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Cell-type-specific interactions at regulatory motifs in the first intron of the lamin A gene

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Abstract Lamins A, C and C2 are alternatively spliced products of the *LMNA* gene; lamins A and C are expressed in differentiated somatic cells, whereas lamin C2 is expressed in germ cells. We have analyzed a segment of the first intron of the *LMNA* gene for cell-type-specific regulatory elements. We identified a 420-bp fragment that increased promoter activity in lamin A-expressing cells but repressed activity in undifferentiated cells. DNase I footprinting and electrophoretic mobility shift assays revealed two binding motifs, footprinted region A (FPRA) and FPRB. The hepatocyte nuclear factor-3β was bound to FPRA only in somatic cell extracts and this motif had an inhibitory effect on promoter activity. The retinoic X receptor β, RXRβ, bound near FPRB with extracts from lamin A- or C2-expressing cells, and this site enhanced promoter activity. We have, thus, identified two novel binding sites for transcription factors in a region likely to function as an important regulatory element for the cell-type-specific transcription of A-type lamins.

Keywords: Nuclear lamina; Lamin A promoter; A-type lamin; Lamin C2; Intronic regulatory element; Gene expression

1. Introduction

The lamins are components of the nuclear lamina, a filamentous network of proteins underlying the inner nuclear membrane. The lamina is considered to be an important determinant of interphase nuclear architecture [1–3], and is involved in the spatial organization of nuclear processes such as DNA replication, transcription and RNA splicing [4–7]. Two major kinds of lamins are present in higher vertebrates. The B-type lamins (B1 and B2) are found in nearly all somatic cells, whereas the A-type lamins (A and C) are expressed primarily in differentiated cells, in a tissue- and stage-specific manner. However, certain cells such as those of the hemopoietic lineage do not express lamins A and C. Lamins A and C are alternatively spliced products of the *LMNA* gene, whereas lamins B1 and B2 are coded by separate genes. Germ cells contain additional splice variants of the lamin A and B2 genes termed C2 and B3, respectively [8–10]. The differential regulation of lamin expression in different tissues has important implications for lamin function, since mutations in the human *LMNA* gene lead to complex disease phenotypes, mainly affecting muscle, cardiac, adipose and bone tissues, and also cause premature ageing (reviewed in [11]).

The expression of the *LMNA* gene is primarily under transcriptional control and is induced upon cell differentiation, being undetectable in embryonic cells [12–14]. We have previously reported the characterization of the proximal promoter of the rat *LMNA* gene which harbors two important motifs, a GC box at −101, which can bind to Sp1/Sp3 transcription factors, and an AP-1 binding motif at −7 [15–17]. The 5′ flanking sequences of the mouse and human *LMNA* genes are highly homologous to the rat sequences, with total conservation of the GC box, TATA-box and AP-1 motif in all three species [18,19].

The developmental regulation of the lamin A promoter is likely to involve a complex series of events that occur in a cell-type-specific manner, and would require specific regulatory motifs such as enhancers and silencers, which might be situated further upstream of the 2.2-kb region that has already been analyzed. Regulatory elements might also be present in the large first intron (∼14 kb) of the gene, as observed with other members of the intermediate filament superfamily [20]. The first intron of the lamin A locus harbors the transcription initiation site for the male germ-cell-specific lamin C2 transcript, after ∼10 kb of intronic sequence [18], which is expressed in pachytene spermatocytes (PS) while lamin A and C transcripts are downregulated during spermatogenesis [8,9]. Although the somatic and germ cell lamins have a different first exon and are likely to be regulated by unique promoters, lamins A, C and C2 share the exons 2–9. Lamin A has distinct exons 10–12 due to 3′ alternate splicing.

As an initial approach towards understanding the cell-type-specific regulation of the rat A-type lamin promoter, we have analyzed a 1.5-kb segment upstream of the lamin C2 translation initiation site for regulatory elements by reporter gene assays of deletion constructs of this region. We have delineated a 420-bp fragment which enhances promoter activity in two differentiated cell lines, but acts as a repressive element in an undifferentiated embryonal carcinoma cell line. DNase I footprinting analysis revealed two protected regions: an AT-rich fragment which harbored a binding site for hepatocyte nuclear factor-3β (HNF-3β) from the winged helix family of transcription factors and another fragment which displayed interactions with the retinoic X receptor (RXR) family of proteins (primarily RXRβ) in electrophoretic mobility shift

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Abbreviations: EMSA, electrophoretic mobility shift assay; HNF-3β, hepatocyte nuclear factor-3β; HFH-1, HNF-3/Fkh homolog 1; NFY, nuclear factor Y; RAR, retinoic acid receptor; RXR, retinoic X receptor; USF, upstream stimulatory factor

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assays (EMSAs). The importance of these motifs was confirmed by mutational analysis.

2. Materials and methods

2.1. Plasmid constructs

A 452-bp fragment encompassing the upstream region of the lamin C2 first exon (−444 to +8, with the translation start site ATG numbered as +1) was amplified by PCR of rat genomic DNA, using primers derived from the mouse sequence [18], and was found to have 90% identity with the mouse sequence. This fragment was then used as a probe to obtain a larger clone of 1.5 kb from a lambda FIX II genomic library (Stratagene, La Jolla, CA). A 420-bp segment spanning −444/−24 and shorter fragments of this 420-bp segment as well as the 1.5-kb region were subcloned into the pGL3-Promoter vector, upstream of the SV40 promoter or downstream of the luciferase (LUC) reporter gene. Mutant constructs were made by PCR with the appropriate mutant primers and verified by DNA sequence analysis.

2.2. Cell culture and DNA transfections

CRL-1600 rat hepatoma cells, NIH 3T3 mouse fibroblasts and PCC-4 mouse embryonal carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections of reporter plasmids and a β-galactosidase expression vector (pCH110 for NIH 3T3 and PCC-4 cells and pCMVSPORT β-gal for CRL-1600 cells) were carried out as described [15,17]. Cells were lysed and aliquots were assayed for LUC activity using a kit from Promega Corporation (Madison, Wisconsin) and for β-galactosidase activity as described [21]. The values for LUC activity were normalized to the β-galactosidase activity as an internal control. Each experiment was repeated at least three times.

2.3. DNase I footprinting analysis

DNase I footprinting analysis was carried out with two overlapping segments spanning −444 to −166 and −222 to +8, which were amplified in separate PCRs using one end-labeled primer and one unlabeled primer for each reaction, so as to provide fragments in which the sense and antisense strands were exclusively labeled. Nuclear extracts of CRL-1600, NIH 3T3 and PCC-4 cells were isolated as described [22]. Pachytene spermatocytes were obtained from rat testicular cells by centrifugal elutriation by a standard procedure, spermatocyte nuclei were isolated as described [23], and extracts were prepared as above. Approximately 2×10^4 cpm (20 ng) of probe and 0–20 μg of nuclear extract were incubated in a total volume of 40 μl of binding buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM DTT, 50 mM NaCl, 4% glycerol and 50 μg/ml of poly(dI–dC)–poly(dI–dC), for 30 min at ambient temperature. Then, 40 μl of 5 mM $CaCl_2$, 10 mM $MgCl_2$ and 0.3 U of DNase I were added. After 1 min at room temperature, the reaction was stopped with 90 μl of stop solution containing 20 mM NaCl, 30 mM EDTA, 1% SDS and 10 μg of yeast tRNA. The DNA probe was then purified and analyzed on an 8% sequencing gel. A Maxam-Gilbert A + G sequencing reaction [21] was electrophoresed in parallel with the DNase I-treated probe to map the location of the footprinted region.

2.4. Electrophoretic mobility shift assays

EMSAs were performed with nuclear extracts of CRL-1600, NIH 3T3 and PCC-4 cells, and labeled double-stranded oligonucleotides. The synthetic oligonucleotides used are listed in Figs. 4A and 6A and were made on an ABI 394 synthesizer. Consensus oligonucleotides for known transcription factor binding sites corresponded to those available commercially (Santa Cruz Biotechnology, Santa Cruz, CA), except as mentioned in Section 3. A typical binding reaction was carried out with 10 000 cpm of probe (0.01 pmol) and 5 μg of nuclear extract in 20 μl buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT, 4% glycerol and 50 μg/ml poly(dI–dC)–poly(dI–dC) or poly(dA–dT)–poly(dA–dT) for 20 min at room temperature. In competition experiments, 50–100-fold molar excess of unlabeled competitor was preincubated with the extracts for 10 min at room temperature before the labeled oligonucleotide was added. For antibody incubation assays, 1 μg of antibody was added to the binding reaction and incubated for 60 min at 4 °C. HNF-3β antibody (clone 4C7) was from the Developmental Studies Hybridoma Bank, Iowa City, IA and Sp4 antibody (clone V-20) was from Santa Cruz Biotechnology. Mouse monoclonal antibodies to RXRα, β and γ were a kind gift from Prof. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). The complexes were resolved in non-denaturing 6% polyacrylamide gels in 45 mM Tris-borate, pH 8.3, 1 mM EDTA buffer and gels were autoradiographed overnight at −70 °C.

3. Results

3.1. Identification of regulatory region in the first intron of LMNA

The region upstream of the lamin C2 translation start site was analyzed for the presence of regulatory elements. The 1.5-kb and 420-bp (−444/−24) genomic fragments were cloned upstream of the SV40 promoter in the LUC reporter vector pGL3-Promoter. Transient transfections were carried out in three cell lines: CRL-1600 rat hepatoma and NIH 3T3 mouse fibroblast cells, which are examples of differentiated cell types that express lamins A and C, and PCC-4 mouse embryonal carcinoma cells which are undifferentiated cells that do not express lamins A and C. All values for LUC activity were expressed as percentage of pGL3-Promoter vector which was taken as 100 to calculate the extent of activation or repression by the different constructs. The actual values for the pGL3-Promoter vector in the different cell lines, expressed as fold activation over the promoterless vector pGL3-Basic, are given in the legend to Fig. 1. The 1.5-kb and 420-bp fragments

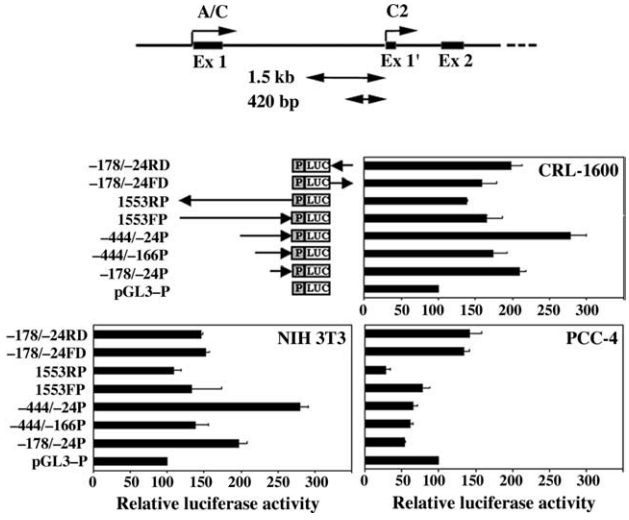

Fig. 1. Deletion analysis of the lamin C2 upstream region. The line diagram depicts the lamin A genomic region spanning the first exons (Ex 1, 1′) for lamins A/C and C2, and the common second exon (Ex 2), and the positions of the 1.5-kb and 420-bp fragments corresponding to the −1553/+4 and −444/−24 segments. The −178/−24 construct was cloned downstream of the LUC gene in the pGL3-Promoter vector (pGL3-P) in the forward (−178/−24FD) and reverse (−178/−24RD) orientation. The −1553/+4 region was cloned upstream of the SV40 promoter (P) in the forward (1553FP) and reverse (1553RP) direction. The −444/−24P, −444/−166P and −178/−24P constructs were cloned upstream of the SV40 promoter. Transient transfections were carried out in the indicated cell lines and LUC activities were measured. The results represent the mean of at least three experiments (±S.E.). Actual values for pGL3-P (relative to pGL3-Basic) are 40.5 (CRL-1600), 80 (NIH 3T3) and 41 (PCC-4).

did not show any promoter activity in somatic cells when cloned into the pGL3-Basic vector (data not shown).

The 420-bp fragment (−444/−24P construct) exhibited nearly 3-fold activation in CRL-1600 and NIH 3T3 cells. Deletions of this fragment as well as the longer 1.5-kb construct gave lower values of activation (1.5–2.0-fold) in these cells. On the other hand, the trend with these constructs in PCC-4 cells was that of repression of the SV40 promoter. The constructs −178/−24P, −444/−166P and −444/−24P reduced SV40 promoter activity by 30–50%, whereas the 1553RP clone reduced SV40 promoter activity by about 70%, suggesting that the silencing activity was orientation independent. When the −178/−24 fragment was analyzed in a position downstream of the reporter gene, it gave 1.5–2.0-fold activation in both orientations in all the cell lines. A construct harboring the 1.5-kb fragment cloned upstream of the lamin A/C promoter (inserted at −740 with respect to the lamin A transcription initiation site) did not exhibit any distinct regulatory effects (data not shown).

3.2. DNase I footprinting of regulatory region

As the transfection experiments revealed cell-type-specific differences in the regulatory activity of the lamin C2 upstream region, DNase I footprinting experiments were carried out to localize the elements that might be important for this activity in the 420-bp segment. Overlapping probes spanning −444/−166 and −222/+8 were assayed separately for protein binding. Only the regions that showed distinct footprints are discussed here. On analyzing the sense strand of probe −222/+8 for protein binding, distinct protection was observed in the region extending from −174 to −195 with nuclear extracts from all the three cell lines as shown in Fig. 2A. The antisense strand of this region gave protection from −201 to −174 (data not shown). The protected segment from −201 to −174 was referred to as FPRA (for footprinted region A). The region from −204 to −172 is highly AT-rich, and there is a continuous AT stretch from −201 to −179. Another protected region extending from −66 to −62 was observed with CRL-160 and NIH 3T3 extracts but not with PCC-4 extracts with the antisense strand of the probe −222/+8 as shown in Fig. 2B. This region was referred to as FPRB. FPRA and FPRB are indicated in the sequence of the −444/+8 segment in Fig. 3. Pachytene spermatocyte extracts gave FPRB but there was negligible protection at FPRA. Hence, distinct differences were observed in the DNase I footprinted regions between lamin A-expressing and non-expressing cells, as well as somatic and germ cells.

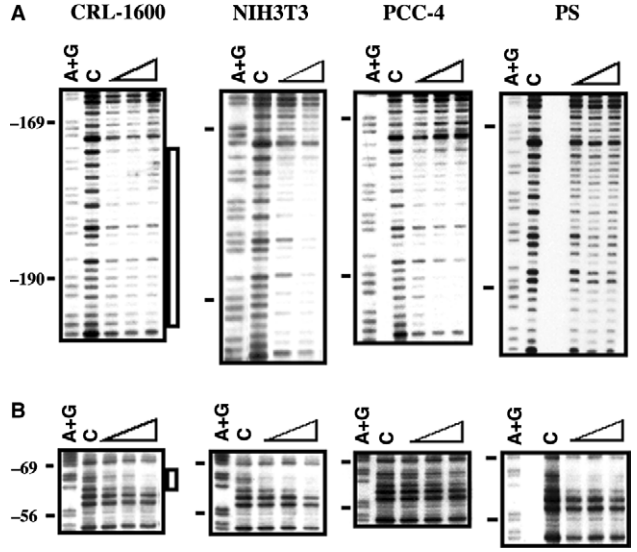

Fig. 2. DNase I footprinting analysis of (A) sense strand and (B) antisense strand of −222/+8 probe. Footprinting reactions were carried out in the absence (C) or presence of increasing concentrations (5–20 μg) of nuclear extracts of the indicated cell lines and PS. An A + G sequencing reaction was run alongside as a marker. The FPRA region was protected in (A) and the FPRB region was protected in (B) (shown by open boxes on the right side of the CRL-1600 footprints).

3.3. Binding interactions at FPRA

In order to study the DNA–protein interactions at the footprinted regions and to localize the precise bases responsible for protein binding, EMSAs were carried out using synthetic oligonucleotides spanning the footprinted regions. Analysis of the FPRA sequence by MatInspector and TFSEARCH yielded putative consensus binding sites for the following transcription factors (sites are indicated in Fig. 4A): HNF-3, site I/II, HNF-3/Fkh homolog 1 (HFH-1, site I/II) and nuclear factor Y (NFY, Y/CCAAT box binding factor, site III). To analyze this region by EMSAs, a 41-bp oligonucleotide extending from −207 to −168 was used. Since this oligonucleotide has a high AT content (78%), poly(dA–dT)–poly(dA–dT) was used as the non-specific competitor instead of poly(dI–dC)–poly(dI–dC) as initial experiments with the latter had resulted in more non-specific bands. Mutant oligonucleotides with mutations in the above consensus sites were used in competitions with the wild-type oligonucleotide at 100-fold molar excess. On incubating CRL-1600, NIH 3T3 or PCC-4 nuclear extracts with the labeled 41-mer probe, one major complex was obtained which was specific as it was competed out with 100-fold excess of unlabeled probe (see Fig. 4B–D). Mutant oligonucleotides AM1 and AM2, that have 2-bp mutations in site I, competed for binding with the wild-type probe. An oligonucleotide with a mutation in site III (AM3), which is a motif for the CCAAT box, also competed for binding. Hence, sites I and III could be ruled out as being important for complex formation. The mutant oligonucleotide AM4 with mutations at −182 and −183 in site II also competed; however, AM5, with mutations at −180 and −181 in site II did not compete with the wild-type probe at 100-fold excess. Hence, the residues −180 and −181 are important for forming the complex.

Further competition reactions were carried out with consensus oligonucleotides for the above transcription factors. The consensus HNF-3 sequence was based on a strong affinity HNF-3 site in the transthyretin promoter [24] and the HFH-1 sequence was based on the consensus binding sequence for purified HFH-1 derived by Overdier et al. [25]. A consensus

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-444 ATTCATGTCTACATAATTAAGATAAAGGAAACTGCATTGTTAGTGGAAAA
-394 AAAAAAATTGGGCCTGGGAATGTAGCTGGCCTAGCACTTAGGAACTTTGG
-344 GGTTTGGGTTTGATCCCAGCATCCCATAAACCACATATAATCCCGGCACT
-294 TGGAAGGTAGAGGCAGGAGGATCAGAACTTTAAGGTCATTCTTGACTACT
-244 TAGCAAATTCGAGGCACCCCAGGATACCTGAGACTCTGTCATGAATAAAA
-194 ATAAATATTAAATAAACCAATATGGGTCTAGGTGGAAAGGCAGCTTGGGC
-144 AGGGTAGGGCCGGAAAAGAGAGATAGAGGGTTGTGGTCTCAGGGGGATCT
 -94 GGGAGCAGCACCTCAGCACCCTTCCCAGTCACAGGGGTCACCGATCTTTC
 -44 CGGAACTCCCGAAGGCTCGAGGCCTTGCTCTTTCTGGCCCAGCCATGGGGAA
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Fig. 3. Sequence of lamin C2 upstream fragment from −448 to +13. FPRA and FPRB are in bold. The lamin C2 transcription initiation sites at −30 and −41 are shown by asterisks, while the ATG translation initiation codon at +1 is in italics.

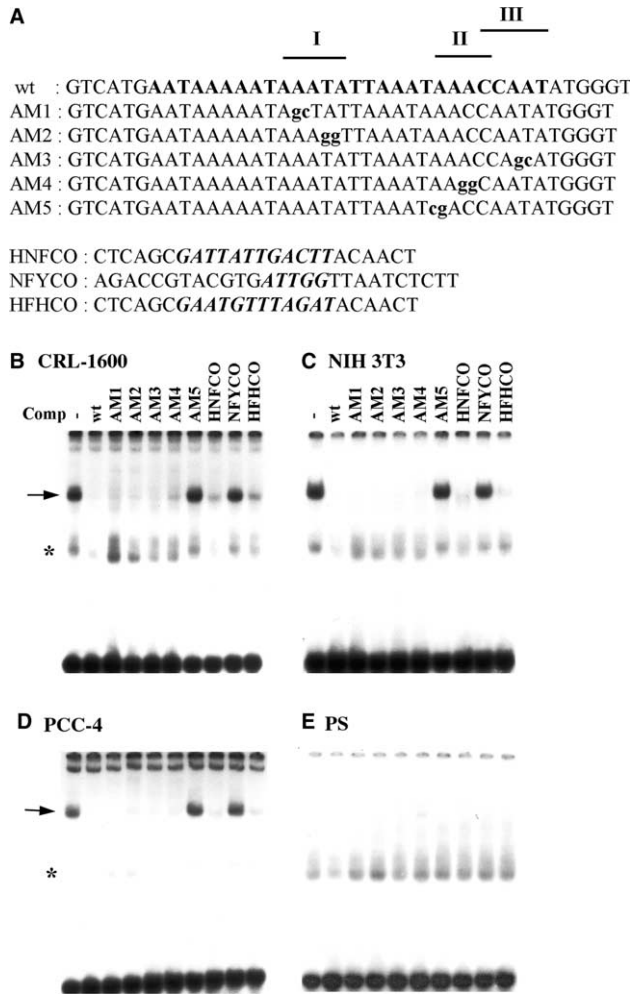

Fig. 4. EMSAs of the FPRA segment. The sequences listed are the FPRA-containing oligonucleotide extending from −207 to −168 (wt, FPRA in bold and sites I, II and III indicated), mutants (AM1–AM5, mutated bases in lower case) and consensus oligonucleotides (HNFCO, NFYCO and HFHCO, binding motifs in bold italics). EMSAs were carried out with nuclear extracts from (B) CRL-1600, (C) NIH 3T3, (D) PCC-4 and (E) PS, with the competitor oligonucleotides (Comp) listed in (A) or with no competitor (−). The major specific complex formed is indicated by an arrow. A non-specific band is shown by an asterisk.

oligonucleotide for NFY corresponded to the commercially available sequence. The NFY consensus did not compete with the wild-type probe, which was consistent with the results obtained for competitions with the mutant oligonucleotides, and suggested that the CCAAT box was unlikely to be involved in complex formation. However, both HNF-3 and HFH-1 consensus oligonucleotides competed with the wild-type probe. As both these factors have overlapping specificities [26], they can theoretically bind at site I or II. Since the competition data with mutant oligonucleotides suggested that site II was important for complex formation, it is likely that the HNF-3/HFH-1 classes of transcription factors bind to site II. No specific complex formation was observed with spermatocyte extracts (Fig. 4E), in keeping with the absence of a footprint at FPRA with these extracts.

To identify the factor forming a complex at site II, EMSAs were carried out in the presence of antibody to the candidate factor HNF-3β [27] as well as Sp4 antibody as a non-specific control. As shown in Fig. 5, the specific complex formed with extracts from CRL-1600, NIH 3T3 and PCC-4 cells was depleted with HNF-3β antibody but Sp4 antibody did not have any effect. This confirms that the complex formed with FPRA is composed of HNF-3β.

3.4. Binding interactions at FPRB

The footprinted region designated as FPRB was further analyzed by EMSAs. A MatInspector search for transcription factor sites in the −70/−45 region yielded two putative consensus sites, one of which was a partial consensus for the CANNTG motif that binds to upstream stimulating factor (USF) and the other was the consensus site for the retinoic acid receptor (RAR) family of transcription activators. A 26-mer double-stranded oligonucleotide extending from −70 to −45 was assayed for protein binding using nuclear extracts from CRL-1600, NIH 3T3 and PCC-4 cell lines, as well as PS extracts. Consensus oligonucleotides containing the binding sites for USF, RAR or RXR were used as competitors. The RAR oligonucleotide has 5 bp between the two direct repeats required for binding, whereas the RXR oligonucleotide has 1 bp.

With CRL-1600 and NIH 3T3 extracts, one major complex 'a' was observed which was specific and showed a similar pattern of competitions with mutant oligonucleotides (Fig. 6). A minor complex 'b' showing similar behavior with mutant competitors as complex 'a' was faintly detectable. Also a minor non-specific band was detectable just above the major complex 'a'. Oligonucleotides containing mutations in the footprinted region or the USF binding site (BM1-4) competed for the formation of complexes 'a' and 'b'. BM6, carrying a 6-bp mutation in bases upstream of BM5, also competed for complexes 'a' and 'b'. Hence, these sequences were not involved in the formation of the complexes. On the other hand, BM5 which had mutations in the bases −59 to −54 did not compete for binding. Thus, this sequence is likely to be important for complex formation. A reexamination of the footprint in this region indicated that this sequence did not get digested efficiently by DNase I even in the absence of nuclear extracts, and hence was not scored as a protected region. Competition ex-

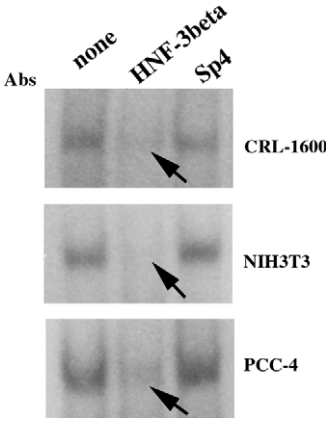

Fig. 5. EMSAs with the FPRA fragment with antibody preincubation. EMSAs were carried out with labeled FPRA oligonucleotide and nuclear extracts from CRL-1600, NIH 3T3 and PCC-4 cells that had been preincubated with HNF-3β or Sp4 antibodies. The immunodepleted complex is marked by arrows. The amount of free probe (not shown) was equivalent in all lanes.

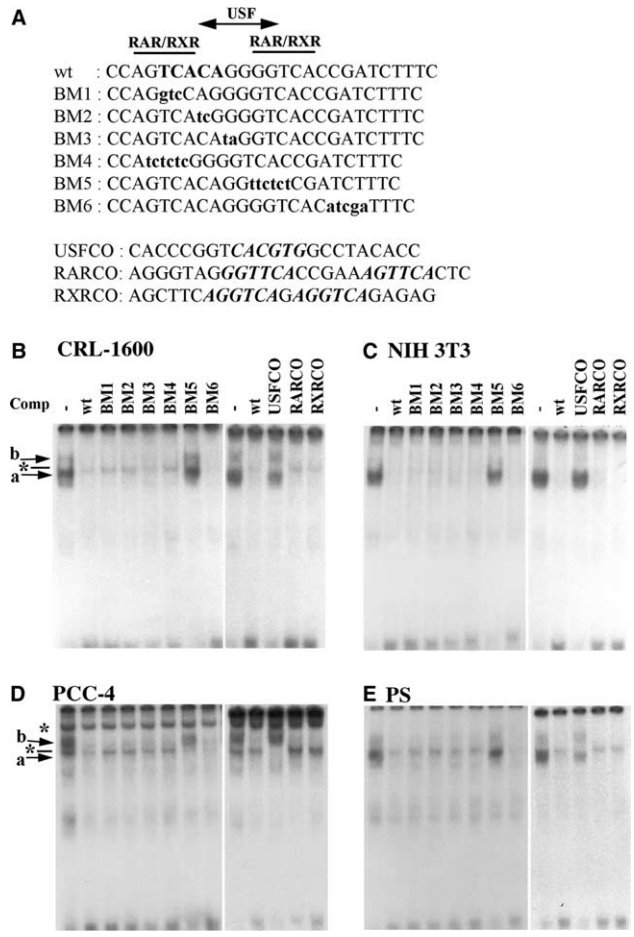

Fig. 6. EMSAs of the FPRB segment. The sequences listed are the FPRB-containing oligonucleotide extending from −70 to −45 (wt, FPRB in bold), mutants (BM1-6, mutated bases in lower case) and consensus oligonucleotides (USFCO, RARCO and RXRCO, binding motifs in bold italics). EMSAs were carried out with nuclear extracts from (B) CRL-1600, (C) NIH 3T3, (D) PCC-4 and (E) PS, with the competitor oligonucleotides (Comp) listed in (A), or with no competitor (−). The major specific complexes 'a' and 'b' are marked by arrows. Non-specific bands are shown by asterisks.

periments were also carried out with consensus oligonucleotides for USF, RAR and RXR. Competition with 100-fold excess of RAR and RXR oligonucleotides but not USF oligonucleotide abolished complexes 'a' and 'b'. Hence, complexes 'a' and 'b' are likely to be formed due to binding of a member of the RAR family. Complex formation with spermatocyte extracts was similar to that observed with CRL-1600 and NIH 3T3 extracts.

A different pattern of complex formation was observed with PCC-4 extract. Complex 'b' was the major complex formed, whereas complex 'a' could not be detected. The competition patterns with the mutant oligonucleotides of both the bands were identical to those observed for CRL-1600 and NIH 3T3 extracts. Also, the competition patterns with the consensus oligonucleotides for USF, RAR and RXR were similar. However, a non-specific band between 'a' and 'b' was more intense in PCC-4 extracts. Only this non-specific band between 'a' and 'b' was competed out by the USF consensus oligonucleotide, suggesting that this band might be formed due to low affinity binding of USF to the CANNTG motif in the −70/−45 oligonucleotide, whereas USF may bind with high affinity to its consensus oligonucleotide. The increased intensity of the complex might be due to a higher amount of USF in PCC-4 cells. A strong non-specific complex was observed just below the wells of the gel in several batches of PCC-4 extracts but its origin could not be ascertained. These data suggest that there are differences in the composition of the complexes formed in lamin A-expressing and non-expressing cell types, though both are likely to involve members of the RAR/RXR families of transcription factors.

To identify the factors binding to this site, EMSAs were carried out with preincubation with specific antibodies. As the short distance between the RAR/RXR half-sites in the FPRB region was more similar to that for the RXR consensus, these assays were carried out with antibodies to the RXR family of transcription factors, specifically RXRα, β and γ [28] as well as Sp4 as a negative control. Antibodies to RXRβ depleted the complex formed with extracts from CRL-1600 and NIH 3T3 cells and PS as shown in Fig. 7. Depletion was also observed with antibody to RXRα with NIH 3T3 extract. However, these antibodies did not affect the complex obtained with PCC-4 extracts.

3.5. Functional analysis of mutant constructs

To ascertain the functional significance of the sequences identified to be important by DNase I footprinting and EMSAs, deletion- and mutant-reporter gene constructs were made and assayed by transient transfections. As the −178/−24P

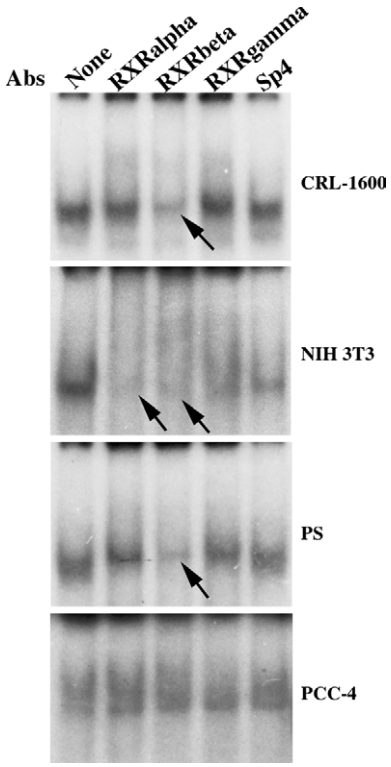

Fig. 7. EMSAs with the FPRB fragment with antibody preincubation. EMSAs were carried out with labeled FPRB oligonucleotide and nuclear extracts from CRL-1600, NIH 3T3, PS and PCC-4 cells that had been preincubated with RARα, β, γ or Sp4 antibodies. Immunodepleted complexes are marked by arrows. The amount of free probe (not shown) was equivalent in all lanes.

construct, which had been analyzed by transient transfections, did not contain the AT-rich FPRA region, a longer clone containing this sequence (from −220 to −24) was obtained by PCR and cloned into pGL3-Promoter vector, to give the −220/−24P construct. This was resorted to as PCR-based mutagenesis of the AT-rich FPRA segment was unlikely to succeed. To analyze the segments identified to be important in protein binding to the FPRB region, PCR-based site-directed mutagenesis of the −444/−24 clone was carried out with the BM4 and BM5 mutant oligonucleotides (by inverse PCR) and the mutated sequences were cloned into pGL3-Promoter. Sequence analysis indicated that in −444/−24BM5P, the wild-type sequence encompassing the downstream RXR half site, GGTCAC was mutated to TTCTCT. In −444/−24BM4'P, the wild-type sequence containing the upstream RXR half-site, GTCACA was mutated to GTCTC. The LUC activities of the control plasmid (pGL3-Promoter) and the above constructs were analyzed by transfection into CRL-1600, NIH 3T3 and PCC-4 cell lines (Fig. 8).

FPRA analysis. In CRL-1600 cells, activation by the −220/−24P construct (1.2-fold) was less than with −178/−24P (2.2-fold). Similar results were obtained with NIH 3T3 cells (1.3 and 1.8-fold). In PCC-4 cells, where the 5′ region of C2 shows general repression, the trend was similar though lower in magnitude. Hence, in all the three cell lines the addition of the AT-rich region has a repressive effect. This raises the possibility that there is an activation motif between −220 and −444. However, this has not been examined further as a distinct footprint was not obtained in this region.

FPRB analysis. Compared to the 3.2-fold activation exhibited by the wild-type −444/−24P construct in CRL-1600 cells, the −444/−24M5P mutant construct showed only 2.0-fold activation of the SV40 promoter. The mutant construct −444/−24M4'P, on the other hand, activated the promoter to a similar extent as the wild-type construct. Similar results were obtained with NIH 3T3 cells. Hence, with both these cell lines, mutation of the downstream RXR site reduced activation levels, indicating that this motif acts as an activator. A similar though smaller effect was observed in PCC-4 cells.

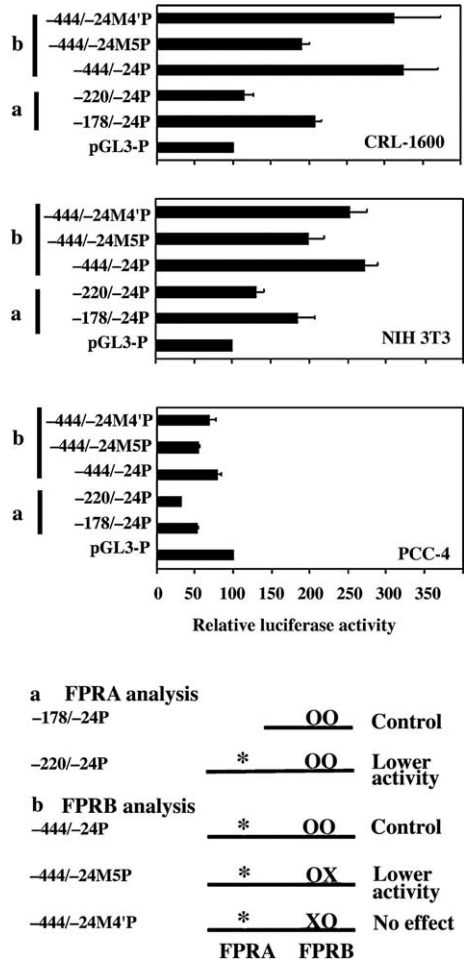

Fig. 8. Functional analysis of FPRA and FPRB segments. The indicated constructs were transfected into CRL-1600, NIH 3T3 and PCC-4 cells, and LUC activities were measured. The results represent the mean of at least three experiments (±S.E.). A summary of the comparative activities is shown; the 'a' and 'b' sets of constructs were used for the analysis of FPRA (*) and FPRB (OO, a mutated half site is shown as OX or XO), respectively.

4. Discussion

We have identified a 420-bp segment in the first intron of the lamin A gene with cell-type-specific regulatory activity. This segment enhanced activity in differentiated cells that express lamins A/C, increasing SV40 promoter activity by almost 3-fold in hepatoma and fibroblast cell lines. On the other hand, in the embryonal carcinoma cell line PCC-4, which does not express A-type lamins, this 420-bp fragment downregulated SV40 promoter activity.

Two major protected regions were identified in the 420-bp fragment by DNase I footprinting analysis. FPRA was protected by nuclear extracts from CRL-1600, NIH 3T3 and PCC-4 cells but not by PS extracts. FPRA contains consensus sites for binding of proteins belonging to the HNF-3/fkh homolog family (also termed the winged helix or forkhead box/Fox family) that includes at least 20 proteins in rodents [29]. Winged helix proteins are important regulators of cellular differentiation and metabolism, and act as transcriptional activators as well as repressors. The target binding site for the winged helix family of transcription factors is AT-rich and has the 7-bp recognition motif 5′ (G/A)(T/C)(C/A)AA(C/T)A. The bases flanking this motif contribute to the binding specificity of various members of the family [25,26]. In EMSAs, a specific complex was formed by CRL-1600, NIH 3T3 and PCC-4 extracts but not by PS extracts. The binding site for the complex was narrowed down to site II, which is a consensus sequence for binding of HNF-3 or HFH-1 classes of transcription factors. EMSAs with a specific antibody indicated that HNF-3β was bound at this motif with extracts from CRL-1600, NIH 3T3 and PCC-4 cells. Functional analysis of this sequence suggested that it had the properties of a repressor motif in all three cell lines. The occupancy of this site by proteins from somatic cells but not PS has important implications, since the lamin C2 promoter is repressed or inactive in somatic cells.

In EMSAs of the FPRB-containing oligonucleotide with CRL-1600, NIH 3T3 and PS extracts, a major complex 'a' was formed at the sequence −59 to −54 downstream of FPRB. On the other hand, in PCC-4 extracts complex 'b', which was of slower mobility, was the major complex formed at this sequence. Both the complexes 'a' and 'b' were competed out by

RAR and RXR oligonucleotides. Members of this nuclear hormone receptor superfamily are known to be differentially regulated during cellular differentiation [28,30]. Most members of this family, including RAR and RXR, bind as homodimers or heterodimers to direct/inverted/everted repeats of two half-sites with the core motif RGGTCA. The spacing between the two half-sites determines the specificity of binding [31]. In this study, the complexes formed in EMSAs appeared to involve the sequence from −59 to −54, that is a single half-site, and the EMSA result was supported by the functional analysis of the mutants. EMSAs with specific antibodies indicated that the complexes from CRL-1600, NIH 3T3 and PS extracts were primarily composed of RXRβ with inclusion of RXRα in the complex from NIH 3T3 cells. The complex formed in PCC-4 cells did not appear to be composed of RXRα, β or γ proteins. This could be due to the lower amounts of RXR factors in undifferentiated cells [28], or binding of other members of the RAR/RXR families of factors. The possibility that this region is a retinoic-acid responsive element has important implications, since expression of lamins A and C is induced upon differentiation and retinoic acid is a well known inducer of differentiation and lamin A expression [32]. A GT-rich motif in the lamin A proximal promoter, that can bind to the Sp1 family of transcription factors [16], is also responsive to retinoic acid [33].

The presence of a regulatory region upstream of the lamin C2 transcription initiation site (multiple sites mapped to −40, −41 and −45 in the mouse [18], and to −30 and −41 in the rat, our unpublished data) has interesting implications. As the FPRA segment acts as a repressor in somatic cells, we suggest that this segment can downregulate the lamin C2 promoter in non-germ cell lineages. This would need to be analyzed in transgenic mouse models, as a functional characterization of the lamin C2 promoter has not been feasible in primary germ cell cultures (our unpublished work). The FPRB regulatory motif, which contains a RAR/RXR binding site, may be required to upregulate promoter activity upon embryonic cell differentiation. The 1.5-kb segment did not alter lamin A promoter activity although we cannot rule out an effect on the lamin A promoter by other intronic sequences. An activating domain has been reported within the first 3 kb of the intron, which enhances promoter activity in a mammary carcinoma cell line, but its functional motifs have not been analyzed [34]. The regulation of expression of the individual lamin isoforms, which are splice variants, might also be exerted at the level of transcript processing. Thus, this genomic locus is subject to complex regulatory controls in different cell types.

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