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An Opportunistic Promoter Sharing Regulatory Sequences with either a Muscle-Specific or a Ubiquitous Promoter in the Human Aldolase A Gene

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The human aldolase A gene is transcribed from three different promoters, pN, pM, and pH, all of which are clustered within a small 1.6-kbp DNA domain. pM, which is highly specific to adult skeletal muscle, lies in between pN and pH, which are ubiquitous but particularly active in heart and skeletal muscle. A ubiquitous enhancer, located just upstream of pH start sites, is necessary for the activity of both pH and pN in transient transfection assays. Using transgenic mice, we studied the sequences controlling the muscle-specific promoter pM and the relations between the three promoters and the ubiquitous enhancer. A 4.3-kbp fragment containing the three promoters and the ubiquitous enhancer showed an expression pattern consistent with that known in humans. In addition, while pH was active in both fast and slow skeletal muscles, pM was active only in fast muscle. pM activity was unaltered by the deletion of a 1.8-kbp region containing the ubiquitous enhancer and the pH promoter, whereas pN remained active only in fast skeletal muscle. These findings suggest that in fast skeletal muscle, a tissue-specific enhancer was acting on both pN and pM, whereas in other tissues, the ubiquitous enhancer was necessary for pN activity. Finally, a 2.6-kbp region containing the ubiquitous enhancer and only the pH promoter was sufficient to bring about high-level expression of pH in cardiac and skeletal muscle. Thus, while pH and pM function independently of each other, pN, remarkably, shares regulatory elements with each of them, depending on the tissue. Importantly, expression of the transgenes was independent of the integration site, as originally described for transgenes containing the β-globin locus control region.

Multiple promoters are often used to express one or several related proteins from a single gene (44). For example, the human aldolase A gene is transcribed from three tandem promoters, pN, pM, and pH, generating mRNAs with different promoter-specific 5′ noncoding exons but a common coding sequence (31, 34). pM, which is highly specific to adult skeletal muscle, lies in between pN and pH, which are ubiquitous but particularly active in heart and skeletal muscle, with pN-derived mRNAs always being approximately 10- to 20-fold less abundant than pH-derived mRNAs (23, 34). In contrast, the mouse and rat aldolase A genes are transcribed from only two promoters; no functional utilization of a pN-like promoter has been detected in these species (37, 49). Interestingly, in rats, pM-derived mRNAs accumulate to a high level in muscles with a high, fast myofiber content, which predominantly have a glycolytic metabolism, and accumulate to a low level in muscles with a low, fast myofiber content, which predominantly have an oxidative metabolism (46). In mice, in situ hybridization studies also suggest that pM is active only in fast myofibers while pH is active in both fast and slow myofibers (13). In humans, myofiber specificity of the pM promoter has not been examined. The study of gene expression specific to either fast or slow myofibers will help in the understanding of the maturation of myofibers into these different types. Genetic influences, but also neural, hormonal, and other environmental influences, have been proposed to play a role in the maturation process (36). However, nothing is known yet about their transcriptional effectors.

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The start sites of the three promoters are all clustered within a small, 1.6-kbp DNA domain. The mutual proximity of the three promoters raises interesting questions about their relations to one another. The human aldolase A gene, therefore, constitutes an excellent model for the study of muscle gene expression and for the study of interactions between multiple promoters.

Using transient transfection assays, we have recently located a strong ubiquitous enhancer upstream of pH start sites which is necessary for the activity of both ubiquitous promoters, pN and pH (14). We were, therefore, interested in determining its role in the activity of the third promoter, pM. In the absence of cell culture assays for fiber type-specific expression, transgenic mice provide a unique system in which to analyze the expression in skeletal muscle of the human aldolase A gene and to investigate the functional relations between the three promoters and the ubiquitous enhancer. In transgenic mice, indeed, expression of a construct can be examined in all cell types, and it can be followed throughout development. In such studies, however, transgene expression can be affected by sequences at the integration site, and several lines carrying an identical construct usually need to be analyzed before significant conclusions can be drawn (40). Three different constructs were injected into mouse fertilized eggs, and for each of them, expression was analyzed in several transgenic lines. A construct driven by the three promoters and the ubiquitous enhancer showed an expression pattern which was consistent with that known for humans. Expression from the human pM promoter was found to be restricted to fast skeletal muscle. In a construct lacking the pH enhancer/promoter region, expression from the muscle-specific pro-

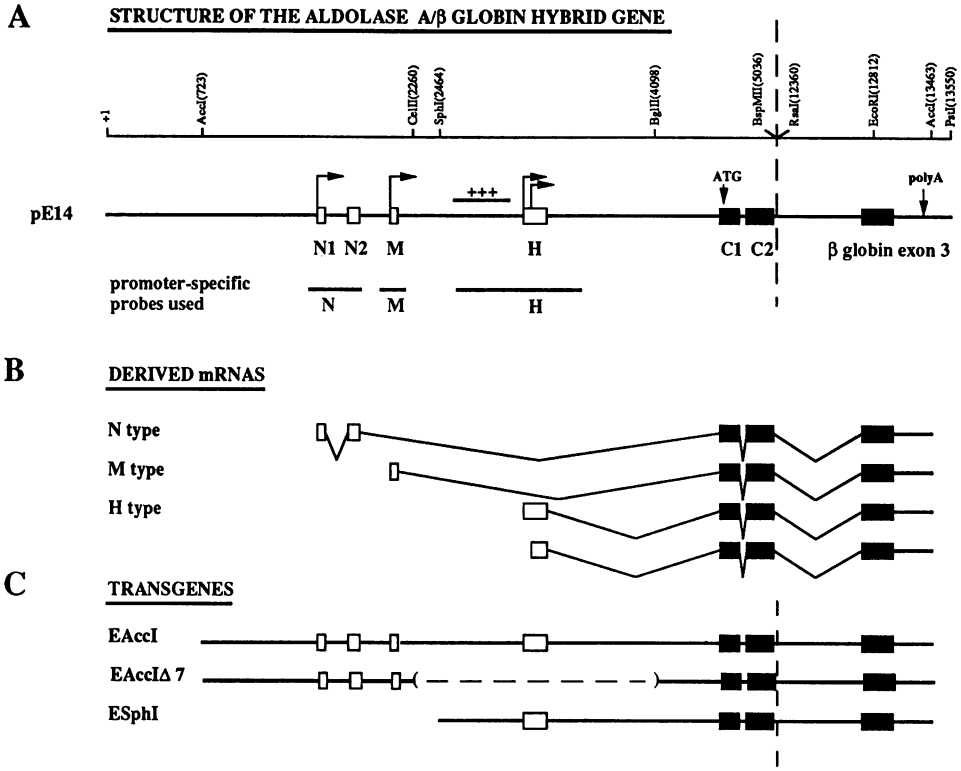

FIG. 1. Structure of the hybrid aldolase A/β-globin transgenes and mRNAs. (A) Structure of the hybrid aldolase A/β-globin gene. The pE14 plasmid contains a fusion of the 5′ region of the human aldolase A gene to the 3′ region of the human β-globin gene. The vertical dashed line represents the border between the human aldolase A and β-globin sequences. White and black boxes represent noncoding and coding exons, respectively. Arrows indicate start sites of transcription. The start sites of pN are very heterogeneous and are represented by a single arrow for the sake of clarity. The restriction sites used to derive the constructs injected from pE14 are indicated, together with their nucleotide coordinates (following the numbering in the published sequences [34, 41]). The DNA fragments used as probes to specifically detect the mRNAs transcribed from the different promoters are also represented (see Material and Methods). (B) Structure of the mRNAs expressed from the hybrid aldolase A/β-globin gene. (C) Structure of the transgenes. Fragments EAccI, ESphI, and EAccIΔ7 were derived from the hybrid gene as described in Materials and Methods by using the indicated restriction enzymes.

moter pM was unaltered, whereas the ubiquitous promoter pN remained active only in fast skeletal muscle. Finally, in a last construct, the pH enhancer/promoter region was sufficient to bring about high-level expression of the pH promoter in heart and skeletal muscle. Moreover, expression from the transgenes was independent of the integration site, suggesting that the sequences injected contained elements which conferred dominance on the integration site.

MATERIALS AND METHODS

Construction of human aldolase A/β-globin fusion genes and preparation of DNA for microinjection. Standard recombinant techniques (5) were used to produce an aldolase A/β-globin hybrid gene subcloned in a PEMBL19 plasmid frame (16). The resulting plasmid, named pE14, was taken as the source of the constructs studied (Fig. 1). In pE14, the 5′ region of the human aldolase A gene, extending from position +1 of the published sequence (34) to the *Bsp*MII (+5036) site located in the intron between the C2 and C3 coding exons, was fused to the 3′ end of the human β-globin gene, extending from the *Rsa*I (+12360) site in the second intron to the *Pst*I (+13550) site located beyond the polyadenylation signal (41). Digestion of pE14 by *Acc*I (cutting at position +723 in the human aldolase A gene and at position +13463 at the end of the human β-globin gene) generated a 5,416-bp-long fragment named EAccI. A pE14Δ7 plasmid was derived from pE14 by deletion of a *Cel*II (+2260)-*Bgl*II (+4098) internal fragment containing the pH enhancer/promoter region. Cutting this plasmid with *Acc*I generated a 3,678-bp-long fragment named EAccIΔ7, delimited by 5′ and 3′ ends identical to those of the EAccI fragment. Digestion of pE14 by *Sph*I (cutting at position +2464 in the aldolase A gene, in between the M and H noncoding exons, and in the plasmid polylinker, downstream of the aldolase A/β-globin hybrid gene) generated a 3,768-bp-long fragment named ESphI. Following separation from the plasmid sequences by electrophoresis, the different fragments were purified by binding to glass powder (29).

Production and detection of transgenic mice. Fertilized pronuclear-stage eggs from mating B6D2 F1 mice were isolated from oviducts, and a few hundred copies of the foreign DNA were injected into one of the pronuclei (29). Transgenic mice were detected by Southern analysis of 5 μg of DNA prepared from either placentas, when testing fetuses, or tail biopsies (29). To estimate transgene copy number in the different transgenic lines, standards, equivalent to 1, 5, or 10 transgene copies integrated per mouse genome, were made by mixing adequate amounts of the injected fragment with 5 μg of nontransgenic mouse tail DNA. The injected fragment labelled by random priming (with a kit purchased from Amersham) was used as a probe

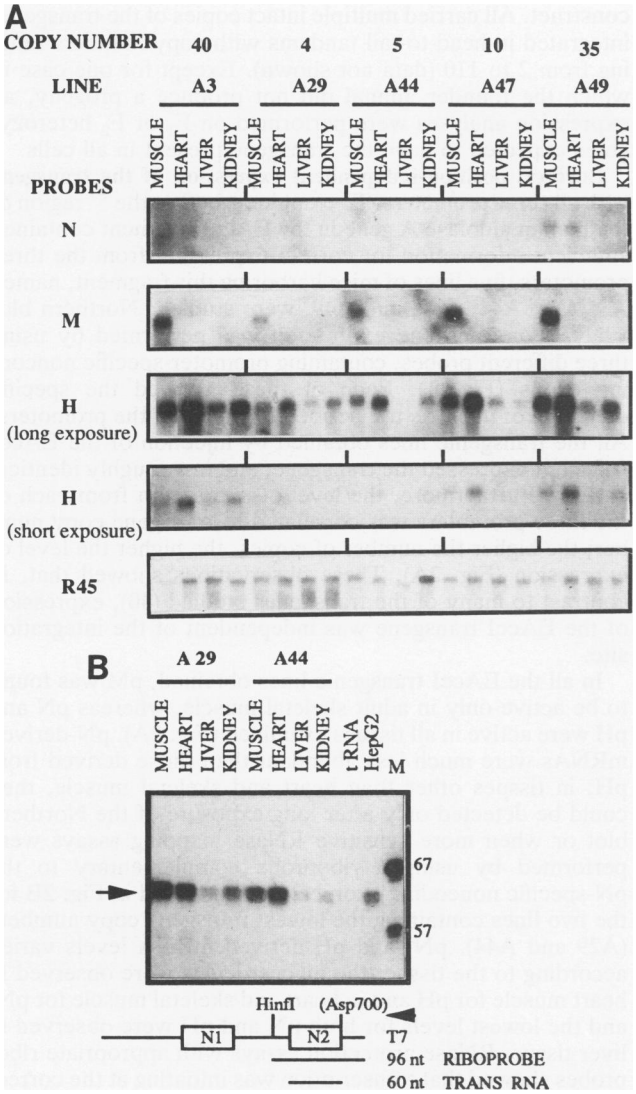

FIG. 2. (A) Northern blot analysis of expression from the three transgenic promoters in EAccI lines. Total RNAs (5 μg) from various tissues of mice from the indicated EAccI transgenic lines were analyzed for expression from the different promoters by Northern blot. Hybridizations were performed successively with the probes indicated from top to bottom. The autoradiograms shown correspond to 64-, 16-, and both 3 (short)- and 16 (long)-h exposures for the N, M, and H probes, respectively. The number of transgene copies integrated was approximately estimated by densitometric scanning of Southern blots containing identical amounts of DNA from the various EAccI lines and known amounts of the injected EAccI fragment (unpublished data) and is indicated for each line. The migration of the transgenic mRNAs compared with that of 18S and 28S RNAs suggested that they were approximately 700 to 800 bp long, as expected from a correct splicing of primary transcripts of the aldolase A/β-globin gene. With the exception of the pN-specific hybridization, in which a smear was occasionally observed in the upper part of the gel, no signal was observed on the parts of the gel that are not shown. In particular, there was no cross-hybridization with the mouse aldolase A mRNAs at the experimental conditions used. The R45 probe, which detected 18S RNA, allowed for standardization. (B) RNase mapping analysis of expression from the pN promoter in EAccI lines with low copy numbers. Total RNAs (5 μg) isolated from the indicated tissues of transgenic mice of the A29 and A44 lines were analyzed by RNase mapping with the riboprobe shown at the bottom, which allowed for the detection of pN-derived mRNAs as 60-nucleotide-long fragments. The position of such fragments is indicated along the autoradiogram. Lane HepG2, positive control with RNAs from human HepG2 hepatoma cells; lane tRNA, negative control with yeast tRNAs; lane M, radiolabelled molecular weight markers (size shown in nucleotides).

for specific hybridization at +68°C. Final washing was at the same temperature in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% (wt/vol) sodium dodecyl sulfate (SDS). In these conditions, no cross-hybridization with the endogenous aldolase A gene was observed. Positive founders were outbred to establish transgenic lines.

RNA analysis. Total RNA was prepared from various mouse tissues by the guanidium thiocyanate procedure (11). RNA samples (5 μg) previously denaturated in 10 mM methyl mercuric hydroxide were electrophoretically separated on a 1.5% agarose formaldehyde gel and transferred to Hybond N^+ nylon membranes (Amersham) with 20× SSC. For the detection of the different aldolase A/β-globin mRNA species, DNA restriction fragments encompassing the N_1 and N_2 exons (*Hin*dII [+1471]-*Asp*-700 [+1852]), the M exon (*Bam*HI [+2003]-*Apa*I [+2155]), or the H exon (*Pst*I [+2697]-*Fsp*I [+3454]) were labelled by random priming, yielding the probes called N, M, and H, respectively (Fig. 1A). Prehybridization and hybridization were performed at 65°C in 3× SSC, 1% (wt/vol) SDS, 1% (wt/vol) glycine, 0.2% (wt/vol) Ficoll, 0.2% (wt/vol) polyvinylpyrrolidone, 5% (wt/vol) polyethylene glycol 6000, and 100 μg of denatured DNA from salmon sperm per ml. Final washing was at 65°C in 0.2× SSC–1% (wt/vol) SDS. Under these conditions, no cross-hybridization with endogenous mouse mRNAs was observed. After hybridization with a first specific probe, the blots were stripped of probe by washing twice in a boiling 0.1× SSC–0.1% (wt/vol) SDS bath and reprobed with another labelled fragment. For standardization, the blots were finally probed with a mouse β-actin cDNA probe (a gift of S. Alonso, Pasteur Institute, Paris, France) or a R45 cDNA probe (corresponding to a fragment of human 18S rRNA, a gift of J.-P. Hugnot, ICGM, Paris, France). Mouse probes encompassing the M and H mouse exons were a gift from M. Colbert (Duke University) (13). Relative amounts of specific mRNAs were measured by scanning appropriate autoradiogram exposures of Northern (RNA) blots with a Shimadzu densitometer.

RNase protection assays were performed as previously described (14), by using antisense riboprobes spanning the N2, M, or H exons.

RESULTS

Generation of aldolase A/β-globin transgenic mice. In order to examine the expression from the three promoters of the human aldolase A gene, a hybrid gene was constructed by fusing the 5′ region of the human aldolase A gene, cloned in the laboratory, with the 3′ region of the human β-globin gene, containing the third exon and the polyadenylation signal (14). Three different constructs were then derived from this hybrid gene (Fig. 1). EAccI was a construct containing a 5′ region from the human aldolase A gene with the three promoters and the ubiquitous enhancer. EAccIΔ7 was a construct containing a 5′ region lacking the pH enhancer/promoter region and, therefore, driven only by pN and pM promoters. Finally, ESphI was a construct which was driven by the pH enhancer/promoter region alone. Several founder transgenic mice were obtained for each

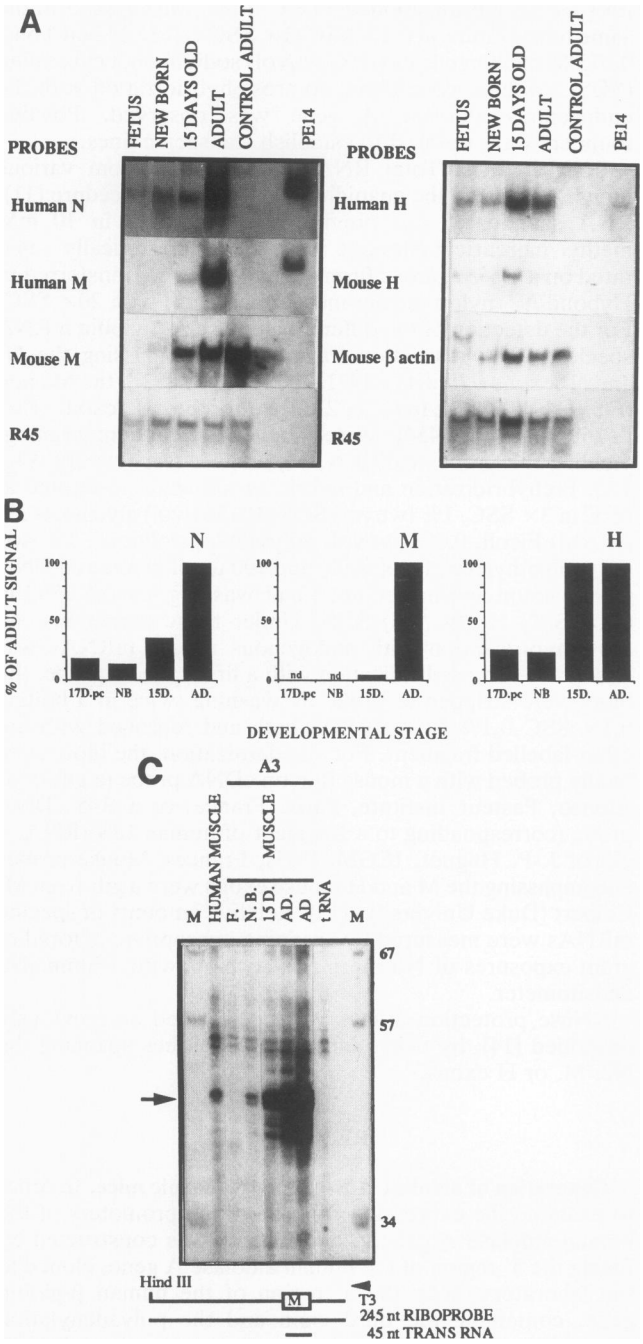

FIG. 3. Expression from the three transgenic promoters during muscle development in line A3. (A) Total RNA from hindlimb muscle of A3 transgenic mice was prepared at the indicated stages of muscle development, and 5-μg samples were analyzed by Northern blot. CONTROL ADULT, total muscle RNA from a nontransgenic mouse. Two identical Northern blots, on each side of the figure, were successively hybridized to the probes indicated, from top to bottom. All the probes being of the same specific activity, the autoradiograms shown correspond to 70-, 24-, and 16-h exposures for the N, M, and H human probes, respectively. The autoradiograms for the mouse probes were exposed for the same period as for the corresponding human probes. The mouse β-actin probe hybridized with β-actin and with skeletal α-actin mRNAs, these isoforms being alternatively expressed during myogenesis. The R45 probe, which detected 18S RNA, allowed for standardization. The PE14 lane contains *Pst*I-cut pE14 plasmid, which contains a single copy of the aldolase A/β-globin hybrid gene, and therefore, the different construct. All carried multiple intact copies of the transgene, integrated in head-to-tail tandems with copy numbers ranging from 2 to 110 (data not shown). Except for one case in which the founder animal did not produce a progeny, all expression analyses were performed on F_1 or F_2 heterozygotes expected to have the transgene present in all cells.

Correct, position-independent expression of the transgene with all three promoters. To examine whether the 5′ region of the human aldolase A gene in the EAccI fragment contained sufficient information for correct expression from the three promoters, five lines of mice harboring this fragment, named A3, A29, A44, A47, and A49, were studied. Northern blot analysis of transgene expression was performed by using three different probes, containing promoter-specific noncoding exons (Fig. 1). Each of these allowed the specific detection of mRNAs transcribed from one of the promoters. All the transgenic lines obtained by injection of the EAccI fragment expressed the transgene, and in a roughly identical pattern. Furthermore, the level of expression from each of the three promoters was correlated to transgene copy number; the higher the number of copies, the higher the level of expression (Fig. 2A). These observations showed that, in contrast to many of the transgenes studied (40), expression of the EAccI transgene was independent of the integration site.

In all the EAccI transgenic lines obtained, pM was found to be active only in adult skeletal muscle, whereas pN and pH were active in all tissues examined (Fig. 2A). pN-derived mRNAs were much less abundant than those derived from pH; in tissues other than heart and skeletal muscle, they could be detected only after long exposure of the Northern blot or when more sensitive RNase mapping assays were performed by using a riboprobe complementary to the pN-specific noncoding exon N2, as illustrated in Fig. 2B for the two lines containing the lowest transgene copy numbers (A29 and A44). pN- and pH-derived mRNA levels varied according to the tissue; the highest levels were observed in heart muscle for pH and in heart and skeletal muscle for pN, and the lowest levels for both pN and pH were observed in liver tissue. RNase protection assays with appropriate riboprobes showed that transcription was initiating at the correct positions in the pM promoter (see below) and in the pH promoter (data not shown). The start sites of pN, which

target sequences of the N, M, and H probes in a 1:1 ratio. When taking into account the relative lengths of the probes and specific exons, this allowed for a comparison of the relative abundancy of the different types of transcripts. (B) For each developmental stage [(17D.pc, 17-day fetus; NB, newborn; 15D, 15-day-old mice; AD, adult), three separate samples were analyzed, each corresponding to one individual or to a pool from several individuals, depending on the stage. Hybridization signals obtained with human promoter-specific probes were quantitated by densitometric scanning of appropriate exposures of the Northern blots. After standardization, the average mRNA level was estimated and expressed relative to that observed at the adult age, which was arbitrarily set at 100%. nd, not detectable. (C) pM activity was analyzed in hindlimb muscle of A3 transgenic mice at the indicated stages of development by RNase mapping with the riboprobe shown at the bottom of the figure. Correctly initiated pM-derived mRNAs are detected as 45-nucleotide-long fragments, which are indicated by an arrow alongside the autoradiogram. Lane HUMAN MUSCLE, positive control with RNAs (2 μg) from human skeletal muscle; lane tRNA, negative control with yeast RNAs, which allows distinguishing of specific signals from the background; lane M, molecular weight markers (size shown in nucleotides).

were expected to be extremely heterogeneous (23), were not examined. The EAccI transgene, therefore, contained sufficient information to bring about activity of the three promoters in a pattern consistent with that known in humans (23, 34, 35). In addition, although no functional pN-like promoter activity could be detected in the mouse (49), the human pN promoter was active in the EAccI transgenic mice.

Expression from the three promoters during myogenesis in line A3. To determine how the three promoters are regulated during myogenesis, total RNA was prepared from hindlimb muscle obtained from 17-day fetuses, from newborn mice, from 15-day-old mice, and from 2-month-old mice (adults), which all came from the A3 transgenic line, harboring the EAccI transgene. Promoter-specific mRNA levels were determined by Northern blot at each stage. Figure 3A shows representative autoradiograms obtained by successively hybridizing two identical Northern blots with the indicated probes. Figure 3B shows the mRNA levels determined at each stage, expressed relative to that found at the adult age. pM-derived mRNAs, by this assay, were first detected at 15 days of age, and their level was found to have increased 25-fold by the adult age. In contrast, the level of pH-derived mRNAs, already detected in fetuses, increased fourfold between birth and 15 days of age but did not vary significantly afterwards. Finally, pN showed an intermediary behavior, as pN-derived mRNAs, found at the same level in the fetuses and at birth, increased progressively from birth to the adult age (a 2-fold increase between birth and 15 days of age, much like that of pH, and a 2.5-fold increase between 15 days of age and the adult age, like that of pM, although much less important). These results show that the ubiquitous pH and muscle-specific pM promoters were differently regulated during myogenesis, while pN regulation had characteristics in common with each of the other two promoters.

Expression of the transgene during myogenesis was also compared with that of the endogenous mouse gene. As previously described (13), mouse pM activity could first be detected at birth, and then it increased progressively until the adult age. The human pM activation, in this Northern blot analysis, seemed to be delayed, as it was first detected only at 15 days of age. However, as shown in Fig. 3C, when more sensitive RNase protection assays were performed, with a riboprobe complementary to the M exon, human transgenic pM promoter activity could also be detected at birth. Therefore, the difference in mouse and transgenic pM promoter activity was essentially quantitative. Finally, while human pH activity showed a marked increase between birth and 15 days of age, remaining at a high level in the adult, mouse pH activity, which showed only a moderate increase during the same period, had decreased by the adult age, returning to the level observed in the newborn mice (13) (Fig. 3A). These results demonstrate similarities but also reveal significant differences between the mouse and human transgene expression during myogenesis.

A comparison of the levels of mRNAs derived from each of the three transgenic promoters was performed. For this purpose, the Northern blot contained a lane with a low amount of linearized pE14 plasmid. Because the plasmid contained target DNA sequences for each of the promoter-specific probes in a 1:1 ratio, the signals detected in the pE14 lane were used to standardize the signals detected in the lanes containing the RNA samples (see the legend to Fig. 3 for further details). pN, pM, and pH were estimated to account for approximately 5, 20, and 75%, respectively, of aldolase A/β-globin mRNAs in adult skeletal muscle. These relative levels were consistent with the relative levels of pN-, pM-, and pH-derived aldolase A mRNAs previously found in human adult skeletal muscle (5, 35, and 60% respectively) (23). In particular, this confirmed that the relative strengths of the ubiquitous promoters, pN and pH, were similar to those found in humans.

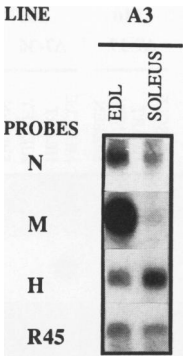

FIG. 4. Fiber specificity of the pM promoter in adult skeletal muscle in line A3. Expression from the pN, pM, and pH promoters was analyzed in two different hindlimb muscles, known to differ in their fiber-type composition. EDL, extensor digitorum longus. Probes and experimental conditions were the same as described in the legend to Fig. 2.

The human transgenic pM promoter was specific to fast-type myofibers. Given that the mouse (13) and rat (46) muscle-specific pM promoters are specific to fast skeletal muscle, we were interested in determining whether it would also be the case with the human transgenic pM. pM-, pN-, and pH-derived mRNA levels were, therefore, compared in two muscles of A3 transgenic mice with a different fiber-type content: extensor digitorum longus, a white muscle composed predominantly of fast fibers, and soleus, a red muscle composed predominantly of slow fibers (3, 17, 30). Figure 4 shows that pM-derived mRNAs accumulated essentially in the fast skeletal muscle, extensor digitorum longus, while pH-derived mRNAs accumulated in both the fast and slow muscles, but to a higher level in the latter than in the former. Interestingly, pN-derived mRNAs were also found in both types of muscle but, in contrast to pH-derived mRNAs, accumulated to a higher level in the fast muscle than in the slow muscle. The human pM and pH transgenic promoters thus behaved as the mouse endogenous ones. These results extended our previous finding that the human ubiquitous pH and muscle-specific pM promoters were differently regulated during myogenesis, while pN regulation had characteristics in common with each of the other two promoters.

Muscle-specific expression from both pN and pM promoters in the transgene lacking the pH enhancer/promoter region. In order to examine the role of the pH enhancer/promoter region in pN and pM activity in vivo, four lines of mice harboring the EAccIΔ7 fragment, named Δ7-16, Δ7-17, Δ7-36, and Δ7-41, were studied. In all four lines the transgene was expressed in an identical pattern, and the level of expression from each promoter was roughly correlated to the number of transgene copies, as observed in the EAccI lines (Fig. 5A). pN and pM were active only in skeletal muscle. For line Δ7-16, which had a low copy number, pN activity could not be visualized by Northern blot, but RNase mapping assays, with a riboprobe complementary to the N2 exon, showed that pN was active only in skeletal muscle (data not shown). Further analysis in line Δ7-17 showed that pN and pM activities were, in fact, restricted to fast skeletal

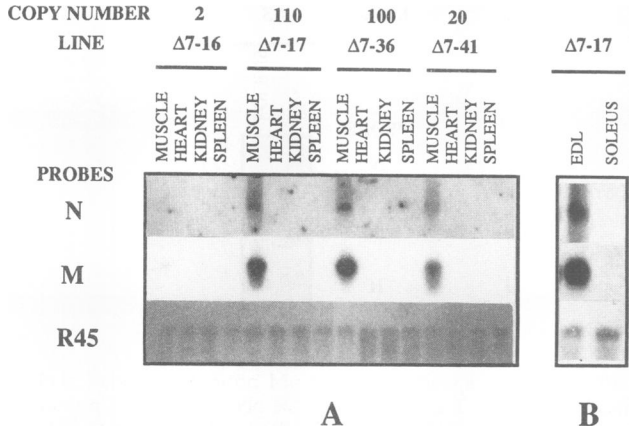

FIG. 5. Northern blot analysis of expression from the transgenic pN and pM promoters in EAccIΔ7 lines. (A) Northern blot analysis was performed in the indicated adult tissues as described in the legend to Fig. 2. EDL, extensor digitorum longus. The autoradiograms shown correspond to 16- and 4-h exposures for the N and M probes, respectively. The number of transgene copies integrated in each line is also indicated. (B) Expression from the pN and pM promoters was further analyzed, with the same conditions as above, in two different adult hindlimb muscles from the Δ7-17 line.

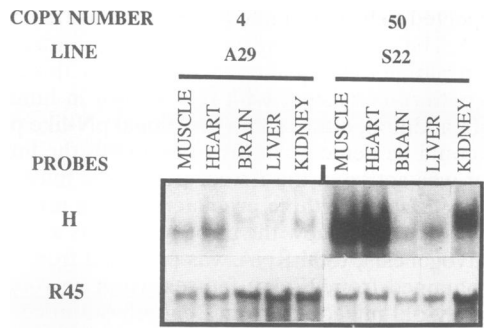

FIG. 6. Northern blot analysis of expression from the transgenic pH promoter in lines A29 and S22 was performed as described in the legend to Fig. 2 in the indicated adult tissues. The number of transgene copies integrated in each line is also indicated.

muscle (Fig. 5B). Moreover, when compared between EAccI and EAccIΔ7 lines, pM activity seemed to be of similar strength, for a similar transgene copy number (data not shown). Therefore, pM activity was unaltered when the pH enhancer/promoter region was deleted, and pM appeared to function independently of pH.

Surprisingly, in all EAccIΔ7 lines, pN was active only in fast skeletal muscle. This finding suggests that the 2.4-kb aldolase A sequences in the EAccIΔ7 fragment contained a fast skeletal muscle-specific enhancer acting on both pN and pM. On the other hand, in tissues other than fast skeletal muscle, pN was inactive. The ubiquitous enhancer lacking in the EAccIΔ7 fragment was, therefore, necessary to pN activity in vivo in all the tissues tested other than fast skeletal muscle, which confirmed and extended our findings in transient transfection assays with cultured cells (14). The regulation of the pN promoter was, therefore, remarkable in the way that pN shared regulatory sequences with the other two promoters, depending on the tissue.

High-level expression from the transgene driven by the pH enhancer/promoter region alone in heart and skeletal muscle. We previously demonstrated that the pH enhancer/promoter region is active in multiple cultured cell lines (14, 23). To examine whether it would also be active in vivo, independently of other sequence elements, one line of mice harboring the ESphI fragment, named S22, was studied. Expression from the pH promoter was compared between line S22 and line A29, the latter harboring the EAccI fragment (Fig. 6). The relative levels of expression in the various tissues were roughly similar in the two lines. In skeletal muscle, however, pH activity was identical to that in heart muscle in line S22 while it was fivefold lower than in heart muscle in line A29. Furthermore, in tissues other than skeletal muscle, the difference in the level of expression between the two lines was related to the difference in transgene copy number. An identical expression pattern was observed in tissue samples from a different founder animal containing the ESphI fragment, and the differences in the level of expression were, again, correlated to transgene copy number. Therefore, the ESphI fragment, containing 1.6 kbp of aldolase A sequences, was sufficient to bring about ubiquitous expression from the pH promoter in vivo, which was particularly high in heart and skeletal muscle.

DISCUSSION

We have studied the muscle-specific promoter, pM, and the two ubiquitous promoters, pN and pH, of the human aldolase A gene in transgenic mice. The use of transgenic technology enabled us to examine the activity of the three promoters during myogenesis and in different types of adult skeletal muscle, fast or slow, and several conclusions regarding each of them and their relations to one another could be drawn. We showed that, while pH and pM were independent of each other, pN, remarkably, shared regulatory sequences with either pH or pM, depending on the tissue. Moreover, we found that expression of the transgenes studied was independent of the integration site.

Expression from the transgenes studied is independent of integration site. We studied 11 lines of transgenic mice harboring aldolase A/β-globin transgenes. For each construct, all the lines obtained expressed the transgene and did so in an identical pattern. Moreover, the level of expression of each of the promoters present on a given construct was correlated to transgene copy number; the higher the copy number, the higher the level of expression. (Fig. 2 and 5, and data not shown). This is unusual in studies using transgenic mice, even when transgene expression is driven by ubiquitous promoters (1, 4, 22, 39, 40, 45). The level of transgene expression is generally unrelated to transgene copy number, and the pattern of expression frequently varies between different lines harboring an identical transgene. These features are attributed to the influence of sequences at the integration site, rather than to modifications in the transgene during integration (2). In contrast, few studies have described position-independent, copy number-dependent expression of transgenes (7, 10, 24, 26, 28). These properties were first reported for human β-globin transgenes (26) and have been assigned to upstream sequences termed locus control regions, which are thought to maintain transgenes in an open chromatin configuration. Therefore, the transgenes described in this report are likely to contain one or several locus control regions.

Expression from the pM promoter in skeletal muscle is independent of the ubiquitous pH enhancer/promoter region. We examined the sequences involved in expression from the muscle-specific promoter pM in adult skeletal muscle. The

EAccIΔ7 fragment, lacking the pH enhancer/promoter region, contained sufficient information for transgene expression from pM in adult skeletal muscle (Fig. 5). pM can, therefore, function independently of the ubiquitous pH enhancer/promoter region. Moreover, a similar level of expression was observed from pM in an EAccIΔ7 line and an EAccI line with a similar transgene copy number; it is, therefore, likely that the enhancer does not significantly stimulate pM in adult skeletal muscle. Such a conclusion can be drawn because the expression levels of the two transgenes were copy number dependent. Several hypotheses can be proposed to explain the lack of strong activity of the ubiquitous enhancer on the muscle-specific promoter. pM may be unable to interact with the ubiquitous enhancer, for instance, because of the absence of an appropriate TATA box sequence (53) or because a particular *trans*-acting factor is required for an efficient interaction (43). Alternatively, pM may be able to interact with the enhancer but is prevented from doing so by a dominant negative element nearby (48, 50), or else because the two ubiquitous promoters trap all enhancer activity.

Expression from the human pM promoter in skeletal muscle is specific to fast fibers. In both EAccI and EAccIΔ7 transgenic lines, we observed that the transgenic pM promoter was predominantly active in fast skeletal muscles such as extensor digitorum longus and gastrocnemius—its activity was only faintly detected in a slow muscle such as soleus (Fig. 4 and 5B) (36a). Starting from the smaller 2.5-kbp EAccIΔ7 fragment, analysis of sequences involved in the fiber-specific activity of the pM promoter will be interesting to perform. Although many myogenic transcriptional factors have been recently characterized and/or cloned (38), their possible contribution to the differentiation of myofibers into fast and slow types remains unclear (36). The characterization of factors that regulate pM activity should be useful in examining this issue as, to our knowledge, pM is the only promoter, other than the quail troponin I (27) and mouse MLC1 promoters (17), for which fiber-specific expression has been shown in vivo by using transgenic mice.

Although human transgenic pM promoter activity was very similar to that of the endogenous promoter in adult muscle, it seemed weaker at earlier stages of development than that in the adult age. These quantitative differences could be due to species-specific regulation of the human aldolase A gene during muscle development. Or, they could be due to the absence of sequences required for proper regulation of the human gene in the transgenes studied.

The latter hypothesis is supported by the recent identification of an enhancer in the mouse gene which accounted for 80% of mouse pM activity in C2C12-cultured myogenic cells and whose potential human homolog was not present in the injected fragments (48). pM could be stimulated by separate *cis*-acting elements during muscle development. An enhancer, absent from our transgenes, could be required for strong activity in immature muscle in vivo and in C2C12 myogenic cultured cells, and another enhancer, present in our transgenes, could be required for activity in adult fast skeletal muscle. Such a regulation by separate enhancers during development has been previously evoked for the albumin promoter (8). In fetal liver tissue, the albumin promoter is stimulated by an enhancer shared with the alpha fetoprotein promoter, and later in the adult liver, it is stimulated by a second enhancer as well. More generally, regulation by separate enhancers in order to achieve expression of a promoter in different tissues has been frequently described (6, 18, 20, 32, 51). Our data show, however, that an enhancer acting in immature muscle was not necessary for correct fiber-specific activity in adult skeletal muscle.

pH is regulated independently of pM in skeletal muscle. In EAccI mice, the pH promoter displayed high-level activity in heart and skeletal muscle compared with that observed in other tissues (Fig. 2). Developmental regulation of pH activity in skeletal muscle followed a different pattern from that of the muscle-specific promoter pM. It increased markedly between birth and 15 days of age and remained roughly the same afterwards, while pM activity increased 25-fold between 15 days of age and the adult age. In addition, unlike pM, it was not fiber specific (Fig. 4). In ESphI mice, in the absence of pM and of the sequences homologous to the weaker mouse enhancer (48), the pH promoter still displayed high-level activity in skeletal muscle. Taken together with the independent activity of pM in EAccIΔ7 mice, these results strongly suggest that in skeletal muscle the pH and pM promoters were regulated independently of each other, under the control of separate *cis*-acting sequences. The pH promoter was controlled by the ubiquitous enhancer, while the pM promoter was controlled by a fast skeletal muscle-specific enhancer. The ubiquitous enhancer, therefore, seems to show tissue preference for skeletal and heart muscle. Regulatory sequences known to be ubiquitously active also frequently show tissue preference when examined in transgenic mice (39, 45). At this stage, however, we cannot formally exclude the possibility that an element residing in the sequences common to the EAccIΔ7 and ESphI fragments (Fig. 1C) contributed to activity of both promoters in fast skeletal muscle.

As with the muscle-specific promoter, pM, comparison of

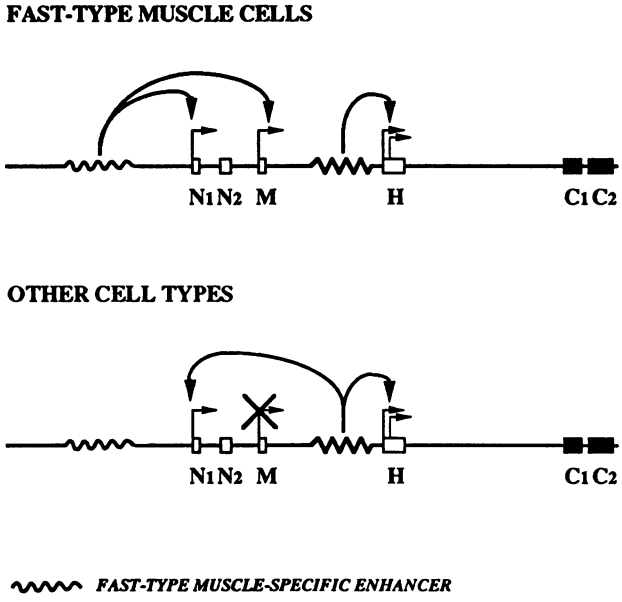

FIG. 7. Enhancer/promoter interactions in the human aldolase A gene as found in this study. Arrows point to the target promoters stimulated by the two enhancers. The ubiquitous enhancer was located in studies using transient transfection assays (14). The fast skeletal muscle-specific enhancer, whose existence was deduced from the analysis of the transgenic mice carrying the EAccIΔ7 fragment, was putatively located upstream of pN start sites.

the mouse and human transgenic pH promoters' activity levels in skeletal muscle showed significant differences. In particular, human pH activity was high in the adult, whereas that of mouse pH was low, being similar to that observed in the newborn. However, when examined in more detail, the human transgenic pH promoter activity was found to vary between different muscles (Fig. 4) (36a). It seemed to be inversely proportional to the level of pM activity, suggesting that the muscle-specific pM activity has a negative effect on pH. Consistent with this notion, pH promoter activity in skeletal muscle relative to heart was found to be higher in ESphI lines, lacking the pM promoter, than in EAccI lines, containing the pM promoter (Fig. 6). Several mechanisms, such as transcriptional interference (15, 42) or competition for a common fast skeletal muscle-specific enhancer (12, 21), which could account for the negative effect of pM on pH, are currently being investigated.

The human pN promoter is active in transgenic mice. The mouse (49) and rat (37) aldolase A genes are transcribed from only two promoters, while the human gene is transcribed from three (34). Although the N1 and N2 noncoding exons (Fig. 1A) are conserved to a high degree in the three species, including at the exon/intron boundaries (37, 49), no mRNA species containing such exons could be detected in mice and rats. Because we observed human pN promoter activity in transgenic mice (Fig. 2), differences in *cis*-acting elements (the promoter or the enhancers), rather than in *trans*-acting factors, must account for the lack of pN activity in the rodent species. This is similar to studies of the α1 antitrypsin gene (33). When placed in mice, human α1 antitrypsin transgenes showed an expression pattern characteristic of humans rather than of mice. Transgenic studies which had bearing upon the evolution of regulatory sequences have been recently reviewed (9).

The pN promoter shares regulatory sequences with either of the other two promoters, the ubiquitous pH or the muscle-specific pM, depending on the tissue. We recently showed that the ubiquitous enhancer is necessary for pN activity in cultured cells (14). Consistent with these findings, in EAccIΔ7 mice lacking a 1.8-kbp region containing the ubiquitous enhancer, pN activity could no longer be detected in tissues other than fast skeletal muscle. Because pM, also, was active only in this tissue, we suggest that fast skeletal muscle-specific regulatory sequences are shared by these two promoters. This is also supported by the thorough expression studies performed in the EAccI line A3, which showed that pN regulation had many characteristics in common with pM. Thus, while the ubiquitous pH promoter and the muscle-specific pM promoter were independent of one another, the third one, the ubiquitous pN, behaved as an opportunistic promoter, sharing regulatory sequences with either of the other two promoters, depending on the tissue.

Original enhancer/promoter interactions therefore take place in a small region of the human aldolase A gene (Fig. 7). Further studies should shed light on this important, but poorly understood, aspect of transcriptional regulation. Indeed, specificity in enhancer/promoter interactions is expected to be essential for appropriate regulation of genes with multiple promoters (44) and, more generally, of gene clusters containing multiple genes and regulatory sequences, such as the globin (19, 21, 52) and the homeobox (47) loci and others (25).

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