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Aphidicolin-Resistant Polyomavirus and Subgenomic Cellular DNA Synthesis Occur Early in the Differentiation of Cultured Myoblasts to Myotubes

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Small DNA viruses have been historically used as probes of cellular control mechanisms of DNA replication, gene expression, and differentiation. Polyomavirus (Py) DNA replication is known to be linked to differentiation of many cells, including myoblasts. In this report, we use this linkage in myoblasts to simultaneously examine (i) cellular differentiation control of Py DNA replication and (ii) an unusual type of cellular and Py DNA synthesis during differentiation. Early proposals that DNA synthesis was involved in the induced differentiation of myoblasts to myotubes were apparently disproved by reliance on inhibitors of DNA synthesis (cytosine arabinoside and aphidicolin), which indicated that mitosis and DNA replication are not necessary for differentiation. Theoretical problems with the accessibility of inactive chromatin to *trans*-acting factors led us to reexamine possible involvement of DNA replication in myoblast differentiation. We show here that Py undergoes novel aphidicolin-resistant net DNA synthesis under specific conditions early in induced differentiation of myoblasts (following delayed aphidicolin addition). Under similar conditions, we also examined uninfected myoblast DNA synthesis, and we show that soon after differentiation induction, a period of aphidicolin-resistant cellular DNA synthesis can also be observed. This drug-resistant DNA synthesis appears to be subgenomic, not contributing to mitosis, and more representative of polyadenylated than of nonpolyadenylated RNA. These results renew the possibility that DNA synthesis plays a role in myoblast differentiation and suggest that the linkage of Py DNA synthesis to differentiation may involve a qualitative cellular alteration in Py DNA replication.

The polyomavirus (Py) family is highly dependent on cellular mechanisms for DNA replication and transcription. Although large T antigen (T-Ag) is active in controlling viral DNA replication and transcription, all other factors, such as DNA and RNA polymerases with their accessory proteins, chromatin proteins, and *trans*-acting factors, are of cellular origin (20, 22, 50, 74). A common view is that because of sequence-specific binding at the viral origin of replication, large T-Ag is a dominant *trans*-activator of viral transcription and viral DNA replication as well as cellular DNA synthesis. However, with mouse Py, it is well established that wild-type viral DNA replication is often, if not generally, also linked to the terminal differentiation of permissive embryonal, neuroblast, erythroblast, and myoblast cells (for an early review, see reference 2). Recent evidence suggests that even in vivo, Py DNA replication may be typically linked to cellular differentiation (4). Cell-type-specific replication of Py DNA is typically *cis* restricted and requires enhancer sequences that bind active (or do not bind repressive) *trans*-acting factors present in the specific cell type. Thus, as a *trans*-acting factor, large T-Ag is unable to compel the replication of wild-type Py replicons, unless viral DNA is also *cis* linked to an appropriate enhancer. This *cis* restriction has been shown by mixed infection experiments both in culture (21, 67, 68, 71) and in vivo (55–57). Since it is known that in vitro replication from naked simian virus 40 and Py DNA templates is not affected by enhancer sequences (54, 77), whereas replication from chromatin is (13, 39), it appears therefore that chromatin stability may limit the accessibility of large T-Ag to viral origins of replication in a cell-specific manner (for a review, see reference 19).

If the problem of access of large T-Ag to binding sites on DNA is considered typical of other cellular *trans*-acting factors, two unresolved questions follow: exactly how and when do *trans*-acting factors gain access to their binding sites, and how is this accomplished during differentiation? Clearly chromatin must be accessible to *trans*-acting factors during major gene commitment changes, such as a terminal differentiation process. Because de novo assembly of active and repressed chromatin occurs during DNA replication (10, 66, 75), early proposals that DNA replication was required to reset chromatin and gene commitment appeared to provide the needed access of *trans*-acting factors to DNA-binding sites (63, 64). Such views also appeared consistent with the long history of an apparent relationship between cell mitosis and cell differentiation (36, 37). However, subsequent observations challenged these early views because efficient inhibition of mitosis and DNA synthesis failed to prevent differentiation (6, 14, 53, 69, 83). Still, the method by which factors gain access to chromatin has remained unknown, even as the details of *trans*-acting factors and their binding to naked DNA have become increasingly well known.

Strong evidence now suggests that resident histones occlude promoter activity, and this has led to increased discussion of the need for possible mechanisms of chromatin clearing in gene activation (see, for example, references 24, 42, and 64). Currently, however, little experimental evidence exists for alternative chromatin-clearing mechanisms, other than DNA replication (although some *trans*-acting factors may bind without nucleosome displacement [3]). Even if

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problems of decondensation of inactive chromatin are ignored, the stability of active chromatin itself presents problems for gene control. Most in vitro analyses have failed to show displacement of nucleosomes (or histone H1 [15]) from a chromatin template by *trans*-acting factors (for reviews, see references 24, 28, 42, 64, and 78). In vitro transcription experiments (with either RNA polymerase II or III) show that *trans*-acting factors assemble into stable committed structures, that factors must be added prior to nucleosomes to assemble transcriptionally active template, and that, once assembled, these complexes are refractory to subsequent competition or nucleosome repression (10, 16, 46, 47, 70, 79–82). A similar chromatin stability is observed for the *trans*-acting-factor-mediated activation of simian virus 40 DNA replication (13); thus, large T-Ag also fails to displace previously assembled repressive chromatin. In vivo experiments by Han and Grunstein with yeast mutants showed that depletion of the histone H4 production (by a repressible promoter) blocks formation of new nucleosomes and dramatically activates many previously silent genes (29). Taken together, these results appear to indicate that nucleosomes must be removed before *trans*-acting factors can bind and activate promoters and that the same *trans*-acting factors cannot displace repressive nucleosomes. Also relevant to this conclusion are correlations of actively transcribing genes with early-S-phase DNA replication (10, 26, 30, 32, 35), which further imply that replication timing within the S phase may be important for the assembly of active or inactive chromatin.

These biochemical and genetic studies seem consistent with that portion of earlier models (10, 36, 75) which proposed a crucial role for DNA synthesis in allowing the access of factors and resetting of chromatin to new patterns of gene commitment. However, these models presumed that the DNA synthesis would consist of full genomic replication (perhaps from a special S phase of a quantal cell cycle, a proposed special cell cycle which would necessarily precede changes in gene commitment). Other studies, especially some with myoblast terminal differentiation to myotubes in culture, have examined the necessity of DNA replication for a differentiation process and have shown that the quantal cell cycle model cannot be strictly accurate since cells can change their patterns of gene expression despite full inhibition of mitosis and nearly full inhibition of DNA synthesis with cytosine arabinoside (ara-C) or aphidicolin (6, 14, 53, 83).

To examine events which immediately follow induced differentiation of myoblasts in culture, we use Py DNA replication as a probe of DNA replication control. The Py genome has been reported to act as a sensitive cell-specific replicon, which becomes activated for viral DNA replication and viral gene expression during very early stages of myoblast differentiation (23, 48). Like cellular DNA replication, Py DNA replication is also highly sensitive to specific inhibitors (ara-C and aphidicolin) of alpha-like DNA polymerases (alpha, delta, and epsilon) in both in vivo and in vitro replication systems (18, 20, 43) and provides a sensitive system to examine DNA replication during myoblast differentiation in the presence or absence of aphidicolin or ara-C. We report here defined differentiation conditions for observing a distinct type of aphidicolin-resistant Py DNA replication.

We also reexamine the role of DNA synthesis (not mitosis or genomic DNA replication) in myoblast differentiation and how this relates to the permissiveness of these cells to Py DNA replication. This article examines a prediction of a general model for gene control concerning the linkage of DNA synthesis and transcriptional control (72), i.e., that Py and a subfraction of cellular DNA should undergo a special (amitotic and aphidicolin- and ara-C-resistant) form of DNA synthesis occurring early during the myoblast terminal-differentiation process.

We now present evidence for a novel mode of cellular DNA synthesis, specific to an early time window (prior to differentiation-specific gene expression changes) during induced differentiation of myoblasts in culture, which is also resistant to the alpha-like DNA polymerase inhibitors. This DNA synthesis does not lead to mitosis, may be subgenomic, and involves sequences which hybridize more strongly to polyadenylated RNA sequences than the repeated genes of poly(A)$^-$ sequences. These results renew the possibility that DNA synthesis (in the absence of mitosis) plays a role in the differentiation of myoblasts and their permissiveness for Py replication.

MATERIALS AND METHODS

Cell lines. All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and either fetal bovine serum or other serum supplementation as specified. The C2C12 myoblasts were obtained from the American Type Culture Collection. This subclone of C2 cells (84) was derived by H. Blau (62) and was grown in 10% fetal bovine serum–DMEM under continually subconfluent conditions. Only low passage numbers (less than 12) from the American Type Culture Collection seed stock were used for these studies. Differentiation to myoblasts was induced by a change to 2% horse serum–DMEM (differentiation medium) at confluence or at specific degrees of subconfluence. The Aza-C1B myoblast cell line (obtained from P. Jones [31]) was maintained and induced to differentiate in identical fashion. Murine 3T3 and 3T6 fibroblasts were maintained in 5% FBS–DMEM.

Viral stocks. The Py A2 strain was used as the wild type. Viral stocks were grown by low multiplicity infection of 3T6 cells, and titers were determined by plaque assay on 3T6 cells. The multiplicities of infection used in myoblast experiments were typically 10 to 20, unless otherwise stated.

DNA polymerase inhibitors. Aphidicolin and ara-C were obtained from Sigma. An analysis of the concentration dependence of DNA synthesis-inhibitory activities of these two alpha-like polymerase inhibitors showed that for both, 2 μg/ml was more than required for maximal effect in the cell lines used (and was similar to concentrations used in many previous myoblast studies [14, 53, 69]). Exceptions to this are the aphidicolin resistance conditions described in the Results section, and under these conditions similar results were observed with 10-fold-higher inhibitor levels (20 μg/ml). In all cases (even when resistance was seen), the inhibitors rapidly stopped all cell mitosis.

RNA dot blot hybridization. Cytoplasmic RNAs were isolated by cell lysis with nonionic detergent in the presence of urea as described previously (12). Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography (59). For RNA dot blot hybridization analysis, indicated quantities of RNA [total, poly(A)$^+$, or poly(A)$^-$ RNA] were bound to Zeta-Probe nylon membrane (Bio-Rad) under alkaline annealing conditions (manufacturer's protocol) and hybridized with ^{32}P-labeled DNA. For myoblast differentiation markers (Fig. 1), the probes were in vitro labeled by random priming (59) of myosin light chain or MyoD DNA fragments from the

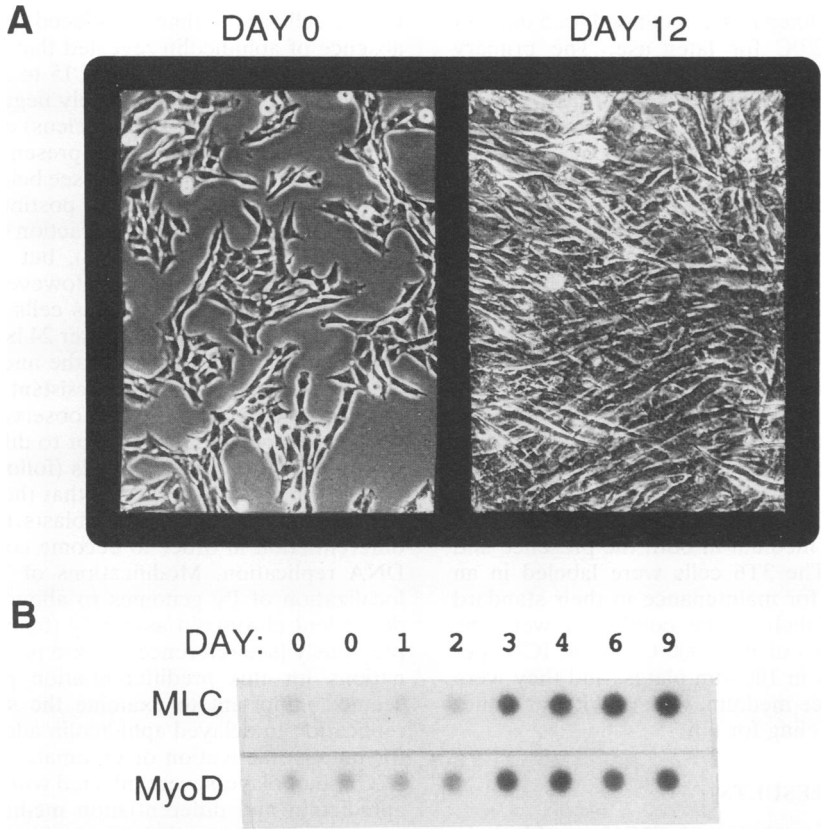

FIG. 1. C2C12 myoblast differentiation in culture. (A) Phase-contrast micrographs of rapidly dividing myoblasts (day 0) and fused multinucleate myotubes (day 12). Differentiation was induced at day 1 (for day 0, $T = 0$ h, in duplicate; for day 1, $T = 24$ h, at confluence) by changing to 2% horse serum–DMEM. (B) Total RNA (2 μg) from the indicated times of differentiation was hybridized with DNA probes for the differentiation marker myosin light-chain (MLC) and MyoD genes.

plasmids pVZLC2 and pVZc11α, respectively, provided by H. Weintraub (University of Washington, Seattle). For the RNA dot blots shown in Fig. 7B, the in vivo-labeled probes were purified ^{32}P-labeled genomic DNAs from either 3T6 or C2C12 myoblast cells, under conditions as described in the figure legends. DNA purification included detergent extraction and RNase A and proteinase K treatment followed by multiple phenol extractions and ethanol precipitations. A portion of this DNA was then analyzed by restriction analysis and electrophoresis on 1% agarose–Tris-borate-EDTA gels and then by gel drying and direct exposure to X-ray film. For hybridization purposes, the relevant in vivo-labeled genomic DNAs from 3T6 or C2C12 plus aphidicolin cells were sheared by sonication to an average size of 300 to 800 bp before equivalent numbers of counts were hybridized (45°C; 50% formamide; 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) to 3T6 or C2C12 RNA dot blots, respectively.

Southern analysis of Py DNA. Episomal Py viral DNA was quantitated by Southern analysis following viral infection of C2C12 cells with aphidicolin either not added or added at specific times. At 48 h postinfection, low-molecular-weight DNA was isolated by Hirt extraction (34) and purified as described previously (11, 34). Aliquots of low-molecular-weight extracts from equivalent numbers of cells were cut with *Bam*HI and subjected to Southern analysis. Briefly, electrophoresis on 1% agarose–Tris-borate-EDTA gels was followed by capillary transfer to Zeta-Probe nylon membranes and UV cross-linking. Hybridization was to random-primed, ^{32}P-labeled Py DNA (50°C; 50% formamide; 6× SSC), and exposure was to Kodak XAR-5 X-ray film with Cronex Lightning-Plus intensifying screens. Exposures in the linear range of grain density were scanned with a densitometer and calibrated against reconstructed copy number controls included on each Southern blot. Additional matched sets of cell culture samples were analyzed by Southern blotting, Py DNA in situ hybridization, and by immunofluorescence for the Py large T-Ag protein (under slightly varying cell growth and confluency conditions) many times with similar results.

In situ hybridization. Py DNA was detected in individual cells as described previously (49) by using a Py DNA probe directly conjugated to horseradish peroxidase (custom probe made by DiGene Diagnostics, Silver Spring, Md.) and by histochemical visualization with color development with diaminonobenzedine and hydrogen peroxide. Tissue culture cells were grown on glass slides, rinsed with saline or phosphate-buffered saline (PBS), fixed in 95% ethanol at 4°C for 5 min, and stored at −20°C until later hybridization. Denaturation, hybridization, and washing conditions followed the manufacturer's recommendations, and uninfected control samples routinely gave virtually undetectable background signal.

Indirect immunofluorescence. Py T-Ag was detected with the use of a rat polyclonal antibody provided by W. Eckhart (Salk Institute, La Jolla, Calif.). Cells grown on glass slides

were rinsed with PBS and fixed in 4°C methanol for 5 min, air dried, and stored at −20°C for later use. The primary antibody was diluted 1:50 in PBS with 3% bovine serum albumin (BSA) (PBS-BSA) and absorbed overnight at 23°C in a humid chamber. Samples were briefly washed for 15 min in PBS-BSA and again in PBS. The secondary antibody (fluorescein isothiocyanate–goat anti-rat immunoglobulin G; Sigma) was diluted per the manufacturer's advice and absorbed for 1 h and washed as described above. Slides were mounted in PermaFluor mounting medium (Immunon, Inc.) and photographed on a Nikon Optiphot-2 microscope.

Aphidicolin effect on [^{3}H]thymidine and ^{32}P incorporation in 3T6 and C2C12 cells. C2C12 cells on six-well plates (Corning) were incubated in differentiation medium for 3 h, pretreated (or mock treated) with aphidicolin for another hour, and treated with [^{3}H]thymidine at 0.1 μCi/ml (6.7 Ci/mmol; ICN) with aphidicolin for the indicated times (either 2 or 6 h) before harvest to label DNA synthesis. Later time points are not shown, since label incorporation into the C2C12 cells dropped rapidly to near zero after 10 h in the low-serum differentiation medium in both the presence and absence of aphidicolin. The 3T6 cells were labeled in an identical manner, except for maintenance in their standard growth media. For ^{32}P labeling, the conditions were the same, except that 0.35 mCi of P_i (7,000 Ci/mmol; ICN) per ml was used to label cells in 100-mm plates, and they were incubated in phosphate-free medium, with or without aphidicolin, for 1 h prior to labeling for 6 h.

RESULTS

Timing of induced differentiation of C2C12 myoblasts in culture. The C2C12 myoblasts (and later 5-aza myoblasts) were induced to differentiate at confluence, which allows fusion and formation of multinucleate myotubes (Fig. 1A). RNA dot blot analysis (Fig. 1B) for differentiation-specific genes (myosin light chain and the MyoD regulatory transcription factor genes) shows a significant increase in these RNA levels after 1 to 2 days following differentiation induction.

Py DNA replication in differentiating C2C12 myoblasts: timing and aphidicolin sensitivity. To establish that addition of aphidicolin normally blocks Py DNA replication in permissive cells, 3T3 cells were infected with a high multiplicity of infection of Py in the presence of concentrations of aphidicolin of either 2 or 20 μg/ml. Essentially, all Py DNA replication was blocked, even when the drug was added 12 h postinfection (Fig. 2A through C). The observed sensitivity of DNA synthesis to aphidicolin agrees with previous reports (18, 20, 43, 50) and also establishes both that Py DNA replication was not detectable at 12 h postinfection and that aphidicolin is rapidly taken up at this time, preventing the onset of significant Py DNA replication.

C2C12 cells were also infected with a high multiplicity of Py and induced to differentiate in the presence of 2 (or 20) μg of aphidicolin per ml. Under these conditions (simultaneous infection and aphidicolin addition), Py DNA replication was completely blocked, as determined by in situ hybridization (data not shown) or Southern analysis (Fig. 3, lane 2) for Py DNA. Noninfected C2C12 controls exposed to aphidicolin (or ara-C) and differentiation medium at confluence decreased [^{3}H]thymidine incorporation by 93% within 1 h of drug addition without apparent inhibition of subsequent cellular differentiation (data not shown), in accord with previous reports (7, 14, 53, 69).

By in situ hybridization analysis, however, Py infection of C2C12 cells at the time of induced differentiation and in the absence of aphidicolin revealed that intense Py DNA replication occurred in only about 15 to 20% of the nuclei. The remaining cells were completely negative for Py DNA (sensitivity, 10 to 20 copies per nucleus) despite a high multiplicity of infection and despite the presence of T-Ag (detected by indirect immunofluorescence [see below]) in the large majority of cells. An increase in the postinfection incubation time to 96 h usually increased the fraction of Py-replicating nuclei about twofold (to 30 to 40%), but the majority of nuclei always remained refractory. However, if subconfluent, rapidly dividing C2C12 myoblasts cells were infected with Py and induced to differentiate after 24 h or more, we could find Py replication in nearly all of the nuclei (20a).

Conditions for aphidicolin-resistant Py DNA replication in differentiating myoblasts. Our observations that infection of nonpermissive myoblasts (prior to differentiation) increased the number of permissive cells (following induced differentiation) led us to hypothesize that the Py genome may need to be present in cycling myoblasts for some time prior to differentiation in order to become configured for high-level DNA replication. Modifications of T-Ag, specific nuclear localization of Py genomes to allow replication, and time-dependent chromatin assembly (for which we have argued previously [see reference 72]) are possible theoretical explanations for this predifferentiation phenomenon. Thus, it seemed important to examine the sensitivity of Py DNA replication to delayed aphidicolin addition to allow a period for putative activation or chromatin assembly. If confluent C2C12 monolayers were infected with Py and the addition of aphidicolin and differentiation media was delayed for between 9 and 11 h postinfection, a small and variable fraction (1 to 0.1% or less) of cells was observed with a distinctive punctate nuclear pattern of Py DNA synthesis. By varying the culture conditions and timings of Py infection, cellular differentiation, and aphidicolin addition, it was observed that low (fewer than 12)-passage C2C12 myoblasts were found to reliably show aphidicolin-resistant Py DNA replication in about 15% of cells. Infecting cycling, 50% subconfluent cells and then inducing them to differentiate and treating them with aphidicolin after a 9- to 11-h delay reproducibly gave the most aphidicolin-resistant Py DNA synthesis (Fig. 2F and I). It should be noted that induction of differentiation in subconfluent myoblasts is less efficient than confluent induction, with a variable fraction of myoblasts, typically 10 to 30%, becoming positive for differentiation markers when induced under similar subconfluent conditions (20a, 33, 44). In general, the aphidicolin-resistant Py DNA replication showed a distinctive subnuclear pattern, not seen in 3T6 cells, that is reminiscent of cellular DNA replication centers. In contrast, if C2C12 cells were infected at ~100 or 90% confluence, we saw only a small fraction of Py-replicating nuclei in the presence of aphidicolin, even with delayed drug addition (Fig. 2D and E). The kinetics showing an increasing number and intensity of aphidicolin-resistant Py DNA replication centers is presented later in Fig. 5. Thus, the ability of C2C12 cells to replicate Py DNA in the presence of aphidicolin corresponds to a very early event in the induced differentiation of these cells, even though Py replication, once it is started, can continue over several days.

Southern analysis of aphidicolin-resistant Py DNA replication. Southern analysis of Py DNA replicated in the presence of aphidicolin is shown in Fig. 3. In the positive (drug-free) controls, infected myoblasts (induced to differentiate at 11 h) had an average copy level of about 27,000 Py genomes per cell, and only about one-third of the cells were Py positive by

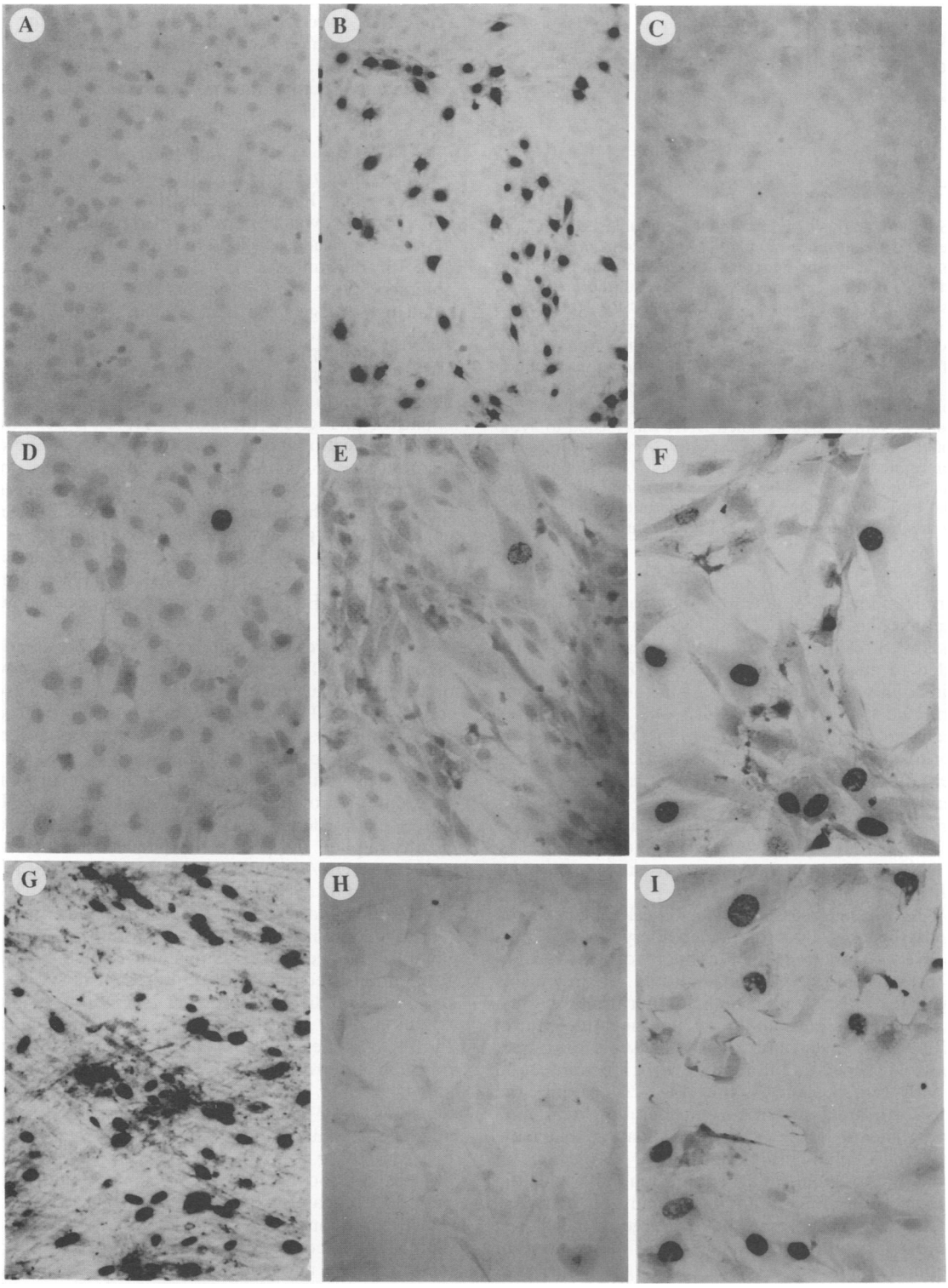

FIG. 2. Histochemical in situ hybridization analysis of Py DNA replication. All panels (except A) show cells infected with Py, harvested at 48 h postinfection (except H), and hybridized with a Py full-length probe. Shown are 3T3 fibroblasts that are either uninfected (A), infected in the absence of inhibitors (B), or treated with 2 μg of aphidicolin per ml at 12 h postinfection (C); C2C12 myoblasts treated with aphidicolin and differentiation medium at 11 h after Py infection of cells at either ~100% (D), ~90% (E), or ~50% (F) confluence; C2C12 cells infected at ~50% density and induced with differentiation medium at 11 h (G to I). Cells shown in panels G and H received no aphidicolin and differ only in harvest time (48 [G] and 11 [H] h postinfection). Cells shown in panel I received aphidicolin at 11 h postinfection, as did those shown in panel F.

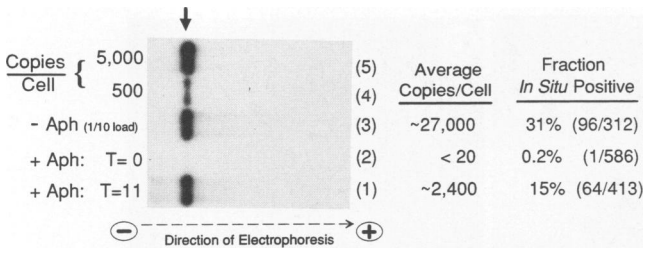

FIG. 3. Southern analysis of differentiating myoblasts replicating Py in the presence and absence of aphidicolin. Matched monolayers of C2C12 cells (from the same experimental set shown in Fig. 2G to I) were used to quantitate episomal Py DNA by Southern analysis. The arrow shows the position of 5.3-kb linearized Py genomes, as seen in the reconstructed copy number control lanes. In all cases, conditions were exactly as described in the legend to Fig. 2, with Py infection of 50% confluent monolayers, differentiation medium added at 11 h postinfection, and aphidicolin added (+Aph) at the indicated hours postinfection or not added (−Aph). In lane 3, the no inhibitor positive control, 1/10 as much DNA was loaded to avoid saturation of the membrane-binding capacity. Average copies per cell were determined by densitometric scanning. The fractions that were in situ positive for Py DNA were determined by counting randomly chosen fields from the matched experimental monolayers analyzed by in situ hybridization (as in Fig. 2G through I).

in situ hybridization (Fig. 3, lane 3). A control culture which received aphidicolin at time (T) = 0 h (simultaneous with infection) showed residual input inoculum viral DNA which corresponded to about 20 copies per cell (Fig. 3, lane 2). When aphidicolin was added at T = 11 h, the average copy level determined by Southern analysis was approximately 2,400 per cell, and 15% of the cells were Py DNA positive by in situ hybridization (Fig. 2I and 3, lane 1). By combining these observations, we can deduce that the average copy number per permissive cell is over 15,000 Py genomes. This level of drug-resistant Py DNA synthesis is, therefore, a large net increase in Py episomal DNA explainable by full-length DNA replication and is not consistent with repair DNA synthesis.

Py large T-Ag is expressed in most C2C12 cells. Because the large T-Ag is the only Py protein necessary for Py DNA replication, we used indirect immunofluorescence to examine T-Ag production in parallel cultures of myoblasts matched with the hybridization and Southern analysis just described. Figure 4A shows an uninfected, negative control field of C2C12 myoblasts, and Fig. 4B shows a drug-free, positive control induced to differentiate at 11 h postinfection (compare with Fig. 3, lane 3, and Fig. 2G). While the intensity of T-Ag staining varies considerably, nearly all cells show significant levels of T-Ag, indicating that most cells are infected. Aphidicolin added at T = 0 (the time of infection) did not prevent T-Ag expression, although the levels are lower (Fig. 4C). In addition, many of the T-Ag-positive nuclei were observed to have a large or ballooning appearance. Aphidicolin added at 11 h after the addition of differentiation medium shows that the large majority of nuclei are positive, with more than half having high levels of T-Ag (Fig. 4D and E), even though only about 15% were apparently replicating Py DNA under these conditions (compare with Fig. 2I). A higher magnification of the cells treated with aphidicolin for 11 h shows patches of intense subnuclear localization for T-Ag (superimposed on a more diffuse pattern of T-Ag [Fig. 4E]), reminiscent of the pattern seen for aphidicolin-resistant Py DNA replication (Fig. 4F).

Ara-C-resistant replication, Aza-C1B myoblasts, and Py replication centers. Since ara-C is also a specific inhibitor of alpha-like polymerases and has been often used to inhibit DNA synthesis, we also examined whether Py DNA would replicate in differentiating myoblasts in the presence of ara-C under conditions which allow aphidicolin-resistant Py DNA replication. In addition, we examined a separate line of myoblasts, the Aza-C1B line (derived from 10T1/2 cells by 5-aza cytidine treatment [31]) to determine whether aphidicolin-resistant Py DNA replication was specific to C2C12 cells. Py DNA was observed to also replicate in the presence of ara-C (at either 2 or 20 μg/ml) under these conditions (Fig. 5A). In addition, the Aza-C1B myoblast cell line exhibits aphidicolin (or ara-C)-resistant Py DNA replication in a small percentage (less than 5%) of the cells and shows a similar punctate pattern of nuclear localization by in situ hybridization (Fig. 5B).

Closer examination of these aphidicolin-resistant Py replication centers in C2C12 cells at various times postinfection is shown in Fig. 5C through F. By 19 h postinfection, Py DNA replication becomes detectable as numerous small, discrete sites of intense signal, uniformly distributed over some of the nuclei (Fig. 5C). At later times, there is a general increase in both the average number and size of replication centers, although some pattern heterogeneity is also seen (Fig. 5E and F). By 57 h, many of the nuclei appear to have intensified and merged signal in the large majority of the positive cells (Fig. 5D), consistent with continued Py DNA replication.

Aphidicolin-resistant cellular DNA synthesis in differentiating myoblasts. The experiments described above were based on the view that Py DNA replication might be a useful model for examining DNA replication control during cellular differentiation. Using Py, we have defined conditions during which there appears to exist a distinct aphidicolin resistance process of viral DNA replication that occurs early in the differentiation of C2C12 myoblasts. Since Py DNA replication is heavily dependent on cellular replication proteins, our results implied the possible existence of a distinct cellular process of DNA synthesis occurring early in the differentiation of C2C12 myoblasts. Previously, others have reported that both aphidicolin and ara-C are efficient inhibitors of [^{3}H]thymidine incorporation (in various cell lines, including myoblasts). Therefore, we examined whether cellular DNA from uninfected myoblast cultures also shows a distinct period of aphidicolin-resistant DNA synthesis during differentiation under conditions analogous to those determined for Py DNA synthesis. Representative tritiated thymidine incorporation patterns for either 2- or 6-h labeling periods are shown in Fig. 6. With cultures at the indicated initial densities, the 3T6 cells showed the expected near-complete aphidicolin inhibition of [^{3}H]thymidine incorporation; however, in strong contrast, the C2C12 cells newly induced to differentiate showed only a very slight inhibition during this period in repeated experiments under a variety of subconfluent conditions (Fig. 6), even though mitosis was completely blocked. In undifferentiated C2C12 myoblasts, [^{3}H]thymidine incorporation was often extremely sensitive to aphidicolin (Fig. 6), as reported by others. Although this sensitivity was frequently observed, there was some variability in these undifferentiated C2C12 cells. At times, we saw aphidicolin-resistant [^{3}H]thymidine incorporation (averaging about 30%) in some cultures. After extensive repetition and controls to rule out artifactual explanations, such as differences in C2C12 culture conditions or lineages, aphidicolin stocks, or experimental protocols, we have surmised

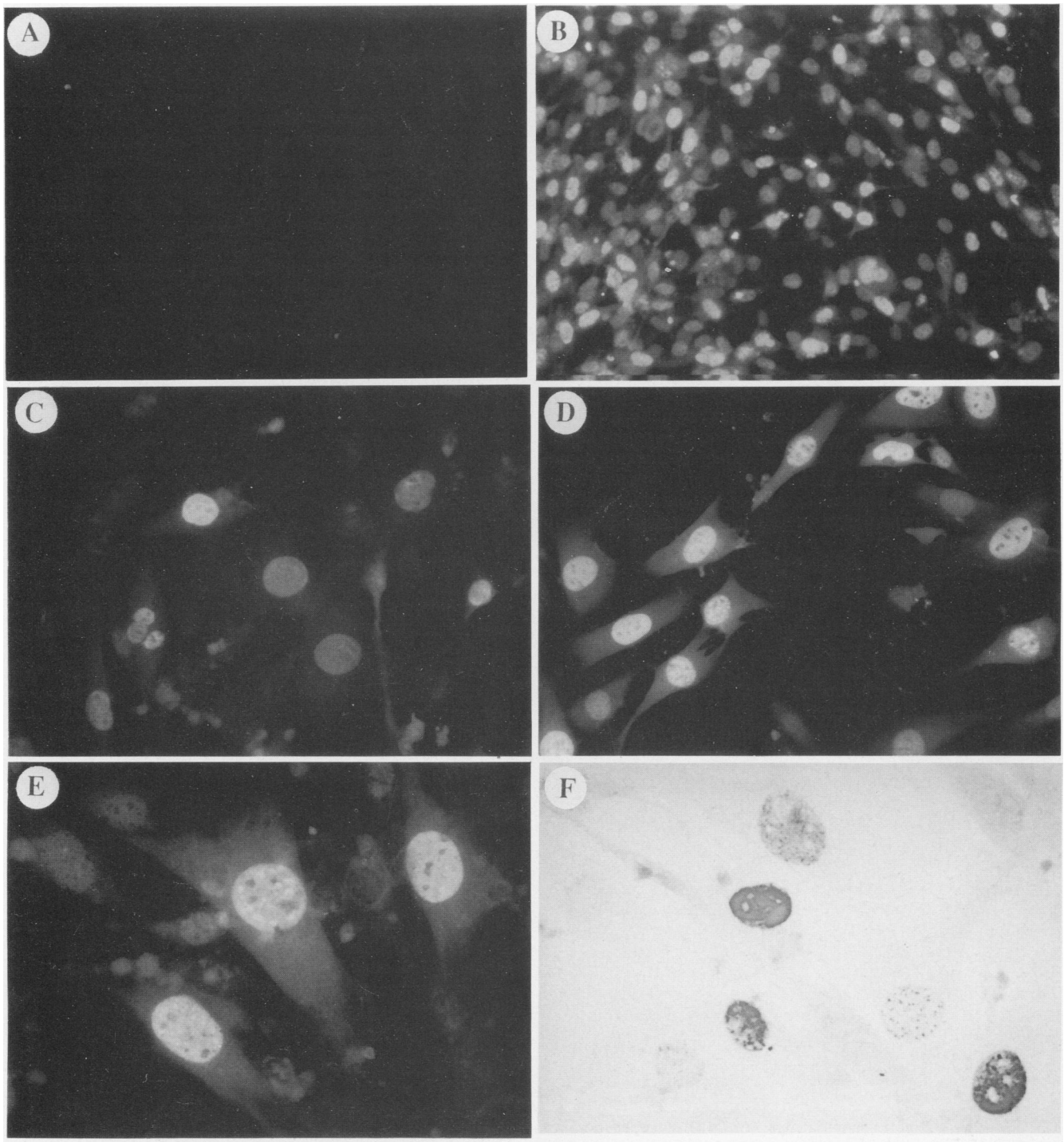

FIG. 4. Py T-Ag immunofluorescence and comparison with Py DNA subnuclear localization. (A through E) Matched C2C12 monolayers (from the experimental set shown in Fig. 2 and 3) were incubated with a polyclonal antibody to Py T-Ag and stained with a fluorescein-conjugated secondary antibody. (A) Uninfected control; (B) inhibitor-free, Py-infected positive control. Aphidicolin addition was at either $T = 0$ (C) or $T = 11$ (D and E) h postinfection. (F) Py DNA localization by in situ hybridization under conditions and magnification matched to those described for panel E.

that the persistent variability is probably due to variable fractions of C2C12 myoblasts which have spontaneously committed to differentiation. In summary, our result indicates a defined period, very early in the induction of myoblast differentiation and prior to the time of expression of differentiation marker genes, during which aphidicolin-resistant cellular DNA synthesis is occurring which does not lead to mitosis.

Distinctive properties of in vivo-labeled differentiating myoblast DNA. To further investigate this aphidicolin-resistant

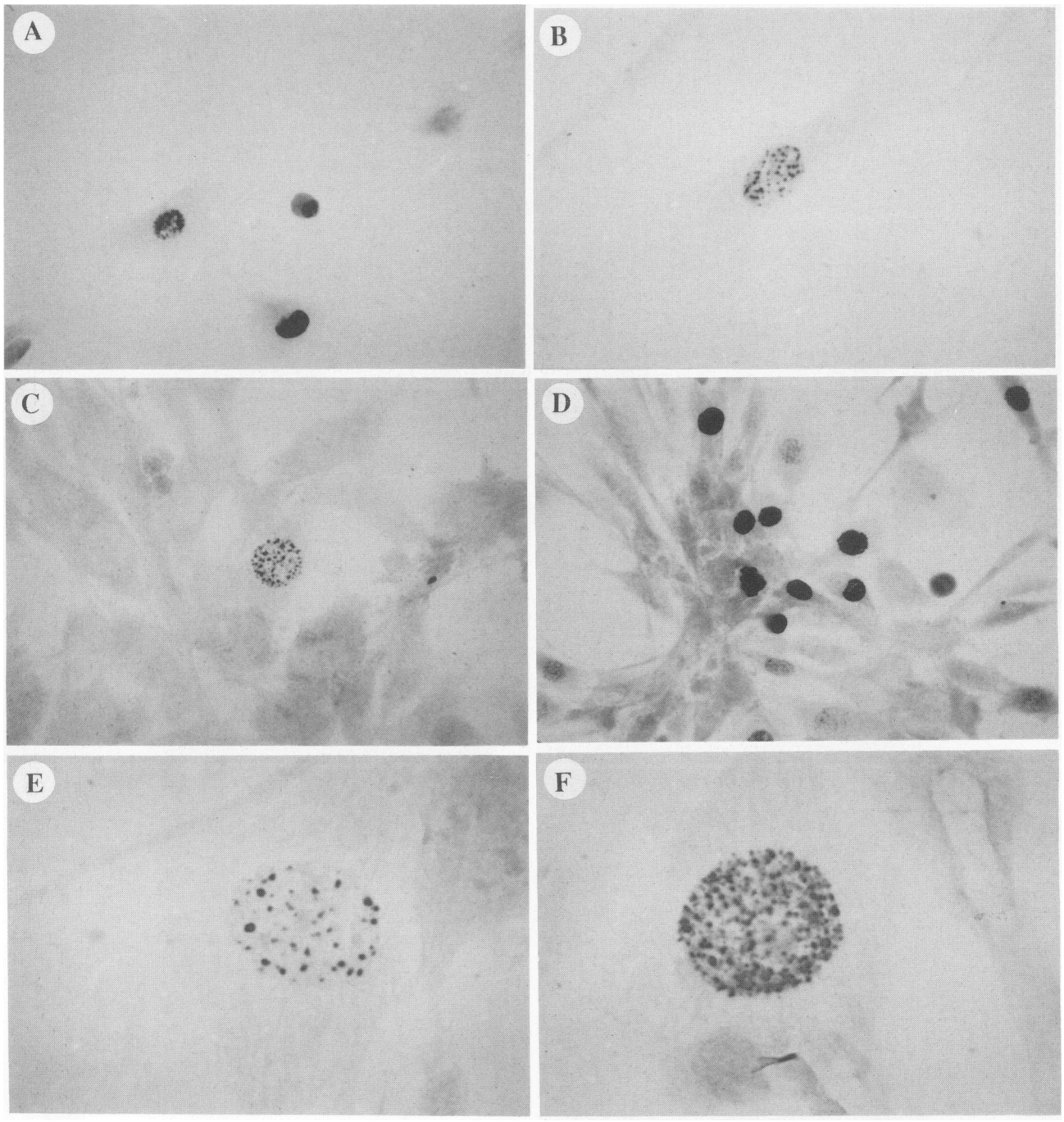

FIG. 5. In situ hybridization analysis of Py DNA replication in the presence of ara-C or aphidicolin and in Aza-C1B as well as C2C12 myoblasts. Differentiation medium and either aphidicolin or ara-C were added at 8 to 11 h postinfection in all cases. (A) Ara-C (2 μg/ml) added to C2C12 cells; (B) Aza-C1B myoblasts treated with aphidicolin; (C through F) Py DNA subnuclear localization in C2C12 cells at specified times between 19 (C) and 57 (D) h postinfection. Panels E and F represent lighter versus heavier patterns seen at 48 h postinfection, under 3.3× higher magnification (oil immersion objective).

C2C12 cellular DNA synthesis, we labeled either control 3T6 and undifferentiated or differentiating C2C12 myoblast DNA in vivo with $^{32}P_i$ for 6 h, in the presence or absence of aphidicolin. Genomic DNA was purified and used for restriction analysis. Figure 7A shows autoradiography patterns of this *Eco*RI- or *Kpn*I-cut DNA. A highly repetitive band (~1.4 kb) which is characteristic of mouse genomic DNA is visible in all *Eco*RI-cut lanes. The relative lack of aphidicolin inhibition of DNA synthesis in the myoblasts that were newly induced to differentiate (but not in undifferentiated myoblasts or 3T6 cells) was again apparent since the intensity of the signal remained relatively high in the presence of the drug. The surprising result was that the *Eco*RI-cut DNA from differentiating myoblasts gave a strikingly banded

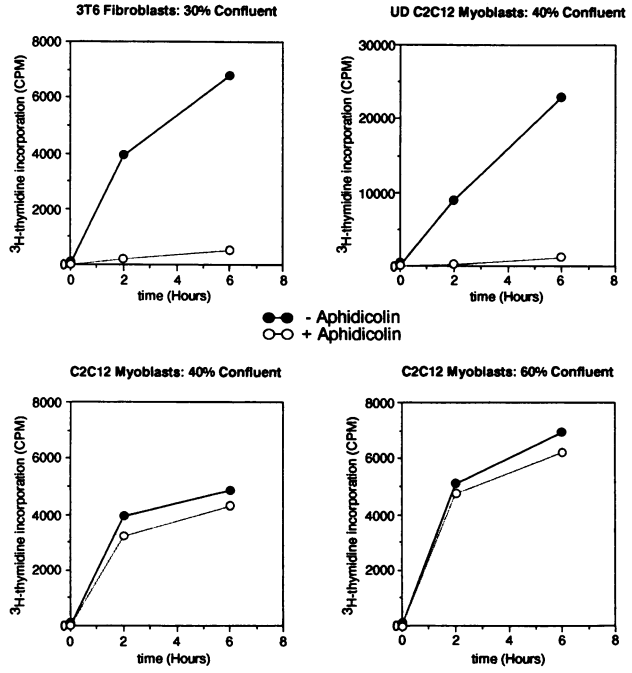

FIG. 6. [^{3}H]thymidine incorporation in 3T6 cells versus differentiation-induced myoblasts. The 3T6 fibroblasts and undifferentiated (UD) C2C12 myoblasts were maintained under subconfluent conditions in their normal growth medium. The differentiating C2C12 cells were changed to 2% horse serum–DMEM (differentiation medium) at the specified cell densities. Aphidicolin was either added or not added at 3 h post-differentiation induction (or mock induction), and [^{3}H]thymidine was added beginning at 4 h postinduction (T = 0 on the y axis).

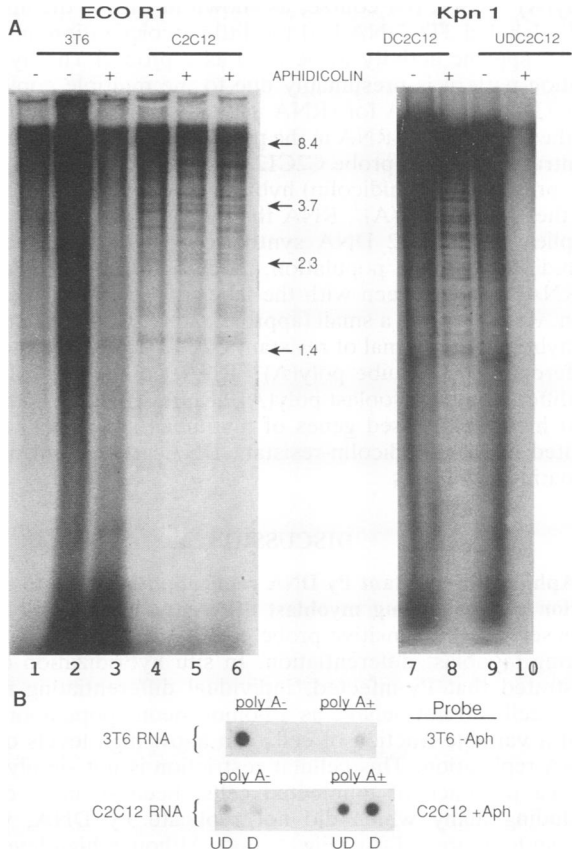

FIG. 7. Restriction and dot blot analysis of in vivo ^{32}P-labeled DNAs from 3T6 versus differentiating C2C12 cells. (A) Autoradiographs of purified genomic DNAs, in vivo labeled for 6 h in the presence or absence of aphidicolin, cut with *Eco*RI or *Kpn*I (separate labeling experiments), and run on a 1% agarose gel. Lanes 4, 5, and 6 are undifferentiated C2C12 cells. Undifferentiated C2C12 cells were labeled in normal high-serum growth medium, as described in the legend to Fig. 6. In lanes 2 through 10, approximately 1/50 of the DNA isolated from each 100-mm plate was analyzed. (Lane 1 is a 1/10 load of the overexposed sample in lane 2.) Lane 5 and 6 are from duplicate, independent in vivo labeling experiments. Arrows indicate molecular weight marker band positions (in kilobases). (B) RNA dot blot analysis with probes (3T6 cells without aphidicolin [−Aph] or C2C12 cells with aphidicolin [+Aph]) labeled in vivo (as described for panel A) which were hybridized with 2-μg spots of the indicated RNAs. UD and D, undifferentiated and differentiated C2C12 cell RNAs, respectively.

pattern instead of a uniform smear characteristic of whole genomic DNA replication, as seen with the 3T6 DNA. In some experiments, this banded DNA pattern was virtually unchanged by the addition of aphidicolin (Fig. 7A, lanes 5 and 6 versus 4), whereas in other experiments there was a reduction in the background smear of incorporation, while the restriction enzyme specific banded patterns remained relatively unchanged. This pattern was reproducible in differentiating myoblasts and sometimes faintly seen in undifferentiated myoblasts (which suggests to us a subfraction of committing cells). This banded pattern was not seen in DNA from 3T6 or other cell lines, and it appears to suggest that specific regions of the myoblast DNA become labeled in differentiating myoblasts. Although this myoblast DNA labeling pattern showed resistance to aphidicolin, it was observed in the absence of aphidicolin, suggesting that such a pattern is not induced by drug treatment. Restriction enzymes other than *Eco*RI were also used, including *Kpn*I (Fig. 7A), *Pst*I, *Hin*dIII, and others (data not shown), and each gave a distinctive characteristic banded pattern visible in the autoradiographs (but never in ethidium bromide-stained gels) from DNA labeled in myoblasts newly induced to differentiate. DNase treatment eliminated all high-molecular-weight radioactive signal, including all traces of the banded pattern (data not shown).

Preferential hybridization of labeled DNA to polyadenylated RNA. We hypothesized that DNA for active genes, or those being newly activated, rather than random or highly repetitive DNA, for example, might become labeled in the differentiating myoblasts. To test this, RNA dot blots were performed with probe DNA which had been labeled in the presence of aphidicolin in differentiating C2C12 cultures. As a control, in vivo-labeled probe DNA from 3T6 cells minus aphidicolin was used. As illustrated in Fig. 6, 3T6 DNA synthesis is highly sensitive to aphidicolin, and the resulting low specific activity precluded its use as a control probe. RNAs for the dot blot were isolated from 3T6 cells and from either undifferentiated myoblasts or 12-day-differentiated myotubes, and they were selected on oligo(dT) cellulose for poly(A)$^-$ and poly(A)$^+$ fractions. The probe DNAs were sheared by sonication and then hybridized and washed at conditions for high stringency. The results displayed in Fig. 7B show that the probe 3T6 without aphidicolin hybridized much more strongly to the poly(A)$^-$ RNA than to the

poly(A)$^+$ RNA. (Of course, as shown in Fig. 6, the aphidicolin-labeled 3T6 DNA had too little incorporation and too low a specific activity to be used as a probe.) The hybridization pattern is presumably due to the multiple copies in the 3T6 probe DNA for rRNA and tRNA genes hybridizing to the predominant RNA in the poly(A)$^-$ fraction. In striking contrast, the DNA probe C2C12 with aphidicolin (labeled in the presence of aphidicolin) hybridized much more strongly to the C2C12 poly(A)$^+$ RNA than to poly(A)$^-$ RNA. This implies that C2C12 DNA synthesized in the presence of aphidicolin is, as a population, much better represented in mRNA than that seen with the labeled 3T6 DNA. In addition, we also found a small (approximately twofold) increase in hybridization signal of resistant C2C12 probe DNA to the differentiated myotube poly(A)$^+$ RNA, compared with the undifferentiated myoblast poly(A)$^+$ RNA. This may indicate that highly expressed genes of myotubes are better represented in the aphidicolin-resistant DNA probe from differentiating myoblasts.

DISCUSSION

Aphidicolin-resistant Py DNA replication localized to replication centers during myoblast differentiation. Initially, Py was served as a sensitive probe of DNA replication control during myoblast differentiation. In situ hybridization demonstrated that Py-infected, individual differentiating myoblast cells do not behave as a homogeneous population but that a variable fraction of cells can allow high levels of Py DNA replication. This cellular restriction is not simply due to the presence of uninfected cells, because most cells, including many which did not replicate Py DNA, were shown to express T-Ag (Fig. 4) (4a). Although high-level Py DNA replication is dependent on myoblast differentiation (23), it appears that some additional aspect of cell differentiation is involved, since only cells infected prior to differentiation were uniformly and highly permissive. These observations led us to explore various predifferentiation conditions for the replication of Py DNA in the presence of aphidicolin and allowed us to define an 8- to 11-h period postinfection which must occur prior to addition of aphidicolin to achieve maximally drug-resistant Py DNA replication. In addition, subconfluent cells recently induced to differentiate by the addition of mitogen-poor medium were also necessary for high levels of aphidicolin-resistant Py DNA replication. We have not further explored the basis of this delayed aphidicolin action but propose that it could be due to a time requirement for the expression and/or modification of T-Ag or replication-linked assembly of an active viral chromatin. We favor this latter proposal, because we hypothesize that the Py genome may need to replicate during an early window of myoblast differentiation in order to assemble for aphidicolin-resistant synthesis; because of asynchronous induction of cellular differentiation, only a fraction of cells will tend to become permissive for Py replication.

Aphidicolin-treated myoblasts had centers of Py DNA hybridization signal beginning as small, intense, uniformly distributed nuclear foci, which increased in number and size with time, eventually merging by 3 days postinfection in many cells. Such a pattern of Py DNA synthesis is distinct from that seen with permissive 3T6 cells and is a characteristic of aphidicolin-treated (but not untreated) differentiating C2C12 and Aza-C1B myoblast cells (4a). This replication pattern is also very similar to the punctate replication centers seen with bromodeoxyuridine pulse-labeling on entry into S phase of cultured rat fibroblasts (38) and somewhat similar to patterns reported for simian virus 40, herpesvirus, and adenovirus (17, 60, 73, 76).

It seemed that a subpopulation of differentiating myoblasts could be defective in the uptake of aphidicolin and ara-C. Selection for aphidicolin-resistant mutant cells usually results in isolation of cell strains containing elevated levels of intracellular pools of deoxynucleotide triphosphates, or sometimes in mutations in alpha polymerase (for reviews, see reference 25). However, the aphidicolin-resistant Py DNA replication that we observed does not appear to be due to such indirect reasons. Such arguments explain neither why a delay time (after Py infection but before aphidicolin-resistant replication is observed) was required nor the need for the induction of cellular differentiation. Also, the observation that the level and pattern of resistant Py DNA synthesis are affected (subnuclear punctate) by the addition of aphidicolin and the observation that all cellular mitosis was prevented suggest that the drug did enter and affect even these resistant cells. Similarly, the near-complete inhibition of both Py and cellular DNA synthesis under most other conditions tested also argues that whatever the mechanism of aphidicolin-resistant Py DNA replication, it is peculiar to a very early period in the induced differentiation of myoblasts.

The combined Southern analysis and in situ hybridization established that 15,000 copies of full-size Py DNA per permissive cell were made in the presence of aphidicolin under the optimal conditions. Since the residual level of unreplicated input DNA was far lower (20 copies per cell remained at the times of the assay), considerable net full-length Py DNA synthesis, and not repair, must have occurred. Although high in individual cells, this net synthesis is still below that seen in the absence of aphidicolin (about 80,000 copies per permissive cell), implying a contribution of alpha-like DNA polymerases to the highest levels of Py DNA synthesis.

Aphidicolin-resistant cellular DNA synthesis in differentiating myoblasts. Under most circumstances that we examined, aphidicolin treatment resulted in an inhibition of [^{3}H]thymidine incorporation into cellular DNA that was greater than 90%, in agreement with prior reports. However, our results with Py DNA replication led us to examine cellular synthesis under conditions defined for aphidicolin-resistant Py DNA replication (subconfluent conditions and at early times after induced differentiation). Under these conditions, we found that C2C12 cellular DNA synthesis was only slightly inhibited during a 6-h labeling period soon after induced differentiation (from between 4 and 10 h following addition of differentiation medium), although subsequent cellular DNA synthesis and all mitosis were completely blocked. One possibility is that this aphidicolin-resistant cellular DNA synthesis involves a gene-specific repair rather than a net synthesis process. Gene-specific repair (which in adipocytes is strongly activated by terminal differentiation) has been reported elsewhere (5, 8). A second possibility is that net cellular (but perhaps not complete genomic) DNA synthesis is also occurring, which could also account for our observation that Py episomes under analogous conditions undergo net synthesis. Although we favor this idea, we have not observed a significant net increase in active cellular gene DNA following C2C12 differentiation by Southern analysis (data not shown). Although this result establishes that high-level amplification of transcriptionally active cellular DNA is not occurring, Southern analysis was insufficiently sensitive to exclude a low-level net synthesis (0.5- to 2-fold) which

could be needed to reassemble gene-specific chromatin. By whatever mechanism (including repair), the short period of distinctive aphidicolin-resistant DNA synthesis that we describe at a very early time following the induced differentiation of C2C12 cells could be involved in clearing chromatin and allowing access to new *trans*-acting factors.

*Eco*RI restriction analysis of the C2C12 DNA made in the presence of aphidicolin showed an unusual pattern of numerous densely banded fragments rather than the homogeneous smear expected (and observed in 3T6 cells) for the synthesis of total cellular DNA. One possible explanation for this is that specific regions of cellular DNA are labeled in differentiating myoblasts, perhaps corresponding to specific origins of replication. When this drug-resistant C2C12 DNA was used as a probe for complementary poly(A)$^+$ and poly(A)$^-$ RNA from undifferentiated and differentiated C2C12 cells and compared with the hybridization of labeled 3T6 DNA to poly(A)$^+$ and poly(A)$^-$ 3T6 RNA, it appeared that DNA complementary to polyadenylated RNA from C2C12 cells is preferentially synthesized in the presence of aphidicolin and is slightly better represented in differentiated myotube polyadenylated RNA. In contrast, the 3T6 DNA probe preferentially hybridized to nonpolyadenylated 3T6 RNA. This result suggests that subgenomic DNA synthesis corresponding to active genes may occur in differentiating C2C12 cells in the presence of aphidicolin. Although it could be more informative to physically isolate and specifically identify the aphidicolin resistance C2C12 DNA sequences, density gradient or immune precipitation isolation with bromodeoxyuridine labeling has proven unfeasible because bromodeoxyuridine blocks terminal differentiation of myoblasts and other cell types (9, 27, 65).

Prior experiments did not result in observations of inhibitor-resistant cellular DNA synthesis. Usually, more efficient induction of myoblast differentiation is achieved with dual triggers of confluence and mitogen-poor differentiation medium, although either alone can start the differentiation process (31). Confluence also allows cell contact and efficient fusion to form multinucleate myotubes. Accordingly, most myoblast differentiation studies have applied polymerase inhibitors to confluent or nearly confluent myoblasts and have seen strong inhibition of [^{3}H]thymidine incorporation (as we also often observed). Therefore, it seems likely that the resistant DNA synthesis that we report here would have already occurred in most prior experiments.

If the replication that we observe is related to gene commitment, this replication occurs before changes in gene expression. To partially address such a possibility, Chiu and Blau (14) used individual cell analysis of populations of heterokaryon cell fusions between murine C2C12 myoblasts and human fibroblastic cells to show that a complete round of DNA replication was not necessary for subsequent gene expression changes (from the fibroblast-derived nuclei in the fused cells). Using ara-C and autoradiography to measure [^{3}H]thymidine incorporation levels in individual cells, they estimated that less than one-fifth of a round of DNA replication had occurred. As noted, this result could not rule out the possibility of subgenomic-limited DNA synthesis, highly specific for the muscle genes; however, this was regarded as unlikely, since "no precedent for localized DNA synthesis in the activation of genes has been described" (14). However, localized DNA synthesis does have precedents in eukaryotes. During the terminal differentiation of chorion genes undergoing endoreduplication in *Drosophila melanogaster* (51, 52, 58) and in processes involving the yeast mating type loci (1, 40), locally replicated DNA appears to become configured for active gene expression. Also, gene-specific DNA synthesis (repair) during adipocyte differentiation has been established previously (5).

Is there a role for alternative DNA polymerases (other than alpha or delta) in differentiation? Although polymerases alpha and delta had been accepted as responsible for all genomic synthesis (excepting repair), there have been increasing suggestions of essential but unknown roles for other polymerases (see reference 45 and references therein). It is possible that a repair-like DNA synthesis process (without mitosis) utilizing aphidicolin-resistant polymerase beta (25, 41) is involved in the resistant DNA synthesis that we have observed and in gene-specific DNA repair (8). Also of interest are reports of aphidicolin-resistant DNA replication (proposed to utilize polymerase beta) in murine cells in vivo during terminal differentiation and endoreduplication of the giant trophoblast cells of the mouse blastocyst (61). Although a specific inhibitor of polymerase beta (i.e., dideoxythymidine) could test this issue, the poor in vivo phosphorylation of this nucleotide analog severely limits its utility (43). Therefore, it also seems possible that beta-like polymerases are participating in the drug-resistant and punctate-localized replication of Py DNA during myoblast differentiation; however, this conclusion will require further biochemical investigation.

The short period of aphidicolin-resistant DNA synthesis that we observed and the requirement for induced differentiation of myoblasts, as well as the banded pattern and unusual hybridization properties of the labeled probe [more representative of poly(A)$^+$ RNA], all appear consistent with the possibility that a newly defined type of subgenomic, amitotic DNA synthesis has been identified. Although we have not established a causal relationship of such synthesis to gene commitment, the timing of this synthesis and the representation of poly(A)$^+$ RNA suggest such an involvement and now question the prevailing view that DNA synthesis cannot be important for myoblast gene commitment. It also appears that such a mode of amitotic, differentiation-linked DNA synthesis may be involved in the replication of Py DNA.

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