Identification of avian leukemia virus subgroup J-associated acutely transforming viruses carrying the v-src oncogene in layer chickens

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To elucidate the molecular basis for the rapid oncogenicity of an acutely transforming avian leukemia virus (ALV), isolated from fibrosarcomas in Hy-Line Brown commercial layer chickens infected with ALV subgroup J (ALV-J), the complete genomic structure of the provirus was determined. In addition to ALV-J replication-complete virus SDAU1102, five proviral DNA genomes, named SJ-1, SJ-2, SJ-3, SJ-4 and SJ-5, carrying different lengths of the v-src oncogene were amplified from original tumours and chicken embryo fibroblasts (CEFs) infected with viral stocks. The genomic sequences of the SJ-1–SJ-5 provirus were closely related to that of SDAU1102 but were defective. The results of Western blot analysis and immunohistochemical staining also showed overexpression of the p60 v-src protein in infected CEFs and tumour tissue. To the best of our knowledge, this is the first report of the isolation and identification of acutely transforming viruses carrying the v-src oncogene with ALV-J as the helper virus. It also offers insight into the generation of acutely transforming ALVs carrying the v-src oncogene.

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INTRODUCTION

Avian leukemia virus (ALV) is an oncogenic retrovirus known to induce the formation of several types of tumours in chickens and is divided into seven subgroups (A–J), according to the envelope protein, of which subgroup J (ALV-J) is the most pathogenic and widespread (Payne & Nair, 2012). Since it was first isolated in the UK in 1991 (Payne et al., 1991), ALV-J has spread rapidly worldwide. Moreover, the pathogenicity of ALV-J has increased in China in recent years and has induced a greater diversity of tumours compared with the ALV-J prototype virus, HPRS-103, such as myeloid leukosis, haemangioma, histiocytic sarcoma, fibrosarcoma and erythroblastoma in both broilers and layers (Cheng et al., 2010; Gao et al., 2010, 2012; Ji et al., 2012; Lai et al., 2011; Pan et al., 2011; Qu et al., 2012; Shi et al., 2011). Although a series of programmes monitoring ALV-J surveillance and control have been conducted in China, the epidemic status is still particularly serious among indigenous Chinese chicken breeds (Dong et al., 2015).

It has been demonstrated that the ALV-J genome does not contain any oncogenes, suggesting that ALV induces tumour formation by insertional mutagenesis, which requires a relatively long period of about 20 weeks (Rubin, 2011; Vogt, 2012). Some ALV strains can obtain cellular oncogenes (c-onc) from host cells by homologous recombination events during infection to become ‘acutely transforming viruses’ (ATVs), which have the ability to rapidly induce tumour formation in chickens over a period of only a few weeks and can transform some specific cell types in vitro (Maeda et al., 2008). However, most ATVs are replication defective and can replicate only when replication-competent viruses are present to serve as ‘helper viruses’. There have been few reports of the isolation and identification of ALV-J-associated ATVs; however, it was demonstrated recently that the ATV strain 966 carrying a v-myc oncogene, isolated from an ALV-J-induced tumour, could induce a rapid onset of tumours in chickens (Chesters et al., 2001; Payne et al., 1993).

During 2009–2012, we received a large number of ALV-J-infected chickens from different layer farms in China. During autopsy examinations, fibrosarcoma lesions were observed in different percentages (0.1–7.6 %) of sick chickens suffering from ALV-J myelocytomas and haemangiomas. Subsequently, a series of experiments was...
performed, demonstrating that these fibrosarcomas were caused by ATVs (Li et al., 2012, 2013; Liu et al., 2011; Wang et al., 2012). In a previous study, ALV-J–associated ATVs named Fu-J1–Fu-J5 carrying the v-fps oncogene were isolated and identified from fibrosarcomas in ‘817’ hybrid broiler chickens (Chen et al., 2012), and chimeric sequences consisting of the v-src oncogene and viral env gene were also amplified from a fibrosarcoma in a 240-day-old ALV-J-infected Hy-Line Brown commercial layer chicken. Furthermore, the complete sequences of the proviral genomes named SJ-1, SJ-2, SJ-3, SJ-4 and SJ-5 were determined from original fibrosarcoma DNA and infected chicken embryo fibroblast (CEF) DNA. In this study, the genomic structures of SJ-1–SJ-5 were deciphered and compared with those of SDAU1102, the helper virus, and other ATVs carrying the v-src oncogene.

RESULTS

Full-length genome sequencing of the replication-complete helper virus SDAU1102

The full-length proviral genome of SDAU1102 was sequenced by PCR amplification, using both original fibrosarcoma DNA and viral stock–infected CEF DNA. Sequence analysis demonstrated that the full-genome sequence of SDAU1102 was 7652 bp, with a typical retroviral genome structure required for complete virus replication (Fig. 1a). Phylogeny evolution analysis of the GP85 glycoprotein with other reference strains confirmed that SDAU1102 belonged to subgroup J ALV (ALV-J). Multiple alignments of the SDAU1102 genome and its deduced amino acid sequences with other ALV-J reference strains are listed in Table 1. The results indicated that the whole proviral genome of SDAU1102 had the closest phylogenetic relationship with the JS09GY6 strain (98.0 %), while the identity with other reference ALV-J strains ranged from 93.8 to 95.6 %.

Viral genomes SJ-1–SJ-5 carry different lengths of the v-src oncogene

Different chimeric fragments consisting of the v-src oncogene and the ALV viral gene were amplified from both original fibrosarcoma DNA and viral stock–infected CEF DNA by PCR using primers 5’LTR-F/src-R, 3’pol-F/src-R, and src-F/3’LTR-R, respectively (Table 2). Five defective viral genomes designated SJ-1, SJ-2, SJ-3, SJ-4 and SJ-5 were assembled. All of the SJ-1–SJ-5 viruses were found to be replication defective because they obtained the v-src oncogene at the expense of losing essential viral genes that are indispensable for virus replication. The SJ-1–SJ-5 viral genomes were described according to the available information as follows: 5’LTR-A gag-s src-D env-3’LTR or 5’LTR-gag-D pol-s src-D env-3’LTR (Fig. 1a). Sequence analysis also revealed that SJ-1–SJ-5 were derived from SDAU1102 with the 5’LTR and residual gag and pol sequences having 94.2–96.1 % sequence identity, and the 3’ LTR and residual env sequences having 97.0 % sequence identity. Therefore, it could be concluded that SDAU1102 co-existed with SJ-1–SJ-5 to function as a helper virus. Furthermore, the viral stocks were named as follows: SJ-1 (SDAU1101), SJ-2 (SDAU1101), SJ-3 (SDAU1101), SJ-4 (SDAU1101) and SJ-5 (SDAU1101), respectively, according to international nomenclature convention.

Sequence analysis showed that the genomic structures of SJ-1–SJ-5 were similar and that all contained the same right-hand src-env junction, with the sequence 5’-GACAG-C CCCGATA-3’, in which nine out of the 13 bp were identical between the SDAU1102 genome and the 3’ region of the c-src gene (Figs 1a and 2b). The formation of this right-hand site yielded a termination codon (TAG) upstream from the 3’ terminal of the c-src gene, located at a position corresponding to the sequence code for Tyr527 of the p60c-src protein (Fig. 2b). However, the left-hand recombination junctions of SJ-1–SJ-5 demonstrated heterogeneity compared with the homogeneity of the right-hand recombination junction (Fig. 2a). SJ-1 and SJ-2 contained the complete v-src ORF and upstream non-coding sequences with splice acceptor sites. SJ-3 contained the complete v-src ORF, without any of the non-coding sequences, while SJ-4 and SJ-5 contained only a partial v-src sequence with a deletion mutation in the 5’ terminal. Moreover, it should be noted that, as for SJ-3, SJ-4 and SJ-5, the number of nucleotide bases from the initiation codon of pol or env to the initiation codon of the v-src oncogene was not an integral multiple of three, which indicated that the Pol–Src or Env–Src fusion proteins were non-functional due to erroneous coding.

Sequence alignment of viral genomes SJ-1–SJ-5 and other ATVs carrying the v-src oncogene

Sequence analysis revealed that the viral genomic sequence of SJ-1 was similar to that of S-1 and S-2 (Hagino-Yamagishi et al., 1984), which were isolated from naturally occurring sarcomas, and the recovered avian sarcoma virus (rASV) PR2257 (Nehyba et al., 1988). Each had left-hand junctions located between the gag and v-src non-coding sequences but with different recombination positions (Fig. 1b). The main difference between SJ-1, S-1, S-2 and PR2257 was the diverse right-hand junctions. As mentioned above, the right-hand junction of SJ-1 was located between the 3’ terminal of v-src and env, which introduced a deletion mutation in the sequences encoding Tyr527 and downstream sequences coding for p60c-src. The viral genomes of S-1 and S-2 contained src-env or src-pol right-hand junctions, respectively. The v-src oncogenes of both S-1 and S-2 contained deletion mutations, upstream from the stop codon of the c-src gene at different positions; therefore, they expressed Src–Env and Src–Pol fusion proteins, respectively. The right-hand junction of PR2257 was located between the v-src non-coding sequence and the 3’ terminal of env. However,
Fig. 1. Viral genomic structure of SJ-1, SJ-2, SJ-3, SJ-4 and SJ-5 and their helper virus SDAU1102 compared with other ATVs carrying v-src oncogene. (a) The genomes of SJ-1–SJ-5 had the same 3’ right-hand junction but different 5’ left-hand junctions. All were replication-defective viruses derived from ALV-J strain SDAU1102, carrying different lengths of the v-src oncogene. (b) Comparison of genomic sequences of SJ-1, S-1, S-2 and PR2257 revealing similar genomic structures but different gag–src junctions. (c) Comparison of genomic sequences of SJ-2 and other ATVs carrying the v-src oncogene revealing that the genomic structure of SJ-2 is similar to those of BH-RSV and SR-RSV, which are the replication-defective viruses that had the shortest passage times of all RSV strains. SR-RSV and PR-C are replication-competent viruses carrying the v-src oncogene. In this figure, green boxes represent the gag region, pink boxes represent the pol region, blue boxes represent the env region, red boxes represent the v-src coding region and grey boxes represent the v-src non-coding regions. Dots represent stop codons.
A C inserted in the 3’ v-src sequence upstream from the stop codon of c-src changed the residues after Phe525 of p60 c-src. A deletion or substitution mutation to Tyr527 was therefore observed in each case.

It was determined that SJ-2, SJ-3 and SJ-4 had genomic sequences similar to those of Rous sarcoma virus (RSV) strains 29 (RSV-29) and Bryan high-titre (BH-RSV) (Dutta et al., 1985; Lerner & Hanafusa, 1984) with left-hand junctions between pol and the v-src oncogene but at different positions as shown in Fig. 1(c). The main difference between them was the location of the right-hand junctions. The right-hand junctions of SJ-2, SJ-3 and SJ-4 were located between the v-src oncogene and env, while those of strains RSV-29 and BH-RSV were located between the v-src oncogene and non-coding regions of the helper ALV. Notably, strains RSV-29 and BH-RSV are replication-defective viruses, which had the shortest passage times of all the RSV strains. The genomic sequence of SJ-2, SJ-3 and SJ-4 indicated that the original RSV strain may also have been replication defective, and replication-competent viruses carrying the v-src oncogene like SR-RSV and PR-C were generated during serial passage later.

The homology of the v-src sequence of SJ-1 was also compared with the c-src and v-src sequences in other ATVs. Other than the 21 bp deletion in the 3’ terminal of the v-src gene in SJ-1, sequence analysis revealed that

Table 1. Multiple alignments of the SDAU1102 genome and deduced amino acid sequence with other ALV-J reference strains

<table>
<thead>
<tr>
<th>SDAU1102</th>
<th>Known ALV-J strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPRS103</td>
</tr>
<tr>
<td>LTR (325 bp)</td>
<td>92.9</td>
</tr>
<tr>
<td>Leader sequence (296 bp)</td>
<td>91.0</td>
</tr>
<tr>
<td>gag (2106 bp; 701 aa)</td>
<td>96.0</td>
</tr>
<tr>
<td>pol (2622 bp; 873 aa)</td>
<td>97.4</td>
</tr>
<tr>
<td>gp85 (921 bp; 307 aa)</td>
<td>98.5</td>
</tr>
<tr>
<td>gp37 (591 bp; 197 aa)</td>
<td>93.8</td>
</tr>
<tr>
<td>DRI</td>
<td>87.9</td>
</tr>
<tr>
<td>E element (146 bp)</td>
<td>92.6</td>
</tr>
</tbody>
</table>

Table 2. Specific primers used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Location (nt)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>CACCACATTGGTGTGCACCTGGGT</td>
<td>239–262</td>
<td>gag sequence</td>
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<tr>
<td></td>
<td>gag-R</td>
<td>GAAGGGGCGCAGGGTCAATGCAA</td>
<td>2800–2823</td>
<td></td>
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<tr>
<td>2</td>
<td>pol-F</td>
<td>GAGATTGTCTGTGACCGCCCTAGGCT</td>
<td>2684–2708</td>
<td>pol sequence</td>
</tr>
<tr>
<td></td>
<td>pol-R</td>
<td>TGGCAAGCACGGGTGCTCTTCCG</td>
<td>5399–5421</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>env-F</td>
<td>GAGGTGACTAAGAAAGATGAGGCGA</td>
<td>5280–5304</td>
<td>env sequence</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>4†</td>
<td>3’ UTR-F</td>
<td>GGCTTCCGTTGTACGGGATAGGA</td>
<td>7131–7154</td>
<td>LTR circular</td>
</tr>
<tr>
<td></td>
<td>5’ UTR-R</td>
<td>CTTCCAAGACCCCTCTGCTGTCG</td>
<td>510–534</td>
<td>sequence</td>
</tr>
<tr>
<td>5</td>
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<td>TGTAGCTTATGCAATAACTCTTATGTA</td>
<td>1–27</td>
<td>LTR-gag-src</td>
</tr>
<tr>
<td></td>
<td>3’pol-F</td>
<td>TAATATATCTATGACGGCAGCAGGG</td>
<td>4529–4554</td>
<td>LTR-pol-src</td>
</tr>
<tr>
<td></td>
<td>src-R</td>
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<td>6</td>
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<td>3’LTR-R</td>
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<td>7627–7652</td>
<td>sequence</td>
</tr>
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</table>

*Primers were designed according to the proviral genome sequence of ALV-J JS09GY6 (GenBank accession no. GU982310) isolated from commercial layers in China.

†The ALV genome can form a circular structure during replication in host cells; the 3’ UTR-F and 5’ UTR-R primers were designed to target this sequence.

‡Primers were designed according to chicken v-src oncogene (GenBank accession no. NM_205457).
the nucleotide identity between the coding regions of v-src in SJ-1 and c-src was as high as 99.6 % with seven nucleotide mutations (G96A, T210C, T609C, G809A, A903C, T1044C and C1581G), and the amino acid similarity was 99.9 % with only one amino acid mutation (G270E). Sequence analysis also revealed that the v-src gene in SJ-1 had the highest homology with the c-src of all ATVs carrying v-src oncogenes. This may be because the SJ-1–SJ-5 viruses were isolated from original fibrosarcoma detected in natural cases with the shortest passage time compared with the other ATVs.

p60\textsuperscript{v-src} protein expression in infected CEFs and tumour cells

In our study, p60\textsuperscript{v-src} protein expression in tumour cells was detected by immunohistochemical analysis (IHC), using mouse anti-Src monospecific serum as the primary antibody. The results showed positive staining for the Src protein in almost all of the tumour cells, and this was located predominantly in the cytoplasm (Fig. 3b). To determine the molecular size of the Src protein, the total protein in CEFs infected with viral stocks, in CEFs infected with the helper virus SDAU1102 and in untreated

![Diagram](image-url)
CEFs was extracted. Western blot analysis was also performed using mouse anti-Src monospecific serum to detect expression of p60v-src protein (Fig. 3a). The results showed that a specific band with a molecular mass of approximately 60 kDa was detected in the SJ (SDAU1102)-infected CEFs when using mouse anti-Src monospecific serum as an antibody, while no bands were detected in the CEFs infected with SDAU1102 or in the untreated CEFs.

**DISCUSSION**

The transduction of oncogenes is a common feature of retroviruses. ALV, a member of the genus Alpharetrovirus of the family Retroviridae, can also acquire cellular oncogenes by recombination events during the infection process, producing ATVs with the ability to induce transformation in some specific types of cultured cells and to rapidly induce tumour formation in chickens within 2 weeks (Maeda et al., 2008; Vogt, 2012). To date, dozens of naturally occurring ATVs coding for different viral oncogenes – such as src, fps, myc, myb, jun and ros – have been isolated (Martin, 2001; Vogt, 2012; Wang & Hanafusa, 1988). In general, ATVs lose genes that are essential for virus replication or infection during the process of transduction and acquire cellular oncogenes. Therefore, most ATVs, other than some specific RSV strains, are replication-defective viruses. Consequently, these viruses can only replicate when the replication-competent viruses that function as helper viruses co-exist to provide viral gene products. Most of the isolated ATVs are currently subgroup A/B/C/E-associated replication-defective viruses, with only 966 and Fu-J using ALV-J as their helper virus (Chen et al., 2012; Chesters et al., 2001). Another ATV strain was isolated recently from fibrosarcomas obtained from a 240-day-old ALV-J-infected Hy-Line Brown commercial layer chicken in China. Both the fibrosarcoma cell-free filtrate and the supernatant of the infected CEFs induced similar fibrosarcomas in chickens, indicating transduction of oncogenes (Wang et al., 2012). In this study, the genomic structures of SJ-1–SJ-5, assembled from original fibrosarcoma DNA and infected CEF DNA, were deciphered and compared with those of SDAU1102, the helper virus, and other ATVs carrying the v-src oncogene.

There are currently three different types of ATVs known to carry v-src oncogenes: RSV, ASV S1 and S2, and ASV PR2257 (Schwartz et al., 1983). To date, all RSVs, including RSV-29, BH-RSV, PR-C and SR-SRV, have been derived from the original tumours collected by Rous (1910). Most of these viruses are replication defective, with the exception of some specific strains, such as PR-C and SR-SRV, which carry v-src oncogenes downstream of the env gene. The chronological pedigree and sequence analysis reported previously suggested that non-replication-defective RSV strains were derived from a parent replication-defective RSV strain (Dutta et al., 1985). In this study, SJ-1–SJ-5 variants were isolated from original tumours in chickens infected with ALV-J, which provided direct evidence that the early RSV strains were most likely replication-defective viruses and precursors of...
non-defective RSV. In addition, to the best of our knowledge, this is the first report of ATV carrying the v-src oncogene with ALV-J as a helper virus.

It is generally recognized that ATVs are generated by two recombination events (Swanstrom et al., 1983; Vogt, 2012; Wang, 1987). Initially, ALV proviral DNA was inserted into the host genome upstream of cellular oncogenes and viral–cellular chimeric RNA was transcribed by the viral LTR promoter. Next, a second recombination event occurred between this viral–cellular chimeric RNA and genomic RNA of the helper virus, leading to the generation of replication-defective viral genomes carrying different oncogenes. As described above, SJ-1–SJ-5, five different quasi-species variants, were isolated from the original tumours, which demonstrated the diversity in the early stage of ATVs generated by naturally occurring recombination events. In fact, similar phenomena have been described in several previous studies. A series of td109-derived rASVs containing deletions to the env, pol and gag genes were replaced by various lengths of v-src oncogenes (Wang et al., 1984). A similar event occurred in ALV-J strain 966, which is an ATV carrying the v-myc oncogene isolated from chicken bone marrow infected with HRPS-103 (Chesters et al., 2001). A range of viral genomes with structures similar to that of ALV-J strain 966 was identified by PCR amplification in tumours induced by HRPS-103. A more recent example is the ALV-J strains Fu-J1–Fu-J6 isolated in China from a fibrosarcoma that carries the v-fps oncogene (Chen et al., 2012). It appears that different variants are generated during the early stage of recombination between ALV and host cellular oncogenes, which may have resulted from the ambiguous homology required in this replication process.

Our results showed that SJ-1–SJ-5 proviral genomes or viral genomes could be detected in infected CEFs or cellular supernatant (data not shown), indicating that they all exist in the form of infectious virions. The main differences found among the SJ-1–SJ-5 genomes were the insertion site and the length of the v-src oncogene. The v-src oncogenes in SJ-1 and SJ-2 encoded a p60v-src protein with a deletion mutation of Tyr527 and downstream amino acids compared with p60v-src, which appeared to be crucial for transforming activity, as described previously (Martin, 2001, 2004; Wyke, 1983). Sequence analysis also revealed that the SJ-3, SJ-4 and SJ-5 viral genomes did not directly express the Src oncoprotein. Moreover, Western blot analysis showed that only a 60 kDa band could be detected in the total protein of viral stock-infected CEFs using mouse anti-Src monospecific serum as antibody. In contrast, no band was detected in the protein of SDAU1102-infected CEFs using the same serum. This implied that the SJ-3, SJ-4 and SJ-5 viral genomes probably did not express Src protein or expressed it at a very low level that could not be detected. The protein produced from the v-src ORF in the SJ-4 and SJ-5 viral genomes, assuming that they could encode mutant Src oncoproteins, was insufficient to induce tumour formation. The N-terminal glycine and its 10–14 downstream amino acids of p60v-src are essential for myristoylation and membrane-associated function, which are necessary for cell transformation (Cross et al., 1984; Kaplan et al., 1988; Linder & Burr, 1988; Shoji et al., 1989). Unfortunately, the p57v-src encoded by an ORF in SJ-4 and p51v-src encoded by an ORF in SJ-5 only contain deletion mutations within this region. Nevertheless, research to determine whether SJ-1–SJ-5 viral genomes transcribe independent v-src mRNA and to analyse their tumorigenicity should be further investigated by reverse genetics techniques in the future.

METHODS

Virus preparation and cell culture. The viral stock of ATV SJ (SDAU1102) strain (containing helper virus SDAU1102) was prepared from the cell-free filtrate of fibrosarcomas in a 240-day-old ALV-J-infected Hy-Line Brown commercial layer chicken (Wang et al., 2012). The viral stock of SJ (SDAU1102) or cellular supernatant of infected CEFs could rapidly induce similar fibrosarcomas in specific-pathogen-free leghorn chickens or broilers inoculated intraperitoneally or subcutaneously (Wang et al., 2012). The helper virus strain SDAU1102 was isolated from cultures exposed to viral stocks diluted beyond the end point of transformed focus formation. CEFs were prepared from 11-day-old chicken embryos, according to classical methods, and cultured in Dulbecco’s modified Eagle’s medium supplemented with 5 % newborn calf serum, as described previously (Cui et al., 2009).

Extraction of DNA from tumour tissues, PCR and sequencing. DNA was extracted from the original fibrosarcomas and CEFs infected with viral stocks after digestion with protease K, phenol/chloroform extraction and ethanol precipitation. Six pairs of primers were designed to amplify the proviral genome of helper virus SDAU1102 and the SJ strains (Table 2). Among these, the first four pairs of primers (nos 1–4) were used to amplify the genome of the helper virus, and the last two pairs of primers (nos 5 and 6) were used to amplify v-src and viral gene chimeric sequences. Proviral genome sequences were amplified using a routine PCR method with LA Taq polymerase (TaKaRa Biotechnology) in accordance with the manufacturer’s protocol. All PCR amplifications were performed using the following parameters: 94 °C initial denaturation for 7 min, and 32 cycles of initial denaturation at 94 °C for 40 s, annealing at 59–62 °C for 40 s and extension at 59–62 °C for 1–3 min, followed by a 10 min extension at 72 °C. The PCR products were run on an agarose gel and purified using a QIAquick Gel Extraction kit (Qiagen), cloned into the PMD-18T vector (TaKaRa Biotechnology) and sequenced by Shanghai Sangon Company. The proviral genome sequences were assembled and aligned using the CLUSTAL W program (http://www.genome.jp/tools/clustalw/) and MEGALIGN sequence alignment software (DNASTAR).

Western blotting assay, immunohistochemical analysis and ELISA. For Western blotting analysis, CEFs infected with viral stocks, CEFs infected with SDAU1102 and untreated CEFs were lysed with RIPA buffer (Beyotime Biotechnology). The protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology). The proteins were denatured by heating and separated by SDS-PAGE (10 % acrylamide) and transferred to a nitrocellulose membrane (EMD Millipore), which was blocked with 5 % skimmed milk in PBS containing 0.1 % Tween 20 (PBST) for 1 h at room temperature and incubated with mouse anti-Src monospecific serum at 4 °C overnight. Notably, the mouse anti-Src monospecific serum was prepared by immunization subcutaneously with a prokaryotically
expressed recombinant src oncogene. This monospecific serum only recognized the Src protein, with no cross-reaction between the Gag, Pol or Env protein. The blots were washed three times with PBST and incubated with goat anti-mouse HRP-conjugated secondary antibody (Sigma-Aldrich) for 1 h at room temperature. The blots were washed as described above, and positive reactions were detected using an enhanced chemiluminescence detection system (Beyotime Biotechnology). For IHC analysis, tumour tissues were fixed in formalin, non-specific reactions were blocked with normal sheep serum and the tumours were probed with mouse anti-Src monospecific serum prepared in our laboratory. After the primary antibodies had been washed away with PBS buffer, the fixed tissue was stained with HRP-conjugated goat anti-mouse antibody (Sigma-Aldrich) and an enhanced tetramethylbenzidine substrate kit (TransGen). Production of the ALV p27 antigen of cultured cell supernatants was determined by ELISA (Idexx Laboratories), according to the manufacturer’s protocol.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences analysed in this study are: RSV Schmidt-Ruppin A, L29199; duck-adapted RSV (Pr-C), X51861; avian sarcoma virus PR2257T, X51863; RSV-29, A0002315 and M11114; RSV Schmidt-Ruppin B, AF052428; chicken c-src mRNA, S43613 and NM_205457; ALV HPRS103, Z46390; ALV ADOL-7501, A027920; ALV NX0101, DQ115805; ALV SD07LK1, FJ216405; ALV JS09GY6, GU982310.

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