Species-specific *in vitro* synthesis of DNA containing the polyoma virus origin of replication

(eukaryotic DNA replication/papovavirus host permissiveness/DNA polymerase α /simian virus 40)

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ABSTRACT *In vitro* replication of DNA containing the polyoma (Py) virus origin of replication has been carried out with cell-free extracts prepared from mouse FM3A cells. The *in vitro* system required the Py virus-encoded large tumor (T) antigen, DNA containing the Py virus origin of replication, ATP, and an ATP-regenerating system. The replication reaction was inhibited by aphidicolin, suggesting the involvement of DNA polymerase α in this system. Simian virus 40 (SV40) T antigen could not substitute for the Py T antigen. Cell extracts prepared from HeLa cells, a source that replicates SV40 DNA in the presence of SV40 T antigen, replicated Py DNA poorly. The addition of purified DNA polymerase α -primase complex isolated from FM3A cells enabled HeLa cell extracts to replicate Py DNA with the same efficiency as FM3A cell extracts. Complementary experiments have shown that FM3A cell extracts do not support SV40 DNA replication unless supplemented with DNA polymerase α -primase complex from HeLa cells [Murakami, Y., Wobbe, C. R., Weissbach, L., Dean, F. B. & Hurwitz, J. (1986) *Proc. Natl. Acad. Sci. USA* 83, 2869-2873]. These results indicate that the host-cell source of the DNA polymerase α -primase complex plays an important role in discriminating between SV40 T antigen- and Py T antigen-dependent replication of their homologous DNA *in vitro*. This may explain the host-range specificity of these viruses *in vivo*.

Different cell types are permissive for replication of the two papovaviruses simian virus 40 (SV40) and polyoma (Py). Mouse cell lines support the replication of Py virus but not of SV40, whereas monkey and human cells will replicate SV40 but not Py. Cells fully permissive for both SV40 and Py have not yet been described. Hybrids formed between permissive and nonpermissive cells yield infectious viral progeny (1-3) suggesting that one or more trans-dominant factors present only in permissive cells interact in a unique way with viral components, leading to viral replication. Early-region viral mRNAs and their protein products, the tumor antigens, are synthesized in a wide variety of infected permissive and nonpermissive cells. However, significant extrachromosomal papovavirus DNA replication occurs only in permissive cells. It is therefore possible that the factor(s) controlling host permissiveness is involved in viral DNA replication.

Cell-free extracts of monkey and human cells that replicate SV40 DNA have been described (4-7). The *in vitro* replication of SV40 DNA requires, in addition to suitably prepared cell extracts, circular DNA containing the viral replication origin (ori) and purified SV40 large tumor (T) antigen. Since SV40 T antigen and DNA containing the SV40 replication origin are the only viral components required, this system should be useful for identifying and characterizing the eu-

karyotic proteins involved in DNA replication. In previous reports (7-9), it was shown that DNA polymerase α (pol α) and DNA primase are essential for the replication of SV40 DNA *in vitro* and that the source of these enzymes is important in determining whether SV40 DNA replication will occur. In contrast to extracts of human cells, mouse cell extracts supplemented with SV40 T antigen did not support replication of DNA containing the SV40 origin unless supplemented with the pol α -primase complex from HeLa cells.

In this report we describe the establishment of a system that replicates Py DNA *in vitro*; it requires mouse cell extract, the Py replication origin, and Py T antigen. In addition, we have confirmed and further defined the important role played by pol α -primase in determining the species specificity of papovavirus DNA replication.

MATERIALS AND METHODS

Purification of Py T Antigen. Py T antigen was purified from CV-1 cells infected with the helper-dependent recombinant adenovirus vector Ad-SVR587 (10) as follows. Cells were infected with wild-type adenovirus [2-5 plaque-forming units (pfu) per cell] and Ad-SVR587 recombinant virus (a gift from S. Mansour, T. Grodzicker, and R. Tjian) (5-10 pfu per cell). After incubation for 36 hr at 37°C, cells were scraped from thirty 150-mm plates into ≈ 3 ml of cold Dulbecco's phosphate-buffered saline (PBS: Ca^{2+} - and Mg^{2+} -free), washed twice with cold PBS, suspended in 10 volumes of pH 9.0 buffer [20 mM Tris-HCl, pH 9.0/0.2 M NaCl/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol/1% (vol/vol) Nonidet P-40 (NP-40)/1 mM phenylmethylsulfonyl fluoride (PMSF)], and lysed on ice for 10 min. The lysate was centrifuged for 10 min at 2000 rpm in a Sorvall H-6000A rotor, and the supernatant was then centrifuged for 10 min at 20,000 rpm in a Sorvall SS34 rotor. The supernatant was mixed with 0.5 volume of pH 6.8 buffer (0.1 M Tris-HCl, pH 6.8/1 mM EDTA/1 mM dithiothreitol/10% glycerol/1% NP-40/1 mM PMSF) and then loaded onto an anti-Py T antigen immunoaffinity column [4 ml; equilibrated with pH 8.0 buffer (20 mM Tris-HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol/0.1 M NaCl/1% NP-40/10% glycerol)] at a rate of 3 ml/hr. The immunoaffinity column was prepared by crosslinking 10 ml of anti-mouse IgG-agarose beads (Hyclone) with 300 ml of supernatant from F4 hybridoma cells (which produce antibody to Py T antigen; the hybridoma supernatant was a gift from C. Schley and E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as previously described (11). The column was washed with 12 ml of pH 8.0 buffer and

Abbreviations: SV40, simian virus 40; Py, polyoma; SV40 T antigen, SV40-encoded large tumor antigen; Py T antigen, Py virus-encoded large tumor antigen; RFI, superhelical circular duplex DNA; RFII, circular duplex DNA containing at least one single-strand break; pol α , DNA polymerase α ; bp, base pair(s).

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then with 12 ml of buffer B (20 mM Tris-HCl, pH 8.5/1 mM EDTA/1 mM dithiothreitol/10% glycerol) containing 0.5 M NaCl. Py T antigen was then eluted with buffer B containing 0.5 M NaCl and 50% ethylene glycol. The fractions that bound DNA (5' ends labeled with 32 P) containing the Py replication origin to nitrocellulose filters were pooled and dialyzed against 1 liter of buffer (10 mM Hepes-KOH, pH 7.5/5 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol/1 mM PMSF/50% glycerol). The retentate was used as purified Py T antigen. This solution contained 0.1 mg of protein per ml and on NaDodSO₄/polyacrylamide gel electrophoresis showed one major band at 92 kDa, the monomeric size of the Py T antigen. This band represented $\approx 60\%$ of the protein applied to the gel.

In Vitro Replication of SV40 ori⁺ DNA and Py ori⁺ DNA. The reaction conditions and methods used for the preparation of extracts of HeLa cells (7) and SV40 T antigen (12) have been described. Cell-free extracts from FM3A cells [mouse mammary carcinoma cell line (13)] were prepared the same way as extracts from HeLa cells. Reaction mixtures (50 μ l) contained 80 mM Tris-HCl (pH 7.8); 40 mM creatine phosphate; 7 mM MgCl₂; 0.5 mM dithiothreitol; 4 mM ATP; 200 μ M each CTP, UTP, and GTP; 100 μ M each dATP, dCTP, and dGTP; 25 μ M [methyl- 3 H]dTTP (150–300 cpm/pmol); 1 μ g of creatine kinase; superhelical circular duplex (RFI) plasmid DNA [0.3 μ g of pSV01 Δ EP3 (SV40 ori⁺) or 0.5 μ g of p373A2 (Py ori⁺)]; 0.2–0.4 mg (protein) of HeLa extract or FM3A extract; and 0.5–1 μ g of SV40 or Py T antigen. The SV40 ori⁺ DNA contained the 311-base-pair (bp) origin fragment (the *Eco*RII fragment G of SV40 DNA inserted into a 2481-bp pBR322 derivative) as described (7). The Py ori⁺ DNA contained the entire Py sequence (5292 bp) as a *Bam*HI insert cloned in a pBR322 derivative (nucleotides 1727–2349 were deleted from pBR322). Reaction mixtures were incubated at 37°C, and the radioactivity incorporated into acid-insoluble material was measured. When products formed in the replication reaction were examined, [α - 32 P]dCTP (7000–8000 cpm/pmol) was used instead of [3 H]dTTP, and analyses were carried out by agarose gel electrophoresis followed by autoradiography as described (7).

Purification of pol α -Primase Complex. FM3A and HeLa pol α -primase complex were assayed and purified as described (8) from 10¹⁰ cells. The specific activities (per μ g of protein) of these enzymes were as follows: preparations from HeLa cells, 0.20 unit (u) of pol α and 0.50 u of primase; preparations from FM3A cells, 0.60 u of pol α and 0.41 u of primase.

Restriction Endonuclease Digestion. Restriction endonucleases were from New England Biolabs and were used according to the supplier's instructions, with the exception that incubation with *Dpn* I was carried out in the presence of 0.2 M NaCl; at lower NaCl concentrations, hemimethylated as well as fully methylated DNA were cut by this enzyme (7).

RESULTS

In Vitro Replication of Py DNA. Previously, it was reported (8) that FM3A (mouse) cell extracts support the replication of DNA containing the SV40 origin in the presence of HeLa cell pol α -primase complex. This suggested that the FM3A cell extracts contained all the replication factors required for SV40 DNA replication *in vitro* except for a competent pol α -primase complex. This result prompted us to develop an *in vitro* system using FM3A cell extracts that replicated Py DNA. Like the T antigen encoded by SV40, Py T antigen is necessary for DNA replication *in vivo* (14). To develop a Py replication system, we utilized the Ad-SVR587 vector, which expresses the Py T antigen (10). Py T antigen was isolated by an immunoaffinity purification procedure similar to that

developed for SV40 T antigen, using the F4 monoclonal antibody specific for Py T antigen.

In the presence of immunoaffinity-purified Py T antigen, FM3A cell extracts catalyzed extensive incorporation of deoxyribonucleotides, whereas extracts from HeLa cells were virtually inactive (Fig. 1A). Incorporation was absolutely dependent on Py T antigen, and SV40 T antigen could not replace it. DNA synthesis with Py ori⁺ DNA was observed only when the concentration of Py T antigen was greater than 0.5 μ g per reaction (Fig. 1B), a phenomenon also observed in the SV40 DNA replication system *in vitro*. Replication was completely dependent on added Py T antigen, Mg²⁺, exogenous Py ori⁺ DNA, and an ATP-regenerating system (Table 1). In the presence of creatine phosphate and creatine kinase, omission of ATP reduced incorporation by a factor of 4. Omission of CTP, UTP, and GTP reduced incorporation by a factor of 2, whereas omission of dATP, dCTP, and dGTP reduced incorporation by an order of magnitude. Aphidicolin, a specific inhibitor of pol α (16), completely inhibited incorporation, suggesting the involvement of pol α in this system. The system was sensitive to the addition of RNase A, suggesting a requirement for RNA in this system. These findings are similar to observations made with the SV40 *in vitro* DNA replication system.

Analysis of Replication Products. Products formed in the Py T antigen-dependent reaction described above were analyzed by agarose gel electrophoresis with or without treatment with restriction endonuclease *Dpn* I (Fig. 2). In the absence of Py T antigen, replication products were not detectable, whereas in its presence, products formed were mainly RFII (circular duplex with at least one single-strand break), topoisomers, and slower migrating products. These DNA products resulted from semiconservative replication, based on their resistance to digestion by *Dpn* I. Both *Dpn* I and *Mbo* I act at the sequence -GATC-, the site of action of *Escherichia coli* Dam methylase. *Dpn* I cuts at this site if adenine residues on both strands are methylated, whereas *Mbo* I cuts only if both strands are devoid of methylated adenine. The ori⁺ plasmid was isolated from a Dam⁺ strain of *E. coli* (HB101) and was efficiently digested by *Dpn* I (data not shown). Replication

FIG. 1. (A) Py DNA replication in FM3A or HeLa cell extract. Various amounts of extract from FM3A cells (●, ○) or HeLa cells (■, □) was added to reaction mixtures (50 μ l) containing 1.2 μ g of Py T antigen (closed symbols) or no T antigen (open symbols). Incubation was carried out for 120 min at 37°C, and acid-insoluble radioactivity was determined. Other additions were as described in *Materials and Methods*. (B) Influence of Py and SV40 T antigens on Py DNA replication. Various amounts of Py T antigen (●) or SV40 T antigen (■) were added to reaction mixtures (50 μ l) containing FM3A cell extract (0.3 mg of protein). Both T antigens were immunoaffinity-purified using Pab419 (SV40, see ref. 12) or F4 (Py) antibody (see *Materials and Methods*). Incubation was carried out as in A.

Table 1. Requirements for replication of Py DNA *in vitro*

Component omitted (–) or added (+)	dTMP incorporated, pmol
None	65.7
– extract	<1
– DNA	<1
– Mg ²⁺	<1
– DNA, + ori [–] DNA	<1
– T antigen	<1
– ATP	17.0
– creatine phosphate	<1
– CTP, GTP, and UTP	36.3
– dATP, dCTP, and dGTP	6.9
+ aphidicolin (0.2 mM)	<1
+ RNase A (50 µg/ml)	<1

Reaction mixtures (50 µl) were as described in *Materials and Methods*, with 0.5 µg of p373A2 (ori⁺) DNA, 300 µg (protein) of FM3A cell extract, and 1.2 µg of Py T antigen. After incubation at 37°C for 120 min, acid-insoluble radioactivity was measured. The ori[–] DNA was the deletion mutant 3027 (15).

products formed after various lengths of incubation were subjected to *Mbo* I restriction (Fig. 3). Labeled fragments were observed after 2 hr of incubation (Fig. 3), suggesting that some of the DNA molecules underwent multiple rounds of replication.

Replication Proceeds Bidirectionally from the Origin Site. After various periods of incubation, replication products were isolated, digested with *Hinc*II and *Pst* I and analyzed by electrophoresis in agarose gel (Fig. 4). After 15 min, only the origin-containing 1422-bp fragment was labeled. After 30 min of incubation, adjacent fragments (1872 and 2336 bp) were

FIG. 2. Analysis of Py DNA replication products with restriction endonuclease *Dpn* I. Reaction mixtures (50 µl), as described in *Materials and Methods*, with 20 µM [α -³²P]dCTP (7400 cpm/pmol) and 300 µg of FM3A cell extract, were incubated for 30, 60, and 120 min. Length of incubation and the addition of Py T antigen (TAg) are indicated above each lane. Such reaction mixtures resulted in the incorporation of 4, 12, and 20 pmol of dCMP, respectively. DNA products were treated with proteinase K (1 mg/ml) extracted with phenol/chloroform (1:1), incubated either with (+) or without (–) restriction endonuclease *Dpn* I, and electrophoresed in 0.5% agarose gel. The positions of supercoiled (RFI) and nicked (RFII) DNA were determined by ethidium bromide staining. An autoradiogram of the dried gel is shown.

FIG. 3. Analysis of replication products with restriction endonuclease *Mbo* I. Reaction mixtures (all containing Py T antigen) were prepared as described in the legend to Fig. 2 and were incubated at 37°C for 60 or 120 min. Replication products were processed as described for Fig. 2 and were analyzed in a 1.5% agarose gel after incubation with (+) or without (–) restriction endonuclease *Mbo* I. p373A2 DNA digested with *Hinc*II and *Pst* I was used as size markers (fragment lengths in bp at right). Positions of RFI, RFII, and marker DNAs were determined by ethidium bromide staining. An autoradiogram of the dried gel is shown.

labeled. The most distal fragment (1236 bp) was labeled to the same extent as the origin-containing fragment after 1 hr. These results suggest that replication starts at the Py origin and proceeds bidirectionally. However, these results would also reflect a site-specific initiation reaction followed by a random unidirectional movement of the replication fork on different DNA molecules.

Specificity of DNA Replication *in Vitro*. Both the origins of replication and the T-antigen polypeptides of SV40 and Py virus have similar sequences, suggesting that these viruses have similar modes of replication. However, replication of SV40 occurs in primate and human cells, whereas Py virus replicates in mouse cells (15, 17, 18). Since we had both the SV40 and Py DNA replication systems operational, we tested the specificity of DNA, T antigen, and extract in each system (Table 2). With SV40 ori⁺ DNA, only the combination of HeLa extract and SV40 T antigen was active. With Py ori⁺ DNA, the combination of FM3A extract and Py T antigen catalyzed efficient incorporation, whereas the combination of HeLa extract and Py T antigen was <10% as active.

It was previously reported (8) that FM3A extracts catalyzed the incorporation of deoxynucleotides into SV40 ori⁺ DNA when supplemented with the HeLa pol α -primase complex. The amount of incorporation depended upon the amount of pol α -primase activity added (8). To test whether pol α -primase complex was responsible for the permissiveness in the Py DNA replication *in vitro*, we examined the activation of HeLa cell extracts (which worked poorly in the Py DNA replication system *in vitro*) by purified FM3A pol α -primase complex. As shown in Fig. 5, the purified pol α -primase complex isolated from FM3A cells activated HeLa cell extracts, whereas the addition of HeLa pol α -primase complex had little effect. The activation was dependent upon the presence of Py T antigen. These findings and previous results (8) suggest that the pol α -primase

FIG. 4. Bidirectional replication starting from the Py origin. (A) Reaction mixtures were prepared as described for Fig. 2. At 15, 30, and 60 min, replication products were isolated and processed as described for Fig. 2, digested completely with *HincII* and *Pst* I, and electrophoresed in a 1.5% agarose gel. An autoradiogram of the dried gel is shown. Size (bp) of *HincII*/*Pst* I-digested fragments are indicated at right. (B) Map of the p373A2 *HincII*/*Pst* I restriction fragments, with the Py replication origin (ori) indicated within the 1422-bp fragment. Solid triangles indicate *HincII* sites; open triangles indicate *Pst* I sites.

complex plays an important role in determining which cell extract supports SV40 and Py DNA replication *in vitro*.

DISCUSSION

The general features of Py DNA replication resemble those observed previously for SV40 DNA replication *in vitro* (4–8), including specificity in its requirement for mouse cell factors, Py T antigen, and DNA containing the Py replication origin. Thus, extracts prepared from FM3A cells supported the efficient replication of Py DNA, while extracts from HeLa cells worked poorly. SV40 T-antigen preparations that supported SV40 DNA replication were unable to replace Py T antigen in the Py replication system. Moreover, the Py replication system specifically required the intact Py replication origin; origin-defective Py DNA [Py deletion mutant 3027 (14)], SV40 ori⁺, and pBR322 DNAs were all inactive.

Table 2. SV40 and Py DNA replication: Role of the replication origin, source of extract, and T antigen

Source of preparation			dTTP incorporated, pmol
DNA	Extract	T antigen	
Py	FM3A	Py	75.7
Py	HeLa	Py	5.0
Py	FM3A	SV40	<1
Py	HeLa	SV40	3.0
SV40	HeLa	SV40	93.1
SV40	FM3A	SV40	2.7
SV40	HeLa	Py	<1
SV40	FM3A	Py	<1
pBR322	FM3A	Py	<1
pBR322	SV40	SV40	<1

Reaction mixtures (50 μ l) were as described in *Materials and Methods*, with 0.5 μ g of DNA (p373A2 as Py DNA, pSV01 Δ EP as SV40 DNA), 1.2 μ g of Py or SV40 T antigen (immunoaffinity-purified), and 300 μ g (protein) of mouse FM3A or HeLa extract. After incubation at 37°C for 120 min, acid-insoluble radioactivity was measured.

The products of the *in vitro* reaction contained a newly replicated strand hydrogen-bonded to a parental template strand. *Mbo* I restriction analysis indicated that some of the newly formed DNA had participated in multiple rounds of replication. The Py system was inhibited by aphidicolin and RNase A, as was observed in the SV40 *in vitro* DNA replication system.

In a previous report (8), it was shown that the source of pol α -primase complex plays a key role in determining whether cell extracts replicate SV40 DNA. Only pol α -primase complex from permissive primate cells worked efficiently. FM3A extracts, normally inactive in the SV40 system, replicated SV40 DNA when supplemented with HeLa pol

FIG. 5. Activation of HeLa cell extracts with purified pol α -primase complex. Various amounts of purified pol α -primase complex from FM3A (●, ○) or HeLa (■, □) cells were added to HeLa extract (300 μ g of protein). Reactions were carried out in the presence (1.2 μ g) or absence of Py T antigen (solid symbols and open symbols, respectively). After 2 hr of incubation at 37°C, acid-insoluble radioactivity was determined.

α -primase fractions. In this report, we have provided further evidence that host-cell specificity for papovavirus DNA replication *in vitro* can be determined by fractions that are highly enriched in pol α -primase complex. HeLa cell extracts normally inactive in Py DNA replication can replicate Py DNA when supplemented with fractions containing FM3A pol α -primase. More recent studies have been carried out with the immunoaffinity-purified HeLa pol α -primase complex. Mouse cell extracts supplemented with this complex supported SV40 T antigen-dependent replication. Immunoaffinity-purified pol α , devoid of primase activity, isolated from HeLa cells did not activate mouse cell extracts (unpublished results).

Although Py and SV40 DNA replication *in vitro* follow a similar overall pattern in their requirements for T antigen and the viral replication origin, there are both similarities and differences in the properties of these viral components. SV40 and Py T antigens are fairly homologous proteins (15) that display high-affinity, sequence-specific DNA binding, particularly to sequences containing repeats of the consensus pentanucleotide GRGGC (14, 17), and exhibit ATPase activity (18). However, SV40 T antigen, but not Py T antigen, binds to the cellular p53 protein and is capable of inducing the transformed phenotype in both permissive and nonpermissive cells (ref. 2; for review, see ref. 1). The viral origins of SV40 and Py share common features, in that both contain palindromes adjacent to A+T-rich regions, as well as binding sites for T antigen. In the case of Py virus, the binding sites within the ori region are substantially weaker than those adjacent to this region (14, 17). The viral ori region of Py lacks early-region promoter elements, in contrast to SV40 (19). A recent report (20) has revealed an important role for enhancer elements in promoting Py viral DNA replication *in vivo*. Mutants that contain altered viral origin and enhancer sequences have been described (21–25). The role played by these sequences in replication can now be examined *in vitro*.

The SV40 *in vitro* DNA replication system has already been important for analyzing the process of eukaryotic DNA replication. Previous reports have described the separation of this replication system into multiple fractions (9), the existence of a pre-elongation step that most likely includes pre-elongation-complex formation (26), and the role of pol α and primase in determining host specificity for viral replication (8). Various replication mutants, including those with conditional lesions in pol α , have been isolated from a variety of rodent cell lines (27–30). Thus, both biochemical and genetic approaches using the *in vitro* Py replication system should contribute further to the understanding of DNA replication in animal cells.

Note Added in Proof. Both polyoma and SV40 T-antigen preparations, isolated by immunoaffinity procedures, contain ATPase and ATP-dependent DNA helicase activities. The latter activity was monitored by the displacement of 5'-³²P-labeled oligonucleotide

(≈60-mer) from a complex that was hydrogen-bonded to single-stranded circular DNAs (5–6 kilobases).

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