cis-Acting Regulatory Elements within *gag* Genes of Avian Retroviruses

SALVATORE ARRIGO, MARY YUN,[†] AND KAREN BEEMON*

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Received 4 June 1986/Accepted 16 September 1986

A *cis*-acting enhancer element has been detected within the *gag* gene of several avian retroviruses, including Rous sarcoma virus, Fujinami sarcoma virus, and the endogenous Rous-associated virus-0. A consensus enhancer core sequence, GTGGTTTG, is present in all of these viral genomes, approximately 900 bases downstream from the site of initiation of transcription. When an internal fragment derived from the *gag* gene of any of these viruses (spanning nucleotides 533 to approximately 1149) was inserted into a plasmid containing the chloramphenicol acetyltransferase (*cat*) gene under control of the simian virus 40 promoter, 9- or 21-fold enhancement of CAT expression was observed after transfection into mouse L cells and chicken embryo fibroblasts, respectively. This enhancement was not dependent on the position of insertion of the *gag* fragment into the plasmid. However, there was a strong dependence on orientation, with higher levels of CAT expression in constructs in which the 5' end of the *gag* fragment was nearest to the promoter, suggesting a possible negative regulatory element at the 3' end of this fragment. Deletion of the 3' end of the insert resulted in a *gag* fragment, containing nucleotides 533 to 1017, which enhanced expression equally in either orientation. When the *gag* fragment was inserted into a plasmid containing the *cat* gene under the control of an intact Rous sarcoma virus long terminal repeat, it induced a two- to threefold increase in CAT activity and CAT mRNA levels. Translation of the *gag* fragment did not appear to be necessary for the observed enhancement, since two insertional mutations resulting in frameshifts in the *gag* insert did not affect CAT expression. However, deletion of a 330-base internal fragment from the *gag* insert restored a basal level of CAT activity. These results suggest that retroviruses have regulatory elements within their genes distinct from those in the long terminal repeats that flank the genes.

Previous work on the regulation of retroviral gene expression has focused on the long terminal repeats (LTRs) at both ends of the integrated proviral DNA. The LTRs contain sequences regulating both initiation and termination of transcription (46). Both transcriptional enhancers and promoters have been characterized within the unique 3' (U3) region of retroviral LTRs (6, 13, 24, 25, 28, 46). In addition to promoting transcription of viral genes, the LTRs of a number of different retroviruses are capable of activating the transcription of contiguous cellular genes, sometimes resulting in neoplasia (16, 32). When the strengths of different avian retroviral LTR enhancers are compared, the LTR of the endogenous avian virus Rous-associated virus-0 (RAV-0) is less than 10% as active as the LTR of Rous sarcoma virus (RSV), whereas the LTR of Fujinami sarcoma virus (FSV) has an intermediate level of expression (5, 7, 44, 45, 51).

There are several suggestions that sequences within the retroviral genome may also be important in regulating viral gene expression. For example, sequences in the 3' noncoding region of the RSV genome augment the enhancer activity of the 3' LTR (25, 28). Furthermore, footprinting analysis of the murine mammary tumor virus genome with the glucocorticoid receptor reveals several sites of receptor-DNA interaction both within the LTR and within the transcribed region of the viral genome (33).

The possibility of an enhancer sequence within the *gag* gene of avian viruses is particularly intriguing because determinants of disease tropism, congenital virus transmis-

sion, gene expression, and transforming potential have been mapped either within the *gag* gene or in the *gag-pol* region (26, 35–37, 44, 45). A transcriptional activator has also been proposed to be encoded by the 5' half of the RSV *gag* gene, in an alternate reading frame (2).

We tested a portion of the *gag* gene including a consensus enhancer core sequence (GTGGTTTG) (22, 52) for enhancer activity initially by introducing it into plasmid pSV1cat. This plasmid contains the *Escherichia coli* chloramphenicol acetyltransferase (*cat*) gene under control of the simian virus 40 (SV40) promoter but lacks most of the SV40 enhancer (14). In transient expression assays in both mouse L cells and chicken embryo fibroblasts (CEFs), we observed 9- to 21-fold enhancement of CAT activity in plasmids containing the *gag* gene fragment. The same effect was seen whether the *gag* fragment was derived from RSV, FSV, or RAV-0. Enhancement was independent of the position of the *gag* insert in the pSV1cat plasmid. However, there was a pronounced orientation dependence with a *gag* fragment containing nucleotides 533 to 1189 (FSV) or 533 to 1149 (RSV). This orientation dependence was not observed when a slightly smaller FSV insert was used (533 to 1017). Furthermore, the *gag* enhancer appears to act with the RSV LTR enhancer; constructs having both the LTR and *gag* enhancer synthesized two- to threefold more CAT mRNA than those with the LTR alone. These data suggest that avian retroviruses may have multiple *cis*-acting enhancer elements, one of which is within the *gag* gene.

(This research was initiated by M. Yun in partial fulfillment of the requirements for a B.A. with Honors in Biology from the Johns Hopkins University.)

* Corresponding author.

[†] Present address: University of Michigan School of Medicine, Ann Arbor, MI 48109.

FIG. 1. Construction of plasmids containing various *gag* fragments inserted into pSV1cat. (A) Diagram of the 5' region of an avian retrovirus genome and the *gag* fragments that were cloned into *cat* plasmids for use in transient expression assays. FSV656 spanned 656 bp (*Bam*HI to *Pvu*I) of FSV *gag*. FSV656+2 and FSV656+10 contained frameshift insertions of 2 and 10 bp, respectively, at the *Nar*I site. FSV656-330 was deleted for 330 bp (from *Ava*I to *Ava*I) and retained 326 bp of FSV *gag*-specific sequences. FSV484 spanned 484 bp of FSV *gag* (*Bam*HI to *Nae*I). Pr-C, SRA, and RAV-0 were similar *gag* fragments from other avian retroviruses (*Bam*HI to *Bcl*I). (B) Structure of pSV1cat, into which the *gag* fragments were inserted. Inserts at the *Nde*I site were denoted as N⁺ or N⁻ and inserts at the *Bam*HI site were denoted as B⁺ or B⁻ depending on the transcriptional orientation of the fragment with respect to the SV40 early promoter and the *cat* gene.

MATERIALS AND METHODS

Construction of recombinant plasmids. pSV2cat was obtained from B. Howard (14). To remove the bulk of the SV40 enhancer, this plasmid was cleaved at the *Acc*I and *Sph*I sites at bases 198 and 520, respectively. After treatment with the Klenow fragment of DNA polymerase I to generate blunt ends, the plasmid was religated. The resulting plasmid, pSV1cat, is shown in Fig. 1B. Plasmid pRSVcat, constructed by Gorman et al. (13), contains the *cat* gene under the control of a Schmidt-Ruppin strain D (SRD) RSV LTR enhancer and promoter.

The FSV656 *gag* fragment (Fig. 1A) was obtained from a subclone of FSV, pBR-F26, which was provided by M. Shibuya and H. Hanafusa (41). A 656-base-pair (bp) *Bam*HI-*Pvu*I fragment, mapping from nucleotide 533 to 1189 on the FSV genome (40), was first inserted at a unique *Bam*HI site in pSV1cat. After an initial ligation between *Bam*HI cohesive ends present in the vector and insert, blunt ends were

generated and ligated. Restriction endonuclease mapping of resultant clones showed that the constructs obtained had the *gag* fragment inserted in both the positive (sense) and negative (antisense) transcriptional orientations relative to the SV40 early promoter and the *cat* gene (Fig. 1B). These constructs were termed pFSV656B⁺ and pFSV656B⁻, respectively. The same FSV *gag* fragment was also inserted into pSV1cat at a unique *Nde*I site by blunt end ligation to generate pFSV656N⁺ and pFSV656N⁻ (Fig. 1B).

The FSV484 *gag* fragment, a 484-bp *Bam*HI-*Nae*I fragment spanning bases 533 to 1017 of FSV, was also obtained from pBR-F26. This fragment was inserted into the *Bam*HI and *Nde*I sites of pSV1cat in the same way that the FSV656 constructs were generated. The positive-orientation constructs at the *Bam*HI and *Nde*I sites of pSV1cat were termed pFSV484B⁺ and pFSV484N⁺, respectively. The negative-orientation construct at the *Nde*I site was termed pFSV484N⁻.

The pFSV656+2 frameshift mutant was derived from pFSV656B⁺. This clone was cut at the unique *Nar*I site at base 789 of FSV; blunt ends were generated with the Klenow fragment of DNA polymerase I, and the plasmid was religated. The mutated region of the FSV656+10 frameshift mutant was derived in a similar way, with 8-bp *Bam*HI linkers (New England Biolabs) included during ligation. The pFSV656+10 construct was sequenced by the dideoxynucleotide method for sequencing plasmids (49) to ensure that only a single 8-bp linker was inserted. The pFSV656-330 deletion mutant was also derived from pFSV656B⁺ by restriction digestion with *Ava*I, which cuts twice within the insert at bases 631 and 961 of FSV. Blunt ends were generated and religated.

The Pr-C *gag* fragment (Fig. 1A) was derived from pATV8, a full-length clone of the Prague C (Pr-C) strain of RSV, obtained from R. Guntaka (21). A 617-bp *Bam*HI-*Bcl*I fragment, mapping from nucleotide 533 to 1149 on the Pr-C genome (39), was isolated. The Schmidt-Ruppin strain A (SRA) and RAV-0 *Bam*HI-*Bcl*I *gag* fragments (Fig. 1A) were likewise isolated from pSRA-2 (8), obtained from J. M. Bishop, and from an infectious clone of pRAV-0, obtained from P. Tsichlis, respectively. The *Bam*HI site is conserved among all four avian retroviruses used in this study, while the *Bcl*I site is not present in FSV (39, 40, 43, 48). These *gag* fragments were inserted into the *Bam*HI site of pSV1cat. Restriction endonuclease mapping of the resultant clones indicated that both positive (B⁺) and negative (B⁻) orientations were obtained for all inserts.

To generate pRSV(gag)cat, the SRA *gag* fragment was inserted into the *Bam*HI site of pRSVcat (13). This was accomplished by digesting both pSRAB⁺ and pRSVcat with *Bam*HI and *Nde*I. Both enzymes cut each plasmid once (Fig. 1B), so that religation of a mixture of the two digested plasmids led to construction of the chimeric plasmid pRSV(gag)cat. In this construct, the SRA *gag* fragment is positioned downstream of the *cat* gene in a positive (sense) orientation with respect to the RSV LTR promoter and *cat* gene. The chimeric plasmid differs from pRSVcat only by the addition of the SRA *gag* fragment at the *Bam*HI site.

pRSV-E(gag)cat was derived from pRSV(gag)cat by digestion with *Mst*II and *Sph*I, generation of blunt ends with T4 polymerase, and subsequent ligation. The resultant plasmid, pRSV-E(gag)cat, lacks the SRD LTR enhancer but contains the promoter regions (6). Since it was derived from pRSV(gag)cat, it retains the SRA *gag* fragment downstream of the *cat* gene. pRSV-Ecat was constructed by removal of the SRA *gag* fragment from pRSV-E(gag)cat. This was

accomplished by digesting pRSV-E(gag)cat and pRSVcat with *Bam*HI and *Nde*I. Religation of a mixture of the two digested plasmids led to the isolation of pRSV-Ecat.

Cell culture and DNA transfection. Secondary CEFs were cultured in medium 199 (Gibco Laboratories) supplemented with 1% calf serum, 1% chicken serum, and 2% tryptose phosphate broth (Gibco). Prior to transfection, they were maintained in Dulbecco modified Eagle medium (DME) containing 5% fetal calf serum (both from GIBCO). Mouse Ltk⁻ cells were obtained from G. Scangos and cultured in DME supplemented with 5% fetal calf serum. Cells were allowed to achieve 50 to 95% confluence on 6-cm dishes prior to transfection. DNA transfections into both CEFs and Ltk⁻ cells were carried out in the presence of DEAE-dextran (200 µg/ml), followed 4 to 6 h later by a 1.5-min shock with 10% dimethyl sulfoxide as described by Lopata et al. (27). Eight micrograms of purified plasmid DNA from the plasmids driven by the SV40 promoter or 2 µg of the RSV LTR constructs, in 1.5 ml of DME without serum, was transfected onto each 6-cm plate.

CAT assay. The CAT assay was performed by the protocol of Gorman et al. (14) approximately 40 h after transfection. Half the cells on each plate were used for DNA quantitation. The remaining cells were lysed by freeze-thawing for the CAT assay. Reactions were performed for 1 h at 37°C. Acetylation of [¹⁴C]chloramphenicol (New England Nuclear Corp.) was monitored by chromatography on silica gel thin-layer plates (E. M. Reagents) and subsequent scintillation counting of the acetylated and nonacetylated forms of [¹⁴C]chloramphenicol.

DNA quantitation. CAT assay results were normalized to levels of intracellular plasmid DNA on the same plate of transfected cells. The amount of intracellular plasmid DNA present 40 h after transfection was determined by a modification of the procedure of Alwine (1). The cells were incubated with 50 µg of pancreatic DNase (Sigma Chemical Co.) per ml in DME lacking serum for 1 h at 37°C and washed with phosphate-buffered saline. The cells were then scraped from the plate and divided into two equal portions. One half of the cells was used for the CAT assay and the other half for DNA quantitation. To quantitate intracellular plasmid DNA, cells were pelleted by low-speed centrifugation and suspended in 50 mM Tris hydrochloride (pH 7.4)–10 mM EDTA, and plasmid DNA was isolated as described by Hirt (18). DNA was linearized with *Hind*III and electrophoresed on a 0.8% agarose gel. After blotting onto nitrocellulose (42), hybridization was performed with an *Hind*III-*Eco*RI *cat* gene-specific probe, radiolabeled by the method of Feinberg and Vogelstein (12). The amount of intracellular plasmid DNA in each sample was quantitated either directly by scintillation counting of the radioactive bands or by densitometry of the resultant autoradiogram.

RNA isolation and mapping. Two riboprobe templates were constructed which spanned the 5' and 3' ends of the expected CAT mRNA from pRSVcat. The 5'-specific template contained the 344-bp *Eco*RI fragment of pRSVcat (see Fig. 4A) cloned into the *Eco*RI site of pGEM-2 (Promega Biotech). The 3'-specific template contained the 851-bp *Hpa*II-*Bam*HI fragment of pRSVcat (see Fig. 4A) cloned into the *Acc*I-*Bam*HI site of pGEM-2; this DNA template was digested with *Hin*fI to define a 542-base SP6 polymerase-directed riboprobe. Transcription of plasmids with SP6 (or T7) polymerase and hybridization of the resulting riboprobes to total cellular RNA was performed as previously described (30). Plates (9 cm) of CEFs were transfected with 18 µg of DNA by the DEAE-dextran procedure (27). At

TABLE 1. Comparison of CAT activity of plasmids driven by the SV40 promoter and containing either the SV40 enhancer or the FSV 656-bp *gag* fragment

Plasmid	Enhancer	Relative CAT activity ^a	
		Ltk ⁻ cells	CEF cells
pSV1cat	None	1.0	1.0
pSV2cat	SV40	6.8	104
pFSV656B ⁺	FSV656 <i>gag</i>	9.0	20.6

^a Averages of three or more experiments, normalized with respect to intracellular plasmid DNA levels. All activities are presented relative to that of pSV1cat (= 1.0). Similar relative activities were observed in 10 additional experiments which were not normalized to intracellular levels of plasmid DNA.

48 h after transfection, the cells were treated with DNase as described above and scraped from the plates. One-sixth of the cell suspension was removed for CAT and DNA analyses. RNA was isolated from the remaining cells by lysis in guanidinium isothiocyanate buffer and pelleting through a 5.7 M CsCl cushion (3). Total recovered RNA was hybridized to either the 5' or 3' riboprobe. After digestion with RNases A and T₁ and ethanol precipitation, the protected fragments were denatured in 95% formamide for 5 min at 90°C and fractionated on a 4% acrylamide-urea sequencing gel (29).

RESULTS

Increase in CAT activity induced by an FSV *gag* fragment.

To test whether the *gag* gene of FSV contained a transcriptional enhancer element, a 656-bp *gag* fragment, FSV656 (containing nucleotides 533 to 1189 from the 5' cap), was cloned into pSV1cat at the *Bam*HI site (Fig. 1A). Transcription of the *cat* gene in pSV1cat is driven by the SV40 promoter, but the enhancer has been deleted at the *Sph*I site (14). The resulting plasmid, pFSV656B⁺, contained the 5' end of the *gag* fragment proximal to the 3' end of the *cat* gene of pSV1cat but downstream of the *cat* mRNA polyadenylation site (Fig. 1B). This orientation resembles the native configuration of the *gag* fragment within the retroviral genome—it is downstream and in a sense orientation with respect to the promoter.

pFSV656B⁺ was transfected into Ltk⁻ cells and into CEFs in parallel with pSV1cat, which lacks an enhancer. Control transfections were also performed with pSV2cat, which contains the SV40 enhancer (14). One half of the cell suspension from each plate was assayed for CAT activity, and the other half was used to quantitate the amount of intracellular plasmid DNA. The results are presented in Table 1 as CAT activity normalized to intracellular plasmid DNA levels. Relative to pSV1cat, pFSV656B⁺ expressed high levels of CAT enzymatic activity in both cell types: its activity was elevated 21-fold in CEFs and 9-fold in Ltk⁻ cells. Furthermore, the activity of the construct with the *gag* fragment was slightly higher than the activity induced by pSV2cat in Ltk⁻ cells and approximately 20% of the activity of pSV2cat in CEFs.

Quantitation of intracellular plasmid DNA in DEAE-dextran-transfected cells. To correct for any variation in transfection efficiencies which might affect CAT enzyme levels, the amount of intracellular plasmid DNA was measured in a portion of the cell suspension from each transfected plate of cells. Alwine has demonstrated that the amount of total intracellular plasmid DNA detected by Southern analysis of a Hirt supernatant is proportional to the

FIG. 2. Comparison of plasmid DNA extracted from transfected Ltk⁻ cells with and without prior DNase treatment. Identical plates of cells were transfected with 5 or 10 µg of pFSV656 DNA. At 40 h posttransfection, cells were treated with DNase I or scraped from the plate without treatment. Hirt supernatant DNA was linearized with *Hind*III, electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized to a *cat*-specific probe. The major band is the linear plasmid DNA.

amount of plasmid DNA in the nucleus (1). Furthermore, he found no effect of prior DNase treatment on levels of plasmid DNA measured 22 h or later after transfection by calcium phosphate precipitation (1).

Since we used the DEAE-dextran transfection procedure, we needed to determine whether this method leads to retention of plasmid DNA on the cell surfaces. Five or ten micrograms of a pFSV656cat construct was transfected in duplicate by the DEAE-dextran method onto Ltk⁻ cells. After 40 h, half the plates were treated with DNase and the other half were not. Hirt supernatant DNA was subsequently isolated and analyzed by the method of Southern (18, 42) (Fig. 2). The samples which were not treated with DNase contained a much higher level of full-length and degraded DNA than did the DNase-treated samples. This suggested that a large amount of plasmid DNA remained on the cell surface 40 h after transfection with DEAE-dextran. Similar results were obtained by DEAE-dextran-mediated transfection of CEFs (data not shown). Consequently, in all CAT experiments, the cells were treated with DNase prior to quantitation of intracellular plasmid DNA.

CAT activity in Ltk⁻ cells transfected with from 4 to 20 µg of pFSV656B⁺ DNA per 6-cm plate was assayed and compared with levels of intracellular plasmid DNA in the same cells (Fig. 3). Approximately 0.02% of the input DNA was detected intracellularly 40 h after transfection. A proportional increase in both CAT activity and intracellular DNA levels was observed over this DNA concentration range, so that the CAT activity per nanogram of intracellular DNA was nearly constant (Fig. 3). Therefore, it appears that the DEAE-dextran transfection efficiency is proportional to the level of plasmid DNA in the Hirt supernatant. Similar results

TABLE 2. Orientation dependence of FSV656 *gag* fragment enhancement of CAT activity

Plasmid	pSV1cat cloning site	FSV656 fragment orientation ^a	Relative CAT activity ^b
pFSV656B ⁺	<i>Bam</i> HI	+	9.0 ± 1.7
pFSV656B ⁻	<i>Bam</i> HI	-	2.4 ± 0.8
pFSV656N ⁺	<i>Nde</i> I	+	2.2 ± 0.6
pFSV656N ⁻	<i>Nde</i> I	-	8.7 ± 0.3

^a With respect to the *cat* gene and SV40 early promoter (see Fig. 1).

^b Transient expression assays were performed in Ltk⁻ cells. Cat activities, normalized for intracellular plasmid DNA levels, are presented relative to the activity of pSV1cat (= 1.0). The mean and variance from the mean are presented for three experiments.

have been obtained with pRSV constructs in CEFs (data not shown). Moreover, we did not observe saturation of CAT expression with input DNA concentrations of up to 20 µg per 6-cm dish. In contrast, Weintraub et al. (53) observed saturation of CAT activity but not of DNA uptake at 2 µg per 10-cm tissue culture dish. Our failure to see saturation of expression may reflect differences in transfection procedures. After DEAE-dextran transfection, we subjected cells to a shock with 10% dimethyl sulfoxide, whereas Weintraub et al. (53) treated cells with chloroquine.

Orientation dependence of FSV656 constructs. To determine whether the FSV *gag* fragment behaved like a typical enhancer element, exerting its effect regardless of its position or orientation relative to the promoter (22), four different FSV656cat constructs were generated and transfected into Ltk⁻ cells. Two of these, pFSV656B⁺ and pFSV656B⁻, contained the FSV656 *gag* fragment inserted into the *Bam*HI site of pSV1cat, and two others, pFSV656N⁺ and pFSV656N⁻, contained the FSV656 *gag* fragment in the *Nde*I site of pSV1cat, which is upstream of the promoter (Fig. 1). At 40 h after transfection, the cells were assayed for CAT activity, which was normalized to intracellular plasmid DNA levels (Table 2). Depending on the orientation of the FSV656 fragment relative to the promoter, a fourfold varia-

FIG. 3. Correlation of levels of intracellular plasmid DNA with CAT activity in transfected Ltk⁻ cells. Identical plates of cells were transfected with 4 to 20 µg of pFSV656B⁺ DNA (input DNA) by the DEAE-dextran method. One half of each plate was assayed for intracellular DNA levels, which were quantified by scintillation counting of bands excised from nitrocellulose in comparison to 0.2- and 2-ng DNA standards. The other half of each plate of cells was assayed for CAT activity. The two monoacetylated forms (3Ac and 1Ac) as well as nonacetylated chloramphenicol (Cm) are indicated.

TABLE 3. CAT activity of plasmids containing 484-bp subclones of the FSV *gag* fragment

Plasmid	pSV1cat cloning site	FSV484 fragment orientation ^a	Relative CAT activity ^b
pFSV484B ⁺	<i>Bam</i> HI	+	5.6 ± 0.6
pFSV484N ⁺	<i>Nde</i> I	+	5.3 ± 0.5
pFSV484N ⁻	<i>Nde</i> I	-	5.6 ± 0.4

^a Orientation with respect to the pSV1cat *cat* gene and the SV40 early promoter (see Fig. 1).

^b Assays were performed as described for Table 2, footnote *b*. Activity is relative to that of pSV1cat (= 1.0).

tion in the level of CAT activity was observed. An 8.9-fold increase in CAT activity was seen when the 5' end of the *gag* insert was proximal to the *cat* gene and the SV40 promoter, regardless of the pSV1cat restriction site into which it was inserted (pFSV656B⁺ and pFSV656N⁻). However, only an approximately 2.3-fold increase over the basal CAT activity of pSV1cat was seen when the insert was in the opposite orientation (pFSV656B⁻ and pFSV656N⁺). Therefore, CAT activity enhancement mediated by the *gag* fragment was not dependent on the position of the insert in the plasmid, but a pronounced orientation dependence was observed at each position. When the *gag* fragment was inserted at the *Bam*HI site, downstream of the polyadenylation site for CAT mRNA, the preferred orientation of the *gag* fragment was the sense orientation. This orientation and position of *gag* are most like those in the native virus.

Localization of the FSV *gag* enhancer element. To begin localization of the putative enhancer element within the 656-bp *gag* fragment, a set of subclones and a deletion mutant were constructed. To create subclones of the FSV *gag* fragment, a 484-bp fragment containing nucleotides 533 to 1017 was cloned into the *Bam*HI and *Nde*I sites of pSV1cat (Fig. 1). Three of four possible constructs, pFSV484B⁺, pFSV484N⁺, and pFSV484N⁻, were isolated and transfected into Ltk⁻ cells. The results of CAT assays done on these subclones are shown in Table 3. All three subclones showed 5.3- to 5.6-fold increases in relative CAT activity without exhibiting the marked orientation dependence of the FSV656cat constructs. Although the orientation dependence had been eliminated in these constructs, there appeared to be a 40% overall reduction in the maximal level of CAT activity produced by pFSV484 constructs compared with pFSV656 constructs. Enhancement with the pFSV484 constructs demonstrated the independence from effects of position and orientation which is characteristic of enhancer elements (22).

pFSV656-330 was derived from pFSV656B⁺ by deletion of a 330-bp fragment (nucleotides 631 to 961) which includes the consensus enhancer core sequence. This deletion mutant, which retains approximately 300 bases of FSV *gag*-specific sequences, was transfected into Ltk⁻ cells. The CAT activity level induced by this mutant was comparable to that of pSV1cat (Fig. 3A). Therefore, an essential element(s) of the enhancer is localized between nucleotides 631 and 961.

We also deleted the region from the *Bam*HI to the *Nar*I site (nucleotides 533 to 789) in the *gag* insert of pSRAB⁺. Transfection of this plasmid into CEFs resulted in a substantial decrease in CAT activity relative to its parental plasmid (data not shown).

A *trans*-activating protein is not responsible for the increased CAT activity in pFSV656cat constructs. Broome and Gilbert have proposed that a transcriptional activator protein

is encoded in an alternate reading frame within the *gag* gene of RSV (2). The FSV *gag* fragment used in our study includes the body of the sequence encoding this proposed activator protein, and its nucleotide sequence is highly homologous to that of RSV throughout this region (39, 40). It was considered possible that the *gag* inserts in the *cat* plasmids might lead to synthesis of an activator protein either by bidirectional transcription from the SV40 promoter (47) (in pFSV656N⁻) or by readthrough of the pSV1cat polyadenylation signal (in pFSV656B⁺).

To assess the possibility that such a transcriptional activator protein might be acting to enhance CAT levels in pFSV656cat constructs, frameshift mutations of +2 and +10 were constructed at a unique *Nar*I site within the *gag* fragment of pFSV656B⁺ to create pFSV656+2 and pFSV656+10, respectively (Fig. 1A). A +2 frameshift at the same site in the RSV *gag* gene was shown by Broome and Gilbert to significantly reduce the activity of the *trans*-activating protein in RSV, while a +10 frameshift at this site eliminated it (2). After transfection into Ltk⁻ cells, neither of our frameshift mutants demonstrated a reduction in CAT activity relative to its parent plasmid (Fig. 4). Therefore, the enhanced CAT activity in pFSV656B⁺ does not appear to be mediated by a *trans*-activating protein. We conclude that the enhanced CAT activity is the result of a *cis*-acting element in the *gag* fragment.

FSV *gag* enhancer element common to other avian retroviruses. To investigate the possibility that other avian retroviruses might contain orientation-dependent enhancer elements within their *gag* sequences, similar *gag* fragments were isolated from three other cloned avian retroviruses: the Pr-C and SRA strains of RSV and the endogenous virus RAV-0. These *gag* fragments contained the same 5' *Bam*HI restriction site as the FSV constructs and approximately 40 bp less than the FSV fragment in pFSV656 at their 3' ends; they were cloned into the *Bam*HI site of pSV1cat (Fig. 1). CAT constructs which contained the *gag* fragment in a positive orientation with respect to the *cat* gene and promoter were designated pPrCB⁺, pSRAB⁺, and pRAV-0B⁺, and those which contained the *gag* fragment in the opposite orientation were designated pPrCB⁻, pSRAB⁻, and pRAV-0B⁻, respectively. After transfection into Ltk⁻ cells, an orientation-dependent enhancement of CAT activity nearly identical to that of pFSV656B⁺ and pFSV656B⁻ was seen with all of the new constructs (Table 4).

This experiment indicates that the *gag* enhancer activity is not specific to FSV but is found also in RSV, as was expected from the strong nucleotide sequence homology between the FSV and RSV *gag* genes (39, 40). More surprising was the finding that the RAV-0 *gag* gene had enhancer activity at a level indistinguishable from that of exogenous viruses, both in mouse L cells (Fig. 4) and in CEFs (A. Lin and K. Beeman, unpublished results). Furthermore, since the RSV and RAV-0 *gag* inserts lacked 40 nucleotides present at the 3' end of the FSV656 *gag* inserts, the results suggest that these 3'-terminal nucleotides are not required either for enhancer activity or for determination of orientation dependence.

***gag* fragment-induced increase in CAT activity is consistent with the level of CAT mRNA.** To examine CAT mRNA levels within transfected cells, the SRA *gag* fragment was cloned into pRSVcat. There were two main advantages to using this construction. In pRSV(gag)cat (Fig. 5A), the SRA *gag* fragment is positioned downstream and in the same orientation as the SRD LTR. In this respect, it closely resembles the configuration of the *gag* fragment in the intact virus. The

FIG. 4. Representative CAT assays performed in Ltk⁻ cells. (A) Demonstration of the increase in CAT activity induced by the FSV *gag* fragment (pFSV656B⁺). pFSV656+2 is identical to pFSV656B⁺ except for a 2-bp insertion at the *Nar*I site in the *gag* fragment. pFSV656-330 contains the 656-bp *gag* fragment with a 330-bp internal deletion. The small apparent increase in pFSV656-330 CAT activity relative to that of pSV1cat was due to an increased level of intracellular plasmid DNA. pSV2cat and pRSVcat were included as positive controls; pSV1cat was included as a negative control. (B) Demonstration that pFSV656+10, containing a 10-bp insertion at the *Nar*I site of pFSV656B⁺, does not decrease the level of CAT activity. See the legend to Fig. 3 for abbreviations.

other advantage is the high level of transcription of pRSVcat when transfected into CEFs (13). This high level of CAT expression facilitated our detection of CAT mRNA. pRSV(gag)cat, pRSVcat, and pSV1cat DNAs were transfected into CEFs, and total RNA was prepared. pSV1cat was included as a negative control since it expressed a low level of CAT activity in CEFs.

Quantitative mapping of the 3' termini of these RNAs demonstrated a 2.9-fold increase in pRSV(gag)cat CAT mRNA relative to pRSVcat (Fig. 5B). Both of these mRNAs were shown to have the correct 3' termini. pSV1cat did not show a detectable level of CAT mRNA. CAT assays showed a 2.5-fold increase in CAT activity of pRSV(gag)cat relative to pRSVcat (Fig. 5C), while the amounts of intracellular plasmid DNA were nearly identical (Fig. 5D). In a similar experiment with a 5'-specific riboprobe, correct initiation of pRSV(gag)cat, as well as a 2.2-fold increase in CAT mRNA relative to pRSVcat, was observed (Fig. 5B). CAT assays for this experiment indicated a 2.1-fold increase in the CAT activity of pRSV(gag)cat relative to pRSVcat while compa-

table levels of intracellular plasmid DNA were observed (data not shown).

The CAT mRNA of pRSV(gag)cat appears to be unaltered from that of pRSVcat; that is, the 5' and 3' termini of the mRNA were identical to those of pRSVcat. Therefore, the increase in levels of CAT mRNA cannot easily be explained in terms of differential mRNA stability or insertion of a new promoter. Since the increased amounts of pRSV(gag)cat mRNA were proportional to the increased levels of CAT enzymatic activity, we conclude that the *gag* fragments are exerting their effects at the level either of transcription or of stabilization of the primary transcript.

***gag* enhancer element directs a fivefold increase in CAT mRNA levels from an RSV promoter.** The SRA *gag* enhancer was shown above to induce a two- to threefold increase in the level of CAT mRNA and CAT enzymatic activity when inserted into pRSVcat. We were also interested in determining the effect of the *gag* fragment on the RSV LTR promoter in the absence of the LTR enhancer. Therefore, an *Mst*II-*Sph*I fragment was deleted from the SRD LTR of pRSVcat and pRSV(gag)cat; deletion of a similar fragment from the SRB strain of RSV results in removal of the LTR enhancer sequences while leaving promoter sequences intact (6). The two constructs lacking the LTR enhancer, as well as the enhancer-containing parental plasmids, were transfected into CEFs. CAT assays and DNA analyses were performed (Table 5).

Transfection of plasmid pRSV-E(gag)cat, which contains the *gag* enhancer and the RSV LTR promoter, was found to result in five times more CAT enzymatic activity than pRSV-Ecat, which has the RSV LTR promoter alone. pRSVcat, which has an intact LTR, directed the synthesis of more than 20 times the CAT activity of pRSV-Ecat. In pRSV(gag)cat, which has an intact LTR and a *gag* insert, the CAT activity observed was 50 times that of pRSV-Ecat.

The 5' and 3' ends of the CAT mRNA synthesized from the pRSV-E(gag)cat and pRSV-Ecat templates were also mapped and shown to be identical to those of pRSVcat (Fig. 6). While the levels of pRSV-Ecat and pRSV-E(gag)cat

TABLE 4. CAT activity of constructs containing other avian retrovirus *gag* fragments

Plasmid	<i>gag</i> fragment orientation ^a	Relative CAT activity ^b (%)
pPrCB ⁺	+	108 ^c
pPrCB ⁻	-	21 ^c
pSRAB ⁺	+	90
pSRAB ⁻	-	19
pRAV-0B ⁺	+	99
pRAV-0B ⁻	-	24 ^c

^a Fragments were inserted into the *Bam*HI site of pSV1cat. Orientation is determined relative to the *cat* gene.

^b CAT assays were carried out as described for Table 2, footnote b. Data are presented relative to the activity of pFSV656B⁺ (= 100%).

^c DNA quantitation was not performed in these cases; the data represent the average of three experiments.

mRNAs detected were too low for accurate quantitation, we observed the following hierarchy of RNA levels in four different experiments: pRSV-Ecat < pRSV-E(gag)cat < pRSVcat < pRSV(gag)cat (Fig. 5 and 6).

The 3' termini of CAT mRNA synthesized from pFSV656B⁺ and pSV1cat templates transfected into Ltk⁻ cells were also determined to be the same as those of pRSVcat mRNA (Fig. 6). Although the RNA signals were difficult to detect from these constructs, there was clearly more CAT mRNA synthesized from the plasmid having the *gag* insert (pFSV656B⁺) than from pSV1cat (Fig. 6). In summary, these experiments indicate that the *gag* enhancer

TABLE 5. CAT activity of constructs containing the LTR or *gag* enhancer or both

Plasmid	LTR enhancer	<i>gag</i> enhancer	Relative CAT activity ^a
pRSV-Ecat	—	—	1
pRSV-E(gag)cat	—	+	5
pRSVcat	+	—	23
pRSV(gag)cat	+	+	52

^a DNA was transfected into CEFs. CAT activity was assayed and normalized to intracellular DNA levels. Data are presented relative to pRSV-Ecat (= 1.0).

is capable of acting alone, with either the RSV or SV40 promoter, or in concert with the RSV LTR enhancer.

DISCUSSION

We have identified a fragment of DNA within the *gag* region of four avian retroviruses which is capable of acting as a transcriptional enhancer in transient expression assays in mouse and chicken cells. Under direction of the SV40 promoter, 9- to 21-fold increases in CAT activity were induced by insertion of the *gag* fragment at two different sites in the plasmid pSV1cat. Under the direction of the RSV promoter, the *gag* fragment alone was about one-fourth as active as the LTR enhancer in CEFs. However, when the *gag* fragment was introduced into pRSVcat, which has an intact LTR promoter and enhancer, it was capable of elevating the high levels of CAT activity of pRSVcat an additional two- to threefold.

This is the first characterization of an enhancer element within a transcribed region of a vertebrate retrovirus. However, the recent observation of similar enhancers within the epsilon region of the *Saccharomyces cerevisiae* Ty transposable elements (11, 38), which are structurally related to retroviruses (4), suggests that they may be a conserved feature present in other retroviruses.

A number of different enhancer sequences, including those of SV40, polyomavirus, the Moloney murine sarcoma virus LTR, and the mouse immunoglobulin heavy-chain gene, share a short consensus enhancer "core" sequence, GTGGAAAG (22, 52). In the SV40 enhancer, specific mutations in this sequence lead to decreased levels of enhance-

FIG. 5. Effects of the SRA *gag* fragment on CAT mRNA transcription, CAT enzymatic activity, and intracellular DNA levels in transfected CEFs. The same plate of cells was used for panels B (3'), C, and D. (A) Restriction endonuclease map of pRSV(gag)cat and pRSVcat, showing the insertion site of the SRA *gag* fragment into pRSVcat and the riboprobes used to quantitatively map 5' and 3' ends of the CAT mRNA. Also shown are the expected sizes (in bases) of the protected fragments resulting from correctly initiated and terminated CAT mRNA. Abbreviations: R, *Eco*RI; H, *Hpa*II; F, *Hin*II; B, *Bam*HI. (B) Results of quantitative RNA mapping with 5' and 3' antisense riboprobes and RNA extracted from transfected CEFs. Lanes: a, pRSV(gag)cat; b, pRSVcat; c, pSV1cat; d, no RNA; M, DNA molecular weight markers; P, 542-base 3' riboprobe. RNA levels transcribed from pRSV(gag)cat were determined by densitometric scanning to be an average of 2.4 times higher than those from pRSVcat in four independent experiments. Sizes are indicated (in bases). (C) Results of CAT assays of transfected CEFs. Lanes: a, pRSV(gag)cat; b, pRSVcat; c, pSV1cat. See the legend to Fig. 3 for abbreviations. (D) Levels of intracellular plasmid DNA. Equal portions of *Hind*III-digested Hirt supernatant DNA from transfected CEFs were electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized to a *cat*-specific probe. Lanes: a, pRSV(gag)cat; b, pRSVcat; c, pSV1cat.

FIG. 6. 5' and 3' mapping of CAT mRNA transcripts. Probes identical to those in Fig. 5 were used to quantitatively map the 5' and 3' ends of CAT mRNA from transfected CEFs. Lanes: a, no RNA; b, pSV1cat; c, pFSV656B⁺; d, pRSV-Ecat; e, pRSV-E(gag)cat; f, pRSVcat. *, Undigested probe. Sizes are indicated (in bases).

ment (17, 52, 55). This consensus sequence is also present in the LTRs of some avian retroviruses, such as Y73 avian sarcoma virus (ASV) (23). The sequence is imperfectly represented in the Pr-C RSV LTR (GTGGTACG) (39), however, and the closest fits to the core sequence in the FSV and RAV-0 LTRs are GTGGCGTG and GTGGCGAC, respectively (19, 40). In contrast to these relatively poor fits, we observed GTGGTTTG, a perfect match with the consensus sequence, within the *gag* genes of RSV, FSV, and RAV-0 at positions ranging from 900 to 912 nucleotides downstream from the 5' termini of the viral RNAs (39, 40, 43, 48). While our attention initially was drawn to this region of the *gag* gene by the presence of this consensus enhancer core sequence (52), we have not yet established whether this sequence is necessary for the enhanced CAT expression observed. It is interesting that the Ty enhancer shares this consensus sequence (4, 11, 38).

DNase I-hypersensitive sites (10), which are frequently associated with enhancers, have been mapped within the *gag* genes of avian retroviruses at sites approximately 1 kilobase from the site of transcription initiation, as well as in LTR sequences. Groudine and Conklin observed such sites in the *gag* gene of the active endogenous virus ev-3 in chicken sperm chromatin (15), while Dyson et al. mapped similar hypersensitive sites within the *gag* gene of the RSV provirus in transformed rat cell lines (9).

Although the presence of the 656-bp FSV *gag* fragment led to an increase in CAT activity regardless of its position relative to the SV40 promoter in pSV1cat, the amount of that increase was orientation dependent. The elevation was maximal when the 5' end of the *gag* fragment was proximal to the *cat* gene and SV40 promoter. After deletion of 172 bp at the 3' end of the *gag* fragment, this orientation dependence was lost and there was a slight decrease in the maximal level of enhancement. One possible explanation is that there is a negative regulatory element closely associated with the *gag* enhancer. This putative negative element would substantially decrease transcriptional activation when placed between the *gag* enhancer element and the promoter, resulting in an enhancer which appears to be orientation dependent. Similar effects of orientation have been observed with other enhancer elements, particularly when a second promoter is placed between an enhancer and the first promoter (50). However, there is no obvious CAT or TATA box in this region of the *gag* gene that would suggest the presence of a promoter. Experiments are presently being performed to determine directly whether a negative regulatory element is present in the sequences at the 3' end of the 656-bp *gag* fragment. In the intact provirus, this putative negative element might restrict the *gag* enhancer to elevating transcription from the 5' LTR.

Broome and Gilbert have proposed that RSV encodes a transcriptional activator within its *gag* gene from a second reading frame (2). We have mapped an enhancer element to roughly the same region of the *gag* gene. Our insertional mutagenesis experiments suggest that we have identified a regulatory element distinct from that of Broome and Gilbert, since the same mutations which inactivated the *trans* activity in their system did not affect the *cis* activity observed by us.

We were surprised to find a second enhancer element within the retroviral genome, because the LTR enhancer is very powerful and capable of acting alone (5, 13, 25, 28, 46, 51). Nevertheless, in most replication-defective avian retroviruses that have been well characterized, including FSV, MC29, and Y73, a significant portion of the *gag* gene is retained, including the sequence in p10 homologous to the

enhancer core (22, 34, 40). However, in the case of UR2 ASV, the *gag-ros* junction is within the p19 coding sequence, 830 nucleotides from the 5' RNA terminus (31). The recently characterized S1 and S2 ASVs have retained only 18 and 122 bases, respectively, of the *gag* gene (20). Therefore, the presence of the *gag* enhancer we have characterized is not obligatory for ASVs. There could be additional internal enhancers that play a role like that of the *gag* enhancer.

There are several suggestions of possible biological significance of the *gag* enhancer (26, 35–37, 44, 45). Stoltzfus and co-workers (personal communication) observed that plasmids containing Pr-A RSV genomes that were deleted from nucleotide 630 to 6576 did not efficiently transform mouse or chicken cells, even though the *src* gene and LTRs were intact. *gag* gene sequences mapping from nucleotide 630 to 1140 were also needed for efficient transformation of mouse NIH 3T3 cells. While this construct transformed CEFs, a 10-fold-higher transformation efficiency was observed when sequences from the 5' terminus to nucleotide 1659 were included. These experiments suggest that sequences downstream from nucleotide 630, where the *gag* enhancer is located, are needed for high-level transformation by integrated RSV provirus.

Studies with recombinants between RAV-0 and an exogenous virus have mapped exogenous virus determinants of both congenital transmission and disease tropism to the vicinity of the *gag* gene (35–37, 44, 45). For example, exogenous virus sequences near the 5' end of the genome determine its ability to induce osteopetrosis (37). Congenital transmission of exogenous viruses, but not of RAV-0, in chickens segregates with a p27^{gag} marker (35). While these properties map near the *gag* enhancer sequence, we have not observed any difference in the activity of the *gag* enhancer between RAV-0 and exogenous viruses in transient expression assays in CEFs and mouse L cells. If tissue-specific differences exist between the *gag* enhancer of RAV-0 and that of exogenous viruses, this enhancer may be responsible for differences between these viruses in growth or disease induction that have been localized to the *gag* gene (35–37, 45).

Avian leukosis virus (ALV) induction of bursal lymphomas usually involves integration of a deleted ALV provirus upstream of the *c-myc* coding region and transcription of the *myc* gene from the 3' LTR promoter (16). In many tumors, both ALV LTRs are present, but a region of the provirus including the 5' half of the *gag* gene has been deleted (36; M. M. Goodenow and W. S. Hayward, personal communication). A similar deleted region was present in a tumor in which ALV was inserted upstream of *myc* but in the opposite orientation (54).

In contrast to the variation in activity of the LTR enhancers of RSV, FSV, and RAV-0 (5, 7, 44, 45, 51), we have observed an enhancer within the *gag* gene of all of these viruses that is invariant in activity in transient expression assays in chicken and mouse fibroblasts. The *gag* enhancer may play a more important role in the replication of FSV and RAV-0, which have relatively weak LTR enhancers, than in the replication of RSV. To assess the significance of the *gag* enhancer sequence for viral replication or disease induction, site-directed mutations within this region of the viral genome are needed.

ACKNOWLEDGMENTS

We thank Alison Lin, Tom Lee, and Eileen Claar for their assistance with experiments and Kristen Carlberg, Janice Clements,

and Susan Kane for their comments on the manuscript. We also thank B. Howard, M. Shibuya, H. Hanafusa, R. Guntaka, J. M. Bishop, P. Tschlis, and P. Shank for generously providing plasmids.

This work was supported in part by Public Health Service grant CA-33199 from the National Cancer Institute, Basil O'Connor Starter Research Grant 5-399 from the March of Dimes Birth Defects Foundation, and National Institutes of Health Biomedical Research Support Grant S07 RRD 7031. K.B. is the recipient of a Faculty Research Award from the American Cancer Society. S.A. was supported by National Institutes of Health predoctoral training grant HD07013.

LITERATURE CITED

- Alwine, J. C. 1985. Transient gene expression control: effects of transfected DNA stability and *trans*-activation by viral early proteins. *Mol. Cell. Biol.* 5:1034-1042.
- Broome, S., and W. Gilbert. 1985. Rous sarcoma virus encodes a transcriptional activator. *Cell* 40:537-546.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
- Clare, J., and P. Farabaugh. 1985. Nucleotide sequence of a yeast Ty element: evidence for an unusual mechanism of gene expression. *Proc. Natl. Acad. Sci. USA* 82:2829-2833.
- Cullen, B. R., K. Raymond, and G. Ju. 1985. Transcriptional activity of avian retroviral long terminal repeats directly correlates with enhancer activity. *J. Virol.* 53:515-521.
- Cullen, B. R., K. Raymond, and G. Ju. 1985. Functional analysis of the transcriptional control region located with the avian retroviral long terminal repeat. *Mol. Cell. Biol.* 5:438-447.
- Cullen, B. R., A. M. Skalka, and G. Ju. 1983. Endogenous avian retroviruses contain deficient promoter and leader sequences. *Proc. Natl. Acad. Sci. USA* 80:2946-2950.
- DeLorbe, W. J., P. A. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop. 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. *J. Virol.* 36:50-61.
- Dyson, P. J., P. R. Cook, S. Searle, and J. A. Wyke. 1985. The chromatin structure of Rous sarcoma proviruses is changed by factors that act in trans in cell hybrids. *EMBO J.* 4:413-420.
- Elgin, S. C. R. 1981. DNase I-hypersensitive sites of chromatin. *Cell* 27:413-415.
- Errede, B., M. Company, J. D. Ferchak, C. A. Hutchison, and W. S. Yanell. 1985. Activation regions in a yeast transposon have homology to mating type control sequences and to mammalian enhancers. *Proc. Natl. Acad. Sci. USA* 82:5423-5427.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- 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.
- 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.
- Groudine, M., and K. F. Conklin. 1985. Chromatin structure and de novo methylation of sperm DNA: implications for activation of the parental genome. *Science* 228:1061-1068.
- Hayward, W. S., B. Neel, and S. Astrin. 1981. Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature (London)* 290:475-480.
- Herr, W., and J. Clarke. 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. *Cell* 45:461-470.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cultures. *J. Mol. Biol.* 26:365-369.
- Hughes, S. H. 1982. Sequence of the long terminal repeat and adjacent segments of the endogenous avian virus Rous-associated virus 0. *J. Virol.* 43:191-200.
- Ikawa, S., K. Hagino-Yamagishi, S. Kawai, T. Yamamoto, and K. Toyoshima. 1986. Activation of the cellular *src* gene by transducing retrovirus. *Mol. Cell. Biol.* 6:2420-2428.
- Katz, R. A., C. A. Omer, J. H. Weis, S. A. Mitsialis, A. J. Faras, and R. V. Guntaka. 1982. Restriction endonuclease and nucleotide sequence analyses of molecularly cloned unintegrated avian tumor virus DNA: structure of large terminal repeats in circle junctions. *J. Virol.* 42:346-351.
- Khoury, G., and P. Gruss. 1983. Enhancer elements. *Cell* 33:313-314.
- Kitamura, N., A. Kitamura, K. Toyoshima, Y. Hirayama, and N. Yoshida. 1982. Avian sarcoma virus Y73 genome sequence and structural similarity of its transforming gene product to that of Rous sarcoma virus. *Nature (London)* 297:205-208.
- Laimins, L. A., G. Khoury, C. Gorman, B. G. Howard, and P. Gruss. 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. *Proc. Natl. Acad. Sci. USA* 79:6453-6457.
- Laimins, L. A., P. Tschlis, 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.
- 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.
- 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 dimethylsulfoxide or glycerol shock treatment. *Nucleic Acids Res.* 12:5707-5717.
- 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.
- Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
- 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.
- Neckameyer, W. S., and L.-H. Wang. 1985. Nucleotide sequence of avian sarcoma virus UR2 and comparison of its transforming gene with other members of the tyrosine protein kinase oncogene family. *J. Virol.* 53:879-884.
- Payne, G. S., J. M. Bishop, and H. E. Varmus. 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. *Nature (London)* 295:209-214.
- Payvar, F., D. DeFranco, G. L. Firestone, B. Edgar, O. Wrangle, S. Okret, J.-A. Gustafsson, and K. R. Yamamoto. 1983. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. *Cell* 35:381-392.
- Reddy, E. P., R. K. Reynolds, D. K. Watson, R. A. Schultz, J. Lautenberger, and T. S. Papas. 1983. Nucleotide sequence analysis of the proviral genome of avian myelocytomatosis virus (MC29). *Proc. Natl. Acad. Sci. USA* 80:2500-2504.
- Robinson, H. L., and R. Eisenman. 1984. New findings on the congenital transmission of avian leukosis viruses. *Science* 225:417-419.
- Robinson, H. L., and G. C. Gagnon. 1986. Patterns of proviral insertion and deletion in avian leukosis virus-induced lymphomas. *J. Virol.* 57:28-36.
- Robinson, H. L., S. S. Reinsch, and P. R. Shank. 1986. Sequences near the 5' long terminal repeat of avian leukosis viruses determine the ability to induce osteopetrosis. *J. Virol.* 59:45-49.
- Roeder, G. S., A. B. Rose, and R. E. Pearlman. 1985. Transposable element sequences involved in the enhancement of yeast gene expression. *Proc. Natl. Acad. Sci. USA* 82:5428-5432.
- Schwartz, D. E., R. Tizard, and W. Gilbert. 1983. Nucleotide

- sequence of Rous sarcoma virus. *Cell* 32:853-869.
40. Shibuya, M., and H. Hanafusa. 1982. Nucleotide sequence of Fujinami sarcoma virus: evolutionary relationship of its transforming gene with transforming genes of other sarcoma viruses. *Cell* 30:787-795.
41. Shibuya, M., L.-H. Wang, and H. Hanafusa. 1982. Molecular cloning of the Fujinami sarcoma virus genome and its comparison with sequences of other related transforming viruses. *J. Virol.* 42:1007-1016.
42. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
43. Swanstrom, R., H. E. Varmus, and J. M. Bishop. 1982. Nucleotide sequence of the 5' noncoding region and part of the *gag* gene of Rous sarcoma virus. *J. Virol.* 41:535-541.
44. Tsichlis, P. N., and J. M. Coffin. 1980. Recombinants between endogenous and exogenous avian tumor viruses: role of the C region and other portions of the genome in the control and replication of transformation. *J. Virol.* 33:238-249.
45. Tsichlis, P. N., L. Donehower, G. Hager, N. Zeller, R. Malavarca, S. Astrin, and A. M. Skalka. 1982. Sequence comparison in the crossover region of an oncogenic avian retrovirus recombinant and its nononcogenic parent: genetic regions that control growth rate and oncogenic potential. *Mol. Cell. Biol.* 2:1331-1338.
46. Varmus, H., and R. Swanstrom. 1982. Replication of retroviruses, p. 369-512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. Vigneron, M., H. A. Barrera-Saldana, D. Baty, R. E. Everett, and P. Chambon. 1984. Effect of the 21-bp repeat upstream element on in vitro transcription from the early and late SV40 promoters. *EMBO J.* 3:2373-2382.
48. Vogt, V. M., R. B. Pepinsky, and L. E. Southard. 1985. Primary structure of p19 species of avian sarcoma and leukemia viruses. *J. Virol.* 56:31-39.
49. Wallace, R. B., M. J. Johnson, S. V. Suggs, K. Miyoshi, R. Bhatt, and K. Itakura. 1981. A set of oligodeoxyribonucleotide primers for DNA sequencing in the plasmid pBR322. *Gene* 16:21-26.
50. Wasylyk, B., C. Wasylyk, P. Augereau, and P. Chambon. 1983. The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. *Cell* 32:503-514.
51. Weber, F., and W. Schaffner. 1985. Enhancer activity correlates with the oncogenic potential of avian retroviruses. *EMBO J.* 4:949-956.
52. Weiher, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. *Science* 219:626-631.
53. Weintraub, H., P. F. Cheng, and K. Conrad. 1986. Expression of transfected DNA depends on DNA topology. *Cell* 46:115-122.
54. Westaway, D., G. Payne, and H. E. Varmus. 1984. Proviral deletions and oncogene base-substitutions in insertionally mutagenized *c-myc* alleles may contribute to the progression of avian bursal tumors. *Proc. Natl. Acad. Sci. USA* 81:843-847.
55. Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon. 1986. Multiple sequence motifs are involved in SV40 enhancer function. *EMBO J.* 5: 387-397.