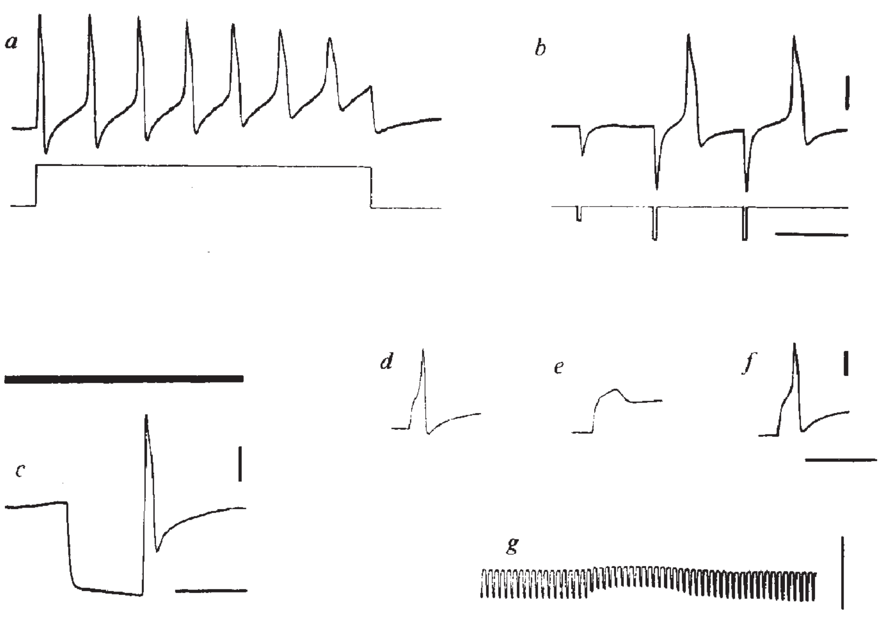

**Fig. 2** Intracellular recordings from salivary gland cells of *H. ghilianii*. *a*, *b*, Action potentials, which may exceed 90 mV, are initiated by depolarizing current (*a*) and also following hyperpolarization by inward current (*b*; 100-ms pulses were applied). *c*, A hyperpolarizing current pulse in one salivary cell (lower trace) produces rebound excitation but there is no sign of electrical responses in an adjacent cell, recorded simultaneously at high gain on the upper trace. Experiments of this type indicate that the gland cells are not electrically coupled. *d*–*f*, Effect of 5 mM $Co^{2+}$ added to the bathing solution. *d*, Control impulse in response to depolarizing current; *e*, 5 min after addition of $CoCl_2$ the action potential is abolished (a delayed rectification is apparent); *f*, recovery. This indicates that $Ca^{2+}$ is the major current carrier for generation of action potentials. *g*, Brief application of $10^{-5}$ M serotonin produces a depolarization and increase in membrane conductance (indicated by reduction in amplitude of constant-current hyperpolarizing pulses). Voltage scales (vertical bars), 25 mV (1 mV in *c*, upper trace); time scales (horizontal bars), 2 s.

The natural stimulus for action potential generation, whether neural and/or hormonal, is unknown. Several putative neurotransmitters, however, (dopamine, serotonin and acetylcholine) were found to depolarize the gland cells, with an accompanying decrease in membrane resistance (Fig. 2*g*) and occasionally the production of no more than four impulses. Interestingly, in the presence of dopamine, applied depolarizing current was sometimes found to produce repetitive firing which was not simply a consequence of the depolarization produced by the drug. If the impulse provides a trigger for secretion, it seems unusual that the cells are normally so difficult to activate. Feeding, however, occurs very infrequently (every few months) and an action such as that of dopamine may mimic a natural process of bringing the gland into secreting condition.

In mammals, salivary and other exocrine gland cells are electrically inexcitable, producing graded potential changes (often hyperpolarizations) which may or may not be related to secretory function[10]. The *H. ghilianii* salivary cells are similar in their electrical excitability to mammalian endocrine cells such as those in the pancreas[12], adenohypophysis[13] and adrenal gland[14] (some molluscan exocrine glands produce action potentials[15]). This similarity extends to the anode-break excitation[8] shown by chromaffin[14] and anterior pituitary cells[13].

We have also found the *H. ghilianii* salivary gland to be suitable for molecular genetics because the cells have a very large ramifying nucleus that displays gene amplification of $\sim 10^6$ times; this should allow precise questions to be asked about the relationship between secretion and transcription/translation of identified genes. Thus, the *H. ghilianii* salivary gland, with its unusual combination of properties, represents a simple, accessible preparation with distinct experimental advantages for cellular studies of glandular secretion.

This specialized leech has been generally unavailable because in its natural habitat it is restricted to Amazonia. We have developed techniques for breeding this species and a facility has been set up by Biopharm to supply hementin to interested researchers.

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# Production of transgenic rabbits, sheep and pigs by microinjection

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**Direct microinjection has been used to introduce foreign DNA into a number of terminally differentiated cell types as well as embryos of several species including sea urchin[1], *Candida elegans*[2], *Xenopus*[3], *Drosophila*[4,5] and mice[6–11]. Various genes have been successfully introduced into mice including constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes. Transgenic mice harbouring such genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA in several organs, substantial quantities of the foreign growth hormone in serum and enhanced growth[12,13]. In addition, the gene is stably incorporated into the germ line, making the phenotype heritable. Because of the scientific importance and potential economic value of transgenic livestock containing foreign genes, we initiated studies on large animals by microinjecting the fusion gene, *MT–hGH*[13], into the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. We report here integration of the gene in all three species and expression of the gene in transgenic rabbits and pigs.**

Studies with mouse ova indicated that integration of a gene into host chromosomes is much more efficient with nuclear than with cytoplasmic injection[14]. On this basis, we reasoned that nuclear injection would be an appropriate first approach with other species. The first problem encountered was visualization

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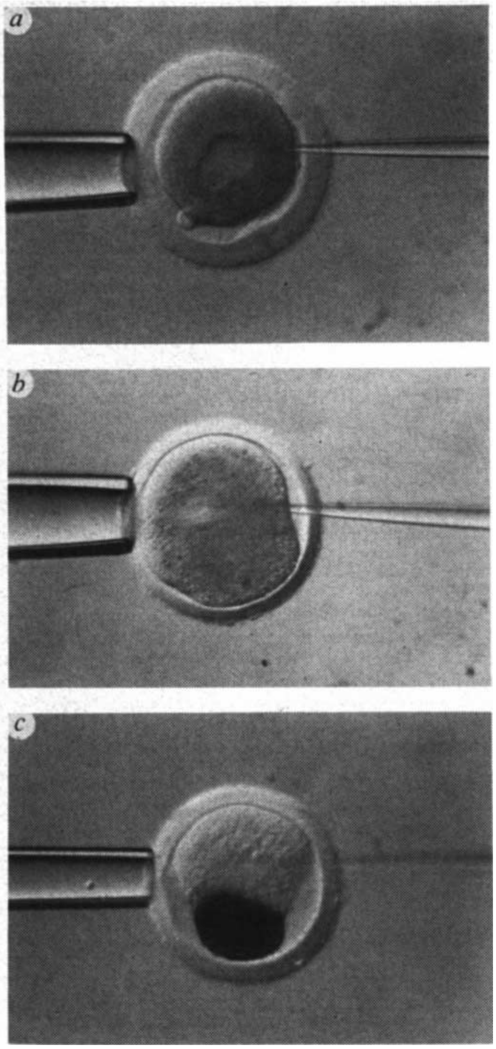

**Fig. 1** Interference-contrast photomicrographs of one-cell fertilized ova from rabbit (*a*), sheep (*b*) and pig (*c*) following microinjection. Ova are held by a blunt holding pipette (diameter $\sim$50 $\mu$m); an injecting pipette (diameter $\sim$1.5 $\mu$m) has penetrated the zona pellucida, plasma membrane and pronuclear envelope. The tip is seen within the nucleoplasm immediately following injection of buffer containing DNA. The porcine ova (*c*) has been centrifuged at 15,000*g* for 3 min to reveal the normally obscure pronuclei[15]. Visualization of nuclear structures is aided by the use of interference-contrast optics, and microinjection is carried out under ×250 magnification using a Leitz microinjecting apparatus[10,14]. Injection was monitored by observing the diameter of the pronuclus or nucleus, which was expanded $\sim$50%.

of the pronuclei or nuclei in the ova. Rabbit nuclear structures are readily seen (Fig. 1*a*). However, pronuclei and nuclei in sheep ova are difficult to locate and can only be seen by fluorescent microscopy using DNA specific fluorochromes (Hoechst, 33258) or by interference contrast (IC) microscopy (Fig. 1*b*). The combination of stain and ultraviolet light is damaging to the ovum (data not shown), so we used IC microscopy for microinjection. Fluorescent analysis indicated that IC microscopy is an effective method for pronuclear localization in approximately 80% of fertilized sheep eggs. Pig ova are opaque and no nuclear structures can be seen even with IC microscopy, but we found that centrifugation of pig ova at 15,000*g* for 3 min stratifies the cytoplasm (Fig. 1*c*), leaving the pronuclei or nuclei visible[15].

Once the nuclei could be visualized, microinjection was performed as described previously[10,14]. A few hundred copies of a 2.6 kilobase (kb) linear fragment containing the *MT–hGH* gene (see Fig. 2) were injected. Approximately 5,000 ova were injected and subsequently transferred to foster animals; about 500 of these resulted in fetuses or neonates (Table 1). The frequency of *MT–GH* integration was similar in the rabbit (12.8%) and the pig (11.0%) and low in sheep (1.3%). These integration efficiencies are probably accurate for the techniques being used because they are based on a large number of animals. The reasons for the lower integration frequency in these species compared to the mouse where it is $\sim$27% are unknown but could be related to factors such as the concentration of DNA, buffer composition, age of the ovum and the structure of the chromosomes[14].

The number of copies of the *MT-hGH* gene that integrated was estimated by quantitative dot hybridization. Figure 2*d* shows the quantitation method as applied to transgenic pigs. Gene copy numbers ranged from 1 to 490 copies per cell (Table 2). The DNA from some of the transgenic animals was also analysed by restriction enzyme digestion of the chromosomal DNA followed by agarose gel electrophoresis and Southern blotting. Figure 2*a* shows the results obtained when the DNA was restricted with *Eco*RI, an enzyme that cuts once within the injected DNA. The probe detects two prominent bands in several rabbits and pigs. One band is close to the length of the injected DNA fragment (2.6 kb) and probably represents a tandem, head-to-tail array of the *MT–hGH* genes as is typically observed in transgenic mice[10,12]. The other band is approximately twice that length and might represent a head-to-head dimer, but further analysis will be required to test that possibility. When the DNA was restricted with *Sst*I (Fig. 2*b*), an enzyme that cuts twice within the injected DNA, two bands of the expected size were observed in all of the pigs and rabbits. Analysis of the sheep sample with *Eco*RI (not shown) and *Sst*I (Fig. 2*c*) revealed bands that were inconsistent with an intact *MT–hGH* gene, suggesting that the DNA had been trimmed or rearranged prior to integration.

Expression of the integrated genes was examined by quantitating *MT–hGH* mRNA by solution hybridization (Table 2). Only 4 of 16 rabbits analysed had any detectable *MT–hGH* mRNA in the liver, but the level was substantial in one of these. In mice, the frequency of expression of this gene is close to 70% (ref. 13). In pigs, mRNA levels were measured only in tail or ear samples because we did not want to risk adverse consequences of liver biopsy. Although tail and ear tissues are not primary sites of *MT* gene expression, we detected low levels of *MT–hGH* mRNA in several of the transgenic pigs (Table 2).

Plasma samples taken from pigs at birth and $\sim$1 month later were analysed for hGH by radioimmunoassay. At birth, 11 of 18 pigs had detectable levels of hGH, ranging between 2 and 730 ng ml$^{-1}$ (Table 2). One month later, hGH exceeded 300 ng ml$^{-1}$ in three pigs. One rabbit also had a high level of hGH. Serum hGH as high as 64,000 ng ml$^{-1}$ has been detected in transgenic mice, but accelerated growth rate was observed at levels of 20 to 80 ng ml$^{-1}$ (ref. 13). None of these animals were exposed to high levels of zinc, a treatment that has been shown to activate *MT–hGH* gene expression $\sim$10-fold in mice[13].

The effects of hGH on the growth of rabbits cannot be evaluated at present because only one live rabbit had detectable serum hGH and unfortunately it had malocclusion that impaired normal food consumption. Early indications are that the levels of hGH found in these transgenic pigs do not increase body weight dramatically. This may not be surprising considering that daily injections of bacterially synthesized hGH had no effect[16], and exogenous, highly purified porcine GH only stimulated growth by 10% when delivered during the major growth phase of the pig[17]. Transgenic offspring and littermate controls will need to be raised on normal and zinc-supplemented diets to determine precisely the effects of hGH on growth rate and other nutritional as well as endocrine parameters.

These experiments demonstrate that foreign genes can be introduced into several large animal species by microinjection of ova. Furthermore, expression of MT-hGH was obtained in rabbits and pigs. We used a fusion gene that has worked well in mice to demonstrate the feasibility of such techniques, and we are now trying several modifications in an effort to improve the level of expression and physiological response.

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**Table 1** Efficiency of producing *MT–hGH* transgenic rabbits, sheep and pigs by microinjection

| Species | Transferred injected ova | Recipients | Integration frequency (%) | Expression frequency *MT–hGH* mRNA | Expression frequency Serum or plasma hGH |
|---|---|---|---|---|---|
| Rabbit* | 1,907 | 73 | 28/218 (12.8) | 4/16 | 1/1 |
| Sheep† | 1,032 | 192 | 1/73 (1.3) | ND | ND |
| Pig‡ | 2,035 | 64 | 20/192 (10.4) | 11/20 | 11/18 |

Integration frequency is the number of animals (fetuses, stillborns and neonates) that retained the injected DNA/total number of animals resulting from injected ova. Six gilts bearing only injected eggs farrowed, producing 52 neonates, 5 of which retained DNA. In 31 gilts that farrowed, 204 fertilized control ova were transferred along with 859 injected ova to ensure sufficient embryos at implantation to maintain pregnancy. If survival of injected eggs to fetuses (16.4%) was similar for both groups, then injected eggs resulted in 140 of 252 fetuses and piglets produced, 15 of which retained injected DNA. We combined the data from the two groups to estimate integration efficiency. Expression frequency is the number of fetuses or neonates containing *MT–hGH* mRNA or plasma hGH per total number of animals examined. ND, not determined.

* Fertilized one-cell rabbit ova were flushed from the oviducts of superovulated New Zealand White (NZW) females 19 h after mating[20]. For microinjection the ova were placed in the well of a depression slide containing ~100 µl modified BMOC culture medium[21] with the NaHCO$_3$ replaced by 25 mM HEPES[22] and covered by silicone oil. Microinjection of embryos (1,857 one-cell and 50 two-cell) was performed as described for mouse ova[10,14]. Following injection, the ova were washed in fresh modified BMOC and surgically transferred to the oviducts of synchronized pseudopregnant rabbits[23].

† Rambouillet ewes were superovulated after exhibiting at least one prior oestrus period. On about day 10 of the oestrous cycle, progestagen-impregnated vaginal sponges (6α-methyl-17α-acetoxy progesterone, 60 mg; from Dr J. Lauderdale, Upjohn) were inserted and left for 12 days. Gonadotropin treatment (porcine follicle stimulating hormone, Burns) began three days before sponge removal and was continued twice daily (2.5 mg per injection, intramuscular) until the day following sponge removal[24]. At the onset of oestrus, ewes were either hand mated to fertile rams or inseminated *in utero* with 0.2 ml per horn of washed ram semen; 72 h after sponge removal, one-cell fertilized ova and cleaved ova were surgically collected from the reproductive tracts of anaesthetized ewes by flushing 6 ml Ham's F-10 medium containing 10% heat-inactivated fetal calf serum (FCS) from the utero-tubal junction through the cannulated infundibular end of each oviduct. The flushings were collected in sterile Petri dishes, and ova were removed under a dissecting scope. Ova were transferred to fresh Ham's F-10 containing 10% FCS and transported (~2.5 h) to Philadelphia in temperature-controlled containers. Microinjection of embryos (641 one-cell, 375 two-cell and 16 four-cell) was performed as previously described[10,14]. After embryos were injected, they were washed and transported to Beltsville. Embryos were aspirated into a glass micropipet tip with 10 µl Ham's F-10 and expelled 1–3 cm into the fimbriated end of the oviduct in synchronized recipient ewes. To assess the effects of transport and microinjection of DNA on egg development, a number of recipients bearing control and injected eggs were flushed 8 days following transfer. In recipients in which eggs were recovered, 26% of transported, uninjected and 10% of injected sheep eggs developed to blastocysts. Because of the high mortality of transported, injected eggs and the concern of multiple births, 5 or 6 embryos were transferred per recipient.

‡ Mature gilts were superovulated and bred as previously described[15]. At 18 to 27 h after the expected time of ovulation, gilts were anaesthetized and one cell fertilized the oviduct with 20 ml modified BMOC[21]. Ova were transferred to fresh BMOC and transported to Philadelphia. Microinjection of embryos (316 one-cell, and 1,719 two-cell) was performed as previously described[10,14]. The obscured pronuclei or nuclei or one- and two-cell pig ova were visible after centrifugation for 3 min at 15,000 g. Centrifugation of pig ova at this force and length of time has no detectable effect on development[15]. After embryos were injected, they were transported to Beltsville and transferred to the oviducts of recipient gilts as previously described[15]. To assess the effects of transport and microinjection of DNA on egg development, recipients bearing control and injected eggs were flushed 5 days following transfer. Approximately 52% of transported, uninjected and 23% of injected pig eggs developed to blastocysts. The pregnancy rate in recipients bearing only injected eggs was 50% while in recipients bearing both injected and control eggs 58% farrowed.

**Table 2** Characteristics of transgenic rabbits, sheep and pigs

| Species | Animal and sex | Gene copy (no. per cell) | *hGH* mRNA (molecules cell$^{-1}$) | Immuno-assayable hGH (ng ml$^{-1}$) | Species | Animal and sex | Gene copy (no. per cell) | *hGH* mRNA (molecules cell$^{-1}$) | Immuno-assayable hGH (ng ml$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Rabbit | 59–3* | 20 | 0 | | Pig | 100–3* | 4 | 0 | ND |
| | 64–2♀ | 18 | 0 | | | 163–4* | 140 | 0 | ND |
| | 68–3† | 28 | 39 | ND | | 3–2♂ | 330 | 26 | Neg. |
| | 68–4♀ | 24 | 0 | | | 3–6♂ | 490 | 53 | 17 |
| | 122–9♀ | 11 | 0 | | | 7–3♀ | 90 | 12 | 80 |
| | 131–8† | 88 | 0 | | | 10–4♀ | 23 | 0 | Neg. |
| | 157–1♀ | 3 | 0 | | | 11–2♂ | 1 | 0 | 40 |
| | 163–3♂ | 3 | 15 | 250 | | 16–3♀ | 3 | 0 | Neg. |
| | 167–5♀ | 10 | 0 | | | 16–8♀ | 10 | 18 | 53 |
| | 179–1♀ | 16 | 0 | | | 16–9♂ | 1 | 5 | 65 |
| | 179–2♂ | 36 | 0 | | | 17–4♀ | 3 | 0 | Neg. |
| | 179–5♀ | 6 | 0 | | | 18–3♀ | 3 | 6 | 60 |
| | 200–3* | 8 | 920 | ND | | 20–2♂ | 2 | 4 | 40 |
| | 221–1* | 40 | 140 | ND | | 20–8♂ | 110 | 1 | 2 |
| | 223–4* | 5 | 0 | | | 21–4♂ | 1 | 2 | Neg. |
| | 223–5* | 40 | 0 | | | 21–5♀ | 1 | 0 | Neg. |
| Sheep | 693–1♀ | 1 | ND | ND | | 22–1♀ | 50 | 24 | 56 |
| | | | | | | 23–8♀ | 7 | 41 | 730 |
| | | | | | | 25–2♀ | 17 | 0 | 108 |
| | | | | | | 25–4♀ | 2 | 0 | Neg |

A 2.6-kb linear fragment of the fusion gene *MT-hGH*[13] containing the mouse MT-I regulator/promoter fused to hGH[19] was injected into fertilized one-cell and two-cell rabbit, sheep and pig eggs as described for mouse ova[10,14]. The male or female pronuclei of one-cell ova and both nuclei of two-cell ova were microinjected with a 3 ng µl$^{-1}$ solution of DNA in Tris-EDTA buffer[14]. The ova were transferred into the oviducts of recipients at the same stage post oestrus as the donors (see Table 1). Animals without identified gender were either killed as fetuses(*) or were stillborn(†). The number of foreign fusion genes per cell was estimated by extracting total nucleic acids from a piece of fetal liver, neonatal ear or tail samples and performing quantitative dot hybridization with a 1.0-kb *Pvu*II probe spanning most of the *hGH* structural gene[19] (see Fig. 2). *MT–hGH* mRNA was measured by solution hybridization with a $^{32}$P-labelled oligonucleotide (21-mer)[25]. For rabbits, either a partial hepatectomy was performed or fetal liver was used. For pigs, the *MT–hGH* mRNA content of ear or tail samples was quantitated. The concentration of *hGH* was measured in pig plasma obtained shortly after birth and serum from a 9-month-old rabbit. Samples were assayed in duplicate at 2.5 and 10 µl by radioimmunoassay using a hGH kit provided by Dr Raiti (National Hormone and Pituitary Program). The assay did not cross-react with porcine GH but required extra normal rabbit serum and anti-rabbit gammaglobulin to quantify hGH in rabbit samples. Pigs with hGH values less than 2 ng ml$^{-1}$ at birth were designated negative for hGH. At about one month of age, these pigs were also negative for hGH. ND, not determined; Neg, negative for hGH.

The key element in our success was the ability to visualize pronuclei and nuclei. Microinjection of the *MT–hGH* gene into the cytoplasm of 485 pig ova failed to produce DNA integration in 42 fetuses. Separate techniques for sheep and pigs were necessary because the opacity of the eggs differed. Although both contain dense cytoplasm, centrifugation did not help visualize pronuclei of sheep eggs and IC microscopy did not allow nuclear localization in pig ova. Preliminary work indicates

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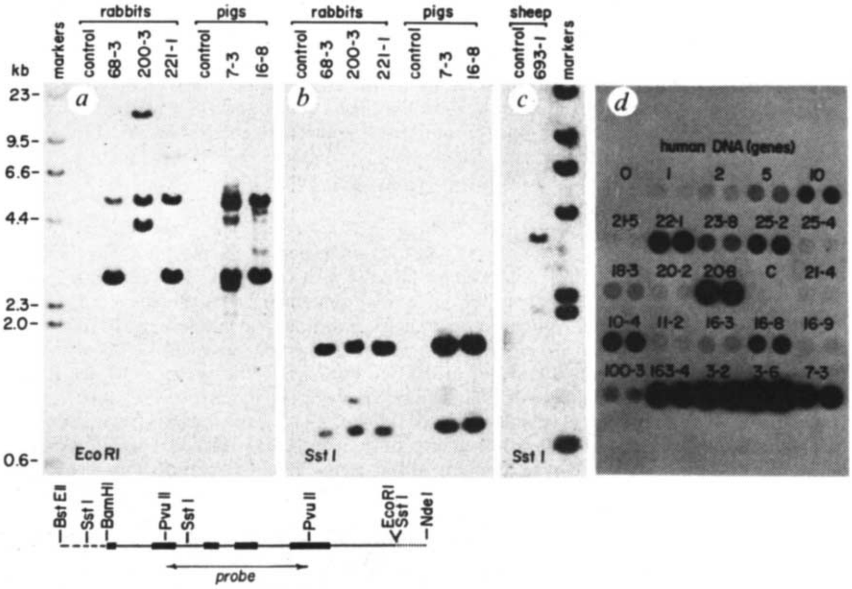

**Fig. 2** Analysis of *MT-hGH* DNA introduced into rabbits, pigs and sheep. The diagram at the bottom shows the 2.6-kb *Bst*EII/*Eco*RI DNA fragment isolated from *MT-hGH* gene plasmid 111 that was microinjected[13]; the mouse MT-I promoter region is dashed, the *hGH* gene is solid, with the exons indicated as boxes, and the residual pBR322 sequences are dotted. The internal *Pvu*II fragment was isolated, nick-translated and used as a probe for quantitation of genes and Southern blots. Panels *a*, *b* and *c*: DNA (5 μg for controls and transgenic animals 200-3, 16-8 and 693-1; 1 μg mixed with 4 μg of control DNA for 68-3, 221-1 and 7-3) were digested with the indicated restriction enzymes (10 units, 6 h), electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated probe, washed and autoradiographed as described previously[18]. *a* and *b*, Exposure was 4.5 h; *c*, 24 h. For quantitation of gene copy number, 5 μg of DNA was spotted in duplicate onto nitrocellulose along with standards of 0, 0.5, 1, 2 and 5 μg of human DNA mixed with control DNA to make a total of 5 μg. *d* Shows the visualization *MT-hGH* gene copy number in transgenic pigs. After exposure, the spots were cut out and the radioactivity determined in a scintillation counter. Gene copy numbers were calculated from the standards assuming that the genome size of pigs and humans are comparable and that diploid human cells contain 10 genes homologous to the *Pvu*II fragment used as probe[19]. The results are shown in Table 2.

that these two techniques can be used for ova of other species; for example, IC microscopy allowed visualization of pronuclei in goat ova (unpublished observations) and centrifugation allowed localization of pronuclei in cow ova[15]. Although improvements in integration efficiency should be possible, the techniques have immediate application for both scientific and practical purposes.

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# Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells

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**Haemophilia B, or Christmas disease, is an inherited X-chromosome-linked bleeding disorder caused by a defect in clotting factor IX and occurs in about 1 in 30,000 males in the United Kingdom[1]. Injection of factor IX concentrate obtained from blood donors allows most patients to be successfully managed. However, because of impurities in the factor IX concentrate presently in use, this treatment involves some risk of infection by blood-borne viruses such as non-A, non-B hepatitis and the virus causing acquired immune deficiency syndrome (AIDS)[2]. Because of the recent concern about the increasing incidence of AIDS amongst haemophiliacs, a factor IX preparation derived from a source other than blood is desirable. Here, we report that after introduction of human factor IX DNA clones[3] into a rat hepatoma cell line using recombinant DNA methods, we were able to isolate small amounts of biologically active human factor IX.**

Factor IX is a plasma glycoprotein which has an essential role in the middle phase of the intrinsic clotting pathway[4] where, in an activated form, IXa, it interacts with factor VIII, phospholipid and calcium ions to form a complex that converts factor X to Xa. Factor IX is synthesized in liver hepatocytes where it undergoes three distinct types of post-translational modification before secretion into the bloodstream as a 415-amino-acid-long, highly modified protein. These modifications are the vitamin K-dependent γ-carboxylation of 12 glutamic acid residues[5], the addition of several carbohydrate residues[6] and the β-hydroxylation of a single aspartic acid residue[7]. The first two modifications are known to be required for activity of factor IX[5,6]. Because of the complex and specialized nature of these modifications, it seemed probable that the expression of active factor IX, derived from factor IX DNA clones, would be most likely to succeed in a hepatic cell or a transformed cell line derived from a hepatocyte. None of the standard mammalian hepatoma cell

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