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JOINING OF SIMIAN VIRUS 40 DNA MOLECULES AT ENDONUCLEASE R *Eco* R_I SITES BY POLYNUCLEOTIDE LIGASE AND ANALYSIS OF THE PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

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Summary

DNA molecules cut with endonuclease R *Eco* R_I can be joined at *Eco* R_I cleavage sites by incubation with polynucleotide ligase. In order to define the optimum conditions for this reaction, linear Simian Virus 40 DNA molecules (SV40(L_{R_I})) produced by endonuclease R *Eco* R_I cleavage of SV40 form I DNA were joined using polynucleotide ligases specified by bacteriophage T4 and *Escherichia coli*. We have determined that the concentration of the substrate DNA molecules is the most important factor determining the distribution of covalently joined product molecules into a variety of circular and linear monomeric and oligomeric species.

Introduction

The *Eco* R_I restriction endonuclease cleaves DNA molecules containing the base sequence

(3'-5')C-T-T-A-A-G

(5'-3')Ġ-Ā-Ā-Ŧ-Ŧ-Ĉ

between the A and G residues to yield fragments having the sequence

(3'-5')C-T-T-A-Ap

(5'-3')Ġ

at each end [1] (except for the two terminal fragments in linear molecules). The T-T-A-Ap single-stranded segments are complementary to each other and, at low temperature, stable duplexes can be formed from these ends [2]. Thus DNA fragments cut by *Eco* R_I can interact intramolecularly, to form circular molecules, or intermolecularly, to form linear or circular oligomeric molecules [2]. Molecules which have been linked by duplex formation at *Eco* R_I cleavage

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sites can then be joined covalently to one another by the action of polynucleotide ligase [2,3,4]. This procedure for forming new kinds of DNA molecules from *Eco* R_I-cleaved DNA fragments from different sources has been used to construct a variety of chimeric DNA molecules in which DNA fragments have been inserted into bacterial plasmid DNA molecules [2,5–9] or bacteriophage λ DNA molecules [10–13]. Since any selfreplicating DNA molecule, such as a bacterial plasmid, can in principle be joined to any other DNA molecule by this method, this technique has become a powerful tool for the cloning and amplification of specific genes from a variety of sources.

Although several laboratories have reported the construction of recombinant DNA molecules employing DNA ligase and *Eco* R_I ends [2,7,8,9], no detailed study has been made of the effect of different reaction conditions on the variety of ligase reaction products that are formed. The conditions under which the DNA molecules are joined by polynucleotide ligase are critical in determining the yield and distribution of the products formed. Since any DNA fragment with two *Eco* R_I cleaved ends can either circularize or interact with any other *Eco* R_I-cleaved molecule, a very complex mixture of products can be formed. It is important that one be able to choose the proper reaction conditions to favor the formation of the desired recombinant molecule. We report here experiments undertaken to determine the optimum conditions for ligase joining of DNA molecules at *Eco* R_I cleavage sites to yield product molecules containing only two *Eco* R_I generated fragments. In order to investigate the joining reaction, we have used a simple model system containing only one species of reactant, the unit-length linear molecule generated by *Eco* R_I cleavage of circular SV40 DNA at its unique *Eco* R_I cleavage site [14,15]. We have employed as joining enzymes the polynucleotide ligases either from *E. coli* [16] or from bacteriophage T4-infected *E. coli* cells [17], but have studied the T4 enzyme reaction more intensively because it is currently commercially available. The specification of the optimum conditions for joining one DNA molecule covalently to another at *Eco* R_I sites should facilitate experiments in which it is desired to join DNA molecules of different kinds to form chimeric recombinant DNAs. The ability to separate many of the species of product molecules on agarose gels will be useful in purifying recombinant DNAs for which no strong biological selection is available.

Materials and Methods

DNA

The covalently closed circular form of SV40 DNA (SV40(I)) was extracted from CV-1 cells infected with plaque-purified SV40 by the method of Hirt [18]. SV40 [³H]- or [¹⁴C]DNA was prepared by growing the infected cells in Dulbecco's modification of Eagle's medium containing 10% dialyzed calf serum and 10 μCi per ml of either [³H]thymidine (25 Ci/mmol, Nuclear Dynamics) or sodium [¹⁴C]formate (50 Ci/mol, Schwarz-Mann). SV40 DNA was purified by centrifugation to equilibrium in CsCl-ethidium bromide gradients [19], digestion of the isolated SV40(I) band with RNAase, followed by preparative sedimentation in neutral sucrose gradients. Monomeric and dimeric SV40(I) were isolated from this step. The relaxed circular form of SV40 DNA (SV40(II)) was

generated by radioactive decay events in the DNA or by limited digestion of the SV40(I) with pancreatic DNAase I in the presence of 10 mM MgCl₂.

Advgal 120 clone B DNA [20,21] was purified from *E. coli* strain DB866 (*Advgal* 120), clone B, as previously described [21]. λ [³²P]DNA was prepared according to Yoshimori [22].

Enzymes

E. coli endonuclease R *Eco* R_I was purified according to Yoshimori [22]. A portion of the enzyme used was the kind gift of Kathleen Blessing. One unit of *Eco* R_I is the amount of enzyme which cleaves 1 pmol of phosphodiester bond in 1 min at 37°C [23]. In this paper, we employ the following relationships relating to SV40 DNA and *Eco* R_I site concentration: 1 pmol of SV40 DNA is 3.4 μ g and contains 2 pmol of *Eco* R_I cleavage sites giving rise to 2 pmol of *Eco* R_I termini in a limit digest. The T4 polynucleotide ligase [17], prepared from phage T4-infected *E. coli* B cells, was obtained from Miles Laboratories, Inc., Kankakee, Ill. (control No. 39-6-625). One unit of T4 ligase is the amount of enzyme which will form 1 nmol of phosphodiester bond in 20 min at 37°C. The *E. coli* polynucleotide ligase [16] was the kind gift of Dr. I.R. Lehman and had no detectable endonuclease or exonuclease activity. Its units are as defined by Modrich and Lehman [16].

Enzyme reaction conditions

Eco R_I. Digestion with *Eco* R_I enzyme was performed for 30 min at 37°C in a reaction mixture containing 10 mM Tris · HCl, pH 7.4, 10 mM MgCl₂, 10 μ g/ml autoclaved gelatin, and 0.6–60 nM *Eco* R_I sites (approx. 1–100 μ g/ml SV40 DNA). Sufficient *Eco* R_I enzyme was added to effect complete cleavage at all *Eco* R_I sites, this amount having been determined in experiments of the type illustrated in Fig. 2. Reactions were stopped by addition of EDTA to a concentration of 20 mM. When required, DNA was concentrated by ethanol precipitation. The designation (L_{R_I}) appended to a DNA indicates that the molecule is a linear generated by *Eco* R_I cleavage. The preparations of *Eco* R_I used in these experiments produced SV40(L_{R_I}) which sedimented as a single homogeneous 16 S peak in alkaline sucrose gradients. This result demonstrates that *Eco* R_I makes only one break in each SV40(I) molecule under these conditions. The SV40(L_{R_I}) also had a specific infectivity on CV-1P cells ranging from $5 \cdot 10^5$ to $1 \cdot 10^6$ p.f.u./ μ g, or about 5–10% that of SV40(I) under our conditions. This result is similar to that reported by Mertz and Davis [2], and suggests that there was little exonuclease activity in the *Eco* R_I preparation under these conditions.

T4 polynucleotide ligase. The T4 ligase reaction mixture [17] contained 66 mM Tris · HCl, pH 7.4, 10 mM MgCl₂, 0.066 mM ATP, 10 mM dithiothreitol, 50 μ g/ml autoclaved gelatin, and amounts of DNA and ligase as indicated in the figure legends. Incubation was at 16°C for the times indicated, and was terminated by the addition of EDTA to a concentration of 20 mM. Under these conditions the T4 ligase preparation was tested for endonuclease activity by incubating SV40(I) DNA (6.25 μ g/ml) with 0.42 units of T4 ligase per ml of reaction mixture. Reaction products were detected by agarose gel electrophoresis as described below. After 5 h of incubation in the presence of ligase

without ATP, 3% more SV40(I) DNA was converted to SV40(II) than in a control without enzyme, indicating the presence of trace amounts of endonucleolytic activity in the T4 ligase preparation. Exonuclease activity in the T4 ligase preparation was determined by the incubation of (5'-³²P)-labelled oligonucleotide (pT)₅ with 1.7 units of T4 ligase per ml of reaction mixture in the presence of ATP. Digestion products were detected by homochromatography on a DEAE-cellulose thin layer and subsequent autoradiography [24]. After 6 h of incubation about 5% more (5'-³²P)-labelled (pT)₅ was digested than in a control without enzyme and ATP. This indicates the presence of a trace of exonuclease contaminant in the T4 ligase preparation.

E. coli polynucleotide ligase. The *E. coli* ligase reaction mixture contained 20 mM Tris · HCl, pH 8.1, 10 mM MgCl₂, 10 mM (NH₄)₂SO₄, 0.052 mM NAD, 1 mM EDTA [16], and amounts of DNA and ligase as indicated. Incubation was as for T4 ligase.

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out essentially as described by Helling et al. [25]. All gels used in these experiments contained 0.7% agarose (Sigma). Both gels and electrophoresis buffer (40 mM Tris · HCl, pH 8.2, 5 mM sodium acetate, 1 mM EDTA) contained 10 or 20 µg/ml ethidium bromide. The DNA samples were applied to 15-cm gels in volumes of 20–60 µl and were run for 5 h at 100 V (3.5–4 mA/gel). In some instances 10 cm gels were employed, and these were run for 2 h. To destain the gels, they were soaked overnight in electrophoresis buffer containing 1 µg/ml ethidium bromide. Gels were examined by illumination from a long wave length ultraviolet light (C50 transilluminator, Ultraviolet Products, San Gabriel, Calif.) and the DNA bands were visualized by fluorescence of the intercalated ethidium bromide [26]. Gels were photographed either with Kodak Highspeed Ektachrome film (ASA 160, aperture *f*1.2, exposure time 1/8 or 1/4 s) using a yellow filter (Kodak no. 9 Wratten gelatin filter) or with a Polaroid camera (Polaroid MP-3 Land camera) using a red filter (Kodak no. 23A Wratten gelatin filter) and Polaroid type 55 positive/negative film (exposure time 60 s).

Quantitation of amount of DNA per band in agarose gels. To quantitate the amount of DNA in each band, DNA labelled with ³H or ¹⁴C was electrophoresed and the gel segments containing the bands of interest were excised with a razor blade, placed in small glass vials (15 × 45 mm packer vials, Brockway Glass, Parkersburg, W. Va.), covered with 2 ml of Aquasol (New England Nuclear), agitated for 18–24 h at 37°C on a reciprocating shaker, and counted in a Nuclear Chicago Mark II scintillation spectrometer. The number of cpm detected in a given gel segment under these conditions becomes constant after 15–18 h at 37°C. This method allows [³H]DNA to be counted at relatively high efficiency and gives a linear correspondence between the amount of DNA in a band and the amount of radioactivity detected in the excised gel slice (see Fig. 1).

Results

Quantitation of DNA in bands on agarose gels

Electrophoresis of DNA in 0.7% agarose gels separates the DNA molecules into bands differing in the size or conformation of the DNA molecules contain-

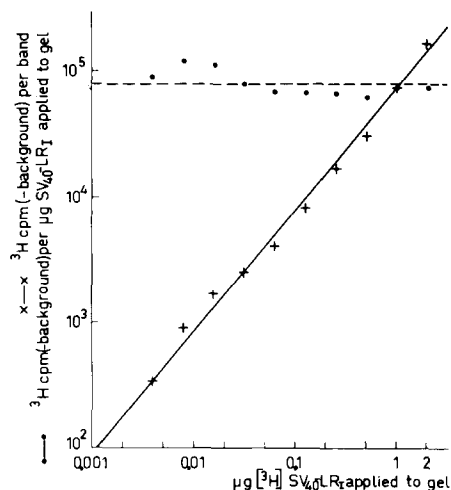


Fig. 1. Quantitation of SV40[³H]DNA in 0.7% agarose gels. ³H-labelled SV40(L_{R1}), spec. act. $8.8 \cdot 10^4$ cpm per µg when counted in Aquasol, was prepared at a concentration of 100 µg/ml, and was diluted through 9 successive two-fold dilutions in electrophoresis buffer. 20 µl of each dilution ranging from 2.0 µg to 3.9 ng of ³H-labelled SV40 (L_{R1}), was applied to 15 cm 0.7% agarose gels and was electrophoresed for 3 h at 100 V. The SV40(L_{R1}) bands were visualized and processed for counting as described in Materials and Methods. The cpm per band (X) and the cpm per µg of DNA applied to the gel (○) were plotted against the log of the µg of DNA applied to the gel. The dotted line represents the mean of the 10 values for cpm per µg. The standard deviation about this mean is $\pm 16\%$.

ed in them [25]. We wished to develop a simple procedure for quantitating radioisotopically labelled DNA in such bands, one which was suitable for use with ³H-labelled DNA and which did not require that the entire gel be sliced and counted. The procedure described in Materials and Methods satisfies these criteria. To determine the relationship between the amount of DNA placed on the gel and the radioactivity found after excising the band and counting it, an experiment was performed in which successive two-fold dilutions of SV40(L_{R1}) [³H]DNA were electrophoresed on different gels and the bands were excised and counted as described in Materials and Methods. The results of this experiment are shown in Fig. 1. It is apparent that there is a linear correlation between the amount of SV40(L_{R1}) [³H]DNA placed on the gel and the cpm found in the excised bands. Thus this procedure can be used to quantitate the amount of ³H-labelled DNA in a band. Additional experiments have demonstrated that the size of the gel slice (between 1 and 6 mm) does not affect these results, that the procedure works equally well for ¹⁴C-labelled DNA, and that the efficiency of counting of SV40[³H]DNA under these conditions is 29% (91% of that for counting the free DNA in Aquasol). The standard deviation of the mean specific radioactivity calculated for the SV40(L_{R1}) DNA from the ten points in Fig. 1 is $\pm 16\%$.

An example of this quantitation method as applied to a previously characterized system is shown in Fig. 2. From a study of the time course of *Eco* R₁ cleavage of SV40(I) DNA, Mulder and Delius [15] have shown that the reaction occurs in two steps, with SV40(II) as an intermediate between SV40(I) and the limit digestion product, SV40(L_{R1}). We performed an experiment in which SV40(I) DNA was incubated with increasing amounts of *Eco* R₁ enzyme

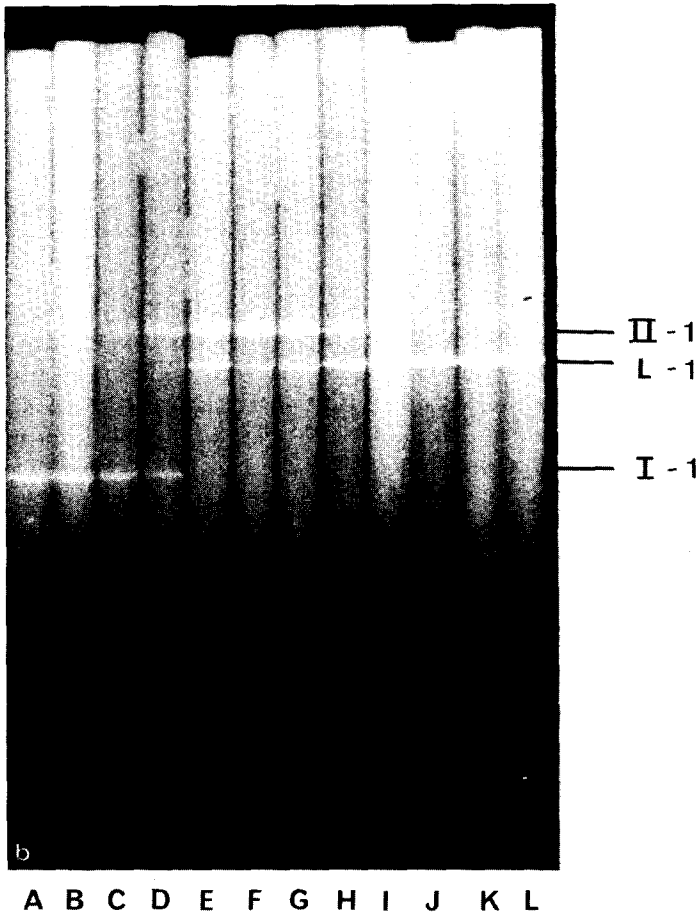
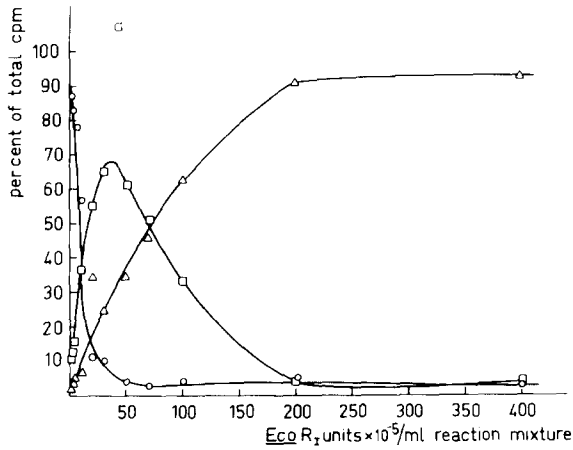


Fig. 2. Cleavage of SV40(I) DNA by endonuclease R *Eco* R_I. (a and b) SV40(I) DNA (200 ng) was incubated with increasing concentrations of endonuclease R *Eco* R_I in a volume of 50 μ l for 30 min at 37°C and the reaction products were analyzed on 10 cm 0.7% agarose gels with 10 μ g/ml ethidium bromide as described under Materials and Methods; in all gels migration was from top to bottom. Units of enzyme $\times 10^{-5}$ per ml of the reaction mixtures analyzed on gels A-K are 0 (A), 2 (B), 5 (C), 10 (D), 20 (E), 30 (F), 50 (G), 70 (H), 100 (I), 200 (J) and 400 (K). Gel L contains SV40(L_RI). The radioactivity per band was plotted as a percentage of the total radioactivity found in all bands for each gel. \circ — \circ , SV40(I) monomer; \square — \square , SV40(II) monomer; \triangle — \triangle , SV40(L_RI) monomer; I-1, SV40(I) monomer; II-1, SV40(II) monomer; L-1, SV40(L_RI) monomer.

and the reaction products were electrophoresed as described in Materials and Methods. Fig. 2a and b illustrates the results of the gel electrophoresis and the results obtained after excising the bands and counting them in Aquasol. Several points can be noted. First, although it is apparent from inspection of both the photograph and the plot of the radioactivity per band that SV40(II) is an intermediate in the conversion of SV40(I) to SV40(L_{RI}) it is only from the plot that quantitative conclusions can be drawn. One such conclusion is that *Eco* R_I attacks SV40(I) preferentially to the SV40(II) intermediate that it generates. That this is so can be inferred from the fact that the rate of disappearance of SV40(II) as a function of enzyme concentration is much slower than the rate of disappearance of SV40(I) under conditions where each represents the predominant species in solution. In approximation the inequality of the slopes of the apparently straightly decreasing sections following the maximum values of the curves for SV40(I) and SV40(II) was tested using Student's *t*-test after arc sine transformation of the observed percent values. The result was significant at the 0.01 level of significance. Secondly, the fact can be noted that SV40(L_{RI}) appearance is an approximately linear function of *Eco* R_I concentration over a 10–20-fold range of enzyme concentration, which makes this procedure a useful assay for quantitating *Eco* R_I enzyme. Greene et al. [23] have described a similar assay which is, however, restricted to [³²P]DNA. The results of the experiment shown in Fig. 2 also define the order and position of migration of the forms of SV40 monomers under these conditions as SV40(I) (fastest), SV40(L_{RI}), SV40(II).

Relative mobilities of conformers of monomeric and dimeric SV40 DNA in 0.7% agarose gels

To identify the products of ligase joining of SV40 (L_{RI}), the positions at which the various conformers of monomeric and dimeric SV40 DNA migrate on 0.7% agarose gels were determined. When purified SV40(I), SV40(II) and SV40(L_{RI}) DNA are electrophoresed on agarose gels as described in Materials and Methods, the pattern shown in Fig. 3 (gels A–D) is obtained. A similar series is obtained when the I, II, and L forms of dimeric SV40 DNA are electrophoresed under these conditions (Fig. 3, gels E–H). The mobilities of dimeric SV40(II) and SV40(L) relative to dimeric SV40(I) are the same as those of monomeric SV40(II) and SV40(L) relative to monomeric SV40(I). In gel I, the position of migration of λ *dvgal* 120 clone B (L) DNA, mol. wt. $10.6 \cdot 10^6$ [21], serves to identify the approximate position at which trimeric SV40(L), mol. wt. $10.2 \cdot 10^6$, will migrate.

The order of migration of these six species of SV40 DNA is constant over a wide range of voltages and extents of migration. The quantitative value of the mobility of one species relative to another does vary somewhat, however, if the concentration of ethidium bromide is changed; for example, the position of SV40(I) dimer migration is shifted to slightly behind that of SV40(II) monomer in the absence of ethidium bromide.

Joining of SV40(L_{RI}) molecules with T4 ligase

When SV40(L_{RI}) molecules are joined by polynucleotide ligase, it would be expected that temperature, DNA concentration, ligase concentration, and time of incubation would all affect the final composition of the mixture of product

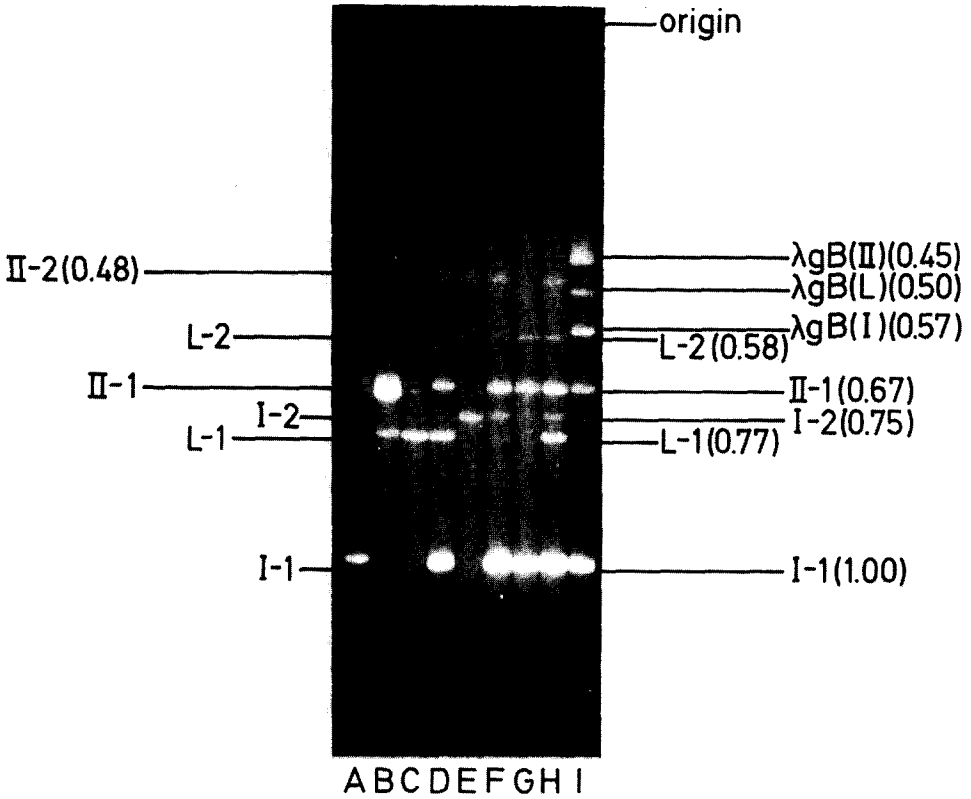


Fig. 3. Mobilities of the conformers of SV40 DNA monomers and dimers and of $\lambda dvgal$ 120 clone B DNA in 0.7% agarose gels. Gels were run for 4.5 h as described in Materials and Methods. I-1, II-1, L-1 are monomeric SV40(I), (II), and (L_{R1}) respectively, I-2, II-2, and L-2 are dimeric SV40(I), (II), and (L) respectively. $\lambda g B(I)$, $\lambda g B(II)$, $\lambda g B(L)$ are $\lambda dvgal$ 120 clone B forms I, II and L respectively. Gel A, I-1; B, II-1; C, L-1; D, I-1 plus II-1 plus L-1; E, I-2 plus II-2; F, I-1 plus II-1 plus I-2 plus II-2; G, I-1 plus II-1 plus L-2; H, I-1 plus II-1 plus L-1 plus I-2 plus II-2 plus L-2; I, I-1 plus II-1 plus $\lambda dvgal$ 120 clone B forms I, II, and L. Numbers in brackets denote relative mobilities (SV40(I) monomer = 1.00). Under the conditions used the migration distance of SV40(I) monomer in 1.15 cm gel is 11.1 cm.

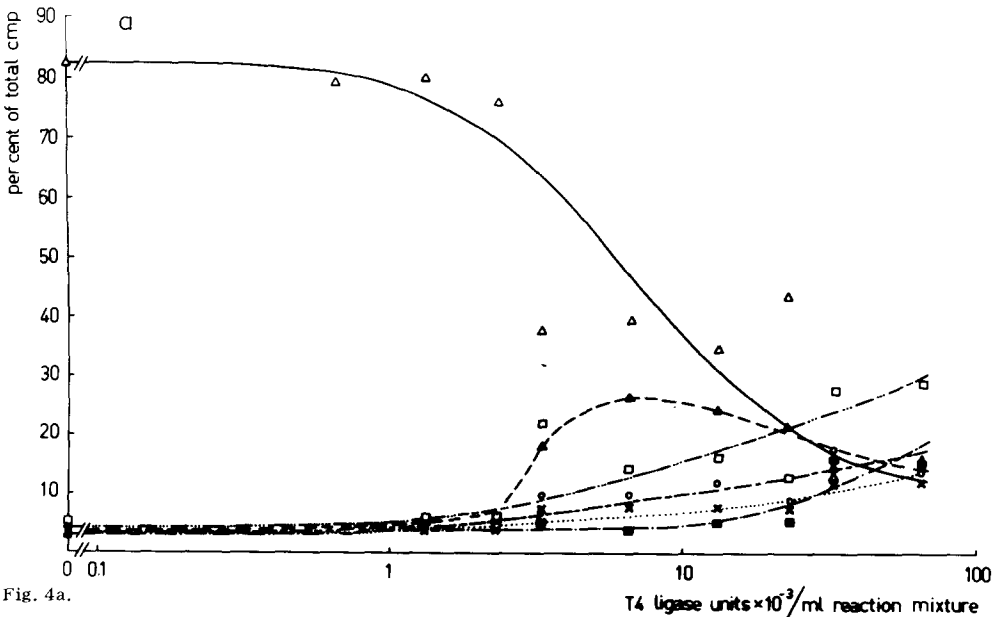


Fig. 4a.

T4 ligase units $\times 10^3$ / ml reaction mixture

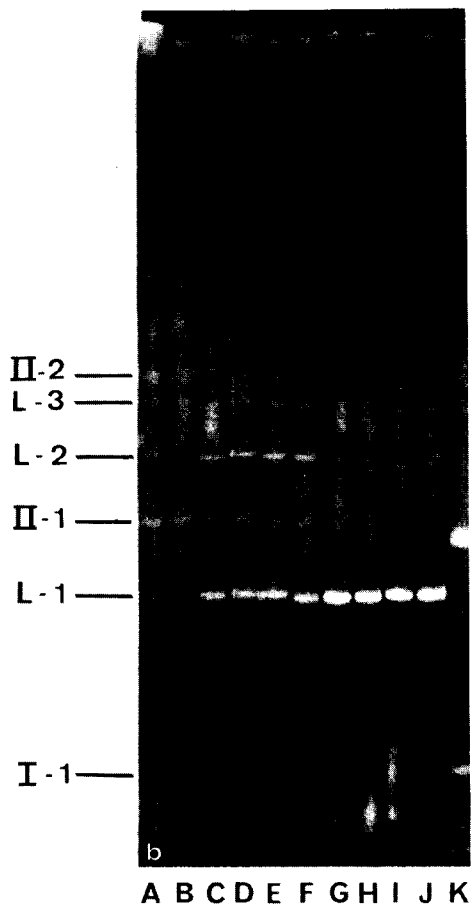


Fig. 4. Joining of SV40(L_{R1}) molecules at various concentrations of T4 ligase. SV40(L_{R1}) DNA (13 $\mu\text{g/ml}$) was incubated with T4 ligase for 6 h and the reaction mixtures, each containing 400 ng of DNA, were analyzed on 15 cm 0.7% agarose gels with 10 $\mu\text{g/ml}$ ethidium bromide as described under Materials and Methods; in all gels migration was from top to bottom (see b). For symbols in b see legend of Fig. 3; L-3, SV40(L) trimers. Units of enzyme $\times 10^{-3}$ per ml of the reaction mixtures analyzed on gels A-J are 660 (A), 330 (B), 230 (C), 132 (D), 66 (E), 33 (F), 23 (G), 13.2 (H), 6.6 (I) and 0 (J). Gel K contains monomeric SV40(I) and (II). (a) The radioactivity per band was plotted as a percentage of the total radioactivity found in all bands for each gel. \circ — \circ , SV40(I) monomer; \square — \square , SV40(II) monomer; \triangle — \triangle , SV40(L_{R1}) monomer; \blacksquare — \blacksquare , SV40(II) dimer; \blacktriangle — \blacktriangle , SV40(L) dimer; \times — \times , SV40(L) trimer.

molecules. When SV40(L_{R1}) molecules are joined to give covalently closed monomeric or oligomeric circular molecules, these circular molecules are relaxed and thus have the same conformation as the covalently open SV40(II) circles. To separate SV40(I') (covalently closed relaxed circles) from SV40(II) molecules by electrophoresis in agarose gels, we have incorporated ethidium bromide in the gels and electrophoresis buffer to convert SV40(I') molecules to a superhelical conformation. An ethidium bromide concentration of 10 $\mu\text{g/ml}$ is sufficient to cause SV40(I') DNA to co-migrate with SV40(I) under these conditions (DeLeys, R.J. and Jackson, D.A., unpublished).

An incubation temperature of 16°C was chosen on the basis of preliminary

experiments on ligase joining at *Eco* R_I sites at 0, 4, 16, 22 and 37°C. Incubation at the lower temperatures did not give significantly greater yields of joined products and did require either more time or more enzyme. Incubation at the higher temperatures reduced the yields of joined products.

The results of an experiment to determine the effect of increasing ligase concentration on the composition of the joined product molecules is shown in Fig. 4. As would be expected at these relatively low DNA concentrations, which favor intramolecular and lower order intermolecular interactions (see below), only monomers, dimers, and linear trimers are formed in detectable quantities. Dimeric SV40(L) and monomeric SV40(II) DNA are formed preferentially at very low ligase concentrations. As ligase concentration is increased, dimeric SV40(L) becomes a smaller proportion of the total DNA species present and monomeric SV40(I'), dimer SV40(II), and trimeric SV40(L), all species which require a minimum of two ligase joinings for their formation, increase in concentration. These observations are consistent with the expectation that dimeric SV40(L) will be a precursor of dimeric SV40(II) and trimeric SV40(L), and that monomeric SV40(II) is a precursor of monomeric SV40(I').

A study of the time course of formation of the various products formed from SV40(L_{R_I}) upon incubation with T4 ligase is shown in Fig. 5. Monomer SV40(II) and dimer SV40(L), those species requiring a single ligase joining event for formation, are seen early in the reaction, while those requiring two joining events, monomer SV40(I'), dimer SV40(II), and trimer SV40(L), appear later at approximately the same rate.

The preceding experiments have led to the conclusion that efficient joining of SV40(L_{R_I}) at a DNA concentration of 10–20 µg/ml (6–12 nM *Eco* R_I ends) occurs at T4 ligase concentrations of 0.2–0.5 units per ml after 2–4 h incubation at 16°C. Higher ligase concentrations favor formation of covalently closed molecules.

Joining of SV40(L_{R_I}) molecules at various DNA concentrations

The principal variable which affects the distribution of SV40 DNA species in the joined product molecules is the concentration of the SV40(L) DNA in the reaction. At low concentration of SV40(L_{R_I}), it would be expected that intramolecular joinings, leading to monomeric circles, would be favored over intermolecular joinings leading to oligomeric species. Conversely, at high SV40(L_{R_I}) concentrations, intermolecular joinings leading primarily to linear oligomers should be favored over intramolecular interactions. The results shown in Fig. 6 bear out this expectation. SV40(L_{R_I}), at concentrations of *Eco* R_I termini ranging from 0.14 to 132 nM, was incubated with T4 ligase and the products were then analyzed by gel electrophoresis. At the lowest concentrations of SV40(L_{R_I}) (0.14–0.54 nM *Eco* R_I termini), monomeric SV40(II), dimeric SV40(L), and small amounts of monomeric SV40(I') are produced. At intermediate concentrations (1.12–8.8 nM), more complex but still well-defined species such as dimeric SV40(II) and trimeric SV40(L) appear, along with small amounts of dimeric SV40(I'). At high concentrations (16.6–132 nM), more and more of the DNA is incorporated into very large oligomeric molecules which are not resolved in the gels. At concentrations greater than 70 nM *Eco* R_I termini, nearly

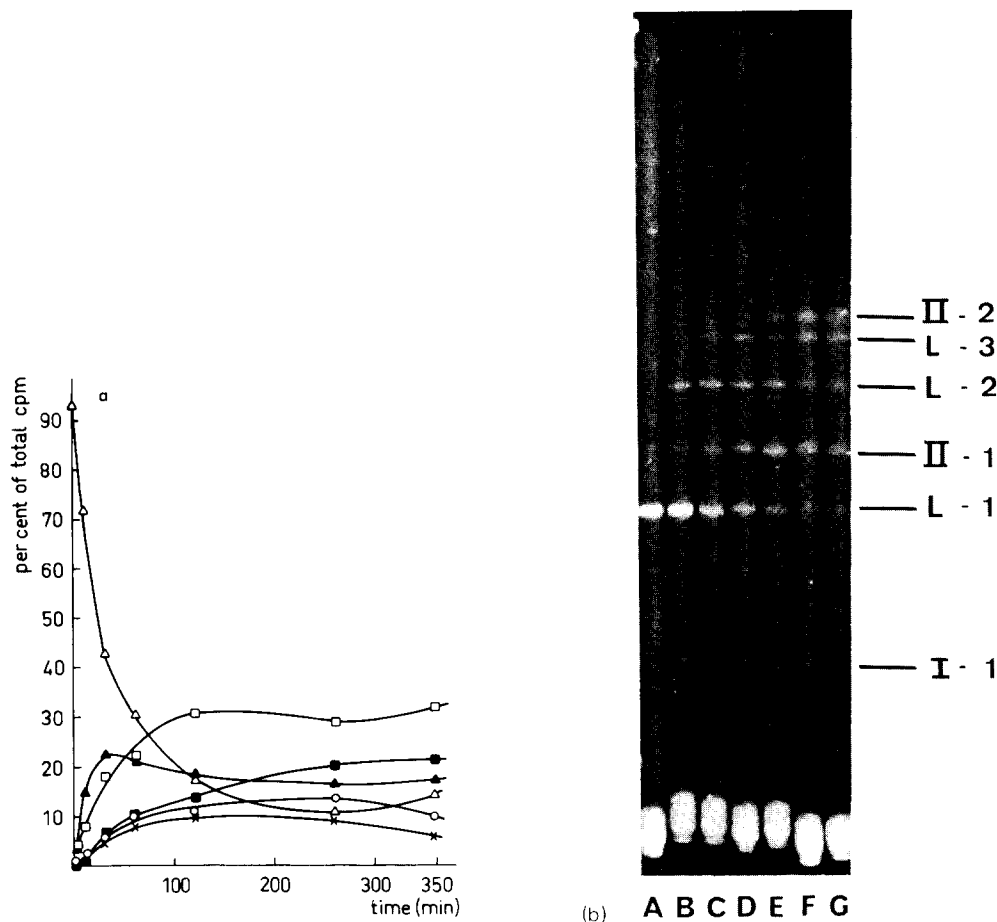


Fig. 5. Joining of SV40(L_{RI}) molecules after various times in the presence of T4 ligase. SV40(L_{RI}) DNA (20 $\mu\text{g/ml}$) was incubated with T4 ligase (0.33 units/ml). Samples containing 600 ng of DNA were taken at the times indicated and were analyzed (see b) as described in the legend of Fig. 4. Times (in min) of incubation at 16°C for the samples analyzed on gels A–G are 0 (A), 10 (B), 30 (C), 60 (D), 120 (E), 260 (F) and 350 (G). (a) The radioactivity per band was plotted as a percentage of the total radioactivity found in all bands for each gel. For symbols see legends to Figs. 3 and 4.

all the SV40(L_{RI}) molecules have been incorporated into these very large structures (see Fig. 6).

The results of this experiment indicate that the most favorable concentration of *Eco* R_I termini for the joining of linear molecules into predominantly dimer and trimer species is in the range of 2 to 10 nM *Eco* R_I termini.

Joining of SV40(L_{RI}) molecules with *E. coli* ligase

Experiments similar to those presented above were performed with polynucleotide ligase from *E. coli*. Results similar to those found for T4 ligase were obtained in terms of the distribution of the molecular species formed at various enzyme concentrations. Fig. 7 shows that *E. coli* ligase promoted somewhat higher yields of covalently closed products than did T4 ligase. In particular, at high *E. coli* ligase concentrations, significant amounts of dimeric and trimeric

SV40(I') were detected. The increased efficiency of covalent closure is probably due to the absence in the *E. coli* ligase preparation of traces endonuclease and exonuclease contaminants which were present in the T4 ligase (see Materials and Methods). Other experiments demonstrated that maximum joining occurred within 3 h when SV40(L_{R_I}) at a concentration of 5.6 $\mu\text{g/ml}$ was incubated with 120 units/ml of *E. coli* ligase (data not shown). Our results thus demonstrate that both T4 and *E. coli* ligase can be used for joining DNA molecules at *Eco* R_I cleavage sites.

Discussion

Studies were undertaken to analyze the products which are formed when linear DNA elements are joined, both intermolecularly and intramolecularly, at their *Eco* R_I generated cohesive ends in the presence of either T4 or *E. coli* ligase. SV40(L_{R_I}) molecules were used as a model system to study the covalent joining reaction over a range of times, enzyme concentrations, and DNA concentrations. The conditions determined in this paper for optimization of the joining of SV40(L_{R_I}) were as well applicable to linear DNA molecules in the range of $2-20 \cdot 10^6$ daltons, when we constructed a series of chimeric bacterial plasmids containing various *Eco* R_I fragments (Collins, C.J., Jackson, D.A. and De Vries, F.A.J., unpublished).

In terms of the distribution of the molecular species formed under the var-

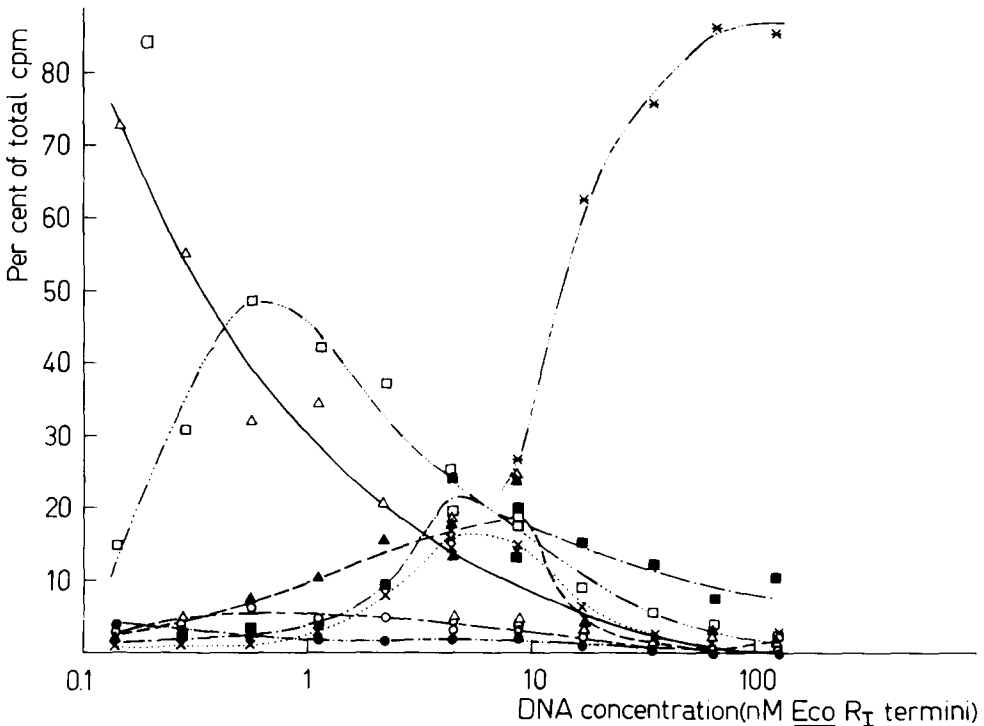


Fig. 6a.

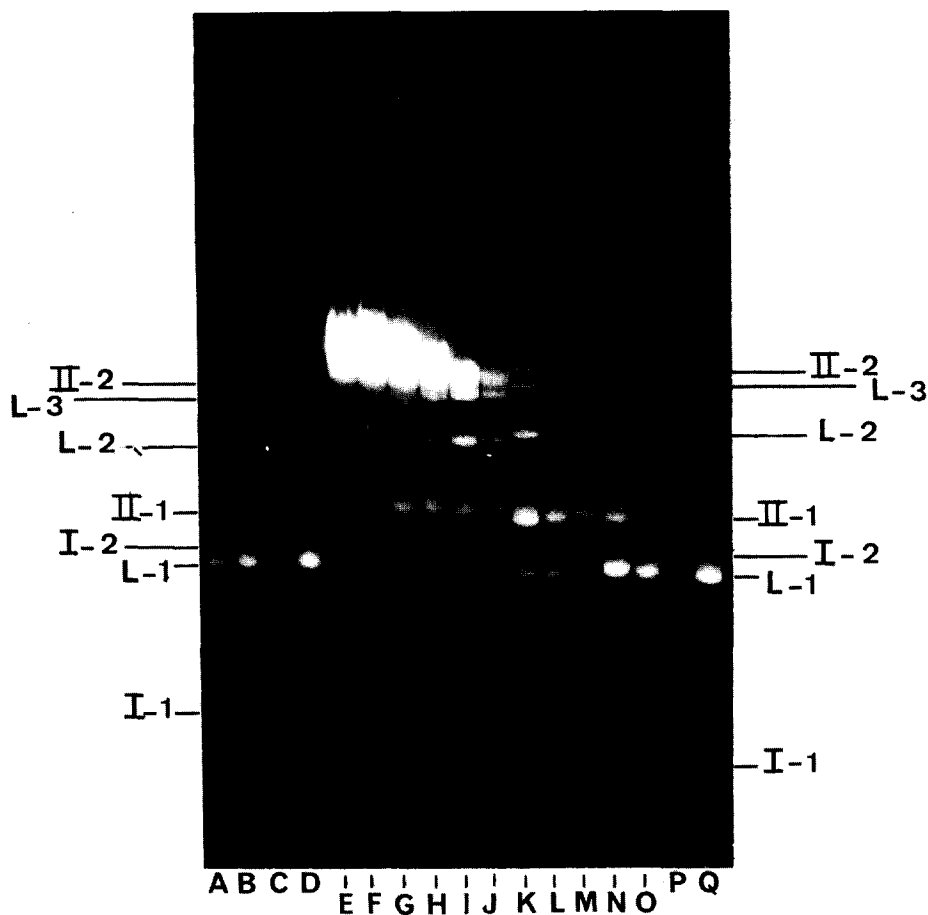


Fig. 6. Joining of SV40(L_RI) molecules by T4 ligase at various DNA concentrations. SV40(L_RI) DNA at various concentrations was incubated with T4 ligase ($33 \cdot 10^{-3}$ units/ μ g of DNA) for 4 h at 16°C and the reaction mixtures, each containing 400–750 ng of DNA, were analyzed (see b) as described in the legend of Fig. 4. Gels A–J were run in 10 μ g/ml ethidium bromide for 4.5 h and gels K–Q were run in 20 μ g/ml ethidium bromide for 5 h. Gels A–D, P and Q contain marker DNA species. Concentrations of DNA in nM *Eco* R_I termini for the reaction mixtures analyzed on gels E–O are 132 (E), 70.6 (F), 35.3 (G), 17.6 (H), 8.8 (I), 4.4 (J), 2.24 (K), 1.12 (L), 0.56 (M), 0.28 (N) and 0.14 (O). (a) The radioactivity per band was plotted as a percentage of the total radioactivity found in all bands for each gel. For symbols see legends to Figs. 3 and 4. Additional symbols: ○—○, SV40(I) dimer; *—*, SV40 oligomers larger than trimers.

ious conditions, we obtained essentially identical results with T4 and *E. coli* ligases. At low enzyme concentrations and short incubation times, those species which required only one ligase joining event (e.g., dimeric SV40(L) and monomeric SV40(II)) were formed preferentially. At high enzyme concentrations and longer incubation times, products requiring additional joining events appeared (e.g., SV40(I) monomer and SV40(L) trimer). Under any conditions, however, the most important determinant of the composition of the product mixture was the initial concentration of SV40(L_RI). We found concentrations between 2 and 10 nM *Eco* R_I termini to be the most favorable for formation of lower order oligomer products (dimers and trimers).

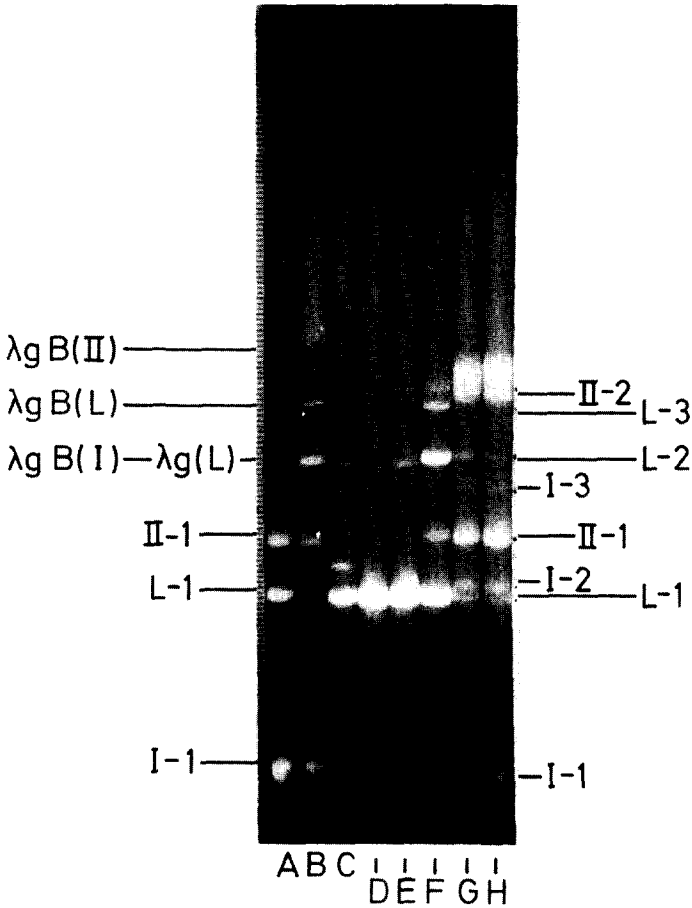


Fig. 7. Joining of SV40(L_{R1}) molecules at various concentrations of *E. coli* ligase. SV40(L_{R1}) DNA (5.6 $\mu\text{g}/\text{ml}$) was incubated with *E. coli* ligase for 14 h at 16°C and the reaction mixtures, each containing 280 ng of DNA, were analyzed as described in the legend of Fig. 4. Gels A, B and C contain marker DNA species. Units of enzyme per ml of the reaction mixtures analyzed on gels D–H are 4 (D), 12 (E), 40 (F), 120 (G) and 400 (H). For symbols see legend of Fig. 3. I-3, SV40(I) trimers; L-3 SV40(L) trimers; λg (L), $\lambda\text{dvgal(L}_{R1})$, mol. wt. $6.7 \cdot 10^6$ [21].

It is clear from our results that it is possible to exert a significant degree of control over the distribution of the molecules formed from random associations at *Eco* R₁ termini. We have defined conditions under which a majority of the joined products contain only two or three monomeric units. It is under such conditions that it should be possible to construct dimeric DNA molecules in which a significant proportion of the joined molecules contain two different monomeric species. If, in addition, the desired chimeric DNA can be isolated (as is shown here by separation on agarose gels), it should be possible to achieve a specificity in the joining of two different DNA molecules to give a specific mixed dimer. This has previously been possible only by significantly more complex biochemical manipulations of the precursor DNA molecules [27]. Such specificity should be especially useful in preparing chimeric molecules (e.g., bacterial genes linked to SV40 DNA) for introduction into mammalian cells in

culture, where it is in general not known to be possible to select for entry and stabilization of the desired genes in the way that is generally possible in bacterial cells.

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