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Analysis of upstream regulatory regions required for the activities of two promoters of the rat aldolase A gene

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Rat aldolase A gene has 2 promoters with different tissue specificities (M- and AH promoters). The M promoter is active only in adult skeletal muscle and induced during myogenesis, whereas the AH promoter is active ubiquitously in many tissues, including various cancer cells. Regulatory sequences for these promoters were investigated through assays for transient expression after introduction into myogenic and nonmyogenic cells. When M promoter–CAT fusion genes were transfected into primary cultures of chicken myoblasts, expression of CAT activity was drastically induced during myotube formation. The region comprising 202 to 85 base pairs (bp) upstream from the transcription initiation site was found to be necessary for the induction and an enhancer activity whose region includes the AT-rich recognition sequence (MEF-2 binding site). On the other hand, 2 upstream regions were found to be responsible for AH promoter activity expressed in HepG2 cells. The distal region (−280 to −260) of the promoter includes the AP1 binding sequence, whereas the proximal region (−207 to −180) contains a novel inverted repeat consisting of 22 bp but does not contain known promoter and enhancer sequences.

Aldolase A; Promoter; CAT assay; Gene expression; Rat

1. INTRODUCTION

Aldolase A is one of 3 isozymic forms of fructose 1,6-bisphosphate aldolase and exists ubiquitously and abundantly in many tissues, especially in skeletal muscle [1,2].

Recently we found that a single gene of rat aldolase A has multiple promoters to allow the tissue-specific generation of mRNA differing in their untranslated regions by alternative usage of the leading exons [3]. The first exon, designated M1, encodes the 5′ untranslated sequence specific for M-type mRNA, whereas the second exon, AH1, encodes the 5′ untranslated sequences specific for AH-type mRNAs. Distribution of the 2 types of mRNAs should be primarily determined by tissue-specific activities of the 2 promoters of the leading exons, which are designated as M- and AH promoters, respectively. M-type mRNA is expressed exclusively and highly abundantly in adult skeletal muscle [4,5]. This expression was found to be induced during myotube formation from myoblasts of established cell lines of rat and mice (unpublished results and [6]). On the other hand, AH-type mRNAs are widely expressed to produce aldolase A in many tissues such as brain, spleen, and heart, and in cancer cells [4,5].

In order to elucidate the regulatory mechanisms for tissue-specific usages of the 2 promoters in a single gene, we have begun to identify *cis*-acting regulatory elements in the promoter regions. This communication describes that a 300 base pair (bp) upstream region of the AH promoter where 2 positive elements are identified is sufficient for the promoter activity in HepG2 cells, while the region from −202 to −85 of the M promoter is responsible for dramatic activation during the differentiation of chicken primary myoblast cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Chicken primary myoblast cultures were prepared from breast muscles of 11-day-old chicken embryos essentially as described [7]. Human hepatoma cell line, HepG2 [8], was grown in Eagle's minimal essential medium (Nissui) containing 5% fetal calf serum.

2.2. *Construction of CAT fusion genes*

pM–CAT and pAH–CAT fusion genes were constructed by inserting either of the promoter fragments of the rat aldolase A genomic gene into pOA/CAT, a promoterless CAT plasmid [9], at *Bam*HI and/or *Hin*dIII sites just upstream of the CAT coding region. 5′ deletion mutants of pM–CAT fusion genes were constructed by digesting the promoter fragment with appropriate restriction endonucleases. For pM85.a+CAT and pM85.a–CAT, the fragment spanning from −85 to −202 was inserted into the *Aat*II site in the pBR322 region of pM85CAT in either orientation. 5′ deletion mutants of pAH-CAT were generated by digesting the 5′ end of the promoter fragment with *Bal*31 exonuclease and ligating the digested end to the vector with *Bam*HI-linker. Linker-scanning and internal deletion mutants of pAH293CAT were constructed by combining a pair of 5′ and 3′ deletion mutants of pAH-CAT having the appropriate deletion ends.

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2.3. *DNA transfection*

DNA transfection was performed by the method of calcium phosphate co-precipitation [10]. The CAT fusion gene plasmid was co-transfected with pCH110, an expression plasmid of β-galactosidase [9] as an internal control. For chicken myoblasts, cells were allowed to grow for about 18–20 h after plating and were then transfected with the fusion gene DNAs. The cells were still myoblasts within 24 h after transfection and fused to form myotubes during the next 24 h. Thus, the cells were harvested 20 h and 55 h after transfection and were regarded as myoblasts and myotubes, respectively.

3. RESULTS

3.1. *Activation of the expression of the M-type aldolase A mRNA in myogenesis*

M-type aldolase A mRNA is expressed predominantly in skeletal muscle of the adult rat. This suggests that, as reported for many other muscle-specific genes, the expression of the mRNA could be activated during differentiation of skeletal muscle. S1 nuclease analysis was performed to analyze the M-type mRNA in fetal leg muscle differentiation. In early fetal stages aldolase A mRNA was almost exclusively of the AH-type. The expression of M-type mRNA was drastically induced immediately before birth, continued to increase after birth with concomitant decrease of AH-type mRNA, and finally became a sole mRNA species of aldolase A in skeletal muscle of adult rat (unpublished results and [6]).

3.2. *Induction of M promoter fusion gene expression in chicken primary myoblast cells*

To analyze the change in M promoter activity during myogenesis, pM663CAT was constructed by inserting the M promoter fragment spanning between 663 base pairs upstream (−663 bp) and 41 bp downstream (+41 bp) of the transcription initiation site into the upstream site of the CAT coding sequence in plasmid pOA/CAT (Fig. 1A). The plasmid (pM663CAT) was transfected into primary myoblasts prepared from breast muscle of 11-day-old chicken embryos. After transfection there was very low expression of CAT activity in myoblasts but this increased drastically (about 20-fold) in myotubes (Fig. 1B and C). Essentially the same result was obtained with a fusion gene construct having a longer upstream region (about 3000 bp) from the promoter (data not shown).

To identify the regulatory element(s) responsible for this induction, a series of unidirectional 5′ deletion mutants were constructed (Fig. 1A) and their CAT expression in both cells was determined (Fig. 1B and C). pM202CAT also showed a drastic increase (17.5-fold) during myogenesis, while pM85CAT showed only a slight increase (3-fold) during the same culture period, although both pM202CAT and pM85CAT showed nearly the same CAT activities in myoblast cells. pM33CAT expressed very low levels of CAT activity in both types of cells.

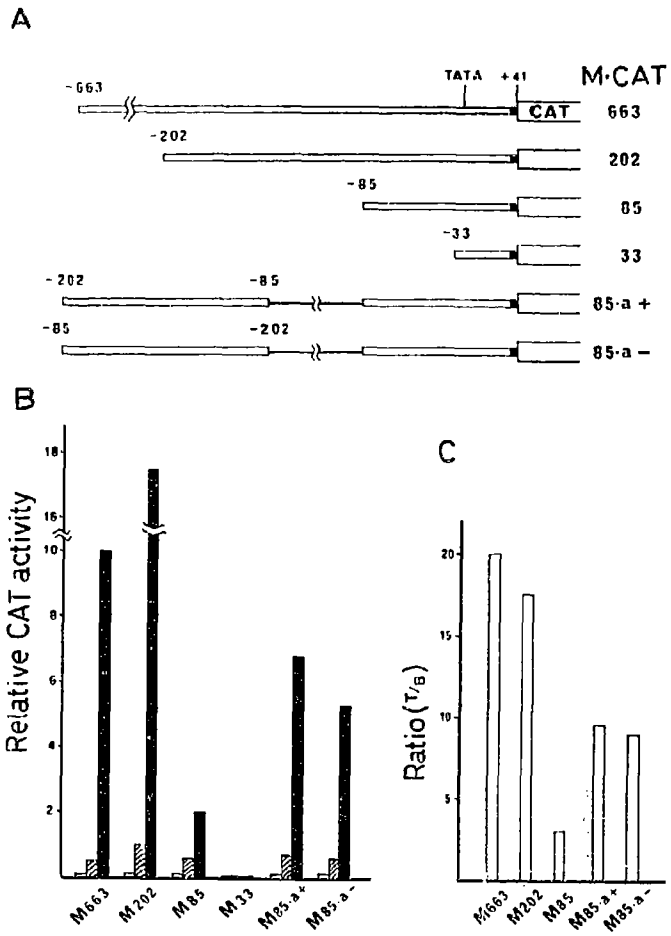

Fig. 1. Induction of expression of M promoter-CAT fusion genes during differentiation of primary chicken myoblast cultures. (A) Structures of M promoter-CAT fusion genes. M promoter sequence in all constructs starts from 41 bp downstream of the transcription initiation site. The upper four are 5′ deletion mutants and are designated as pMxxCAT, where xx indicates the size of the 5′ upstream region in bp. The lower two, pM85.a+CAT and pM85.a−CAT, are derivatives of pM85CAT with the fragment from −85 to −202 inserted into the vector region about 2700 bp from the M promoter region in either orientation. (B) CAT activities expressed from M-CAT fusion genes. 2.5 μg of pM–CAT was cotransfected with 5 μg of pCH110 DNA. CAT activities expressed in chicken myoblasts (hatched bars), in chicken myotubes (filled bars) and in HepG2 (open bars) were quantified and were compared for equal activities of β-gal. CAT activities are presented in relative values assuming the CAT activity from pM663CAT in myotube to be 10. (C) The ratio of CAT activities in myotubes to those in myoblasts. Activities of pM33CAT in both cells were very low and were, thus, omitted.

Fusion gene plasmids, pM85.a+CAT and pM85.a−CAT, also showed the induction of CAT activities during myogenesis (about 10-fold) (Fig. 1B). When the pM-CAT plasmids were transfected into HepG2, the activities from the plasmids in HepG2 cells were negligible (Fig. 1B). This suggests that the activity of the M promoter in these fusion genes is cell type-specific.

3.3. *Identification of* cis*-regulatory elements in AH promoter with 5′ deletion mutants*

When the whole rat aldolase A gene was transfected

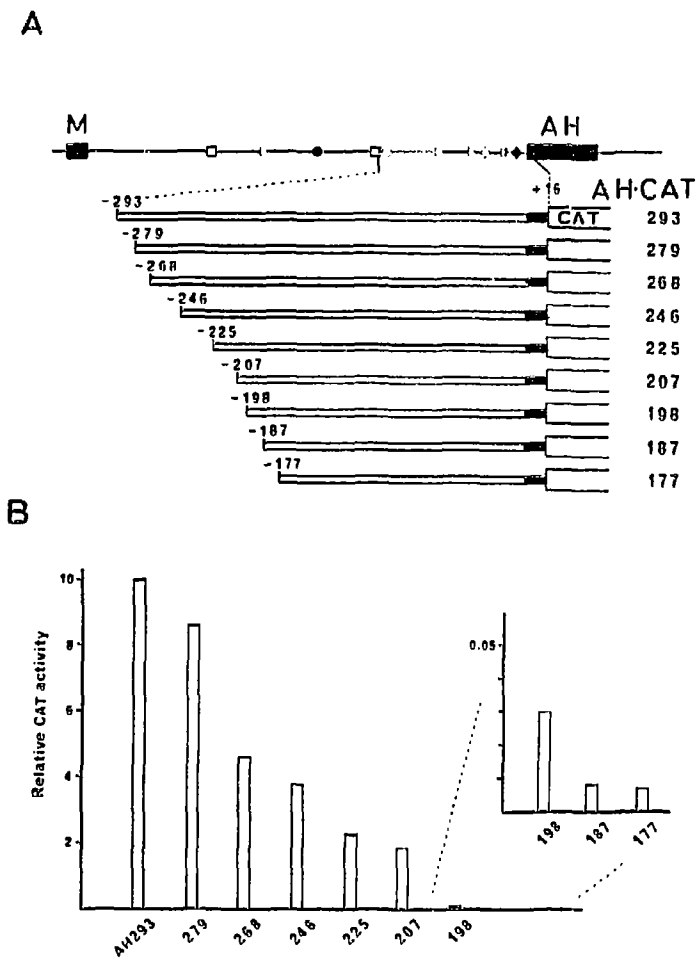

Fig. 2. Expression of 5′ deletion mutants of AH promoter–CAT fusion genes. (A) Structures of AH–CAT fusion genes. The genomic region from exon M to exon AH is shown schematically with identified promoter and enhancer elements, such as TATA box (♦), CAT box (◇), Sp1-binding site [13] (▷), AP1-binding site [11] (○), CRE [22] (●), and NF1-binding site [23] (□) in the above. AH promoter sequence in all AH–CAT fusion genes starts at a site 16 bp downstream from the transcription initiation site of mRNA III and as a result, the initiation site of mRNA II is excluded [3]. AH–CAT fusion genes were designated as pAHxxCAT, the same as M–CAT fusion genes. (B) Expression of the fusion genes in HepG2 cells. CAT activities are presented in values relative to that of pAH293CAT, which is considered as 10. Transfection efficiency was normalized to the β-gal activity expressed from co-transfected pCH110.

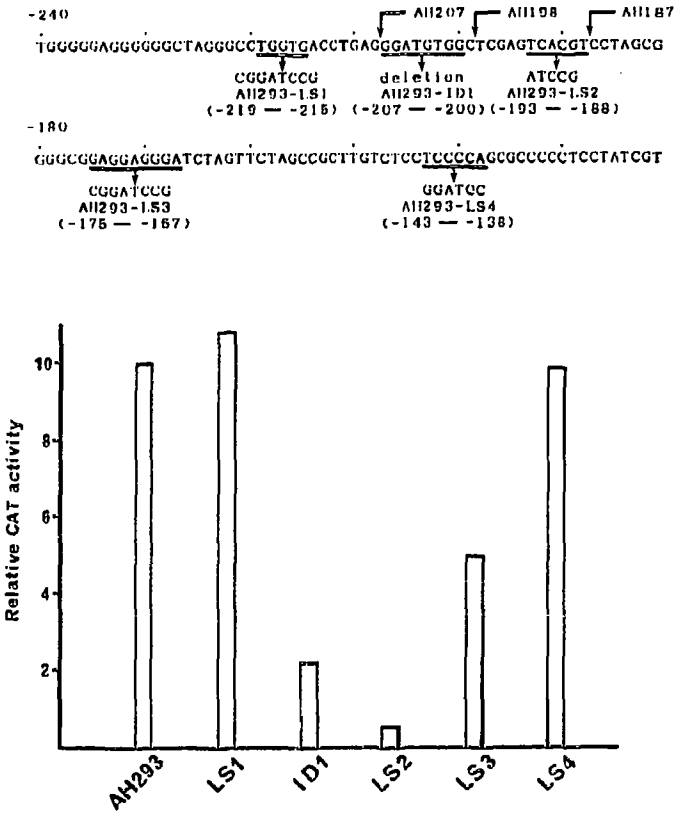

Fig. 3. Expression of internal mutants of AH–CAT fusion genes. Five mutations are described in the sequence between −240 bp and −121 bp of AH promoter. All mutants were constructed from pAH293CAT. The position of the mutated sequence is indicated in parentheses under the mutant. The deletion end points of the three 5′ deletion mutants are also shown above the sequence. CAT activities were normalized to β-gal and are presented in relative values as in Fig. 2.

into HepG2, AH-type mRNA starting from the authentic initiation sites was detected by RNase mapping, and the expression level was not affected by shortening the upstream region of the AH exon from 800 bp to 300 bp (unpublished data). Thus we focused our efforts on analyzing the 300 bp upstream region of the AH exon by constructing AH promoter–CAT fusion genes.

To determine the promoter activities, a series of 5′ deletion mutants were constructed (Fig. 2). As seen in Fig. 2B, deletion of 2 regions was found to decrease the CAT activity; one between −279 bp and −268 bp and the other between −207 bp and −198 bp. Deletion of the distal region caused about a 2-fold decrease in the CAT activity (pAH279CAT to pAH268CAT), while deletion of the proximal region resulted in a 60- to 70-fold decrease of the CAT activity (pAH207CAT to pAH198CAT). The AP1-binding sequence was found to locate exactly in the distal region (−276 to −270, see Fig. 4B) [11]. Thus this *cis*-element could be responsible for the AH promoter activity in HepG2 cells. Further deletion caused a greater decrease of about 3-fold (pAH198CAT to pAH177CAT). However, in this region no previously reported regulatory elements were found [12]. Therefore, this region was analyzed in detail by construeting linker scanning (LS) mutants and an internal deletion (ID) mutant.

3.4. *Analysis of sequences required for AH promoter activity in the proximal region*

Four LS mutants and an ID mutant were constructed by introducing local changes of short sequences into the −220 to −140 region of pAH293CAT (Fig. 3). Mutations in positions −219 to −215 (pAH293-LS1CAT) and −143 to −138 (pAH293-LS4CAT), which locate outside the proximal regions, were found to show no effect on the promoter activity. On the other hand, 3 mutants, pAH293-LS2CAT (−193 to −180), pAH293-LS3CAT

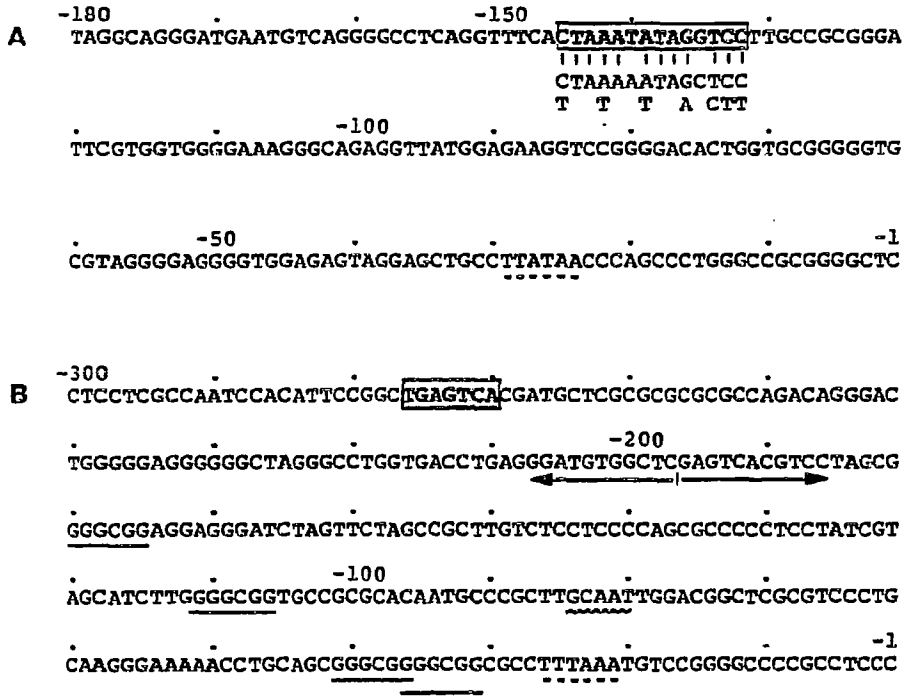

Fig. 4. Nucleotide sequence of M and AH promoter regions. (A) The sequence between 180 bp to 1 bp upstream of exon M is shown. Shaded area is the putative MEF-2 binding site. A consensus sequence for MEF-2 binding is shown below [16,17]. Bars indicate identical nucleotides. TATA box is broken line. (B) The sequence between 300 bp to 1 bp upstream of exon AH1 is shown. Shaded region is the AP1-binding site. Inverted repeat sequence is indicated with a pair of arrows. Four Sp1-binding sites are underlined. CAT box and TATA box are wavy and broken lines, respectively.

(−175 to −167), and pAH293-ID1CAT (−207 to −200), showed decreased expression of CAT (Fig. 3). In particular, the mutations in pAH293-LS2CAT and pAH293-ID1CAT showed remarkable effects on the AH promoter activity (20- and 5-fold decrease, respectively), supporting the premise that the proximal region is important for promoter activity. On the contrary, pAH293-LS3CAT showed only a moderate effect on promoter activity (2-fold decrease). Since the mutation destroyed one of four Sp1-binding sites [13] in the 300 bp promoter region as follows, GGGCGG to GGGCGC (Fig. 3), it is likely that a loss of Sp1-binding to the site caused the decrease of promoter activity in LS3 mutant.

4. DISCUSSION

4.1. *A muscle-specific enhancer in the M promoter*

M-CAT fusion genes, pM663CAT and pM202CAT, showed a dramatic appearance of expression in association with myogenesis when they were transfected into chicken primary myoblasts. These results strongly suggest that the induction of M-type mRNA in rat fetal leg muscle resulted from transcriptional activation of the M promoter. Our results showed that a 202 bp upstream region of the M1 exon is sufficient for the promoter activation coordinated with myogenic differentiation (Fig. 1B and C). Deletion of the sequence between −202 bp and −85 bp greatly decreased the induction efficiency during myogenesis but did not affect the expression in myoblasts. This indicated that a sequence(s) in this region does not function in myoblasts but functions positively on the expression in myotubes. This putative upstream regulatory element functioned independently of orientation and distance from the promoter, and therefore exhibited the properties of an enhancer. In this region no Myo D1 binding sequence [14] or CArG box [15] was found. Instead an AT-rich sequence, so-called MEF-2 binding sequence [16,17], was observed (Fig. 4A). Thus, this element could be responsible for the M promoter induction in myotube.

4.2. *Regulatory elements in AH promoter*

A 293 bp upstream sequence of the transcription initiation site was found to be sufficient for the promoter activity. Two regions required for the activity were identified in this flanking sequence; distal region (−279 to −268) and proximal region (−207 to −198). A sequence for AP1-binding was within the distal region (−276 to 270, see Fig. 4B). Thus the decrease in the promoter activity should be due to the loss of AP1-binding to the promoter.

Another region, the proximal region, was identified by a deletion from −207 bp to −198 bp. As a result of this deletion, the 5′ end of the proximal element seemed to be located between −207 and −198. In this region we could not find any of the promoter or enhancer core sequences previously reported but identified an almost complete inverted repeat sequence, a pair of 11-bp se-

quences with 2 mismatches (see horizontal arrows in Fig. 4B). When the inverted repeat is assumed to be required for the promoter activity, it becomes clear that the promoter activities of ID als LS mutants AH–CAT fusion genes showed good correlation with the extent of destruction of the inverted repeat sequence. These results thereofore strongly suggest that the inverted repeat is important for the promoter activity.

The human aldolase A gene has also been cloned, and its structure was analyzed by several laboratories including ours [18–20]. The homology of these exons between rat and human is very high. A high homology is also found between the upstream regions of the exons, especially between −300 bp regions (data not shown). Although Izzo et al. [21] observed a major decrease (about one third) of promoter activity when the upstream region between −384 and −262 (corresponding to the distal region in ours) was deleted, no remarkable decrease in expression was observed by deleting the region corresponding to the proximal region of the rat promoter. The inverted repeat found in the rat promoter was also found in the corresponding position in the human promoter, although the human repeat carries 2 additional mismatches.

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