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Transient Gene Expression Control: Effects of Transfected DNA Stability and *trans*-Activation by Viral Early Proteins

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The effects of *trans*-acting factors and transfected DNA stability on promoter activity were examined with chloramphenicol acetyl transferase (CAT) transient expression analysis. With cotransfection into CV-1P and HeLa cells, simian virus 40 T antigen, adenovirus E1a, and herpes-virus IE proteins were compared for their ability to *trans*-activate a variety of eucaryotic promoters constructed into CAT plasmids. T antigen and the IE protein were promiscuous activators of all the promoters tested [the simian virus 40 late promoter, the adenovirus E3 promoter, the α2(I) collagen promoter, and the promoter of the Rous sarcoma virus long terminal repeat]. Conversely the E1a protein was specific, activating only the adenovirus E3 promoter and suppressing the basal activity of the other promoters. This specificity of activation by E1a contrasted with the high activity generated by all of the promoter-CAT plasmids when transfected into 293 cells, which endogenously produce E1a protein. Examination of transfected 293 cells determined that they stabilized much greater amounts of plasmid DNA than any other cells tested (CV-1P, COS, NIH-3T3, KB). Thus the high activity of nonadenovirus promoter-CAT plasmids in 293 cells results from the cumulative effect of basal promoter activity from a very large number of gene copies, not from E1a activation. This conclusion was supported by similar transfection analysis of KB cell lines which endogenously produce E1a protein. These cells stabilize plasmid DNA at a level comparable to that of CV-1P cells and, in agreement with the CV-1P cotransfection results, did not activate a nonadenovirus promoter-CAT plasmid. These results indicate that the stability of plasmid DNA must be considered when transient gene expression is being compared between cell lines. The use of relative plasmid copy numbers for the standardization of transient expression results is discussed.

Early viral proteins produced in cells infected by several DNA viruses have in common the function of activating viral genes expressed during the later phases of the temporally regulated lytic life cycle (5, 7, 8, 25, 26, 30, 32, 38). These proteins, simian virus 40 (SV40) T antigen, adenovirus E1a protein, and the herpesvirus immediate early (IE) proteins, are the products of genes containing very efficient promoters activated by cis-acting elements (4, 9, 18, 19, 39), although herpesvirus IE gene expression is additionally stimulated by a component of the virion particle (3, 8, 33, 38). These strong *cis*-activated promoters ensure the efficient initiation of the viral infection. In a permissive cell the subsequent *trans*-activating function of each protein would then activate the expression of other viral genes to permit the virus to progress through its lytic cycle. The *trans*-activating function of each of these proteins has been demonstrated in its respective viral system (5, 7, 8, 25–27, 30, 32, 38). Additionally, transient expression experiments with the E1a and the IE proteins (17, 23) indicate that these proteins may also *trans*-activate promoters that are not homologous to their respective viral systems; in some cases E1a appears to repress some promoters (6; A. Velcich and E. Ziff, Cell, in press; see below). Considering that these proteins, as well as T antigen, have been implicated in molecular events that contribute to transformation (36), it seems possible that many, if not all, of the phenotypic changes characteristic of transformation may be due to pleiotropic effects mediated by the *trans*-activating function of these early viral proteins.

In the present communication, the chloramphenicol acetyl transferase (CAT) transient expression system (13, 14) was utilized to study the ability of T antigen, the E1a protein, and the IE protein to activate a variety of eucaryotic promoters [the SV40 late promoter, the adenovirus E3 promoter, the α2(I) collagen promoter, and the promoter of the Rous sarcoma virus (RSV) long terminal repeat (LTR)]. In these experiments the *trans*-acting proteins were supplied by expressing plasmids cotransfected with the promoter-CAT plasmids into CV-1P or HeLa cells. T antigen and IE protein were found to be promiscuous, activating CAT expression from all of the promoter-CAT plasmids. However, E1a protein was more specific, activating only its homologous adenovirus E3 promoter and exhibiting an apparent suppressive effect on the basal activity of the other promoters tested.

The inability of E1a protein to activate nonhomologous promoters in the cotransfection experiments was in sharp contrast to the results obtained when the promoter-CAT plasmids were transfected into the 293 human cell line, which endogenously produces the adenovirus E1a and E1b proteins (16). This cell line has been used in many transfection experiments to demonstrate the effect of the E1a protein on promoter activity. In these cells all of the promoter-CAT plasmids generated very high CAT activity. Thus it would appear that all of the promoters tested were activated by E1a protein in 293 cells, whereas E1a-mediated activation was specific for the E3 promoter in the cotransfection experiments in CV-1P or HeLa cells. However, the data indicate that the high CAT activity observed in 293 cells is not due to activation by E1a, but is due to a greatly enhanced stability of transfected plasmid DNA in these cells as compared with other cell lines we have analyzed (CV-1P, COS, NIH-3T3, KB). Thus the high CAT activity generated by the promoter-CAT plasmids in 293 cells can be accounted for by the cumulative effect of low or basal promoter activity from a very large number of gene copies, rather than by E1a activation. Additional experiments are presented with se-

lected KB cell lines, which, like 293 cells, endogenously produce E1a and E1b proteins, both separately and together (2). In these cells, plasmid DNA did not have the stability observed in 293 cells; correspondingly, CAT activity from the nonadenovirus promoters was not enhanced. In addition to supporting the observation that E1a protein may be specific in its promoter activation function, the KB cell data also suggest that the stability of plasmid DNA in 293 cells is not imparted directly to these cells solely by the production of E1a or E1b or both.

Finally, the data presented herein show that the amount of plasmid DNA maintained by a transfected cell can be variable, resulting in different levels of transfected gene activity due to copy number differences. This source of experimental variation must be considered in the interpretation of comparative transient expression results. The data indicate that such variations can be corrected by determining the amount of transfected plasmid DNA and using this as a standardization parameter. The utilization of relative plasmid DNA copy number as a means of standardizing for transfection variations is discussed.

MATERIALS AND METHODS

Cells and cell culture. The following cells were utilized: NIH-3T3 cells; the established African green monkey kidney line CV-1P and the SV40 transformed derivative of this line, COS (10), which endogenously produces SV40 T antigen; 293 cells, human cells transformed by the E1a-E1b coding region of adenovirus (16), which endogenously produce these viral proteins; and a collection of selected human KB cell lines that produce adenovirus E1a or E1b proteins or both (2). The KB lines were the gift of H. Ginsberg and colleagues and are described below. All cells were grown in a 37°C CO_2 incubator on Falcon tissue culture plastic in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics.

Plasmids and plasmid DNA preparation. The promoter-CAT plasmids used in these experiments included the following: pL2-cat and pL2n-cat (26), which have the CAT gene under the regulation of the SV40 late promoter either with (pL2-cat) or without (pL2n-cat) a functional viral origin of replication (the replication defect in pL2n-cat has been introduced by a 6-base-pair deletion at the *Bgl*I site within the origin of replication [11]); pSVO-cat (13), the parent CAT plasmid containing no eucaryotic promoter regulating the CAT gene; pkcat-23 (39), a gift from N. Jones, which has the CAT gene under the control of the adenovirus E3 promoter; pCOL-cat (20, 37), a gift from B. de Crombrugghe, which has the CAT gene under the control of the chicken α2(I) collagen promoter; pRSV-cat (12), a gift from B. Howard and C. Gorman, which has the CAT gene under the control of the promoter within the RSV LTR.

The plasmids that produce the *trans*-acting proteins include the following: p1A (34), a gift of E. Ruley, which produces the adenovirus E1a protein in human, monkey, and mouse cells; p6-1dl, a derivative of p6-1 (11), which produces T antigen in human, monkey, and mouse cells; pIE (22), a gift of T. Ben-Porat, which produces the herpesvirus (pseudorabies virus [PrV]) immediate early protein.

All plasmids were maintained in *Escherichia coli* strain HB101 and isolated by standard procedures (29). Chloramphenicol amplification of CAT-containing plasmids was generally ineffectual, since a moderate amount of CAT enzyme is produced from the plasmids during growth in bacteria. Isolated plasmids were purified through two rounds of ethidium bromide-CsCl gradient centrifugation before they were used for transfections.

Transfections. Cells to be transfected were plated on the day before transfection at a density of 3×10^5 cells per 60-mm dish. At 4 h before transfection, the medium was removed, and 4 ml of fresh medium was added to each plate. Calcium phosphate precipitates of the plasmid DNA were prepared as previously described (13, 15, 26) and added to the medium in a volume of 0.5 ml. Each transfection contained 5 μg of plasmid DNA per 60-mm dish. Where cotransfections were performed, 5 μg of each plasmid was used. The pSVO-cat plasmid, containing no promoter, was utilized as a inactive control plasmid and as a filler plasmid to adjust plasmid concentrations in cotransfections. After 4 h the transfection medium was removed, and the cells were washed four times with serum-free medium and then fed with 5 ml of medium containing 10% fetal bovine serum and incubated for 48 h at 37°C. Transfections were done multiple times with different plasmid preparations to control for transfection efficiency variations. In addition, the results were often standardized by determining the amount of plasmid DNA in the cells at the time of harvest. This is discussed below and in the text.

CAT extraction and assay. CAT extractions and assay were performed as previously described (13, 26). CAT activities were converted to units by comparison to standard curves generated by known amounts of purified CAT (P-L Biochemicals Inc.). One unit of CAT catalyzes the acetylation of one nanomole of chloramphenicol per minute at 30°C and pH 7.8. The enzyme activity is not adversely affected by the assay in very high or very low protein concentrations; this makes it possible to accurately determine enzyme activities over a 1,000-fold range.

Isolation and analysis of plasmid DNA from transfected cells. At various times after transfection cells were harvested and assayed for plasmid DNA levels. Two plates were harvested at each time point, one with DNase treatment and one without. When no DNase was used, the cells were lysed by the method of Hirt (21). The Hirt supernatants were phenol extracted twice, chloroform extracted once, and ethanol precipitated. The ethanol precipitates were centrifuged, the pellets were washed twice with 80% ethanol, and the dry pellets were dissolved in 0.15 ml of 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl. When the DNase treatment was used, the cell monolayers were washed with serum-free medium and then treated with 5 ml of serum-free medium containing 50 μg of pancreatic DNase I per ml for 1 h at 37°C. After the DNase treatment the medium was removed, and the cells were suspended by trypsinization for counting in a hemocytometer. The cells were then sedimented by centrifugation and suspended in Hirt lysis buffer, and the DNA was prepared as described above.

Equivalent portions of each sample were digested with a combination of *Bam*HI and *Mbo*I or with *Bam*HI alone. The digests were electrophoresed on 1% agarose gels and blotted to nitrocellulose (35), and plasmid DNA was visualized by hybridization with ^{32}P-labeled, nick-translated pSVO-cat DNA. After autoradiography, the resultant bands were quantitated by densitometer scanning and comparison with the intensities of bands of known amounts of plasmid DNA.

CAT immunofluorescence analysis of transfected cells. Cells grown on cover slips in 60-mm plates were transfected as described above. After 48 h the cover slips were washed three times with standard phosphate-buffered saline and fixed with 95% methanol for 5 min. The cover slips were air dried and then hydrated with phosphate-buffered saline for

treatment. A 5-μl sample of goat anti-CAT antibody (the kind gift of B. Howard and C. Gorman), appropriately diluted in phosphate-buffered saline, was applied to a small area of cells and incubated at room temperature for 45 min. The cells were washed three times with phosphate-buffered saline, and appropriately diluted fluorescein-labeled staphlococcus protein A was added to the treated cells. After 45 min at room temperature the cells were washed three times with phosphate-buffered saline and examined.

RESULTS

Activation of promoters by T antigen, E1a protein, and IE protein. To compare the *trans*-activation function of these proteins, their effect was tested on CAT transient expression vectors (13) in which the CAT gene was regulated by one of the following: the SV40 late promoter, pL2n-cat (26); the adenovirus E3 promoter, pKcat23 (39); the chicken α2(I) collagen promoter, pCol-cat (20, 37); or the promoter of the RSV LTR, pRSV-cat (12). In addition, as a negative control the effect of each viral protein was tested on pSVO-cat (13), which contains no eucaryotic promoter region before the CAT gene. None of these plasmids can replicate under the conditions used; this includes the SV40 late promoter plasmid, pL2n-cat, which contains a nonfunctional origin of replication (26) (see above). For the experiment shown in Table 1, these promoter-CAT plasmids were transfected into CV-1P or HeLa cells, either alone or cotransfected with a plasmid that could express one of the following viral early proteins: SV40 T antigen, p6-1dl (see above); adenovirus E1a protein, p1A (34); or herpesvirus (PrV) IE protein, pIE (22). Gene expression was measured 48 h after transfection by assaying CAT activity. The data are expressed as microunits of CAT activity per 10^5 cells transfected. The control experiments, using pSVO-cat, show that no CAT activity is generated by this promoterless plasmid, even in the presence of the viral proteins. This argues that CAT activity, and any stimulation of CAT activity, from the promoter plasmids results from utilization of the inserted eucaryotic promoter sequences. This is supported by substantial RNA analysis (using the promoter plasmids described above) from this laboratory (27, unpublished observations) and others (6, 12, 13, 20, 23, 26), which indicate that (i) known transcriptional start sites are utilized within the eucaryotic promoter sequences inserted in the plasmid constructs, and (ii) CAT enzyme activity varies with its mRNA concentration. For these reasons the CAT gene activity assayed is presented as a measure of promoter activity.

TABLE 1. Comparison of *trans*-activation[a]

Promoter	Activator	μU of CAT/10^5 cells	
		CV-1P cells	HeLa cells
SV40 late (pL2n-cat)	None	351 (1)	32 (1)
	SV40 T Antigen	9,011 (26)	491 (15)
	Adenovirus E1a	55 (0.2)	34 (1)
	PrV IE	1,648 (4.7)	NT
Adenovirus E3 (pKcat-23)	None	2 (1)	90 (1)
	SV40 T Antigen	940 (470)	2,050 (23)
	Adenovirus E1a	1,100 (550)	3,500 (39)
	PrV IE	841 (420)	NT
α2(I) collagen (pCol-cat)	None	40 (1)	NT
	SV40 T Antigen	580 (14)	NT
	Adenovirus E1a	35 (1)	NT
	PrV IE	520 (13)	NT
RSV LTR (pRSV-cat)	None	4,515 (1)	NT
	SV40 T Antigen	22,575 (5)	NT
	Adenovirus E1a	1,638 (0.36)	NT
	PrV IE	11,740 (2.6)	NT
No promoter (pSVO-cat)	None	<2	<2
	SV40 T Antigen	<2	<2
	Adenovirus E1a	<2	<2
	PrV IE	<2	<2

[a] CAT activity was assayed from cells transfected for 48 h with the various promoter-CAT plasmids indicated (see the text). Plasmids were transfected alone (none) or cotransfected with a plasmid that could supply one of the viral activator proteins (see the text). The plasmid pSVO-cat contains no eucaryotic promoter sequences and is inactive in expressing CAT in eucaryotic cells. It was used in the control experiments and as the filler plasmid in the transfections where no activator plasmid was included. CAT activity was assayed and converted to microunits as described in the text. NT, Not tested.

The data in Table 1 indicate that SV40 T antigen and the PrV IE protein clearly stimulate CAT gene expression from each promoter-containing plasmid. This includes the RSV LTR promoter, which has a very efficient *cis*-acting enhancer element. The numbers within parentheses indicate the fold stimulation with the basal (no activator, denoted None in Table 1) activity of each promoter plasmid as unity. Surprisingly, the adenovirus E1a protein showed activation of CAT expression only with the plasmid containing its homologous adenovirus promoter and no activation with the other promoter plasmids. Furthermore, in repeated experiments with the SV40 late promoter plasmid and the RSV LTR plasmid, the E1a protein has consistently shown a repression of the basal CAT activity. These results agree with recent results (6; Velcich and Ziff, in press) that show repression, but contrast other reports that used transient expression analysis to determine that E1a protein has a stimulatory effect on the human β-globin promoter (17). Since the β-globin experiments utilized human cells (17), the possibility was considered that E1a might function in a species-specific manner. Therefore, some of the experiments in Table 1 were repeated in HeLa cells. The results show that the effects of E1a protein on the various promoter-CAT plasmids are qualitatively similar in CV-1P and HeLa cells, arguing against a species specificity for E1a protein function. It must be pointed out that the data in Table 1 should be compared in a qualitative manner only. The CAT activity of each promoter plasmid alone can be directly compared with the activity resulting from that plasmid in the presence of one of the viral proteins. Thus the data indicate stimulation or repression. However, quantitative comparisons of the effects of different viral proteins on a specific promoter plasmid may not be valid. It is unknown whether comparably active levels of each activator protein are synthesized during transient expression. It is possible that activation or repression of a specific promoter by E1a is mediated by the active concentration of the E1a protein in the cell.

Overall, the data in Table 1 suggest that SV40 T antigen (in CV-1P and HeLa cells) and IE protein (in CV-1P cells) are promiscuous activators in that they activate a variety of promoters; in contrast, a level of adenovirus E1a protein that can adequately stimulate expression from an adenovirus promoter-CAT plasmid has no activating effect, but rather an apparent suppressive effect on some of the other promoter-CAT plasmids tested.

Promoter activities in 293 cells. The above results with E1a protein were further tested by introducing the promoter-CAT plasmids into 293 cells (Table 2), a human cell line transformed by the E1a-E1b-coding region of adenovirus

TABLE 2. Activity of promoter-CAT plasmids in 293 and CV-1P cells[a]

Promoter	Activator	μU of CAT/10^5 cells	
		293 cells	CV-1P cells
SV40 late (pL2n-cat)	None	25,000	150
	SV40 T Antigen	25,000	10,000
	Adenovirus E1a	25,000	60
Adenovirus E3 (pKcat23)	None	28,000	18
	Adenovirus E1a	28,000	1,500
No promoter (pSVO-cat)	None	0	0
	SV40 T Antigen	0	0
	Adenovirus E1a	0	0

[a] Plasmids, transfection proceedures, and CAT assays were as described in footnote *a* of Table 1.

DNA (16). This cell line constitutively produces the E1a and E1b proteins and can rescue adenoviruses mutated in these genes (16). When tested in 293 cells, all of the promoter-CAT plasmids gave similar results; therefore, the SV40 late promoter-CAT plasmid, pL2n-cat, was chosen as the representative for extensive characterization. Table 2 shows that when this plasmid was introduced alone, very high CAT activity was generated in the 293 cells, compared with the low activity in CV-1P cells, as note previously (26) and in Table 1. Several controls, similar to experiments presented in Table 1, were repeated in the Table 2 experiment so that the data could be compared. Again it is noted that T antigen activated the SV40 late promoter in CV-1P cells, and E1a protein did not, although E1a protein could activate its homologous adenovirus promoter. The activator proteins had no apparent additional effect on the activity of the promoter-CAT plasmids in 293 cells. Additionally, the control plasmid pSVO-cat, containing no promoter, gave no activity in either 293 or CV-1P cells, indicating that the activities noted are promoter dependent (see above).

Thus the data obtained with 293 cells appear to be inconsistent with the data generated by cotransfection experiments in CV-1P and HeLa cells. The E1a protein does not activate the SV40 late promoter-CAT construct in CV-1P cells, yet this plasmid shows extremely high activity when transfected into 293 cells. An explanation for this inconsistency was found by an examination of the amount of plasmid DNA in the cells at the time of harvest for the CAT assays. Figure 1 shows Southern blot analysis (35) of the plasmid DNA extracted from the transfected cells described in Table 2. The DNAs were digested with *Bam*HI and *Mbo*I. With these restriction endonucleases the plasmids will be linearized by the *Bam*HI cleavage, and plasmids that have been methylated by growth in bacteria will be resistant to *Mbo*I unless they have replicated in the eucaryotic cell. The first lane (CV-1, nonreplicative) is the DNA from the CV-1P cells in Table 2 that were transfected with the nonreplicative plasmid pL2n-cat (band A is pL2n-cat; band B is pSVO-cat added to compensate for differences in DNA amounts between transfections). Here the plasmids are linearized by *Bam*HI, but resistant to *Mbo*I, as expected, because the plasmid is nonreplicative. This same result is seen when T antigen is supplied (data not shown), again because of the replication defect. However, if the replication defect is removed by using the replicative counterpart of pL2n-cat, pL2-cat (26; see above), replication occurs, in the presence of T antigen, as demonstrated in the second lane (CV-1, replicative). Here the replication has resulted in nonmethylated genomes, which are sensitive to *Mbo*I. The third lane indicates the level of pL2n-cat DNA contained in the 293 cells whose CAT activity is given in Table 2. The DNA shown in this lane was derived from the same number of transfected cells as the DNA in the first lane. Thus the two lanes can be quantitatively and qualitatively compared; clearly much more plasmid DNA is present in the 293 cells. The last lane is a short exposure of the third lane to show that the band pattern is the same as in the first lane. The restriction pattern clearly indicates that the plasmid DNA in 293 cells is resistant to *Mbo*I; therefore, the high amount of pL2n-cat DNA in 293 cells cannot be accounted for by unexpected replication. Thus the data indicate that a substantial difference in plasmid copy number exists between the two cell lines. Since the DNA for the experiment in Fig. 1 was derived from the transfection experiment discussed in Table 2, the quantity of DNA and the CAT activity can be compared directly, and CAT activity can be normalized for the relative differences in plasmid copy number. Such normalization indicates that the high amount of CAT activity generated from promoter-CAT plasmids in 293 cells can be accounted for by the cumulative effect of a low or basal level of late promoter activity occurring from many genomes and is not necessarily the effect of activation by the endogenous E1a protein. This is analyzed further below.

Analysis of plasmid DNA uptake and stability. The results of Figure 1 indicate that transfected 293 cells contain much more transfected DNA than CV-1P cells. Several mechanisms could be responsible for this increased amount of DNA: (i) a higher proportion of the 293 cells may be competent to take up DNA, whereas only a small proportion of CV-1P cells may be competent at any given time; (ii) competent 293 cells may initially take up more DNA than CV-1P cells; or (iii) the transfected DNA may be more stable in the 293 cells.

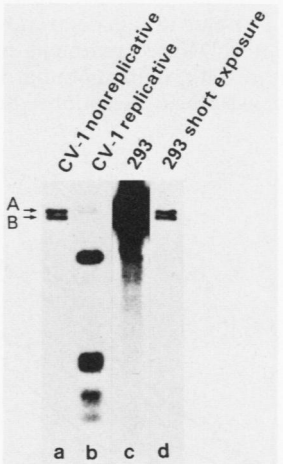

FIG. 1. Analysis of transfected plasmid DNA in CV-1P and 293 cells. Plasmid DNA was extracted from the cells at 48 h after transfection; these were the same cells assayed for CAT activity in Table 1. The DNA was prepared as described in the text, digested with the restriction enzymes *Bam*HI and *Mbo*I, and then analyzed by Southern blot analysis (18). Lanes: a, CV-1P cells transfected with the nonreplicative plasmid pL2n-cat (band A) and the inactive plasmid pSVO-cat (band B); b, CV-1P cells transfected with the replicative plasmid pL2-cat and p6-1d1, which supplies T antigen (this is the pattern obtained when the plasmid is replicated); c, 293 cells transfected with pL2n-cat and pSVO-cat (this lane is quantitatively comparable to the lane a); d, a short exposure of lane c.

The first possibility was tested by immunofluorescence analysis with goat anti-CAT serum (the kind gift of B. Howard and C. Gorman) to determine the percentage of cells in pL2n-cat-transfected cultures that are producing CAT (T antigen was supplied by cotransfection when necessary for high CAT gene expression). This is a reasonable indication of the proportion of cells initially transfected (28). For these experiments, transfected 293, CV-1P, COS, and NIH-3T3 cells were compared. The COS cell line is an SV40-transformed CV-1 cell line that produces relatively high levels of endogenous T antigen (10). Thus, like 293 cells, COS cells are transformed and produce a viral regulatory protein. In each cell line approximately 20 to 25% of the cells showed CAT immunofluorescence, although the analysis of 293 cells was somewhat difficult due to their loose adherence to plastic or glass and their clumped growth characteristics. Overall the data indicated no large differences in the proportions of competent cells in the various cultures and cannot account for the very high levels of transfected DNA in 293 cells.

DNA uptake and stability studies were also analyzed in 293, CV-1P, COS, and NIH-3T3 cells. The cells were transfected with pL2n-cat, and cultures were harvested for DNA at 0, 22, 46, and 72 h after transfection. The 0-h time point was taken at the time of removal of the calcium phosphate-DNA precipitate from the cells (see above). Figure 2 shows growth curves of the transfected cells over the 72-h period. The data indicate that there are no substantial differences in growth rates of the cells; in particular, the growth rates of CV-1P and 293 cells are quite similar over the course of the experiment. Thus comparisons of plasmid DNA uptake and stability will not be biased by differences in cell growth rate. For each time point in the stability study, cultures were harvested as described above. Briefly, after thorough washing of the monolayers, they were treated with DNase I to remove any DNA that had not been internalized and then were extracted by the Hirt procedure (21). Details of results without the initial DNase treatment are discussed below; it should be noted at this point, however, that similar DNA analysis after DNase treatment of suspended cells gave comparable results, indicating that DNA not internalized is freely accessible to digestion when cells are maintained as attached monolayers (data not shown). The extracted plasmid DNA was linearized by digestion with *Bam*HI and analyzed by Southern blotting (35) under quantitative conditions. After hybridization and autoradiography, the band intensities were quantitated by densitometer tracing and compared with band intensities generated by known amounts of plasmid DNA. Figure 3 shows a comparison of the amounts of DNA initially taken up by the cells. These amounts are calculated from the 0-h samples (i.e., 4 h after calcium phosphate-DNA precipitate was added to the cells; see above) and represent the amount of plasmid DNA associated with an equivalent number of transfected cells. The results indicate moderate variation in the uptake of plasmid DNA among the various cell lines; clearly 293 cells took up more than the others. However, the uptake is no more than 2 times the uptake of CV-1P cells and cannot account for the discrepancy in Fig. 1.

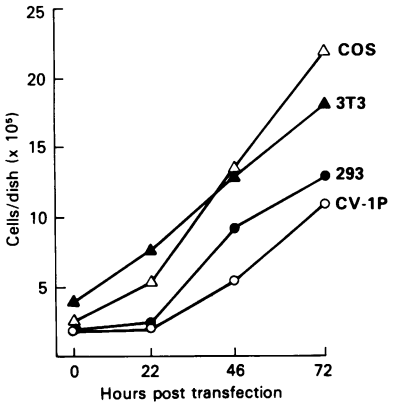

FIG. 2. Growth curves of transfected CV-1P, COS, 293, and NIH-3T3 cells. At each time point the transfected cultures were detached from the culture dish by treatment with 1 ml of standard trypsin solution and then diluted in 3 ml of medium containing 10% fetal bovine serum; 0.1 ml of the sample was removed for counting in a hemocytometer, and the remainder was sedimented and extracted for DNA (see the text).

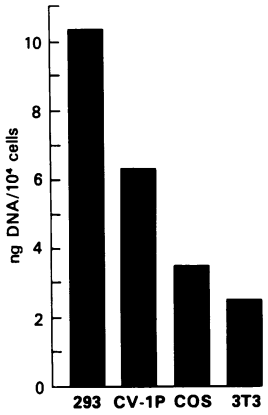

FIG. 3. Initial uptake of transfected plasmid DNA by CV-1P, COS, 293, and NIH-3T3 cells. Immediately after the removal of the transfection medium, the cell monolayers were treated with DNase I, and the DNA was prepared and analyzed as described in the text. The quantities of DNA determined are the 0-h points in Fig. 4 and are standardized to reflect equivalent cell numbers for each sample.

The stability of the transfected DNA is analyzed in Fig. 4. The data are presented as the amount of transfected DNA remaining in cultures plotted as a function of time after transfection. The 293 cells clearly maintain a much higher proportion of the transfected plasmid DNA than do the other cell lines. Within the first 22 h after transfection more than 98% of the transfected plasmid DNA is lost from CV-1P, COS, and NIH-3T3 cells. In the comparable time frame, 293 cells lose, at most, 30 to 40% of the transfected DNA. The decay of the remaining plasmid DNA in CV-1P, COS, and NIH-3T3 cells in much slower between 22 and 72 h. On a log plot the slope of the decay rates between 22 and 72 h for these cells is comparable to the slope of the curve generated over the entire time course, 0 to 72 h, for 293 cells. This implies that DNA entering CV-1P, COS, and NIH-3T3 cells encounters an initial period of instability that does not occur in 293 cells.

It could be argued that the stability of DNA seen in 293 cells is not specific, but may be a phenomenon common to human cells. This does not seem to be the case, since plasmid DNA stability in HeLa and 143B cells (data not shown), as well as KB cells (see below), is similar to that observed in CV-1P, COS, and NIH-3T3 cells. In addition, the stabilization of plasmid DNA in 293 cells does not appear to be related to the specific plasmid used in these experi-

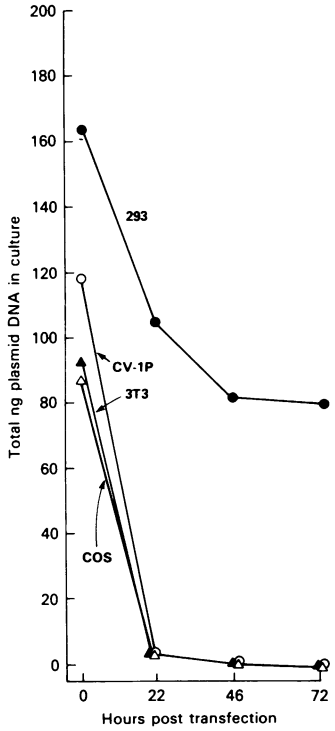

FIG. 4. Stability of transfected plasmid DNA in CV-1P, COS, 293, and NIH-3T3 cells. The plasmid DNA from DNase-treated cells was analyzed and quantitated as described in the text. The amount of DNA is plotted as the total DNA detected in the culture at the specific time point. The use of total DNA avoided considerations of dilution by the increase in cell number as the cultures divided with time.

ments. Similar results have been noted with unrelated plasmids, some containing no eucaryotic DNA (data not shown).

All of the experiments discussed above were done by analyzing plasmid DNA extracted from whole cells. Cell lines were also tested to determine whether there were any differences in the proportion of plasmid DNA that was actually in the nucleus at the time of assay. To determine this, cells transfected with pL2n-cat for 48 h were lysed with Nonidet P-40 and deoxycholate (1); the nuclear and cytoplasmic fractions were separated by centrifugation, and plasmid DNA was prepared from each fraction by the Hirt procedure (21). Hirt extractions of total cell DNA were also prepared. All samples were quantitated by Southern blot analysis as described above. In all of the cells tested, the total amount of plasmid DNA recovered in the nuclear and cytoplasmic fractions was equivalent to the amount detected in the total cell extracts, indicating little or no loss during the fractionation. Although the total amount of DNA maintained by the different cells varied as shown above, Table 3 shows that the relative proportion of the intracellular plasmid DNA contained within the nucleus was very similar, between 16 and 20%, among all of the cell lines. We cannot rule out possible nuclear leakage during fractionation; however, the fractionation procedure has been used successfully for the quantitative isolation of nuclear RNA, including small RNAs (1). In addition, the high-molecular-weight DNA pellets from the Hirt extractions were extensively reextracted to determine that large or disproportionate amounts of plasmid DNA were not being lost in this fraction. In all cases a relatively constant proportion of 15 to 20% of the plasmid was found to precipitate with the high-molecular-weight DNA (data not shown).

Overall, the data indicate that the proportion of transfected plasmid DNA in the nuclei of recipient cells is relatively constant among the cells tested; therefore the conclusions based on the relative differences in amount of plasmid DNA in the total DNA fraction are representative of relative differences in the nuclear DNA fraction.

Analysis of late promoter activity in human KB cells that endogenously produce E1a and E1b proteins. The data presented above strongly suggest that transfected plasmid DNA is more stable in 293 cells than in other cells. This increased gene copy number can account fully for the apparent high gene expression activity from the promoter-CAT plasmid. To further test this, human KB cell lines were obtained which endogenously produce adenovirus early proteins (2). To prepare these cells, Babiss et al. (2) transfected KB cultures with plasmids containing adenovirus type 2 DNA encoding E1a and E1b (0 to 15.5 map units) as well as the selectable *E. coli* XGPRT gene. Isolates selected as Gpt^+ were then tested for adenovirus protein production (2). The cell lines used in the present studies include KB7, a control cell line which is Gpt^+, but produces no adenovirus RNA or proteins; KB8, which produces E1a, but not E1b; KB16, which produces both E1a and E1b and thus is analogous to 293 cells; and KB18, which produces E1b, but not E1a. Other than immunofluorescence analysis (2), an exact comparison of the amounts of adenovirus proteins present in these cells with respect to 293 cells has not been done. However, Babiss et al. (2) have shown that, like 293 cells, the specific KB cell lines can rescue adenoviruses mutated in the corresponding early genes; mutant rescue by KB16 is quite comparable to that in 293 cells. In addition, Babiss et al. (2) have demonstrated by Northern blot analysis that, in comparison to 293 cells, cell line KB16 produces approximately 25% as much E1a mRNA and equivalent amounts of E1b mRNA. After establishing that the growth rates of the KB cell lines and 293 cells were very similar (data not presented), the stability of pL2n-cat DNA was tested in the KB cell lines in comparison to 293 cells. The results (Fig. 5) indicate that plasmid DNA is no more stable in any of the KB cell lines than in CV-1, COS, or NIH-3T3 cells (Fig. 4), whereas the stability of plasmid DNA in 293 cells is again high. Finer analysis of the amounts of plasmid DNA in the KB cell lines shows that those cells containing E1b (KB16 and KB18) contain approximately 4 times more plasmid DNA than the other KB cells. However, this small difference is insignificant compared with the amount of plasmid DNA in 293 cells (Fig. 5). Tempered by the caveats discussed above regarding possible differences in levels of E1a in the cell lines, these data suggest that stabilization of

TABLE 3. Percentage of transfected plasmid DNA in the nuclear fraction[a]

Cell line	% Nuclear
CV-1P	20
COS	19
NIH-3T3	19
293	20
KB7	18
KB8	16
KB16	16
KB18	20

[a] The nuclear and cytoplasmic fractions of cells transfected for 48 h were separated, and DNA was analyzed as described in the text.

transfected plasmid DNA in 293 cells, is not conferred directly on cells due to the production of E1a or E1b proteins or both.

In Table 4 the CAT gene expression, generated by pL2n-cat after 48 h of transfection, is shown for the KB cell lines and 293 cells. The activity is again very high in 293 cells, but not in the KB cells, in which the gene copy number is dramatically lower. KB16 and KB18 show slightly higher activity than KB7 and KB8. As discussed above, these cells containing E1b appeared to retain or stabilize 4 times more plasmid DNA than the other KB cell lines. Standardization of CAT activity for relative differences in genome copy number (Table 4) strongly suggests that neither E1a or E1b activates the SV40 late promoter. Due to the low basal activity of the promoter-CAT plasmid in the KB cell lines, no conclusions can be made regarding E1a repression.

Use of plasmid DNA concentration as a method of standardizing transfection assays. A recurring problem with transient expression analysis has been inconsistencies in the efficiency of transfections. Occasionally, identical transfections will result in very different results. As indicated above, such occurrences undermine valid comparisons of the data. To deal with this problem, a variety of internal standardization techniques have been attempted. These involve the transfection of a second assayable gene, under the control of a known promoter (often the SV40 early promoter). The activity of this gene should vary in proportion to the transfection efficiency. These standard genes are introduced as cotransfecting plasmids or constructed into the same plasmid with the test gene. This approach suffers from at least three problems. First, it is difficult to predict what effect the presence of the control gene promoter will have on the test gene promoter. Second, the presence of activator proteins may affect the expression of the control gene in such a way that it is no longer independent of the experimental variables (Table 1). Third, the standard gene assays can be tedious and often so much less sensitive than the CAT assay that the results are barely useful.

An alternate approach to standardize the transfections, suggested by the studies presented above, is to quantitate the amount of plasmid DNA in the cells at the time of harvest for the assay. This can be done relatively simply by Southern or dot blot analysis. This approach has been used successfully in this laboratory; for example, in cases where variations in CAT activity have occurred between identical transfected cultures, the difference could be routinely corrected by normalization to the relative amounts of transfected DNA. The validity of this method seems proven by experience; however, it has been questioned due to the inadequate knowledge of whether the plasmid DNA detected is (i) within the cells or partially adhered to the outside and (ii) representative of the plasmid DNA in the nucleus. Figure 6 shows an example of a Southern blot of the DNA extracted from the transfected CV-1P cells described in Fig. 4. The four time points are shown where the DNA was extracted from cells directly (no DNase treatment) or

TABLE 4. Activity of pL2n-cat in E1a and E1b-producing cells[a]

Cell line	Adenovirus protein	μU of CAT/10^5 cells	
		Uncorrected	Corrected
293	E1a, E1b	20,000	60
KB7		30	30
KB8	E1a	50	27
KB16	E1a, E1b	285	75
KB18	E1b	293	77

[a] The various cell lines (described in the text) were transfected with pL2n-cat for 48 h and then assayed for CAT activity. The amount of plasmid DNA was determined. In the right-hand column the CAT activity is corrected for relative differences in copy number; the amount of DNA assayed in the KB7 cell samples was designated unity for the calculations.

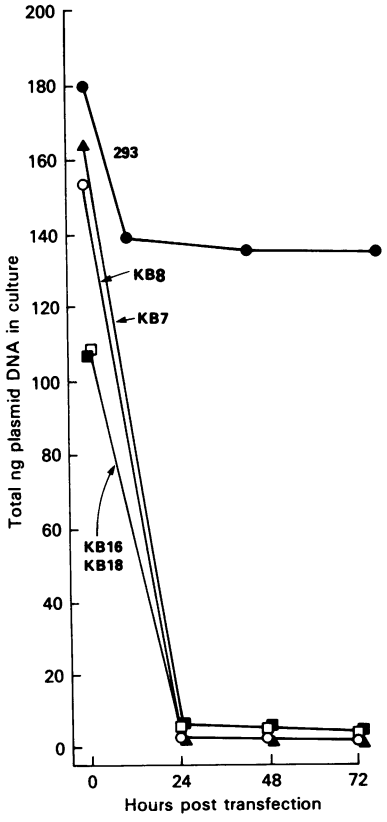

FIG. 5. Stability of transfected plasmid DNA in 293 cells and KB cell lines that produce adenovirus early proteins. Analysis was as in Fig. 4. Cell lines: KB7, control, produces no adenovirus proteins; KB8, produces E1b only; KB16, produces E1a and E1b; KB18, produces E1a only (see the text).

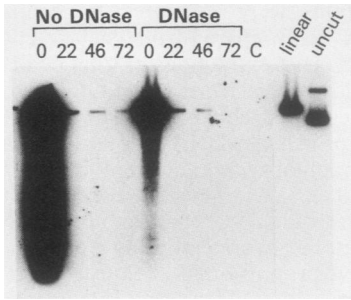

FIG. 6. Comparison of the plasmid DNA extracted from transfected cells with or without prior DNase treatment. Cells identically transfected with pL2n-cat were extracted for plasmid DNA at the times shown (hours). One set of cells was extracted directly (no DNase); the other was treated with DNase I (DNase) on the monolayer as described in the text. The extracted plasmid DNA was digested with *Bam*HI and quantitated by Southern blot analysis. Lane C is DNA extracted from mock-transfected cells. The lanes marked linear and uncut are samples of pL2n-cat plasmid either intact (uncut) or after linearization by cleavage with *Bam*HI (linear).

after DNase treatment of the monolayer as described above. Clearly the DNase treatment removed a substantial amount of extracellular DNA in the 0-h time point; however, at the later times the DNase treatment made little or no difference. This was true for all of the cell lines tested, implying that extracellular DNA is naturally degraded during the initial 22 h of the transfection. This result indicates that the transfected plasmid DNA quantitated at later times represents intracellular plasmid DNA. The data in Table 3, showing that the DNA detected is representative of the nuclear fraction, have already been discussed.

Overall, these studies indicate that quantitation of the amount of plasmid DNA in transfected cells is a valid means of standardizing experiments for variations in transfection efficiency and variations in cell line-dependent stabilization of transfected DNA. It should be noted that this conclusion is true only for transfections by calcium phosphate precipitation with or without glycerol boost (31). The results may be different with the DEAE-dextran procedure, in which charge interactions between DEAE-dextran and cell surfaces may maintain extracellular DNA for an extended period.

DISCUSSION

The promoter activation studies (Table 1) indicate that the SV40 T antigen and herpesvirus (PrV) IE protein are promiscuous gene expression activators, whereas the adenovirus E1a protein is more specific. The E1a protein activated only its homologous adenovirus promoter and had an apparent suppressive effect on the other promoters tested. Repression is most clearly demonstrated in the case of the SV40 late promoter and the promoter of the RSV LTR (Table 1), and selective activation by E1a is indicated cumulatively, by all of the experiments presented. Repression by the E1a protein has also been noted in recent experiments, indicating that the E1a protein can suppress promoters that contain *cis*-acting enhancers (6; Velcich and Ziff, in press). It has been suggested that repression is mediated by interactions between the E1a protein and the enhancer (6). In agreement with these data, E1a repression of the enhancer-activated promoter of the RSV LTR is shown in Table 1. However, the basal activity of the SV40 late promoter, which contains no enhancer, also appears to be repressed by E1a; thus other mechanisms of E1a repression, not requiring enhancers, are indicated.

In contrast to the effect of E1a, T antigen and the IE protein activated the RSV LTR promoter-CAT plasmid (Table 1). This is interesting considering that the promoter is already activated by efficient *cis*-acting enhancer elements (12). Several previous reports have demonstrated that promoters of genes such as β-globin (17) or the adenovirus E2 gene (23) can be activated by either *trans*-activating proteins or by the insertion of *cis*-activating enhancer elements. The data presented here suggest an additional factor, that *trans*-activating proteins can increase the expression of genes already activated by enhancer elements. This raises the intriguing possibility that *trans*-activating proteins may be able to stimulate expression not only of cellular genes, but also of endogenous retroviruses. In addition, the apparent promiscuity of SV40 T antigen and herpesvirus IE protein suggests that these proteins may function in transformation by turning on silent genes or by augmenting the expression of active genes. In contrast, the selective activating and repressing effects noted in the case of the adenovirus E1a protein indicate that proteins acting in *trans* may be widely used in the positive and negative control of normal cellular gene expression. This idea is supported by the suggestion that E1a may have a cellular counterpart (24).

The observation that E1a is specific in its effects required verification, since no specificity of gene activation was apparent when the promoter-CAT plasmids were transfected into the E1a-E1b-producing cell line, 293. In these cells all of the promoter-CAT plasmids generated very high CAT activity. Examination of 293 cells determined that transfected plasmid DNA is much more stable in these cells than in any other cell line tested. In all cells tested except 293 cells, the transfected plasmid DNA was extremely unstable during the first 22 h of transfection. The lack of such a period in 293 cells allows the maintenance of much more plasmid DNA. Thus the high activity of CAT plasmids containing promoters such as the SV40 late promoter in 293 cells is not the result of promoter activation by E1a protein, but can be accounted for by a cumulative basal level of promoter activity from many gene copies. In cell line KB16 (which also produces endogenous E1a and E1b proteins) the SV40 late promoter-CAT plasmid does not appear to be activated (Table 4); predictably, the plasmid DNA is not stabilized in these cells (Fig. 5). The latter result suggests that the endogenous production of the E1a and E1b proteins per se does not confer upon a cell the ability to stabilize plasmid DNA.

These findings cast doubt on the interpretation of any transfection experiments done in 293 cells, unless some consideration has been made for the unusual stability of the plasmid DNA. Clearly the stability of plasmid DNA must be considered when different cell lines are used in comparative transfection studies of gene expression. However, the data indicate that variations in results caused by differences in transfected DNA levels can be corrected by determining the relative quantities of transfected plasmid DNA in a given number of cells and standardizing the gene activity to equivalent copy numbers. This DNA standardization procedure has been particular useful in this laboratory in correcting for plate-to-plate variations in transfection efficiency when testing plasmids in a single cell line.

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