An Alu Element in the Myeloperoxidase Promoter Contains a Composite SP1-Thyroid Hormone-Retinoic Acid Response Element*

(Received for publication, December 29, 1995, and in revised form, March 14, 1996)

F. JavieR Piedrafita, Rachel B. Molander, Gordon Vansant, Elena A. Orlova, Magnus Pfahl, and Wanda F. Reynolds§

From the Sidney Kimmel Cancer Center, San Diego, California 92121

An Alu element preceding the myeloperoxidase gene (MPO) contains four hexamer motifs related to the consensus recognition sequence for nuclear hormone receptors (AGGTCA), arranged as direct repeats with spacing of 2, 4, and 2 nucleotides (DR-2-4-2). Gel shift experiments and transient transfection assays demonstrate that these sequences include binding sites for retinoic acid and thyroid hormone receptors and function in vivo to activate transcription of a chloramphenicol acetyltransferase reporter gene. The first DR-2 elements of the series do not bind known receptors but do bind the SP1 transcription factor. Two alleles of the series do not bind known receptors but do bind the SP1 to activate transcription of a chloramphenicol acetyltransferase reporter gene. The first DR-2 elements of the series do not bind known receptors but do bind the SP1 transcription factor. Two alleles of the MPO gene exist that differ at one position within this element, resulting in one allele with and one without a strong SP1 binding site. The element with the SP1 site activates transcription by 25-fold in transient transfection assays, while the alternative allele confers severalfold less transcriptional activity. Most cases of acute myelocytic leukemia are homozygous for the allele with the SP1 binding site, suggesting this element plays an important role in regulating the MPO gene in myeloid leukemias. This MPO-Alu is a representative of an Alu subclass numbering 400,000 copies, suggesting many genes may be regulated by such elements.

The human genome contains up to one million copies of a sequence element known as the Alu repeats, comprising ~5% of the DNA. These elements are potentially functional class III genes, transcribable by RNA polymerase III, originally derived from a 7SL gene. During the preceding 30–50 million years of primate evolution, Alu transcripts were converted into cDNA by a reverse transcription mechanism and inserted randomly throughout the genome in retropon fashion. An evolutionary lineage of highly conserved Alu source genes is thought to have provided the transcripts, which were converted into retroposons (1, 2). The Alu elements can be grouped into several subfamilies, each representing the progeny of one Alu source gene. The degree of sequence divergence within an Alu subfamily is a measure of the time elapsed since those Alu retroposons were introduced into the genome, with subgroups I–IV representing the oldest to the most recent retroposition events.

There has long been suggestive evidence that Alus or other polymerase III genes may function in gene regulation (3–6).

More recent evidence implicates specific Alu elements as influencing the expression of nearby genes (7–12). We reported that a major class of Alu elements (subclass III–IV) has evolved to encode a composite hormone response element (HRE) overlapping the internal promoter of the Alu (7). These several hexamer sequences are related to a consensus binding site, AGGTCA, recognizable by members of the nuclear receptor superfamily of ligand-activated transcription factors, including receptors for retinoic acid (RA) (RAR), thyroid hormone (T3) (TR), vitamin D (VDR), steroids, and a number of orphan receptors for which a ligand has not yet been identified (13–16). Most of these nuclear receptors recognize two adjacent hexamer half-sites that can be arranged as direct repeats (DR), palindrome, or inverted palindromes. The spacing and orientation of the half-sites is a major determinant of receptor binding specificity (17–19). Receptors can bind DNA as monomers to a single half-site, as homodimers to two adjacent half-sites, or as heterodimers with the retinoid X receptors (RXRs). The RAR/1RX family requires heterodimerization with RXRs for effective DNA binding and function (20–24).

An Alu preceding the human keratin K18 gene was shown to contain four half-sites oriented as direct repeats and spaced by 2 base pairs (DR-2) (7), consistent with the binding characteristics of RAR-RX. DNA binding and translocation studies confirmed that these sites included two overlapping retinoic acid response elements (RARE), which were recognized by RAR-RX heterodimers and functioned as positive activators of CAT gene expression in transient transfection assays. Those findings and previous studies (25) implicated this Alu-RARE in the regulation of the keratin K18 gene. Alu elements with encoded HREs could thus represent mobile enhancers containing recognition sites for RARs or other types of nuclear receptors. Random insertion of hundreds of thousands of these elements during primate evolution is likely to have affected the regulation of numbers of genes and may have represented an important source of genomic plasticity. To obtain evidence as to the extent to which Alu-HREs regulate neighboring genes, a literature search was performed that revealed a number of Alu elements with potential regulatory function, one being upstream of the human myeloperoxidase gene (MPO) (26). The MPO gene is expressed specifically in the myeloid lineage (27). The encoded myeloperoxidase enzyme is localized in lysosomal vesicles and catalyzes the reaction between hydrogen peroxide and chloride ions to form hypochlorous acid, a potent antibacterial agent and generator of oxidizing free radicals (28, 29). The MPO gene is transcribed in the early stages of myeloid cell

*This study was funded by National Institutes of Health Grant RR09118-09 (to W. R.). 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.

†Supported by a postdoctoral fellowship from the Spanish Ministry of Education and Science.

§To whom correspondence should be addressed: Sidney Kimmel Cancer Center, 3099 Science Park Rd., Suite 200, San Diego, CA 92121. Tel.: 619-450-5990 (ext. 236); Fax: 619-450-3251.

The abbreviations used are: HRE, hormone response element; RA, retinoic acid; RAR, retinoic acid receptor; TR, thyroid hormone; CAT, chloramphenicol acetyltransferase; AML, acute myelocytic leukemia(s); APL, acute promyelocytic leukemia(s); TRE, T3 response element; RXR, retinoid X receptor; RARE, retinoic acid receptor element; PCR, polymerase chain reaction; DR, direct repeats; bp, base pair(s).
was assayed by a standard phase extraction method using \[3H\]acetyl
dase gene is expressed in all six subtypes of AML, the levels of
through M6, distinguishable by cytochemical, morphological,
among the major leukemias and are subdivided into six subtypes, M1
or monocytes/macrophages (27, 30–33).

Acute myelocytic leukemias (AML) account for 46% of all
major leukemias and are subdivided into six subtypes, M1
MPO

A single A/G base transition within the Alu element preced-
ing the MPO gene was previously reported to be associated
with most cases of AML (26). We noted that this base difference
was within the HRE region, and the AML-associated residue
resulted in an improved fit to the half-site consensus sequence.
This raised the possibility that this base transition enhanced
binding by a nuclear receptor or other transcription factor,
altering MPO gene expression in a way that might potentiate
the development of AML. To investigate this possibility, we
tested the ability of several nuclear receptors to bind these
elements and activate transcription in vivo. Our results indi-
cate that the MPO-associated Alu includes a RARE as well
as a T3 response element (TRE). Interestingly, a strong binding
site for the general transcription factor SP1 is created by the
single base difference associated with acute myelocytic
leukemia.

RESULTS

The Alu Preceding the MPO Gene Contains a Composite HRE
with Four Half-sites Arranged as Direct Repeats—The DNA
sequences preceding the MPO gene (26, 46) include a 300-bp
Alu element situated between positions −200 and −505 (Fig.
1A). A previous report suggested that a G residue at position
−463 was associated with AML more often than an A residue
(Fig. 1B). That site is within a region of four potential hexamer
half-sites, related to the consensus AGGTC(A), located between
positions −436 and −467, with the fourth half-site overlapping
the B box internal promoter element of the Alu gene (Fig. 1C).
All four half-sites are oriented as direct repeats with the first
two separated by two base pairs (DR-2), typical of RARE; the
second and third half-sites are DR-4, consistent with TRE, and
the third and fourth half-sites are again DR-2 (Fig. 1C). The
third and fourth half-sites fit the consensus HRE sequence
(Fig. 1D), while the first and second half-sites are nonconsen-
sus at positions four and three, respectively. The G/A base
difference associated with AML is at position 5 of the first
potential half-site, and the G residue creates a better fit to the
HRE consensus (19).

Acute Myelocytic Leukemias Are Predominantly Homozygous
for a G at Position −463—The A/G transition at position −463
in acute myelocytic leukemias has been suggested to arise by
somatic mutation (26). Alternatively, this could represent
allelic polymorphism in the population, with a preference for
the G allele in leukemic cells. To distinguish between these
possibilities, we analyzed the sequences of DNA isolated from
lymphocytes from 18 AML patients and 44 control blood donors.
Nested oligonucleotide primers were designed to allow PCR
amplification of sequences extending from −829 to +310 of the
MPO gene. Sequence analysis showed that 50% of the normal
donors (22 of 44) were homozygous for the G residue at position
−463, 18 were heterozygous, and 4 were homozygous for the A
residue, indicating an allelic polymorphism. Examination of
the DNA sequences from the AML samples revealed that 13 out
of 18, or 72%, were homozygous for the G residue at −463,
indicating a preference for that allele. This preference was

MATERIALS AND METHODS

DNA Plasmids—The oligonucleotides encoding the different re-
sponse elements from the Alu sequence were annealed and inserted
upstream of the CAT gene in the pBluescript vector between the Sall
and XbaI sites (41). One copy of each response element was present
as verified by DNA sequencing. The receptor expression vectors used in
these studies, pECE-RARα, pECE-RXRα, and pECE-T3Rα, have been de-
scribed elsewhere (Ref. 20 and references therein).

Cell Culture and Transient Transfection—CV-1 cells (106 cells/well)
were plated in 96 well plates in Dulbecco’s modified Eagle’s medium
supplemented with 10% fetal calf serum, 2 mM glutamine, and penicil-
lin/streptomycin. The cells were transfected 24 h later by the calcium
phosphate precipitation method. The transfection mixture contained
24 h and 50-

When antibodies were used, proteins were mixed with 1 μg of non-
immune serum, polyclonal α-RXR (kindly donated by A. Lombardo
and K. Ely), or α-Sp1 (Santa Cruz) for 30 min on ice before incubation
with the DNA. In the competition experiments, the indicated amounts
of nonlabeled oligonucleotides were added to the binding reaction
with the poly(dI-dC).

Polymerase Chain Reaction (PCR)—PCR was performed with 200 ng
of genomic DNA isolated from peripheral blood lymphocytes or bone
marrow lymphocytes, with 0.5 μg of each primer in a 50-μl reaction
volume containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl2,
200 μM nucleotides, and 2.5 units of Taq polymerase (Perkin Elmer
Cetus). Nested primers (Life Technologies, Inc.) were synthesized to
amplify a region extending from position −310 within the myeloperox-
dase gene to position −829 bp. The first primer set was 5′-CTTGGTC-
CTGGCCCAACAGTCCC-3′ and 5′-TCCACCTTTGGAACGTACAC-
CTG-3′. A previous report suggested that a G residue at position
−463 was associated with AML more often than an A residue
(Fig. 1B). That site is within a region of four potential hexamer
half-sites, related to the consensus AGGTC(A), located between
positions −436 and −467, with the fourth half-site overlapping
the B box internal promoter element of the Alu gene (Fig. 1C).
All four half-sites are oriented as direct repeats with the first
two separated by two base pairs (DR-2), typical of RARE; the
second and third half-sites are DR-4, consistent with TRE, and
the third and fourth half-sites are again DR-2 (Fig. 1C). The
third and fourth half-sites fit the consensus HRE sequence
(Fig. 1D), while the first and second half-sites are nonconsen-
sus at positions four and three, respectively. The G/A base
difference associated with AML is at position 5 of the first
potential half-site, and the G residue creates a better fit to the
HRE consensus (19).

Acute Myelocytic Leukemias Are Predominantly Homozygous
for a G at Position −463—The A/G transition at position −463
in acute myelocytic leukemias has been suggested to arise by
somatic mutation (26). Alternatively, this could represent
allelic polymorphism in the population, with a preference for
the G allele in leukemic cells. To distinguish between these
possibilities, we analyzed the sequences of DNA isolated from
lymphocytes from 18 AML patients and 44 control blood donors.
Nested oligonucleotide primers were designed to allow PCR
amplification of sequences extending from −829 to +310 of the
MPO gene. Sequence analysis showed that 50% of the normal
donors (22 of 44) were homozygous for the G residue at position
−463, 18 were heterozygous, and 4 were homozygous for the A
residue, indicating an allelic polymorphism. Examination of
the DNA sequences from the AML samples revealed that 13 out
of 18, or 72%, were homozygous for the G residue at −463,
indicating a preference for that allele. This preference was
The M3 subclass produces the highest levels of most pronounced in the M3 and M4 subclasses (6 of 7, or 85%).

**Fig. 1.** The promoter region of the MPO gene contains a potential hormone response element embedded in an Alu sequence. A, schematic representation of promoter sequences preceding the MPO gene (26). An Alu sequence extends from position −505 to −200 and includes four potential half-sites, related to the RAR/TR binding motif, located between positions −467 and −436 (HRE). B, the allele correlated with acute myelocytic leukemia contains a G residue at position −463 and is designated AML. The allele designated MPO contains an A residue at that position (*). C, dinucleotides used in this study. MPO and AML refer to dinucleotides containing the four potential binding sites (numbered 1–4) and differing by a single G/A transition (shadowed) at position 5 of the first motif. The arrows show the position and orientation of the AGGCTA-like motifs, which appear in bold. These are arranged as DR-2 or DR-4 as indicated. MPO34 and MPO23 contain the 3′-DR-2 and the central DR-4, respectively. The 5′-DR-2 elements are referred to as MPO12 or AML12 and contain the G/A base difference. The artificial restriction sites are indicated in lower case letters. The B box promoter element is underlined, overlapping the fourth motif. D, the consensus sequence recognized by nuclear hormone receptors as deduced from natural sites and in vitro binding studies using synthetic dinucleotides (19).
thyroid hormone receptor (TRα) and RXR in the presence or absence of thyroid hormone (T3). Under these conditions, the MPO23 element activated transcription by 7-fold in the presence of TR/RXR and T3, indicating the MPO23 element can function as a positive TRE (Fig. 2B).

RAR/RXR and TR/RXR Bind to the Alu Sequences—We analyzed the binding of several nuclear hormone receptors to the MPO-Alu sites using a gel retardation assay. In vitro translated proteins were incubated with a 32P-labeled oligonucleotide containing all four MPO half-sites in the presence or absence of in vitro synthesized RXRα with the 32P-labeled oligonucleotides MPO (A), MPO23 (B), or MPO34 (C). As control for nonspecific binding, non-programmed reticulocyte lysate (rrl) was used in all the experiments. Heterodimeric complexes of RXR with TR or RAR were observed (indicated with a bracket in A). ARP-1 bound DNA independently of RXR. The arrow indicates the position of TRα monomer, which increased slightly in mobility and intensity in the presence of T3. D, RAR-RXR do not bind to the 5'-DR-2, MPO12, or AML12. Binding of RAR-RXR was detected with the AML sequence containing all four half-sites (arrow) but not with AML12 or MPO12. Arrowheads indicate nonspecific binding. The film was overexposed in an attempt to observe binding to MPO/AML12.

found with the oncoprotein v-ErbA, a mutated form of TRα, but not with the TRα variant, TRα2. Binding by these receptors was observed with the presence of RXR, consistent with previous studies (18, 20, 47). The ARP-1 receptor, bound independently of RXR and TRα monomer, was also found. Binding to AML1234 was also investigated, and identical patterns were observed (data not shown).

To determine which of the DR elements are recognized by these various receptors, the elements were tested as individual dimer sites in gel retardation experiments. The central DR4 element, MPO23, formed a strong complex with TRα, TRβ, and v-ErbA, all requiring RXR (Fig. 3B). TRα also formed a rela-
tively weak monomer complex in the absence of RXR, and the mobility and intensity of this complex increased in the presence of 100 nM T3. ARP-1 bound independently of RXR, while RARα and TRα2 failed to bind (Fig. 3B). Conversely, the DR2 element, MPO34, formed a complex with all three isoforms of RAR, all requiring RXR, but did not bind TRα (Fig. 3C). The ARP-1 receptor bound in the presence or absence of RXRα.

These findings are consistent with the known binding preferences of RAR-RXR for DR-2 elements and TR-RXR complexes for DR-4 elements.

Surprisingly, the DR2 elements, AML12 and MPO12, did not form a complex with in vitro synthesized RARα-RXR (Fig. 3D) nor with the other isoforms, RARβ and RARγ (data not shown). This was unexpected since the AML12 element is a strong transcriptional activator of the CAT reporter gene in transfection assays and produced the highest level of transactivation in the presence of RAR-RXR expression vectors and RA (Fig. 2A). Other nuclear hormone receptors (ARP-1, TRs) were assayed and also failed to bind this potential DR-2 element (data not shown).

Nuclear Extracts Contain Proteins Which Bind to the AML12 Element—Because AML12, and to a lesser extent MPO12, activates transcription of a CAT reporter gene in transient transfection assays (Fig. 2A), transcription factors recognizing these elements presumably exist in CV-1 cells. To test for such factors, nuclear extracts were prepared from cell lines including CV-1, NB4, CaSki, and HeLa, treated or untreated with 1 μM RA (Fig. 4A). These extracts were incubated with 32P-labeled AML12 and MPO12 elements, and the protein-DNA complexes were analyzed by electrophoretic mobility shift assays. Four complexes, termed I–IV, were observed with the MPO12 element, most clearly seen with the CaSki nuclear extracts. With AML12, only three of these complexes, I, II, and IV, were observed. The amount of complexes I, II, and IV formed was severalfold greater with the AML12 element than with MPO12, indicating the single base difference in the first half-site results in higher affinity binding. This single base difference also results in specific binding by complex III to the MPO12 element. Treatment with RA had no effect on the efficiency of complex formation with extracts from CV-1, CaSki, and HeLa cells. However, in the AML-M3-derived cell line, NB4 (42), RA treatment resulted in a strong induction of complex III to MPO12, and also binding of complexes I, II, and IV to AML12 was increased.

To test whether complexes I, II, III, and/or IV contain RAR or RXR, competition studies were performed. Binding of HeLa nuclear proteins to the AML12 element was competed by a 250-fold excess of unlabeled oligonucleotides containing AML12 or MPO12 sequences (Fig. 4B), but binding was not competed by the MPO34 sequence, which binds RAR-RXR heterodimers (Fig. 4B and 3C). This argues that complexes I, II, and IV do not contain RAR-RXR heterodimers. Incubation of the labeled AML12 element with HeLa nuclear extracts produced a complex with mobility equivalent to that of in vitro synthesized RARα and RXRα. This complex was not competed by an excess of unlabeled AML12 or MPO12, further indicating these elements do not bind RAR-RXR. As further evidence, we investigated the effect of several antibodies raised against RXRα, RARαβγ, TRαβ, and COUP. The binding of nuclear extracts to AML12 and MPO12 was not affected by incubation with any of these antibodies (data not shown), further confirming the absence of those receptors in the complexes observed with nuclear extracts.

The AML12 Element Contains an SP1 Binding Site—Examination of the AML12 sequence revealed a perfect match to a 10-base pair consensus binding site for the general transcrip-
To gain further evidence for the presence of SP1 in complex I, we tested the effect of anti-SP1 antibody on binding by purified SP1 protein and HeLa nuclear extracts (Fig. 5B). In both cases, the αSP1 antibody inhibited complex I formation and produced a supershifted complex. Complexes II and IV were also inhibited, although not as completely, suggesting these complexes may also contain an SP1-like protein. As controls, an antibody directed against RXR and non-immune serum failed to inhibit complex formation. These findings indicate that proteins in complex I, and to a lesser extent complexes II and IV, are antigenically related to SP1. Complex III, which specifically binds to MPO12, did not immunoreact with the anti-SP1 antiserum (data not shown).

As another means to assay for the presence of SP1 protein in complexes I, II, and IV, the complexes were competed with unlabeled oligonucleotides containing the AML12 sequence or the consensus SP1 binding site (Fig. 5C). The SP1 oligonucleotide specifically and completely competed with proteins in complex I as well as complexes II and IV. The SP1 consensus was in fact a more efficient competitor than the AML12 sequence. A nonspecific oligonucleotide, with no nuclear receptor or SP1 binding sequences, was unable to compete for binding of any of these complexes (not shown). As further evidence that the MPO12-specific complex III is not SP1 related, binding was not competed by an excess of cold SP1 oligonucleotide (data not shown). These findings indicate that proteins in complexes I, II, and IV, but not complex III, are SP1-like in their DNA binding preference. Complexes II and IV may be degradation products or other related proteins, since several SP1-related proteins have been identified.

To further compare the binding characteristics of nuclear proteins in complexes I, II, and IV with SP1 protein, mutations were introduced into the AML12 and MPO12 elements (Fig. 6A). In the mutant oligonucleotide A1m, four nucleotides within the first potential half-site and the SP1 core consensus were changed from GGCG to TTAT. This resulted in complete loss of binding by the purified SP1 protein and loss of complexes I, II, and IV from HeLa and NB4 nuclear extracts. In mutant A2m, changing four bases within the second half-site, outside the SP1 binding site, did not alter complex formation by SP1 or the nuclear extracts. Mutant A3m contained two base changes, one within the core SP1 site and one in the second half-site of the DR-2, converting the first two potential half-sites to the consensus HRE sequence; these base changes abolished binding by both SP1 and nuclear extracts. Another mutant, A4m, had one base change in the spacer between the two half-sites and within the 10-base pair SP1 consensus. This mutation severely reduced binding by both SP1 and nuclear proteins. The observation that this series of mutations similarly affected binding efficiency of SP1 and complexes I, II, and IV argues that the latter contain SP1 or proteins related to SP1.

To test whether abolition of SP1 binding activity correlates with loss of AML12 transcriptional enhancer activity, we tested the A1m mutant in transient transfection experiments using CV-1 cells (Fig. 6B). The four base changes in the core SP1 binding site in mutant A1m, which abolished SP1 binding, also negated activation of the CAT reporter gene. This finding suggests that SP1 plays a major role in the transcriptional activation conferred by the AML12 element.

The Response Elements in the MPO-Alu Activate Transcription in Myeloid Cells—Since the myeloperoxidase gene is specifically activated by guest on July 22, 2018http://www.jbc.org/ Downloaded from
Specifically expressed in myeloid cells, we assayed the ability of the AML response element to activate gene expression in the acute promyelocytic leukemia cell line, NB4 (42) (Fig. 7). When NB4 cells were transfected with the different CAT reporters in the absence of cotransfected receptors, AML12 increased CAT gene expression by 6-fold, indicating that endogenous factor(s), possibly SP1, are present and capable of activating transcription through this element. These findings are consistent with gel shift experiments (Fig. 4A) indicating apparently similar, SP1-related complexes formed on AML12 with nuclear extracts from NB4 cells as well as CV-1 and other cell types. Curiously, RA treatment of NB4 cells eliminates the transcriptional activation through AML12, in contrast to results obtained with CV-1 cells (see Fig. 2A). This finding is consistent with the observation that MPO gene expression is down-regulated in differentiating myeloid cells (28–33) and in RA-treated NB4 cells. The unusual and strong RA response of NB4 cells is typical of APL and is thought to be mediated through the aberrant PML-RAR fusion protein. Unlike AML12, the MPO12 element had no significant effect on CAT gene expression with or without RA treatment, indicating the A/G transition in AML12 increases transcriptional activation in myeloid cells, as in CV-1 cells. As for the other Alu response elements, MPO34 activated transcription by 6-fold in the presence of RA and in the absence of cotransfected RARs, suggesting the presence of endogenous RAR and RXR in these myeloid cells. Similarly, MPO23 activated transcription by 6-fold in the presence of T3 and in the absence of cotransfected TRs, indicating the presence of endogenous TRs. These findings demonstrate that the response elements AML12, MPO23, and MPO34 function as transcriptional enhancers in myeloid cells and thus may potentially contribute to the regulation of the MPO gene during myeloid cell development.

**DISCUSSION**

The MPO-associated Alu is of the evolutionarily intermediate subgroup II, which includes the majority of Alu repeats, numbering ~400,000 copies (1, 2, 51). The previously reported keratin K18-associated Alu (7) was of the evolutionarily recent subgroup III–IV and contained a series of three overlapping DR-2 elements (DR-2-2-2), while subgroup II Alus have a series of DR-2-4-2 elements. As for the other Alu response elements, MPO34 activated transcription by 6-fold in the presence of RA and in the absence of cotransfected RARs, suggesting the presence of endogenous RAR and RXR in these myeloid cells. Similarly, MPO23 activated transcription by 6-fold in the presence of T3 and in the absence of cotransfected TRs, indicating the presence of endogenous TRs. These findings demonstrate that the response elements AML12, MPO23, and MPO34 function as transcriptional enhancers in myeloid cells and thus may potentially contribute to the regulation of the MPO gene during myeloid cell development.

**FIG. 6.** Mutations similarly affect binding by SP1 and nuclear extract proteins. A, two concentrations of purified SP1 protein (0.4 and 0.8 µl) or HeLa and NB4 nuclear extracts (5 and 10 µg) were incubated with the indicated 32P-labeled oligonucleotides for 20 min at room temperature, and the complexes were analyzed by gel electrophoresis. The sequences of the different mutated oligonucleotides are shown at top. Half-sites are indicated by arrows and bold letters. Small letters correspond to the artificial restriction sites flanking the natural sequence, and the A/G transition is marked (*). The SP1 site is shadowed in AML12. Mutations in the consensus SP1 site significantly and similarly affected binding by SP1 and the nuclear extract proteins. MPO34 was used as a comparison for the nuclear extract and as a negative control for the SP1 protein. B, mutation of the SP1 site abolishes transcriptional activation by the A12 element. CV-1 cells were cotransfected with 100 ng of CAT reporter constructs containing the indicated response elements along with cotransfected RARα (12 ng) and RXRα (6 ng) expression vectors. After 24 h, cells were treated with 1 µM RA for an additional 24 h. Extracts were then prepared and assayed for CAT activity. The data shown and the S.E. were calculated from three different experiments.
The SP1 motif significantly reduces binding. Consistent with the binding studies, the AML12 site provides a strong 25-fold transcriptional enhancement to a reporter CAT gene in transient transfection assays, while the MPO12 element is severalfold less effective.

The subgroup II Alu sequences can be thought of as mobile cassettes of regulatory elements, containing RARE and TRE, in some cases overlapping an SP1 site. These sites are present in the Alu consensus sequences thought to represent the source gene sequence (1-3), implying these RARE/TRE sites existed prior to dispersal of the progeny retrotransposons, thus prior to the insertion of the Alu upstream of the MPO gene. Since the RARE/TRE/SP1 sites did not arise in response to selective pressures related to MPO gene regulation, these sites may not all contribute significantly to the regulation of the MPO gene. The SP1 site probably has important influence on MPO gene regulation because this sequence has improved to become a 100% match for the 10-bp SP1 consensus, as compared to 8 or 9 out of 10 matches in most Alu elements of this subfamily. Also, two alleles, with significantly different SP1 binding capability, are differentially associated with cases of acute myelocytic leukemias, suggesting this SP1 site contributes to MPO gene regulation and does so in a manner conducive to development of this leukemia.

Two alleles of the MPO gene differ at one position within the first half-site in the upstream Alu-HRE. Most AML patients are homozygous for the AML allele, which has a G residue at position five of the first half-site. This single base transition creates the strong SP1 binding consensus within AML12, which is correlated with a 25-fold transcriptional enhancement of a CAT reporter gene in transient transfection assays. The other allele has an A residue at position 5, which negates the SP1 binding site, and this element (MPO12) confers a much weaker transcriptional advantage to a reporter CAT gene. Since SP1 is a positive transcription factor, this suggests that the AML allele enhances MPO gene expression, and this expression somehow potentiates the leukemic phenotype. The MPO gene is normally transcribed in early myeloid cells (myeloblasts and promyelocytes). MPO gene expression ceases when these cells differentiate into monocytes or granulocytes, although the MPO enzyme remains stored in cytoplasmic vesicles (27, 30-33). AML cells represent clonal expansions of early myelocytes, which have lost the ability to differentiate in response to normal cellular signals, and thus continue to express the MPO gene. Clearly, the SP1 site in the AML allele could promote MPO gene expression, but it is unclear why enhanced MPO gene expression should be linked to the leukemic state. The MPO enzyme, when released by granulocytes or monocytes, catalyzes the reaction of chloride and hydrogen peroxide to yield hypochlorous acid, a strong oxidant. In the presence of superoxide, released by macrophages, hypochlorous acid generates hydroxyl radicals (29), which react with most biological molecules, creating secondary radicals of variable reactivity. There is evidence that production of oxygen radicals by monocytes inhibits the natural killer cell immune response (52). If so, higher expression of the MPO enzyme by leukemic cells carrying the AML allele might enable those cells to preferentially escape immune surveillance, which could explain why fewer AML patients carry the MPO allele. Another possibility is that free radicals produced by the MPO pathway result in DNA damage leading to the leukemia; myeloperoxidase has been linked to inflammation-associated cancers through DNA damage (53, 54) and the production of carcinogens (55, 56).

With the exception of the myeloid leukemic cell lines NB4 and HL60, it would be difficult to obtain isolates of early myeloid stages representing the in vivo stages of these leukemic cells. Therefore, it is difficult to show directly whether the potent RARE represented by M34 influences MPO gene expression in vivo in these early myeloid stages. There are, however, several lines of evidence indicating that RARα plays an important role in myeloid cell differentiation and in the acquisition of acute myelocytic leukemia. First, retinoic acid induces NB4 (M3 class) and HL60 (M2 class) cells to differentiate. A retinoic acid-resistant variant of HL60 cells was found to have a point mutation in the ligand binding domain of the RARα gene (57). Introduction of wild type RARα allowed this mutant cell line to differentiate in response to RA (58). Second, a chromosomal breakpoint in the RARα gene causes M3-AML or APL, presumably mediated by the PML-RARα fusion protein. Treatment of APL cells with RA induces rapid differentiation and loss of cellular proliferation (40). As further evidence for the involvement of RARα in the acquisition of AML, a second translocation t(11;17), which causes AML, also interrupts the RARα gene, fusing it to the Kruppel-related gene, PLZF (59). Thus, disruption of RARα function is linked to the inability to differentiate, resulting in maintenance of the undifferentiated myeloid state in which the MPO gene is expressed.

The AML12 element is not an RAR recognition site but does enhance transcription of the CAT gene most effectively in CV-1 cells in the presence of cotransfected RAR-RXR and RA, apparently by indirect means. In contrast, in NB4 cells, RA treatment results in loss of transcriptional activation by AML12, coincident with loss of MPO gene expression3 and loss of cellular proliferation. These findings suggest that AML12 enhances transcription of MPO, in a manner influenced by RA and its receptors, and abrogated by the PML-RAR induced differentiation process.

AML12 does not appear to represent a binding site for PML-RAR. Complexes I, II, and IV, which form on AML12, are observed not only in nuclear extracts of NB4 cells but also in extracts from CV-1, HeLa, and CaSki cells, which lack the PML-RAR fusion protein (Fig. 4A). As further evidence, antisera against RARα, which recognizes the PML-RAR fusion protein, failed to supershift or inhibit binding of complexes I, II, III, or IV to either the AML12 or MPO12 elements (data not shown).

An understanding of the complex, overlapping nuclear receptor/SP1 binding sites in the Alu elements will be important for determining which of the highly abundant Alu elements is contributing to the regulation of nearby polymerase II genes. Presumably, most Alu elements will have inserted too distant from genes to have an effect, while other Alu inserts may have had a negative effect, bringing a gene under control of nuclear receptors or SP1 in a way that was deleterious to the organism, and individuals carrying such Alu inserts would have been deleted from the gene pool. Conversely, some Alu insertions may have benefited the organism by bringing particular genes under control of nuclear receptors or SP1, and individuals carrying those insertions would have been retained in the population with a selective advantage. The question then is: which of the hundreds of thousands of Alu inserts are contributing to the regulation of nearby genes, and which are without significant effect?

Acknowledgments—We thank Michelle Lanotte for kindly providing the NB4 cell line and Heli Collins, Robert Sobol, and Fred Saleh for providing blood samples from patients with AML. We also thank A. Lombardo and K. Ely for the anti-RXR antiserum.

REFERENCES

1. Deininger, P. L., Batzer, M. A., Hutchison, C. A., III, and Edgell, M. H. (1992) Trends Genet. 8, 307-311

3 E. A. Orlova and W. F. Reynolds, unpublished results.
An Alu Element in the Myeloperoxidase Promoter Contains a Composite SP1-Thyroid Hormone-Retinoic Acid Response Element
F. Javier Piedrafita, Rachel B. Molander, Gordon Vansant, Elena A. Orlova, Magnus Pfahl and Wanda F. Reynolds

J. Biol. Chem. 1996, 271:14412-14420.
doi: 10.1074/jbc.271.24.14412

Access the most updated version of this article at http://www.jbc.org/content/271/24/14412

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 24 of which can be accessed free at http://www.jbc.org/content/271/24/14412.full.html#ref-list-1