MOLECULAR AND CELLULAR BIOLOGY, Dec. 1988, p. 5257–5267
0270-7306/88/125257-11$02.00/0

Vol. 8, No. 12

Stepwise Activation of the Mouse Acetylcholine Receptor δ- and γ-Subunit Genes in Clonal Cell Lines

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Received 20 July 1988/Accepted 12 September 1988

We used the DNase I-hypersensitive sites around the mouse acetylcholine receptor δ-subunit gene as a guide toward the cloning and sequencing of δ and γ transcriptional regulatory regions and as a means to assess chromatin structural activation of the δ- and γ-subunit genes during myogenesis. Genomic cloning of hypersensitive sites downstream of the δ-subunit gene revealed the presence of the γ-subunit gene approximately 5 kilobases away; the hypersensitive sites mapped to the 5′ end of the γ-subunit gene. Sequence comparison of restriction fragments containing hypersensitive sites in analogous locations at the 5′ ends of the δ- and γ-subunit genes uncovered little overall homology between the two genomic fragments; however, an 11-of 13-base-pair match between the two sequences was found. Homologs to this sequence were also found to be present in the upstream regions of the chicken α- and mouse β-subunit genes. By RNase protection and primer extension analyses, the δ-subunit gene transcription start site was mapped to 56 base pairs upstream of the initiator ATG codon. Clonal cell lines with various potentials to differentiate to the skeletal muscle phenotype were examined for their hypersensitive site pattern within the δ-γ locus. Only remote hypersensitive sites flanking the locus appear in pluripotential mesodermal cells. A cell line of determined but inducible myoblasts expressed only one more intergenic site, while in permissively differentiating myoblasts hypersensitive sites were already present at the 5′ ends of the δ and γ genes prior to differentiation. Terminal differentiation resulted in an identical pattern of hypersensitive sites in all muscle cell lines examined so far, with an intergenic site near the γ-subunit gene being the only site specific to the differentiated muscle phenotype.

The skeletal muscle acetylcholine receptor (AchR) is a multisubunit protein whose expression is regulated at least in part by transcriptional mechanisms (1, 7, 8, 18, 36, 69). The mechanisms that regulate transcription are almost certainly complex given that receptor subunit gene expression must be coordinate, tissue and developmentally specific, and modulated by nerve-muscle interaction.

Normally undetectable in nonskeletal muscle tissues, subunit mRNAs are accumulated in significant quantities only after terminal differentiation of myoblasts into embryonic myotubes (1, 8, 18, 36, 69). In the C2 cell line, this accumulation of subunit mRNAs is associated with a 5- to 10-fold increase over the background level in the transcription rates of the mouse α- and δ-subunit genes (7); similar nuclear run-on assays have recently been completed with β- and γ-subunit probes with similar results (A. Buonanno and J. P. Merlie, unpublished). In developing embryonic muscle, AchR mRNA levels decrease dramatically (41; J. P. Merlie, unpublished data). This decrease presumably results from motor innervation since denervation of adult muscle stimulates a rapid and dramatic increase in subunit mRNA levels (18, 21, 33, 38) that can be reversed or inhibited by direct electrical stimulation of the denervated muscle (22). Interestingly, the very small amount of AchR mRNA found in adult innervated muscle is concentrated near synaptic sites (19, 39), suggesting a regulatory mechanism that must operate at the subcellular level. Finally, with few exceptions (18, 26), all of these alterations in mRNA levels are coordinate, in that all four subunit mRNAs vary in a parallel manner.

Despite this complex and interesting set of regulatory phenomena, relatively little is known about the molecular mechanisms involved. Klarsfeld et al. (34) reported the isolation of a fragment at the 5′ end of the chicken α-subunit gene that conferred differentiation specificity to a fusion gene when transfected into the C2 mouse muscle cell line. Although this is a considerable advance toward defining *cis* regulators of receptor subunit genes, examination of only a short stretch of upstream sequence may ignore important regulatory regions, particularly given the diverse signals that affect receptor subunit gene expression. Initially, we took a less focused approach by examining large regions of chromatin around the mouse δ-subunit gene for DNase I-hypersensitive (DH) sites (13). DH sites are 100 to 300 base pairs (bp) of chromatin that are exquisitely sensitive to DNase I (28). Results of a large body of experiments indicate that DH sites reside in chromatin that are devoid of normal nucleosomes and that this perturbation is the result of the binding of nonhistone proteins (for reviews, see references 10 and 58). As markers of DNA-nonhistone protein interactions, DH sites are extremely useful in locating transcriptional regulatory regions, particularly when they are remote (24, 56). In addition, DH sites provide a means to monitor DNA-protein binding events in situ as they occur during the normal activation sequence of a given gene (9, 17, 20, 53, 56, 70). One significant caveat of hypersensitivity studies is that not all DH sites are involved with transcriptional regulation; origins of replication (60), transposons (43), and centromeres (2), all of which are tightly associated with DNA-binding proteins, have been found within hypersensitive domains. Thus, establishing the cell type specificity of DH sites is essential.

We previously mapped six DH sites flanking the δ-subunit gene, three sites 5′ to the gene and three sites downstream (13). The sites were designated, from 5′ to 3′, X_1, X_2, D_1, D_2 (now called X_3), G_1, and G_2. The X sites are not specific to muscle; the D and G sites are muscle specific. The D_1 site

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mapped 5′ to the δ-subunit gene. We speculated, for reasons given below, that the G sites were likely to be near the γ-subunit gene. In this study we used these hypersensitive sites as a guide toward cloning putative δ-γ regulatory regions and for assessing activation of the δ-γ genes during myogenesis.

MATERIALS AND METHODS

Cloning. The λL47.1 library was derived from C57 black mouse DNA that was partially digested with *Mbo*I and was generously donated by the laboratory of Stephen Weaver (University of Illinois, Chicago). The Charon 28 library was derived from BALB/c mouse DNA that was partially digested with *Mbo*I and was generously donated by the laboratory of Phil Leder (Harvard Medical School, Boston, Mass.). Approximately 3×10^5 plaques from each library were screened with the δ-subunit cDNA insert 6H (36). A single type of 6H-positive clone was purified from the L47.1 library, and six different overlapping clones were purified from the Charon 28 library.

Sequencing. Fragments were subcloned into mp18/19 and sequenced on both strands by the dideoxy method (50). Sequencing of γHH2.3 was aided by a set of overlapping deletions obtained by the method of Dale et al. (14). Sequences were compared both by eye and with the NUCALN program (63).

RNase protection. Fragments containing exons 1 and 2 and exon 1 of the δ gene were subcloned into pGEM1 (Promega Biotech, Madison, Wis.) and transcribed in the presence of [α-^{32}P]CTP by Promega transcription protocol 1. The radiolabeled RNA probes (specific activity, 10^9 dpm/μg) were hybridized to 10 μg of BC3H-1 or mouse brain total RNA or to 10 μg of wheat germ tRNA (Sigma Chemical Co., St. Louis, Mo.) and subsequently digested by Promega recommendations. The sizes of RNase-protected products were determined on a sequencing gel and visualized by autoradiography.

Primer extension. Oligonucleotide primers were end-labeled with T4 kinase to a specific activity of 5×10^6 dpm/pmol and hybridized to 2 μg of poly(A)$^+$ RNA from differentiated BC3H-1 or mouse brain RNA or to 2 μg of wheat germ tRNA in 10 mM Tris (pH 7.5)–1 mM EDTA at 10°C below the $T_m = 4(G+C) + 2(A+T)$. Primers were extended with reverse transcriptase in 10 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$–5 mM dithiothreitol–250 μM deoxynucleoside triphosphates for 30 min at 42°C. The sizes of primer-extended products were determined on a sequencing gel and visualized by autoradiography.

DNase I hypersensitivity experiments. BC3H-1, C2, F3, and C3H10T1/2 cells were cultured as described previously (13). Undifferentiated BC3H-1 and C2 cells were harvested in high levels of serum (10% fetal bovine serum, 10% newborn calf serum) 2 days after they were plated at approximately 10% of confluent density. Differentiated BC3H-1, C2, and F3 cultures were harvested 3 days after they were switched to cultures with reduced levels of serum (10% horse serum), which was 7 days after they were plated. C3H10T1/2 cells (48) were harvested when they reached confluency, which was 7 days after plating. C2-inducible (C2i) cells were cultured as described by Pinset et al (47a). C2i cells were maintained as myoblasts by the presence of 20% fetal bovine serum. Differentiation of confluent C2i myoblasts was induced by replacement of all serum with an insulin-transferrin-containing medium (47a). The degree of muscle differentiation was routinely examined in all cell lines by Northern blotting analysis of α-actin and AchR-subunit mRNA levels and by quantitation of α-bungarotoxin-binding sites. Nuclei preparation, DNase I digestions, DNA purification, restriction digests, Southern blots, and hybridizations were performed as described previously (13).

RESULTS

Cloning of the δ-γ locus. At the onset of this study, we had several reasons to believe that the mouse γ-subunit gene lay downstream of the δ-subunit gene. Nef et al. (44) found that the δ- and γ-subunit genes were separated by only 900 bp in the chicken genome; another report (54) anecdotally mentioned a human genomic clone that contained both δ- and γ-subunit gene sequences. Furthermore, we demonstrated that there is a close genetic linkage between the δ and γ subunits in the mouse genome (27). Finally, the mapping of muscle-specific, developmentally regulated hypersensitive sites approximately 5 kilobases (kb) 3′ of the δ gene (13) suggested that the γ-subunit gene might lie closely downstream.

To confirm our hypothesis that the DH sites downstream of the δ-subunit gene were near the γ-subunit gene, we set out to clone the mouse δ-subunit gene along with the downstream sequence. Toward this end, two mouse genomic λ phage libraries (see above) were screened with the δ-subunit cDNA insert 6H (36). Screening of the L47.1 library yielded only one type of clone, which is denoted in Fig. 1 as L47.1δA-1. Although A-1 included the entire δ gene and approximately 5 kb downstream, it did not cross-hybridize with the full-length γ cDNA γ18 (8). A second library, the Charon 28 library, was then screened yielding the six types of clones shown in Fig. 1. A restriction enzyme map was constructed for each clone by using *Bam*HI-, *Hin*dIII-, and *Bam*HI-*Hin*dIII-digested DNA analyzed by Southern blotting. These DNAs were hybridized with full-length γ or δ cDNA probes or with exon-specific synthetic oligonucleotides. The seven overlapping clones were thus shown to map to a single locus, with approximately 5 kb separating the δ and γ genes. The DH sites within the δ-γ locus are also denoted in Fig. 1. The linkage of the δ- and γ-subunit genes placed the G_1 and G_2 DH sites near the 5′ end of the γ-subunit gene, and by their proximity it is suggested that the sites are more likely to be associated with the γ rather than the δ cistron.

Hypersensitivity experiments with a γ-subunit probe. A more precise localization of DH sites G_1 and G_2 was obtained by probing these sites with a γ cDNA. With such a probe, distances from the internal *Bam*HI site of 4.8 and 3.5 kb for G_1 and G_2, respectively, were measured with a precision of ±100 bp. G_2 is centered 300 bp upstream of the γ structural gene; G_1 lies 1,300 bp farther in the 5′ direction. Two of the blots used for these measurements are reproduced in Fig. 2. The two γ-subunit gene *Bam*HI fragments comigrated on 1% agarose gels and thus appeared as a single band. The tightly regulated C2i muscle cell line (see below) was used in these experiments to examine G_1 and G_2 in both undifferentiated and differentiated muscle cells. The blots were hybridized with both full-length (Fig. 2A) and 3′ (Fig. 2B) γ-specific probes. Besides the more precise localization of G_1 and G_2, two novel results were obtained from these blots. First, a DH site labeled X_4, which was not previously seen with the δ-subunit probes, was detected with both full-length and 3′-specific γ-subunit probes. X_4 was present in both undifferentiated and differentiated C2i muscle cells and was also detected in mouse liver tissue (data not shown).

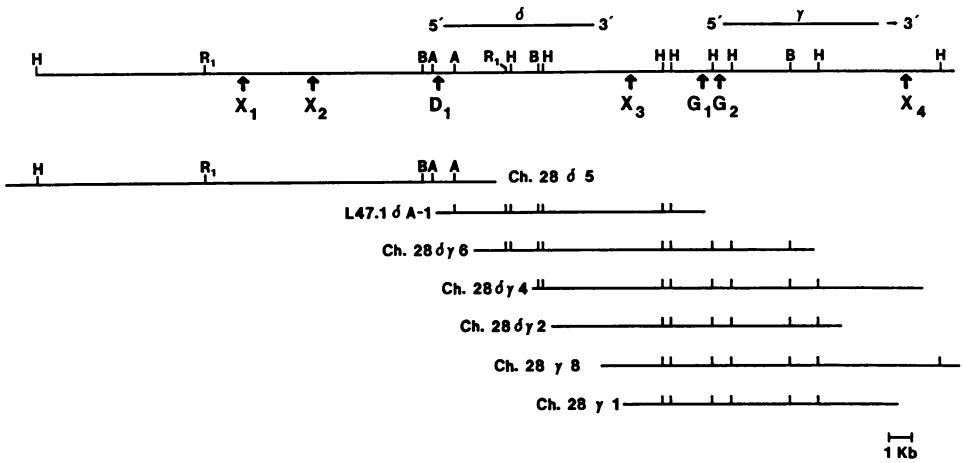

FIG. 1. The δ- and γ-subunit genes are tightly linked in the mouse genome. Linkage was demonstrated by isolating several individual clones that hybridized to both δ- and γ-subunit gene probes. The extent and orientation of the δ and γ genes are shown overlying the restriction map of the entire genomic region. The locations of the hypersensitive sites in the δ-γ locus are drawn below. The restriction map of the clones obtained from screening a λL47.1 C57BL mouse genomic library and a λCharon 28 (Ch. 28) BALB/c mouse genomic library with a δ-subunit cDNA probe is given at the bottom. Abbreviations: H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI; A, *Acc*I.

Thus, X_4 represents a relatively constitutive DH site at or distal to the 3′ end of the γ-subunit gene.

Both G_1 and G_2 were induced with differentiation of the C2i myoblasts. The restriction of G_1 and G_2 to differentiated C2i differed from their specificity in the other differentiating muscle cell lines C2, BC3H-1, and F3 (13), in which G_2 was fully expressed in myoblasts and only G_1 was induced with differentiation. This specificity difference is discussed at length in conjunction with Fig. 7 and 8 (see below).

Sequence of the δ-γ hypersensitivity regions. As asserted above, DH sites are the result of the binding of nonhistone proteins (58). In order to examine probable protein-binding sites within the δ-γ DH sites, we sequenced both strands of restriction fragments containing the D_1, G_1, and G_2 DH sites.

Figure 3 gives the sequence of the 970-bp *Bam*HI-*Acc*I fragment that was derived from the 5′ end of the L47.1δA-1 clone; D_1 mapped within this fragment. The center of D_1 was estimated to lie about 75 bp upstream of the initiator codon.

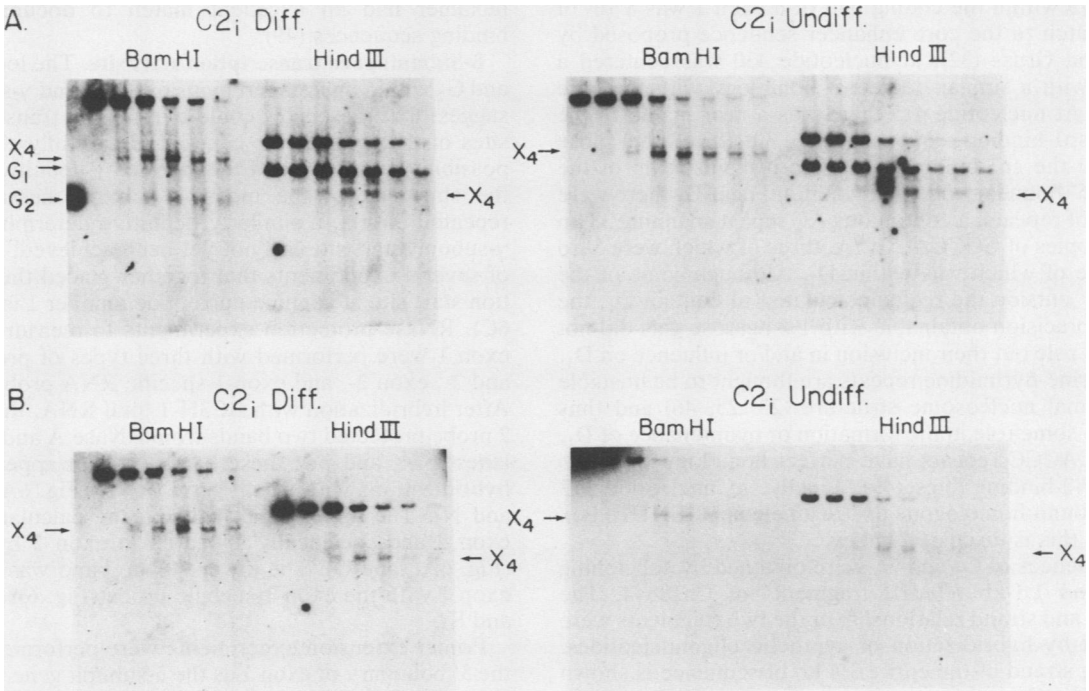

FIG. 2. γ-Subunit gene DH sites in differentiated and undifferentiated C2i cells. Southern blots of *Bam*HI- and *Hin*dIII-restricted DNAs from differentiated and undifferentiated C2i cells were probed with a full-length γ cDNA (A) and the 3′-most 390 bp of the γ cDNA (B). The DNase I concentration increased from left to right, from 0 to 30 U/mg of DNA. The X_4 site is labeled; the X_3 band, which is faintly present beneath the comigrating *Bam*HI parent fragments at 8 kb, hybridized only with the full-length probe. X_3 was better visualized with the δ-subunit probe and is discussed in conjunction with Fig. 7 and 8.

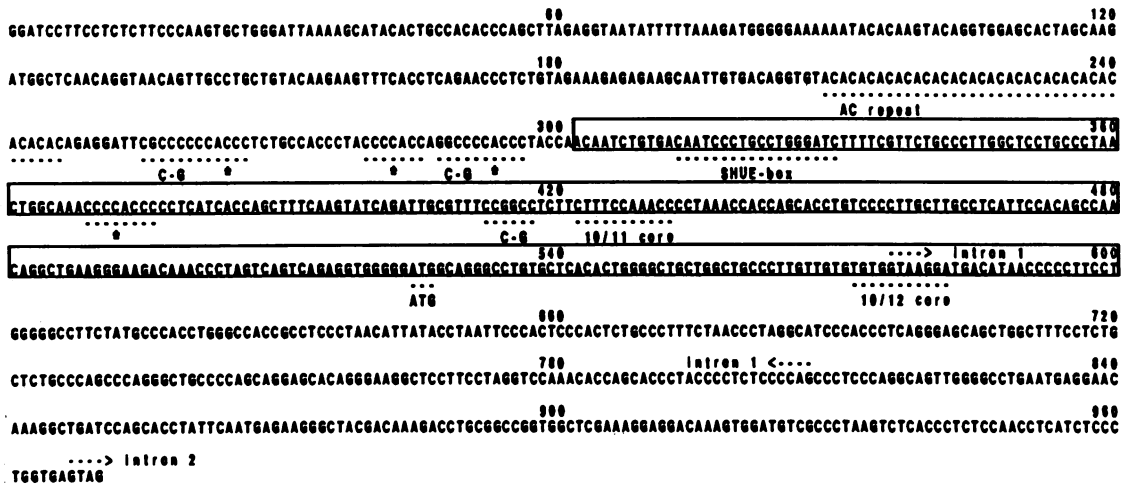

FIG. 3. Sequence features of the δ *Bam*HI-*Acc*I fragment. Homologies to sp1-binding sites (C-G) and to the core enhancer sequence are delineated. Two repeats, a 38-nucleotide AC repeat and a CCCCACCC/A repeat (designated by asterisks and repeated 4 times), are underscored with a dashed line. The SHUE box, an 18-nucleotide sequence (nucleotides 313 to 330) homologous to upstream sequences in all four subunits, is also underscored with a dashed line. The probable extent of the DH site is denoted by the boxed sequence.

This estimate is based on careful measurements of the distance from the 5′ *Bam*HI site to the next 3′ *Hin*dIII site in the L47.1 clone and on the size of the D_1-*Hin*dIII subfragment in whole genomic Southern blots. From multiple experiments, we estimated the maximum error in placing D_1 to be ±100 bp. The width of the DH subband appeared to be no more than 100 bp, a typical size for a DH site. Thus, D_1 is depicted as a 300-bp region that, as demonstrated below (see Fig. 6), is centered around the δ transcription start site.

Within the D_1 region there were several notable sequences, some or all of which were likely to be involved in the creation or maintenance of the D_1 site. Centered at position 575 within the coding half of δ exon 1 was a 10- of 12-base match to the core enhancer sequence proposed by Khoury and Gruss (32); at nucleotide 430 was centered a sequence with a similar degree of homology with the core sequence. At nucleotide 415 there was a near match to the canonical sp1-binding sequence (5, 16, 30). Two other close matches to the sp1-binding site were just upstream of the estimated 5′ boundary of D_1. In addition, near D_1 there were two types of repeats, a continuous AC repeat spanning 38 bp and four copies of CCCCACCC/A, three of which were 5′ to D_1 and one of which was within D_1. Although some of the repeats lay outside the region calculated to contain D_1, the degree of precision obtainable with 1% agarose gels did not allow us to rule out their inclusion in and/or influence on D_1. In fact, purine-pyrimidine repeats are thought to be unstable in the normal nucleosome structure (23, 25, 46) and thus could have some role in the formation or maintenance of D_1. The CCCCACCC repeats have perfect homology with two known AP-2-binding sites (49). Finally, at nucleotide 313 was the subunit homologous upstream element (SHUE) box homology; this is discussed below.

The sequences of G_1 and G_2 were obtained by subcloning the 2.3- and 1.1-kb *Hin*dIII fragments of Ch28δγ4. The colinearity and strand relationship of the two fragments were established by hybridization of synthetic oligonucleotides. The coding strand of the entire 3.4 kb of sequence is shown in Fig. 4. The G_1 and G_2 sites were placed on the sequence in the same manner as D_1 was placed. Approximately centered within the G_1 site was a 10- of 12-base match with the core enhancer sequence. The G_2 site included two remarkable types of sequences. A nearly perfect (22 of 24 bases) tandem repeat separated by 7 bp spanned the middle portion of G_2. The other sequence denoted as the SHUE box, is a stretch of 13 bp that has high homology with a sequence within the D_1 region of the δ-subunit upstream sequence. Alignment of the SHUE box with the two other AchR-subunit genes whose upstream sequences were available, chicken α (34) and mouse β (unpublished data), uncovered close matches in these genes as well. The alignments are shown in Fig. 5. The relation of the SHUE box to transcription start sites is only known with certainty for the δ- and α-subunit genes. The sequence CCCTGG/C was conserved in all four subunits. Notably, the conserved hexamer had an excellent match to documented AP-2-binding sequences (49).

δ-Subunit gene transcription start site. The locations of D_1 and G_2 within upstream regions of the δ- and γ-subunit genes suggest that these sites could overlap the transcription start sites of their respective genes. We carefully examined this possibility in the case of the D_1 site by definitively mapping the start site of the mouse δ-subunit message. Despite repeated efforts, a similarly definitive determination of the γ-subunit start site has not yet been achieved. A composite of several experiments that together placed the δ transcription start site at adenine nucleotide number 1 is shown (Fig. 6C). RNase protection experiments to measure the size of exon 1 were performed with three types of probes: exon 1 and 2-, exon 2-, and exon 1-specific RNA probes (Fig. 6A). After hybridization with BC3H-1 total RNA, the exon 1 and 2 probe protected two bands from RNase A and T_1 (Fig. 6A, lanes I, K, and M); these bands did not appear in control hybridizations with wheat germ tRNA (Fig. 6A, lanes J, L, and N). The upper band matched the calculated size of δ exon 2 and specifically appeared in exon 2 hybridizations (Fig. 6A, lane O). The lower 107-bp band was identified as exon 1 with the exon 1-specific probe (Fig. 6A, lanes Q, R, and S).

Primer extension experiments were performed to identify the 5′ boundary of exon 1 as the δ-subunit gene transcription start site (Fig. 6B). Two different oligonucleotide primers, one directed to δ exon 2 and one beginning at the 3′ boundary of exon 1, extended distances that mapped to the 5′ boundary of exon 1; the extended products were specific to skeletal muscle RNA templates.

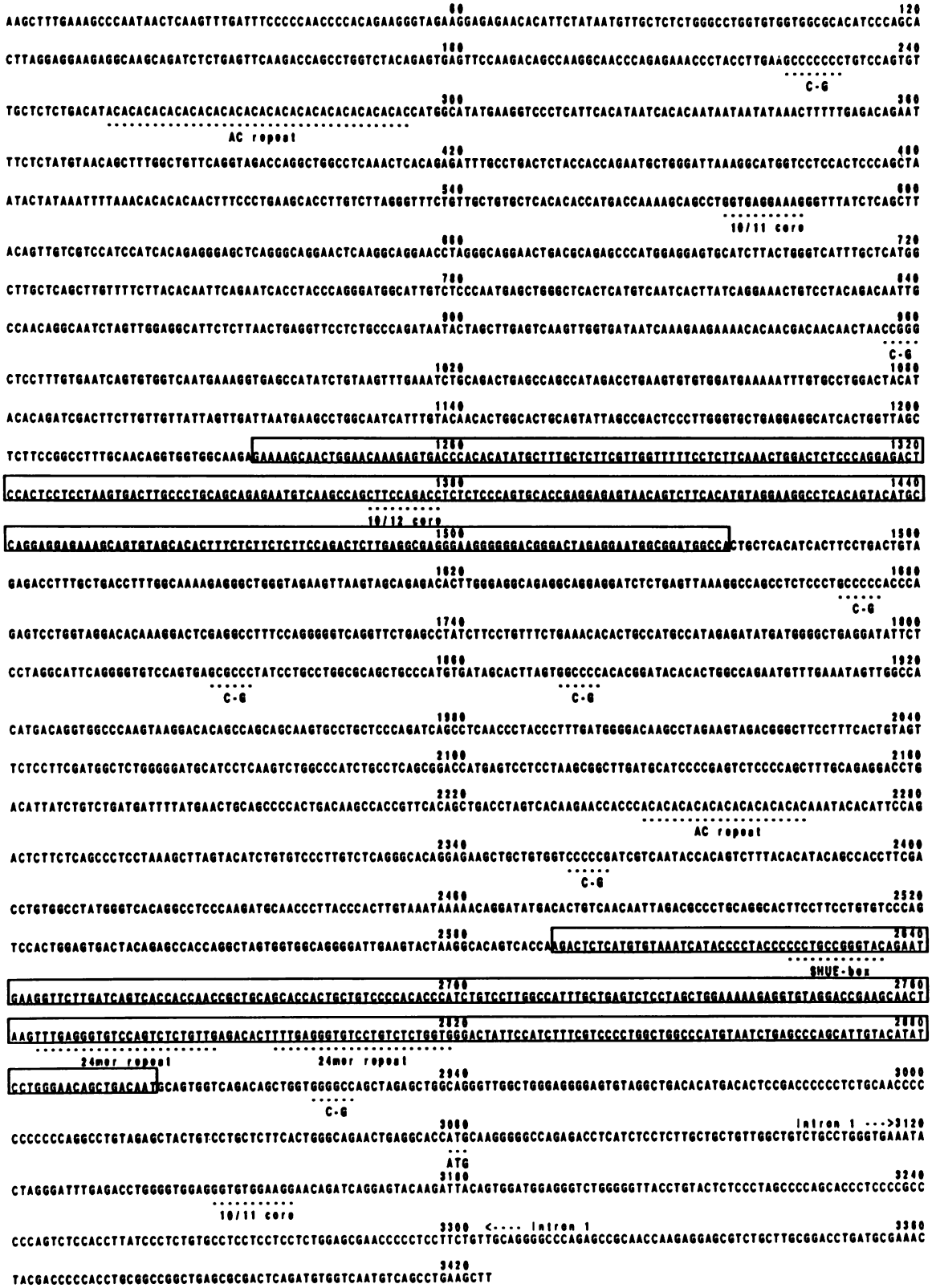

FIG. 4. Sequence features of γHH2.3 and γHH1.1 (symbols and details are as described in the legend to Fig. 3). The G_1- and G_2-hypersensitive sites are boxed. In addition, two 24-mer repeats that differed in only two positions are underlined.

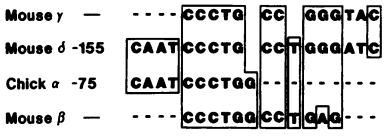

FIG. 5. Alignment of AchR-subunit homologous upstream element. Locations of the sequence relative to the transcription start site are given for the subunit genes for which it is known. Although the transcription start sites of the β- and γ-subunit genes have not been defined, both the β- and γ-subunit SHUE boxes lie upstream of the ATG codon. Fractional homologies were as follows: γ-δ, 11 of 13 bases; γ-α, 5 of 6 bases; γ-β, 9 of 10 bases; δ-α, 9 of 10 bases; δ-β, 10 of 11 bases; α-β, 6 of 6 bases.

Upstream of the δ subunit start site was a TA-rich sequence that was positioned appropriately for serving as the δ gene TATA box (Fig. 6C). The putative δ-subunit TATA box does not have perfect homology to the TATA consensus sequence (4, 12); however, similarly poor matches have been shown to function as TATA elements for other promoters (29, 42, 59). Homologies to other promoter elements were present farther upstream with a 5- of 6-base match to the CCAATC box (4, 12) sequence at positions −60 to −65 on the noncoding strand and with a CG-rich region from positions −50 to −55. The SHUE box homology was also delineated (δ-α, β, and γ homology).

Developmental activation of the δ-γ locus. We have described previously (13) the regulation of the δ-γ DH sites during differentiation of BC3H-1 cells. The differentiation of BC3H-1 is accompanied by the induction of two DH sites, G_1 and X_3; the remainder of the sites are present prior to differentiation of BC3H-1. In order to better define the role of each site in myogenic activation of the δ-γ genes, we subsequently examined muscle cell lines that had different requirements for differentiation. Upon reaching confluency, C2 myoblasts spontaneously fuse without a change in the culture medium, although a reduction of mitogens does hasten differentiation. A variant subclone of C2, C2i, does not differentiate in the presence of serum, even in confluent cultures (47a). The C2i line was developed and kindly donated by Christian Pinset and co-workers (Institut Pasteur, Paris, France). We made use of the different differentiation properties of the two muscle cell lines along with the premuscle lineage cell line C3H10T1/2, to examine closely the association of each DH site with muscle differentiation.

Figure 7 gives the results of this study. The Southern blots were derived from *Bam*HI or *Hin*dIII restriction of DNase I-digested DNA. The blots were probed with the δ cDNA 6H; every DH site within the δ-γ locus except X_4 was detected with this combination of enzymes and probe. C3H10T1/2 cells are pluripotential precursors to committed mesodermal lineages, including that of muscle (35, 55). In confluent cultures of C3H10T1/2 cells, only DH sites X_1 and X_2 were detected with the 6H probe. The two sites lay distantly 5′ to the δ gene (Fig. 1). One more site, X_3, was expressed in the committed but undifferentiated C2i myoblasts. The X_3 site lay in the intergenic region about 2 kb 3′ to δ exon 12 (Fig. 1). Unlike the remainder of the DH sites in the δ-γ locus, this site did not fit into a scheme in which the number of hypersensitive sites correlated with the proximity of a cell to the differentiated muscle phenotype. X_3 was expressed in mouse liver cells (data not shown), but was not expressed in C3H10T1/2 or undifferentiated BC3H-1 cells. Interestingly, the DH sites near the 5′ ends of the δ and γ genes did not appear in confluent C2i myoblasts. However, the spontaneously differentiating C2 myoblasts did express both D_1 and G_2 prior to detectable transcription of the δ-γ gene. The X or nonmuscle-specific sites were present in both C2i and C2 myoblasts. The rightmost myoblast blot in Fig. 7 blot shows the DH site pattern characteristic of all differentiated muscle cells examined so far. Thus, in C2i, after a switch to serumfree medium, sites D_1, G_1, and G_2 are induced, while in spontaneously differentiating muscle cell lines like C2, only G_1 remains to be created at the time of differentiation.

DISCUSSION

Eucaryotic gene regulation is achieved by a complex interplay of *cis*-acting sequences and *trans*-acting proteins in the context of the regional chromatin structure. Chromatin structure has been the initial focus of our studies of the mechanisms of AchR-subunit gene regulation. Although it is perhaps an indirect means of analyzing regulatory mechanisms, this approach has led us to several discoveries that were not as easily obtainable by other methods.

Linkage of the δ- and γ-subunit genes. The evolutionary and functional significance of the tight δ-γ linkage is unknown. Ballivet and co-workers (44) have pointed out the high degree of sequence homology and identical exon-intron organization shared by the chicken δ- and γ-subunit genes; they and others (44, 45) have suggested that the δ- or γ-subunit gene is derived from a relatively recent tandem duplication. As for functional significance, the proximity of the two genes, in theory, allows for *cis*-coordinate regulation. Although it is the rule in procaryotes, this *cis* coordination is rare in higher organisms, with the β-globin locus perhaps being an example (24). Candidates for coordinate regulatory regions such as the G_1 or the X sites will allow for a directed examination of *cis*-coordinate regulation of the δ- and γ-subunit genes.

Remote regulatory elements. In general, hypersensitive sites are markers of transcriptional regulatory regions; however, this generalization is not absolute. Therefore, one must ask whether every DH site (mapped above) is part of the δ-γ transcriptional regulatory mechanisms. The proximal DH sites D_1, G_1, and G_2 almost certainly are involved, given their muscle and developmental specificity and, at least in the case of δ, given that they overlap with the promoter region. However, the remote sites X_1, X_2, X_3, and X_4 are not muscle specific; moreover, X_2 and X_4 have been expressed in every cell type examined so far. Thus, the regulatory role, if any, of the remote sites is uncertain. These sites might be markers of replication origins, although their presence does not correlate with the mitotic activity of the cell types examined. Localization with transposons is unlikely since no *Bam*HI, *Hin*dIII, or *Eco*RI fragment-length polymorphisms have been observed in the multiple cell types that we examined. Chromosomal mapping of the mouse δ-γ locus excludes proximity to a centromere (27). In terms of gene regulation, these sites could represent the binding of proteins, perhaps ubiquitous transcription factors, that are shared by liver and muscle. Although the proximal sites are the current focus of our search for δ-γ-regulatory elements, inclusion of one or more of the distal sites in minilocus constructs may prove essential for insertion site-independent, properly regulated, and high-level expression of the δ-γ locus. This scenario has recent precedence in the human β-globin locus, which requires inclusion of remote, nondevelopmentally regulated DH sites for proper expression of the β-globin gene (24).

δ-γ sequence homology. In order to form a functional receptor, the expression of all four receptor subunits must be

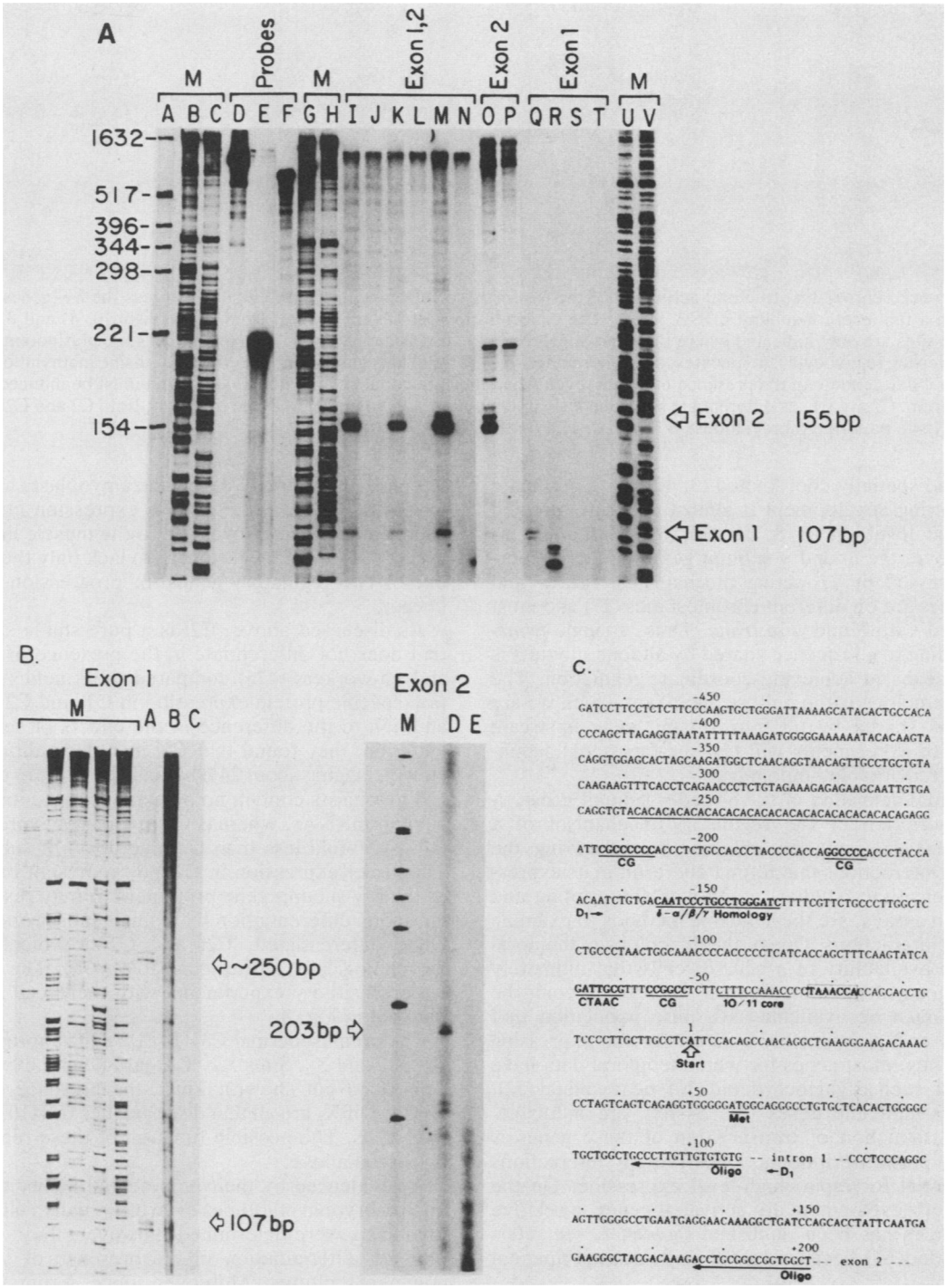

FIG. 6. Transcription start site mapping of the δ subunit gene. (A) RNase protection with δ exon 1 and 2 (D)-, δ exon 2 (E)-, and δ exon 1 (F)-specific probes. Probes were hybridized with 10 μg of BC3H-1 total RNA (lanes I, K, M, O, Q, R, and S) with 10 μg of wheat germ tRNA (lanes J, L, N, and P) or with 10 μg of mouse brain total RNA (lane T). Hybridizations with the δ exon 1 and 2 probe were performed at three different temperatures: 45°C (lanes I and J), 55°C (lanes K and L), and 65°C (lanes M and N). Hybridizations with δ exon 2 and δ exon 1 probes were performed at 55°C. δ Exon 1 and 2 and δ exon 2 hybrids were digested with RNase A/T_1 for 30 min. δ Exon 1 hybrids were digested for 5 min (lane Q), 30 min (lanes R and T), or 90 min (lane S). Size markers (lanes A, B, C, U, V) were *Hin*fI-cut pBR322 and mp18, sequenced with universal primer. (B) Primer extension with exon 1- and exon 2-specific oligonucleotides. Location of the oligonucleotides is shown in Fig. 2C. Radiolabeled primers were hybridized to 2 μg of poly(A)$^+$ BC3H-1 RNA (lanes A and D), 2 μg of mouse brain poly(A)$^+$ RNA (lane B), or 2 μg of wheat germ tRNA (lanes C and E). Primers were extended for 30 min at 42°C with avian myeloblastosis virus reverse transcriptase. (C) The 107-bp exon 1 and the 203-bp exon 2 primer extension products mapped to the same adenosine residue as the δ exon 1 RNase-protected fragment. The sequence TAAACCA at positions −33 to −27 relative to the transcription start site presumably serves as the TATA box for the δ gene. A CCAATCT box homology was present on the opposite strand at positions −61 to −66. The SHUE box was centered at position −150. CG-rich sequences were centered at positions −55, −180, and −210.

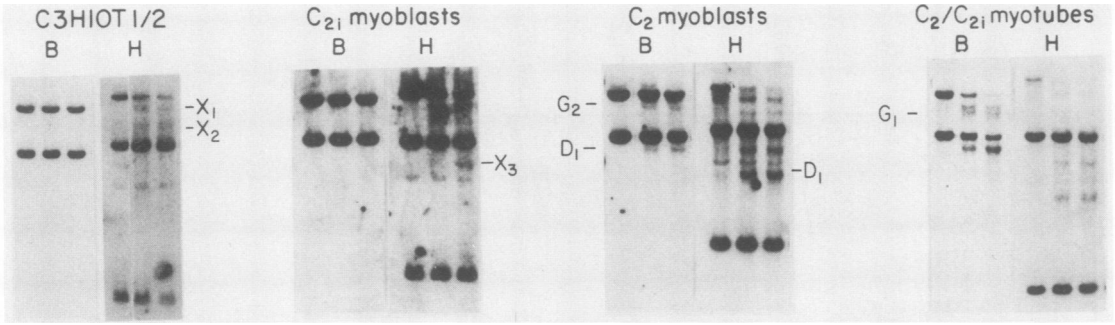

FIG. 7. Degree of chromatin structural activation of the δ-γ locus correlates with the potential to express the δ-γ genes. All blots were performed with a full-length δ-subunit cDNA probe. The concentrations of DNase I were, from left to right, 0, 4, and 8 U/mg of DNA. Hypersensitive sites are only indicated where they first appeared in the myogenic pathway. C3H10T1/2 cells are pluripotential cells that are capable of converting to myocytes, adipocytes, or chondrocytes (mesodermal lineages) when treated with cytosine methylation inhibitors. C2i myoblasts do not differentiate in the presence of serum, even when they are confluent and nondividing, and must be induced to differentiate by removing serum. C2 myoblasts differentiate spontaneously into myotubes, even in the presence of serum. Both C2 and C2i myotubes have an identical DH site pattern. Abbreviations: B, *Bam*HI; H, *Hin*dIII.

temporally and spatially coordinated (3, 40, 62). Coordinate expression during development is almost certainly directed at the genetic level (1, 7, 8, 18, 36, 69). Although, as discussed above, the δ- and γ-subunit genes could be coordinately expressed by *cis*-acting mechanisms, the α- and β-subunit genes are on different chromosomes (27) and must be coregulated with δ and γ in *trans*. Thus, a single *trans*-activator binding to a sequence shared by all four subunits is an obvious means of achieving coordinate regulation. The SHUE box homology is the only sequence, of which we are aware, that is shared by all four subunit gene upstream regions. Future experiments will test the functional significance of this remarkable homology.

Developmental activation of the δ- and γ-subunit genes. A temporal description of the regulatory mechanism of a particular gene can only be obtained by observing the DNA-protein interactions that ultimately result in its expression. Hypersensitivity studies, along with footprinting and gel retardation assays, are the available methods to examine DNA-protein interactions. One of the difficulties of temporal studies is the availability of precursor cells that ultimately express the gene of interest. Even if such a cell could be identified, it must be available as a pure population that quantitatively and reproducibly converts to the expressing phenotype. Thus, most genes for which temporal data have been obtained, such as glucocorticoid (70), heavy metal (52), or heat shock-inducible genes (31, 64–68), are inducible. Interestingly, induction of transcription of these genes is preceded by pretranscriptional DNA-protein interactions that are essential for rapid, high-level expression. On the other hand, for developmentally activated genes, a relative dearth of studies has been published (however, see references 6, 11, and 61) because of the difficult experimental constraints.

In lieu of tissue from developing mice, we used permanent cell lines that had properties consistent with different stages of myogenesis. Our conclusions from the hypersensitivity studies follow directly from the properties of these cell lines.

C3H10T1/2 cells are derived from C3H mouse embryos and are best described as a pluripotential mesodermal precursor. C3H10T1/2 cells are biochemically and morphologically undifferentiated, but when they are treated with the cytosine methylation inhibitor 5-azacytidine, C3H10T1/2 cells transform to each of the mesodermal lineages, chondrocytes, adipocytes, and myocytes (55). No ecto- or endodermal cell types are detectable after this treatment (37). Recently, C3H10T1/2-derived azamyoblasts have been used to isolate cDNA clones whose expression in C3H10T1/2 is sufficient for their conversion to the muscle lineage (15, 47). Thus, C3H10T1/2 cells appear to lack only the expression of one or two regulatory genes for progression to the muscle lineage.

As discussed above, C2i is a pure stable subclone of C2 that does not differentiate in the presence of serum. Pinset and co-workers (47a) compared the kinetics of differentiation-specific protein expression in C2i and C2 myoblasts. In addition to the difference in the effects of serum on differentiation, they found that C2i myoblasts differentiate more slowly, lagging about 24 h behind C2. Prior to differentiation, C2i myoblasts contain no detectable concentration of AchR subunit mRNAs, whereas C2 myoblasts express a low level (10- to 50-fold less than C2 myotubes) of subunit mRNAs. This leaky expression in C2 may represent basal activation of the δ-γ-subunit gene promoters or may result from asynchronous differentiation by a small subset of C2 myoblasts. Once differentiated, C2i and C2 are biochemically and morphologically indistinguishable (47a). The results of our hypersensitivity experiments with these cell lines are summarized in Fig. 8.

The premesodermal C3H10T1/2 cells expressed only sites X_1, X_2, and X_4. Sites X_2, X_3, and X_4 were expressed in liver cells (data not shown). Thus, the presence of X_1 and the absence of X_3 are all that distinguishes C3H10T1/2 cells from liver cells. The possible function of these remote sites was discussed above.

As evidenced by their unipotential ability to differentiate into embryonic myotubes even after many cell divisions, C2i myoblasts were determined; however, they were incompetent for differentiation in the presence of serum even in confluent cultures. Moreover, once serum was removed, differentiation was slow. These unique properties of C2i myoblasts were reflected in their DH site pattern at the δ-γ locus. The only detectable difference between C3H10T1/2 cells and C2i myoblasts was the expression in the latter of X_3 (Fig. 7). Site X_3 lies in the δ-γ intergenic region and is present both in muscle and liver cells. Thus, X_3 is not a specific marker of early myogenic determination. Perhaps, more than one factor, one specific to liver and one specific to the muscle lineage, is capable of creating X_3. The alternative offered above is that X_3 results from the binding of a factor that is common to both liver and muscle. A third alternative is that X_3 is created by a common liver and muscle factor but

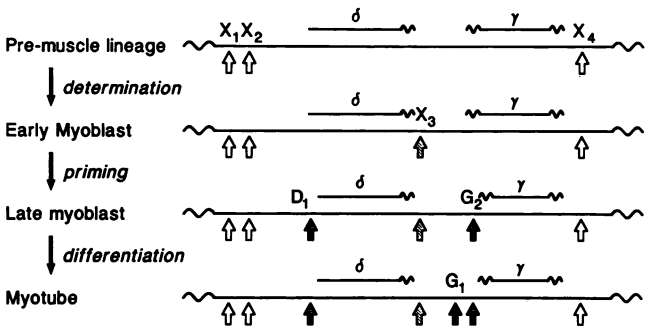

FIG. 8. Stepwise activation of the δ-γ locus during myogenesis. Activation of the δ-γ locus in clonal cell lines is modeled as a three-step process of determination, priming, and differentiation. These stages of activation can be distinguished by their pattern of hypersensitive sites. Priming, the unique step in this model, is characterized by the appearance of DH sites at the 5′ ends of the δ- and γ-subunit genes. The primed chromatin conformation of the δ-γ genes, although not essential to the determined myogenic state, correlates with rapid and permissive myoblast differentiation and δ- and γ-subunit gene activation.

additional muscle-specific factors subsequently bind to the X_3 site. Regardless of the etiology of the X_3 site, it should serve to isolate DNA-binding proteins that are likely to be involved in early activation of the δ-γ locus.

Spontaneously differentiating myoblasts such as C2, F3, and BC3H-1 differ strikingly from C2i by the presence of DH sites D_1 and G_2 (13) (Fig. 7). The sites are near the 5′ ends of the δ and γ structural genes; D_1, in fact, overlaps the δ promoter region. We propose that D_1 and G_2 are homologous DH sites that represent the binding of transcription factors to the δ and perhaps γ promoters, thus priming the promoters for the influx and subsequent action of differentiation-specific factors. This priming hypothesis could serve to explain the slow onset of δ-γ expression in unprimed C2i. A similar priming hypothesis is suspected for a number of other genes (9, 51, 53, 61, 70) and is well established for *Drosophila melanogaster* heat shock-inducible genes (31, 57, 64–68). Alternatively, or in addition to priming, the D_1 and G_2 sites may be associated with the basally activated state of the δ-γ locus, thus explaining their expression in C2 myoblasts.

The differentiated muscle phenotype is characterized by the DH site pattern shown at the bottom of Fig. 8. Site G_1 is the only site specific to differentiated muscle cells. The types of muscle cell lines that we examined are diverse and include not only C2 and C2i but also the azamyotube cell line F3 and the nonfusing BC3H-1 cell line. The differentiation specificity of G_1 in each of these lines implicates it as having some role in the late activation of the δ-γ locus. The lack of a detectable G_1 analog 5′ to the δ-subunit gene indicates either that G_1 serves in the activation of both δ and γ or that the δ analog is hidden within or near D_1.

The developmental model proposed in Fig. 8 is not meant to suggest that these intermediate cell types and activation states necessarily occur during in vivo mouse muscle development, but only that through these cell lines we were able to isolate different stages of chromatin structural activation of the δ-γ locus and can predict possible functions for the DH sites from the properties of the cell lines.

ACKNOWLEDGMENTS

This study was dependent on the excellent technical assistance of Jacque Mudd, Vandana Shah, and Jon Kornhauser. We thank Andrew Lassar for the gift of C3H10T1/2 and F3 cells and Christian Pinset for the C2i cells. We are grateful to Tom Dietz and Liu lin Thio for help in cloning and mapping the δ-γ locus. We also appreciate the advice of Jim Krause on primer extension protocols and Sarah Elgin on hypersensitivity studies. Finally, we appreciate the help of Anne Dillon and the Washington University computer graphics facility.

C.M.C. was supported by National Research Service Awards 5T32 GM07200 and 5T32 HL07275. The laboratory was supported by funds from the Senator Jacob Javits Center of Excellence in Neurosciences and by grants to J.P.M. from the National Institutes of Health, the Muscular Dystrophy Association of America, and Monsanto Co.

LITERATURE CITED

1. **Baldwin, T. J., C. M. Yoshihara, K. Blackmer, C. R. Kintner, and S. J. Burden.** 1988. Regulation of acetylcholine receptor transcript expression during development in *Xenopus laevis*. J. Cell Biol. **106**:469–478.
2. **Bloom, K. S., E. Amaya, J. Carbon, L. Clarke, A. Hill, and E. Yeh.** 1984. Chromatin conformation of yeast centromeres. J. Cell Biol. **99**:1559–1568.
3. **Blount, P., and J. P. Merlie.** 1988. Native folding of an acetylcholine receptor α subunit expressed in the absence of other receptor subunits. J. Biol. Chem. **263**:1072–1080.
4. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
5. **Briggs, M. R., J. T. Kadonaga, S. P. Bell, and R. Tjian.** 1986. Purification and biochemical characterization of the promoter-specific transcription factor, SP1. Science **234**:47–52.
6. **Bryan, P. N., J. Olah, and M. L. Birnstiel.** 1983. Major changes in the 5′ and 3′ chromatin structure of sea urchin histone genes accompany their activation and inactivation in development. Cell **33**:843–848.
7. **Buonanno, A., and J. P. Merlie.** 1986. Transcriptional regulation of nicotinic acetylcholine receptor genes during muscle development. J. Biol. Chem. **261**:11452–11455.
8. **Buonanno, A., J. Mudd, V. Shah, and J. P. Merlie.** 1986. A universal oligonucleotide probe for acetylcholine receptor genes. J. Biol. Chem. **261**:16451–16458.
9. **Burch, J. B. E., and H. Weintraub.** 1983. Temporal order of chromatin structural changes associated with activation of the major chicken vitellogenin gene. Cell **33**:65–76.
10. **Cartwright, I. L., M. A. Keene, G. C. Howard, S. M. Abmayr, G. Fleischmann, K. Lowenhaupt, and S. C. R. Elgin.** 1982. Chromatin structure and gene activity: the role of nonhistone chromosomal proteins. Crit. Rev. Biochem. **13**:1–86.
11. **Choi, O.-R., and J. D. Engel.** 1986. A 3′ enhancer is required for temporal and tissue specific transcriptional activation of the chicken adult β-globin gene. Nature (London) **323**:731–734.
12. **Corden, J., B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, and P. Chambon.** 1980. Promoter sequences of eukaryotic protein-coding genes. Science **209**:1406–1414.
13. **Crowder, C. M., and J. P. Merlie.** 1986. DNase I-hypersensitive sites surround the mouse acetylcholine receptor δ-subunit gene. Proc. Natl. Acad. Sci. USA **83**:8405–8409.
14. **Dale, R. M. K., B. A. McClure, and J. P. Houchins.** 1985. A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing corn mitochondrial 18 S rDNA. Plasmid **13**:31–40.
15. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**:987–1000.
16. **Dynan, W. S., and R. Tjian.** 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature (London) **316**:774–778.
17. **Emerson, B. M., J. M. Nickol, P. D. Jackson, and G. Felsenfeld.** 1987. Analysis of the tissue-specific enhancer at the 3′ end of the chicken adult β-globin gene. Proc. Natl. Acad. Sci. USA **84**: 4786–4790.
18. **Evans, S., D. Goldman, S. Heinemann, and J. Patrick.** 1987.

Muscle acetylcholine receptor biosynthesis. J. Biol. Chem. **262:** 4911–4916.
19. **Fontaine, B. F., D. Sassoon, M. Buckingham, and J.-P. Changeux.** 1988. Detection of the nicotinic acetylcholine receptor α-subunit mRNA by *in situ* hybridization at neuromuscular junctions of 15-day-old chick striated muscles. EMBO J. **7:**603–609.
20. **Fritton, H. P., T. Igo-Kemenes, J. Nowock, U. Strech-Jurk, M. Theisen, and A. E. Sippel.** 1984. Alternative sets of DNase I-hypersensitive sites characterize the various functional states of the chicken lysozyme gene. Nature (London) **311:**163–165.
21. **Goldman, D., J. Boulter, S. Heinemann, and J. Patrick.** 1985. Muscle denervation increases the levels of two mRNAs coding for the acetylcholine receptor α-subunit. J. Neurosci. **5:**2553–2558.
22. **Goldman, D., H. R. Brenner, and S. Heinemann.** 1988. Acetylcholine receptor α-, β-, γ-, δ-subunit mRNA levels are regulated by muscle activity. Neuron **1:**329–333.
23. **Gross, D. S., S.-Y. Huang, and W. T. Garrard.** 1985. Chromatin structure of the potential Z-forming sequence $(dT\text{-}dG)_n$-$(dC\text{-}dA)_n$. J. Mol. Biol. **183:**251–265.
24. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51:**975–985.
25. **Hamada, H., M. Seidman, B. H. Howard, and C. M. Gorman.** 1984. Enhanced gene expression by the poly (dT-dG):poly (dC-dA) sequence. Mol. Cell. Biol. **4:**2622–2630.
26. **Harris, D. A., D. L. Falls, R. M. Dill-Devor, and G. D. Fischbach.** 1988. Acetylcholine receptor-inducing factor from chicken brain increases the level of mRNA encoding the receptor α subunit. Proc. Natl. Acad. Sci. USA **85:**1983–1987.
27. **Heidmann, O., A. Buonanno, B. Geoffroy, B. Robert, J.-L. Guenet, J. P. Merlie, and J.-P. Changeux.** 1986. Chromosomal localization of muscle nicotinic acetylcholine receptor genes in mouse. Science **234:**866–868.
28. **Igo-Kemenes, T., W. Horz, and H. G. Zachau.** 1982. Chromatin. Annu. Rev. Biochem. **51:**89–121.
29. **Ishi, S., G. T. Merlino, and I. Pastan.** 1985. Promoter region of the human Harvey ras proto-oncogene: similarity to the EGF receptor proto-oncogene promoter. Science **230:**1378–1380.
30. **Kadonaga, J. T., K. A. Jones, and R. Tjian.** 1986. Promoter-specific activation of RNA polymerase II transcription by Spl. Trends Biochem. Sci. **11:**20–23.
31. **Keene, M. A., V. Corces, K. Lownhaupt, and S. C. R. Elgin.** 1981. DNase I hypersensitive sites in *Drosophila* chromatin occur at the 5′ ends of regions of transcription. Proc. Natl. Acad. Sci. USA **78:**143–146.
32. **Khoury, G., and P. Gruss.** 1983. Enhancer elements. Cell **33:** 313–314.
33. **Klarsfeld, A., and J.-P. Changeux.** 1985. Activity regulates the levels of acetylcholine receptor α-subunit mRNA in cultured chicken myotubes. Proc. Natl. Acad. Sci. USA **82:**4558–4562.
34. **Klarsfeld, A., P. Daubas, B. Bourachat, and J.-P. Changeux.** 1987. A 5′-flanking region of the chicken acetylcholine receptor α-subunit gene confers tissue specificity and developmental control of expression in transfected cells. Mol. Cell. Biol. **7:**951–955.
35. **Konieczny, S. F., and C. P. Emerson.** 1984. 5-Azacytidine induction of stable mesodermal stem cell lineages from $10T_{1/2}$ cells: evidence for regulatory genes controlling determination. Cell **38:**791–800.
36. **LaPolla, R. J., K. Mayne, and N. Davidson.** 1984. Isolation and characterization of a cDNA clone for the complete protein coding region of the δ subunit of the mouse acetylcholine receptor. Proc. Natl. Acad. Sci. USA **81:**7970–7974.
37. **Lassar, A. B., B. M. Paterson, and H. Weintraub.** 1986. Transfection of DNA locus that mediates the conversion of $10T_{1/2}$ fibroblasts to myoblasts. Cell **47:**649–656.
38. **Merlie, J. P., K. E. Isenberg, S. D. Russell, and J. R. Sanes.** 1984. Denervation supersensitivity in skeletal muscle: analysis with a cloned cDNA probe. J. Cell Biol. **99:**332–335.
39. **Merlie, J. P., and J. R. Sanes.** 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. Nature (London) **317:**66–68.
40. **Mishina, M., T. Kurosaki, T. Tobimatsu, Y. Morimoto, M. Noda, T. Yamamoto, M. Terao, J. Lindstrom, T. Takahashi, M. Kuno, and S. Numa.** 1984. Expression of functional acetylcholine receptor from cloned cDNAs. Nature (London) **307:**604–608.
41. **Mishina, M., T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. Methfessel, and B. Sakmann.** 1986. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature (London) **321:**406–411.
42. **Nandi, A., G. Das, and N. P. Salzman.** 1985. Characterization of surrogate TATA box promoter that regulates in vitro transcription of the simian virus 40 major late gene. Mol. Cell. Biol. **5:** 591–594.
43. **Nasmyth, K. A.** 1982. The regulation of yeast mating-type chromatin structure by SIR: an action at a distance affecting both transcription and transposition. Cell **30:**567–578.
44. **Nef, P., A. Mauron, R. Strader, C. Alliod, and M. Ballivet.** 1984. Structure, linkage, and sequence of the two genes encoding the δ and γ subunits of the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA **81:**7975–7979.
45. **Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, Y. Furutani, T. Hirose, H. Taskashima, S. Inayama, T. Miyata, and S. Numa.** 1983. Structural homology of *Torpedo californica* acetylcholine receptor subunits. Nature (London) **302:**528–532.
46. **Nordheim, A., and A. Rich.** 1983. The sequence $(dC\text{-}dA)_n$: $(dT\text{-}dG)_n$ forms left-handed Z-DNA in negatively supercoiled plasmids. Proc. Natl. Acad. Sci. USA **80:**1821–1825.
47. **Pinney, D. F., S. H. Pearson-White, S. F. Konieczny, K. E. Latham, and C. P. Emerson, Jr.** 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. Cell **53:**781–793.
47a. **Pinset, C., D. Montarras, J. Chenevert, A. Minty, P. Barton, C. Laurent, and F. Gros.** 1988. Characterization of two myoblast subclones, permissive and inducible C2 cells, which exhibit different patterns of control of muscle gene expression. Differentiation **38:**28–34.
48. **Reznikoff, C., D. Brankow, and C. Heidelberger.** 1973. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. **33:**3231–3238.
49. **Roesler, W. J., G. R. Vandenbark, and R. W. Hanson.** 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. **263:**9063–9066.
50. **Sanger, F., S. Nicklen and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
51. **Scheidereit, C., S. Geisse, H. M. Westphal, and M. Beato.** 1983. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. Nature (London) **304:**749–752.
52. **Senear, A. W., and R. D. Palmiter.** 1983. Expression of the mouse metallothionein I gene alters the nuclease hypersensitivity of its 5′ regulatory region. Cold Spring Harbor Symp. Quant. Biol. **47:**539–547.
53. **Shermoen, A. W., and S. K. Beckendorf.** 1982. A complex of interacting DNAase I-hypersensitive sites near the *Drosophila* glue protein gene, SGS4. Cell **29:**601–607.
54. **Shibahara, S., T. Kubo, H. J. Perski, H. Takahashi, M. Noda, and S. Numa.** 1985. Cloning and sequence analysis of human genomic DNA encoding γ subunit precursor of muscle acetylcholine receptor. Eur. J. Biochem. **146:**15–22.
55. **Taylor, S. M., and P. A. Jones.** 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell **17:**771–779.
56. **Theisen, M., A. Stief, and A. Sippel.** 1986. The lysozyme enhancer: cell-specific activation of the chicken lysozyme gene by a far-upstream DNA element. EMBO J. **5:**719–724.
57. **Thomas, G. H., and S. C. R. Elgin.** 1988. Protein/DNA architecture of the DNase I hypersensitive region of the *Drosophila* hsp26 promoter. EMBO J. **7:**2191–2201.
58. **Thomas, G. H., E. Siegfried, and S. C. R. Elgin.** 1985. DNase I hypersensitive sites: a structural feature of chromatin associ-

ated with gene expression, p. 77–101. *In* G. Reeck, G. Goodwin, and P. Puigdomenech (ed.), Chromosomal proteins and gene expression. Plenum Publishing Corp., New York.

59. **Tunnacliffe, A., J. E. Sims, and T. H. Rabbitts.** 1986. T3δ pre-mRNA is transcribed from a non-TATA promoter and is alternatively spliced in human T cells. EMBO J. **5**:1245–1252.
60. **Varshavsky, A. J., O. Sundin, and M. Bohn.** 1979. A stretch of "late" SV40 viral DNA about 400 bp long which includes the origin of replication is specifically exposed in SV40. Cell **16**:453–466.
61. **Weintraub, H., H. Beug, M. Groudine, and T. Graf.** 1981. Temperature-sensitive changes in the structure of globin chromatin in lines of red cell precursors transformed by ts-AEV. Cell **28**:931–940.
62. **White, M., K. Mayne, H. Lester, and N. Davidson.** 1985. Mouse-*Torpedo* hybrid acetylcholine receptors: functional homology does not equal sequence homology. Proc. Natl. Acad. Sci. USA **82**:4852–4856.
63. **Wilbur, W. J., and D. J. Lipman.** 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA **80**:726–730.
64. **Wu, C.** 1980. The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. Nature (London) **286**: 854–860.
65. **Wu, C.** 1984. Two protein-binding sites in chromatin implicated in the activation of heat-shock genes. Nature (London) **309**:229–234.
66. **Wu, C.** 1985. An exonuclease protection assay reveals heat-shock element and TATA box DNA-binding proteins in crude nuclear extracts. Nature (London) **317**:84–87.
67. **Wu, C., P. M. Bingham, K. J. Livak, R. Holmgren, and S. C. R. Elgin.** 1979. The chromatin structure of specific genes. I. Evidence for higher domains of defined DNA sequence. Cell **16**: 797–806.
68. **Wu, C., Y.-C. Wong, and S. C. R. Elgin.** 1979. The chromatin structure of specific genes. II. Disruption of chromatin structure during gene activity. Cell **16**:807–814.
69. **Yu, L., R. J. LaPolla, and N. Davidson.** 1986. Mouse muscle nicotinic acetylcholine receptor γ subunit: cDNA sequence and gene expression. Nucleic Acids Res. **14**:3539–3555.
70. **Zaret, K. S., and K. R. Yamamoto.** 1984. Reversible and persistence changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. Cell **38**: 29–38.