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Sequence-nonspecific replication of transfected plasmid DNA in poxvirus-infected cells

(tumorigenic poxvirus/calcium phosphate transfection/autonomous plasmid replication/replication origin)

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ABSTRACT **A system in which transfected plasmid DNA replicates in the cytoplasm of poxvirus-infected cells is described. A variety of recombinant plasmids was introduced into poxvirus-infected cells by transfection, and replication of input plasmid DNA was monitored by (*i*) digestion with restriction enzymes that discriminate between input methylated plasmid DNA and unmethylated DNA produced by replication in mammalian cells; (*ii*) amplification of intracellular plasmid DNA; and (*iii*) density shift analysis in the presence of BrdUrd. Replication of plasmid DNA was observed in the cytoplasm of cells infected with the tumorigenic leporipoxviruses Shope fibroma virus (SFV) and myxoma, and less extensively with the orthopoxvirus vaccinia, but not in uninfected cells. Unexpectedly, all input plasmids tested, including pBR322, pUC13, polyoma, PM2, ϕX174 replicative form (RF), and M13 RF, replicated with equal efficiency in SFV-infected cells, indicating that no specific replication origin sequence is required. The transfected plasmid DNA was replicated concomitantly with the infecting poxviral DNA and by 24 hr post-transfection, it resided predominantly in high molecular weight *Dpn* I-resistant head-to-tail tandem repeats. The failure to detect unreplicated *Dpn* I-sensitive plasmid concatemers early in replication together with the absence of significant levels of integrated plasmid sequences in the poxviral genome suggest that replication of the transfected plasmid DNA is not the consequence of nonhomologous recombination of concatemeric plasmid DNA into the poxvirus genome, but rather of an autonomous process that is dependent on *trans*-acting replication factors produced during virus infection, and that does not require a specific origin sequence on the substrate plasmid DNA.**

Poxviruses replicate their double-stranded linear genomes in factories or "micro-nuclei" within the cytoplasm of infected cells (1–3). The observation that these viruses are capable of replication in enucleated cells suggests that poxvirus DNA synthesis is a relatively autonomous process and may not require host functions (4–6). Electron microscopic observation of the replicating vaccinia genome (7–9) and *in vivo* labeling experiments (10–12) suggest that DNA replication starts near the genomic termini, but definitive evidence that a specific poxviral origin sequence exists is lacking. To further localize and examine the role of viral sequences during replication, we have utilized the transfection of exogenous plasmids into virus-infected cells, an approach that has previously been used with success to identify and characterize various functional viral DNA domains, including replication origins (13–18).

In this communication, we report on the fate of transfected plasmids with and without viral DNA inserts after their introduction into cells infected with either vaccinia virus or the tumorigenic poxvirus, Shope fibroma virus (SFV). Although preliminary experiments using standard calcium phosphate ($CaPO_4$) precipitation protocols had indicated that some, but not all, of these plasmids were capable of autonomous replication, under the optimized transfection conditions described here it was unexpectedly observed that cytoplasmic replication of a broad variety of circular input plasmids can be detected in cells infected with either SFV or vaccinia, although to a lesser extent in the latter. In fact, all input circular DNAs tested were found to replicate into high molecular weight head-to-tail tandem arrays, and we present evidence indicating that the exogenous plasmid DNA is autonomously replicated in a process that does not require a specific origin sequence on the replicating plasmid DNA.

MATERIALS AND METHODS

Cells and Viruses. The monkey cell lines BSC1, VERO, and BGMK (obtained from S. Dales, Department of Microbiology and Immunology, University of Western Ontario), the rabbit cell line SIRC (American Type Culture Collection), and primary rabbit kidney (RK) cells (Flow Laboratories) were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum. Conditions of infection with SFV strains Kasza, Boerlage, and Patuxent; myxoma strain Lausanne; and vaccinia strain IHD-W have been described (19, 20).

Transfection Protocol. Confluent cell monolayers ($2–3 \times 10^6$ cells) grown in 6-well multidishes (COSTAR) were infected at a multiplicity of 1.0 plaque (or focus) -forming unit per cell. At 2 hr (for SFV and myxoma), or 0.5 hr (for vaccinia) after a 1-hr adsorption period, $CaPO_4$-precipitated plasmid DNA was added as follows (21): 5–125 ng of plasmid DNA plus, when it was included, 2 μg of carrier calf thymus DNA, was precipitated in a total vol of 200 μl of $CaPO_4$ and added to the infected monolayer in 2 ml of DMEM with 5% fetal calf serum. Unexpectedly, carrier DNA was found to be not required for optimal uptake into the infected cells and subsequent cytoplasmic replication. At 3 hr after the addition of the $CaPO_4$ precipitate, the cells were washed once with phosphate-buffered saline (PBS; 145 mM NaCl/4 mM KCl/10 mM phosphate, pH 7.3) and once with PBS/5 mM EDTA, and fresh medium was added. For density shift experiments the medium was supplemented with 5-bromodeoxyuridine (BrdUrd) plus 5-fluorodeoxyuridine at 3 μg/ml and 250 ng/ml, respectively. For experiments in which viral DNA replication was inhibited, the medium was supplemented at 6 hr post-transfection with 300 μg of phosphonoacetate per ml. The cells were harvested at various times after the addition of the $CaPO_4$ precipitate by washing the monolayers sequentially with cold PBS/5 mM EDTA, and then with PBS, followed by addition of 0.5 ml of 10 mM Tris·HCl, pH 7.5/10

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Abbreviations: SFV, Shope fibroma virus; kb, kilobase(s); RF, replicative form.

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mM EDTA/0.5% $NaDodSO_4$/self-digested Pronase (0.5 mg/ml) (Calbiochem). This mixture was incubated at 37°C overnight, extracted with phenol and phenol/chloroform, and then precipitated with alcohol. The DNA was pelleted, washed once, lyophilized, resuspended in 10 mM Tris·HCl, pH 8/0.1 mM EDTA, and digested with RNase A (100 μg/ml) for 2 hr at 50°C.

DNAs and Enzymes. Plasmids pUC13, pBR322, pKBI^T, and M13 mp8 RF DNA were isolated by a scaled-up rapid alkaline procedure (22); ϕX174 replicative form (RF) DNA, PM2 DNA (obtained from A. R. Morgan), and polyoma DNA (obtained from J. S. Colter) were isolated by cesium chloride/ethidium bromide isopycnic centrifugation. Restriction endonucleases *Dpn* I, *Bcl* I, and *Bgl* II were purchased from Boehringer Mannheim; and *Mbo* I, *Pst* I, *Sst* I, and *Eco*RI were from Bethesda Research Laboratories. Restriction fragments of polyoma obtained with *Sst* I and *Pst* I digestions, and varying in size from 0.6 to 3.0 kilobases (kb), were purified by preparative agarose gel electrophoresis and ligated into circles at low DNA concentrations (<1 μg/ml).

Agarose Gel Electrophoresis and Hybridization. Electrophoresis of DNA in 0.7% agarose, transfer to nitrocellulose paper by Southern blotting, hybridization with nick-translated ^{32}P-labeled probes, and exposure to films with intensifier screens were as described (23). For dot blot analysis, DNA was denatured in 0.25 M NaOH (20 min at room temperature), neutralized with HCl, adjusted to 6× SCC (1× SSC = 0.15 M NaCl/0.015 M Na citrate), and applied to nitrocellulose. Drying, baking, and hybridization methods were identical to those used for Southern blot analysis. The fluorograms of the dot blots, including appropriate concentrations of plasmid standards, were quantitated with a Joyce–Loebl Chromoscan 3 microdensitometer.

RESULTS

Replication of Exogenous Plasmid DNA in SFV- and Vaccinia-Infected Cells. In experiments designed to screen for plasmids capable of autonomous replication in virus-infected cells, vector pBR322 and various plasmids with poxvirus DNA inserts were transfected singly and simultaneously in SFV-infected rabbit (SIRC) cells. At 24 hr after transfection with plasmid DNA, the majority of the detectable vector sequences were located in high molecular weight DNA (>40 kb): since no differences were detected between pBR322 and any of its derivative plasmids, only those data with pBR322 are presented in Fig. 1. To check whether these high molecular weight plasmid sequences are the result of replication in the transfected cells, DNA was digested with the restriction enzyme *Dpn* I, which cleaves input methylated plasmid DNA (i.e., DNA that had been propagated in HB101, a *dam*$^+$ host) but will not cut unmethylated DNA that has been replicated in mammalian cells (24). At 3 hr after transfection into either virus-infected or mock-infected cells, all of the intracellular pBR322 was sensitive to *Dpn* I digestion, as would be expected for unmodified input bacterial DNA (Fig. 1). However, after 24 hr in SFV-infected cells, the majority of pBR322 DNA was present as a *Dpn* I-resistant high molecular weight species; similar *Dpn* I-resistant pBR322 DNA sequences were also observed, although to a lesser extent, in vaccinia-infected cells. The appearance of this high molecular weight *Dpn* I-resistant pBR322 DNA is observed specifically in cytoplasmic extracts and is clearly dependent on poxvirus infection, since no replication was detected in uninfected cells (Fig. 1). In addition, it was observed that three strains of SFV (Kasza, Boerlage, and Patuxent), as well as myxoma virus, which are all of the leporipoxvirus genus, consistently produced much higher quantities of replicated product than the orthopoxvirus vaccinia. Surprisingly, the efficiency of transfection and the degree of amplification were found to be completely independent of carrier DNA in the $CaPO_4$ precipitation step, even at input levels as low as 1 ng per 10^6 cells.

That the *Dpn* I-resistant high molecular weight plasmid DNA was indeed fully demethylated was further demonstrated by its sensitivity to *Mbo* I (Fig. 2, lane b) and *Bcl* I (data not shown), enzymes that do not digest DNA methylated by *dam*$^+$ bacteria. In these digests, methylated input DNA was untouched, whereas all high molecular weight plasmid DNA detected at 24 hr post-transfection was completely digested. To ensure that the observed alterations of methylation were due to *bona fide* replication and not to a novel poxvirus-specific demethylase, two criteria were used. First, dot blot analysis was used to demonstrate an overall amplification of intracellular plasmid sequences. As shown in Fig. 3, this amplification could be readily observed at moderate to low levels of input plasmid (e.g., 5 ng per 10^6 cells) and coincided with the time of maximal viral DNA synthesis between 8 and 24 hr post-transfection (also see below). In uninfected cells no amplification was ever observed. In addition, phosphonoacetate, an inhibitor of poxviral DNA polymerase (25), blocked amplification of plasmid DNA in SFV-infected SIRC cells (Fig. 3). The second criterion relied on the ability to detect incorporation of BrdUrd into plasmid DNA during the replication phase (Fig. 4). By 24 hr post-transfection, virtually all the detectable pBR322 DNA was found to be fully substituted in both strands with BrdUrd, as evidenced by its buoyant density in CsCl (1.765 g/ml). At this time, all of the viral DNA was also fully substituted with BrdUrd, as

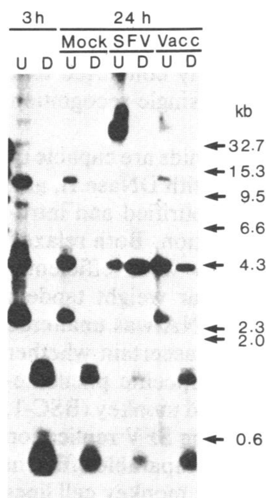

FIG. 1. Fate of plasmid pBR322 DNA in SFV- and vaccinia-infected cells. Monolayers of SIRC cells were infected with SFV or vaccinia (Vacc) or were mock-infected, and 125 ng of $CaPO_4$-precipitated pBR322 DNA was added to 2–3 × 10^6 cells. Total cellular DNA was isolated after 3 and 24 hr, and 1.5 μg of undigested (U) or *Dpn* I/*Eco*RI-digested (D) samples were electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with pBR322 probe. The size standards used were λ-digested with *Hin*dIII or *Sal* I. The *Eco*RI digestion converted the high molecular weight pBR322 sequences to unit length (4.3 kb).

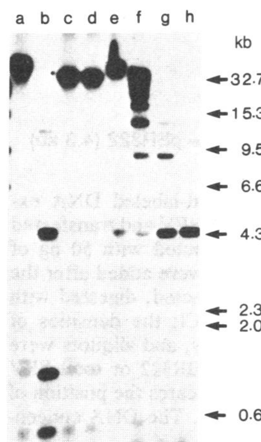

FIG. 2. Restriction enzyme analysis of *Dpn* I-resistant pBR322 DNA extracted from SFV-infected cells. Total cellular DNA from SFV-infected pBR322 transfected SIRC cells (see Fig. 1) was isolated 24 hr after transfection, digested with various restriction enzymes, and analyzed by Southern blotting using pBR322 DNA as probe. Samples of DNA (1.5 μg) were digested with *Dpn* I (lane a), *Mbo* I (lane b), *Dpn* I/*Bcl* I (lane c), *Dpn* I/*Bgl* II (lane d), undigested (lane e), *Dpn* I/partial *Eco*RI (lanes f and g), and *Dpn* I/*Eco*RI (lane h). The size standards are as in Fig. 1.

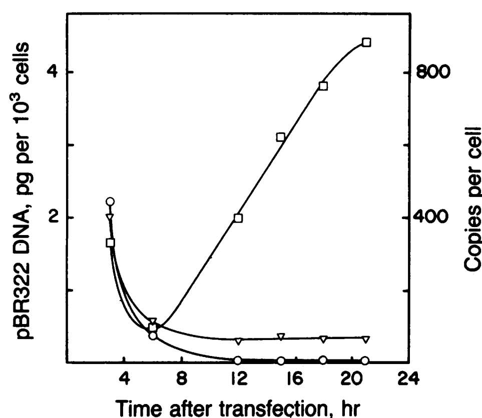

FIG. 3. Amplification of exogenous plasmid DNA in SFV-infected SIRC cells. Monolayers of SIRC cells were infected with SFV (□) and transfected with 5 ng of $CaPO_4$-precipitated pBR322 DNA. To ensure removal of all extracellular DNA, sequential washes with PBS, PBS/5 mM EDTA, and PBS were performed 3 and 6 hr post-transfection. At various times after transfection, cells were washed and total cellular DNA was harvested, digested with *Eco*RI, and analyzed by dot blot hybridization. Amplification was not observed in uninfected cells (○) or when 300 μg of phosphonoacetate per ml was added to the medium 6 hr post-transfection (▽).

expected, but the cellular DNA was either hybrid (1.73 g/ml) or fully light (<1.70 g/ml).

Replicated Plasmid DNA Is Maintained as Head-To-Tail Concatemers. To further characterize the high molecular weight plasmid DNA from SFV-infected cells, a partial digestion was carried out with *Eco*RI, which cuts pBR322 once. The resulting laddered series of fragments (Fig. 2, lanes e–h) is characteristic of head-to-tail tandem repeats (*Dpn* I was included to digest all unreplicated plasmid DNA). To determine whether these tandem arrays might be incorporated in the replicating viral genome, total DNA was digested with the restriction enzymes *Bcl* I and *Bgl* II (lanes c and d), neither of which cuts pBR322, but which cleave SFV DNA to fragment sizes <20 kb and <13 kb, respectively. The inability of these enzymes to digest any portion of the high molecular weight plasmid DNA indicates that at least lower oligomers (such as dimers, trimers, and tetramers) are not integrated in the viral genome. These results do not, however, rigorously exclude the selective integration of only larger head-to-tail plasmid oligomers into the replicating viral genome.

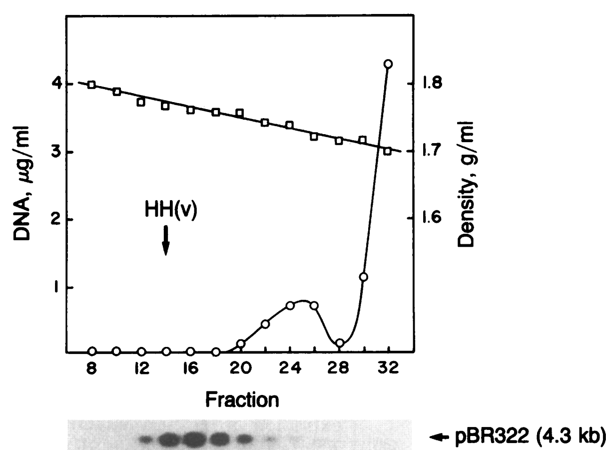

FIG. 4. Isopycnic centrifugation of BrdUrd-labeled DNA extracted from SIRC cells that were infected with SFV and transfected with pBR322. SFV-infected cells were transfected with 50 ng of pBR322, and BrdUrd plus fluorodeoxyuridine were added after the transfection step. After 24 hr, DNA was extracted, digested with *Eco*RI, and centrifuged to equilibrium in CsCl; the densities of fractions (□) were determined by refractometry, and aliquots were analyzed by Southern blotting, using either pBR322 or total SFV DNA as probes. The arrow denoted HH(v) indicates the position of fully replicated (i.e., heavy/heavy) SFV DNA. The DNA concentrations (○) were determined by fluorimetry.

Kinetics of Replication of Plasmid DNA in SFV-Infected Cells. To determine whether the time course of replication of pBR322 might differ from that of recombinant plasmids containing viral DNA sequences, SFV-infected cells were cotransfected with a mixture of pBR322 and various plasmids with inserts that span the 12.2-kb terminal inverted repeat of SFV. As an example, cotransfection of pBR322 and $pKBI^T$, which contains the terminal 3.6-kb *Bam*HI I^T fragment of SFV (19), resulted in the appearance of *Dpn* I-resistant plasmid DNA within 8 hr after transfection, and a steady increase for at least the next 12 hr (Fig. 5*A*). In each case, recombinant plasmid and pBR322 vector DNA replicated with approximately equal efficiency. To compare the timing of replication of plasmid DNA and viral DNA, the blot shown in Fig. 5*A* was subsequently probed with the viral DNA fragment E^{-B} (Fig. 5 *B* and *C*). The initiation of replication of both plasmid and viral DNA appears to coincide between 4 and 8 hr post-transfection. This observation strongly suggests that plasmid replication in SFV-infected cells is under viral control. In an attempt to distinguish between autonomous plasmid replication and the possibility that tandemly repeated plasmid sequences were incorporated, presumably by nonhomologous recombination, into the replicating viral genome (see also previous section), we have probed DNA extracted at times when viral DNA replication has just commenced (8–16 hr post-transfection) for high molecular weight methylated (i.e., input DNA) or hemimethylated plasmid sequences. Digestion with *Mbo* I, which cuts neither methylated nor hemimethylated DNA, failed to reveal any high molecular weight plasmid intermediates, even after prolonged exposures of the blots (not shown), suggesting that all high molecular weight plasmid DNA was fully demethylated. These results strongly argue against significant levels of integration of concatemeric plasmid DNA in the viral genome and indicate an autonomous mechanism of replication for the transfected plasmids.

Lack of Specific Sequence Requirement for Replication of Circular Plasmids in SFV-Infected Cells. Even though no specific viral DNA sequence is required for plasmid replication in SFV-infected cells, it is possible that a fortuitous sequence on pBR322 acts as a replication origin. We therefore transfected the prokaryotic plasmids, pUC13, ϕX174 RF, M13 mp8 RF, PM2, a circular eukaryotic virus genome, polyoma, and eight circularized polyoma restriction fragments (see *Materials and Methods*) into SFV-infected cells, and we monitored the presence of head-to-tail concatemers. All these plasmids were efficiently replicated to high molecular weight DNA, which was quantitatively converted to a monomer fragment upon digestion with a single-recognition restriction enzyme (not shown).

To determine whether only circular plasmids are capable of replication, supercoiled, relaxed (nicked with DNase I), and *Eco*RI-linearized forms of pBR322 were purified and introduced into SFV-infected cells by transfection. Both relaxed and supercoiled circular plasmid DNAs were efficiently converted to demethylated high molecular weight tandem arrays of vector DNA, while the linear DNA was unaltered in both uninfected and infected cells. To ascertain whether the observed replication might be a cell-specific phenomenon, several types of rabbit (SIRC, RK) and monkey (BSC-1, VERO, BGMK) cells capable of supporting SFV replication were tested. Both rabbit cells permitted comparable efficient replication of the transfected DNA but the monkey cell lines

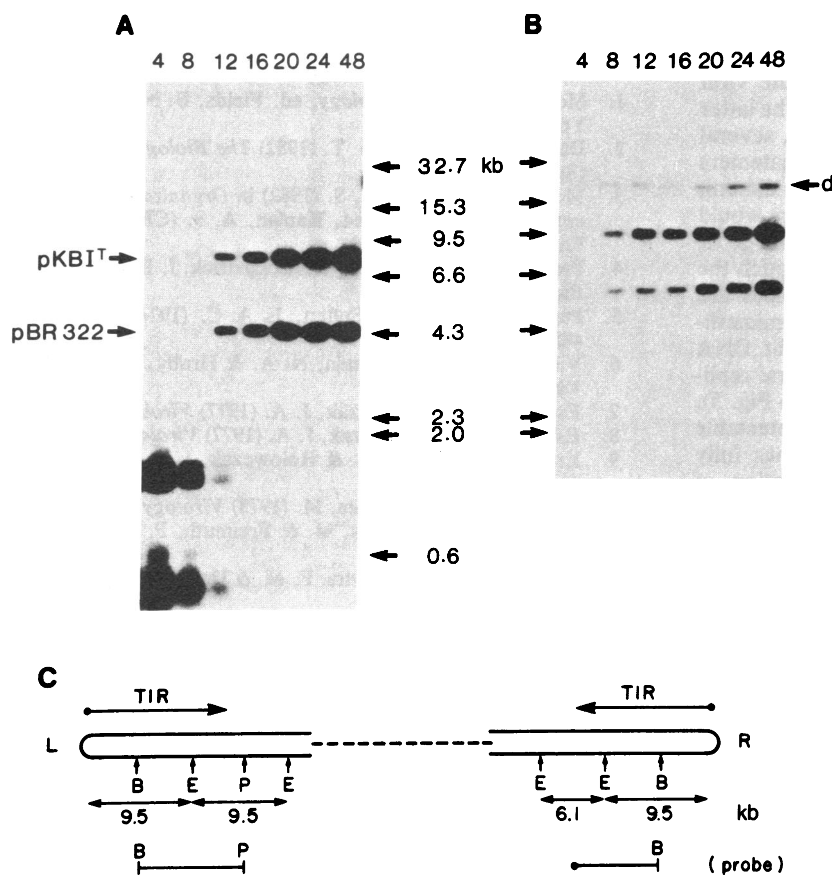

FIG. 5. Time course of replication of plasmids pBR322 and pKBI^T, and of viral DNA in SFV-infected cells. SFV-infected SIRC cells were transfected with 125 ng each of plasmids pBR322 and pKBI^T, and total DNA was harvested at various times after transfection (indicated in hours). DNA (1.5 μg) was digested with *Dpn* I/*Eco*RI, electrophoresed on 0.7% agarose gel, blotted onto nitrocellulose and probed with pBR322 (*A*) or the viral restriction fragment E^{-B} (*B*). In *A*, faint plasmid bands were detected at the 8-hr time point upon longer exposures of the fluorogram. The location of E^{-B} on the viral genome, and fragment sizes expected to hybridize with E^{-B} probe, are shown in *C*. Since part of E^{-B} falls within the terminal inverted repeat (TIR), restriction fragments from both the left end (9.5-kb terminal and 9.5-kb near-terminal) and the right end (9.5-kb terminal and 6.1-kb near-terminal) hybridize to the probe. In addition, a faint band at 19 kb (denoted d) represents the dimeric terminal palindromic replicative intermediate fragment (2 × 9.5-kb terminal fragment). E, *Eco*RI; B, *Bam*HI; P, *Pvu* II.

in general supported significantly less replication than did the rabbit cells.

DISCUSSION

We have demonstrated that sequence-nonspecific replication of transfected plasmid DNA takes place in the cytoplasm of poxvirus-infected cells. Analogous systems using nuclear viruses have a stringent requirement for the presence of viral replication origin sequences on the replicating plasmid DNA (reviewed in ref. 26). Evidence of extensive replication of plasmid pBR322 in SFV-infected cells, and somewhat lesser amounts in vaccinia-infected cells, was provided by (*i*) the rapid conversion of methylated (i.e., sensitive to *Dpn* I) to demethylated (i.e., resistant to *Dpn* I) DNA; (*ii*) an overall amplification of the intracellular plasmid DNA; (*iii*) suppression of amplification by phosphonoacetate, which inhibits poxvirus DNA replication, and (*iv*) the conversion of plasmid sequences to the fully heavy/heavy density in the presence of BrdUrd. A fortuitous poxviral replication origin on pBR322 was ruled out by the observation that five different circular DNA genomes (pUC13, ϕX174, PM2, M13, and polyoma) were similarly replicated in SFV-infected cells. In addition, the replication of circularized polyoma restriction fragments as small as 0.6 kb long strongly suggests that DNA replication in this system initiates in a sequence-nonspecific fashion. The replicated *Dpn* I-resistant plasmid sequences are observed exclusively in head-to-tail concatemers in a fashion similar to that described for autonomous replication of plasmids carrying a HSV replication origin (15–17). Several lines of evidence indicate that the plasmid replication reported here is under poxvirus control: (*i*) the absolute dependence on poxvirus infection; (*ii*) the approximate synchrony of initiation of plasmid and poxvirus replication; and (*iii*) the cytoplasmic location of replicated plasmid DNA.

One unexpected observation of this study was that replication of plasmid DNA in SFV-infected cells was not affected by omission of carrier DNA from the $CaPO_4$ precipitate, even when very low amounts of input DNA were used. This observation contrasts the previously reported requirement of carrier DNA for optimal marker rescue in another poxvirus, vaccinia (27, 28). The difference is likely not due to differential uptake into the cells, because $CaPO_4$ precipitates enter the cytoplasm of cells with great efficiency, even in the absence of DNA (29). It is plausible that carrier DNA facilitates the integration of plasmid sequences in the poxvirus genome, as it is thought to do in nuclear DNA (26).

The replication system described here differs markedly from a transient gene expression system in vaccinia-infected cells recently described by Cochran *et al.* (30). Optimal transcription of transfected plasmids requires multiplicities of infection and input plasmid DNA several orders of magnitude higher than we have observed to be needed for optimal plasmid replication. The inability of Cochran *et al.* to detect plasmid DNA replication in vaccinia-infected cells implies that conditions used in the gene expression system were not optimal for the detection of plasmid replication. Differences in transfection protocol, target cell, and DNA isolation methods may all have contributed to the apparent discrepancy. For example, we have detected vastly different amounts of plasmid replication in different cell lines. In addition, the use by Cochran *et al.* (30) of the Hirt procedure of DNA isolation would allow detection of replicated molecules if plasmid DNA were replicated by the Cairns mode, but selects against the high molecular weight concatemeric plasmid DNA described here.

The replicated *Dpn* I-resistant pBR322 sequences are arranged in head-to-tail concatemers with at least 4–5 units per tandem repeat and probably many more. This type of tandem array could, in theory, result from either autonomous

replication or by extensive recombination of unreplicated input DNA into concatemers followed by integration, presumably by nonhomologous recombination, into the viral genome. Even though we cannot rigorously rule out the latter mechanism of replication in poxvirus-infected cells, several observations make this unlikely. First, if large concatemers integrate into the viral genome, why are monomers, dimers, and other lower multimers excluded? Such oligomers would have been detected by enzymes such as *Bcl* I and *Bgl* II, which cleave the viral DNA frequently but do not touch the input plasmid (Fig. 2). Second, if plasmid sequences are constantly being integrated, both methylated and hemimethylated plasmid sequences in high molecular weight DNA should have been observed during periods when viral replication was first detected (e.g., 8- to 16-hr period in Fig. 5). Digests with *Dpn* I and *Mbo* I clearly show that the detectable high molecular weight plasmid DNA is at all times fully demethylated. Third, a model proposing integration of unreplicated plasmid DNA into the replicating viral genome would have to account for approximately equal frequencies of homologous and nonhomologous recombination, because mixed transfections with pBR322 and recombinant plasmids containing viral inserts produce no preferential replication of either plasmid. We therefore favor the model in which transfected plasmids in poxvirus-infected cells are replicated autonomously, possibly by a rolling circle mechanism, or alternatively by a θ type mechanism in combination with unusually active recombination, which in theory could convert the replicated monomers into high molecular weight tandem arrays.

The observed lack of specific sequence requirement for plasmid replication in poxvirus-infected cells is reminiscent of a similar lack of sequence specificity for plasmids microinjected into *Xenopus* eggs (31, 32). It has been suggested that, as the consequence of amplification of components required for cell proliferation in *Xenopus* eggs, the constraint of initiation at specific sequences is relaxed so as to be mainly controlled by the binding of the DNA to the nuclear matrix or another cellular structure (32, 33). Later in development, when the rate of DNA replication decreases, specific replication origins could then be activated. Similarly, the sequence-nonspecific replication of plasmids in poxvirus-infected cells may reflect a mechanism that is only used by the virus under certain circumstances or at a specific stage during the replication cycle, and it does not necessarily preclude the existence of specific origin sequences on the poxvirus genome itself. The results do indicate, however, that poxviruses can induce replication of heterologous intracellular plasmid DNA, which suggests that DNA templates such as molecules of endogenous small polydisperse circular DNA (35) or quiescent circular viral genomes could be similarly triggered into rounds of replication by a supervening poxvirus infection. Finally, the replication system described here provides a sensitive system to study the role of *trans*-acting viral proteins in viral DNA replication and recombination, including such specialized events as the resolution of terminal hairpin telomeres (34).

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