JOURNAL OF VIROLOGY, May 1981, p. 409-419
0022-538X/81/050409-11$02.00/0

Vol. 38, No. 2

Characterization of the Oncogene (*erb*) of Avian Erythroblastosis Virus and Its Cellular Progenitor

S. SAULE, M. ROUSSEL, C. LAGROU, AND D. STEHELIN*

Institut National de la Santé et de la Recherche Médicale U 186, Oncologie Moléculaire, Institut Pasteur, 59019 Lille, Cedex, France

Received 27 October 1980/Accepted 4 February 1981

Avian erythroblastosis virus (AEV) induces primarily erythroblastosis when injected intravenously into susceptible chickens. In vitro, the hematopoietic target cells for transformation are the erythroblasts. Occasional sarcomas are also induced by intramuscular injection, and chicken or quail fibroblasts can be transformed in vitro. The transforming capacity of AEV was shown to be associated with the presence of a unique nucleotide sequence denoted *erb* in its genomic RNA. Using a simplified procedure, we prepared radioactive complementary DNA ($cDNA_{aev}$) representative of the *erb* sequence at a high yield. Using a $cDNA_{aev}$ excess liquid hybridization technique adapted to defective retroviruses, we determined the complexity of the *erb* sequence to be 3,700 ± 370 nucleotides. AEV-transformed erythroblasts, as well as fibroblasts, contained two polyadenylated viral mRNA species of 30 and 23S in similar high abundance (50 to 500 copies per cell). Both species were efficiently packaged into the virions. AEV-transformed erythroblasts contained additional high-molecular-weight mRNA species hybridizing with $cDNA_{aev}$ and $cDNA_{5'}$ but not with cDNA made to the helper leukosis virus used ($cDNA_{rep}$). The nature and the role, if any, of these bands remain unclear. The *erb* sequence had its counterpart in normal cellular DNA of all higher vertebrate species tested, including humans and fish (1 to 2 copies per haploid genome in the nonrepetitive fraction of the DNA). These cellular sequences (c-*erb*) were transcribed at low levels (1 to 2 RNA copies per cell) in chicken and quail fibroblasts, in which the two alleged domains of AEV-specific sequences corresponding to the 75,000- and 40,000-molecular-weight proteins seemed to be conserved phylogenetically and transcribed at similar low rates.

Among avian retroviruses, avian defective leukemia viruses (DLVs) form a group of highly oncogenic RNA viruses inducing acute leukemias with a short period of latency in susceptible birds and also occasional sarcomas or carcinomas (8).

Recently, the seven available independent isolates of DLVs have been assigned to three subgroups based on the types of malignant hemopathy that they induce in vivo and on the differentiation phenotype of hematopoietic cells that they transform in infected bone marrow cells in vitro (3, 8). (i) Avian erythroblastosis virus (AEV) causes erythroblastosis in vivo and induces the proliferation of erythroblast-like cells. It also induces sarcomas at the site of injection (24). (ii) The four avian myelocytomatosis-type viruses (MC29, CMII, OK10, and MH_2) induce the proliferation of macrophage-like transformed cells, and some of them induce carcinomas in vivo (10, 19, 20). (iii) The avian myeloblastosis viruses (AMV and E26) induce myeloblastosis in vivo and transform myeloblast-like cells in vitro (6). Studies on the genetic content of DLVs have documented the following common properties. All DLVs contain avian leukemia virus (ALV)-related nucleotide sequences in different amounts (28 to 77%) (25) but are deleted in genes necessary for viral replication (31) and therefore are replication defective and require a helper ALV for their propagation in cultured cells (8). None of these viruses contain sequences homologous to the transforming gene *src* of avian sarcoma virus (ASV) (4, 25, 27, 28). Instead, they contain one of three different sequences inserted within the ALV-related sequences, which correlate with the capacity of a given virus strain to transform erythroblasts, macrophage-like cells, or myeloblasts (25, 31). These sequences have been termed *erb*, *mac*, and *myb*, respectively. As published previously (25, 26, 31), these unique sequences have their counterpart in the normal cellular DNA. Thus, DLVs seem to be recombinants between an

ALV-related vector and normal chicken cellular DNA sequences which confer to these viruses their oncogenicity.

In this paper we describe extensively the selection and properties of a radioactive DNA complementary to the specific sequences (*erb*) of AEV ($cDNA_{aev}$). We established the complexity of the $cDNA_{aev}$ to be 3.7 ± 0.37 kilobases, using a method adapted from Young et al. (37).

This cDNA enabled us to determine unambiguously the size of the AEV genomic RNA and its products of transcription in some AEV-infected cells. Our results are consistent with the hypothesis that the viral *erb* sequences contain two different genes, one coding for the 75,000-molecular-weight (75K) *gag-erb* polyprotein (12, 15, 23; H. Beug, G. Ramsay, S. Saule, D. Stehelin, M. J. Hayman, and T. Graf, *in* B. Fields, R. Jaenisch, and F. Fox, ed., *Animal Virus Genetics, ICN-UCLA Symposium on Cell and Molecular Biology*, vol. 18, in press) and the other coding for a 40K protein as shown previously by in vitro transcription of AEV RNA (15, 21, 36). AEV-transformed erythroblasts contain additional mRNA species hybridizing with $cDNA_{aev}$ and related to ALV sequences that are packaged into the virions. These species are not seen in AEV-transformed uncloned fibroblasts or erythroblasts and may represent occasional read-through molecules extending into cellular sequences.

Normal avian DNA contains sequences (c-*erb*) related to $cDNA_{aev}$ (25). The moieties in $cDNA_{aev}$ corresponding to both the 75K and the 40K proteins are conserved in quail DNA and transcribed at similar levels. It is unclear that this is the case in more distantly related species. Nevertheless, $cDNA_{aev}$-related sequences are at least partially present in the DNAs of humans and fish at a similar frequency of 1 to 2 copies per haploid genome.

MATERIALS AND METHODS

Cells and viruses. Viruses were grown on C/E chicken fibroblasts prepared from 11-day fertile eggs (Brown Leghorn; Institut Gustave Roussy, Villejuif, France). AEV nonproducer NP 75 chicken fibroblasts (11) and AEV (Rous-associated virus type 2 [RAV-2])-producing 6C2 and 9D4 erythroblasts and nonproducing NP A6C1 erythroblasts, prepared with *ts*34 AEV (7), were obtained from T. Graf and H. Beug, AEV nonproducer NP ATla rat fibroblasts (22) were obtained from K. Quade. The transformation-defective Prague B strain (*td* PrB) of ASV was obtained originally from R. Junghans. All other viruses used in these studies have been described elsewhere (29).

RNA extraction. Viral 50 to 70S RNA was extracted from viruses harvested from culture medium clarified from cell debris. Viruses were suspended in STE buffer (0.1 M NaCl, 0.001 M EDTA, 0.02 M Tris) containing 200 μg of proteinase K per ml and 1% sodium dodecyl sulfate (SDS). After 5 min of digestion at 37°C, the RNA was extracted twice with an equal volume of STE buffer-saturated phenol. RNA was precipitated from the aqueous phase by the addition of 2 volumes of 95% ethanol at −20°C, collected by centrifugation (10,000 × *g*, 10 min, 4°C), and dissolved in STE buffer before preparative sucrose gradient centrifugation. The 50 to 70S RNA was separated from rRNA by sedimentation through a 15 to 30% sucrose gradient made in STE buffer in an SW41 rotor at 40,000 rpm for 3.5 h at 4°C. The 50 to 70S peak was detected by UV absorption at 260 nm, pooled, and precipitated by the addition of 2 volumes of cold ethanol.

Cellular RNAs were extracted as previously described (28).

DNA extraction. Chicken embryos were homogenized in 10 ml of TE buffer (0.02 M Tris-hydrochloride [pH 7.5], 0.01 M EDTA) per embryo at 0°C for 60 s with an Ultra-Turrax homogenizer (Scientific Instruments Ltd., London, England). The mixture was then adjusted to 1% with SDS and 50 μg/ml with proteinase K. Tissue culture cells (10^6 cells per ml) were suspended in TE buffer containing 50 μg of proteinase K per ml and 1% SDS. After incubation overnight at 37°C, the DNA was extracted twice with STE buffer-saturated phenol. The final aqueous phase was made 0.2 M in sodium acetate, and the DNA was precipitated by 2 volumes of 95% ethanol. The DNA was spooled out, washed with 95% ethanol, and air dried. This DNA was dissolved overnight in TE buffer at a concentration of 1 to 2 mg/ml and then treated with pancreatic RNase (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.). After 1 h at 28°C, the DNA was sonicated and incubated overnight in 0.3 M NaOH at 37°C. The mixture was adjusted to neutral pH by the addition of 3 M HCl in the presence of phenol red indicator and extracted by STE buffer-saturated phenol as described above. The DNA was then ethanol precipitated and dissolved in TE buffer (5 to 10 mg/ml) before UV spectral analysis. The ratio of absorbance at 260 nm to that at 280 nm was more than 1.9 for all DNAs tested.

Preparation of virus-specific cDNA's. Representative radioactive cDNA's were synthesized on a 70S viral RNA template with fragmented calf thymus DNA primer (34), using the exogenous AMV reverse transcriptase (J. Beard, Life Science Inc., through the auspices of the Office of Program Resources and Logistics, National Cancer Institute). The reaction mixture (for ^3H, 1 ml; for ^{32}P, 100 μl) contained the following: 20 μg of viral RNA per ml, 0.05 M Tris (pH 8.1), 0.02 M dithiothreitol, 8 mM $MgCl_2$, 0.05 M KCl, 100 μg of actinomycin D (Rhône-Poulenc) per ml, 2.5 mg of DNA primer per ml, 280 U of AMV polymerase per ml, 0.4 mM concentration each of dGTP, dATP, and dCTP, and 0.15 mM [^3H]TTP (60 Ci/mmol, 10 mCi; International Chemical Nuclear, Irvine, Calif.) or 0.002 mM [^{32}P]TTP (350 Ci/mmol, 0.5 to 1 mCi; New England Nuclear Corp., Boston, Mass.). The reaction was allowed to proceed for 45 min at 41°C and was terminated by the addition of 1% SDS (final concentration). After the reaction mixture had been digested with 100 μg of proteinase K per ml for 15 min at 37°C,

200 μg of yeast RNA per ml was added. The nucleic acids were then extracted once with phenol-STE buffer and precipitated with 2 volumes of ethanol at −20°C. The specific activities of the probes complementary to the *td* PrB genome ($cDNA_{rep}$) were as follows: [^{32}P]cDNA, 5×10^8 cpm/μg; [^{3}H]cDNA, 4.8×10^7 cpm/μg. The cDNA's were sonicated and sedimented through 5 to 20% (wt/vol) sucrose gradients made in 0.9 M NaOH–1 M NaCl–0.01 M EDTA for 24 h at 37,000 rpm in an SW41 rotor at 24°C. The cDNA's ranged in size from 3 to 10S, with a peak at 5S. A size pool of 4 to 7S was used throughout the studies described below. $cDNA_{sarc}$ was prepared as described previously (29), $cDNA_{5'}$ (strong stop) was prepared by the method of Friedrich et al. (5), and $cDNA_{gpC}$ was prepared by the method of Tal et al. (33).

Nucleic acid hybridization. Standard hybridization reaction mixtures (stringent conditions) contained 0.6 M NaCl, 2 mM EDTA, 0.02 M Tris (pH 7.4), 500 μg of calf thymus DNA per ml as carrier, 2,000 cpm (0.04 ng) of [^{3}H]cDNA or 2,000 cpm (0.005 ng) of [^{32}P]cDNA, and appropriate RNAs or DNAs in large excess. Hybridizations were conducted in glass capillaries at 68°C, and the extent of annealing was analyzed by digestion with S1 nuclease (17), prepared by the method of Sutton (32).

Determination of T_m. For the determination of thermal denaturation (T_m), erythroblast 6C2 DNA or normal chicken DNA (final DNA concentration of 10 mg/ml) was hybridized with AEV-specific [^{3}H]cDNA to reach a C_0t of 2×10^4 mol·s liter^{-1}. The samples were diluted to a final concentration of 0.3 M NaCl, and 20,000 cpm of [^{32}P]$cDNA_{rep}$, preannealed with normal chicken DNA, was added as an internal control. Samples were then divided into 10 aliquots and incubated in a water bath at different temperatures for 10 min. The percentage of cDNA remaining annealed to the particular cellular DNA at each defined temperature was determined by S1 nuclease digestion. The self-annealing of the cDNA was not subtracted (1 to 3%), and all hybridization values were normalized by using a correction factor corresponding to 100% of plateau hybridization of the cDNA with its homologous RNA.

Complexity of $cDNA_{aev}$. We determined the complexity of $cDNA_{aev}$ by using a method adapted from Young et al. (37), based on the fact that the complexity of a given radioactive cDNA can be defined experimentally by the $C_0t_{1/2}$ of the hybridization kinetics established between this cDNA in excess and its homologous nonradioactive RNA. Under these conditions, the $C_0t_{1/2}$ depends only on the complexity of the cDNA and is independent of any RNA heterogeneity in the hybridization reaction. One essential condition is to be in cDNA excess. To control this absolute requirement, we set the experimental procedure as follows. The hybridization kinetics were established by varying the time of hybridization of samples containing each, in addition to [^{3}H]cDNA in excess and cold RNA homologous to [^{3}H]cDNA, [^{32}P]cDNA representative of the RNA genome 100 times less concentrated than the [^{3}H]cDNA.

Thus, with [^{3}H]cDNA, we constructed a C_0t curve, and with [^{32}P]cDNA as an internal control, we constructed a C_rt curve which allowed us to estimate accurately the RNA concentration in the hybridization reaction.

We standardized our system by determining the complexity of a known [^{3}H]$cDNA_{gpC}$ prepared and purified by the method of Tal et al. (33), except rd Bryan ASV 60 to 70S RNA was used to select the single-stranded cDNA (ss-cDNA). We similarly used [^{3}H]$cDNA_{rep}$.

Hybridizations were conducted in capillaries at 68°C in 0.6 M NaCl. See the legend to Fig. 2 for details.

Gel electrophoresis of RNA. Polyadenylated RNA was prepared from AEV-transformed cells as described elsewhere (35). The RNA was treated with glyoxal and submitted to electrophoresis on agarose gels as previously described (16, 18, 31). RNAs separated by size were then transferred to activated diazobenzyloxymethyl paper by the method of Alwine et al. (1), and the northern blots were hybridized with different [^{32}P]cDNA's as described previously (1, 16, 18, 31).

We used an original washing procedure of ours (unpublished data). After a 10-min wash in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt buffer, the blots were cleaned of cDNA's by mild sonication in 0.1× SSC–0.1% SDS during 1 to 3 min (Pons sonicator, 100 W, 40 kHz; Bioblock, Strasbourg, France).

The hybrids were scored by autoradiography, using Kodak XR-1 X-ray film with Du Pont Lightning-Plus screens at −70°C.

RESULTS

Preparation of $cDNA_{aev}$. We adopted the following substractive hybridization strategy to purify $cDNA_{aev}$. [^{3}H]DNA complementary to the 50 to 70S RNA of AEV (RAV-2)-producing 6C2 cloned chicken erythroblasts was synthesized as described above. The 4 to 7S cDNA size pool (30% of the total cDNA, 9.6 μg) obtained after centrifugation through an alkaline sucrose gradient was hybridized to 78 μg of RAV-2 70S RNA under nonstringent conditions (1.2 M NaCl, 60°C) to reach a C_rt of 25 mol·s liter^{-1} and a C_0t of 4 mol·s liter^{-1} (Table 1). The ss-cDNA was separated from hybrids by chromatography on hydroxyapatite. The ss-cDNA eluted from the column (7 μg) was completely sensitive to S1 nuclease as tested on an aliquot, did not hybridize to helper 70S RAV-2 RNA ($C_rt \geq 10$ mol·s liter^{-1}), but hybridized to AEV (RAV-2) RNA ($C_rt \geq 11$ mol·s liter^{-1}) with a plateau at 50% hybridization, indicating that 50% of the ss-cDNA was not virus-hybridizable cDNA (Table 1).

The ss-cDNA was then hybridized to AEV (RAV-2) 50 to 70S RNA under stringent conditions (0.6 M NaCl, 68°C; final C_rt = 4.5 mol·s liter^{-1}), and the hybrids were treated preparatively with S1 nuclease (15 min, 50°C) to digest the ss-cDNA which was not AEV specific. Proteinase K (100 μg/ml), EDTA (0.01 M), and SDS

TABLE 1. *Purification of cDNA$_{aev}$*[a]

cDNA	g × 10^{-6}	cpm × 10^{-9}	% of total cDNA	S1 resistance (%) after hybridization with:	
				AEV (RAV-2)	RAV-2
Total	32	1.6	100		
4 to 7S	9.6	0.48	30	67	37
ss after hydroxyapatite[b]	7	0.35	21.8	50	<3
ss after S1 digestion[c]	3.5	0.176	11	88	<3

[a] The data represent recoveries during the preparation of the [^{3}H]cDNA$_{aev}$ that was used in our experiments. Similar results were obtained for the preparation of [^{32}P]cDNA$_{aev}$ that was used in experiments shown in Fig. 3.

[b] A total of 9.6 μg of cDNA was hybridized to 78 μg of RAV-2 70S RNA in 1.2 M NaCl at 60°C at a final C_rt of 25 mol·s liter^{-1}, and ss-DNA was separated from hybrids by chromatography on hydroxyapatite (see text).

[c] A total of 7 μg of cDNA was hybridized to 33 μg of AEV (RAV-2) 50 to 70S RNA in 0.6 M NaCl at 68°C at a final C_rt of 4.5 mol·s liter^{-1}. ss-DNA was digested with S1 nuclease (see text), and the cDNA was recovered from hybrids by treatment with 0.3 N NaOH (12 h at 37°C to hydrolyze RNA) and ethanol precipitation after neutralization with HCl. During the procedure, the selected cDNA's were tested for their hybridization capacities at plateau C_rt values to AEV (RAV-2) RNA or RAV-2 RNA, as scored by the percentage of radioactivity rendered resistant to S1 nuclease.

(1%) were then added, and the mixture was incubated at 37°C for 15 min. The hybrids were phenol extracted once, treated with alkali (0.3 N NaOH for 12 h at 37°C) to eliminate the viral RNA, neutralized with HCl, and ethanol precipitated (−20°C, 12 h). After this step, 11% of the starting cDNA was recovered as ss-cDNA (Table 1) and was denoted cDNA$_{aev}$.

Specificity of cDNA$_{aev}$. The specificity of cDNA$_{aev}$ was documented more precisely by hybridizing cDNA$_{aev}$ and cDNA$_{rep}$ to viral 50 to 70S RNAs. No residual hybridization of cDNA$_{aev}$ with RAV-2 RNA could be detected (Fig. 1a), indicating the efficiency of the selection procedure. On the contrary, the kinetics with AEV (RAV-2) RNA from the 6C2 erythroblasts was as expected (Fig. 1b), showing that cDNA$_{aev}$ had maintained its hybridization qualities, with a maximum plateau value of 88%. A similar curve was obtained with cDNA$_{rep}$, indicating a favorable ratio of AEV RNA to RAV-2 RNA of near 1:1 for the viruses produced by these cells.

A possible drawback to our strategy for preparing cDNA$_{aev}$ was that we might coselect for cellular sequences specific to erythroblasts that could be encapsidated with the virus or unidentified viral sequences unrelated to AEV and present in this clone. This possibility would predict that some sequences in cDNA$_{aev}$ would not hybridize with the viral RNA in another nonerythroblastic AEV-transformed cell clone. Thus, we tested the cellular RNA from an independently prepared clone (NP 75) of chicken fibroblasts nonproductively transformed by AEV. Figure 1c shows that, indeed, the same plateau value was reached with cDNA$_{aev}$, confirming the purity of this probe, whereas the curve obtained with cDNA$_{rep}$, with similar kinetics, indicated less than 30% homology of the AEV genome with cDNA$_{rep}$.

We further analyzed the distribution of cDNA$_{aev}$-related sequences among a variety of avian retroviruses, using stringent hybridization conditions at high C_rt values (≥4 mol·s liter^{-1}). None of the viruses tested, except AEV (RAV-2), showed any annealing above background (Table 2). Thus, we concluded that cDNA$_{aev}$ was specific for viral nucleotide sequences found so far only in AEV.

Complexity of cDNA$_{aev}$. Complexities of cDNA probes are usually determined by measuring the percentage of labeled viral RNA rendered resistant to RNase by the cDNA present in excess. The precision of this method becomes unsatisfactory when the complexity of the cDNA represents only a small part of the viral RNA, which is often the case for a defective retrovirus obtained with sometimes a severalfold excess of helper virus. We thus used an alternative method to determine the complexity of cDNA$_{aev}$. The validity of this method is first documented here by using cDNA probes of known complexities.

Following the experimental procedure of using C_0t curves in cDNA excess as described by Young et al. (37), modified for better accuracy as we described above, we determined the complexities of well-characterized probes such as cDNA$_{rep}$ and cDNA$_{gpC}$ as a test for the precision of this method. Figure 2a shows the kinetics between [^{3}H]cDNA$_{rep}$ in slight excess and *td* PrB ASV RNA, on the one hand (C_0t curve), and [^{32}P]cDNA$_{rep}$ in a negligible amount with the same RNA, on the other hand (control C_rt curve). Figure 2 clearly shows that these conditions yielded a $C_0t_{1/2}$ value of 1.6×10^{-2} mol·s liter^{-1}, corresponding to the expected complexity of 8,000 ± 800 nucleotides of the viral RNA and matching the $C_rt_{1/2}$ value. The almost equimolar ratio of viral RNA and [^{3}H]cDNA$_{rep}$ would predict a plateau value of the C_0t curve of near 90%. In fact, the observed value was lower (60%). This was probably due to the fact that the RNA might have been slightly degraded upon long incubation times; it also could mean that [^{3}H]cDNA$_{rep}$ was not absolutely representative or not able to hybridize all along the RNA molecules. Nevertheless, the $C_0t_{1/2}$ value does not seem to be affected detectably by these

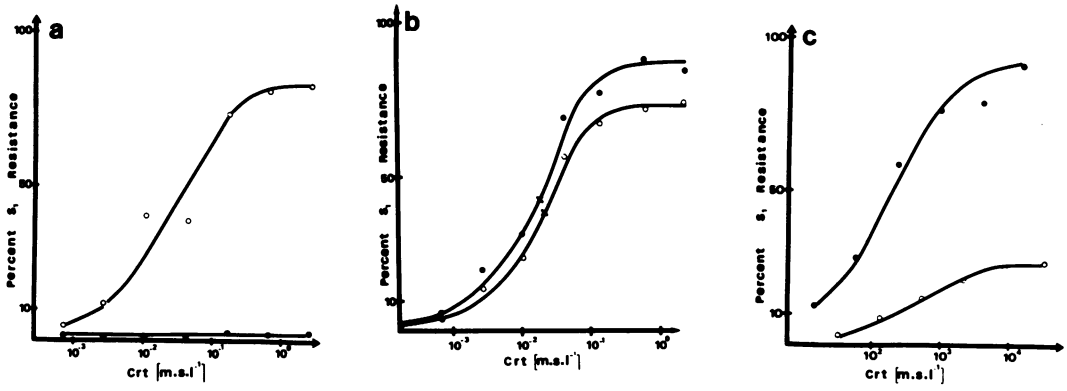

FIG. 1. *Hybridization of cDNA$_{aev}$ after the S1 nuclease selection step. [^{3}H]cDNA$_{aev}$ (●) and [^{32}P]cDNA$_{rep}$ (○) were hybridized under stringent (0.6 M NaCl) conditions to dilutions of viral RNAs in a volume of 8 μl for 15 h at 68°C. The extent of hybridization was measured by digestion with S1 nuclease. (a) Hybridization with RAV-2 RNA; (b) hybridization with AEV (RAV-2) RNA; (c) dilutions of total cellular RNA extracted from a clone (NP 75) of chicken fibroblasts nonproductively infected with AEV were hybridized to both probes in 0.6 M NaCl at 68°C for 25 h in 7 μl (0.075 mg of RNA at the highest C$_r$t). Hybridization was measured by resistance of the cDNA's to hydrolysis with S1 nuclease.*

TABLE 2. *Hybridization of viral 50 to 70S RNAs to cDNA$_{rep}$ and cDNA$_{aev}$*[a]

	Viral RNAs	Hybridization of cDNA (%) cDNA rep	cDNA aev
ALV	RAV 0	91	3
	RAV 1	79	4
	RAV 2	80	3
	RAV 49	80	3
	RAV 50	86	3
	td.B77	83	2
	td.Pr B	100	3
ASV	nd.B77	94	4
	nd.Pr B	100	3
	CZ	86	4
	rd Bry	70	2
DLV	AEV (RAV 2)	86	100 (88)
	MC 29 (RAV 2)	89	5
	MH 2 (RAV 3)	89	6
	OK 10 (RAV 2)	100	3
	CM II (CM-AV)	76	3
	AMV	84	3
	E 26 (RAV 2)	89	4

[a] Hybridization reactions were performed in solution under conditions of RNA excess as described in the text. [^{32}P]cDNA$_{rep}$ was included in each reaction mixture as a standard (final C$_r$t ≥ 4 mol·s liter^{-1}). The values for the percent cDNA's in hybrids were normalized to 100% for the final extent of reaction with a homologous RNA: 80% for cDNA$_{rep}$ with *td* PrB and 88% for cDNA$_{aev}$ with AEV (RAV-2).

parameters and proves the validity of this method, provided that the ^{3}H probes have been selected to exclude small pieces (<4S) that would affect the kinetics. Similar experiments are shown for [^{3}H]cDNA$_{gpC}$ in excess over *td* PrB RNA in Fig. 2b. The C$_0$t$_{1/2}$ value found was 4.5×10^{-3} mol·s liter^{-1} and thus corresponded to a complexity of 2,250 ± 200 nucleotides, a value which agrees with other determinations (13, 33). As expected, the C$_0$t$_{1/2}$ value did not vary when the ratio of cDNA to RNA was increased (Fig. 2c), but the plateau value was then lower and the precision was somehow affected.

Using this procedure for [^{3}H]cDNA$_{aev}$, we found (Fig. 2d) a C$_0$t$_{1/2}$ value of 7.5×10^{-3} mol·s liter^{-1}, which corresponded to a complexity of 3,700 ± 370 nucleotides. If one adds the 2,200 ± 200 nucleotides of ALV-related sequences detected in the AEV genomic RNA (25) to the 3,700 ± 370 nucleotides of the specific sequence, the AEV genomic RNA should be 5,900 ± 600 nucleotides long. These numbers are in good agreement with the size of the AEV genomic RNA as determined by northern blotting (30S) and described below and with the results obtained by analysis of heteroduplex molecules formed between a long cDNA (cDNA PrC) and the AEV genomic RNA (14).

Sizes of AEV-specific RNAs in infected cells. The strategy of transcription of the AEV provirus was analyzed to determine whether we could detect a difference in the relative level of transcription or in the sizes of viral transcripts in erythroid versus fibroblastic transformed cells.

We thus performed northern blots with polyadenylic acid-containing total cell RNA from the following AEV-transformed cells: a chicken erythroblast clone (9D4) producing AEV (RAV-2) (the virus produced here was also tested); a chicken erythroblast nonproducer clone (NP

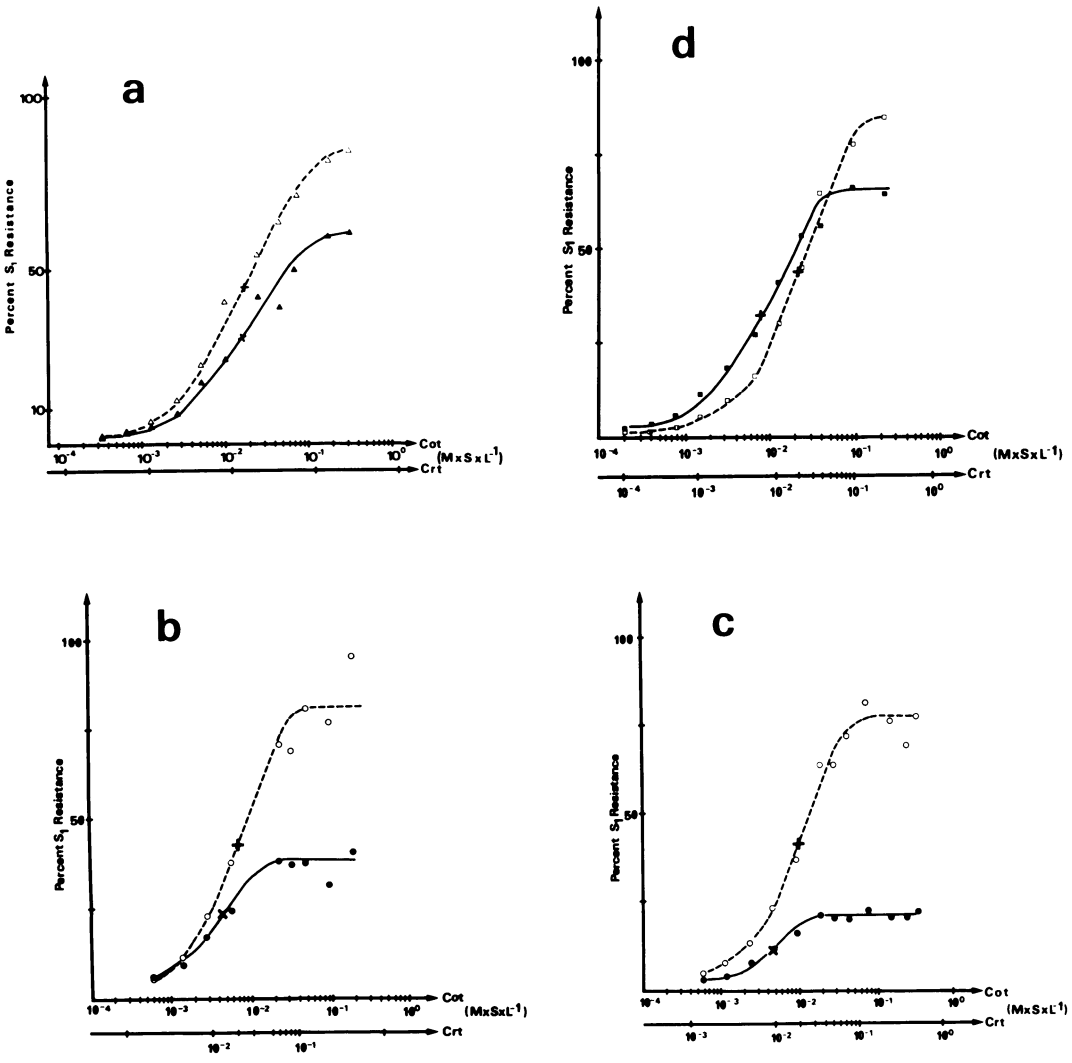

FIG. 2. *Determination of cDNA complexities. (a) Complexity of $cDNA_{rep}$. A total of 39,000 cpm (0.82 ng) of [^{3}H]$cDNA_{rep}$ (▲) and 2,000 cpm (0.005 ng) of [^{32}P]$cDNA_{rep}$ (△) were hybridized to 0.73 ng of td PrB RNA in each sample (10 μl). The times of incubation ranged from 210 s to 64 h. Hybridization was measured by resistance to S1 nuclease. (b and c) Complexity of $cDNA_{gpC}$ at two different values of cDNA excess. (b) A total of 24,000 cpm (0.37 ng) of [^{3}H]$cDNA_{gpC}$ (●) and 2,000 cpm (0.005 ng) of [^{32}P]$cDNA_{rep}$ (○) were hybridized as in (c) to 1 ng of td PrB RNA in each sample (2 μl) (gpC = 25% of viral RNA sequences). (c) A total of 17,000 cpm (0.31 ng) of [^{3}H]$cDNA_{gpC}$ (●) and 2,000 cpm (0.005 ng) of [^{32}P]$cDNA_{rep}$ (○) were hybridized to 0.29 ng of td PrB RNA in each sample. The times of incubation ranged from 225 s to 32 h for each curve. Hybridization was measured by resistance to S1 nuclease. All values were normalized to a final extent of hybridization of 75% for both ^{3}H and ^{32}P probes (factor, ×1.33). (d) Complexity of $cDNA_{aev}$. The C_0t curve was constructed as follows. Samples contained 44,000 cpm (0.92 ng; ■) of [^{3}H]$cDNA_{aev}$, 2,000 cpm (0.005 ng; □) of [^{32}P]$cDNA_{rep}$, and 0.8 ng of AEV (RAV-2) RNA in 10 μl (0.6 M NaCl, 68°C). The C_0t values were obtained by varying the time of incubation from 225 s to 37 h. Percent values were corrected by the factors ×1.14 for [^{3}H]$cDNA_{aev}$ and ×1.18 for [^{32}P]$cDNA_{rep}$.*

A6C1); and two nonproducer fibroblast clones of chicken (NP 75) and rat (NP AT1a). The results are shown in Fig. 3. For clone 9D4, three mRNA species of 34, 30, and 23S were detected with [^{32}P]$cDNA_{rep}$ (Fig. 3, lane a). The 30 and 23S species also hybridized with [^{32}P]$cDNA_{aev}$ (Fig. 3, lane b), but the 34S species that corresponded to the RAV-2 helper RNA did not. In addition, two other RNA species of high molecular weight (31 and 32S) were detected wtih [^{32}P]$cDNA_{aev}$.

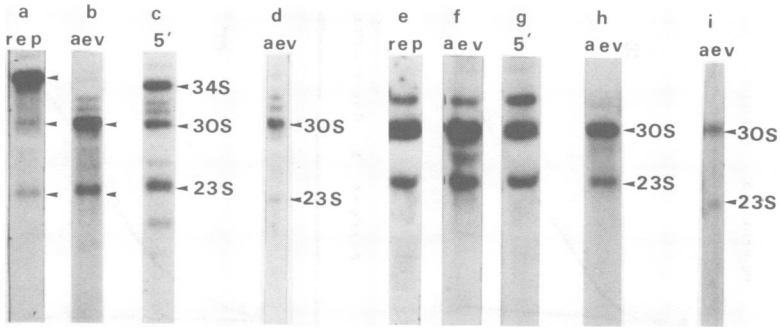

FIG. 3. *Sizes of AEV-coded RNAs. Total cellular polyadenylic acid-containing RNAs were run on 1% agarose gels, transferred to activated diazobenzyloxymethyl paper, and hybridized with [^{32}P]cDNA$_{rep}$ (lanes a and e), cDNA$_{aev}$ (lanes b, f, h, and i), or cDNA$_{5'}$ (lanes c and g). The following AEV-transformed cells were tested: chicken erythroblast clone 9D4 (lanes a, b, and c) and the AEV (RAV-2) that it produces in the culture fluid (lane d, with cDNA$_{aev}$); chicken erythroblast nonproducer clone NP A6C1 (lanes e, f, and g); chicken fibroblast clone NP 75 (lane h); and rat fibroblast clone NP AT1a (lane i).*

These bands also hybridized to cDNA$_{5'}$ (Fig. 3, lane c), indicating their viral origin or viral induction. Interestingly, all of these sequences were found in the viral RNA from the culture fluid (Fig. 3, lane d), indicating that they were probably efficiently packaged into the virions. This matter was not analyzed further.

The complicated pattern of cDNA$_{aev}$-related mRNA species in clone 9D4 raised the question of their relevance to erythroblast transformation by AEV. We thus analyzed another clone of similar origin but a nonproducer, NP A6C1. Only three mRNA species (23, 30, and 33S) were seen with cDNA$_{rep}$ (Fig. 3, lane e), cDNA$_{aev}$ (Fig. 3, lane f), or cDNA$_{5'}$ (Fig. 3, lane g). The band at 27S (Fig. 3, lane f) could not be reproduced in subsequent blots and thus represented an artifact. Thus, only the 30 and 23S species are shared between the two erythroblast clones tested, and it is unclear whether the other species observed play any role in the transformation of erythroblasts by AEV. Certainly, the cDNA$_{aev}$-related 30 and 23S species seemed to be sufficient to transform chicken or rat fibroblasts (Fig. 3, lanes h and i, respectively), and no obvious variation in the relative abundance of these species was observed in the four AEV-transformed clones tested, although the rat cells contained about 10-fold-fewer AEV-related RNA copies per cell (unpublished data of K. Quade and us).

Cellular origin of the nucleotide sequences related to cDNA$_{aev}$. We have shown previously that cDNA$_{aev}$ annealed under stringent reaction conditions (0.6 M NaCl, 68°C) to DNA from normal chickens, as well as to DNA from other birds phylogenetically diverged from chickens and to DNA from mammalian species and fish (25, 31). For the latter species, the percent annealing was low, and the copy number per cell was not known. We thus decided to analyze more closely the kinetics of these annealings. The C_0t curves constructed (Fig. 4) with the DNAs of human placenta (Fig. 4a) and salmon sperm (Fig. 4b) indicated that in both cases 1 to 2 copies of cDNA$_{aev}$-related sequences were present per haploid genome ($C_0t_{1/2} = 3.5 \times 10^3$ mol·s liter^{-1}). The hybridization kinetics allowed us to exclude the possibility of nonspecific hybridization, since in the same experiment, the cDNA$_{rep}$ used as a negative control did not hybridize (Fig. 4). The plateau value observed (7%) reflected the phylogenetic distance of mammals and fish from chickens, but significant annealings were detected even under our stringent conditions (0.6 M NaCl, 68°C, S1 nuclease treatment).

***T_m* studies.** We previously reported that AEV-specific sequences (v-*erb*) are transduced from normal cell DNA (c-*erb*) and might represent a cellular gene involved in the normal hematopoietic cell differentiation process (9, 25, 31).

To establish the relatedness between cellular AEV-related sequences and viral AEV-specific sequences, we determined the T_m curve of the cDNA$_{aev}$ hybridized with the normal c-*erb* sequences in chicken DNA and the T_m curve of the cDNA$_{aev}$ hybridized with the viral AEV sequences present in the DNA of 6C2 chicken erythroblasts. In each experiment, [^{32}P]cDNA$_{rep}$ preannealed with normal chicken DNA was added as an internal standard. As shown in Fig. 5, the v-*erb* sequences and their alleged progenitor c-*erb* sequences are not totally homologous. These results are very similar to those found previously for the *src* gene of ASV (30) and the *mac*-specific sequences of MC29 and their cellular equivalents (26).

Transcription of cellular AEV-related sequences in normal cells. The RNA of unin-

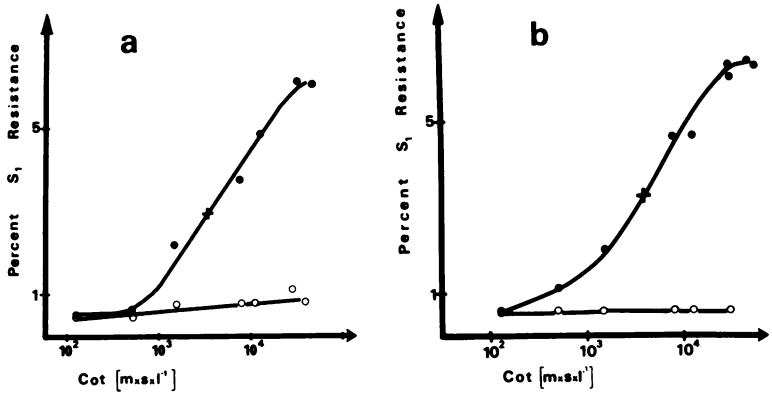

FIG. 4. *AEV-specific sequences present in the DNAs of humans (a) and salmon (b). A total of 200 μg (4 to 6S) of denatured DNA was hybridized (25 μl) with 2,000 cpm of cDNA's per point (^3H, 0.04 ng; ^{32}P, 0.005 ng) under stringent conditions (0.6 M NaCl, 68°C, S1 nuclease treatment) at increasing Cot values to a final Cot of 40,000 mol·s liter^{-1}. Symbols: ●, [^3H]cDNA$_{aev}$; ○, [^{32}P]cDNA$_{rep}$.*

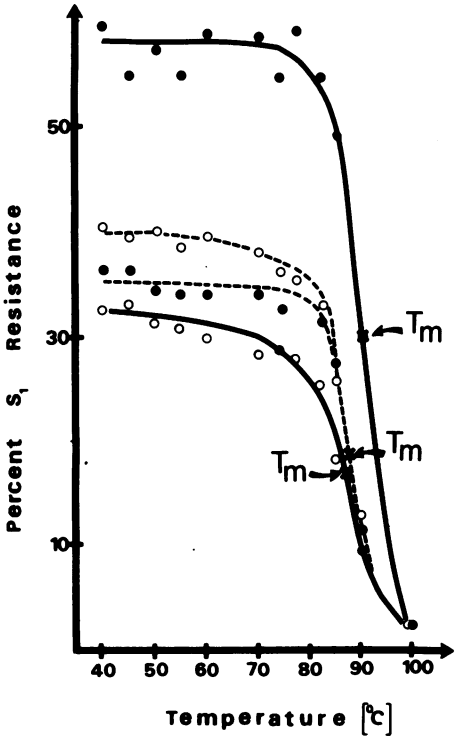

FIG. 5. *T_m profiles. The DNAs were annealed to [^3H]cDNA$_{aev}$, the T_m values were determined as described in the text, and the values were corrected for the final extent of reaction for each probe with a homologous RNA. A duplex preformed between [^{32}P]cDNA$_{rep}$ and normal chicken DNA was included in each T_m series as an internal control. Symbols: ○——○, cDNA$_{aev}$ to normal chicken DNA; ○- - -○, control; ●——●, cDNA$_{aev}$ to AEV-infected erythroblast DNA; ●- - -●, control.*

fected chicken fibroblasts contains 1 to 2 copies per cell of cDNA$_{aev}$-related sequences (25). In these experiments, the plateau value of the C$_r$t curves was near 100%, indicating that most or all of these sequences were present in the chicken DNA and transcribed. We now know that cDNA$_{aev}$ detects probably two different moieties of the specific AEV sequences corresponding to the 75 and 40K virus-coded proteins (12, 21, 36). This raised the questions of whether both domains were conserved phylogenetically and whether they were transcribed at similar frequencies. We thus conducted a similar experiment in quail.

RNA extracted from normal quail embryo fibroblasts was hybridized to different cDNA's under stringent reaction conditions. The kinetics of hybridization are shown in Fig. 6. cDNA$_{aev}$ showed extensive homology (over 90%) to the quail fibroblast RNA, with kinetics similar to those presented here for a comparison with cDNA$_{src}$. In contrast, cDNA$_{rep}$ did not hybridize to more than 5%. We thus concluded that both domains represented in cDNA$_{aev}$ are conserved in quail DNA and that both are transcribed at a frequency of 1 to 2 copies per cell in quail, as well as in chicken, fibroblasts.

DISCUSSION

Viral *erb* sequence. We have been able to prepare a cDNA (cDNA$_{aev}$) representing the specific, ALV-unrelated nucleotide sequences contained in the AEV genome. An efficient and simplified purification procedure that produces cDNA$_{aev}$ in only two selection steps, allowing an optimized yield (11% of the starting radioactivity) with no detectable residual hybridization

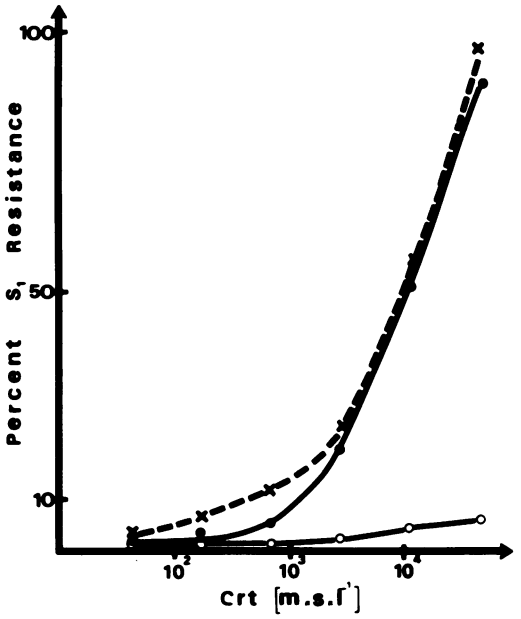

FIG. 6. *Transcription of the cellular AEV-related specific sequences in normal quail fibroblasts. Total RNA was extracted from normal quail embryo fibroblasts and hybridized (30 μl) at increasing C,t values (varying the RNA concentration up to 10 mg/ml) under stringent conditions (0.6 M NaCl, 68°C) with different cDNA's (2,000 cpm each per point). The extent of hybridization was measured by S1 nuclease digestion. Symbols: ●, [³H]cDNA$_{aev}$; ○, [³²P]-cDNA$_{rep}$; ×, [³H]cDNA$_{sarc}$.*

with ALV RNA, has been designed.

Complexity measurements of cDNA's representing parts of viral genomes have used the protection of labeled viral RNA by cDNA excess liquid hybridization (27). Such determinations are not accurate for defective viral genomes. Indeed, the specific RNA annealed represents only a small fraction of the labeled RNA, which consists mostly of helper RNA usually in severalfold excess over the defective genomic RNA. We thus adapted the method of Young et al. (37) to deduce the complexity of the specific [³H]cDNA directly from the $C_0t_{1/2}$ value determined in cDNA excess liquid hybridization. Under these conditions, the measure is then independent of any helper RNA excess, provided that the cDNA is driving the reaction kinetics. Such conditions were conveniently monitored by including in the reaction mixture a control cDNA ([³²P]cDNA$_{rep}$) made to the helper RNA by using a different radioactive label. The concentration range of viral RNA can then be carefully determined to ensure hybridization kinetics in which $C_0t_{1/2} < C_rt_{1/2}$ and $C_0t_{1/2}$ is indicated by the ³H counts and $C_rt_{1/2}$ is indicated by the ³²P counts. If such conditions are fulfilled, the plateau value of the C_0t curve is lower than the maximum expected under RNA excess conditions, an additional internal control for the cDNA excess requirement in such studies.

The validity of our modification was tested by determining the complexities of [³H]cDNA's prepared to whole ALV RNA and also to the well-characterized *env* gene of an ALV. Measured complexities were 8.0 ± 0.8 and 2.25 ± 0.2 kilobases, respectively, values that are in agreement with published data (13, 33). The complexity of 3.7 ± 0.37 kilobases that we determined for cDNA$_{aev}$ agrees with the value of 3.25 ± 0.33 kilobases for the continuous stretch seen by Lai et al. (14) with heteroduplex mapping. Thus, the adapted method indicated here, although somehow complicated to describe but in fact easy to use, has proven to be a method of choice for determining more accurately the complexities of cDNA probes made to specific parts of defective viral genomes. It remains to be documented whether a highly unusual guanine-plus-cytosine content in such sequences would significantly modify the precision of such determinations.

Among all of the avian retroviruses tested (ALVs, ASVs, and DLVs), only AEV contains the *erb* sequences. Other viruses have been reported to induce occasionally erythroblastosis (8); thus, they must contain other transforming genes or act by a different mechanism. If other viruses that cause acute erythroid leukemias are isolated in the future, it will be of interest to determine whether their genomes show relatedness with cDNA$_{aev}$.

Two sizes of AEV-coded RNAs. Total polyadenylic acid-containing cellular RNA was tested in different types of cells by northern blotting for AEV-related sequences. RNAs from AEV nonproducer chicken fibroblasts show two distinct *erb*-related species: a 30S genomic RNA and a 23S subgenomic RNA. Such species are also found in mammalian fibroblastic cells nonproductively transformed by AEV, as well as in the RNA of chicken erythroblast clones productively or nonproductively transformed by AEV. Both species were also detected by cDNA$_{rep}$ and cDNA$_{5'}$, indicating that the 23S species probably represents a spliced viral mRNA. Surprisingly, both species are packaged efficiently into mature virions.

These results have several important implications. (i) There are two AEV-coded mRNA's in all tested AEV-transformed cells. (ii) Their sizes are identical as tested by northern blottings, indicating a similar, if not identical, processing in mammalian versus avian fibroblasts on the one hand and in hematopoietic target cells

versus nonhematopoietic cells on the other hand. (iii) The 23S species could correspond to a spliced mRNA containing the 5′ leader linked to the 3′ half of the *erb* sequence next to the Δ*env*-c-polyadenylic acid-containing ALV-related sequences seen by heteroduplex mapping (14) and in our studies (31; unpublished data). This virion-packaged mRNA is a good candidate for the AEV-specific 40K polyprotein reported by other investigators (15, 21, 36) by in vitro translation of AEV RNA, in addition to the *gag* fusion 75K polyprotein.

Additional virus-coded bands are seen with $cDNA_{aev}$ and $cDNA_{5'}$ in some infected cells. They may represent read-through transcripts into cellular sequences due to a leaky termination signal at the 3′ right-end long terminal redundancy, as recently suggested by Benz et al. (2). Such additional bands seem to be more abundant in clones producing virus (this study and our unpublished data) and could be explained by new postcloning intergrations of complete or partial viral DNA copies. The role, if any, of such bands in the transformation process of erythroblasts has not been investigated further in our studies. We also cannot exclude the alternate possibility of viral promoters inducing the transcription of some c-*erb* sequences.

c-*erb* sequence. We previously showed that normal cellular DNAs of avian and mammalian species contain nucleotide sequences (c-*erb*) related to $cDNA_{aev}$ (25, 31). A cellular origin of the c-*erb* sequences was indicated by a hybridization plateau decreasing with the phylogenetic distance from chickens to the species tested, as was found also for the cellular equivalents of the specific sequences of other transforming viruses (25, 26).

The small number of hybrids observed with nonavian DNA, though, demanded closer studies. In this paper, we showed that indeed there was a significant slope in the C_0t curve obtained with $cDNA_{aev}$ and the DNA of humans or fish (Fig. 4), indicating the presence of 1 to 2 copies of these sequences per haploid genome, as shown previously in chicken DNA (25). The important implication of these results is that it should now be possible, using $cDNA_{aev}$, to isolate human molecular clones from a human DNA library inserted in a λ phage.

It is most likely that AEV was formed by recombination of an ALV-related vector virus with the c-*erb* sequence from chickens or a species close to chickens. This is indicated by the small value of mismatching observed between the v-*erb* and the chicken c-*erb* sequences (3 to 4°C in our experiments) compared with similar experiments performed with other avian DNAs. These results are similar to those obtained previously for the *src* gene of ASV (30) and the *mac* sequences of MC29 (26) and their cellular equivalents. Such a divergence of a low percentage of bases probably reflects the increased capacity of the viruses to accumulate mutations because nonlethal mutations are efficiently selected in the viral progeny. Repeated cycles of infection and transformation could lead to the selection of mutations that would enhance the transformation activity of the *erb* gene or could extend its target cell specificity. In such a way, the structural comparison of normal cellular genes and their equivalents acquired by retroviruses could represent a suitable experimental approach to the problem of eucaryotic gene evolution.

Transcription experiments performed with total cellular RNA extracted from normal quail embryo fibroblasts and hybridized with $cDNA_{aev}$ show that most or all of the sequences of $cDNA_{aev}$ are found in the cellular RNA, although at a very low level (1 or 2 copies per cell). Similar results were obtained previously for chicken fibroblasts (25) and indicate that: (i) the c-*erb* sequences corresponding to the specific genetic information coding for both the p75 and the p40 viral polyproteins are transcribed in normal cells; (ii) similar mechanisms of transcription are operating in chicken and quail fibroblasts; (iii) these sequences are tightly controlled by the cellular machinery and kept to a low level of expression. Whether the c-*erb* sequences have any role to play in fibroblasts or in any other non-hematopoietic or hematopoietic normal cell functions remains to be shown.

ACKNOWLEDGMENTS

We thank T. Graf and H. Beug for having made available to us lines 6C2, 9D4, and NP A6Cl and K. Quade for supplying line NP AT1a; M. Boccara, A. Sergeant, and J. Svoboda for helpful advice and discussion; M. B. Raes for technical assistance; and N. Devassine for patient typing.

This work was supported by Institut National de la Santé et de la Recherche Médicale (ASR 1-018, CRL 77.5.054.2, and CRL 79.5.060.2), Centre National de la Recherche Scientifique (AI and ERA 225), Délégation Générale à la Recherche Scientifique et Technique (77.7.1879), and the Pasteur Institute of Lille.

LITERATURE CITED

1. **Alwine, J. C., J. K. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
2. **Benz, E., R. Wydro, B. Nadal-Ginard, and D. Dina.** 1980. Moloney murine sarcoma proviral DNA is a transcriptional unit. Nature (London) **288**:665–669.
3. **Beug, H., A. von Kirchbach, G. Doderlein, J. F. Conscience, and T. Graf.** 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell **18**:375–390.
4. **Chen, J. H., M. G. Moscovici, and C. Moscovici.** 1980.

Isolation of complementary DNA unique to the genome of avian myeloblastosis virus (AMV). Virology **82**:431–448.

5. **Friedrich, R., H. Kung, B. Baker, H. Varmus, H. Goodman, and M. Bishop.** 1977. Characterization of DNA complementary to a nucleotide sequence at the 5′ terminus of the ASV genome. Virology **79**:198–215.
6. **Gazzolo, L., C. Moscovici, M. G. Moscovici, and J. Samarut.** 1979. Response of hemopoietic cells to avian acute leukemia viruses: effects on the differentiation of the target cells. Cell **16**:627–638.
7. **Graf, T., N. Ade, and H. Beug.** 1978. Temperature sensitive mutant of avian erythroblastosis virus suggests a block of differentiation as mechanism of leukemogenesis. Nature (London) **275**:496–501.
8. **Graf, T., and H. Beug.** 1978. Avian leukemia viruses. Interaction with their target cells in vivo and in vitro. Biochim. Biophys. Acta **516**:269–299.
9. **Graf, T., H. Beug, A. von Kirchbach, M. J. Hayman.** 1980. Three new types of viral oncogenes in DLVs: biological, genetical and immunochemical evidence. Cold Spring Harbor Symp. Quant. Biol. **44**:1225–1234.
10. **Graf, T., N. Oker-Blom, T. Todorov, and H. Beug.** 1979. Transforming capacities and defectiveness of avian leukemia viruses OK10 and E26. Virology **99**:431–436.
11. **Graf, T., B. Royer-Pokora, G. E. Schubert, and H. Beug.** 1976. Evidence for a multiple oncogenic potential of cloned leukemia virus: in vitro and in vivo studies with avian erythroblastosis virus. Virology **71**:423–433.
12. **Hayman, M. J., B. Royer-Pokora, and T. Graf.** 1979. Defectiveness of avian erythroblastosis virus: synthesis of a 75K gag related protein. Virology **92**:31–45.
13. **Hu, S. S. F., M. M. C. Lai, and P. K. Vogt.** 1978. Characterization of the *env* gene in avian oncoviruses by heteroduplex mapping. J. Virol. **27**:667–676.
14. **Lai, M. M. C., S. S. F. Hu, and P. K. Vogt.** 1979. Avian erythroblastosis virus: transformation specific sequences form a contiguous segment of 3.25 kb located in the middle of the 6 kb genome. Virology **97**:366–377.
15. **Lai, M. M. C., C. J. Neil, and P. K. Vogt.** 1980. Cell free translation of avian erythroblastosis virus RNA yields two specific and distinct proteins with molecular weights of 75,000 and 40,000. Virology **100**:475–483.
16. **Lee, J. S., H. E. Varmus, and M. J. Bishop.** 1979. Virus specific messenger RNAs in permissive cells infected by avian sarcoma virus. J. Biol. Chem. **254**:8015–8022.
17. **Leong, J. A., A. C. Garapin, N. Jackson, L. Fanshier, W. Levinson, and J. M. Bishop.** 1972. Virus-specific ribonucleic acid in cells producing Rous sarcoma virus: detection and characterization. J. Virol. **9**:891–902.
18. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835–4838.
19. **Mladenov, Z., V. Heine, D. Beard, and J. W. Beard.** 1967. Strain MC29 avian leukosis virus. Myelocytoma, endothelioma and renal growths: pathomorphological and ultrastructural aspects. J. Natl. Cancer Inst. **38**: 251–285.
20. **Moscovici, C., R. W. Alexander, M. G. Moscovici, and P. K. Vogt.** 1978. Transforming and oncogenic effects of MH-2 virus, p. 45–56. *In* S. Barlati and C. De Giuli (ed.), Avian RNA tumor viruses. Piccin Books, Padua, Italy.
21. **Pawson, T., and G. S. Martin.** 1980. Cell-free translation of avian erythroblastosis virus RNA. J. Virol. **34**:280–284.
22. **Quade, K.** 1979. Transformation of mammalian cells by avian myelocytomatosis virus and avian erythroblastosis virus. Virology **98**:461–465.
23. **Rettenmier, C. W., S. M. Anderson, M. W. Riemen, and H. Hanafusa.** 1979. *gag*-related polypeptides encoded by replication-defective avian oncoviruses. J. Virol. **32**:749–761.
24. **Rothe-Meyer, A., and J. Engelbreth-Holm.** 1933. Experiementelle Studien über die Beziehungen zwischen Hühnerleukose und Sarkom an Hand eines Stammes von übertragbarer leukose-sarkom Kombination. Acta Pathol. Microbiol. Scand. **10**:380–427.
25. **Roussel, M., S. Saule, C. Lagrou, C. Rommens, H. Beug, T. Graf, and D. Stehelin.** 1979. Three new types of viral oncogene of cellular origin specific for hematopoietic cell transformation. Nature (London) **281**:452–455.
26. **Sheiness, D., and J. M. Bishop.** 1979. DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. J. Virol. **31**:514–521.
27. **Sheiness, D., L. Fanshier, and J. M. Bishop.** 1978. Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. J. Virol. **28**:600–610.
28. **Stehelin, D., and T. Graf.** 1978. Avian myelocytomatosis and erythroblastosis viruses lack the transforming gene src of avian sarcoma viruses. Cell **13**:745–750.
29. **Stehelin, D., R. V. Guntaka, H. E. Varmus, and J. M. Bishop.** 1976. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. J. Mol. Biol. **101**:349–365.
30. **Stehelin, D., M. Roussel, and M. Gardes.** 1978. The transforming gene of avian sarcoma viruses, p. 134–144. *In* S. Barlati and C. De Giuli (ed.), Avian RNA tumor viruses. Piccin Books, Padua, Italy.
31. **Stehelin, D., S. Saule, M. Roussel, C. Lagrou, and C. Rommens.** 1980. Three new types of viral oncogenes in DLVs: viral nucleotide sequences correlating with distinct phenotypes of virus transformed cells. Cold Spring Harbor Symp. Quant. Biol. **44**:1215–1223.
32. **Sutton, W. D.** 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochim. Biophys. Acta **240**:522–531.
33. **Tal, J., D. J. Fujita, S. Kawai, H. E. Varmus, and J. M. Bishop.** 1977. Purification of DNA complementary to the *env* gene of avian sarcoma virus and analysis of relationships among the *env* genes of avian leukosis-sarcoma viruses. J. Virol. **21**:497–505.
34. **Taylor, J. M., R. Illmensee, and J. Summers.** 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta **442**: 324–330.
35. **Verma, I. M.** 1978. Genome organization of RNA tumor viruses. I. In vitro synthesis of full-genome-length single-stranded and double-stranded viral DNA transcripts. J. Virol. **26**:615–629.
36. **Yoshida, M., and K. Toyoshima.** 1980. In vitro translation of avian erythroblastosis virus RNA: identification of two major polypeptides. Virology **100**:484–487.
37. **Young, B. D., P. R. Harrison, R. S. Gilmour, G. D. Birmie, S. Annahell, S. Humphries, and J. Paul.** 1974. Kinetic studies of gene frequency. II. Complexity of globulin complementary DNA and its hybridization characteristics. J. Mol. Biol. **84**:555–568.