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α ACTIN GENE EXIST IN AN ACTIVE STRUCTURAL CONFIGURATION IN THE PROLIFERATING MYOBLASTS AS WELL AS IN DIFFERENTIATED MYOTUBES OF THE L6 LINE

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SUMMARY : Single-stranded DNA (ssDNA) mainly consisting of transcription sites was probed with cDNA actin clones for studying the expression of actin specific genes during myogenesis in the L6 line of rat myogenic cells. As compared with total nuclear DNA, ssDNA from myoblasts and myotubes was found greatly enriched in sequences complementary to both muscular and non muscular actin sequences. In contrast, ssDNA from spleen, hepatocytes or hepatoma cells was found enriched only in sequences complementary to non muscle actin cDNA. Actin specific sequences accumulated in the ssDNA fraction are almost entirely derived from the coding DNA strand. The DNAase I sensitivity of the actin genes sequences in myogenic and non myogenic cells correlated the data obtained with the ssDNA fraction. It is concluded that the muscle specific actin genes is either transcriptionally active or at least exist in an active configuration in the proliferating myoblasts as well as in the terminally differentiated myotubes.

INTRODUCTION : The L6 line of rat myogenic cells (1) provides a model for investigating the differentiation of skeletal muscle under standard conditions of tissue culture. Stepwise changes in the mRNA populations (2) and in the synthesis of muscle proteins (3) culminate in the formation of myotubes (4). One of the well studied proteins is the muscle specific α actin which differs from isoform polypeptides that will be designated as non muscle specific which are cytoskeleton constituents (5). All those actins are encoded by the same multigenic family (6). Myogenesis appearts to involve the switching on of α actin genes (7) but it is not known whether these genes are already activated in proliferating myoblasts or become activated at a latter stages of the differentiation process. Previous studies have shown that a minor single-stranded DNA, component (ssDNA) mainly originating from active chromatin regions, amounting to 1-2% of the total nuclear DNA, could be reproducibly isolated with the use of improved method of hydroxylapatite chromatography. Up to 80%

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of ssDNA consist of non self-reassociating sequences, a major part of which can be hybridized to homologous mRNAs and most of which can be hybridized to non repetitious nuclear DNA. It was further shown that a great part of the DNA chains complementary to ssDNA sequences, consisted of single-stranded stretches, attached to the double-stranded molecules of nuclear DNA (8). Moreover, changes in the mRNA populations that were found to occur during the differentiation of L6-cell (2) were correlated with changes in the ssDNA fractions (9). In the present work we have used ssDNA as a tool for comparing the expression of muscle-specific and non muscle-specific actin genes in proliferatif L6-myoblasts, in terminally differentiated L6-myotubes and non-muscular rat cells. The results are confronted with those obtained by treating purified cell nuclei with DNAase I which is known to degrade preferentially active gene in situ.

MATERIAL AND METHODS

Cell cultures : The myogenic L6-cells were cultured as previously described and collected either at the stage of proliferating, or at the stage of terminal differentiation when the great majority of cells form multinucleated myotubes (10). Cultured hepatocytes from young adult rats were kindly provided by Dr Gueguen-Guillouzo.

Isolation of ssDNA : Total nuclear DNA was purified by the previously described conventional procedure and ssDNA was isolated by the improved method of hydroxylapatite chromatography using phosphate buffer pH 7.85 at 56°C (8).

Cloned probes for the α and β actin gene and β globin gene : The actin specific probes obtained from Dr A.J Minty (11) consisted of two Pst I recombinant cDNA inserts integrated into pAM 91 plasmids. The first insert, 1150 bp long corresponds to a coding sequence of the α actin gene. Under standard annealing conditions it is hybridizable to both muscle-specific and non muscle-specific actin sequences. The second insert 185 bp long, which corresponds to the non coding 3' sequence of the muscle specific α actin gene, has been recloned in the PBR 322 plasmid(p91-1). The β globin probe (obtained from Dr P. Leder) is a 1.6 Kb EcoRI-Bam H1 fragment from λ gt-WES-M β G2 which has been subcloned in PBR 322.After restriction endonuclease digestion the inserts were purified by agarose gel electrophoresis and electroelution, they were labelled by nick-translation with ^{32}P d-CTP (800 Ci/mM) and ^{32}P d-TTP (800Ci/mM) to specific radioactivities of $2\text{-}3\times10^8$ cts/min/ug.

Hybridization in liquid : Hybridization were performed either as described previously (9) or at 37°C in 0.4 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.4, 50% formamide, because the latter medium was found better for the 185bp insert. The amounts hybridized were estimated by the use of S1 nuclease.

Isolation of non-muscle-specific actin sequences that encode poly(A) rich-mRNAs : One samples of the ^{32}P-labelled 1150 bp-insert of 2.10^6 cpm (about 0.01 µg) was allowed to hybridize with 15,000 fold greater amount of poly(A) rich-mRNA from L6 myoblasts until a R_0t value of 50 was attained. The solution was treated with ribonuclease A (25 µg/ml) and T1 (10 units/ml) for 30 min at 37°C, brought to 1% sarcosyl, mixed with an equal volume of satured Cs_2SO_4 to yield a starting density of 1.5g/cm^3 and centrifuged at 39,000 rev/min for 65 h at 20°C in a Ti 50 Beckman rotor. Fractions were collected automatically and aliquots were assayed for acid-precipitable radioactivity. The fractions corresponding to the RNA-DNA hybrids were pooled, treated with 0.3 N NaOH for 1 h at 65°C, neutralized and dialyzed against 1 mM Tris-HCl pH 7.4, 0.1 mM EDTA overnight at 4°C.

Nuclease digestion, blotting, hybridizations : Cell nuclei, were purified in the presence of protease inhibitors as previously described (12), then treated with DNAase I, and the extent of digestion was estimated by measuring the percentage of DNA rendered acid-soluble, following the usual procedures (13). Control samples were incubated under the same conditions with absence of enzyme.

Following nuclease treatment, DNA was purified, digested with restriction endonuclease(s) and analyzed by the method of Southern (14). Electrophoresis was performed on horizontal 0,8% agarose gels in 1mM EDTA, 40 mM Tris-acetate buffer pH 7.6. The gel was stained with ethidium bromide and visualized under UV light to check for complete digestion. Blots were prehybridized for 2-3 h at 42°C in 20-50 ml of 5 x SSC, 50 mM sodium phosphate pH 7.0, containing 50% deionized formamide, 0.1% SDS, 5 x Denhardt medium (0.1% bovine serum albumine fraction V, 0.1% polyvinyl pyrolidone-360, Ficoll 400 and 100 µg/ml of denatured, sonicated Salmon sperm DNA. Dot blots were monitored following the procedure of Thomas (15). Hybridizations were performed for 16-24 h under the same conditions with 0.1-0.2 µg of highly labelled nick-translated probes. Blots were then washed for 3 x 15 min with 250 ml of 2 x SSC, 0.5% SDS with gentle shaking at room temperature, and then were given a final wash in 0.1 x SSC, 0.1% SDS at the temperatures indicated in the legends to figures.

For Northern blot hybridizations cytoplasmic RNA from myogenic and non myogenic cells was purified as described (2), and analyzed as described by Thomas (15).

RESULTS

Specific enrichment of the ssDNA fraction in transcribing actin-gene sequences . The characteristics of ssDNA isolated from L6 myoblasts or L6 myotubes have been previously described (9). Aliquots of the muscle-specific 185 bp actin probe and non muscle-specific 1150 bp-actin probe, highly-labeled with ^{32}P, were annealed in liquid to the bulk DNA or ssDNA from proliferating myoblasts, terminally differentiated myotubes or non muscular rat cells (normal spleen, normal hepatocytes and Zajdela ascites-hepatoma cells). In all assays, results comparable to those shown in fig 1 were obtained. At least 90% of the 1150 bp insert and 70-75% of the 185 bp insert could be hybridized to high excess amount of bulk DNA with respective $C_o t\ \frac{1}{2}$ values of about 300 and 3,000 as compared with about 5,000 for the self reassociated bulk DNA. These results suggested that the diploid rat genome contains 15-16 copies of actin-related genes of which 1-2 copies represent muscle-specific α actin genes. This was confirmed by preliminary results of Southern blotting hybridizations (not shown)

Using low excess amounts of DNA no more than 15-20% of both actin specific inserts could be hybridized to the bulk DNA whereas $C_o t$ saturation plateaus, corresponding to 55-58% hybridization for the 1150 bp-insert and 45 - 50% for the 185 bp-insert, were obtained.

Futhermore, the approximate $C_o t\ \frac{1}{2}$ values indicated that the ssDNA fraction appears to be 40-50 fold enriched in actin gene sequences as compared with the bulk DNA. The saturation levels close to 50% suggested than onlyone DNA trand of both actin specific probes, can be hybridized to ssDNA, pre-

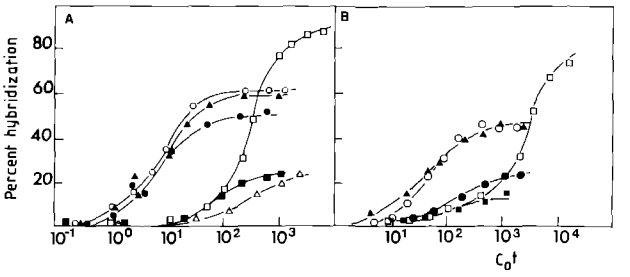


Fig 1 Hybridization of the non muscle-specific (1A) 1150 bp insert and the muscle-specific (1B) 185 bp insert with ssDNA and bulk DNA. Aliquots of about 25,000 cts/min of the 1150 bp insert (Fig 1A) and of the 185 bp insert (Fig 1B) were annealed with high excess ($8x10^6$ fold) of total nuclear DNA (nDNA) from myoblast (□——□) or with a low excess ($3.2x10^5$ fold) of myoblast nDNA (■——■)myoblast-ssDNA (○——○), myotube-ssDNA (▲——▲) or hepatocytes ssDNA (●——●). The fraction of the 1150 bp insert, consisting of sequences that encode cytoplasmic mRNAs was isolated from 1150 bp DNA-myoblast RNA hybrids as described in the text. Aliquots of about 25,000 cts/min were further hybridized to 10^6-fold excess amounts of myoblast-ssDNA (△——△). In all assays, control radioactive samples were incubated in the presence of equivalent yeast tRNA excess. At each indicated C_0t values (Moles-sec/liter) the percentages hybridized were determined by treating with S1 nuclease and substracting the control figures wich attained a maximum of 8-10% at C_0t 10^4.

sumably the anti-coding strand, since coding DNA sequences accumulate into ssDNA (see 8, and references quoted therein). This was confirmed, using the fraction of the 1150 bp insert, prehybridized to L6-myoblast RNAs. Only low percentages of this fraction could be reassociated with ssDNA whereas the largest part was hybridizable to the bulk DNA.

As shown in fig 1B only low percentages of the muscle-specific 185 bp insert could be hybridized to ssDNA from non-myogenic cells, whereas equivalent percentages of the non-muscle specific 1150 bp insert were hybridized to the bulk DNA and to the ssDNA from both myogenic and non-myogenic cells. When comparing the terminal melting curves of the hybrids (determined as previously described in low ionic strength 0.1 x SSC) the same Tm values of about 66°C were obtained with all hybrids involving the 1150 bp-insert. The Tm values were of 57°C for hybrids between the 185 bp-insert (probably because of its relatively lond dA-dT tails) and either bulk DNA from muscular and non-muscular cells or ssDNA from myoblasts or myotubes. In contrast the Tm values were reduced to about 51°C for hybrids between 185 bp-insert and ssDNA from spleen cells, normal hepatocytes or hepatoma cells. This confirms that the ssDNA from non-myogenic cells contained few sequences, remotely related if at all, to muscle-specific actin genes.

Effects of DNAase I

A large body of evidence indicates that in purified nuclei the active genes are much more sensitive than the silent genes to different nucleases,

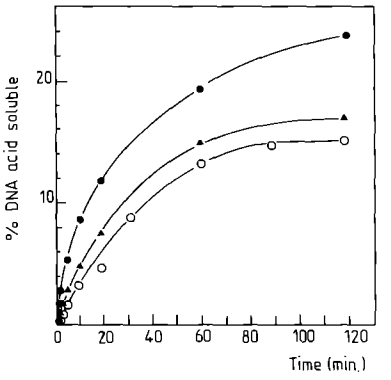


Fig 2 Kinetics of DNAase I digestion in nuclei from myogenic and hepatic cells. Nuclei from proliferating L6-myoblasts (O——O), myotubes (▲——▲) or hepatocytes (●——●) were incubated at 37°C for increasing times with DNAase I (15µg per mg DNA). At each indicated time the percentage of DNA rendered acid-soluble was measured in aliquots. The same DNA preparations were utilized in all experiments illustrated by Fig 3,5.

in particular DNAase I (16,17 and references quoted therein). We used the latter enzyme to confirm the results obtained with ssDNA.

In first series of assays we have compared the effects of DNAase I, in nuclei from L6-myoblasts, L6-myotubes and normal hepatocytes in culture, by measuring the fraction of DNA rendered acid-soluble in the course of time. As shown in Fig 2, liver-cell nuclei were markedly more sensitive to DNAase I than L6-myoblast nuclei. These differences were taken into account for normalizing the experimental conditions.

Aliquots of DNA from DNAase I-treated or untreated nuclei were digested at completion with EcoRI and analyzed by Southern blot hybridizations with the actin-specific probe : 1150 bp long which corresponds to the coding region of α actin gene but contain sequences shared with the cytoskeleton β actin genes (11,18). The β globin-specific 1,600 bp-insert was used as internal control. We had previously verified that L6-cells and hepatocytes contained no detectable amount of RNA hybridizable to this probe but the use of Northern blots (data not shown). The Southern blots obtained with untreated nuclei from L6-myoblasts, L6-myotubes and hepatocytes exhibited identical autoradiographic patterns but the number of autoradiographic bands depended on the washing conditions applied. After low-stringent washing (0.1 x SSC, 45°C), 20-25 distinct EcoRI fragments were hybridized to the 1150 bp-insert, including the 5.7 Kb and 8 Kb fragments corresponding to α actin gene and β actin gene respectively (19,20) (data not shown). After medium stringent washing (0.1 x SSC, 65°C) the 5.7 Kb and 8 Kb fragments still represented major radioactive bands (Fig 3) and were the only ones that remained significantly labelled after high-stringent washing (0.1 SSC, 75°C). As shown in Fig 3A the 8 Kb fragment was progressively degraded at comparable rates by DNAase I in nuclei from the 3 cell types. In

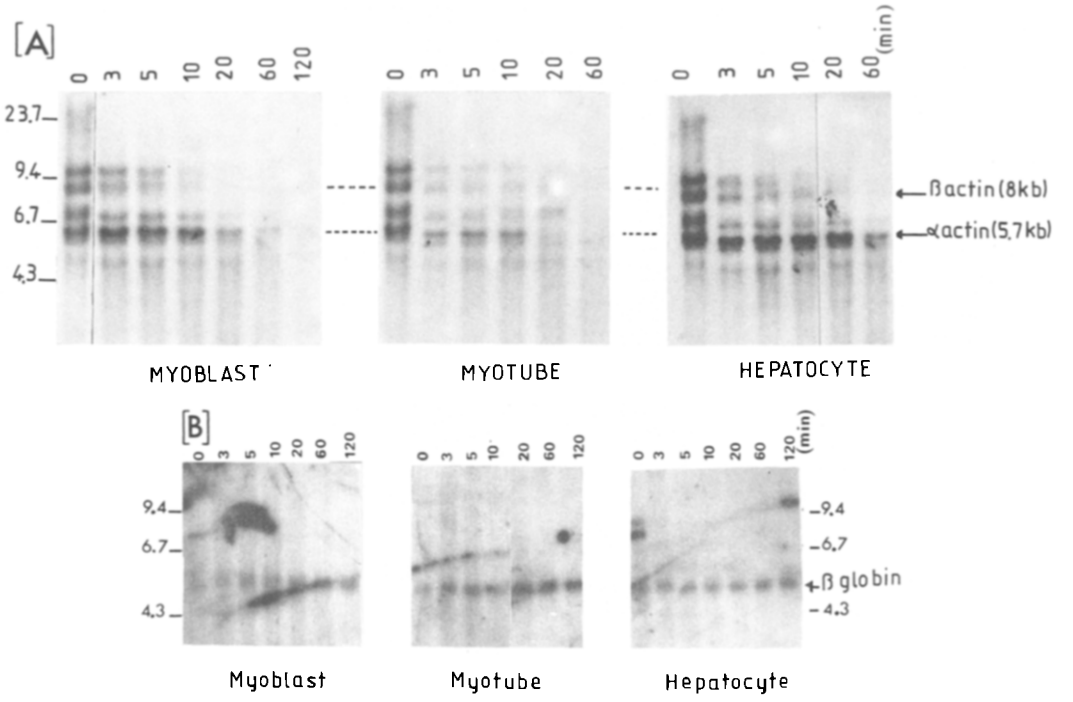
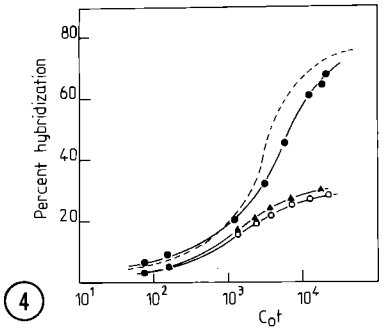


Fig 3 Southern blots showing the effects of DNAase I on the muscle-specific and non muscle-specific actin gene sequences in nuclei from L6-myoblasts, L6-myotubes and hepatocytes. Duplicate samples (10µg) of DNA from untreated nuclei (lane 0) or from nuclei treated with DNAase I for the indicated periods of time were hybridized with the radiolabelled 1150 bp insert (panel A) or with the β globin insert (panel B) as described in the text. Thereafter the blots were washed in 0.1xSSC at 65°C and autoradiographed for 2 days. Hin III λ DNA fragments were included in each gel as molecular size markers (in Kb).

contrats the 5.7 Kb fragment resisted to DNAase I in liver-cell nuclei (even after digestion of at least 20% of the total nuclear DNA) but was similarly attacked by this enzyme in nuclei from both L6-myoblasts and L6-myotubes in which it became hardly detectable after digestion of 4-5% of the nuclear DNA , as compared with 2-2,5% for the 8 Kb fragment. The latter difference indicates that the non-muscle specific actin gene is more sensitive than the muscle actin gene to the effects of DNAase in nuclei from myogenic cells. The control Southern blots (Fig 3B) obtained with the 1,600 bp-insert clearly demonstrated that the globin genes were totally resistant to DNAase in the 3 cell types , even after digestion of 15-20% of the total nuclear DNA. The finding that α actin gene is similarly accessible to DNAase I in committed stem cells, still in the process of multiplication and in terminally differentiated muscle fibers was confirmed by the results of various assays which were performed with the 185 bp-insert : Southern blot hybridization (data not shown similar to those ob tained with the 1150 bp probe hybridized to the 5.7 Kb EcoRI fragment of



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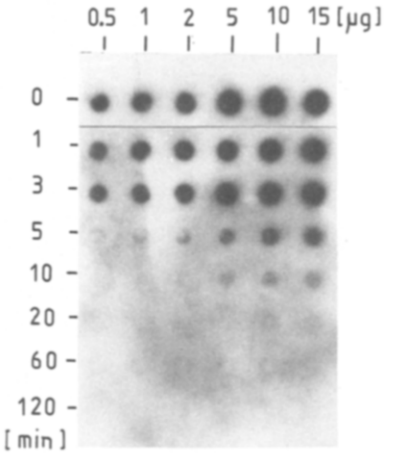

⑤ 185 bp probe

Fig 4 Hybridization kinetics in liquid medium of the 185 bp insert specific for the α actin of skeletal muscle with total DNA from untreated nuclei and from DNAase I treated nuclei. Nuclei from proliferating myoblasts (O—O) myotubes (▲——▲) and hepatocytes (●——●) were treated with DNAase I until 4-5% of the DNA was rendered acid-soluble (see Fig 2). Aliquots of about 25,000 cts/min of the 185 bp probe were annealed with high excess ($8x10^6$ fold) of DNA. At each indicated C_0t value the percentage hybridized was determined as described in Fig 1. The dashed line shows hybridization with DNA from untreated myoblast nuclei (Fig 1B).

Fig 5 Dot blot hybridizations showing the degradation with DNAase I of muscle-specific α actin gene in L6-myoblast nuclei. Samples of DNA from myoblast-nuclei pretreated or not with DNAase I for increasing periods of time were purified and spotted onto nitro cellulose paper. Various amounts of each DNA preparation were hybridized with the muscle-specific 185 bp insert. The blots were washed in 0.1xSSC at 65° and autoradiographed for 2 days. In control assays (data not shown) duplicate DNA samples were hybridized with the globin specific probe. No effect of DNAase I treatment was observed.

cellular DNA), hybridzation in liquid medium to high DNA excess (Fig 4) or dot blot hybridization (Fig 5).

DISCUSSION

The results obtained with the ssDNA from L6-myoblasts and L6-myotubes confirm and extend our previous data showing that in all the cell types studied, this DNA subfraction was mainly derived from the coding DNA strand of transcription-active chromatin regions (see 8 and references quoted therein). The significance of this finding was discussed in light of various other data showing that DNA helix openings, ranging in size from 70 to 2000 bp only occur in transcribed euchromatin regions (21) and that active genes are muche more sensitive than silent genes to the effects of exogenous nucleases purified cell nuclei (22,23). In particular the micrococcal nuclease liberates nucleosome oligomers that are greatly enriched in transcribed DNA sequences (17, 24 ,25) and we have previously found a great extent of homology between the oligomeric

fragments obtained from micrococcal nuclease treated nuclei, and the ssDNA isolated from the untreated cell nuclei (25). We also demonstrated that the single-strand-specific S1 nuclease, mimics the effects of micrococcal nuclease in purifed cell nuclei (13) . Furthermore it was recently shown that the transcription-active DNA is preferentially nicked by endogenous nuclease(s) in animal cell nuclei incubated in the absence of exogenous enzymes(26,27). All these evidences supported the hypothesis that the formation of ssDNA results of a selective enzymatic breakage which probably occurs at an early stage of the DNA preparation procedure. However, it was reported that a mammalian DNA unwinding protein which(at least in vitro) operates as a nicking-closing enzyme, is selectively located in transcription-active chromosomal regions (28). This led us to suggest that the formation of ssDNA could be initiated during cell life, through selective nicks probably related to the transcriptional process, and thereafter completed by artificial breakage (8). This hypothesis is indirectly supported by the recent isolation,of a minor nucleoprotein com-plex from L6-cell nuclei, mainly consisting of transcribed sequences, a large part of which are single-stranded and tightly bound to a small number of non-histone protein species (29).

Whatever its formation mechanism, ssDNA provides a relaible tool for evaluation the activation of specific genes. As shown above the ssDNA from both L6-myoblasts and L6-myotubes was found 40-50 fold enriched in sequences specific for the α actin genes, as compared with the total nuclear DNA,while the ssDNA from spleen or liver-cells contained practically no α actin gene . These results, consistent with those obtained with the use of DNAase I, support the conclusion that muscle-specific actin gene becomes activated in muscular stem cell at a stage preceding the appearence of differentiated functions and the synthesis of specific proteins. This seems to contradict a previous report which claimed that α actin gene was not sensitive to DNAaseI in L8-myoblasts another line of rat myogenic cells (20). It is possible that this contradiction simply reflects different developmental stages of L6 and L8 cell lines. Using Northern blot hybridization we found no detectable amount of α actin-related RNA species in L6-myoblasts. The possibility that the actin genes were nonetheless transcribed but their transcripts were undetectable, because of a fast degradation, cannot excluded. Another possibility is that an altered chromosomal configuration which renders specific genes more accessible to nucleases, precedes the actual expression of these genes and may, in fact, be required before this expression can occur (17). This could be invoked to explain how globin genes are sensitive to DNAase I in non differentiated Friend erythroleukemia cells (30) and how the same is true for the adult-globin genes in embryonic erythroblastoïd cells (16,31). Finally the present work provides new clearcut evidence supporting the concept that

cellular genes which are programmed to be expressed during myogenesis do become structurally activated at early stages in the differentiation programms (2,32,33).

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