Evidence for intracellular generation of angiotensin II in rat juxtaglomerular cells

Chantal Mercure[1,a], Djamel Ramla[2,a], Raul Garcia[b], Gaétan Thibault[a], Christian F. Deschepper[a], Timothy L. Reudelhuber*

[a] *MRC Multidisciplinary Research Group on Hypertension, Clinical Research Institute of Montreal, 110 Pine Ave. West, Montreal, Que. H2W 1R7, Canada*
[b] *Laboratory of Experimental Hypertension and Vasoactive Peptides, Clinical Research Institute of Montreal, 110 Pine Ave. West, Montreal, Que. H2W 1R7, Canada*

Received 12 January 1998

Abstract The formation of the vasoactive peptide angiotensin II (AII) is dependent on the sequential action of two enzymes, renin and angiotensin converting enzyme (ACE), on the substrate angiotensinogen. Although the renin-producing cells of the kidney do not express angiotensinogen, they contain large amounts of AII in the same storage granules that contain renin. When renin expression is suppressed in these cells, AII also disappears. In the current study, we have tested whether the renin-associated disappearance of AII in renal juxtaglomerular (JG) cells is due to a renin-dependent down-regulation of granule biosynthesis and whether receptor-mediated internalization of AII could account for its concentration in these cells. Our results support a model whereby AII peptides are generated within JG cells, presumably by a mechanism which involves the action of endogenous renin on internalized, exogenous angiotensinogen.

Key words: Angiotensin II; Renin; Juxtaglomerular cell; Angiotensinogen

1. Introduction

The renin-angiotensin system (RAS) plays a critical role in the regulation of blood pressure and fluid balance in mammals. Active renin is secreted from storage granules of the renal juxtaglomerular (JG) cells of the kidney. Within the circulation, renin catalyzes the cleavage of the decapeptide angiotensin I (AI) from the amino-terminus of the hepatic glycoprotein angiotensinogen. AI is subsequently converted to the potent vasopressor angiotensin II (AII) by angiotensin converting enzyme (ACE) present on the membranes of endothelial cells throughout the vasculature.

Somewhat surprisingly, the renin-containing storage granules in the JG cells of some animals also contain significant amounts of immunoreactive AII [1–3], in spite of the fact that JG cells are not known to express the substrate for the RAS, angiotensinogen [2,4]. Two possibilities have been proposed to explain the presence of AII in JG cell granules. First, JG cells might capture angiotensinogen and re-route the protein to the secretory pathway where it is converted to AII by resident renin and ACE. Support for this model comes from experiments in which rats were treated with an ACE inhibitor, leading to the appearance of AI (the peptide precursor of AII) in JG cell granules [5]. A second possibility is that AII is concentrated in the lysosome-like granules of JG cells by a receptor-mediated internalization mechanism. JG cells do indeed contain AII receptors of the AT_1 subtype [6–8] and the AII AT_1 receptor mediates at least a portion of its biological activity through receptor internalization [9]. Somewhat inconsistent with the AII internalization model are experiments using a rat model of renovascular hypertension caused by partial ligation of the renal artery leading to one of the two kidneys. In this so-called Goldblatt or 2 kidney, 1 clip (2K, 1C) model of hypertension, the clipped kidney becomes ischemic and dramatically increases its expression of renin [10], while the non-clipped kidney represses renin expression [11,12] presumably due to either the resulting increase in blood pressure and/or increases in circulating AII [13,14]. AII staining appears to follow that of renin in the non-clipped kidney, gradually disappearing after artery ligation [11]. However, one possible explanation for this finding is that the JG cells of the non-clipped kidney have simply lost their storage granules concomitant with the repression in renin expression. Indeed, mice in which the expression of the Ren-1 renin gene is eliminated by homologous recombination show an absence of JG cell secretory granules [15].

In the current study, we have investigated the effect of renin repression and AII receptor blockade on the presence of AII in JG cells of the rat. Our results demonstrate that renin is not requisite for maintenance of secretory granules in rat JG cells and that AII receptor blockade does not eliminate AII in JG cell secretory granules, lending support to the model of intracellular generation of AII by a JG cell-mediated internalization of angiotensinogen.

2. Materials and methods

2.1. Animals

All of the experiments were carried out with male Sprague-Dawley rats (250–300 g). Animals were given regular rat chow and free access to water. At the end of the treatment period, the animals were killed by decapitation and blood was collected on EDTA for renin assay (see below). All of the experimental protocols were reviewed and approved by an institutional animal ethics committee.

For generation of the 2K, 1C hypertension model (Goldblatt model), rats were divided into two groups of 16 animals (test and sham-operated groups). In the test group, the rats were anesthetized (50 mg/kg sodium pentobarbital i.p.) and the left renal artery was partially occluded using a silver clip with an internal diameter of 0.2 mm as previously described [16]. The animals were returned to their cages for 5 weeks and systolic blood pressures were determined by the tail cuff method once per week. For the current protocol, animals were chosen

*Corresponding author. Fax: (1) (514) 987 5717.

[1] The first two authors contributed equally to this work.

[2] Present address: Département de Chimie Biologique, UQTR, CP500, Trois Rivières, Qué. G9A 5H7, Canada.

PII S0014-5793(98)00052-0

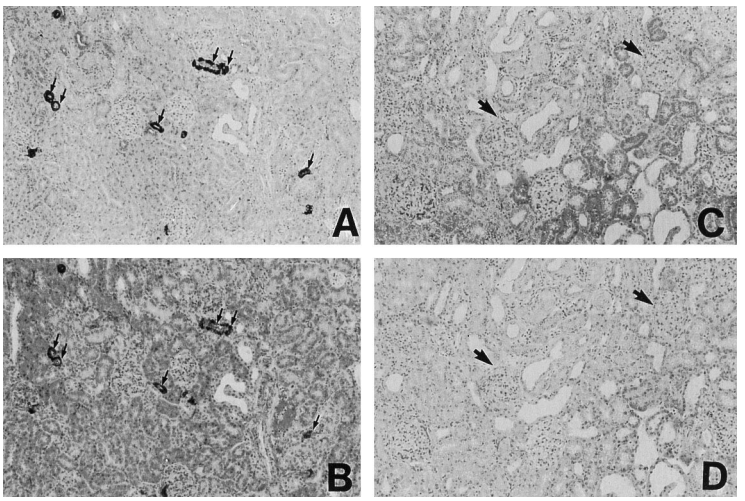

Fig. 1. Distribution of renin and AII immunoreactivity in the kidneys of the 2K, 1C rat. Serial sections from either the clipped kidney (A,B) or the non-clipped kidney (C,D) were stained with either anti-renin antiserum (A,C) or an antiserum to AII (B,D). Small arrows (left panel) indicate corresponding JG cell clusters in serial sections stained by both renin and AII antibodies. Large arrows (right panel) indicate the position of non-staining JG cells in the non-clipped renal cortex incubated with the two antisera.

that were hypertensive (systolic BP >180 mm Hg) for at least 3 weeks and had significantly elevated ($>10\times$ normal) plasma renin activity.

To study the effect of angiotensin AT_1 receptor antagonism, 12 male Sprague-Dawley rats were divided into four groups of three animals (control untreated and treated with three doses of losartan). The treated groups received losartan (losartan potassium, Dup753; a generous gift from E.I. DuPont Nemours and Co., Wilmington, DE) at doses of either 10, 30 or 60 mg/kg/day, administered by gavage for 3 days. In our experience the lowest dose (10 mg/kg/day) is sufficient to normalize blood pressure in the 2K, 1C hypertensive rat (data not shown).

2.2. Renin assay

Trunk blood (500 μl) was collected on EDTA at decapitation and was cleared by centrifugation. Plasma was stored at −20°C until assayed. Plasma renin activity (PRA) was determined by the AI generation assay as previously described [16].

2.3. Immunohistochemistry

Kidneys were quickly removed and sliced into 3–4 mm sections. Kidney slices were rinsed twice in Tris-buffered saline (TBS; 50 mM Tris pH 7.4, 0.9% NaCl) and fixed overnight at 4°C in Bouin's fixative. Tissues were imbedded in paraffin and 5 μm sections were mounted onto gelatin coated slides for immunohistochemistry according to standard protocols. Slides were pre-incubated for 30 min in TBS containing 1% normal goat serum followed by incubation overnight with the primary antibody diluted in TBS containing 5% powdered milk (Carnation). Rabbit anti-recombinant human renin/prorenin antiserum was used at a dilution of 1/400. Rabbit anti-angiotensin II (CD4) antiserum was used at a dilution of 1/250. Rabbit anti-rat angiotensinogen antiserum was used at a dilution of 1/200. Immune complexes were stained by the avidin-biotin peroxidase complex method using a commercial kit (Vectastain ABC kit, Vector laboratories, Mississauga, Ont.) according to the manufacturer's instructions. Sections were either counter-stained with methyl green and Ehrlich's hematoxylin (BDH Inc., Toronto, Ont.) or were photographed without counter-staining using Nomarski optics (Fig. 3).

2.4. Electron microscopy

Kidneys were rapidly removed after decapitation and were cut into 1 mm^2 blocks. These were rinsed in PBS and fixed for 3 h in cacodylate-buffer (0.1 M sodium cacodylate, pH 7.1) containing 2% glutaraldehyde. Samples were post-fixed for 1 h in cacodylate buffer containing 1% osmium tetroxide and were included in LX112 resin (Ladd Research Industries, Burlington, VT) after dehydration. Sections (90 nm) were mounted on copper grids, counterstained with uranyl acetate and lead citrate and were examined in a Joel JEM 1200 EX microscope.

3. Results

3.1. Juxtaglomerular cell AII in the 2K, 1C model

Confirming previous reports [11,12], both renin and AII are readily detected in JG cells of the clipped kidney from rats subjected to unilateral renal artery constriction (Fig. 1A,B) while there is a near complete elimination of staining for both in the non-clipped kidney from the same animal (Fig. 1C,D). Both the clipped and the non-clipped kidneys showed abundant and equivalent staining for angiotensinogen in proximal tubules, but no staining in JG cells (data not shown). To compare the granulation of the JG cells in both the clipped and non-clipped kidneys, a portion of the same kidneys shown in Fig. 1 was fixed and embedded for electron microscopy. Fig. 2 shows that JG cells from both kidneys show abundant granulation. These results are typical of those found in several rats examined and are representative of all of the JG apparatuses examined. Higher magnification of the granules reveals a difference in the morphology of their content (Fig. 2, insets). On the clipped side (panel A), the granules are frequently distorted and display the typical rhomboid paracrystalline structures typical of renin-containing JG cells [17] while on the non-clipped side (panel B) the granules are more uniformly round and display a very regular granular pattern, devoid of paracrystalline structures. These data demonstrate that the loss of staining for renin and AII in the non-clipped kidney is explained by a loss of granules in the JG cells of that kidney.

3.2. AII receptor antagonism

To test whether the AII present in JG cells may be derived from internalization via the AII AT_1 receptors present on these cells [6–8], we treated adult rats for 3 days with losartan, a specific antagonist of this receptor subtype [18], and tested whether we could see a qualitative change in the AII staining of JG cells in treated animals versus control. Results (Fig. 3) show that JG cells stain for AII in all of the animals tested, even at the highest dose of losartan used. Moreover, in analyzing over 60 individual sections from 12 different animals, we did not find any consistent variation in the staining inten-

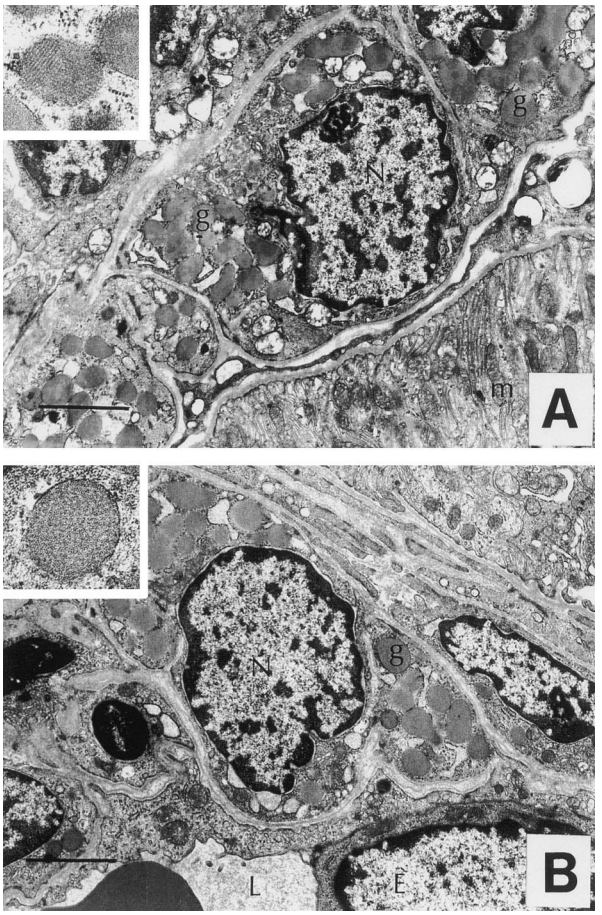

Fig. 2. Electron micrograph of JG cells from the clipped (A) and non-clipped (B) kidneys of the 2K, 1C rat. g, secretory granule; m, mitochondria; N, nucleus; L, lumen of the afferent arteriole; E, endothelial cell. Bar = 2 μm. Insets in upper left of each figure show a higher magnification of typical secretory granules in the corresponding JG cells.

sity of JG cells between treated and untreated animals. Taken together, these results suggest that AII internalization via the AT_1 receptor is not the major factor responsible for the appearance of AII in JG cell granules.

4. Discussion

In the current study we have sought to examine the mechanism by which AII is stored in the secretory granules of the JG cells in rats. Our studies confirm that AII content in JG cell granules is strictly dependent on the presence of renin as the cellular distribution of positively stained cells for both renin and AII is identical (Fig. 1, compare panels A and B) and both disappear simultaneously with the feedback down-regulation of renin expression in the non-clipped kidney. High levels of renin are secreted by the JG cells of the kidney, resulting in the formation of paracrystalline structures containing prorenin in the immature secretory granules [17]. Thus, with the loss of renin expression seen in the non-clipped kidney, secretory granule generation might be impaired, thereby simply removing the physical entity in which AII is found in JG cells. However, our results (Fig. 2) clearly demonstrate that a non-renin-expressing kidney still contains abundant secretory granules, thereby ruling out the loss of JG cells granules as the cause for the loss of AII staining in the non-clipped kidney. It is apparent that the morphology of these granules differs from that in the renin-expressing JG cells (Fig. 2, compare insets), confirming the suggestion that renin is responsible for the appearance of the paracrystalline structures typically seen in the granules of these cells. Rather, in the absence of immuno-detectable renin, JG cell granules are filled with an amorphous material (Fig. 2B, inset). The nature of this material is not clear at this time, although staining of the non-renin-containing kidney does not reveal granule storage of angiotensinogen (data not shown) as might be expected if these cells capture angiotensinogen and are unable to convert it to angiotensin I in the absence of renin. However, previous studies have shown that JG cell granules contain a number of lysosomal proteases including cathepsins B, H, and L [19]. It is possible, therefore, that in the absence of renin, angiotensinogen is degraded by alternative pathways of non-specific proteolysis. It is also somewhat remarkable that the abundance of secretory granules in JG cells does not appear to be decreased by the profound down-regulation of renin in the non-clipped kidneys of these adult rats (Fig. 2B). Granule biogenesis has previously been shown to be dependent on the expression of atrial natriuretic factor in the atria of mice in which the gene for ANF was deleted by homologous recombination [20]. Likewise, granules are absent from the JG cells of mice in which the Ren-1 gene is deleted [15]. The current experiments would suggest that either JG cell granule biogenesis differs between mice and rats or that the lack of JG cell granules seen in the 'knock-out' mice is due to an effect of the lack of renin during development, leading to defects in JG cell differentiation.

Blockade of the AT_1 subtype of the AII receptors with a specific antagonist (losartan) did not lead to a qualitative difference in the staining of AII in the JG cell clusters. It is also notable that the kidney of adult rats does not express the only other known receptor for AII, the AT_2 subtype [21,22], making it highly unlikely that AII is internalized in JG cells by any of the known receptors. One possible explanation for our failure to see any differences with losartan could be that we did not completely block the AT_1 receptors with the doses used. However, even the lowest dose of losartan used in our treatment regimen was sufficient to lead to a greater than 10-fold increase in plasma renin activity in the treated rats and has previously been shown to be adequate to normalize blood pressure in the 2K, 1C Goldblatt hypertensive model (data not shown). Another possibility is that AII enters JG cells by a mechanism other than AT_1 receptor internalization. Indeed, Taugner and colleagues have demonstrated that JG cells are capable of internalizing several exogenous proteins, including peroxidase and ferritin [23]. However, both the 2K, 1C model and losartan treatment result in dramatic increases in circulating AII which should have had the effect of increasing AII content in JG cells if trapping were non-specific. Liquid trapping would also be unlikely to account for the localization of AII only in JG cells containing renin (Fig. 1A,B).

In conclusion, our results are most consistent with an intracellular model of generation of AII, as was originally proposed by Naruse et al. [5]. In this instance, the pinocytotic activity of the JG cells might be used to capture circulating angiotensinogen which would be routed to the lysosomal-like granules of the JG cells [24]. Upon encountering renin, angio-

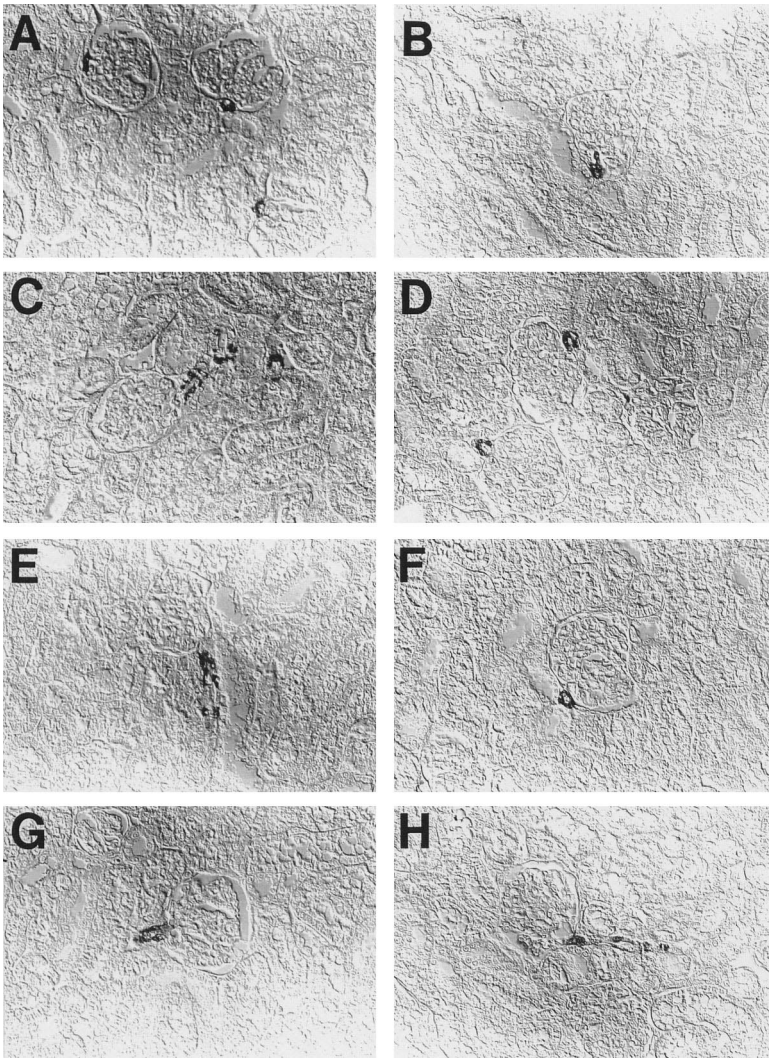

Fig. 3. Immunostaining of serial sections for renin (A,C,E,G) and AII (B,D,F,H) in the kidney JG cells of control rats (A,B) or rats treated with 10 mg/kg/day losartan (C,D), 30 mg/kg/day losartan, (E,F) or 60 mg/kg/day losartan (G,H).

tensin peptides would be generated and would presumably be released concomitantly with renin. Indeed, renal lymph and renal tubular fluid have been reported to contain high levels of angiotensin peptides [25], which could conceivably originate in the JG cells. Such locally synthesized AII has been proposed to regulate electrolyte homeostasis in the renal tubules [25] and may play a role in the development of the kidney [26,27].

Acknowledgements: The authors would like to express their gratitude to Vivianne Jodoin for secretarial aid and Susanne Diebold for technical support. This work was supported by research grants from the Medical Research Council of Canada.

References

[1] Celio, M.R. and Inagami, T. (1981) Proc. Natl. Acad. Sci. USA 78, 3897–3900.
[2] Rightsel, W.A., Okamura, T., Inagami, T., Pitcock, J.A., Takii, Y., Brooks, B., Brown, P. and Muirhead, E.E. (1982) Circ. Res. 50, 822–829.
[3] Taugner, R., Mannek, E., Nobiling, R., Buhrle, C.P., Hackenthal, E., Ganten, D., Inagami, T. and Schroder, H. (1984) Histochemistry 81, 39–45.
[4] Ingelfinger, J.R., Schunkert, H., Ellison, K.E., Pivor, M., Zuo, W.M., Pratt, R. and Dzau, V.J. (1990) Pediatr. Nephrol. 4, 424–428.
[5] Naruse, K., Inagami, T., Celio, M.R., Workman, R.J. and Takii, Y. (1982) Hypertension 4, 70–74.
[6] Gasc, J.M., Monnot, C., Clauser, E. and Corvol, P. (1993) Endocrinology 132, 2723–2725.
[7] Paxton, W.G., Runge, M., Horaist, C., Cohen, C., Alexander, R.W. and Bernstein, K.E. (1993) Am. J. Physiol. 264, F989–995.
[8] Kakinuma, Y., Fogo, A., Inagami, T. and Ichikawa, I. (1993) Kidney Int. 43, 1229–1235.
[9] Inagami, T. (1995) Curr. Opin. Nephrol. Hypertens. 4, 47–54.
[10] Goldblatt, H., Lynch, J., Hanzal, R.F. and Summerville, W.W. (1954) J. Exp. Med. 59, 347–378.
[11] Taugner, R., Marin-Grez, M., Keilbach, R., Hackenthal, E. and Nobiling, R. (1982) Histochemistry 76, 61–69.
[12] Morishita, R., Higaki, J., Okunishi, H., Tanaka, T., Ishii, K., Nagano, M., Mikami, H., Ogihara, T., Murakami, K. and Miyazaki, M. (1991) J. Hypertens. 9, 187–192.
[13] Schunkert, H., Ingelfinger, J.R., Jacob, H., Jackson, B., Bouyounes, B. and Dzau, V.J. (1992) Am. J. Physiol. 263, E863–869.
[14] Matsusaka, T., Nishimura, H., Utsunomiya, H., Kakuchi, J., Niimura, F., Inagami, T., Fogo, A. and Ichikawa, I. (1996) J. Clin. Invest. 98, 1867–1877.
[15] Clark, A.F., Sharp, M.G.F., Morley, S.D., Fleming, S., Peter, J. and Mullins, J.J. (1997) J. Biol. Chem. 272, 18185–18190.

[16] Li, J.S., Knafo, L., Turgeon, A., Garcia, R. and Schiffrin, E.L. (1996) Am. J. Physiol. 271, H88–93.
[17] Taugner, R., Kim, S.J., Murakami, K. and Waldherr, R. (1987) Histochemistry 86, 249–253.
[18] Timmermans, P.B. and Smith, R.D. (1996) Blood Pressure Suppl. 2, 53–61.
[19] Matsuba, H., Watanabe, T., Watanabe, M., Ishii, Y., Waguri, S., Kominami, E. and Uchiyama, Y. (1989) J. Histochem. Cytochem. 37, 1689–1697.
[20] John, S.W., Krege, J.H., Oliver, P.M., Hagaman, J.R., Hodgin, J.B., Pang, S.C., Flynn, T.G. and Smithies, O. (1995) Science 267, 679–681.
[21] Shanmugam, S., Llorens-Cortes, C., Clauser, E., Corvol, P. and Gasc, J.M. (1995) Am. J. Physiol. 268, F922–930.
[22] Amiri, F. and Garcia, R. (1996) Am. J. Physiol. 270, E810–815.
[23] Taugner, R., Hackenthal, E., Rix, E., Nobiling, R. and Poulsen, K. (1982) Kidney Int. Suppl. 12, S33–43.
[24] Taugner, R. and Hackenthal, E. (1988) Int. Rev. Cytol. 110, 93–131.
[25] Dzau, V.J. and Ingelfinger, J.R. (1989) J. Hypertens. 7, S3–S8.
[26] Krege, J.H., John, S.W., Langenbach, L.L., Hodgin, J.B., Hagaman, J.R., Bachman, E.S., Jennette, J.C., O'Brien, D.A. and Smithies, O. (1995) Nature 375, 146–148.
[27] Kim, H.S., Krege, J.H., Kluckman, K.D., Hagaman, J.R., Hodgin, J.B., Best, C.F., Jennette, J.C., Coffman, T.M., Maeda, N. and Smithies, O. (1995) Proc. Natl. Acad. Sci. USA 92, 2735–2739.