Late Transcription and Simultaneous Replication of Simian Adenovirus 7 DNA as Revealed by Spreading Lytically Infected Cell Cultures

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SUMMARY

Miller's technique of spreading DNA was applied to monkey cells productively infected with simian adenovirus 7. This permitted the visualization of cellular DNA transcription, both nucleolar and non-nucleolar, and of late transcription and replication of virus. Virus double-stranded DNA, thin fibres with very few nucleosome-like particles, were observed carrying either transcription or replication complexes. In addition, both RNP transcripts and replication forks were found on some virus duplex DNA. Virus single-stranded DNA replicative intermediates were identified on the basis of their increased thickness and contrast which results from the presence of a DNA binding protein.

INTRODUCTION

The techniques developed by Miller & Beatty (1969) and Miller & Bakken (1972) have been widely used to spread transcription complexes in various cell systems characterized by a high transcriptional activity. More recently, several papers from this laboratory have shown that it is possible to study transcription in somatic mammalian cells by Miller's technique (Puvion-Dutilleul et al. 1977a, b, c, 1978), providing that transcriptional activity is high. We have now employed this approach to study adenovirus-infected mammalian cells which are the site of very active transcription and replication of virus DNA. We chose as the model simian adenovirus 7 (SA 7) because one of us had previously studied the localization of its transcription and replication in ultrathin tissue sections by cytochemical procedures (Moyne et al. 1978).

The interpretation of our results has been facilitated by the work of Kedinger et al. (1978 b). These authors described the special aspect of spread replicative intermediates of adenovirus type 2 which are made up of single-stranded DNA molecules coated with a binding protein. The protein coat explains the thickened configuration of the replicative intermediates. We report here on observations of virus replication and transcription, and demonstrate that both events may occur simultaneously on the same molecule. Miller's technique so far seems to be the only way to demonstrate that these events occur simultaneously. The limited number of such events that we could find restricted this work to a qualitative rather than a quantitative study.

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**METHODS**

*Cells and virus.* Kidney cells of the green monkey, *Cercopithecus aethiops,* were obtained in suspension from the Institut Mérieux (Lyons, France). After cultivation in glass flasks for 10 days at 37 °C in Eagle’s MEM supplemented with 10% calf serum, the cultures were trypsinized. The secondary cultures were grown in 5% CO₂ in 5 cm plastic Petri dishes seeded with 5 x 10⁵ cells in 5 ml of the same medium. After 72 h, the cultures were infected with SA 7 (10 p.f.u./cell) in MEM containing 1% calf serum. The SA 7 strain was initially obtained from Dr F. Rapp (Baylor University, Houston, Texas).

*Autoradiography.* The cells were grown on cover-slips in Leighton’s tubes. Infected and control cells were pulsed for 10 min with 10 μCi/ml ³H-5-uridine (sp. act. 30 Ci/mM, Commissariat à l’Energie Atomique, France) in culture medium. The incubation was ended by treating the cells for 1 min in 0.1 mg/ml non-radioactive uridine in the culture medium. After acetic alcohol fixation and 3 x 10 min washes with 5% trichloracetic acid in water, the cells were dehydrated and the cover-slips were coated with Ilford K2 emulsion. After an exposure of 8 days, the autoradiograms were developed in Kodak D19 and finally stained with May–Grunwald solution.

*Spreading technique and observation.* The cultures were rinsed twice with ice-cold distilled water adjusted to pH 8.8 with borate. The following steps of the spreading procedure were as previously described by Puvion-Dutilleul et al. (1977c), according to Miller & Bakken (1972). After drying in air, the grids were either stained for 1 to 2 min in alcoholic phosphotungstic acid and rinsed in 95% ethanol or were shadow-cast with platinum on a rotating disk at an angle of 6 to 8°. The grids were observed in a Siemens Elmiskop IA electron microscope. Magnifications were checked with a carbon grating replica (Fullam). Measurements of transcription lengths and statistical computations were done as previously published (Puvion-Dutilleul et al. 1978).

**RESULTS**

A preliminary experiment based on light microscope autoradiography was carried out to determine the moment of maximum incorporation of tritiated uridine, considered as a measurement of the transcriptional activity. Control and infected cells were pulsed and autoradiographed as described in the Methods every 2 h from 6 h post-infection (p.i.) until 24 h p.i. The highest activity was observed in cells 20 h p.i. (Fig. 1). The subsequent observations described in this paper were therefore carried out on cells 20 h p.i.

*General aspect of the spread preparations*

Our observations on control, uninfected cells confirmed previous work: the contents of mammalian cell nuclei by Miller’s technique display innumerable DNP fibres extending from the residual, unspread, nuclear constituents. The transcriptionally active DNP fibres appear either as ‘Christmas-trees’, which represent nucleolar transcription, or as transcription units carrying fewer RNP fibrils of irregular lengths, which are interpreted as non-nucleolar transcription. The DNA of the extranucleolar units is generally folded in nucleosomes. Both types of transcription units were visible in control as well as in SA 7-infected cells. In these latter preparations, we observed, in addition, three structural features not visible in control cells: (1) electron opaque granules interpreted to be virus particles; (2) DNP fibres of limited length, entirely or almost entirely devoid of nucleosome-like structures, carrying some RNP fibrils. These molecules were interpreted as double-stranded virus DNA; (3) thick filaments of limited length, interpreted in the light of recent biochemical results (Kedinger et al. 1978b) as single stranded virus DNA.
Fig. 1. Light microscope autoradiographs of \(^3\)H-uridine incorporation. (a) Control cells, (b) cells 20 h p.i. The infected cells are much more heavily labelled than the control cells. Magnification × 720.

A quantitative approach was impossible owing to the small quantity of virus DNA deposited on the grids in comparison with the abundant cellular chromatin. The unambiguous identification of virus DNA required the observation of free ends of the molecule. This criterion could be met only in a few regions of the grid where the density of DNA molecules was low enough to permit an easy tracing of the path of the molecules. It is thus probable that a notable part of the virus DNA was not recognized in areas containing too many
Fig. 2. Transcription of cellular DNA in cells 20 h p.i. Platinum-shadowed preparation. (a) Typical ribosomal transcription unit. The transcribed DNA, covered by RNP fibres is 3.5 μm long. The transcript density is about 29 RNP fibres/μm DNP. (b) Non-ribosomal transcription unit. Note the variable spacing of the transcripts. The arrowhead points to an RNA polymerase molecule, at the base of a nascent RNP fibril. Numerous nucleosomes (arrow) are visible between the transcripts. (c) Higher magnification of a non nucleolar transcription unit. The localized disappearance of the nucleosomes between neighbour transcription complexes is evident. Closely packed nucleosomes are visible on the right side of the micrograph (arrow). The arrowheads show RNA polymerase molecules, larger and more contrasted than the nucleosomes.
molecules. The number of observable virus molecules was also likely to be decreased by the absence of sedimentation of most free virus DNA molecules not mechanically retained by the cellular chromatin.

Transcription complexes of cellular DNA

‘Christmas-tree’-like transcription units characterize transcription of nucleolar ribosomal genes. They were observed in control as well as in infected cells (Fig. 2a) and were morphologically identical in both types of cells. As described in other works, the RNP-covered DNA was always smooth, without nucleosomes, whereas the spacer intercepts occasionally displayed a ‘beads-on-a-string’ appearance. Quantitative differences between ribosomal DNA of normal and infected cells could not be investigated because of the retention of most nucleolar transcription units in the vicinity of the unspread residual nucleus. In this region, DNP fibres are so numerous that analysis of the ‘Christmas-trees’ becomes impossible.

Transcription complexes of cellular non-ribosomal DNA were observed on very long DNP molecules of indeterminable length carrying nucleosomes. In both control and infected cells these transcription units were comprised of a single or several RNP fibrils, either linear or twisted with a bush-like appearance, as already described in other mammalian cells. The base of the transcript was marked by a molecule of RNA polymerase, easily recognized by its high contrast and by its size, characteristically larger than that of nucleosomes (Fig. 2b, c). Here again, we saw no difference between control and infected cells.

Identification and replication of virus DNA

In the infected cells, apart from the very long molecules of cellular nucleosomal DNA, we observed a new type of double-stranded DNA never seen in control cells. These molecules were of limited length (8 to 12 μm) with both extremities easily identified and seemed to be less rigid than the cellular DNA which, in general, is linearly stretched. These molecules were almost entirely smooth, with 0 to 10 particles of nucleosome size per DNA duplex. Their diameter was identical to that of smooth double-stranded cellular DNP molecules. Such results were in favour of a virus origin for the short molecules, but did not prove it. A more stringent demonstration was made by the observation of replication forks carried by these molecules (Fig. 3a). Replication forks were seen neither in control uninfected cells nor on very long nucleosomal DNA molecules in infected cells. The forks were characterized by the presence of a thick filament in contact with a double-stranded smooth DNA molecule. This puzzling structure could be understood owing to the work of Miller & Hodge (1975) and Kedinger et al. (1978b). Thick filaments represent a replicative intermediate containing a single-stranded virus DNA molecule, thickened and shortened by a binding protein playing a presumably protective role. The single-stranded DNA was homogeneously coated by the binding protein and displayed a constant thickness (10.6 ± 2.5 nm after positive staining) along its entire length, starting from the replication site. Free single-stranded molecules were also observed (Fig. 3b) as well as linear molecules containing a transition from single-stranded to double-stranded DNA (Fig. 4b). These latter linear molecules were two or three times less numerous than the DNA molecules containing the replication forks. In some instances, more complex situations occurred (Fig. 4a) in which two replication forks and a prolonged thick filament were carried by the same molecule. The free single-stranded molecules had a mean length of 6.2 ± 3.2 μm.
Fig. 3. Virus DNA; positive stain with alcoholic PTA. (a) A double-stranded virus DNA molecule is characterized by its smooth appearance, without nucleosomes. One of its ends is free (curved arrow). It carries a replication fork from which extends a thickened single-stranded copy (arrowhead). The DNA polymerase is not visible. The open arrow points to a virus RNP fibril with a markedly contrasted polymerase. A cellular DNP fibre covered with nucleosomes crosses the field (double arrow). (b) Free single-stranded virus DNA, 8.5 μm long.
Fig. 4. Replication of virus DNA; positive stain with alcoholic PTA. (a) Complex replication pattern. The synthesis of the DNA strand complementary to a thick single-stranded virus DNA molecule is nearly achieved. The remaining single-stranded end (1.34 μm long), not yet replicated, is marked by the straight arrow. The double-stranded molecule just completed (curved arrow) is immediately replicated in two sites. The arrowheads point to the thickened single-stranded molecules (2.8 and 5.7 μm long) issued from the replication forks. The open arrows indicate the replication sites; no DNA polymerase is recognizable. (b) The replication of a virus DNA molecule is shown on this micrograph. The single-stranded part of the molecule (arrow) is thickened by its coating protein, while the double stranded completed copy looks thinner in the absence of the coating protein (curved arrow). The double arrow points to a nucleosome covered cellular DNP fibre.
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Fig. 5. Transcription of virus DNA; platinum-shadowed preparation (a) Both ends of a double-stranded virus DNA molecule are visible (curved arrow). They carry a few nucleosome-like particles (arrowheads). Typical RNP fibres are being transcribed. The straight arrow points to an RNA polymerase. (b) This schematic drawing interpretative of (a) shows the path of the virus DNA molecule (solid line).

Fig. 6. Transcription of virus DNA; platinum-shadowed preparation. A virus double-stranded DNA molecule 10.05 μm long is being transcribed. The curved arrows signal the free ends of the molecule, punctuated by scarce nucleosome-like granules (arrowhead). The basis of the RNP transcripts display an obvious polymerase (straight arrow). The length of the RNP fibrils increases regularly; the least squares method reveals a significant correlation (t = 13.4, p < 10^-5) between the increasing length of the RNP fibre and the length of the DNP fibrils as measured from the smallest transcript (equation of the regression line: y = 0.13x + 0.12). A polysome of cytoplasmic origin is visible in the upper part of the photograph.
Fig. 6. For legend see facing page.
Transcription complexes of virus DNA

The transcription complexes carried by virus DNA were similar to cellular complexes with respect to the size of the polymerases and the appearance of the RNP. However, they were carried by smooth DNA molecules of limited length, with a transcript density near that of non-ribosomal cellular transcription units. These properties identified a kind of transcription unit not detected in control cells, in general unknown in non-infected mammalian cells (Fig. 5). Fig. 6 shows this type of unit where the RNP display a regular length gradient; however, they are obviously smaller than the corresponding part of the DNA molecule presumed to have been transcribed. This situation is very common in transcription units visualized by Miller's technique. The bush-like aspect of some RNP fibrils is easily seen in Fig. 6 and 7.

Transcription and replication complexes carried by the same virus duplex DNA were also observed. Fig. 8 shows a typical micrograph displaying a replication fork and two transcripts on the same double-stranded molecule. Shorter transcripts were often seen on replicating molecules as in Fig. 3(7.)

DISCUSSION

In this work we demonstrate, for the first time to our knowledge, electron micrographs of transcription and of replication, occurring both individually and simultaneously on the same molecule, of an adenovirus DNA late in the lytic cycle. The virus under study, SA 7, has not yet been the object of detailed biochemical investigations; therefore, we have based our interpretations on data from other adenoviruses since all the members of this virus family studied so far display marked analogies. Our spreading technique did not allow
Fig. 8. Simultaneous replication and transcription of virus DNA; platinum-shadowed preparation. A virus double-stranded DNA molecule displays a replication fork (open arrow), from which extends a single-stranded thickened DNA (arrowhead); the DNA polymerase is not visible. The same double-stranded molecule comprises two RNA polymerases (straight arrow) carrying nascent irregular RNP fibrils. A nucleosome-covered cellular DNP fibre is shown on the upper left of the photograph.
quantitative analysis of the results; however, many micrographs lend themselves to an unambiguous interpretation and justify our qualitative approach.

Virus DNA from infected cells was identified by the presence of molecular species not observed in control cells: duplex DNA of limited length, almost smooth, carrying replication forks and RNP fibrils. Each one of these features was sufficient to characterize morphologically a novel kind of DNP. The replication forks pointed out the presence of virus DNA since the single-stranded molecules displayed the peculiar thick structure first described by Miller & Hodge (1975) and in more detail by Kedinger et al. (1978b). It is noteworthy that both works contain essentially similar observations, albeit obtained by very different techniques. Miller & Hodge (1975) spread whole cells whereas Kedinger et al. (1978b) observed purified virus DNA. In agreement with the latter authors, we noted that the thickening of the single-stranded DNA was absolutely regular throughout the length of the molecule, starting from the replication fork or from the duplex DNA in the case of a replicative intermediate being completed (Fig. 4). Again like Kedinger et al. workers, we could not identify a structure corresponding to the DNA polymerase. However, the observation of numerous replication forks demonstrates that the single-stranded molecule remains bound to the DNA duplex during the spreading procedure, presumably by the intermediary of the DNA polymerase molecule. The thickness of the single-stranded DNA is attributable to coating of the molecule by a DNA binding protein isolated in human adenoviruses type 2 (Kedinger et al. 1978b) and type 5 (Van der Vliet & Levine, 1973; Carter & Blanton, 1978). In both cases this protein has a mol. wt. of 72,000. In contrast to Girard et al. (1977), we observed neither circular virus DNA nor any structure that could correspond to the 55 K protein, thought to be necessary to complete the circular form of the DNA.

The replication complexes visualized in our material were similar to those described in 1972 by Sussenbach et al. and later by Lechner & Kelly (1977) in spite of the different techniques employed. Three main forms of molecules can be distinguished: Y-shaped molecules (replication forks), linear molecules with a transition from single- to double-strandedness and finally linear molecules entirely single- or double-stranded. Here again, the limitations of the spreading technique do not allow a discussion of the localization of the initiation sites or of the displacement of a parental DNA strand (Sussenbach et al. 1972; Lechner & Kelly, 1977).

Frequently, we observed a pronounced discrepancy between our measurements of the SA 7 genome length and its theoretical value estimated from the mol. wt. of a linear, double-stranded molecule (approx. 22 × 10^8 according to Burnett & Harrington, 1968). Various states of spreading or artefactual breaks of the virus DNA molecules may explain the frequent shortening of the SA 7 genome after Miller's technique. However, a compaction of the DNA by proteins cannot be excluded. With respect to the nucleosomes, we should like to underline the striking difference observed between the virus DNA, which was almost smooth, and the non-nucleolar DNA, which carries numerous nucleosomes. However, rare beads of nucleosome size are occasionally seen on virus DNA (Fig. 5). If these particles are actually nucleosomes, we are not able to determine whether they are artefacts or whether they exist in vivo. These observations are similar to those of Kedinger et al. (1978b). On the other hand, it is well known that adenovirus DNA is not combined with histones in the virus particles (Philipson & Lindberg, 1974) and that the synthesis of histones is repressed during adenovirus infection (Ginsberg et al. 1967; Russell, 1971; Tallman et al. 1977).

We observed no difference in morphology between transcription of adenovirus DNA and transcription of non-nucleolar mammalian cell DNA as studied by Miller's technique. The RNP transcripts are always shorter than the corresponding transcribed DNP fibre, either
SA 7 simultaneous transcription and replication

ribosomal or not (Miller & Bakken, 1972; Foe et al. 1976; Puvion-Dutilleul et al. 1978). There are three possible explanations, namely. (a) early cleavage of the nascent RNA, (b) artefactual cleavage of the transcript by the spreading procedure, (c) packing of the RNA molecule in coils within the RNP transcript. Since it is known that during late transcription, the whole adenovirus genome is transcribed as a single giant RNA molecule (Green et al. 1970; McGuire et al. 1972; Wall et al. 1972; Bachenheimer & Darnell, 1975; Goldberg et al. 1978; Nevins & Darnell 1978) which can be readily isolated, the possibility of early cleavage of portions of the RNA molecule during transcription is excluded and the interpretation of the short transcripts in our micrographs representing coiled, packed molecules is supported. Artefactual cleavages seem unlikely because in some cases where the paths of both DNP and RNP were traceable we were able to demonstrate a regular gradient of the RNP fibrils (Fig. 6). On the contrary, a high packing ratio of the RNA would satisfactorily explain the particulate structure which is a constant aspect of RNP as visualized by Miller's technique (Oda et al. 1977; Puvion-Dutilleul et al. 1978), even in the absence of detergent (Angelier & Lacroix, 1975). It is also in agreement with findings about the HnRNP structure (Stevenson et al. 1976).

In a cytochemical study on ultrathin sections of SA 7 infected cells (Moyne et al. 1978) one of us demonstrated that virus transcription and replication were located in the same nuclear region characterized by a special ultrastructure. This paper confirms this finding by extending it to the molecular level with the demonstration of both replication forks and RNP transcripts occurring simultaneously on the same DNA duplex. McKnight & Miller (1977) have previously observed simultaneous replication and transcription in another system characterized by a high nucleic acid synthesizing activity, the blastoderm of *Drosophila melanogaster* embryos. However, it should be mentioned that in nuclei of HeLa cells infected with adenovirus type 2, Kedinger et al. (1978a) did not find simultaneous replication and late transcription. The generality of this phenomenon is therefore still to be demonstrated.

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