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env Gene of Chicken RNA Tumor Viruses: Extent of Conservation in Cellular and Viral Genomes

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The *env* gene of avian sarcoma-leukosis viruses codes for envelope glycoproteins that determine viral host range, antigenic specificity, and interference patterns. We used molecular hybridization to analyze the natural distribution and possible origins of the nucleotide sequences that encode *env*; our work exploited the availability of radioactive DNA ($cDNA_{gp}$) complementary to most or all of *env*. *env* sequences were detectable in the DNAs of chickens which synthesized an *env* gene product (chick helper factor positive) encoded by an endogenous viral gene and also in the DNAs of chickens which synthesized little or no *env* gene product (chick helper factor negative). *env* sequences were not detectable in DNAs from Japanese quail, ring-necked pheasant, golden pheasant, duck, squab, salmon sperm, or calf thymus. The detection of sequences closely related to viral *env* only in chicken DNA contrasts sharply with the demonstration that the transforming gene (*src*) of avian sarcoma viruses has readily detectable homologues in the DNAs of all avian species tested [D. Stehelin, H. E. Varmus, J. M. Bishop, and P. K. Vogt, Nature (London) **260**:170-173, 1976] and in the DNAs of other vertebrates (D. Spector, personal communication). Thermal denaturation studies on duplexes formed between $cDNA_{gp}$ and chicken DNA and also between $cDNA_{gp}$ and RNAs of subgroup A to E viruses derived from chickens indicated that these duplexes were well matched. In contrast, $cDNA_{gp}$ did not form stable hybrids with RNAs of viruses which were isolated from ring-necked and golden pheasants. We conclude that substantial portions of nucleotide sequences within the *env* genes of viruses of subgroups A to E are closely related and that these genes probably have a common, perhaps cellular, evolutionary origin.

The *env* gene of avian leukosis-sarcoma viruses codes for the envelope glycoproteins which are responsible for viral host range, antigenic specificity, and interference patterns. In a previous report (29) we described the purification and properties of $cDNA_{gp}$, a DNA complementary to *env* gene sequences of chicken leukosis-sarcoma virus RNAs. Because subgroup specificity of avian leukosis-sarcoma viruses is a property determined by the viral envelope glycoproteins (15), it was conceivable that differences in nucleic acid sequences of the *env* gene would exist between viruses of different subgroups. Such differences, if large enough, might be detectable in molecular hybridization tests with $cDNA_{gp}$. Major differences were in fact found between the *env* genes of viruses of pheasant origin (subgroup F viruses and golden pheasant virus [GPV]) (6, 12) and those of chicken origin (subgroups A to E). In particular, there was little or no hybridization of $cDNA_{gp}$ to viral RNAs of subgroup F viruses and GPV, whereas we observed extensive hybridization to the RNAs of subgroup A to E viruses (29). These results suggested that the *env* genes of chicken viruses of subgroups A to E were closely related to each other and only distantly related, if at all, to the *env* genes of the subgroup F viruses and GPV.

In the present report we used $cDNA_{gp}$ in molecular hybridization studies with a variety of avian DNAs to examine the natural distribution and possible origins of viral *env* genes. Our results indicate that nucleotide sequences closely related to $cDNA_{gp}$ are present in chicken DNA, but not in other avian DNAs tested or in calf thymus and salmon sperm DNAs. These results are in marked contrast to our experience with the transforming gene (*src*) of avian sarcoma viruses (ASV), for which homologues have been demonstrated in the DNAs of all avian species tested (28) and also in the DNAs of other vertebrates (D. Spector, personal communication).

env gene sequences were found to be present

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in the DNAs of both chick helper factor-positive (chf^+) and chick helper factor-negative (chf^-) chicken cells, at a level of one copy per haploid genome. The presence of *env* gene sequences in chf^- DNA suggests that the chf^- phenotype does not result from a major deletion of *env* gene sequences and is consistent with the existence of mechanisms for the regulation of *env* gene expression.

We also employed thermal denaturation studies to further examine the homology between the *env* genes of representatives of viral subgroups A to E. Our results indicate that the hybrids formed between $cDNA_{gp}$ and RNAs of subgroup A to E viruses are closely matched. This suggests that a substantial portion of the *env* genes of viruses of subgroups A to E consists of shared, closely related sequences and that these genes probably have a common evolutionary origin.

MATERIALS AND METHODS

Cells and viruses. All viruses used in this study were propagated in C/E or C/BE chicken fibroblasts which score as negative in chf assays (35). These fibroblasts were prepared from fertile eggs obtained from H & N Farms, Redmond, Wash. XC cells were provided by J. A. Levy. We have previously described the sources of our viral stocks and our procedures for propagation and purification of virus, extraction of viral RNA, and fractionation of viral RNA by rate-zonal sedimentation (2, 27, 29).

Preparation of cellular DNAs. Chicken, duck, and quail DNAs were prepared from embryonated eggs after 12 to 16 days of gestation. Ring-necked pheasant, golden pheasant, and squab DNAs were prepared from adult livers. In each case, the tissues were homogenized for 30 s in a Waring blender in DNA buffer (0.1 M NaCl, 0.01 M EDTA, 0.05 M Tris-hydrochloride, pH 8), incubated overnight at 37°C in the presence of 500 μg of Pronase per ml and 1% sodium dodecyl sulfate (SDS), and then extracted three times with phenol. After ethanol precipitation, the samples were suspended in TE buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.01 M EDTA) and treated with 100 μg of RNase per ml for 1 h at 37°C, followed by incubation with 500 μg of Pronase per ml–0.5% SDS for 1 h at 37°C. After two additional phenol extractions, the DNA was ethanol precipitated and resuspended in TE buffer. The DNA was then boiled in 0.3 M KOH for 20 min, neutralized with HCl, and ethanol precipitated. This procedure shears the DNA by depurination to average chain lengths of 300 to 400 nucleotides (9).

Molecular hybridization. Standard conditions were 68°C and 0.6 M NaCl (in 0.02 M Tris-hydrochloride, pH 7.4–0.01 M EDTA), as previously described (29). Hybridization of labeled DNA was measured as resistance to hydrolysis by S1 nuclease (18).

Conditions of lowered stringency, allowing the formation of stable duplexes between nucleic acid chains having partially mismatched sequences, were 59°C and 1.5 M NaCl (in 0.02 M Tris-hydrochloride, pH 7.4–0.001 M EDTA) (23). Analysis of these duplexes was performed by chromatography at 50°C on hydroxylapatite columns that contained at least a 1-ml packed volume of hydroxylapatite per 100 μg of total DNA in the sample. Single-stranded DNA was eluted with 5 column volumes of 0.14 M sodium phosphate, pH 6.8, and double-stranded DNA was eluted with 5 column volumes of 0.4 M sodium phosphate, pH 6.8. The eluted fractions were precipitated at 4°C with 5% trichloroacetic acid after addition of 80 μg of calf thymus carrier DNA. Precipitates were trapped on glass fiber filters, dried, and then eluted to minimize quenching of radioactivity by incubation in 1 ml of Protosol (New England Nuclear Corp.) in screw-capped glass counting vials for at least 8 h at 60°C. A 10-ml amount of Omnifluor solution (New England Nuclear Corp.) was added to each sample, and the mixtures were allowed to remain at room temperature for 24 h before counting in a Beckman scintillation counter.

All hybridization mixtures involving normal cell DNA and radioactive viral DNA synthesized in vitro (cDNA) included 600 μg to 1 mg of cell DNA and 700 to 1,000 cpm (0.03 to 0.05 ng) of labeled cDNA per experimental point. Cell DNA concentrations were adjusted to 4 to 5 mg/ml.

Thermal denaturation studies. Method 1. Hydroxylapatite columns. After a period of incubation adequate to permit maximal levels of annealing, hybridization mixtures were diluted into 0.12 M sodium phosphate, pH 6.8, and applied at 55°C to hydroxylapatite columns containing at least a 1-ml packed volume of hydroxylapatite per 100 μg of total nucleic acid in the tested sample. The columns were washed extensively at 60°C, and the temperature was raised stepwise at 4°C intervals, monitored by thermometers placed inside control columns in the same water bath. At each interval, the columns were washed with 5 column volumes of prewarmed 0.12 M sodium phosphate. Radioactivity was measured by using the protocol described above. T_m was defined as the temperature at which 50% of the duplex structures containing a radioactive cDNA had denatured, as measured by elution of radioactive cDNA from the column. An internal standard consisting of duplexes formed between either [^{32}P]$cDNA_{B77}$ and XC cell DNA or [^{32}P]$cDNA_{B77}$ and the RNA of the Prague C strain (Pr-C) of ASV was added to hybridization mixtures before loading on the columns.

Method 2. S1 nuclease assay. Hybridization mixtures, prepared as described for method 1, were diluted to a final concentration of 0.18 M NaCl in 0.02 M Tris-hydrochloride, pH 7.4–0.01 M EDTA, containing 20 μg of calf thymus carrier DNA per ml, in a volume of approximately 4 ml. Portions (300 μl) were distributed to glass tubes and stored briefly on ice until use. Thermal denaturation curves were obtained by adjusting the temperature of a circulating water bath to an appropriate temperature, incubating a tube for 5 min, and then rapidly chilling the mixture in an ice water bath. The starting temperature was 55 or 60°C, and the temperature was raised at 4°C intervals up to a temperature of greater than 90°C, at which complete denaturation was observed. The amount of label re-

maining in duplex structures at each temperature was measured by resistance to hydrolysis by S1 nuclease, and the extent of denaturation was calculated by comparison with control values of S1 resistance of samples heated at 55°C for 5 min before S1 nuclease treatment. T_m was defined as the temperature at which 50% of the radioactivity originally in duplexes remained S1 resistant.

Preparation of virus-specific single-stranded DNA. Procedures for synthesizing radioactive cDNA and for purification of $cDNA_{gp}$ specific for *env* gene sequences of avian leukosis-sarcoma viruses have been described previously (29). In brief, radioactive cDNA was synthesized with purified ASV, 0.08% Nonidet P-40 (Shell Chemical Co.), actinomycin D (100 to 200 μg/ml; Calbiochem), [^{3}H]TTP (55 Ci/mmol) at 5.5×10^{-5} M, and the other deoxynucleoside triphosphates at 10^{-4} M. Single- and double-stranded products were separated by hydroxylapatite fractionation, as previously described (27).

$cDNA_{gp}$ was prepared from DNA synthesized using purified virions of either the subgroup A Schmidt-Ruppin (SR-A) strain of ASV ($cDNA_{gpA}$) or Pr-C ASV ($cDNA_{gpC}$). Selection of $cDNA_{gp}$ involved hybridization of single-stranded cDNA to a limited excess (less than twofold) of 70S ASV RNA, recovery of the hybridized cDNA by hydroxylapatite chromatography and alkali digestion, and rehybridization of the cDNA to at least an eightfold mass excess of 60 to 70S RNA prepared from SR-A NY8, a variant having an *env* gene deletion of ca. 2,000 nucleotides (16, 29). The single-stranded fraction ($cDNA_{gp}$) was recovered by hydroxylapatite chromatography.

[^{32}P]$cDNA_{B77}$ was synthesized using detergent-disrupted virions of the subgroup C B77 strain of ASV as previously described (5). This DNA contained sequences complementary to most or all of the ASV genome (8), although a large portion represented transcripts complementary to the 5′ terminus of the ASV genome (5).

Preparation of ^{14}C-labeled unique-sequence chicken DNA. Chicken embryo fibroblasts were seeded onto three 100-mm petri dishes at a density of 5×10^6 cells per dish and incubated at 38°C in medium 199 containing 5% calf serum (Pacific Biologicals). After 3 h, the medium was replaced with 5 ml of fresh medium containing 2 μCi of [*methyl*-^{14}C]thymidine per ml. After an additional 54 h, each monolayer was washed with Tris-glucose and removed from plates by the addition of Pucks-EDTA saline solution for 10 min at 37°C. The pooled cells were washed by centrifugation at 1,000 rpm for 5 min, followed by resuspension in 4 ml of TE buffer. The suspension was incubated for 30 min at 37°C in the presence of 0.5% SDS–500 μg of Pronase per ml and then extracted with phenol-chloroform (2:1, vol/vol). Nucleic acids were ethanol precipitated, resuspended in 3 ml of TE buffer, and treated for 20 min at 100°C in 0.3 M KOH.

The DNA was neutralized with HCl, ethanol precipitated, resuspended in standard hybridization buffer (0.6 M NaCl, 0.02 M Tris-hydrochloride, pH 7.4, and 0.01 M EDTA), and incubated at 68°C to a C_0t of 200. At this C_0t, virtually all of the DNA remaining single stranded (67% of the labeled material) represented unique-sequence DNA. The single-stranded DNA was separated from double-stranded DNA by hydroxylapatite (Bio-Rad Laboratories) column fractionation at 60°C (4) and precipitated with cetyltrimethylammonium bromide (22) in the presence of 120 μg of yeast RNA carrier per ml. The single-stranded unique-sequence DNA had a specific activity of 3.9×10^4 cpm/μg.

RESULTS

Hybridization of $cDNA_{gp}$ to normal avian DNAs. Previous reports from this laboratory have indicated that nucleotide sequences related to the *src* gene of ASV were detectable in the DNAs of all avian species examined (28) by molecular hybridization studies using a labeled cDNA specific for *src* (27). This finding implied that the *src* gene of avian tumor viruses evolved from a cellular sequence that has been highly conserved in all avian species. The preparation of $cDNA_{gp}$, a cDNA specific for the *env* gene of avian tumor viruses (29), has now allowed us to examine whether the *env* gene evolved in a similar fashion or, alternately, whether *env* gene nucleotide sequences were confined to a more limited number of avian species.

We examined this question by using $cDNA_{gp}$ in annealing experiments with DNAs from a variety of normal avian species. Results obtained with stringent annealing conditions of 0.6 N NaCl and 68°C are shown in Table 1, column A. These results were obtained at C_0t values greater than 10^4, which are at least 10 times higher than the $C_0t_{1/2}$ value required for the renaturation of single-copy DNA. There is appreciable hybridization of labeled $cDNA_{gp}$ to chicken (*Gallus gallus*) DNA (39%), but little or no hybridization to DNAs from Japanese quail (*Coturnix coturnix japonica*), golden pheasant (*Chrysolophus pictus*), Pekin duck (*Anas platyrhynchos*), squab (*Columba livia*), or calf thymus. Therefore, sequences closely related to *env* are readily detectable in chicken DNA, but not in the DNAs of the other species tested.

We then asked whether the avian species contained nucleotide sequences that were related to sequences in $cDNA_{gp}$, but formed extensively mismatched hybrids that were unstable under our annealing conditions. We lowered the stringency of the hybridization conditions to permit an increased level of hybridization between nucleotide sequences that were mismatched (23). In these experiments, DNA from ring-necked pheasants (*Phasianus colchicus torquatus*) was also included. Typical results, obtained using hybridization conditions of 1.5 M NaCl, 59°C, and analysis on hydroxyapatite at 50°C, are shown in Table 1, column B. A slight increase was observed in the level of $cDNA_{gp}$ hybridization to chicken DNA under these conditions, but

TABLE 1. *Hybridization of $cDNA_{gp}$ to normal avian DNAs*

Cell DNA	Stringent hybridization conditions[a] (extent of hybridization of $cDNA_{gp}$ [%]) (A)	Relaxed hybridization conditions[a]		Phylogenetic distance from chicken (years × 10^{-6})[b]
		Extent of hybridization of $cDNA_{gp}$ (%) (B)	Extent of hybridization of chicken unique-sequence DNA (%) (C)	
XC (Pr-C provirus)	48			
Chicken	39	45–56	90	0
Golden pheasant	1	3	80	20–40
Ring-necked pheasant		3	72	20–40
Japanese quail	6			20–40
Squab	7	5	43	40–80
Duck	5	4	58	80
Salmon		<4	7	>300
Calf thymus	1	<4		>300

[a] Hybridization was performed as explained in the text to values of C_0t greater than 10,000, except for XC DNA, which was hybridized to a C_0t of 1,400. "Stringent" conditions involved hybridization at 68°C in 0.6 M NaCl, assayed with S1 nuclease at 50°C. "Relaxed" conditions involved hybridization at 59°C in 1.5 M NaCl, assayed on hydroxylapatite at 50°C.

[b] From references 21 and 25.

no increase in the level of hybridization was observed with other avian DNAs. These conditions would permit the detection of hybrids that were at least 15% mismatched, as demonstrated in previous studies (23, 28; D. Spector, personal communication). A similar pattern of results are also shown in Fig. 1, which includes experimental points obtained at several different C_0t values, in addition to those values included in Table 1.

^{14}C-labeled unique-sequence chicken DNA was also annealed to these DNAs to obtain an indication of the total nucleotide sequence homology existing between the single-copy genes

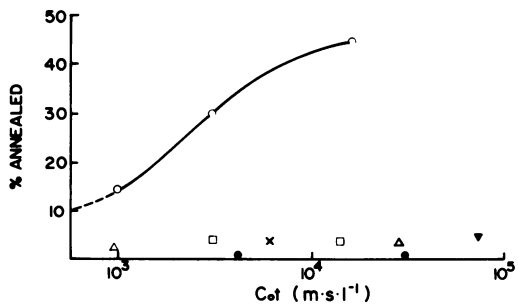

FIG. 1. *Annealing of [^3H]$cDNA_{gpC}$ to normal avian DNA. [^3H]$cDNA_{gpC}$ was annealed to various cellular DNAs at 59°C in 1.5 M NaCl–0.02 M Tris-hydrochloride, pH 7.4, as described in the text. Annealing was assayed by chromatography on hydroxylapatite at 50°C, as described in the text. Some of the values appearing in this figure have been incorporated in Table 1, column C. Symbols: ○, chicken DNA; □, ring-necked pheasant DNA; △, golden pheasant DNA; ×, duck DNA; ▼, squab DNA; ●, salmon sperm DNA.*

of chickens and other avian species. These results (Table 1, column C) reveal extensive homology between total single-copy nucleotide sequences in chicken DNA and DNAs of the other avian species tested. In general, the extent of annealing of chicken [^{14}C]DNA to other avian DNAs was consistent with other estimates of the evolutionary relatedness between chickens and the other species tested (Table 1 columns C and D).

We conclude from these results that only chicken DNA contained nucleotide sequences closely related to *env* sequences of subgroup A to E sarcoma-leukosis viruses. *env* gene nucleotide sequences in other avian DNAs, if present at all, were only distantly related to those in $cDNA_{gp}$ and did not hybridize appreciably to $cDNA_{gp}$ under the two different hybridization conditions that we used. The results are consistent with the observed lack of homology between $cDNA_{gp}$ and *env* gene sequences in the RNAs of ring-necked pheasant virus (RPV) and GPV (Table 2), viruses which presumably possess *env* genes derived from ring-necked and golden pheasant cells (6, 12, 13; Fujita et al., manuscript in preparation). On occasion, we observed slightly higher levels of hybridization of $cDNA_{gp}$ to DNAs of avian species other than chickens, but in these instances the background levels of $cDNA_{gp}$ eluting in the high-salt (0.4 M PO_4) wash in the presence of salmon or calf DNA were also correspondingly higher.

***env* genes in chf$^+$ and chf$^-$ chicken DNAs.** All chicken cells are thought to harbor genes of the endogenous virus RAV-0 (11, 33, 34) and nucleotide sequences related to the *src* gene of

TABLE 2. *ENV nucleotide sequences in avian leukosis-sarcoma virus RNAs*

Subgroup	Virus	Description	Hybridization to $cDNA_{gp}$ (%)[a]
A	SR-A	Exogenous chicken virus	100
B	RAV-2	Exogenous chicken virus	80
C	B77	Exogenous chicken virus	100
	Pr-C	Exogenous chicken virus	100
D	SR-D	Exogenous chicken virus	88
E	RAV-0	Exogenous chicken virus	70–75
F	RPV[b]	Endogenous ring-necked pheasant virus[b]	3–10
PV	GPV[c]	Endogenous golden pheasant virus	3–10

[a] Data were compiled from our results and those previously cited in Table 4 of reference 29. At least 40 ng of viral 70S RNA was hybridized with 500 to 800 cpm (0.02 to 0.04 ng) of $[^3H]cDNA_{gpA}$ at 68°C to C,t values of ≥2. The extent of hybridization was measured by resistance to S1 nuclease (18).

[b] RPV may be a recombinant virus, possessing some genetic material from leukosis viruses of both chicken and ring-necked pheasant cells.

[c] GPV, originally classified as a subgroup G avian leukosis virus (6), has recently been assigned to a new class of viruses known as pheasant viruses, or PV (3, 13).

ASV (28). The *env* gene product is readily detectable in certain types of chicken cells, designated as chf^+, but not in other types of cells, designated as chf^- (14; B. Baker, personal communication). Presently available evidence suggests that the synthesis of the endogenous *env* gene product is regulated either by transcriptional control or post-transcriptional processing. However, the alternate possibility remained of an *env* gene deletion in the DNA of chf^- cells. We tested this possibility directly by hybridizing $cDNA_{gp}$ to DNAs obtained from chf^+ and chf^- chicken embryos. These results are shown in Fig. 2. ^{14}C-labeled unique-sequence chicken DNA was included in these tests to define annealing kinetics typical of single-copy genes. It is apparent from the curves depicting hybridization of $cDNA_{gp}$ to chf^+ and chf^- chicken DNAs (Fig. 2A and B) that *env* gene nucleotide sequences are present in both chf^+ and chf^- DNAs. Initial data points were also redrawn in the form of second-order rate plots (36), in which the reciprocal of the fraction of labeled DNA remaining unhybridized was plotted against the time of reaction (Fig. 2C and D). The number of envelope gene copies per cell was computed from the ratio of the slopes of the lines depicting annealing of $[^3H]cDNA_{gp}$ and chicken unique-copy $[^{14}C]$DNA to a given type of chicken DNA. By this method, both chf^- DNA (Fig. 2C) and chf^+ DNA (Fig. 2D) were found to have 1.7 copies of the *env* gene per cell. This suggests that there are probably two copies of the *env* gene per cell (one per haploid genome) and provides evidence that an extensive deletion of the *env* gene is not the basis for the chf^- phenotype. Similar conclusions have been reported by Hayward and Hanafusa (14), using an *env* gene probe made from a subgroup B virus, RAV-2.

env genes in normal chicken DNA are closely related to those of exogenous chicken viruses. We tested the thermal stability of duplexes formed between $cDNA_{gp}$ and endogenous *env* gene nucleotide sequences in normal chicken DNA to determine the degree of homology between these sequences. The extent of mispairing of bases in a duplex can be estimated by the ΔT_m, or difference in thermal denaturation temperatures (T_m) observed between the duplex being tested and a standard homologous duplex. Each increment of 1°C in ΔT_m reflects 0.7 to 1.5% mispairing of bases (31). Such information may provide clues concerning the structure and evolutionary origins of viral *env* genes.

The results of thermal denaturation tests involving $cDNA_{gp}$ annealed to chf^- chicken DNA or to XC cell DNA are shown in Fig. 3A and B. XC DNA contains multiple integrated copies of ASV proviral sequences, of which at least some encode a subgroup C *env* product (19). Thus, XC DNA serves as a convenient source of subgroup C *env* gene sequences. Duplexes formed between $[^{32}P]cDNA_{B77}$ and XC DNA were included in each denaturation experiment as an internal standard. In these experiments, performed on hydroxylapatite column, the T_m for the duplexes formed between $cDNA_{gp}$ and chicken DNA was 77°C (Fig. 3A), as was the T_m for the duplex formed between $cDNA_{gp}$ and XC DNA (Fig. 3B). In both cases, the T_m for the internal standard duplexes formed between $[^{32}P]cDNA_{B77}$ and XC DNA exhibited a T_m of 78°C. Therefore, within the limits of sensitivity of our assay conditions (approximately 1 to 2°C), there was no mismatching in duplexes formed between $cDNA_{gp}$ and chicken DNA. We conclude from these results that at least a substantial portion of an *env* gene present in uninfected chicken DNA contains nucleotide sequences that are closely related to the exogenous avian virus *env* gene sequences present in XC DNA.

Some *env* gene nucleotide sequences in RNAs of exogenous and endogenous chicken virus appear to be highly conserved. We have demonstrated previously that extensive homology existed between $cDNA_{gpA}$ or $cDNA_{gpC}$ and envelope gene sequences in RNAs of avian sarcoma-leukosis viruses of subgroups A, B, C, D, and E (29). These and other results based on the extent of hybridization of $cDNA_{gp}$ to viral RNAs are summarized in Table 2. Complete (100%) hybridization was observed in tests

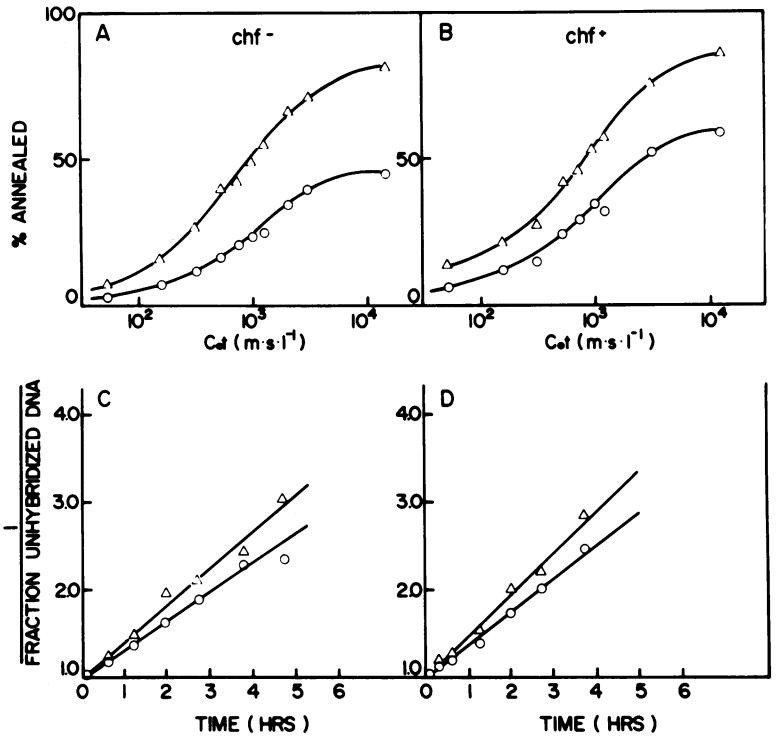

FIG. 2. *Annealing of [^{3}H]cDNA$_{gpA}$ to DNA from chf$^-$ and chf$^+$ chickens. Annealing of [^{3}H]cDNA$_{gpA}$ to chf$^-$ and chf$^+$ chicken DNA was performed at 68°C in 0.9 M NaCl-0.02 M Tris-hydrochloride, pH 7.4, as described in the text. ^{14}C-labeled chicken unique-sequence DNA was included in each reaction. Annealing was assayed by chromatography on hydroxylapatite at 50°C, as described in the text. Symbols: ○, [^{3}H]-cDNA$_{gpA}$; △, ^{14}C-labeled unique-sequence chicken DNA. (A) Annealing to chf$^-$ DNA. (B) Annealing to chf$^+$ DNA. (C) Second-order rate plot (36) of data from (A). Hybridization values were standardized such that maximal plateau values in (A) represented 100% hybridization; other values were corrected to this scale. (D) Second-order rate plot (36) of data from (B). Hybridization values were standardized such that maximal plateau values in (B) represented 100% hybridization; other values were corrected to this scale.*

involving cDNA$_{gpA}$ or cDNA$_{gpC}$ with RNA from either subgroup A or C viruses. These data suggested that the nucleotide sequences of *env* genes of subgroup A and C viruses shared extensive homology. Slightly less, but yet extensive, hybridization was observed with subgroup B, D, and E RNAs (80, 88, and 70 to 75%, respectively). Presumably, these lower levels of hybridization reflected subgroup-specific differences in *env* gene nucleotide sequences. In contrast, little or no hybridization was observed with RNAs of RPV and GPV, viruses of pheasant origin. This pattern of hybridization suggested the existence within the chicken viruses of a family of related envelope genes, distinct from those present in either RPV or GPV.

The high, but incomplete, level of hybridization between cDNA$_{gp}$ and RNA from the subgroup E virus RAV-0 was of particular interest because RAV-0 is the endogenous virus of chickens (33). Extensive homology between *env* gene nucleotide sequences present in RNAs of the exogenous subgroup A to D chicken viruses and those in the RNA of the endogenous subgroup E chicken virus RAV-0 would provide evidence that the exogenous and endogenous viral genes shared a common evolutionary history. For this reason, it was of interest to determine the extent of mispairing in hybrids formed between cDNA$_{gp}$ and RAV-0 RNA through the use of thermal denaturation studies.

A control experiment demonstrating that virtually no mismatching existed between the *env* gene sequences in cDNA$_{gp}$ and viral RNA of subgroups A or C is shown in Fig. 4. Figure 4A shows the results of thermal denaturation experiments involving the hybrids cDNA$_{gpA}$:SR-A RNA and cDNA$_{gpA}$:Pr-C RNA. These hybrids had identical T_m values of 72°C under our experimental conditions. Figure 4B shows the results of parallel experiments, involving the hybrids formed between cDNA$_{gpC}$ and either SR-A RNA or Pr-C RNA. These hybrids had T_m values of 72 and 73°C, respectively. All of the

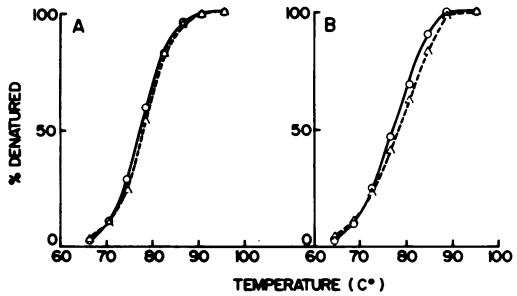

FIG. 3. *Thermal denaturation on hydroxylapatite of duplexes formed between [^{3}H]cDNA$_{gpC}$ and chf chicken or XC DNA. Appropriate DNAs were annealed to [^{3}H]cDNA$_{gpC}$ at 68°C in 0.6 M NaCl-0.02 M Tris-hydrochloride, pH 7.4, to C$_0$t values in excess of 10,000. T_m was determined by thermal elution of duplexes from hydroxylapatite columns, as described in the text. As an internal control, a duplex formed between [^{32}P]cDNA$_{B77}$ and XC DNA was included in each experiment. A total of 825 cpm of [^{3}H]cDNA$_{gpC}$ and 1,867 cpm of [^{32}P]cDNA$_{B77}$ were present in hybrids shown in (A). A total of 1,626 cpm of [^{3}H]-cDNA$_{gpC}$ and 1,968 cpm of [^{32}P]cDNA$_{B77}$ were present in hybrids shown in (B). (A) Thermal denaturation of hybrids formed between: [^{3}H]cDNA$_{gpC}$ and chicken DNA (○; T_m = 77°C); [^{32}P]cDNA$_{B77}$ and XC DNA (△; T_m = 78°C). (B) Thermal denaturation of hybrids formed between: [^{3}H]cDNA$_{gpC}$ and XC DNA (○; T_m = 77°C); [^{32}P]cDNA$_{B77}$ and XC DNA (△; T_m = 78°C).*

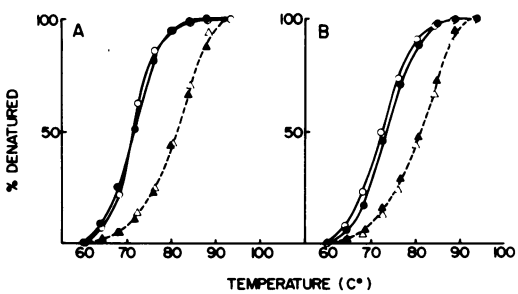

FIG. 4. *Thermal denaturation on hydroxylapatite of hybrids formed between cDNA$_{gpA}$ or cDNA$_{gpC}$ and SR-A or Pr-C RNA. Viral RNAs were hybridized to [^{3}H]cDNA$_{gp}$ at 68°C in 0.6 M NaCl-0.02 M Tris-hydrochloride, pH 7.4, to C$_r$t values of >2. T_m was determined by thermal elution of hybrids from hydroxylapatite columns, as described in the text. As an internal control, a duplex formed between [^{32}P]-cDNA$_{B77}$ and XC DNA was included in each thermal denaturation experiment. At least 986 cpm of [^{3}H]-cDNA$_{gp}$ and 998 cpm of [^{32}P]cDNA$_{B77}$ were present in hybrids shown in (A). At least 719 cpm of [^{3}H]-cDNA$_{gp}$ and 743 cpm of [^{32}P]cDNA$_{B77}$ were present in hybrids shown in (B). (A) Thermal denaturation of hybrids formed between: [^{3}H]cDNA$_{gpA}$ and SR-A RNA (○; T_m = 72°C); [^{3}H]cDNA$_{gpA}$ and Pr-C RNA (●; T_m = 72°C); [^{32}P]cDNA$_{B77}$ and XC DNA (△ and ▲; T_m = 81°C). (B) Thermal denaturation of hybrids formed between: [^{3}H]cDNA$_{gpC}$ and SR-A RNA (○; T_m = 72°C); [^{3}H]cDNA$_{gpC}$ and Pr-C RNA (●; T_m = 673°C); [^{32}P]cDNA$_{B77}$ and XC DNA (▲ and △; T_m = 81°C).*

thermal denaturation experiments shown in Fig. 4 were performed by the hydroxylapatite column method and included an internal standard consisting of a duplex formed between [^{32}P]-cDNA$_{B77}$ and XC DNA, which exhibited a T_m of 81°C. The close similarity in thermal stabilities of duplexes formed between cDNA$_{gpA}$ or cDNA$_{gpC}$ and subgroup A or subgroup C RNAs indicated that there was very little mismatching between *env* gene sequences present in cDNA$_{gpA}$ or cDNA$_{gpC}$ and those in viral RNAs of subgroup A or C. The largest difference observed in these instances was the 1°C difference in T_m (ΔT_m = 1°C) exhibited between duplexes formed with cDNA$_{gpC}$ and subgroup C RNA (T_m = 73°C) and duplexes formed with cDNA$_{gpC}$ and subgroup A RNA (T_m = 72°C). Therefore, the extent of mismatching detectable in our experiments between subgroup A and C enveloped gene sequences involved less than 1.5% of the nucleotide pairs in the hybrids tested. Because of the absence of detectable major differences between subgroup A and C *env* gene sequences in these experiments, cDNA$_{gpC}$ and cDNA$_{gpA}$ were frequently used interchangeably in subsequent studies.

Thermal denaturation curves obtained with hybrids formed between cDNA$_{gpA}$ and RAV-0 RNA (subgroup E) or Pr-C RNA (subgroup C) are shown in Fig. 5A and B. As an internal control, a [^{32}P]cDNA$_{B77}$:Pr-C RNA hybrid, exhibiting a T_m of 76°C, was included in both cases, and the thermal denaturations were performed by the hydroxylapatite method. The T_m observed for the duplex formed between cDNA$_{gpA}$ and RAV-0 RNA was 72.5°C, and the T_m for the duplex formed between cDNA$_{gpA}$ and Pr-C RNA was 73°C. Therefore, although the extent of hybridization of cDNA$_{gp}$ to RAV-0 (subgroup E) RNA was limited to about 75%, the sequences that were represented in these hybrids were well matched and exhibited a ΔT_m of less than 1°C, relative to the homologous hybrids formed between cDNA$_{gpA}$ and SR-A RNA or between cDNA$_{gpC}$ and Pr-C RNA. This result is consistent with the close homology observed between cDNA$_{gp}$ and chicken DNA, which contains the proviral DNA form of RAV-0. Similar results indicating a level of less than 1.5% mismatching were obtained using hybrids formed between subgroup D ASV RNA and cDNA$_{gpA}$ (data not shown).

Additional results which confirmed these conclusions were obtained in an independent series of thermal denaturation experiments, using the S1 nuclease method (Fig. 6). Thermal denatur-

ations of the hybrids formed between $cDNA_{gpC}$ and RAV-0 RNA and between $cDNA_{gpC}$ and Pr-C RNA are shown in Fig. 6A and B. In each case, the T_m (81°C) was indistinguishable from that of the homologous hybrid formed between $cDNA_{gpC}$ and Pr-C RNA. The absolute T_m values obtained with the S1 method in these particular experiments were higher than those obtained with the hydroxylapatite method, a pattern that has been consistently observed throughout these studies. We conclude from these data that hybrids formed between $cDNA_{gp}$ and RAV-0 RNA are not sufficiently mismatched to produce a detectable reduction in thermal stability.

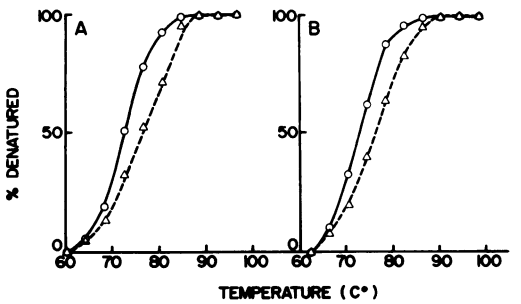

FIG. 5. *Thermal denaturation on hydroxylapatite of hybrids formed between [^{3}H]$cDNA_{gpA}$ and RAV-0 or Pr-C RNA. Conditions were as described in the legend to Fig. 4. A hybrid formed between [^{32}P]$cDNA_{B77}$ and Pr-C RNA was included in each thermal denaturation as an internal control. At least 687 cpm of [^{3}H]$cDNA_{gpA}$ and 616 cpm of [^{32}P]$cDNA_{B77}$ were present in hybrids. (A) Thermal denaturation of hybrids formed between: [^{3}H]$cDNA_{gpA}$ and RAV-0 RNA (○; T_m = 72.5°C); [^{32}P]$cDNA_{B77}$ and Pr-C RNA (△; T_m = 76°C). (B) Thermal denaturation of hybrids formed between: [^{3}H]$cDNA_{gpA}$ and Pr-C RNA (○; T_m = 73°C); [^{32}P]$cDNA_{B77}$ and Pr-C RNA (△; T_m = 76°C).*

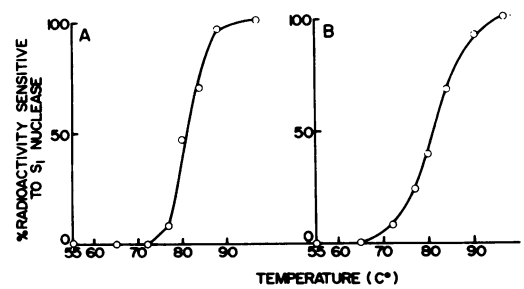

FIG. 6. *Thermal denaturation, using S1 nuclease assay, of hybrids formed between [^{3}H]$cDNA_{gpC}$ and RAV-0 or Pr-C RNA. Experimental conditions were as described in the text. At least 1,050 cpm of $cDNA_{gpC}$ was present in hybrids. ^{32}P-labeled control duplexes were not included in these experiments, which were performed simultaneously under identical conditions in the same water bath. (A) Thermal denaturation of hybrid formed between [^{3}H]$cDNA_{gpC}$ and RAV-0 RNA (○; T_m = 81°C). (B) Thermal denaturation of hybrid formed between [^{3}H]$cDNA_{gpC}$ and Pr-C RNA (○; T_m = 81°C).*

The existence of extensive regions of well-matched nucleotide sequences in the *env* genes of the viral subgroups tested here (A, C, D, E) suggests that a substantial portion of the *env* gene is conserved among these subgroups and presumably codes for a region of the *env* glycoproteins that is common to viruses of these subgroups.

DISCUSSION

Distribution of *env* gene nucleotide sequences in normal avian DNAs. $cDNA_{gp}$ hybridized substantially to normal uninfected chicken DNA, but not to DNAs from ring-necked pheasant, golden pheasant, quail, squab, and duck. This pattern of hybridization was observed even under conditions that allowed formation of duplexes with moderate mismatching of base pairs (23). These results were consistent with the high levels of $cDNA_{gp}$ hybridization observed with RNAs of viruses derived from chickens (subgroups A to E) and the absence of hybridization observed with RNAs of viruses derived from pheasants (RPV and GPV). The simplest interpretation of these data is that the *env* genes of subgroup A to E viruses (env_{A-E}) have a common origin. It is possible that these viral genes evolved from a normal cellular gene of chickens, as has been proposed for retrovirus genes in general (30), and the *src* gene of ASV in particular (28, 32). Alternatively, a viral *env* gene may possibly have been introduced into the germ line of chickens or a closely related species through exogenous infection. Evidence for horizontal infection of animal gametes by retroviruses has been obtained for several mammalian species (1).

The pattern of hybridization of $cDNA_{gp}$ to cellular DNAs contrasted sharply with the pattern exhibited by $cDNA_{sarc}$ (complementary to part of all of *src*), which hybridized substantially to all avian DNAs tested (28). This suggested that if the env_{A-E} alleles evolved from a normal cellular gene, the cellular progenitor of *env* has evolved much more rapidly than either the progenitor of *src* or the average single-copy DNA of chickens (Table 1, column C). Moreover, the cellular progenitor of *src* and the endogenous RAV-0 provirus, presumably containing the *envE* gene, are physically unlinked in the chicken genome (20), and their expression is independently regulated (26a).

It seems likely that different groups or "families" of envelope genes exist among, and perhaps

even within, avian species. The *env* genes of the chicken viruses of subgroups A to E appear to belong to one such group; the *env* genes of the pheasant viruses RPV and GPV share little nucleic acid homology with this (A–E) group and probably belong to one or more other groups. We have not tested whether the *env* genes of RPV and GPV are related to each other, but recent studies by Keshet and Temin suggest that they are unrelated (17).

Our results are based on hybridization tests with cDNA's specific for the subgroup A and C *env* genes. Therefore, we cannot exclude the possibility that slightly different patterns of hybridization to various avian DNAs might be observed if the *env* probe were prepared with a virus of subgroup B, D, or E. However, the extensive cross-homology that we and others (14) have observed between the *env* genes of subgroup A to E viruses suggests that such differences would probably be small. Ideally, in studies of the evolution of *env* sequences in normal avian DNAs, the cDNA should be prepared with the subgroup E endogenous chicken virus RAV-0. However, because of technical limitations and the absence of appropriate deletion mutants, the purification of a subgroup E *env* gene probe is not yet possible.

Conserved nucleotide sequences in viral *env* genes. $cDNA_{gpA}$ and $cDNA_{gpC}$ hybridized to high levels (75 to 100%) with the RNAs of viruses of the avian-sarcoma virus subgroups A to E. Thermal denaturation experiments indicated that the hybrids formed between $cDNA_{gp}$ and RNAs from viruses representing subgroups A, C, D, and E exhibited little, if any, mispairing (less than 1.5%), even in the case of subgroup E RNA which hybridized only to about 75% of the labeled sequences in $cDNA_{gp}$. These results suggested that at least 75% of the labeled sequences in $cDNA_{gp}$ represented nucleotide sequences that have been highly conserved during the evolution of the *env* genes of the chicken leukosis-sarcoma viruses. Although we have not evaluated the stability of hybrids formed between $cDNA_{gp}$ and subgroup B RNA, the high level of hybridization (80%) of $cDNA_{gp}$ to subgroup B RNA is consistent with the presence of highly conserved *env* gene sequences in subgroup B RNA also. Similarly high levels of hybridization have been observed by Hayward and Hanafusa (14) between a cDNA specific for the subgroup B *env* gene and RNAs from subgroup A, B, C, and E viruses.

It is possible that the highly conserved nucleotide sequences that hybridize to $cDNA_{gp}$ code for structural regions common to all glycoproteins of viral subgroups A to E. The existence of such common regions is indicated by the ability of antiserum prepared against gp85 of a specific viral subgroup to recognize and precipitate gp85 molecules of different subgroup specificities (10, 24). It is possible that certain other *env* gene nucleotide sequences code for "variable" regions which are responsible for the antigenic and subgroup specificities exhibited by viral *env* proteins. The existence of such highly variable regions would provide an explanation for the observed incomplete levels of hybridization between $cDNA_{gpA}$ and $cDNA_{gpC}$ and RNAs of viral subgroups B, D, and E. In these respects, our data conform to previous suggestions that *env* glycoproteins may contain two or more different domains (7).

Alternatively, it may be possible that only a very small change in nucleotide sequence is necessary to confer a difference in subgroup specificity. This possibility cannot be easily discounted, especially if the nature or structure of the added carbohydrate moieties is of importance in determining these specificities. Very little evidence is currently available regarding the role of carbohydrate moieties of avian tumor virus envelope glycoproteins in determining host range or antigenic specificities. However, recent studies with Friend murine leukemia virus have concluded that the carbohydrate moieties of the gp71 have little, if any, role in determining viral antigenic or host range specificities (26).

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