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Scavenger receptor A gene regulatory elements target gene expression to macrophages and to foam cells of atherosclerotic lesions

ANDREW HORVAI*, WULF PALINSKI†, HONG WU*, KAREN S. MOULTON*‡, KRISTIN KALLA*, AND CHRISTOPHER K. GLASS*†§

*Division of Cellular and Molecular Medicine and †Division of Endocrinology and Metabolism, Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0651

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ABSTRACT **Transcription of the macrophage scavenger receptor A gene is markedly upregulated during monocyte to macrophage differentiation. In these studies, we demonstrate that 291 bp of the proximal scavenger receptor promoter, in concert with a 400-bp upstream enhancer element, is sufficient to direct macrophage-specific expression of a human growth hormone reporter in transgenic mice. These regulatory elements, which contain binding sites for PU.1, AP-1, and cooperating ets-domain transcription factors, are also sufficient to mediate regulation of transgene expression during the *in vitro* differentiation of bone marrow progenitor cells in response to macrophage colony-stimulating factor. Mutation of the PU.1 binding site within the scavenger receptor promoter severely impairs transgene expression, consistent with a crucial role of PU.1 in regulating the expression of the scavenger receptor gene. The ability of the scavenger receptor promoter and enhancer to target gene expression to macrophages *in vivo*, including foam cells of atherosclerotic lesions, suggests that these regulatory elements will be of general utility in the study of macrophage differentiation and function by permitting specific modifications of macrophage gene expression.**

Among their repertoire of cell surface receptors, macrophages express a set of scavenger receptors (SRs) that are capable of binding and internalizing a relatively broad spectrum of polyanionic macromolecules. Several structurally unrelated proteins have been identified that possess SR activity, including the class A SRs (1–3), CD36 (4), and a 94- to 95-kDa protein that binds oxidized low density lipoprotein (ox-LDL) (5, 6). SRs have generally been considered to play physiological roles in the clearance of damaged proteins, cells, and other debris at sites of inflammation and tissue damage (3). Because of the abilities of the class A SRs to bind and internalize ox-LDL (7), they have been proposed to provide an important pathway for the progressive accumulation of cholesterol by arterial wall macrophages and the formation of macrophage foam cells that characterize early atherosclerotic lesions.

Expression of class A SRs is generally restricted to macrophage-like cells (8) and becomes markedly upregulated during the process of monocyte to macrophage differentiation (9). SR expression is positively regulated by macrophage colony-stimulating factor (M-CSF) and granulocyte–macrophage-CSF (9–11), factors that promote the proliferation and differentiation of macrophage progenitor cells in bone marrow (12). To identify transcription factors that regulate SR expression, genomic sequences containing the SR promoter and upstream regulatory regions have been isolated and functionally characterized in the THP-1 human monocytic leukemia cell line (13–15). Treatment of THP-1 cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) induces a program of macrophage differentiation and a marked upregulation of the SR gene (13). Based on transient transfection assays, three functionally distinct cis-acting regulatory regions were defined (14, 15). A proximal promoter, which extends from −245 to +46 bp from the major transcriptional start site, was found to be sufficient to mediate cell-type-specific expression. Second, an enhancer region, located from −4.1 to −4.5 kb, was required for maximal induction by PMA. Third, a distal silencer region (−4.5 to −6.5 kb) was found to suppress promoter activity in undifferentiated THP-1 cells.

Cell-specific expression of the −245-bp promoter was, in part, dependent on PU.1/Spi-1 (14), an ets-domain transcription factor that has been implicated to regulate the expression of a number of macrophage and B-cell-specific genes (ref. 16 and references therein). Transcriptional activation of the SR gene in response to PMA was found to be mediated by AP-1 and cooperating ets-domain transcription factors that bound to regulatory elements in both the promoter and distal enhancer (15). Because these factors are downstream targets of the Ras signaling pathway that is activated in response to M-CSF (17–19), we speculated that macrophage-specific activation of the SR^- gene may depend on the combinatorial interactions of PU.1, AP-1, and ets-2 (14). To test this model *in vivo*, and to evaluate the utility of the SR promoter to direct macrophage-specific expression of transgenes in experimental animals, we have investigated the function of the human SR gene promoter and upstream regulatory elements in transgenic mice.

MATERIALS AND METHODS

Construction of SR–Human Growth Hormone (hGH) Transgenes and Generation of Transgenic Mice. The suppressor (S), enhancer (E), and promoter (P) regions of the human SR used in these studies are illustrated in Fig. 1*A* and were detailed by Wu *et al.* (15). The hGH gene from pØGH (Nichols Institute, San Juan Capistrano, CA) was used as a reporter gene by insertion downstream of the SR regulatory sequences to generate the SEP-H, EP-H, and EPmPU.1-H transgenes. Microinjection of DNA into 0.5-day-old (B6 × D2)F_1/J (The Jackson Laboratory) mouse embryos was performed by standard techniques (20). Founder animals were screened by dot blot of tail DNA as described (21) and copy number was determined by Southern blotting. Transgenic lines were es-

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Abbreviations: LDL, low density lipoprotein; AcLDL, acetyl LDL; SR, scavenger receptor; M-CSF, macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; diI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; hGH, human growth hormone; apoE, apolipoprotein E.

‡Present address: Division of Cardiology, Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.
§To whom reprint requests should be addressed.

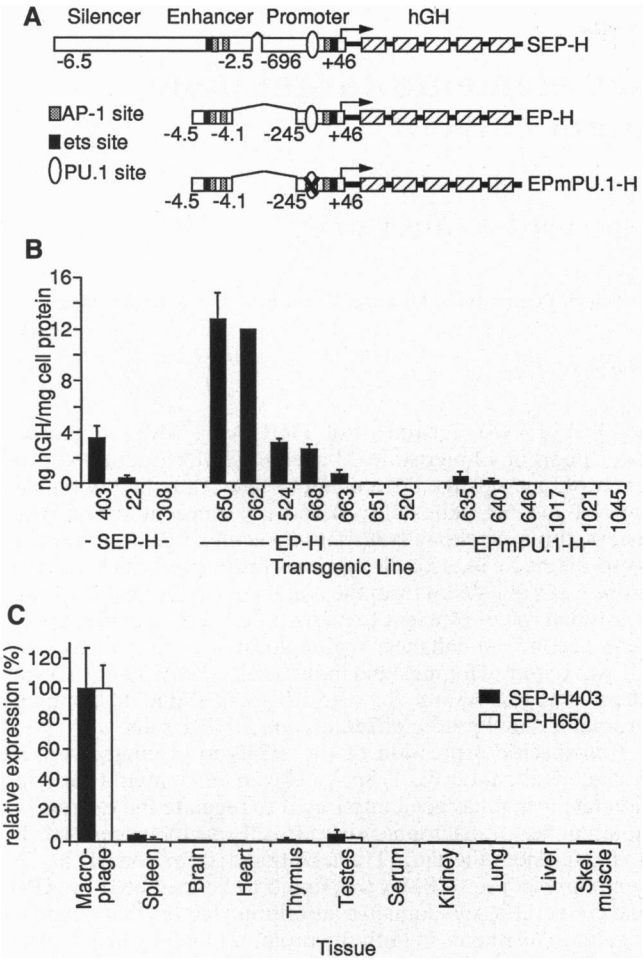

FIG. 1. SR regulatory elements direct transgene expression in peritoneal macrophages. (*A*) SR genomic sequences used to direct expression of the hGH gene in transgenic mice. Locations of the silencer, enhancer, and promoter are given relative to the major transcriptional start site of the human SR. The EPmPU.1-H construct is identical to EP-H except for an 8-bp mutation in the PU.1 binding site at −194 bp. (*B*) hGH content of mouse peritoneal macrophages of transgenic mice. Numbers associated with each construct correspond to mouse lines derived from independent integration events. Values are averages of three to eight individuals in all cases, except EP-H662 where only the founder was analyzed, and are standardized to total macrophage lysate protein. Bars are ± SEM. (*C*) Tissue specificity of SR-hGH transgene expression. The hGH content in tissue lysates of SEP-H403 and EP-H650 mice was corrected for total tissue protein and is represented as a fraction of the hGH content in the peritoneal macrophages of each line. The value for each tissue represents the average of tissues from three to seven animals ± SEM.

tablished from all founder animals except ESH662, which did not transmit the transgene. Levels of hGH expression were determined by RIA (Nichols Institute) as described (22).

Cell Culture. Resident or thioglycolate-induced mouse peritoneal macrophages and splenic lymphocytes were obtained by established methods (23). Bone marrow cells were isolated and purified as described in detail elsewhere (24). After initial purification, adherent cells were removed by culture for 1–2 h on tissue culture dishes. The nonadherent fraction (15–20 × 10^6 cells per mouse) was assayed or cultured in bone marrow medium (25) with or without added recombinant human M-CSF (20 ng/ml) or granulocyte CSF (20 ng/ml) (R & D Systems).

Determination of SR Activity. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diI) acetyl (Ac)LDL was prepared as described (26). Cell uptake of diI AcLDL was measured as outlined by Dejager *et al.* (27). HeLa and CV-1 cells were used as negative controls. Degradation of ^{125}I-AcLDL (5 μg/ml) was measured according to established methods (28). Native LDL and fucoidan (50 μg/ml each) were used as competitors.

RNA Analysis. Total RNA was isolated by the guanidium thiocyanate method (29). RNase protection assays were performed as described (13). The antisense RNA probe for the murine SR corresponded to codons 84–158 that are common to both types I and II SRs. The antisense probe for hGH corresponded to the *Bgl* II/*Sma* I fragment of exon 5 of the hGH gene.

Generation of Animals for Analysis of hGH Expression in Apolipoprotein E (apoE)-Deficient Mice. Mice of the SEP-H403 line were crossed with mice homozygous for a disrupted apoE allele (from a colony established from breeders generously provided by Jan Breslow, Rockefeller University, New York). The SEP-H403 F_1 animals were then backcrossed to produce apoE$^{-/-}$; SEP-H403$^+$ F_2 progeny. F_2 animals were fed a Western-type diet containing 0.15% cholesterol (30) for 3 months prior to analysis of hGH expression in peritoneal macrophages and atherosclerotic lesions.

Immunohistochemistry. Tissues and peritoneal macrophages were fixed with Omnifix (Con-Sciences, New York), paraffin embedded, and sectioned as described (31). Immunostaining for the endogenous SR gene was performed by using a guinea pig antiserum raised against a purified recombinant protein corresponding to the α-helical and coiled-coil domains of the mouse scavenger receptor at a dilution of 1:200. Immunostaining for hGH was performed with guinea pig antiserum raised against hGH at a dilution of 1:200 (Arnell Products, New York). Antibodies bound to the tissue were detected with an avidin-biotin alkaline phosphatase system as described (31). Competitive immunostaining was performed by incubating the antiserum to hGH with an excess of hGH (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda) for 1 h at 25°C.

Statistical Analysis. Because the underlying distribution of expression was not known, the Mann–Whitney test was used to test the hypothesis that hGH expression did not differ between the group of EP-H and the group of EPmPU.1-H transgenic lines.

RESULTS

Elements of the SR Promoter Direct Macrophage-Specific Expression of hGH Transgenes. Based on our definition of functional genomic regulatory elements in THP-1 monocytic leukemia cells, we prepared the three SR-hGH constructs illustrated in Fig. 1*A* for analysis in transgenic mice. The SEP-H construct contained the SR silencer and enhancer sequences within a 4-kb fragment (extending from −6.5 to −2.5 kb upstream of the major transcriptional start site) linked to SR promoter sequences from −696 to +46 bp. EP-H consisted of the enhancer (−4.5 to −4.1 kb) and core promoter (−245 to +46 bp) and corresponds to the minimal information required for cell-specific expression and PMA responsiveness in THP-1 cells. Finally, EPmPU.1-H consisted of the EP-H construct with a mutated PU.1 binding site at −194 bp.

As measured by intracellular hGH content, five of the seven lines of mice generated with the EP-H construct and two of the three lines generated with SEP-H expressed the transgene in peritoneal macrophages (Fig. 1*B*). Mutation of the PU.1 site in the SR promoter (EPmPU.1-H) nearly abolished transgene expression in peritoneal macrophages as compared to the otherwise identical EP-H construct (P = 0.02 for the two-tailed Mann–Whitney test; Fig. 1*B*). The majority of mice examined for each independent line of EPmPU.1-H exhibited no detectable hGH expression, consistent with a crucial role of PU.1 in establishing SR expression. The levels of hGH expres-

sion in various tissues of SEP-H403 and EP-H650 mice relative to the expression of hGH in thioglycolate-elicited peritoneal macrophages are illustrated in Fig. 1*C*. SEP-H22 and EP-H524 mice demonstrated a similar pattern of expression, although at lower absolute levels (data not shown). Among these tissues, the highest levels of hGH expression were observed in spleen and testes. In each case, the levels of hGH were <5% of the levels present in peritoneal macrophages. No hGH expression was observed in purified splenic lymphocytes (data not shown), indicating appropriate restriction of transgene expression from this population of cells. Very little hGH expression was observed in the liver for either the SEP or EP transgenic animals, in spite of previous observations that liver Kupffer cells exhibit SR activity (8). Whether this observation reflects a lack of sensitivity of the radiometric assay at the whole organ level or indicates that additional regulatory sequences are required for Kupffer cell expression was not investigated.

To determine whether SR regulatory elements could direct the expression of transgenes to foam cells of atherosclerotic lesions, SEP-H403 mice were bred to apoE-deficient mice (30). Transgenic mice homozygous for the disrupted apoE allele were fed a Western-type diet to enhance the development of atherosclerosis. The specificity of immunostaining for hGH in these animals was first examined in resident peritoneal macrophages. Strong immunostaining for hGH was observed in most, but not all, macrophages (Fig. 2*C*). Specificity of staining was confirmed by preabsorbing with excess purified hGH (Fig. 2*D*). The percentage of cells staining for hGH was roughly similar to the percentage of cells that were stained with an antiserum directed against the mouse scavenger receptor (Fig. 2*B*). Macrophages for nontransgenic animals exhibited no staining for hGH (Fig. 2*A*). We next examined the pattern of hGH expression in atherosclerotic lesions. Specific immunostaining for hGH was observed in macrophage-rich areas of lesions (Fig. 2*E*) in a distribution similar to immunostaining for the endogenous SR (Fig. 2*F*). The more diffuse pattern of staining for hGH in lesions may reflect the fact that GH is secreted, in contrast to the SR. In addition to foam cells, some staining for hGH was observed in adjacent smooth muscle of the heart (Fig. 2*E*) but not in the media of the aorta. No staining for hGH was observed in macrophage-rich lesion areas of nontransgenic animals (data not shown). Staining for hGH varied among different lesions, with some lesions showing little or no staining (data not shown).

Regulated SR and Transgene Expression During Macrophage Differentiation. To examine SR expression during the process of macrophage differentiation, bone marrow progenitor cells were isolated from nontransgenic mice and cultured in the presence of M-CSF. Upon initial isolation, purified bone marrow cells represented a very heterogeneous population of hematopoietic progenitors (Fig. 3*A*). These cells were found to possess little or no SR activity as detected by degradation of ^{125}I-AcLDL (Fig. 4*A*). Furthermore, none of the progenitor cells were found to be capable of taking up dil-conjugated AcLDL, including cells exhibiting morphologic characteristics of monocytic precursors (Fig. 3*B*). However, treatment of bone marrow progenitor cells with M-CSF for 5 days resulted in the proliferation and differentiation of a population of adherent macrophages (Fig. 3*C*) with marked SR activity as measured by both dil AcLDL uptake and ^{125}I-AcLDL degradation (Figs. 3*D* and 4*A*, respectively). In contrast, no increase in SR activity was observed in the resultant granulocyte population after treatment of progenitor cells with granulocyte CSF. Untreated bone marrow cells did not acquire SR activity in culture (Fig. 4*A*) and the majority of progenitor cells did not survive beyond ≈5 days in the absence of CSFs. We next examined the expression of the hGH transgene in purified bone marrow progenitor cells. Progenitors from either SEP-H403 or EP-

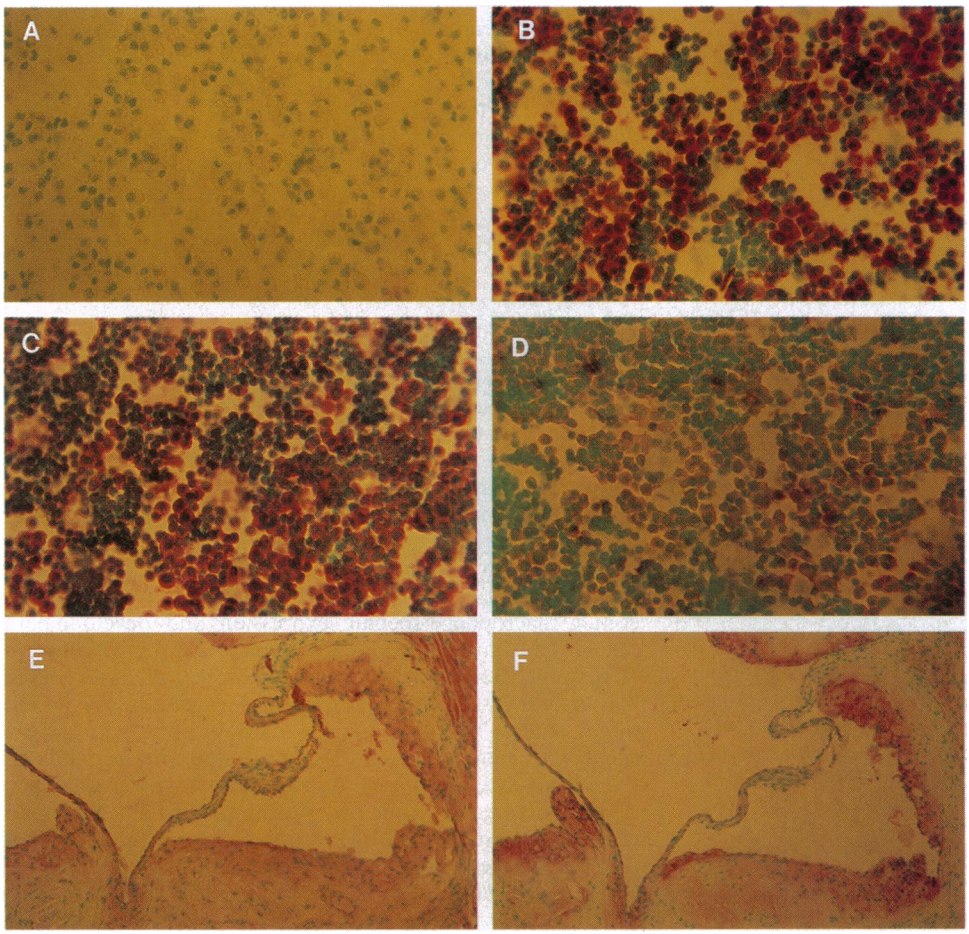

FIG. 2. Expression of the SR and hGH in peritoneal macrophages and atherosclerotic lesions in SEP-H403 apoE$^{-/-}$ mice. (*A*) Macrophages from a nontransgenic mouse stained for hGH and counterstained with methyl green. (*B*) Immunostaining of resident peritoneal macrophages obtained from a SEP-H403 apoE$^{-/-}$ mouse using a guinea pig anti-mouse SR antiserum. (*C*) Immunostaining of SEP-H403 apoE$^{-/-}$ macrophages with anti-hGH antiserum. (*D*) Immunostaining of SEP-H403 apoE$^{-/-}$ macrophages with anti-hGH antiserum preabsorbed with excess hGH. (*E*) Cross-section of the aortic root at the level of the aortic valve of a SEP-H403 apoE$^{-/-}$ mouse fed a Western style diet for 3 months. The section is immunostained for hGH and illustrates macrophage-rich lesions in the vicinity of one of the aortic valve leaflets. (*F*) Immunostaining of an adjacent serial section for the endogenous SR. (*A–D*, ×279; *E* and *F*, ×111.)

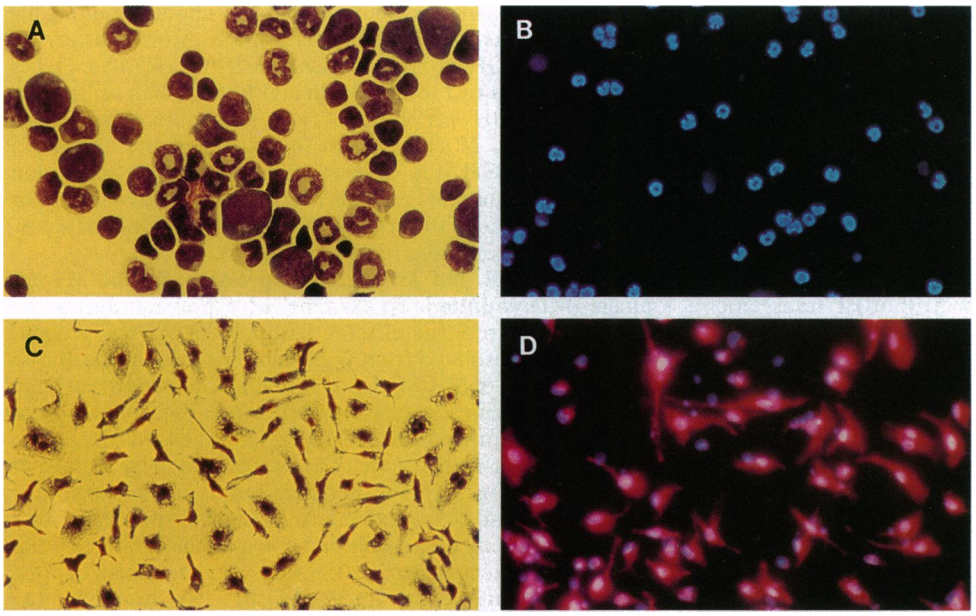

FIG. 3. Induction of SR activity during macrophage differentiation of bone marrow progenitor cells. (*A*) Purified bone marrow progenitor cells treated with Wright–Giemsa stain. Field illustrates a heterogeneous population of cells that includes lymphoid, erythroid, and myelomonocytic precursor cells. (×837.) (*B*) Bone marrow progenitor cells lack SR activity. Progenitor cells incubated for 5 h with dil AcLDL show no rhodamine fluorescence. Nuclei are marked by 4′,6-diamidino-2-phenylindole (DAPI) counterstain. (×334.) (*C*) Wright–Giemsa stain of bone marrow progenitor cells after 5 days of M-CSF illustrates the characteristic macrophage morphology of the resulting cell population. (×334.) (*D*) Rhodamine fluorescence represents specific uptake of dil AcLDL by the SR in nearly every cell. Nuclei were counterstained with DAPI. (×502.)

H650 transgenic mice exhibited near background levels of hGH (Fig. 4*B*). Treatment with M-CSF resulted in a >20-fold induction of the transgene, similar to the induction observed for the endogenous SR activity. Both SEP-H22 and EP-H524 lines also demonstrated similar induction of transgene expression with M-CSF (data not shown), indicating that the patterns of expression observed for SEP-H403 and EP-H650 were not artifacts of the integration site.

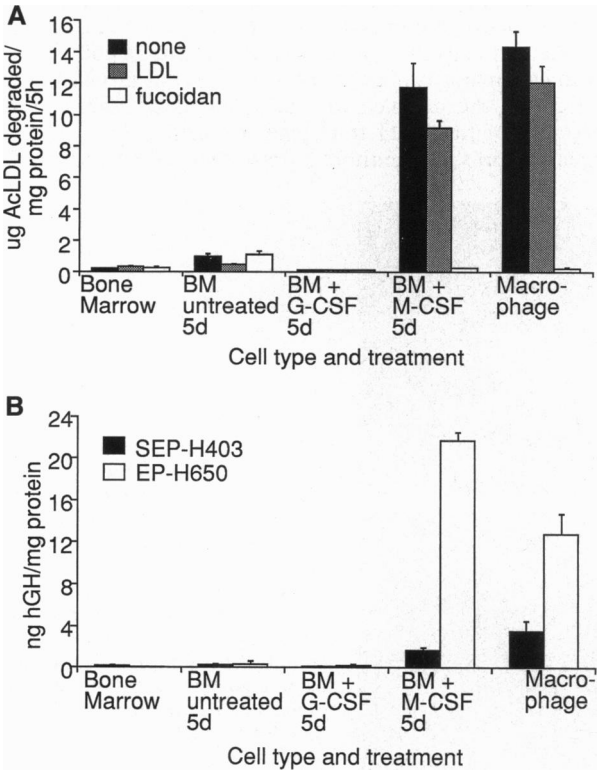

FIG. 4. Regulation of SR and transgene expression by M-CSF. (*A*) SR activity as measured by ^{125}I-AcLDL degradation in peritoneal macrophages, bone marrow (BM) progenitor cells, and progenitor cells cultured with no added factors, with granulocyte CSF (G-CSF) (20 ng/ml) for 5 days, or with M-CSF (20 ng/ml) for 5 days. Each point represents the average of three to six assays ± SEM. Degradation was standardized to total cell protein for each assay condition: none, no competitor; LDL, 50 μg of native LDL per ml; fucoidan, 50 μg of fucoidan per ml. (*B*) SR regulatory elements confer M-CSF responsiveness to the hGH transgene in bone marrow progenitor cells. hGH content of bone marrow progenitor cells derived from SEP-H403 or EP-H650 mice and of progenitor cells cultured with no added factors, with G-CSF (20 ng/ml) for 5 days, or with M-CSF (20 ng/ml) for 5 days is shown. Values represent average of three to six animals ± SEM.

To assess the relative levels of expression of hGH and the native SR in peritoneal macrophages and in bone marrow progenitor cells subjected to various differentiation conditions, RNase protection assays were performed. When corrected for probe specific activity, hGH mRNA levels were 7- and 25-fold greater than the mRNA levels for the endogenous SR in macrophages derived from the SEP-H403 and EP-H650 animals, respectively (Fig. 5*A*). Because the EP-H construct was sufficient to confer M-CSF responsiveness, we analyzed the EPH-650 line in greater detail. A comparison of the mRNA levels corresponding to the endogenous SR and the EPH-650 transgene demonstrated similar, although not identical, temporal responses to M-CSF, with the endogenous SR mRNA appearing somewhat sooner than hGH mRNA (Fig. 5*B*).

DISCUSSION

The macrophage SR gene is representative of a class of genes that are upregulated during monocyte to macrophage differentiation and, in part, specify the macrophage phenotype. The SR gene therefore serves as a useful model system for the study of transcription factors and signal transduction mechanisms that mediate macrophage differentiation. The present studies have demonstrated that the combination of the SR promoter, extending from −245 to +46 bp from the major transcriptional start site, and a 400-bp upstream enhancer is sufficient to confer macrophage-specific expression and developmental regulation of a hGH transgene in mice. The EP-H construct lacking the silencer element did not express in either nonhematopoietic tissues or bone marrow progenitor cells. Additional studies will be required to determine whether the silencer element influences SR expression *in vivo*. Although the SR has been localized to macrophages of virtually every tissue by immunohistochemistry using specific anti-SR monoclonal antibodies (8, 32), only spleen and testes, in addition to peritoneal macrophages, were found to consistently express detectable levels of hGH. These results most likely reflect the fact that macrophages did not constitute a sufficiently large

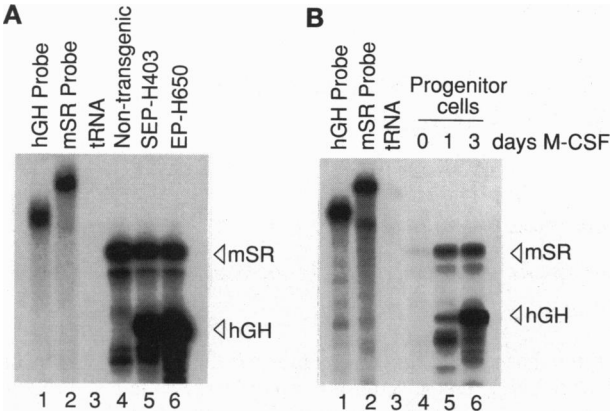

FIG. 5. Relative levels of murine (m) SR and hGH mRNA as determined by RNase protection assay. (*A*) Analysis of mSR and hGH mRNA in peritoneal macrophages. Intact antisense hGH and mSR RNA probes are illustrated in lanes 1 and 2, respectively. To simultaneously measure mSR and hGH mRNA levels, the probes were combined, hybridized with 20 μg of total RNA, and digested with RNase A. The predicted 220-nt fragment corresponding to the protected SR probe was obtained with total RNA obtained from peritoneal macrophages from nontransgenic (lane 4), SEP-H403 (lane 5), and EP-H650 (lane 6) mice. The predicted 164-nt fragment corresponding to the protected hGH probe was present only in SEP-H403 and EP-H650 mice. (*B*) Expression of the endogenous SR gene and the EP-H transgene in bone marrow progenitor cells after treatment with M-CSF. RNase protection assay showing undigested hGH probe (lane 1), mSR probe (lane 2), tRNA control (lane 3), bone marrow progenitor cell RNA (lane 4), and progenitor cells with M-CSF (20 ng/ml) for 1 and 3 days (lanes 5 and 6, respectively). β-Actin mRNA levels were approximately equal in each total RNA preparation as determined by parallel RNase protection experiments.

percentage of these tissues to permit detection of the transgene when analyzed at the whole organ level.

The observation that the minimal SR promoter and enhancer were sufficient to mediate an appropriate developmental response to M-CSF *in vivo* is consistent with the hypothesis that the SR gene is a direct target of Ras-dependent signal transduction pathways activated by the M-CSF receptor (15). Activation of Ras in response to M-CSF is likely to result in upregulation of SR transcription through the composite AP-1/ets factor binding sites present in the promoter and enhancer. The AP-1 and ets factors that bind to these elements are apparently insufficient to activate the gene in the context of a mutation in the PU.1 binding site present in the proximal promoter, however. These observations support the idea that PU.1 plays a crucial role in the initial activation of the SR gene and suggest that PU.1 acts synergistically with AP-1 and cooperating ets-domain factors.

The ability of SR regulatory elements to target transgene expression to macrophages *in vivo*, including macrophage foam cells within atherosclerotic lesions, raises the possibility of using these sequences to specifically alter macrophage gene expression in physiological or pathological settings. For example, enzymes, growth factors, or other regulatory molecules could be overexpressed in the macrophage to determine their influence on the development of atherosclerosis in susceptible strains of mice. Alternatively, SR regulatory elements might be used to direct the expression of a sequence-specific recombinase, such as the cre recombinase (33, 34), to mediate macrophage-specific disruption of appropriately targeted genes. Such approaches are likely to lead to insights into the molecular mechanisms that control macrophage differentiation and function.

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