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Efficient Transformation by Prague A Rous Sarcoma Virus Plasmid DNA Requires the Presence of *cis*-Acting Regions within the *gag* Gene

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A region in addition to and outside the long terminal repeats (LTRs) in the *gag* gene of the Prague A strain of Rous sarcoma virus was found to be essential in *cis* for efficient cell transformation by cloned viral DNA. Transformation in chicken embryo fibroblasts, which requires infectious virus production and reinfection, was facilitated in *cis* by sequences between nucleotides 630 and 1659. Efficient transformation of NIH 3T3 cells in which secondary spread of virus is not necessary (as it is in chicken embryo fibroblasts) required sequences between nucleotides 630 and 1149. A *src* cDNA clone which also lacks this region demonstrated low transformation efficiency, indicating that the role of the *cis* element cannot be attributed to interference with RNA splicing. The *gag* gene segment required in *cis* for transformation, between nucleotides 630 and 1149, could substitute for the simian virus 40 enhancer in either orientation, and cells transfected with Rous sarcoma virus LTR-driven plasmids containing the *gag cis* element had a two- to threefold increase in steady-state viral RNA levels compared with plasmids lacking this region. Thus, additional *cis*-acting regulatory elements located outside the viral LTRs may modulate viral gene expression and contribute to the efficiency of cell transformation.

cis-Acting elements affecting transcription of the Rous sarcoma virus (RSV) DNA are located in the ~300-base long terminal repeats (LTRs) flanking the provirus genome (13, 21, 26). The known elements include a promoter, an enhancer, and a polyadenylation signal. The LTR element has been widely used to achieve high levels of expression of heterologous genes in a variety of cell types (13, 18, 26, 30). Transformation of both mammalian and avian fibroblasts by RSV DNA requires the transcription of integrated virus-specific DNA initiated in the 5′ LTR and splicing of the RNA to form *src* mRNA (for a review, see reference 40). Transformation of mammalian cells with the *src* gene DNA by transfection occurs by direct integration of the DNA (9); thus, the only *cis*-acting sequences expected to be necessary, in addition to the *src* gene, for transformation of mammalian cells are the LTR elements and the splice sites. In contrast, stable transformation of avian cells by transfecting DNA requires transcription of the proviral DNA, virion RNA packaging, and spread of virus to adjoining cells (10). Therefore, *cis*-acting encapsidation sequences on the viral DNA are also necessary, in addition to *cis* elements required for transcription and splicing (20, 24, 31, 36).

As part of ongoing studies on *cis*-acting sequences required for the regulation of viral RNA expression and processing, we constructed a set of derivatives of an infectious Prague A (PrA) RSV plasmid, pJD100, with deletions and, thus, variable amounts of the *src* gene intron sequences. Surprisingly, we found that elements besides the LTR and the known packaging sequences (20, 24, 31, 36) are required in *cis* for efficient transformation of both avian and murine fibroblasts by plasmid DNA. We have localized these elements in the 5′ region of the *gag* gene between nucleotides 630 and 1659. A DNA fragment from this region also contains a *cis*-acting element with the properties of a transcriptional enhancer.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Chicken embryo fibroblasts (CEF) were prepared from C/E chf$^-$ gs$^-$ embryonated eggs obtained from SPAFAS, Inc. (Norwich, Conn.) and were grown in SGM, medium 199 with 10% (vol/vol) tryptose phosphate broth and 5% (vol/vol) calf serum. NIH 3T3 cells were maintained in Dulbecco minimum Eagle medium containing 10% (vol/vol) calf serum. Infectious PrA RSV plasmid pJD100 and infectious Rous-associated virus-1 plasmid pRAV10R (35) were generously provided by J. Thomas Parsons, Department of Microbiology, University of Virginia, Charlottesville, Va., and by Linda Sealy, Department of Molecular Physiology, Vanderbilt University School of Medicine, Nashville, Tenn., respectively.

Transfection procedures and focus-forming assays. Transfections of both CEF and NIH 3T3 cells were carried out by the calcium phosphate precipitation method (15) as described previously (39). For CEF transfections, plasmid DNA (2 μg) and sheared salmon sperm DNA (20 μg), as well as pRAV10R helper DNA (100 ng) when appropriate, were added to 0.48 ml of 0.25 M $CaCl_2$ solution. To this solution was added 0.5 ml of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline, pH 7.05, and the mixture was incubated for 30 min at room temperature. The mixture was then added to 2×10^6 CEF cells per 100-mm plate. After 15 min at room temperature, 10 ml of SGM containing 1% (vol/vol) heat-inactivated chicken serum was added, and the cells were incubated for 3 to 4 h at

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37°C. The cultures were treated with 3 ml of 30% (vol/vol) dimethyl sulfoxide in 1× HEPES-buffered saline for 4 min, washed with 3 ml of SGM containing 1% (vol/vol) chick serum, and overlaid with 10 ml of SGM. When helper transformation-defective B77 RSV was used, it was added at this point in the procedure. After 48 h, the cells were passaged 1:3, and on the following day, they were overlaid with SGM containing 0.5% (vol/vol) agar. Foci were counted on duplicate plates approximately 7 days later. To analyze progeny virus in the foci, the cells in the foci were withdrawn with a Pasteur pipette and cultures of uninfected cells were inoculated.

Transfection of NIH 3T3 cells was carried out in a similar manner by using 1.0 μg of cloned plasmid DNA per dish. Plasmid DNA was linearized with *Hin*dIII before transfection. After exposure to the DNA precipitate for 4 h, the cells were treated with 1× HEPES-buffered saline containing 20% (vol/vol) glycerol for 4 min. The cells were washed once, and the medium was changed at 2- to 3-day intervals. After approximately 3 or 4 weeks, foci of transformed cells were counted on duplicate plates.

Cloning procedures and restriction enzyme digests. Cloning techniques were carried out according to the standard methods described by Maniatis et al. (27). Plasmids used for transformation assays in this paper were constructed as shown in Fig. 1. The plasmids containing the chloramphenicol acetyltransferase (*cat*) gene were constructed by ligation of PrA RSV *Bam*HI-*Bcl*I fragment (nucleotides 532 to 1149) or *Xho*I-*Bcl*I fragment (nucleotides 630 to 1149) into a unique *Bgl*II site of a pSV1*cat* derivative 3′ to the *cat* gene (18). Restriction enzyme digestions were carried out according to the specifications of the suppliers.

Northern blot analysis of RNA. Isolation of total RNA from cells was carried out according to the method of Strohman et al. (38). Northern (RNA) blot analysis of RNA on formaldehyde-agarose gels was carried out as previously described (27). ^{32}P-labeled probes were prepared according to the nick translation technique of Rigby et al. (32).

CAT assays. Transfection of DNAs into CEF was performed as described above, except that cell cultures were treated with 20% (vol/vol) glycerol, rather than 30% (vol/vol) dimethyl sulfoxide. Transfection of CV-1 cells was carried out according to the procedures described above for NIH 3T3 cells, except that cells were treated with 24% (vol/vol) dimethyl sulfoxide. At 60 h posttransfection, assays for CAT enzyme activity were carried out by previously described methods (18, 25).

Simultaneous viral RNA assay and CAT analysis. Transfection of CEF was carried out with 20 μg of plasmid DNA according to the procedure described above. After 48 h, the cells were harvested. One-tenth of the cells was used for the CAT assays described above, and the remainder was used for total RNA isolation. The amount of viral RNA was determined by slot blotting 7 μg of RNA to a nitrocellulose filter and hybridizing the blot to an ~800-base antisense ^{32}P-RNA probe (28) which spanned the 5′ end of the viral RNA from the *Xho*I site at nucleotide 630 to a *Hin*dIII site at ~−200. The autoradiograms were scanned, and the peak areas were determined. After the background value obtained from the control RNA slot was subtracted, the ratio of peak area to CAT activity was determined for each sample and normalized to the value of pJDΔ11-3′CAT, which was set at 1.00.

Materials. ^{32}P-labeled deoxyribonucleotides (2,000 Ci/mmol), ribonucleotides (800 Ci/mmol), and [^{14}C]chloramphenicol (54 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or New England BioLabs, Inc. (Beverly, Mass.).

RESULTS

Transformation of CEF with pJD100 plasmids with deletions and helper virus and helper virus DNA. The plasmids used in this study and their constructions are shown in Fig. 1 and are derived from an infectious PrA RSV plasmid, pJD100. In all of these plasmids, the *pol* gene is completely deleted and the *env* gene is deleted to various extents; pJDΔ5-14 contains most of the *env* gene, whereas *env* is completely deleted in pJDΔ5-25 and pJDΔ5. The *gag* gene is also deleted to different extents in the plasmids. pJDΔ5 does not contain *gag* sequences and also lacks the donor splice junction at nucleotide 398 (5, 17). pJDΔ5-25 and pJDΔ5-14 both contain *gag* sequences to nucleotide 630, pJDΔ9 contains *gag* to nucleotide 1149, and pJDΔ11 contains *gag* to nucleotide 1659 (the nucleotide numbering system in this paper follows that of Schwartz et al. [34]). pJS2 was constructed by cloning a *Sac*I-*Ava*II fragment from a cDNA clone of *src* mRNA which spans the splice junction together with both an *Ava*II-*Bgl*II fragment (nucleotides 7155 to 7736) from pJD100 and with pJD100 cleaved to completion with *Bgl*II and *Sac*I. It also lacks *gag* sequences, except for the first five amino acids of P19 (5, 17).

The deleted pJD100 derivatives shown in Fig. 1 were transfected into CEF with appropriate amounts of transformation-defective B77 RSV to supply the *trans*-acting functions required for replication of the defective genomes. Foci were consistently observed only in cells transfected with pJDΔ11 (Table 1). Several preparations of cloned plasmid DNA were used with similar negative results for pJDΔ5-14, pJDΔ5-25, and pJDΔ5. Transformed foci were obtained with pJDΔ9 at approximately 10-fold reduced efficiency compared with pJDΔ11. Thus, the ability to transform cells in this assay correlated with the additional *gag* gene sequences (nucleotides 1149 to 1659) present in pJDΔ11.

Individual foci of pJDΔ11-transfected cells were picked and transferred to uninfected CEF. After several cell passages, the total *src* gene-specific RNA from infected cells was examined on formaldehyde-agarose gels by Northern blotting and compared with RNA isolated from purified virions. (Helper virus RNA was not detected with this probe.) We expected to generate virions containing only 4.6-kilobase (kb) RNA, whereas in the cell, both unspliced 4.6-kb RNA and spliced 2.7-kb *src* mRNA were expected (Fig. 1). The results of these analyses for two different foci (Δ11-1 and Δ11-3) are given in Fig. 2, and both show the expected RNA patterns. A small amount of 9.3-kb RNA was also present in the Δ11-1- but not the Δ11-3-infected cells (presumably as a result of the presence of recombinants) in which the deleted genomes have recombined with the helper virus sequences to generate a full-length genome RNA. We concluded from these results that pJDΔ11 contains all the sequences required in *cis* to be propagated as a viral genome, and the sequences necessary in *cis* to maintain pools of spliced and unspliced RNAs are present in pJDΔ11. Thus, we concluded that nucleotides 1659 to 6574, the region deleted in pJDΔ11, are not required in *cis* for replication or transformation of CEF.

To further investigate the reason for the failure of plasmids with deletions in the *gag* gene region to transform CEF in the above assay, we first tested whether we could reconstruct

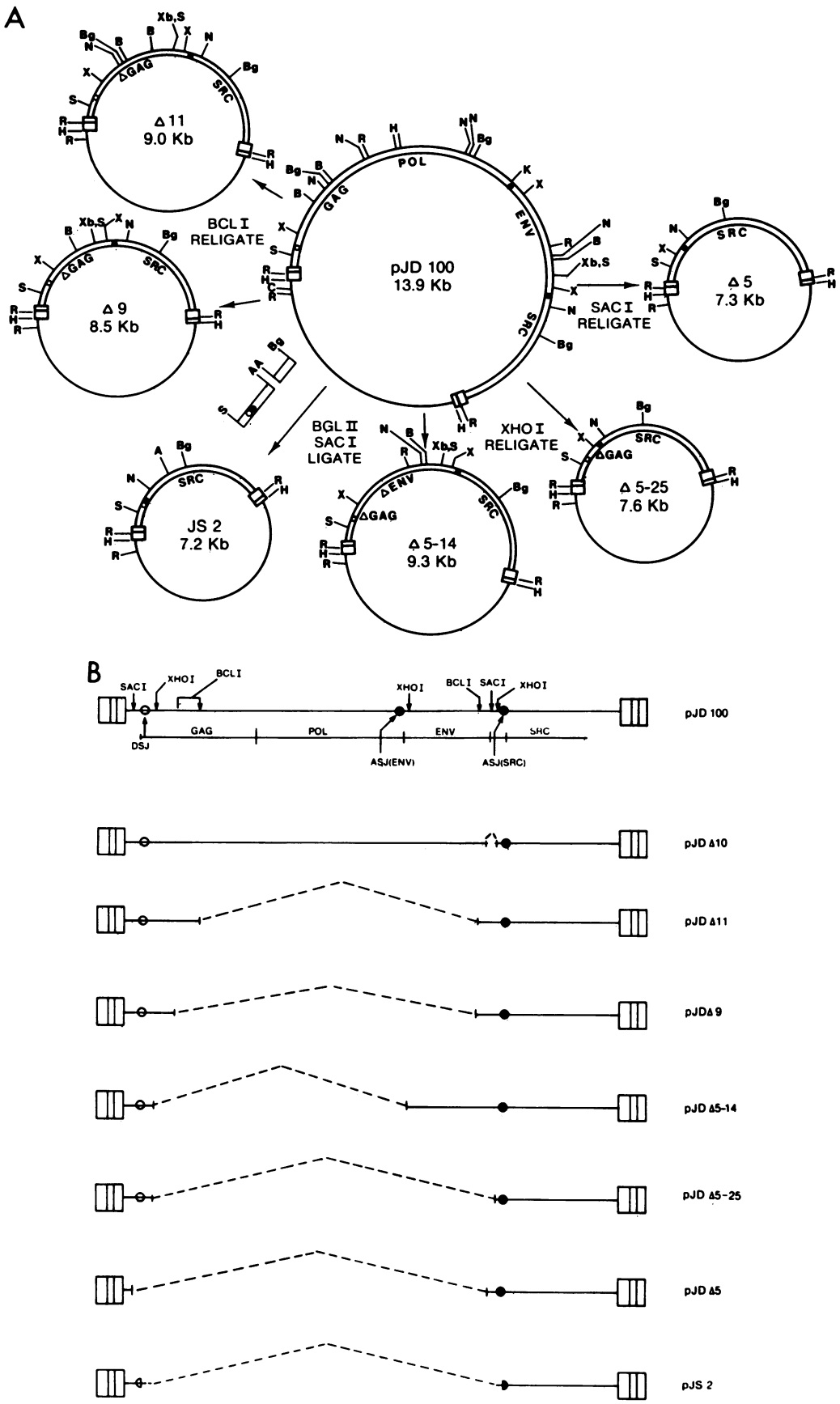

FIG. 1. Structure and construction of pJD100 plasmids with deletions in circular (A) and linear form (B). The following abbreviations were used: A, *Ava*II; B, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; R, *Eco*RI; S, *Sac*I; X, *Xho*I; Xb, *Xba*I; ASJ, acceptor splice junction; Kb, kilobase. Symbols: ○, donor splice site; ●, acceptor splice site; ----, deletion. The cDNA clone pJS2 was constructed by inserting the *Sac*I-*Ava*II fragment from the *src* mRNA cDNA clone spanning the *src* splice junction (5). (Not all the *Ava*II sites in pJD100 are shown.) In panel A, viral sequences are shown by double lines; pBR322 sequences are shown by single lines.

TABLE 1. Transformation efficiencies of pJD100 plasmids with deletions with helper transformation-defective B77 virus

Plasmid	Deletion (bp)[a]	FFU[b]/pmol (10^3)	Relative infectivity
Δ11	1659–6574	0.2	1.0
Δ9	1149–6574	0.03	0.1
Δ5-14	630–5258	<0.01	<0.05
Δ5-25	630–6983	<0.01	<0.05
Δ5	255–6865	<0.01	<0.05

[a] Nucleotide number according to sequence of PrC RSV (31).
[b] FFU, Focus-forming units.

infectious plasmids from pJDΔ5 and pJDΔ5-25 by inserting the appropriate missing restriction fragments. These plasmids transformed like wild-type pJD100 did (data not shown). It appeared therefore that there were no mutations in the coding region of the *src* gene which occurred during cloning and which would block its expression in the plasmids with deletions.

The deleted *src* plasmids were also contransfected with DNA from an infectious Rous-associated virus-1 clone, pRAV10R (35), and the results are given in Table 2. A greater than 10-fold increase in the transformation efficiency of pJDΔ11 was obtained (compare with results in Table 1). Futhermore, the plasmids which were negative or low for transformation in the previous experiments (i.e., pJDΔ9, pJDΔ5-14, pJDΔ5-25, and pJDΔ5) demonstrated transformation efficiencies on the order of 10 to 30% that of pJDΔ11. As in the first assay, transformation was dependent upon the presence of virus helper. Individual foci were picked from the transfected cells as described above and transferred to uninfected CEF. Total RNA was isolated from infected cells, electrophoresed on formaldehyde-agarose gels, and examined by using a *src*-specific probe. Foci derived from pJDΔ9, pJDΔ5-14, pJDΔ5, and pJDΔ5-25 in all cases demonstrated a wild-type virus RNA profile, i.e., a prominent band at 9.3 kb (Fig. 3). Several foci from each plasmid transfection were examined with similar results. Foci derived from pJDΔ11, on the other hand, demonstrated a mixture of 4.6- and 2.7-kb RNAs and little or no 9.3-kb RNA, as was observed above, when helper virus rather than helper DNA was used (Fig. 2). These results suggest that recombination between the deleted genomes and the helper DNA occurred at high frequencies either during the transfection procedure itself or during the passage of the infected cells. It suggested that, without the *gag* gene sequences included in pJDΔ11, viral genomes were poorly replicated and therefore that nondefective *src*-containing recombinants were selected at high frequency during virus passage.

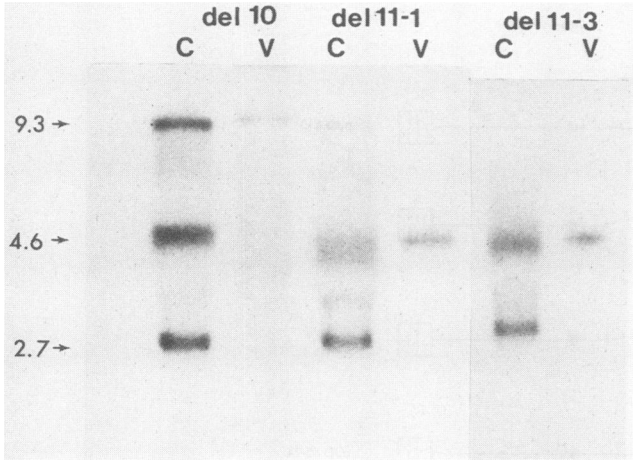

FIG. 2. Formaldehyde-agarose gel electrophoresis of RNA from cells infected with pJDΔ11-derived virus and from purified virions. CEF were transfected with pJDΔ11 or pJDΔ10 and infected with helper transformation-defective B77 as described in Materials and Methods. pJDΔ10 (del 10) is a pJD100 derivative with a 21-base deletion at the *Sac*I site at nucleotide 6865 and serves as a control for this experiment. Foci from the transfected plates were isolated and transferred to uninfected CEF. After the cells exhibited extensive transformation, total RNA was isolated by the methods described in Materials and Methods. RNA was isolated from purified virions by previously described methods (37). Aliquots of infected-cell RNA (20 μg) (C) and appropriate amounts of purified virion RNA (V) were electrophoresed on a formaldehyde-agarose gel, blotted onto nitrocellulose, and hybridized according to procedures described by Maniatis et al. (27). The ^{32}P-labeled hybridization probe was prepared by nick translation of a 364-bp *Ava*II-*Ava*II *src* gene-specific restriction fragment (nucleotides 7281 to 7645). The specific activity of the probe was approximately 3×10^7 cpm/μg of DNA. Autoradiography was carried out for 7 days. The numbers to the left of the gel indicate the size (in kilobases) of RNA fragments.

Transformation of NIH-3T3 cells with pJD100 plasmids with deletions. Transformation of NIH 3T3 cells by *src*-containing DNA, in contrast to CEF, does not require a round of replication before the establishment of transformation, and the DNA is thought to integrate directly in the transfected cell (9, 10). Therefore, helper virus or helper viral DNA is not required to establish transformation. The results, given in Table 3, indicated that the transformation efficiencies of pJDΔ11 and pJDΔ9, in contrast to the results in CEF, were not significantly different in this assay. However, the transformation efficiencies of pJDΔ5-14, pJDΔ5-25, pJDΔ5, and pJS2 (a *src* cDNA clone [Fig. 1]) were less than 10% that of pJDΔ11. We concluded from these results that in NIH 3T3 cells, the presence of the *gag* gene region between the *Xho*I site at nucleotide 630 and the *Bcl*I site at nucleotide 1149 was correlated with high transformation efficiency. These same observations were made in a number of inde-

TABLE 2. Transformation efficiencies of pJD100 plasmids with deletions with pRAV10R DNA

Helper and plasmid[a]	FFU[b]/pmol (10^3)		Relative infectivity	
	Expt 1	Expt 2	Expt 1	Expt 2
No helper				
Δ11	<0.009		<0.003	
Δ9	<0.008		<0.003	
Δ5-14	<0.009		<0.003	
Δ5-25	<0.006		<0.002	
Δ5	<0.006		<0.002	
JS2	<0.006		<0.002	
None	<0.006		<0.002	
pRAV10R				
Δ11	3.3	0.5	1.0	1.0
Δ9	0.4	0.2	0.1	0.3
Δ5-14	1.1	0.09	0.3	0.2
Δ5-25	0.7	0.003	0.2	0.01
Δ5	0.3	0.009	0.1	0.02
JS2	0.2	ND[c]	0.08	ND
None	<0.006	<0.003	<0.002	<0.01

[a] In each assay, 2 μg of the indicated plasmid was used. When present, 0.1 μg of pRAV10R was used in each assay.
[b] FFU, Focus-forming units.
[c] ND, Not done.

TABLE 3. Transformation efficiencies in NIH 3T3 cells of pJD100 plasmids with deletions

Plasmid	FFU[a]/pmol (10^2)				Relative infectivity[b]			
	Expt 1	Expt 2	Expt 3		Expt 1	Expt 2	Expt 3	
			Without pJF11107[c]	With pJF11107			Without pJF11107	With pJF11107
Δ11	3.3	2.5	8.3	4.5	1.0	1.0	1.0	1.0
Δ9	2.9	1.7	3.1	6.1	0.9	0.7	0.4	1.3
Δ5-14	0.06	0.06	0.6	1.7	0.02	0.02	0.07	0.4
Δ5-25	0.02	0.17	0.3	0.5	0.01	0.07	0.04	0.1
Δ5	0.02	0.04	0.2	0.1	0.01	0.02	0.02	0.02
JS2	ND[d]	0.04	<0.05	<0.05		0.02	<0.01	<0.01

[a] FFU, Focus-forming units.
[b] Relative to pJDΔ11 (1.0). Plasmids were linearized with *Hin*dIII before transfection.
[c] pJD100 derivative with a deletion from nucleotide 5594 to 8662. This plasmid would be expected to supply required *trans*-acting factors.
[d] ND, Not done.

pendent experiments as shown in Table 3 and with several preparations of plasmid DNA.

We next tested for the possibility that the region between nucleotides 630 and 1149 coded for a *trans*-acting factor which enhanced the efficiency of transformation. Cells were cotransfected with the *src*-deleted plasmid and another plasmid, pJF11107, which is a pJD100 derivative with a deletion from nucleotides 5594 to 8662. Thus, most of the *src* gene is deleted in this plasmid, but required *trans*-acting factors from the *gag* gene should be expressed. The only plasmid whose transformation efficiency was significantly increased in this assay was pJDΔ5-14 (an approximately sixfold increase) (Table 3). This is also the only plasmid in which wild-type genomes can be regenerated by a single crossover with pJF11107 in a region of *env* gene homology (336 base pairs [bp]). In view of the lack of a significant effect on the low-efficiency plasmids, we surmised that the singular effect on transformation by pJDΔ5-14 was due to homologous recombination with the coinfecting DNA which can occur in transfected DNA at high frequencies. Therefore, the region increasing transformation efficiency in these experiments (nucleotides 630 and 1149) acted in *cis*.

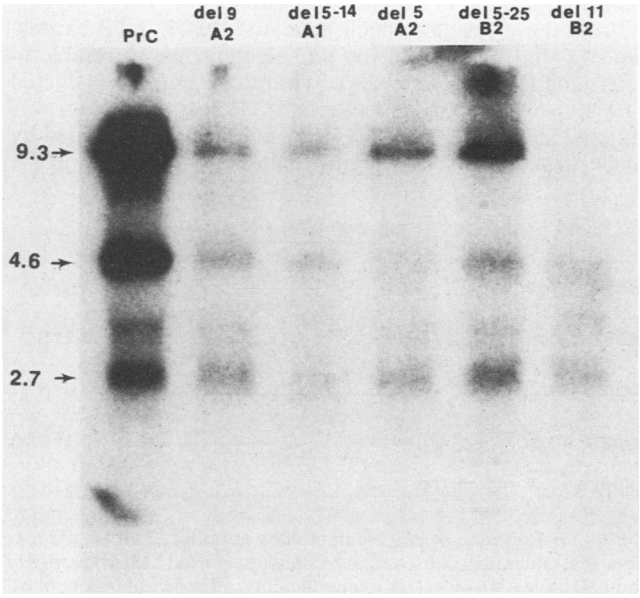

FIG. 3. Formaldehyde-agarose gel electrophoresis of RNA from cells infected with virus derived from cotransfection of pJD100 plasmids with deletions and pRAV10R DNA. CEF were transfected with plasmids with various deletions as shown and cotransfected with pRAV10R DNA as described in Materials and Methods. RNA was isolated and analyzed as described in the legend to Fig. 1. Exposure to X-ray film was for 10 days. Control lane (PrC) is RNA from Prague C RSV-infected cells. (The minor band between the 4.6- and 2.7-kb RNA species was also present in uninfected control cells and is presumably c-*src* mRNA.)

***cis*-Acting elements in the *gag* gene region with the characteristic of an enhancer.** A possible explanation for the data would be that the PrA RSV genome contains an additional transcriptional enhancer element within the *gag* gene whose presence would act to increase the transformation efficiency. Such enhancers have recently been reported to be present in several avian retrovirus genomes (1). To examine this possibility, a 617-bp fragment extending from the *Bam*HI site at nucleotide 532 to the *Bcl*I site at nucleotide 1149 was cloned into a plasmid (pSV1*cat*) which contains the simian virus 40 (SV40) early promoter upstream from the *cat* gene and SV40 polyadenylation site but does not contain the SV40 72-bp enhancer (14). These plasmids, as well as the enhancerless pSV1*cat* and the clone pSV2*cat* (which contains the SV40 72-bp enhancer), were transfected into CEF and CV-1 monkey cells. The structures of the plasmids and representative data are given in Fig. 4. In CEF, the plasmid pMS151*cat*, in which the orientation of the fragment relative to the SV40 promoter is the same as it is in the viral genome (sense orientation), expressed CAT enzyme activity at a level sixfold greater than pSV1*cat* did. In the opposite antisense orientation, the plasmid pMS152*cat* expressed CAT enzyme activity at a somewhat lower level but still demonstrated a threefold increase compared with pSV1*cat*. The CAT activity induced by pSV2*cat*, containing the SV40 enhancer, was 22-fold greater than was that of pSV1*cat* in CEF. In CV-1 monkey cells, pMS151*cat* demonstrated a 29-fold enhancement compared with pSV1*cat*, whereas pMS152*cat*, in the antisense orientation, yielded an enhancement of 14-fold. Another set of plasmids (pMS101*cat* and pMS102*cat*) was constructed in which the 519-bp fragment from the *Xho*I site at nucleotide 630 to the *Bcl*I site at nucleotide 1149 was cloned into pSV1*cat* in both orientations. These clones demonstrated similar activities in CV-1 cells (Fig. 4), i.e., enhancement was approximately 10- to 20-fold and was orientation dependent, with higher expression obtained when the RSV sequences were inserted in the sense orientation. The results of this experiment were confirmed by the direct measurement of *cat* gene-specific RNA in transfected cells. Cells were transfected with pMS152*cat*, the cells were harvested at approximately 48 h after transfection, and RNA from the cells was hybridized to a 448-base ^{32}P-RNA probe

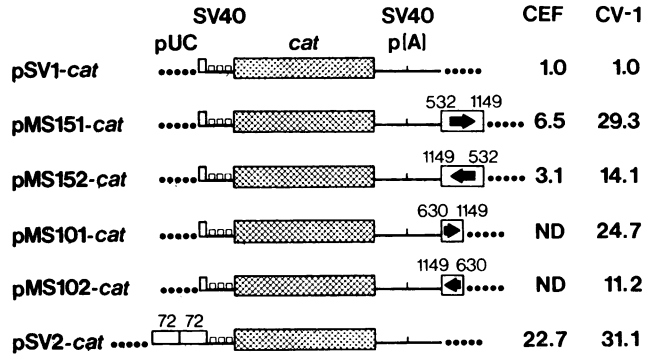

FIG. 4. CAT activities of PrA *gag*-containing constructs in CEF and CV-1 cells. The structures of the relevant portions of the plasmids are shown, and the region of *gag* included is indicated by the nucleotide numbers. Transfections and CAT activities in CEF and CV-1 cells were determined as described in Materials and Methods and in reference 25. For each transfection in CEF, 500 ng of plasmid DNA was used; in CV-1 cells, 6 μg of plasmid was used. For assays in CEF, 30 μl of cell extract was used and a 45-min incubation was used at 37°C. In CV-1 cells, 40 μl of cell extracts was used with a 180-min incubation. The percent conversion to acetylated [^{14}C]chloramphenicol was determined by measuring the radioactivity of the appropriate spots in a scintillation counter. The activity was normalized to the activity of pSV1*cat*. ND, Not done; p[A], SV40 early RNA polyadenylation signal.

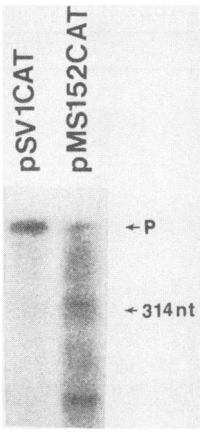

FIG. 5. Analysis of RNA from CV-1 cells transfected with *cat* plasmid derivatives. Plasmids pSV1*cat* and pMS152*cat* were transfected into CV-1 cells according to the procedure given in Materials and Methods. After 48 h, the cells were harvested and the total RNA was purified. The RNA was hybridized to a 448-nucleotide ^{32}P-labeled antisense RNA probe which spanned the region from an *Eco*RI site in the *cat* gene to an *Sph*I site in the SV40 upstream regulatory region. The RNase-protected RNAs were analyzed according to the procedures of Melton et al. (28) on a denaturing 5% polyacrylamide gel. RNA initiated from the SV40 promoter protected a 314-nucleotide ^{32}P-labeled fragment (314nt) as shown. Band P represents undigested probe.

which was complementary to the 5′ end of the SV40-*cat* RNA. A specific fragment of ~314 nucleotides was protected by the RNA from the pMS152*cat*-transfected cells but not by RNA isolated from pSV1*cat*-transfected cells (Fig. 5), which was expected for transcripts initiated at the SV40 early promoter. We concluded from these results that a *cis* regulatory element with the properties of a transcriptional enhancer exists in the *gag* gene region between nucleotides 630 and 1149.

Effect of the *gag* region on steady-state viral RNA levels and downstream LTR expression. The previous experiments indicated that the region of *gag* between nucleotides 630 and 1149 had the properties of a transcriptional enhancer when it was introduced in both orientations downstream from the SV40-*cat* transcriptional unit, and they confirmed for PrA RSV the results of Arrigo et al. (1). We next determined the effect of this region on the levels of transcription from both the 5′ and 3′ LTR in the context of the RSV genome. For this purpose, we constructed the plasmids shown in Fig. 6, in which the deleted genomes were placed 5′ or 3′ from a promoterless *cat* gene. By simultaneously measuring steady-state viral RNA levels and CAT activity in CEF transfected by these constructs, we could determine both the steady-state viral RNA levels initiated in the 5′ LTR and *cat* expression, which results from transcription initiated in the 3′ LTR.

We first found that the presence or absence of the *gag* region did not significantly affect the amount of CAT enzyme expressed from the 3′ LTR-driven transcripts after 48 h of transfection. The data, given in Fig. 6, were based on a number of separate transfection experiments in which the expression of clones pΔ11-3′CAT (which contains the *gag* *cis*-acting element) and pJS2-3′CAT (which does not contain the element) were compared. The *cat* expression of these two clones was not significantly different. The deletion of the 5′ LTR containing the viral promoter, as well as sequences from *gag* (plasmid pd3-3′CAT), resulted in an approximate 20-fold increase in *cat* expression. These results are consistent with a previous model which suggested that transcription from an intact 5′ LTR into the 3′ LTR region interferes with the initiation of RNA synthesis in the 3′ LTR (12). Placement of the *cat* gene upstream and in the opposite orientation from the 5′ LTR (i.e., pΔ11-5′CAT and pJS2-5′CAT) resulted, as expected, in little or no *cat* expression.

From these results, which indicated that 3′ LTR expression was independent of the *gag* element, we were able to determine the relative levels of viral RNA in the transfected CEF by slot blot analysis (Table 4). This transcription was initiated in the 5′ LTR at the RSV cap site as determined by single-strand nuclease mapping (data not shown). The RNA

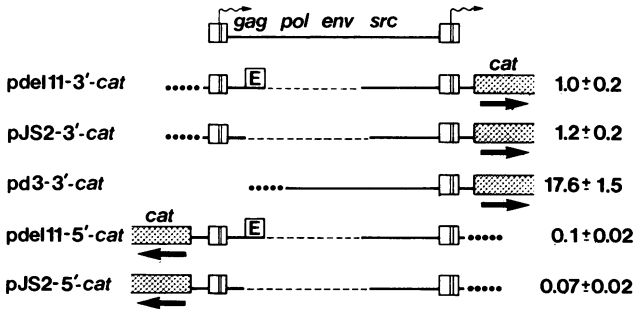

FIG. 6. Presence of *gag* element does not affect 3′ LTR expression. Plasmids were constructed by cleaving pJDΔ11 (pdel11), pJS2, and pJD100 with *Hin*dIII, isolating the appropriate viral fragment by gel electrophoresis, and ligating the fragment into a *Hin*dIII-cleaved pUC18-based plasmid both upstream and downstream from the promoterless *cat* gene as shown. The presence of the region of *gag* shown to have enhancer activity (E) is indicated. Assays for *cat* expression in CEF were carried out and the percent conversions of [^{14}C]chloramphenicol were determined as described in the legend to Fig. 4. CAT enzyme activities were normalized to the value in cells transfected with pΔ11-3′CAT (pdel11-3′-*cat*). Plasmid pd3-3′CAT (pd3-3′-*cat*) contains the pJD100 *Hin*dIII fragment from nucleotides 2867 to ~9400 at the boundary of the 3′ LTR.

levels were normalized to the amount of CAT activity to correct for different transfection efficiencies. Note that the normalized RNA levels in CEF transfected by pJS2-3'CAT and pΔ5-14-3'CAT, which do not contain the *gag* element, were two- to fourfold lower than in cells transfected by pΔ11-3'CAT and pΔ9-3'CAT, which do contain the *gag* elements. These results are therefore consistent with the hypothesis that the region between nucleotides 630 and 1149 contains a *cis*-acting element that increases the level of transcription from the 5' LTR.

TABLE 4. Relative steady-state viral RNA levels in transiently transfected CEF cells[a]

Plasmid	RNA[b]		CAT activity[c]		Relative RNA levels[d]		
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Avg
pΔ11-3'CAT	5.93	2.45	3.04	1.37	1.00	1.00	1.00
pΔ9-3'CAT	2.61	2.96	1.65	2.28	0.81	0.73	0.77
pΔ5-14-3'CAT	3.34	2.76	5.30	3.32	0.32	0.46	0.39
pJS2-3'CAT	1.48	0.81	2.62	1.93	0.29	0.24	0.27

[a] Plasmids were constructed as shown in Fig. 6. Simultaneous viral RNA assays by slot blot analysis and CAT analysis were carried out as described in Materials and Methods. Two independent experiments (1 and 2) were carried out as shown and, for each experiment, two determinations of RNA levels and CAT activities were performed. The results were then averaged.

[b] Areas under the curve were determined by densitometric scanning of the autoradiogram from the slot blots.

[c] CAT activities were determined as described in the legend to Fig. 4 and are expressed in terms of percent conversion of [^{14}C]chloramphenicol.

[d] Relative RNA levels were determined by dividing the numbers in columns 1 and 2 by the numbers in columns 3 and 4, respectively. These values were then normalized such that pΔ11-3'CAT = 1.00.

DISCUSSION

In this report, we have defined a *cis* element in the *gag* gene of RSV which increases the transformation efficiency of transfected plasmid RSV DNA. This element, which affects transformation of both CEF and NIH 3T3 cells, is located between nucleotides 630 and 1149 in the p19-p10 region of the *gag* gene. This sequence acts in *cis* since we have shown that coinfecting helper virus or DNA does not increase transformation by plasmids with deletions in *trans*. This was demonstrated in transfections of NIH 3T3 cells in which coinfection with helper virus is not required for transformation as it is in CEF (9, 10). Deletion of the *gag* segment reduced transformation efficiency by a factor of 10- to 50-fold. Cotransfection with plasmids expected to express the putative RSV *trans*-acting factor described previously (3) did not significantly increase the transformation efficiencies of the defective plasmids. Plasmid pJDΔ5-14 was the single exception; however, this plasmid contains 336 bp from *env*, which would allow homologous recombination with the cotransfecting DNA in a single crossover event. Genetic recombination between defective *src* clones and helper virus interfered with the interpretation of the focus-forming efficiencyresults in CEF. This was shown by the fact that cells infected with several individual virus clones derived from plasmids pJDΔ9, pJDΔ5-25, pJDΔ5-14, and pJDΔ5 contained abunda.. quantities of recombined full-size 9.3-kb RSV RNA (Fig. 3).

Another *src*-containing recombinant plasmid, p*src*11, lacking *gag* sequences between nucleotides 630 and 1149, has been shown by others to induce NIH 3T3 foci at a comparatively high efficiency (42). This clone, derived from the Schmidt-Ruppin strain D RSV genome, had a deletion from the *Xho*I site in *gag* at nucleotide 630 to the *Sal*I site in *env* at nucleotide 6058. It is not clear whether the presence of additional *gag* sequences in p*src*11 would influence transformation. The p*src*11 clone differed in a number of ways from the pJD100-derived plasmids used in our study. Transcription of the p*src*11 clone could be augmented in *cis* by an additional proviral DNA segment linked upstream to the 5' LTR. These proviral sequences, derived from the region between *src* and U3 of the downstream LTR, have been shown to potentiate the effects of the LTR enhancer in some transient expression experiments (21, 26). These sequences are not present upstream to the 5' LTR in pJD100. Therefore, the pJD100 constructions more closely mimic the structure of an integrated DNA provirus. Furthermore, the activity of the LTR enhancer elements themselves may differ between Schmidt-Ruppin D RSV and PrA RSV. In addition, there may be *cis*-acting elements in the Schmidt-Ruppin D *env* gene that are not present in the PrA *env* gene and which may influence expression. Further experiments will be required to understand the reasons for the different behavior of the two types of recombinant plasmids.

One explanation for our results would be that the deletions impair viral RNA splicing. Although we cannot completely rule out this hypothesis, it is unlikely to explain our results for several reasons. The plasmid pJS2, a *src* cDNA clone which does not require splicing to express *src* mRNA, transforms NIH 3T3 cells at a low frequency similar to that of the plasmids with deletions, pJDΔ5-25, pJDΔ5-14, and pJDΔ5. Furthermore, our results indicate that correctly spliced v-*src* mRNA is present in transiently transfected cells from both plasmids containing and lacking the *cis* element affecting transformation (M. Stoltzfus and S. Fogarty, unpublished observations). The efficiency of splicing may differ in cells transfected with the various plasmids with deletions, and this possibility is currently under investigation.

The most attractive hypothesis to explain the requirement for a *cis*-acting sequence in the *gag* gene is that there is a transcriptional enhancer outside the LTR. We have shown that a *cis*-acting element with the characteristics of a transcriptional enhancer exists in the PrA RSV *gag* gene segment between nucleotides 630 and 1149. This DNA fragment can substitute for the SV40 enhancer when located downstream of the transcriptional unit, in both the sense and antisense orientations. The presence of the element in the deleted PrA RSV clones was correlated with an increase in the steady-state level of viral RNA, suggesting that the element enhances transcription initiated by the 5' LTR promoter in the context of the viral genome. However, since the deleted region is also part of the RNA transcripts, we cannot rule out an effect of these sequences on the stability of mRNA as well. The fact that the element, when placed outside an SV40 transcriptional unit, increases the level of specific RNA (Fig. 5) would suggest that in this context it is increasing transcription. Enhancers in the *gag* region have recently been reported in a number of avian retroviruses including PrC and Schmidt-Ruppin strain A RSV, Fujinami sarcoma virus, and the avian endogenous virus Rous-associated virus-1 (1).

The *cis* element coincides with the location of a secondary DNase I-hypersensitive site in an integrated RSV provirus in a transformed RAT-1 cell line, in addition to the major hypersensitive site within the LTR (6). Spontaneous expression of the endogenous avian retrovirus *ev*-1 is also associated with the appearance of DNase I-hypersensitive sites in both the LTR and the *gag* gene (8). Futhermore, a major S1 nuclease-sensitive site is found around nucleotide 720 in Schmidt-Ruppin strain A RSV plasmid (L. Karnitz, L.

Sealy, and R. Chalkley, Nucleic Acids Res., in press), and S1 nuclease-hypersensitive sites have been demonstrated in regions where *cis*-acting transcriptional elements are located (7, 19, 22, 29, 33). The RSV genome may therefore have several such *cis* elements, some of which lie outside the LTR.

Since the LTR possesses a strong enhancer (13, 21, 26), what would be the purpose of an additional enhancer(s) within the unique sequences, and how would it (they) increase the efficiency of transformation? It is known that only a small fraction of the RSV proviruses which are integrated into nonpermissive mammalian cells are transcriptionally active (2, 39). Cell lines containing such silent proviruses can be readily obtained (2, 39). Therefore, many integration sites apparently do not allow the expression of viral RNA, presumably as a result of the effects of *cis*-acting inhibitory cellular sequences (4, 11). The presence of multiple enhancers in the viral genome may counteract this negative effect and provide a selective advantage in the evolution of the viral genome. Deletion of the positive elements would result in a reduced probability that the integrated viral DNA would be expressed. Conversely, rearrangements of proviral DNA which reduplicate putative internal regulatory sequences may favor expression of viral transcripts and transformation. In the laboratory of Wyke and co-workers, duplications of viral DNA 5′ to the complete integrated provirus in RSV-transformed rat cells have been found (16, 23). In one cell line, this duplicated DNA included the viral *src* gene (23). Expression of the duplicated DNA segment by transformation assays was greatly influenced by the presence in *cis* of sequences from the *gag-pol* region between nucleotides 630 and 2740. Surprisingly, expression of this DNA in transformed cells obtained by transfection was not influenced by the presence of an RSV LTR, and RNA was initiated from cryptic promoter sites in the region between *env* and *src*. These results suggest that the expression of the *src* gene in this case is increased by the presence of *cis*-acting elements outside the LTR and apparently within the *gag-pol* region.

The region between nucleotides 1149 and 1659, whose deletion impairs transformation in CEF, is not required in NIH 3T3 cells (compare Tables 1, 2, and 3). Since transformation in CEF depended on virus replication (9, 10) and the steady-state levels of RNA in CEF were not decreased significantly when this region was absent (Table 4), this segment may play an as yet undefined role at a step in virus replication beyond transcription. The coinfecting wild-type virus supplies all *trans*-acting functions for virion production. Therefore, the retained *gag* sequences in the defective genomes would appear to be also acting in *cis*. The fact that pJDΔ9 DNA transformed CEF to a low level in the presence of transformation-defective B77 virus (Table 1) when the other more extensive deletions were completely negative in this assay suggests that the region is not absolutely required. Repeated serial undiluted passage of PrB RSV in permissive avian cells results in the selection of highly defective variants (41). These variants lost, in addition to the *src* gene, much of the *gag*, *pol*, and *env* genes. The 5′ endpoints of these deletions mapped to nucleotide 1930 in *gag*. Since these deletions occurred relatively rapidly and then were maintained through subsequent passages, deleted viral genomes which retain these sequences may have a selective replication advantage over those with a more extensive defect.

Our data as well as other observations discussed here show that the expression of the RSV genes may be more complex than previously thought. It is clear that the enhancer elements in the RSV LTR are sufficient to ensure high levels of transcription in transient expression experiments in a variety of cell types (13, 18, 26, 30). It is possible, however, that additional *cis*-acting viral elements may be necessary to efficiently express integrated RSV genomes.

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LITERATURE CITED

1. **Arrigo, S., M. Yun, K. Beemon.** 1987. *cis*-Acting regulatory elements within *gag* genes of avian retroviruses. Mol. Cell. Biol. **7**:388–397.
2. **Boettiger, D.** 1974. Virogenic nontransformed cells isolated following infection of normal rat kidney cells with B77 strain Rous sarcoma virus. Cell **3**:71–76.
3. **Broome, S., and W. Gilbert.** 1985. Rous sarcoma virus encodes a transcriptional activator. Cell **40**:537–546.
4. **Catala, F., and P. Vigier.** 1979. Infectivity of proviral DNA from avian sarcoma virus-transformed mammalian cells. J. Virol. **29**: 833–839.
5. **Chang, L.-J., and C. M. Stoltzfus.** 1985. Cloning and nucleotide sequences of cDNAs spanning the splice junctions of Rous sarcoma virus mRNAs. J. Virol. **53**:969–972.
6. **Chiswell, D. J., D. A. Gillespie, and J. A. Wyke.** 1982. The changes in proviral chromatin that accompany morphological variation in avian sarcoma virus-infected rat cells. Nucleic Acids Res. **10**:3967–3980.
7. **Chung, S., V. Folson, and X. Wooley.** 1983. DNase I-hypersensitive sites in the chromatin of immunoglobulin K light chain genes. Proc. Natl. Acad. Sci. USA **80**:2427–2431.
8. **Conklin, K. F., J. M. Coffin, H. L. Robinson, M. Groudine, and R. Eisenman.** 1982. Role of methylation in the induced and spontaneous expression of the avian endogenous virus *ev*-1: DNA structure and gene products. Mol. Cell. Biol. **2**:638–652.
9. **Cooper, G. M., N. G. Copeland, A. D. Zelenetz, and T. Krontiris.** 1979. Transformation of NIH-3T3 mouse cells by avian retroviral DNAs. Cold Spring Harbor Symp. Quant. Biol. **44**:1169–1176.
10. **Cooper, G. M., and S. Okenquist.** 1978. Mechanism of transfection of chicken embryo fibroblasts by Rous sarcoma virus DNA. J. Virol. **28**:45–52.
11. **Cooper, G. M., and H. M. Temin.** 1976. Lack of infectivity of the endogenous avian leukosis virus-related genes in the DNA of uninfected chicken cells. J. Virol. **17**:422–430.
12. **Cullen, B. R., P. T. Lomedico, and G. Ju.** 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis. Nature (London) **307**:241–245.
13. **Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA **79**:6777–6781.
14. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
16. **Green, A. R., S. Searle, D. A. F. Gillespie, M. Bissell, and J. A. Wyke.** 1986. Expression of integrated Rous sarcoma viruses:

DNA rearrangements 5′ to the provirus are common in transformed rat cells but not seen in infected but untransformed cells. EMBO J. 5:701–711.
17. **Hackett, P. B., R. Swanstrom, H. E. Varmus, and J. M. Bishop.** 1982. The leader sequence of the subgenomic mRNA's of Rous sarcoma virus is approximately 390 nucleotides. J. Virol. **41:** 527–534.
18. **Haugen, T., T. P. Cripe, G. D. Ginder, M. Karin, and L. P. Turek.** 1987. Trans-activation of an upstream early gene promoter of bovine papilloma virus-1 by a product of the viral E2 gene. EMBO J. **6:**145–152.
19. **Herbonel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39:**653–662.
20. **Kawai, S., and T. Koyoma.** 1984. Characterization of a Rous sarcoma virus mutant defective in packaging its own genomic RNA: biological properties of mutant TK15 and mutant-induced transformants. J. Virol. **51:**147–153.
21. **Laimins, L. A., P. Tsichlis, and G. Khoury.** 1984. Multiple enhancer domains in the 3′ terminus of the Prague strain of Rous sarcoma virus. Nucleic Acids Res. **12:**6427–6442.
22. **Larsen, A., and H. Weintraub.** 1982. An altered DNA conformation detected by S1 nuclease occurs at specific regions in active chicken globin chromatin. Cell **29:**609–622.
23. **Levantis, P., D. A. F. Gillespie, K. Hart, M. J. Bissell, and J. A. Wyke.** 1986. Control of expression of an integrated Rous sarcoma provirus in rat cells: role of 5′ genomic duplications reveals unexpected patterns of gene transcription and its regulation. J. Virol. **57:**907–916.
24. **Linial, M., E. Medeiros, and W. S. Hayward.** 1978. An avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: biological and biochemical characterization. Cell **15:**1371–1381.
25. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethylsulfotide or glycerol shock treatment. Nucleic Acids Res. **12:**5707–5717.
26. **Luciw, P. A., J. M. Bishop, H. E. Varmus, and M. R. Capecchi.** 1983. Location and function of retroviral and SV40 sequences that enhance biochemical transformation after microinjection of DNA. Cell **33:**705–716.
27. **Maniatis, T. E., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
29. **Mills, F., M. Fisher, R. Kurda, A. Ford, and H. Gould.** 1983. DNase I hypersensitive sites in the chromatin of human μ immunoglobulin heavy chain gene. Nature (London) **306:** 809–812.
30. **Pasleau, F., M. J. Tocci, F. Leung, and J. J. Kopchick.** 1985. Growth hormone gene expression in eukaryotic cells directed by the Rous sarcoma virus long terminal repeat or cytomegalovirus immediate-early promoter. Gene **38:**227–232.
31. **Pugatsch, T., and D. W. Stacey.** 1983. Identification of a sequence likely to be required for avian retroviral packaging. Virology **128:**505–511.
32. **Rigby, P. W. J., M. Dieckman, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113:** 237–251.
33. **Schon, E., T. Evans, J. Welsch, and A. Efstradiatis.** 1983. Conformation of promoter DNA: fine structure mapping of S1-hypersensitive sites. Cell **35:**837–848.
34. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32:**853–869.
35. **Sealy, L., M. L. Privalsky, G. Moscovivi, C. Moscovivi, and J. M. Bishop.** 1983. Site-specific mutagenesis of avian erythroblastosis virus: *erb*-B is required for oncogenicity. Virology **130:**155–178.
36. **Sorge, J., W. Ricci, and S. H. Hughes.** 1983. *cis*-Acting RNA packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. J. Virol. **48:**667–675.
37. **Stoltzfus, C. M., and P. N. Snyder.** 1975. Structure of B77 sarcoma virus RNA: stabilization of RNA after packaging. J. Virol. **16:**1161–1170.
38. **Strohman, R., P. Moss, J. Micou-Eastwood, D. Spector, A. Przybyla, and B. Peterson.** 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. Cell **10:**265–273.
39. **Turek, L. P., and H. Oppermann.** 1980. Spontaneous conversion of nontransformed avian sarcoma virus-infected rat cells to the transformed phenotype. J. Virol. **35:**466–478.
40. **Varmus, H., and R. Swanstrom.** 1982. Replication of retroviruses, p. 369–512. *In* R. Weiss, W. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Voynow, S. L., and J. M. Coffin.** 1985. Evolutionary variants of Rous sarcoma virus: large deletion mutants do not result from homologous recombination. J. Virol. **55:**67–78.
42. **Yaciuk, P., and D. Shalloway.** 1986. Features of the pp60$^{v\text{-}src}$ carboxyl terminus that are required for transformation. Mol. Cell. Biol. **6:**2807–2819.