Testing the eukaryotic promoters for efficient expression of exogenous genes in chondrocytes and synoviocytes

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Received 2 September 1996

Abstract To identify suitable promoters for expressing exogenous genes in arthritic joints, the constitutive, simian virus 40 (SV 40) and IL-1 or metal inducible, human stromelysin and metallothionein (MT) gene promoters were tested for their activity in chondrocytes and synovial fibroblasts. Transient transfection with plasmids containing the reporter chloramphenicol acetyltransferase (CAT) gene attached to these promoters showed that SV40, stromelysin and MT promoters drove CAT expression with different strengths in primary bovine chondrocytes. The MTI-F and MT-IG gene promoters were also functional in human chondrocytes. The SV40, IL-1 inducible stromelysin-1 and MT-IG driven CAT activity was also detectable in human synoviocytes. Therefore, chondrocytes and synoviocytes have the *trans*-acting factors necessary for transcription from the respective promoters which may be conserved in bovine and human cells. These promoters could be useful for expressing potentially therapeutic anti-inflammatory and anti-erosive genes in arthritic joints.

Key words: Chondrocyte; Osteoarthritis; Promoter; Synoviocyte; Transfection

1. Introduction

Osteoarthritic (OA) is a common disorder of joints with unknown etiology. Factors such as joint overuse, injuries, obesity, aging, hormones (more women affected), life-style and mutations in the cartilage-specific type II collagen gene in heritable forms of the disease have been implicated in OA [1]. A related but more aggressive disease, rheumatoid arthritis (RA), has more auto-immunological etiology and much pronounced synovial inflammation and hyperplasia [2]. A common manifestation of RA and OA is the progressive loss of articular cartilage and release of extracellular matrix (ECM) components in synovial fluid and plasma by activated matrix metalloproteinases (MMPs) [3]. This leads to exposure of underlying bone and disability. Currently, these diseases are treated with non-steroidal anti-inflammatory drugs (NSAIDs), steroids, gold compounds, methotrexate and by surgical means with limited success. With a better understanding of the molecular mechanisms of the diseases, a recent alternative to conventional therapy is to target the genes involved in the disease either by blocking them with antisense nucleic acids or by overexpression of poorly expressed or defective genes.

Compared to the diseases of other organs, progress in gene therapy research for joints has been very slow. The prospects for such treatment were proposed recently by Bandara et al. [4]. It was suggested that joints are poor targets of conventional drugs administered by intravenous, oral and intramuscular means. The intra-articularly injected drugs have a short half-life. Two approaches for treating arthritis with genes were proposed. The antiarthritic genes could be introduced directly in synoviocytes by means of the viral vectors. Alternatively, synovium could be removed, synoviocytes grown in culture, genetically modified and transplanted back into synovium. The second approach was utilized to introduce *E. coli* markers; an easily assayable β-galactosidase (lacZ) and neomycin antibiotic resistance genes. These lacZ$^+$ cells persisted in joints for 3 months. Attempts at direct gene transfer into synovium did not succeed as retroviral vectors need dividing cells to transduce their DNA [4]. These investigators subsequently reported retrovirus-mediated ex vivo transduction of the markers and interleukin 1-receptor antagonist protein (IRAP) into rabbit synoviocytes which after reinjection colonized the synovium and expressed elevated levels of biologically active IRAP for 5 weeks. After recombinant IL-1β injection, the knee joints expressing IRAP, were protected from the leukosis [5]. The knees of control rabbits developed leukocyte infiltration in the joint space, synovial thickening, hypercellularity and loss of proteoglycans from cartilage. These symptoms were inhibited by IRAP [6]. This important study demonstrated the potential of gene therapy for arthritis.

Arthritis is a complex multi-factorial group of diseases and these authors have noted that the only anti-inflammatory IRAP may not have all the antiarthritic properties. In another report, the lacZ gene under the control of cytomegalovirus early promoter placed in an adenovirus vector was directly injected in rabbit joints. The expression and high efficiency transfer was demonstrated by in situ β-galactosidase (production of blue color), immunohistochemical staining and electron microscopy in type A and B synoviocytes. Expression lasted for 8 weeks after infection [7]. In a recent study, cells transfected with an anti-inflammatory cytokine, IL-4 cDNA, over-produced this protein and upon injection in mice, resulted in some improvement in collagen induced arthritis compared to joints with non-transfected cells [8]. All the cited studies have targeted the inflammatory aspect of arthritis. However, there is a clear need to target other aspects such as the degradation of cartilage by metalloproteinases originating from synoviocytes, inflammatory cells and chondrocytes. In addition, techniques of gene therapy can in principal be applied to inhibit undesirable gene products and to stimulate repair processes. Basic research is needed to test the efficiency

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PII S0014-5793(96)01037-X

of different eukaryotic promoters, and to develop methods and vectors for DNA delivery in matrix-rich tissues like cartilage. The proper choice of promoters is an important factor for successful therapeutic gene transfer and efficient expression of the exogenous genes. This study was aimed at testing the functionality of human metallothionein, stromelysin and simian virus 40 promoters in chondrocytes and synoviocytes.

2. Materials and methods

2.1. Chondrocyte and synovial fibroblast culture

Human cartilage and synovium were obtained immediately after total knee or hip replacement surgery of patients with OA. Bovine articular cartilage was obtained from the femoral heads, condyles and tibial plateaus of freshly slaughtered normal adult animals through a local slaughterhouse. The cartilage-containing bones were dipped briefly in 1% proviodine (Rougier Inc., Chambly Quebec) for sterilization and washed extensively with 0.9% NaCl. The slices of cartilage were dissected out, kept for 1–2 h at 4°C in 5×antibiotic-antimycotic solution and washed five times with large volumes of phosphate-buffered saline (PBS) containing 5×penicillin-streptomycin and 1×fungizone (Gibco-BRL Burlington, Ontario). Chondrocytes were released from cartilage following digestion with pronase (1 mg/ml) for 90 min and clostridial collagenase (Sigma type II) for 12 h in DMEM alone or supplemented with 10% fetal calf serum (Gibco BRL) at 37°C. Viability was about 80% as determined by trypan blue exclusion test. For primary cultures, cells were passed through a sterile microsieve to remove debris. The cells were pelleted at 2000 rpm for 15 min and washed three times with PBS and plated at high density. The cells were first allowed to attach to the plates in DMEM alone for 4 h [9] and then supplemented with 10% serum for confluent growth (up to 3 days). Synovial membranes were dissociated with trypsin for 1 h and with collagenase for 6 h and adherent fibroblast-enriched cultures selected after several passages.

2.2. Plasmids, transient transfection and CAT assay

The three well characterized human MT gene promoter vectors named p630 MT-IG-CAT, p437 MT-IF-CAT [10] and hMT-IIA CAT [11] cloned upstream of the bacterial CAT gene in pGEM-2 (Promega) based vector were utilized. The MT-IG-CAT contains nucleotides −630 to +65 of the gene and includes a TATAAA motif and four metal responsive elements (MREs). The MT-IF-CAT contains sequences −437 to +71 comprising a TATCA box, four MREs and several SP-1 binding sites. The hMT-IIA (−770 to +75) promoter contains a positive glucocorticoid responsive element (GRE) besides MREs and is therefore inducible by both metal salts and dexamethasone in the human hepatoma (HepG2) cell line [11]. The pCAT Basic (Promega) plasmid has no promoter cloned upstream of the CAT gene. The pCAT control vector has CAT gene under the control of the SV 40 promoter. The vector p+4CAT contained CAT gene under the control of the human stromelysin-1 −1303 to +4 promoter region [12]. This fragment has 480 base pairs of 5′ region which has most of the elements responsible for induction by interleukin-1 and phorbol esters. However, the sequences upstream from −480 were recently found to be rearranged in this construct and an additional 1 kb fragment was characterized [13]. The role of this 1 kb segment in regulation of this gene is not known.

For transfections, cells were plated at a density of 100 000 per 35 mm (or in 6-well plates for human cells) plate. Prior to transfection, cells were given fresh medium. Plasmids (20 μg) were isolated by Qiagen column purification and precipitated (500 μl) using the calcium phosphate method [14] and added to the plates. After 4 h of transfection, the medium was removed, washed once with PBS and cells glycerol (15%) shocked for 1 min, washed and incubated with medium for 24 h. Cells were then treated with interleukin-1β (20 ng/ml) for 24 h or zinc chloride for 8 h and collected by scraping, and lysed in 200 μl of 0.25 M Tris-HCl (pH 7.8) by sonication. The protein content of the lysate was measured by the Bradford method using Bio-Rad reagents [15]. CAT assay were performed by adjusting 20 μg of protein extract to 120 μl with Tris, combined with 20 μl of [^{14}C]chloramphenicol (Cm) (0.133 μCi) (Dupont-NEN Canada) and 20 μl of 4 mM acetyl-CoA (0.5 mM final). After 8 h incubation at 37°C, Cm was extracted with ethyl acetate, solvent evaporated by speed vac and resuspended in 30 μl of ethyl acetate. The acetylated forms of Cm were separated by ascending thin-layer chromatography. Autoradiography was performed to detect CAT activity by localizing different forms of Cm and percent acetylation determined by scintillation counting of the spots.

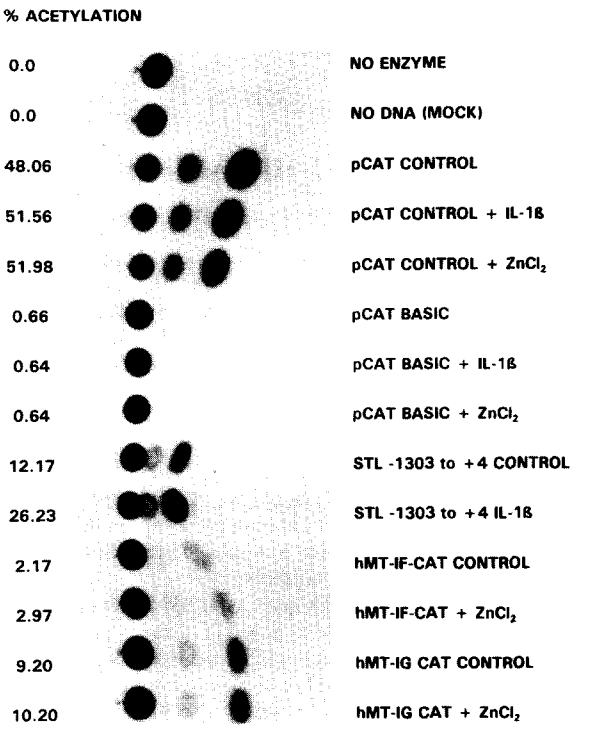

Fig. 1. Transfection of primary bovine chondrocytes with SV40, stromelysin and metallothionein promoters. CAT assay of protein extracts (20 μg) normalized with the SV40-driven β-galactosidase activities from primary bovine chondrocytes transfected transiently with $CaPO_4$ precipitate only (no DNA), 20 μg of a vector with CAT under the SV40 early promoter (pCAT control), a vector with CAT but without a promoter (pCAT basic), human stromelysin-1 (STL) promoter (−1303 to +4) in pGCAT-C and human MT-IG and MT-IF-CAT vectors. The inductions were performed 60 h post-transfection to permit cell recovery. IL-1 (20 ng/ml) and $ZnCl_2$ (100 μM) treatment was for 24 h in serum-free medium or 8 h in serum-containing medium, respectively. The film was exposed for 24 h. The 'no enzyme' lane contains all the assay reagents but no protein extract.

2.3. β-Galactosidase assay

Along with the test plasmids, 10 μg of pSV-β-galactosidase control vector (Promega Corp.) was co-transfected in bovine chondrocytes and human synoviocytes. 20 μg of protein extract was assayed for β-galactosidase enzyme with the assay system of Promega according to their instructions [16]. The extract was incubated for 30 min with reaction buffer containing *o*-nitrophenyl-β-D-galactopyranoside as substrate, the reaction terminated with sodium bicarbonate and the absorbance of the yellow colored *o*-nitrophenol measured at 420 nm with a spectrophotometer.

3. Results

To test the functionality of different eukaryotic promoters in bovine and human chondrocytes and synoviocytes, plasmid constructs with the bacterial chloramphenicol acetyl transferase (CAT) gene placed downstream of different eukaryotic promoters were obtained or constructed (see Section 2). For monitoring transfection efficiencies, cells were co-transfected with a vector expressing β-galactosidase enzyme under SV 40

promoter. Transient transfection by the calcium phosphate precipitation method into the primary monolayer cultures of bovine chondrocytes and CAT assay normalized with β-galactosidase activity revealed that the SV 40 early promoter was unresponsive to $ZnCl_2$ or IL-1 but was most active constitutively in these cells. The promoterless vector alone or with inducing agents had negligible CAT activity. The stromelysin-1 promoter was clearly able to drive the expression of the CAT gene at basal level whose activity was approx. 2-fold inducible with interleukin-1β. MT-IF and MT-IG transfected cells had considerable basal CAT activity which was higher for the latter promoter. MT-IG promoter was 3–4-fold stronger than MT-IF. Induction with Zn in both cases was not very obvious (Fig. 1).

The metal (Cd, Zn) inducible, metallothionein promoters have been a popular choice in numerous transgene expressions and stable transfections of foreign genes [17,18]. Transient transfection of these vectors in human OA chondrocytes and CAT assay depicted in Fig. 2 revealed that MT-IG promoter was functional at basal levels and could be induced to slightly higher levels by metal salt treatment. In Zn-treated cells, MT-IG promoter appeared to be stronger than MT-IF while MT-IIA was inactive in three experiments (results not shown). Other controls such as MT-IF-CAT (uninduced) could not be tested due to the limited availability of human OA chondrocytes.

In synovial fibroblasts, the promoterless vectors alone or with inducing agents had very little CAT activity (Fig. 3). As in bovine chondrocytes, the constitutive SV 40 promoter was the most active promoter in human synovial fibroblasts, followed by stromelysin, MT-IG and MT-IF; the latter being the weakest. The MT-IG and MT-IF promoters were also functional at the basal level but could not be induced further with $ZnCl_2$ as evident from the β-galactosidase normalized CAT activity depicted as percent acetylation normalized with β-galactosidase activity. The MT-IG promoter had 3-times higher activity than MT-IF. The basal stromelysin promoter activity could be induced 1.6-fold by interleukin-1β treatment in these cells (Fig. 3).

4. Discussion

The utilization of appropriate promoters is an important factor for successful therapeutic gene transfer and its high-level expression in target tissue [19]. In this report, we have

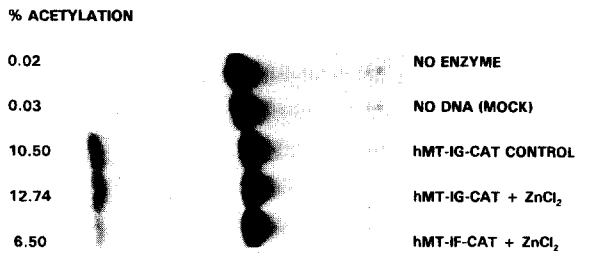

Fig. 2. Activity of human metallothionein IG and F promoters in human chondrocytes. Autoradiogram of CAT assay of protein extract from human OA chondrocytes (1st passage) transfected with a vector where human metallothionein-IG and F promoters are fused with the bacterial CAT gene. The cells were either untreated or treated with 100 μM $ZnCl_2$ for 7 h in the presence of 10% serum after transfection. Other denotations as in Fig. 1. Time of film exposure was 48 h.

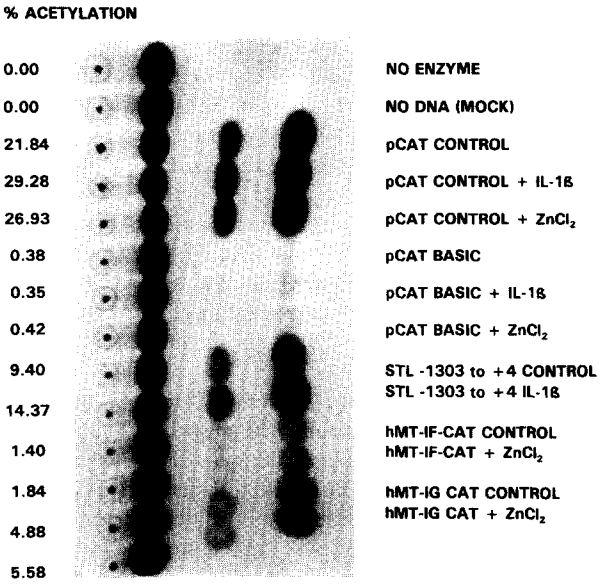

Fig. 3. Demonstration of SV40, human stromelysin and metallothionein IG and F promoter driven CAT activity in human synovial fibroblasts. The CAT assays with 20 μg of protein extract (normalized with the SV40-driven β-galactosidase activity) show successful transfection and activity of SV40 (pCAT control) and MT promoters. The inducibility of the stromelysin promoter with IL-1 is also shown. Conditions were the same as in Fig. 1. Time of autoradiography was 24 h.

tested some eukaryotic promoters and demonstrated their activity in human and bovine connective tissue cells. The bovine cartilage ex vivo model system, because of its abundant availability, is a widely used and well-recognized system in orthopaedic and cartilage matrix research.

The activity of SV40, stromelysin and MT promoters in bovine chondrocytes demonstrated that *cis*-acting factors of these promoters are recognized by the bovine transcription factors. These may include AP-1 (fos/jun) and PEA-3 factors which are responsible in part for regulation of the stromelysin gene by phorbol esters in human cells [20]. The 480 base pairs in the 1.3 kb promoter segment most likely contain all the sequences necessary for promoter activity in bovine cells. This conservation is also extended to the protein-coding part of the gene where human stromelysin probes cross-hybridize with corresponding bovine mRNA (our unpublished results).

The activity of metallothionein promoters in bovine and human chondrocytes is consistent with our earlier observations that the MT genes are expressed in bovine [21] and human [22] chondrocytes, and on these grounds, the respective promoters are likely to function in in vivo gene transfer experiments. MT regulatory factors are therefore present in cartilage and to a lesser extent in synovium [23]. The higher activity of MT-IG over MT-IF promoter in chondrocytes and synoviocytes may be related to the presence of a canonical TATAAA in the former and an altered TATCAA in the latter promoter which were in part responsible for differential expression of these genes in HepG2 cells [24]. Surprisingly, hMT-IIA, the strongest of all the human MT promoters in other cell types, was not active in our cells, suggesting a lack of certain regulatory factors specific to this gene. Of the three MT promoters tested so far, the MT-IG promoter seemed to

be most active. Our inability to detect clearly strong induction of MT promoters by zinc in some experiments may be due to high basal levels of metal salts in commercially available media which may saturate the system from further induction. Alternatively, the maximal induction time point could have been reached earlier than the 8 h utilized in our study.

The in vivo utility of the tested promoters remains to be examined as the inducing agents such as metals (for MT) and IL-1 (for stromelysin) have to be utilized for achieving maximal activities. Zinc being a physiological agent may not have many adverse effects and has been utilized for expression of numerous transgenes [17]. IL-1, a pro-inflammatory cytokine, may invoke undesired side-effects.

Arthritis is a major group of multifactorial diseases affecting millions of people around the world. While efforts at understanding the mechanism of OA and RA continue, there is a need to develop tools for gene transfer and expression in joint cells. Recent evidence suggests mutated collagen genes as a possible cause of familial OA [25]. It may be necessary to treat these ailments by expressing normal versions of these genes at high level under the control of strong promoters. A progressive in vitro, ex vivo and in vivo approach would represent an important step in this direction. Parallel developments in chondrocyte growth, phenotype maintenance, cryopreservation and cartilage tissue engineering will complement these approaches [26]. Further, proper anti-arthritic genes and methods of gene delivery in matrix-rich connective tissue have to be identified and developed respectively [27].

In summary, the metallothionein, SV40 and stromelysin promoters are functional in connective tissue cells and could serve as potentially useful tools for expressing antiarthritic genes in ex vivo gene transfer or in experimental therapy of animal models of arthritis with appropriate vectors.

Acknowledgements: This study was supported in part by the Medical Research Council of Canada, the Comité d'attribution des fonds internes de recherche (CAFIR) de l'Université de Montréal and Fonds de la Recherche en Santé du Québec (FRSQ). M.Z. acknowledges research scholarships of the FRSQ and Arthritis Society. We are indebted to Dr. Markku Kurkinen (Wayne State University, Detroit, MI) for the human stromelysin promoter, Dr. Alfred Assalian and Dr. Afzal Mehdi (Ophthalmology) for permitting us to use their cell culture facilities, Dr. Randy Foster and Dr. Nicholas Shworak for constructing human metallothionein plasmids, Mr. Jean Maher, (Abattoir les Cèdres) for bovine cartilage and Ms. Anna Chelchowska for processing this manuscript and preparing figures.

References

[1] Hamerman, D. (1989) New Engl. J. Med. 320, 1322–1330.
[2] Schumacher, H.R. Jr. (1988) Primers on Rheumatic Diseases, 9th edn., Arthritis Foundation, Atlanta, GA.
[3] Poole, A.R. (1994) Ann. Rheum. Dis. 53, 3–5.
[4] Bandara, G., Robbins, P.D., Georgescu, H.I., Mueller, G.M., Glorioso, J.C. and Evans, C.H. (1992) DNA Cell Biol. 11, 227–231.
[5] Bandara, G., Mueller, G.M., Galea-Lauri, J., Tindal, M.H., Georgescu, H.I., Suchanek, M.K., Hung, G.L., Glorioso, J.C., Robbins, P.D. and Evans, C.H. (1993) Proc. Natl. Acad. Sci. USA 90, 10764–10768.
[6] Hung, G.L., Galea-Laurie, J., Mueller, G.M., Georgescu, H.I., Larkin, L.A., Suchanek, M.K., Tindal, M.H., Robbins, P.D. and Evans, C.H. (1994) Gene Therapy 1, 64–69.
[7] Roessler, B.J., Allen, E.D., Wilson, J.M., Hartman, J.W. and Davidson, B.L. (1993) J. Clin. Invest. 92, 1085–1092.
[8] Boissier, M.-C., Bessis, N., Roth, C., Fradelizi, D. and Fournier, C. (1993) Arthritis Rheum. 36, S45–38.
[9] Pearson, D. and Sasse, J. (1992) J. Biol. Chem. 267, 25364–25370.
[10] Foster, R. and Gedamu, L. (1991) J. Biol. Chem. 266, 9866–9875.
[11] Jahroudi, N., Foster, R., Price-Haughey, J., Beitel, G. and Gedamu, L. (1990) J. Biol. Chem. 265, 6506–6511.
[12] Quinones, S., Saus, J., Otani, Y., Harris, E.D. Jr. and Kurkinen, M. (1989) J. Biol. Chem. 264, 8339–8344.
[13] Borden, P., Song, K. and Heller, R.A. (1994) Gene 151, 315–320.
[14] Gorman, C.M., Moffat, L.D. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044–1051.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Rosenthal, N. (1986) Method. Enzymol. 152, 704–720.
[17] Palmiter, R.D. and Brinster, R.L. (1986) Annu. Rev. Genet. 20, 465–499.
[18] Palmiter, R.D., Sandgren, E.P., Koeller, D.M. and Brinster, R.L. (1993) Mol. Cell Biol. 13, 5266–5275.
[19] Rettinger, S.D., Kennedy, S.C., Wu, X., Saylors, R.L., Hafenrichter, D.G., Flye, M.W. and Ponder, K.P. (1994) Proc. Natl. Acad. Sci. USA 91, 1460–1464.
[20] Buttice, G. and Kurkinen, M. (1993) J. Biol. Chem. 268, 7196–7204.
[21] Zafarullah, M., Su, S. and Gedamu, L. (1993) Exp. Cell Res. 208, 371–377.
[22] Zafarullah, M., Martel-Pelletier, J., Cloutier, J.M., Gedamu, L. and Pelletier, J.P. (1992) FEBS Lett. 306, 169–172.
[23] Zafarullah, M., Martel-Pelletier, J., Cloutier, J.M., Gedamu, L. and Pelletier, J.P. (1993) OA Cartilage 1, 151–156.
[24] Shworak, N.W., O'Conner, T., Wong, N.C.W. and Gedamu, L. (1993) J. Biol. Chem. 268, 24460–24466.
[25] Jimenez, S.A. and Dharmavarm, R.M. (1994) Ann. Rheum. Dis. 53, 789–797.
[26] Langer, R. and Vacanti, J.P. (1993) Science 260, 920–926.
[27] Evans, C.H. and Robbins, P.D. (1994) J. Rheum. 21, 779–782.