

DNA POLYMERASE ACTIVITY IN MUSCLE CULTURES

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ABSTRACT

Nuclei within myotubes do not synthesize DNA for replication. Accordingly, cultures of myotubes display low levels of DNA polymerase activity. The coincidental decline in DNA polymerase activity and increased formation of multinucleated myotubes during culture does not prove that the loss of capacity to synthesize DNA is a consequence of fusion. The experiments described demonstrate that myogenic cells prevented from fusing have low levels of DNA polymerase activity. This is consistent with the notion that, in myogenic cultures, there is a population of mononucleated cells, the myoblasts, which have withdrawn from the mitotic cycle before fusion.

KEY WORDS myogenesis · cultures · DNA polymerase · myoblasts · differentiation

Studies of *in vitro* myogenesis by O'Neil and Strohmman (20) and Stockdale and O'Neil (24) show a rapid decline in DNA polymerase activity coinciding with fusion. The experiments of O'Neil and Strohmman (20) reveal that the DNA polymerase activity in postfusion cultures is almost completely associated with the mononucleated population. Other workers (3, 16) have concluded that the loss of capacity to synthesize DNA is thus a consequence of fusion and a property restricted to the nuclei in myotubes. However, according to Holtzer and co-workers (11, 14), there are two classes of mononucleated myogenic precursor cells. The first, the presumptive myoblasts, are capable of division but do not possess any of the characteristics of terminally differentiated muscle. The second is the terminally differentiated myoblast which Holtzer and co-workers propose has irreversibly withdrawn from the cell cycle and is in a state of G_0 and should display low levels of DNA polymerase activity. The data obtained by O'Neil and Strohmman do not rule out the possibility that, within the mononucleated

population, which as a whole possesses relatively high levels of polymerase activity, there exists a subpopulation which have withdrawn from the cell cycle. This subpopulation would be difficult to demonstrate in standard myogenic cultures in view of the rapidity with which myoblasts fuse, and it is likely that this transient population would represent a small proportion of the total mononucleated cells examined by O'Neil and Strohmman (20).

In this communication, we report the levels of DNA polymerase measured in fusion-arrested ethylene glycol-bis(β -aminoethyl ether) N,N,N',N' -tetraacetate (EGTA)-treated myogenic cultures (21) and show that these fusion-competent mononucleated myoblasts in fact display low levels of DNA polymerase, of the order seen in pure myotube cultures.

RESULTS AND DISCUSSION

To determine which cell types in the standard complex myogenic culture display the characteristic loss in DNA polymerase activity, the following kinds of cultures were assayed: (i) *Standard complex myogenic cultures, 1 day after inoculation.*

These cultures were prepared as described by Bishchoff and Holtzer (2). During the first 24 h, these cultures consist of only mononucleated cells, the majority of which (circa 80%) are replicating. (ii) *Standard complex myogenic cultures at the end of day 5*. In these cultures, approx. 40% of the nuclei are in myotubes, and the remaining 60% are in mononucleated cells. Most of the mononucleated cells are capable of replicating. (iii) *Pure myotube cultures*. When standard myogenic cultures are exposed to cytosine arabinoside (1 $\mu\text{g}/\text{ml}$), from day 2 to day 4 virtually all replicating mononucleated cells are killed, except those cells that did not enter S or ones that were postmitotic. As a consequence, on day 5 such cultures consist almost exclusively of multinucleated myotubes (22). (iv) *Definitive myoblasts*. These cells are prepared by treating standard cultures with the Ca^{++} chelating agent EGTA (1.75 μM) from day 0 to day 5 to suppress fusion, plus cytosine arabinoside (1 $\mu\text{g}/\text{ml}$) from day 2 to day 4. In excess of 90% of these mononucleated myoblasts have been shown to be synthesizing the definitive myosin heavy and light chains (4, 5). When fed with standard medium, many of these myoblasts fuse to form myotubes within a short period. (v) *Untreated fibrogenic cultures*. When standard 5-day myogenic cultures are repeatedly subcultured, four or more passages, a mononucleated population that is operationally indistinguishable from authentic fibroblasts is obtained. These cells secrete large quantities of hyaluronic acid, synthesize Type I collagen chains, and, if labeled with [^3H]thymidine and mixed with fresh myogenic cells, will not be incorporated into myotubes (1). (vi) *Treated fibrogenic cells*. These are fifth-passage fibrogenic cultures which are exposed to EGTA (1.75 μM) from day 0 to day 5 and to cytosine arabinoside (1 $\mu\text{g}/\text{ml}$) from day 2 to day 4. It has been shown elsewhere that 70% of these cells are killed during the 2-day exposure to cytosine arabinoside (29). However, after removal of the drug, the surviving cells replicate and can be cultured for many generations without displaying any myogenic capacity.

The DNA polymerase activity in the six types of culture described above was assayed in sonicated extracts according to the method of Holmes et al. (10). When three nucleoside triphosphates, α -ATP, α -GTP and α -CTP, were omitted from the assay mixture, negligible counts above background were incorporated into DNA. The omis-

sion of activated calf thymus DNA from the assay mixture produced counts comparable to reaction blanks, i.e., reactions terminated at zero time. The results shown in Table I indicate that most of the DNA polymerase activity in day-5 standard myogenic cultures (culture ii), can be attributed to the replicating mononucleated population, since cultures which contain only myotubes and from which most of the mononucleated cells have been removed (culture iii) show little activity. On the other hand, on a per protein basis, the myogenic culture (culture ii), and the fibrogenic culture (culture v) display comparable activity. In a culture in which fusion is blocked, and in which the replicating mononucleated cells are killed leav-

TABLE I
Comparison of DNA Polymerase Activities in
Myogenic and Fibrogenic Cultures

Cultures	DNA polymerase activity cpm/mg protein
(i) Standard myogenic (day 1)	1,450 (5)
(ii) Standard myogenic (day 5)	1,235 (3)
(iii) Pure myotube (+Ara-C and EGTA) (day 5)	215 (3)
(iv) Myoblasts (+Ara-C and EGTA) (day 5)	316 (3)
(v) Fibrogenic (day 5)	1,321 (2)
(vi) Fibrogenic (+Ara-C and EGTA) (day 5)	1,833 (2)

The cultures used were prepared as described in the text. The assay system contained 15 μmol of Tris-HCl pH 7.5, 1.25 μmol of MgCl_2 , 25 μmol each of dATP, dGTP, dCTP and [^3H]dTTP, 0.25 μmol of dithiothreitol, 50 μg of activated calf thymus DNA and 125 μg of bovine serum albumin. Sonicated muscle extracts or fibroblast extracts were added to start the reaction. Total volume of reaction mixture was 250 μl . The incorporation of [^3H]dTTP was shown to be linear for up to 60 min; therefore, incubations at 37°C were carried out routinely for 40 min, then stopped by the addition of 50 μl of 10% TCA. Precipitates were solubilized in 500 μl of 0.5 M NaOH at 80°C for 10 min, then chilled in ice and mixed with 2.5 ml of cold TCA. DNA is then absorbed onto Whatman's glass fiber filters, washed with cold 10% TCA (5 ml) followed by washes with 95% EtOH. Filters were then dried, and radioactivity was determined by liquid scintillation counting. Blanks incorporated in all determinations ranged from 150 to 250 cpm. Determinations were carried out in duplicate. The number of cultures used is shown in brackets. Reproducibility of duplicates was $\pm 10\%$, and within groups of cultures, $\pm 15\%$. Proteins were determined by the method of Lowry et al. (17).

ing only mononucleated cells that have been shown to be synthesizing the definitive muscle proteins (culture iv) (4, 5), the level of DNA polymerase activity approaches that found in pure myotube cultures (culture iii). This result suggests that either myoblasts before fusion already have low levels of DNA polymerase, or that the treatment of cells with EGTA and cytosine arabinoside depresses the level of polymerase in the cells. The finding that fibrogenic cells treated with EGTA and cytosine arabinoside (culture vi) do not show depressed levels of the enzyme suggests that the second alternative is unlikely. Paterson and Strohman (21) have reported that fibroblast morphology, mobility, and capacity to divide remained unaltered in EGTA-treated cultures (21).

The results of this study show that fusion competent mononucleated myoblasts display decreased levels of DNA polymerase activity comparable to that observed in "pure" myotube cultures. It has been previously shown that DNA polymerase activity determined by this assay correlates closely with cell proliferative activity of fetal hepatocytes in the neonatal period (30). Thus, the low level of DNA polymerase activity in the myoblasts suggests that they are a nondividing population, i.e. in a state of G_0 or that they are in an extended G_1 . The evidence is not definitive insofar as it has not been established that a particular DNA polymerase is directly involved in DNA replication.

Primary myogenic cultures established from 10-day-old chick embryos consist of a complex population of cells. The majority of cells are replicating myogenic cells which belong to the various compartments of the myogenic lineage (14). These cells do not synthesize the muscle specific heavy or light chains of myosin (4, 14), or the muscle specific forms of creatine kinase or aldolase (26). Indirect evidence suggests that these replicating myogenic cells constitute between 40% and 60% of the original inoculum (5). The remainder includes a fraction of cells in the fibrogenic lineage and a fraction, possibly as high as 20%, which has been shown by clonal analysis to be ancestral to both myogenic and fibrogenic lineages (1). After about 48 h in culture, many cells are observed to (a) fuse to form multinucleated myotubes, (b) cease synthesis of DNA, and (c) commence synthesis of the definitive muscle proteins.

According to Holtzer and co-workers (11, 14), myogenic precursor cells, presumptive myoblasts,

divide to yield terminally differentiated mononucleated myoblasts. It is proposed that, by virtue of this cell division, the myoblasts acquire the following characteristics: (a) They can synthesize myosin and assemble thick and thin filaments, and (b) they become postmitotic and irreversibly withdraw from the cell cycle.

The first proposal (13) implies that fusion is not a prerequisite event for initiating the synthesis of muscle contractile proteins. This was not initially supported by subsequent studies (9, 21, 23). However, more recently, a substantial number of laboratories have shown that myogenic cells blocked from fusions by Cytochalasin B (12, 15), phospholipase C (19, 25), EGTA (7, 27), or low Ca^{++} (18) do in fact synthesize the definitive myosins and assemble hexagonally arrayed thick and thin filaments.

The second proposal implies that these cells become incapable of division before fusion rather than as a consequence of fusion. Furthermore, experiments reported elsewhere (5, 6, 8, 14, 30) suggest that these cells have permanently withdrawn from the cell cycle and are in a state of G_0 . These EGTA-derived myoblasts when cultured at low densities conducive to cell proliferation do not divide and incorporate tritiated thymidine. When calcium ions are restored to the medium, these cells fuse without further division (5, 6, 8, 15). However, the possibility that these cells are in an extended G_1 (and can thus re-enter the cell cycle) of a duration greater than the period under observation in the experiments cannot be ruled out. The low level of DNA polymerase in these cells is consistent with either proposition, i.e., reversible or irreversible withdrawal from the cell cycle. This observation together with other characteristics of these myoblasts (4-8, 13, 15, 18, 19, 21, 22, 26, 27, 30) suggests that they constitute a stable class of cells in the myogenic lineage. The central issue in myogenesis is to elucidate the mechanisms responsible for the transition of replicating presumptive myoblasts that have high levels of DNA polymerase activity and synthesizing "constitutive" contractile protein (4, 14) to myoblasts that have withdrawn from the cell cycle, possessing low levels of DNA polymerase activity and the ability to synthesize the definitive muscle proteins.

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