Mannose Receptor–Mediated Regulation of Serum Glycoprotein Homeostasis

Sena J. Lee,[1] Stefan Evers,[2] Daniel Roeder,[3] Albert F. Parlow,[4] Juha Risteli,[5] Leila Risteli,[5] Y. C. Lee,[6] Ten Feizi,[7] Hanno Langen,[2,3] Michel C. Nussenzweig[1,8]*

Carbohydrates are thought to function as tags that mark circulatory glycoproteins for rapid clearance. To examine the role of the mannose receptor (MR) in glycoprotein clearance, we generated mice genetically deficient in MR. $MR^{-/-}$ mice were defective in clearing proteins bearing accessible mannose and *N*-acetylglucosamine residues and had elevated levels of eight different lysosomal hydrolases. Proteomic analysis of $MR^{-/-}$ and control mouse sera showed that an additional 4 out of 52 proteins identified were elevated in $MR^{-/-}$ serum. Each of these is up-regulated during inflammation and wound healing. Thus, MR appears to operate as an essential regulator of serum glycoprotein homeostasis.

The idea that membrane lectins remove senescent circulating glycoproteins was first proposed by Ashwell and Morell (*1*). In support of this hypothesis, they showed that removing the sialic acid caps on complex *N*-glycans, and thereby exposing the penultimate galactose residues, altered the serum survival of glycoproteins, and this was attributed to the asialoglycoprotein receptor (ASGPR). However, ASGPR-deficient mice do not accumulate endogenous serum proteins with terminal galactose residues (*2*), suggesting other mechanisms of asialoglycoprotein clearance. To date, essential clearance receptors for glycoproteins have not been identified.

The mannose receptor (MR) is an endocytic receptor for glycans expressed in a number of tissues, including the hepatic sinusoidal endothelium. It is composed of 10 extracellular domains: an NH_2-terminal cysteine-rich (CR) domain, a fibronectin type II repeat domain, and

[1]Laboratory of Molecular Immunology, The Rockefeller University, 1230 York Avenue, Box #220, New York, NY 10021, USA. [2]Infectious Diseases, [3]Genetics, F. Hoffmann-La Roche, CH-4070 Basel, Switzerland. [4]National Hormone and Pituitary Program, Harbor–University of California–Los Angeles Medical Center, Torrance, CA 90509, USA. [5]Department of Clinical Chemistry, University of Oulu, Oulu FIN-90014, Finland. [6]Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA. [7]Glycosciences Laboratory. Imperial College of Science Technology and Medicine, Faculty of Medicine, Northwick Park Institute for Medical Research, Harrow, HA1 3UJ, UK. [8]Howard Hughes Medical Institute, New York, NY 10021, USA.

*To whom correspondence should be addressed. E-mail: nussen@mail.rockefeller.edu

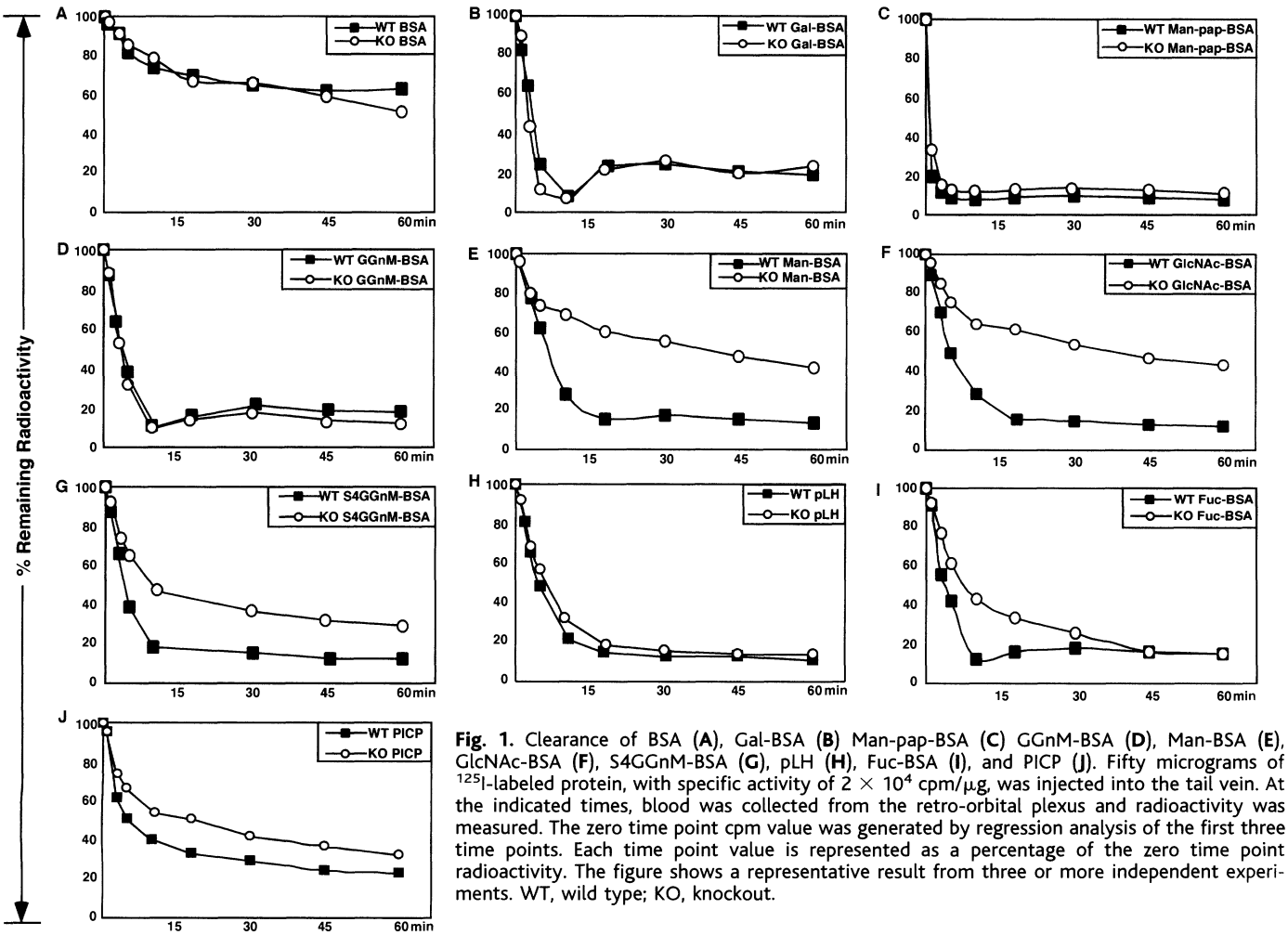

Fig. 1. Clearance of BSA (**A**), Gal-BSA (**B**) Man-pap-BSA (**C**) GGnM-BSA (**D**), Man-BSA (**E**), GlcNAc-BSA (**F**), S4GGnM-BSA (**G**), pLH (**H**), Fuc-BSA (**I**), and PICP (**J**). Fifty micrograms of ^{125}I-labeled protein, with specific activity of 2×10^4 cpm/μg, was injected into the tail vein. At the indicated times, blood was collected from the retro-orbital plexus and radioactivity was measured. The zero time point cpm value was generated by regression analysis of the first three time points. Each time point value is represented as a percentage of the zero time point radioactivity. The figure shows a representative result from three or more independent experiments. WT, wild type; KO, knockout.

eight tandem carbohydrate recognition domains (CRDs). The CRDs bind terminal mannose (Man), fucose (Fuc), and *N*-acetylglucosamine (GlcNAc) residues (*3*), and the CR domain binds sulfated sugar moieties, including terminal sulfated *N*-acetylgalactosamine (SO_4-GalNAc), chondroitin sulfates A and B, and sulfated Lewis^a and Lewis^x groups (*4*, *5*). A panoply of functions has been attributed to this receptor, including pathogen pattern recognition with a role in mediating innate immunity (*6*), attachment of sperm to oocyte (*7*), endocytosis for antigen presentation (*6*), and clearance of glycoproteins (*5*, *8*).

To determine whether the MR is a nonredundant receptor for glycoprotein clearance, we examined the ability of wild-type and $MR^{-/-}$ mice (*9*) to clear ^{125}I-labeled neoglycoproteins, including candidate MR ligands [Man–bovine serum albumin (BSA), GlcNAc-BSA, Fuc-BSA, and S4GGnM-BSA] and controls [BSA, galactose-BSA (gal-BSA), gal-GlcNAc-Man-BSA (GGnM-BSA), and Man-para-amino-phenyl-BSA) (Man-pap-BSA)] (*10*, *11*). BSA, Gal-BSA, Man-pap-BSA, and GGnM-BSA control clearance was indistinguishable between wild-type and $MR^{-/-}$ mice (Fig. 1, A to D, and Table 1). In contrast, removal of Man-BSA and GlcNAc-BSA from serum was markedly delayed in $MR^{-/-}$ mice (Fig. 1, E and F). In wild-type mice, clearance of Man-BSA and GlcNAc-BSA was associated with rapid accumulation of radioactivity in the liver and spleen (*9*). No such differential hepatic and splenic accumulation was found in $MR^{-/-}$ mice, indicating that the MR is the only mannose- and GlcNAc-specific clearance mechanism (*9*). Clearance of S4GGnM-BSA, a ligand for the CR domain of MR (*4*, *5*), was also delayed in $MR^{-/-}$ mice (Fig. 1G) but to a lesser extent than either Man-BSA and GlcNAc-BSA (Table 1). In addition, there was differential hepatic accumulation of S4GGnM-BSA in $MR^{-/-}$ mice, indicating the existence of a second specific receptor in liver (*9*). This sulfated carbohydrate recognition has been implicated in the regulation of serum luteinizing hormone (LH) levels (*4*, *12*); however, there was little difference in half-life of porcine LH between $MR^{-/-}$ mice and controls (Fig. 1H) (*9*). Finally, we found only small differences in the clearance of the Fuc-BSA, consistent with reports of a second fucose receptor in liver (*13*) (Fig. 1I). In conclusion, the MR is essential for normal clearance of mannose, S4GGnM, and GlcNAc, but not fucose.

Among native glycoproteins, MR has been proposed as a clearance receptor for β-glucuronidase, one of several lysosomal enzymes bearing high-mannose glycans (*8*). In agreement with this idea, we found that the circulating levels of β-glucuronidase were about fourfold higher in $MR^{-/-}$ mice than in wild-type controls (Fig. 2A). In contrast, there was no significant difference in tissue enzyme levels, suggesting that intracellular stores of the enzyme were normal (Fig. 2A). Similar results were obtained with seven of eight other lysosomal hydrolases tested (Fig. 2, B to I). Our experiments show that the MR is essential for regulating the steady-state serum levels of most lysosomal hydrolases.

In search of other circulatory proteins regulated by the MR, we carried out proteomic analysis of sera from wild-type and $MR^{-/-}$ mice. More than 350 spots were visualized by two-dimensional immobilized pH gradient (IPG)/SDS–polyacrylamide gel electrophoresis (PAGE), and identification by mass spectrometry or MS-MS sequencing was attempted on each spot (*14*). Fifty-two unique proteins were identified, and three (spots 1, 2, and 3) of those were consistently elevated and none were consistently decreased in the $MR^{-/-}$ mice (Fig. 3A). An additional species (spot 4) was elevated in all $MR^{-/-}$ samples but could not be identified. The three identified proteins are the COOH-terminal propeptide domains of the pro-alpha 1 and 2 chains of type 1 procollagen and the pro-alpha 1 chain of type III procollagen. Circulatory levels of these by-products of collagen synthesis are elevated during wound healing (*15*), and these peptides are modified with high-mannose glycans (*16*).

To determine whether these proteins indeed contain accessible mannose residues, we performed lectin blotting with concanavalin A (Con A). The spots that correspond to procollagen α1(I) and collagen α2(I) COOH-terminal peptides were bound by Con A and elevated in $MR^{-/-}$ mice, thus confirming the results of SyproRuby staining (Fig. 3B). Three additional Con A–positive spots were observed to be present only on $MR^{-/-}$ blots. These were identified as different glycoforms of fetuin-B (Fig. 3B, spot 5). Fetuin-B is a recently discovered member of the cystatin superfamily (*17*), whose expression level changes during inflammation (*18*).

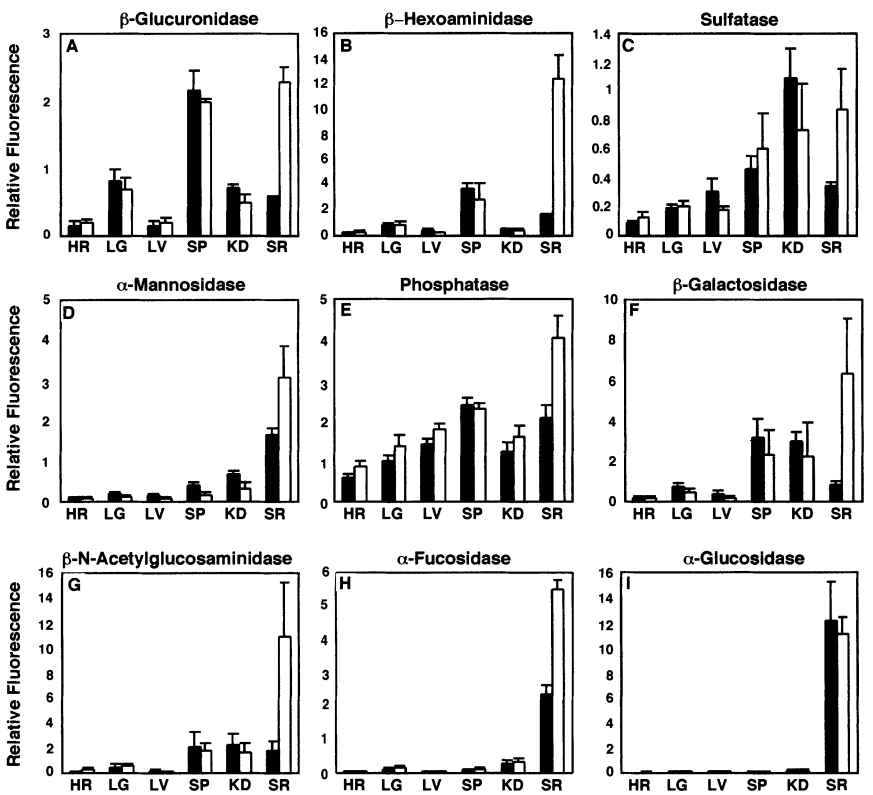

Fig. 2. Measurement of serum lysosomal enzyme levels. (**A** to **I**) Serum or tissue lysate samples were incubated with 4-methylumbelliferyl (4-MU) enzyme substrates (*27*). Solid bars, wild-type mice; open bars, $MR^{-/-}$ mice. Relative fluorescence was calculated by dividing the mean values from four mice per group by the blank controls (substrates only) and adjusting for total protein amount (μg) or serum volume (μl). Error bars indicate +1 SD. HR, heart; LG, lung; LV, liver; SP, spleen; KD, kidney; SR, serum.

Table 1. Half-life of glycoproteins in serum. Half-life values were calculated from the clearance curves of three or more mice per group and then averaged. SD (±1) values are also indicated.

Protein	Wild type (min)	Knockout (min)
BSA	>60	>60
Man-BSA	6.4 ± 0.8	39 ± 2.4
GlcNAc-BSA	4.8 ± 0.5	40 ± 10
Fuc-BSA	4.0 ± 0.4	8.0 ± 0.7
GGnM-BSA	4.9 ± 0.9	12 ± 1.7
GGnM-BSA	4.0 ± 1.1	3.4 ± 0.6
Gal-BSA	3.1 ± 0.5	4.0 ± 0.4
pLH	4.1 ± 0.7	6.1 ± 1.1
PICP	7.4 ± 1.8	15 ± 3.6

To confirm that the elevated procollagen peptide levels in the serum of $MR^{-/-}$ mice is due to altered clearance, we measured the clearance of ^{125}I-labeled propeptide of human type 1 procollagen (PICP) (*19*). We found that the half-life of PICP increased from 7.4 min in wild-type mice to 15 min in $MR^{-/-}$ mice ($P = 0.03$; Fig. 1J and Table 1). We conclude that the accumulation of procollagen peptides in serum of $MR^{-/-}$ mice is due to delayed clearance.

Because the MR-regulated serum proteins identified are normally elevated in inflammation and wound healing (*15*, *18*), we examined whether $MR^{-/-}$ mice show an increase in serum levels of these proteins during inflammation. After stimulation with intraperitoneal injection of thioglycollate, we found a disproportionate increase in serum β-glucuronidase levels in $MR^{-/-}$ mice: 1.53 units per μl in $MR^{-/-}$ mice, compared with 0.55 units per μl in wild type (*9*). However, there were no differences in the numbers or types of inflammatory cells in the peritoneal cavity, tissue levels of β-glucuronidase, or serum amyloid protein A (an acute phase reactant). Thus, the MR is required for rapid clearance of a subset of mannose bearing serum glycoproteins that are normally elevated during inflammation, but it does not appear to regulate the initiation of inflammation.

Our results provide in vivo evidence of lectin regulation of serum glycoprotein levels. The MR appears to remove serum glycoproteins for degradation and subsequent excretion because neither liver nor spleen retains the initially accumulated radioactivity. This is consistent with the idea that MR ligands are delivered to lysosomes for degradation and the finding of catabolites in the peripheral tissues in the form of a single amino acids and free iodine (*20*). We conclude that MR provides an efficient and essential clearance and degradation mechanism for its serum glycoprotein ligands.

The glycoproteins whose steady-state levels are elevated in the sera of $MR^{-/-}$ mice are all known inflammatory proteins. Inducers of inflammation, such as lipopolysaccharide, phorbol esters (*21*), and interferon-γ (*22*), down-regulate the expression of the MR, whereas dexamethasone, an immunosupressor, up-regulates MR expression (*23*). Thus, MR expression is repressed in the early stages of inflammation and increased in the resolution phase, which is consistent with a role for this receptor in clearing inflammatory agents. Our findings suggest that high-mannose and other MR-binding glycans convey the transient resident status of inflammatory proteins in circulation and that the mannose receptor recognizes this glycan signal. On the basis of the 52 proteins identified in our proteomic analysis, out of which 4 were MR-regulated, we expect that clearance of a fraction of serum proteins is dependent on this receptor. The finding that the MR plays an essential role in clearance of serum glycoproteins offers an opportunity to manipulate this glycobiological code and thereby specifically regulate serum levels of important bioactive proteins in health and disease.

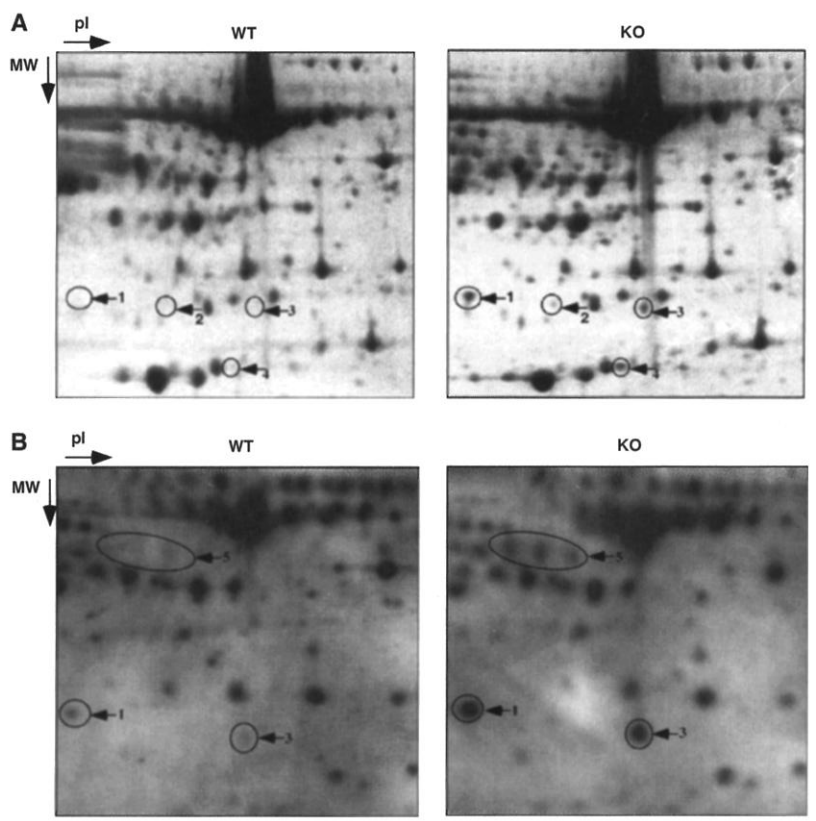

Fig. 3. Proteomic analysis of total serum proteins. (**A**) Two-dimensional PAGE of serum proteins (*28*). For spot quantification, gels were stained with SYPRO Ruby and scanned on a laser fluorescence scanner. Left, wild type; right, $MR^{-/-}$. (**B**) Detection of accessible mannose residues on serum proteins (*29*). Two-dimensional blots were incubated with Con A–HRP in binding buffer, plus 10 mM α-methyl-glucopyranoside to diminish Con A binding to glycoproteins with complex-type N-glycans. Blots were treated with chemiluminescent HRP substrate before exposure on film. Left, wild type; right, $MR^{-/-}$. pI, isoelectric point; MW, molecular weight.

References and Notes

1. G. Ashwell, A. G. Morell, *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 99 (1974).
2. S. Ishibashi, R. E. Hammer, J. Herz, *J. Biol. Chem.* **269**, 27803 (1994).
3. M. E. Taylor, K. Drickamer, *J. Biol. Chem.* **268**, 399 (1993).
4. D. J. Fiete, M. C. Beranek, J. U. Baenziger, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2089 (1998).
5. C. Leteux *et al.*, *J. Exp. Med.* **191**, 1117 (2000).
6. P. D. Stahl, R. A. Ezekowitz, *Curr. Opin. Immunol.* **10**, 50 (1998).
7. S. Benoff *et al.*, *Fertil. Steril.* **59**, 854 (1993).
8. P. Stahl, P. H. Schlesinger, J. S. Rodman, T. Doebber, *Nature* **264**, 86 (1976).
9. Supplemental data are available on *Science* Online at www.sciencemag.org/cgi/content/full/295/5561/1898/DC1.
10. D. Fiete, V. Srivastava, O. Hindsgaul, J. U. Baenziger, *Cell* **67**, 1103 (1991).
11. Y. C. Lee, C. P. Stowell, M. J. Krantz, *Biochemistry* **15**, 3956 (1976).
12. J. U. Baenziger, S. Kumar, R. M. Brodbeck, P. L. Smith, M. C. Beranek, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 334 (1992).
13. G. W. Hoyle, R. L. Hill, *J. Biol. Chem.* **263**, 7487 (1988).
14. Identification of proteins was carried out by peptide mass fingerprinting as described (*24*). As a criterion for protein identification, five peptides had to match the theoretical values. If identification could not be achieved by this means, internal sequencing by nano-spray-tandem mass spectrometry on a quadrupole time-of-flight mass spectrometer (Qstar Pulsar 1; ABI/MDS Sciex, Toronto, Canada) was performed.
15. K. Haukipuro, L. Risteli, M. I. Kairaluoma, J. Risteli, *Ann. Surg.* **206**, 752 (1987).
16. C. C. Clark, *J. Biol. Chem.* **254**, 10798 (1979).
17. E. Olivier *et al.*, *Biochem. J.* **350** (part 2), 589 (2000).
18. W. M. Brown, N. R. Saunders, K. Mollgard, K. M. Dziegielewska, *Bioessays* **14**, 749 (1992).
19. J. Melkko, S. Niemi, L. Risteli, J. Risteli, *Clin. Chem.* **36**, 1328 (1990).
20. M. E. Taylor, M. S. Leaning, J. A. Summerfield, *Am. J. Physiol.* **252**, E690 (1987).
21. V. L. Shepherd, R. Abdolrasulnia, M. Garrett, H. B. Cowan, *J. Immunol.* **145**, 1530 (1990).
22. R. A. Ezekowitz, M. Hill, S. Gordon, *Biochem. Biophys. Res. Commun.* **136**, 737 (1986).
23. V. L. Shepherd, M. G. Konish, P. Stahl, *J. Biol. Chem.* **260**, 160 (1985).
24. B. Grunenfelder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4681 (2001).
25. I. Sohar, D. Sleat, C. Gong Liu, T. Ludwig, P. Lobel, *Biochem. J.* **330**, 903 (1998).
26. B. Bjellqvist, C. Pasquali, F. Ravier, J. C. Sanchez, D. Hochstrasser, *Electrophoresis* **14**, 1357 (1993).
27. Organs were homogenized in 1:10 (w/v) volume of homogenization buffer [25 mM tris-HCl (pH 7.2), 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride] and sonicated for 30 s on ice. Lysosomal

enzyme activities in serum and tissue lysates were measured as described (*25*).

28. Plasma samples were concentrated and buffer was exchanged with IEF sample buffer [7 M urea, 2 M thiourea, 50 mM tris (pH 7.5), 2% CHAPS, and 0.4% dithioerythritol] by diafiltration with 10-kD cutoff centrifugal filters (Millipore, Bedford, MA). Two-dimensional SDS-PAGE was carried out as described (*26*).
29. Two-dimensional blots were treated with 0.6% H_2O_2 in methanol for 30 min and blocked overnight at 4°C with 0.25% BSA (RIA grade, Sigma) in binding buffer (50 mM tris, 150 mM NaCl, and 4 mM $CaCl_2$, pH 7). Blots were incubated at room temperature with Con A–horseradish peroxidase (HRP) (EY Laboratories) at 1.8 μg/ml concentration in binding buffer, plus 10 mM α-methyl-glucopyranoside for 90 min. Blots were washed and then incubated with Chemiluminescence Reagent Plus (NEN Life Science) for 1 min before exposure on film.
30. We are grateful to E. Besmer, C. Nathan, J. Ravetch, and R. Steinman for discussions and suggestions; Y. Combarnous and O. Hindsgaul for pLH and S4GGnM- and GGnM-BSA, respectively; and Y. Hu and H. Nagaoka for assistance. This work was supported in part by Programme Grant G9601454 from the U.K. Medical Research Council (T. F), NIH Medical Scientist Training Program grant GM07739 (S.J.L.); and NIH and the Howard Hughes Medical Institute (M.C.N.).

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Coordination of PIC Assembly and Chromatin Remodeling During Differentiation-Induced Gene Activation

Evi Soutoglou and Iannis Talianidis*

We analyzed the ordered recruitment of factors to the human α_1 antitrypsin promoter around the initial activation of the gene during enterocyte differentiation. We found that a complete preinitiation complex, including phosphorylated RNA pol II, was assembled at the promoter long before transcriptional activation. The histone acetyltransferases CBP and P/CAF were recruited subsequently, but local histone hyperacetylation was delayed. After transient recruitment of the human Brahma homolog hBrm, remodeling of the neighboring nucleosome coincided with transcription initiation. The results suggest that, at this promoter, chromatin reconfiguration is a defining step of the initiation process, acting after the assembly of the Pol II machinery.

Because packaging of DNA into chromatin causes a general repression of gene activity, reconfiguration of chromatin has been postulated to be mandatory for preinitiation complex (PIC) formation and transcriptional initiation (*1*–*3*). This is achieved by the adenosine triphosphate (ATP)–driven remodeling complexes, which act by altering nucleosome conformation, and by histone acetyltransferases (HATs), which covalently modify nucleosomal core histones (*4*–*6*). The current view suggests that these two types of proteins are first recruited by sequence-specific transcription factors to establish a local chromatin structure that is permissive for the subsequent assembly of an active PIC, including RNA pol II at the promoters (*1*, *2*). The in vivo sequence of nucleosome acetylation and remodeling events relative to transcriptional activation has been studied in two types of promoters. In the cell cycle–regulated yeast HO promoter, the Swi5p activator recruits the SWI/SNF remodeling complex, which then recruits the SAGA HAT complex. These two factors then facilitate the binding of a second activator (SBF) and the recruitment of the SRB/mediator complex, and these are followed by the Cdk1 activation-dependent association of RNA pol II with the promoter and transcription initiation (*7*–*9*). In the case of the virus-induced nucleosome-free interferon-β promoter, transient H4 hyperacetylation is required for remodeling of the neighboring nucleosome, which is essential for the general transcription factor TFIID recruitment and transcription initiation (*10*).

Because most active eukaryotic promoters are not nucleosome-free but are organized in precisely positioned nucleosomal arrays, we decided to study the relation between chromatin reconfiguration and PIC formation on the differentiation-induced α_1-antitrypsin ($\alpha_1$1-AT) gene. Our studies were conducted on CaCo-2 cells, which, upon reaching confluence, spontaneously differentiate from cryptlike to villuslike enterocytes and express several marker genes, including α_1-AT (*11*, *12*). Transcriptional activation of the α_1-AT gene requires the synergistic action of hepatocyte nuclear factors HNF-1α and HNF-4α (*13*, *14*).

The earliest time point when α_1-AT mRNA can be detected by reverse transcription–polymerase chain reaction (RT-PCR), nuclear run-on, or S-1 nuclease protection assays is at day 5.5 of the CaCo-2 cell-differentiation program (Fig. 1A). The ab-

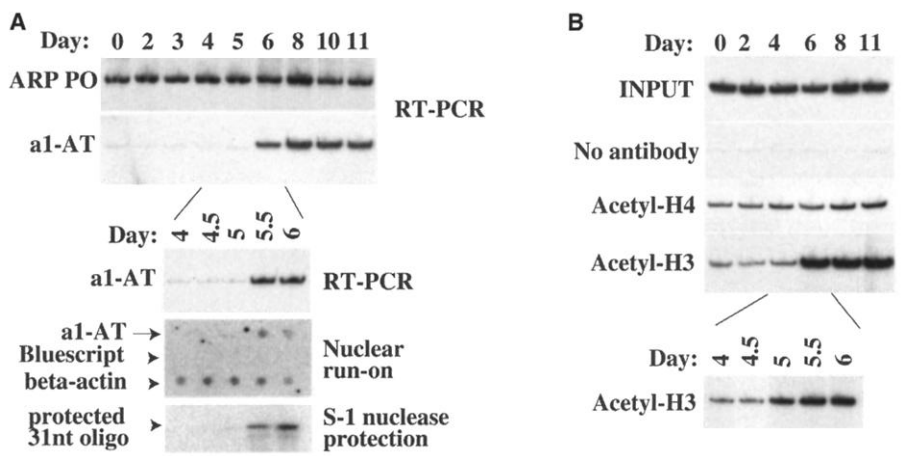

Fig. 1. Activation of the α_1-AT gene during enterocyte differentiation. (**A**) CaCo-2 cells were grown as described (*16*), and total RNA was prepared at the indicated days after the cells reached confluence (at day 0). Semiquantitative RT-PCR was performed with primers (*16*) spanning the α_1-AT or the acidic ribosomal phosphoprotein (ARP-PO) coding region. S-1 nuclease protection analysis with an antisense oligonucleotide corresponding to the +4 to +35 nt region of the α_1-AT gene and nuclear run-on assays with α_1-AT cDNA, bluescript vector, and beta-actin controls were performed essentially as in (*26*). (**B**) At the indicated time points, soluble chromatin from formaldehyde–cross linked CaCo-2 cells was prepared and immunoprecipitated with antibodies to acetyl-H3 or acetyl-H4 (Upstate Biotechnology), and the amounts of α_1-AT promoter–containing DNA in the immunoprecipitates were estimated by radioactive PCR (*16*). Aliquots (1/200) of total chromatin DNA before immunoprecipitation were processed similarly (INPUT).

Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, Post Office Box 1527, 711 10 Herakleion, Crete, Greece.

*To whom correspondence should be addressed. E-mail: talianid@imbb.forth.gr