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Enhancer activity correlates with the oncogenic potential of avian retroviruses

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Avian retroviruses lacking an oncogene, such as Rous-associated virus 1 (RAV-1), RAV-2, and *td* mutants of Rous sarcoma virus (RSV), can nevertheless cause leukemias and other neoplastic diseases. During this process, viral DNA integrates near a cellular proto-oncogene, such as c-*myc*, and thus de-regulates its expression. The virus RAV-0, on the other hand, is known to be non-oncogenic even in long-term *in vivo* infections of domestic chickens. The major difference between oncogenic and non-oncogenic viruses is found within the U3 region of the long terminal repeat (LTR) which is known to harbor the promoter and enhancer elements. We therefore wanted to see whether viral oncogenicity was correlated with enhancer activity. Using a variety of techniques (including the SV40 'enhancer trap' from which we obtained RSV-SV40 recombinant viruses), we demonstrate that a strong enhancer exists within the LTRs of both RSV and RAV-1. In contrast, no enhancer is present in RAV-0, although RAV-0 has functional promoter elements. Our data therefore strongly support a concept of oncogenesis by enhancer insertion.

Key words: oncogenesis by enhancer insertion/enhancer trap/retrovirus/long terminal repeat

Introduction

A cancer cell is thought to be generated in a multi-step process resulting in aberrant regulation of genes controlling cell proliferation and communication. Several mechanisms of oncogenesis have been identified: (i) transformation by virus-encoded oncogenes (reviewed in Tooze, 1981; Weiss *et al.*, 1982), and (ii) activation of cellular proto-oncogenes (reviewed in Bishop, 1983; Klein, 1983). This can either be the result of mutations affecting protein structure (e.g., Tabin *et al.*, 1982) or of a de-regulation at the level of transcription (and possibly also RNA processing and mRNA translation), for example, leading to constitutive rather than cell cycle-dependent gene expression (Kelly *et al.*, 1983).

Retroviruses of the avian sarcoma virus-avian leukosis virus (ASV-ALV) group which lack a viral oncogene can be divided into oncogenic and non-oncogenic viruses. Oncogenic viruses like RAV-1, RAV-2, and *td* mutants of Rous sarcoma virus (RSV) (Weiss *et al.*, 1982) are able to induce leukemias and other neoplastic diseases. A crucial event in this process is the integration of viral DNA near a cellular proto-oncogene (e.g., c-*myc*) and apparent de-regulation of its expession (Fung *et al.*, 1981; Hayward *et al.*, 1981; Neel *et al.*, 1981; Payne *et al.*, 1981, 1982; Cullen *et al.*, 1984). There are, on the other hand, non-oncogenic viruses like RAV-0 (Critten *et al.*, 1979; Hughes, 1982; Tsichlis *et al.*, 1982; Coffin *et al.*, 1983). The endogenous RAV-0 virus can be rescued from the chicken genome so that it can multiply and infect other avian cells. Nevertheless, it does not induce neoplastic diseases even in long-term infections of domestic chickens.

Two hypotheses have been proposed to explain the lack of oncogenicity of the RAV-0 like group: RAV-0 is unable to insert near a cellular oncogene or, alternatively, it can integrate, in principle, in the same sites as the oncogenic avian leukaemia viruses (ALV), but fail to activate a nearby proto-oncogene.

Detailed comparison of RAV-0 and RSV by restriction analysis and sequencing has revealed clear differences in the U3 region of the long terminal repeats (LTRs) and only minor differences in other parts of the viral genomes (Hughes, 1982; Robinson *et al.*, 1982). In fact, RAV-0 becomes oncogenic when its own U3 region is replaced by that of RSV (Robinson *et al.*, 1982; Tsichlis *et al.*, 1982). Furthermore, it has been demonstrated that the LTR of a tumorigenic retrovirus (RAV-2) gives rise to a 10-fold higher level of *env* gene transcription than the RAV-0 LTR (Cullen *et al.*, 1983). Thus, the U3 region of the LTR, which harbors the promoter components and, as shown for RSV (Luciw *et al.*, 1983; Laimins *et al.*, 1984) a transcription enhancer, seems to determine both the level of viral transcription and the oncogenic potential of avian retroviruses.

Enhancers are regulatory DNA elements, usually ~100–200 bp long, which are able to stimulate transcription of linked genes in *cis* in either orientation (Banerji *et al.*, 1981; Moreau *et al.*, 1981), over large distances of many kilobase pairs (kb) and from positions 5′ or 3′ to the promoter (Banerji *et al.*, 1981). They play an important role in early gene expression in viruses from different families (for reviews, see Gluzman and Shenk, 1983; Picard, 1985). Enhancers have also been identified within murine and avian retroviruses. Most, if not all, of the enhancer activity is present within the LTR (Laimins *et al.*, 1982, 1984; Luciw *et al.*, 1983). Enhancers with a strict cell type specificity have also been found in association with cellular genes, notably within immunoglobulin genes (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984) and 5′ to the insulin, chymotrypsin, and a class II histocompatibilty gene (Walker *et al.*, 1983; Gillies *et al.*, 1984).

Because of this unique potential of activating genes over long distances, we reasoned that it is the enhancer activity present within the LTR which determines whether a retrovirus can activate nearby cellular oncogenes.

We have previously used a linear, enhancerless, non-viable SV40 molecule (the so-called enhancer trap) to isolate viral enhancers from short, random DNA fragments (Weber *et al.*, 1984). The enhancer trap is co-transfected with sonicated viral DNA into monkey CV-1 cells; viable virus which has integrated the heterologous enhancer is thereby rescued. We have now used this system in an extended experimental approach to test whether the LTR sequences of the RAV-0 and the Schmidt-Ruppin A strain of RSV harbor significant enhancer activity. In addition a transient expression assay was used to test LTR se-

quences of RSV, RAV-1, and RAV-0 for enhancer activity. In both tests, RAV-0 did not have detectable enhancer activity whereas the RAV-1 and RSV LTR sequences were strongly enhancer-positive. Here we extend several independent lines of evidence to show conclusively that enhancer activity correlates with the oncogenic potential of the studied avian retroviruses.

Results

RSV but not RAV-0 LTR sequences harbor an enhancer

We have used the SV40 enhancer trap (Weber *et al.*, 1984) to determine whether the RSV and RAV-0 LTRs harbor transcriptional enhancers. Cloned LTR sequences of both viruses were fragmented by extensive sonication resulting in fragments of ~300 bp in length. Variable amounts of the sonicated LTR DNAs were mixed with 1 μg of linearized enhancer trap DNA and the total amount of DNA was kept constant by adding carrier DNA. The DNA was transfected into duplicate plates of monkey kidney CV-1 cells using the calcium phosphate co-precipitation method. As a most rapid test for enhancer activity, cells in one plate were fixed after 2 days and stained for T-antigen production by immunofluorescence. Relatively small numbers of stained nuclei were found in transfections using either the SV40 enhancer trap DNA alone, or together with mixed-in sonicated RAV-0 LTR (410 positive per 90 000 cells and 395 per 83 000 cells, respectively). Significantly greater numbers of stained nuclei were found when we co-transfected the enhancer trap DNA with either sonicated RSV LTR DNA or SV40 enhancer DNA (1190 positive per 57 000 cells and 2290 positive per 50 000 cells, respectively). Even more obvious than the numerical differences was the staining intensity: without enhancer and with mixed-in RAV-0 the staining was very dull, whereas brilliantly fluorescent nuclei were seen with the RSV and SV40 enhancers (not shown; for illustration of staining difference see also Figure 3).

The duplicate plates were checked for virus production, which allows recovery of the selected enhancer (Weber *et al.*, 1984). No virus growth was observed during prolonged incubation of the cells transfected with enhancer trap DNA alone or together with RAV-0 LTR DNA. In all cases where RSV LTR or SV40 enhancer DNA was present, however, viable virus was produced (not shown). These preliminary studies therefore suggested the presence of an enhancer within the RSV LTR.

In further enhancer trap experiments we wanted to determine the lower limit of RSV LTR DNA necessary to obtain viral growth. Plates transfected with 1 μg of enhancer trap DNA (= 1/3 pmol) and at least 1/30 pmol of sonicated RSV-LTR lysed within 5 weeks after transfection (Table I). Again no rescue of viable virus could be observed in plates transfected with various amounts of sonicated RAV-0 LTR DNA together with enhancer trap DNA, or with the enhancer trap alone. The lowest concentration of RSV DNA in which the cells lysed (10 ng RSV DNA per 10 μg carrier DNA) would correspond to ~10 000 RSV enhancer copies per mammalian genome.

Table I. Enhancer trap experiments

Amount of sonicated LTR clone added[a]	Lysis within 5 weeks (triplet plates)					
	RSV LTR			RAV-0		LTR
3 pmol	+	ND	ND	−	−	ND
1 pmol	+	+	+	−	−	−
1/3 pmol	+	+	+	−	−	−
1/10 pmol	+	+	+	−	−	−
1/30 pmol	−	+	−	−	ND	ND
1/100 pmol	−	−	ND	−	ND	ND
1/300 pmol	−	ND	ND	−	ND	ND
1/1000 pmol	−	ND	ND	−	ND	ND

[a]1 μg of 'enhancer trap' DNA was mixed with the appropriate amount of sonicated LTR DNA and adjusted to 10 μg total DNA with sonicated salmon sperm carrier DNA.

Isolation of SV40-RSV recombinants

To identify the RSV sequences contained within the rescued viruses, viral DNA from a plate lysate obtained after co-transfecting the SV40 enhancer trap with sonicated RSV-LTR DNA was extracted and cloned in a bacterial plasmid. To exclude complementation-dependent viruses, individual virus clones were excised from plasmid DNA, re-transfected into monkey CV-1 cells, and tested for their ability to express viral antigens. Three of four cloned viral DNAs, called SVR1, SVR2 and SVR3, expressed both early (T-antigen) and late (V-antigen) viral genes. Data for SVR2 and SVR3 are presented in Table II. T-antigen as well as V-antigen production of SVR3 is somewhat higher than with SVR2. In addition, the infectivity of these two viral clones was examined in CV-1 cells. Both SVR2 and SVR3 gave rise to viable virus resulting in cell lysis, although the time of lysis was significantly delayed in comparison with wild-type SV40.

These viruses were not only tested in a transient assay but also in stable transformation. Mink lung cells (Owen and Diggelmann, 1983) are particularly suitable for transformation studies since the background of spontaneous cell foci is zero, that is every focus expresses T-antigen (W.Schaffner, unpublished). Both recombinants SVR2 and SVR3 produced transformed foci (Table IIIA) although less efficiently than wild-type SV40. As in the immunofluorescence and growth studies, SVR3 was more effective than SVR2.

Sequence analysis of viral recombinants

SVR2 and SVR3 were further analyzed by DNA sequencing (Figure 1). They were each found to contain inserts of RSV LTR DNA which had become integrated into the SV40 enhancer trap by intracellular ligation/repair processes. Both of the inserts are derived from the 5′ part of the LTR (U3 region). They are pre-

Table II. Growth properties of SV40 variants

DNA clone[a]	Cells per 8 x 8 mm area[b]	Positive for T-antigen	Positive for V-antigen	First signs of infection at day	Lysis at day
SV40	15 000	817	105	11	15
SVR2	14 000	402	36	17	25
SVR3	14 300	516	48	14	19

[a]5 μg of cloned DNA was reclaimed from the plasmid vector by *Bam*HI digestion and used to transfect CV-1 cells of one 60 mm tissue culture plate by the calcium-phosphate co-precipitation method, and this plate was split into three 35 mm plates after 24 h. 48 h and 60 h post-transfection, plates were stained for T-antigen and V-antigen, respectively. The third plate was incubated until lysis.
[b]Counting of the cells and immunofluorescence was done as described in Banerji *et al.* (1981, 1983).

sent, however, in opposite orientation to one another in the enhancer trap.

Since the sequence of the SVR3 enhancer is contained within the insert of SVR2, it is unexpected that the shorter insert of SVR3 is somewhat more effective both in transient expression as well as long-term transformation assays (Table III and Figure 2). Minor sequence differences at the junction of the RSV and SV40 DNA could possibly explain this finding. Alternatively, the enhancer could exhibit a slight orientation dependence. The SVR3 enhancer, which gives higher T-antigen expression than SVR2, is present in its 'natural' orientation with respect to the SV40 early transcription unit.

The SVR3 insert has true enhancer activity

The RSV LTR insert in SVR3 appears to be able to replace functionally the SV40 enhancer in both short-term expression and long-term transformation assays as shown above. To test whether the selected Rous sequences have all the properties attributed to enhancers, we have subcloned the *Hind*III C fragment of SVR3, which contains the RSV insert, downstream of the rabbit β-globin gene and tested it for its ability to enhance β-globin transcription. We have previously used this assay to identify other viral and cellular enhancers (Banerji *et al.*, 1981; de Villiers and Schaffner, 1981; Weber *et al.*, 1984). The SVR3 insert is able to enhance strongly the synthesis of authentic β-globin transcripts (Figure 2). The number of transcripts is estimated to be about one third of that obtained with the SV40 enhancer fragment, which is ~50 times higher than without enhancer. This indicates that the SVR3 insert has true enhancer activity though it appears to be weaker than the SV40 enhancer also in HeLa cells.

Lack of enhancer activity within the RAV-O LTR

In all experiments using monkey CV-1 and human HeLa cells the RAV-0 LTR did not show any enhancer activity. This negative result was substantiated further. It could have been that the RAV-0 LTR had no enhancer activity in primate cells but was active in other cells such as chicken (or mink) cells. Instead of using a selection system, we directly cloned the enhancer region, sequences from upstream of the TATA box to the 3′ end of the LTR of RSV into the SV40 enhancer trap. In parallel, the corresponding upstream regions of RAV-0 and RAV-1 (a transforming oncogeneless retrovirus) were similarly subcloned into the enhancer trap molecule (for further details see Materials and methods). These clones, called pSVRAV-0, pSVRAV-1 and pSVRSV were transfected into human HeLa cells as well as into chicken embryo fibroblasts. After 2 days the cells were fixed and stained for T-antigen by indirect immunofluorescence. Figure 3

Table III. Mink transformation

(A) Experiment 1

DNA clone	Amount of DNA	Foci per plate
pSV40 (SV40 wild-type)	10 μg	76
pSVR2 (SV40-RSV recombinant)	10 μg	34
pSVR3 (SV40-RSV recombinant)	10 μg	57

(B) Experiment 2

DNA clone	Amount of DNA	Foci per plate[a]
pET-1 (SV40 without enhancer)	10 μg	0, 1
	2 μg	0
pSVRAV-0 (RAV-0 upstream region)	10 μg	1, 0
	2 μg	0, 0
pSVRSV (RSV upstream region)	10 μg	31
	2 μg	6
pSVRAV-1 (RAV-1 upstream region)	10 μg	21, 23
	2 μg	5, 6
pSVR3 (SV40-RSV recombinant)	10 μg	42
	2 μg	6

[a]Some transfections were done in duplicate.

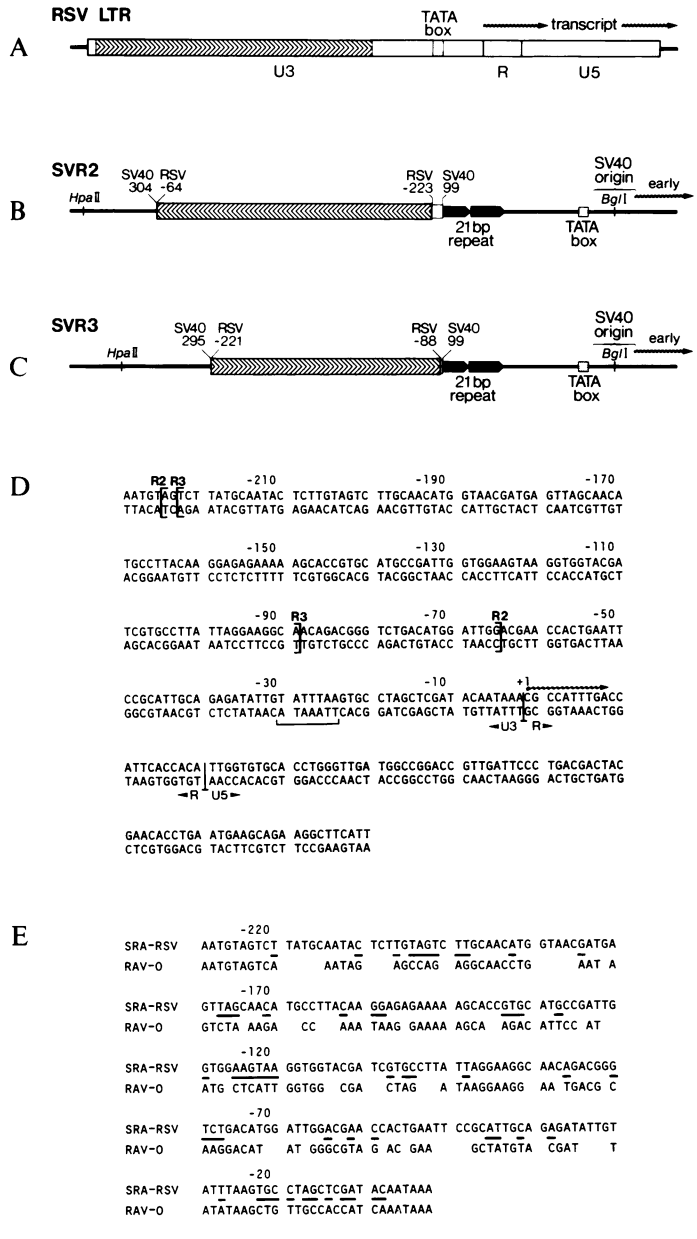

Fig. 1. Structure of SV40-RSV recombinants. (**A**) Schematic map of the SRA-RSV LTR deduced from the nucleotide sequence of Hughes (1982). The region of the sequences integrated into SV40 recombinant viruses is hatched by arrows. (**B**) and (**C**) Maps of the enhancer region of the recombinants SVR2 and SVR3. The numbering of SV40 wild-type sequences is according to Tooze (1981). Position 99 in the SV40 sequence corresponds to −106 and −112 from the two major early cap sites. The numbering of the RSV sequence is with respect to the start site of the viral transcript, that is to the 5′ end of the R region (Gilmartin and Parsons, 1983). Note that the inserts in SVR2 and SVR3 are in opposite orientation. (**D**) Sequence of the SRA-RSV LTR. Important landmarks are indicated. The areas represented in the recombinants SVR2 and SVR3 are marked by brackets. (**E**) Sequence comparison of the U3 regions of the Schmidt-Ruppin A strain of RSV and RAV-0 (according to Hughes, 1982). The sequences were aligned wherever it was possible. Gaps indicate missing nucleotides, mismatches are indicated by bars.

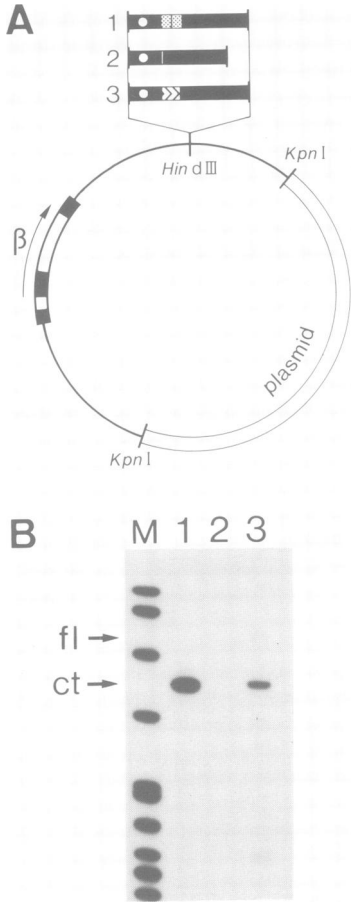

Fig. 2. Comparative analysis of enhancer strength, measured by S1 nuclease assay. (**A**) Enhancer-β-globin recombinants used. To test the strength of the enhancers, different DNA fragments were inserted downstream of the genomic rabbit β-globin gene (Maniatis *et al.*, 1978) in clone pβG (de Villiers *et al.*, 1982). (**1**) pβGHCSV; the *Hind*III C fragment of SV40 wild-type virus was inserted at the *Hind*III site in pβG. (**2**) pβGHCΔE; contains the *Hind*III C fragment of a reclosed viral enhancer trap DNA lacking enhancer sequences. (**3**) pSVHCR3; contains the *Hind*III C fragment of the SVR3 virus. (**B**) A β-globin gene lacking the first intervening sequence (IVS1; Weber *et al.*, 1981) was used as a radioactive probe (for further details see Rusconi and Schaffner, 1981). DNA, end-labeled at the *Bam*HI site, was hybridized to 20 μg cytoplasmic RNA from transfected HeLa cells, digested with S1 nuclease, and then denatured, fractionated by gel electrophoresis, and autoradiographed. The autoradiograph shows the results of hybridization to RNA from cells transfected with the following β-globin recombinants: **Lane 1**: pβGHCSV (SV40 enhancer). **Lane 2**: pβGHCΔE (no enhancer). **Lane 3**: pSVHCR3 (RSV enhancer). fl: indicates the size of full-length probe (453 nucleotides). ct: correct terminus (354 nucleotides). M: marker DNA fragments.

shows that the enhancerless SV40 DNA as well as pSVRAV-0 do not give rise to substantial T-antigen production in both chicken and human cells, whereas pSVR3, (the plasmid clone of SVR3), pSVRSV and pSVRAV-1 give large numbers of brilliantly stained T-antigen-positive nuclei.

All of these constructs were also tested in stable transformation experiments using mink lung cells. Whereas RAV-1 and RSV upstream sequences boosted the frequency of transformed cell foci, RAV-0 was again negative (Table III and Figure 4).

Although we could not detect any enhancer activity in the LTR of RAV-0 in many independent enhancer assays performed, RAV-0 nevertheless has retained functional promoter components. We linked the entire LTR of RAV-0 5′ to the coding sequence of the SV40 T-antigen, thus replacing the entire SV40 early promoter by the LTR. After transfection of this recombinant into human HeLa cells we found 6% of the cells to be positive for T-antigen. This value is significantly higher than the ones obtained with transcription from the enhancerless SV40 promoter (for the latter see, e.g., Figure 3) but, as expected, much lower than the value of 35% positive cells obtained with a similar recombinant containing the RSV LTR (not shown). These data are similar to the ones of Cullen *et al.* (1983) who found 10% as much *env* gene transcription from the RAV-0 LTR as compared with the RAV-2 LTR.

The relatively high level of transcripts from the RAV-0 LTR in absence of an enhancer is best explained by the presence of efficient proximal promoter components such as a consensus TATA box (Figure 1E). This is different from the SV40 early promoter which harbors a 'weak' TATA box (Vigneron *et al.*, 1984) and is very inefficiently transcribed in the absence of the enhancer.

Discussion

Selection of enhancers

We have used a variety of approaches to demonstrate enhancer activity within the RSV LTR and, at the same time, the lack of enhancer activity within the RAV-0 LTR. The SV40 enhancer trap (Weber *et al.*, 1984), consisting of a linear, enhancerless SV40 molecule, was co-transfected with small fragments of LTR DNA. When RSV LTR DNA was used, infectious virus was generated by intracellular ligation/repair processes. This rescue of viable virus is an enhancer-concentration and enhancer-strength dependent event that takes place whenever the trapped DNA restores sufficient T-antigen expression and thus allows for replication of the SV40 DNA. In fact, T-antigen expression can be measured by immunofluorescence 2 days after transfection, thus being the fastest assay for the presence of an enhancer in a mixture of DNA fragments. The number and intensity of stained nuclei correlates with both the strength and the concentration of an enhancer present in the co-transfected DNA.

Obviously, the assay only works with enhancers that are active in monkey kidney CV-1 cells. It seemed possible, though not likely, that we might have missed RAV-0-SV40 recombinants because the RAV-0 enhancer, in contrast to the RSV enhancer, would not work in CV-1 cells. Although there are viral enhancers with a pronounced host cell preference, notably polyomavirus (de Villiers *et al.*, 1982), Moloney mouse sarcoma virus (Laimins *et al.*, 1982; Levinson *et al.*, 1982), murine leukemia virus (Celander and Haseltine, 1984) and lymphotropic papovavirus (LPV), among these only the LPV enhancer is completely inactive in CV-1 cells (Mosthas *et al.*, 1985). The RSV enhancer seems less active than, or at most as active as, the SV40 enhancer in mouse (Luciw *et al.*, 1983) monkey, mink and human cells (see Results) and seems to work best in chicken cells (Laimins *et al.*, 1984; and our data). Nevertheless the enhancers from SV40, human and mouse cytomegalovirus, herpes saimiri and also RSV are generally active not only in mammalian cells but also in avian cells and even frog kidney cells (Boshart *et al.*, 1985; Schirm *et al.*, 1985; K.Dorsch-Häsler, G.Keil, U.Koszinowski and W.Schaffner, unpublished).

The LTR of RAV-0 does not contain enhancer activity and we consider it unlikely that sequences outside of the LTR would substitute for the missing enhancer. In RSV some of the enhancer activity in the second LTR may extend into upstream viral sequences (Luciw *et al.*, 1983; Laimins *et al.*, 1984). This upstream non-LTR sequence, however, seems to have no enhancer activi-

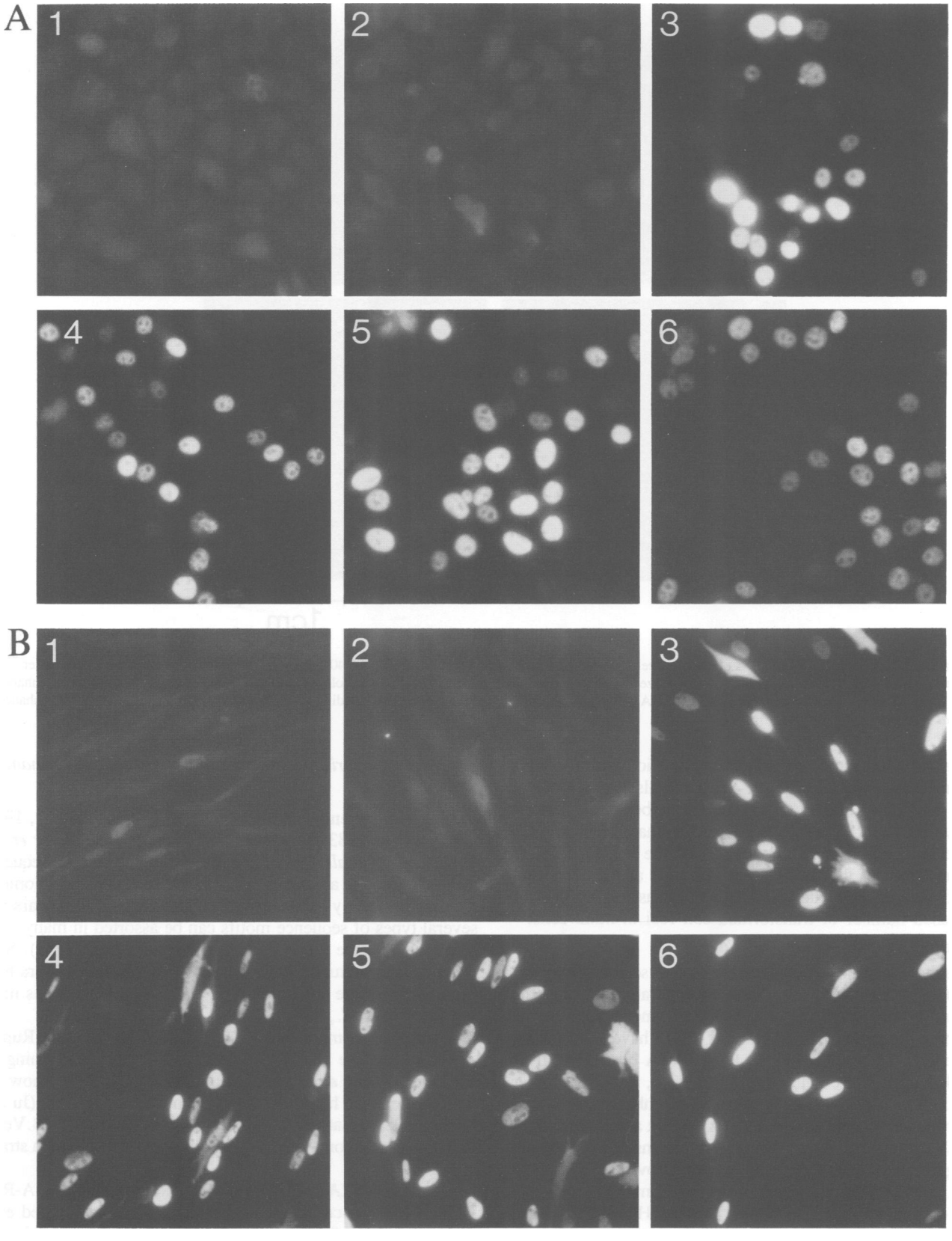

Fig. 3. Enhancer-dependent T-antigen expression in human HeLa and chicken embryo fibroblast cells. (**A**) Photographs of HeLa cells. The cells were transfected with the following DNA clones and after 48 h fixed and stained for T-antigen by immunofluorescence. The DNAs used for transfections were: (**1**) pSVΔE, (**2**) pSVRAV-0, (**3**) pSVRAV-1, (**4**) pSVRSV, (**5**) pSVR3 and (**6**) pSV15-(SV40 wild-type like virus, see Weber *et al.*, 1984). (**B**) Photographs of chicken embryo fibroblasts. The DNAs were the same as in (**A**).

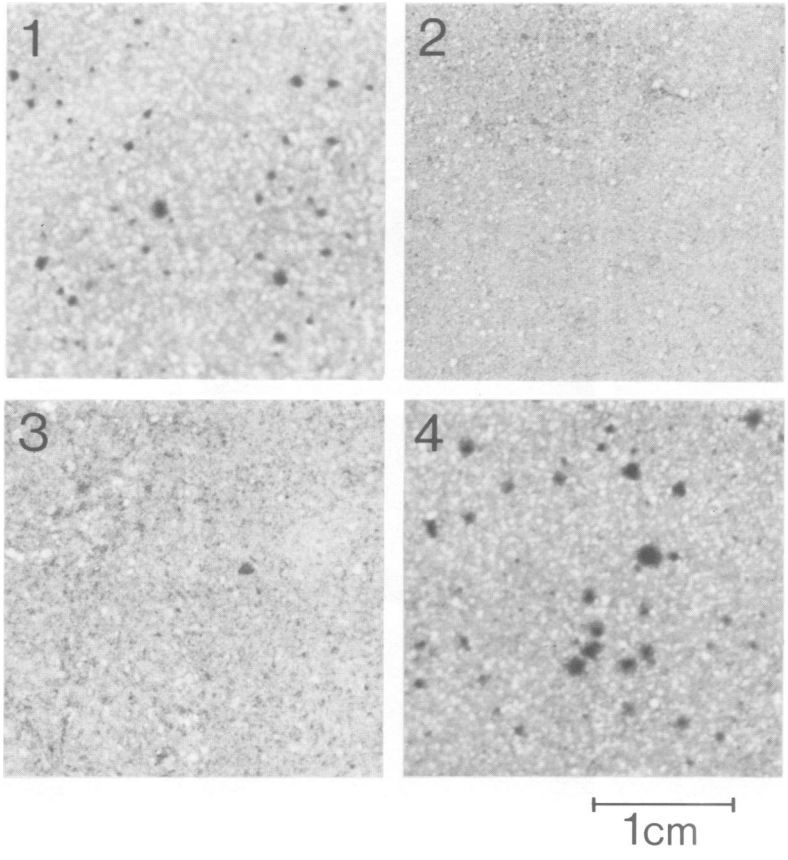

Fig. 4. Enhancer-dependent transformation of cultured cells. Mink lung cells were transfected with SV40 recombinant clones. The cells were fixed after 3 weeks and stained with 0.25% amido black to visualize the clumps (foci) of transformed cells. The following DNAs were used: (**1**) pSVR3 (with enhancer from RSV); (**2**) pSVRAV-0 (with upstream region of RAV-0 virus); (**3**) pET-1 (without enhancer; yielding a single focus); (**4**) pSV15- (with SV40 enhancer; Weber *et al.*, 1984).

ty *per se* and manifests itself only in conjunction with the LTR enhancer (Laimins *et al.*, 1984). We have also ruled out the unlikely possibility that the RAV-0 LTR harbors an enhancer which, unlike the RSV enhancer, would be inactive in primate cells. To this end we have directly cloned the RAV-0 LTR 5′ region upstream of the SV40 T-antigen gene and did not find enhanced expression in chicken embryo fibroblasts, nor was there an increased number of transformed cell foci in mink cells.

Why is the TATA box always missing?

In our enhancer trap experiments, a strong selecton may act against those molecules which have incorporated not only the enhancer but also proximal promoter components such as the TATA box. Recombinants SVR2 and SVR3 have inserts starting 44 and 58 bp upstream of the RSV TATA box, respectively (−64 and −88 from the cap site). Also, in all the other recombinant viruses obtained with the SV40 enhancer trap, proximal promoter sequences with the TATA box are always missing, with the cut off points being at positions −118 (human cytomegalovirus), −90 (mouse cytomegalovirus) and −63 (mouse metallothionein I gene) upstream from the gene's cap site (Boshart *et al.*, 1985; K.Dorsch-Häsler, G.Keil, U.Koszinowsky, F.Weber and W.Schaffner, unpublished; E.Serfling and W.Schaffner, unpublished). The consistent lack of proximal promoter components in our enhancer trap is not due to a size limitation for inserts in the enhancer trap since DNA fragments of up to 350 bp can be accommodated. Rather it appears that proximal promoter components are excluded since they would interfere with the regulation of SV40 transcription.

Sequence comparison between enhancing and non-enhancing DNA

In spite of some encouraging results (Hearing and Shenk, 1983; Lusky *et al.*, 1983; Nordheim and Rich, 1983; Weiher *et al.*, 1983; Boshart *et al.*, 1985), it seems difficult to detect a sequence motif common to all enhancers. Rather, scattered short homologies are shared by subgroups of enhancers, and it appears that several types of sequence motifs can be assorted in many combinations to make up an enhancer (Banerji *et al.*, 1983). Such elements can mutually replace each other since enhancers have been found where any given type of 'consensus' motif is missing (Weber *et al.*, 1984).

A sequence comparison of the LTRs of the Schmidt-Ruppin A strain and the Prague strain of RSV, both containing an enhancer (Luciw *et al.*, 1983; Laimins *et al.*, 1984), show extensive sequence homology. Also, the LTR of RAV-2 (Ju and Skalka, 1980), that is very similar to the RAV-1 LTR (B.Vennström, personal communication), is closely related to both strains of RSV.

Although the RAV-0 LTR is 55 bp shorter than the SRA-RSV LTR, the sequences are quite similar and can be aligned even though the RAV-0 LTR has suffered numerous small deletions (Hughes, 1982; Scholl *et al.*, 1983). Some, if not all, of these small deletions may be responsible for the lack of enhancer activity in RAV-0, since it seems difficult, if not impossible, to render an enhancer non-functional by small sequence alterations (Weiher *et al.*, 1983; Queen and Stafford, 1984; Boshart *et al.*, 1985; T.Grundström and P.Chambon, personal communication).

All of these studies suggest that the enhancer effect is the result of an interplay of various sequence motifs that are distributed throughout the entire enhancer element and may not be attributed exclusively to some type of 'consensus box'.

The concept of oncogenesis by enhancer insertion

All of our experiments indicate the absence of enhancer activity within RAV-0 LTR sequences and the presence of an enhancer within homologous sequences of RSV and related viruses. Therefore the RAV-0 virus seems to be a naturally occurring 'enhancer-minus' mutant. Since RAV-0 is an endogenous virus which also has the ability to multiply in avian cells, it might be that the loss of enhancer activity evolved to reduce oncogenicity without losing viability.

Robinson *et al.* (1982) and Tsichlis *et al.* (1982) have demonstrated that replacing the U3 region of the RAV-0 LTR with the homologous region of *td*-RSV restores oncogenicity *in vivo*. Thus, the U3 region determines the potential of a virus to induce malignant diseases such as lymphomas, carcinomas, chondrosarcomas, fibrosarcomas and osteopetrosis (Tsichlis *et al.*, 1982; Robinson *et al.*, 1982). Additional viral sequences determine the relative frequency of particular tumors and may be responsible for specific targeting of the virus (Robinson *et al.*, 1982). Viral gene expression, however, is not required for the maintenance of the cancerous state of a cell (Payne *et al.*, 1981), which is supported by recent findings using the mouse system where an LTR of Moloney leukemia virus is sufficient to induce transformation of NIH3T3 cells (Müller and Müller, 1984).

Insertion near a cellular proto-oncogene (e.g., c-*myc*) and deregulation of its expression apparently is a crucial event in the generation of leukemias and other neoplastic diseases by retroviruses of the ALV group. On the basis of this finding, it was proposed that a 'promoter insertion' mechanism operates during ALV-induced oncogenesis. The LTR promoter, inserted near a cellular proto-oncogene such as c-*myc*, would give rise to high levels of readthrough transcripts containing both viral and cellular sequences and as a result enhanced expression of the cellular proto-oncogene (Payne *et al.*, 1981; Neel *et al.*, 1981; Hayward *et al.*, 1981; Cullen *et al.*, 1984; see also Nusse and Varmus, 1982).

However, Payne *et al.* (1982) have analyzed different configurations of the integrated provirus relative to c-*myc* in a number of lymphomas and found some configurations which are incompatible with the promoter insertion hypothesis. They suggested that an ALV provirus, independent of its configuration, can affect the transcriptional activity of adjacent cellular DNA.

Because enhancers are able to stimulate a nearby gene from a 5′ or 3′ position in either orientation and over relatively large distances they have all the properties required to explain an activation of a cellular proto-oncogene by LTR enhancer sequences. While this concept appears relatively simple, there are also more complex situations in tumors of both viral and non-viral origin. In many lymphomas, c-*myc* is linked by a chromosome translocation to a strong constitutive transcription unit, namely an immunoglobulin heavy or light chain gene (reviewed in Klein, 1983). Although in the majority of these configurations the immunoglobulin gene enhancer is no longer present (reviewed by Robertson, 1983), it may nevertheless be indirectly responsible for c-*myc* activation. In terminally differentiated cell lines the enhancer activity seems to be dispensable for immunoglobulin gene transcription (Wabl and Burrows, 1984; Klein *et al.*, 1984). Our most recent findings (S.Klein, T.Gerster, A. Radbruch and W.Schaffner, unpublished) suggest that the enhancer is only required in early stages of differentiation, probably to organize the chromatin in a region of the gene into stable transcription complexes. Along these lines we hypothesize that such a stably organized chromatin domain de-regulates a proto-oncogene translocated into its vicinity.

While these chromosome translocations must await further analyses for their elucidation, the situation with retroviral oncogenicity appears more obvious: previous findings (Payne *et al.*, 1981, 1982; Neel *et al.*, 1981; Hayward *et al.*, 1981; Cullen *et al.*, 1984; Corcoran *et al.*, 1984; see also Nusse and Varmus, 1982) together with the detailed analysis provided in this paper, where we show that the oncogenic potential of the examined viruses correlates with their enhancer activity, support a model of oncogenesis by enhancer insertion.

Materials and methods

Cell growth

Mammalian cells were cultured in Dulbecco's modified Eagle's minimal essential medium (Gibco), containing 2.5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Chicken embryo fibroblasts were grown in DMEM containing 1% chicken serum, 1% fetal calf serum, 2% Tryptose-phosphate broth, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded 1 day before transfection; at the time of transfection HeLa and CV-1 cells were 80% confluent and chicken embryo fibroblasts 60% confluent.

DNAs

All DNA constructs were made by standard recombinant DNA techniques (Maniatis *et al.*, 1982). In typical experiments 1 μg of *Xba*I- and *Kpn*I-digested enhancer trap DNA (clone pET-1, Weber *et al.*, 1984) was mixed with 10 μg of sonicated DNA. The conditions for sonication (ultrasonics W-lo) were chosen such that the main fraction of the DNA was ~300 bp in size. Clones were constructed as follows.

pSVRSV. Clones of the LTRs of SRA-RSV and RAV-0 (kindly provided by J.Sorge and S.H.Hughes) contain a *Cla*I linker 8 bp and 16 bp, respectively, upstream of the LTRs. The 190-bp *Cla*I to *Eco*RI (position −53, see Figure 1) fragment of the RSV LTR was cloned into the *Xba*I site of an SV40 enhancer deletion mutant clone. This deletion clone (kindly provided by Y.Gluzman and modified by J.Banerji) contains an *Xba*I linker at the position of the deleted enhancer.

pSVRAV-0. The 150-bp *Cla*I to *Alu*I (position −42, Hughes, 1982) was cloned as described for pSVRSV.

pSVRAV-1. The 1-kb *Eco*RI to *Hind*III fragment of RAV-1 (Weiss *et al.*, 1982), containing U3 and LTR upstream sequences was cloned similarly.

Viral DNA was extracted from infected CV-1 cells by the procedure of Hirt (1967). The viral DNAs were linearized with *Bam*HI and cloned into the *Bam*HI site of pBR327 (Covarrubias *et al.*, 1981). The DNA of the enhancer region was sequenced according to Maxam and Gilbert (1980).

Transfection

The calcium phosphate transfection protocol was that of Graham and van der Eb (1973) and of Wigler *et al.* (1979) with the modifications described in Weber *et al.* (1984).

RNA analysis was performed as described (Weaver and Weissmann, 1979; Rusconi and Schaffner, 1981; de Villiers and Schaffner, 1983).

Immunofluorescence assays were performed as described earlier (Banerji *et al.*, 1981, 1983).

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