Infrequent Involvement of *c-fos* in Avian Leukosis Virus-Induced Nephroblastoma

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To determine whether *c-fos* is involved in avian leukosis virus-induced nephroblastoma, 28 tumors from chickens were analyzed for novel *fos* fragments. DNA from 1 of 16 clonal outgrowths (in chicken 6561) contained novel *fos*-related *Eco*RI and *Kpn*I fragments which hybridized to both *v-fos* and viral probes. Oncogenicity tests using filtered 6561 tumor cell homogenates did not reveal a tumor-inducing transduction of *c-fos*. We conclude that *c-fos* is only an occasional target for proviral insertions or new transductions in avian leukosis virus-induced nephroblastoma. The results also identify a polymorphism in *c-fos* in K28 chickens and demonstrate that unintegrated viral DNA is not a general characteristic of avian leukosis virus-induced nephroblastoma.

Avian leukosis viruses (ALVs) can cause cancer when proviral insertions convert a proto-oncogene to an oncogene or when proto-oncogene sequences are incorporated into the viral genome. ALV-induced B-cell lymphomas are associated with proviral insertions into *c-myc* or *c-myb* (7, 8, 10, 13, 14, 17), whereas cases of erythroblastosis are associated with insertions into *c-erbB* (6, 11, 15). An occasional lymphoma is associated with the incorporation of *c-myc* sequences into a viral genome (17). In contrast, up to 50% of the cases of erythroblastosis can contain new *erbB*-transducing viruses (3, 9, 11, 16). A target gene for ALV-induced nephroblastomas has not been identified. In a series of 10 nephroblastomas, one insertion which up-regulated *c-ras* was observed (23). Recently, a *c-fos*-transducing virus was isolated from a nephroblastoma that occurred in an old chicken on a poultry farm (12). To test whether *c-fos* might be a frequent target for insertions or transductions in ALV-induced nephroblastomas, tumors from 28 chickens have been examined by DNA blots for novel *c-fos* fragments.

Nephroblastomas. The nephroblastomas were collected during a series of oncogenicity tests initiated by intravenous inoculation of either $\sim 10^6$ infectious units of virus into day-old K28 or $(15_1 \times \text{K28}) \times \text{K28}$ chicks (5, 11) or 10^5 infectious units into 10 day-old SC or B-14 embryos (20). Inoculated ALVs included two naturally occurring subgroup A viruses, Rous-associated virus-1 (RAV-1) (19) and University of Rochester-2-associated virus (UR2AV) (22); one naturally occurring subgroup B virus, myeloblastosis-associated virus-2-O (MAV-2-O) (21); and recombinants between these viruses and the subgroup E endogenous virus of chickens (RAV-0 [5]), the subgroup A RCAN ALV vector (5), and a subgroup F virus recovered from Rous sarcoma virus-infected pheasant cells (20). The incidence of nephroblastoma in K28 chickens ranged from a high of 20% (RAV-1 induced) to a low of 5% (WF180 induced) (5). In the tests initiated in 10-day-old SC and B-14 embryos, MAV-2-O, UR2AV, and pEU induced, respectively, a 10 to 20, 5 to 10, and 2% incidence of nephroblastoma. Tumors were classi-

fied as nephroblastomas on the basis of histologic analysis revealing abnormal growth of epithelial as well as mesenchymal cells. All tumors contained aberrant tubular arrangements of epithelial cells. The mesenchymal cells in the tumors exhibited various stages of differentiation, with some nephroblastomas containing mesenchymal cells that ranged from primitive mesenchyme (blastema) to fully differentiated cartilage and bone and others containing only blastema or blastema and fibroblasts. Some tumors in K28 chicks containing only blastema or blastema and fibroblasts had previously been classified as renal-cell carcinoma (5). For discussion and photomicrographs of the differential diagnosis of avian nephroblastoma and renal-cell carcinoma, see Beard et al. (1).

DNA blot analyses. Tumors induced by proviral insertions arise from clonal outgrowths of cells that have a mutation or accumulated mutations in proto-oncogenes. To determine whether DNA extracted from the nephroblastomas represented DNA from a malignant expansion of a founder cell (as opposed to DNA from surrounding or intermingled normal or dysplastic tissue), DNA was extracted from tumors and analyzed on Southern blots for novel virus-host junction fragments. Because retroviruses integrate at many sites, Southern blots of restriction endonuclease-digested tumor DNA detect novel viral-host junction fragments only when the tumor has arisen from a clonal or oligoclonal outgrowth.

The clonality of the tumors in K28 chickens was tested by hybridizing blots of *Eco*RI-digested DNA with a probe for the ~ 165 bases 3' to the *Eco*RI site in the RAV-1, WF170, WF180, and WF190 viral long terminal repeats (probe 165) (Fig. 1e) (5, 18). Hybridization of probe 165 to *Eco*RI-digested DNA revealed a 2.3-kilobase (kb) internal ALV fragment that is present in all ALV-infected tissue, the 16- and 8.5-kb junction fragments of *ev-1* (the only endogenous virus present in K28 chickens), and 3' viral-host junction fragments which result from clonal or oligoclonal outgrowths of infected cells (Fig. 1b and e). The clonality of the tumors in SC and B-14 chickens was tested by hybridizing blots of *Kpn*I-digested tumor and control (spleen) DNA with RAV-1 sequences. UR2AV, EU8, and MAV-2-O have a single *Kpn*I

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FIG. 1. Tests for novel *c-fos* fragments in nephroblastomas. (a through c) Autoradiographs of DNA blots. Designations at the tops of panels indicate restriction endonucleases used to digest DNAs and the chicken (number) and tissue (RBC, red blood cells; neph, nephroblastoma; spl, spleen) from which each DNA was extracted. Designations at the bottoms of panels indicate hybridization probes. Numbers on the sides are sizes in kilobases, with asterisks designating novel *fes*-related fragments. (d and e) Schematics of *c-fos* and RAV-1 sequences indicating restriction endonuclease sites and hybridization probes used for DNA blot analyses. (f) Schematics indicating restriction endonuclease sites and hybridization patterns of novel *fes* fragments in the nephroblastoma in chicken 6561. E, *EcoRI*; K, *KpnI*; D, *DraI*; C, *ClaI*.

site in *pol* similar to that in RAV-1 (Fig. 1e) (2, 20). The DNA blots revealed a number of endogenous viruses segregating in the SC and B-14 pedigrees as well as 5' and 3' viral-host junction fragments that had resulted from clonal or oligoclonal outgrowths of infected cells (data not shown). DNAs from 16 of the 28 tumors examined revealed the presence of novel virus-host junction fragments. Data on these 16 clonal outgrowths are summarized in Table 1.

Abnormal growth of a tissue in response to ALV infection does not necessarily involve viral mutation of a proto-oncogene sequence. The best example of this is ALV-induced osteopetrosis, a nonclonal outgrowth of improperly differentiating osteoblasts. In osteopetrosis, the diseased tissue is associated with the presence of 5 to 10 copies of unintegrated viral DNA (18). Since unintegrated viral DNA has been reported in some cases of nephroblastoma (4), the tumor DNAs were tested for the presence of unintegrated

viral DNA by using blots of undigested or *KpnI*-digested DNA (data not shown). Two tumors, both induced by UR2AV, revealed low levels (~one copy per cell) of unintegrated DNA. One of the tumors was a clonal outgrowth (chicken 298). Thus, detectable levels of unintegrated DNA are not a general characteristic of ALV-induced nephroblastoma.

The 16 tumors that had been demonstrated to be clonal or oligoclonal outgrowths (DNAs which represented tumor DNA) as well as the 12 tumors that did not appear to be clonal outgrowths (DNAs which may or may not have represented tumor DNA) were next tested for the presence of novel *fes* fragments. DNAs from nonclonal tumors were included in these analyses, since new transductions of a proto-oncogene can cause a nonclonal tumor (11). Tests for novel *fes* fragments used DNA blot analyses of *EcoRI*- or *KpnI*-digested tumor DNA hybridized with *v-fos* or *c-fos*

TABLE 1. Clonal or oligoclonal nephroblastomas analyzed for insertions or transductions or both of *c-fos*

Chicken pedigree	Virus (subgroup)	Chicken no.	No. of novel junction fragments	Presence of novel <i>fos</i>
K28	RAV-1 (A)	5533	9	—
		5541	2	—
		6547	8	—
		6561	4	+
		7097	6	—
		7101	1	—
	WF170 (A)	1659	2	—
	WF180 (A)	2187	2	—
	WF190 (E)	8483	2	—
(15 ₁ × K28) × K28	RAV-1 (A)	4435	(1) ^a	—
		4582	(3) ^a	—
B-14	MAV-2-O (B)	RS86-34	(5) ^a	—
SC	UR2AV (A)	271	5	—
		298	5 ^b	—
		322	5	—
	pEU (A)	RS89-14	5	—

^a Tumors in pedigrees in which multiple endogenous viruses were present and for which control tissue from the bird with the tumor was not tested. For these tumors, fragments common to more than one tested tissue were considered to be of endogenous origin and are not included in the tally.

^b Tumor with unintegrated viral DNA. Number of novel fragments does not include those representing unintegrated DNA.

probes. The *Eco*RI digests tested *c-fos* as well as approximately 15 kb of upstream sequences for ALV insertions, and the *Kpn*I digests tested *c-fos* as well as approximately 7 kb of downstream sequences for ALV insertions (Fig. 1d). The *v-fos* probe was from pNK171 (12). The *c-fos* probe was prepared by subcloning the 1.8-kb *Kpn*I fragment of pfos706 (genomic clone of chicken *c-fos*) (12) into Bluescribe (Stratagene, La Jolla, Calif.).

When *Eco*RI-digested DNA blots were hybridized with *v-fos*, DNA from 13 of 15 K28 chickens revealed an ~23-kb as well as an ~19-kb band (chickens 5533 and 6547; Fig. 1a). The ~23-kb fragment appears to reflect a polymorphism segregating in K28 chickens, since it was present in normal as well as tumor tissues. Cleavage of both the ~23- and ~19-kb *Eco*RI fragments to a single ~15-kb fragment by *Eco*RI plus *Cla*I or *Dra*I digests suggests that the first *Eco*RI site downstream of *c-fos* determines this polymorphism (Fig. 1d; data not shown). The ~23-kb *c-fos* fragment was not observed in SC or B-14 chickens.

Only one of the nephroblastomas, a clonal outgrowth in chicken 6561, contained novel *fos* fragments. *Eco*RI-digested DNA from chicken 6561 contained three novel fragments, with sizes of approximately 5.8, 4.0, and 3.1 kb, that hybridized with *v-fos* as well as with probe 165 sequences (Fig. 1b). The 5.8-, 4.0-, and 3.1-kb *Eco*RI fragments did not hybridize with the *c-fos* probe (Fig. 1d; data not shown), suggesting that the novel fragments do not contain the first exon of *c-fos*. *Kpn*I-digested chicken 6561 DNA revealed a novel ~5.2-kb fragment that hybridized with the *v-fos*, *c-fos*, and 165 probes (Fig. 1c; data not shown).

To map *Eco*RI fragments with respect to other sites in chromosomal DNA, double digests were done with *Eco*RI plus *Dra*I and *Eco*RI plus *Cla*I (Fig. 1b and d). The 5.8-kb *Eco*RI fragment was digested by both *Cla*I and *Dra*I to an ~5.2-kb fragment that hybridized with *v-fos* and probe 165. The relative positions of *Eco*RI, *Cla*I, and *Dra*I sites and the

TABLE 2. Incidence of kidney tumors and novel *fos* fragments after inoculation of filtered homogenates into day-old K28 chicks^a

Expt	Source of filtered homogenate (chicken no.)	Chicks with kidney tumors/no. inoculated	Presence of novel <i>fos</i> in induced tumors ^b
1	6561	1 ^c /6	—
2	6561 10388	0/10	NA
		0/5	NA
3	6561 10388	0/18	NA
		0/17	NA

^a Chicks were sacrificed and autopsied between 2 and 3 months after inoculation.

^b NA, Not applicable.

^c No. 10388.

hybridization patterns of the 5.8- and 5.2-kb fragments suggest that the 5.8-kb fragment is bounded at its 5' end by an *Eco*RI site in viral sequences and at its 3' end by an *Eco*RI site of unknown origin. The 4.0- and 3.1-kb *Eco*RI fragments were not digested by *Cla*I or *Dra*I, indicating that both of these fragments also contained 5' as well as 3' mutations in *c-fos* sequences. Mapping data for the novel *fos* fragments are summarized in Fig. 1f.

Tests for transducing viruses. Because all of the novel *fos* fragments appeared to contain 5' as well as 3' mutations in chromosomal DNA, the tumor in chicken 6561 could have been caused by a transduction or transductions of *c-fos* sequences. To test for transducing viruses, filtered 6561 tumor homogenates were inoculated into 34 1-day-old K28 chicks (Table 2). One of the 34 chicks (no. 10388) developed a nephroblastoma within 2 months of inoculation. Southern blot analyses of the DNA from this tumor revealed a clonal outgrowth that did not contain novel *fos* fragments (data not shown). Inoculation of 22 1-day-old K28 chicks with filtered tumor homogenates from chicken 10388 did not result in the induction of rapid-onset nephroblastoma (Table 2). This suggests that the nephroblastoma in chicken 10388 was caused by RAV-1, the virus that induced the tumor in chicken 6561 (Table 1). RAV-1 causes a 3% incidence of nephroblastoma within the first 3 months of infection in day-old-inoculated K28 chicks (5). The inability to detect a tumor-inducing transduction of *c-fos* in a test group of 34 chicks is in sharp contrast to results with tumor homogenates containing candidate transductions of *c-erbB* or *c-myc*. Such transductions cause rapid-onset tumors in ~50% of inoculated chicks (11; unpublished data). Thus, we consider it unlikely that the novel fragments in the nephroblastoma in chicken 6561 represented a tumor-inducing transduction of *c-fos*.

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