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DNAase I sensitivity of genes expressed during myogenesis

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ABSTRACT

Cultures of a rat myogenic cell line were used to examine the question of whether in proliferating precursor cells genes which are programmed to be expressed later in development, in the same cell lineage, differ in DNAase I sensitivity from genes which are never expressed in these cells. Nuclei isolated from proliferating mononucleated myoblasts, differentiated cultures containing multinucleaged fibers, and rat brain, were treated with DNAase I. The sensitivity of the genes coding for the muscle-specific α-actin, myosin light chain 2 and the nonmuscle β-actin was measured by blot hybridization of nuclear DNA with the corresponding cloned cDNA and genomic DNA probes. The sensitivity of these genes was compared to that of a gene not expressed in the muscle tissue. The results showed that in the muscle precursor cells, the potentiality of tissue-specific genes to be expressed is not reflected in DNAase I sensitivity. The changes which render these genes preferentially sensitive to DNAase I take place during the transition to terminal differentiation. The results showed also that the region of DNAase I sensitivity of the α-actin gene in the differentiated cells ends between 40 to 700 bp 5' to the structural gene. No DNAase I hypersensitive site was detected 5' to the α-actin gene.

INTRODUCTION

Studies using a variety of cell types have shown that active genes are organized in chromatin in a conformation which renders them more susceptible to mild digestion with DNAase I than genes which are not active in the same tissue (1-5). Some findings suggested that preferential sensitivity to nuclease digestion may reflect the potentiality of genes to be expressed in a cell rather than their actual transcriptional activity (see Ref. 6 for review). A number of investigators have examined the question of whether in precursor cells genes which are programmed to be expressed later in development are arranged in an altered chromatin structure which renders them preferentially sensitive to nuclease digestion. Stalder et al. (7) addressed this question using embryonic chicken erythroid cells. They found that in primitive erythroblasts (present in the embryonic circulation at 2-5 days), producing only embryonic β-globin chains, both embryonic and adult β-globin genes are

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sensitive to DNAase I. However, in a recent publication, Groudine and Weintraub (8) tested the DNAase I sensitivity of globin genes in erythroid progenitor cells from an earlier stage (20-23 h embryos), which synthesize neither embryonic nor adult globin mRNA. They found that in those cells the whole β-globin gene complex is DNAase I insensitive. As was pointed out by the investigators, due to the nature of this system they could not prove unequivocally that the cells isolated from 20-23 h embryos were indeed the precursors of the globin-producing cells in which this gene was found to be preferentially DNAase I sensitive. Miller et al. (9) tested the DNAase I sensitivity of the globin gene in Friend erythroleukemia cell lines. In these cells the expression of the globin gene at the mRNA level increased as much as 45-fold following induction of terminal differentiation by DMSO. Yet, the globin gene sequences were preferentially sensitive to DNAaseI digestion even before the induction of differentiation. However, since these cells produced a small amount of globin mRNA even without DMSO treatment, the interpretation of the results is rather difficult.

In view of the possible significance of the changes in chromatin, which are reflected in DNAase sensitivity, it was of interest to test this question in another differentiating cell type. In the present study we examined the DNAase I sensitivity of genes expressed during myogenesis in cloned cell populations of a myogenic cell line. Mononucleated cells of this line can be kept in log phase for many generations in standard culture conditions. When the cultures are allowed to reach confluency, or as a response to switching to nutritional medium which stimulates cell fusion, these cells cease dividing and fuse into multinucleated fibers. Cell fusion is associated with the onset of synthesis of a number of muscle-specific proteins (10-16). Hybridization of mRNA extracted from differentiating cultures with cloned DNA probes specific for myosin light chain 2 or skeletal muscle actin mRNAs showed that the mRNAs for the proteins are undetectable in the proliferating mononucleated cells but accumulate rapidly during the process of cell fusion (17). Using the myogenic cell line, it is possible to test the DNAase I sensitivity of genes programmed to be expressed later in development, in the same cell lineage, and to determine at which stage of cellular differentiation the conformational change in the organization of the genes in chromatin occurs.

The investigation shows that in the proliferating myogenic cells the genes coding for the muscle-specific proteins α-actin and myosin light chain 2 are indistinguishable in their DNAase I sensitivity from genes which are not expressed in the muscle tissue. They become preferentially sensitive to

DNAase I during the transition of the cells to terminal differentiation.

MATERIALS AND METHODS

Cell cultures

Proliferating mononucleated cells from the rat myogenic line L8 (18), were grown in 15-cm plates in Waymouth medium supplemented with 10% fetal calf serum. When the cultures approached confluence the medium was changed to Eagle's medium supplemented with 2% horse serum and 0.1 µg/ml insulin. The phase of rapid cell fusion started 30 h after the change of medium. Myoblasts were harvested when the cultures approached confluence. Differentiated cultures, consisting mostly of multinucleated fibers, were harvested 100 h after the change of medium.

Preparation of nuclei

The method used to prepare nuclei from muscle cells was a modification of that described by Jacquet et al. (19). The nutritional medium was removed by aspiration and the culture plates were washed with ice-cold 0.15 M NaCl - 10 mM Tris, pH 7.6 and incubated for 10 min at 4^{o} in 10 mM HEPES-KOH, pH 7.6, 5 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA (hypotonic medium), 10 ml medium per plate. The cells were then scraped with a rubber policeman, collected and disrupted by 20 strokes in a Dounce homogenizer in the presence of 0.5% Nonidet P-40. Nuclei were collected after centrifugation for 5 min at 1000 xg, washed with hypotonic medium containing 25% glycerol and precipitated by centrifugation at 2000 xg for 10 min. The nuclei were suspended in the hypotonic medium and centrifuged through a cushion of hypotonic medium containing 25% glycerol.

Nuclei were also prepared from 1-month old Wistar rat brains. The brains were homogenized with a teflon-glass homogenizer in hypotonic medium containing 1% Nonidet P-40. Nuclei from the 1000 xg pellet were purified as described above.

All types of nuclei were suspended in the buffer described by Wu et al. (20) and washed several times by sedimentation and resuspension. The final preparations were examined under a phase contrast microscope for homogeneity and purity. Nuclei were used immediately after purification.

DNAase I digestion

DNAase I (E.C.3.1.4.5) was purchased from Worthington. All digestions were performed on intact nuclei (1 mg DNA/ml) at $37^{o}C$ for 10 min in the buffer described by Wu et al., (20). Aliquots were removed and the extent of DNA digestion was determined by perchloric acid precipitation as described by

Lohr et al., (21).

Preparation of DNA

DNA was extracted from treated and untreated nuclei as described by Bellard et al. (22).

Restriction enzyme digestion, separation of DNA fragments and blotting

Restriction enzymes were used as described by the manufacturers. All enzymes were purchased from Bio-Lab, except for ECoRI which was prepared as described by Greene et al., (23). The restricted DNA fragments in 10 mM Tris HCl, pH 7.4, 5 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% bromphenol blue were separated by electrophoresis on horizontal 1% agarose (Seakem) slab gels, using the Tris-acetate -EDTA buffer system of Hayward and Smith (24) supplemented with 0.5 µg/ml ethidium bromide. Fifteen micrograms of DNA were used per slot. After the run, the gels were photographed under UV illumination.

The DNA was transferred to nitrocellulose sheets according to Southern (25).

Cloned probes for myosin light chain 2, α- and β-actin, and immunoglobin genes

Plasmid 103 contains a cDNA insert of 274 bp which hybridizes specifically with skeletal muscle myosin light chain 2 mRNA (26,27); it has 213 nucleotides coding for the 71 amino acids at the C terminal end of the protein plus 61 nucleotides within the 3' untranslated region (Calvo et al., unpublished). Plasmid 749 contains a cDNA insert of 567 bp coding for amino acids 171 to 360 at the C terminal end of the skeletal muscle α-actin (27). In the standard hybridization conditions, it hybridizes with muscle as well as with nonmuscle actin sequences. Under very stringent conditions, it hybridizes specifically with the skeletal muscle actin genes (28). Plasmid 72 contains a cDNA insert of about 250 bp (29) hybridizing specifically to β-actin mRNA (28). Plasmid B1 contains a cDNA insert of 380 bp which hybridize specifically to the constant kappa chain region (C_K) of the immunoglobulin gene (I. Schechter, unpublished).

Preparation of ^{32}P DNA probes

All inserts were excised with PstI isolated on 1% agarose gels and electroeluted into dialysis bags. DNA was radiolabelled by nick-translation, as described by Maniatis et al (30).

Hybridization of restricted DNA with cloned probes

Filters were prehybridized for 2-4 h at 42° in a mixture of 50% deionized formamide, 5-fold strength SSC (1 SSC is 0.15M NaCl, 0.015 M trisodium citrate), 5-fold strength Denhardt reagent (31), 50 mM sodium phosphate buffer, pH 6.5, and 200 µg/ml sonicated denatured salmon sperm DNA (Sigma). Filters

were then hybridized overnight at 42° in the same medium supplemented with 10% sodium dextran sulfate (32) and with ^{32}P-labeled probes. Filters were then washed in 1 xSSC -0.1% SDS 65° (low stringency) or in 0.1 xSSC-0.1% SDS at 72° (high stringency). Labeled DNA fragments were detected by exposing the filters to x-ray film in the presence of an intensifying screen (Dupont).

RESULTS AND DISCUSSION

DNAase I sensitivity of myosin light chain 2 (MLC2) and actin genes

Using specific cloned probes for MLC2 (plasmic p103), α-actin (plasmid p749) and β-actin (plasmid p72), we examined the differentiation-related changes in the chromatin conformation of the genes specifying these proteins as revealed by their susceptibility to DNAase I. A probe for a rat immunoglobulin constant region gene (plasmid B1) was used as an internal control to measure the DNAase I sensitivity of a gene which is not expressed in the muscle tissue.

Nuclei were isolated from cultured proliferating myoblasts of the line L8. Parallel cultures from the same cell line were induced to differentiate by changing the nutritional medium (as described in Materials and Methods) and the nuclei were isolated 100 h later. At this stage, the great majority of the cells have fused into multinucleated fibers. Nuclei were also isolated from rat brain to provide information on the DNAase I sensitivity of muscle-specific genes in a non-muscle tissue. The isolated nuclei were incubated with increasing concentrations of DNAase I. The extent of DNA digestion was monitored by measuring the degree of DNA solubilization. The isolated DNA was digested with ECoR1. The fragments were separated by electrophoresis, transferred onto nitrocellulose sheets, hybridized with the radiolabeled DNA probes and exposed to x-ray films (Figs. 1 and 2). The autoradiograms were also scanned and the preferential sensitivity of the muscle-specific genes quantitated as described in Fig. 3. It was found that nuclei isolated from the different sources differed considerably in their sensitivity to DNAase I. Therefore, the DNAase I concentrations were matched to give comparable degrees of DNA digestion (Table 1).

As can be seen in Fig. 1, plasmid B1 hybridized to a single 6.8 kb DNA fragment and p103 hybridized to a 3.0 kb fragment. It is evident that the chromatin region containing the MLC2 gene is much more sensitive to the nuclease in the differentiated than in undifferentiated muscle cells (Figs. 1 and 3). At high DNAase I concentrations, the precursor cell DNA loses its capacity to hybridize to p103 and plasmid B1 in a similar manner (not shown),

Table 1. Extent of digestion of DNA in DNAase 1 treated nuclei.

Source of nuclei	Sample number	DNAase I units/mg DNA	Acid soluble DNA (%)
Mononucleated cells	1	0	0
	2	3.0	0.5
	3	6.0	1.2
	4	10.0	2.0
	5	15.0	2.7
	6	19.6	3.4
	7	22.0	3.8
Multinucleated fibers	1	0	0
	2	0.5	0.2
	3	1.3	0.5
	4	1.9	0.8
	5	2.6	1.1
	6	5.2	1.7
	7	10.5	3.2
	8	13.0	4.0
Brain	1	0	0
	2	0.8	0.2
	3	1.7	0.4
	4	5.0	0.6
	5	10.8	1.0

Nuclei isolated from cultures of proliferating myogenic cells, from differentiated cultures containing multinucleated fibers and from rat brain, were incubated for 10 min at 37° with increasing concentrations of DNAase I (1 mg DNA/ml). The degree of solubilization of the DNA was determined as described by Lohr et al. (21). The DNA extracted from the same DNAase I-treated nuclei was used in the experiments shown in Figs. 1-6.

thus indicating no preferential degradation of the MLC2 gene. For some unknown reasons, the MLC2 gene seemed to be mildly DNAase I sensitive in brain tissue (Fig. 3), although expression of this gene at the RNA level was undetectable in this tissue (26).

The DNAase I sensitivity of the α- and β-actin genes in nuclei of proliferating and differentiated cells was examined by the same method (Fig. 2). Blots containing the same DNA samples as those used in the experiments illustrated in Fig. 1 were hybridized to a single 5.7 kb ECoR1 fragment which has been shown to contain the α-actin gene (28); p72 hybridized to two ECoR1 fragments of 8.0 and 6.9 kb. The 8.0 kb fragment has been shown to contain the structural gene of the nonmuscle cytoplasmic β-actin (28). The identity of the gene contained in the 6.9 kb fragment is still unknown. It was found that the DNA fragment containing the cytoplasmic β-actin gene is extremely

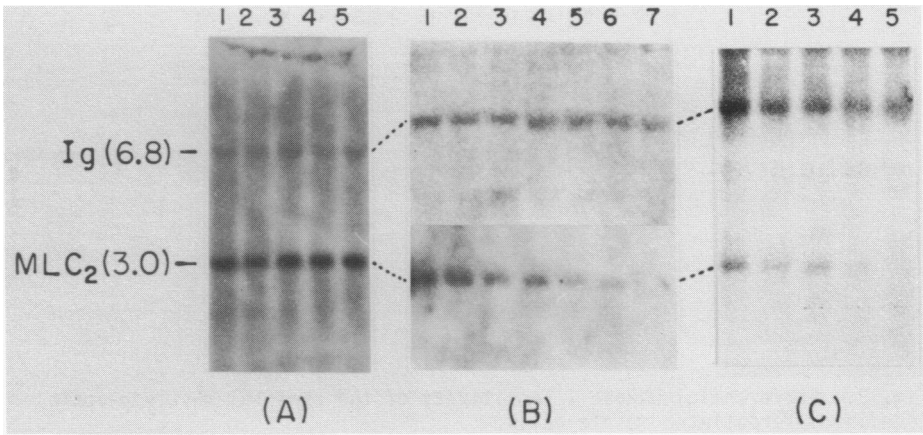

Fig. 1. Preferential DNAase I sensitivity of the myosin light chain 2 gene in differentiated muscle cells.
The DNA samples described in Table 1 were restricted with ECoR1, run on a 1% agarose gel, blotted and hybridized with the probe specific for sequences of an immunoglobulin gene (Ig) and for the myosin light chain 2 gene (MLC2). After hybridization, the blots were washed at high stringency and autoradiographed.
(A) Mononucleated cells; (B) differentiated cultures; (C) brain. The sample numbers are identical to those shown in Table 1. Numbers on the left indicate fragment size (kb). The blots in A and C were hybridized with a mixture of the 2 probes; blot B was hybridized with pB1 (Ig), washed in alkali and then hybridized with p103 (MLC_2).

sensitive to DNAase I in all three cell types (Fig. 2, A-C). However, the DNA fragment which hybridized to the skeletal muscle α-actin probe is DNAase I sensitive only in nuclei isolated from differentiated muscle cells (Fig. 2B). In the mononucleated myoblasts as well as in the brain, this DNA fragment is as stable as the immunoglobulin gene fragment (Figs. 1 and 3). The 6.9 kb fragment is DNAase I sensitive in all three cell types and may perhaps contain another nonmuscle actin gene.

In all experiments, the ECoR1 fragment containing the β-actin gene was more sensitive to DNAase I than the fragment containing the α-actin gene. This might be due to some intrinsic differences between the chromatin regions containing these genes, or to the fact that the β-actin gene is active in all cell types while the α-actin gene is active only in multinucleated fibers and not in the mononucleated cells which are always present in small amounts in these differentiated cultures.

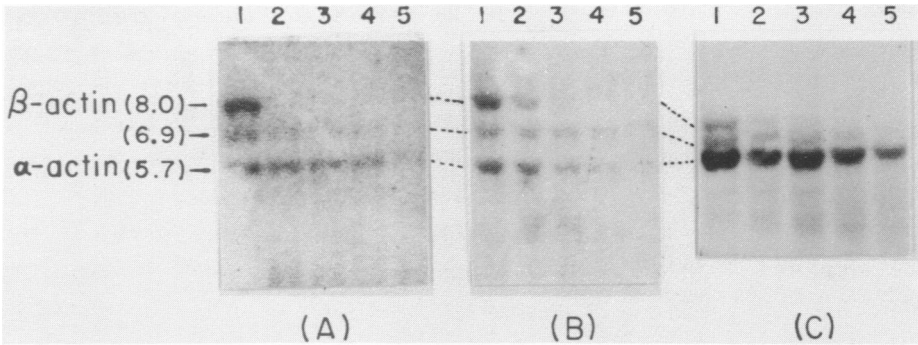

Fig. 2. Preferential DNAase I sensitivity of the skeletal muscle α-actin gene in differentiated muscle cells.
The same DNA preparations as in Fig. 1, restricted with ECoR1 and run on a 1% agarose gel, were blotted and hybridized with a mixture of probes specific for the β-actin gene (p72) and the α-actin gene (p749). After hybridization, the filters were washed at high stringency and autoradiographed. See also legend to Fig. 3.

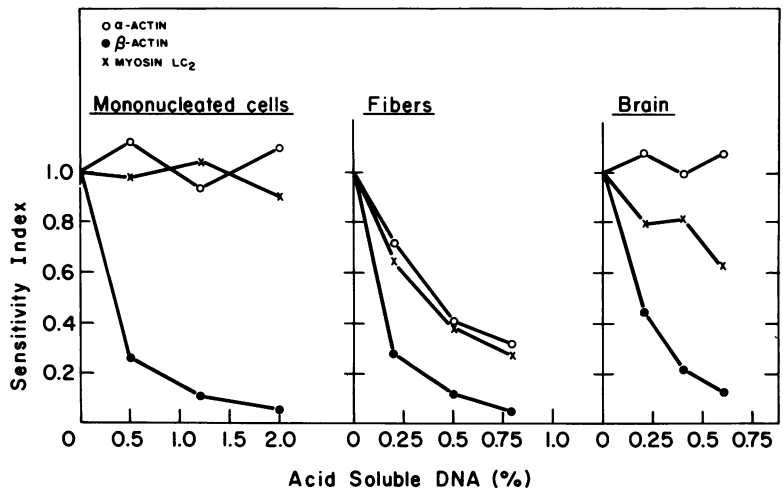

Fig. 3. Quantitation, by scanning of autoradiograms, of DNAase I sensitivity of the α-actin, β-actin and myosin light chain 2 genes.
Autoradiograms of blots of the same DNA samples as described in Table 1 were scanned in a Gilford 2400s spectrophotometer. The intensity of the bands was recorded at 590 nm. The DNAase I sensitivity index was determined by dividing the intensity of the bands corresponding to the fragments containing the α-actin, β-actin and myosin light chain 2 genes in each track by the value of the immunoglobin gene band in the same sample. The values are normalized; the ratios obtained for DNA from untreated nuclei = 1.

DNAase I insensitivity of the 5' flanking region of the skeletal muscle actin gene

In a number of genes it was found that in addition to the preferential sensitivity to DNAase I of the entire gene region, there exists a very defined small region hypersensitive to DNAase I. This site is usually located 5' to the structural gene (33-37 and reviewed in 38). By using the right combination of restriction enzymes and probes, it was possible to detect the appearance of a DNA fragment which was bordered by the DNAase I hypersensitive site and by a cut produced by the restriction enzyme. It has been suggested that this site is associated with the regulation of the activity of the gene. In the β-globin gene system, the hypersensitive sites were found only in cells which express the globin gene (38). The hypersensitive site 5' to the Drosophila heat shock protein gene was found also in cells which were not induced to synthesize these proteins (33). It was therefore of interest to test the DNAase I sensitivity of regions upstream to a gene expressed during myogenesis.

We have isolated from a rat genomic DNA library a recombinant lambda phage containing the skeletal muscle actin gene (28). Restriction mapping and DNA sequencing have shown that the insert contains the structural gene located at the 3' end, and 11 kb flanking the gene on its 5' side (Fig. 4). Fragments of this insert were used to probe the DNAase I sensitivity of the 5' region flanking the skeletal muscle actin gene.

Blots containing DNA isolated from DNAase-treated nuclei and digested with Hind III were hybridized to nick-translated fragment A (see Fig. 4). In agreement with the previous experiment (Fig. 2), it was found that the 10.4 kb Hind III fragment which contains the α-actin structural gene is DNAase I sensitive in differentiated muscle cells but not in proliferating myoblasts (Fig. 5). To define the limits of the region sensitive to DNAase I, DNA from DNAase I-treated nuclei isolated from differentiated cultures was digested with ECoRI. ECoRI cuts 0.7 kb upstream from the 5' end of the structural gene (Fig. 4). The 1 kb ECoRI fragment which hybridized to probe A was not preferentially sensitive to DNAase I in nuclei of differentiated cultures (Fig. 6). Essentially the same results were obtained using fragments B, C and D as probes (results not shown). These probes hybridize to fragments upstream from the ECoRI 1 kb fragment A. Thus, the region of preferential sensitivity does not extend beyond 0.7 kb 5' to the transcribed region of the α-actin gene.

To determine the DNAase I sensitivity of the region extending from

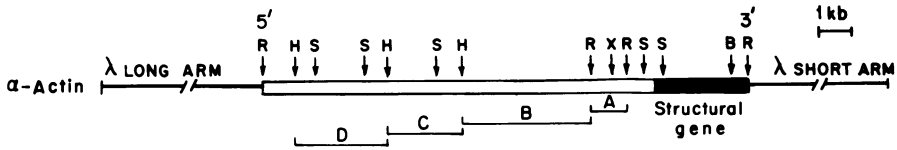

Fig. 4. Restriction sites in the insert of the recombinant phage Act. 15, containing the skeletal muscle α-actin gene and its 5' flanking region. Solid bar: the structural gene; open bar: flanking region, 5' of the α-actin gene. R: ECoR1: H: Hind III; S: SacI. The bars A, B, C, D mark the fragments used as hybridization probes. The ECoR1 site bordering the insert on the 3' end was constructed during the preparation of the library and is not present in the genome.

the 5' end of the structural gene to the ECoR1 site 0.7 kb upstream to it, we digested DNA from DNAase I-treated nuclei with the restriction enzyme Sac I. Restriction mapping (Fig. 4) and DNA sequencing data (Zakut et al., unpublished) showed that this enzyme cuts the DNA in a CG rich region 39 bp upstream from the CAP site and 6 bp 5' to a TATAAA sequence (Goldberg-Hogness

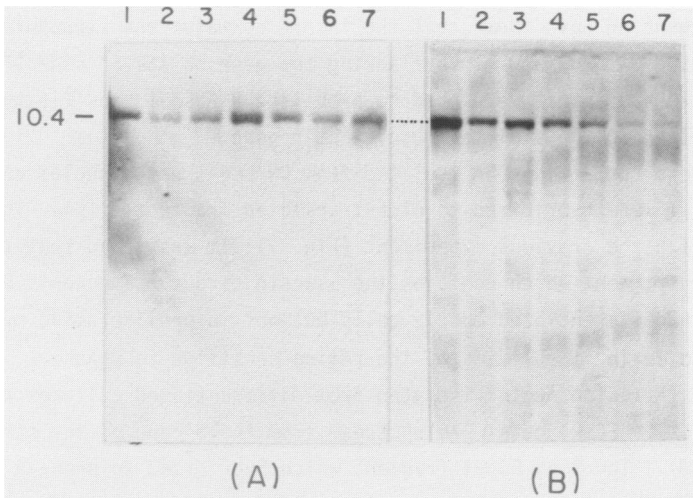

Fig. 5. DNAase I sensitivity of the Hind III 10.4 kb fragment containing the structural α-actin gene.
The fragment contains 6.2 kb flanking the actin gene on its 5' side and extend to a Hind III site 0.5 kb 3' to the constructed ECoR1 site in the 3' end of the insert of Act 15 (Fig. 4). DNA from DNAase I treated nuclei isolated from proliferating cells (A) and fibers (B) was restricted with Hind III and the fragments separated by electrophoresis on a 1% agarose gel. The blots were hybridized with the 1 kb fragment A described in Fig. 4. The samples were identical to those in Table 1.

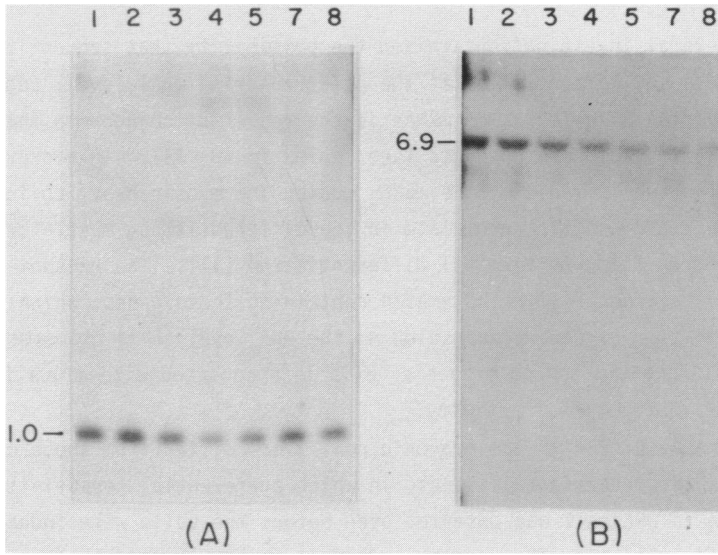

Fig. 6. The DNAase I sensitive region of the α-actin gene does not extend more than 0.7 kb 5' to the structural gene.
DNA from DNAase I treated nuclei isolated from differentiated cultures was digested with ECoR1 (A) or with Sac I (B). After electrophoresis on a 1% agarose gel and blotting, the DNA was hybridized with fragment A. DNA samples were the same as used in Table 1.

box; 39). As can be seen in Fig. 6B, the hybridization with probe A produced a 6.9 kb fragment which is DNAase I sensitive in differentiated cells. Thus the DNAase I sensitive region ends between 40 - 700 bp upstream from the structural gene. It should be mentioned that in the β-globin gene, the hypersensitive site extends from 60 to 260 base pairs upstream to the structural gene (38). The absence of a new band, produced by DNAase I treatment, in all the experiments described above indicates the lack of a defined sharp border to the DNAase I sensitive region and the absence of a hypersensitive site 5' to the α-actin gene. If a sharp border of the sensitive region, or a defined hypersensitive site existed 5' to the genes, probe A, B, C or D would have lit up a DNA fragment formed by the DNAase I cut and the restriction enzyme cut 5' to it.

The fact that the DNAase I sensitivity region ends close to the 5' end of the α-actin gene may perhaps indicate the absence of other genes expressed in muscle, in close proximity 5' to the α-actin gene.

Conclusions

Several investigations raised the possibility that sequential expression of genes may be controlled at the posttranscriptional level, and that the accumulation of cytoplasmic mRNAs is the result of changes in the processing or stability of gene products (see review in 40, 41). In a previous investigation, we showed that the mRNAs coding for myosin heavy chain, myosin light chain 2 and α-actin accumulate in the differentiating muscle cells during the transition to terminal differentiation (17). The present investigation shows a change in the chromatin containing these genes, which happens close to the time of their expression on the RNA level. This indicates that terminal differentiation of myogenic cells is associated with a qualitative change in transcriptional activity.

The results with the myogenic cell lines differ from those obtained with the erythroleukemia cell lines, in which preferential sensitivity of the globin gene to DNAase I was observed even before the cells were induced by DMSO to differentiate. They are in principle in agreement with those reported by Groudine and Weintraub (8) for the embryonic erythroid progenitor cells. As mentioned in the introduction, in an earlier study Stalder et al. (7) tested the DNAase I sensitivity of globin genes in embryonic red blood cells which produced embryonic β-globin but not adult β-globin. In these cells, both the embryonic and the adult globin genes were DNAase I sensitive. The sensitivity of the unexpressed globin gene might have been due to its close proximity to the active embryonic globin gene (34). Alternatively, it may indicate the existence of a transient state of differentiation, which was not detected in the muscle cell system, in which the genes, although not phenotypically expressed are already DNAase I sensitive.

In the muscle cell culture system, cell fusion was induced by a change in the composition of the nutritional medium. As a response to this treatment, most cells complete one division and fuse (42). Therefore, the change in DNAase I sensitivity must occur in this system within the last cell cycle preceding fusion. It would be of interest to determine whether within this period the change in DNAase I sensitivity coincides with or shortly precedes the expression of the genes at the RNA level. The fact that in proliferating myogenic cells, genes which are programmed to be expressed during terminal differentiation are indistinguishable in their DNAase I sensitivity from genes which are not expressed in this cell lineage indicates that conformational changes of these genes, which are reflected in the preferential sensitivity to DNAase I, are not associated with the stable maintenance of the

latent program of gene expression. This, however, does not rule out the possibility that other differences, not manifested in the DNAase I sensitivity, do exist. It is, of course, also possible that other genes which control the activation of the genes involved in the phenotypic expression of myogenesis, do become DNAase I sensitive much earlier in development.

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