# A membrane cofactor protein transgenic mouse model for the study of discordant xenograft rejection

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## Abstract

***Background:*** **In recent years, interest has been revived in the possibility of transplanting organs into humans from a phylogenetically disparate species such as the pig (xenotransplantation). Such discordant xenografts, however, are subject to hyperacute rejection (HAR) and activation of host complement plays a major role in this rejection. This problem may be solved through the use of transgenic technology by providing the grafted tissue with molecules that down-regulate the action of host complement.**

***Results:*** **Transgenesis with a yeast artificial chromosome (YAC) was used to produce transgenic mice with the complete genomic gene of the human complement regulator membrane cofactor protein (MCP). Transgenic mice were obtained that exhibit full regulation of MCP as normally observed in humans. Hearts from these mice were shown to be significantly protected from HAR caused by human serum in an *in vivo* experimental procedure.**

***Conclusions:*** **We conclude that MCP can protect discordant xenografts from HAR caused by human serum and that transgenic mice can be used effectively as *in vivo* models for the study of the role of human complement regulatory molecules in xenotransplantation.**

## Introduction

Membrane cofactor protein (MCP, CD46) is an extracellular, membrane-bound regulatory protein of the complement system. It acts on the complement components C3b and C4b and serves as a cofactor for their proteolytic inactivation by the serum protein factor I (reviewed in Liszewski *et al.* 1991). The gene for MCP is located on chromosome 1 at q32, a region known as the regulators of complement activation (RCA) gene cluster, because it contains, over a 1.5 megabase area, the genes for several other soluble and membrane-bound proteins that regulate complement at the C3 level (Hourcade *et al.* 1992). The MCP gene extends over a length of ≈50 kb and contains 14 exons and 13 introns (Post *et al.* 1991; and Fig. 1). The structure of the gene includes exons encoding four of the short concensus repeat units (SCRs) that are characteristic of all the RCA proteins, three exons encoding a serine–threonine–proline rich domain, two exons encoding the hydrophobic transmembrane domain, and two exons encoding the cytoplasmic part of the protein. While the four SCRs are always present in the protein, differential splicing among the three exons of the serine–threonine–proline rich domain and the two exons of the cytoplasmic domain, results in various mRNA isoforms that contribute to differences in the protein of different cell lines and different tissues (Johnstone *et al.* 1993). MCP is very widely distributed on epithelial and endothelial cells, fibroblasts, platelets, lymphocytes, granulocytes, placental tissue and sperm, haemopoietic cells and peripheral blood cells except erythrocytes (reviewed in Liszewski *et al.* 1991); the

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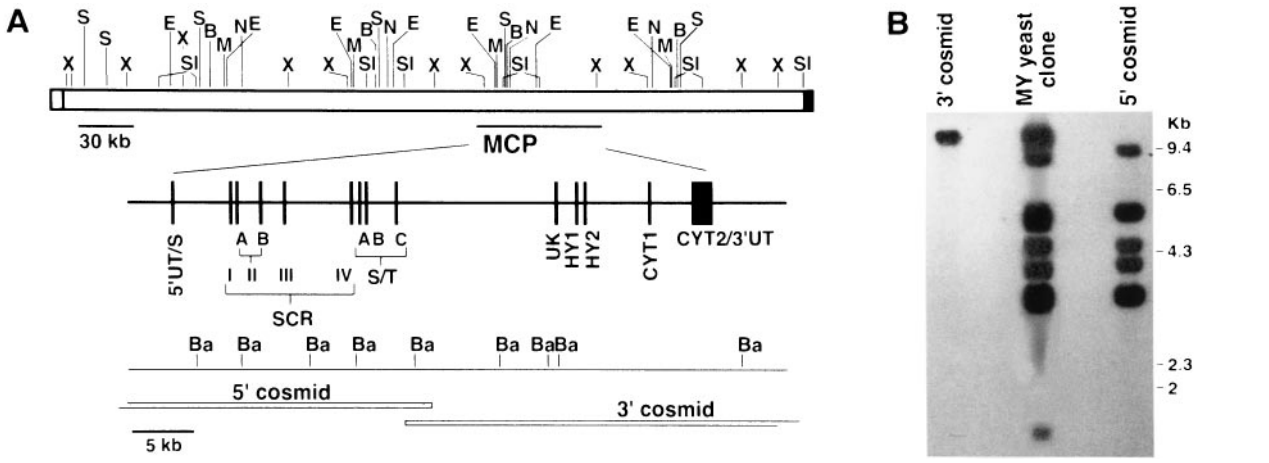


**Figure 1** (A) The MCP YAC and the MCP gene. The restriction sites for the YAC are: BssHII (B), EagI (E), MluI (M), NruI (N), *Sal*I (Sl), SfiI (Sf), *Sad*II (S), *Xho*I (X). The positions of the sites on the YAC are approximate. On the gene are shown the exons for the 5′ untranslated-signal peptide sequence (5′ UT/S), the short concensus repeats (SCR I, IIA, IIB, III, IV), the serine-threonine rich region ($S/T^{A, B, C}$), a sequence of unknown significance (UK), the transmemrane domain (HY1 and 2) and the alternate cytoplasmic regions (CYT1, CYT2). Below the gene are shown the BamHI sites (Ba) and two cosmids that were used to test the integrity of gene. (B) Southern blot of BamHI restriction pattern of DNA from the 5′ and 3′ cosmids, and the yeast clone containing the MCP YAC (MY yeast clone).

functional significance of the different isoforms and the different levels of MCP expression in different tissues is unknown.

Among complement regulatory proteins, such widespread distribution is paralleled only by the RCA regulator, decay accelerating factor (DAF, reviewed in Lublin & Atkinson 1990), and by CD59. The latter is unrelated to the RCA molecules and acts to inhibit complement activation, not at the C3 level, but at the final steps of the lytic pathway (reviewed in Davies & Lachmann 1993). The phenomenon of 'homologous restriction', i.e. the efficiency of complement in lysing heterologous rather than homologous species cells, has been proposed to be due to the species-specificity of the interaction between complement and its regulatory molecules (Atkinson & Farries 1987; Rollins *et al.* 1991). In recent years the idea has been developed that complement attack is the major factor in the hyperacute rejection of organs transplanted into a phylogenetically discordant host species (xenografting, reviewed in Platt & Bach 1991). Initial work with mouse and rat cell lines transfected with cDNAs of these molecules showed significant protection of the transfected cells to the effects of human serum (White *et al.* 1992; Oglesby *et al.* 1992; Walsh *et al.* 1991). This was followed by experiments with transgenic mice and pigs that confirm the role of these molecules in protecting heterologous animal tissue from human serum attack, even in the context of *ex vivo* and *in vivo* models of xenotransplantation (McCurry *et al.* 1995; Fodor *et al.* 1994; Cozzi & White 1995; White *et al.* submitted). While this work provides important proof of function for the use of these molecules in discordant xenotransplantation, it remains short of providing complete animal models for study. A major reason for this is that the gene constructs were based on cDNAs which are difficult to express at the appropriate levels and in the right tissues. However, it is important to produce good animal models for the study of these molecules, in view of the fact that complement, in addition to its lytic function, is also involved in the processing of immune complexes, in the promotion of binding and phagocytosis of C3b-coated particles and in several types of immune responses, including the inflammation and thrombosis that are major pathological symptoms of xenograft rejection (Dierich *et al.* 1988; Matis & Rollins 1995; Morgan 1995; Bach *et al.* 1995). How the diversity of complement regulatory molecules and the variability of their expression relates to any one of these functions is presently unknown. Current research in xenotransplantation focuses on the endothelial lining of donor organs that are immediately vascularized after recipient blood flow is established following transplantation. In hyperacute xenograft rejection (HAR),

 

which occurs in minutes to hours, fixation of complement leads to retraction of the endothelial cells from each other, exposure of subendothelial surfaces, inflammation, and thrombosis. Although the pathology of the rejection has been described in considerable detail (Bach *et al.* 1995), it is not yet clear how the fixation of complement initiates these events. Similarly, it is not clear whether it is the lytic function of complement, or the participation of complement in the inflammatory processes and in leukocyte recruitment around the xenograft, that is of critical importance in the rejection.

The complexity of the events in the rejection process, combined with the multiplicity of immune responses in which complement participates, makes it difficult to obtain physiologically relevant data from isolated *in vitro* assays or transgenic models with limited regulation of the transgenes. Questions such as: why is complement so closely regulated by so many different molecules with distinct functions and with variable expression in different tissues? what is the relative importance of each of these regulators in the rejection process? and which combination of regulators might offer the best protection of a xenograft in humans? can only be clearly evaluated in animal models that ensure correct regulation of the expression of these molecules in the different tissues where they are normally expressed. Such evaluation can best be done in experimental systems that give the opportunity of analysing the different aspects of xenograft rejection.

Here we report the generation of transgenic mice that contain a yeast artificial chromosome (YAC) with the complete genomic gene of human MCP. These mice exhibit fully regulated MCP expression on different tissues, comparable to the expression on human tissues. We also show that transgenic mouse hearts are protected from hyperacute rejection caused by human serum, in an *in vivo* model that makes possible the study of xenograft rejection on mouse organs.

## Results

To obtain the full genomic gene for MCP flanked by substantial length of 5′ and 3′ flanking sequences we screened the YAC reference library of the Imperial Cancer Research Fund (ICRF, London, UK), and a clone containing a YAC of ≈420 kb in length was recovered (Fig. 1). Restriction mapping of the YAC reveals a highly repetitive sequence structure with virtually every restriction site (or combination of sites like the two *Sal*I sites, see Fig. 1) repeated after ≈70–100 kb; furthermore, fluorescence *in situ* hybridization

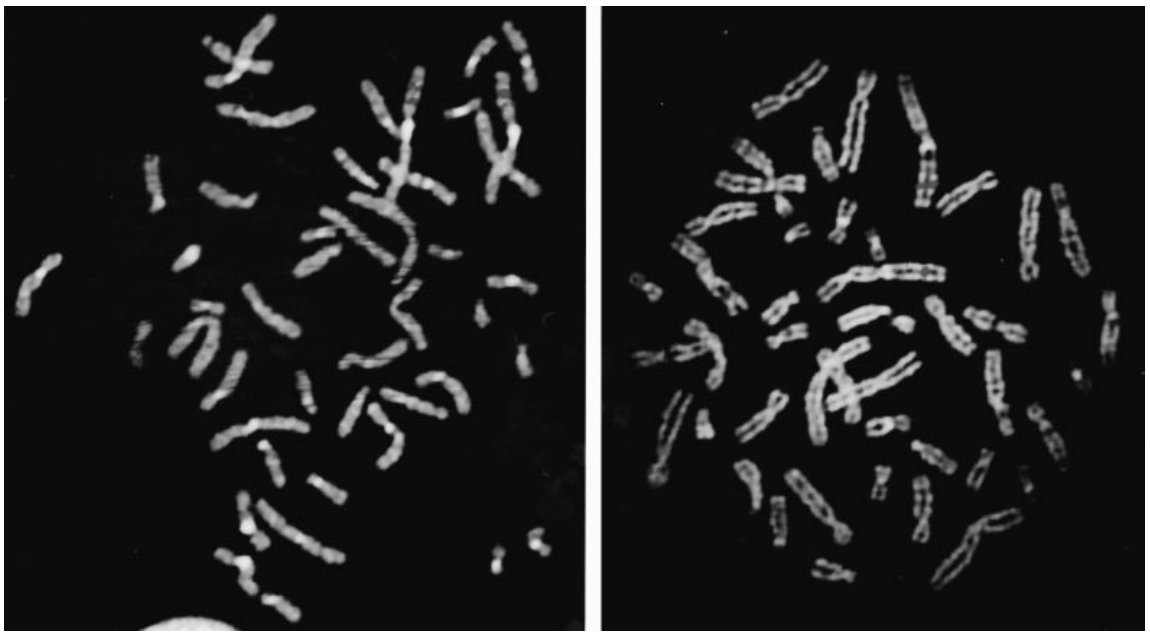

**Figure 2** Fluorescence *in situ* hybridization of human metaphase chromosomes to labelled total DNA from the yeast clone containing the MCP gene (left panel) or a YAC clone containing the DAF gene (right panel).

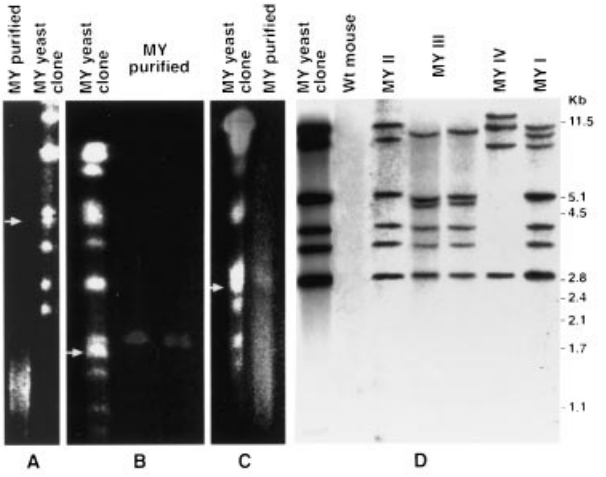


**Figure 3** (A–C) Three purification preparations of the MCP YAC (MY). ≈1/5 to 1/10 of the total volume of the purified YAC is visualised on ethidium bromide stained gels vs. the yeast clone from which it was purified. The arrow indicates the 420 kb position of the intact YAC in the MY yeast clone. The yeast clone DNA is run into the gel from agarose plugs, while the purified MY DNA is loaded into the gel slots in solution. The position of the intact purified YAC is always displaced upwards relative to the intact YAC in the yeast clone (verified by Southern blot of the gels shown here). (D) Southern blot of BamHI restriction pattern of DNA from transgenic mouse lines (MY I, MY II, MY III, MY IV) obtained by microinjection of the purified MY preparations shown in the adjacent panels, compared to the pattern of DNA from the MY yeast clone; non-transgenic mouse (wild-type, Wt mouse) DNA is the negative control.

(FISH) of the YAC on human chromosomes shows hybridization at the proper locus at chromosome 1q32.3 but also at position 1q32.1, several megabases centromeric to the MCP gene (Fig. 2). Although these data demand more detailed analysis of the YAC, as discussed below, Southern blot analysis of the YAC DNA, compared with two cosmids that span the 5′ and 3′ regions of the gene, showed that the complete MCP gene was present (Fig. 1B).

To generate transgenic mice with the MCP YAC (MY), we isolated the MY after size-fractionation of yeast chromosomes by pulsed field electrophoresis on low-melt agarose gels. Slicing of the MY out of the gel was followed by melting and agarase treatment. NaCl and spermine and spermidine were added in the buffer in which the YAC-containing agarose slice was equilibrated after gel fractionation, and they remained with the DNA throughout the rest of the procedure in order to protect the YAC, by compaction, against random shearing (Gnirke *et al.* 1993). All manipulations were carried out very gently (pipetting with wide-bore tips, no vortexing or shaking of the DNA in solution, etc.). The YAC-containing slice, after melting and agarasing, was placed in an 8-mL collodion dialysis bag with a molecular cut-off point of 75 kDa, and vacuum dialysed to an approximately eightfold higher concentration, as described in detail in the Experimental procedures section. In our hands this procedure of YAC purification worked well, although it did not completely solve the inherent problems associated with obtaining the YAC intact and in satisfactory amounts in a consistent manner. The problems are reflected by the purification and microinjection results shown in Fig. 3. The injection of small amounts of a completely intact YAC (Fig. 3B) resulted in a very low transgenesis rate (no transgenic mice were obtained out of 60 offspring born). In contrast, large amounts of the isolated YAC broken to fragments with an average size of 100 kb (Fig. 3A), resulted in three transgenic mouse lines out of 28 offspring born, none of which on Southern blot analysis, however, showed the correct restriction pattern for the intact gene (MY I, MY III, MY IV, Fig. 3D). Finally, it was microinjection of the YAC purification shown in Fig. 3C, with the DNA partly intact and in an amount that was intermediate between the two previous cases, that gave one transgenic mouse line out of 30 offspring born (MY II, Fig. 3D) which clearly contains the intact gene, including the 5′ and 3′ flanking regions as defined by the BamHI restriction sites of the two cosmids shown in Fig. 1; further restriction analysis of line MY I (not shown) shows that the integrated gene is broken at the 3′ untranslated region. Lines MY I, MY III and MY IV are single-copy, while line MY II is high-copy (10–12 copies, data not shown). Lines MY III and MY IV did not express any RNA (Fig. 4) while MY II expressed the proper mRNA message of ≈4.5–4.8 kb and lower size transcripts in all the tissues examined; line MY I also gave the same pattern of expression as MY II but the transcripts were slightly shorter. Most importantly, the expression levels seemed to correlate roughly with the number of copies when the levels of the MY I and MY II mice were compared with the human expression levels.

Tissue sections from spleen, kidney, heart, liver and lung of MY I and MY II mice probed with a mouse monoclonal anti-CD46 antibody, clearly reveal the presence of the MCP protein on the tissues of the MY II mouse, as shown in Fig. 5, for heart, spleen and liver. Comparison with the corresponding human sections shows that the pattern of expression in mouse tissues closely compares with expression in humans, with high

 

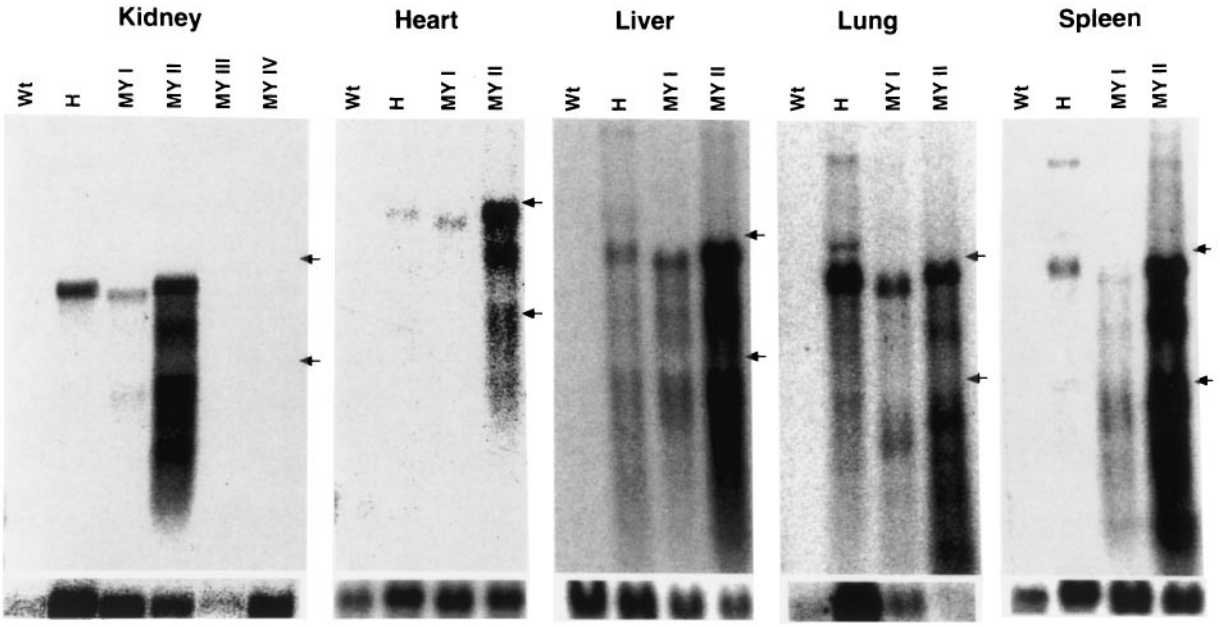


**Figure 4** Northern blot analysis of the MCP transgenic mouse lines, MY I, MY II, MY III and MY IV on total RNA from the tissues indicated. Wt is non-transgenic mouse RNA, and H is human RNA. The position of the 28S and 18S RNA is indicated by arrows. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (lower panel) was used as loading control.

levels on the endothelial lining of capillaries and blood vessels in general, and the sinusoid structures in the liver (in addition, high expression in the glomeruli and the proximal and distal tubules of the kidney, and in the epithelial lining of bronchiolar structures of the lung, data not shown). FACS analysis of spleen suspensions shows that mouse MY II exhibits 10-fold more surface protein than the human (van Binnendijk, personal communication), which is reflected in the higher intensity of all the MY II vs. the human tissue section staining.

To test whether the presence of the MCP on the surface of the mouse tissues offers protection against the action of human serum in the context of an *in vivo* assay involving a whole organ, rather than an *in vitro* cell assay, we developed a model based on the fact that the phylogenetically concordant species of mouse and rat do not exhibit hyperacute xenograft rejection: when a mouse heart is heterotopically transplanted into a rat, it is viable for 3 days or more (Gannedahl *et al.* 1994). In our model the mouse heart was heterotopically grafted onto the rat aorta directly below the origin of the renal arteries, but it was perfused with human serum. Perfusion of non-transgenic mouse hearts with 1.5 mL of human serum, over a period of 1.5 min, made the hearts slow down immediately, resulting in white discoloration and complete cessation of heart beat within 3–5 min in all eight control hearts tested. These hearts never resumed beating during an observation period of 30 min. Perfusion of non-transgenic hearts with decomplemented human plasma ($n = 6$), led to a similar slowing down of the heart beat and discoloration of the hearts in a similar manner to that after injection of normal human plasma; however, the hearts regained their normal colour after 3–5 min, and they resumed beating again at a similar or slightly reduced pace as before perfusion. The hearts remained functioning over an observation period of 45 min. Ten MY II transgenic hearts were transplanted and perfused with human serum in the same way. All hearts experienced the immediate discoloration and reduction in heart frequency as described above. Three hearts did not recover during the observation period of 30 min and were scored as 'rejected'. Seven out of the 10 hearts resumed functioning after about 3–5 min, in a similar fashion as that following perfusion with decomplemented plasma. All seven hearts that recovered were beating normally again within 15 min and remained so during the observation period of 45 min, after which time the experiment was terminated (Table 1).

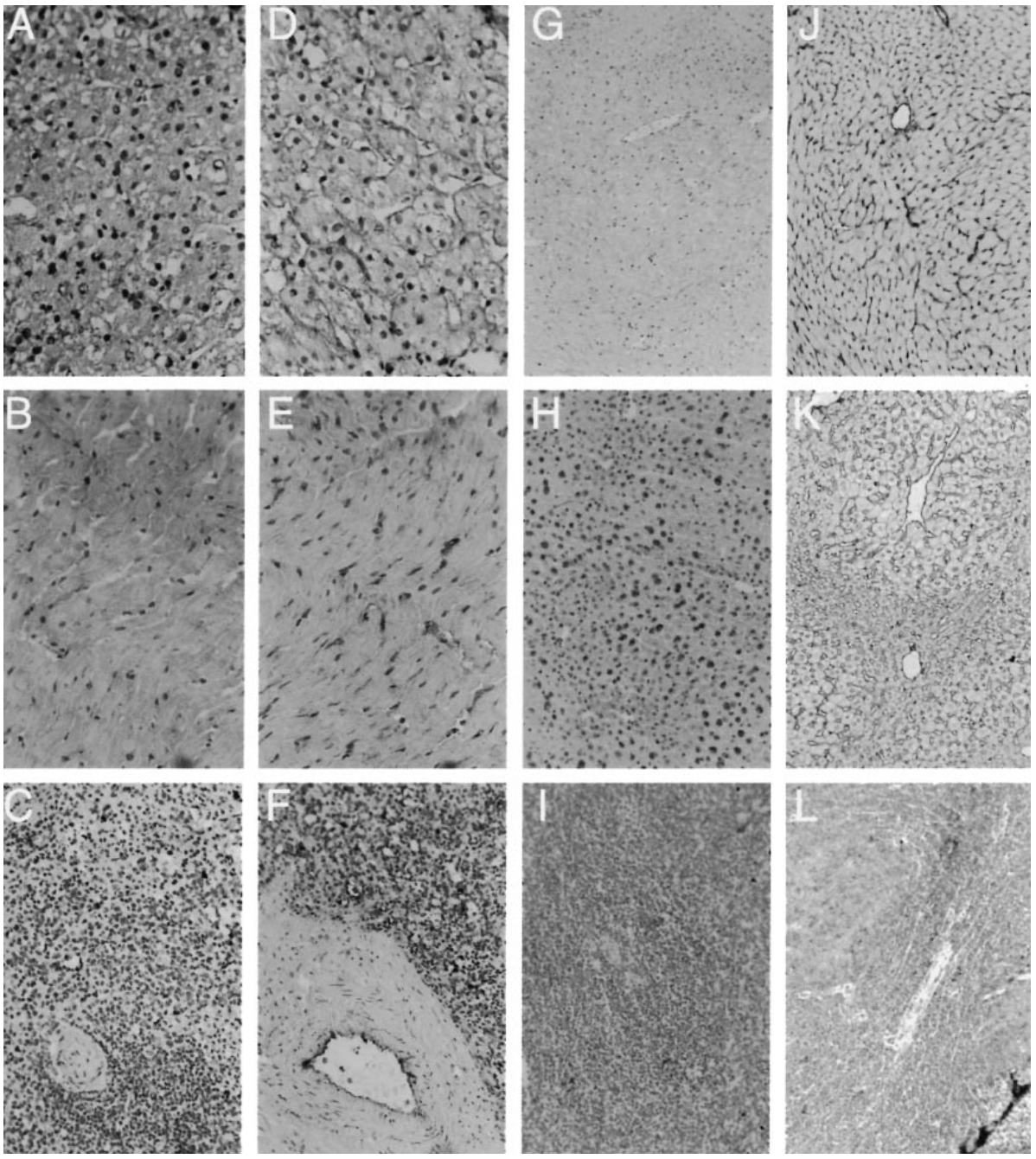


**Figure 5** Immunostaining of human and mouse frozen sections with a biotinylated anti-CD46 monoclonal antibody. The negative control (nc) for the human tissue is the exclusion of anti-CD46 antibody from the staining protocol, while the negative control for the mouse is staining of wild-type (Wt) mouse sections with the anti-CD46 antibody included in the staining protocol. (A) Human liver, nc. (B) Human heart, nc. (C) Human spleen nc. (D) Human liver. (E) Human heart. (F) Human spleen. (G) Wt heart. (H) Wt liver. (I) Wt spleen. (J) MY II heart. (K) MY II liver. (L) MY II spleen.

**Table 1** Survival of the MY II transgenic mouse hearts in the mouse-to-rat model

| Group | Survival over 45 min (number of experiments) | Rejection within 5 min (number of experiments) |
|---|---|---|
| Wild-type mouse + decomplemented human serum | 6 | 0 |
| Wild-type mouse + human serum | 0 | 8 |
| MY II transgenic mouse + human serum | 7 | 3 |

## Discussion

In recent years, interest in complement research has been revived in conjunction with its role in the hyperacute rejection of xenografts. Transgenesis with regulatory molecules such as MCP, into animals such as the pig—which shares anatomical and physiological similarities with man—may make possible the utilization of transgenic pigs as donors of organs for transplantation into humans (Cooper *et al.* 1991; Rooney *et al.* 1993). Despite recent advances concerning our understanding of hyperacute rejection, a great deal of basic research is still required to clarify the role and the appropriate use of complement regulatory molecules in xenotransplantation.

The regulation of complement involves a large number of regulatory proteins that act at different stages of the complement cascade and exhibit great or subtle differences of activity, tissue distribution and levels of expression (reviewed in articles edited by Cruse & Lewis 1993 and Davies & Lachmann 1993). The great variation of expression of MCP (and DAF) among tissues implies that complement regulation depends on parameters that are significantly different from tissue to tissue. It is conceivable that the overall functional profile of even the individual molecules in such a system is defined only in the context of all the others, their relative levels of expression in different tissues, the location and exposure of a particular tissue to serum, and the cooperation among different complement regulators. To date, however, no transgenic animals exhibiting fully regulated expression of MCP, or any of the other regulators, have been described.

Current work to produce transgenic animals with human regulators focuses mainly on DAF, MCP and CD59: (a) because they are membrane-bound, and will therefore be carried on the xenograft, (b) because of their wide tissue distribution, which argues for their prominent role in protecting autologous tissue, but also implies that different organs from the same transgenic animal could be utilized in xenografting, and (c) because they represent different routes and different stages of complement inactivation that might prove to act synergistically for optimal effect. MCP is a cofactor for the enzymatic proteolysis of C3b by the serum factor I, while DAF accelerates the dissociation of the convertase by binding C3b and preventing it from binding to the other components of the convertase (Lublin & Atkinson 1990), and CD59 inhibits the formation of the final attack complex by binding to C8 (Rollins *et al.* 1991). All three genes for these molecules have coding sequences that are dispersed over a 45–50 kb area (Lublin & Atkinson 1990; Tone *et al.* 1992), which implies that a satisfactory transgene for any one of them would probably have to cover a considerably larger locus to ensure inclusion of all the regulatory sequences necessary for full regulation in different tissues. To obtain the genes in their natural state, with all the introns and a significant length of 5′ and 3′ sequences, we decided to utilize YAC technology, and we have presented here the first transgenic mice with a fully regulated expression of MCP in different tissues, according to the human pattern. We have also utilized the same technology to generate transgenic mice with the full genomic DAF and CD59 genes. They also express the genes according to the patterns observed in humans (work in progress) and can therefore serve as animal models for study and the functional comparison with the MCP transgenic mice described here. It will be interesting to eventually produce a single mouse model that will carry all three of these important human complement regulators, either by mating the existing transgenic mice or combining the three genes on one single YAC. This would be of considerable use in producing transgenic pigs with all three regulators.

The introduction of YACs into the mouse genome has made possible the study of DNA loci of much greater size than regular transgenes (Cappechi 1993). The most direct route of accomplishing this, i.e. isolation of the YAC and microinjection into cells or eggs, presents two basic areas of difficulty that distinguish it from regular transgenesis. One is the purification and concentration of the YAC, intact and in microinjectable form, and the other is the detailed analysis of the integrated fragments in the transgenic mice obtained. The general logic of isolating YACs from agarose via agarase treatment and the protection from random shearing by compaction of the DNA, as we have followed here, has been described in detail by Gnirke *et al.* (1993) and Schedl *et al.* (1993). There are differences, however, in the methods followed for concentrating and purifying the YAC DNA into microinjection buffer. Schedl *et al.* (1993) concentrated the DNA prior to melting of the agarose, by embedding the YAC-containing slice in a second agarose gel and electrophoresing at right angles to the direction of the pulse-field fractionation, until the DNA was collected at one end of the slice; the slice end is cut off and, after melting and agarasing, the YAC solution is purified by dialysis on filter paper discs. Gnirke *et al.* (1993) first melted and agarase treated the gel slice, after which they concentrated the DNA by centrifugation through a filter unit. We have presented here a third possible way of concentrating and purifying the YAC DNA—through vacuum dialysis in collodion bags—which compares favourably with the other two and which we also utilized to obtain the DAF and CD59 transgenic mice mentioned above.

The problem of analysing the integration of the microinjected YAC in the transgenic mice obtained greatly depends on the available information regarding the DNA that surrounds the gene on the YAC. We are currently subcloning parts of the MCP YAC in order to define its different areas in more detail, and to facilitate shortening of the YAC around the MCP gene. This will help to define the areas of the gene that are required for efficient regulation of the transcription, and will also help in clarifying the FISH data shown in Fig. 2. This hybridization of the YAC to two adjacent areas within 1q32 could be due to the ligation of two non-contiguous fragments during the creation of the library, to produce a chimeric YAC. It is also interesting to note that the RCA locus has arisen by extensive inter- and intra-genic duplication events (Hourcade *et al.* 1992), so that recombination events resulting in elimination by looping-out of large fragments of DNA are probable. However, the possibility that sequences present on our YAC simply cross-hybridize with sequences distant from the MCP gene cannot be excluded at this point. Further manipulation and study of the MCP YAC, as well as that of the DAF and CD59, can perhaps be facilitated by the use of amplifiable YAC vector arms (Smith *et al.* 1990; Schedl *et al.* 1993).

The role of human complement regulatory molecules during the hyperacute rejection of xenografts by human serum cannot be easily studied in transgenic mouse models, because methods to reproduce the physiological context in which mouse tissues would react to human blood or serum are not easily available. We are currently involved in developing such *in vivo* and *ex vivo* methods and we have presented here an experimental procedure by which the function of MCP can be studied in the context of the function of a whole mouse organ rather than in isolated *in vitro* cell assays. Our effort in this direction aims at methods that will not only enable us to observe that the transgenes are functional, i.e. that they downregulate human complement, but will also enable us to correlate such findings with whole organ function. This is necessary, since HAR is a characterized by several interconnected processes, some apparently independent of complement activation (Bach *et al.* 1995).

Our experiment shows that human complement is responsible for the rejection of the non-transgenic mouse hearts, since no rejection is observed when decomplemented human serum is injected. It also shows that MCP significantly protects against this rejection, presumably by inactivating human complement. There are, however, several questions to be answered. It is possible that components of the complement cascade from rat and human can act co-operatively to abrogate the concordance between rat and mouse. It will also be interesting to evaluate the species-specificity of the interaction between MCP, C3b (or C4b), and factor I, by injecting serum or components from different sources. The overall phenomenon of homologous restriction observed in the action of complement might be a result of the combined activity of different regulators with varying degrees of species-specificity in their interaction with complement components. Furthermore, the evolutionarily conserved structure of both the complement components and the RCA molecules implies that any species-specificity exhibited in their interaction is based on relative degrees of affinity between components from different species. Rabbit C3 can bind human MCP (Manthei *et al.* 1988), and *in vitro* studies concerning the interaction of other human RCA molecules with C3s of different species (reviewed by Lambris 1993) show extensive but variable binding cross-reactivity. There is, in addition, controversy

 

surrounding the physiological relevance of the species-specificity of CD59, as it has been defined in different *in vitro* experimental systems (Rollins *et al.* 1991; Van den Berg & Morgan 1995). Such questions concerning MCP, as well as the general pathology of hyperacute rejection, can be studied using our *in vivo* model in comparison with results concerning DAF and CD59, as numbers of transgenic mice produced by microinjection of the DAF and CD59 YACs become available. We are also developing an *ex vivo* perfusion model that eliminates the presence of rat serum and will therefore be complementary to the *in vivo* one presented here in the analysis of xenograft rejection and the role in it of complement and its regulation. The production of transgenic pigs by microinjection of the MCP YAC presented here is in progress.

## Experimental procedures

The Imperial Cancer Research Fund (ICRF, London UK) reference YAC library (Lehrach *et al.* 1990) was screened with an MCP cDNA kindly provided by Dr Atkinson's laboratory, as were the cosmids used in the Southern blot analysis of the YAC. The YAC clone containing the MCP gene has the ICRF designation AM2: 6C10. The vector used in the generation of this library is pYAC4 and the host yeast strain AB1380.

**The general culture and manipulation of yeast clones** and mapping of the YAC was carried out according to the manual issued by the 6th Wellcome Summer School: *DNA Related Methods in Human Genetics: YAC cloning in Genome Analysis, 5th July–13th July 1991*, and according to Cox *et al.* 1993.

**Isolation of the YAC** was performed as follows. Agarose blocks of yeast cells were made by mixing a 2% low-melt agarose solution (containing 1 M sorbitol, 20 mM EDTA, pH 8.0, 14 mM mercaptoethanol) with an equal volume of cells resuspended in the same solution supplemented with 1 mg/mL zymolyase (without agarose). The agarose blocks were first incubated in a 1 mg/mL zymolyase solution (as above, also Tris pH 7.5), at 37 °C, for 2 h and then in lysis buffer (1% lithium dodecyl sulphate, 0.1 M EDTA, 10 mM Tris, pH 8.0), at 37 °C, overnight. The agarose blocks were extensively washed in 10 mM Tris pH 8.0, 1 mM EDTA, pH 8.0 and the DNA was run from them into a 1% low-melt preparative agarose gel and size-fractionated on a Biometra Rotaphor system for at least 36 h with a pulse of 35–5 s in logarithmic ramp (log), at an angle of 120°–110° in linear ramp (lin), voltage of 180–160 (log), rotor speed 7 (moving with the power off) and temperature at 13 °C. The position of the MCP containing YAC (420 kb) was located after the run by ethidium bromide staining of side strips cut from the sides of the gel. The band was cut out of the unstained gel and equilibrated for at least 1 h in commercially provided Gelase buffer (Epicentre Technologies, 1 × concentrations are 40 mM NaCl, 40 mM bis(2-hydroxyethyl)-imino-tris (hydroxymethyl) methane pH 6.0, 1 mM EDTA), supplemented with 0.1 M NaCl, 0.75 mM spermidine and 0.30 mM spermine. The agarose slice was melted, in Eppendorf tubes, at 68–70 °C for 5–7 min and agarased with 2–3-fold excess of Gelase (Epicentre Technologies) at 45 °C for 45 min to 1 h. Generally, a 10 min spin at full speed in an Eppendorf centrifuge showed no residual undigested agarose after this treatment. The agarased DNA solution was transferred into an 8-mL collodion dialysis bag (Ultra thimble, Schleicher & Schuell) with a molecular cut off point of 75 kDa, and vacuum dialysed from ≈5–8 mL to 200–800 μL. The dialysis buffer contained 0.2 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA pH 8.0 and 17–20% polyethylene glycol (PEG). Attempts to utilize this method without vacuum, by dialysing against 17–20% PEG-containing buffer, proved too slow and unproductive for the desired concentration range. Vacuum was therefore applied during this step in the procedure, but 17–20% PEG was still added, because we found that it was helpful both in accelerating the concentrating process and in the recovery of more intact YAC preparations. The collodion bag with the concentrated YAC solution was then dialysed without vacuum and without PEG against 1 L of microinjection buffer (10 mM Tris pH 7.5, 0.1 mM EDTA) for several hours. To protect the DNA from shearing, 0.1 M NaCl was also extensively added to this final step through the tip of the microinjection needle. No spermine or spermidine were added at this final dialysis, since excess of these agents seemed to create problems during microinjection (blocking of the needle, decreased survival of the injected eggs); in contrast, 0.1 M NaCl did not affect either the survival of the eggs or optimal microinjection conditions in general. Liquid samples of the purified YAC, ranging from 1/10 to 1/5 of the total preparation were run and visualized by ethidium bromide staining on pulse-field gels run at similar conditions as above for 16–18 h and probed on Southern blots with the MCP cDNA.

**Microinjection** of the purified YAC into fertilized mouse eggs was performed according to the methods of Hogan *et al.* (1994), and the strains utilized were (CBA×C57BL10) F1 (for the generation of transgenic lines MY I, MY III and MY IV) and FVB (for transgenic line MY II).

**Fluorescence *in situ* hybridization (FISH)**. Total yeast DNA from the MCP YAC containing clone was labelled by nick-translation with biotin-11-dUTP (Boehringer) and 1 μg was used to probe metaphase chromosomes prepared from normal male lymphocytes cultured with BrdU, as described in Pinkel *et al.* (1988).

**General molecular techniques** were performed acording to Sambrook *et al.* (1989). Genomic DNA preparations were done according to Laird *et al.* (1991). Total RNA from human tissues was obtained from Clontech laboratories, Inc. Total RNA from mouse tissues was prepared according to the LiCl/Urea method (Auffray & Rougon 1980) and electrophoresed through gels containing formaldehyde as described in Sambrook *et al.* (1989). Southern and Northern blots (including high molecular weight DNA fractionations by pulse field electrophoresis on agarose gels) were performed by capillary transfer to nylon membranes (Fluka or Hybond N+ from Amersham) and hybridized, under stringent conditions, to $^{32}$P-labelled DNA probes obtained using a random primer labelling kit

(Stratagene), according to the manufacturers' instructions or protocols described in Sambrook *et al.* (1989).

**Immunostaining**. Mouse Organs were embedded in OCT-compound (LabTek products) and cryosections of 5 $\mu$m thickness, fixed in acetone, were incubated with biotin blocking system and normal mouse serum from DAKO according to the manufacturers' instructions. After staining with biotinylated monoclonal antibody, the sections were incubated with ABComplex (DAKO). Peroxidase activity was developed with DAB substrate (Zymed) and the sections were counterstained with Gill's haematoxylin (Polysciences, Inc). Biotinylated mAb J4-48 (IgG1) and help with the procedure was kindly provided by Joanne Horsley (Papworth Hospital, UK).

**Mouse-to-rat heart transplantation** was performed heterotopically using microsurgical techniques as described in a previous paper (Van Bekkum *et al.* 1969). Briefly, donor mice were anaesthetized and heparinized, their hearts were exposed and the ascending aorta and pulmonary vein were cut close to the first branch and bifurcation, respectively. The caval and pulmonary veins were ligated in one ligature and the heart was removed and placed in RPMI medium at 4 °C. Recipient rats were anaesthetized with ether, their abdomen was opened via a midline incision and the abdominal aorta, and vena cava were exposed by dividing the overlying peritoneum. The infrarenal aorta and caval vein were clamped together with a curved haemostat. Longitudinal openings of about 1–1.5 mm were cut into both vessels and the aorta and pulmonary artery of the donor heart were anastomosed end-to-side by continuous suturing using 10.0 nylon (Ethicon). After removal of the clamp the hearts started beating immediately. About 5 min after transplantation, when the hearts were beating regularly, a catheter was introduced into the right renal artery and pushed into the aorta up to the aorta anastomosis. A bulldog clamp was placed on the recipient aorta, just below the anastomoses, and 0.5–1.5 mL of human plasma was gently injected through the catheter, resulting in perfusion of the transplanted heart with almost undiluted plasma over a fixed period of 1.5 min. By increasing the amount of plasma step-wise with 0.5 mL per group of four animals, it was observed that 100% rejection (complete and lasting cessation of heart beating) took place at a dose of 1.5 mL. This dose was used in the current experiments. Fresh frozen plasma from a single donor (blood group A+), was obtained from the blood bank of the Dijkzigt Hospital, Rotterdam. The plasma was stored in samples of 10 mL at −20 °C. Decomplementation of the plasma was achieved by heating at 56 °C for 1 h.

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