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PROSPECTS

The Prospects for Domesticating Milk Protein Genes

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Abstract It is possible to convert milk glands of transgenic animals into bioreactors producing heterologous proteins such as scarce human pharmaceuticals. To predictably and successfully engineer the milk gland, we will need a thorough understanding of its physiology. Expression studies in transgenic animals have located mammary specific and hormone inducible transcription elements in the promoter/upstream regions of milk protein genes, and transfection studies in cell lines or primary cells have identified constitutive and hormone inducible elements. Most importantly, it appears that in addition to individual promoter based transcription elements structural features of milk protein chromosomal loci may contribute to the tight developmental and hormonal regulation.

I will discuss milk protein gene regulation with emphasis on regulatory differences between genes and species, and the possibility that transcription elements function only properly within genetically defined chromatin domains. Novel strategies to build mammary expression vectors and to test their functionality without pursuing the standard transgenic route will be presented. Finally, I will discuss homologous recombination with the goal to target milk protein genes. Only through the domestication of milk protein genes will we be able to use their full potential in the mammary bioreactor.

Key words: mammary gland, milk protein genes, bioreactor, biotechnology, gene regulation

A plethora of milk protein genes from several species have been isolated. These include α-lactalbumin genes from rat [Qasba and Safaya, 1984], human [Hall et al., 1987], bovine [Vilotte et al., 1989], guinea pig [Laird et al., 1988], and caprine [Soulier et al., 1992], whey acidic protein (WAP) genes from mice and rats [Campbell et al., 1984] and rabbits [Thepot et al., 1990], the β-lactoglobulin gene from sheep [Ali and Clark, 1988], β-casein genes from mice [Yoshimura and Oka, 1989], rats [Jones et al., 1985], bovine [Gorodetsky et al., 1988] and goats [Persuy et al., 1992], and α-casein genes from rat [Jones et al., 1985] and bovine [Meade et al., 1990].

Three levels of milk protein gene regulation have been studied; mammary specificity, induction during pregnancy and lactation, and stimulation by steroid and peptide hormones.

MAMMARY SPECIFICITY

Promoter upstream sequences of the WAP [Andres et al., 1987; Gordon et al., 1987; Pittius et al., 1988a], β-lactoglobulin [Archibald et al., 1990], α-casein [Meade et al., 1990], β-casein [Greenberg et al., 1991; Lee et al., 1988], and α-lactalbumin [Stinnakre et al., 1991] gene direct expression of heterologous genes to mammary tissue in transgenic animals. However, in the context of the whole organism no mammary specific element has been isolated yet. Using transgenic animals transcription elements have been identified in the bovine α-lactalbumin gene between nucleotides −475 and −220 [Soulier et al., 1992], but it is not clear whether they are mammary specific and/or of general nature. With respect to the WAP gene, constitutive and hormone inducible elements have been identified in transfected tissue culture cells between −2500 and −450 [Doppler et al., 1991] and between −6300 and −3000 in transfected primary cells [Devinoy et al., 1991]. Transgenic experiments are being needed to resolve the apparent differences observed in the two assay systems.

Sequences in the WAP [Lubon and Hennighausen, 1987], α-lactalbumin [Lubon and Hennighausen, 1988], β-casein [Schmitt-Ney et al., 1991], and β-lactoglobulin [Watson et al., 1991] gene promoter bind nuclear proteins, some of them mammary specific, but the functional significance of most of these binding sites re-

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mains to be determined. A correlation between protein binding and functional activity has been made for the rat β-casein gene promoter which shows transcriptional activity in some immortalized mammary cell lines. The binding site for an apparently mammary specific nuclear protein was necessary for basal and hormone induced transcriptional activity [Schmitt-Ney et al., 1991]. However, it is not clear that every milk protein gene contains a mammary specific transcription element in the classical sense. The WAP gene, for example, is transcribed in vitro in nuclear extracts from a variety of non-mammary cells [Lubon et al., 1989], suggesting that mammary specificity may be imposed through a mammary specific access of general transcription elements to the chromatin template. In support of this the WAP gene can be expressed upon stable integration in some cell lines, but not in transient systems [Doppler et al., 1991].

As expected, β-casein [Lee et al., 1988] and α-lactalbumin [Soullier et al., 1992; Maschio et al., 1991; Vilotte et al., 1989] transgenes from rats, bovine, goat, and guinea pigs were expressed in transgenic mice which contain equivalent genes. However, it was surprising that regulatory elements from the mouse WAP gene were recognized efficiently in transgenic pigs [Shamay et al., 1991; Wall et al., 1991] which do not have an identifyable WAP gene. Similarly, the sheep β-lactoglobulin gene was expressed at high levels in transgenic mice [Simons et al., 1987] which also do not contain an endogenous counterpart. These experiments suggest that the molecular basis of mammary-specific gene expression is conserved between species and between different milk protein genes. In addition, expression levels may be higher when species boundaries are crossed [Shamay et al., 1991; Velander et al., 1991b].

DEVELOPMENTAL REGULATION

Although milk protein genes are subject to tight mammary specificity, striking differences have been noticed between their developmental and hormonal regulation [Hobbs et al., 1982]. Most noticable, while accumulation of WAP [Pittius et al., 1988a,b] and α-lactalbumin [Soulier et al., 1992] RNA occurs just prior to parturition, high levels of β-casein [Harris et al., 1991; Hennighausen et al., 1988; Shamay et al., 1992a; Gaye et al., 1986] and β-lactoglobulin [Devinoy et al., 1988; Harris et al., 1991; Shamay et al., 1992a] mRNA can already be detected in early pregnancy. The timing of expression is probably not due to differences in the concentration of lactogenic hormones, such as insulin, hydrocortisone, and prolactin, or their respective receptors, but may rather be caused by differences in the access of chromatin to individual signaling pathways. Evidence for this comes from transgenic studies with the WAP gene. Whereas the endogenous WAP gene is only induced late in pregnancy, timing of transgene expression is highly position dependent and in general seen already in early pregnancy [Burdon et al., 1991a,b; Pittius et al., 1988a,b; Shamay et al., 1992a]. Less position dependent regulation has been observed with the sheep β-lactoglobulin gene in transgenic mice [Harris et al., 1991]. Activation of endogenous and transgenic milk protein genes in the intact animal can be achieved in the absence of pregnancy and lactation. Treatment of immature virgin pigs with estrogen and progesterone resulted in the accumulation of β-casein and β-lactoglobulin mRNA, the latter to almost lactational levels [Shamay et al., 1992a]. Developmental and hormonal induction patterns obtained with WAP transgenes varied substantially with the integration site [Shamay et al., 1992a] further emphasizing the role of chromatin for regulated expression. Critical chromatin components may be matrix attachment regions (MAR) which appear to anchor genes to the nuclear matrix and thereby establish independent genetic domains [McKnight et al., 1992b].

HORMONAL INDUCTION

Hormonal induction of cloned rat β-casein and mouse and rabbit WAP genes has been studied in transiently and stably transfected cell lines, in transiently transfected organoids, and in transgenic animals. Because of differences in the test systems the results in some cases are not directly comparable, or may even be contradictory. Based on extensive mutagenesis studies with the rat β-casein gene promoter in HC11 cells it has been suggested that several elements, which bind a total of at least five nuclear proteins, participate in milk protein gene regulation [Schmidt-Ney et al., 1991]. A mammary element conferred cell type specificity, and hormone induced transcription was mediated by a relief of repression and displacement of repressor proteins from a separate element. Moreover, transcription was synergistically induced by glucocorticoids and prolactin, and activation by glu-

cocorticoids appeared to be not through the direct binding of steroid receptors to the promoter but rather indirectly [Doppler et al., 1990]. Whereas cell culture experiments have placed the key β-casein gene regulatory elements within 175 bp of promoter sequence [Schmidt-Ney et al., 1991], studies with primary mammary cells have placed additional elements further upstream [Yoshimura and Oka, 1990]. Surprisingly, the presence of these elements, however, did not permit efficient activation of the β-casein gene in transgenic mice [Lee et al., 1988]. Critical elements for in vivo expression appear to be located in a 14 kb goat β-casein gene which was expressed at high levels in transgenic mice [Persuy et al., 1992].

Insight into hormonal activation in the context of the whole organism has been obtained with the WAP gene. Activation of the endogenous mouse [Pittius et al., 1988a,b] and rabbit [Puissant and Houdebine, 1991] WAP genes in mammary organ cultures prepared from pregnant animals was dependent upon the synergistic presence of insulin, hydrocortisone, and prolactin. Activation of a mouse WAP transgene in mice [Burdon et al., 1991a] and swine [Shamay et al., 1992a] and in stably transfected cell lines [Doppler et al., 1991] was not any longer dependent upon prolactin, suggesting that prolactin response elements may be located outside the 2.5 kb promoter region employed. However, hormone requirements, in particular those for prolactin, varied between lines of animals, suggesting that flanking chromatin modified the response of transcription elements to hormonal pathways. This hypothesis was supported by experiments in which WAP transgenes were insulated with MAR elements and accurate prolactin response was observed [McKnight et al., 1992b].

EXPRESSION OF TRANSGENES IN MAMMARY TISSUE

Genetic control elements from the mouse and rabbit WAP, bovine α-casein, bovine α-lactalbumin, sheep β-lactoglobulin, and the rabbit and rat β-casein genes have been used to direct production of foreign proteins in the milk of transgenic mice, rabbits, sheep, and pigs [for references see Wilmut et al., 1991; Ebert et al., 1991; Wright et al., 1991; Hennighausen, 1990]. Several lessons have been learned from those experiments. In general, genomic clones are expressed better than cDNAs, specific introns may be necessary for high activity, expression levels are dependent upon the reporter gene, and expression of a given hybrid gene can greatly vary between different species.

A wealth of data accumulated with WAP (Fig. 1) and β-lactoglobulin based transgenes provided insight into the dynamics of transgene expression in the mammary gland. Most interestingly, regulatory elements from the mouse WAP gene appear to drive expression much more efficiently in transgenic pigs than in transgenic mice [Wall et al., 1991; Shamay et al., 1991; McKnight et al., 1992a], suggesting that it may be of advantage to use cross species regulatory elements for mammary expression. From the production point of view this has been confirmed with human protein C. A hybrid gene consisting of the mouse WAP gene and a protein C cDNA inserted into the first WAP gene exon (Fig. 1) was expressed in transgenic pigs at 1 mg of protein C per ml of milk [Velander et al., 1992b] and at lower levels in transgenic mice [Velander et al., 1992a]. Cross species expression differences may be unique for the WAP gene because it has not been seen with β-lactoglobulin based transgenes [Harris et al., 1991; Archibald et al., 1990; Wright et al., 1991].

Expression of milk protein gene promoter or genomic sequences linked to cDNA sequences encoding non-milk proteins, although in general milk specific, have been overall disappointingly low, suggesting that additional intronic sequences within the cDNA part may be required. Genomic sequences linked to milk protein promoter elements (Fig. 1) showed greatly improved expression levels [Archibald et al., 1990; Sandgren et al. and M.L. Houdebine, personal communications] supporting the hypothesis that intron sequences are of benefit. High activity of intron containing transgenes may be due to splicing as proposed earlier [Brinster et al., 1988; Palmiter et al., 1991; Choi et al., 1991] and to specific introns containing regulatory elements which interact with promoter based transcription elements [Whitelaw et al., 1991]. Since sequences encoding the protein to be produced are frequently only available as cDNAs, or the genomic sequences are too large to be easily manipulated, we decided to analyze whether WAP introns, if located 3′ of a heterologous *cDNA*, could confer high level expression. In a collaborative effort between the American Red Cross, Virginia Polytech, and the NIH a cDNA encoding human protein C was inserted into the first

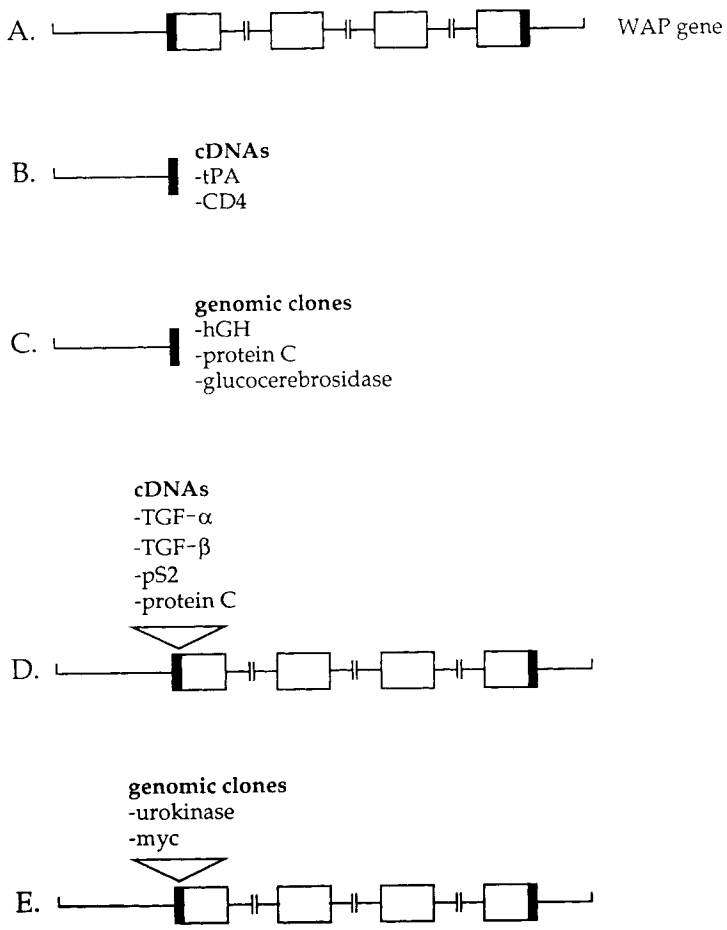

Fig. 1. WAP based transgenes. **A:** Structure of a 7 kb mouse WAP gene including 2.5 kb 5′ and 1.6 kb 3′ flanking sequence [Campbell et al., 1984]. The rectangles represent exons, with the solid parts encoding untranslated regions. The reporter genes were linked to WAP gene sequences via a KpnI site located at nucleotide +24 within the 5′ untranslated region of the first exon. Genomic sequences spanning the mouse and rat WAP gene have been expressed in transgenic mice [Bayna and Rosen, 1990; Burdon et al., 1991a,b], and the mouse WAP gene has been expressed in transgenic swine [Wall et al., 1991; Shamay et al., 1991, 1992a,b] and sheep [Rexroad et al., unpublished]. **B:** The human tPA cDNA was under the control of a 2.5 kb WAP promoter fragment [Gordon et al., 1987; Pittius et al., 1988a] and the human cD4 cDNA was controlled by a 1.8 kb promoter fragment [Shu-Hua et al., 1989]. **C:** Human growth hormone [Reddy et al., 1991; Günzburg et al., 1991] and glucocerebrosidase [Ginns et al., unpublished] genomic sequences were fused to the 2.5 kb mouse WAP gene promoter fragment. The human growth hormone gene was also expressed under a 6.3 kb promoter fragment from the rabbit WAP gene. Expression levels in 6 lines were about 10 mg per ml of milk [Houdebine et al., unpublished]. **D:** cDNAs encoding TGF-α [Sandgren et al., unpublished], TGF-β [Merlino et al., unpublished], pS2 [Tomasetto et al., 1989], and human protein C [Velander et al., 1992a,b] were cloned into the KpnI site of the 1st exon. The cDNAs contained protein start and stop codons. **E:** Genomic sequences encoding human myc and mouse urokinase [Sandgren et al., unpublished] were cloned into the KpnI site of the 1st exon.

exon of the WAP gene, and high level expression was obtained in transgenic pigs [Velander et al., 1992b]. This and other experiments clearly show that high gene activity can be obtained with introns positioned in the WAP gene located 3′ to various cDNAs (Fig. 1).

The combination or arrangement of DNA sequences in the hybrid gene may also influence expression levels, tissue specificity, and regulation. For example, whereas human α1-antitrypsin, sheep β-lactoglobulin, and human factor IX encoding cDNAs when integrated into the first exon of the sheep β-lactoglobulin gene were expressed in a range of ng to μg of protein per ml of milk [Whitelaw et al., 1991], TGF-α, pS2, and protein C encoding cDNAs inserted into the first exon of the WAP gene (Fig. 1) were expressed at μg to mg amounts per ml of milk. Expression of some transgenes, such as the urokinase gene inserted into the first exon of the mouse WAP

gene, or the human growth hormone gene linked to the rabbit WAP gene promoter (Fig. 1), actually exceeded that of the endogenous WAP gene. The size of the transgene may also influence its expression levels [Dale et al., 1992], suggesting that regulatory elements may cooperate to activate transgenic loci containing several transgene copies.

RESEARCH OPPORTUNITIES

Mammary Regulatory Domains

There is evidence to suggest that genetic units are located in topologically constrained loop domains which are flanked and defined by matrix attachment regions (MARs) [for review and references see Bonifer et al., 1991]. The presence of such MARs appears to permit local diversity of function, and prevent 'spill-over' to neighboring domains. In general, expression levels and accuracy of regulation of milk protein transgenes are highly dependent upon the integration site, suggesting that the transgenes did not encompass complete genetic units, and probably lacked insulator sequences to shield their regulatory elements from influences of flanking chromatin. Evidence that milk protein genes may contain MAR like sequences to insulate regulatory elements comes from transgenic experiments with WAP transgenes. Without MAR sequences expression and regulation of WAP transgenes is highly position dependent, but it appears to be accurately regulated in the presence of heterologous MARs from the chicken lysozyme locus [McKnight et al., 1992b]. Alternatively, milk protein genes may contain locus control regions (LCR) which are cis-acting elements, and probably enhancers which confer position independent expression to genes in transgenic loci [Grosveld et al., 1987].

The isolation of MAR like sequences or LCRs from milk protein genes will be necessary not only for studies on the hierarchy of milk gene regulation, but also for biotechnological purposes. From a pragmatic point of view, significant benefits may be realized by including either homologous or heterologous MARs with transgene constructs used in transgenic livestock. Though construct dependent, the proportion of transgenic large animals that express their transgene is approximately 60%. Given that the cost of producing transgenic sheep and pigs is in the tens of thousands of dollars, and production of transgenic cattle may be an order of magnitude higher, the use of MARs could substantially reduce production costs.

Transient Expression System

Expression of milk protein genes is probably controlled by a network of regulatory circuits which are linked to the intact developing mammary gland and homeostasis during puberty, pregnancy, lactation, and involution. The analysis of putative regulatory elements and of hybrid genes in tissue culture cells may therefore result in a simplified view of regulatory events occurring in the gland itself, and may not be transferrable to the intact organism. On the other hand, analyses of hybrid genes in transgenic mice are tedious and, further, the results may not reflect the situation in farm animals [Wall et al., 1991; McKnight et al., 1992a]. It will therefore be necessary to develop a predictive screening test for hybrid genes. A means of testing hybrid genes may be through the physical introduction of DNA via jet injection into mammary epithelial cells of a living lactating animal. We are currently pursuing a promising route by injecting solubilized DNA with a jet injection gun into mammary tissue of livestock [P.A. Furth and L.H., in preparation]. The jet accelerated gene (JAG) technology should facilitate the introduction of DNA into mammary epithelial stem cells. The generation of somatic transgenics for the purpose of establishing a mammary bioreactor in cows would cut the lead time by several years.

Mammary Expression Vectors

At present about 50% of the transgenic animals express a given transgene, and expression is highly variable and position dependent. High level and regulated expression in all animals may be achieved with milk protein derived MAR or LCR elements. Alternatively regulatory elements from other genes, such as MARs from the chicken lysozyme gene, which appear to function with heterologous genes [Stief et al., 1989; Phi-Van et al., 1990] may be used. It is also not necessary to built the elements into the respective expression vector, because the coinjection of MARs with hybrid genes normally results in cointegration in the same transgenic locus. The inclusion of such insulator sequences in a transgenic locus may also result in proper transgene regulation [McKnight et al., 1992b] which may be of particular importance in the light of cytotoxic effects potentially imposed by proteins. For example precocious expression of WAP in both

transgenic mice [Burdon et al., 1991b] and swine [Shamay et al., 1992b] resulted in impaired mammary development during pregnancy and a *milchlos* phenotype. Since DNA fragments coinjected into fertilized oozytes will in many cases integrate into the same transgene locus, and will thus be under the same regulatory control [Burdon et al., 1991a], it should be possible to generate transgenic animals that secrete several different proteins into their milk.

Inserting DNA Into Milk Gene Loci

Transgenic animals have advanced our understanding of milk gene regulation and have permitted the development of the mammary bioreactor, but this technology has its limitations in that the sites of transgene integration into the genome appear to be random. Although some of the scientific and biotechnological problems associated with position effects may be bypassed by employing MAR like insulator or LCR sequences, it would be very helpful to direct transgene integration to specific sites within the genome. The tools of homologous recombination in embryonic stem cells [for review see Bradley et al., 1991] and the Cre recombination system [Odell et al., 1990; Dale and Ow, 1991] will greatly enhance our abilities to manipulate mammary expression systems. With respect to homologous recombination the 'hit-and-run' technology makes it is possible to alter control elements within a locus. This allows the analysis of control elements in the context of the endogenous locus. Homologous recombination in combination with the Cre/lox recombination system will also be critical for the mammary bioreactor concept. Upon introduction of a the 34 bp lox sequence either into the endogenous locus of a milk protein gene or into a locus permitting high level regulated expression, it should be possible to use this site as an entry port for hybrid genes. Thus Cre mediated integration of microinjected genes into the host genome should result in reproducible high level production of foreign proteins.

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