

The jaagsiekte sheep retrovirus envelope gene induces transformation of the avian fibroblast cell line DF-1 but does not require a conserved SH2 binding domain

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Ovine pulmonary adenocarcinoma, caused by jaagsiekte sheep retrovirus (JSRV), is a naturally occurring retrovirus-induced pulmonary neoplasm of sheep. We report here that expression of the JSRV *env* gene is sufficient to transform an avian embryo fibroblast cell line, DF-1. DF-1 cells transfected with an avian sarcoma-leukaemia retroviral expression vector containing the JSRV *env* gene [pRCASBP(A)-J:*env*] exhibited changes consistent with transformation, including contraction and rounding of cells with formation of dense foci. Transfection with a reporter construct expressing the green fluorescent protein did not induce morphological changes in DF-1 cells, eliminating the possibility that the vector, the transfection protocol or culturing techniques were responsible for the transformed phenotype. When pRCASBP(A)-J:*env*-transfected cells were inoculated into nude mice, tumours formed, verifying that the DF-1 cells were tumorigenic. Analysis of the JSRV *env* gene revealed a conserved tyrosine (597) and methionine (600) residue in the cytoplasmic tail within the transmembrane domain of the envelope, which creates a known binding site of SH2 domains in the p85 subunit of phosphatidylinositol 3-kinase. However, when this tyrosine residue was mutated to serine or alanine, transformation was not affected. Furthermore, mutation of the methionine residue to valine or leucine also failed to eliminate JSRV *env*-mediated transformation. These results are in contrast to mutational analysis performed in JSRV *env*-transformed murine NIH-3T3 cells in which both the tyrosine and methionine residues are necessary for transformation. These findings suggest that more than one mechanism may be involved in JSRV *env*-mediated transformation.

Introduction

Ovine pulmonary adenocarcinoma (OPA) is a naturally occurring retrovirus-induced pulmonary neoplasm of sheep, which shares certain characteristics with human bronchioalveolar carcinoma (BAC) (DeMartini & York, 1997; Palmarini *et al.*, 1997; Palmarini & Fan, 2001). Both OPA and BAC are well-differentiated tumours of alveolar type II (ATII) or non-ciliated bronchiolar (Clara) cells exhibiting multifocal growth and a peak occurrence in adults (Bonne, 1939; Liebow, 1960; Perk & Hod, 1982; Palmarini & Fan, 2001; Platt *et al.*, 2002). The incidence of BAC, which is only weakly associated

with smoking, has dramatically increased since 1955 and now represents 24% of lung cancers, or as many as 40 000 deaths annually (Wu *et al.*, 1988; Barsky *et al.*, 1994; Ginzburg *et al.*, 1996). While the aetiology of BAC is unknown, OPA is one of only a few epithelial cancers associated with a retrovirus aetiology (Morris & Cardiff, 1987). Thus, OPA is an excellent model with potential to contribute significantly to our understanding of retroviral oncogenesis as well as pulmonary biology.

Classified as a betaretrovirus (type B/D chimeric retrovirus), jaagsiekte sheep retrovirus (JSRV) clone JSRV_{JS7} has been shown to cause OPA (York *et al.*, 1992; Palmarini *et al.*, 1999; DeMartini *et al.*, 2001). JSRV is considered a simple retrovirus and contains no obvious oncogene within its genome although an alternative open reading frame of unknown function within the *pol* gene has been identified and

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is referred to as *orfX* (Bai *et al.*, 1999; Palmarini *et al.*, 1999). Although JSRV does not contain an obvious oncogene, OPA pathogenesis is reminiscent of an acute transforming retrovirus in that OPA lesions can be seen in experimentally inoculated lambs within weeks to months after inoculation (Sharp *et al.*, 1983; DeMartini *et al.*, 1987; Rosadio *et al.*, 1988). Recently, it has been reported that the JSRV *env* gene is capable of transforming murine NIH-3T3 cells as well as a rat fibroblast cell line, F208 (Maeda *et al.*, 2001; Rai *et al.*, 2001). The purpose of this study was to test the hypothesis that the JSRV_{JS7} *env* gene was sufficient to transform an avian embryo fibroblast cell line, DF-1, and that the transformed cells would form tumours in mice. In addition, we examined the possible role of a conserved SH2 binding domain in the cytoplasmic tail within the transmembrane domain (TM-CD) of the envelope protein.

To examine JSRV genomic components for oncogenic effects, we used a series of avian sarcoma-leukaemia retroviral (ASLV)-derived vectors [pRCASBP(A)] to express JSRV sub-genomic components in the cell line DF-1 (Federspiel & Hughes, 1997; Schaefer-Klein *et al.*, 1998; Fisher *et al.*, 1999). The DF-1 cell line is a non-transformed, immortalized avian cell line that efficiently supports replication of ASLV-derived vectors and has been used for the study of oncogenic transformation (Himly *et al.*, 1998). In addition, the DF-1 cell line was derived from an EV-0 embryo, which was free of endogenous retroviral sequences with high homology to RAV-0, thus eliminating the possibility of recombination with the introduced recombinant virus (Schaefer-Klein *et al.*, 1998). The pRCASBP(A) vectors were constructed by removing the *src* gene from the virus genome and replacing it with a multiple cloning region (Federspiel & Hughes, 1997). JSRV_{JS7} *env*, *orfX* and the surface glycoprotein (SU) component of the *env* gene were each cloned separately into pRCASBP(A) vectors and their effect on DF-1 cells was monitored.

We report here that only the *env* gene of JSRV_{JS7} is necessary for transformation of DF-1 cells. Furthermore, in contrast to recent data suggesting that a conserved SH2 binding domain in the TM-CD of the envelope protein is necessary for transformation in murine NIH-3T3 cells (Palmarini *et al.*, 2001), the elimination of this binding site did not abrogate JSRV *env*-induced transformation of DF-1 cells. These findings suggest that more than one mechanism may be involved in JSRV *env*-induced transformation *in vitro* and in neoplastic events in OPA.

Methods

Cell culture. DF-1 cells (ATCC CRL-1203) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS; Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (DMEM) at 39 °C in 10% CO₂.

Plasmid construction and nomenclature. All plasmid propagation, ligations and PCR amplifications were performed as described in Sambrook *et al.* (1989). The vector pGreen-Lantern (Gibco BRL) was used

as a template for amplification of the gene for green fluorescent protein (GFP). The vector pRCASBP(A) was generously supplied by Galen H. Fisher (NIH) and has been described (Federspiel & Hughes, 1997; Fisher *et al.*, 1999). Primers used to create the pRCASBP(A)-based vectors are listed in Table 1. Primers were designed to create a 5' *NotI* site and a 3' *Clal* site for cloning into the pRCASBP(A) expression vector. Primers used for PCR were: TA-64F/65R for pRCASBP(A)-J:*orfX*, TA-66F/67R for pRCASBP(A)-J:*env*, TA-56F/57R for pRCASBP(A)-J:SU, TA-66F/105R for pRCASBP(A)-J:(-99) and TA-48F/49R for pRCASBP(A)-J:*gfp*. PCR amplification cycles for each primer set consisted of: 94 °C for 2 min (1 cycle); 94 °C for 15 s, 55 °C for 30 s, 72 °C for 45 s (30 cycles); and 72 °C for 10 min (1 cycle).

Transfection of the DF-1 cell line. All transfections were performed using LipofectAmine, as described by the manufacturer (Life Technologies). Briefly, DF-1 cells were plated on 60 mm tissue culture plates followed by incubation for ~ 15 h or until the cells were ~ 70% confluent. Two µg of plasmid was then mixed with 18 µl LipofectAmine in 400 µl serum-free DMEM (SFM) and allowed to incubate for 40 min at room temperature followed by the addition of 1.6 ml of SFM. DF-1 cells were washed twice with SFM and the transfection mixture was added directly on to cells. Cells were incubated for 5 h at 39 °C in a 10% CO₂ incubator followed by the addition of 2 ml DMEM containing 20% FBS. Cells were incubated for ~ 15 h followed by removal of the transfection mixture and replacement with DMEM.

Infection of DF-1 cells. Tissue culture supernatants from pRCASBP(A)-J:*env*-transfected, JSRV *env* mutant-transfected and pRCASBP(A)-J:*gfp*-transfected DF-1 cells were harvested and tested for reverse transcriptase (RT) activity. DF-1 cells were plated on 60 mm tissue culture plates and allowed to grow overnight so that they would be ~ 70% confluent at the start of the experiment. Virus-containing supernatants were then added to the DF-1 cells and virus was allowed to adsorb to the cells for ~ 5 h at 39 °C. Cells were then washed twice in DMEM and maintained for the duration of the experiment in DMEM at 39 °C in 10% CO₂.

RT activity. RT activity was monitored by measuring bromodeoxyuridine incorporation into an immobilized template (Lenti-RT; Cavid Tech) as described by the manufacturer.

RNA isolation and Northern analysis. RNA was isolated from DF-1 cells and from mouse tissue using the RNeasy RNA isolation kit (Qiagen). Northern blots were prepared as described (Sambrook *et al.*, 1989). Probes used for Northern blot analysis were created using digoxigenin-11-uridine 5'-triphosphate as described by the manufacturer (Roche). Primers used to produce probes were: TA-48F/49R for *gfp*, TA-64F/65R for *orfX*, TA-56F/57R for SU and TA-60F/61R for *env* (Table 1).

RT-PCR. RT-PCR was performed using a one-step RT-PCR method as described by the manufacturer (Life Technologies). Conditions for cDNA production involved incubation at 50 °C for 30 min. Subsequent PCR amplification cycles for each primer set consisted of: 94 °C for 2 min (1 cycle); 94 °C for 15 s, 55 °C for 30 s, 72 °C for 45 s (35 cycles); and 72 °C for 10 min (1 cycle). Primers (Table 1) for production of cDNA and PCR amplification were the same as those used for creation of the pRCASBP(A) plasmids listed above with the addition of TA-88F/91R for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

DNA isolation and PCR. DNA from DF-1 cells and mouse tissue was isolated using a DNeasy DNA purification kit (Qiagen) as described by the manufacturer. DNA PCR amplification cycles and primer sets were

Table 1. List of primer sequences

Primer	Sequence (5' → 3')	Gene
TA-64F	GCGGCCGCATGCAGCCCGAAATCCCAT	<i>orfX</i>
TA-65R	ATCGATTTAATAATTGATGTTTAAGGCAT	<i>orfX</i>
TA-66F	GCGGCCGCATGCCGAAGCCCGCGCTGG	<i>env</i>
TA-67R	ATCGATTCACGGTTCGTCCTCCCGCATCTC	<i>env</i>
TA-56F	GCGGCCGCATGCCGAAGCGCCGCGCTGG	SU
TA-57R	ATCGATTCTTTAGGGCGGCTAAGAGCC	SU
TA-48F	ATGAGCAAGGGCGAGGAACTGTTCACTGG	<i>gfp</i>
TA-49R	TCACTTGACAGCTCGTCCATGCCATGTGT	<i>gfp</i>
TA-88F	ACTGGTGTCTTACCACCATGGAGAAGG	<i>gapdh</i>
TA-91R	TCCACAATGCCAAAGTTGTCATGGATGAC	<i>gapdh</i>
TA-60F	ATGGGCCTGAGCCTGATTATTCTG	TM
TA-61R	TCACGGGTCGTCCTCCCGCATCTCC	TM
TA-105R	TGTATCGATTCAAACCTCATCTTTAGAAAGTC	<i>env</i>
TA-118F	GCTGCATATGAAAGCTAGAAATATGTTACAGCACC	<i>env</i>
TA-119R	GGTGTGTAACATATTTCTAGCTTTCATATGCAGC	<i>env</i>
TA-120F	GCTGCATATGAAATCTAGAAATATGTTACAGCACC	<i>env</i>
TA-121R	GGTGTGTAACATATTTCTAGATTTTCATATGCAGC	<i>env</i>
TA-122F	GCTGCATATGAAATATAGAAATGTTACAGCACCAAC	<i>env</i>
TA-123R	GTTGGTGTGTAACACATTTCTATATTTTCATATGCAGC	<i>env</i>
TA-124F	GCTGCATATGAAATATAGAAATCTGTTACAGCACCAAC	<i>env</i>
TA-125R	GTTGGTGTGTAACAGATTTCTATATTTTCATATGCAGC	<i>env</i>

Table 2. Morphological changes observed with RCASBP(A)-J:*env* and pRCASBP(A)-J:*env*

Raised, scattered raised areas of densely packed cells; Round, contraction and rounding of cells; Foci, microscopic foci of piled cells and increase in metabolic activity; Macro, macroscopically visual foci and dramatic increase in metabolic activity.

Experiment	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 12
Transfection								
Expt #1		Raised		Round			Foci	Macro
Expt #2			Raised		Round		Foci	Macro
Expt #3		Raised		Round		Foci		Macro
Infection								
Expt #1	Raised	Round			Foci		Macro	
Expt #2	Raised	Round			Foci		Macro	
Expt #3	Raised	Round	Foci		Macro			

the same as described above for RT-PCR except that 30 cycles were performed instead of 35.

■ **Limiting dilution analysis.** Tissue culture supernatant from cells in culture 20 days after transfection with pRCASBP(A)-J:*env* was added to DF-1 cells plated 24 h earlier on 60 mm tissue culture plates (in triplicate). At the time of inoculation, cells were ~ 70% confluent. A series of tenfold dilutions of the virus-containing supernatant stock in a final volume of 2 ml DMEM was added to cells and the virus was allowed to adsorb for ~ 5 h at 39 °C. Cells were then washed in DMEM and cultured at 39 °C with 10% CO₂ in DMEM. Cells were trypsinized and diluted (1:5) on days 3, 5 and 7 post-transfection. Cell monolayers were monitored daily for changes in morphology and phenotypic behaviour.

Supernatant collected on day 9 was tested for RT activity as described above. Cell cultures were monitored for a minimum of 13 days to ensure sufficient time to observe JSRV *env*-related results.

Experiments designed to test the relative efficiency of transformation of the JSRV *Env* mutants produced using site-directed mutagenesis (see below) were performed essentially as described above with the following modifications. Virus stocks used for the limiting dilution assay were collected from transfected cells on day 15. RT assays (see above) on the various viral stocks were performed to ensure that equal titres of virus were used in each experiment. A series of twofold dilutions were used (in triplicate) to determine the end-point dilution at which no phenotypic changes consistent with transformation were observed. Supernatants

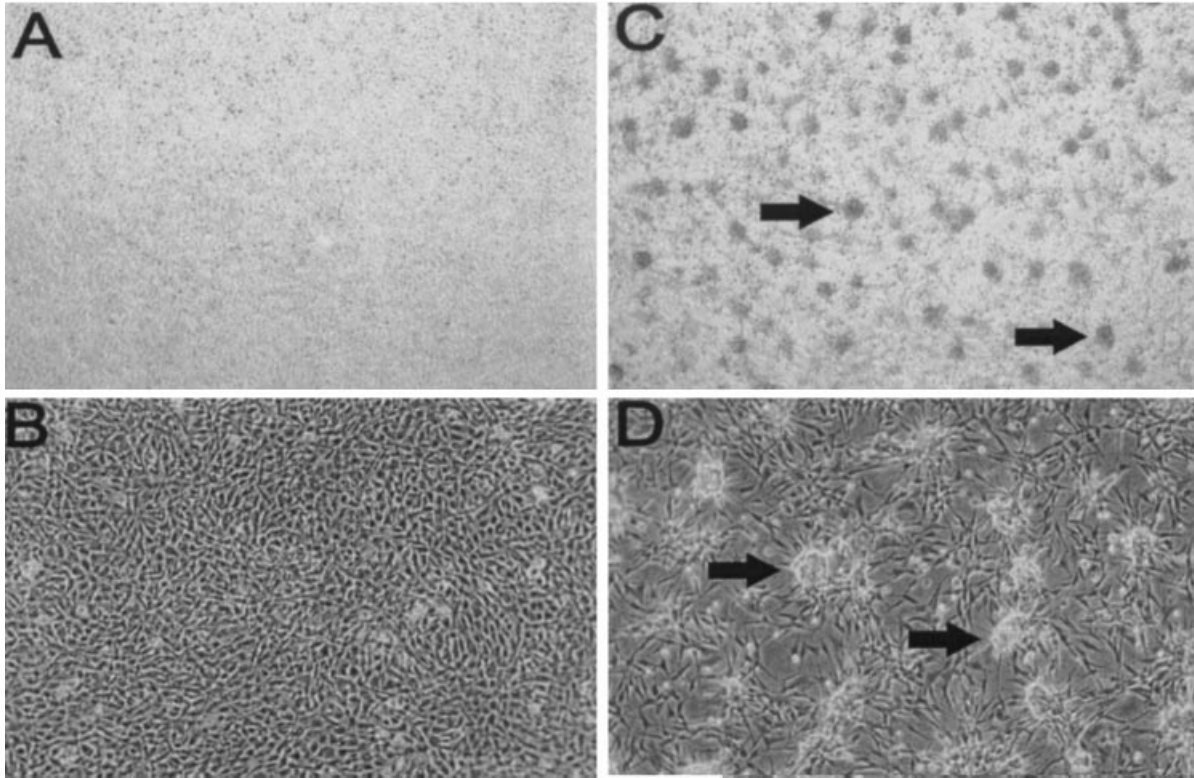


Fig. 1. JSRV_{JS7} *env*-induced morphological changes in DF-1 cells. (A) (magnification 20 ×) and (B) (40 ×) represent DF-1 cells at day 12 post-transfection with pRCASBP(A)-*gfp*. No changes in morphological or growth characteristics were seen in these cells. (C) (20 ×) and (D) (40 ×) represent DF-1 cells at day 12 post-transfection with pRCASBP(A)-J:*env*. Indications of altered growth characteristics and morphological changes can be seen. Cells appear rounded and have contracted into obvious foci containing multi-layered cells. Arrows highlight typical cellular foci.

collected from infected cells were tested for RT activity on both day 9 and day 13. Finally, cell cultures were monitored for 17 days to ensure sufficient time to observe JSRV *env*-related results. The Kärber method was used to estimate the 50% endpoint for transformation (Payment & Trudel, 1993).

■ **Tumor induction in nude mice and histology.** To determine the capacity of transfected DF-1 cells to form tumours *in vivo*, three female athymic nude mice (Harlan) were inoculated subcutaneously at separate sites with 10^5 , 10^6 and 10^7 pRCASBP(A)-J:*env*-transfected DF-1 cells and two mice were inoculated with the same concentrations of DF-1 cells transfected with pRCASBP(A)-*gfp*. For inoculation, transfected cells were harvested 20 days after transfection, washed in PBS and resuspended in PBS. Cell suspensions (200 μl) containing the indicated cell concentration were inoculated subcutaneously into nude mice and the sites of implantation were observed for 40 days after which the mice were sacrificed. Tissue masses were processed by fixation in 4% paraformaldehyde for 24 h followed by paraffin embedding. Following sectioning (5 μm), slides were stained with eosin and haematoxylin. Additional sections of tumour tissue were stained by histochemistry using the Alcian blue technique for acid mucopolysaccharides and Masson's trichrome technique for collagen. Statistical relevance of tumour formation in mice was analysed using Fisher's Exact Test.

■ **Site-directed mutagenesis.** Mutation of the JSRV_{JS7} *env* gene was performed by site-directed mutagenesis using a PCR-based, commercially available kit (Quikchange XL; Stratagene) as described

by the manufacturer. Primers used for introducing mutations were: TA-118F/119R for pRCASBP(A)-J:*env*(YΔA), TA-120F/121R for pRCASBP(A)-J:*env*(YΔS), TA-122F/123R for pRCASBP(A)-J:*env*(MΔV), TA-124F/125R for pRCASBP(A)-J:*env*(MΔL). Temperature cycling involved 94 °C for 1 min (1 cycle); 94 °C for 50 s, 60 °C for 50 s, 68 °C for 12 min (18 cycles); and 68 °C for 7 min (1 cycle).

Results

Transfection of DF-1 cells

To examine whether expression of JSRV subgenomic components is capable of inducing cellular changes, we developed a series of pRCASBP(A) plasmid-based viral vectors to express JSRV_{JS7} subgenomic components individually in the avian embryo fibroblast cell line, DF-1 (Federspiel & Hughes, 1997; Schaefer-Klein *et al.*, 1998). The pRCASBP(A) vector was created by removing the *src* gene of the virus and replacing it with a multiple cloning region flanked by a 5' *NotI* site and a 3' *Clal* site (Federspiel & Hughes, 1997). Vectors for the expression of JSRV_{JS7} *orfX*, *env* and the SU portion of *env* were created by PCR amplification as described in Methods (Table 1). In addition, a vector containing the gene for GFP was created to act as a control for JSRV non-specific events.

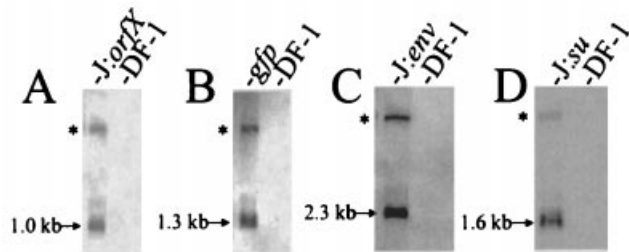


Fig. 2. Northern blot analysis of DF-1 cells transfected with (A) pRCASBP(A)-J:*orfX*; (B) pRCASBP(A)-J:*gfp*; (C) pRCASBP(A)-J:*env*; and (D) pRCASBP(A)-J:*su*. Approximate sizes of transcripts are denoted. The symbol (*) identifies the full-length virus genome containing the indicated gene. The Northern blot analysis demonstrates that the transfected cells contain similar levels of transcripts of the introduced gene.

Plasmid-based viral constructs were transfected into DF-1 cells and transfection efficiencies determined (data not shown). Although initial transfection efficiencies were low, the robust production of virus and subsequent infection of surrounding cells compensated for the inefficiencies. For each pRCASBP(A) construct, production of recombinant virus was confirmed by testing the cell supernatant for RT activity (data not shown). To monitor virus spread and expression of GFP, cells transfected with pRCASBP(A)-*gfp* were monitored by fluorescence (data not shown).

JSRV_{JS7} envelope gene induces changes in DF-1 cells consistent with transformation

Cells transfected with the pRCASBP(A) vectors were monitored for changes in growth characteristics. Whereas no changes were seen with pRCASBP(A)-J:*orfX*, *gfp* and the SU component of *env*, dramatic changes were observed with pRCASBP(A)-J:*env* (Table 2 and Fig. 1). Between day 5 and day 6 post-transfection, scattered cells throughout the culture appeared to be raised from the monolayer and densely packed. Between days 7 and 8, indications of rounding, contraction and multi-layering of cells were observed. By day 10, all pRCASBP(A)-J:*env*-transfected cultures displayed microscopic dense cellular foci and a striking decrease in the pH of the cell culture medium compared with that of control cells, suggesting an increase in metabolic activity (data not shown). By 12 days post-transfection, macroscopic foci were observed in all pRCASBP(A)-J:*env*-transfected cell cultures (Fig. 1).

To confirm that RNA transcripts for RCASBP(A)-J:*orfX*, *su*, *env* and RCASBP(A)-J:*gfp* were present in transfected cells, Northern blot hybridization analysis was performed. In each case, a band of the correct size was detected (Fig. 2). No corresponding bands were observed with non-transfected DF-1 cells (Fig. 2). To confirm the production of recombinant virus and to test for the ability of the recombinant virus to transform DF-1 cells, tissue culture supernatant from transfected cells was used to infect freshly plated DF-1 cells. Morphological changes

in DF-1 cells infected with RCASBP(A)-J:*env* virus were qualitatively equivalent to cells transfected with the pRCASBP(A)-J:*env* plasmid vector (Table 2). However, changes were seen at earlier time points, presumably due to rapid widespread infection of the virus carrying the JSRV_{JS7} *env* gene. As in the transfection experiments, no morphological changes were observed with RCASBP(A)-*gfp* virus.

To quantitate the transforming effect of RCASBP(A)-J:*env*, a limiting dilution strategy was employed. Cultures of DF-1 cells were exposed to virus-containing supernatant that was serially diluted to achieve an endpoint dilution where no JSRV *env*-induced events were observed. Transformation occurred in all cultures at dilutions of 10^{-5} in each of three independent experiments. However, at dilutions of 10^{-6} , no transformation was detected in any culture. To confirm the presence of RCASBP(A)-J:*env* virus in cultures exhibiting the transformed phenotype, RT activity in infected cultures was assayed. Only cultures that displayed a transformed phenotype contained RT activity in tissue culture supernatant (data not shown). These results demonstrate a direct and consistent correlation between the presence of the RCASBP(A)-J:*env* virus and the transformation of DF-1 cells.

Tumorigenicity of the JSRV_{JS7} envelope gene

To test for tumorigenicity of DF-1 cells transfected with the pRCASBP(A)-J:*env* vector, three mice were inoculated subcutaneously with cells from DF-1 cultures at 20 days post-transfection. As a control, two mice received the same number of cells from pRCASBP(A)-J:*gfp*-transfected DF-1 cells, also collected at 20 days post-transfection. After a few days, the initial swelling observed in all mice resolved. Subsequent development of subcutaneous nodules was then observed in all mice inoculated with 10^7 cells transfected with the pRCASBP(A)-J:*env* vector. In two of the three mice, nodules 3–4 mm in diameter were seen at the site of cell implantation at 19 days post-implantation. These grew progressively to 6–7 mm in diameter by 40 days post-implantation, when the experiment was terminated. At the time of termination, the third mouse inoculated with pRCASBP(A)-J:*env*-transfected cells had developed a 2 mm nodule at the site of cell implantation. In addition, one of the mice developed a lesion at a site inoculated with 10^6 cells that also progressively grew over 40 days to 6–7 mm in diameter. No growth was observed at any site inoculated with cells transfected with pRCASBP(A)-J:*gfp*. Using the Fisher Exact Test, tumour formation induced by cells transfected with pRCASBP(A)-J:*env* was found to be statistically significant ($P < 0.04$) when compared with controls.

At necropsy, white–grey tissue masses were found in the subcutis below the panniculus carnosus muscle at the site of the gross lesions in mice inoculated with pRCASBP(A)-J:*env*-transfected cells. Histologically, the masses were generally similar, consisting of well-circumscribed cellular masses ex-

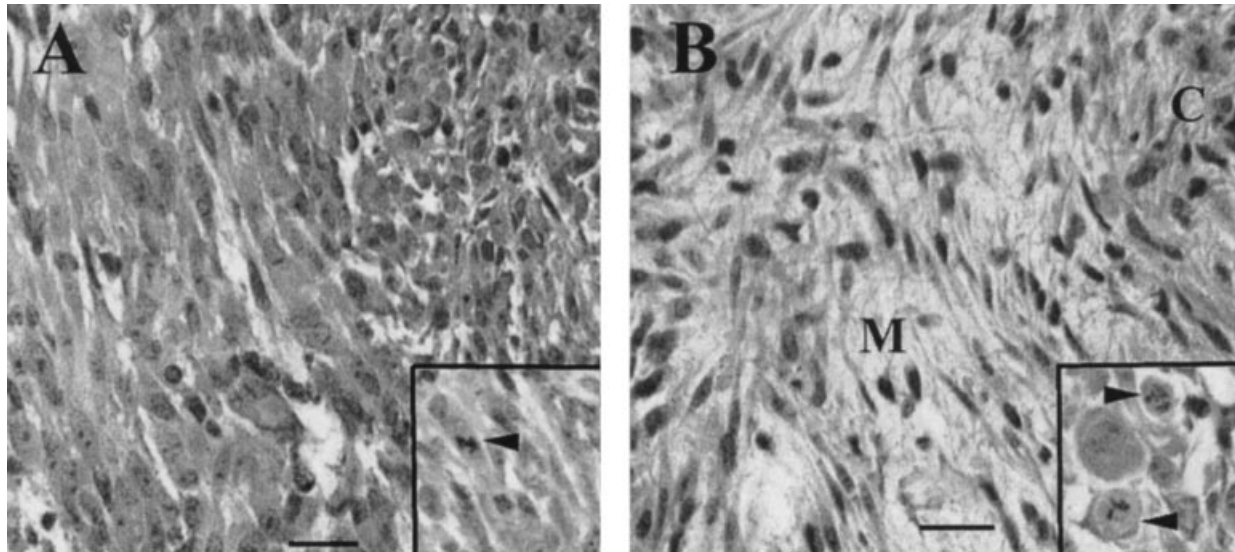


Fig. 3. Histopathology of a subcutaneous nodule from a nude mouse. Two areas of one mass are shown. (A) Densely cellular area consisting of spindle cells with oval nuclei and prominent nucleoli, and occasional mitotic figures (arrowhead, inset). (B) Loosely arranged central area consisting of spindle cells separated by mucopolysaccharide matrix (M) and collagen (C), and larger, anaplastic cells with two mitotic figures (arrowheads, inset). Bar, 50 μ m.

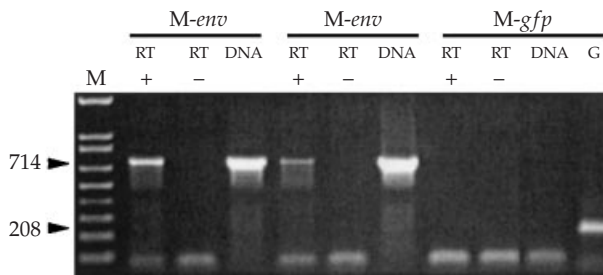


Fig. 4. Confirmation of transcription and integration of the JSRV_{J57} *env* gene in cells isolated from mouse tumours. DNA and RNA was isolated from tumour masses from two mice inoculated with transformed DF-1 cells (M-*env*). Liver tissue was used to isolate DNA and RNA from a mouse inoculated with pRCASBP(A)-*gfp*-transfected DF-1 cells (M-*gfp*). Primers for the TM component (714 bp) of the JSRV_{J57} *env* gene were used for each analysis. RT, lanes depicting RT-PCR analysis; + and -, RT-PCR experiments with or without RT; DNA, lanes containing samples from DNA PCR analysis. Only the mice that received transformed DF-1 cells were shown to contain both integrated JSRV_{J57} *env* and the transcript of the gene. The lane marked G represents a control DNA PCR for GAPDH (208 bp) to confirm the quality of DNA from the control mouse.

hibiting dense cellularity in the peripheral areas and a loose arrangement in the central areas (Fig. 3). The cells were spindle-shaped to polygonal with moderate amounts of cytoplasm and anisokaryotic nuclei, often containing prominent nucleoli. A few multinucleate or binucleate cells were seen. The mitotic index averaged 1–3 per high power field (40 \times objective). In the central areas, the stellate cells were separated by a myxomatous matrix, revealed by histochemistry to contain acid mucopolysaccharides, and some areas of eosinophilic

fibrillar material revealed by histochemistry to contain collagen (data not shown).

To confirm the presence of the JSRV_{J57} *env* gene in the tumour tissue, DNA PCR was performed using primers specific for the TM component (Table 1). In the two mice tested, PCR amplification demonstrated the presence of the *env* gene (Fig. 4). The presence of JSRV *env* RNA also was confirmed by RT-PCR analysis for the JSRV_{J57} *env* transcript (Fig. 4).

Deletion mutant analysis

Comparison of the predicted envelope protein sequence from four JSRV and three endogenous sheep retroviruses related to JSRV (ESRV) revealed that the greatest dissimilarity occurred in the C terminus of the TM domain of the envelope protein (Fig. 5A) (Palmarini *et al.*, 2000). This region also contains the cytoplasmic tail of the envelope protein, a site of potential cellular protein interactions. To determine if this variable region plays a role in mediating JSRV *env* transformation, a series of three deletion mutants were constructed that removed sequential, non-overlapping coding sequences starting from the 3' end of the *env* gene, each corresponding to a deletion of 33 amino acids per mutant construct (Fig. 5A).

In three independent experiments, DF1 cells were transfected individually with each of the plasmid constructs. In each case, no transformation occurred. Thus, analysis focused on construct pRCASBP(A)-J:(-99), which contained the full-length *env* gene with the last 99 nucleotides of the 3' end of the gene removed. To ensure that the loss of the transformed phenotype was not due to reduction in transcription efficiency, Northern blot hybridization analysis was performed. Tran-

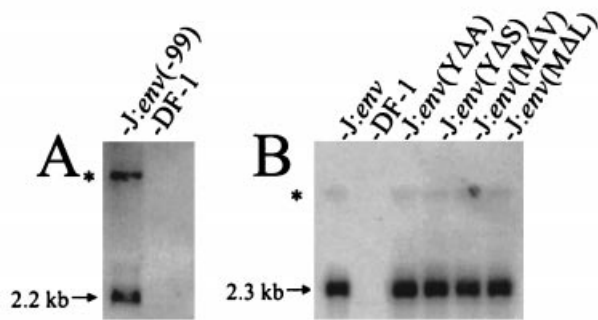


Fig. 6. Northern analysis of JSRV mutants. (A) RNA transcription levels of the deletion mutant, pRCASBP(A)-J: (-99), in DF-1 cells 9 days after transfection with the plasmid construct. No band is seen in untransfected controls (DF-1). (B) RNA transcription analysis of JSRV mutants created by site-directed mutagenesis of the Y-X-X-M sequence, a known binding site of SH2 domains in the p85 subunit of PI3-K. Transcription levels for each mutant are comparable with cells transfected with the non-mutated counterpart, pRCASBP(A)-J:env. No signal is seen in untransfected controls. The symbol (*) identifies the full-length virus genome containing the indicated gene.

not shown). These results demonstrate a direct and consistent correlation between the presence of the RCASBP(A) viral construct expressing the JSRV *env* gene and the transformation of DF-1 cells.

Discussion

The production of pRCASBP(A)-based viral vectors permitted experiments to test the cytopathic effects of subgenomic components of JSRV_{JS7} in DF-1 cells. In these experiments, only the JSRV_{JS7} *env* gene induced observable changes consistent with transformation (Fig. 1). In addition, the ability of DF-1 cells transfected with the *env* gene to induce tumours when transplanted into mice confirmed that the cells were tumorigenic (Shin *et al.*, 1975). It is interesting to note that the SU component of the *env* gene did not by itself cause transformation. This result suggests that the TM domain is either directly responsible for transformation or is required for functional presentation of the SU protein, possibly by influencing protein folding, modification and/or trafficking. Experiments designed to determine directly a role for the TM protein in transformation using our system were inconclusive since little TM mRNA was observed in DF-1 cells transfected with constructs designed to overexpress this protein.

Clinical and pathological features of JSRV-induced OPA are consistent with both an acute and non-acute transforming virus. In experimentally induced disease, OPA lesions are seen weeks to months after inoculation (DeMartini *et al.*, 1987; Rosadio *et al.*, 1988). The rapid onset and multifocal nature of the lesions suggest that JSRV may act by directly inducing cell proliferation, thus resembling an acute transforming virus. However, in naturally occurring cases, OPA is seen almost exclusively in adults, and lesions are typically a single lung

tumour mass (DeMartini & York, 1997). In this way, JSRV may resemble a non-acute transforming virus. Since it can be assumed that younger sheep are exposed to the virus, the more frequent occurrence of OPA in adult sheep suggests a long latent period during which virus replication and cellular proliferation may be followed by JSRV-induced insertional mutagenesis. Genetic changes in the target cells may then lead to expansion of a monoclonal or oligoclonal cell population and tumour formation. The recent description by our laboratory of an integration event into the SP-A surfactant gene of an ATII cell line isolated from a natural occurring tumour is of interest, especially since a hallmark feature of OPA is the abundant production of lung fluid that is rich in SP-A (Sharp *et al.*, 1983; DeMartini *et al.*, 1987). Work is in progress to determine whether a common integration site can be found in OPA tumours. The diverse manifestations of OPA suggest that JSRV-induced OPA may be an example of multistep carcinogenesis (Morris & Cardiff, 1987; Fan, 1997). However, the evidence in this report supports the hypothesis that the JSRV *env* gene product may be sufficient to cause neoplasia.

With the observation that the *env* gene causes transformation and tumour production, attention turns to the possible mechanism of action. There are few precedents of a retroviral structural protein directly causing neoplasia. Most acute transforming retroviruses contain within their genome an oncogene that is responsible for rapid development of tumours (Kung *et al.*, 1991; Kung & Liu, 1997). These viruses are usually replication defective and require a non-defective 'helper virus' to replicate and spread (Kung *et al.*, 1991; Kung & Liu, 1997). For example, the spleen focus-forming virus directly causes the initial proliferative stage of erythro-leukaemia in infected mice (Fang *et al.*, 1998). The interaction of the gp55 component of the *env* gene with the erythropoietin cell surface receptor triggers massive erythroid proliferation (Fang *et al.*, 1998). In contrast, the avian hemangioma retrovirus is a replication-competent virus whose *env* gene has been recently shown to cause proliferation of NIH-3T3 cells directly, although the mechanism of action is unknown (Alian *et al.*, 2000).

The presence of a conserved SH2 binding motif in the predicted cytoplasmic tail of the JSRV envelope suggests a role for phosphatidylinositol 3-kinase (PI3-K) in the transforming properties of this protein. This domain is present in all isolates of JSRV but not in the related endogenous proviruses, ESRV (Palmarini *et al.*, 2000). Recent work in murine NIH-3T3 cells has implicated a role for PI3-K and AKT kinase in mediating transformation (Palmarini *et al.*, 2001). In that report, mutational analysis of the Y-X-X-M motif demonstrated that both the tyrosine and methionine residues were necessary to induce transformation. In addition, AKT kinase was activated in transformed cells. Proteins of the PI3-K/AKT kinase cascade have been implicated as determinants in many human cancers (Moore *et al.*, 1998; Datta *et al.*, 1999; Kobayashi *et al.*, 1999; Shayesteh *et al.*, 1999). For example, AKT genes are

overexpressed or amplified in breast, pancreatic, ovarian and prostate cancers (Datta *et al.*, 1999). Relevant to retroviruses, the avian sarcoma virus 16, which induces hemangiosarcomas in chickens, has been shown to contain an oncogene that is derived from the cellular catalytic subunit of PI3-K (Chang *et al.*, 1997). This gene has been shown to be a potent transforming factor in chicken embryo fibroblast cells and to activate AKT kinase (Chang *et al.*, 1997). However, the results presented in this report do not support a direct role for this motif in DF-1 transformation since elimination of either the tyrosine (597) or the methionine (600) residues contained within this motif did not abrogate transformation. Nevertheless, the elimination of the last 33 amino acids of the TM-CD of the Env protein does eliminate transformation, suggesting a role for the cytoplasmic domain in transformation, although this role may be as trivial as influencing the correct folding and/or trafficking of the protein.

The discrepancy in observed results regarding the SH2 binding domain between the DF-1 system and the NIH-3T3 system is intriguing. A possible explanation for the observed differences is that PI3-K is activated in both DF-1 and NIH-3T3 cells, but PI3-K does not directly interact with the JSRV Env protein. Alternatively, it is possible that at least two distinct mechanisms are involved in transformation. Recent efforts in our laboratory have focused on the development of JSRV Env-specific antibodies, which should prove invaluable to the further study of Env-induced transformation. Of particular interest is the examination of the phosphorylation state of the tyrosine in the Y-X-X-M motif, a prerequisite for direct interaction with PI3-K.

The DF-1 cell system using pRCASBP(A) vectors should prove to be a useful model for dissecting the molecular mechanism involved in JSRV tumorigenesis. It should be noted, however, that the events observed in this report involve an avian fibroblast cell line, whereas the predominant cell type transformed in OPA is an epithelial cell of the lung, the ATII cell. While it seems unlikely that the transformation event seen in this report is specific to DF-1 cells, confirmation of these results will need to be made in the ovine system. Recently, our laboratory has demonstrated the ability to isolate and culture ovine primary ATII cells (T. Allen, unpublished results), which will allow for a direct examination of JSRV *env*-induced events in the predominant neoplastic cell type found in OPA. By analysing and comparing the mechanism of JSRV *env*-induced DF-1 cell transformation with *env*-induced effects on ATII cells, a more thorough examination of the role that JSRV *env* plays in OPA will be possible.

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