Post-transcriptional Processing of Simian Virus 40 Late Transcripts in Injected Frog Oocytes

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The capacity of fully grown Xenopus oocytes to process messenger RNA precursors has been assessed using transcripts synthesized from simian virus 40 (SV40) DNA microinjected into the oocyte nucleus. In oocytes, stable transcripts of the SV40 virion protein genes have undergone at least four post-transcriptional maturation steps: cleavage at 3' splice sites, formation of a mature 3' terminus, addition of poly(A), and selective intraacellular partitioning, such that only those RNAs with a mature 3' terminus and poly(A) are located in the cytoplasm. Apparently unspliced transcripts with mature 3' termini are transported into the oocyte cytoplasm.

A prominent transcript of roughly the full length of SV40 DNA, bearing a 5' terminus in the same region as late mRNA and confined to the nucleus, is found in oocytes injected with SV40 DNA. The possibility that this transcript may serve as a precursor to late mRNA is discussed.

1. Introduction

During oogenesis, each Xenopus oocyte accumulates about 1,000,000 copies of each of 20,000 different messenger RNA species (Davidson & Hough, 1971; Perlman & Rosbash, 1978). This massive amount of mRNA (80 ng/cell) accumulates early in oogenesis and does not increase thereafter (Rosbash & Ford, 1974; Golden et al., 1980). Paradoxically, fully grown oocytes, which no longer accumulate mRNA, nonetheless contain lampbrush chromosomes in which RNA polymerase II actively transcribes many single-copy genes (reviewed by Callan, 1982).

Two resolutions of this paradox may be considered. Firstly, perhaps mRNA is produced in mature oocytes, but is degraded sufficiently rapidly that it does not increase the total amount of mRNA which the oocyte contains. This hypothesis is consistent with the measured rate of mRNA synthesis (Anderson & Smith, 1977; Dolecki & Smith, 1979). Alternatively, mature oocytes might be deficient in some
mRNA processing step, such that nuclear transcripts do not give rise to mature mRNA. For instance, Diaz et al. (1981) have shown that termination of histone gene transcription in lampbrush chromosomes is inefficient, and Anderson et al. (1982) have proposed that incompletely processed mRNA precursors are transported to the cytoplasm.

In the experiments reported here, we have asked which post-transcriptional processes involved in mRNA maturation are carried out in fully grown oocytes. To examine this question using endogenous oocyte genes is difficult, since each gene is present only once or a few times per genome and since the genes transcribed in lampbrush chromosomes have already produced, earlier in oogenesis, a substantial background of cytoplasmic mRNA. In this paper the post-transcriptional capacities of oocytes are assessed using transcripts synthesized from simian virus 40 (SV40) DNA microinjected into the oocyte nucleus. Since many copies of the viral genes are injected and since the oocyte contains no endogenous viral transcripts, the difficulties encountered in studying the oocyte’s own genes are avoided.

Previous microinjection experiments have shown that at least some transcripts synthesized from injected DNA templates must be spliced, since oocytes synthesize proteins from genes whose protein-coding sequence is interrupted (Rungeger & Turler, 1978; Wickens et al., 1980). The amount of mRNA translated in such coupled transcription–translation experiments is a small fraction of the RNA transcribed (J. B. Gurdon & M. P. Wickens, unpublished results), suggesting that processing of primary transcripts into functional mRNA is inefficient. However, direct measurements of mRNA maturation steps have not been reported except in the case of transport of histone gene transcripts (Probst et al., 1979): only those histone gene transcripts with proper 5' and 3' termini enter the cytoplasm. Since histone mRNAs do not undergo RNA splicing, cleavage to form their 3' termini, or polyadenylation, these studies do not bear on these common mRNA maturation steps, nor on their relationship to transport.

SV40 is a tumour virus with a genome containing 5243 base-pairs of double-stranded DNA (reviewed by Tooze. 1981). SV40 infection of monkey cells is divided into early and late phases, separated by the onset of viral replication. Late in infection, RNA synthesized from the “late” strand is processed to form “late” mRNA. Among the maturation steps required for late mRNA formation is the generation of the 3' mRNA terminus by cleavage of a precursor RNA extending 1000 or more nucleotides beyond that site (Ford & Hsu, 1978; Lai et al., 1978), polyadenylation, RNA splicing, and the transport of mRNA from nucleus to cytoplasm. This paper assesses each of these maturation steps in mature oocytes injected with SV40 DNA.

2. Materials and Methods

(a) Oocytes and microinjection

The microinjection technique and oocyte culture conditions have been published (Gurdon, 1977). A total of 35 nl. containing 5 to 10 ng supercoiled SV40 DNA, was injected into each oocyte. When labelled RNA was required, 0.5 to 2 μCi of [32P]CTP or [35P]GTP per oocyte were mixed with the DNA sample before injection. Oocytes were incubated for 1 to 2 days.
RNA was prepared essentially as described by Probst et al. (1979). Ten to 30 oocytes were homogenized quickly in 1 ml of homogenization buffer (2% (w/v) sodium dodecyl sulphate, 0.3 M NaCl, 50 mM Tris, pH 8, 1 mM EDTA) and extracted with buffer-saturated phenol/chloroform (1:1, v/v). After removing the aqueous phase, the interphase and organic phases were re-extracted with 1 ml of homogenization buffer. The 2 aqueous phases were pooled, precipitated with ethanol, redissolved in a small volume of 50 mM Tris (pH 8), 2.5 mM EDTA, extracted again with phenol/chloroform and precipitated with ethanol a second time.

The purification of template-specific RNA by hybridization to single-stranded DNA bound to derivatized paper followed the procedure described by Stark & Williams (1979) with minor modifications which have been described (Wickens et al., 1980). RNA was not denatured before hybridization. Hybridization was carried out for 5 h at 42°C. After washing the filters, template-specific transcripts were eluted from the paper-bound DNA using 3 successive washes with 90% (v/v) formamide (deionized), 50 mM HEPES (pH 8), 10 mM EDTA at 42, 50, and 60°C.

Poly(A)+ and poly(A)- oocyte RNA fractions were isolated by poly(U)-Sepharose chromatography (Buell et al., 1978). Immediately before chromatography, RNA was heated in binding buffer without salt at 70°C for 10 min.

Poly(A)-containing RNA from CV1 cells late in viral infection was a kind gift from Peter Rigby.

(c) Experiments involving separated nuclei and cytoplasm

Experiments involving the separation of the germinal vesicle from its surrounding cytoplasm used the same techniques as those involving whole oocytes, with the following exceptions. Only 10 nl were injected per oocyte in order to avoid rupturing the germinal vesicle. Germinal vesicles and cytoplasts were separated by opening the oocyte in Callan's medium D (Callan & Lloyd, 1960) with 2 pairs of watchmaker's forceps and washing the germinal vesicle by passing it in and out of a pipette. Single isolated germinal vesicles and cytoplasts were immediately transferred into 0.4 ml of homogenization buffer containing 1 mg proteinase K/ml and 100 µg transfer RNA/ml as carrier.

The extent to which nuclear RNAs artifactualy leak into the cytoplasm, monitored by the presence of labelled ribosomal precursor RNA in the cytoplasmic fraction, varies from oocyte to oocyte. It was therefore essential to analyse RNA from each oocyte separately.

(d) Mapping transcripts using S, nuclease digestion of RNA : DNA hybrids

The technique of Berk & Sharp (1977) was used with minor modification. DNA probes were prepared by labelling the termini of SV40 DNA cleaved with the appropriate restriction enzyme. 5' Termini were labelled using T4 polynucleotide kinase and [γ-32P]ATP; 3' termini were labelled using the Klenow fragment of DNA polymerase I and [α-32P]deoxyribonucleoside triphosphates (Maxam & Gilbert, 1980).

Single-stranded probes were prepared by annealing a labelled restriction fragment to an excess of single-stranded bacteriophage M13 carrying sequences complementary to the RNA to be analysed. Hybridizations were carried out in 0.1 M NaCl, 20 mM MgCl2, 20 mM Tris (pH 7.5) at 65°C for 1 h. The hybridization mixture was then subjected to electrophoresis on a non-denaturing polyacrylamide gel. The labelled fragment of the same sense as that cloned in the phage was still single-stranded, as its complementary strand had annealed to the M13. The single-stranded probe fragment was eluted electrophoretically (McDonnell et al., 1977).

Hybridizations involving single-stranded probes were done in 15 µl of 50% formamide (deionized), 0.4 M NaCl, 20 mM PIPES (pH 6.4), 2.5 mM EDTA, and 0.1% sodium dodecyl sulphate (Stark & Williams, 1979), for 3 h, at 30°C to 42°C, depending on the base composition of the probe. Hybridizations involving double-stranded probes were done in 15 µl of 80% formamide (deionized), 0.4 M NaCl, 20 mM PIPES (pH 6.4), 2.5 mM EDTA for
3 h at 49°C (Casey & Davidson, 1977; Berk & Sharp, 1977). After hybridization, 200 μl of ice-
cold S1 buffer (0·25 M-NaCl, 50 mM-sodium acetate, pH 4·5, 1 mM-ZnSO4) containing 2000 to
4000 units of S1 nuclease (Miles) were added. S1 digestions were incubated at 37°C for 30 min,
then extracted once with phenol/chloroform (1:1, v/v) and precipitated with ethanol.

From 0·2 to 3 oocytes' worth of RNA was analysed in each hybridization. A 6-h exposure
of the autoradiogram was typical for an experiment involving a DNA probe of specific
activity 10⁶ cts/min per μg and one oocyte's worth of RNA.

The sequencing ladder in Fig. 5 was generated as follows. Single-stranded DNA from an
M13 phage carrying the early strand of the BamHI to HindIII fragment of SV40 (positions
2533 to 3476 ; Tooze, 1981) was sequenced by the dideoxynucleotide substitution method
(Sanger et al., 1977). The products of the sequencing reaction, which have the sequence in
the sense of late mRNA, were then digested with BamHI to form a group of fragments with 5'
termini at the same residue as the hybridization probe.

(e) Gel electrophoresis

RNAs were analysed either by electrophoresis in 1·2% (w/v) agarose gels containing
10 mM-methyl mercuric hydroxide (Bailey & Davidson, 1976) or by electrophoresis in
sodium phosphate-buffered agarose gels following treatment of the RNA with glyoxal and
dimethyl sulphoxide (McMaster & Carmichael, 1977). RNAs were analysed by
electrophoresis on alkaline agarose gels (McConnell et al., 1977) or by electrophoresis on thin
polyacrylamide gels containing 7 M-urea (Sanger & Coulson, 1978).

(f) Construction of recombinant DNAs

Purified HindIII 1, 2 and 3 fragments of SV40 DNA (also called HindIII C, A and B
fragments, respectively; see the legend to Fig. 2 for map positions) were cloned into M13mp8
cleaved with HindIII. The identity of the inserted DNA and its orientation with respect to
the vector were determined by sequencing using the method of Sanger et al. (1977).

3. Results

(a) The size distribution and polarity of SV40 transcripts synthesized in oocytes

In this section, the length and polarity of stable transcripts of SV40 DNA are
described. This information is essential for the design of subsequent experiments in
which their detailed structure and intracellular partitioning is examined.

(i) Size distribution

A mixture of SV40 DNA and [32P]GTP was injected into the oocyte nucleus
(germinal vesicle). After the oocytes had been incubated for two days. RNA was
extracted. SV40 transcripts were then purified from this crude RNA preparation by
hybridization to and elution from paper filters bearing large amounts of denatured
SV40 DNA. The size of the purified 32P-labelled SV40 transcripts was determined
by electrophoresis on agarose gels containing methyl mercury as a denaturant. As
controls, transcripts from a second group of oocytes injected with [32P]GTP only
(no DNA) were analysed in parallel (Fig. 1).

Transcripts of SV40 in oocytes include two prominent RNAs of discrete size: one
of about 5300 bases and the other of about 2200 bases (19 S RNA), and a group of
transcripts larger than 28 S ribosomal RNA and heterogeneous in size (Fig. 1, lanes
4 and 5). These are all absent from RNA purified from control oocytes. which
Fig. 1. Prominent transcripts of SV40 synthesized in oocytes. RNA was prepared from oocytes injected with 10 ng SV40 DNA and $[^{32}P]GTP$ (lanes 2, 4 and 5) or from oocytes injected with $[^{32}P]GTP$ alone (lanes 1 and 3). Lanes 1 and 2 are total RNA samples before hybridization to paper filters. Labelled RNA was then hybridized to paper filters bearing immobilized SV40 DNA. SV40-specific transcripts were eluted from each filter and analysed by electrophoresis on a 1.5% agarose gel containing 10 mM methyl mercuric hydroxide. Lanes 3, 4 and 5 are filter-bound RNAs. Lane 5 contains more of the same RNA sample as lane 4, to make minor transcripts visible. The positions of prominent SV40 transcripts (5300, 2200 (19 S), and upper brackets) in lanes 1, 4 and 5 are indicated, as are the positions of rRNA and its precursors (pre) in lanes 2 and 3. The small arrowheads and lower brackets indicate minor, promoter-proximal transcripts and their approximate lengths in nucleotides.

instead consist of only a small amount of rRNA not removed by the washing procedure (lane 3). The 19 S SV40 transcript is, in fact, not homogeneous, but consists of a sharp band and some trailing RNA which migrates slightly slower. We shall refer to this group of RNAs as 19 S RNA because, as shown in subsequent sections, their structure is similar to that of the 19 S RNAs produced late in viral infection. Crude RNA preparations, prior to hybridization to SV40 DNA, contain predominantly ribosomal RNA (18 S and 28 S) and its 40 S precursor, whether or
not the oocytes have been injected with SV40 (lanes 1 and 2). However, oocytes injected with SV40 DNA also clearly contain the 5300 base transcript and large, heterogeneous transcripts as well (lane 2): the 19.8 transcript is obscured by the background. These data are consistent with published reports that 10^9 to 20^9 of the total newly synthesized RNA in SV40-injected oocytes is virus-specific (Mertz & Gurdon, 1977; Wickens et al., 1980) and with the sedimentation coefficients of viral transcripts in sucrose gradients (Laskey et al., 1978).

Minor transcripts of about 1000, 900, and 100 to 300 bases also are present (lane 5, open arrowheads). These are all promoter proximal (not shown). In particular, the 100 to 300 base group of transcripts includes several of discrete size which hybridize exclusively to the HindIII-1 fragment of SV40 DNA indicated in Figure 2. These short RNAs may correspond to the prematurely terminated late transcripts in infected cells (Hay et al., 1982). None of these minor transcripts will be considered further in this paper.

(ii) Polarity

All the stable SV40 transcripts identified in Figure 1 are from the same strand of the template, the so-called late strand whose transcripts predominate late in viral infection of cultured monkey cells. This is demonstrated by the experiment presented in Figure 2. 32P-labelled RNA from oocytes injected with SV40 was hybridized to six different paper filters. These filters bore single-stranded DNA of either the late strand (L) or the early strand (E) of three different HindIII fragments of SV40 (1, 2 or 3), purified by cloning in the single-stranded bacteriophage vector, M13mp8 (see the restriction map and the legend to Fig. 2 for details). After hybridization, SV40-specific RNAs were eluted and subjected to electrophoresis. From the results, two general conclusions may be drawn. Firstly, oocytes transcribe predominantly the late strand of SV40. Only filters bearing the late strand of SV40 purify any transcripts (lanes 1L, 2L and 3L); those bearing the early strand fragments show only a small amount of residual rRNA not removed by the washing procedure (lanes 1E, 2E and 3E). (These data do not show that the early strand is not transcribed at all; indeed it must be transcribed, since oocytes injected with SV40 DNA synthesize T-antigen (Rungger & Turler, 1978). Rather, our data demonstrate that transcripts of the late strand are more abundant than those from the early strand. Early strand transcripts are not detected because they form RNA:RNA duplexes with the more abundant late strand transcripts and so cannot hybridize to immobilized DNA. Indeed, when transcripts are denatured...
before hybridization, early strand transcripts are detected, and constitute roughly
15% of the total viral RNA.) The second conclusion is that 19S RNA is purified
only by fragments contained within the region of SV40 which encodes late
messenger RNA (lanes 1L and 2L), not by the fragment which contains regions
beyond the 3' terminus of late mRNA (lane 3L). In contrast, both the large
heterogeneous transcripts and the 5300 base transcript are complementary to at
least part of the late strand of all three fragments tested (1L, 2L and 3L).

Fig. 3 (a), (b)
The general structure of SV40 transcripts synthesized from the late strand during viral infection of cultured monkey cells is depicted in Figure 3(a) (for details, see Tooze, 1981). The 16S mRNAs, which encode the major virion protein VP1, and the 19S mRNAs, which encode the minor virion proteins VP2 and VP3, both consist of a "body" segment spliced to one of a heterogeneous family of "leader" segments. The body of VP2 and VP3 mRNAs is identical and contains within it the shorter body segment of VP1 mRNA. Thus, the 5' boundaries of the two body segments differ, but the 3' boundaries of all late mRNAs coincide precisely. Leader segments are heterogeneous at both their 5' and 3' termini. Their 5' termini span roughly 250 base-pairs. Note that the 5' boundary of a body segment is, by definition, the 3' splice site used to splice a leader segment to a body segment. An unspliced 19S RNA also has been identified (Ghosh et al., 1978; Lai et al., 1978).

To determine whether the 5' termini of oocyte transcripts coincide with the 5' termini produced in infected cells, and to establish which, if any, of the 3' splice sites are used in oocytes, unlabelled RNA from SV40-injected oocytes was annealed to an EcoRI/TaqI SV40 DNA probe which had been 5' end-labelled at the EcoRI site, and the hybrids treated with the single strand-specific nuclease, S1 (Fig. 3(a) and (b)). The length of labelled DNA fragments remaining after nuclease digestion

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Fig. 3. 5' Termini and 3' splice sites. (a) The structure of SV40 late transcripts in infected cells. Shaded boxes indicate exons (present in mRNA); thin black lines indicate intervening sequences (missing in spliced mRNAs). Note that late transcripts initiate at multiple sites, as indicated by broken lines. For simplicity, the diagram indicates only one of the several possible 19S and 16S 5' splice sites and only those restriction sites relevant to this experiment. (b) Predicted hybrids between the RNAs shown in (a) and an EcoRI/Taq1 SV40 DNA probe 5' end-labelled at the EcoRI site. Shaded boxes indicate the structures of 3 mRNAs; blank lines indicate the EcoRI/Taq DNA probe, with a star to represent the 5' label at the EcoRI site. Arrowheads indicate those sites of 17 cleavage which lie closest to the labelled EcoRI site. The products of S1 nuclease digestion are indicated to the right, bp. base pairs. (c) Results of S1 mapping experiments. Lanes 1 to 3 and 7 to 10, unlabelled RNA from SV40-injected oocytes was hybridized to either, in lanes 1 to 3, an EcoRI/Taq1 SV40 DNA fragment (positions 1782 to 4739; Tooze, 1981) 5' end-labelled at the EcoRI site, or, in lanes 7 to 10, an EcoRI/HpaII fragment (positions 1782 to 346; Tooze, 1981) 5' end-labelled at the same EcoRI site. Hybrids were treated with S1 nuclease and analysed by electrophoresis through 1.5% agarose gel containing 10 mM methyl mercuric hydroxide. Lane M (leftmost), DNA markers obtained by digesting the probe fragment with the indicated restriction enzyme (Bgl is BglI, Hpa is HpaII, and Hae is HaeIII). Lane 1, total RNA from SV40-injected oocytes, displaying a splicing pattern in which little cleavage at the 16S 3' splice site is detectable; lane 2, total RNA from a second group of SV40-injected oocytes, showing a high level of cleavage at the 16S 3' splice site; lane 3, poly(A)-containing RNA from SV40-injected monkey (CV 1) cells; lane 7, total poly(A)-containing RNA from SV40-injected oocytes; lane 8, oocyte 5300 base RNA, purified by gel electrophoresis; lane 9, oocyte 19S RNA purified by gel electrophoresis; lane 10, no RNA. Lanes 4 to 6, markers (M) are HpaII fragments of pBR322 labelled at their 3' terminus using Klenow DNA polymerase I. Lane 4, total oocyte RNA hybridized to a 700 base HindIII/HpaII DNA fragment (position 1046 to 346; Tooze, 1981) 5' end-labelled at the HindIII site, digested with S1 nuclease and electrophoresed on a 2% polyacrylamide gel containing 7 M urea. The 3' splice site of 19S RNA from infected cells lies at position 557 (Piatk et al., 1981). Lanes 5 and 6, RNA from oocytes (lane 5) or from SV40-infected monkey cells (lane 6) was hybridized to an EcoRI/HpaI DNA fragment (positions 1782 to 1381) 5' end-labelled at the EcoRI site, digested with S1 nuclease and analysed as described above. The 3' splice site of 16S RNA from infected cells lies at position 1461 (Gross & Khoury, 1980).
was determined by gel electrophoresis (Fig. 3(c)). Each transcript depicted in Figure 3(a) should produce a characteristic RNA:DNA hybrid structure with the DNA probe. The three structures can be distinguished by the length of the labelled DNA fragment which will be resistant to S1 nuclease (Fig. 3(b)).

Whereas RNA from control oocytes protects no probe (Fig. 3(c), lane 10), RNA from oocytes injected with SV40 typically protects prominent fragments of 1230 and 1600 bases (lane 1). Lengths of protected fragments were determined by comparison to the DNA markers (lane M on left), each of which is a restriction digest of the same end-labelled DNA fragment used as probe. The 1600 base fragment corresponds to an unspliced RNA having a 5' terminus in approximately the same region as in infected cells. The 1230 base band corresponds to the 3' splice site of 19 S RNA from infected cells, as confirmed in lane 4, in which a shorter HindIII/HpaII DNA probe, 5' end-labelled at the HindIII site (see Fig. 3(a)), has been used. The length of the protected fragment is 495 (±5) bases, corresponding well with the 493 bases between the labelled HindIII site and the 19 S 3' splice site.

As expected, the full length probe of 700 bases also is protected. We conclude that the 19 S 3' splice site has been accurately cleaved in oocytes injected with SV40 DNA.

Cleavage at the 16 S 3' splice site can also occur accurately in oocytes; however, the amount of cleavage at this position is generally low, and is variable. The experiment in lane 1, already described, is typical: the amount of the 325 base band, diagnostic of cleavage at the 16 S 3' splice site, is only 5% of the amount of the 1230 base band. In lane 2 is shown the result of an identical experiment using RNA from another group of oocytes, in which the amount of the 325 base band is atypically high, being roughly 55% of the amount of the 1230 base band. The 325 base band seen in this experiment does indeed represent RNAs cleaved precisely at the 16 S splice site (lane 5), as shown by hybridization to a shorter probe, again 5' end-labelled at the EcoRI site: the 325 band protected by oocyte RNA (lane 5) precisely comigrates with the fragment protected by RNA from SV40-infected monkey cells (lane 6), and is 325 (±5) bases long, coinciding with the 325 bases between the labelled EcoRI site and the 16 S 3' splice site. The full length probe of 394 bases is also protected, as expected.

The apparent variability in the amount of the 325 base band is not an artifact of our S1 mapping technique. Our methods properly quantitate the 16 S/19 S ratio, since RNA from infected monkey cells reproducibly yields the same ratio of about three (lane 3) as has been reported by Villareal et al. (1979). Furthermore, the typical inefficiency of the 16 S cleavage in oocytes is independently corroborated by the fact that 19 S RNA, but not 16 S RNA, is prominent among the stable viral transcripts in oocytes (Figs 1 and 2). We conclude that the 16 S 3' splice site can be used in oocytes, but that it is generally cleaved less efficiently than the 19 S 3' splice site.

Both the 5300 base transcript and 19 S RNA include transcripts which have been cleaved at the 19 S 3' splice site. Isolated 19 S RNA, purified by gel electrophoresis, protects a 1230 base fragment of a 5' end-labelled EcoRI/HpaII hybridization probe (lane 9), as does an isolated 5300 base transcript (lane 8). The protected 1436 base fragment corresponds to unspliced RNA spanning the entire probe: thus, the
Figure 4.
ratio of cleaved to uncleaved species is higher with 19 S RNA than with the 5300 base transcript.

Taken together, these data permit two main conclusions. First, late strand transcripts possess a 5' terminus in approximately the same region as is found in infected cells. The resolution of our measurements is not sufficient to establish that the 5' termini of oocyte and cultured cell transcripts coincide precisely. Second, both the 19 S and 16 S 3' splice sites can be accurately cleaved in oocytes, though the ratio of 16 S to 19 S 3' splice sites used is typically much lower in oocytes than in SV40-infected monkey cells.

(c) Accurate 3' termini are produced in oocytes

The formation of the 3' termini of late SV40 mRNAs involves post-transcriptional cleavage of a longer precursor RNA (Ford & Hsu, 1978; Lai et al., 1978), not transcription termination. Since the 19 S oocyte transcript hybridizes to the late strand of fragments 1L and 2L, but to neither strand of fragment 3 (Fig. 2), it seemed likely that the 3' end of this RNA coincided with the 3' end of late transcripts from infected cells. This was shown to be the case by the experiments in Figures 4 and 5.

Unlabelled transcripts were purified from oocytes injected with SV40 DNA. These were then hybridized to a HindIII/HphI DNA fragment of 1530 bases which spans the 3' terminus of late mRNAs and which had been 3' end-labelled at the HindIII site (see Fig. 4 for details). Hybrids were treated with S1 nuclease and analysed by gel electrophoresis (Fig. 4). Unfractionated oocyte RNA protects two fragments, one of 1530 bases (corresponding to the intact probe) and the other of approximately 970 bases (Fig. 4, lane 1), in a molar ratio of about 1.5:1 (1530:970). The distance from the labelled HindIII site to the 3' end of SV40 late transcripts in infected cells is 968 bases, in excellent agreement with the length of the smaller protected fragment. The 1530 base protected fragment represents transcripts which contain sequences beyond this polyadenylation site, not the self-annealed DNA probe, since purified 19 S RNA (lane 3) and RNA from oocytes which have not been injected with SV40 (lane 4) do not yield this band. Furthermore, the protection is due to RNA, not to the injected DNA, since both bands are abolished by pretreating the RNA with alkali (Fig. 7, lane 4) or with DNase-free RNase (not shown).

The same experiment was then repeated using unlabelled 19 S or 5300 base RNA purified by gel electrophoresis. The 5300 base transcript contains all sequences present in the probe fragment (Fig. 4, lane 2). In contrast, 19 S RNA terminates at

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Fig. 4. 3' Termini. RNA purified from oocytes injected with SV40 was hybridized to a HindIII/HphI double stranded fragment of SV40 end-labelled at the HindIII site (position 1708 to 3235; Tooze, 1981). Hybrids were treated with S1 nuclease and analysed by electrophoresis on a 1% agarose gel containing 10 mM-methyl mercuric hydroxide. The structures of hybrids, and the predicted products of S1 digestion, are indicated using the symbols described for Fig. 3. Lane 1. total RNA; lane 2. purified 5300 base RNA; lane 3. purified 19 S RNA; lane 4. RNA from uninjected oocytes. The positions of various DNA markers, each produced by digestion of SV40 DNA, are shown to the right.
the same position as late mRNA from infected cells, i.e. about 970 bases from the *Hind*III site labelled in the probe (Fig. 4, lane 3). The uncertainty in such a measurement is roughly 25 bases.

At higher resolution, it becomes clear that 19 S RNA terminates at precisely the correct base (Fig. 5). Unlabelled RNA from oocytes injected with SV40 was hybridized to a shorter, end-labelled DNA fragment such that the exact length of the protected fragment could be determined by comparison with a sequencing ladder of the same fragment (Fig. 5, lanes G, T, C and A). In RNA from infected cells, the third A residue of the poly(A) tract replaces the C residue arrowed in Figure 5 (Fitzgerald & Shenk, 1981). The 3' end of oocyte 19 S transcripts lies at precisely the same residue (lanes 1 to 3). No probe is protected in the absence of RNA (lane 4) or in the presence of RNA from uninjected oocytes (lane 5). We conclude that oocytes contain whatever factors are essential for the production of accurate 3' termini from the late region of SV40.

(d) Transcripts with mature 3' termini are polyadenylated

32P-labelled SV40-specific RNA was passed over a poly(U)-Sepharose column after the RNA had been heated at 70°C for ten minutes to disrupt aggregates. RNA which bound was eluted with 90% formamide, precipitated with ethanol and then passed over a second poly(U)-Sepharose column. Material which did not bind the second time and material which had bound twice were both precipitated with ethanol. These two samples and the RNA which had not bound to the first column were then subjected to electrophoresis under denaturing conditions (Fig. 6). Roughly 80% of 19 S RNA, and about 20% of the 5300 base and heterogeneous transcripts are polyadenylated. Three aspects of the results substantiate the assay's validity: (1) 95% of the RNA which bound the first time bound again the second time (compare lanes 2 and 3); (2) 28 S rRNA did not bind; (3) 19 S RNA in the twice bound fraction (lane 2) is slightly larger than that in the unbound fraction (lane 1), consistent with its containing additional nucleotides. Although the proportion of SV40 RNA which is retained by the column varies somewhat between experiments, such that as little as 10% of the large RNAs may be retained, at least 60% of the 19 S transcript has been retained in all experiments. These data strongly support the conclusion that oocytes polyadenylate a large proportion of those transcripts which have a mature 3' terminus.

Fig. 5. 19 S transcripts terminate at the correct base. RNA purified from oocytes injected with SV40 was hybridized to a single-stranded BamHI to *Hind*III (positions 2533 to 3476; Tooze, 1981) fragment of SV40 DNA. Hybrids were treated with S1 nuclease and analysed by electrophoresis on a 6% acrylamide gel containing 7 M urea. A sequencing ladder produced as described in Materials and Methods provides a precise sequencing ladder with which to compare the protected fragment. The structures of hybrids and the predicted products of S1 digestion are shown using the symbols described for Fig. 3. Lanes G, T, C and A, sequencing ladder of anti probe fragment (see Materials and Methods): the sequence is indicated to the left (hyphens omitted for clarity). The position of the C residue which is replaced by an A in polyadenylated mRNA from infected cells is big. dark. and indicated by an arrow. Hybrids (lanes 1 to 5) were treated with 2000 (lane 1), 5000 (lane 2), or 12,500 (lanes 3 to 5) units of S1 nuclease/ml. Lanes 1 to 3, RNA from oocytes injected with SV40: lane 4, no RNA added: lane 5, RNA from uninjected oocytes.
Fig. 6. Poly(U)-Sepharose chromatography of labelled SV40 transcripts. RNA prepared from oocytes injected with SV40 DNA and [32P]GTP was passed over a poly(U)-Sepharose column as described in Materials and Methods and in the text. RNA which bound once was eluted, precipitated with ethanol and passed over a second column. Lane 1: RNA which did not bind to the first column (poly(A)'); lane 2: RNA which bound twice (poly(A)''); lane 3: RNA which bound once but did not rebinding.

(e) Only RNAs with a mature 3' terminus and poly(A) are found in the cytoplasm.

To evaluate the partitioning of SV40 transcripts between nucleus and cytoplasm, oocytes were injected with SV40 DNA and incubated for two days. Nuclei and cytoplasms were then isolated manually. RNA was prepared from the separated
nucleus and cytoplasm of single oocytes. This RNA was then hybridized to a
\textit{HindIII}/\textit{HphI} DNA probe 3' end-labelled at the \textit{HindIII} site (position 1708; Tooze, 1981) and the hybrids treated with S\textsubscript{1} nuclease, exactly as shown in Figure 4. The results are presented as a nucleus and cytoplasm pair from each oocyte (Fig. 7).

Whereas RNA from uninjected oocytes protects no probe (Fig. 7, oocyte E), RNA from oocytes which have been injected with SV40 does (oocytes A to D). The fragments protected by nuclear and cytoplasmic RNAs differ. Nuclear RNA yields both the 1530 base fragment, which represents transcripts containing all of the sequence in the probe, and the 970 fragment, which represents transcripts with mature 3' termini. Nuclear RNA also protects fragments of heterogeneous length (between 400 and 1530 bases) and a fragment of 900 bases which is probably artifactual, resulting from S\textsubscript{1} digestion in a 26 base-pair region containing 22 A·T base-pairs (positions 2593 to 2618; Tooze, 1981). In contrast, the pattern from cytoplasmic RNA is simple: only the 970 base fragment is present. The proportion of RNA with a proper 3' terminus which is cytoplasmic ranges from 30 to 80\% among oocytes. These results demonstrate that the only abundant, stable transcripts present in the cytoplasm are those which have 3' termini at the same site as the 3' termini of late mRNA from infected cells.

This conclusion rests on the nuclear retention of transcripts containing sequences beyond the mature 3' terminus. The crucial 1530 base fragment does indeed represent nuclear RNA, not DNA, for the following reasons. Nuclear RNA from whole oocytes in which DNA had been injected into the cytoplasm and was therefore not transcribed, does not protect any probe (Fig. 7 controls, lane 2), nor does RNA from uninjected oocytes mixed with 10 ng SV40 DNA before hybridization (lane 3). Thus, protection of the 1530 base fragment is not the result of annealing with injected DNA. Furthermore, if the RNA is first treated with alkali (lane 4) or DNase-free RNase (not shown), then hybridized, protection of the probe (lane 1) is eliminated, even though the probe itself is intact (lane 5). Thus, the protection, by nuclear RNA, of the entire probe and of fragments of heterogeneous length, reflects selective nuclear partitioning of transcripts which do not bear a mature 3' terminus. The following experiments using \textsuperscript{32}P-labelled RNA corroborate this conclusion and, in addition, show that stable transcripts in the cytoplasm are polyadenylated, while non-polyadenylated transcripts are exclusively nuclear.

A mixture of SV40 DNA and \textsuperscript{32}P\textsubscript{GTP} was injected into oocyte nuclei. After two days' incubation, nuclei and cytoplasms were isolated manually, and the labelled RNA from each compartment of a single oocyte was analysed by gel electrophoresis. As controls, oocytes which had been injected with \textsuperscript{32}P\textsubscript{GTP} alone were analysed in parallel. The results are again presented as a nucleus and cytoplasm pair from each oocyte (Fig. 8).

Labelled RNA from an uninjected oocyte consists primarily of rRNA and its precursors (Fig. 8, oocyte A). The 40 S and 32 S ribosomal RNA precursors are exclusively nuclear, while the 28 S and 18 S ribosomal RNAs are mostly cytoplasmic (open arrowheads). RNA from oocytes injected with SV40 DNA (oocytes B through E) contain all of the RNAs present in the controls; in addition, however,
Fig. 7. Only RNA with a mature 3' terminus is transported into the oocyte cytoplasm. RNA was prepared from the nucleus (n) and cytoplasm (c) of single oocytes (A to E). Oocytes A to D had been injected with SV40 DNA; oocyte E was not injected. Each RNA fraction was then analysed by $S_{1}$ mapping exactly as described for Fig. 4, using the same HindIII/HphI probe. Results are presented as a nucleus and cytoplasm pair from each oocyte. Controls: the following RNA samples were analysed by the same $S_{1}$ mapping method. Lane 1. total RNA from oocytes injected with SV40 DNA; lane 2. RNA from oocytes in which DNA was injected into the cytoplasm; lane 3. RNA from un.injected oocytes mixed with 10 ng DNA immediately before hybridization; lane 4. RNA from injected oocytes, treated with alkali immediately before hybridization; lane 5. probe treated with alkali (in parallel with lane 1 sample). Alkali treatment involved incubation in 0.2 M NaOH, 10 mM EDTA at 37°C for 2 h.
FIG. 8. Only transcripts with a mature 3' end and poly(A) are found in the cytoplasm. RNA was prepared from the separated nucleus and cytoplasm of single oocytes which had been injected with SV40 DNA and [32P]GTP. Each fraction was then analysed by electrophoresis in a 1.2% agarose gel containing 10 mM methyl mercuric hydroxide. The results are presented as a nucleus (n) and cytoplasm (c) pair for each oocyte (A to E). Oocytes B to E received SV40 DNA, while oocyte A did not. The positions of endogenous transcripts are indicated by open arrowheads in oocyte A. The positions of SV40-specific transcripts are labelled and indicated by filled arrowheads in oocyte B (40S, 5300, and poly(A) and poly(A)- 19S RNA). In oocytes B to E, the same bands are indicated, again using open arrowheads for endogenous transcripts and filled arrowheads for SV40 RNAs. Poly(U)-Sepharose chromatography (lanes 1 to 4). Nuclear RNA from oocytes injected with [32P]GTP (lanes 1 and 2) or with [32P]GTP and SV40 DNA (lanes 3 and 4) was passed over a poly(U)-Sepharose column. Bound (lanes 2 and 4) and not bound (lanes 1 and 3) fractions were recovered by precipitation with ethanol and subjected to electrophoresis in a 1.2% agarose gel containing 10 mM methyl mercuric hydroxide.
virus-specific transcripts also are seen, and are selectively partitioned. The large heterogeneous RNA (40 S) and the 5300 base transcript are exclusively nuclear (oocyte B). The distribution of 19 S transcripts is more complex: nuclear RNA contains a viral transcript of discrete size in this region (poly(A)^+, 19 S), while both the nucleus and cytoplasm contain a heterogeneous group of RNAs of slightly greater molecular weight (poly(A)^+. 19 S).

We have shown (Fig. 6) that 60 to 80% of 19 S RNA from whole oocytes is retained by poly(U)-Sepharose, and that this polyadenylated 19 S RNA migrates slightly slower during electrophoresis than does non-polyadenylated 19 S RNA. The data in Figure 8 demonstrate that polyadenylated and non-polyadenylated 19 S RNAs are selectively partitioned: such that the heterogeneous, more slowly migrating group of polyadenylated 19 S RNAs are found both in the nucleus and in the cytoplasm, while the discrete, non-polyadenylated RNA is located only in the nucleus. Thus, in nuclear RNA, the heterogeneous 19 S RNAs are retained by poly(U)-Sepharose and the discrete band is not (Fig. 8, lanes 3 and 4). Note that the non-polyadenylated transcript comigrates with the fastest migrating species in the polyadenylated group, as expected. Nuclear RNA from control oocytes contains neither transcript (Fig. 8(b), lanes 1 and 2). Analogous experiments with cytoplasmic RNA confirm that the heterogeneous transcripts in the cytoplasm are also polyadenylated (not shown).

Taken together, Figures 7 and 8 show that only RNAs with both the mature 3' terminus and poly(A) are found in the cytoplasm; transcripts which lack poly(A) (or have poly(A) so short that it will not bind to the poly(U)-Sepharose) are found exclusively in the nucleus.

(f) Unspliced RNAs are transported

In light of the proposal that oocytes transport incompletely processed RNAs into the cytoplasm (Anderson et al., 1982), it was of interest to establish whether unspliced 19 S RNAs were transported. Nuclear and cytoplasmic RNAs from the oocytes A to D depicted in Figure 7 were pooled to form a single nuclear fraction and a single cytoplasmic fraction. Both RNAs were hybridized to an EcoRI/TaqI SV40 DNA probe 5' end-labelled at the EcoRI site, treated with S1 nuclease, and analysed by electrophoresis, exactly as shown in Figure 3(a). The resulting bands are identified by comparison to the DNA markers (M) and to the fragments protected by RNA from SV40-infected monkey cells (CV-1). Recall that these same preparations of nuclear and cytoplasmic RNAs displayed partitioning of transcripts on the basis of 3' termini (Fig. 7). In contrast, the results of the present experiment, shown in Figure 9 (lanes N and C) and summarized in Table 1, demonstrate that both unspliced RNA (1600 base fragment) and RNA spliced at the 19 S 3' splice site (1230 base fragment) enter the cytoplasm (C). Thus, roughly the same proportion of the 1600 base band (26%) and the 1230 base band (33%) are derived from cytoplasmic RNA (Table 1). Full length probe is protected by nuclear RNA (N), not by cytoplasmic RNA (C), confirming that large (40 S) heterogeneous RNAs are confined to the nucleus (Figs 7 and 8). We conclude that splicing, unlike the generation of 3' termini and polyadenylation, is not correlated
with the partitioning of SV40 transcripts between the oocyte nucleus and cytoplasm.

4. Discussion

Fully grown oocytes injected with SV40 DNA accumulate stable late region transcripts which have been processed by at least four mRNA maturation steps: cleavage at 3' splice sites, formation of a 3' terminus, polyadenylation, and specific
transport into the cytoplasm. These results demonstrate, qualitatively, that mature oocytes contain those factors which are essential for each of these steps. The data do not permit a quantitative assessment of the efficiency of each step, in terms of what proportion of the total RNA transcribed undergoes each modification, since we have assayed stable, rather than newly synthesized RNA. Each processing step is considered in detail below.

(a) mRNA maturation in oocytes

(i) Splicing

Although the existence of mRNA splicing activities in oocytes has been inferred from the synthesis of proteins from genes containing introns (Rungger & Turler, 1978; Wickens et al., 1980), previous studies have not analysed RNA directly. Our results show that late transcripts of SV40 can be cleaved accurately at both the 19 S and 16 S 3' splice sites (Fig. 3). We have not assayed tRNA ligation after cleavage, nor cleavage at 5' splice sites, since the 5' splice sites of the SV40 late mRNAs are heterogeneous (Piatak et al., 1981).

The proportion of stable transcripts which are cleaved, typically about 50%, (Fig. 3, lanes 1 and 2), is comparable to the proportion of tRNA precursors which are spliced in oocytes injected with yeast tRNA genes (Melton et al., 1979). If mRNA splicing activities are confined to the oocyte nucleus, as are the activities which splice tRNA precursors in oocytes (Melton et al., 1979; De Robertis et al., 1981), then those unspliced SV40 transcripts which enter the cytoplasm (Fig. 9) would become unavailable for subsequent splicing. Thus, the apparent inefficiency of cleavage at 3' splice sites might reflect the cytoplasmic location of unspliced transcripts, as it does with tRNA precursors (Melton et al., 1979).

(ii) The formation of 3' termini and polyadenylation

Roughly half of the stable transcripts of SV40 which are synthesized in oocytes possess the same 3' terminus as transcripts formed in infected monkey cells (Figs 4

<table>
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<tr>
<th>Protected fragment</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
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<tr>
<td>2286</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>1600</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>1230</td>
<td>67</td>
<td>33</td>
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Microdensitometry of autoradiograms like that shown in Fig. 9 (lanes X and C) was used to determine the percentage of each S1-protected fragment which is derived from nuclear versus cytoplasmic RNA. The average of 2 experiments is shown, each of which involved 4 oocytes which clearly displayed partitioning on the basis of 3' termini (Fig. 7). The range of percentages is indicated to the right. The 2 preparations were typical, in that 45 and 52% of the RNA had been cleaved at the 19 S 3' splice site, and less than 10% at the 16 S 3' splice site (see Fig. 3 and text).
and 5). In infected cells, the formation of the SV40 late mRNA 3' terminus involves cleavage of a precursor RNA extending at least 1000 bases beyond the polyadenylation site (Ford & Hsu, 1978; Lai et al., 1978). We assume, but have not proved, that the same post-transcriptional processing steps are involved in the formation of the late mRNA 3' terminus in oocytes.

The 3' terminus of eukaryotic mRNAs can be formed not only by processing, as with SV40 late genes and the adenovirus late (Nevins & Darnell, 1978) and mouse β-major globin (Hofer & Darnell, 1981) genes, but also by transcription termination, as with histone (Birchmeier et al., 1982) and ovalbumin (Tsai et al., 1980) genes. As shown here, oocytes accurately form the 3' terminus of SV40 late transcripts, presumably by RNA processing. In contrast, oocytes fail to terminate transcription at the 3' end of histone genes in newt lampbrush chromosomes (Diaz et al., 1981) and terminate only inefficiently at the 3' end of injected sea urchin histone H3 genes (Hentschel et al., 1980). The possibility therefore arises that oocytes may possess factors responsible for the formation of 3' termini by processing, but be deficient in at least some factors required for termination.

(iii) **Partitioning of transcripts between nucleus and cytoplasm**

Although SV40 late transcripts clearly are partitioned on the basis of their 3' termini, such that only those RNAs with a mature 3' terminus are located in the cytoplasm, late transcripts are not significantly partitioned with respect to whether or not they have been spliced. This result cannot be rationalized as leakage of nuclear RNA into the cytoplasm, either in vivo or during isolation, since the same preparations of RNA which show no partitioning on the basis of splicing do show partitioning on the basis of 3' termini. The results are analogous to those observed with tRNA precursors and tRNA: tRNA precursors which have 3' flanking sequences are confined to the nucleus, while precursors with a mature 3' end enter the cytoplasm, whether or not they contain an intervening sequence (Melton et al., 1979).

We have not proved that the observed partitioning is due to specific transport of only those RNAs with mature 3' termini; the formal possibility remains that all transcripts are transported, but that only RNAs with mature 3' termini are stable in the cytoplasm.

The presence of unspliced viral RNAs in the cytoplasm may reflect the transport of incompletely processed RNAs in oocytes, as proposed by Anderson et al. (1982). However, this interpretation of our data is complicated by the controversy over whether unspliced 19 S RNA also reaches the cytoplasm in infected monkey cells. Some workers have argued that unspliced 19 S RNA is transported to the cytoplasm of infected cells (Pataki et al., 1981), while others, using isotonic nuclear isolation procedures which minimize nuclear leakage, contend that over 95% of unspliced RNA is nuclear (Villereal & Jovanovich, 1980). Further experiments, involving the microinjection of genes encoding precursors known to be confined to the nucleus of somatic cells, will be needed to clarify whether the presence of unspliced RNA in the oocyte cytoplasm is a general property of the oocyte, or is a peculiarity of unspliced viral 19 S RNA.
(b) The 5300 base transcript

The significance of the 5300 base transcript is enigmatic. It seemed possible that it is a late mRNA precursor which is processed to generate the 3' terminus of late mRNA. The 5300 base transcript does appear before 19 S RNA, consistent with its being a precursor. Oocyte RNA labelled for less than ten hours contains the large species but not the small one, while RNA labelled for one day or more contains roughly equal amounts of both (not shown). Similarly, Miller, Stephens & Mertz (unpublished results) have demonstrated that pulse-labelled SV40 late transcripts synthesized in oocytes are large and non-polyadenylated, while stable transcripts include a polyadenylated 19 S species. However, radioactivity present in the 5300 base RNA cannot be "chased" into 19 S RNA (Wickens & Gurdon, unpublished results): if oocytes are allowed to accumulate the 5300 base transcript, are then injected with α-amanitin to stop further synthesis and incubated for an additional 15 hours, the large transcript is not converted to the smaller one. Thus, the significance of the 5300 base RNA has not yet been unambiguously established.

The 3' end of the primary transcript in infected monkey cells is unknown, since this precursor is very unstable (Ford & Hsu, 1978). The 3' terminus of the 5300 base transcript synthesized in oocytes probably lies in the HindIII-C fragment (positions 5171 to 1046; Tooze, 1981) for the following reasons: its 5' terminus lies in the same region as the 5' terminus of late mRNAs from infected cells (not shown), and it is about 5300 bases long, having an electrophoretic mobility very similar to linear SV40 DNA on denaturing agarose gels; it is complementary to sequencers 2000 bases beyond the poly(A) site (Figs 2 and 4) and contains all of the sequences in the TaqI/BglII fragment which immediately precedes the SV40 origin (not shown). Our preliminary S1 mapping experiments indicate that its 3' end probably lies near the termination site described by Hay et al. (1982), in which case the 5300 base RNA may correspond, at least in structure, to the unit length primary transcript hypothesized by Lebowitz & Weissmann (1979).

(c) Gene expression during oogenesis

Qualitatively, the structure of stable viral transcripts indicates no gross deficiency in the post-transcriptional activities of oocytes: mRNA precursors are cleaved accurately at splice sites, are processed to form the same 3' mRNA terminus as in infected cells, are polyadenylated, and are specifically partitioned between nucleus and cytoplasm. Those aspects in which late mRNA synthesis in oocytes differs from that in infected cells are predominantly quantitative: thus, the relative amounts of cleavage at the 16 S and 19 S 3' splice sites differ, and the proportion of stable RNA which possesses the mature, polyadenylated 3' terminus is lower in oocytes. By examining pulse-labelled RNA, Mertz and colleagues (personal communication) have concluded that the formation of SV40 late transcripts in oocytes proceeds by a processing pathway very similar to that used in infected monkey cells. The simplest extension of our results is that a gross deficiency in a processing activity is unlikely to explain why, in mature oocytes, the level of mRNA does not increase even though nuclear transcripts continue to be
synthesized in lampbrush chromosomes. Rather, we favour the hypothesis of Dolecki & Smith (1979) that mature, cytoplasmic mRNA is formed in oocytes but is degraded rapidly enough so that it does not increase the amount of mRNA which the oocyte contains.

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