Inhibition of Myogenesis in Mouse C2 Cells by Double-stranded Phosphorothioate Oligodeoxynucleotides containing mef-1 Sequence*

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Phosphorothioate oligonucleotides containing the muscle creatinine kinase enhancer sequence (mef-1) and a mutant of the enhancer sequence (mmef-1) were tested for their ability to block muscle differentiation in mouse C2 cells in culture. Maximum inhibition of fusion of myoblasts was observed at 10 μM concentration of mef-1 oligomer. No appreciable inhibition of fusion was observed with the mmef-1 oligomer at the same concentration was observed. Synthesis of myogenin, muscle creatinine kinase, and myosin heavy chain polypeptides were reduced in mef-1 oligomer-treated cells. In contrast, no significant reduction in the synthesis of these polypeptides in mmef-1-treated cells was detected. The overall protein synthesis was not affected. These results suggest that muscle differentiation may be disrupted by competition of the oligomer with the endogenous promoter for specific transcription factor(s).

Analysis of the role of transcription factors is important for examining the molecular basis of cellular differentiation. Alteration of the normal function of transcription factors is essential for the elucidation of their in vivo role in cellular differentiation. Inactivation of the gene for a transcription factor by homologous recombination is one approach to examine its function (1). An alternative approach is to utilize enhancer/promoter competition for transcription factor binding by introducing a high copy number of the enhancer sequence. This can be achieved by integrating multiple copies of this sequence in the genome of the target cell by transfection (1, 2). This approach could produce inviable cells, and the high copy number of the plasmid may not be universally maintained (3). Promoter/enhancer competition by oligonucleotide decay provides a simple alternative method to examine the in vivo role of the target transcription factor. Oligonucleotides with phosphodiester backbone modifications, such as methyl phosphonate, phosphoramidate, or phosphorothioate, can be synthesized in large quantities and show increased resistance to nuclease digestion (3). Therefore, the double-stranded (ds) oligomer with modified backbone may be used for an extended period to block the activity of the target transcription factor. It has been shown in vitro that a ds modified oligonucleotide containing the binding sequence of the transcription factor can specifically compete with DNA for binding (4, 5). Studies in vivo have also shown that these modified oligonucleotides accumulate within the cell and can alter target gene expression in a specific manner by competing for binding of transcription factors (6, 7). Conversion of proliferating myoblasts to terminally differentiated myotubes in culture during myogenesis is a good model to study the role of specific transcription factors in cellular differentiation. Several transcription factors, which include MyoD, myogenin, myf-5, and MRF-4 (also known as myf-6 and herculin), are involved in myogenesis (8–10). These factors all show homology in a sequence that has a basic region, that binds DNA, and a helix-loop-helix motif that accomplishes dimerization, and therefore belong to a family of proteins called the basic helix-loop-helix family (11–14). It is not precisely known how the MyoD family of factors turns on all of the muscle proteins required for terminal differentiation, but the binding of these factors to variants of the consensus sequence CANNTG, called an E-box, has been shown to be important for muscle-specific gene expression (1, 7). It has also been shown that many muscle proteins have these transcription factor binding consensus sequences in their enhancers. One such gene that has been well characterized is the muscle creatinine kinase gene of mice. In the upstream region of this gene, multiple E-boxes have been found (8).

It has been shown that MyoD and myogenin are both necessary and sufficient for myogenesis (9). The basis of our work is to try to alter gene expression by competing for binding of specific transcription factors (namely MyoD and myogenin) in vivo and thereby block terminal differentiation of C2C12 mouse muscle cells in culture. We have used one of the E-box sequences found in the muscle creatinine kinase enhancer, designated mef-1 (10), which has been shown to bind both MyoD and myogenin, to synthesize a 25-base pair ds oligonucleotide with a fully phosphorothioate-modified backbone (PS oligomer) that included the mef-1 sequence (Fig. 1A).

EXPERIMENTAL PROCEDURES

Oligomer Treatment—Differentially proliferating myoblasts were treated with the oligomer. Once the proliferating cells were 50–60% confluent, the culture medium was changed from growth medium (Dulbecco's modified Eagle's medium and 10% fetal bovine serum) to low mitogen differentiation medium (Dulbecco's modified Eagle's medium and 10% horse serum). The ds oligomer was added at concentrations between 5 and 10 μM. Control cells received no oligomer. The treatment lasted for 6 days, with a change of medium and oligomer every 2 days. The reversed treatment consisted of the 6-day oligomer treatment, followed by a 4-day recovery in differentiation medium lacking oligomer.

Oligomer Uptake—Oligomers were 5'-end labeled using γ-32P ATP and T4 polynucleotide kinase. Uptake was determined by incubating labeled oligomer (1 × 107 cpm) at a concentration of 5 μM for the times indicated (Fig. 1). The cells were lysed, ds oligomer was purified by phenol-chloroform extraction, and the presence of radioactivity in the aqueous phase was measured (15).

Microscopy of Cells—Confocal microscopy was done on the Bio-Rad MRC-600 laser scanning confocal microscope using a × 60 objective. An average of five optical slices was taken and Kalman filtered. Images were merged with Nexus (Bio-Rad), and false color was added with
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RESULTS AND DISCUSSION

To examine how efficiently ds oligomers are transported in differentiating myoblasts, cells were treated with 5 μM of either PS or phosphodiester (PO) oligomers for various times. Results show that the PS oligomer accumulates in cells by passive diffusion better than a unmodified ds oligomer (PO) of the same length and sequence (Fig. 1B). Uptake of the ds oligomer is similar to that of single-stranded oligomers (data not shown). The increase in the cellular uptake over time of the PS oligomer compared to the PO oligomer could be attributed to its improved intracellular stability. To confirm the cellular uptake of the ds oligomer, a rhodamine-tagged deoxyuridine was incorporated in the oligomer during synthesis (Oligos Etc., Wilsonville, OR) and after uptake, the cells were examined with a confocal microscope (Fig. 2). It can be observed that although fluorescent-labeled oligomer was detected in the nucleus, most of the oligomer was located in the cytoplasm of treated cells (Fig. 2, A and B). It is difficult to judge the potential effectiveness of the oligomer from this distribution pattern, but since the transcription factors are being made in the cytoplasm, it may be possible that the oligomer could interfere with their localization to the nucleus as well as their binding pattern in the nucleus. The distribution pattern was studied at various time points, and there seems to be no significant change in distribution pattern between a 12-h (Fig. 2A) and a 4-day (Fig. 2B) oligomer treatment.

It has been reported previously that ds oligonucleotides that had a fully PS-modified backbone show high levels of nonspecific binding to proteins (21). Therefore, to examine whether the PS oligomer binds to the same factors as the PO oligomer, without significant nonspecific interactions, a series of electrophoretic mobility shift assays were performed. Results (Fig. 3) show that PO oligomer produced one myotube-specific band (Fig. 3, arrow). Formation of this nucleoprotein complex was competed equally by both unlabeled PO and PS oligomer with mef-1 sequence (Fig. 3, lanes 3–6). Studies using radiolabeled PO oligomer showed formation of similar complexes (Fig. 3, compare lanes 2 and 7). The myotube-specific band was formed when the PS oligomer was used; in fact, the PS oligomer produced a better binding pattern than those observed with the PO oligomer. This may be attributed to the greater nuclease resistance of the PS oligomer. Therefore, in contrast to previously published results (21), we did not find an increase in nonspecific binding of the PS oligomer modified. It is likely that the increase in nonspecific binding by the PS oligomer may depend on the DNA sequence used for electrophoretic mobility shift studies.

If the oligomer was indeed binding to myogenin and MyoD in the nucleus, then we should be able to block in vivo terminal differentiation of C2C12 cells in culture. Initial studies were performed to examine if the oligomer could block morphological differentiation by preventing myotube formation. We observed that the mef-1 oligomer-treated cells failed to fuse and form myotubes (Fig. 4C). Less than 10% of the nuclei in treated cells was found in multinucleated myotubes as compared to greater than 90% in untreated control myotubes (Fig. 4A). Mutant oligomer-treated (Fig. 4B) cells had greater than 80% of nuclei in myotubes. We have also tested if the inhibition of myotube formation by mef-1 oligomer was reversible. Following 6 days of oligomer treatment, cells were allowed to differentiate in absence of the oligomer for 4 days. Results (Fig. 4D) show that the

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2 The MF20 hybridoma was obtained from the Developmental Studies hybridoma maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the National Institute of Child Health and Human Development.
The inhibitory effect of the mef-1 oligomer was reversible, and greater than 80% of nuclei in these cells was found in myotubes.

The effect of these oligomers on biochemical aspects of differentiation was also examined. Cellular proteins were labeled with [35S]methionine during the final 4 h of oligomer treatment.

Results (Fig. 5, A and B) show that the synthesis of a large number of polypeptides were not markedly affected by the mef-1 oligomer. Furthermore, the overall pattern of protein synthesis resembled that of the proliferating myoblast (Fig. 5, compare B and C). Synthesis of the majority of non-muscle proteins including β/γ-actin and non-muscle myosin light chain was not reduced, whereas synthesis of α-actin, α and β tropomyosin, and myosin light chains 1 and 2 was reduced in mef-1 oligomer-treated cells (Fig. 5, A and B). In several experiments, synthesis of the muscle proteins, mentioned above, in treated cells was only 40–60% of control myotube levels. In contrast, the synthesis of a number of non-muscle proteins examined from the autofilograms varied from 80–110% of the control.

To examine more accurately the effect of the mef-1 oligomer, the synthesis levels of specific muscle proteins were analyzed by immunoprecipitation. Radioactively labeled cellular proteins were precipitated using antibodies against myosin heavy chain, muscle creatinine kinase, and myogenin (Fig. 6). Each of
these three proteins showed decreased synthesis levels in mef-1-treated cells. The level of decrease in specific protein synthesis was dependent on the concentration of the mef-1 oligomer. Treatment of cells with 10 μM mef-1 oligomer showed an approximate 72% reduction in myogenin synthesis (Fig. 6A), whereas similar treatment with the mutant mef-1 (mmef-1) showed an approximately 15% reduction in myogenin synthesis. Reversing the mef-1 treatment, by changing to fresh differentiation medium, without the oligomer, for 4 days, resulted in increased myogenin synthesis. However, the level of myogenin synthesis was approximately only 30% of untreated control myotubes. This may be due to the continual presence of the oligomer in previously treated cells. Similar levels of inhibition was also observed on the synthesis of muscle creatine kinase (Fig. 6B) and myosin heavy chain (Fig. 6C) in mef-1 oligomer-treated cells, whereas mutant oligomer treatment had no marked effect on the synthesis of these polypeptides. Reversion of the oligomer treatment showed a much greater recovery in synthesis of muscle creatine kinase and myosin heavy chain than that observed for myogenin production. Reversion of the treatment resulted in greater than 80% of control (untreated) levels of synthesis for these proteins.

Our results on the uptake of the ds oligomer show that even after prolonged treatment, only 2% of the input material can be found in the cell. Attempts were made, therefore, to improve the uptake. Short treatment, with 1 μM oligomer using a cationic liposome (Lipofectin; Life Technologies, Inc.) for 16 h followed by 5 days of treatment without the oligomer, did not result in marked inhibition of myotube formation. Therefore, passive diffusion appears to be the best available means for long term oligomer treatment to block differentiation of C2C12 myoblasts. Results of our studies show that 90% of the mef-1 oligomer-treated cells fail to form myotubes. Visual observation of the process revealed that a small number of cells (10–20%) appeared to fuse after 3 to 4 days of treatment but were later lost from the culture. This indicates that terminally differentiated cells may have undergone apoptosis when synthesis of muscle-specific proteins were blocked by the oligomer.

Our results suggest that the ds oligomer can block the biological function of a specific transcription factor in cell culture. It is possible that any transcription factor can be targeted using this method so long as its binding sequence is well characterized. Another attractive feature is the transience of the effect of the oligomer. The blockage of the transcription factor function lasts only as long as the oligomer treatment, and the cells return to normal after removal of the oligo. Further enrichment of the observed effect could include the addition of multiple binding sites per oligomer and further improvement of the cellular uptake and intracellular stability of the oligomer.

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