REPAIR DNA SYNTHESIS IN DIFFERENTIATED EMBRYONIC MUSCLE CELLS

FRANK E. STOCKDALE and MICHAEL C. O'NEILL

From the Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

The differentiation of embryonic skeletal muscle cells is closely coupled with the cessation of normal DNA replication. Once these cells begin to differentiate, they normally never undergo semiconservative replication of DNA during the entire life time of the muscle cell. Cessation of DNA synthesis has been shown to be accompanied by a loss of 80–90% of the replicative DNA polymerase activity of these cells. Despite this loss the studies reported here demonstrate that muscle cells retain the ability to synthesize DNA of a repair type after UV irradiation. These results suggest that the control exercised over semiconservative DNA synthesis during differentiation of these cells does not extend to repair synthesis after UV irradiation.

INTRODUCTION

Embryonic multinucleated skeletal muscle cells are formed by the fusion of many mononucleated cells which, before cell fusion, divide every 12 hr (1, 16). As the process of differentiation begins, those cells which fuse never again enter the DNA synthetic phase of the cell cycle, and this change is accompanied by a loss of 90% of the DNA polymerase activity coincidentally with cell fusion (24, 14, 15, 22). However, after UV irradiation, every nucleus (99%) within differentiated muscle cells incorporates tritiated thymidine after UV light treatment (23). This paper reports experiments which demonstrate that the previous radioautographic result (23) does indeed represent DNA synthesis of a repair nature (18) induced by UV irradiation in differentiated skeletal muscle cells.

MATERIALS AND METHODS

Cell Culture

White leghorn 11 or 12 day chick embryo breast muscle was used in all experiments (16). Breast muscle was removed, cleaned of adhering connective tissue, and minced. After a 20 min incubation in 0.2% trypsin (Difco 1:250) (Difco Laboratories, Inc., Detroit, Mich.) in Saline G (19), a suspension of mononucleated cells was obtained by repeated pipetting and filtering through lens paper and nylon mesh with an average pore size of 10 μ. The cell suspension was allowed to settle at 37°C for 10 min on collagen-coated dishes and the unattached cells were removed, counted in a Coulter Counter (27), and plated at a concentration of 3.33×10^5/ml on Falcon 60-mm dishes (3 ml/dish) (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) previously treated with collagen (Worthington Biochemical Corp., Freehold, N.J., crude collagen) (8). All cultures were grown at 37°C in a CO_2 incubator in Eagle's Minimal Essential Medium with Earle's Salts (MEM)[1]:horse serum:11 day

[1] *The following abbreviations are used in this paper*: BudR, 5 bromodeoxyuridine; MEM, Eagle's Minimal Essential Medium with Earle's Salts; POPOP, *p*-Bis-[2-(5-Phenyloxazolyl)]-Benzene (Scintillation Grade); PPO, 2,5-Diphenyloxozole (Scintillation Grade); SLS, sodium lauryl sarcosine; SSC, 0.15 M sodium

embryo extract (88:10:2) which was replaced every third day.

Thymidine Incorporation

For determinations of thymidine incorporation, culture medium was removed from the dishes and replaced with a medium (MEM:horse serum, 90:10) containing 5–10 μCi/ml of methyl-labeled tritiated thymidine (SA 19.9–6 Ci/mmole, Schwarz Bio Research Inc., Orangeburg, N.Y. or New England Nuclear Corp., Boston, Mass.). In those experiments where DNA synthesis was inhibited, hydroxyurea (0.3 M) was added to MEM:horse media at the indicated times. After incubation at 37°C the dishes were washed repeatedly with cold Hanks' balanced salt solution and incorporation was stopped by the addition of 1 ml of 5% cold Trichloroacetic acid (TCA). The cells were scraped from each dish, collected on glass fiber filters (Whatman GF/C), and washed with 5% TCA and finally with ether:ethanol (1:4). The filters were placed in plastic counting vials, to which was added 5 ml of toluene: 2,5-Diphenyl-oxazole (PPO): *p*-Bis[2-(5-Phenyloxazolyl)]-Benzene (POPOP) (1000 ml:6 g:0.5 g), and counted in a Mark I Nuclear-Chicago Spectrometer (Nuclear-Chicago, Des Plaines, Ill.).

Equilibrium Density Centrifugation

Cultures to be used for equilibrium density centrifugation were grown for two to four cell cycle times (24–48 hr) in 16 μM 5 bromodeoxyuridine (BUdR) (Nutritional Biochemicals Corporation, Cleveland, Ohio). For studies on repair DNA synthesis, cultures were exposed to varying doses of UV light (General Electric Lamp, G25T8, which delivers 30 ergs/mm^2 per sec). During these brief exposures the medium was completely removed from the dishes. After irradiation, MEM:horse serum (90:10) containing 10 μCi of tritiated BUdR per ml of medium (SA 9.4 Ci/mmole, New England Nuclear Corp.) was added to each dish. After incubation at 37°C the cells were removed from the dishes and lysed in 1 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate [pH 7.4]) containing 0.25% sodium lauryl sarcosine (SLS) and diluted to 5.5 ml with 1 × SSC. This solution was dialysed overnight against two changes of 1 × SSC. A portion of this solution was added to cesium chloride to give a refractive index of 1.4020–1.4023. In some experiments, the cells were scraped from the dishes, homogenized in a Dounce homogenizer in 6% *p*-amino-salicylic acid, freeze thawed, and then extracted with phenol according to the method of Souleil and Panijel (21). The supernatant was treated at 37°C with ribonuclease A (50 μg/ml, Worthington Biochemical Corp.) for 30 min and then pronase (500 μg/ml, Calbiochem, Los Angeles, Calif.) for 60 min, and reextracted with phenol and precipitated with ethanol. The DNA was dissolved in 1 × SSC and added to cesium chloride. Equilibrium density centrifugation results were the same whether the dialysed cell homogenates were placed directly in cesium chloride or were first extracted with phenol. Alkaline cesium gradients (pH 12.5) were centrifuged in a Spinco 40 rotor at 37,000 rpm at 20°C for 38–39 hr.

Fractions were collected with a fraction collector from the bottom of the tube and precipitated with cold 10% TCA after adding 25 μg of casein to each fraction. The precipitate was collected on glass fiber filters (Whatman GF/C) and washed with 5% TCA and ether:ethanol (1:4) for counting.

chloride, 0.015 M sodium citrate (pH 7.4); TCA, trichloroacetic acid.

RESULTS

In this report, we wish (*a*) to reduce the qualitative radioautograph result to a quantitative result by showing that there is thymidine incorporation into DNA specifically as a result of UV irradiation and (*b*) to show that such synthesis is occurring in postmitotic differentiated muscle cells and is of a repair nature. When mononucleated cell suspensions are plated in a monolayer, virtually every cell divides once or twice during the first 2 days of incubation. By the third day, 70–80% of the daughter cells fuse into long multinucleated differentiated cells called myotubes (24, 11, 2, 1, 16). In all cultures a population of mononucleated undifferentiated cells remains, and, as noted above, these are the only cells which synthesize DNA. These observations were confirmed under the conditions used here by incubating 3-day cultures with tritiated thymidine for 2 hr. Radioautographs reveal that there are no more grains over myotube nuclei than over equal areas of cytoplasm in the same cells. However, in parallel cultures exposed to UV irradiation the average grain count per nucleus increases 6- or 7-fold above the cytoplasmic level (Fig. 1 and Table I).

Despite the increase in the total number of nuclei which incorporate label after irradiation, there is a substantial decrease in total acid-precipitable counts incorporated. UV irradiation under these conditions stops 75–90% of normal DNA synthesis relative to unirradiated controls. Since it has been shown that hydroxyurea inhibits semiconservative DNA synthesis while not preventing repair synthesis (20, 28, 4, 17), it was

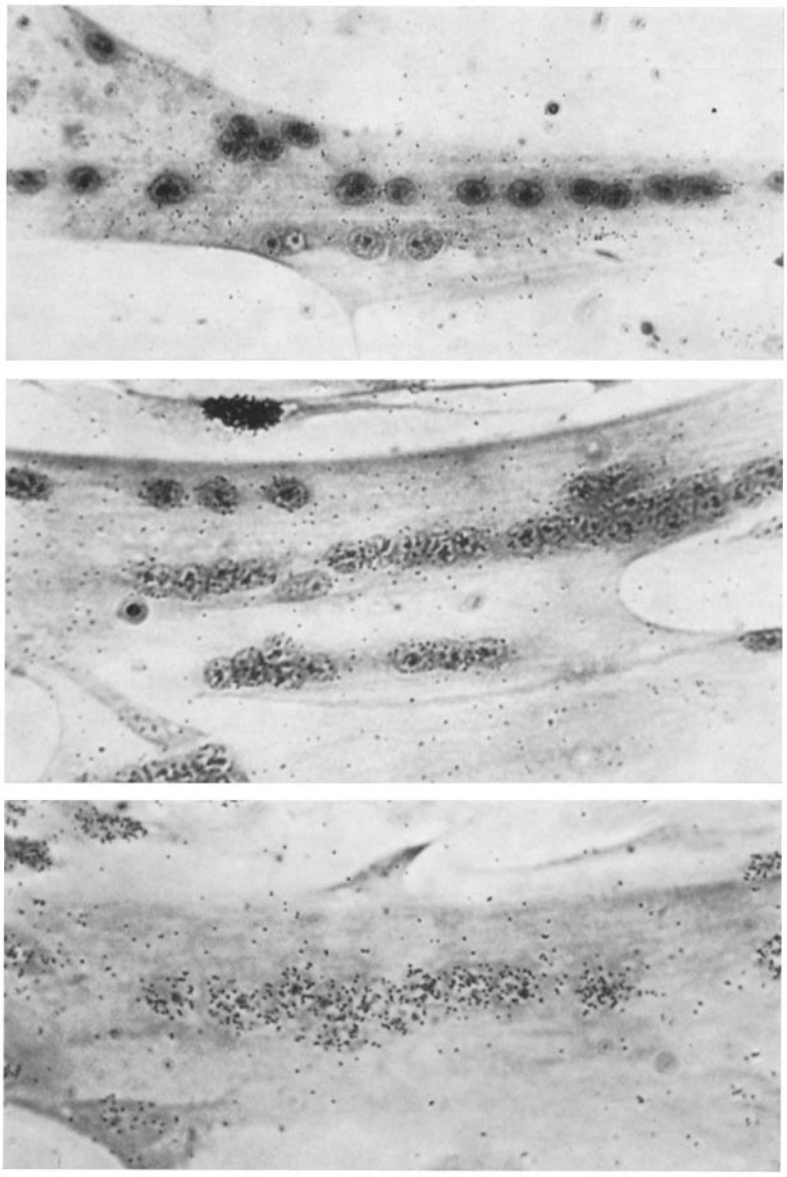

FIGURE 1 Photomicrographs of radioautographs of 5-day embryonic muscle cultures. Muscle cell cultures after 5 days of incubation were incubated in tritiated thymidine medium (10 μCi/ml) for 2 hr, followed by 200 ergs of UV light and a 4 hr incubation in tritiated thymidine (10 μCi/ml, SA 19 Ci/mmole). They were fixed and radioautographs were prepared. Top and middle radioautographs were exposed for 2 wk, and bottom radioautograph for 8 wk. Radioautographs prepared as in Reference 16. Top, No UV light treatment. × 400. Middle, Replicate culture treated with UV light. Note the increase in grains over myotube nuclei after irradiation. × 400. Bottom, This radioautograph of a myotube was exposed four times longer than the above two, to make the grains over the nuclei more apparent. Focus is on the grains in the emulsion. × 700.

TABLE I

Effect of UV Irradiation on Thymidine-^3H Labeling of Myotubes

Area counted	Number of grain ± SEM*	
	No UV light	UV light
Noncellular	97 ± 7	71 ± 7
Cytoplasm	140 ± 12	147 ± 14
Nucleus	117 ± 12	695 ± 67

4-day cultures were incubated for 2 hr in tritiated thymidine (10 μCi/ml, SA 19 Ci/mmole) and then irradiated with 200 ergs per mm^2 of UV light. After an additional 4 hr of incubation in the tritiated-thymidine medium the cells were fixed and radioautographed. Radioautography was performed with NTB II liquid emulsion (16). Radioautographs were exposed for 2 wk, and 1000–1800 μ^2 of area were examined in each case.

* Number of radioautographic grains over 1000 μ^2 determined at a magnification of 1800.

TABLE II

Inhibition of Tritiated Thymidine Incorporation by Hydroxyurea

Time in hydroxyurea	Control	
min	*cpm*	%
None	533	100
30	82	15
60	90	17
120	45	9

4-day cultures were incubated in 0.3 M hydroxyurea for the times indicated and then incubated in tritiated thymidine for 15 min. The cells were washed and precipitated with cold 5% TCA. The acid-precipitable counts incorporated were determined in duplicate.

hoped, by using this agent to block normal DNA synthesis, that we might be able to detect an increase in incorporation relative to unirradiated controls after UV irradiation. Incubation of fully fused cultures with 0.3 M hydroxyurea for 2 hr before irradiation reduced tritiated thymidine incorporation to less than 10% of that in untreated controls (Table II). Using cultures preincubated with hydroxyurea, it was then possible to show an increase in tritiated thymidine incorporation even after low doses of UV irradiation (Fig. 2). This incorporation was linear for 4 hr and was about 1–4% of the amount of incorporation into control cultures not treated with hydroxyurea and UV light (Fig. 3, Table III).

Experiments were then performed to determine if this enhanced tritiated thymidine incorporation was in DNA, and, if so, the nature of the synthesis involved and whether the synthesis was occurring in multinucleated cells rather than exclusively in undifferentiated mononucleated cells. Because of the postmitotic nature of these differentiated cells, the only DNA to become heavy during exposure of the cultures to BUdR is that of the undifferentiated mononucleated cells. The DNA in the myotube nuclei remains light. Cultures were plated and, after 3 days when the cells were maximally differentiated, BUdR was added for 48 hr. Under these conditions, 98% of the undifferentiated cells replicate their DNA one or more times (25). This was confirmed under the conditions of these experiments by incubating cultures for 48 hr with tritiated BUdR, and fixing them for radioautography. The radioautographs show that 98% of the labeled nuclei were in undifferentiated cells and that 97% of all the mononucleated cells in the culture had become labeled with BUdR (Table IV). In such cultures (grown in BUdR for four cell cycle times: days 3–5) the only double-stranded DNA with a normal buoyant density should be in differentiated cells. Fig. 4 *b* shows the results of equilibrium density centrifugation of DNA extracted from a culture incubated from 3 day–day 5 in 16 μM BUdR and for the last 4 hr in tritiated BUdR. The optical density is due to added normal chick DNA. The tritiated BUdR incorporated into DNA bands at densities greater than that of the marker and the DNA synthesized by mononucleated cells in the presence of tritiated thymidine (Figs. 4 *a* and 4 *b*). The more dense component in fractions 6–8 corresponds to double-stranded DNA with both strands partially replaced with BUdR, and the less dense component in fractions 10–11 to hybrid molecules in which there is one light strand and one heavy strand (see Fig. 6). This pattern of tritiated BUdR incorporation is consistent with incorporation into double-stranded DNA replicating by a semiconservative mechanism (12). The radioautographic experiments show that this incorporation of tritiated BUdR into replicating nuclear DNA is exclusively in mononucleated cells.

After UV irradiation a different result is found. Fig. 4 *c* shows that there is incorporation into an additional component of DNA with a normal

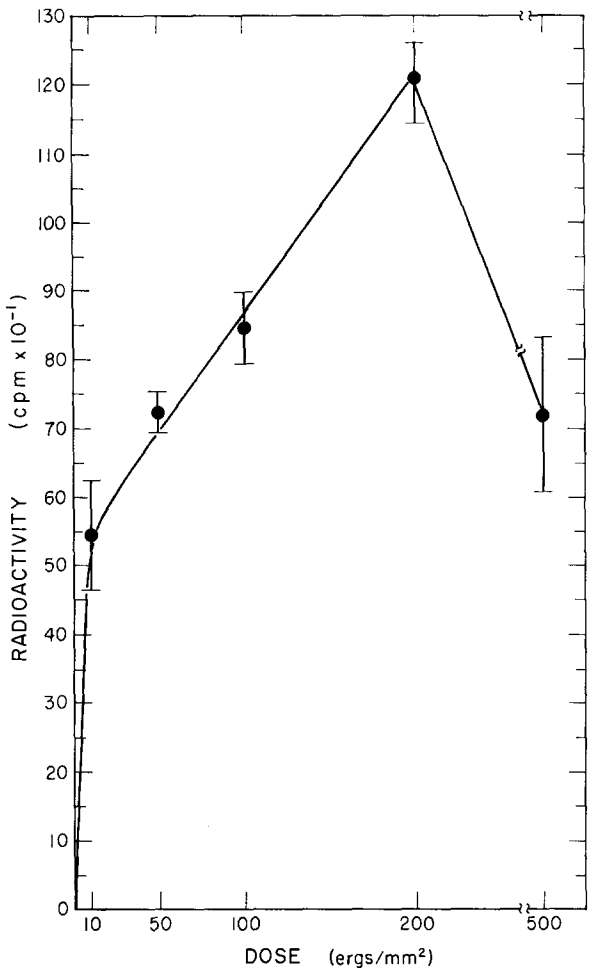

FIGURE 2 Incorporation of thymidine-^{3}H into muscle cell cultures irradiated with varying doses of UV irradiation. 3-day, fully differentiated cultures of muscle cells were incubated for 2 hr in 0.3 M hydroxyurea in MEM:horse serum (90:10) and then exposed to various dosages of UV light. After UV light treatment, the cultures were incubated in 0.3 M hydroxyurea and tritiated thymidine (5 μCi/ml, SA 18.3 Ci/mmole) for 2 hr. Points represent the average of duplicate determinations from which the control value has been subtracted.

buoyant density. This is more striking when hydroxyurea is used to inhibit 90% of semiconservative synthesis (Fig. 5). The normal density of this new fraction suggests that only a small amount of BUdR has been incorporated into DNA molecules, a pattern consistent with a repair synthesis mechanism (18). To confirm this, radioactive DNA from the hybrid region and that from the normal density region were rebanded in alkaline cesium chloride gradients (Fig. 6). In control cultures, the labeled single-stranded DNA from both regions banded near the bottom of the gradient, a result consistent with semiconservative synthesis. The radioactive, normal density DNA which appeared after UV irradiation, however, banded in the light region of the alkaline gradient and corresponded in density to a normal chick single-stranded DNA marker. Therefore, the DNA synthesis in myotube nuclei after UV irradiation is not of the semiconservative type but is consistent with the repair type DNA synthesis.

DISCUSSION

Regulatory mechanisms are evident in the control of both the rate at which the chromosomal DNA is replicated at different stages of embryonic

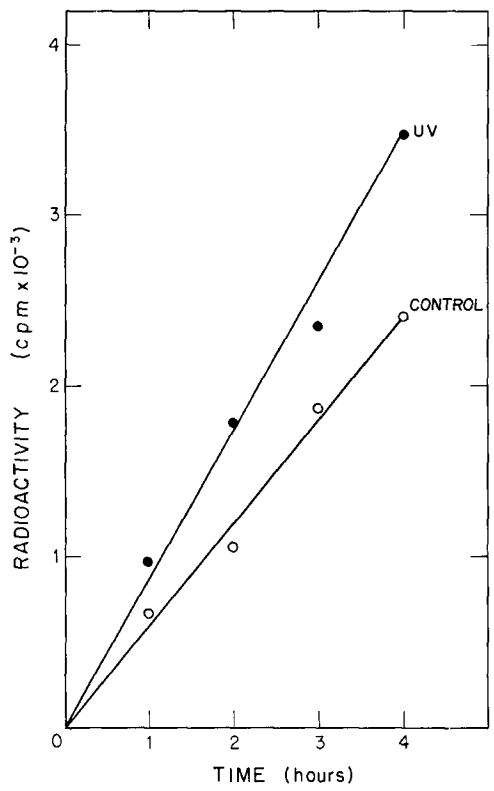

FIGURE 3 Kinetics of thymidine-^{3}H incorporation into muscle cell cultures after UV irradiation. 7-day, fully differentiated cultures of muscle cells treated as in Fig. 2. 85 ergs per mm^2 of UV light was used in each case. Each point is the average of duplicate determinations.

development and the frequency with which DNA synthesis is initiated in differentiating eukaryotic cells (5). In general, as cells differentiate *in situ*, they enter DNA synthesis less and less frequently, and, in a few cell types (for example, skeletal muscle, mammalian red blood cells, and neurons), the process of differentiation is associated with a complete failure to enter DNA synthesis. In the case of the muscle cell, the explanation for this is not readily apparent for, unlike the red blood cell, these cells do retain a nucleus. It is clear, though, that the failure to synthesize DNA is first demonstrable at the time of maximal cell fusion and that, once two or more nuclei are within a common muscle cytoplasm, DNA synthesis has not been demonstrated. Regulation of semiconservative DNA synthesis within muscle cells could occur at a number of testable levels: initiation on the template; polymerization of the triphosphates; and availability of substrates. Any or all of the listed mechanisms could be operative in controlling DNA synthesis during skeletal muscle cell differentiation, and the results of these experiments bear on some of these possibilities.

The control of DNA synthesis at the level of the DNA template, whether this involves conforma-

TABLE III

Incorporation of Tritiated Thymidine During Repair DNA Synthesis

Dose	TdR-^{3}H incorporated	Repair	Repair
ergs/mm²	*cpm ± SD*	*cpm*	*%*
0 minus HU	28,638 ± 1351	—	—
0 plus HU	2050 ± 42	0	0
10 plus HU	2596 ± 81	546	1.9
50 plus HU	2774 ± 61	724	2.5
100 plus HU	2893 ± 57	843	2.9
200 plus HU	3256 ± 53	1206	4.2

3-day, fully differentiated cultures were incubated in hydroxyurea as in Fig. 2, treated with UV light, and then incubated for 4 hr in MEM:horse serum (90:10) containing 10 μCi of tritiated thymidine per ml (SA 10 Ci/mmole) and 0.3 M hydroxyurea (HU). One set of controls was not treated with hydroxyurea or UV light. The cells were washed and precipitated with cold 5% TCA and collected on glass filters as described in Material and Methods. Per cent repair is the ratio of thymidine incorporated during repair to thymidine incorporated into cells exposed to neither UV irradiation nor hydroxyurea. Values are the average of triplicate determinations.

TABLE IV

BUdR Incorporation into Muscle Cell Cultures

Cell type	Total labeled nuclei	Specific nuclei labeled
	%	%
Mononucleate	98	97
Myotube	2	—

3-day cultures were incubated for 48 hr in 1 μCi of BUdR (SA 9.3 Ci/mmole) and the cells were fixed and radioautographed. The percentage of labeled nuclei in the two cell types was determined at a magnification of 400, and a minimum of 500 cells was counted. The 2% of labeled myotube nuclei represent the cells which had replicated their DNA as mononucleated cells and then fused with existing myotubes. Radioautographs were prepared as in Reference 16.

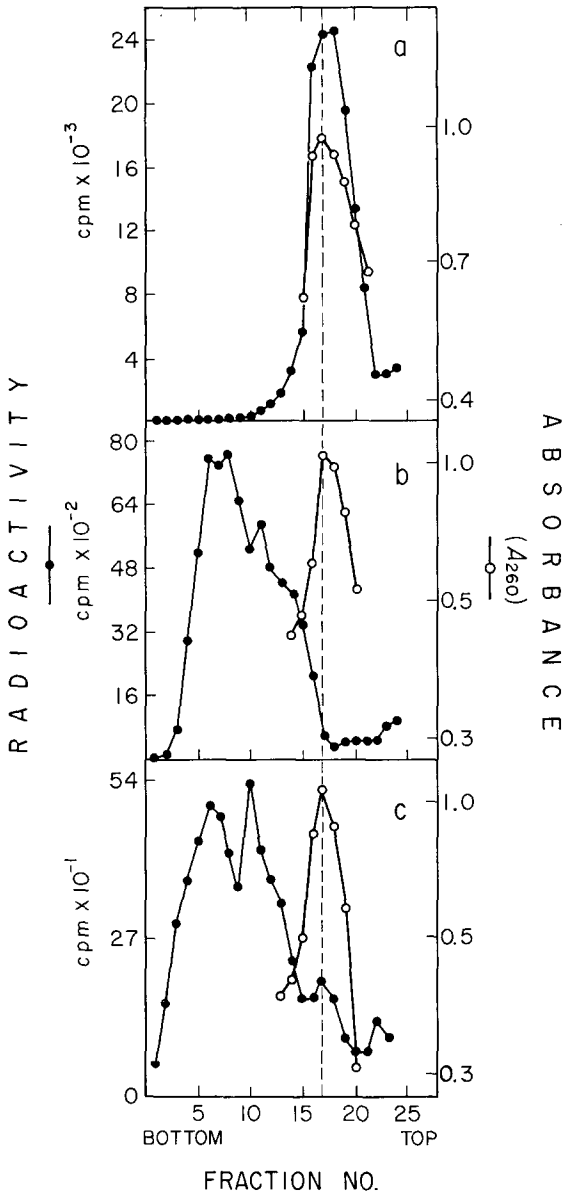

FIGURE 4 CsCl equilibrium centrifugation of ^{3}H-labeled DNA from muscle cell cultures. Fig. 4 *a*, Differentiated cell cultures were incubated for 4 hr in tritiated thymidine (10 μCi/ml) on day 5 of culture. Figs. 4 *b* and *c*. Differentiated cell cultures were incubated for 48 hr from the 3rd to the 5th day in 16 μM BUdR. One half of the cultures were then exposed to 150 ergs/mm^2 of UV light (Fig. 4 *c*). Both sets of BUdR-treated cultures were incubated with tritiated BUdR (1.1 μM) for 4 hr. All dishes were exhaustively washed with Hanks' balanced salt solution, and the cells were lysed in 1 × SSC and 1% SLS and dialysed, as in Material and Methods, before addition of cesium chloride. The gradients were centrifuged at 37,000 rpm for 39 hr in a 40 rotor at 20°C using a Spinco L2 centrifuge, and fractions were collected from the bottom. An optical density marker of chick embryo DNA was added to each sample before centrifugation.

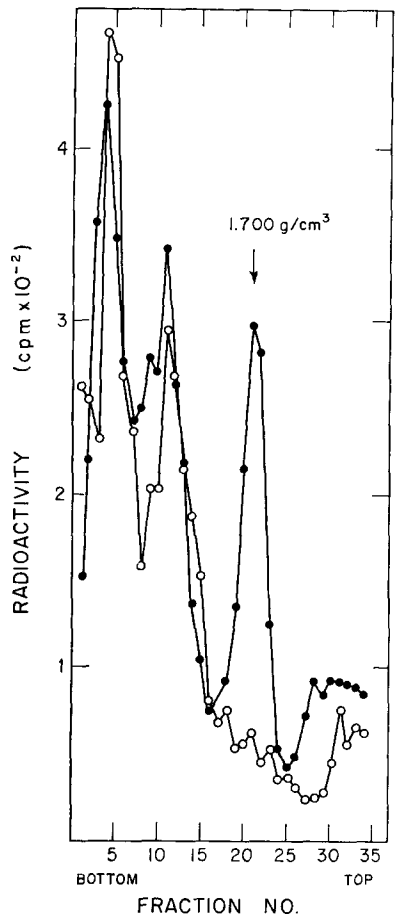

FIGURE 5 CsCl equilibrium centrifugation of ^{3}H-labeled DNA formed by hydroxyurea-inhibited muscle cell cultures. Differentiated cultures were incubated for 48 hr, from the 3rd to the 5th day of incubation, in 16 μM BUdR. All cultures were then incubated for 2 hr in 0.3M hydroxyurea in normal medium. Half the cultures were irradiated with 150 ergs/mm^2 of UV light, and all dishes were incubated in MEM:horse serum (90:10) containing 0.3 M hydroxyurea and 10 μCi/ml of tritiated BUdR for 4 hr. The cells were washed 3 times in Hanks' balanced salt solution and lysed in 1 ml of SSC containing 1% SLS. This solution was diluted to 4.4 ml, and 5.73 g of cesium chloride was added. Centrifugation was carried out for 38 hr as in Fig. 3. The cpm for separate gradients are plotted in the same figure and were normalized by refractive index. (—●—), UV. (—○—), no UV control.

tional changes of the chromosomes through alteration of their histone or associated protein composition or cleavage of phosphodiester bonds, is unknown. The fact that increasing doses of UV irradiation result in increased rates of repair and increased percentages of nuclei which synthesize DNA suggests that initiation could be a limiting step in the control of DNA synthesis in differentiating muscle.

Although it is recognized that the requirements of substrates for repair synthesis are small relative to those required for normal semiconservative DNA synthesis, it is unlikely that regulation of DNA synthesis during myogenesis is occurring at the level of substrate availability, since all the nuclei in differentiated muscle cells can carry out repair DNA synthesis.

Although it is generally thought that DNA

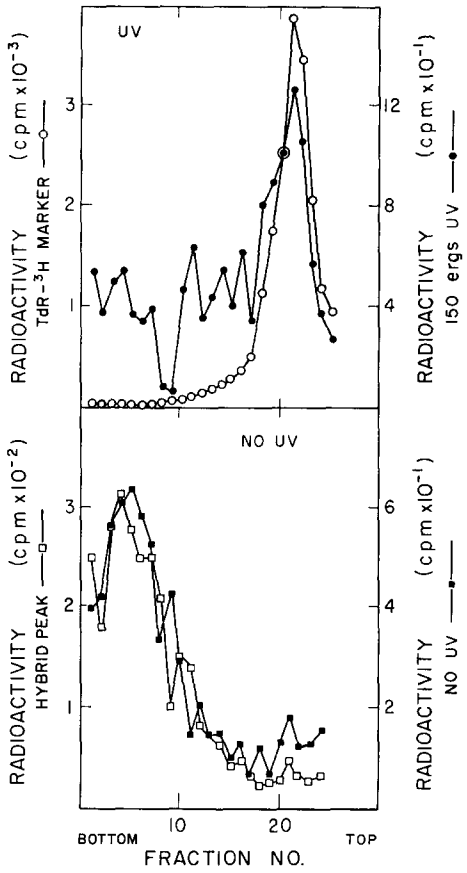

FIGURE 6 Alkaline CsCl rebanding of muscle cell DNA from neutral CsCl gradients. Top: the normal density region (—●—) from neutral cesium chloride gradients of UV light-treated cells (from Fig. 5) was rebanded in alkaline cesium chloride (pH 12.5). A marker of tritiated thymidine-labeled normal chick DNA is included (—○—). Bottom: the hybrid (—□—) and normal density (—■—) regions from neutral cesium chloride gradients from muscle cells not exposed to UV light (Fig. 5) were rebanded in alkaline cesium chloride (pH 12.5). Separate gradients are plotted in the same figures and normalized by refractive index.

polymerase is not involved in the regulation of DNA synthesis in eukaryotic cells, recent work on the control of DNA synthesis in the amphibian egg after fertilization suggests that this enzyme may play such a role (6). One could postulate such a role in developing embryonic muscle cells as well, since the activity of this enzyme increases as mononucleated myoblasts proliferate in vitro and decreases to less than 20% of this activity over a 12 hr period during cell fusion and the cessation of DNA synthesis (14).

If a single DNA polymerase is involved in both normal semiconservative and repair DNA synthesis in differentiating muscle it is surprising that an 80–90% loss of enzymatic activity does not markedly affect repair DNA synthesis. Previous observations, however, do suggest that muscle cells are less able to repair DNA after a given dose of UV irradiation after cell fusion than before fusion (23). Observations by Hahn and coworkers (7) indicate as well that repair of monovalent alkalating agent damage may also be changed after cell fusion. It remains to be determined if these results support a single enzyme system for the two types of synthesis or distinct enzymatic systems as suggested in prokaryotic systems (3, 9, 13, 10). It is possible that the 20% of enzyme activity remaining after differentiation is a different DNA polymerase than that responsible for normal replication, especially in the light of recent demonstration that there are two molecular size classes of DNA polymerase in *Tetrahymena*, one of which increases in amount after UV irradiation (26).

These experiments indicate that the regulatory mechanisms for controlling normal DNA synthesis in differentiating muscle cells do not extend to repair DNA synthesis. This may be because separate enzymatic systems are involved in the two processes or simply because differentiation in these cells involves the loss of the initiating step of DNA synthesis.

These investigations were supported by a National Science Foundation Grant (GB 6618X). Frank E. Stockdale is the recipient of a Career Development Award from the United States Public Health Service (AM 35252), and Michael C. O'Neill is the recipient of a United States Public Health Service postdoctoral fellowship (1-FO2 CA 28855). We thank Elaine Davis and Arlene Korn for their excellent technical assistance.

Received for publication 20 September 1971, and in revised form 8 November 1971.

REFERENCES

1. BISCHOFF, R., and H. HOLTZER. 1969. *J. Cell Biol.* **41**:188.
2. COLEMAN, J. R., and A. W. COLEMAN. 1968. *J. Cell Physiol.* (Suppl. 1.) **72**:19.
3. DE LUCA, P., and J. CAIRNS. 1969. *Nature (London).* **224**:1164.
4. EVANS, R., and A. NORMAN. 1968. *Radiat. Res.* **36**: 287.
5. GRAHAM, C. F., and R. W. MORGAN. 1966. *Develop. Biol.* **14**:439.
6. GURDON, J. B., and V. A. SPEIGHT. 1969. *Exp. Cell Res.* **55**:253.
7. HAHN, G., D. KING, and S. J. YANG. 1971. *Nature (London).* **230**:242.
8. HAUSCHKA, S. D., and I. R. KONIGSBERG. 1966. *Proc. Nat. Acad. Sci. U. S. A.* **55**:119.
9. KELLY, R., H. ATKINSON, J. HUBERMAN, and A. KORNBERG. 1969. *Nature (London).* **224**:495.
10. KNIPPERS, R., and W. STRÄTLING. 1970. *Nature (London).* **226**:713.
11. KONIGSBERG, I. R. 1963. *Science (Washington).* **140**:1273.
12. MESELSON, M., and F. W. STAHL. 1958. *Proc. Nat. Acad. Sci. U. S. A.* **47**:671.
13. MOSES, R., and C. C. RICHARDSON. 1970. *Biochem. Biophys. Res. Commun.* **41**:1565.
14. O'NEILL, M., and R. STROHMAN. 1969. *J. Cell Physiol.* **73**:61.
15. O'NEILL, M., and R. STROHMAN. 1970. *Biochemistry* **9**:2832.
16. O'NEILL, M., and F. E. STOCKDALE. 1972. *J. Cell Biol.* **52**:52.
17. PAINTER, R. B., and J. E. CLEAVER. 1969. *Radiat. Res.* **37**:451.
18. PETTIJOHN, D., and P. C. HANAWALT. 1964. *J. Mol. Biol.* **9**:395.
19. PUCK, T. T., S. J. CIECIURA, and A. ROBINSON. 1958. *J. Exp. Med.* **108**:945.
20. SINCLAIR, W. K. 1965. *Science (Washington).* **150**: 1729.
21. SOULEIL, C., and J. PANIJEL. 1970. *Nature (London).* **227**:456.
22. STOCKDALE, F. E. 1970. *Develop. Biol.* **21**:462.
23. STOCKDALE, F. E. 1971. *Science (Washington).* **171**: 1145.
24. STOCKDALE, F. E., and H. HOLTZER. 1961. *Exp. Cell Res.* **24**:508.
25. STOCKDALE, F. E., D. OKAZAKI, M. NAMEROFF, and H. HOLTZER. 1964. *Science (Washington).* **146**:533.
26. WESTERGAARD, O. 1970. *Biochim. Biophys. Acta.* **213**:36.
27. YAFFEE, D. 1968. *Proc. Nat. Acad Sci. U. S. A.* **61**:477.
28. YOUNG, C. W., G. SHACHETAM, and D. KARNOFSKY. 1967. *Cancer Res.* **27**:526.