.

Summarizing recent data, the author reports arguments favouring the idea that the therapeutic use of recombinant antibodies is greatly increasing with the result that production capacity is becoming quite insufficient.

8. Ferrantelli F, Ruprecht RM: Neutralizing antibodies against HIV — back in the major leagues? *Curr Opin Immunol* 2002, 14:495-502.
9. Quadri SM, Lai J, Mohammadpour H, Vriesendorp HM, Williams JR: Assessment of radiolabeled stabilized F(ab′)2 fragments of monoclonal antiferritin in nude mouse model. *J Nucl Med* 1993, 34:2152-2159.
10. Webber D: Lymphoma Mabs rivalry continues. *Nat Biotechnol* 1998, 16:1000-1001.
11. Baluna R, Coleman E, Jones C, Ghetie V, Vitetta ES: The effect of a monoclonal antibody coupled to ricin A chain-derived peptides on endothelial cells ***in vitro***: insights into toxin-mediated vascular damage. *Exp Cell Res* 2000, 258:417-424.
12. Poncet P, Panczak A, Goupy C, Gustafsson K, Blanpied C, Chavanel G, Hirsch R, Hirsch F: Antifection: an antibody-mediated method to introduce genes into lymphoid cells ***in vitro*** and ***in vivo.*** *Gene Ther* 1996, 3:731-738.
13. Hudson PJ: Recombinant antibody fragments. *Curr Opin Biotechnol* 1998, 9:395-402.
14. Rader C, Ritter G, Nathan S, Elia M, Gout I, Jungbluth AA, Cohen LS, Welt S, Old LJ, Barbas CF III: The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies. *J Biol Chem* 2000, 275:13668-13676.
15. Huls GA, Heijnen IA, Cuomo ME, Koningsberger JC, Wiegman L, Boel E, van der Vuurst de Vries AR, Loyson SA, Helfrich W, van Berge Henegouwen GP *et al.*: A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. *Nat Biotechnol* 1999, 17:276-281.
16. Lo D, Pursel V, Linton PJ, Sandgren E, Behringer R, Rexroad C, Palmiter RD, Brinster RL: Expression of mouse IgA by transgenic mice, pigs and sheep. *Eur J Immunol* 1991, 21:1001-1006.
17. Weidle UH, Lenz H, Brem G: Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs. *Gene* 1991, 98:185-191.
18. Lorenzen N, Cupit PM, Einer-Jensen K, Lorenzen E, Ahrens P, Secombes CJ, Cunningham C: Immunoprophylaxis in fish by injection of mouse antibody genes. *Nat Biotechnol* 2000, 18:1177-1180.
19. Heppner FL, Musahl C, Arrighi I, Klein MA, Rulicke T, Oesch B, Zinkernagel RM, Kalinke U, Aguzzi A: Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. *Science* 2001, 294:178-182.
20. Jakobovits A: Production of fully human antibodies by transgenic mice. *Curr Opin Biotechnol* 1995, 6:561-566.
21. Mendez MJ, Green LL, Corvalan JR, Jia XC, Maynard-Currie CE, Yang XD, Gallo ML, Louie DM, Lee DV, Erickson KL *et al.*: Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat Genet* 1997, 15:146-156.
22. Tomizuka K, Yoshida H, Uejima H, Kugoh H, Sato K, Ohguma A, Hayasaka M, Hanaoka K, Oshimura M, Ishida I: Functional expression and germline transmission of a human chromosome fragment in chimaeric mice. *Nat Genet* 1997, 16:133-143.
23. Kost TA, Condreay JP: Recombinant baculoviruses as expression vectors for insect and mammalian cells. *Curr Opin Biotechnol* 1999, 10:428-433.

24. Andersen DC, Krummen L: Recombinant protein expression for
• therapeutic applications. *Curr Opin Biotechnol* 2002, 13:117-123.
The authors describe possible modifications of the CHO cell culture method to optimize its capacity to produce recombinant antibodies at an industrial scale.

25. Berdoz J, Blanc CT, Reinhardt M, Kraehenbuhl JP, Corthesy B: ***In vitro*** comparison of the antigen-binding and stability properties of the various molecular forms of IgA antibodies assembled and produced in CHO cells. *Proc Natl Acad Sci USA* 1999, 96:29-34.

26. Andersson R, Mynahan R: The protein production challenge: ***in vivo***.
•• *The Business and Medicine Report* 2001, May 1-5.
The authors are Arthur D Little consultants who compared the capacity of the different production systems for recombinant antibodies. They also evaluated the total potency of these systems and the amplitude of the market in the coming years.

27. Larrick JW, Yu L, Naftzger C, Jaiswal S, Wycoff K: Production of secretory IgA antibodies in plants. *Biomol Eng* 2001, 18:87-94.

28. Stoger E, Sack M, Fischer R, Christou P: Plantibodies: applications, advantages and bottlenecks. *Curr Opin Biotechnol* 2002, 13:161-166.

29. Houdebine LM: Transgenic animal bioreactors. *Transgenic Res* 2000, 9:305-320.

30. Pollock DP, Kutzko JP, Birck-Wilson E, Williams JL, Echelard Y,
•• Meade HM: Transgenic milk as a method for the production of recombinant antibodies. *J Immunol Methods* 1999, 231:147-157.
The authors have prepared more than 10 recombinant antibodies in mouse or goat milk. This article summarizes the different problems they faced and how they achieved success.

31. Wright A, Morrison SL: Effect of glycosylation on antibody function: implications for genetic engineering. *Trends Biotechnol* 1997, 15:26-32.

32. Faye L, Lerouge P, Gomord V: Perfect mammalian glycoproteins in plants come of age. In *Molecular Farming*. Edited by Toutant JP, Balazs E. INRA Editions Paris: Springer-Verlag, Berlin; 2001, 187-195.

33. Chargelegue D, Vine ND, Van Dolleweerd CJ, Drake PM, Ma JK: A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. *Transgenic Res* 2000, 9:187-194.

34. Ma JK, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB, Lehner T: Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nat Med* 1998, 4:601-606.

35. Bouquin T, Thomsen M, Nielsen LK, Green TH, Mundy J, Dziegie MH: Human anti-rhesus D IgG1 antibody produced in transgenic plants. *Transgenic Res* 2002, 11:115-122.

36. Lubon H: Transgenic animal bioreactors in biotechnology and production of blood proteins. *Biotechnol Annu Rev* 1998, 4:1-54.

37. Raju TS, Briggs JB, Borge SM, Jones AJ: Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* 2000, 10:477-486.

38. Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE: Engineered
• glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 1999, 17:176-180.
In this study, it is reported that CHO cells overexpressing β(1,4)-*N*- acetylglucoseaminyltransferase III produce recombinant antibodies harbouring the bisecting GlcNac monosaccharide. These antibodies induce ADCC. The same approach could be extended to transgenic animals to obtain fully active antibodies in milk.

39. Weikert S, Papac D, Briggs J, Cowfer D, Tom S, Gawlitzek M,
• Lofgren J, Mehta S, Chisholm V, Modi N *et al.*: Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. *Nat Biotechnol* 1999, 17:1116-1121.
CHO cell lines overexpressing human β1,4-galactosyltransferase or α2,3-sialyltransferase secrete a higher proportion of recombinant antibodies containing sialic acid. This approach could be extended to transgenic animals in order to improve glycosylation of recombination antibodies secreted in milk.

40. Sola I, Castilla J, Pintado B, Sanchez-Morgado JM, Whitelaw CB, Clark AJ, Enjuanes L: Transgenic mice secreting coronavirus neutralizing antibodies into the milk. *J Virol* 1998, 72:3762-3772.

41. Castilla J, Pintado B, Sola I, Sanchez-Morgado JM, Enjuanes L: Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. *Nat Biotechnol* 1998, 16:349-354.

42. Limonta J, Pedraza A, Rodriguez A, Freyre FM, Barral AM, Castro FO, Lleonart R, Gracia CA, Gavilondo JV, de la Fuente J: Production of active anti-CD6 mouse/human chimeric antibodies in the milk of transgenic mice. *Immunotechnology* 1995, 1:107-113.

43. Van Kuik-Romeiln P, De Groot N, Hooijberg E, De Boer H: Expression of a functional mouse–human chimeric anti-CD19 antibody in the milk of transgenic mice. *Transgenic Res* 2000, 9:155-159.

44. Newton DL, Pollock D, DiTullio P, Echelard Y, Harvey M, Wilburn B, Williams J, Hoogenboom HR, Raus JC, Meade HM, Rybak SM: Antitransferrin receptor antibody-RNase fusion protein expressed in the mammary gland of transgenic mice. *J Immunol Methods* 1999, 231:159-167.

45. Houdebine LM, Attal J, Vilotte JL: Vector design for transgene expression. In *Transgenic Animal Technology*, Edn 2nd. Edited by Pinkert CA. Academic Press: London, UK; 2002, in press.

46. Recillas-Targa F, Pikaart MJ, Burgess-Beusse B, Bell AC, Litt MD, West AG, Gaszner M, Felsenfeld G: Position-effect protection and enhancer blocking by the chicken β-globin insulator are separable activities. *Proc Natl Acad Sci USA* 2002, 99:6883-6888.

47. Taboit-Dameron F, Malassagne B, Viglietta C, Puissant C, Leroux-Coyau M, Chereau C, Attal J, Weill B, Houdebine LM: Association of the 5′HS4 sequence of the chicken β-globin locus control region with human EF1 α gene promoter induces ubiquitous and high expression of human CD55 and CD59 cDNAs in transgenic rabbits. *Transgenic Res* 1999, 8:223-235.

48. Fujiwara Y, Miwa M, Takahashi R, Hirabayashi M, Suzuki T, Ueda M: Position-independent and high-level expression of human α-lactalbumin in the milk of transgenic rats carrying a 210-kb YAC DNA. *Mol Reprod Dev* 1997, 47:157-163.

49. Stinnakre MG, Soulier S, Schibler L, Lepourry L, Mercier JC, Vilotte JL: Position-independent and copy-number-related expression of a goat bacterial artificial chromosome α-lactalbumin gene in transgenic mice. *Biochem J* 1999, 339:33-36.

50. Rival-Gervier S, Viglietta C, Maeder C, Attal J, Houdebine LM: Position-independent and tissue-specific expression of porcine whey acidic protein gene from a bacterial artificial chromosome in transgenic mice. *Mol Reprod Dev* 2002, 63:161-167.

51. Chadd HE, Chamow SM: Therapeutic antibody expression technology. *Curr Opin Biotechnol* 2001, 12:188-194.

52. Gavin WG: DVM: The future transgenics. *Regulatory Affairs Focus*
•• 2001, May 13-18.
The author gives comments on the official guidelines that need to be followed when producing recombinant proteins in milk. With reference to his own experience, the author considers that this regulatory process is not a limitation to the use of milk as a source of recombinant proteins.

53. Houdebine LM: Transgenic animal bioreactors. *Transgenic Research* 2000, 9:305-320.

54. Harvey AJ, Speksnijder G, Baugh LR, Morris JA, Ivarie R: Expression of exogenous protein in the egg white of transgenic chickens. *Nat Biotechnol* 2002, 20:396-399.

55. Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM: Cloned transchromosomic calves producing human immunoglobulin. *Nat Biotechnol* 2002, 20:889-894.

56. Echelard Y, Meade H: Toward a new cash cow. *Nat Biotechnol* 2002, 20:881-882.

57. Dove A: Uncorking the biomanufacturing bottleneck. *Nat Biotechnol* 2002, 20:777-779.