Biochemical and Biophysical Research Communications **269,** 197–202 (2000)
doi:10.1006/bbrc.2000.2242, available online at http://www.idealibrary.com on IDEAL®

Identification of a Novel Retinoic Acid-Responsive Element within the Lamin A/C Promoter

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Received January 14, 2000

A-type lamins are not present in either early embryos or the embryonal carcinoma (EC) cell line. P19 cells, which are EC cell line, are able to express A-type lamins upon retinoic acid (RA) treatment. Here we report that a novel RA-responsive element, termed lamin A/C-RA-responsive element (L-RARE), is located within the lamin A/C promoter. RA activated the luciferase activity of the reporter which had four tandem repeats of the wild-type L-RARE, while a loss of function mutant, which altered CACCCCC to CACtatC within L-RARE, did not respond. Four specific binding complexes of L-RARE, Complexes-A, -B, -C, and -D, were detected in protein extracts obtained from P19 cells treated with and without RA. Specific antibodies revealed that Sp1 and Sp3 were included in Complex-A and Complexes-B and -C, respectively. Thus, L-RARE was important in the RA-mediated activation of the lamin A/C promoter and was recognized by DNA binding proteins.

Key Words: **retinoic acid; transcription; transcription factor; lamin; differentiation; Sp1; Sp3; embryonal carcinoma cell line; retinoic acid-responsive element; promoter.**

The nuclear lamina consists of a protein meshwork that lines the inner side of the nuclear membrane, and its disassembly is a critical step during mitosis (1). The major components of the nuclear lamina are the A- and B-type lamins, which are members of the intermediate filament protein family (2, 3). A- and B-types of lamin are distinguished by a number of criteria. A-type lamin, which subdivided into A and C, binds to chromosome surfaces with specific DNA sequences (4–6). Lamin A might contribute to scaffold of nuclear membrane (7). Moreover, A-type lamin binds to the retinoblastoma gene product, which controlled cell cycle and gene expression (8). On the other hand, B-type lamin, which is subdivided into B1 and B2, mainly binds to the nuclear membrane (9) and associates with replicating chromatin in mammalian cells (10, 11). Lamin B1, in particular, associates with matrix attachment regions, which mediate interaction between matrix and chromatin DNA (12). In addition, the B-type lamin co-localizes with Oct-1, a transcription factor, at the nuclear periphery and might repress the activity of Oct-1 (13). Therefore, A- and B-types of lamin have distinct functions in the nucleus (14).

In most mammalian somatic cells, both types of lamins are expressed. However, type-A lamin is not expressed in early vertebrate embryos (15–18), and the amounts of lamin A and C transcripts are either very low or undetectable in mouse embryonal carcinoma (EC) cells, which are pluripotent stem cells of malignant teratocarcinomas, and a number of murine and human tumor cell lines (18–22). In the presence of retinoic acid (RA) or DMSO, mouse EC cells, such as P19 and F9 cells, differentiate *in vitro* into a variety of cell types (23, 24). P19 and F9 cells express lamin B in both the undifferentiated and differentiated states, whereas lamins A and C, which are alternative splice variants expressed from a single lamin A/C promoter (25, 26), are only expressed in the differentiated state, 48 h after RA treatment (20). The regulation of lamin A/C gene expression is certainly regulated in a differentiation-dependent manner. However, the mechanism and the regulatory factors of lamin A gene expression in RA-induced differentiation of P19 cells are not known.

In this study, we analyzed the promoter region of the lamin A/C gene in P19 cells. Our results show that the lamin A/C promoter has a novel RA-responsive element that binds transcription factors, included Sp1 and Sp3. Thus, we propose that a novel RA-responsive element are involved in regulation of the lamin A gene expression during RA treatment.

MATERIALS AND METHODS

Cell cultures and retinoic acid treatment. P19 mouse embryonal carcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were then were

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0006-291X/00 $35.00

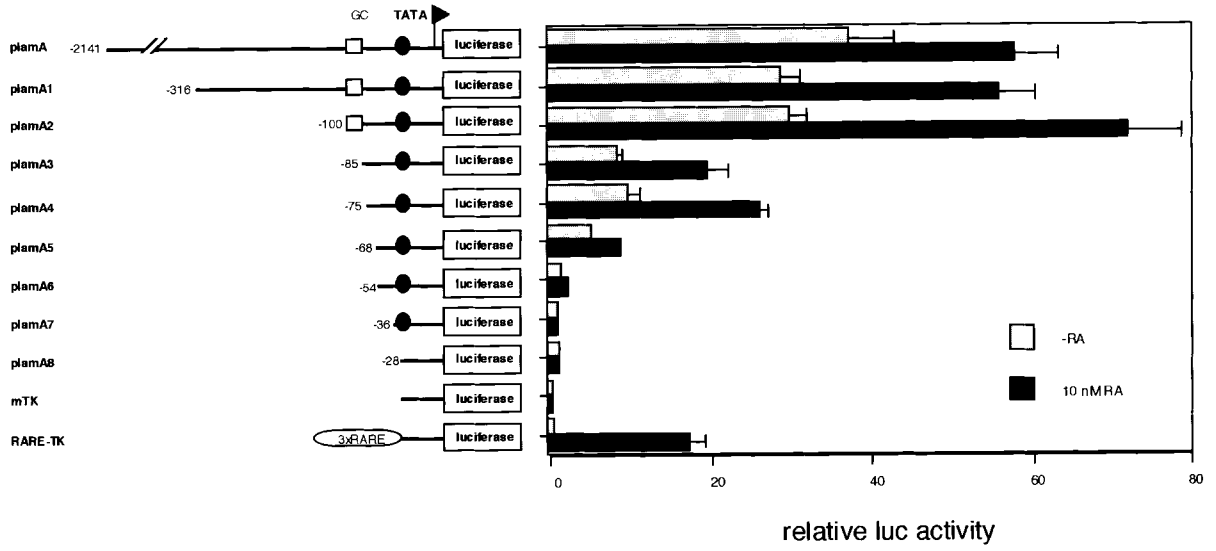

FIG. 1. Deletion analysis of the lamin A/C promoter. The lamin A/C promoter deletions were fused to the luciferase reporter gene. TATA represents the lamin A/C TATA box, located 27 bp upstream from the transcriptional start site (defined as +1). pTK-luc and p3xRARE-luc were used as negative and positive controls, respectively. Transfection analyses in P19 cells were carried out with various reporter constructs and with pRL-TK, which expresses Renilla luciferase, as an internal expression control. These cells were cultured with (black bar) or without (gray bar) RA treatment for 48 h. Relative luciferase activity is indicated in arbitrary units normalized to the activity of pRL-TK. Means and standard deviations for six independent experiments are shown.

exposed to 10 nM retinoic acid, solvent (ethanol) or 0.5% DMSO for 48 h.

Transfection and luciferase assays. The various luciferase (luc) reporters, which were fused to lamin A promoter deletion mutants, were constructed (Fig. 1). p4xL-RARE-, p4xM1-, and p4xM2-TK-luc were derived from pTK-luc, which has a tk minimal promoter (27). p3xRARE-luc, which has three RARβ binding sequences, was used as a positive control for RA treatment (28). The expression plasmid of the dominant negative RARβ was from Dr. K. Umesono (29). For transient transfection, P19 cells (5×10^4 cells per well in a six-well plate) were seeded the day before transfection. Cells were incubated with a mixture of DNA and Lipofectamine-Plus reagents (GIBCO BRL) for 5 h. The DNA mixtures contained both 200 ng luciferase plasmids as a reporter and 10 ng pRL-TK, which has Renilla luciferase, as an internal control. The cell growth media were then changed to complete media containing either all-trans RA (10 nM, Sigma), solvent (ethanol) or 0.5% DMSO, and the cells were incubated for an additional 48 h. The luciferase activity was normalized to the internal control activity.

Electrophoretic mobility shift assay (EMSA). P19 cells (1×10^8) were harvested and centrifuged at 250*g* for 10 min. The cells were washed in 5 volumes of buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF] and were then kept on ice for 10 min. After suspension in 3 volumes of buffer A, Nonidet P-40 (NP-40) were added to a 0.05% concentration, and the cells were homogenized with 20 strokes of a tight fitting Dounce homogenizer. The nuclear pellet was suspended in 1 ml buffer C [5 mM Hepes (pH 7.9), 26% glycerol (v/v), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF] and was incubated on ice for 30 min. After centrifugation, aliquots of the supernatant were used as nuclear extracts for EMSA (30). Double stranded oligonucleotides corresponding to the desired lamin promoter sequences (−64 to −33) were labeled with [^{32}P]dCTP using the Klenow fragment.

L-RARE, 5′-CTAGTGCAGTGGATCCACCCCCTGTAGAGGAGGGCCT-3′

M1, 5′-CTAGTGCAGTGGATCCACCCCCTGTAGCTCGAGGCCT-3′

M2, 5′-CTAGTGCAGTGGATCCACTATCTGTAGAGGAGGGCCT-3′

Binding reactions were performed with 4 μg nuclear extract in a binding buffer [20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg poly(dI–dC), 5% glycerol]. The reaction were incubated for 10 min on ice and then were incubated for an additional 30 min after the addition of 50 fmol of the labeled probe. The reaction mixtures were loaded on 5% polyacrylamide gels in TAE. For competition assays, a 100-fold molar excess of double-stranded oligonucleotides was added to the binding reactions. The GC box oligonucleotide was purchased from Promega. For the supershift experiments, the immuno-reactions were incubated with 1.0 μl of either anti-Sp1, Sp3, or C/EBP antibody (Santa Cruz Biotechnology) for 20 min at 25°C after the binding reactions (30).

RESULTS

RA-responsive element exists in the A-type lamin promoter. The A-type lamin is not present in P19 and F9 cells. However, it is detected in these cells 48 h after RA treatment (20). Using RT-PCR of RNA extracted from P19 cells, we confirmed that the level of lamin A transcripts increased 3.6-fold 48 h after RA treatment. The effect of the RA treatment was also followed using an antibody against Oct3 (data not shown), which has been shown to be expressed only in undifferentiated P19 cells and whose levels decreases upon RA treatment (31).

The functional role of the lamin A/C promoter was investigated by generating truncated versions of the promoter and comparing basal and RA induced activities. We designed a luciferase reporter plasmid with 2.2 kb (+48 to −2141) of the promoter region of lamin A/C, plamA-luc and then made a series of deletion mutants based on that plasmid (Fig. 1). The basal level of luciferase activity was significantly decreased to

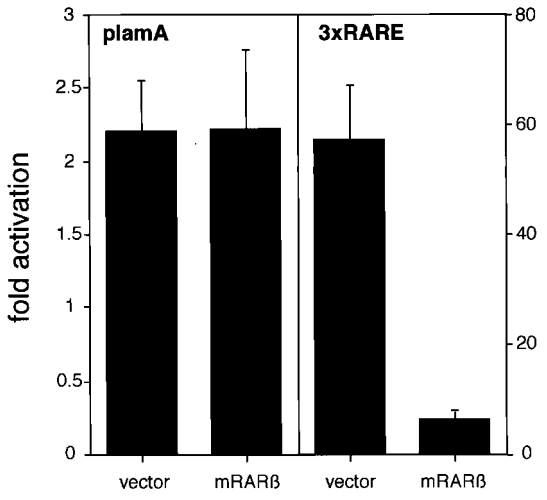

FIG. 2. Effect of the dominant negative mutant of RARβ. The plamA-luc (plamA) and 3xRARE-luc (3xRARE) were transfected with the dominant negative mutant of RARβ (mRARβ). p3xRARE-luc was used as a positive control of mRARβ activity. Transfection analyses in P19 cells were carried out with both reporter constructs and pRL-TK, as an internal expression control. These transfected cells were cultured with (black bar) or without (gray bar) RA treatment for 48 h. Values, which are expressed relative to no RA treatment for each plasmid as 1.0, are expressed as the means (bars represent SD) obtained from three independent experiments.

about 22, 5, and 2% of that of the wild type promoter in the deletion mutants plamA3-luc, plamA6-luc, and plamA7-luc, respectively. In other words, the regions −100 to −85, −68 to −54, and −54 to −36 from the transcriptional start site are control regions for the basal activity of the lamin A/C promoter. The region from −100 to −85 has a GC box, which is potentially recognized by Sp1 family proteins (26). The region from −68 to −54 had no resemblance to previously reported elements. The region from −54 to −36 has a CACCC box, a known Sp1 binding site (30, 32, 33).

Comparison of the level of RA stimulation of promoter activity identified a novel RA response element. In the full-length promoter, there was an approximately 1.6-fold increase from basal promoter activity. Deletion mutants, from plamA1-luc to plamA6-luc, were also stimulated by RA treatment with 1.6- to 2-fold increases in luciferase activity. However, further deletion to plamA7-luc abolished any response to RA, although there were still significant levels of basal expression. Furthermore, dominant negative form of the retinoic acid receptor (RAR) β did not inhibit the induction of plamA with RA treatment, while it strongly repressed the induction of 3xRARE-TK luc, which has RARβ binding sequence (Fig. 2). Thus, the region between nucleotides −54 and −36 appears to be critical for RA stimulation of promoter activity without RARβ and has been designated Lamin A/C-RA-responsive element (L-RARE).

Characterization of the RA-responsive element of the lamin A promoter. To identify the minimum sequence for the RA-response, we constructed point mutations within the plamA2-luc plasmid (Fig. 3). Of four constructs, the basal luciferase activities were significantly decreased by three mutations, which were plamA2-1-luc (changed from CCCGCCCC to CCtcgagC; −97 to −93), plamA2-3-luc (AAGAGCAGT to ActcgAGT; −67 to −64), and plamA2-4-luc (CACCCCC to CACtatC; −51 to −49) and slightly reduced in the case of the plamA2-2-luc mutation (CTTTAAGAG to CTTctcGAG; −70 to −68), in agreement with the results from the deletion mutants (Fig. 1). However, whereas most of the mutants showed a 2.2-fold stimulation by RA treatment, only plamA2-4-luc had reduced RA-inducible activity to 1.3-fold (Fig. 3). Thus, the region from −51 to −49 was critical for RA stimulation.

To further elucidate whether L-RARE is sufficient for RA stimulation, we constructed and analyzed four tandem DNA repeats of the region −65 to −33 fused to the tk minimal promoter (Fig. 4A) along with two mutants, M1 and M2, mutated at regions −42 to −38 and

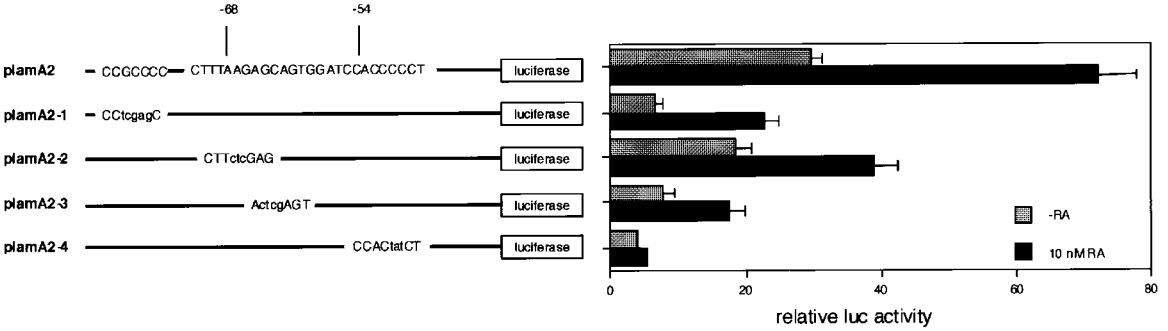

FIG. 3. Mutation analysis of the lamin A/C promoter. The four mutants were identical to the wild type plamA2-luc with the exception of the sequences shown for each mutant construct. Transfection analyses were carried out with various reporter constructs and pRL-TK, as an internal expression control. P19 cells were cultured with (black bar) or without (gray bar) RA treatment for 48 h after transfection. Relative luciferase activity is indicated in arbitrary units normalized to the activity of pRL-TK. Means and standard deviations for four independent experiments are shown.

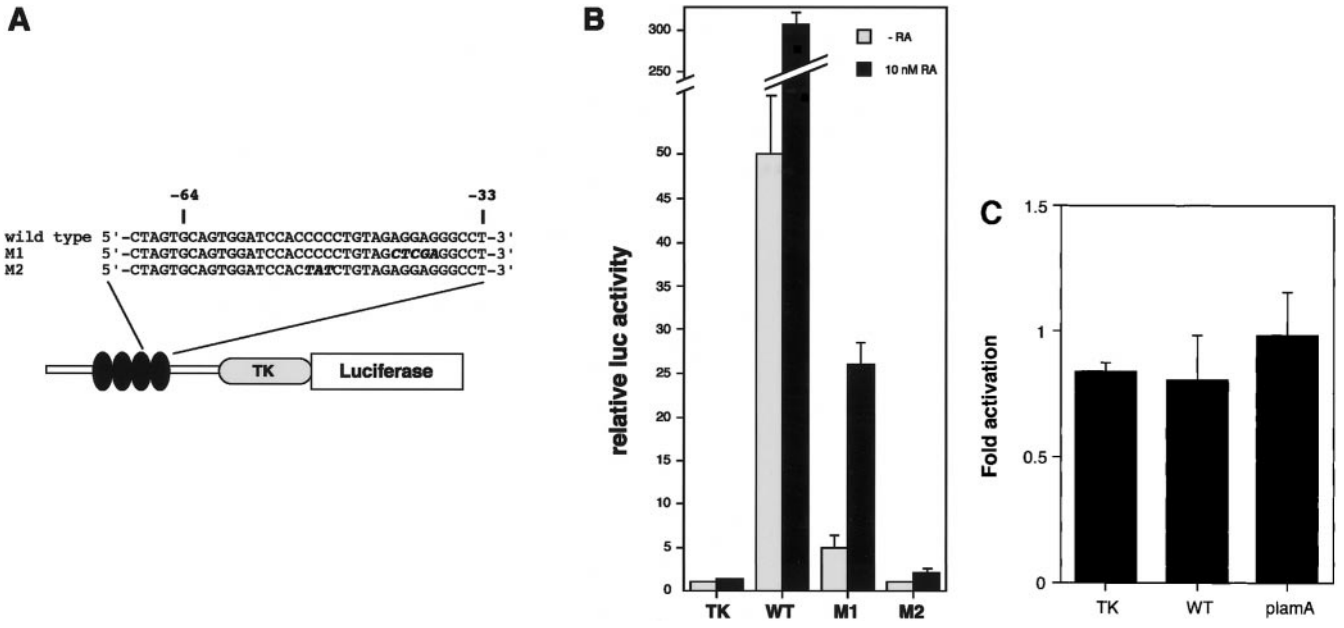

FIG. 4. Identification of the RA-responsive element of lamin A/C. (A) Schematic diagram and insert sequences of luciferase reporter plasmids, which are the tk minimal promoter bearing four tandem repeats of L-RARE or derivatives thereof, L-RARE and its mutants are indicated by closed ellipses. (B) Effect of L-RARE on the tk minimal promoter with RA treatment. The reporter plasmid: pTK-luc (TK), p4xL-RARE-TK-luc (WT), p4xM1-TK-luc (M1), and p4xM2-TK-luc (M2) were transfected with pRL-TK into P19 cells. P19 cells were cultured with (black bar) or without (gray bar) RA treatment for 48 h after transfection. Values, which are expressed relative to no RA treatment for TK plasmid as 1.0, are expressed as the means (bars represent SD) obtained from four independent experiments. (C) Effect of L-RARE with DMSO treatment. pTK-luc (TK), p4xL-RARE-TK-luc (WT) and plamA-luc (plamA) were transfected with pRL-TK into P19 cells. Values, which are expressed relative to no DMSO treatment for each plasmid as 1.0, are expressed as the means (bars represent SD) obtained from three independent experiments.

−51 to −49, respectively. In basal level, the wild type L-RARE activated transcription in comparison with the activity of tk minimal promoter and M1 decreased the activity of L-RARE, while M2 did not show any activity (Fig. 4B). Thus, L-RARE acts as an enhancer in undifferentiated P19 cells.

Whereas the tk promoter alone did not respond to RA, the one containing the wild type L-RARE was stimulated 6-fold upon RA treatment, demonstrating that L-RARE is a RA-responsive element (Fig. 4B). The M1 mutant showed a similar level of activation. However, the lack of response by the M2 mutant clearly shows the importance of the CCC sequence in the region from −51 to −49. Furthermore, the wild type L-RARE reporter was not activated with DMSO treatment (Fig. 4C). Therefore, we concluded that the CCC sequence of L-RARE is critical for activation of lamin A/C promoter with RA treatment.

L-RARE is recognized by specific binding proteins. To identify L-RARE binding proteins, we performed EMSA using nuclear extracts prepared from P19 cells that were either treated or not treated with RA for 48 h. Four shifted bands were detected from both extracts with ^{32}P-labeled L-RARE as a probe (Fig. 5, lane 2) and were named Complexes-A, -B, -C, and -D. To characterize the proteins of the binding complexes, we carried out an EMSA competition analysis. Complexes-A, -B, and -C had decreased intensities in the presence of a 100-fold excess of the unlabeled wild type and M1 forms of L-RARE (Fig. 5, lanes 3, 5, 8, and 10). Interestingly, these three complexes also competed with GC box, which is recognized by Sp1 and Sp1 family proteins (34) (Fig. 5, lanes 6 and 11). Complex-D was competed with both the wild type and M1 formed of L-RARE (Fig. 5, lanes 3, 5, 8, and 10). M2 had no effect on any shifted band (Fig. 5, lanes 4 and 9), indicating that these complexes are specifically binding to the wild-type sequence, in particular to the region including the CCC (−51 to −49) sequence. It is notable that there was no remarkable difference between the intensities of the shifted bands in RA-untreated and -treated extracts.

Sp1 and Sp3 can bind to the CACCCCC element of L-RARE. Since Complexes-A, -B, and -C could be competed off by an Sp1 binding site (Fig. 5) and since CACCC is recognized by Sp1 family member proteins (30, 32, 33), we tested whether these complexes included Sp1 family proteins using supershift assays with Sp1 and Sp3 antibodies (Fig. 6). The C/EBP antibody was used as a negative control in these assays and did not affect the binding of proteins to L-RARE (Fig. 6, lanes 3 and 7). The finding that Complex-A and

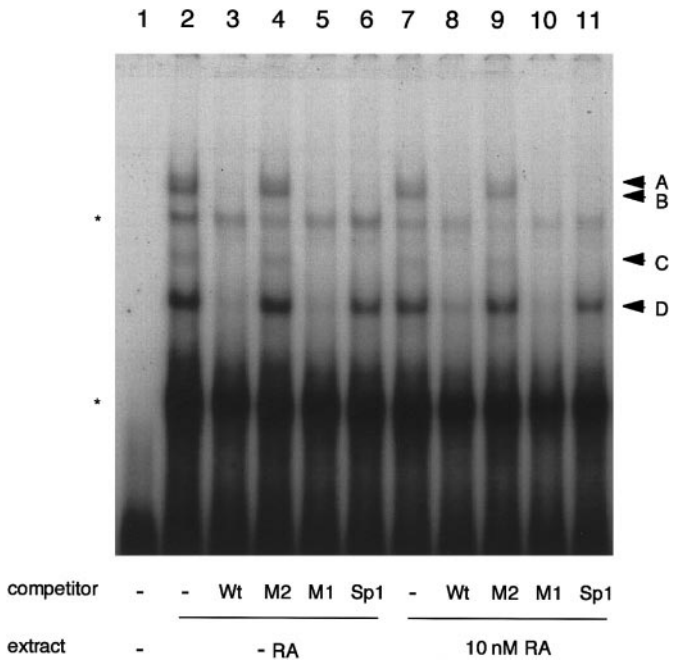

FIG. 5. Comparison of binding proteins prepared from P19 cells with or without RA treatment. Four micrograms of nuclear extracts from RA-treated or untreated P19 cells was incubated with ^{32}P-labeled probes containing L-RARE sequences. The four major bands are indicated as A, B, C, and D. Each competitor was added in a 100-fold excess to each reaction (lanes 3 to 6, and 8 to 11, respectively). Nonspecific complexes are indicated with asterisks on the left side of the panel.

Complexes-B and -C were supershifted by Sp1 and Sp3 antibodies, respectively, indicates that Sp1, Sp3, or related proteins are binding in these complexes. Complex-D was not supershifted by either antibody.

DISCUSSION

In this report, we have characterized the lamin A/C promoter upon RA treatment. We found that the lamin A/C promoter was activated by RA and had a novel retinoic acid-responsive element, which we named L-RARE. Lamin A/C is expressed in most somatic tissues, but not in embryonic cells (15–18). The mechanism of the increase in the lamin A/C mRNA might reflect the use of various regulatory factors related to differentiation. P19 cells are pluripotent cells and can differentiate into various types of cells *in vitro,* after exposure to RA or DMSO (23, 24). By using P19 cells as a model system, it was possible to demonstrate the mechanism underlying the increase in lamin A/C transcription during differentiation. We found that L-RARE, which is located in the region from −54 to −36 on the lamin A/C promoter, responded to RA stimulation but not DMSO stimulation and that the region from −51 to −49 was necessary for this response. This L-RARE sequence, however, is different from any previously described RARE consensus sequence (28). Furthermore, lamin A/C expression does not depend on the activation of retinoic acid receptor (RAR)β, which regulates RA inducible gene expression, because a dominant negative mutant of RARβ had no effect when cotransfected with lamin A/C promoter. Indeed, it is known that the c-jun gene upregulated its gene expression by RAR independent pathway during RA treatment (35). Thus, we conclude that L-RARE is a novel retinoic acid-responsive element, and that the CAC-CCCC region of L-RARE is occupied in the inducible state.

Sp1 family proteins might be important regulators of lamin A/C gene. Four different binding complexes, Complexes-A, -B, -C, and -D, recognized L-RARE in an EMSA. The wild type L-RARE and M1 (mutated in the region from −42 to −38) but not M2 (mutated in the region from −51 to −49) competed with a labeled L-RARE probe for all complexes. Furthermore, using specific antibodies, we determined that Complex-A is composed of Sp1 and Sp1-related proteins, and the

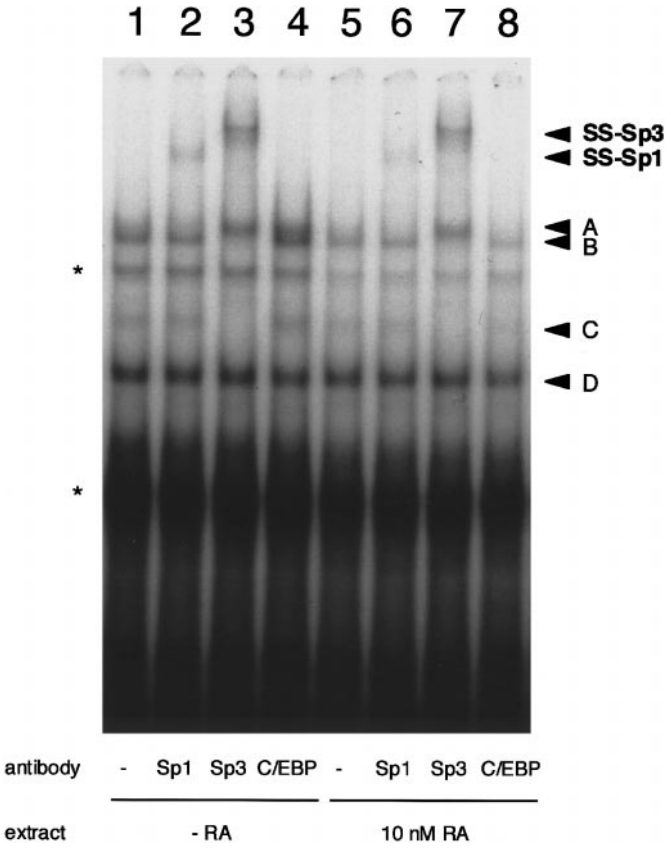

FIG. 6. Involvement of Sp1 family proteins in Complexes-A, -B, and -C. Four micrograms of nuclear extracts from RA-treated or untreated P19 cells was incubated with an end-labeled L-RARE probe. Following the binding reaction, each reaction mixture was incubated with (lanes 2 to 4 and 6 to 8, respectively) or without antibodies to Sp1, Sp3, or C/EBP (lanes 1 and 5). Nonspecific complexes are indicated with asterisks on the left side of the panel.

Complexes-B and -C included Sp3 and Sp3-related proteins. Using reporter assays, we found that the M2 mutant was a loss of function mutant in both with and without RA treatment, and that M1 mutant effected the basal level activity of L-RARE. The M2 mutated region is critical for the DNA binding of four specific complexes and gene expression. Whereas the M1 mutant could not compete with any binding complex in gel shift assays, the function of L-RARE for basal level expression is necessary to the M1 mutated region of L-RARE. The M1 mutated region might have conformational importance for the L-RARE activity in basal level expression. Therefore, these specific complexes, which included Sp1 family proteins, are potentially important factors regulating lamin A/C promoter function with and without RA treatment.

Although the mechanism of the regulation of the lamin A/C promoter in P19 cells stimulated with RA is still not clear, it is clear that L-RARE was important in the RA-mediated activation of the lamin A/C promoter and was recognized by DNA binding proteins *in vitro.*

ACKNOWLEDGMENTS

We thank Drs. H. Hamada and K. Umesono and Mr. Y. Ono for kindly providing some reagents. We also thank Drs. S. Tsutakawa, I. Cann, and T. Yoshinaga for critical reading of the manuscript and helpful discussion. We are very grateful to Dr. Y. Shimura for his constant support and encouragement.

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