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Formation of stable transcription complexes as assayed by analysis of individual templates

(single-cell assay/enhancers/cotransfection)

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ABSTRACT **Conditions were established where transient transfection of two marker genes resulted in the expression of one or the other, but not both, in individual cells as assayed by immunofluorescence. Thus, the expression from a single cell reflects the activity of single active transcription templates. Under these conditions, a vector encoding the simian virus 40 large tumor antigen (SV40 T-Ag) driven by the SV40 enhancer and early promoter was transfected into CV-1, L, or HeLa cells yielding, for all three cell types, about 10–30% T-Ag-positive cells as assayed by immunofluorescence. Similar vectors containing either mutated or deleted SV40 enhancers also gave T-Ag-positive cells, but at about 1/100 the frequency. Quantitative analysis showed that T-Ag-positive cells produced about the same amount of T-Ag whether or not an active enhancer was present. Chloramphenicol acetyltransferase-encoding vectors gave the same result. The data are consistent with the hypothesis that at a low, but finite, probability, fully functional transcription complexes can form on a given active template in the absence of enhancer DNA. Enhancers seem to increase the number of active templates. Subcloning experiments suggest that these transcription complexes can be surprisingly stable.**

As a consequence of binding specific protein factors (1), enhancer DNA sequences dramatically activate gene expression, more or less independent of their orientation and distance from nearby promoters (for review, see ref. 2). A variety of models have been proposed and tested to understand the mechanisms of enhancer activation (for review, see ref. 3). Enhancer-mediated transcription seems to involve the establishment of stable initiation complexes, presumably at the promoter (4, 5). Enhancers also lead to an increase in the total number of active RNA polymerases present on the total population of potential templates as assayed by a nuclear runoff transcription procedure (6, 7). This could occur either by increasing the number of active DNA templates in the population without increasing polymerase density per gene or by increasing the rate at which all active templates are transcribed and, hence, increasing polymerase density per gene.

To distinguish between these two possibilities, conditions were established to transfect one or a few active templates into a single cell and to monitor expression of these templates as it is reflected in single cells. The results show that in the absence of an enhancer, many fewer cells express a given marker [large tumor antigen (T-Ag)], confirming earlier work by Chambon and colleagues (8). Those cells that do express the marker in the absence of an enhancer show nearly as much activity as those that express the marker from an enhancer-driven template. These results are interpreted to mean that enhancers increase the frequency of forming a stable transcription complex at the promoter and, once established, these complexes are equally efficient whether assembled on enhancer-containing or enhancerless templates.

MATERIALS AND METHODS

Cells and Transfections. Procedures for growing cells and performing transfections were as described (9). All transfections were made up to 20 μg per dish with carrier chicken erythrocyte DNA.

T-Ag Staining. Cells were washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS at 22°C for 15 min. Dishes were then washed twice in PBS and the cells were permeabilized in 0.5% Triton X-100 in PBS for 5 min, rinsed twice in PBS, and stained with a 1:200 dilution of anti-T antibody (a kind gift from L. Tack) for 30 min. The cells were then washed three times for 3 min each with antibody wash buffer (1% bovine serum albumin/0.1% Tween 20 in PBS), treated with secondary antibody (rhodamine-labeled rabbit anti-goat antibody) for 30 min, washed three times in antibody wash buffer, and stored in PBS. Fluorescence visualization was with a Zeiss Photo III microscope with spot monitoring, usually with a 20× objective under water. Under these conditions, the time needed to collect a specified number of photons is inversely proportional to the concentration of rhodamine-labeled second antibody (data not shown) for exposure times >2 sec and <40 sec. For fluorescence-activated cell sorter analysis, nuclei were isolated (9) and stained in suspension exactly as described above.

Autoradiography. CV-1 cells were transfected with SV-T or TDPM (triple-double, point mutant) at 1 μg per dish with carrier. After 17 or 48 hr, dishes were washed three times in PBS and fixed in cold methanolacetic acid (3:1); dehydrated in successive ethanol washes; dried; denatured by treatment with 0.3 M NaCl/30 mM sodium citrate at pH 12 for 2 min at room temperature; dehydrated through ethanol; dried; hybridized overnight at 42°C in Stark's buffer [0.75 M NaCl/75 mM sodium citrate/25 mM sodium pyrolidone (M_r 40,000)/250 μg of sonicated salmon sperm DNA per ml/50% deionized formamide] containing 10% dextran sulfate with a [H^3]SV-T probe (≈2 × 10^7 cpm/μg); washed twice in 0.3 M NaCl/30 mM sodium citrate at 65°C for 30 min; and exposed under Kodak NTB2 emulsion for 1–14 days before development with D-19 and rapid fix (Kodak).

Alkaline Phosphatase. The alkaline phosphatase vector is described in ref. 10. Staining was performed according to ref. 10. Alkaline phosphatase antibody was obtained from DAKO (Santa Barbara, CA). Antibody to chloramphenicol acetyltransferase (CAT) was a gift from Parker Antin (Univ. of California, San Francisco Medical Center).

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Abbreviations: SV40, simian virus 40; T-Ag, large tumor antigen; TDPM, triple-double, point mutant; CAT, chloramphenicol acetyltransferase.

RESULTS

Some Cells Transfected with Enhancerless Vectors Produce Normal Amounts of T-Ag. SV-T (or 1 × 72) is a vector that encodes the entire simian virus 40 (SV40) genome with early transcription being driven from the early promoter and a single 72-base-pair (bp) enhancer element (11). When transfected into CV-1 cells, which are permissive for SV40 replication, about 10–30% of the cells become T-Ag positive after 48 hr. In contrast, a derivative of SV-T, DPM 1, 2, 6, contains double point mutants in each of three elements of the enhancer (11). For convenience, this plasmid is referred to as TDPM for triple-double, point mutant.

At a population level, TDPM expression in CV-1 or HeLa cells is about 1/100 that of SV-T (11); however, at the single-cell level, T-Ag fluorescence in CV-1 cells shows only about 0.1–1% of the cells as T-Ag positive (Fig. 1). Surprisingly, by eye, these positive cells are as intense as the cells that were transfected with SV-T. About 1% (relative to SV-T) of T-Ag-positive cells are also observed with TDPM after transfection of HeLa cells, which are semipermissive for replication, or L cells, which are nonpermissive. These T-Ag-positive cells are not staining artifacts since, when examined after 48 hr (one or two cell doublings), they are often present as doublet cells (or much less frequently, as quadruplets), presumably representing daughters (or granddaughters) from an original transfectant. Moreover, and especially in HeLa nuclei, characteristic nuclear staining patterns observed with SV-T are also seen with TDPM (Fig. 2). These results confirm and extend earlier results of Chambon and colleagues (8, 12), showing that in the absence of an enhancer, fewer marker positive cells are observed after either transfection or microinjection. In terms of the percentage of T-Ag-positive cells, maximal levels of expression are achieved as early as 24 hr after transfection with SV-T; however, for TDPM, maximal levels are obtained only after 48 hr.

By using different plasmids, SV2CAT and SV1CAT, which encode the CAT gene driven by the SV40 enhancer and a deletion of the enhancer, respectively, it was observed by staining with an anti-CAT antibody that again the enhancerless

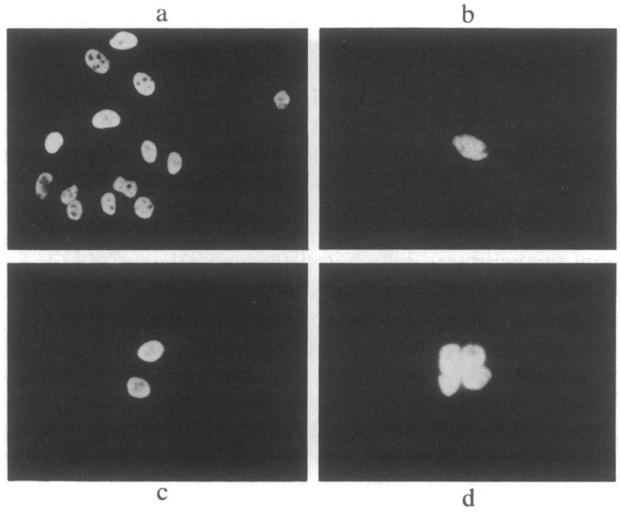


FIG. 1. Enhancers increase the frequency of fully positive T-Ag-producing cells. SV-T (*a*) or TDPM (*b–d*) was transfected (using calcium phosphate) into CV-1 cells at 1 μg per dish with 20 μg of chicken erythrocyte DNA carrier. T-Ag was detected by immunofluorescence after 1 day. (*a*) Almost-confluent CV-1 cells transfected with SV-T (19% positive cells). (*b–d*) Almost-confluent CV-1 cells transfected with TDPM. These are selected fields, most fields showing no straining. (*b*) A mother transfectant. (*c*) Daughters. (*d*) Granddaughters. Exposure times were roughly equivalent for all inserts. (×160.)

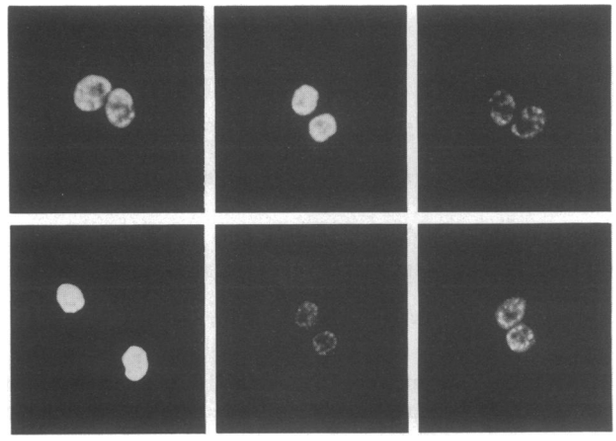


FIG. 2. Specific T-Ag staining morphologies in HeLa cells. HeLa cells were transfected with 1 μg of SV-T (*Upper*) or TDPM (*Lower*) and after 2 days were stained with T-Ag. Each frame shows selected daughter doublets to emphasize some of the "morphologies" observed. Almost all stained cells show absence of nucleolar staining, but the intense staining spots could not be seen by phase microscopy. Daughters tend to stain more like each other; however, this might reflect the fact that daughters express similar amounts of T-Ag. Although not shown, a uniform staining (excluding the nucleoli) occurs in about 30–50% of the transfectants. Also not shown is a smooth nuclear periphery staining in about 10% of transfectants. Subcloning the original HeLa population reveals the same heterogeneity in the cloned populations. Exposure times were roughly equivalent for all inserts. (×270.)

plasmid produced about 1–5% the number of fully positive CV-1 cells or HeLa cells relative to SV2CAT (not shown). This result controls for possible complications arising from T-Ag autoregulation (13) or T-Ag-induced plasmid replication.

A competition assay (9) was used to test whether expression from TDPM could be competitively inhibited by the SV40 enhancer. TDPM (1 μg per plate) was cotransfected with 20 μg per plate of an enhancer-containing plasmid (SV2CAT) or an enhancerless plasmid (SV1CAT). From three transfections, an average of 1.6% of the cells were T-Ag positive with no competitor, 1.1% of the cells were T-Ag positive with SV1CAT competitor, whereas only 0.2% were T-Ag positive with SV2CAT competitor. Hence, at these DNA concentrations enhancer-containing plasmids compete with TDPM for expression in individual cells.

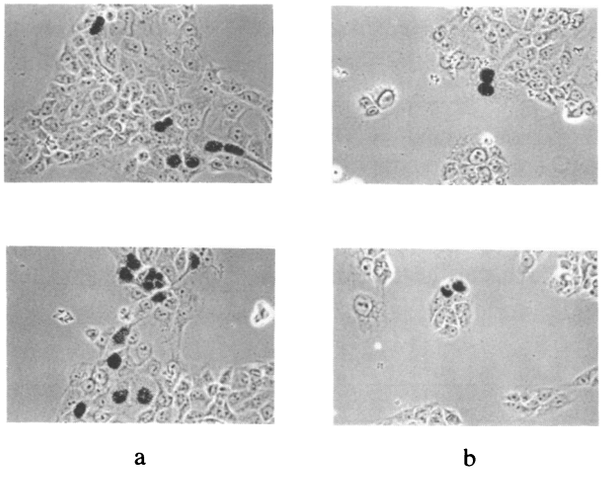
Quantitative Analysis of Expression in Single Cells. Attempts were made to quantitate the fluorescence assay. By using the Zeiss Photo III microscope with spot monitoring, which gives a linear and quantitative measure of fluorescence intensity with exposure times <40 sec (*Materials and Methods*), and measuring the time required to accumulate a given amount of fluorescent light from an isolated nucleus within the collecting spot, the times of exposure for CV-1 cells, L cells, or HeLa cells were very similar for positive cells transfected with SV-T or TDPM: from 100 positive cells assayed 24 hr after transfection, exposure times (mean ± SD) in CV-1 cells were 6.3 ± 1.5 sec for SV-T and 8.8 ± 1.7 sec for TDPM. In all cases the extreme values (high and low) were the same for SV-T and TDPM. In L cells, the differences were somewhat greater, 8.6 ± 2.5 sec for SV-T and 16.2 ± 6 sec for TDPM. Similar results are obtained for HeLa cells (7.2 ± 2 sec for SV-T; 13.1 ± 4 sec for TDPM). In general, the means were closer and the standard deviations were smaller in CV-1 cells as compared to L cells or HeLa cells. Although there is a clear difference in the average intensity between SV-T and TDPM in all three cell lines, the difference, which is 2- to 3-fold, cannot account for the 100-fold difference in overall expression.

A second assay was also used to quantitate fluorescence intensity per cell. Twenty-four hours after transfection nuclei

were isolated from transfected L cells and stained for T-Ag. The population was then assayed by the fluorescence-activated cell sorter. Twelve percent of the cells were positive for SV-T and only 0.4% of the nuclei were positive for TDPM, which had a mean populational fluorescence for the positive cells about half (43%) that of SV-T.

Replication of SV-T and TDPM was also monitored in transfected CV-1 cells, and the replicated DNA was detected by *in situ* hybridization (Fig. 3). This assay represents an indirect measure of T-Ag synthesis, since there is evidence that SV40 DNA replication is dependent on the continued synthesis of T-Ag (14). Depending on the experiment, 10–30% of cells were positive with SV-T transfections, whereas only 1/20–1/100 that number were positive with TDPM. Most important, the positive cells with TDPM had about the same average grain density as those transfected with SV-T. In one experiment (analyzed after short exposure) where 20 random cells were chosen, 160 ± 23 vs. 180 ± 35 grains per nucleus were counted for TDPM and SV-T, respectively. The same relative result was also obtained at 17 hr after transfection and 49 hr after transfection when replication is just beginning and when it is at its peak, respectively.

Can a Small Percentage of Cells Acquire an Active Enhancer by Recombination? The results presented above might be explained if recombination events created a functional enhancer. Recombination could be "internal," by duplication of SV40 elements, or "external," by acquiring cellular sequences. The first possibility is raised by very clear experiments showing that with prolonged growth and multiple passages, revertant SV40 virus can be selected from these point mutants (11). In most cases, these revertants show duplications and rearrangements of the residual mutated enhancer elements. The experiments reported here were performed under very different conditions since comparatively high levels of expression were observed as early as 15 hr after transfection, prolonged growth and multiple passages not being necessary. Nevertheless, the possibility that a mutated enhancer was duplicated was tested by constructing a deletion mutant where the enhancer is removed by deleting enhancer DNA [between *Sph* I (bp 128) and *Kpn* I (bp 294) sites]. In all three cell types, this deletion gave as many fully positive cells as TDPM. A more extreme deletion, removing all enhancer DNA and going 20 bp beyond the *Sph* I site (bp 108), also gave T-Ag-positive cells at the same frequency. However, removal of the 21-bp repeats of the SV40 promoter by a deletion to the *Nco* I site (bp 37) resulted in no expression whatsoever, as detected by the single-cell T-Ag fluorescence assay.



a b

FIG. 3. Normal replication of an enhancerless SV40 plasmid in a fraction of CV-1 cells. One microgram of SV-T (*a*) or TDPM (*b*) was transfected into CV-1 cells, and after 48 hr cultures were fixed and processed for autoradiography. Exposures were for 2 weeks. Quantitative grain densities were determined by using exposures of only a few days. (×110.)

A second type of analysis was also performed to assay directly for possible rearrangements. CV-1 cells were transfected with low levels of TDPM (0.1 μg per dish) and, after 48 hr, the Hirt supernatant was isolated. The DNA was cut twice with *Dpn* I to restrict the analysis to only replicated molecules so that contamination by nonnuclear transfected DNA is prevented. (*Dpn* I cuts only DNA modified on both strands by *Escherichia coli* methylase; when replicated in eukaryotes, this modification is not preserved.) This DNA was then used to transform *E. coli*, where 1000 colonies were obtained. One hundred colonies were picked, and mini-preps were performed on each and analyzed with pairs of a variety of restriction enzymes (*Kpn* I, *Bgl* I, *Hpa* II) that cut near the enhancer. For each of the 100 DNA preparations no evidence for any type of rearrangement was detected.

These results suggest that rearrangement of the TDPM plasmid is not responsible for its full expression in a small percentage of cells. The possibility that TDPM is reactivated by integration into the host genome is also unlikely given the clonal analysis to be discussed below.

Only a Few Active Templates per Cell Are Expressed After Transfection. If, for example, the output from 100 active transfected templates were required to give a fully T-Ag-positive cell with SV-T, then with TDPM, even though the probability of assembly of an active template is 1/100, the few T-Ag-positive cells present would each have to have all 100 templates active. This seems very unlikely and, hence, a strong prediction from these results is that the active cells contain only a few active templates. To test this prediction, increasing amounts of SV-T or TDPM were transfected into L cells (HeLa and CV-1 cells gave similar results) with carrier DNA to give a final DNA input of 20 μg per dish. At low levels of both plasmids the percentage of positive cells increased with dose (Table 1). The increase in T-Ag-positive cells parallels the increased amount of expression with transfected DNA observed at the populational level using the same SV40 enhancer and early promoter (9). Consequently, these results suggest that at these levels there are only a few, and not hundreds of, active templates in each positive cell.

Additional evidence to support the prediction that only a few active and stable transcription complexes form in each transfected cell comes from the following experiments: HeLa cells were transfected simultaneously with two marker vectors, and the frequency of double-labeled cells was determined. One marker was SV-T, which stains nuclei and is detected by using a rhodamine-labeled second antibody; the other is a Rous sarcoma virus, long terminal repeat-driven

Table 1. Transfection with increasing amounts of DNA

DNA, μg per dish	T-Ag$^+$ cells, % of maximum	
	Exp. 1	Exp. 2
	SV-T	
0.1	18	24
1	82	78
5	100	100
	TDPM	
0.1	10	18
1	63	68
5	100	100

L cells were transfected with increasing amounts of SV-T or TDPM DNA, and the percentage of T-Ag-positive cells was determined and normalized to the maximum achieved at 5 μg input. Total DNA was made up to 20 μg per dish with carrier. Assays were performed 24 hr after transfection. In experiment 1, 26% of cells were positive for SV-T at 5 μg and 0.8% were positive TDPM; in experiment 2, 18% were positive for SV-T and 1% were positive for TDPM.

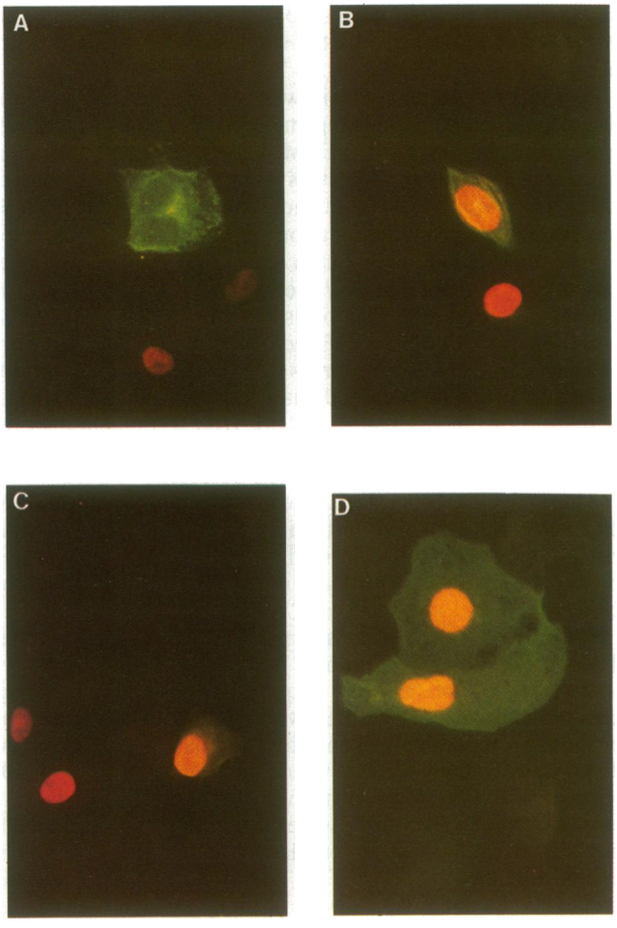


FIG. 4. Transfection of two marker genes. Details of the analysis are given in Table 2 and the text. HeLa cells were cotransfected with 0.5 μg of the SV-T plasmid and 0.5 μg of the alkaline phosphatase plasmid plus carrier DNA. (*a*) Alkaline phosphatase-positive, T-Ag-negative cell near two alkaline phosphatase-negative, T-Ag-positive cells. (*b*) Alkaline phosphatase-positive, T-Ag-positive cell next to alkaline phosphatase-negative, T-Ag-positive cell. (*c*) Alkaline phosphatase-positive, T-Ag-positive cell next to two T-Ag-positive, alkaline-phosphatase-negative cells. (*d*) Two alkaline phosphatase-positive, T-Ag-positive cells.

vector (10) that produces alkaline phosphatase, which is detected on the cell membrane by using a fluorescein-labeled second antibody.

The two vectors were cotransfected at low (0.5 μg per dish) or at high (5 μg per dish) inputs together with carrier DNA to a final DNA concentration of 20 μg per dish (Fig. 4). At low levels, fully positive cells were observed for both vectors; however, the frequency of doubly labeled cells was low. At high levels of input DNA, the overall frequency of positive cells increased and the percentage of doubly labeled cells also increased (Table 2). These data suggest that with low levels of input DNA only a few active templates are present per transfected cell. How transcription becomes restricted to only a few templates remains unanswered.

In contrast to results with HeLa cells, transfection of the two markers into CV-1 cells, where both plasmids will replicate if the SV-T plasmid is expressed (since the alkaline phosphatase plasmid also contains a SV40 origin of replication), results in most cells being doubly positive at all levels of input DNA. These results suggest that when replication occurs, the extremely high levels of both plasmids are able to recruit additional transcription complexes. The results with CV-1 cells imply that both plasmids actually enter cells, even at low concentrations of input DNA, but for HeLa cells only one is usually chosen for transcription.

The double-label analysis can also be used to test whether the small number of positive cells observed after transfection of TDPM occurs because some "exceptional" cells in the population do not need enhancers—e.g., because they produce large amounts of transcription factors or because they produce a special class of such factors that does not need enhancers. Two enhancerless plasmids, SV1CAT and TDPM, were cotransfected into HeLa cells at relatively high concentrations (10 μg each) where most cells would be expected to take up both plasmids (Table 2). For transfections with TDPM alone, about 0.5% of the cells were positive; for SV1CAT alone, about 0.1% of the cells were positive (after staining with an antibody to CAT); for transfections with both SV1CAT and TDPM, of the cells positive for either marker, 19% were positive for SV1CAT alone, 76% were positive for TDPM alone, and 5% were positive for both. These results are most consistent with the independent expression of both plasmids and suggest that expression from an enhancerless vector is not a consequence of exceptional cells in the population.

Active Templates Segregate to Individual Cells After Cloned Growth. Another assay has been used to verify that only a few templates are active per transfected cell. HeLa cells were transfected with the nonreplicating alkaline phosphatase vector. One day after transfection, the cells were replated at low density so that only zero or one isolated cell was present per low-power field. These cells were allowed to grow up into clones and assayed for alkaline phosphatase expression 4 or 14 days after plating. About 10–20% of clones showed positive cells and almost all of these showed positive segregants where only one or a few cells stained positively, either for the 4-day or 14-day growth period. One clone from over several hundred analyzed showed all cells staining (weakly) positive, possibly because the plasmid had integrated. The same type of "sectoring" was seen in HeLa cells with the TDPM and SV-T plasmids and also when the alkaline phosphatase plasmid was transfected into CV-1 cells. These results indicate that only a few active templates are established with transfection and these are stable and become segregated to daughter cells with growth of the clone. To have obtained these results it is also necessary to assume that the mRNA and the protein (for alkaline phosphatase and T-Ag) are rather unstable.

Table 2. Transfection of two markers

Dose	% positive cells		Distribution, %					
			$T\text{-}Ag^+$		AP^+		$AP^+, T\text{-}Ag^+$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0.5 μg of SV-T + 0.5 μg of AP	6	3	52	62	35	32	12	6
5 μg of SV-T + 5 μg of AP	14	18	12	16	2	5	84	79
0.5 μg of SV-T + 5 μg of AP	21		8		62		30	
0.5 μg of AP + 5 μg of SV-T	16		81		0		19	

HeLa cells were used. AP, alkaline phosphatase marker; T-Ag, T-Ag marker.

When the TDPM plasmid was transfected into HeLa cells and the clones were allowed to grow up, segregation of expression to only a few fully positive cells in large colonies was also seen (Fig. 5 *e* and *f*). This last result makes it unlikely that expression of TDPM is a result of an host cell integration event since in this case it is expected that all cells in the clone would be positive.

DISCUSSION

Enhancers activate expression by as much as 100- to 1000-fold or more. The results presented here show that this activation is dramatically reflected in the number of cells expressing the marker, T-Ag, and, much less so, in the amount of T-Ag per cell. A major conclusion is that enhancers increase the frequency with which an active template is formed and that since, under these conditions, there seems to be only a few active templates per cell, the activity of individual templates can, for the most part, be visualized by the activity of individual cells. Although other explanations are possible, the most reasonable assumption that must be made to explain this result is that once formed, either on an enhancer-containing vector or on an enhancerless vector, a transcription complex is stable. This is confirmed by demonstration of the stable expression from only a few progeny cells in large clones (Fig. 5). It is likely that this stability is preserved over many rounds of transcription. In contrast, some promoters, particularly those that are inducible or repressible, might be designed to function with inducible enhancer elements that catalyze the assembly of transcription complexes that are unstable (e.g., see refs. 15–17). The experiments presented here do not address whether the complex is preserved during DNA replication nor do they measure whether very low levels of transcription occur in the large numbers of T-Ag-negative cells that presumably take up the enhancerless plasmid. Consequently, although the results suggest that a fully functional transcription machinery (e.g., see refs. 18 and 19) can assemble at a promoter in the absence of enhancer, the experiments do not exclude the possibility that enhancers can also increase the rate of transcription on putative templates that would normally function at a very low rate.

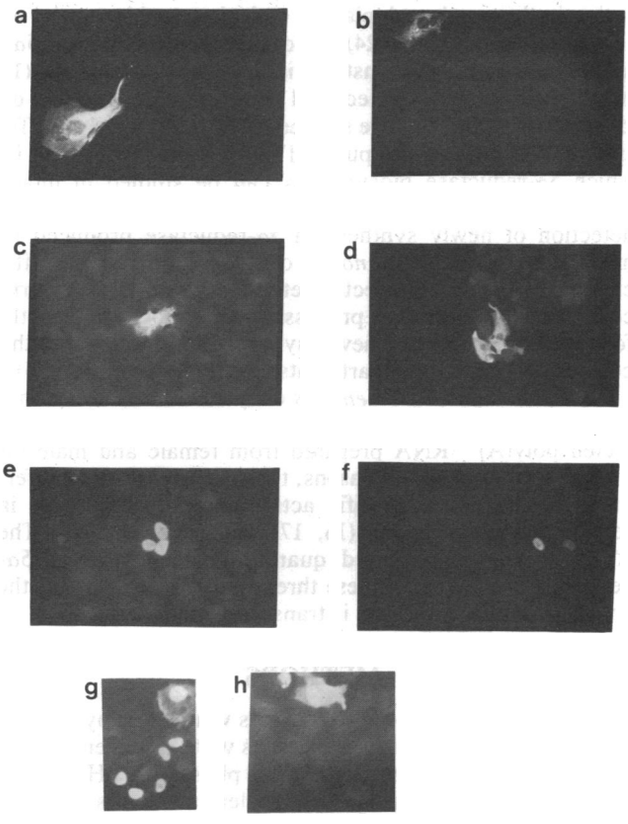


FIG. 5. Clonal analysis of transfected cells. Cells were transfected with either the alkaline phosphatase plasmid or TDPM plasmid and subcultured at clonal density. Clones were allowed to grow up for 4 to 14 days. (*a*) A single alkaline phosphatase-positive CV-1 cell in clone of about 50 cells. (*b*) Two alkaline phosphatase-positive HeLa cells in clone of about 50 cells. (*c*) Alkaline phosphatase-positive HeLa cell in clone of about 200 cells (fluorescence of surrounding cells is due to background). (*d*) Two alkaline phosphatase-positive CV-1 cells in clone of about 200 cells. (*e*) Three T-Ag-positive L cells from a TDPM transfection in clone of about 50 cells. (*f*) Two T-Ag-positive L cells from a TDPM transfection in clone of about 200 cells. (*g*) A cluster of 5 T-Ag-positive cells and 1 T-Ag-positive, alkaline phosphatase-positive cell 2 days after transfection of HeLa cells—transfections contained an excess of SV-T (Table 2). (*h*) A T-Ag-positive cell adjacent to an alkaline phosphatase-positive cell 7 days after transfection of HeLa cells and subsequent cloning. It is suspected that the two plasmids were once in the same cell but later segregated during division. *c*, *d*, *g*, and *h* are overexpressed to show background fluorescence of neighboring cells.

The results are interpreted to mean that there is a finite probability that the transcription machinery can assemble at a promoter; however, enhancer DNA seems to catalyze this process. A clear prediction is that overexpression of a limiting transcription factor should result in less dependence on enhancer DNA sequences. Indeed, this situation might apply to mouse oocytes where the same high level of expression from SV40 occurs in the presence or absence of the SV40 enhancer (ref. 20; see also, refs. 21–23).

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