IRF-2 Is Involved in Up-regulation of Nonmuscle Myosin Heavy Chain II-A Gene Expression during Phorbol Ester-induced Promyelocytic HL-60 Differentiation*

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Transcription of the nonmuscle myosin heavy chain II-A (NMHC-A) gene is regulated by various factors, including cell type, proliferation and differentiation stage, and extracellular stimuli. We have identified an intrinsic region (designated 32kb-150), which is located 32 kb downstream of the transcription start sites in the human NMHC-A gene, as a transcriptional regulatory region. 32kb-150 contains an interferon-stimulated response element (ISRE). By using HeLa and NIH3T3 cells, in which NMHC-A is constitutively expressed, interferon regulatory factor (IRF)-2 was found to be the only major protein, among the IRF family proteins, that bound to the ISRE in 32kb-150 both in vitro and in intact cells. IRF-2, which is known to either repress or activate target gene expression, acts as a transcriptional activator in the context of the 32kb-150 reporter gene. The carboxyl-terminal basic region of IRF-2 serves as an activation domain in this context. This in contrast to its acting as a repressor domain in the context of the synthetic core ISRE. Furthermore, after treatment of promyelocytic HL-60 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), which triggers differentiation into macrophages, both NMHC-A expression and IRF-2 expression were found to be up-regulated with a similar time course. TPA treatment leads to recruitment of IRF-2 to 32kb-150 of the endogenous NMHC-A gene and acetylation of the core histones surrounding this region. In addition, the ISRE in the 32kb-150 reporter gene recruits IRF-2 and mediates TPA-induced activation of a reporter gene in HL-60 cells. Together, these results indicate that IRF-2 contributes to transcriptional activation of the NMHC-A gene via 32kb-150 during TPA-induced differentiation of HL-60 cells.

Myosin is a family of proteins that generate mechanical force by catalyzing hydrolysis of ATP when they interact with actin filaments (1, 2). Conventional myosin (class II) consists of a pair of heavy chains (~200 kDa) and two pairs of light chains (15–20 kDa). Myosin II plays an important role in diverse cellular contractile and motile processes, such as muscle contraction, cytokinesis, cell migration, and cell adhesion in eukaryotic cells. More than 10 genes encode myosin heavy chains II (MHCs)1 in vertebrates, and they are divided into two sub-classes: sarcomeric (skeletal and cardiac muscles) and non-sarcomeric (smooth muscle and nonmuscle) MHC genes, based on homologies of primary sequences. In the human genome, there are three genes for nonmuscle MHC (NMHC), termed NMHC-A (MYH9), NMHC-B (MYH10), and NMHC-C (MYH14; names in parenthesis correspond to nomenclature for human genome) (2–4).

During the last decade, a great deal of progress has been made in understanding the molecular function and pathological implication of the NMHCs. Recent studies demonstrate that NMHC-B is essential for the normal neural cell migration during mouse brain development and neurite outgrowth in cultured neural cells (5–7). NMHC-A plays pivotal roles in phagocytosis, assembly of focal contacts, and cell adhesion (7–10). More recently, mutations in the coding region of the NMHC-A (MYH9) gene have been found to be linked to a number of human disorders, such as May-Hegglin anomaly, Fechtner syndrome, Sebastian syndrome, Epstein and Alport-like syndromes, and non-syndromic hereditary deafness, DFNA17 (11–13). Therefore, the term MYH9 disorder (or syndrome) has been proposed to encompass all of these disorders (14, 15).

The NMHC genes are differentially expressed, and their isoform-specific expression is dependent on cell types and linked to cell proliferation and differentiation. For instance, NMHC-A is expressed abundantly in hematopoietic and lymphopoietic cells and epithelial cells but less abundantly in differentiated neural and muscle cells (4, 16, 17). During differentiation of promyelocytic leukemia HL-60 and U937 cells into the macrophage lineage, expression of NMHC-A increases significantly at the mRNA and protein levels (18), presumably reflecting an important role of this protein in migration and phagocytosis by macrophages. However, regulatory mechanisms controlling NMHC-A gene expression during cell proliferation or differentiation have not been fully explored.

Studies in this laboratory have been focused on characterization of the promoter and enhancers of the human NMHC-A gene (19–21). The promoter region of the NMHC-A gene shows a number of features typical of a housekeeping gene; there is no TATA box and the GC content is high, with multiple GC boxes. The proximal downstream region of the transcriptional initiation sites is involved in cell type-specific activation of this gene via both pre-translational (transcriptional) and translational mechanisms. An enhancer region composed of multiple clustered cis-elements, which is located 23 kb downstream of the

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1 The abbreviations used are: MHC, myosin heavy chains II; NMHC, nonmuscle myosin heavy chains II; IFN, interferon; ISRE, interferon-stimulated response element; IRF, interferon regulatory factor; VCAM, vascular cell adhesion molecule; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; TPA, 12-O-tetradecanoylphorbol-13-acetate; CMV, cytomegalovirus; β-Gal, β-galactosidase; Rn, normalized reporter; a.a., amino acids; CBP, cAMP response element-binding protein-binding protein; PCAF, p300/CBP-associated factor.
promoter in intron 1, has been demonstrated to modulate transcription in cell type- and differentiation state-dependent manners. Sp1 and Sp3 specifically recognize one of the elements in this region (20), and TFE3, which is expressed abundantly in leukocytes, can act as a transcriptional activator via a second element (21). In this study, we describe another enhancer region, designated 32kb-150, which is also located in intron 1. The 32kb-150 enhancer region includes a previously unrecognized interferon (IFN)-stimulated response element (ISRE).

The ISRE is recognized by a family of transcriptional factors, IFN regulatory factors (IRFs), some of which are induced by several types of IFNs (22, 23). This family of proteins has a conserved DNA-binding domain in the N-terminal region, which contains a characteristic repeat of five tryptophan residues. Two well studied members of the IRF family, IRF-1 and IRF-2, have antagonistic roles in IFN-related gene regulation; IRF-1 activates transcription of IFN-inducible genes and IRF-2 represses the activation by IRF-1 (24). On the other hand, IRF-1 and -2 are also involved in modulation of the cell cycle and apoptosis by regulating transcription of other sets of genes. Although IRF-2 was originally described as a transcriptional repressor, this protein can act as a transcriptional activator for some genes, including histone H4 and vascular cell adhesion molecule (VCAM)-1 (25-28). IRF-2 can interact with other members of the IRF family and regulate cell growth- and differentiation-related genes in myeloid and lymphoid cells (29). Some members of this family, such as IRF-4 and IRF-8, are expressed specifically in lymphoid and myeloid cells, whereas other members, including IRF-2, are expressed in most cells. Ubiquitous expression of IRF-2 and the diverse cellular functions involving IRF-2 suggest the existence of additional target genes that have not been described previously.

Herein, we report that IRF-2 acts as a transcriptional activator for the NMHC-A gene via an ISRE in the 32kb-150 enhancer region located in intron 1. Analyses of reporter gene expression, electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays provide strong evidence supporting the idea that IRF-2 and the 32kb-150 region play an important role in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced activation of the NMHC-A gene during differentiation of promyelocytic cells to the macrophage lineage.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction—**Luciferase reporter genes were constructed using two plasmids, pGL2basic and pGL2basic (Promega), as host vectors. The core promoter luciferase reporter gene containing the 173-bp core promoter of the human NMHC-A gene has been described previously (19). The 32kb-150 fragment (for sequence, see Fig. 1B) was generated by PCR using the human NMHC-A genomic clone as a template and inserted upstream of the core promoter. The 32kb-150 fragment with the mutated ISRE (for sequence, see Fig. 2A) was generated by recombinant PCR using appropriate primers, which include the mutated sequences. Three copies of the wild-type and mutated ISRE were prepared by annealing two complementary strands of oligonucleotides. The upper strand of each sequence of oligonucleotides is as follows: wild-type (5’-cATTATCTTCACTGATTCTCA-3’) and mutant (5’-cATTATCTTCACTGATTCTCA-3’). Underlined and lowercase letters represent mutated sequences and adaptor sequences, including restriction enzymes, respectively. DNA probes were generated by 5’-end labeling using T4 polynucleotide kinase and [γ-32P]ATP.

Nuclear extracts from NIH3T3 and HeLa cells were prepared as described previously (20). Whole cell extracts from HL-60 cells not treated or treated with 10 nM TPA or 100 units/ml IFN-γ were prepared by using a lysis buffer containing 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10 mM MgCl₂, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and a protease inhibitor mixture (Sigma). The lysates were centrifuged in a microcentrifuge for 5 min, and the pellets were resuspended in the same buffer as that used for binding reactions (see below) but without poly(dI-dC). Binding reactions were carried out in a 10-μl mixture that contained 3–5 μg of nuclear extracts, 0.5 μg of poly(dI-dC), 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 4% glycerol. For competition experiments, the unblabeled DNA was preincubated for 15 min at room temperature before addition of the probe. For antibody supershift experiments, indicated antibodies were preincubated for 15 min at room temperature before addition of the probe. After additional incubation with the probe for 20 min, the reaction mixtures were analyzed by electrophoresis in a 6% DNA retardation gel (Invitrogen).

**Chromatin Immunoprecipitation (ChIP) Assay and Quantification by Real-time PCR**—ChIP assays were performed using an immunoprecipitation kit (Upstate Biotechnology) according to the manufacturer’s recommendations. In brief, 2–5 × 10⁶ HL-60 cells were treated with 10 nM TPA for the indicated times (see Fig. 9), and the proteins bound to DNA were subsequently cross-linked by addition of formaldehyde to the culture medium to a final concentration of 1% and then incubated for 20 min. The fixed cells were washed with cold phosphate-buffered saline containing a protease inhibitor mixture and resuspended in a SDS lysis buffer. The cell lysates were sonicated for 10 s at 30% of maximum power. After the samples were diluted 10-fold with ChIP dilution buffer, 2 μl of diluted samples were precleared by addition of 80 μl of salmon sperm DNA/protein A agarose-50% slurry. Precleared samples were immunoprecipitated at 4 °C overnight with antibodies specific for IRF-1, IRF-2 (Santa Cruz Biotechnology), acetylated histone H3, or acetylated histone H4 (Upstate Biotechnology). After deproteination with proteinase K digestion and reversal of cross-links, the presence of selected DNA sequences was assessed either by conventional PCR or by real-time PCR. The primers used for amplification of the 200-bp fragment, including the 32kb-150 region of the endogenous NMHC-A gene in human HL-60 cells, were as follows: forward primer 5’TACTTGAGTACGATTCAggatcccg-3’ and reverse primer 5’TACTTTCAGTTTCATCAggatcccg-3’. Underlined and lowercase letters indicate mutated sequences and adaptor sequences, including restriction enzymes, respectively. DNA probes were generated by 5’-end labeling using T4 polynucleotide kinase and [γ-32P]ATP.

For analysis of endogenous protein binding to the 32kb-150 region of the luciferase reporter constructs transfected in NIH3T3 or HL-60 cells, the same ChIP protocol was applied with the exception of PCR primers 5’TGTATCTTATGGTACTGTAAGCT-3’ and 5’-CAGCAGTTGCCTTATTTAGG-3’. This primer set amplifies the 220-bp fragment, which is specific to the reporter sequence, including a part of the vector sequence, but not amplifies the endogenous sequence.
To determine the relative amounts of the co-immunoprecipitated DNA fragments, real-time quantitative PCR was performed using a Quantitech probe PCR kit (Qiagen) in a Prism 7900HT sequence detection system (Applied Biosystems). The primers used were the same as those for the conventional PCR. The amplified PCR products were monitored by a TaqMan minor groove binder probe (Applied Biosystems) specific to the 32kb-150 region, which has a 5'-fluorophore (6-carboxyfluorescein) and a 3' minor groove binder non-fluorescent quencher (5'-6-carboxyfluorescein-TCATTTCCTGAGAGTCCTA-MGB-NFQ-3'). The PCR reactions containing 0.4 μm concentrations of primers at a 0.2 μm concentration of the reporter probe were performed in triplicate under the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of PCR consisting of 15 s for 95 °C and 60 s for 60 °C. The emission intensity of the reporter dye was divided by the emission intensity of the internal passive reference dye, and the resulting value was defined as the Rn (normalized reporter). ΔRn was calculated by subtracting the Rn value of the reaction without template or the Rn value at the early cycles from the Rn value of the reaction with template. The threshold cycle (Ct) value was defined as the PCR cycle number at which PCR amplification reaches the threshold ΔRn value. The Ct value of each experimental sample was converted to the relative DNA amounts by comparison with a standard curve. The standard curve was established for each amplification by inputting serial dilutions of the 32kb-150 reporter gene construct. The relative amounts of the co-immunoprecipitated DNA with specific antibodies were calculated by subtracting the DNA amounts of the negative control sample obtained in the absence of antibodies from those of the sample immunoprecipitated with specific antibodies.

Northern and Western Blot Analyses—Total RNA was isolated from HL-60 cells cultured in the absence or presence of 10 nM TPA using an RNeasy RNA isolation mini kit (Qiagen). Fifteen μg of total RNA was separated by electrophoresis in a 1% agarose gel containing formaldehyde. The gel was blotted onto a nylon membrane, and hybridization was carried out with 32P-labeled probes: the full-length coding region of the human NMHC-A gene, we have searched for regulatory elements in the region extending 20 kb upstream and 40 kb downstream of the transcriptional start sites, which includes the 39 kb intron 1, by reporter gene analysis and followed by data base analysis. A 150 bp region –32 kb downstream from the transcriptional start sites, designated 32kb-150, was found to enhance NMHC-A promoter activity as described below in detail (see Fig. 2). Real-time quantitative PCR was performed using the SuperSignal System (Pierce Chemical Co.) to analyze the level of luciferase activity. The 32kb-150 fragment was inserted upstream to the NMHC-A promoter in a luciferase reporter construct, and the construct was transfected into the mouse embryonic cell line, NIH3T3, and the human epithelial carcinoma cell line, HeLa, in which NMHC-A is highly expressed. As shown in Fig. 2, the 32kb-150 fragment causes 10- and 6.5-fold increases in luciferase activity in NIH3T3 and HeLa cells, respectively. Mutation of the ISRE results in a 2-fold decrease in luciferase activity, compared with wild-type constructs transfected into both cell backgrounds. These results suggest that 32kb-150 is a ubiquitously occurring enhancer for the NMHC-A promoter in mammalian species and that the ISRE is an important element within 32kb-150 for its enhancer activity.

IRF-2 Can Specifically Bind to the ISRE in the 32kb-150 Fragment—To determine whether the ISRE in the 32kb-150 fragment can bind specific protein(s), EMSAs were carried out using nuclear extracts from NIH3T3 and HeLa cells and a labeled DNA probe consisting of the ISRE and a few flanking nucleotides. As shown in Fig. 3, a major probe DNA-protein complex (complex C) is demonstrated with both cell types. A 150 bp region –32 kb downstream from the transcriptional start sites, designated 32kb-150, was found to enhance NMHC-A promoter activity as described below in detail (see Fig. 2). Data base analysis also revealed that this region was highly conserved in sequence among humans, mice, and rats. Fig. 1A shows a comparison of the human NMHC-A genomic sequence with that of mice in the region spanning exon 1 to exon 3, using a Vista program. The peak height represents percentage identity for each 100 bp. In addition to three exonic regions, a number of intronic regions show high homology. Among them, a homology at the region –32 kb downstream of exon 1 is remarkable, having 95% identity. The same homology peak in this region was also observed in a comparison between human and rat sequences (data not shown). This region consists of 156 bp and the sequence alignment by a Blast 2 program is shown in Fig. 1B. This peak region and the 32kb-150 region overlap by 121 bp. It is noteworthy that an ISRE is embedded in the middle of the 32kb-150 region.

The effects of the 32kb-150 fragment on transcriptional activity were examined by reporter gene analysis. A copy of the 32kb-150 fragment was inserted upstream to the NMHC-A core promoter in the luciferase reporter construct, and the construct was transfected into the mouse embryonic cell line, NIH3T3, and the human epithelial carcinoma cell line, HeLa, in which NMHC-A is highly expressed. As shown in Fig. 2, the 32kb-150 fragment causes 10- and 6.5-fold increases in luciferase activity in NIH3T3 and HeLa cells, respectively. Mutation of the ISRE results in a 2-fold decrease in luciferase activity, compared with wild-type constructs transfected into both cell backgrounds. These results suggest that 32kb-150 is a ubiquitously occurring enhancer for the NMHC-A promoter in mammalian species and that the ISRE is an important element within 32kb-150 for its enhancer activity.

RESULTS

The Conserved 32 kb Downstream Intronic Region Containing an ISRE in the NMHC-A Gene Exhibits Enhancer Activity—To understand the transcriptional regulation of the human NMHC-A gene, we have searched for regulatory elements in the region extending 20 kb upstream and 40 kb downstream of the transcriptional start sites, which includes the 39 kb intron 1, by reporter gene analysis and followed by data base analysis. A 150 bp region –32 kb downstream from the transcriptional start sites, designated 32kb-150, was found to enhance NMHC-A promoter activity as described below in detail (see Fig. 2). Data base analysis also revealed that this region was highly conserved in sequence among humans, mice, and rats. Fig. 1A shows a comparison of the human NMHC-A genomic sequence with that of mice in the region spanning exon 1 to exon 3, using a Vista program. The peak height represents percentage identity for each 100 bp. In addition to three exonic regions, a number of intronic regions show high homology. Among them, a homology at the region –32 kb downstream of exon 1 is remarkable, having 95% identity. The same homology peak in this region was also observed in a comparison between human and rat sequences (data not shown). This region consists of 156 bp and the sequence alignment by a Blast 2 program is shown in Fig. 1B. This peak region and the 32kb-150 region overlap by 121 bp. It is noteworthy that an ISRE is embedded in the middle of the 32kb-150 region.

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IRF-2 Can Specifically Bind to the ISRE in the 32kb-150 Fragment—To determine whether the ISRE in the 32kb-150 fragment can bind specific protein(s), EMSAs were carried out using nuclear extracts from NIH3T3 and HeLa cells and a labeled DNA probe consisting of the ISRE and a few flanking nucleotides. As shown in Fig. 3, a major probe DNA-protein complex (complex C) is demonstrated with both cell types. A
100-fold molar excess of unlabeled wild-type DNA, but not mutant DNA, competes efficiently with the labeled probe for protein binding, resulting in disappearance of the labeled complex C (Fig. 3A). These results indicate that complex C is specific to the ISRE.

Several members of the IRF family are known to share the conserved DNA-binding domain and bind to the common DNA target sequences (22, 23). To identify the protein(s) forming complex C, we performed gel supershift assays using antibodies specific for each of the IRF family proteins. As shown in Fig. 3B, only the anti-IRF-2 antibodies, among five anti-IRF antibodies, shift complex C to complex SS in both NIH3T3 and HeLa nuclear extracts (Fig. 3B, lanes 3 and 9). Moreover, anti-IRF-2 antibodies supershift all of complex C, indicating that IRF-2 is the single major protein in the nuclear extracts binding to the ISRE in the EMSA.

We next sought to determine whether the ISRE in the 32kb-150 fragment could recruit IRF-2 in intact cells. For this, the same reporter constructs used for luciferase expression were transfected into NIH3T3 cells, and the DNA-protein complexes were cross-linked by formaldehyde in the cells. After immunoprecipitation of the DNA-protein complexes with specific antibodies, DNAs were recovered and subjected to PCR using primers specific for the 32kb-150 region of the reporter constructs.
The reporter gene construct with the core promoter alone does not contain the amplifiable DNA sequences; therefore, it serves as a negative control. The increase caused by exogenous IRF-2 depends on the DNA contexts as well as cellular contexts. We, therefore, examined how the exogenously expressed IRF-2 affects NMHC-A promoter activity of the reporter gene. Various amounts of the IRF-2 expression construct were co-transfected into HeLa cells with a number of luciferase reporter genes. As controls, the empty expression vector (Vector, lane 2) was co-transfected with the luciferase reporter gene constructs as described above, as well as that containing the core promoter alone (Core, lane 1). Total amounts of DNA transfected were kept constant by addition of the empty vector. Relative luciferase activities normalized by β-galactosidase activities are shown as -fold activation (mean ± S.D., n = 4). The luciferase activity driven by the core promoter alone is represented as 1.

Fig. 4. Binding of endogenous IRF-2 to the ISRE of the reporter gene in intact NIH3T3 cells. ChIP assays were carried out after transfection of the luciferase reporter gene construct, which contains the mutated (MT) or wild-type (WT) ISRE in the 32kb-150 region inserted upstream of the core promoter, or the core promoter alone (Core), into NIH3T3 cells. Cross-linked DNA-protein complexes were immunoprecipitated by antibodies specific to IRF-1 or IRF-2. 10% of total DNA before immunoprecipitation was used for PCR as an input control (Input). A, agarose gel electrophoresis after conventional PCR. B, real-time PCR profiles. Representative plots were traced. Arrows indicate the points where the indicated PCR amplification curves cross the threshold line (ΔRn = 0.2). Cycle number corresponding to each of these points is the CT value for each amplification. C, quantification of the co-immunoprecipitated 32kb-150 fragments. The relative DNA amounts were calculated by a comparison with amplifications of known amounts of DNA using the CT values, and are shown as a bar graph (means ± S.D., n = 3). The reporter gene construct with the core promoter alone does not contain the amplifiable DNA sequences; therefore, it serves as a negative control.

Fig. 5. Transcriptional activation of the NMHC-A promoter by IRF-2. 100, 200, and 500 ng of the expression constructs for IRF-1 or IRF-2 (lanes 3–5 for IRF-1 and lanes 6–8 for IRF-2) were co-transfected with the luciferase reporter gene construct, which contains the 32kb-150 region with the wild-type (WT) ISRE, and pCMV-β-Gal in HeLa cells. As controls, the empty expression vector (Vector, lane 2) was co-transfected with the luciferase reporter gene constructs as described above, as well as that containing the core promoter alone (Core, lane 1). Total amounts of DNA transfected were kept constant by addition of the empty vector. Relative luciferase activities normalized by β-galactosidase activities are shown as -fold activation (mean ± S.D., n = 4). The luciferase activity driven by the core promoter alone is represented as 1.

IRF-2 Activates NMHC-A Transcription and Its C-terminal Region Serves as an Activation Domain—IRF-2 has been reported to either repress or enhance transcription depending on the DNA contexts as well as cellular contexts. We, therefore, examined how the exogenously expressed IRF-2 affects NMHC-A promoter activity of the reporter gene. Various amounts of the IRF-2 expression construct were co-transfected into HeLa cells with a number of luciferase reporter genes. As shown in Fig. 5, exogenous expression of IRF-2 results in a dose-dependent increase in luciferase activity driven by the reporter gene, which contains the wild-type 32kb-150 (lanes 6–8). The increase caused by exogenous IRF-2 depends on the presence of the intact ISRE, because no increase is seen with the reporter gene, which has mutation at the ISRE. In contrast, co-transfection of IRF-1 results in a dose-dependent decrease in luciferase activity. This decrease is observed not only with the wild-type 32kb-150 reporter but also the mutant one (Fig. 5, lanes 3–5) as well as the core promoter alone (data not shown), implying that IRF-1 represses core promoter activity itself. Therefore, these data demonstrate that IRF-2, but not IRF-1, activates transcription of the NMHC-A-luciferase reporter gene in an ISRE-dependent manner. Because ISRE-dependent enhancement of reporter gene expression occurs without exogenous IRF-2 (Fig. 2) and endogenous IRF-2 is recruited to the ISRE of the reporter gene (Fig. 4), it is reasonable to conclude that endogenous IRF-2 also functions as an activator for this reporter system.

The finding that IRF-2 functions as a transcriptional activator, unlike a number of previous reports, in which IRF-2 has been described as a transcriptional repressor, prompted us to...
undertake characterization of the domain in this protein that was responsible for trans-activation of the NMHC-A gene. Expression constructs, which contain full-length IRF-2 cDNA, as well as those lacking the domains shown in Fig. 6A, were prepared. Note that all four constructs include the intact DNA-binding domain. These constructs were co-transfected into HeLa cells with two different luciferase reporter genes: one that includes the NMHC-A core promoter and the 32kb-150 fragment containing the ISRE (Fig. 6C) and a second one that includes the same core promoter and three copies of the 20-bp core ISRE sequence (Fig. 6D). The effects of the deletion mutant proteins differ between the two reporter contexts. The mutant protein ΔB, which lacks the C-terminal basic domain (Δa.a. 300–349), represses the expression of luciferase to a level lower than that caused by co-transfection of the empty vector in the context of the 32kb-150 reporter gene (Fig. 6C, lane 3). This implies that the mutant protein ΔB competes with endogenous IRF-2 for DNA binding and inhibits trans-activation. In contrast, this mutant shows enhanced trans-activation in the context of the 3X ISRE reporter gene (Fig. 6D, lane 3). Therefore, the basic region between a.a. 300 and 349 serves as an activation domain in the 32kb-150 gene context but acts as a repression domain in the 3X ISRE gene context. Further removal of the C-terminal region (ΔAB, Δa.a. 180–349) shows a further decrease in luciferase activity (Fig. 6C, lane 4), and the mutant protein ΔA, which lacks the middle acidic domain (Δa.a. 180–220), shows trans-activation equivalent to the full-length protein (Fig. 6C, lane 5), in the context of the 32kb-150 reporter gene. These results indicate that the region between a.a. 220 and 349 of IRF-2 is required for full trans-activation potency in the 32kb-150 gene context and that the most crucial sequences
Transcriptional Activation of Nonmuscle Myosin II-A by IRF-2

To see whether the elevated IRF-2 level reflects the binding activity of IRF-2 to the ISRE in the 32kb-150, cellular extracts were prepared from the TPA-treated and untreated HL-60 cells and were subjected to EMSA. As shown in Fig. 7C, TPA treatment also causes an increase in formation of the ISRE-IRF-2 complex (C-IRF-2), which can be supershifted by anti-IRF-2 antibodies (Fig. 7C, lanes 1, 3, 4, and 6). To assess the specificity of TPA treatment for the increased binding activity of IRF-2 to the ISRE, the extracts from the IFN-γ-treated cells were also analyzed. IFN-γ treatment induces IRF-1 protein expression (Fig. 7D, lanes 3, 4). IRF-1 is also capable of binding to the ISRE and this complex (C-IRF-1, open arrowhead, lane 9), which is visualized better as a band remaining in the presence of anti-IRF-2 antibodies, migrates just below the complex formed with IRF-2 (closed arrowhead, lane 8), as seen in Fig. 7C. It is noteworthy, however, that the elevated IRF-1

Fig. 7. TPA-induced up-regulation of IRF-2 and NMHC-A expression in HL-60 cells. A, time course analysis of expression of IRF-1, IRF-2, and NMHC-A mRNAs. Total RNAs were isolated from HL-60 cells treated with TPA for indicated times and were subjected to Northern blot analysis. Ethidium bromide-staining gel (EtBr) and blots hybridized with the indicated probes are shown. B, time course analysis of expression of the IRF-1, IRF-2, and NMHC-A proteins. HL-60 cells were harvested after treatment with TPA for indicated times. 20 μg of total cellular proteins were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies specific to the proteins indicated. C, binding of TPA- or INF-γ-induced IRFs to the ISRE. EMSAs were performed using the ISRE as a probe and whole cell extracts prepared from HL-60 cells, which were treated with TPA or IFN-γ for 24 h or remained untreated (No). Specific complexes containing IRF-1 (C-IRF-1) and IRF-2 (C-IRF-2) as well as complexes supershifted (SS) by antibodies (Ab) are indicated. C-IRF-1 migrates slightly faster than C-IRF-2. In the presence of anti-IRF-1 antibodies, the C-IRF-1 complex is supershifted. Therefore, the remaining complex, indicated by the solid arrowhead, is C-IRF-2 (lanes 5 and 8). In the presence of anti-IRF-2 antibodies, the C-IRF-2 complex is shifted; therefore, the remaining complex, indicated by an open arrowhead, is C-IRF-1 (lane 9). Asterisks indicate unidentified complexes. D, increased expression of IRFs in HL-60 cells treated with TPA or IFN-γ. The same cell extracts used for C were subjected to Western blot analysis using antibodies specific to proteins indicated.
expression and the IRF-1 binding to the ISRE induced by IFN-γ are not accompanied by an increase of NMHC-A expression (Fig. 7D, lane 3, bottom) in contrast to treatment with TPA. Taken together, up-regulation of NMHC-A expression is accompanied by an increase in IRF-2 expression as well as an increase in IRF-2 binding activity to the ISRE in HL-60 cells during TPA treatment.

The ISRE in the 32kb-150 Region Recruits IRF-2 and Mediates TPA-induced Activation of the NMHC-A Promoter in HL-60 Cells—The finding that NMHC-A up-regulation is accompanied by IRF-2 up-regulation during TPA treatment prompted us to investigate further the role of the ISRE in the 32kb-150 region and IRF-2 in NMHC-A gene activation. We analyzed whether the reporter gene containing the 32kb-150 could respond to TPA treatment in HL-60 cells. Because HL-60 cells showed poor transfection efficiency and only trace levels of luciferase activity derived from the pGL2 luciferase construct, which had been used for the experiments with HeLa and NIH3T3 cells, a more sensitive reporter gene, pGL3, was used for the following experiments. Among a number of transfection methods we tested, electroporation using an Amaxa Nucleofector provided maximal transfection efficiency and allowed us to measure luciferase activity in HL-60 cells. As shown in Fig. 8, in the absence of TPA, the luciferase activity caused by the reporter gene containing the 32kb-150 fragment with the wild-type ISRE shows a 3-fold higher activity compared with the activity caused by the mutated ISRE or the core promoter alone. When the transfected cells are treated with TPA, the luciferase activity caused by the reporter gene containing wild-type ISRE is further increased ~1.7-fold. In contrast, the luciferase activity of a construct containing a mutation in the ISRE or the core promoter alone is not affected by TPA treatment. Of particular importance is that the TPA-induced increase is absolutely dependent on an intact ISRE in the 32kb-150 fragment, indicating that TPA-induced enhancement of the promoter activity is mediated via the ISRE.

To examine participation of IRF-2 in this TPA-induced increase of the reporter gene expression in HL-60 cells, binding of IRF-2 to the ISRE in the 32kb-150 region of the reporter gene was analyzed using real-time quantitative PCR after co-immunoprecipitation of the cross-linked cellular extracts with anti-IRF-2 antibodies. As seen in Fig. 8B, binding of IRF-2 to the 32kb-150 region containing the wild-type ISRE increases 2.6-fold upon TPA treatment. In contrast, there is no TPA-induced increase in the binding of IRF-2 to the 32kb-150 region containing the mutant ISRE. Therefore, the TPA-induced increase of ISRE-dependent reporter gene expression is accompanied by the TPA-induced increase of IRF-2 binding to the ISRE in the reporter gene. Consistent with the observation that the wild-type reporter gene shows a higher promoter activity than the mutant reporter gene in the untreated cells (Fig. 8A), a higher extent of IRF-2 binding to the wild-type 32kb-150 region is demonstrated in the untreated cells, compared with that of the mutant 32kb-150 region (Fig. 8B). Although real-time PCR analysis detects small amounts of the mutant 32kb-150 fragment associated with the anti-IRF-2 immunocomplexes in both TPA-treated and untreated cells, these levels of interaction of IRF-2 with the mutant 32kb-150 fragment may not be functionally relevant, because the reporter gene with the core promoter alone and that with the mutant 32kb-150 show similar promoter activities (Fig. 8A). Taken together, the above data strongly suggest that the TPA-induced IRF-2 binding to the ISRE in the 32kb-150 region leads to activation of the NMHC-A reporter gene in HL-60 cells.

TPA-induced NMHC-A Up-regulation is Accompanied by Recruitment of IRF-2 to the Endogenous Gene and Histone Acetylation in Intact Cells—Finally, to determine whether IRF-2 would bind to the 32kb-150 region of the endogenous NMHC-A gene in HL-60 cells upon TPA treatment, we carried out ChIP assays targeting the endogenous gene. The ChIP technique can provide information as to whether a specific transcriptional factor truly binds near a specific region of genomic DNA in intact cells. The cells were treated with TPA for 0, 6, and 24 h and directly fixed with formaldehyde to cross-link chromatin DNA-protein complexes. The target DNAs, which recruited IRF-2, were co-immunoprecipitated with anti-IRF-2 antibodies, and were subjected to PCR using a primer set specific to the 32kb-150 region of the endogenous NMHC-A gene. As shown in Fig. 9A, anti-IRF-2 antibodies co-immunoprecipitate much larger amounts of the target DNA containing 32kb-150 from TPA-treated cells (both at 6 and 24 h) than untreated cells. Without antibodies, no PCR products are generated. Quantification by real-time PCR demonstrates a 9- and 22-fold increase after 6 and 24 h of TPA treatment, respectively (Fig. 9A, right). These results indicate that IRF-2 binding to the 32kb-150 region is induced by TPA treatment.

The degree of the TPA-induced increase in the IRF-2-bound 32kb-150 fragment recovered from the endogenous NMHC-A gene (22-fold; Fig. 9A) is larger than that of the IRF-2-bound 32kb-150 fragment from the reporter gene (2.6-fold; Fig. 8B). This may be the result of the difference between the chromosomal endogenous DNA and the epichromosomal reporter DNA in association with chromatin structural proteins such as histones.
indicated antibodies. Immunoprecipitation without antibodies (No Ab) for the indicated times and subjected to ChIP analysis using the antibodies specific for acetylated histones during TPA treatment of HL-60 cells. HL-60 cells were treated with TPA for the indicated times and subjected to ChIP using the antibodies, Immunoprecipitation without antibodies (No Ab) serves as a negative control. As a positive control, 10% of the input sample (Input) before immunoprecipitation was used for PCR. A, increased recruitment of IRF-2 to the endogenous 32kb-150 region. Left, agarose gel electrophoresis after conventional PCR. Right, the relative amounts of the co-immunoprecipitated 32kb-150 region with anti-IRF-2 quantified by real-time PCR (means ± S.D., n = 3). B, changes in interaction of the 32kb-150 region with acetylated histones in intact chromatin. Agarose gel electrophoresis after the conventional PCR is shown.

The 32kb-150 region of the endogenous NMHC-A gene in the chromatin context of the untreated HL-60 cells presumably interacts tightly with histones and, therefore, is less accessible to the sequence-specific DNA binding protein IRF-2. On the other hand, the exogenously introduced reporter gene is not packed in chromatin and is therefore readily available to IRF-2. Changes in chromatin structure surrounding other portions of the NMHC-A gene in TPA-treated cells may also affect the accessibility of IRF-2 to the ISRE in the endogenous 32kb-150 region. A lower degree of IRF-2 binding to the endogenous gene in the untreated cells and/or a higher degree of IRF-2 binding to the endogenous gene in the TPA-treated cells, compared with the reporter gene, is the most likely cause of the greater extent of the TPA-induced increase of the IRF-2 binding to the endogenous gene.

Because active transcription of a given gene is often associated with acetylation of histones surrounding that gene, we also examined the status of histone H3 and H4 acetylation by ChIP assay using antibodies specific for acetylated histones and the PCR primer set for the endogenous 32kb-150 region. As shown in Fig. 9B, after co-immunoprecipitation with anti-acetylated histone H3 and H4 antibodies, larger amounts of the 32kb-150 DNA are detected after 6 h of TPA treatment compared with before treatment. This indicates that histones H3 and H4, which surround the 32kb-150 region, are more acetylated. After 24 h of TPA treatment, the 32kb-150 fragments cross-linked with acetylated histones, especially H4, are somewhat reduced. Interpretation for this decrease will be discussed below. These results demonstrate that IRF-2 is recruited to the 32kb-150 region of the NMHC-A gene in HL-60 cells upon TPA treatment and that IRF-2 recruitment is associated with acetylation of the histones surrounding this region, presumably leading to trans-activation of the NMHC-A promoter. All of the above results collectively support the hypothesis that IRF-2 is responsible, at least in part, for up-regulation of NMHC-A gene transcription during TPA-induced differentiation of HL-60 cells.

**DISCUSSION**

The human NMHC-A gene consists of 41 exons spanning ~110 kb; more than one third of this length is intron 1. At first, we identified the promoter region and have been mapping the regions that are responsible for cell type-dependent or stimulation-induced transcriptional regulation of the NMHC-A gene. Despite our earlier efforts in searching for such regulatory elements in the region extending 20 kb upstream of the promoter using reporter gene analysis, we had found only a weak modulatory region within 2 kb. In contrast, we have found cell type-dependent regulatory regions just downstream from the promoter as well as in a 23 kb downstream region in intron 1 (19, 20). Identification and characterization of cis-elements and trans-acting factors in these regions have already been published (20, 21). Herein we describe another distal region, located 32 kb downstream of the promoter, that is responsible for induction of the NMHC-A expression in the macrophage lineage. Consistent with our experimental findings, a number of regions in intron 1, including the region proximal to the promoter and the regions 32 and 23 kb downstream of the promoter, show sequence conservation among humans, mice, and rats (Fig. 1). Among them, the 32kb-150 region shows the highest conservation. On the other hand, there is poor sequence conservation among mammalian species in the region beyond more than 1.5 kb upstream of the promoter. NMHC-A belongs to class II myosins among a large superfamily of myosins. In the class II MHC genes, NMHC-A, -B, and -C and the smooth muscle MHC genes compose a subclass of the myosin II family (1, 2). These four MHC genes have identical exon-intron organizations and contain a relatively large intron 1. Both the smooth muscle MHC and NMHC-A genes have been characterized in more detail with respect to transcriptional regulation. It is interesting that smooth muscle MHC intron 1 also contains a number of crucial elements required for general smooth muscle-specific expression as well as smooth muscle subtype-selective expression (30, 31). Therefore, the feature that transcriptional regulatory elements are embedded in intron 1 may be common to this subclass of gene family.

To elucidate the roles of the 32kb-150 region in transcriptional regulation of the NMHC-A gene, we have used two types of cells to characterize NMHC-A expression. One cell type includes NIH3T3 and HeLa cells, in which NMHC-A is constitutively expressed under regular culture conditions. The other is HL-60 cells, in which NMHC-A expression is induced in response to extracellular stimuli. First, using HeLa and NIH3T3 cells, we have demonstrated that IRF-2 can bind to the 32kb-150 fragment via the ISRE both in vitro (Fig. 3) and in intact cells (Fig. 4). We have also demonstrated that exogenously expressed IRF-2 (Fig. 5) and the endogenous protein (Fig. 2) trans-activate the 32kb-150 reporter gene in an ISRE-dependent manner. Based on these results, we conclude that IRF-2 binding to the 32kb-150 region results in transcriptional activation.

IRF-2 was originally described as an antagonist of the transcriptional activator IRF-1 for the IFN-β gene (24) and was generally considered to act as a transcriptional repressor in the IFN-responsive promoters, especially in lymphocytes. However, more recent studies demonstrated that IRF-2 activated the promoters of a number of genes, including histone H4, VCAM-1, and gp91phox (25–28). The domain of IRF-2 required for transcriptional activation has been reported in the context of the artificial promoter pc1Btk (HSVtk promoter plus 8 copies of the ISRE) in mouse fibroblast L929 cells (32) and in the context of the VCAM-1 promoter in which an ISRE exists between a TATA box and the transcriptional start site (28). In the context of pc1Btk, it has been shown that the C-terminal basic region (aa. 290–349) has a repressor function and that the central acidic region (aa. 160–220) is required for activation function. On the other hand, no repression domain was detected in the context of the VCAM-1 promoter. However, similar to pc1Btk, the central acidic region (aa. 200–240) was mapped as the crucial region required for trans-activation. Our results obtained with the 3X ISRE reporter gene agree with those reported previously. Of interest, however,
in the context of the 32kb-150 reporter, the C-terminal region is crucial for trans-activation potency, rather than for repression activity (Fig. 6). The apparently opposing effects of removing the C-terminal region on reporter gene expression are caused by a difference in DNA contexts. When IRF-2 is the only factor, which binds to the regulatory region in the 3X ISRE reporter, the C-terminal region may mask intramolecularly its activation potential or recruit a co-repressor molecule (33). However, when other DNA binding proteins are recruited in the immediate vicinity of the IRF-2 in the 32kb-150 reporter, the C-terminal region may interact with other DNA-binding protein(s) or co-activator(s) and this interaction would augment trans-activation of the promoter activity. As seen in Fig. 2, the 32kb-150 region with mutation of the ISRE can enhance reporter gene expression substantially more compared with the core promoter alone. Therefore, the 32kb-150 region is recognized by other DNA-binding protein(s) in addition to IRF-2 and presumably assembles an enhancedosome composed of multiple proteins. Data base search reveals that there are putative binding sites for signal transducer and activator of transcription, nuclear factor of activated T cells, and CCAAT/enhancer-binding protein in the 32kb-150 region. IRF-2 has been reported to interact with DNA-binding proteins such as NFκB as well as co-regulators of transcription (33–36). The C-terminal region of IRF-2 would contribute to such interactions in the context of the 32kb-150 reporter. Further study is required to elucidate what proteins can bind to the 32kb-150 region and how IRF-2 and these proteins interact and activate NMHC-A transcription.

Unlike the VCAM-1, histone H4, and gp91phox promoters, where IRF-2 or -1 activates the promoters (25, 27, 28), exogenously expressed IRF-1 does not activate but represses the promoter activity of the 32kb-150 reporter gene. The IFN-γ-induced increase in IRF-1 expression also leads to repression of the NMHC-A reporter gene expression in NIH3T3 cells (data not shown). Therefore, similar to many other IFN-responsive promoters, IRF-1 and IRF-2 show antagonistic effects on the NMHC-A promoter. However, the mechanism by which IRF-1 and IRF-2 regulate NMHC-A transcription is different from that of other IFN-responsive promoters, where IRF-1 and -2 compete for the same ISRE (37). In the context of the NMHC-A promoter, IRF-1 represses the core promoter independently of the ISRE in the 32kb-150 region, although there is no ISRE in the core promoter region. It has recently been reported that IRF-1 represses transcription by interfering with Sp1-dependent transcriptional activation in the CDK2 promoter (38). The NMHC-A core promoter contains at least three Sp1 binding sites. Therefore, a similar mechanism to that for the CDK2 promoter might be operating for the IRF-1 mediated repression of the NMHC-A promoter.

Using HL-60 cells, we have concluded that IRF-2 is responsible for transcriptional activation of the NMHC-A gene during TPA-induced differentiation of these cells to macrophages, from the following findings. IRF-2 is up-regulated upon TPA treatment with a time course similar to that of NMHC-A up-regulation (Fig. 7). TPA treatment leads to recruitment of IRF-2 to the 32kb-150 region of the endogenous NMHC-A gene, to the accompaniment of an increase in histone acetylation (Fig. 9). The TPA-induced and ISRE-dependent activation of the reporter gene is associated with recruitment of IRF-2 to the ISRE in the 32kb-150 fragment in HL-60 cells (Fig. 8). Although the observation that NMHC-A is up-regulated during macrophage differentiation was described a number of years ago (18), our study provides the first mechanism responsible for this observation.

HL-60 is a promyelocytic cell line that, upon TPA treatment, can differentiate into the macrophage lineage. Hence, it has been used extensively as a model system for examining the factors involved in promyelocytic cell differentiation (39). Another monocyte/myeloid cell line, U937, shows similar properties to HL-60 cells. U937 cells differentiate to macrophages upon TPA stimulation and NMHC-A expression is also increased during this differentiation (18). It has been reported that in U937 cells, TPA treatment leads to acetylation of IRF-2 and restoration of expression of the full-length IRF-2, instead of the truncated IRF-2 (40). IRF-2 has been shown to be a substrate for protein kinase C, protein kinase A, and casein kinase II in vitro and to be phosphorylated in intact cells (41). Therefore, in addition to an increase of the IRF-2 protein amounts demonstrated here, post-translational modifications of IRF-2 would also contribute to TPA-induced transcriptional regulation of the NMHC-A gene via IRF-2. Furthermore, TPA treatment of U937 cells has been reported to induce up-regulation of histone acetylases p300/CBP-associated factor (PCAF) and p300/CAMP response element-binding protein-binding protein (CBP), and IRF-2 has been shown to associate with PCAF and p300/CBP upon its binding to the ISRE (36). Histone acetylation is controlled by various histone acetylases and deacetylases, which are recruited by sequence-specific activators and repressors. Because we see an increase in acetylation of histones H3 and H4 surrounding the 32kb-150 region of the endogenous NMHC-A gene, and because this increase is accompanied by recruitment of IRF-2 in this region (Fig. 9), it is tempting to envision that IRF-2 associates with histone acetylases such as PCAF and/or p300/CBP upon TPA-induced binding to the 32kb-150 region in HL-60 cells. However, the ChIP results shown in Fig. 9 show some discrepancy between the binding of IRF-2 to the 32kb-150 region and the association of the acetylated histones with this DNA region during the time course of TPA treatment. After 6 h of TPA treatment, the 32kb-150 region of the NMHC-A gene is cross-linked with IRF-2 as well as with the acetylated histones H3 and H4 surrounding the 32kb-150 region of the endogenous NMHC-A gene, and because this increase is accompanied by recruitment of IRF-2 in this region (Fig. 9), it is tempting to envision that IRF-2 associates with histone acetylases such as PCAF and/or p300/CBP upon TPA-induced binding to the 32kb-150 region in HL-60 cells. However, the ChIP results shown in Fig. 9 show some discrepancy between the binding of IRF-2 to the 32kb-150 region and the association of the acetylated histones with this DNA region. Although our study does not distinguish these two cases, we favor the latter case. We interpret the above observations as follows: IRF-2 binding to the 32kb-150 region becomes more stabilized over the time course and, presumably, the histone acetylase(s) associated with IRF-2 acetylates the surrounding histones to higher extents. As a consequence, highly acetylated histones dissociate from the 32kb-150 region, consistent with a decrease in cross-linking between acetylated histones and the DNAs. The acetylation state of histones affects the interaction between histones and DNAs and hyperacetylation of histones leads to changes in chromatin structure from a closed (stronger histone-DNA interaction) to a more open (weaker histone-DNA interaction) structure. The extent of histone acetylation affects transcriptional activities of the surrounding genes in the chromatin context (42, 43). Of great interest is the current concept that hyper-acetylation of histones in chromatin reflects active gene transcription, the results of ChIP analysis support our conclusion that the TPA-induced IRF-2 binding to the 32kb-150 region contributes to transcriptional activation of the NMHC-A gene.

A number of enhancers are known to be located far from the promoters. Herein, we have described a 32-kb downstream enhancer that is located in intron 1. A number of models, including the looping model and the facilitated tracking model, have been proposed to explain how distal elements...
can affect assembly of the initiation complex at the promoters (44–46). Although the reporter gene analysis in TPA-treated HL-60 cells shown in Fig. 8 suggests that the complex assembled on the 32kb-150 fragment, which is located upstream near the promoter, is likely to enhance transcriptional initiation, we do not know whether the complex assembled in the 32kb-150 region of the endogenous gene enhances the initiation of transcription. Sequence-specific transcriptional activators can stimulate transcriptional initiation as well as elongation via interaction with other factors. Some activators have stronger effects on initiation and others on elongation (47). The control of transcriptional elongation in the chromatin context is an important step during gene activation (48, 49). Because the 32kb-150 region is located far downstream, in the middle of the gene, this enhancer might contribute to transcriptional elongation by keeping the chromatin structure in an open state.

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REFERENCES

1. Sellers, J. R. (2000) Biochim. Biophys. Acta 1496, 3–22
2. Berg, J. S., Powell, B. C., and Cheney, R. E. (2001) Mol. Biol. Cell 12, 780–794
3. Leal, A., Endele, S., Stengel, C., Huehne, K., Loetterle, J., Barrantes, R., Greenspan, P., and Wintz, A., and Rautenstrauss, B. (2003) Gene 312, 165–171
4. Golomb, E., Ma, X., Jana, S. S., Preston, Y. A., Kawamoto, S., Shoham, N. G., Goldin, E., Conti, M. A., Sellers, J. R., and Adelstein, R. S. (2004) J. Biol. Chem. 279, 2800–2808
5. Ma, X., Kawamoto, S., Hara, Y., and Adelstein, R. S. (2004) Mol. Biol. Cell 15,2568–2579.
6. Bridgman, P., Dave, S., Ames, C. F., Tullio, A. N., and Adelstein, R. S. (2001) J. Neurosci. 21, 6159–6169
7. Wylie, S. R., and Chantler, P. D. (2001) Nat. Cell Biol. 3, 88–92
8. Olazabal, I. M., Caron, E., May, R. C., Schilling, K., Knecht, D. A., and Macheky, L. M. (2002) Curr. Biol. 12, 1413–1418
9. Araki, N., Hatae, T., Furukawa, A., and Swanson, J. A. (2003) J. Cell Sci. 116, 247–257
10. Wei, Q., and Adelstein, R. S. (2000) Mol. Biol. Cell 11, 3617–3627
11. The May-Hegglin/Fechtner Syndrome Consortium (2000) Nat. Genet. 26, 103–105
12. Kelley, M. J., Jawien, W., Ortel, T. L., and Korczak, J. F. (2000) Nat. Genet. 26, 106–108
13. Laulani, A. K., Goldstein, J. A., Kelley, M. J., Lusford, W., Castelein, C. M., and Mhatre, A. N. (2000) Am. J. Hum. Genet. 67, 1121–1128
14. Keath, K. E., Campos-Parra, A., Toren, A., Rouzenfel-Granot, G., Carlsson, L. E., Savige, J., Denison, J. C., Gregory, M. C., White, J. G., Barker, D. F., Greiner, A., Epstein, C. J., Glucksman, M. J., and Martignetti, J. A. (2001) Am. J. Hum. Genet. 68, 1033–1045
15. Seri, M., Pecci, A., Di Bari, F., Canoso, R., Savino, M., Panza, E., Nigo, A., Noris, P., Gangarossa, S., Rocca, B., Gressele, P., Bizzaro, N., Malatesta, P., Koviisto, P. A., Longo, I., Musso, R., Pecoraro, C., Iolascon, A., Magrini, U., Rodriguez Soriano, J., Renieri, A., Ghiggeri, G. M., Ravazzone, R., Balsalducci, C. L., and Savoia, A. (2000) Medicine 82, 203–215
16. Kawamoto, S., and Adelstein, R. S. (1991) J. Cell Biol. 112, 915–924
17. Phillips, C. L., Yamakawa, K., and Adelstein, R. S. (1995) J. Muscle Res. Cell Motil. 16, 379–389
18. Tothaker, L. E., Gonzalez, D. A., Tung, N., Lemons, R. S., Le Beau, M. M., Arnaut, M. A., Clayton, L. K., and Tenen, D. G. (1991) Blood 78, 1826–1833
19. Kawamoto, S. (1994) J. Biol. Chem. 269, 15101–15110
20. Beohar, N., and Kawamoto, S. (1998) J. Biol. Chem. 273, 9168–9178
21. Chung, M. C., Kim, H. K., and Kawamoto, S. (2001) Biochemistry 40, 8887–8897
22. Mamane, Y., Heybrobec, C., Genin, P., Algarte, M., Servant, M. J., LePage, C., DeLuca, C., Kwon, H., Lin, R., and Hiscott, J. (1999) Gene 237, 1–14
23. Taniuchi, T., Ogawara, K., Takasaka, A., and Tanaka, N. (2001) Annu. Rev. Immunol. 19, 623–655
24. Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniuchi, T. (1989) Cell 59, 729–739
25. Yamamoto, H., Aniz, F., van Wijnen, A. J., Wu, S., Harada, H., Taniguchi, T., Soprano, K. J., Stein, J. L., and Stein, G. S. (1995) Nature 377, 362–365
26. Araki, N., Hatae, T., Furukawa, A., and Swanson, J. A. (2003) Mol. Biol. Cell 14, 155–165
27. Madsen, C. S., Rogn, C. P., Hungerford, J. E., White, S. L., Manabe, I., and Owens, G. R. (1998) Circ. Res. 82, 908–917
28. Manabe, I., and Owens, G. K. (2001) J. Biol. Chem. 276, 39076–39087
29. Yamamoto, H., Lamphier, M. S., Fujita, T., Taniguchi, T., and Harada, H. (1994) Oncogene 9, 1423–1428
30. Childs, K. S., and Goodfellow, S. (2003) Nucleic Acids Res. 31, 3016–3026
31. Arai, N., Hatae, T., Fujitaka, A., and Swanson, J. A. (2003) J. Cell Sci. 116, 247–257
32. Drew, P. D., Franzoso, G., Carlson, L. M., Biddison, W. E., Siebenlist, U., and Ozato, K. (1995) J. Neuroimmunol. 63, 157–162
33. Masumi, A., Wang, I. M., Lefebvre, B., Yang, X. J., Nakatani, Y., and Ozato, K. (1999) Mol. Cell. Biol. 19, 1810–1820
34. Tanaka, N., Kawakami, T., and Taniuchi, T. (1993) Mol. Cell. Biol. 13, 4531–4538
35. Xie, R. L., Gupta, S., Miele, A., Shiffman, D., Stein, J. L., Stein, G. S., and van Wijnen, A. J. (2003) J. Biol. Chem. 278, 26589–26596
36. Lu, R., and Pitha, P. M. (2001) J. Biol. Chem. 276, 45491–45496
37. Masumi, A., and Ozato, K. (2001) J. Biol. Chem. 276, 20973–20980
38. Birnbaum, M. J., van Zandert, B., Vaughan, P. S., Whitmarsh, A. J., van Wijnen, A. J., Davis, R. J., Stein, G. S., and Stein, J. L. (1997) J. Cell Biochem. 66, 175–183
39. Jenuwein, T., and Allis, C. D. (2001) Science 293, 1074–1080
40. Rippe, K., von Hippel, P. H., and Langowski, J. (1995) Trends Biochem. Sci. 20, 500–506
41. Blackwood, E. M., and Kadonaga, J. T. (1998) Science 281, 60–63
42. Levine, M., and Tjian, R. (2003) Nature 424, 147–151
43. Blau, J., Xiao, H., McCracken, S., O’Hare, P., Greenblatt, J., and Bentley, D. (1996) Mol. Cell. Biol. 16, 2044–2055
44. Orphanides, G., and Eisen, R. (2000) Nature 407, 471–475
45. Hartung, G. A., Speer, J. L., and Lindstrom, D. L. (2002) Biochim. Biophys. Acta 1577, 276–286