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Cellular Sequences Related to Three New *onc* Genes of Avian Sarcoma Virus (*fps*, *yes*, and *ros*) and Their Expression in Normal and Transformed Cells

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Two *onc* genes of avian sarcoma viruses unrelated to the *src* gene have recently been identified: *fps* of Fujinami sarcoma virus/PRCII/UR1 and *yes* of Y73/Esh sarcoma virus. In the first part of this study we demonstrated that UR2, the most recently isolated avian sarcoma virus, contains in its genome a unique sequence, *ros*, nonhomologous to *src*, *fps*, and *yes* sequences or to transforming genes of avian acute leukemia viruses. Using cDNAs specific to the inserts of avian sarcoma virus genomes, we examined the existence and the transcription of cellular nucleotide sequences related to the three new *onc* genes of avian sarcoma virus (*fps*, *yes* and *ros*) in various cells. The progenitor cellular sequences for these *onc* genes (c-*onc*) were present in uninfected chicken DNA in one or few copies per haploid genome. These c-*onc* sequences were detectable in cellular DNA of a wide variety of vertebrates, and the homology between viral and cellular *onc* was inversely related to the phylogenetic distance of animal species. The pattern of expression of these c-*onc* genes in different tissues of chickens was found to be unique to each gene. The expression of c-*fps* and c-*ros* genes was generally repressed in many tissues, but c-*fps* was expressed at higher levels in bone marrow (2.5 copies per cell) and lung (1.1 copies per cell), whereas c-*ros* was mainly transcribed in kidney (2.5 copies per cell). On the other hand, c-*yes* transcripts were easily detectable in all tissues analyzed and were found at high levels in kidney (26 copies per cell). These c-*onc* expressions were unaffected by infection with avian sarcoma viruses that contained other *onc* genes. In a few cultures of chicken and quail transformed cells derived from tumors induced by chemical carcinogens, we found that the levels of transcription of the four c-*onc* genes remained unaltered, compared with that in normal tissues.

The *src* gene of Rous sarcoma virus (RSV) is known to be responsible for transforming activity of this virus (21, 52) and encodes a phosphoprotein, $p60^{src}$, which carries a protein kinase activity that phosphorylates tyrosine residues of substrate proteins (7, 11, 26, 33). A cellular *src*-related sequence (c-*src*) has been detected in normal cellular DNA of a wide variety of vertebrates (48, 50). The c-*src* appeared to be expressed at low levels in these normal cells (47), and a protein similar to $p60^{src}$ in size and enzymatic activities has been identified (10, 29, 39), although its physiological function is not known.

In addition to RSV, several strains of avian sarcoma viruses (ASVs) have been known or recently isolated. These include Fujinami sarcoma virus (FSV), PRCII, Y73, UR1, UR2, and Esh sarcoma virus (3, 8, 17, 27, 53). These viruses appear to be different from RSV but similar to one another in several characteristics. First, they are all defective in virus replication, because their viral genomes contain unique sequences unrelated to avian C-type retroviruses, in place of sequences required for virus replication (19, 23, 31, 37, 54, 55, 60). Second, their genomes encode unique proteins ranging from 68,000 to 140,000 daltons which are precipitable by antisera against viral structural proteins coded by the *gag* gene of avian retroviruses. These ASV proteins are read-through products of *gag* and unique sequences in their genomes. Third, their unique sequences are nonhomologous to *src*; however, all of these polyproteins in immunoprecipitated forms are associated with an enzymatic activity of protein kinase specific for tyrosine residues similar to $p60^{src}$ of RSV (13, 14, 30, 38, 41, 54). RNA-cDNA hybridization experiments have shown that among these viruses at least two nonhomologous *onc* genes are present. One is *fps*, which is shared with FSV, PRCII, and UR1 (44, 54), and the other is *yes* of Y73 (59). By comparison of tryptic peptides of

transforming proteins, Esh sarcoma virus was shown to be related to Y73 in the unique sequences (19). On the other hand, the unique sequence of UR2 (*ros*) has been suggested to be different from other *onc* genes of ASV and of representative avian acute leukemia viruses (55).

In this paper we describe our finding that the *ros* sequence of UR2 is unrelated to *src*, *fps*, and *yes* sequences by molecular hybridization and thus is apparently a new *onc* gene among ASVs. To examine possible cellular origins of these three new *onc* genes, we carried out a survey for the existence of *onc*-related nucleotide sequences in normal cellular DNA and for the endogenous expression of these *onc*-related sequences in various cells and tissues. We report that cellular sequences related to these *onc* genes are conserved in normal cellular DNA in a wide variety of vertebrates and that their expression is tissue specific. The regulation of the expression of these c-*onc* genes in ASV-transformed or chemically transformed cells is also discussed.

MATERIALS AND METHODS

Cells and viruses. The preparation of chicken embryo fibroblasts (CEF), FSV, and Fujinami-associated virus (FAV) was described previously (23). Other avian retroviruses used were Schmidt-Ruppin RSV, subgroups A and B (SR-A and SR-B), Rous-associated virus-2, PRCII (8), Y73 (27), Y73-associated virus (Y73-AV), UR1 (3), UR2 (3), UR2-associated virus (UR2-AV), and acute leukemia viruses of chickens. The sources of PRCII, Y73, and RNAs of acute leukemia viruses in chickens were described elsewhere (44). Y73-AV was provided by S. Kawai (Institute of Medical Science, University of Tokyo, Tokyo, Japan), and UR2-AV was isolated from original virus stock of UR2 by endpoint dilution. Mammalian retroviruses used were Gardner-Arnstein (18), Snyder-Theilen (45), and McDonough (34) strains of feline sarcoma virus and Abelson murine leukemia virus (1). These mammalian viruses were obtained from J. R. Stephenson (National Cancer Institute).

Source of tissues and transformed cells. Normal cellular DNA was prepared from erythrocytes of endogenous virus-free chickens (2), 14-day-old quail embryos, erythrocytes of ducks, a liver of a cat, HeLa cells, and *Escherichia coli*. High-molecular-weight DNAs of calf thymus and salmon sperm were purchased from Sigma Chemical Co.

For the source of normal cellular RNA, various tissues were obtained from uninfected 2-week-old chickens. Thymus was obtained from 10-day-old chickens. Bone marrow tissues for the analysis of c-*fps* RNA were prepared from both 10-day-old and 2-week-old chickens. Tumor cell cultures (CT4, CT5, CT40, and CT70) were obtained from fibrosarcomas induced in chickens with dimethylbenzanthracene (32). These tumors were originally provided by G. J. Thorbecke (New York University Medical Center, New York, N.Y.) and were maintained in tissue culture by T. Hanafusa (Rockefeller University). QT6 cells which were derived from fibrosarcomas induced in quails with methylcholanthrene were a gift from C. Moscovici (Veterans Administration Hospital, Gainesville, Fla.) (35).

Preparation of RNA and DNA. Purification of virus particles and extraction of viral RNAs were performed essentially as described previously (24). Tissue RNAs for hybridization were extracted as follows: 0.5 to 1 g of fresh tissues was chopped into small pieces and homogenized with a Potter tissue homogenizer in a 20-ml solution containing 1 mg of proteinase K per ml (preincubated at 37°C for 1 h), 10 mM Tris-hydrochloride, pH 7.5, 10 mM disodium EDTA, and 0.5% sodium dodecyl sulfate (SDS). After incubation at 37°C for 2 h, samples were extracted twice with phenol-$CHCl_3$ (1:1) and once with $CHCl_3$. NaCl was added to 0.2 M, and RNA was precipitated with 2 volumes of ethanol at −20°C. The RNA pellet was suspended in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 5 mM $MgCl_2$ and was treated with 2 μg of DNase I (RNase-free, Worthington Diagnostics) per ml at room temperature for 2 h. The samples were again extracted with phenol and precipitated with ethanol. The RNA was suspended in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 1 mM disodium EDTA (TE buffer) and adjusted to a concentration of 25 mg/ml.

For the preparation of cellular DNA, tissues were homogenized in 0.1 M Tris-hydrochloride, pH 7.4, containing 10 mM disodium EDTA, 0.1 M NaCl, 0.5% SDS, and 0.5 mg of pronase per ml (preincubated at 37°C for 1 h). After incubation at 37°C for 7 to 10 h, the samples were extracted with phenol-$CHCl_3$ as above. DNA was spooled up after addition of NaCl to 0.2 M and 2 volumes of ethanol and then was resuspended in TE buffer. The samples were treated with 50 μg of RNase A per ml (pretreated at 95°C for 5 min) at 37°C for 1 h, extracted with phenol, and precipitated with ethanol. DNA was dissolved in TE buffer, and the concentration was examined by absorbance at 260 nm. DNA samples for liquid hybridization were depurinated twice to shear the DNA fragments to about 150 bases long (24).

Preparation of cDNA probes. Four ^3H- or ^{32}P-labeled $cDNA_{onc}$ probes were prepared as described previously (44) with some modifications. Viral genomic RNA was partially purified by sucrose linear gradient centrifugation and then was transcribed into labeled cDNA by use of an in vitro reverse transcription reaction with avian myeloblastosis virus reverse transcriptase (provided by J. Beard, Life Science, St. Petersburg, Fla., through the courtesy of the Resource Program, National Cancer Institute), [^3H]dTTP, unlabeled dCTP, dGTP, and dATP, and calf thymus DNA primer. For selection of $cDNA_{fps}$, $cDNA_{yes}$, and $cDNA_{ros}$, each cDNA sample was hybridized with an excess amount of RNA of the respective helper virus, i.e., FAV, Y73-AV, or UR2-AV. For $cDNA_{src}$ we used SR-B RNA as a template and selected against Rous-associated virus 2 (RAV-2) RNA. After incubation at 50°C, single-strand cDNA specific for unique sequences was isolated by hydroxylapatite column chromatography. cDNA was then hybridized with original ASV RNA for positive selection, and nonhybridizable radioactivity in the cDNA sample was removed by S1 nuclease digestion. Specific activities

of [^{3}H]cDNAs were about 2×10^7 cpm/μg and those of [^{32}P]cDNAs were about 5×10^8 cpm/μg.

^{3}H-labeled chicken single-copy DNA was prepared by hybridization of denatured ^{3}H-labeled chicken DNA to a C_0t (mol·s/liter) of 200 and by elution of single-copy DNA from a hydroxylapatite column at 60°C with 0.14 M phosphate buffer.

Nucleic acid hybridization. Liquid hybridization between cellular DNA and [^{3}H]cDNA probes or ^{3}H-labeled chicken single-copy DNA was performed under the relaxed condition reported by Spector et al. (48) with some modifications. DNA probes (500 cpm) were mixed at 50°C with cellular DNA (final concentration, 10 mg/ml) in 15 μl of 10 mM Tris-hydrochloride buffer, pH 7.4, containing 1.5 M NaCl and 5 mM disodium EDTA. Samples were sealed in 50-μl capillary tubes, preheated at 95°C for 5 min, and then incubated at 60°C for various lengths of time (2 min to 100 h). After incubation, the samples were frozen and stored at −70°C until incubation was completed for all samples in the same series. Extent of hybridization was assayed by S1 nuclease digestion as follows. Samples were diluted into 0.4 ml of 0.025 M potassium acetate, pH 4.4, containing 5 mM $ZnSO_4$, 0.3 M NaCl, 60 μg of double-stranded salmon sperm DNA per ml, and 0.04% SDS. One-half of the diluted samples were kept at 0°C for estimation of total counts and the other half was digested with 10^4 U of S1 nuclease (Boehringer Mannheim Corp.) at 37°C for 2 h. Acid-insoluble material in each half was collected on a glass-fiber filter and assayed in a liquid scintillation counter.

Hybridization between cDNA and either viral or cellular RNA was carried out under conditions of moderate stringency (50°C in 30% [vol/vol] formamide, 0.45 M NaCl, 0.045 M sodium citrate, 5 mM disodium EDTA, and 0.1% SDS), and the extent of hybridization was determined by S1 nuclease digestion (24). When we estimated the extent of hybridization at the highest concentration of cellular RNA (10 to 15 mg/ml), we examined the background (0 to 5%), using a sample without incubation, and subtracted this level from the value of S1 nuclease-resistant fraction obtained after incubation.

The concentration of *onc*-related cellular RNA in normal or transformed cells was estimated by comparing the kinetics of hybridization between cDNA for viral sequences and purified viral RNA with that between $cDNA_{onc}$ and cellular RNA (24, 47). Kinetics of hybridization was shown as a function of C_rt (mol·s/liter) values. The number of copies of *onc*-related cell RNA was calculated as follows: number of copies per cell = ($C_rt_{1/2}$ for viral RNA/$C_rt_{1/2}$ for cellular RNA) × (weight of RNA per avian cell/weight of one molecule of viral RNA), in which $C_rt_{1/2}$ indicates the C_rt value at which half-maximal hybridization is obtained. The amount of avian cellular RNA was assumed to be 10^{-11} g per cell. In a previous paper (44) we showed that the $C_rt_{1/2}$ value of $cDNA_{FAV}$ for purified 35S FAV RNA (4.5×10^{-18} g per molecule) was about 0.02 in our hybridization conditions. Frequently, the extent of hybridization between $cDNA_{onc}$ and cellular RNA was lower than the half-maximal extent of hybridization obtained between $cDNA_{onc}$ and viral RNA even after incubation for 100 h at the highest concentration of RNA. In these cases we compared the C_rt values for viral and cellular RNAs at which the same (less than 50%) extent of hybridization was obtained.

Southern blotting analysis. Twenty-five micrograms of normal cellular DNA obtained from erythrocytes of endogenous virus-free chickens was digested with 25 U of several restriction endonucleases (New England Biolabs), concentrated by ethanol precipitation, and separated by electrophoresis in 0.8% agarose gels in a buffer solution of 40 mM Tris-acetate, pH 7.8, 5 mM sodium acetate, and 1 mM disodium EDTA. The DNA was denatured, neutralized in situ, and transferred onto a nitrocellulose sheet (pore size, 0.22 μm) with the use of 20× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate) as described by Southern (46). The sheet was baked (80°C, in vacuo), preincubated in a prehybridization solution, and then hybridized with [^{32}P]$cDNA_{fps}$ or $cDNA_{ros}$ (10^6 cpm per sheet). The conditions for prehybridization and hybridization were as described previously (43) except for incubation at 37°C instead of 42°C. After hybridization, the sheet was washed once in 2× SSC containing 1× Denhardt's solution (0.02% each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone) at 37°C for 1 h, twice in 0.1× SSC with 0.1% SDS for 40 min at 50°C, and then four times in 0.1× SSC for 1 min at room temperature. The filter was dried and exposed to Kodak XR-5 film at −70°C in the presence of an intensifying screen.

RESULTS

Preparation of cDNAs specific to unique sequences of new ASVs. cDNAs specific to unique sequences of FSV, Y73, and UR2 were prepared by a method similar to that used for $cDNA_{src}$ as reported by Stehelin et al. (49). The specificities of $cDNA_{yes}$ and $cDNA_{ros}$ are shown in Fig. 1. Each probe hybridized to the respective ASV

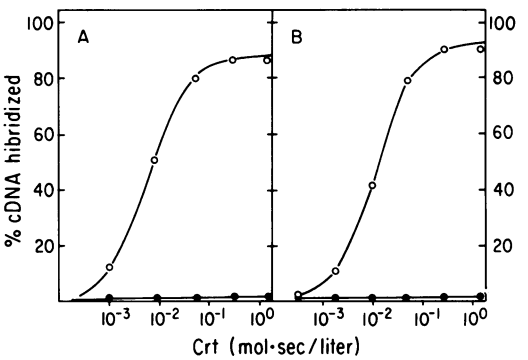

FIG. 1. (A) Hybridization of $cDNA_{yes}$ with RNAs of Y73(Y73-AV) (○) and Y73-AV (●). (B) Hybridization of $cDNA_{ros}$ with RNAs of UR2(UR2-AV) (○) and UR2-AV (●). About 500 cpm of ^{3}H-labeled cDNAs was mixed with 0.08 ng to 2 μg of viral RNA in a solution (10 to 20 μl) containing 30% formamide, 0.45 M NaCl, 0.045 M sodium citrate, 5 mM disodium EDTA, and 0.4% SDS, and the mixture was incubated at 50°C for 20 to 40 h. Extent of hybridization was measured by S1 nuclease digestion. C_rt values of the hybridization with Y73(Y73-AV) and UR2(UR2-AV) RNAs were standardized for the amount of sarcoma virus RNAs.

RNAs (Y73 RNA or UR2 RNA) to the extent of 90%, but failed to hybridize with their helper viral RNA (Y73-AV or UR2-AV; less than 2%). $cDNA_{fps}$ and $cDNA_{src}$ have similar degrees of specificity which have been described elsewhere (44).

***ros* sequence: a new *onc* gene of avian sarcoma viruses.** We have analyzed cross-homology between transforming sequences of various retroviruses, using four cDNA probes specific to unique sequences of ASVs. Viral RNAs were extracted from virions and hybridized to cDNA probes under conditions of moderate stringency. The data summarized in Table 1 include data on the cross-homology of *fps* of FSV to unique sequences of PRCII, UR1, and two strains of feline sarcoma viruses which have been reported elsewhere (44, 54). The absence of homology of *yes* to RSV, FSV, or avian acute leukemia viruses confirms the observations described by Yoshida et al. (59). From the results shown, it is clear that UR2 RNA does not hybridize with any *onc* cDNA other than $cDNA_{ros}$. Conversely, $cDNA_{ros}$ of UR2 does not hybridize with RNAs of RSV, FSV, and Y73 nor with RNAs of representative avian acute leukemia viruses. Thus, ASV UR2 has a unique transforming insert unrelated to any of the several viruses examined, including Abelson murine leukemia virus.

TABLE 1. Homology between unique sequences of various transforming viruses[a]

Viral RNA[b]	Extent of hybridization (%) to the indicated cDNA probe			
	src	*fps*	*yes*	*ros*
Avian				
RSV	[93]	1	1	1
FSV	3	[90]	3	2
UR1	1	[89]	1	3
PRCII	5	[50]	2	3
Y73	3	1	[88]	2
UR2	1	1	1	[90]
RAV-2	1	1	0	1
AEV	5	1	1	1
AMV	4	1	1	2
MC29	2	0	0	1
Mammalian				
GA-FeSV	1	[24]	1	1
ST-FeSV	1	[17]	2	1
SM-FeSV	0	1	1	1
A-MuLV	0	1	1	1

[a] Various amounts (0.05 to 5 μg) of viral RNAs were hybridized with ^{3}H-labeled $cDNA_{onc}$ probes (500 cpm) under the same conditions given in the legend to Fig. 1. The saturation level of hybridization was obtained from at least three different experiments at C_rt values of 1 to 1,000 mol·s/liter. Except for RSV and RAV-2, viral RNAs used contained RNAs of the respective helper viruses. Values in boxes indicate significant degrees of homology.

[b] RAV-2, Rous-associated virus-2; AEV, avian erythroblastosis virus; AMV, avian myeloblastosis virus; MC29, avian myelocytomatosis virus MC29; GA-, ST-, and SM-FeSV, Gardner-Arnstein, Snyder-Theilen, and McDonough strains of feline sarcoma virus; A-MuLV, Abelson murine leukemia virus.

Nucleotide sequences in DNA from uninfected cells related to ASV transforming sequences. The presence of a nucleotide sequence (c-*src*) related to the *src* gene of RSV in normal chicken DNA has been demonstrated (50), and the sequence appears to be highly conserved in a wide variety of vertebrates (48). We examined the presence of nucleotide sequences related to *ros* as well as *fps* and *yes* in normal cellular DNA of various species of vertebrates. Hybridization was carried out under relaxed conditions (48; see Materials and Methods) to facilitate cross-hybridization between cDNAs and DNA sequences obtained from different species. To avoid hybridization with endogenous retrovirus sequences, endogenous virus-free (*ev*-minus) chickens (2) were used as a source of high-molecular-weight cellular DNA. As shown in Fig. 2, cDNA probes specific to *fps*, *yes*, and *ros* hybridized with chicken cellular DNA to the extent of more than 50%, with kinetics quite similar to that between chicken DNA and ^{3}H-labeled chicken single-copy DNA (slowly reassociated sequences; see Materials and Methods), indicating that these transforming sequences are present in normal cells in probably one or few copies per haploid genome. As expected, hybridization of $cDNA_{FAV}$, a probe for helper virus sequences, was negligible to this *ev*-minus chicken DNA.

The nature of cellular sequences related to *fps* and *ros* was further analyzed by comparing DNA fragments generated by digestion with restriction enzymes. *ev*-minus chicken DNA was digested with several restriction enzymes and separated by gel electrophoresis. DNA fragments were transferred to nitrocellulose paper (46) and then hybridized with ^{32}P-labeled $cDNA_{fps}$ or $cDNA_{ros}$. As shown in Fig. 3, hybridization of *Eco*RI or *Bam*HI digests with $cDNA_{fps}$ (lanes 1 and 3) gave a single major band (about 30 kilobases [kb] and 12 kb, respectively) of cellular *fps* (c-*fps*). *Sac*I-digested DNA hybridized with $cDNA_{ros}$ showed a single DNA fragment of about 9 kb for cellular *ros* (c-*ros*) (lane 5). The coding sequences of *fps* and *ros* have been estimated to be approximately 2.7 and 1.3 kb, respectively (23, 31, 54, 55; M. Shibuya, unpublished data). Therefore, if these *onc* sequences are not in tandem in each band of DNA fragments, it is likely that c-*fps* and c-*ros* are present at single loci in the haploid genome of chickens.

These *onc*-related sequences are present in

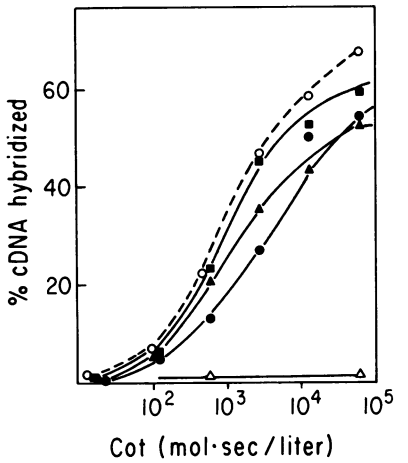

FIG. 2. Hybridization of various $cDNA_{onc}$ probes or chicken single-copy DNA with uninfected chicken DNA. DNA was prepared from erythrocytes of uninfected endogenous virus-free chickens, denatured, and incubated with ^3H-labeled $cDNA_{fps}$ (●), $cDNA_{yes}$ (■), $cDNA_{ros}$ (▲), $cDNA_{FAV}$ (△), and chicken single-copy DNA (○). The hybridization reaction was carried out in 15 μl of 10 mM Tris-hydrochloride buffer, pH 7.4, containing 1.5 M NaCl, 5 mM disodium EDTA, 500 cpm of each probe, and 150 μg of chicken cell DNA at 60°C (relaxed condition; see text), for 2 min to 100 h. Extent of hybridization was determined by S1 nuclease digestion. C_0t values shown are corrected for standard conditions of salt concentration (6).

normal cellular DNA of various species of vertebrates. Figure 4 shows the hybridization kinetics between $cDNA_{fps}$ and various DNAs, and Table 2 summarizes the extent of hybridization with $cDNA_{fps}$, $cDNA_{yes}$, and $cDNA_{ros}$. Substantial hybridization of these ASV-unique sequences was observed with cellular DNA of duck and quail. In addition, less, but significant, hybridization was observed with cellular DNAs of mammalian species. The hybridization of cDNAs with salmon DNA was close to background level, and that with *E. coli* was considered negative. These results demonstrate that, like unique sequences of RSV, acute leukemia viruses, and other RNA tumor viruses (15, 16, 20, 42, 48, 50), cellular sequences related to *fps*, *yes*, and *ros* are present in a wide variety of vertebrates, and the degree of homology correlates with the phylogenetic relationship.

Endogenous expression of cellular *onc*-related sequences in uninfected CEF and in various normal tissues. The expression of cellular *fps*-, *yes*-, or *ros*-related sequences in various cells and tissues was determined by hybridization of cDNAs with total cellular RNA. The hybridization was carried out up to the C_rt values of 1.5×10^4 (mol·s/liter), and the number of RNA copies per cell related to *onc* sequences was calculated from the C_rt curves (see Materials and Methods). As shown in Fig. 5, the expression of four *onc*-related cellular sequences in CEF varied significantly. About 4 copies per cell of c-*src* transcripts were found in CEF, and this level is similar to that reported by others (47, 56). c-*yes* RNA was more abundant at the level of 16 copies per cell. On the other hand, the expression of c-*fps* and c-*ros* was extremely low and only less than 0.3 copy per cell was detected. Levels of *onc*-related transcripts in quail embryo fibroblasts were found to be similar to those in CEF (Table 3).

The levels of endogenous expression of cellu-

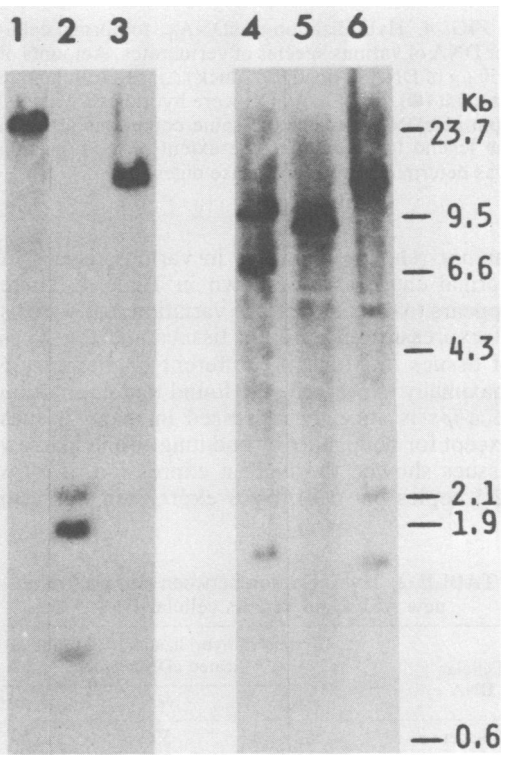

FIG. 3. Detection of nucleotide sequences homologous to unique sequences of FSV and UR2 in uninfected chicken cellular DNA. DNA extracted from erythrocytes of uninfected endogenous virus-free chickens was digested with restriction endonuclease *Eco*RI (lanes 1 and 4), *Sac*I (lanes 2 and 5), or *Bam*HI (lanes 3 and 6). The DNA fragments were separated by electrophoresis in 0.8% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled $cDNA_{fps}$ (lanes 1, 2, and 3). After autoradiography for the detection of c-*fps*, the hybridized ^{32}P probe was removed by incubation in prehybridization solution at 68°C for 30 min; then the nitrocellulose filter was hybridized with [^{32}P]$cDNA_{ros}$ for the analysis of c-*ros* sequence (lanes 4, 5, and 6). The marker DNA fragments, shown at the right, were λ DNA digested with *Hin*dIII.

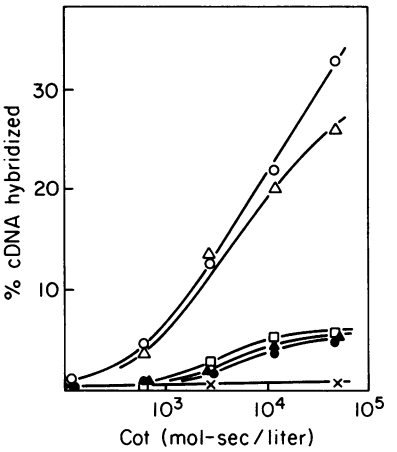

FIG. 4. Hybridization of $cDNA_{fps}$ to normal cellular DNA of various species of vertebrates. Amounts of 150 μg of DNA of quail (○), duck (△), calf (□), human (▲), cat (●), and *E. coli* (X) were hybridized with 500 cpm of $cDNA_{fps}$ under the same conditions given in the legend to Fig. 2, and the extent of hybridization was determined by S1 nuclease digestion.

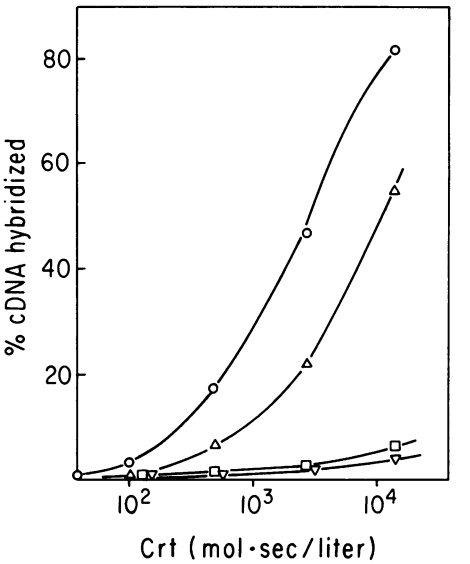

FIG. 5. Cellular *onc*-related RNAs in uninfected CEF. Total cell RNA was extracted from a subconfluent culture of CEF, and various amounts (0.16 to 100 μg) of RNA were incubated with 500 cpm of ^3H-labeled $cDNA_{src}$ (△), $cDNA_{fps}$ (□), $cDNA_{yes}$ (○), and $cDNA_{ros}$ (▽) in 8 to 16 μl of a hybridization solution. The reaction was carried out for 100 h under the same conditions given in the legend to Fig. 1.

lar *onc*-related sequences in various tissues of normal chickens are shown in Table 4. There appears to be considerable variation in the levels of expression in different tissues and the types of tissues in which the different *onc* genes are maximally expressed. We found that expression of c-*fps* is strongly repressed in many tissues except for bone marrow and lung. Bone marrow tissues showed the highest expression of c-*fps* (2.5 copies per cell). c-*ros* expression was also very low in many tissues, but we detected 2.5 copies per cell of *ros* transcripts in kidney. On the other hand, c-*yes* was expressed in all tissues analyzed (1 to 26 copies per cell) and at a remarkably high level in kidney (26 copies per cell).

TABLE 2. Hybridization between *onc* probes of new ASVs and various cellular DNAs[a]

Cellular DNA	Extent of hybridization (%) to the indicated cDNA probe		
	fps	*yes*	*ros*
Chicken	52	59	53
Quail	32	37	46
Duck	30	48	29
Calf	6	5	4
Cat	4	7	6
Human	5	9	5
Salmon	2	1	2
E. coli	1	0	1

[a] Cellular DNAs prepared from various species of animals and *E. coli* were mixed with ^3H-labeled $cDNA_{onc}$ probes (500 cpm). The hybridization reaction was carried out under the same conditions described in the legend to Fig. 2, and the extents of hybridization were determined at a C_0t value (mol·s/liter) of 6×10^4.

TABLE 3. *onc* sequence-specific RNA in normal and ASV-transformed cells[a]

Cells	RNA content (copies per cell)[b]			
	src	*fps*	*yes*	*ros*
CEF	4	<0.3	16	<0.3
QEF	2	<0.3	12	<0.3
CEF, infected with:				
RSV	8,800	<0.3	12	<0.3
FSV	3	500	10	0.7
Y73	2	<0.3	1,600	0.3
UR2	2	<0.3	13	1,400

[a] Total cellular RNA was extracted from uninfected CEF or quail embryo fibroblasts (QEF) and from ASV-transformed CEF. ^3H-labeled $cDNA_{onc}$ probes (500 cpm) were incubated with various amounts (0.06 to 100 μg) of each RNA in 8 to 16 μl for 100 h under the same conditions described in the legend to Fig. 1. The extent of hybridization was determined by S1 nuclease digestion.

[b] Calculation of RNA copies per cell is based on the ratio of C_rt values for hybridization with viral RNA and with cellular RNAs (see text).

TABLE 4. *onc* sequence-specific RNA in tissues of normal chickens[a]

Tissues	RNA content (copies per cell)			
	c-*src*	c-*fps*	c-*yes*	c-*ros*
Bone marrow	0.7	2.5	1.4	0.7
Spleen	1.4	0.7	2.7	<0.3
Thymus	0.3	0.3	1.4	0.3
Lung	1.5	1.1	3.5	0.4
Kidney	0.8	0.3	26	2.5
Bursa	0.6	0.5	3.3	0.7
Muscle	<0.3	<0.3	1.0	<0.3
Heart	<0.3	<0.3	3.1	<0.3
Liver	0.7	0.3	4.1	<0.3
Brain	0.5	0.3	5.0	<0.3

[a] Total cellular RNA was prepared from various tissues of uninfected 10- to 14-day-old chickens and was hybridized with ^{3}H-labeled cDNA$_{onc}$ probes. The hybridization conditions and the calculation of RNA content were the same as in the legend of Table 3. The content were the same as in the footnotes to Table 3. The contents of c-*onc* RNA in bone marrow, spleen, lung, kidney, bursa, and brain were the average values of two samples of these tissues which were prepared separately. Because of the low extent of hybridization, the values of RNA content of less than one copy per cell have an average deviation of ±40%.

Expression of *onc*-related sequences in ASV-transformed or chemically transformed fibroblasts. Infection of CEF with ASV should result in the synthesis of a large number of viral RNA molecules. The precise level of the expression of viral and cellular *onc* sequences was determined in each virus-transformed culture (Table 3). SR-A ASV-infected CEF carried about 10^4 copies of *src*-containing RNA, and FSV-, Y73-, or UR2-transformed CEF contained 500 to 1,600 copies of *fps*, *yes*, or *ros* RNA. Interestingly, infection of CEF with different ASV strains did not result in a change in the level of transcription of endogenous cellular *onc* genes related to other ASV strains, suggesting that the transcription of each c-*onc* gene is regulated independently from the control of other *onc* genes.

Concentration of c-*src* RNA in certain chemically induced tumors has been reported to be unchanged from that in normal fibroblasts (47). The levels of cellular *onc* RNAs in chemically transformed cells were examined. Cell cultures (CT4, CT5, CT40, and CT70) were prepared from several chicken tumors induced by dimethylbenzanthracene (32), and QT6 cells were derived from quail tumors induced by methylcholanthrene (35). As shown in Table 5, c-*src* transcripts in these transformed cells were within 2 to 6 copies per cell and were similar to the levels in normal fibroblasts. These results are consistent with the observation reported by Spector et al. (47). The concentrations of c-*fps* (less than 0.3 copy per cell), c-*yes* (15 to 33 copies per cell), and c-*ros* (less than 0.3 copy per cell) in these cells were also found to be essentially the same as the levels in normal fibroblasts.

DISCUSSION

In the first part of this study, we demonstrated that the unique sequence of UR2, denoted *ros*, is nonhomologous to *src*, *fps*, or *yes* sequences and thus apparently represents a new *onc* gene of ASV. This is consistent with our previous results obtained from fingerprinting analyses of viral RNAs (55). It is of interest to note that all of the gene products of these four *onc* sequences have been reported to be associated with protein kinase activity specific for phosphorylation of tyrosine residues on substrate proteins (13, 14, 30, 38, 41, 54). In addition we showed that none of the cDNAs specific to these four *onc* sequences hybridized with RNA of Abelson murine leukemia virus, whose genome has also been reported to contain a gene for tyrosine-specific protein kinase (57). Therefore, at least five independent genes for functionally related proteins have been identified among avian and mammalian retroviruses.

A considerable body of evidence indicates that the *onc* genes of RNA tumor viruses analyzed so far are derived from homologous cellular sequences by recombination events (12, 15, 16, 20, 42, 44, 48, 50). Furthermore, several cellular *onc* genes have recently been demonstrated to express transforming activity under some special conditions (12, 22, 25, 28, 40). Using liquid hybridization and Southern blotting analyses, we have shown that the sequences of the three new *onc* genes of ASV have their counterpart sequences in normal chicken DNA. Since these c-*onc* sequences are detected in endogenous virus-minus chickens, it is clear that

TABLE 5. *onc* sequence-specific RNA in chemically transformed cells[a]

Cells	RNA content (copies per cell)			
	c-*src*	c-*fps*	c-*yes*	c-*ros*
CT4	5	<0.3	16	<0.3
CT5	3	<0.3	15	<0.3
CT40	3	<0.3	31	<0.3
CT70	2	<0.3	30	<0.3
QT6	6	<0.3	33	<0.3

[a] Total cellular RNA was extracted from four cell cultures induced in chickens (CT4, CT5, CT40, and CT70) with dimethylbenzanthracene and from a quail tumor cell line (QT6) induced with methylcholanthrene. The procedures for the hybridization experiments and the calculation of RNA contents were the same as in the footnotes to Table 3.

they are unrelated to endogenous retrovirus genomes of chickens. Yoshida et al. (59) also found cellular *yes*-related sequences in uninfected chicken DNA. These results suggest that, like other transforming retroviruses, newly characterized ASVs bearing *fps*, *yes*, or *ros* as transforing sequences were generated by a recombination process between prototype *onc*-minus retrovirus sequences and cellular sequences related to viral *onc* genes.

The kinetics of liquid hybridization indicates that these c-*onc* sequences are present in one or few copies per haploid genome. Consistent with this result, major sequences of c-*fps* and c-*ros* are found to be located in single DNA restriction fragments, i.e., a 12-kb *Bam*HI fragment and a 9-kb *Sac*I fragment, respectively. Recently, we molecularly cloned a DNA fragment carrying the c-*fps* gene from a random DNA library of chickens (Shibuya, unpublished data). In this DNA we detected a 12-kb *Bam*HI DNA fragment which contains only one copy of the c-*fps* gene. Therefore, it is most likely that the cell contains a single copy of c-*fps* per haploid genome.

Under the relaxed conditions of hybridization, weak but significant cross-hybridization between the *onc*-specific cDNAs and mammalian DNAs was detected. This suggests that cellular sequences related to *fps*, *yes*, or *ros* are present in DNA of not only avian but also mammalian species. Such a low extent of hybridization has been reported between mammalian DNA and cDNA probes specific to *src* of RSV or to *onc* genes of avian acute leukemia viruses (42, 48). In a previous paper we reported that the *fps* sequence of FSV in chickens is homologous to the *fes* sequence of feline sarcoma virus (44). Here we have shown that $cDNA_{fps}$ hybridizes to the highest extent with chicken DNA among DNAs of various species and hybridizes weakly with cat DNA. In contrast, the *fes* probe was reported to hybridize best with cat DNA but to a considerably lower extent with chicken DNA (16). Therefore, these data are consistent with the view that *fps*-containing ASVs (FSV, PRCII, and UR1) and two strains of feline sarcoma virus were independently generated by a recombination between retroviruses and cognate cellular genes which are evolutionarily diverged in chicken and cat DNAs. The results also show that, despite the divergence of nucleotide sequences, the function of the product of each gene is conserved, since the products of feline sarcoma viruses were also shown to be associated with tyrosine-specific protein kinase activity (4, 51).

Tissue-specific expression of cellular sequences related to several *onc* genes has recently been reported. Chen (9) has described that cellular RNA related to *myb* of avian myeloblastosis virus is relatively abundant in granulopoietic cells. Witte et al. (58) observed that NCP150, a normal cell protein cross-reactive to the polyprotein of Abelson murine leukemia virus is expressed to a greater extent in lymphoid organs compared with other tissues. In this study we found that the expression of cellular *fps*, *yes*, or *ros* sequences is also tissue specific. The highest level of c-*fps* transcripts was observed in bone marrow tissues, whereas c-*ros* transcripts were most abundant in kidney. c-*yes* transcripts were easily detected in many tissues and were particularly high in kidney. The physiological significance of these tissue-specific expressions is still unknown. Recently, a normal cell protein immunologically cross-reactive to a unique portion of FSV polyprotein has been observed in bone marrow tissues (B. Mathey-Prevot, H. Hanafusa and S. Kawai, Cell, in press). These results might suggest that c-*fps* is involved in the process of hematopoiesis or some function in differentiated blood cells of avian species.

CEF transformed by representative ASVs showed different levels of viral *onc* transcripts (Table 3). In RSV-infected cells about 10^4 copies of *src* RNA were detected, whereas severalfold lower amounts of viral *onc* sequences were present in FSV-, Y73-, or UR2-infected cells. Since these infected CEF were morphologically well transformed, it is unlikely that the difference in the amounts of transcripts is due to incomplete virus infection. The difference could be due to relatively small numbers of copies of defective ASV (FSV, Y73, and UR2) proviral DNA integrated in CEF compared with that of nondefective SR-A RSV DNA, because the latter may have a better chance to superinfect cells than the former ASVs which are associated with helper viruses. It is also conceivable that RSV transcription might be intrinsically more efficient than that of other ASVs as the result of a difference in their promoters. Although the basis for the difference in the amounts of transcripts is not clear, the amounts of functional message for the transforming inserts may not be so different. The measurements of *src*-containing RNA include both genomic and subgenomic RNAs. The functional *src* message (21S RNA) has been estimated to be about one-fifth to one-fourth of total viral RNA (24). On the other hand, genomic RNAs of defective ASVs represent the only viral transcripts in transformed cells (54, 55; Shibuya, unpublished data), and they are known to serve as messages for transforming proteins (14).

In these ASV-transformed CEF, the amounts of the cellular *onc* RNAs unrelated to ASV used for infection were found to be essentially the same levels as those in uninfected cells. Thus,

the product of one *onc* gene does not appear to have influence over the expression of other cellular *onc* genes. In a study on morphological reversion of transformed cells, more than 100 copies of *src* RNA were considered to be necessary for cell transformation (5). The levels of *onc* RNAs in virus-transformed cells and in uninfected normal cells shown in this study appear to fall in these ranges. The relatively high expression of *yes* in normal tissues distinguishes it from other *onc*-related genes and might suggest that the expression of this *yes* gene is required for cellular metabolism.

Recent investigations on chicken tumors induced by avian leukosis viruses have demonstrated that an activation of the c-*myc* gene, the cellular homologue of the *onc* gene of MC29, is correlated with the leukemogenesis by these viruses (25, 36). In several fibrosarcomas induced by chemical carcinogens, we were unable to detect elevated expression of the c-*onc* genes related to the four classes of ASV (Table 5). Although c-*yes* RNA was slightly elevated in a few cell lines, the level of the increase (about 15 copies) may be insignificant compared with the more than 1,000 copies of viral *yes* RNA found in Y73 virus-transformed CEF (Table 3). Therefore, we conclude that these c-*onc* genes are not likely to be directly responsible for the formation of these tumors.

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