Tissue-specific regulation of the rabbit 15-lipoxygenase gene in erythroid cells by a transcriptional silencer

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Received July 3, 1995; Revised and Accepted August 18, 1995

GenBank accession nos M33291 and X90860

ABSTRACT

The 15-lipoxygenase (lox) gene is expressed in a tissue-specific manner, predominantly in erythroid cells but also in airway epithelial cells and eosinophils. We demonstrate in this report that the 5' flanking DNA of the 15-lox gene contains sequences which down-regulate its activity in a variety of non-erythroid cell lines but not in two erythroid cell lines. The element has characteristics of a transcriptional 'silencer' since it functions in both orientations. The main activity of the silencer has been mapped to the first 900 bp of 5' flanking DNA, which contains nine binding sites for a nuclear factor present in non-erythroid cells but not in erythroid cells. These binding sites have similar sequences and multiple copies of the binding sites confer tissue-specific down-regulation when attached to a minimal lox promoter fragment. The 5' flanking DNA also contains a cluster of three binding sites for the GATA family of transcription factors.

INTRODUCTION

Studies of the regulation of genes that are highly expressed in erythroid cells, including the globin genes, both by our own group and others, have defined the importance of cis-control regions which function due to the binding of members of the GATA family of transcription factors (particularly GATA-1) in conjunction with other ubiquitous factors (1,2, reviewed in 3 and 4). Recently, we have also presented evidence for a role of members of the Ets family of transcription factors in the up-regulation of the glutathione peroxidase gene in erythroid cells (5).

Evidence has also accumulated for the importance of negative control of transcription of genes in a wide variety of contexts (reviewed in 6–11). In the context of erythroid-specific gene expression in particular, there have been various reports of negative regulation of globin (12–19) and non-globin genes such as the histone H5 gene (20,21), the erythropoietin receptor gene (22) and the glycoporphin B gene (23).

In this report, we have investigated the mechanisms of tissue-specific regulation of the 15-lox gene in erythroid cells.

The LOXs are a family of enzymes which catalyse the dioxygenation of polyenoic acids or their derivatives containing at least one 1,4-cis,cis-pentadiene system (24). Different cells, such as leukocytes, platelets and endothelial cells contain LOXs of different specificities according to the hydroperoxide products produced with arachidonic acid (5,8,11,14-all-cis-eicosatetraenoic acid) as substrate. Three different enzymes have been extensively characterised: the 5-, 12- and 15-LOXs, although other LOX activities (such as 8-specificity) have also been reported. The 15-LOX is expressed predominantly in reticulocytes, where it plays a role in mitochondrial degradation (25), but also in eosinophils and epithelial cells of the bronchial airways (26–28). Expression of the 15-lox gene is regulated both at the level of transcription (29) and mRNA translation during reticulocyte maturation (30). In previous work, we isolated the rabbit 15-lox gene and defined its exon/intron structure and transcription start site in erythroid cells (31).

In this report we demonstrate the importance of negative control mechanisms in the erythroid-specific expression of the 15-lox gene. We show that the 5'-flanking region of the 15-lox gene contains a tissue-specific transcriptional silencer which seems to function by binding of a novel factor present in non-expression tissues that is not the previously described negative regulator, YY1.

MATERIALS AND METHODS

The methods for cell culture, transient transfections and CAT assays, preparation of nuclear protein extracts, DNase I footprinting, electrophoretic mobility shift assays (EMSAs) and generation of site-directed mutations by the polymerase chain reaction (PCR) have all recently been described in detail (5).

Cells and cell culture

Adherent mouse erythroleukemia (MEL) cells (F4-12B2; a gift from W. Ostertag, Hamburg, Germany), mouse epithelial cell line C5 (32), STO mouse fibroblasts, HepG2 human liver cells, HeLa cells, the rabbit skin fibroblast line Rab 9, and the rabbit lung fibroblast cell line R9AB were obtained from the European Collection of Animal Cell Cultures, Porton Down, UK and were grown in a modification of Eagle's minimal essential medium with double concentrations of amino acids and vitamins, 10% fetal calf
Plasmid constructions

Plasmids pCAT19, pCAT-40, pCAT-150 and pHSVβGal were constructed as previously described (31). pCAT-40 and pCAT-150 contain sequences extending from -40 and -150 to +20 bp (with respect to the transcription initiation site), respectively, linked to the bacterial chloramphenicol acetyltransferase gene (CAT) and transcriptional termination sequences of the herpes simplex virus type 2 (HSV-2) immediate-early 5 gene (IE-5). pCAT-2700 was obtained by co-ligation of a 1000 bp SstI-Xbal fragment from pCAT-150 (containing 15-lox sequences from -150 to +20 bp, the CAT gene and the HSV-2 IE-5 transcriptional termination sequences) with a 2550 bp KpnI–SstI fragment of the 15-lox genomic clone (containing sequences from -2700 to -150 bp) into KpnI-Xbal digested pUC19. pCAT-315 contains a 335 bp Smal–BamHI fragment from pCAT-2700 containing 15-lox sequences from -315 to +20 bp inserted into pCAT-19. pCAT-430 contains 15-lox sequences from -430 to +20 bp and was obtained by insertion of a 700 bp EcoRI fragment of pCAT-2700 into EcoRI cut pCAT19. pCAT-930 was obtained by insertion of a 615 bp Smal fragment from pCAT-2700 containing 15-lox sequences between -930 and -315 bp into Smal digested pCAT-315. pCAT-1800 and pCAT-2200 contain 15-lox sequences from -1800 to -2200 bp, respectively. These plasmids were constructed by the isolation of a KpnI–HindIII fragment of pCAT-2700 containing 15-lox 5' flanking sequences and the CAT gene. This fragment was partially digested by TaqI or PstI and fragments containing 1800 or 2200 bp of 15-lox sequence linked to the CAT gene were isolated and cloned into pUC19. The mini-gene construct was obtained by the cloning of a 4600 bp partial Xbal–HindIII fragment from immediately downstream of the 15-lox gene into Xbal–HindIII digested pCAT-2700. pCAT-150(0.78)5' contains a 780 bp SstI fragment from -930 to -150 bp in the reverse orientation and was prepared by cloning this fragment into SstI digested pCAT-930. pCAT-150(0.78)3' and pCAT-150(0.78)3' were prepared by cloning the blunt-ended 780 bp SstI fragment in both orientations into blunt-ended HindIII digested pCAT-150. pCAT-150(2.55)5' contains a 2550 bp SstI fragment of 15-lox sequence from -2700 to -150 bp in the reverse orientation and was prepared by cloning this fragment into SstI digested pCAT-2700. pCAT-150(2.55)3' and pCAT-150(2.55)3' were constructed by cloning the blunt-ended 2550 bp SstI fragment in both orientations into blunt-ended HindIII digested pCAT-150. pCAT-150(Fp 4) and pCAT-150(Fp 5) were prepared by cloning four copies of the Fp 4 binding site (oligonucleotide Fp 4) or two copies of the Fp 5 binding site (oligonucleotide Fp 5) linked head-to-tail in the natural orientation into the blunt-ended SstI site of pCAT-150. Concatamers were prepared using oligonucleotide primers containing a BspMI restriction site as previously described (33). pCAT-150(GS) and pCAT-150(GL) constructs contain 15-lox sequences from -551 to -736 bp and -551 to -930 bp respectively, prepared by PCR (using primers L-2 and L-3 for GS and primers L-1 and L-3 for GL) and cloned into SstI digested pCAT-150. pCAT-150(GS) contains FP 8 and pCAT-150(GL) contains FPs 8–11 linked to the minimal 150 bp 15-lox promoter.

The pGPY289/2.55lox construct contains the 2.55 kb KpnI–SstI fragment of the 15-lox gene from -2700 to -150 bp inserted into the SstI site upstream of the 289 bp glutathione peroxidase (GPX) promoter/CAT construct pGPY289 (5).

The integrity of all constructs was determined by restriction enzyme site mapping and all constructs produced by PCR were sequenced by the dideoxy chain termination method (34).

Oligonucleotide PCR primers

The following oligonucleotides were used in this study (all sequences are written 5'→3'):

- Fp 3(NT-1), AGGCGGGCCTGTGATGGAGCAGGGACGGTACG;
- Fp 4, ATCCGGGAACAGGACGCTGGGAGAGGCT;
- Fp 5, ATGCCAACCGGGGAGGGACGGGACGGGAGGG;
- Fp 7, CCCTTCGTCCTGAGCGCCCGCCAGCCAGCCAGCC;
- oligo M, GAGGGGACGCGGCTGTCGTTTTTTGTTTTTG;
- Fp5M, ATGCCAACCGGGGAGGGACGGGACGGGAGGG;
- G-3, AAGTTTATATATACATATTATATACATAT;
- G-3M, AAGTTTATATATATACATATTATATACATAT;
- YY1, GATCGTITTGACAGCTTTGACAC;
- OCT-1, AATTGTCGCTCTGAGGAGCTCTAAGAGA;
- TCCATGGAATGTGACCGCGCGGATCGACT;
- GATA, GCGGGCAACTGATAAGGTCCCGT;
- L-1, CGGGAGCTCCCGGGGCAGCTACAGAGTCTG;
- L-2, CCGGAGCTCCCGGGGCAGCTACAGAGTCTG;
- L-3, CCGGAGCTCCCGGGGCAGCTACAGAGTCTG;

Isolation of stable transfectants

Stable transfectants were generated by transfecting cells with 20 μg of test plasmid and 0.2 μg of HOMER 6 plasmid containing the neomycin resistance gene (35). Sixteen hours later, 10 ml of fresh medium was applied. Stable transfectants were selected by treatment with genetin (800 μg/ml; Gibco-BRL) for 3 weeks, and colonies (>100) were pooled. Transfectants were induced to differentiate with either 4 mM N,N'-hexamethylenebisacetamide (HMBA) or 1.6% dimethylsulfoxide (DMSO) for up to 7 days.

RESULTS

Identification of cis-control regions

To search for any tissue-specific cis-control regions within the 15-lox genomic DNA regions available, a 'mini-gene' was constructed containing a fragment of 5'-flanking DNA from -2.7 kb to +20 bp linked upstream of the CAT-gene and 4.6 kb of 3'-flanking 15-lox gene DNA from +8 to +12.6 kb linked 3' to the CAT gene. For comparison, constructs containing 5' flanking DNA from -40 to +20 bp (pCAT-40), -150 to +20 bp (pCAT-150) and -2.7 kb to +20 bp (pCAT-2700) linked upstream of the CAT gene were also used (Fig. 1A). Each of these constructs was transfected into mouse erythroid (MEL) or epithelial (CS) cells and the CAT activity measured 48 h later and standardised against the activity of a co-transfected β-galactosidase expression construct, pHSVβGal. The results (Fig. 1A) show that the pCAT-40 had virtually no promoter activity in either cell line, whereas pCAT-150 had similar levels of activity in both cell types [as compared with a 109 bp murine thymidine kinase gene promoter construct (data not shown)]. Both the mini-gene and...
15-lox DNA in pCAT-2700 were constructed and tested by transfection into MEL, C5 and Rab 9 cells as before. The results (Fig. 2A) show that down regulation in non-erythroid cells is gradually lost as the 5' flanking DNA is progressively deleted from -930 to -315 bp.

To test whether the down-regulation was due to a transcriptional silencer, the 2.55 kb fragment from -2.7 kb to -150 bp was cloned in either the natural or opposite orientation 5' or 3' to the CAT gene in pCAT-150 and the effect of promoter activity determined after transfection into MEL or C5 cells. The results in C5 cells showed that CAT activity was reduced -20-fold with 2.55 kb fragment placed 5' in either orientation when compared with pCAT-150 alone (Fig. 2B, lines 5 and 6); when placed 3', the negative effect was lower (2-fold) (Fig. 2B, lines 7 and 8), but still significant. However, the negative effect was not apparent in MEL cells (Fig. 2B, lines 5–8). A reduction in CAT activity in C5 cells was also found with a 780 bp fragment from -150 to -930 bp inserted 5' in either orientation (Fig. 2B, lines 1 and 2), although the effect (5–6-fold) was not as large as with the 2550 bp fragment; again the down-regulation was 2-fold when the 780 bp fragment was placed 3' (Fig. 2B, lines 3 and 4). These results show that the 780 bp fragment of the 15-lox gene contains a transcriptional silencer.

To determine whether the silencer functions in a promoter-specific way, the 2.55 kb 5' 15-lox gene fragment was cloned in its 'natural' orientation upstream of a 289 bp minimal promoter from the glutathione peroxidase (GPX) gene linked to the CAT gene in plasmid pGPY289 (5) to give pGPY289/2.55lox. We have previously shown that the minimal 289 bp GPX promoter works equally well in both MEL and C5 cells (5). Silencer function was then assessed by comparing the promoter activity of pGPY289/2.55lox with pGPY289 after transfection into erythroid cells (MEL) or non-erythroid cells (C5 or Rab9 cells); the results were then compared with those using the pCAT-2700 and pCAT-150 15-lox gene constructs transfected in parallel experiments. The results (Fig. 2C) show that the 15-lox silencer functions much more strongly with its homologous promoter than with the GPX promoter.

Nuclear proteins binding to the silencer

To locate any binding sites for nuclear factors in the silencer region, the first 930 bp of 5'-flanking DNA was subjected to DNase I footprinting, using the eight end-labelled fragments shown in Figure 3A. All the footprints (FPs) detected are displayed against the 15-lox gene sequence in Figure 4A. Eleven reproducible FPs were detected in the region from the transcription start site to -930 bp (Fig. 3B–F, summarised in Fig. 4A).

FPs in the minimal promoter. The minimal 150 bp promoter fragment used in the functional experiments contains three binding sites for nuclear factors present in both erythroid (MEL, K562) and non-erythroid (C5, HeLa) cell types (FPs 1–3, Fig. 3B); these correspond to the consensus binding sites for Sp1, AP-2 and NF1, respectively (Fig. 4A). The AP-2 site appears not to be footprinted in Rab9 cells or in HepG2 cells which have previously been shown not to express AP-2 (36). The presence of the AP-2 site probably explains why the minimal promoter construct pCAT-150 is inducible by forskolin and TPA after transfection into MEL cells (data not shown).
Figure 2. Defining the negative control element in the 5'-flanking DNA. (A) Deletion mapping. Constructs containing different 5'-flanking regions upstream of the CAT gene were transfected into MEL cells (open boxes), C5 cells (hatched boxes) or Rab9 cells (filled-in boxes) and the CAT activity measured 48 h later. The constructs pCAT-2700, pCAT-2200, pCAT-1800, pCAT-930, pCAT-315 and pCAT-150 contained sequences from +20 to -2700 bp, -2200, -1800, -930, -430, -315 and -150 bp, respectively, inserted upstream of the CAT gene. The results are expressed relative to the levels with the minimal promoter construct pCAT-150, after subtraction of the slight background CAT activity of the promoter-less CAT vector. (B) Evidence that the 5'-flanking DNA contains a transcriptional silencer. The 780 bp fragment of the $15\lox$ gene from -150 to -930 bp was inserted in either the natural (+) or reverse (-) orientation 5' to the 150 bp minimal promoter in pCAT-150 to give pCAT-150(0.78)5'+ or pCAT-150(0.78)5'-, or 3' to the CAT gene in pCAT-150 to give pCAT-150(0.78)3'+ and pCAT-150(0.78)3'-, respectively. Similar constructs were made by inserting the 2.55 kb fragment from -150 bp to -2.7 kb into pCAT-150 instead of the 0.78 kb fragment. Each of the constructs was transiently transfected into MEL, C5 or Rab9 cells and CAT activities expressed relative to pCAT-150. The key to the cell types and other details are given in (A). (C) Testing the silencer with a heterologous promoter. The 2.55 kb fragment of the $15\lox$ promoter was inserted in the natural orientation 5' to a minimal promoter fragment of the mouse cytosolic glutathione peroxidase (GPX) gene (containing sequences from -289 to +10 bp, hatched box) linked to the CAT gene [pGPY289 (5)] to give pGPY289/2.55$lox$+. pGPY289 and pGPY289/2.55$lox$ were then transiently transfected into MEL, C5 or Rab9 cells [see (A) for key to cell types]. The effect of the 2.55 kb fragment on the GPX promoter can be compared with its effect on the 150 bp $lox$ promoter in (B), line 5.

Non-erythroid-specific factor(s) binding to the silencer fragment. FPs 4-7 and 10 (Fig. 3C-E) within the silencer fragment from -150 to -930 bp are only found with non-erythroid (C5) nuclear extracts. In fact, the sequences of the FP4, 5, 6 and 7 regions (Fig. 4A) are remarkably similar (Fig. 4B) and oligonucleotides from the FP4 or FP5 regions compete the binding to FPs 7 and 10 strongly (Fig. 3D and F), suggesting that FPs 4-7 and 10 all bind the same C5 cell factor. This is also confirmed by EMSA experiments showing that at least two specific retarded complexes are formed with an oligonucleotide from the FP4 region with three different non-erythroid cell extracts (C5, HeLa and Rab9) (Fig. 5A, bands 1 and 2); these FPs are competed by oligonucleotides from the FP4, 5, 7 and 10 regions (Fig. 5B), but not by an oligonucleotide containing a mutated version of FP5 (FP5M; see Fig. 4), nor by oligonucleotides containing binding sites for the NF1 or Oct-1 factors (Fig. 5B). Interestingly, another oligonucleotide [M, (Fig. 4A)] from the 15-lox flanking region containing sequences similar to the FP4-6, 10 strongly (Fig. 3D and F), suggesting that FPs 4-7 and 10 all bind the same C5 cell factor. This is also confirmed by EMSA experiments showing that at least two specific retarded complexes are formed with an oligonucleotide from the FP4 region with three different non-erythroid cell extracts (C5, HeLa and Rab9) (Fig. 5A, bands 1 and 2); these FPs are competed by oligonucleotides from the FP4, 5, 7 and 10 regions (Fig. 5B), but not by an oligonucleotide containing a mutated version of FP5 (FP5M; see Fig. 4), nor by oligonucleotides containing binding sites for the NF1 or Oct-1 factors (Fig. 5B). Interestingly, another oligonucleotide [M, (Fig. 4A)] from the 15-lox flanking region containing sequences similar to the FP4-6, 10 consensus also competes with FP5 in EMSAs (Fig. 5B), although this region and another region, N (Fig. 4A), showed only weak binding in footprinting experiments (data not shown). The faster migrating complexes in some tracks appear to be non-specific since they are not competed by a 200-fold excess of unlabelled FP4 oligonucleotide in Rab9 extracts (Fig. 5A). The nuclear factor binding to the 15-lox silencer elements is not the well characterised negative regulator known as YY1, δ, NF-E1 or UCRBP (37-40),
Figure 3. Footprinting of the 15-lox 5' flanking region from the transcription initiation site to -930 bp. The fragments shown in (A) were end-labelled, incubated either with no extract (0) or nuclear extracts from the cell types stated and then partially digested with DNase I before electrophoresis of the digestion products. The lanes marked A+G show the results of chemical cleavage of the probes at adenosine and guanosine residues. (B) Probe 1; (C) probe 2; (D) probe 3; (E) probe 5; (F) probe 6. The other probes shown in (A) were used to confirm the location of the footprints (data not shown). The competitors used in (D) and (E) were oligonucleotides from the FP4 or FP5 regions (Fp4 and Fp5, respectively) or an oligonucleotide containing the binding site for the nuclear factor NF1 (see Materials and Methods for details).
since an oligonucleotide containing the FP4 binding site does not compete for nuclear proteins binding to an oligonucleotide containing the YY1 binding site (Fig. 5C).

The footprint 8 region. The footprinting experiment shown in Figure 3E shows that both erythroid and non-erythroid cells contain factors capable of binding to this region in vitro. Since inspection of the 15-lox gene sequence in the FP8 region revealed three potential binding sites for GATA-1 (Fig. 4A), the nature of the factors binding to this region in erythroid and non-erythroid cells was investigated further by EMSA (Fig. 6). These experiments clearly show the same series of multiple retarded complexes (Fig. 6, bands 2–5) with both MEL and C5 nuclear extracts, except for an extra complex (Fig. 6, band 1) found only with MEL nuclear extracts. Competition experiments show that the slowest migrating complex (Fig. 6, band 1) found only with MEL extracts is competed with a GATA-1 binding site from the mouse α-globin promoter, whereas the other retarded complexes are not (Fig. 6). Conversely, an FP8 oligonucleotide mutated in each of the three GATA sites competes out bands 2–5 common to both MEL and C5 extracts, but not band 1 (Fig. 6). The erythroid-specific complex is also preferentially competed by an oligonucleotide from the FP8 region mutated in the regions between the three GATA sites (data not shown). These data show that both erythroid and non-erythroid cells contain factor(s) binding to sequences in the FP8 region between the GATA sites, but this does not prevent the GATA-1 in erythroid cells binding to the GATA sites, at least in vitro. The factor(s) responsible for these non-tissue-specific retarded complexes are not the same as those binding to FPs 4–7 and 10 since an FP4 oligonucleotide does not compete (Fig. 6).

Other binding sites. Two other FPs were found. One, FP9, is only found with MEL extracts (Fig. 3E and F), but the nature of the erythroid factor binding is unknown: the sequence of the binding site bears no obvious similarity to those of other erythroid-specific factors. The other, FP11, is found with both MEL and C5 extracts (Fig. 3E) and is almost certainly due to binding of NF1,

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**Figure 4.** (A) Sequence of the 15-lox 5′-flanking DNA. The positions of the FPs found in Figure 3 are marked: underlined (solid line, non-erythroid cell-specific; dashed line, ubiquitous) or over-lined (dashed line, erythroid-specific) with the conserved binding sites for non-erythroid factor(s) boxed. The positions of consensus binding sites for GATA-1, NF1, AP2 and Spl are also marked. The 15-lox gene sequence is available in under GenBank accession numbers M33291 and X90860. (B) Comparison of the sequences in FPs 4–7 and 10 and the mutated version of the consensus sequence (FP5M) used in Figure 5.

**Figure 5.** (A) An oligonucleotide from FP4 was incubated with nuclear extracts from MEL, K562, C5, HeLa and Rab9 cells and then analysed by EMSA. (B) Competition experiment in which the binding a labelled FP5 oligonucleotide to HeLa cell extracts is competed by a 200-fold excess of oligonucleotides from the FP4, FP5, FP7, FP10 or oligo M regions, the mutated FP5M oligonucleotide (see Fig. 4) or oligonucleotides containing binding sites for the nuclear factors NF1 or Oct1. (C) A labelled oligonucleotide containing the YY1 binding site was incubated with MEL or C5 nuclear extracts, with or without unlabelled competitors (YY1, oligonucleotides containing the FP4 or FP5 regions, or a GATA-1 binding site) and analysed by EMSA.
The experimental results indicated that the tandem copies of the FP4 and FP5 regions act as tissue-specific negative regulators. This conclusion was reached through the analysis of nuclear factors binding to the FP8 region, followed by the functional analysis of tandem copies of sequences from the FP4 and FP5 regions linked upstream of the minimal 15-lox promoter. Oligonucleotides containing four copies of the FP4 region or two copies of the FP5 region were inserted upstream of the 150 bp 15-lox minimal promoter in pCAT-150, transiently transfected into MEL, C5, or Rab9 cells, and their effect on promoter activity was determined by assays of CAT activity. The levels of CAT activity were expressed relative to the values for pCAT-150. Results for the 930 bp promoter fragment, containing the complete silencer (pCAT-930), are included for comparison.

**DISCUSSION**

The major conclusion from our functional studies is that the 15-lox gene contains multiple copies of negative elements distributed throughout at least 1 kb of 5' flanking DNA that only function in non-erythroid cells and collectively have the properties of a transcriptional silencer. These elements bind nuclear factor(s) [repressor(s)] present in cells in which the 15-lox gene is not expressed (various fibroblast and epidermal cell lines of mouse, rabbit, and human origin). All the sites appear to bind the same factor, as judged by the fact that the retarded complexes formed with each of the sites in EMSA experiments migrate at the same position and are competed by oligonucleotides containing the other sites. In contrast, the 15-lox gene repressor is not present, or at least cannot bind, in cells from the erythroid lineage in which the 15-LOX is expressed (mouse and human erythroid cell lines). We have therefore been unable to obtain evidence that the GATA and FP9 regions have a functional role in the regulation of the 15-lox gene.

**Figure 6.** Analysis of nuclear factors binding to the FP8 region. A labelled oligonucleotide from the FP8 region was incubated with MEL or C5 nuclear extracts with or without the unlabelled competitors indicated, and analysed by EMSA. Key to competitors used: G-3, FP8 oligonucleotide; G-3M, the FP8 oligonucleotide with each of the three GATA sites mutated (as indicated in Materials and Methods); GATA, an oligonucleotide containing the GATA site from the mouse α-globin gene promoter; FP4, an oligonucleotide from the FP4 region.

**Multiple copies of the FP4 or FP5 regions alone act as tissue-specific negative regulators**

The nuclear protein binding experiments described above strongly suggest that the binding of a nuclear factor to the FP4–7 and 10 regions is involved in the function of the 15-lox silencer. To test this directly, four tandem copies of the FP4 sequence, or two copies of the FP5 sequence, were cloned upstream of the 15-lox minimal promoter in pCAT-150 and their effects on promoter activity assayed after transfection into MEL or C5 cells. The results (Fig. 7) show an 80% reduction of promoter activity in C5 and Rab9 cells, whereas only a 10% reduction is evident in MEL cells. This indicates that tandem copies of the FP4 and FP5 sequences act in isolation as tissue-specific negative regulators of the minimal 15-lox promoter.

**The role of the FP8 triplet GATA region?**

The transient transfection experiments shown in Figure 2 show that the 930 bp promoter fragment containing the GATA sites gives only a small increase (20%) in erythroid cells compared with the minimal 150 bp promoter. To determine whether the GATA region might function late in erythroblast differentiation, stable transfectants were generated containing the pCAT-150, pCAT-930, pCAT-2700 or mini-gene constructs and the promoter activity measured after induction of differentiation by treatment for 5 days with 4 mM HMBA or 1.6% DMSO. To control for the effects of position of integration site in individual transfectants, pools of at least 100 transfectant clones were analysed. However, promoter activity was not increased for any of the constructs during MEL cell differentiation (data not shown). Similarly, stable MEL transfectants containing the FP8 or FP8–11 regions linked directly to the minimal 150 bp lox promoter [pCAT-150(GS) and pCAT-150(GL)] did not show increased promoter activity on induction of differentiation. One hypothesis concerning the role of the triplet GATA region is that binding of the ubiquitous factors to this region repress the 15-lox gene but GATA-1 releases this repression. This seems not be correct since a CAT construct containing the FP8 region linked to the −150 bp lox promoter [pCAT-150(GS)] does not show down-regulated expression in non-erythroid C5 cells in which GATA-1 is not expressed (data not shown). Alternatively, activation of transcription by GATA-1 could be repressed by the binding of the non-tissue-specific factors at the adjacent sites: these factors might only disappear from erythroid cells at late stages of erythroid maturation. To examine whether the factors binding to the non-tissue-specific sites decrease during MEL differentiation relative to the GATA-1 level, EMSA experiments with the FP8 oligonucleotide were performed using extracts from MEL cells at different stages of erythroid maturation. However, even after 5 days of induction with 1.6% DMSO the relative levels of erythroid and ubiquitous factors remain constant (data not shown). We have therefore been unable to obtain evidence that the GATA and FP9 regions have a functional role in the regulation of the 15-lox gene.
expressed (airway epithelial cells and eosinophils) due to the lack of suitable cell lines. Also, it has not been possible to analyse endogenous 15-LOX gene expression in the cell lines used in this study since the rabbit probes do not cross-hybridize with mouse and human genomic DNA or mRNA even at low stringency (data not shown).

Evidence has recently accumulated for the importance of negative control of transcription of genes in a wide variety of contexts (reviewed in 6–11). In general terms, the 15-LOX silencer elements resemble the tissue-specific silencers involved in the lineage-specific regulation of the immunoglobulin κ (41) and T cell receptor (42) genes which may involve Oct-2 (43). Silencers have also recently been defined upstream of the human γ and ε globin genes (13,15,44) and the well-characterized zinc-finger protein known as YY1, 5, NF-E1 or UCRBP (37–40) has been implicated in their activity. YY1 is of considerable interest since it can act either as an activator (40,45–47) or as a repressor (15,37,38,40) of transcription, depending on the context and the other nuclear factors with which it interacts (reviewed in 6). However, YY1 appears not to be the factor binding to the 15-LOX silencer, since this factor is tissue-specific, whereas YY1 is ubiquitous, and an oligonucleotide containing the 15-LOX silencer repressor binding site does not compete for the binding of factors to the YY1 binding site in EMSA experiments. Thus, the identity of the factor binding to the multiple 15-LOX silencer elements remains unknown.

Positive control of LOX expression?

Positive control of erythroid-specific expression, mediated at least in part by members of the GATA family of transcription factors, has been a major feature of the regulation of globin genes and a variety of other genes expressed in an erythroid-specific manner (1,2,3,5,18,23). The 5′-flanking region of the 15-LOX gene contains a cluster of three binding sites for GATA proteins, presumably GATA-1, at about −750 bp. These are the only motifs conforming to the GATA-1 binding site consensus sequence (WGATAR) present in the 10 kb of 15-LOX gene sequence presently available (31). However, using approaches that we have found to be successful in identifying positive cis-control regions involved in the erythroid-specific expression of other genes (1,2,5), we have failed to detect such regions by functional experiments with the 15-LOX genomic fragments available, which contain 2.7 and 4.6 kb of 5′ and 3′ flanking DNA, respectively. None of the reporter constructs containing any or all of these 15-LOX gene regions, including the ‘mini-gene’ (Fig. 1A), have much greater promoter activity than the minimal 150 bp 15-LOX promoter when introduced into erythroid cells in transient transfection experiments (a maximum of 2-fold for the mini-gene construct); nor do the constructs function more efficiently when the promoter when introduced into erythroid cells in transient constructs); nor do the constructs function more efficiently when the promoter when introduced into erythroid cells in transient

References


