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Independence and Interdependence of the Three Human Aldolase A Promoters in Transgenic Mice

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The human aldolase A gene is transcribed from three alternative promoters, clustered in a small 1.6-kb DNA domain. In transgenic mice, the upstream pN and the downstream pH promoters are ubiquitous, whereas the pM promoter, located between pN and pH, is activated specifically in fast skeletal muscles. A strong ubiquitous enhancer, lying upstream of the pH promoter, is necessary for both pN and pH ubiquitous activities, whereas a fast-muscle-specific enhancer, located upstream of the pM promoter, is required for pM-specific activation. In the present study, we use the transgenic mice model to further investigate the contribution of these two regulatory elements to the overall control of these three promoters. We confirm that the pM and pH promoters are activated independently of each other and, in particular, we show that the activation of pM in fast muscle is not responsible for the downregulation of the downstream pH in this tissue. By contrast, the pN promoter needs the presence of both enhancers to reproduce its correct pattern of activity and is unable to function autonomously in vivo.

Aldolase A gene Multiple-promoter system Ubiquitous enhancer
Fast-muscle-specific enhancer Shared regulatory sequences

FOR an increasing number of genes, it is found that expression in different cell types and at different developmental stages relies on the use of alternative promoters. Transcription of a single gene from multiple promoters can lead to the production of one or several related proteins, thus conferring quantitative and/or qualitative flexibility in the control of expression (34).

Over the past decade, whereas numerous studies have dealt with the analysis of the *cis*-acting DNA elements involved in the tissue-specific transcription of a given gene promoter, very few have considered the higher degree of complexity that represents the control of closely spaced promoters driving overlapping transcriptional units. The existence, in the same locus, of several promoters endowed with common and/or different cell specificities and developmental profiles raises interesting questions about the nature and organization of the *cis*-acting elements contributing to their activity and about their mutual interactions.

The human gene coding for the glycolytic enzyme aldolase A provides a paradigm for such a complex organization: it is transcribed from three different promoters, pN, pM, and pH, which generate mRNAs with different 5′ ends but with a common coding sequence (Fig. 1A, B). The initiation sites of these promoters are all clustered in a short 1.6-kb DNA domain (19,23). Two of these promoters, the upstream pN and the downstream pH, direct transcription of ubiquitous mRNA species that are found in most fetal and adult tissues, but are particularly abundant in adult heart and skeletal muscles, with pN activity always being

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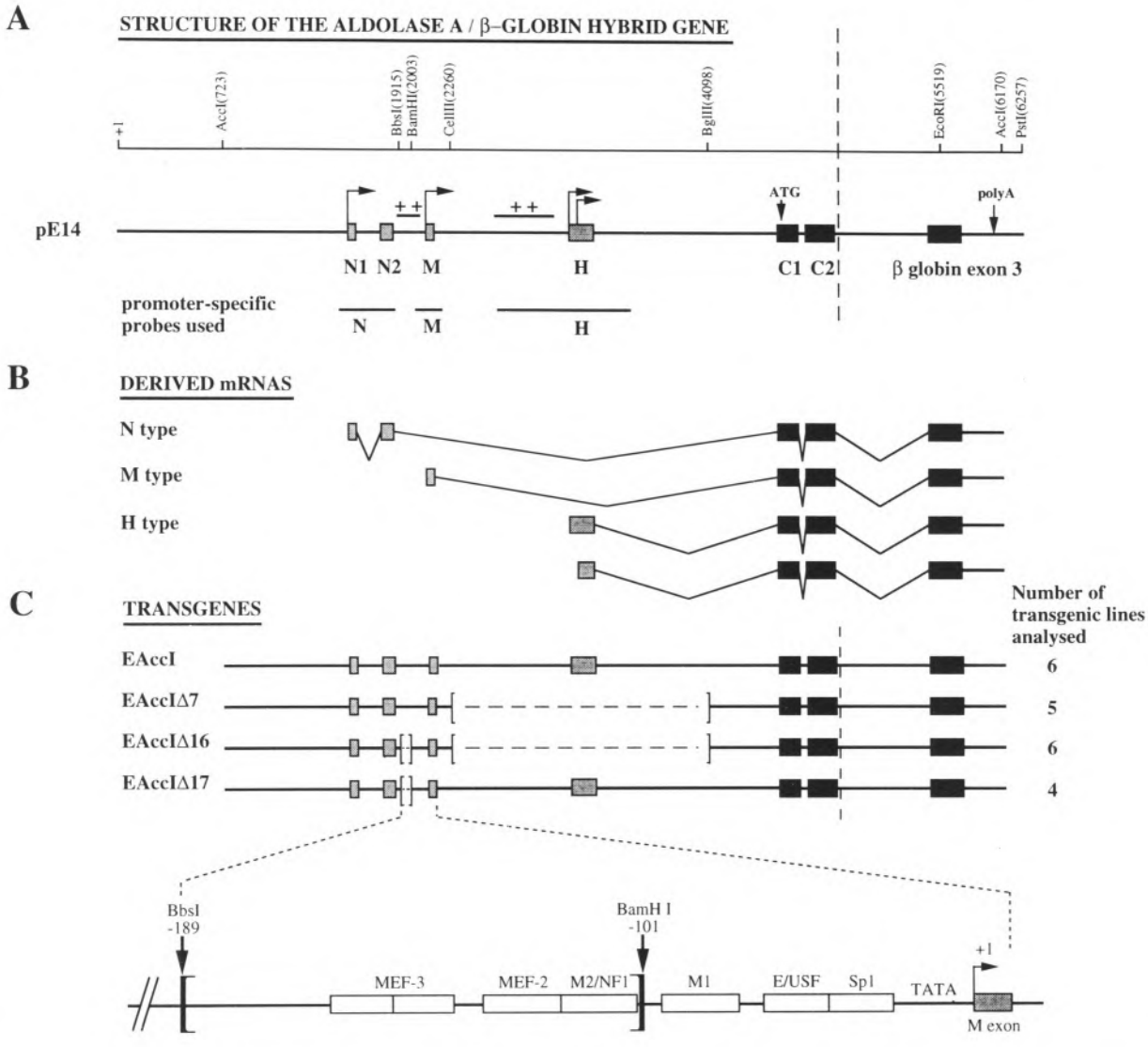

FIG. 1. Structure of the hybrid aldolase A/β-globin transgenes and mRNAs. (A) Structure of the hybrid aldolase A/β-globin gene. The previously described pE14 plasmid (7) contains a fusion of the 5′ region of the human aldolase A gene to the 3′ region of the human β-globin gene, the border between both genes being indicated by a vertical dashed line. Arrows indicate the main transcription start sites and gray and black boxes represent noncoding and coding exons, respectively. The + + symbols indicate the two previously described enhancers. The restriction enzymes used to generate the different transgenes are indicated at the top, with the numbering derived from previous publications (29). The DNA fragments used as probes in Northern blot analysis to specifically detect the different promoter-derived mRNAs are also represented at the bottom. (B) Structure of the mRNAs expressed from the different promoters of the hybrid aldolase A/β-globin gene. (C) Structure of the transgenes. Deleted regions are represented in brackets by dashed lines. The number of independent transgenic lines analyzed for each construct is given on the right (for the EAccIΔ16 transgene, we analyzed in fact two independent lines and four independent founder mice). At the bottom, an enlargement of the pM fast-muscle-specific enhancer/promoter region is shown. Open boxes represent the protein binding DNA elements (not drawn to scale) previously detected by in vitro binding assays (30). The positions of the restriction sites used to delete part of the pM upstream regulatory sequences in EAccIΔ16 and EAccIΔ17 trangenes are indicated with arrows, with another numbering relative to the pM cap site set at +1.

weaker than that of pH (14,23). The third promoter, pM, located between pN and pH, gives rise to transcripts that are highly specific to adult skeletal muscles composed mainly of glycolytic fast-twitch fibers (6,29).

We have shown (6) that a 4.3-kb fragment of the human aldolase A gene including the three promoters, linked to the last coding exon of the human β-globin gene (EAccI transgene, see Fig. 1C), is sufficient to reproduce the proper tissue-specific and developmental regulation of all three human promoters in transgenic mice. Within this 4.3-kb fragment, two main regulatory sequences have been identified (Fig. 1A): a strong ubiqui-

tous enhancer located upstream of the pH promoter, necessary for both pN and pH ubiquitous activities in transient transfection assays (7,14) as well as in transgenic mice (6), and a fast-muscle-specific enhancer located upstream of the pM promoter, necessary for pM activation during myoblast differentiation in cell cultures (30) and for its specific activation in fast muscles of transgenic mice [(29,31), F. Spitz et al., in preparation]. Therefore, because of the close proximity of these three promoters and two enhancers, the human aldolase A gene constitutes a choice model to study their mutual relations. As the transgenic mice model allows studies in all cell types throughout development, it is particularly suited to the investigation of such a promoter system.

In this model, previous results suggested that, in skeletal muscle, the pM and pH promoters are activated independently of each other by their own upstream enhancers (6,29). In contrast, pN seemed to share regulatory sequences with either of the two other promoters, depending on the tissue. In particular, our results suggested that pN could share some regulatory elements with pM for its activation in fast muscles (6). However, in transient transfection assays, it has been reported that some elements located upstream of pN could regulate it independently of the other two promoters in hepatoma cells (9). In the present study, using the transgenic mice model, we further investigate the relative autonomy of the three promoters. Particularly, as we have now localized a fast-muscle-specific enhancer upstream of pM [(29–31), F. Spitz et al., in preparation], we analyze the precise contribution of the two characterized enhancers to the control of pN. We show that the pN promoter needs the presence of both the ubiquitous and the fast-muscle-specific enhancers to reproduce the correct human pattern of activity. When both enhancers are deleted, the pN promoter becomes either totally inactive or prone to position effects, which are due to the influence of the surrounding chromatin at the integration site, showing that the pN promoter is unable to function autonomously in vivo.

In addition, our previous results suggested that the activation of the pM promoter in fast skeletal muscles could result in the subsequent occlusion of the downstream pH promoter. We proposed several hypotheses, such as competition between pM and pH for a common regulatory element or transcriptional interference from the upstream pM on the downstream pH, raising the possibility that such mechanisms could thus play a role in the sequential use of these promoters during myogenesis. Here we show that the complete inactivation of pM is without any consequence on the pH promoter activity, demonstrating that neither competition with pM nor transcriptional interference are the mechanisms implicated in the downregulation of pH in fast muscles.

MATERIALS AND METHODS

Plasmid Constructions and Preparation of Transgenes

Plasmid DNA manipulations were done according to standard recombination techniques (2,32). The aldolase A/β-globin hybrid plasmids pE14 and pE14Δ7 were previously described (6). A pE14Δ16 plasmid was derived from pE14Δ7 by deletion of a BbsI (+1915)-BamHI (+2003) internal fragment in the fast-muscle-specific enhancer. The CellI (+2260)-BglII (+4098) fragment containing the pH enhancer/promoter region was reintroduced in this pE14Δ16 plasmid at its original position and orientation to obtain the pE14Δ17 plasmid (Fig. 1A, C).

The EAccI, EAccIΔ7, EAccIΔ16, and EAccIΔ17 transgenes (Fig. 1C) were obtained by digestion of the pE14, pE14Δ7, pE14Δ16, and pE14Δ17 plasmids with the AccI restriction enzyme, cutting at positions +723 and +6170 (Fig. 1A). Following separation from the plasmid sequences by electrophoresis, the different transgenes were purified by binding to glass powder (18).

Production and Detection of Transgenic Mice

Production of transgenic mice was performed as previously described (6). Detection of transgenic founder mice and of heterozygous offspring and quantification of transgene copy number were done by Southern blot analysis of genomic tail DNA (or placenta DNA when testing fetuses) as previously described (6).

In the case of direct analysis of founder mice, the presence of the transgene in all tested tissues was verified by Southern blot analysis of genomic DNA prepared from biopsies of each tissue.

RNA Analysis

For each transgenic line and each developmental stage, at least two to three different mice were tested. Total RNA was prepared from several tissues of adult mice and from hindlimb muscle at different developmental stages by the guanidium

thiocyanate procedure (3) or by the single-step isolation method (4).

Northern blot analysis was done as previously described (6), using specific probes encompassing the N1 and N2 exons, the M exon, or the H exon (Fig. 1A). For standardization, the blots were finally probed with a R45 ribosomal cDNA probe (6). Relative amounts of specific mRNAs were measured by scanning appropriate autoradiogram exposures of the blots with a Shimadzu densitometer.

For some transgenic lines, an additional reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to detect the pN- or pM-derived mRNAs. The cDNA was synthesized from 5 μg of total RNA, using the random hexamer primer procedure (26), in the presence of 40 units of RNAsin (Promega) and 500 units of M-MLV reverse transcriptase (Gibco-BRL), for 1 h at 37°C. The reverse transcriptase was then inactivated for 5 min at 95°C. The pN- and pM-derived cDNAs were amplified from cDNA aliquots corresponding to 500 ng of total RNA with 200 ng of each primer. The pN-derived cDNA was amplified with forward primer N2–18 (5′-CCAAGGGCCTCCGTCTGG-3′) located in the N2 exon and reverse primer aβ19 (5′-AGCCA CACCAGCCACCACT-3′) located in the β-globin exon, generating a 496-bp amplified spliced product. The pM-derived cDNA was amplified with forward primer M18c (5′-TCCTTC GGCCTCGCCGCA-3′) located in the M exon and reverse primer aβ19, generating a 465-bp amplified spliced product. The cDNA samples were normalized by amplification of the β-actin cDNA, from a cDNA aliquot corresponding to 300 ng of total RNA, with 100 ng of each primer RBACT5 (forward: 5′-CGTGGGCCGCCCTGGCACCA-3′) and RBACT3 (reverse: 5′-TTGGCCTTA GGGTTCAGGGGGG-3′), generating a 240-bp amplified product. In each case, the PCR buffer contained the previous oligonucleotides and 250 μM dNTP, 1.5 mM $MgCl_2$, and 1.25 units of *Taq*-polymerase (Gibco-BRL), in a total volume of 100 μl. The samples were amplified for 25 cycles in a Thermal Cycler (Perkin Elmer Cetus), each cycle consisting of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and primer extension at 72°C for 1 min. Southern blot analysis was then performed: 16-μl aliquots of the amplified products were electrophoretically separated on a 1.2% (w/v) agarose gel and transferred to Hybond N+ nylon membranes (Amersham) in 20 × SSC (3 M NaCl, 0.3 M trisodium citrate). Filters were first fixed in 0.4 N NaOH, and prehybridization and hybridization were then performed at 42°C in the same hybridization buffer [0.2% (w/v) Ficoll 400, 0.2% (w/v) polyvinyl-pyrrolidone 40, 0.2% (w/v) glycin, 6 × SSC, 0.2% (w/v) SDS] containing 100 μg/ml of denatured DNA from salmon sperm. For N and M amplification products, the blots were hybridized with an internal oligonucleotide C1–24 (5′-GACTCATCTGCAGCCAGGATGCCC-3′) located in the aldolase C1 exon. For β-actin amplification products, the blots were hybridized with the PCR oligonucleotide RBACT5. Then 200 ng of each oligonucleotide probe was labeled with 10 units of T4 polynucleotide kinase (Gibco-BRL) and 3 μl of [γ-^{32}P]ATP at >5000 Ci/mmol (Amersham), for 30 min at 37°C, and purified on a Sephadex G-50 column (Pharmacia). Final washing of blots was at 42°C in 0.2 × SSC, 0.1% (w/v) SDS.

RESULTS

The pN and pM Promoters Are Activated in a Parallel Way During Myogenesis in a Transgene Lacking the pH Enhancer/Promoter Region

The previous analysis of six lines harboring the EAccI transgene (Fig. 1C) has shown that pN activity parallels that of pH in most tissues except in fast skeletal muscles [(6), see also Fig. 3A]. Here we quantify the pN- and pH-derived mRNAs in different skeletal muscles of transgenic mice from several EAccI lines (Table 1), showing that whereas pH is 5-fold less active in fast muscles (gastrocnemius and vastus lateralis) than in a slow one (soleus), pN is found 2.5-fold more active in fast muscles than in soleus. When a 1.8-kb fragment including the pH enhancer/promoter region was deleted in the EAccIΔ7 transgene (Fig. 1C), pN was no longer active in any of the tissues of the five transgenic lines analyzed, except in fast skeletal muscles where it remained active together with pM (6). In addition, the analysis of one EAccI line during muscle development showed that pN-derived mRNAs accumulate in a pattern intermediary between those of pM and pH [(6), see also Fig. 2A). Taken together, these results suggested that pN and pM promoters could share common regulatory elements present in the EAccIΔ7 construct. Therefore, it was of interest to compare the regulation of pN and pM during myogenesis in EAccI and EAccIΔ7 transgenic lines.

For this purpose, we performed Northern blot analysis of RNA prepared from total hindlimb muscles taken at different developmental stages, from fetal to adult stage. Figure 2 shows the accu-

TABLE 1
COMPARISON BETWEEN pN AND pH PROMOTERS RELATIVE EXPRESSION IN DIFFERENT SKELETAL MUSCLES OF EAccI AND EAccIΔ17 TRANSGENIC MICE

mRNA Type	Transgenes	Soleus	Gastrocnemius	Vastus Lateralis
N	EAccI	100	248 ± 133	262 ± 133
	EAccIΔ17	100	55 ± 7	56 ± 15
H	EAccI	100	22 ± 8	18 ± 5
	EAccIΔ17	100	36 ± 16	39 ± 22

Samples (5 μg) of total RNAs prepared from different adult hindlimb skeletal muscles (soleus: slow muscle; gastrocnemius and vastus lateralis: fast muscles) of transgenic mice were analyzed by Northern blot. For each EAccI or EAccIΔ17 transgene, four to six separate samples of each muscle type, corresponding to individuals from two to three independent lines, were analyzed. The blots were successively hybridized to the three human promoter-specific probes (Fig. 1A), and finally to a 18S rRNA probe (R45) for standardization (6). Hybridization signals obtained with N- and H-specific probes and with the R45 probe were measured by densitometric scanning of appropriate exposures of the blots. After standardization, the average mRNA level from each N or H type and in each muscle type was calculated and expressed relative to that obtained for soleus muscle, which was arbitrarily set at 100%.

mulation patterns of the various promoter-specific mRNAs during myogenesis, measured in one representative line for each transgene: line A3 for EAccI and line Δ7-17 for EAccIΔ7. The expression from the three promoters in line A3 has already been reported (6). Here we present a more extensive study, including additional developmental stages, to facilitate the comparison with the results obtained at the same stages in line Δ7-17. Contrary to what is observed in line A3, pN- and pM-derived mRNAs accumulate similarly in line Δ7-17: undetectable at the fetus stage, they are first weakly detected at birth and then their level increases mostly after 16 days of age and reaches the maximal at the adult stage.

These results show that in the absence of the pH enhancer/promoter region, pN and pM are coregulated during myogenesis, strengthening the idea that in fast skeletal muscles, pN and pM could share some regulatory sequences, possibly located in the fast skeletal muscle-specific enhancer.

Moreover, it may be noticed that pM-derived mRNAs accumulate in slightly different ways in A3 and Δ7-17 lines: they are first faintly detected at the fetus stage in line A3 (Fig. 2A), whereas they are never detectable at this stage in line Δ7-17, in which they become detectable only at birth (Fig. 2B). That fits with our previous observation that pM activity can be detected in slow skeletal muscles in EAccI lines, but not in EAccIΔ7 lines (6,29). Therefore, in the context of the whole regulatory region, this weak pM activity in fetuses, as well as in adult slow skeletal muscles, could depend on the presence of the ubiquitous enhancer.

The pN and pH Promoters Show a Similar Activity Pattern in Different Muscles in a Transgene Lacking 87 bp in the Fast-Muscle-Specific Enhancer Sequence

To examine the contribution of the muscle-specific enhancer to the activity pattern of the pN promoter, we created the EAccIΔ17 construct (Fig. 1C), consisting of a short 87-bp deletion in the fast-muscle-specific enhancer. This deletion removes part of the pM upstream regulatory sequences (29), and particularly an overlapping binding site for NF1 and MEF-2 factors and a MEF-3 motif (Fig. 1C) required for pM fast-muscle-specific activity (31). Four transgenic lines were obtained with this EAccIΔ17 construct, which all exhibited an identical expression pattern from the three promoters.

Figure 3B shows a Northern blot analysis of expression from pN, pM, and pH promoters in several muscles (composed of diverse proportions of the different fast or slow fibers) and various other tissues of one representative EAccIΔ17 line (line Δ17-55). For comparison, the expression pattern observed in a control EAccI line (line A49) is shown in Fig. 3A. In this EAccIΔ17 transgenic line, as in the others, no activity of the pM promoter could be detected by Northern blot analysis in all tested tissues, including fast skeletal muscles as gastrocnemius and vastus lateralis. An addi-

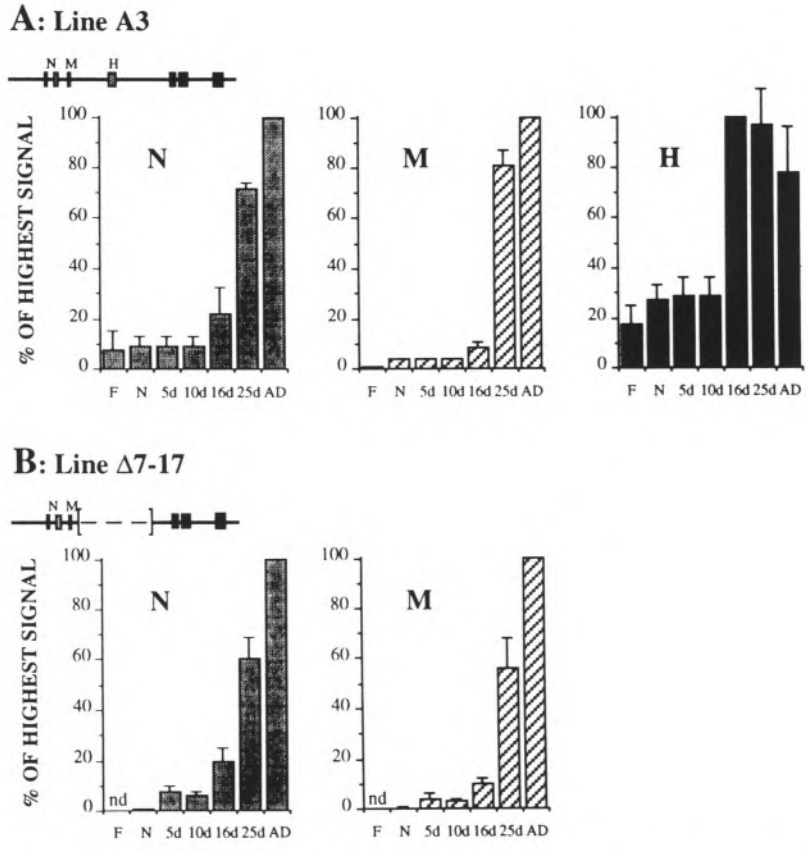

FIG. 2. Expression from the three transgenic promoters during muscle development in lines A3 harboring the EAccI transgene (A) and Δ7–17 harboring the EAccIΔ7 transgene (B). For each developmental stage (F: 17-day postcoitum fetuses; N: 1-day newborn mice; 5d: 5-day-old mice; 10d: 10-day-old mice; 16d: 16-day-old mice; 25d: 25-day-old mice; AD: at least 2-month-old adult mice), three to five separate samples were analyzed by Northern blot, each corresponding to 5-μg sample of total RNAs prepared from total hindlimb muscles of one individual or of a pool from several individuals of transgenic mice from the indicated A3 or Δ7–17 lines. The blots were successively hybridized to the three human promoter-specific probes (Fig. 1A), and finally to a 18S rRNA probe (R45) for standardization (6). Hybridization signals obtained with the promoter-specific probes and with the R45 probe were measured by densitometric scanning of appropriate exposures of at least two independent Northern blots. After standardization, the average mRNA level from each N, M, or H type and at each developmental stage was calculated and expressed relative to the highest level obtained for each mRNA type, which was arbitrarily set at 100%. The histograms show means and standard deviations (sometimes not visible, due to scale) for each stage. nd: not detectable.

tional, more sensitive, RT-PCR analysis, consisting of 25 cycles amplification followed by hybridization with an internal oligonucleotide, also failed to allow detection of any pM-derived mRNAs (data not shown), thus confirming that the deleted fragment includes core sequences necessary for pM activity. This result also shows that, in the absence of these sequences, pM cannot be activated by the strong adjacent ubiquitous enhancer.

By contrast, pH activity is unchanged when compared to that of the EAccI line (Fig. 3A, B), showing that the fast-muscle-specific enhancer is totally dispensable for pH activity. Moreover, this result shows that the complete inactivation of the pM promoter is without significative consequence on the activity of the downstream pH promoter in fast skeketal muscles (Table 1).

In EAccIΔ17 lines, the expression pattern of the pN promoter is unchanged in most tissues when compared to EAccI lines, but clearly modified in fast skeletal muscles (Fig. 3A, B; Table 1): whereas in EAccI lines, pN is found 2.5-fold more active in fast muscles than in a slow muscle (such

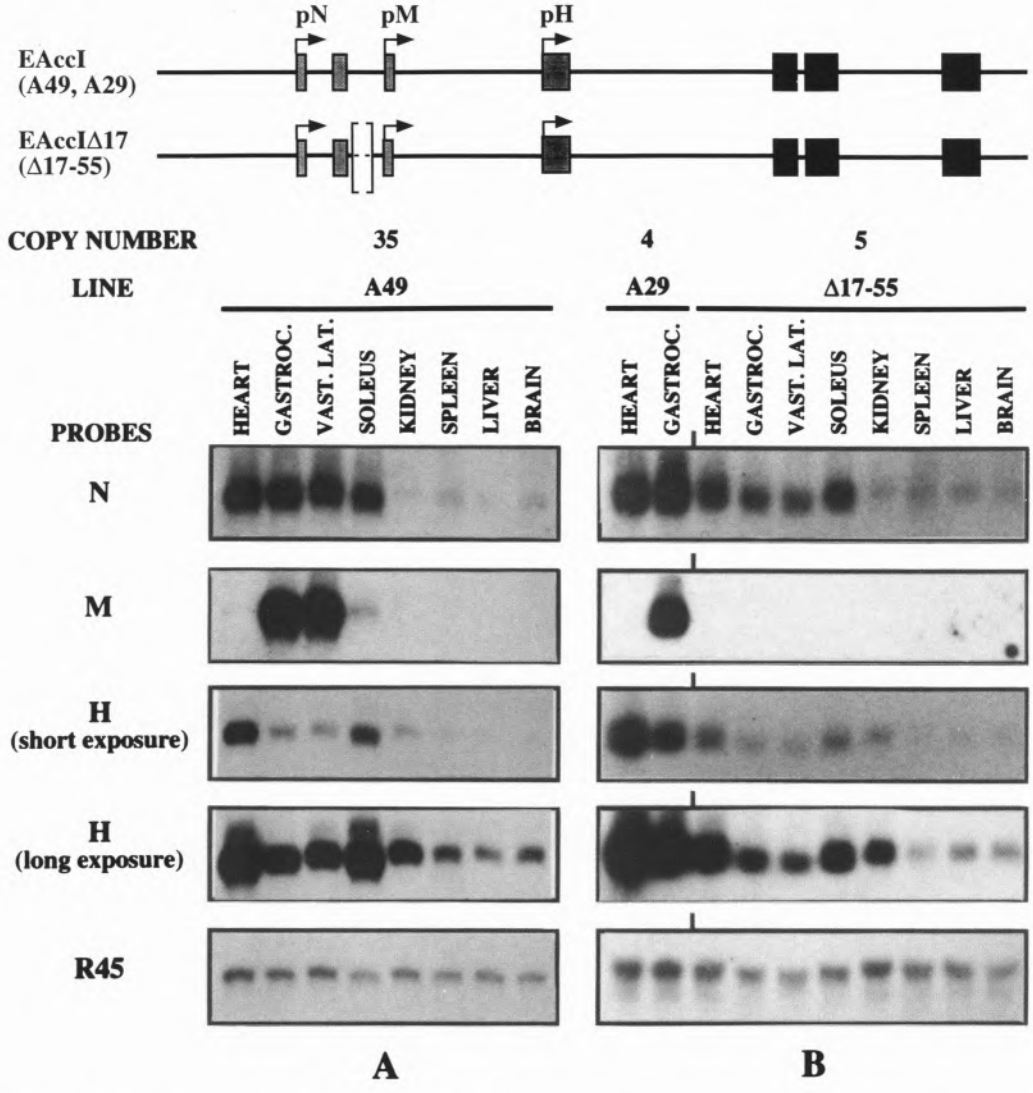

FIG. 3. Northern blot analysis of expression from the three transgenic promoters in lines A49 harboring the EAccI transgene (A) and Δ17–55 harboring the EAccIΔ17 transgene (B). Samples (5 μg) of total RNAs prepared from various tissues, including different muscle types [heart: a nonskeletal muscle; gastroc. (gastrocnemius) and vast. lat. (vastus lateralis): fast skeletal muscles; soleus: a slow skeletal muscle], of transgenic mice from the indicated lines were analyzed for expression from the three transgenic promoters by Northern blot. The blots were successively hybridized to the three human promoter-specific probes (Fig. 1A), and finally to a 18S rRNA probe (R45) for standardization (6). (A) and (B) show two independent Northern blots. The autoradiograms shown for the different probes and in (A) and (B) correspond to various exposures of the blots, and do not allow comparison of the relative abundancy of the transcripts. The number of transgene copies integrated, approximately estimated as previously described (6), is indicated for each line. Two muscle tissues from an EAccI line (line A29) were included on the blot presented in (B) to attest hybridization particularly with the M probe.

as soleus), in EAccIΔ17 lines, pN is 2-fold less active in fast muscles than in soleus. In fact, in this context, the expression pattern of pN becomes very similar to that observed for pH.

Taken together, these results confirm that pH functions independently of pM and show that in the absence of the fast-muscle-specific enhancer, pN and pH are coregulated by the ubiquitous enhancer in all tissues examined, including fast muscles.

Absence of Activity or Position-Dependent Activity of the pN Promoter in a Transgene Deleted in Both the Muscle-Specific Enhancer and the pH Enhancer/Promoter Regions

To examine the possibility of an autonomous activity of the pN promoter due to upstream sequences as previously reported (9), we created the EAccIΔ16 construct (Fig. 1C), combining the two previous deletions of the 87-bp fragment in the

pression in some tissues, depending on the transgenic line considered.

DISCUSSION

The appropriate temporal and spatial control of a gene with multiple promoters is still a poorly understood aspect of transcriptional regulation. In many respects, the questions raised resemble those encountered when considering gene clusters in which pairs or groups of genes are situated near each other and share some aspects of regulation as tissue specificity. Multiple mechanisms, such as sharing of a common regulatory region (10,15, 20,24,35), with sometimes competition between gene promoters for the shared enhancer element (12) or transcriptional interference (40), have been described. Moreover, in these clusters, some of the genes show the same tissue specificity but are nonetheless independently regulated, each by its own tissue-specific and/or timing regulatory sequences (10,28,39).

A remarkable feature of the human aldolase A gene regulatory region is the coexistence, in a small 1.6-kb DNA domain, of three promoters and two enhancers endowed with different temporal and cell specificities. This mutual proximity should allow multiple interactions between promoters and enhancers, with shared or autonomous regulations, and might also lead to competition or transcriptional interference between the promoters. In addition, mechanisms that maintain the specificity of these enhancer/promoter interactions are expected to take place, to ensure the proper expression of the three promoters. In the present study, we have further investigated the mutual relations between these three promoters and two enhancers, with a special look at the most upstream pN promoter, which displayed characteristics in common with both other promoters: pM and pH.

The 87-bp Fragment From the Muscle-Specific Enhancer, Including a MEF-3 Binding Site and an Overlapping Binding Site for NF1 and MEF-2 Factors, Is Necessary for In Vivo pM Activity

We have recently shown that the isolated proximal sequences of the human aldolase A pM promoter are able to target very efficiently the expression of a CAT reporter gene in fast skeletal muscles of transgenic mice (29,31). The sequences necessary and sufficient for this specific expression were delimited to a 209-bp fragment extending from base pairs −164 to +45 relative to the pM transcription start site. To further examine the role of this fast-muscle-specific enhancer in the activities of the three promoters, and to see whether pM could be activated by other upstream or downstream sequences in the context of the whole regulatory region, we created the EAccIΔ17 construct (Fig. 1C), consisting of a short 87-bp deletion (from base pairs −189 to −101 relative to the pM transcription start site) in the fast muscle-specific enhancer. This deletion removes a MEF-3 binding site and an overlapping binding site for NF1 (M2 element) and MEF-2 factors, but retains the M1 element, the USF-binding E box, and the Sp1 site (Fig. 1C). The individual importance of these different motifs has been previously tested in the context of the isolated pM enhancer/promoter region, both ex vivo in myogenic cells (30) and in vivo in the transgenic mice model (31).

Here we show that in all four EAccIΔ17 transgenic lines, when a 87-bp fragment including the MEF-3 and the MEF-2-M2/NF1 regions is deleted in the natural context of the three promoters (Fig. 1C), no pM-derived mRNAs could be detected in any of the tissues tested, even when using a sensitive RT-PCR technique. Moreover, whereas in EAccIΔ7 lines, harboring a transgene deleted from the pH enhancer/promoter region, pM was found fully active in fast skeletal muscles (6), in EAccIΔ16 lines, in which the transgene combines both deletions, no activity of the pM promoter could be detected. Taken together, among the 10 transgenic lines harboring the EAccIΔ17 or EAccIΔ16 constructs deleted from the 87-bp fragment in the fast-muscle-specific enhancer, none of them expresses pM.

In the EAccIΔ17 transgene, pM, which lies in between the other two promoters, is situated in an ubiquitously transcribed region. In particular, transcription starting at the upstream pN promoter and proceeding through pM is supposed to create a loose chromatinian structure. However, this open environment is unable to compensate for the absence of the deleted fragment in the EAccIΔ17 transgene, because pM is totally inactive. These results confirm that the deleted fragment includes sequences required for pM muscle-specific activity, and show that no other elements present in the transgene are able to activate pM. Especially, in spite of their individual importance shown both ex vivo (30) and in vivo (31), the remaining proximal elements of the muscle-specific enhancer (the M1 element, the USF-binding E box, and the Sp1 binding site, see Fig. 1C), even

associated with the remaining distal elements, are not sufficient to confer any activity on the pM promoter.

pM Behaves as a "Locked" Promoter in Tissues Other Than Skeletal Muscle

Despite the close proximity of the downstream ubiquitous enhancer, the pM promoter, which is under the control of its own upstream regulatory sequences, is specific to skeletal muscle and is not activated in other tissues. However, we have recently reported that the deletion of the pH enhancer/promoter region makes pM more specific to fast skeletal muscles: when this region is deleted, pM activity becomes barely detectable in a slow muscle such as the soleus (29). These results suggested that in vivo pM expression could in fact result from the combined effects of the upstream enhancer specific to the fast-twitch glycolytic IIB fibers [(29), M. Salminen et al., in preparation], with downstream sequences, located in the ubiquitous enhancer, extending its specificity to other IIX and/or IIA fast muscle fibers, which are the only fast fibers present in the slow soleus muscle beside type I slow fibers (11). Thus, pM seems not to escape totally from the influence of the strong ubiquitous enhancer. In good accordance with these observations, here we show that during myogenesis, pM-derived mRNAs accumulate later when the pH enhancer/promoter region is deleted (Fig. 2A, B), suggesting that the ubiquitous enhancer could also exert its influence on pM in fetal skeletal muscle. So, pM may be influenced by the ubiquitous enhancer, but this effect is restricted to skeletal muscle, suggesting that in other tissues, pM is in a relatively "locked" configuration, thus escaping the enhancer effects.

Moreover, here we show that in EAccIΔ17 transgenic lines, although the ubiquitous enhancer is present in this construct, no pM activity could be detected in any tested tissue. These results demonstrate that even when its privileged enhancer partner is not functional, the pM promoter remains independent from the influence of the ubiquitous enhancer.

Interestingly, when both enhancers are deleted in the EAccIΔ16 transgene, although some nonreproducible expression could be sometimes observed from the pN promoter, we never observed any expression from the pM promoter. These two promoters being likely exposed to the same position effects, due to the chromatinian environment of the transgene at its integration site in the mouse genome (1), once more pM seems to be resistant to the influence of other regulatory elements located in its neighborhood.

Several hypotheses can be proposed to explain the lack of activity of the ubiquitous enhancer on the pM promoter. Some incompatibility phenomena between enhancers and promoters have been described, for instance, in the case of the closely spaced *Drosophila gooseberry* and *gooseberry neuro* genes (21). The absence of an appropriate TATA box (38) or of particular elements of the transcription initiation complex (17), or of muscle-specific *trans*-acting factors required for efficient interaction (33), could prevent pM from interacting with the ubiquitous enhancer. Transcriptional repression by a "quenching" mechanism, whereby a repressor blocks the ability of the activators to contact the transcription complex in nonmuscle tissues, is also conceivable (16). Another hypothesis is that this interaction could be hindered by a dominant negative element as described in the immunoglobulin μ gene, in which the nuclear matrix attachment regions (MARs) that flank the μ enhancer may participate in the repression of enhancer activity in non-B lymphoid cells (13). Another mechanism involving a negative control element has been proposed in the case of the M promoter of the mouse aldolase A gene (36): a so-called "M Sequestering Element," located between -1100 and -350 bp of the M promoter, seemed to prohibit inappropriate activation of the M promoter by the ubiquitous enhancer, because deletion of this element resulted in a strong stimulation of the M promoter activity by downstream sequences both in myoblasts and myotubes. In the case of the human gene, we have previously shown that deleting upstream sequences up to -305 bp of pM does not alter its specificity in transgenic mice (29). Therefore, if such a sequestering mechanism occurs in the human gene, the implicated sequences would be located downtream of position -305 bp and would not involve the 87-bp regulatory fragment deleted in EAccIΔ16 and EAccIΔ17 transgenes.

The Downregulation of pH in Fast Skeletal Muscles Is Not Due to Competition or Transcriptional Interference Mechanisms

We have previously observed that pH promoter activity varies inversely to that of the upstream pM promoter in different muscles: pH activity is higher in slow muscles in which pM is poorly active and lower in fast muscles where pM is highly

activated [(6), see also Fig. 3A and Table 1]. By contrast, when isolated from upstream promoters, pH was found as active in all muscle types (6). This observation suggested that pM activity could have a negative effect on pH, either due to competition for a common regulatory element (12) or to a transcriptional interference mechanism as already described in tandemly arranged promoters (8) or genes (27). The hypothesis that initiation from the distal pM promoter may result in the subsequent repression of the proximal pH promoter by transcriptional read-through was also suggested for the mouse aldolase A gene (5,36).

Here we show that the complete inactivation of pM observed in the EAccIΔ17 transgenic lines is without any consequence on the downstream pH activity profile in various muscles. This result demonstrates that pH is controlled independently of pM and that neither competition between pM and pH nor transcriptional interference are the mechanisms implicated in the downregulation of pH in fast muscles. We therefore suggest the existence of an upstream negative regulatory element that would inhibit specifically pH promoter activity in fast skeletal muscles.

The pN Promoter Is Unable to Function Autonomously In Vivo

The particular organization of the human aldolase A 5′ region raised the possibility of shared regulatory sequences between the three promoters. We have previously shown that the ubiquitous enhancer is necessary for both pN and pH ubiquitous activities in cultured cells (7) and in transgenic mice (6), and suggested that pN could also share regulatory sequences with the muscle-specific pM promoter (6).

Nonetheless, since then, it was reported that the genomic region upstream from the human pN promoter efficiently directs transcription of a reporter CAT gene after transient transfections in human hepatoma cells, suggesting that it could be per se an active promoter under the control of its own regulatory elements located within 800 bp upstream from the initiation site (9). Moreover, we had previously shown that in cultured human lymphocytes, only the pN promoter, and not pH, exhibits a strong response to serum, suggesting that pN and pH are differently regulated in these cells (14). Taken together, these results suggested

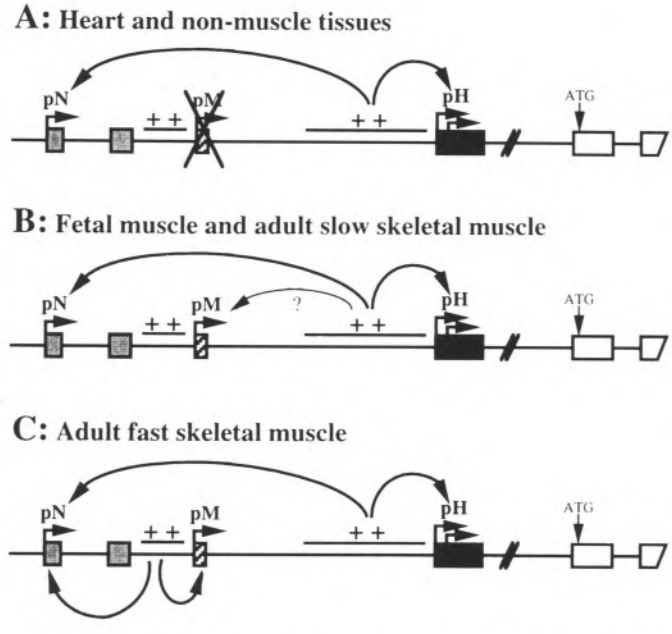

FIG. 5. Model of the different enhancer/promoter interactions occurring in the human aldolase A gene in different tissues and at different developmental stages. Arrows point to the target promoters activated by the two enhancers, represented by the + + symbol. In (A), the pM promoter is crossed out to indicate that it is not active in these tissues. In (B), the question mark under the arrow means that the sequences activating the pM promoter in these muscles, which are indeed located in the pH promoter/enhancer region deleted in the EAccIΔ7 transgene, are only putatively corresponding to the ubiquitous enhancer.

that the pN promoter could have some own regulatory regions able to activate it, in particular cell types ex vivo.

However, we show in this article that the pN promoter is substantially inactive in transgenes lacking both the ubiquitous and the muscle-specific enhancers, indicating that it seems devoid of regulatory elements allowing it to function autonomously. In the absence of any contiguous enhancer, pN seems to be prone to weak position effects, as suggested by its very low activity in muscle and brain of some EAccIΔ16 lines. It should be noticed that pN activity seems to be more or less specific to humans, because no N-type aldolase A transcripts have been found in any rodent tissue in vivo (25,37). However, a pN activity has been detected in some myogenic and glial rodent cell lines (22), suggesting that in these species pN, although silent under normal conditions, remains activable under some circumstances. Therefore, we cannot exclude that, while being controlled by both contiguous enhancers in humans (and not in rodents), the pN promoter can also be activated by distinct regulatory sequences active only under certain conditions (e.g., in some cultured cell lines or in activated lymphocytes) in humans as well as in rodents. According to this hypothesis, subjection of pN to ubiquitous and muscle-specific enhancers would be specific to humans, but not the intrinsic activability of this promoter. However, the physiological relevance of pN promoter activity remains obscure.

In conclusion, the present study confirms the dependence of the pN promoter on contiguous ubiquitous and muscle-specific enhancers, according to the tissue and the developmental stage (Fig. 5), contrasting with the strong autonomy of pM and pH promoters. In spite of the presence of the strong ubiquitous enhancer in the vicinity, pM is only active in fast skeletal muscles. pM activation and pH inhibition in fast muscles appear to be concomitant but independent mechanisms, because pH inhibition is observed in the absence of any activation of pM when the muscle-specific enhancer is partly deleted.

Thus, in a small DNA domain, different modes of promoter regulation have evolved, leading to distinct activation and inhibition mechanisms of these promoters in muscles. This remarkable organization of the regulatory domain of the human aldolase A gene makes it an excellent model to further investigate the mechanisms that maintain the specificity of interactions between promoters and enhancers.

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