The basic helix-loop-helix (bHLH)\textsuperscript{1} transcription factors E12 and E47 regulate cellular differentiation and proliferation in diverse cell types. While looking for proteins that bind to E12 and E47 by the yeast interaction trap, we isolated the rat (r) homologue of the human (h) polymyositis-scleroderma autoantigen (PM-Scl), which has been localized to the granular layer of the nucleolus and to distinct nucleocytoplasmic foci. The rPM-Scl and hPM-Scl homologues are 96% similar and 91% identical. We found that rPM-Scl mRNA expression was regulated by growth factor stimulation in cultured rat aortic smooth muscle cells. rPM-Scl bound to E12 and E47 but not to Id3, Gax, Myb, OCT-1, or Max. The C terminus of rPM-Scl (amino acids 283–353) interacted specifically with a 54-amino acid domain in E12 that is distinct from the bHLH domain. Finally, cotransfection of rPM-Scl and E47 specifically increased the promoter activity of a luciferase reporter construct containing an E box and did not affect the basal activity of the reporter construct. rPM-Scl appears to be a novel non-HLH-interacting partner of E12/E47 that regulates E2A protein transcription.

\textsuperscript{1}This work was supported in part by a grant from Bristol-Myers Squibb. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{‡‡}To whom correspondence should be addressed: Cardiovascular Biology Laboratory, Harvard School of Public Health, Boston, Massachusetts 02115, the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the Cardiac Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Cardiovascular Division, Brigham and Women’s Hospital, Boston, Massachusetts 02115

** Plasmids and Strains—Plasmid DNAs and the yeast strain EGY48 (MATa trp1 ure3 his3 LEU2::LexAop6-LEU2) used in the interaction trap assays were provided by Dr. Roger Brent and colleagues (Massachusetts General Hospital) and used as described (22, 23). The Eco\textsuperscript{R}ichia coli K-12 strain KC8 (pyrF-Tn5, hsdR, leuB60, trpC8830, lacD74, strA, galK, hisB436), the gift of Dr. Kevin Struhl (Harvard Medical School, Boston, MA), was used to rescue cDNA plasmids from yeast. The E12 bait plasmid used in the interaction trap was constructed by cloning a 0.53-kilobase pair cDNA fragment encoding amino acids 477–654 of human E12 (E12-(477–654)) (24), which had been obtained by polymerase chain reaction amplification of pGEM3E12 (kindly provided by Dr. David Baltimore, Massachusetts Institute of Technology, Cambridge) into the Eco\textsuperscript{RI} and XhoI-digested yeast expression vector pEG202 (23). The pEG202 plasmid is used to constitutively express a LexA fusion or bait protein.

** This work was supported in part by a grant from Bristol-Myers Squibb. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Cardiovascular Biology Laboratory, Harvard School of Public Health, Boston, Massachusetts 02115, the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the Cardiac Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Cardiovascular Division, Brigham and Women’s Hospital, Boston, Massachusetts 02115

** Plasmids and Strains—Plasmid DNAs and the yeast strain EGY48 (MATa trp1 ure3 his3 LEU2::LexAop6-LEU2) used in the interaction trap assays were provided by Dr. Roger Brent and colleagues (Massachusetts General Hospital) and used as described (22, 23). The *Eco*\textsuperscript{R}ichia coli K-12 strain KC8 (pyrF-Tn5, hsdR, leuB60, trpC8830, lacD74, strA, galK, hisB436), the gift of Dr. Kevin Struhl (Harvard Medical School, Boston, MA), was used to rescue cDNA plasmids from yeast. The E12 bait plasmid used in the interaction trap was constructed by cloning a 0.53-kilobase pair cDNA fragment encoding amino acids 477–654 of human E12 (E12-(477–654)) (24), which had been obtained by polymerase chain reaction amplification of pGEM3E12 (kindly provided by Dr. David Baltimore, Massachusetts Institute of Technology, Cambridge) into the *Eco*\textsuperscript{RI} and XhoI-digested yeast expression vector pEG202 (23). The pEG202 plasmid is used to constitutively express a LexA fusion or bait protein.

** Plasmids and Strains—Plasmid DNAs and the yeast strain EGY48 (MATa trp1 ure3 his3 LEU2::LexAop6-LEU2) used in the interaction trap assays were provided by Dr. Roger Brent and colleagues (Massachusetts General Hospital) and used as described (22, 23). The *Eco*\textsuperscript{R}ichia coli K-12 strain KC8 (pyrF-Tn5, hsdR, leuB60, trpC8830, lacD74, strA, galK, hisB436), the gift of Dr. Kevin Struhl (Harvard Medical School, Boston, MA), was used to rescue cDNA plasmids from yeast. The E12 bait plasmid used in the interaction trap was constructed by cloning a 0.53-kilobase pair cDNA fragment encoding amino acids 477–654 of human E12 (E12-(477–654)) (24), which had been obtained by polymerase chain reaction amplification of pGEM3E12 (kindly provided by Dr. David Baltimore, Massachusetts Institute of Technology, Cambridge) into the *Eco*\textsuperscript{RI} and XhoI-digested yeast expression vector pEG202 (23). The pEG202 plasmid is used to constitutively express a LexA fusion or bait protein.
The DNA sequences coding for the bait and interactant proteins were generated by polymerase chain reaction and reverse transcription polymerase chain reaction and cloned between the unique EcoRI and XhoI sites of pEG202 and pJG4-5 (23), respectively. The various deletion mutants of E47 were the gifts of Dr. Lennart Philipson (New York University Medical Center, New York, NY). These colonies (33) and introduced them into KC8 cells by electroporation with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 25 mM Tris-HCl, pH 7.4, 1% glucose, and grown to saturation overnight at 30 °C. The next day, cells were diluted 1:50 into medium containing 2% galactose and 1% raffinose and allowed to grow to an A_600 of 1.0–2.0. The A_600 of the plates containing 2% galactose was measured, and the cells were harvested and permeabilized by boiling in SDS-containing sample buffer, fractionated by 12% SDS-PAGE, stained with Coomassie Blue, and exposed to Kodak XAR film.

**RESULTS**

**Isolation of cDNA Clones Encoding E12-interacting Proteins**—A human E12 cDNA spanning amino acids 477–654, containing the basic and helix-loop-helix domains, was fused in-frame with the LexA DNA-binding domain and used in a yeast interaction trap screen (23). This region is devoid of transcriptional activity but retains the ability to interact with MyD and the Id proteins (11). Forty-two cDNAs were isolated and partially sequenced from an adventitia-stripped rat aorta cDNA library. Twenty-nine cDNAs encoded Id3 (12), and five cDNAs were full-length rat Id3 (9), mouse Gax (amino acids 121–303) (25), mouse Myb (amino acids 1–185) (26), human OCT-1 (amino acids 294–429) (27), and full-length rat Max (28). All constructs were sequenced to confirm in-frame expression of fusion proteins. For expression in eukaryotic cells, RPM-ScI and Id1 were cloned into the pcRS vector (InVitrogen), whereas full-length E47 was expressed in the pMGSV plasmid (29). The pGEX4T-1 vector (Pharmacia Biotech Inc.) was used for the expression of glutathione S-transferase (GST) fusion proteins in E. coli host HB101. The E box reporter plasmid pGL2(E box) was generated by cloning an oligonucleotide (30) containing three E box sequences into the minimal SV40 promoter of the pGL2 promoter vector (Promega).

**Yeast Interaction Trap**—We screened a rat aorta cDNA library for E12 interactants by the yeast two-hybrid interaction trap as described (31). Briefly, EGY48 (MATa trpl ura3 his3 LEU2::pLexop6-LEU2) was used as host yeast strain. The bait plasmid was constructed by cloning E12-(477–654) in-frame of the LexA gene contained in the pEG202 plasmid. An oligo(dt) primed cDNA library from rat aorta (CD strain, Charles River Laboratories) was constructed with the yeast galactose-inducible expression plasmid pJG4-5.

The yeast were transformed by the method of Gietz et al. (32) with modification. A total of 4 × 10^6 primary yeast transformants were selected and plated onto Ura His Trp Leu plates containing 2% galactose. After 4 days at 30 °C, Ura Leu colonies appeared, of which 90 showed a galactose-dependent blue color in 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside medium. We prepared plasmid DNAs from 42 of these colonies (33) and introduced them into E. coli cells by electroporation. One of the clones encoded residues 283–353 of rPM-Scl. The pGEX4T-1 vector (Pharmacia Biotech Inc.) was used for the expression of glutathione S-transferase (GST) fusion proteins in E. coli host HB101. The E box reporter plasmid pGL2(E box) was generated by cloning an oligonucleotide (30) containing three E box sequences into the minimal SV40 promoter of the pGL2 promoter vector (Promega).
type in aortic tissue. We performed Northern analysis on total RNA isolated from cultured early passage RASMC to confirm expression of rPM-Scl in this cell type and determine the effect of growth factor stimulation on rPM-Scl expression. rPM-Scl mRNA expression was high in growing subconfluent RASMC and low in quiescent RASMC (Fig. 2, Growing versus 0 h). After the quiescent RASMC had been stimulated with serum, the rPM-Scl mRNA level increased within 6 h and reached a level comparable to that in growing nonconfluent RASMC within 18 h. rPM-Scl expression was also suppressed in quiescent as compared with growing skeletal myoblasts (C2C12), NIH 3T3 fibroblasts, and rat aortic endothelial cells (data not shown).

rPM-Scl Interacts Specifically with E12/E47—To assess the interaction between rPM-Scl and the E12 proteins, we performed in vitro binding assays with GST fusion proteins. The in vitro translation products of full-length E47 and rPM-Scl were retained selectively by GST-E12 in comparison with GST alone (Fig. 3). This result confirmed the binding interaction observed in the yeast two-hybrid interaction trap. The specificity of the E2A-rPM-Scl interaction was confirmed by the dark hatches in the Galactose panel (Fig. 4), which indicate activation of the lacZ reporter gene. Because expression of a C-terminal (283–353) rPM-Scl transcription activation domain (TAD) fusion protein is under the control of the galactose-inducible GAL1 promoter, β-galactosidase activity is observed only in the presence of galactose. The rPM-Scl (283–353) TAD bound both E12 and E47 (Fig. 4) but not five other transcriptional regulators (Id3, Gax, Myb, OCT-1, and Max).

Map of Regions That Interact with E12/E47 and rPM-Scl—Next we used the yeast interaction trap to map the respective binding domains of E12 and rPM-Scl. We generated three additional rPM-Scl TAD hybrids (Fig. 5, top) to map the region of rPM-Scl that interacts with E12. These constructs were transfected into yeast harboring a LexA-E12-(477–654) construct, which contains the basic and HLH domains, and the β-galactosidase reporter plasmid pSH18-34. For each construct, fusion protein biosynthesis was confirmed by Western blot analysis. β-Galactosidase production was directly proportional to the degree of hybrid binding (Fig. 5). The C-terminal region (283–353) of rPM-Scl was necessary and sufficient for interaction with E12. rPM-Scl binding to E12 was not altered by the addition of the rest of the molecule (rPM-Scl construct 1–353), whereas deletion of the C-terminal region abolished...
binding (rPM-Scl constructs 1–288, 1–192, and 1–112). These observations show that the 70 C-terminal amino acids of rPM-Scl are responsible for its interaction with E12.

We similarly mapped the region of E47 that bound rPM-Scl by the yeast interaction trap. Deletion of the E47 basic or HLH domain did not affect rPM-Scl binding (Fig. 6, E47ΔHLH and E47ΔBasic, respectively). However, deletion of amino acids 477–538 (Fig. 6, E47(539–651) construct) reduced the binding interaction to one-fourteenth the value obtained with the E47(477–651) construct. E47(477–530) and E47(477–651) bound rPM-Scl equally well.

rPM-Scl Enhances Transcriptional Activation of E47—To determine whether the binding of rPM-Scl to an E2A protein would affect its function, we studied the effect of rPM-Scl on E47-mediated enhancement of transcription of an E box luciferase reporter plasmid (Fig. 7). Cotransfection of full-length rPM-Scl with the E box reporter plasmid did not alter its basal activity. Cotransfection with full-length E47 did increase the activity of the E box reporter plasmid; however, this increase in activity was prevented when E47 was cotransfected with Id1, in keeping with the antagonistic effect of the Id proteins on E2A protein transcription (11). Although rPM-Scl alone had no effect on the activity of the E box reporter plasmid, rPM-Scl increased activation of the reporter plasmid by E47 by 2.4-fold (Fig. 7). To confirm the specificity of the effect of rPM-Scl on the E box reporter plasmid, we cotransfected the rPM-Scl plasmid with a pGL2 control plasmid (SV40 promoter/enhancer luciferase). rPM-Scl did not enhance luciferase activity mediated by pGL2 (data not shown). The increase in E47-mediated E box activation after cotransfection with rPM-Scl (Fig. 7) substantiates a cellular interaction between the two proteins.

DISCUSSION

hPM-Scl was first identified with autoantibodies from patients with the syndrome for which it is named (20, 38). Immunoelectron microscopy identified prominent hPM-Scl staining in the granular layer of the nucleolus and in distinct nucleoplasmic clusters (21), and anti-hPM-Scl antibodies precipitated a multimeric complex of 11 or more peptides and phosphoproteins (39). This complex is distinct from the ribonucleoprotein, and indirect evidence suggests that it associates with DNA rather than rRNA (21, 39). The corresponding cDNA was cloned by expression screening with antibodies isolated from patients suffering from the polymyositis-scleroderma overlap syndrome (19). hPM-Scl encodes a 355-amino acid protein whose highly acidic C terminus is responsible for a characteristic pattern of aberrant migration at 75 kDa on SDS-PAGE, despite a predicted size of 39.5 kDa. We have found that rPM-Scl also has an aberrant migration pattern on SDS-PAGE owing to its C terminus (Fig. 5, bottom).

The localization of hPM-Scl to the granular layer of the nucleoli and to distinct nucleoplasmic clusters suggests that it is associated with the nuclear matrix. The nuclear matrix is a complex lattice of nonhistone proteins that is resistant to nuclease digestion and high salt extraction (40, 41). The matrix...
BankTM analysis of this 54-amino acid domain revealed con-
not affect the binding interaction of E47 with rPM-Scl. Gen-
epitope binds to a 54-amino acid domain upstream of the E12/
protein binding epitope is in the C terminus of rPM-Scl. This
ing domain.
E47 in binding affinity for rPM-Scl implies a non-bHLH bind-
similar domains that have clearly different affinities for other
structs. A representative of two experiments is shown.
affects transcription by binding to trans-acting factors that
include the steroid receptors (42), ATF (43), OCT-1 (43, 44), and
the retinoblastoma protein (45). Retention of trans-acting fac-
tors in the nuclear matrix can increase or decrease transcription-
al activity. In addition, the nuclear matrix orchestrates the
formation of distinct subnuclear compartments into specific foc-
of transcription (46–48).
To consider the possibility that PM-Scl interacted with dif-
ferent families of trans-acting factors, we tested the specificity
of the rPM-Scl-E12/E47 interaction by the yeast two-hybrid
assay. rPM-Scl did not interact with the HLH protein Id3 or
with the trans-acting factors Max, Gax, Myb, and OCT-1 (Fig.
4). By contrast, rPM-Scl interacted well with E12 and E47. E12
and E47 differ by their HLH domains, which result from dif-
ferential splicing of the E2A gene. The two bHLH exons encode
similar domains that have clearly different affinities for other
HLH domains (2). The absence of a difference between E12 and
E47 in binding affinity for rPM-Scl implies a non-bHLH bind-
ing domain.
We again used the yeast two-hybrid assay to identify the
domains through which rPM-Scl and E12/E47 bind. The E2A
protein binding epitope is in the C terminus of rPM-Scl. This
epitope binds to a 54-amino acid domain upstream of the E12/
E47 bHLH domain. Removal of the basic or HLH domains did
not affect the binding interaction of E47 with rPM-Scl. Gen-
BankTM analysis of this 54-amino acid domain revealed cons-
servation of amino acid sequence between the E2A proteins and
E2-5/ITF-2. This domain is also conserved among the human,
rat, mouse, and guinea pig E2A proteins.
To validate further the cellular interaction between E12/E47
and rPM-Scl, we performed transient transfection assays with
an E box-luciferase reporter plasmid. rPM-Scl increased E47-
dependent luciferase activity without increasing the basal ac-
tivity of an E box-luciferase or an SV40-luciferase (pGL2) re-
porter plasmid (Fig. 7). Although a nonspecific reduction in
reporter plasmid activity is common in cotransfection assays,
an increase in activity indicates a specific interaction. This
observation supports the conclusion that rPM-Scl and E12/E47
are interacting partners within mammalian cells.
The regulated expression of rPM-Scl may indicate that this
nuclear protein is a novel non-HLH regulator of the E2A
proteins. Like PM-Scl, B23 is a nuclear protein that asso-
ciates with a trans-acting factor, YY1. Both B23 and YY1 have
been colocalized to the nuclear matrix and the nuclear rem-
nant (49), and transfection of B23 with YY1 relieves YY1-
induced repression of transcription. In contrast, our cotrans-
fection experiments (Fig. 6) indicate that rPM-Scl enhances
activation of E2A protein transcription. Still, the net effect of
B23 on YY1 and rPM-Scl on E12/E47 is to increase transcription.
We have cloned and characterized UbcE2A (31), a ubiquitin-
conjugating enzyme that binds the same E2A protein domain
as rPM-Scl. UbcE2A antagonizes the functional activity of the
E2A proteins by promoting their degradation. Also, Sloan et al.
(50) have found that B cell differentiation may be regulated by
phosphorylation of E47 serine residues 514 and 529, which are
contained within the rPM-Scl binding epitope. The convergence
in one E2A protein region of these three regulatory mecha-
nisms (degradation, phosphorylation, and possibly nuclear ma-
trix attachment) may indicate an important non-HLH regula-
dory domain.

Acknowledgments—We are grateful to Roger Brent and Russell Fin-
ley (Massachusetts General Hospital) for generous contributions of
reagents and to those named in the text for gifts of plasmids. We thank
Zhengsheng Ye (Rockefeller University, New York) for help in setting
up the yeast interaction trap system, Thomas McVarish for expert
editorial assistance, Bonna Ith for tissue culture assistance, and mem-
bers of the Cardiovascular Biology Laboratory for discussions and
suggestions.

REFERENCES
1. Murre, C., McCaw, P. S., Vaessen, H., Caudy, M., Jan, L. Y., Jan, Y. N.,
Cabrer, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H.,
and Baltimore, D. (1989) Cell 58, 537–544.
2. Sun, X.-H., and Baltimore, D. (1991) Cell 64, 459–470.
3. Roberts, V. J., Steenbergen, R., and Murre, C. (1993) Proc. Natl. Acad.
Sci. U. S. A. 90, 7583–7587.
4. French, B. A., Chow, K.-L., Olsen, E. N., and Schwartz, R. J. (1991) Mol.
Cell. Biol. 11, 2439–2450.
5. Neuhold, L. A., and Wald, R. (1993) Cell 74, 1033–1042.
6. Weintraub, H. (1993) Cell 75, 1241–1244.
7. Bain, G., Robanus Maandag, E. C., Iton, D. J., Amsen, D., Kruisbeek, A. M.,
Weintraub, B. C., Kroop, I., Schlissel, M. S., Feeny, A. J., van Roon, M., van
der Valk, M., te Riele, H. P. J., Berns, A., and Murre, C. (1994) Cell 79,
