Genome sequence analysis of the avian retrovirus causing so-called fowl glioma and the promoter activity of the long terminal repeat.

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So-called fowl glioma is a poorly understood entity characterized by multiple nodular gliomatous growths associated with disseminated nonsuppurative encephalitis (Summers et al., 1995). This disease has been described as glioma (Belmonte, 1935; Jackson, 1954), astrocytoma (Jungherr & Wolf, 1939; Reece, 1997), astroblastoma, mixed gliomas (Biering-Sorensen, 1956), epizootic gliosis and astrocytoma (Wight & Duff, 1964). We had previously reported that avian leukosis virus (ALV) infection causes this disease (Ochiai et al., 1999; Iwata et al., 2002a). The virus, which belongs to subgroup A, definitely shows neurotropic oncogenesis also in ovo inoculation (Tomioka et al., 2003). ALV commonly induces bursal lymphoma (Ewert & de Boer, 1988). Provirus is integrated next to the c-myc proto-oncogene and c-myc is abnormally expressed under the control of sequences in the long terminal repeat (LTR)-associated enhancer (Hayward et al., 1981). To the best of our knowledge, avian retrovirus does not spontaneously induce brain tumours, although some avian sarcoma viruses induce neurogenic tumours following intracerebral inoculation (Vasquez-Lopez, 1936; Beard, 1980). The neuropathology for several ALVs has been previously described, but they caused meningoencephalomyelitis without any nodular gliomatous lesions (Ewert et al., 1990; Whalen et al., 1988). In the present study, we determined the complete nucleotide sequence of the glioma-inducing virus and performed a functional analysis of the LTR to find a clue about the unique oncogenicity in the central nervous system (CNS). Chick embryo fibroblasts (CEF) were prepared from 10-day-old SPF chicken embryos of the WL-M/O (C/O) strain. Fertile eggs of the commercial SPF White Leghorn strain WL-M/O were purchased from Nippon Institute for Biological Science. This strain lacks both chicken helper factor (chf) and group specific (gs)-antigen and is susceptible to ALV subgroups A-E (International registry of poultry genetic stocks, Bulletin 476, March 1988, Ralph G. Somes, Jr, PhD, University of Connecticut, USA). Cell culture and preparation of the viral suspension were performed using the methods described by Iwata et al. (2002a). For sequencing, a 100-fold concentrated culture supernatant was prepared as described previously (Bowles et al., 1996). Total RNA was extracted from the CEF inoculated with this concentrated virus and uninfected CEF using the TRIZOL reagent (Invitrogen). The extracted RNA was treated with 40 U of RNase-free DNase (Roche Diagnostics). Reverse transcription was carried out using random hexamers (Takara) and SuperScript II RNase H− Reverse Transcriptase (Invitrogen). Primers for PCR were designed based on the nucleotide sequence of ALV-A (accession No. M37980) or sequences newly obtained by genome walking. The PCR amplification for sequences more than 1–2 kb in length was performed using LA Taq polymerase (Takara). To obtain short-PCR products of
up to 1-2 kb, amplification was performed using AmpliTaq DNA polymerase (Applied Biosystems). The PCR amplifications were performed using temperature profiles within the following ranges, depending on expected product sizes and primer sequences: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30-60 s, 50-63 °C for 30-60 s, 72 °C for 1-3 min, and a final extension at 72 °C for 10 min. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into the pGEM-T vector (Promega). More than five colonies containing an insert were selected and plasmid DNA was purified by a standard mini-prep method. Sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems) and a model 310 genetic analyser (Applied Biosystems). All computational sequence analysis was done using the GENETYXMAC 10.1.2 package (Software Development) in combination with the BLAST program at GenBank. The sequences (with accession numbers) used in the alignments were: ALV-RSA (M37980), ALV-HPRS103 (Z46390), Rous sarcoma virus (RSV; Prague C; J02342), RSV (Schmidt-Ruppin B; AF052428, Schmidt-Ruppin A; U41731, Schmidt-Ruppin D; D10652), avian carcinoma Mill Hill virus 2 (K02082), avian sarcoma virus CT10 (Y00302), and Y73 sarcoma virus (J02027). The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number AB112960.

The full cDNA sequence of the glioma-inducing virus was 7,448 nucleotides long with a genetic organization typical of replication-competent type C retroviruses lacking viral oncogenes. A schema of the structure of the virus and comparison with other strains mentioned in the following sections are shown in Fig. 1. The gag and pol genes of the ALV (bases 383 to 2485 and 2506 to 5193, respectively) were well conserved with those of other avian leukosis and sarcoma viruses (ALSVs) (which show 96 to 98 % overall identity to the sequences of subgroups A, B, C, D and J). The gag and pol genes of ALV-A and RSV-Pr-C were most closely related to those of the glioma-inducing virus. The sequence encoding the SU (gp85) domain of the virus isolate showed 82, 92, 83 and 87 % overall nucleotide identity to that of subgroup A, B, C and D, respectively, and was most closely related to that of RSV-SR-B (92 %). The SU-encoding sequences had only 47 % identity overall to the corresponding sequences of ALV-J. The sequence encoding the TM (gp37) of the virus isolate showed over 92 % identity to the corresponding sequences of subgroup A, B, C, and D, and 61 % identity to that of ALV-J.

The 3’ untranslated region (3’ UTR) adjacent to the LTR of the virus isolate showed 62, 82, and 59 % overall nucleotide identity to the sequences of ALV subgroup A, B, and J, respectively. The 3’ UTR was 308 nucleotides long, and the 5’ and 3’ ends (approx. 90 nucleotides, respectively) of the sequence were closely related to those of sarcoma virus Y73 (Y73SV; more than 90 % identity) (Fig. 2). The fragment sequence between them was somewhat divergent from that of Y73SV (58 % identity) and was most similar (96 % identity) to that of RSV SR-B. The 3’ UTR of the virus isolate contained a single copy of the direct repeat (DRI) element, which is also found as a single copy in ALVs but as two copies in ASVs. The 5’-leader of the virus isolate had three ATG codons and three short open reading frames, which are conserved with respect to their positions and primer binding site in all ALSVs. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number AB112960.

Fig. 1. Sequence comparison between avian retrovirus causing so-called fowl glioma and other avian leukosis sarcoma viruses. The boxed sequence line shows the structure of the glioma-inducing virus genome. The short lines above indicate viral sequences showing high homology with those regions of the glioma-inducing virus.

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The sequence analysis revealed that the LTR of the glioma-inducing ALV (LTRgli) had a few deletions and several point mutations compared to that of other ALVs. Therefore, we examined the transcriptional activity of the LTRgli in each of three different cell lines: CEF, Vero, and human astrocyte U87MG. Vero cells and U87MG were grown at 37°C in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf medium. For PCR amplification of the full-length LTRs, total DNA was extracted from the CEF infected with the glioma-inducing virus or ALV-A standard strain, RAV-1. The PCR was performed using primers designed based on the 5' part of the U3 region and 3' part of the LTR, with the following thermocycling conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. pLTRgli/CAT or pLTRrav1/CAT were constructed by cloning the full-length of the LTR sequence of the glioma-inducing virus or RAV-1 directly upstream of the chloramphenicol acetyltransferase (CAT) gene in pCAT-Basic (Promega) and prepared for transfection using a standard mini-prep method. Control vector DNAs, pCAT-Basic with no inserts.
and pSV2/CAT (Gorman et al., 1982) containing simian virus 40 (SV40) early gene promoter, were similarly prepared. Cells were plated onto six-well culture plates and incubated until approximately 60% confluent. Plasmid DNA (0·625 pmol) was transfected by using Metafectene (Biontex). Forty-eight hours after transfection, the cells were harvested and cell lysates were prepared. The CAT assay was performed using CAT-ELISA (Roche). All transfections were performed in triplicate, and the mean fold induction and standard deviation were determined.

The results were normalized to a positive control containing the SV40 early gene promoter and enhancer (Fig. 3a–c). Transcriptional activity levels of the LTRgli observed in all cells examined were almost the same as or higher than those of the positive control. The transcriptional activity of the LTRgli in CEF was twelve times of that of the positive control and was higher than that observed in the other cells examined. In addition, the transcriptional activity of the LTRgli was compared with that of LTRrav1 in CEF and U87MG. Promoter activities of these two LTRs were equivalent in CEF (Fig. 3d), whereas the activity of the LTRgli was significantly lower than that of LTRrav1 in U87MG ($P<0·001$, Fig. 3e).

As was expected from the long incubation period in vivo, the glioma-inducing virus was a replication-competent, nonacute transforming ALV. The coding sequences of the virus were well conserved with those of other ALVs, while the noncoding regions were most closely related to replication-defective sarcoma viruses. The nucleotide sequences of the 3′ UTR have been suggested to provide some information on the phylogenies of the ancestral ALSVs (Onuki et al., 1987). The sequence of this region suggests the virus to be combined with several virus strains. Experimentally, recombinant viruses had shown an unexpected

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**Fig. 3.** (a–c) The transcriptional activity regulated by the LTR of glioma-inducing virus. The names of the reporter plasmids are shown at the bottom: Basic, pCAT-Basic; SV40, pSV2/CAT; LTR, pLTR/CAT. The transfected cells are CEF (a), Vero (b), and U87MG (c). CAT activity is represented relative to that obtained in pSV2/CAT-transfected cells. (d), (e) The transcriptional activity regulated by the LTRs of glioma-inducing ALV versus RAV-1. The names of the reporter plasmids are shown at the bottom: Basic, pCAT-Basic; LTRrav1, pLTRrav1/CAT; LTRgli, pLTRgli/CAT. The transfected cells are CEF (d) and U87MG (e). CAT activity is represented relative to that obtained in pLTRrav1/CAT-transfected cells.
and unusual pathogenesis unlike the parent viruses (Kogekar et al., 1987; Aurigemma et al., 1991).

Since the envelope glycoprotein of ALV serves as a ligand for a receptor (Weiss, 1992), the env gene influences the types of cell targets (Brown & Robinson, 1988). ALV-J, which has a distinct env gene (Bai et al., 1995), causes myeloid leukemia rather than lymphoid leukemia (Payne et al., 1992). The specific oncogenicity is suggested to be largely dependent on the viral envelope (Chesters et al., 2002). Unlike ALV-J, the env gene of the glioma-inducing ALV has high homology with that of subgroup A, B, C and D. Also the virus was detected in various organs with the same distribution pattern as other ALVs, indicating little difference among them in terms of viral entry and infection (Iwata et al., 2002b; Tomioka et al., 2003). The SU sequence more closely resembles SR-B than subgroup A, although we have previously reported the ALV causing glioma belongs to subgroup A. Some minor mutations in the sequence may be related to the biological activity. The cell type-specific oncogenesis regulated by the LTR is dependent on subtle differences among the cellular transcriptional factors. Because high expression of c-myc by stable transfection may lead to the elimination of infected pre-B cells before developing lymphoma, the labile, low LTR-enhanced transcription could be important in ALV lymphomagenesis (Ruddell et al., 1989; Bowers et al., 1994). The promoter activity of the LTRgli was significantly lower than that of LTRrav1 in the astrocytic cell line. The subtle sequence differences may be related to the transcriptional activity, which cause the unique capability of transformation.

As involved proto-oncogenes influence the neoplastic phenotype induced by ALV (Hayward et al., 1981; Fung et al., 1983; Kanter et al., 1988), it is also possible that the unique neoplastic phenotype of the glioma-inducing ALV could depend on the integration site. Further examination of these subjects is necessary for elucidation of the determinant of the neurotropic oncogenicity of this virus.

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