Biochemical Method for Inserting New Genetic Information into DNA of Simian Virus 40: Circular SV40 DNA Molecules Containing Lambda Phage Genes and the Galactose Operon of *Escherichia coli*

(molecular hybrids/DNA joining/viral transformation/genetic transfer)

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ABSTRACT   We have developed methods for covalently joining duplex DNA molecules to one another and have used these techniques to construct circular dimers of SV40 DNA and to insert a DNA segment containing lambda phage genes and the galactose operon of *E. coli* into SV40 DNA. The method involves: (*a*) converting circular SV40 DNA to a linear form, (*b*) adding single-stranded homodeoxypolymeric extensions of defined composition and length to the 3′ ends of one of the DNA strands with the enzyme terminal deoxynucleotidyl transferase (*c*) adding complementary homodeoxypolymeric extensions to the other DNA strand, (*d*) annealing the two DNA molecules to form a circular duplex structure, and (*e*) filling the gaps and sealing nicks in this structure with *E. coli* DNA polymerase and DNA ligase to form a covalently closed-circular DNA molecule.

Our goal is to develop a method by which new, functionally defined segments of genetic information can be introduced into mammalian cells. It is known that the DNA of the transforming virus SV40 can enter into a stable, heritable, and presumably covalent association with the genomes of various mammalian cells (1, 2). Since purified SV40 DNA can also transform cells (although with reduced efficiency), it seemed possible that SV40 DNA molecules, into which a segment of functionally defined, nonviral DNA had been covalently integrated, could serve as vectors to transport and stabilize these nonviral DNA sequences in the cell genome. Accordingly, we have developed biochemical techniques that are generally applicable for joining covalently any two DNA molecules.‡ Using these techniques, we have constructed circular dimers of SV40 DNA; moreover, a DNA segment containing λ phage genes and the galactose operon of *Escherichia coli* has been covalently integrated into the circular SV40 DNA molecule. Such hybrid DNA molecules and others like them can be tested for their capacity to transduce foreign DNA sequences into mammalian cells, and can be used to determine whether these new nonviral genes can be expressed in a novel environment.

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‡ Drs. Peter Lobban and A. D. Kaiser of this department have performed experiments similar to ours and have obtained similar results using bacteriophage P22 DNA (Lobban, P. and Kaiser, A. D., in preparation).

MATERIALS AND METHODS

DNA. (*a*) Covalently closed-circular duplex SV40 DNA [SV40(I)] (labeled with [³H]dT, 5×10^4 cpm/µg), free from SV40 linear or oligomeric molecules [but containing 3–5% of nicked double-stranded circles—SV40(II)] was purified from SV40-infected CV-1 cells (Jackson, D., & Berg, P., in preparation). (*b*) Closed-circular duplex λ*dvgal* DNA labeled with [³H]dT (2.5×10^4 cpm/µg), was isolated from an *E. coli* strain containing this DNA as an autonomously replicating plasmid (see ref. 3) by equilibrium sedimentation in CsCl–ethidium bromide gradients (4) after lysis of the cells with detergent. A more detailed characterization of this DNA will be published later. Present information indicates that the λ*dvgal* (λ*dv-120*) DNA is a circular dimer containing tandem duplications of a sequence of several λ phage genes (including C_I, O, and P) joined to the entire galactose operon of *E. coli* (Berg, D., Mertz, J., & Jackson, D., in preparation). DNA concentrations are given as molecular concentrations.

Enzymes. The circular SV40 and λ*dvgal* DNA molecules were cleaved with the bacterial restriction endonuclease RI (Yoshimori and Boyer, unpublished; the enzyme was generously made available to us by these workers). Phage λ-exonuclease (given to us by Peter Lobban) was prepared according to Little *et al.* (5), calf-thymus deoxynucleotidyl terminal transferase (terminal transferase), prepared according to Kato *et al.* (6), was generously sent to us by F. N. Hayes; *E. coli* DNA polymerase I Fraction VII (7) was a gift of Douglas Brutlag; and *E. coli* DNA ligase (8) and exonuclease III (9) were kindly supplied by Paul Modrich.

Substrates. [α-³²P]deoxynucleoside triphosphates (specific activities 5–10 Ci/µmol) were synthesized by the method of Symons (10). All other reagents were obtained from commercial sources.

Centrifugations. Alkaline sucrose gradients were formed by diffusion from equal volumes of 5, 10, 15, and 20% sucrose solutions with 2 mM EDTA containing, respectively, 0.2, 0.4, 0.6, and 0.8 M NaOH, and 0.8, 0.6, 0.4, 0.2 M NaCl. 100-µl samples were run on 3.8-ml gradients in a Beckman SW56 Ti rotor in a Beckman L2-65B ultracentrifuge at 4° and 55,000 rpm for the indicated times. 2- to 10-drop fractions were collected onto 2.5-cm diameter Whatman 3MM discs, dried without washing, and counted in PPO–dimethyl POPOP–toluene scintillator in a Nuclear Chicago Mark II

scintillation spectrometer. An overlap of 0.4% of ^{32}P into the ^{3}H channel was not corrected for.

CsCl–ethidium bromide equilibrium centrifugation was performed in a Beckman Type 50 rotor at 4° and 37,000 rpm for 48 hr. SV40 DNA in 10 mM Tris·HCl (pH 8.1)–1 mM Na EDTA–10 mM NaCl was adjusted to 1.566 g/ml of CsCl and 350 μg/ml of ethidium bromide. 30-Drop fractions were collected and aliquots were precipitated on Whatman GF/C filters with cold 2 N HCl; the filters were washed and counted.

Electron Microscopy. DNA was spread for electron microscopy by the aqueous method of Davis *et al.* (11) and photographed in a Phillips EM 300. Projections of the molecules were traced on paper and measured with a Keuffel and Esser map measurer. Plaque-purified SV40(II) DNA was used as an internal length standard.

Conversion of SV40(I) DNA to Unit Length Linear DNA [SV40(L_RI)] with R_I Endonuclease. [3H]SV40(I) DNA (18.7 nM) in 100 mM Tris·HCl buffer (pH 7.5)–10 mM MgCl₂–2 mM 2-mercaptoethanol was incubated for 30 min at 37° with an amount of R_I previously determined to convert 1.5 times this amount of SV40(I) to linear molecules [SV40(L_RI)]; Na EDTA (30 mM) was added to stop the reaction, and the DNA was precipitated in 67% ethanol.

Removal of 5′-Terminal Regions from SV40(L_RI) with λ Exonuclease. [3H]SV40(L_RI) (15 nM) in 67 mM K-glycinate (pH 9.5), 4 mM MgCl₂, 0.1 mM EDTA was incubated at 0° with λ-exonuclease (20 μg/ml) to yield [3H]SV40(L_RIexo) DNA. Release of [3H]dTMP was measured by chromatographing aliquots of the reaction on polyethyleneimine thin-layer sheets (Brinkmann) in 0.6 *M* NH₄HCO₃ and counting the dTMP spot and the origin (undegraded DNA).

Addition of Homopolymeric Extensions to SV40(L_RIexo) with Terminal Transferase. [3H]SV40(L_RIexo) (50 nM) in 100 mM K-cacodylate (pH 7.0), 8 mM MgCl₂, 2 mM 2-mercapto-ethanol, 150 μg/ml of bovine serum albumin, [α-32P]dNTP (0.2 mM for dATP, 0.4 mM for dTTP) was incubated with terminal transferase (30–60 μg/ml) at 37°. Addition of [32P]dNMP residues to SV40 DNA was measured by spotting aliquots of the reaction mixture on DEAE-paper discs (Whatman DE-81), washing each disc by suction with 50 ml (each) of 0.3 M NH₄-formate (pH 7.8) and 0.25 M NH₄HCO₃, and then with 20 ml of ethanol. To determine the proportion of SV40 linear DNA molecules that had acquired at least one "functional" (dA)_n tail, we measured the amount of SV40 DNA (3H counts) that could be bound to a Whatman GF/C filter (2.4-cm diameter) to which 150 μg of polyuridylic acid had been fixed (13). 15-μl Aliquots of the reaction mixture were mixed with 5 ml of 0.70 M NaCl–0.07 M Na citrate (pH 7.0)–2% Sarkosyl, and filtered at room temperature through the poly(U) filters, at a flow rate of 3–5 ml/min. Each filter was washed by rapid suction with 50 ml of the same buffer at 0°, dried, and counted. Control experiments showed that 98–100% of [3H]oligo(dA)₁₂₅ bound to the filters under these conditions. When the ratio of [32P]dNMP to [3H]DNA reached the value equivalent to the desired length of the extension, the reaction was stopped with EDTA (30 mM) and 2% Sarkosyl. The [3H]SV40(L_RIexo)–[32P]dA or –dT DNA was purified by neutral sucrose gradient zone sedimentation to remove unincorporated dNTP, as well as any traces of SV40(I) or SV40(II) DNA.

Formation of Hydrogen-Bonded Circular DNA Molecules. [32P]dA and -dT DNAs were mixed at concentrations of 0.15 nM each in 0.1 M NaCl–10 mM Tris·HCl (pH 8.1)–1 mM EDTA. The mixture was kept at 51° for 30 min, then cooled slowly to room temperature.

Formation of Covalently Closed-Circular DNA Molecules. After annealing of the DNA, a mixture of the enzymes, substrates, and cofactors needed for closure was added to the DNA solution and the mixture was incubated at 20° for 3–5 hr. The final concentrations in the reaction mixture were: 20 mM Tris·HCl (pH 8.1), 1 mM EDTA, 6 mM MgCl₂, 50 μg/ml bovine-serum albumin, 10 mM NH₄Cl, 80 mM NaCl, 0.052 mM DPN, 0.08 mM (each), dATP, dGTP, dCTP, and dTTP, (0.4 μg/ml) *E. coli* DNA polymerase I, (15 units/ml) *E. coli* ligase, and (0.4 unit/ml) *E. coli* exonuclease III.

RESULTS

General approach

Fig. 1 outlines the general approach used to generate circular, covalently-closed DNA molecules from two separate DNAs. Since, in the present case, the units to be joined are themselves circular, the first step requires conversion of the circular structures to linear duplexes. This could be achieved by a double-strand scission at random locations (see *Discussion*) or, as we describe in this paper, at a unique site with R_I restriction endonuclease. Relatively short (50–100 nucleotides) poly(dA) or poly(dT) extensions are added on the 3′-hydroxyl termini of the linear duplexes with terminal transferase; prior
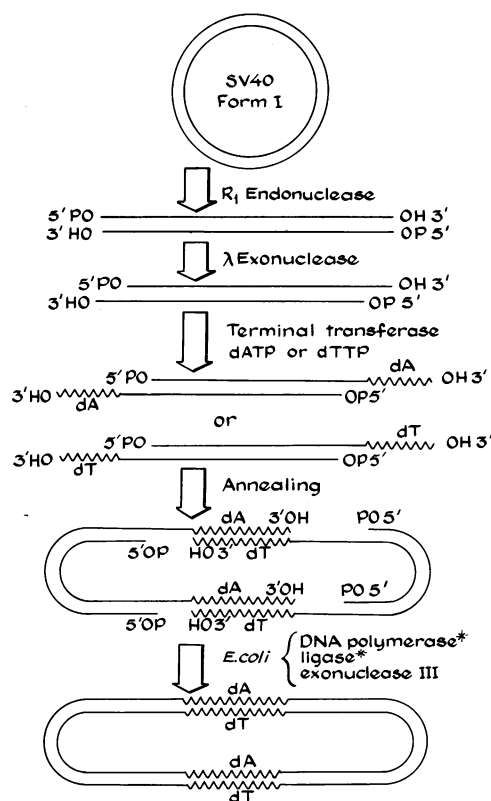

FIG. 1. General protocol for producing covalently closed SV40 dimer circles from SV40(I) DNA.

* The four deoxynucleoside triphosphates and DPN are also present for the DNA polymerase and ligase reactions, respectively.
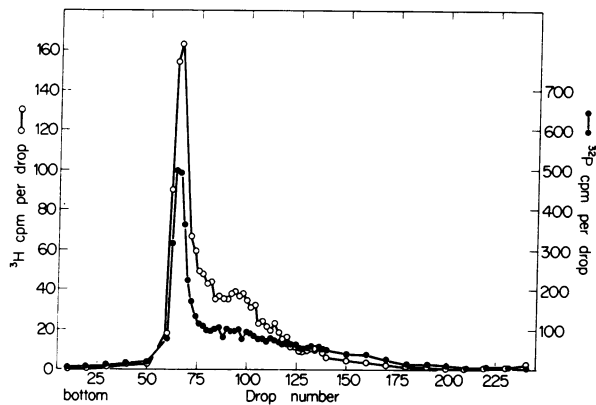
FIG. 2. Alkaline sucrose gradient sedimentation of [³H]SV40-(L_RIexo)-[³²P](dA)₈₀ DNA. 0.16 μg of DNA was centrifuged for 6.0 hr.

removal of a short sequence (30–50 nucleotides) from the 5'-phosphoryl termini by digestion with λ exonuclease facilitates the terminal transferase reaction. Linear duplexes containing (dA)_n extensions are annealed to the DNA to be joined containing (dT)_n extensions at relatively low concentrations. The circular structure formed contains the two DNAs, held together by two hydrogen-bonded homopolymeric regions (Fig. 1). Repair of the four gaps is mediated by *E. coli* DNA polymerase with the four deoxynucleosidetriphosphates, and covalent closure of the ring structure is effected by *E. coli* DNA ligase; *E. coli* exonuclease III removes 3'-phosphoryl residues at any nicks inadvertently introduced during the manipulations (nicks with 3'-phosphoryl ends cannot be sealed by ligase).

Principal steps in the procedure

Circular SV40 DNA Can Be Opened to Linear Duplexes by R_I Endonuclease. Digestion of SV40(I) DNA with excess R_I endonuclease yields a product that sediments at 14.5 S in neutral sucrose gradients and appears as a linear duplex with the same contour length as SV40(II) DNA when examined by electron microscopy [(18); Jackson and Berg, in preparation; see Table 1]. The point of cleavage is at a unique site on the SV40 DNA, and few if any single-strand breaks are introduced elsewhere in the molecule (18); moreover, the termini at each end are 5'-phosphoryl, 3'-hydroxyl (Mertz, J., Davis, R., in preparation). Digestion of plaque-purified SV40 DNA under our conditions yields about 87% linear molecules, 10% nicked circles, and 3% residual supercoiled circles.

Addition of Oligo(dA) or -(dT) Extensions to the 3'-Hydroxyl Termini of SV40 (L_RI). Terminal transferase has been used to generate deoxyhomopolymeric extensions on the 3'-hydroxyl termini of DNA (7); once the chain is initiated, chain propagation is statistical in that each chain grows at about the same rate (12). Although the length of the extensions can be controlled by variation of either the time of incubation or the amount of substrate, we have varied the time of incubation to minimize spurious nicking of the DNA by trace amounts of endonuclease activity in the enzyme preparation; we have so far been unable to remove or selectively inhibit these nucleases (Jackson and Berg, in preparation).

Incubation of SV40(L_RI) with terminal transferase and

either dATP or dTTP resulted in appreciable addition of mononucleotidyl units to the DNA. But, for example, after addition of 100 residues of dA per end, only a small proportion of the modified SV40 DNA would bind to filter discs containing poly(U) (13). This result indicated that initiation of terminal nucleotidyl addition was infrequent with SV40(L_RI), but that once initiated those termini served as preferential primers for extensive homopolymer synthesis.

Lobban and Kaiser (unpublished) found that P22 phage DNA became a better primer for homopolymer synthesis after incubation of the DNA with λ exonuclease. This enzyme removes, successively, deoxymononucleotides from 5'-phosphoryl termini of double-stranded DNA (15), thereby rendering the 3'-hydroxyl termini single-stranded. We confirmed their finding with SV40(L_RI) DNA; after removal of 30–50
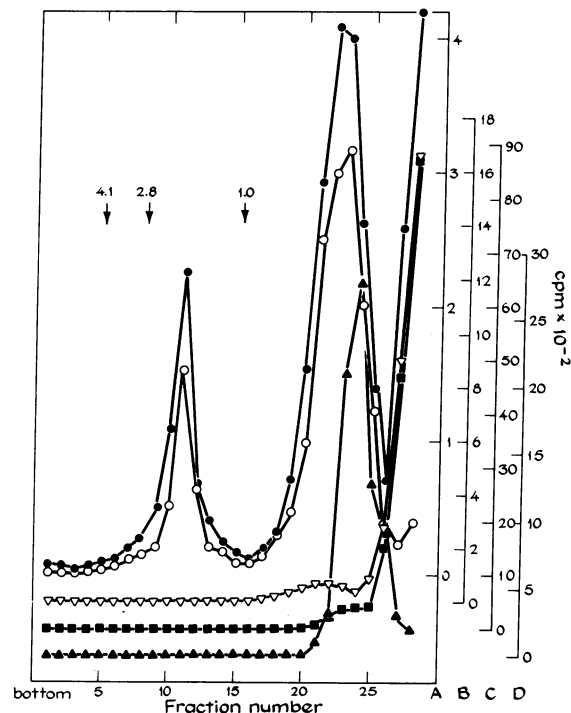

FIG. 3. Alkaline sucrose gradient sedimentation of [³H]-SV40(L_RIexo)-[³²P](dA)₈₀ and −(dT)₈₀ DNA incubated 4 hr with and without addition of *E. coli* DNA polymerase I (*P*), ligase (*L*), and exonuclease III (*III*). Conditions are described in *Methods*. 8-Drop fractions were collected. Samples A, C, and D were centrifuged for 60 min, sample B for 90 min. Line A, dA-ended, plus dT-ended SV40 linears, plus (P+L+III) (³²P, ●; ³H, ○); line B, dT-ended, SV40 omitted, plus (P+L+III) (³²P, ▼); line C, dA-ended SV40 omitted, plus (P+L+III) (³²P, ■); line D, dA-ended plus dT-ended SV40 linears, without (P+L+III) (³²P, ▲). ³H profiles are not shown for lines B, C, and D, but all show that the SV40 DNA sediments in its normal monomeric position. The ³²P and ³H profiles in line A are shifted to a faster-sedimenting position with respect to the ³²P profile in line D because SV40 strands are covalently linked to one another through (dA)₈₀ or (dT)₈₀ bridges in most of the molecules, whether or not covalently closed-circles are formed. Very little ³²P remains associated with the SV40 DNA in lines B and C because tails that remain single-stranded are degraded to 5'-mononucleotides by the 3'- to 5'-exonuclease activity of *E. coli* DNA polymerase I (7).

The *arrows* indicate the position in the gradient of different size supercoiled marker DNAs; the *number* is the multiple of SV40 DNA molecular size (1.0).

nucleotides per 5'-end (see Methods), the number of SV40(L$_{RI}$) molecules that could be bound to poly(U) filters after incubation with terminal transferase and dATP increased 5- to 6-fold. Even after separation of the strands of the SV40(L$_{RI}$exo)-dA, a substantial proportion of the ^{3}H-label in the DNA was still bound by the poly(U) filter, indicating that both 3'-hydroxy termini in the duplex DNA can serve as primers.

The weight-average length of the homopolymer extensions was 50–100 residues per end. Zone sedimentation of [^{3}H]-SV40(L$_{RI}$exo)-[^{32}P](dA)$_{80}$ (this particular preparation, which is described in Methods, had on the average, 80 dA residues per end) in an alkaline sucrose gradient showed that (i) 60–70% of the SV40 DNA strands are intact, (ii) the [^{32}P](dA)$_{80}$ is covalently attached to the [^{3}H]SV40 DNA, and (iii) the distribution of oligo(dA) chain lengths attached to the SV40 DNA is narrow, indicating that the deviation from the calculated mean length of 80 is small (Fig. 2). SV40(L$_{RI}$exo), having (dT)$_{80}$ extensions, was prepared with [^{32}P]dTTP and gave essentially the same results when analyzed as described above.

Hydrogen-Bonded Circular Molecules Are Formed by Annealing SV40(L$_{RI}$exo)-(dA)$_{80}$ and SV40(L$_{RI}$exo)-(dT)$_{80}$ Together. When SV40(L$_{RI}$exo)-(dA)$_{80}$ and SV40(L$_{RI}$exo)-(dT)$_{80}$ were annealed together, 30–60% of the molecules seen by electron microscopy were circular dimers; linear monomers, linear dimers, and more complex branched forms were also seen. If SV40(L$_{RI}$exo)-(dA)$_{80}$ or -(dT)$_{80}$ alone was annealed, no circles were found. Centrifugation of annealed preparations in neutral sucrose gradients showed that the bulk of the SV40 DNA sedimented faster than modified unit-length linears (as would be expected for circular and linear dimers, as well as for higher oligomers). Sedimentation in alkaline gradients, however, showed only unit-length single strands containing the oligonucleotide tails (as seen in Fig. 2).

Covalently Closed-Circular DNA Molecules Are Formed by Incubation of Hydrogen-Bonded Complexes with DNA Polymerase, Ligase, and Exonuclease III. The hydrogen-bonded complexes described above can be sealed by incubation with the E. coli enzymes DNA polymerase I, ligase, and exonuclease III, plus their substrates and cofactors. Zone sedimentation in alkaline sucrose gradients (Fig. 3) shows that 20% of the
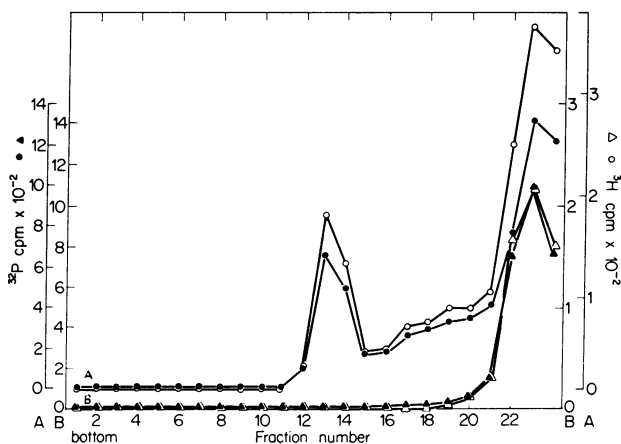

Fig. 4. CsCl–ethidium bromide equilibrium centrifugation of the products analyzed in Fig. 4. Line A, dA-ended, plus dT-ended SV40 linears, plus (P+L+III) (^{32}P, ●; ^{3}H, ○); line B, the same mixture without (P+L+III) (^{32}P, ▲; ^{3}H, △).

TABLE 1. *Relative lengths of SV40 and* λdvgal-120 *DNA molecules*

DNA species	Length ± standard deviation in SV40 units*	Number of molecules in sample
SV40(II)	1.00	224
SV40(L$_{RI}$)†	1.00 ± 0.03	108
(SV40–dA dT)$_2$	2.06 ± 0.19	23
λdvgal-120(I)	4.09 ± 0.14	65
λdvgal-120(L$_{RI}$)	2.00 ± 0.04	163
λdvgal–SV40	2.95 ± 0.04	76
λdv-1	2.78 ± 0.05	13

* The contour length of plaque-purified SV40(II) DNA is defined as 1.00 unit.

† Data supplied by J. Morrow.

input ^{32}P label derived from the oligo(dA) and -(dT) tails sediments with the ^{3}H label present in the SV40 DNA, in the position expected of a covalently closed-circular SV40 dimer (70–75 S). About the same amount of labeled DNA bands in a CsCl–ethidium bromide gradient at a buoyant density characteristic of covalently closed-circular DNA (Fig. 4).

DNA isolated from the heavy band of the CsCl–ethidium bromide gradient contains primarily circular molecules, with a contour length twice that of SV40(II) DNA (Table 1) when viewed by electron microscopy. No covalently closed DNA is formed if either one of the linear precursors is omitted from the annealing step or if the enzymes are left out of the closure reaction. We conclude, therefore, that two unit-length linear SV40 molecules have been joined to form a covalently closed-circular dimer.

Covalent closure of the hydrogen-bonded SV40 DNA dimers is dependent on Mg^{2+}, all four deoxynucleoside triphosphates, E. coli DNA polymerase I, and ligase, and is inhibited by 98% if exonuclease III is omitted (Lobban and Kaiser first observed the need for exonuclease III in the joining of P22 molecules; we confirmed their finding with this system). Exonuclease III is probably needed to remove 3'-phosphate groups from 3'-phosphoryl, 5'-hydroxyl nicks introduced by the endonuclease contaminating the terminal transferase preparation. 3'-phosphoryl groups are potent inhibitors of E. coli DNA polymerase I (14) and termini having 5'-hydroxyl groups cannot be sealed by E. coli ligase (8). The 5'-hydroxyl group can be removed and replaced by a 5'-phosphoryl group by the 5'- to 3'-exonuclease activity of E. coli DNA polymerase I (7).

Preparation of the Galactose Operon for Insertion into SV40 DNA. The galactose operon of E. coli was obtained from a λdvgal DNA; λdvgal is a covalently closed, supercoiled DNA molecule four times as long as SV40(II) DNA (Table 1). After complete digestion of λdvgal DNA with the R$_I$ endonuclease, linear molecules two times the length of SV40(II) DNA are virtually the exclusive product (Table 1). This population has a unimodal length distribution by electron microscopy and appears to be homogeneous by ultracentrifugal criteria (Jackson and Berg, in preparation). The R$_I$ endonuclease seems, therefore, to cut λdvgal circular DNA into two equal length linear molecules. Since one R$_I$ endonuclease cleavage per λdv monomeric unit occurs in the closely related λdv-204 (Jackson and Berg, in preparation), it is likely that λdvgal is cleaved at the
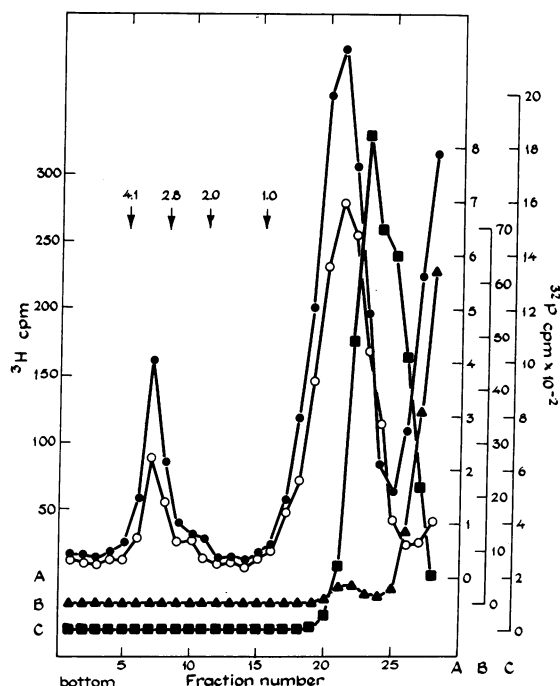
FIG. 5. Alkaline sucrose gradient sedimentation of annealed [^{3}H]SV40(L$_{RI}$exo)–[^{32}P](dA)$_{80}$ and [^{3}H]λ*dvgal–120*) (L$_{RI}$exo)–[^{32}P](dT)$_{80}$ incubated for 3 hr with and without (P+L+III). Centrifugation was for 60 min. Line *A*, dA-ended SV40, plus dT-ended λ*dvgal–120* linears, plus (P+L+III)(^{32}P, ●; ^{3}H, ○); line *B*, dT-ended λ*dvgal–120* linears, plus dT-ended SV40 linears, plus (P+L+III) (^{32}P, ▲); line *C*,dA-ended SV40 linears, plus dT-ended λ*dvgal–120* linears, without (P+L+III) (^{32}P, ■).

The *arrows* indicate the position in the gradient of supercoiled marker DNAs having the indicated multiple of SV40 DNA molecular size.

same sites and, therefore, that each linear piece contains an intact galactose operon.

The purified λ*dvgal* (L$_{RI}$) DNA was prepared for joining to SV40 DNA by treatment with λ-exonuclease, followed by terminal transferase and [^{32}P]dTTP, as described for SV40-(L$_{RI}$).

Formation of Covalently Closed-Circular DNA Molecules Containing both SV40 and λdvgal DNA. Annealing of [^{3}H]-SV40(L$_{RI}$exo)–[^{32}P](dA)$_{80}$ with [^{3}H]λ*dvgal*(L$_{RI}$exo)–[^{32}P]-(dT)$_{80}$, followed by incubation with the enzymes, substrates, and cofactors needed for closure, produced a species of DNA (in about 15% yield) that sedimented rapidly in alkaline sucrose gradients (Fig. 5) and that formed a band in a CsCl–ethidium bromide gradient at the position expected for covalently closed DNA (Fig. 6). The putative λ*dvgal*–SV40 circular DNA sediments just ahead of λ*dv*-1, a supercoiled circular DNA marker [2.8 times the length of SV40(II)DNA], and behind λ*dvgal* supercoiled circles [4.1 times SV40(II)DNA] in the alkaline sucrose gradient. Electron microscopic measurements of the DNA recovered from the dense band of the CsCl–ethidium bromide gradient showed a mean contour length for the major species of 2.95 ± 0.04 times that of SV40(II) DNA (Table 1). Each of these measurements supports the conclusion that the newly formed, covalently closed-circular DNA contains one SV40 DNA segment and one λ*dvgal* DNA monomeric segment.

Omission of the enzymes from the reaction mixture prevents λ*dvgal*–SV40 DNA formation (Figs. 5 and 6). No covalently closed product is detectable (Fig. 5) if λ*dvgal* and SV40 linear molecules with identical, rather than complementary, tails are annealed and incubated with the enzymes. This result demonstrates directly that the formation of covalently closed DNA depends on complementarity of the homopolymeric tails.

We conclude from the experiments described above that λ*dvgal* DNA containing the intact galactose operon from *E. coli*, together with some phage λ genes, has been covalently inserted into an SV40 genome. These molecules should be useful for testing whether these bacterial genes can be introduced into a mammalian cell genome and whether they can be expressed there.

DISCUSSION

The methods described in this report for the covalent joining of two SV40 molecules and for the insertion of a segment of DNA containing the galactose operon of *E. coli* into SV40 are general and offer an approach for covalently joining any two DNA molecules together. With the exception of the fortuitous property of the R$_I$ endonuclease, which creates convenient linear DNA precursors, none of the techniques used depends upon any unique property of SV40 and/or the λ*dvgal* DNA. By the use of known enzymes and only minor modifications of the methods described here, it should be possible to join DNA molecules even if they have the wrong combination of hydroxyl and phosphoryl groups at their termini. By judicious use of generally available enzymes, even DNA duplexes with protruding 5′- or 3′-ends can be modified to become suitable substrates for the joining reaction.

One important feature of this method, which is different from some other techniques that can be used to join unrelated DNA molecules to one another (16, 19), is that here the joining is directed by the homopolymeric tails on the DNA. In our protocol, molecule A and molecule B can only be joined to each other; all AA and BB intermolecular joinings and all A and B intramolecular joinings (circularizations) are prevented. The yield of the desired product is thus increased, and subsequent purification problems are greatly reduced.
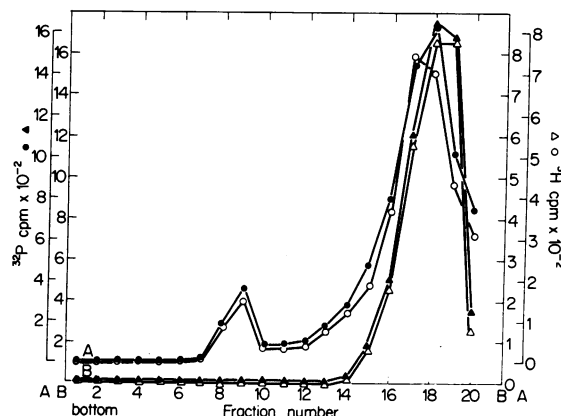

FIG. 6. CsCl–ethidium bromide equilibrium centrifugations of joined [^{3}H]SV40(L$_{RI}$exo)–[^{32}P](dA)$_{80}$ and [^{3}H]λ*dvgal–120*(L$_{RI}$exo)–[^{32}P](dT)$_{80}$ DNA. The samples were those referred to in Fig. 5. Line *A*, dA-ended SV40 linears, plus dT-ended λ*dvgal–120* linears, plus (P+L+III) (^{32}P, ●; ^{3}H, ○); line *B*, the same mixture without (P+L+III) (^{32}P, ▲; ^{3}H, △).

For some purposes, however, it may be desirable to insert λ*dvgal* or other DNA molecules at other specific, or even random, locations in the SV40 genome. Other specific placements could be accomplished if other endonucleases could be found that cleave the SV40 circular DNA specifically. Since pancreatic DNase in the presence of Mn^{2+} produces randomly located, double-strand scissions (17) of SV40 circular DNA (Jackson and Berg, in preparation), it should be possible to insert a DNA segment at a large number of positions in the SV40 genome.

Although the λ*dvgal* DNA segment is integrated at the same location in each SV40 DNA molecule, it should be emphasized that the orientation of the two DNA segments to each other is probably not identical. This follows from the fact that each of the two strands of a duplex can be joined to *either* of the two strands of the other duplex $\left(\text{e.g.,} \begin{smallmatrix} W \frown W \\ C \smile C \end{smallmatrix} \text{ or } \begin{smallmatrix} W \frown C \\ C \smile W \end{smallmatrix} \right)^{\S}$. What possible consequences this fact has on the genetic expression of these segments remains to be seen.

We have no information concerning the biological activities of the SV40 dimer or the λ*dvgal*-SV40 DNAs, but appropriate experiments are in progress. It is clear, however, that the location of the R_I break in the SV40 genome will be crucial in determining the biological potential of these molecules; preliminary evidence suggests that the break occurs in the late genes of SV40 (Morrow, Kelly, Berg, and Lewis, in preparation.

A further feature of these molecules that may bear on their usefulness is the $(dA \cdot dT)_n$ tracts that join the two DNA segments. They could be helpful (as a physical or genetic marker) or a hindrance (by making the molecule more sensitive to degradation) for their potential use as a transducer.

The λ*dvgal*-SV40 DNA produced in these experiments is, in effect, a trivalent biological reagent. It contains the genetic information to code for most of the functions of SV40, all of the functions of the *E. coli* galactose operon, and those functions of the λ bacteriophage required for autonomous replication of circular DNA molecules in *E. coli*. Each of these

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§ The symbols W and C refer to one or the other complementary strands of a DNA duplex, and the "connectors" indicate how the strands can be joined in the closed-circular duplex.

sets of functions has a wide range of potential uses in studying the molecular biology of SV40 and the mammalian cells with which this virus interacts.

1. Sambrook, J., Westphal, H., Srinivasan, P. R. & Dulbecco, R. (1968) *Proc. Nat. Acad. Sci. USA* 60, 1288-1295.
2. Dulbecco, R. (1969) *Science* 166, 962-968.
3. Matsubara, K., & Kaiser, A. D. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 27-34.
4. Radloff, R., Bauer, W., Vinograd, J. (1967) *Proc. Nat. Acad. Sci. USA* 57, 1514-1521.
5. Little, J. W., Lehman, I. R. & Kaiser, A. D. (1967) *J. Biol. Chem.* 242, 672-678.
6. Kato, K., Goncalves, J. M., Houts, G. E., & Bollum, F. J. (1967) *J. Biol. Chem.* 242, 2780-2789.
7. Jovin, T. M., Englund, P. T. & Kornberg, A. (1969) *J. Biol. Chem.* 244, 2996-3008.
8. Olivera, B. M., Hall, Z. W., Anraku, Y., Chien, J. R. & Lehman, I. R. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 27-34.
9. Richardson, C. C., Lehman, I. R. & Kornberg, A. (1964) *J. Biol Chem.* 239, 251-258.
10. Symons, R. H. (1969) *Biochim. Biophys. Acta* 190, 548-550.
11. Davis, R., Simon, M. & Davidson, N. (1971) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 21, pp. 413-428.
12. Chang, L. M. S. & Bollum, F. J. (1971) *Biochemistry* 10, 536-542.
13. Sheldon, R., Jurale, C. & Kates, J. (1972) *Proc. Nat. Acad. Sci. USA* 69, 417-421.
14. Richardson, C. C., Schildkraut, C. L. & Kornberg, A. (1963) *Cold Spring Harbor Symp. Quant. Biol.* 28, 9-19.
15. Little, J. W. (1967) *J. Biol. Chem.* 242, 679-686.
16. Sgaramella, V., van de Sande, J. H. & Khorana, H. G. (1970) *Proc. Nat. Acad. Sci. USA* 67, 1468-1475.
17. Melgar, E. & Goldthwait, D. A. (1968) *J. Biol. Chem.* 243, 4409-4416.
18. Morrow, J. F. & Berg, P. (1972) *Proc. Nat. Acad. Sci. USA* 69, in press.
19. Sgaramella, V. & Lobban, P. (1972) *Nature*, in press.