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**Effect of intron size on splicing efficiency in retroviral transcripts**

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**ABSTRACT**

To study the effect of intron size on splicing efficiency we have varied the size of the avian leukosis virus (ALV) env mRNA intron in a cloned ALV genome. This was accomplished by deletion of ALV sequences or insertion of phage  $\lambda$  DNA. The effect of these modifications on splicing was analyzed by microinjection of the modified clones into RSV(-) chicken cells. Viral env mRNA when transcribed and properly spliced within these cells complemented the RSV(-) env deficiency leading to the production of focus forming units. Using this assay it was shown that deletion of up to 3.7 kb of the 4.68 kb env intron did not inhibit correct splicing nor did insertion of up to 8 kb of phage  $\lambda$  DNA prevent splicing. Our results indicate that intron size can be varied over a wide range without preventing splicing.

**INTRODUCTION**

Most mRNA species synthesized by eukaryotic cells contain intragenic untranslated regions (introns) which must be spliced away from the expressed regions of the mRNA (exons) before transport to the cytoplasm and translation occur (1). Up to 50 introns have been observed in a gene and in several cases the introns make up the majority of the total initial mRNA transcript (2). A small number of naturally occurring genes yield stable functional transcripts which do not require splicing. Work on recombinant genes constructed to lack introns has also indicated that whereas some genes appear to require a splice to yield functional mRNA (3), other genes are able to produce stable functional unspliced transcripts (4). Thus, although several workers have argued for a role for introns in evolution (1,5), the degree of involvement of splicing in the regulation of gene expression remains uncertain.

A clue to the importance of splicing may come from examination of the intronic structure of evolutionarily related genes. The primary sequence of introns, except for splice junctions, is generally found to be highly variable between homologous genes (6). Homologous genes, with a few exceptions (7), nevertheless retain exactly the same intron number and pattern and approximately the same intron size through evolution (8). This appears to be parti-

cularly true of the globin gene family where, despite the extreme age of the  $\alpha$ - $\beta$ -globin gene duplication, the size of the first intron is 116 to 130 bases in all mammalian  $\alpha$  as well as  $\beta$ -globin genes studied so far (9). The second intron in the  $\alpha$ - and  $\beta$  gene families also varies little in length, leading to the suggestion by Van den Berg et al. (6) that intron length, rather than sequence, might be important in some manner for correct globin gene function.

Khoury et al. (10) examined the effect of deletions in the early gene region of SV40 on the differential RNA splicing process which yields mRNAs coding for either the large T or small t antigen. They observed that these deletions affected the relative abundance of the spliced small t mRNA and suggested that the deletions, by changing the conformation of the transcript, affected the location and frequency of splicing events. Should this hypothesis have general validity, intronic structure might be important in the control of gene expression by affecting processing efficiency.

Splicing is known to be involved in the control of gene expression in the case of differential splicing. This occurs in several viral systems such as SV40 (10), adenovirus (11) and avian retrovirus (12) where a given transcript can give rise to multiple mRNA species depending on the choice of differing splicing modes. Differential splicing has also been shown to occur in the case of some cellular genes (13,14). We have chosen one such differential splicing system, synthesis of avian leukosis virus (ALV) env mRNA, to directly analyze the effect of alterations in intron size upon splicing.

ALV codes for at least two mRNAs. The full genome size mRNA transcribed in the nucleus encodes the core proteins (gag) and possibly the reverse transcriptase (pol) (15). We have previously shown that this RNA may be spliced in the nucleus to give a smaller mRNA coding for the envelope glycoprotein (env) or may be transported directly to the cytoplasm (16). The splicing event joins the first 389 nucleotides of the 5' end of the large mRNA to the downstream env gene (12), in the process removing an intron of approximately 4.7 kb which includes gag and pol sequences (17).

We have reported a sensitive assay for env mRNA, which involves the use of cells infected with the env-deficient Bryan strain of Rous sarcoma virus (RSV(-) cells) (15). Cloned retroviral DNA injected into the nucleus of RSV(-) cells is rapidly transcribed to yield a full genomic transcript which may be spliced to produce active env mRNA (18); the env mRNA is quantitated by its ability to compliment the env deficiency of the injected RSV(-) cells to allow release of infectious focus forming units (FFU). Unspliced full genomic ALV transcripts produced after microinjection are also packaged into virus particles. We have

detected such viruses using an indirect assay based on their ability to express the env gene (18,19).

In this report, the size of the env mRNA intron has been altered by deletion or by insertion of exogenous DNA. The effect of these alterations was assessed by analysis of the genetic properties of the virus released following injection of the DNA into RSV(-) cells.

#### MATERIALS AND METHODS

Cell Culture. Chick embryo fibroblast (CEF) preparations and culture conditions have been described (15,16). CEF preparations were negative in the expression of endogenous group specific antigens and endogenous env activity (*gs*<sup>-</sup>, *chf*<sup>-</sup>, SPAFAS). RSV(-) refers to CEF infected with the Bryan strain of RSV in the presence of ultraviolet-light-inactivated Sendai virus. RSV(-) virions produced by RSV(-)-transformed cells contain no helper virus. No virus infectious for CEF (C/E type, which are resistant to infection with subgroup E virus) were released from RSV(-)-transformed cells.

Molecular Cloning. The derivation of retroviral clone  $\lambda$ SRBtd2.2 used in these experiments has been previously described (18,20). Clone  $\lambda$ SRBtd2.2 contains the genome of transformation defective Schmidt-Ruppin B virus inserted into the *S*all site of  $\lambda$  vector Charon 21A. Clone  $\lambda$ LD12 contains the genome of a RAV-2 virus cloned into the *S*all site of  $\lambda$  vector Y2222 (manuscript in preparation).  $\lambda$ SRBtd2.2 contains an approximately equal mixture of DNA molecules containing one or two viral long terminal repeats (LTRs). This is due to the instability of this repeat in this  $\lambda$  vector (20). Clone  $\lambda$ LD12 contains two LTRs.

In order to facilitate our experiments it was necessary to sub-clone the viral DNA inserts into the *S*all site of plasmid vector pBR322. Plasmid clones containing either one or two long terminal repeats (LTRs) were obtained from  $\lambda$ SRBtd2.2. The clone containing a single LTR was named pL39td2.2 while that containing two LTRs was named pL13td2.2. The two repeated copies of the viral LTR were stable in this plasmid clone. The clone obtained from  $\lambda$ LD12 contains two LTR and was named pLD12. Clones pLD12 and pL13td2.2 were chosen for further work. Both express a "B" type envelope gene and are similar in their restriction map (Fig. 1) and biological activity upon injection (results not shown).

Deletion mutants were obtained by cleavage with restriction enzymes and then either treating with *S*1 nuclease followed by ligation using T4 DNA ligase or by ligation in dilute conditions directly after cleavage. The internal *B*glIII fragment of clone pL13td2.2 was removed in this way to yield clone pL13Bg6.

Lambda phage Y2222, a gift from A.M. Skalka, was derived from  $\lambda$ gtWES and

obtained from L. Enquist (unpublished results). BglII digested  $\lambda$ Y2222 DNA fragments were ligated to BglII digested pL13Bg6 using a partial BglII digest of  $\lambda$ 2222 to facilitate recovery of different size fragments.

**Enzymes.** Sall, BglIII, XhoI, EcoRI, HindIII, XbaI, BamHI and KpnI were obtained from New England Bio-Labs, Beverly, Mass., SstII and BstEII were obtained from Bethesda Research Laboratories, Rockville, Maryland, S1 nuclease and T4 DNA ligase was obtained from PL Biochemicals, Milwaukee, Wis.

**Microinjection and Virus Assay.** The technique used for microinjection of retroviral DNA into nuclei of RSV(-) cells has been previously described (18). DNA is microinjected into the nuclei of 250 RSV(-) cells at 20-200 ng/ml to introduce between 100-1,000 retroviral DNA molecules per cell. We have previously (18) shown this to be the optimum level for virus expression.

Injected DNA directs the synthesis of full size genomic RNA and spliced *env* mRNA resulting in the release of infectious virus beginning at 3 to 4 hrs after injection. Injected cell supernatants are collected at 20-22 hrs after injection. This is the latest timepoint that can be taken before the appearance of the progeny of the virus released at 4 hr (18).

Two types of virus particles are produced by injected cells. The majority contain the RSV(-) genome. These are now infectious as they contain envelope glycoprotein synthesized under the direction of the injected DNA. These are measured by focus formation on CEF as previously described (15). Other virus particles contain the full length transcript of the injected retroviral DNA including the *env* gene. These viruses are assayed indirectly based on their ability to express the *env* gene and thus complement the *env* deficiency of RSV(-) cells. The titre of *env*(+) viruses is determined by infecting RSV(-) cells with serial dilutions of the *env*(+) virus containing supernatant. After 72 hours of incubation at 37°C the presence of infectious RSV in these cultures is determined as described above. The titre of *env*(+) virus is deduced from the positive end point of the serial dilution. The presence of recombinant replication competent virus can be determined by assaying for the presence of virus able to be passaged on CEF. Due to the nature of the assays used the number of FFU is reproducible to within approximately 10% while the number of *env*(+) virus is accurate only to within a factor of 2.

## RESULTS

**Biological activity of deletion mutants.** Several viral deletion mutants were constructed; the regions removed are outlined in Fig. 1. Their ability to complement RSV(-) cells was compared to the parental clone (Table 1) using the experimental design outlined in Fig. 2. Clone pL13Bg6 lacks 2.6 kb between the Bgl

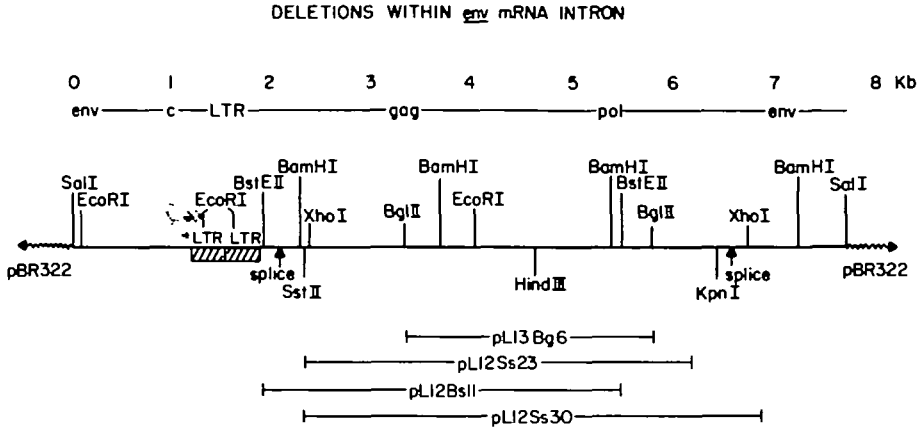


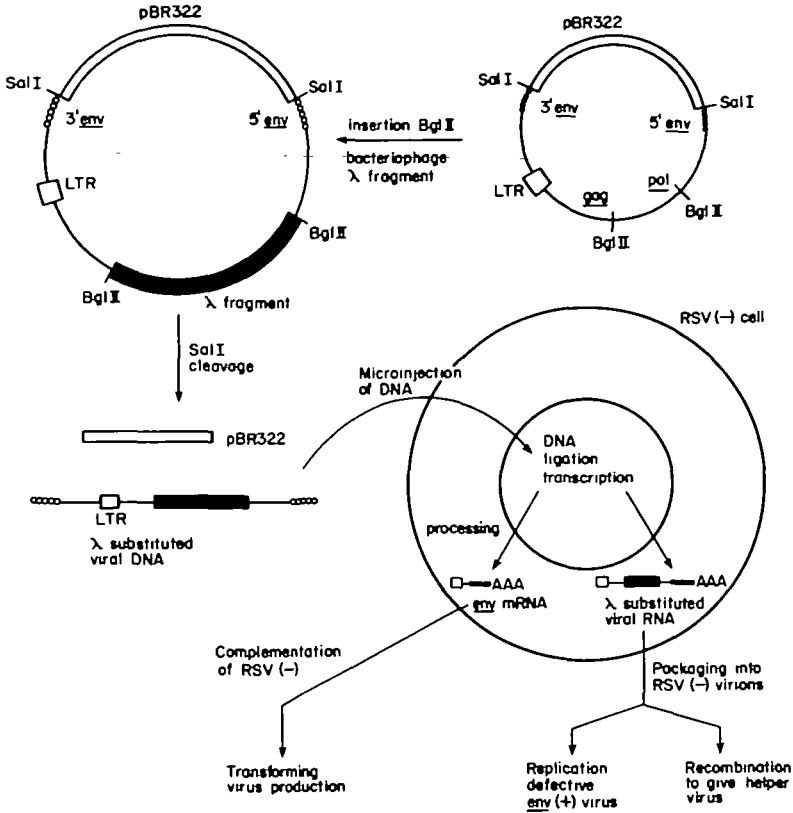
Figure 1. Restriction endonuclease map of clone pL13td2.2. Clone pL13td2.2 is permuted around the unique SalI site in the env gene. The position of the two viral long terminal repeats (LTRs) and the viral genes is shown as well as the approximate position of the splice sites used to produce env mRNA. The location and size of the deletions in the indicated viral clones is shown. Part of this restriction endonuclease map was obtained by Ju et al.(20). Clone pLD12 has a similar restriction endonuclease map.

II sites in the gag-pol region of pL13td2.2. This deletion does not reduce complementation of RSV(-) cells after injection. Clone pL12Ss23 lacks 3.7 kb 3' to the unique SstII site in pLD12. This deletion lies entirely within the env mRNA intron and encompasses the majority of its 4.7 kb length leaving only 3.95 kb of viral sequence. Nevertheless, after injection of this DNA, focus forming unit

Table 1. Biological Activity of Deletion Mutants.

	FFU released at 22hrs	ENV(+) Virus released at 22hrs	Recombinant Helper virus appearance
pL13td2.2	96	217	+
pL13Bg6	132	434	+
pL12Bs11	0	-	-
pL12Ss23	572	434	+
pL12Ss30	0	-	-

Deletion mutants described in the text were injected into RSV(-) cells and the ability of the injected DNA to complement the env deficiency of RSV(-) cells was assayed. The number of FFU and env(+) virus released by the injected cultures was determined at 22 hours after injection. The presence of helper ALV was also determined at three days following injection. The titre of env(+) virus is determined by two-fold serial endpoint dilution and thus differences of two-fold or less are not significant.



**Figure 2.** Experimental design used to test the biological activity of the retroviral clones. The parental ALV clone may be modified by deletion or insertion of DNA fragments between the *Bgl* II sites shown. Prior to injection the retroviral portion of the clone is separated from the plasmid vector by cleavage with *Sal*I to give the permuted linear retroviral DNA molecule. Upon injection into the nucleus of an RSV(-) cell these linear DNA molecules are ligated and used as a template for transcription of viral RNA molecules. These may either be processed to yield *env* mRNA or transported directly to the cytoplasm. Here, *env* mRNA complements the *env* deficiency of the RSV(-) cell allowing release of focus forming units of infectious RSV (18). Additionally, unspliced ALV chimeric or deleted transcripts may be packaged to yield *env*(+) replication defective virus. FFU and *env*(+) virus are assayed separately, added together they yield a figure for total *env* production.

(FFU) production by the RSV(-) cells was consistently higher than after injection of the parental undeleted clone.

Two other deletions were also tested. Clone pL12Bs11 lacks 3.7 kb between the two *Bst*EII sites of pLD12. It retains the cap site of ALV and all of the 5' long terminal repeat containing the transcriptional promoter as well as the entire structural *env* gene. It has, however, lost the splice donor site and is totally unable

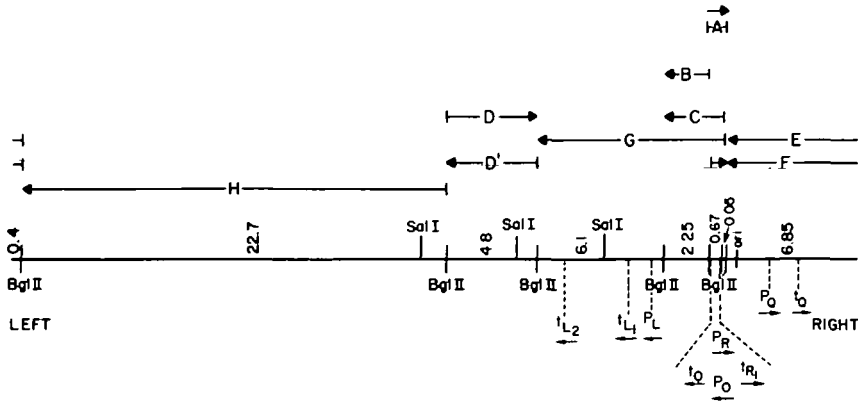
to complement the env deficiency of RSV(-) cells. Clone pL12Ss30 is deleted 5.1 kb 3' to the unique SstII site. We believe this removes the env gene splice acceptor site as well as a portion of the env structural gene. Clone pL12Ss30 is unable to complement RSV(-) cells.

The amount of env mRNA produced from the injected DNA determines the total number of infectious virus released by injected cells. This virus would be of two types. Focus forming units (FFU) represent the packaging of RSV(-) genomic RNA into infectious virions. An unspliced transcript derived from the injected deleted DNA when packaged into virions would be detected by its ability to express the env gene (env(+) virus). This virus would not express viral gag and pol. If viral recombination were to occur between the packaged unspliced transcript and the RSV(-) genome RNA a replication competent virus containing gag, pol and env could be formed. The different possible viruses described and their origin are outlined in Fig. 2.

Injected cultures were assayed for env(+) virus production at 22 hours and for presence of any replication competent ALV (helper virus) at day three. The env mRNA positive clones pL13Bg6 and pL12Ss23 are both able to produce env(+) virus at levels comparable to the wild type clone pL13td2.2. Both clones are also able to recombine with the RSV(-) genome to give helper virus (Table 1). Cells injected with the env mRNA negative clones pL12Bs11 and pL12Ss30 do not release env(+) virus or produce recombinant helper virus. This is particularly noteworthy in the case of clone pL12Bs11 which contains intact long terminal repeats and the full structural env gene but nevertheless is unable to efficiently recombine with RSV(-). This suggests that viral replication is a requirement for recombination to occur in this system.

Clones pL12Ss23 and pL13Bg6 are both able to produce FFU and env(+) virus at levels comparable to or higher than the wild type clone pL13td2.2. This suggests that deletions of up to 80% of the env mRNA intron have little effect on the differential splicing process in retroviruses as long as the splice donor and acceptor sites are unaffected.

**Effect of Inserted Exogenous DNA.** In order to further assess the effect of intron size and conformation on splicing, exogenous DNA was inserted in the intron of the env gene. This exogenous DNA would thus have to be transcribed and then spliced out of the full size RNA transcript as a part of the intron in order to obtain functional env mRNA. We chose to use the unique BglII site of deleted clone pL13Bg6 as an acceptor site for insertion of exogenous DNA as this deletion mutant retains the full env activity of the parental clone. Bacteriophage  $\lambda$  DNA was used as the inserted exogenous DNA in order to minimize the incidence of



**Figure 3.** Origin and transcriptional orientation of the  $\lambda$  DNA sequences integrated into the retroviral clones pL $\lambda$ 13A through pL $\lambda$ 13H. All clones contain contiguous sections of the  $\lambda$  genome with the exception of clone type F which contains two fragments of opposite orientation in tandem. Clones type D and D' contain the same fragment in opposite orientations. Also shown are the position and orientation of some  $\lambda$  transcriptional promoters and terminators.

eukaryotic splicing and control signals in the inserted DNA, which might be recognized by the injected cell.

To maximize the variety of  $\lambda$  DNA fragments inserted into the retroviral clone pL13Bg6, a partial Bgl II digest of  $\lambda$  DNA was prepared and ligated into the BglIII site of clone pL13Bg6. Clones containing  $\lambda$  DNA inserts were isolated and DNA characterized as to origin, size and orientation of the  $\lambda$  inserts (Fig. 3). The clones tested and others obtained fell into 9 groups, designated A to H, with  $\lambda$  DNA inserts increasing in size from 670 bases to 22,700 bases. Several fragments were repeatedly isolated in only one orientation. Independent isolates of each different clone type were injected into RSV(-) cells as outlined in Table 2. Supernatants from the injected RSV(-) cells were sampled at 22 hrs and at later times for focus forming unit (FFU) release and the presence of env(+) virus.

Clone pL13Bg6, which has a 2.6 kb deletion, produced both env(+) virus and FFU at titers comparable to the wild type. Insertion of the 670 base pair  $\lambda$  fragment in clone pL $\lambda$ 13A repressed total env activity somewhat, particularly when one compares env(+) virus release. Insertion of the 2.25 kb fragment in clone pL $\lambda$ 13B still allowed significant env production to occur as did insertion of  $\lambda$  fragments size 7.3 kb and 8 kb in clones pL $\lambda$ 13E and pL $\lambda$ 13F. These latter clones produced very little env(+) virus at 22 hrs. The unspliced transcripts in these clones were 12.0 and 12.7 kb in size respectively, well above the retroviral genome size range (21).



Table 2. Biological Activity of retroviral clones containing exogenous  $\lambda$  DNA inserts.

Clone	$\lambda$ DNA Insert Size	F.F.U. Released	Env(+) Virus Released	Virus Produced as % of Wt.	Helper Virus
pL13td2.2 (wt)	---	244	195	100%	+
pL13Bg6	---	241	160	90%	+
pL $\lambda$ 13A	0.67 kb	168	50	50%	+
pL $\lambda$ 13B	2.25 kb	52	14	15%	+
pL $\lambda$ 13C	2.95 kb	0	0	1%	+/-
pL $\lambda$ 13E	7.3 kb	60	0	14%	+
pL $\lambda$ 13F	8.0 kb	48	0/1	11%	+
pL $\lambda$ 13D	4.8 kb	0/1	0	1%	+/-
pL $\lambda$ 13D'	4.8 kb	0	N.D.	1%	---
pL $\lambda$ 13G	9.1 kb	0	N.D.	1%	---
pL $\lambda$ 13H	22.7 kb	0/1	N.D.	1%	---

Clones in the lower part of the table contain *Sa*II sites in the  $\lambda$  insert and are thus injected as partial digests. The ability of the injected DNA to complement the *env* deficiency of RSV(-) cells was assayed by determining the number of FFU and *env*(+) virus released by the injected culture at 22 hrs. The totals are compared as a percentage of the parental clone's activity. At four days the cultures were assayed for the presence of recombinant helper ALV.

Clone pL $\lambda$ 13C, containing a 2.95 kb  $\lambda$  insert, produced very little *env* mRNA although it is close in size to the 2.6 kb retroviral intron fragment it replaces and consists of fragments A and B in tandem, both of which allow good *env* mRNA production if inserted individually. It may be significant that this  $\lambda$  fragment contains the rho independent  $\lambda$  terminator  $t_{oop}$  in a sense orientation (22).

Clones of class D, D', G and H all contain *Sa*II sites in the  $\lambda$  DNA insert. As the retrovirus must be excised from its plasmid vector using *Sa*II prior to injection, these clones must be injected as a partial *Sa*II digest. Thus a reduced level of activity is expected. Nevertheless, clone pL $\lambda$ 13D and clone pL $\lambda$ 13H gave rise to single foci at 22 hrs. The existence of these foci was confirmed by sub-culture when numerous secondary foci were observed. Foci have never been observed in control plates. Thus it appears that  $\lambda$  DNA inserts of up to 22.7 kb allow at least a small amount of transcription and processing of the substituted retroviral DNA.

In addition to assaying for *env*(+) virus and FFU at 22 hours we assayed for the presence of recombinant helper ALV at four days after injection. All the clones able to yield *env* activity early were able to recombine to give helper virus with the exception of clone pL $\lambda$ 13H. Clone pL $\lambda$ 13C, which did not give any activity at 22 hours, on one occasion gave rise to a recombinant. In the injection experiment in which this occurred recombination was preceded by the release of FFU at day 3 (results not shown).

### DISCUSSION

We have analyzed the effect of deletions and insertions of exogenous DNA in an intragenic region on the production of a spliced messenger RNA from cloned avian leukosis virus (ALV) DNA. The assay we have used, complementation of RSV(-)env deficiency, is a sensitive but indirect assay for mRNA production. We are unable, due to the nature of the experiments, to directly examine the mRNA transcripts synthesized.

Following microinjection the full size transcript derived from the cloned ALV genome may be transported to the cytoplasm and packaged into virions as genomic RNA or may be spliced to remove a 4.7 kb intron containing the viral gag and pol genes (16,17). This splicing of the 5' 389 nucleotides (12) to the 3' env gene yields functional env mRNA. The synthesis of env glycoprotein within injected RSV(-) cells is required for the release of infectious virus. The number of infectious virus released is an accurate measure of the level of env mRNA produced from the cloned injected ALV DNA (15).

We show in this paper that at least 3.7 kb of the 4.7 kb env intron can be deleted without reducing env mRNA production. The splice sites, however, must remain intact. These results are in agreement with previous work by Khoury et al. (10) on the large T antigen gene intron in SV40.

Insertion of exogenous DNA into the env mRNA intron has varying effect.  $\lambda$  DNA inserts from 670 bp to 8000 bp allow between 50 and 10% of normal env glycoprotein synthesis to occur. While one 2.95 kb insert appears to inhibit env production by over 99% a 22.7 kb insert allows a small amount of env synthesis to occur.

Only part of the full length ALV transcript is normally spliced to yield env mRNA before transport into the cytoplasm. In order to assess the total amount of env mRNA produced and to obtain an indication of the ratio of spliced to unspliced transcripts it is necessary to assay the release of env(+) virus, containing the full length ALV transcripts, as well as FFU from the injected RSV(-) cells.

The effect of deletions in the intron of the ALV env gene was first assessed. Deletions which affected the splice donor or acceptor sites prevented env synthesis. Deletions totally within the env gene intron did not inhibit env synthesis. Clone pL13Bg6, containing a 2.6 kb deletion, appears to show about the same level of both FFU and env(+) virus release as the parental clone pL13td2.2. On the other hand clone pL12Ss23 gives a higher level of FFU and a similar level of env(+) virus release than pL13td2.2 upon injection. Clone pL12Ss23 contains a 3.7 kb deletion and is thus only 3.95 kb in length compared to the 7.65 kb parental clone. The observed increase in env activity might be due to the reduction in

intron size or may be caused by an increase in transcripts due to the smaller transcriptional unit in clone pL12Ss23.

Insertion of  $\lambda$  DNA into the env mRNA intron causes a reduction in env activity ranging from 50% to nearly 100%. It is notable particularly that env(+) virus production appears to be decreased to a greater extent than FFU production.  $\lambda$  sequences in packaged chimeric ALV RNA may interfere with the assay of env(+) virus by interfering with reverse transcription or infectivity of the virus. Conversely, chimeric RNA may be spliced more efficiently than normal ALV RNA. Another explanation is that the chimeric RNAs are packaged less efficiently than RSV(-) genomic RNA available in the same cell. This appears particularly likely in the case of clones pL $\lambda$ 13E and pL $\lambda$ 13F where the chimeric RNAs, at 12.0 and 12.7 kb respectively, are larger than the largest known avian retroviral genomes and therefore might be expected to package poorly (21). Nevertheless, they do appear to be packaged at a low level.

It is not possible for us to determine whether the inhibition of env production by  $\lambda$  insert C and to a lesser extent A, B, E, and F is caused by the inability of RNA polymerase II to transcribe through the inserted  $\lambda$  sequences or by difficulty in splicing the resultant transcripts to give active env mRNA. If splicing of the chimeric transcript was inhibited this would presumably lead to the accumulation of unspliced transcripts which should be available for packaging into virions. The low level of env(+) virus released tends to argue against this.

The ability of insert size C to inhibit production of env mRNA is of interest because it consists of fragments A and B in tandem, both of which on their own allow good env production to occur. Fragment C is also close in size to the retroviral sequence it replaces and is smaller than fragments E and F, which allow activity, so that size appears unlikely to be a factor. The most important difference then appears to be in the orientation of the "A" part of clone pL $\lambda$ 13C (Fig. 3) which is opposite to that found in active clones pL $\lambda$ 13A and pL $\lambda$ 13F. In this orientation the  $\lambda$  transcriptional terminator  $t_{oop}$  is in a sense orientation. While a transcriptional terminator occurs in the other orientation of "A" also, this terminator,  $tr_1$ , functions only in the presence of rho in the bacterium while  $t_{oop}$  is a strong rho independent terminator (21). This suggests that a procaryotic terminator may be recognized by a eukaryotic polymerase. Data showing that  $t_{oop}$  acts as a transcriptional terminator for eukaryotic RNA polymerase *in vitro* (W. Hatfield and M. Rosenberg, personal communication) supports this suggestion. Our intention in inserting procaryotic DNA into the env intron was to minimize the occurrence of sequences that would be recognized by the eukaryotic cell. It appears possible that such recognition of a transcriptional terminator in fragment "C"

might be responsible for the inhibition of env synthesis. Similar phenomena, such as the recognition of cryptic splice sites in procaryotic DNA (23), might be responsible for the reduced env synthesis observed with fragments A, B, E and F.

We believe active clones synthesize env mRNA in a manner analogous to the wild type clone by appropriate initiation at the cap site of the retrovirus by RNA polymerase II, transcription through the entire inserted intronic sequence to the env gene and splicing using the correct donor and acceptor sites (24). A second possibility would be that initiation of transcription is occurring within the inserted  $\lambda$  sequences. We believe this is unlikely for the following reasons: (i) Data derived from the sequence of a related avian retrovirus (12) indicates that the first 6 amino acids of the env protein, including the initiation codon, may be encoded in the 5' donor sequence, these would thus be lost if transcription began in the intron; (ii) Retroviral clones containing procaryotic DNA inserts lose their ability to promote synthesis of env glycoprotein if the env splice donor site is deleted (results not shown).

We are unable to directly demonstrate the presence of  $\lambda$  DNA sequences in the released env(+) virions due to the small amounts of virus obtained. It is also impossible to amplify these viruses because they are rapidly lost via recombination with RSV(-). Evidence that chimeric env(+) RNAs were packaged into virions comes from the observation of env(+) replication defective virus. These viruses are able to complement the env deficiency of a second culture of RSV(-) cells in the absence of detectable helper ALV (results not shown). We have previously shown that env mRNA itself is packaged too poorly to account for these env(+) viruses (19).

A second indication that chimeric RNAs were packaged into virions in the injected cultures was the occurrence of recombination. Retroviruses show an extremely high level of recombination during replication. This high level is thought to arise during reverse transcription of the retroviral genome utilizing both the RNA genomes which are packaged into the virion (25,26). Recombination would thus require retroviral replication.

Micro-injected DNA molecules containing the env structural gene but unable to promote env production in the injected RSV(-) cells, such as deleted clone pL12Bs11, are unable to give rise to recombinant ALV helper virus (Table 1,2). All clones able to promote good env production recombine efficiently. If recombination occurs primarily during retroviral replication only chimeric transcripts packaged into the same virion as an RSV(-) genome could recombine to produce helper virus. Recent data on the use of retroviruses as vectors has demonstrated that insertion of exogenous DNA into the retroviral DNA genome

does not prevent packaging of the resultant chimeric RNA transcript into the virion and its subsequent ability to replicate (27,28).

Our results, in total, indicate that deletions within the *env* intron do not reduce, and may in fact slightly increase, *env* mRNA production. Insertion of DNA into the *env* mRNA intron does not, with the exception of fragment "C", prevent splicing but all insertions reduce *env* production to some extent. While these data are not inconsistent with the hypothesis that intron size or structure have some effect on splicing efficiency they indicate that these variables can be varied over a wide range without drastic effect.

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