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Establishment of Composite DNA Derived from L Factor as a Plasmid in Mouse Embryonal Carcinoma (F9) Cells

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We have recently reported a mammalian cell plasmid (L factor) whose structure is related to that of polyomavirus (T. Kusano, H. Uehara, H. Saito, K. Segawa, and M. Oishi, Proc. Natl. Acad. Sci. USA 84:1789–1793, 1987). When composite DNA constructed from L factor and a foreign gene was introduced into mouse embryonal carcinoma (F9) cells by transfection, the DNA was reestablished in the cells as a plasmid. The reestablished plasmid DNA in F9 cells could be rescued in *Escherichia coli*. The plasmid-bearing cells underwent normal in vitro differentiation in response to retinoic acid. The efficiency of plasmid establishment of the L-factor-derived DNA and transcriptional and transient replicational activities were compared with those of similar composite DNA constructed from polyomavirus and an embryonal carcinoma mutant of polyomavirus which is permissive in F9 cells. The results suggest an inverse relationship between the efficiency of the plasmid establishment and the activity of gene expression controlled by the intrinsic enhancer-promoter of the DNA.

Several viruses are known to proliferate as plasmids in mammalian cells. These include bovine papillomavirus (11, 18), Epstein-Barr virus (22), and BK virus (15). Composite DNAs constructed from these virus DNAs and a specific gene can be also established as plasmids in appropriate recipient cells after transfection (23). On the other hand, composite DNAs constructed from simian virus 40 DNA or polyomavirus DNA are generally not stably maintained as plasmids except for a limited number of cases (5, 14). Little is understood about the mechanism of plasmid establishment in mammalian cells.

Recently, we reported a plasmid discovered in a subclone (B822) of mouse L cells (10). The plasmid, named L factor, is stably maintained in the cells with a considerably high copy number, more than 5,000 copies per cell. Two different L factors were present in the subclone, one with 5.3 kilobases (kb) (LF I) and the other with 5.5 kb (LF II). Restriction mapping and DNA sequencing have revealed these two L factors to be almost identical except for a 0.2-kb insertion in LF II, and they are structurally related to polyomavirus. We have found, however, that compared with polyomavirus, substantial differences in base sequences exist in the enhancer region and coding frames for early and late genes, in addition to a number of single-base substitutions which are scattered along the whole genome.

In this paper, we report that composite DNA constructed from L factors and a foreign (bacterial *neo*) gene can be reestablished as a plasmid in mouse embryonal carcinoma (F9) cells without affecting the capacity of the cell to differentiate in vitro. The reestablished plasmid DNA can be rescued in *Escherichia coli*, serving as a plasmid shuttle vector. Furthermore, we investigated the mechanism of the plasmid establishment by employing various composite DNAs constructed from L factor, polyomavirus, or a mutant polyomavirus which is permissive in embryonal carcinoma (EC) cells.

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MATERIALS AND METHODS

Cells and cell culture. Mouse embryonal carcinoma cells (F9) were cultured in gelatin-coated plastic dishes in ES medium (Nissui Co.) supplemented with 10% (vol/vol) fetal calf serum (Sigma Chemical Co.). The cells were incubated at 35°C in a humidified CO_2 incubator unless otherwise specified.

Transfection and selection of plasmid-bearing cells. F9 cells bearing a composite plasmid were obtained after transfection with DNA. F9 cells (7×10^5 cells) cultured at 37°C were transferred to plastic dishes (100-mm diameter); 24 h later they were transfected with composite DNA (20 μg) by calcium phosphate coprecipitation (21), and after 12 h of incubation they were subjected to a dimethyl sulfoxide (25%, vol/vol) pulse for 4 min (9). At 24 h after the pulse, the cells were diluted three times with the fresh medium that contained G418 (0.2 mg/ml, GIBCO Laboratories). The medium was replenished every 5 days. After 2 to 3 weeks, colonies resistant to G418 were selected for further analysis.

Preparation and Southern blot analysis of DNA. Extrachromosomal (low-molecular-weight) DNA was extracted by the procedure of Hirt (8), including phenol treatment. Cells were lysed with sodium dodecyl sulfate, and the total DNA was directly isolated from the lysate after phenol extraction. DNA (from 10^6 cells) dissolved in TE buffer (10 mM Tris hydrochloride [pH 7.6], 1 mM EDTA) was subjected to agarose gel electrophoresis, electrotransferred to a nylon membrane (Biodyne A; Pall Co.), baked, and hybridized with an appropriate probe labeled with [α-^{32}P]dCTP (ICN Pharmaceuticals) by nick translation as described previously (13).

Construction of composite DNA. Composite DNA pLIIN5 consisting of the putative enhancer/*ori*-early region of LF II and the bacterial *neo* (G418) gene was constructed by ligating the 3.8-kb fragment of *Bam*HI and *Sal*I digests of LF II and the 3.6-kb fragment of *Bam*HI and *Sal*I digests of pHSG274. pHSG274 is a ColE1-derived DNA containing a hybrid Tn*5* Km^r/G418^r gene (3). To construct pPy274 and pPyEC274, the first *Hin*cII site (polyomavirus nucleotide 2962) of PyVA2 (20) and PyVN2 (an EC mutant of polyomavirus) (19) DNA was converted to a *Sal*I site by adding a

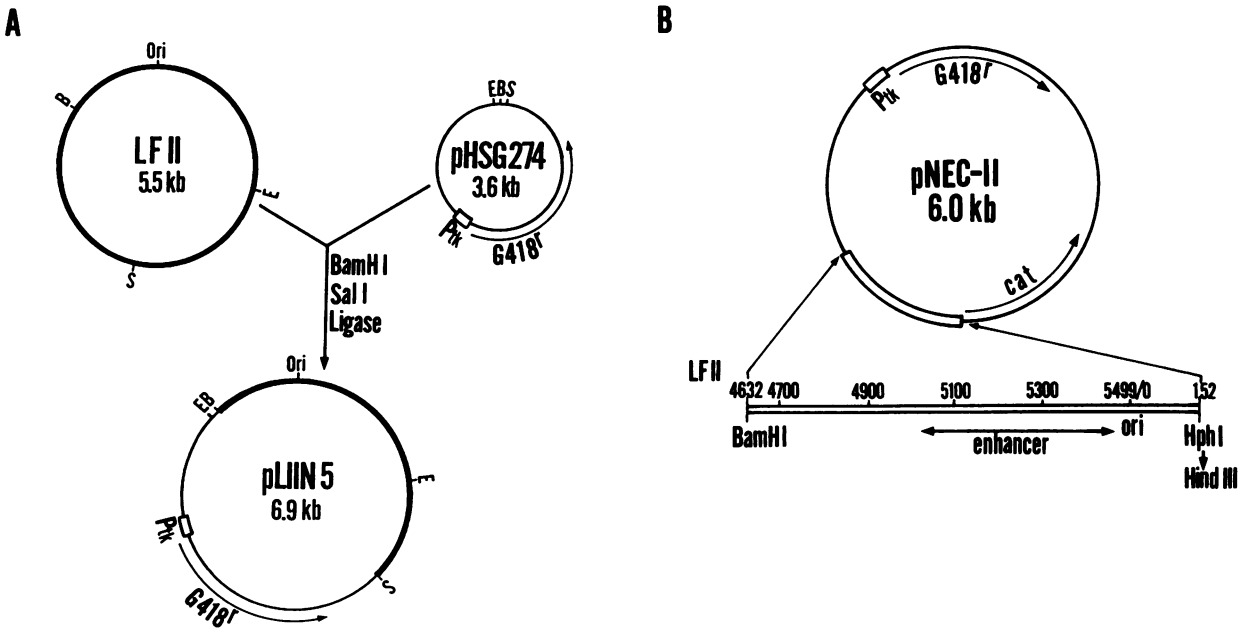

FIG. 1. Construction of (A) the composite plasmid DNA (pLIIN5, pPy274, and pPyEC274) and (B) the CAT expression DNA (pNEC-Ii, pNEC-Py, and pNEC-EC). (A) pLIIN5 was constructed from the early regulatory region of LF II and pHSG274. For pPy274 and pPyEC274, PyVA2 and PyVN2 DNA were used in place of LF II after converting the *Hin*cII site (polyomavirus nucleotide 2962) to the *Sal*I site with a *Sal*I linker before ligation to pHSG274. Abbreviations: Ori, putative origin of DNA replication; Ptk, the site of the *tk* promoter; E, *Eco*RI; B, *Bam*HI; S, *Sal*I. (B) pNEC-II DNA was constructed as follows. pNEC-O, constructed from pHSG274 and pSV2cat, was cleaved by *Bam*HI and *Hin*dIII. To the larger fragment, the enhancer-promoter region of LF II (L-factor nucleotides 4632 to 154 are shown) was ligated to generate pNEC-II. pNEC-Py and pNEC-EC were constructed similarly by using PyVA2 and PyVN2 DNA, respectively, in place of LF II. The site of the *tk* promoter is indicated. For details, see Materials and Methods.

linker. The larger fragment (3.6 kb) of *Bam*HI and *Sal*I digests of these processed DNA was then ligated to pHSG274 as done for the construction of pLIIN5. The construction procedure is summarized in Fig. 1A.

Chloramphenicol acetyltransferase (CAT) expression DNA was constructed as follows. pNEC-O was constructed by ligating the *cat* fragment produced by *Hin*dIII-*Apa*I digestion of pSV2cat DNA (7) to the larger fragment of *Hin*dIII-*Apa*I digests of pHSG274. For pNEC-II, *Hph*I site of the early region of LF II (polyomavirus nucleotide 154) was repaired by T4 DNA polymerase and converted to the *Hin*dIII site by adding a linker. The DNA was digested with *Hin*dIII and *Bam*HI, and the smaller fragment containing the enhancer/*ori*-promoter region was ligated to *Hin*dIII-*Bam* HI-digested pNEC-O. Other CAT expression DNAs used here, such as pNEC-Py and pNEC-EC, were constructed by a procedure essentially identical to that for pNEC-II. The structure of pNEC-II is shown in Fig. 1B.

Assay of tissue plasminogen activator production. The assay of tissue plasminogen activator was performed as described by Nishimune et al. (16) and is described in more detail in the legend to Fig. 5.

Purification of composite DNA used for transfection experiments. Composite DNA used for transfection experiments was purified by a standard procedure (13), including two cycles of CsCl-ethidium bromide centrifugation and gel filtration through Sephacryl S-400 (Bio-Rad Laboratories).

Assay of CAT. Transient expression of the CAT gene was assayed after transfection of F9 cells with CAT expression DNA as described by Gorman et al. (7). The cells (10^6 cells) were transfected with CAT expression composite DNA (20 μg) by the calcium phosphate coprecipitation procedure; 48 h after a dimethyl sulfoxide pulse, the cells were collected by scraping them off the dishes. CAT activity in the extracts (100 μg of protein) was assayed by thin-layer chromatography after the samples were incubated for 2 h at 37°C.

Transient replication of L-factor and related DNA. Transient replication of transfected DNA was assayed as follows. F9 cells (7×10^5 cells) cultured in three plastic dishes (60-mm diameter) were transfected with 30 μg of DNA by calcium phosphate coprecipitation; after 24, 60, and 80 h the cells were trypsinized and rinsed twice with phosphate-buffered saline containing 1% ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and extrachromosomal (low-molecular-weight) DNA was isolated as described previously (8). DNA was then treated with *Dpn*I and *Bam*HI, subjected to agarose gel electrophoresis, electrotransferred to a nylon membrane, and hybridized with an appropriate ^{32}P-labeled probe.

RESULTS

Establishment of composite DNAs derived from L factor as plasmids in F9 embryonal carcinoma cells. To investigate the mechanism of plasmid establishment in mammalian cells and to utilize L factor as an expression plasmid vector for a specific gene during in vitro differentiation, we first examined whether composite DNA constructed from L factor and the bacterial *neo* gene could be established as a plasmid in mouse embryonal carcinoma (F9) cells. F9 cells were transfected with pLIIN5 DNA, a composite DNA constructed from a segment containing the putative enhancer/*ori*-early region of LF II and pHSG274 (ColE1 DNA containing the *neo* gene with herpes simplex virus *tk* promoter) (Fig. 1A). Extrachromosomal (low-molecular-weight) DNA of resulting G418-resistant colonies was then analyzed by Southern

blot hybridization. Throughout several independent experiments, we found that more than 50% of G418-resistant colonies contained extrachromosomal DNA which hybridized with an L-factor probe. Typical patterns of the extrachromosomal DNA in G418-resistant colonies are presented in Fig. 2A. The extrachromosomal DNA migrated in the gel to approximately the same position as the original DNA did (Fig. 2A), suggesting that the DNA structure is preserved during plasmid establishment. The preservation of the original DNA structure was confirmed by restriction endonuclease analysis. The DNA (clone II-7) recovered from *E. coli* after transformation with the extrachromosomal DNA still maintained essentially the same restriction endonuclease (*Hae*III and *Hin*fI) digestion pattern as that observed with the original DNA (Fig. 2B). This also indicates that the composite DNA serves as a plasmid shuttle vector between *E. coli* and at least F9 cells. Although in most cases the extrachromosomal DNA was indistinguishable from the original DNA in the gel, some of the extrachromosomal DNA was apparently rearranged, judging from the migration pattern in electrophoresis (data not shown). Once established, however, the structure and copy number of the extrachromosomal DNA remained relatively constant throughout many generations (more than 6 months) in the presence of G418 (data not shown; see below).

The copy number of the extrachromosomal DNA among

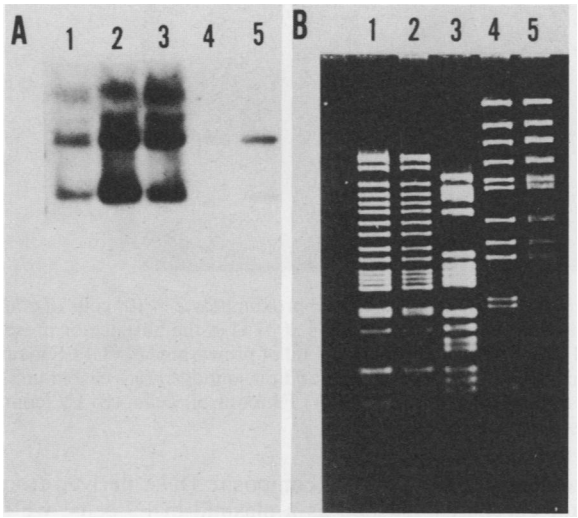

FIG. 2. Analysis of extrachromosomal DNA from G418-resistant clones. (A) Analysis by Southern blot hybridization. F9 cells (7×10^5 cells) were transfected with pLIIN5 DNA (20 μg), and G418-resistant clones were isolated as described in Materials and Methods. Low-molecular-weight DNA from 10^6 cells was electrophoresed through a 0.7% agarose gel, blotted onto a nylon membrane, and probed with nick-translated pLIIN5 DNA. Lanes: 1 to 3, DNA from three independently isolated clones (II-1, II-4, and II-7); 4 and 5, standard plasmid DNA (pLIIN5) equivalent to 2 and 10 copies per cell, respectively. (B) Restriction patterns of pLIIN5 extrachromosomal DNA rescued in *E. coli*. Low-molecular-weight DNA was prepared from a G418-resistant clone (II-7) harboring pLIIN5 by a modified alkaline procedure (1) and used for transformation of *E. coli* (DH5α). From a kanamycin-resistant *E. coli* transformant, low-molecular-weight, covalently closed circular DNA was isolated and digested with *Hae*III or *Hin*fI. The DNA was then electrophoresed through 5% polyacrylamide gel and stained with ethidium bromide. Lanes: 1, rescued DNA digested with *Hae*III; 2, control (pLIIN5) DNA digested with *Hae*III; 3, pBR322 DNA digested with *Hae*III; 4, rescued DNA digested with *Hin*fI; 5, control (pLIIN5) DNA digested with *Hin*fI.

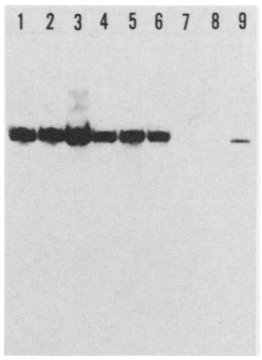

FIG. 3. Southern blot hybridization of total genomic DNA from G418-resistant clones obtained with pLIIN5. F9 cells (7×10^5 cells) were transfected with 20 μg of pLIIN5 DNA, and total genomic DNA was isolated from six independently isolated, G418-resistant clones bearing the extrachromosomal DNA. The DNAs (10 μg of each) were digested with *Bam*HI, electrophoresed through 0.7% agarose gels, blotted onto nylon membranes, and probed with nick-translated pLIIN5 DNA. Lanes: 1 to 6, six independently isolated G418 resistant clones (lane 2 DNA was isolated from clone II-7 described in the text); 7, DNA from control (untransfected) F9 cells; 8 and 9, marker pLIIN5 DNA equivalent to 1 and 4 copies per cell, respectively. For details, see Materials and Methods.

G418-resistant colonies varied considerably from clone to clone, ranging from approximately 10 copies per cell (Fig. 2A, lane 1) to more than 50 copies per cell (Fig. 2A, lanes 2 and 3). To examine whether some of the composite DNA was integrated in the host chromosomes in the cells where the extrachromosomal DNA was found, the total genomic DNA from these cells was digested with *Bam*HI, which gave one cut in the composite DNA, and subjected to Southern blot hybridization after electrophoresis. In Fig. 3, we show the results from six independently isolated G418 clones bearing the extrachromosomal DNA (ca. 25 to 75 copies per cell). No sign of chromosomal integration was observed, except for one clone (lane 5) in which an extra band was detected representing either integrated DNA or rearranged extrachromosomal DNA. These results indicate that most, if not all, extrachromosomal DNAs are maintained in the cells as autonomously replicating units (plasmids) as the original L factors in a subclone (B822) of mouse cells, although we could not exclude the possibility that sometimes the plasmid DNA exists as a polymeric (or rearranged) form of the original DNA. We also found that LF II-derived composite DNAs were more easily established than LF I-derived DNAs as plasmids in F9 cells (data not shown). Thus, the experiments reported here were carried out with LF II-derived composite DNA.

The plasmid present in G418-resistant clones was stably maintained as long as selective pressure was being exerted. A clone bearing pLIIN5 was transferred to a medium with or without G418, and the extrachromosomal DNA was examined after prolonged culture at 35°C and at 37°C. L factors are more stably maintained as plasmids at 35°C than at 37°C (10). At 35°C the copy number of the plasmid in the clone (II-7) remained constant at least for 60 days in the presence of G418, and no indication of integration of the plasmid in chromosomal (high-molecular-weight) DNA was observed (data not shown). Without G418 the copy number of the plasmid seemed to remain unchanged up to 30 days at 35°C but gradually declined thereafter. The decline of the copy number was even more pronounced at 37°C (data not shown). Essentially the same results were obtained with

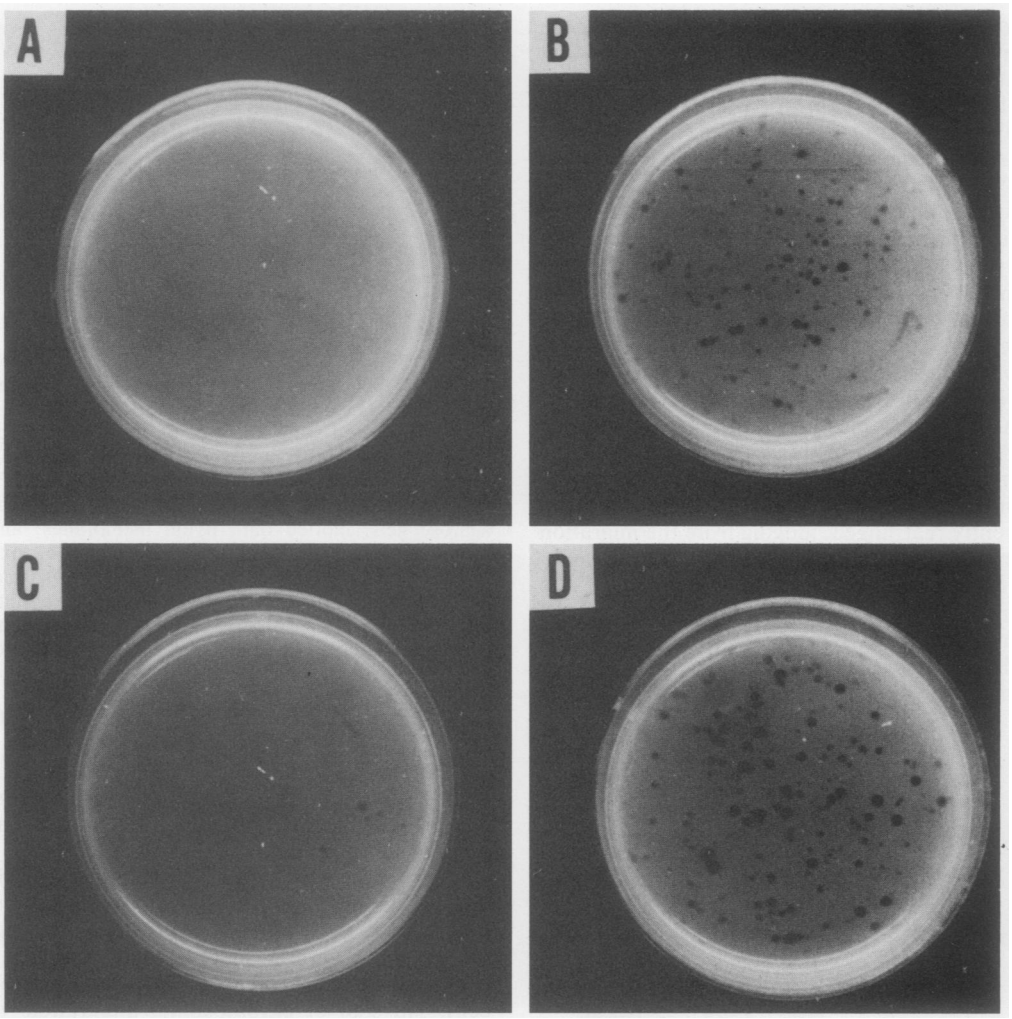

FIG. 4. Induction of tissue specific plasminogen activator in plasmid-bearing cells by retinoic acid. Approximately 2×10^3 cells of control (F-9) and plasmid-bearing (II-7) cells were plated in 60-mm plastic petri dishes and incubated for 4 days at 37°C in the absence or presence of 1 μM retinoic acid. The plates were washed twice with phosphate-buffered saline and covered with 2.5 ml of prewarmed (45°C) ES medium supplemented with Noble Agar (0.75%, wt/vol; Difco Laboratories), skim milk (0.25%, wt/vol; Difco), and plasminogen (0.2 casein unit per ml; Green Cross Co.) (15). The plates were incubated for 12 h at 37°C, and the pictures were taken. (A) F9 (control) cells; (B) F9 (control) cells in the presence of retinoic acid; (C) II-7 cells; (D) II-7 cells in the presence of retinoic acid.

several other G418-resistant clones bearing the plasmid. Once the composite DNA was established as a plasmid in F9 cells, the structure of the DNA seemed to remain intact, without showing a sign of rearrangement or integration into the chromosomes (data not shown).

In vitro differentiation of the plasmid-bearing cells. The plasmid-bearing F9 cells were almost indistinguishable from the original F9 cells in morphology and growth rate (data not shown). We examined whether retinoic acid induces in vitro differentiation in the plasmid-bearing cells as in the original F9 cells. The plasmid-bearing cells (II-7) produced tissue plasminogen activator in response to retinoic acid at an efficiency almost equal to that observed in the original F9 cells (Fig. 4). Furthermore, essentially the same morphological changes as those in the original cells were induced in the II-7 cells by retinoic acid (Fig. 5). These results indicate that the presence of L factor-derived plasmids in F9 cells has no effect and is neutral with respect to the retinoic acid-inducible in vitro differentiation.

Plasmid establishment of composite DNA derived from L factor, polyomavirus DNA, and a mutant polyomavirus DNA. To obtain a clue as to why composite DNA derived from L factor can be established as a plasmid in F9 cells, we first compared the efficiency of plasmid establishment among composite DNA derived from L factor (LF II), polyomavirus (PyVA2), and an EC mutant of polyomavirus (PyVN2) that is permissive in proliferation in F9 cells (19). For this, pHSG274 DNA was ligated to PyVA2 and PyVN2 DNA at the same site as in pLIIN5 to construct composite plasmids pPy274 and pPyEC274, respectively (Fig. 1A) (Materials and Methods). pLIIN5, pPy274, and pPyEC274 were introduced into F9 cells by transfection, and G418-resistant colonies were selected at 35°C. DNA from these resistant colonies was then analyzed with respect to the presence of the DNA in the extrachromosomal fraction (Table 1). Whereas LF II-derived DNA (pLIIN5) gave the highest efficiency in G418-resistant colony formation (10.2×10^{-5}), the polyomavirus EC mutant-derived DNA (pPyEC274) gave the least efficient G418-resistant colony formation (0.1×10^{-5}). Polyomavirus-derived DNA (pPy274) gave an efficiency (1.1×10^{-5}) which was approximately 1/10 of that by pLIIN5.

When we examined the presence of the extrachromosomal

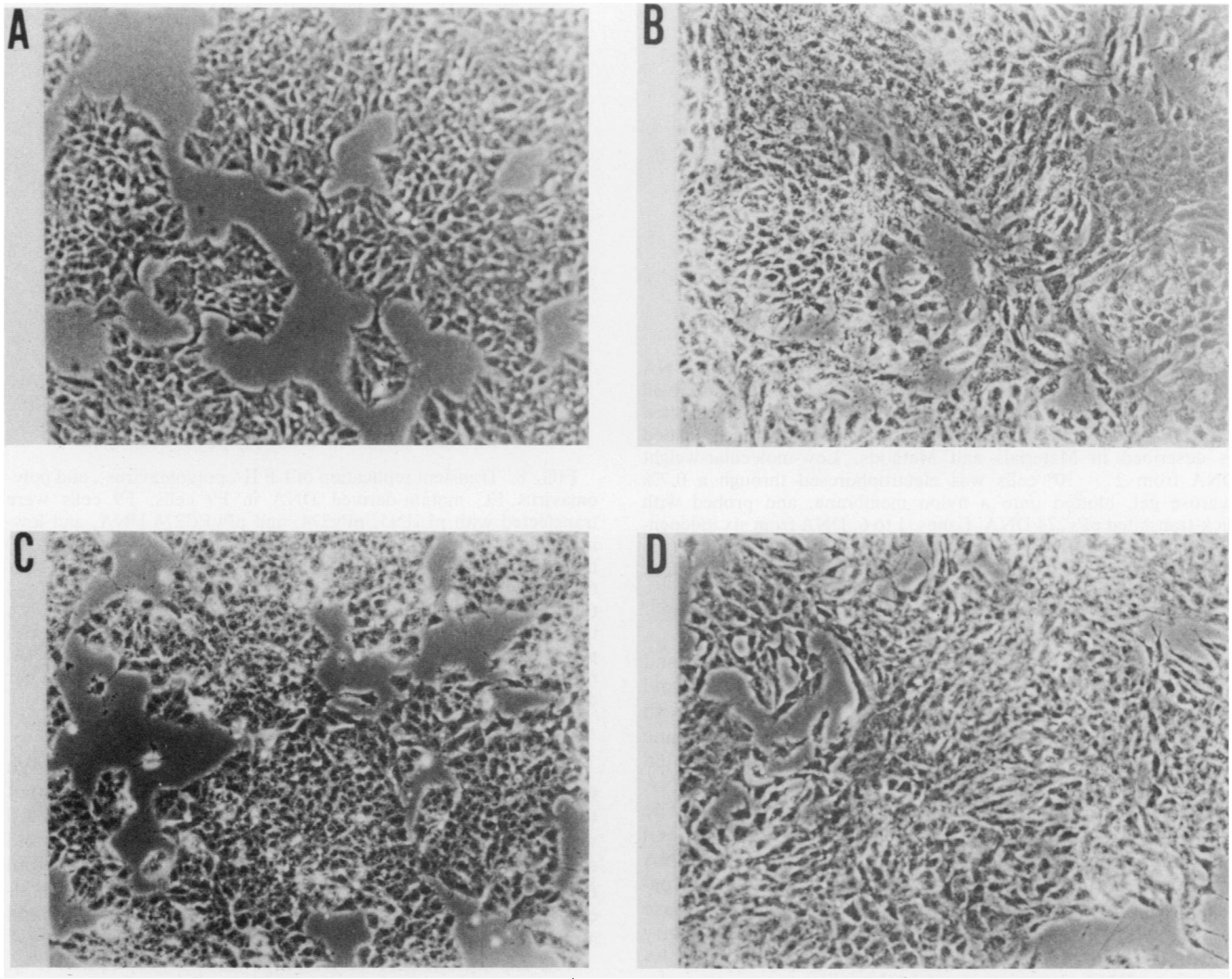

FIG. 5. Changes in cell morphology of plasmid-bearing F9 cells. F9 (control) cells and plasmid-bearing F9 (II-7) cells were cultured in ES medium supplemented with 10% fetal calf serum in 100-mm plastic petri dishes at 37°C. When the cell number reached 10^6 cells per dish, the medium was replaced by the fresh medium with or without 1 μM retinoic acid. After incubation for 5 days at 37°C, photographs were taken under an Olympus phase-contrast microscope (IMT-2) at ×100 magnification. (A) F9 (control) cells; (B) F9 (control) cells in the presence of retinoic acid; (C) II-7 cells; (D) II-7 cells in the presence of retinoic acid.

DNA and analyzed the DNA structure of these G418-resistant colonies, a considerable percentage (55% for pLIIN5, 28% for pPy274) of G418-resistant clones contained the original DNA extrachromosomally, whereas no clones bearing the extrachromosomal DNA were detected among G418-resistant clones obtained with pPyEC274-derived DNA (Table 1). Typical patterns of these extrachromosomal DNAs, especially polyomavirus-derived DNA (pPy274), are shown in Fig. 6. The extrachromosomal DNAs produced after the transfection of polyomavirus-derived composite DNA were either rearranged (Fig. 6, lanes 3 and 6) or contained rearranged DNA in addition to the DNA of the original size (lane 1). We do not know whether these extrachromosomal DNAs are self-replicating (plasmids) as L factor-derived DNAs or excision products of composite polyomavirus DNA integrated in the chromosomes, although circumstantial evidence suggests the former possibility.

Expression of CAT gene in F9 cells. Transcriptional activity controlled by the enhancer-promoter of L factor in F9 cells was compared with those of polyomavirus (PyVA2) and an EC mutant of polyomavirus (PyVN2). We assayed CAT activity expressed after transfection of the composite DNA in F9 cells. Three CAT expression composite DNAs (pNEC-II, pNEC-Py, and pNEC-EC) corresponding to LF II, polyomavirus, and the EC mutant of polyomavirus, respectively,

TABLE 1. Relative efficiency of G418-resistant colony formation and presence of plasmid DNA among G418-resistant colonies[a]

Composite DNA	Efficiency of G418-resistant colony formation	No. of G418-resistant colonies	
		Total no. examined	No. harboring plasmid
pLIIN5	10.2×10^{-5}	20	11
pPy274	1.1×10^{-5}	18	5
pPyEC274	$<0.1 \times 10^{-5}$	7	0

[a] The data are presented as sums of three independent experiments except for pPyEC274, with which eight transfection experiments were performed.

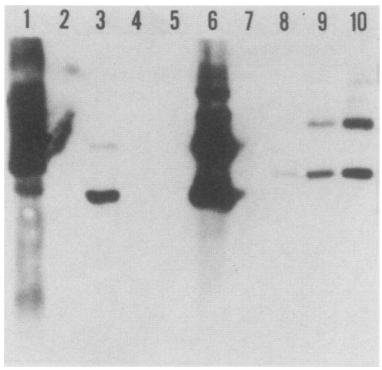

FIG. 6. Southern blot hybridization of extrachromosomal DNA from G418-resistant clones obtained with polyomavirus-derived composite DNA (pPy274). F9 cells (7×10^5 cells) were transfected with 20 μg of pPy274 DNA, and G418-resistant clones were isolated as described in Materials and Methods. Low-molecular-weight DNA from 2×10^6 cells was electrophoresed through a 0.7% agarose gel, blotted onto a nylon membrane, and probed with nick-translated pPy274 DNA. Lanes: 1 to 6, DNA from six independently isolated G418-resistant clones; 7, DNA from control (untransfected) cells; 8 to 10, marker pPy274 DNA equivalent to 1, 4, and 20 copies per cell, respectively.

were constructed for this purpose. (Fig. 1B) (Materials and Methods). F9 cells were transfected with these DNAs, and CAT activities were assayed. The cells transfected with composite DNA derived from LF II (pNEC-II) (Fig. 7, lane 1) and from polyomavirus (pNEC-Py) (lane 2) exhibited much lower CAT activity (lane 3) than composite DNA (pNEC-EC) derived from the permissive EC mutant. The higher CAT activity with the EC mutant DNA had been expected from the nature of the mutant (2, 12). DNA (pNEC-O) which contained no L factor or polyomavirus-related DNA showed almost no activity (lane 4). These results suggest that the putative enhancer-promoter activity of LF II is considerably repressed in F9 cells as in polyomavirus DNA, consistent with the observation that the T-antigen level of L factor-bearing cells (B822) was low (10).

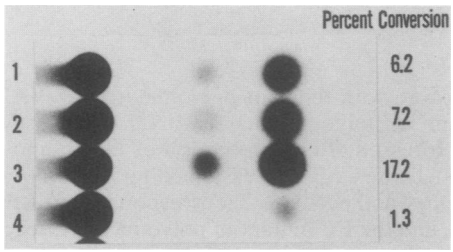

FIG. 7. Expression of CAT gene in transfected F9 cells. F9 cells (10^6 cells) grown in a 100-mm plastic petri dishes were transfected with composite DNA (20 μg) and subjected to a dimethyl sulfoxide pulse after 16 h of incubation at 37°C. At 48 h (at 37°C) after the pulse, the cells were washed with phosphate-buffered saline, suspended in 0.4 ml of 250 mM Tris hydrochloride (pH 7.8), lysed by repeated (three times) freezing and thawing, and centrifuged (12,000 × *g*, 5 min). A portion (100 μg of protein) of the supernatant was used for the CAT assay (6, 7) with [^{14}C]chloramphenicol (0.2 μCi for each assay; 50 Ci/mmol; Amersham Corp.) as the substrate. Lanes: 1, pNEC-II; 2, pNEC-Py; 3, pNEC-EC; 4, pNEC-O. The direction of the thin-layer chromatography was from left to right. Percent conversion was calculated by counting radioactivities which were shifted from the original spots. For details, see Materials and Methods.

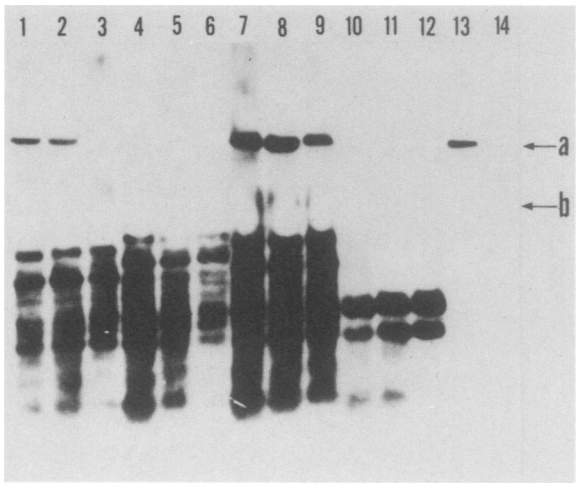

FIG. 8. Transient replication of LF II-, polyomavirus-, and polyomavirus EC mutant-derived DNA in F9 cells. F9 cells were transfected with pLIIN5, pPy274, and pPyEC274 DNA, and low-molecular-weight DNA was isolated at 24 h (lanes 1, 4, 7, and 10), 60 h (lanes 2, 5, 8, and 11), and 80 h (lane 3, 6, 9, and 12) after transfection. Lanes: 1 through 3, DNA transfection with pPy274; 4 through 6, DNA transfection with pLIIN5; 7 through 9, DNA transfection with pPyEC274; 10 through 12, DNA transfection with pHSG274; 13 and 14, *Bam*HI-digested pLIIN5 and pHSG274 DNA, respectively (their positions are indicated by arrows a and b). For details, see Materials and Methods.

Transient replication of composite DNA in F9 cells. We examined the degree of DNA replication after transfection of the composite DNA in F9 cells. The cells were transfected with the DNAs (pLIIN5, pPy274, and pPyEC274), and extrachromosomal DNA was digested with *Dpn*I and *Bam*HI, which distinguish replicated DNA from nonreplicated DNA. The digested DNA was then electrophoresed and analyzed by Southern blot hybridization.

The EC mutant-derived DNA (pPyEC274) replicated (Fig. 8, lanes 7, 8, and 9), as evidenced by the sensitivity of the DNA to *Dpn*I, reflecting a sufficient supply of T antigens which was allowed by the activated enhancer-promoter of the mutant. On the other hand, DNA derived from LF II (pLIIN5) showed only a little, if any, replication (Fig. 8, lanes 4, 5, and 6). The replication of polyomavirus-derived DNA (pPy274) was also low (Fig. 8, lane 1, 2, and 3), but the level was slightly higher than that observed with pLIIN5. This tendency of transient replication among the composite DNAs correlated with that observed in the expression of CAT activity after transfection as described above. From these results, it is clear that the efficiency of plasmid establishment is not directly correlated with the replication capacity (transient replication assay) of the composite DNA. Rather, there seems to be an inverse relationship between the efficiency of plasmid establishment and the replicational (transcriptional) activities which are probably controlled by the intrinsic enhancer-promoter specific to the DNA.

DISCUSSION

In this paper we have demonstrated that composite DNA derived from a polyomavirus-related L factor can be reestablished as a plasmid in mouse embryonal carcinoma (F9) cells after transfection. The reestablished DNA can be rescued in *E. coli*, indicating that the composite DNA serves as a plasmid shuttle vector between *E. coli* and at least F9

cells. Since polyomavirus DNA is not generally established as a plasmid in the virus-infected cells, the stable plasmid maintenance of L factor in F9 cells may provide a clue to the mechanism of plasmid establishment of papovaviruses in mammalian cells. When compared with similar composite DNA constructed from an EC mutant of polyomavirus, the L factor-derived DNA exhibited a suppressed level of gene expression and only a limited transient replication activity in F9 cells, as observed with polyomavirus-derived DNA. In a previous paper, we showed that the level of T antigen (like material) in the L factor-bearing cells (B822) was extremely low, although it was required for replication (10). We suggested that the limited supply of T antigen is one of the necessary conditions for the establishment of papovavirus DNA in mouse cells as a plasmid. The results presented in this paper are consistent with the previous suggestion. Whereas the expression of CAT activity and transient replication by L factor were suppressed in F9 cells in which plasmid establishment of L factor-derived DNA was demonstrated, similar DNA derived from the EC mutant of polyomavirus permissive in F9 cells was not established as a plasmid in the cells. It is possible that the high transcriptional and replicational activities associated with the EC mutant DNA in F9 cells were toxic to the cells and led to the inefficient establishment of plasmids.

We found extrachromosomal DNA, although most of it was rearranged, in F9 cells transfected with composite DNA derived from polyomavirus. Although we have not confirmed whether the DNA was self-replicating, this has raised the possibility that even polyomavirus DNA could be established as a plasmid in the cells where T-antigen expression is suppressed, such as in F9 cells. In this respect, recent experiments by Dandolo et al. also suggest that polyomavirus replicates in the absence of T antigens (4).

So far, in almost all mouse and hamster cells tested we have been able to reestablish various composite DNAs constructed from L factors (LF I and LF II) and foreign genes (herpes simplex virus *tk*, *neo*, etc.) as stable plasmids (17). Probably, the structure of L factor is such that the expression of T antigens is universely suppressed in most murine cells. Total DNA sequencing of L factors has revealed that the structure of L factors differs from that of polyomavirus in the following respects (Yoshimura et al., submitted for publication): (i) shorter coding frames (three amino acid less) for early gene products (T antigens), (ii) premature termination of all three structural proteins (VP_1, VP_2, and VP_3), and (iii) drastic DNA rearrangement in the enhancer region corresponding to the *Pvu*II fragment of polyomavirus. Also, a number of base substitutions are scattered along the whole genome.

Based on these results, one of the obvious approaches to elucidate the mechanism of plasmid establishment would be to construct various artificial DNAs consisting of different segments of L factor and polyomavirus or EC mutant DNA and to locate the DNA sequences crucial for the establishment of plasmids. Preliminary experiments, however, employing composite DNA consisting of various segments of L factors, polyomavirus, or EC mutant DNA have failed to identify a specific block of DNA sequences responsible for the plasmid establishment. It seems that the mechanism of plasmid establishment is rather complex, involving complicated interactions between T antigens, host cell proteins, and different DNA sequences in the enhancer/*ori*-promoter region.

The presence of L factor-derived plasmids in F9 cells did not alter the inducibility of the cell differentiation by retinoic acid. This suggests that an L factor-derived plasmid can be used as an expression vector for a specific gene during in vitro differentiation. In fact, we have observed expression of the plasmidal CAT gene in the composite plasmid under the control of appropriate viral enhancer-promoters to be drastically (over 50-fold) stimulated by retinoic acid (Nishimori et al., manuscript in preparation), raising the possibility that the plasmid expression system can substitute for authentic chromosomal gene expression specific to the differentiation and can become a useful tool for the analysis of the complex intracellular processes leading to mouse embryonal differentiation.

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