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Recombinants Between Endogenous and Exogenous Avian Tumor Viruses: Role of the C Region and Other Portions of the Genome in the Control of Replication and Transformation

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Endogenous retroviruses of chickens are closely related to exogenous viruses isolated from spontaneous tumors in the same species, yet differ in a number of important characteristics, including the ability to transform cells in culture, ability to cause sarcomas or leukemias, host range, and growth rate in cell culture. To correlate these differences with specific sequence differences between the two viral genomes, the genome RNA of transforming subgroup E recombinants between the Prague strain of Rous sarcoma virus, subgroup B (Pr-RSV-B), and the endogenous Rous-associated virus-0 (RAV-0), subgroup E, and seven nontransforming subgroup E recombinants between the transformation-defective mutant of Pr-RSV-B and RAV-0 was examined by oligonucleotide fingerprinting. The pattern of inheritance among the recombinant viruses of regions of the genome in which Pr-RSV-B and RAV-0 differ allowed us to draw the following conclusions. (i) Nonselected parts of the genome were, with a few exceptions, inherited by the recombinant virus progeny randomly from either parent, with no obvious linkage between neighboring sequences. (ii) A small region in the Pr-RSV-B genome which maps in the 5′ region was found in all transforming but only some of the nontransforming recombinants, suggesting that it plays a role in the control of the expression of transformation. (iii) A region of the Pr-RSV-B genome which maps between *env* and *src* was similarly linked to the *src* gene and may be either part of the structural gene for *src* or a control sequence regulating the expression of *src*. (iv) The C region at the extreme 3′ end of the virus genome which is closely related in all the exogenous avian retroviruses but distinctly different in the endogenous viruses is the major determinant responsible for the differences in growth rate between RAV-0 and Pr-RSV-B. This latter observation allowed us to redefine the C region as a genetic locus, *c*, with two alleles c^n (in RAV-0) and c^x (in exogenous viruses).

On the basis of their origin, the avian retroviruses can be divided into two distinct categories: the endogenous viruses which are spontaneously expressed by or can be induced from normal cells of certain chickens (26) and the exogenous viruses which were originally isolated from spontaneously arising chicken neoplasms (1, 2, 12, 17, 28, 29). Close genetic similarity has been observed between the endogenous and the exogenous avian retroviruses (10, 23). In spite of this similarity, there are distinct biological differences between them. All known exogenous viruses are pathogenic for chickens, causing neoplastic or other diseases (3, 25, 28). For example, all strains of avian sarcoma virus both transform chicken embryo cells in culture and induce sarcomas in the appropriate hosts (25), whereas transformation-defective (*td*) mutants of these viruses do not induce transformation or sarcomas but cause leukemias (3, 25). In contrast, endogenous viruses do not transform cells, nor do they induce any known disease (25; L. B. Crittenden, personal communication). Furthermore, they replicate to relatively low titers in cell cultures compared with exogenous viruses (20, 27), and all are classified by host range into subgroup E—a subgroup not found in exogenous viruses (26).

The nucleotide sequence relationship between the two genomes has been studied in our laboratory (10) and elsewhere (23). Comparison of the genomes of Rous-associated virus-0 (RAV-0), a spontaneously expressed replication-competent endogenous virus, with that of the Prague strain of Rous sarcoma virus subgroup B (Pr-RSV-B), a replication-competent transforming virus (Fig. 1E) confirms the close relationship between these viruses. Significant differences in sequence are found only near their 3′ ends. RAV-0 appears to be missing the *src* gene and an

undefined region between *env* and *src*, and the sequence nearest the 3′ end (the C region [37, 38]) is not detectably related to that of exogenous viruses.

The experiments presented here were performed to correlate such nucleotide sequence differences between exogenous and endogenous avian tumor viruses with biological differences between the two. For this purpose, we took advantage of the different host range of the two viruses to select for viruses generated by recombination between the two virus strains. Recombinant viruses were further selected on the basis of their transforming capacity or their ability to grow to high titers. Analysis of the genomes of the recombinant viruses allowed us to infer (i) that two portions of the avian sarcoma virus genome outside of *src* are linked to the ability of the virus to transform cells and (ii) that the major—and perhaps the only—determinant responsible for the difference in growth rate is the C region, which we redefine here as a genetic locus, *c*, with two alleles, c^n (in RAV-0) and c^x (in exogenous viruses).

MATERIALS AND METHODS

Cells and viruses. RAV-0-producing line 100 chicken embryo fibroblasts (VE^+ C/O) were kindly provided by L. B. Crittenden. Turkey embryo fibroblasts (T/BD) were prepared from fertilized turkey eggs that were purchased from Wilmar Poultry Farms, Minnesota. C/E chicken embryo fibroblasts were prepared from fertilized chicken eggs purchased from SPAFAS. The QT6 line of methylcholanthrene-transformed quail cells was a gift from P. Vogt (22). All cells were frozen in liquid nitrogen and thawed as needed.

To prepare the recombinant viruses analyzed in this paper, we started with the same strains of RAV-0, Pr-RSV-B, and *td*-Pr-RSV-B previously described (10). T_1 oligonucleotide fingerprints and maps of RAV-0 and Pr-RSV-B are shown in Fig. 1. The map of *td*-Pr-RSV-B is identical to that of the Pr-RSV-B except near the 3′ end where it lacks oligonucleotides 5, 6A, and 6B.

Preparation and cloning of recombinant viruses. To prepare recombinants between RAV-0 and Pr-RSV-B or *td*-Pr-RSV-B, line 100 cells were infected with one of the two viruses at a multiplicity of infection of about 0.5 to 1 infectious units per cell, a 24-h harvest was collected between 4 and 5 days after infection and used undiluted to infect T/BD cultures which were again harvested at 4 to 5 days after infection to provide the stock for cloning. Each virus clone isolated originated from a different initial infection to avoid analyzing the same recombinant twice. Cloning of the recombinant viruses was carried out on T/BD cells. The selection applied in the case of the transforming recombinants was for RAV-0 subgroup E envelope and transformation (env^E src^+) and, in the case of the nontransforming recombinants, for RAV-0 subgroup E envelope and rapid growth (env^E $growth^+$). The transforming recombinants were cloned by harvesting virus from individual foci of transformed turkey cells grown under a layer of agarose-containing medium. The biological purity of each clone was tested before fingerprinting by its ability to grow on turkey (T/BD) and not on chicken (C/E) embryo fibroblasts. Between two and four cycles of recloning were necessary to obtain biologically pure recombinants. The nontransforming recombinants were cloned by three cycles of endpoint dilution on turkey (T/BD) embryo fibroblasts.

T_1 oligonucleotide fingerprinting of the viral RNA. Large T_1 oligonucleotide fingerprints of viral 70S RNA were prepared by two-dimensional polyacrylamide gel electrophoresis of 70S RNA uniformly labeled with ^{32}P as described elsewhere (4).

T_1 oligonucleotide fingerprints of the extreme 3′ end of the viral genome were prepared as described elsewhere (10). Briefly, ^{32}P-labeled viral 70S RNA was partially hydrolyzed with alkali (0.025 M Na_2CO_3 at 50°C for 15 min) to yield fragments about 300 nucleotides long, and the polyadenylic acid-containing fragments were isolated by chromatography on polyuridylic acid-Sephadex. The fingerprints of the 3′-terminal fragments were run only two-thirds as far as those of total genomes in the second dimension to retain smaller oligonucleotides.

In general, most parental oligonucleotides could be unambiguously identified by their electrophoretic mobility. In some cases RNase T_1 oligonucleotides were eluted from the gels and digested with RNase A. This analysis was always performed with oligonucleotides 2 and 012, 5 and 308, 11 and 014, and 10 and 402. The digestion products were separated by high-voltage ionophoresis on DEAE paper (5). The digestion products obtained from oligonucleotides near the 3′ end of Pr-RSV-B and RAV-0 are shown in Table 1.

RESULTS

Isolation of transforming recombinants. To detect sequences in the Pr-RSV-B genome outside of *src* relevant to the biological differences between endogenous and exogenous avian oncoviruses, a series of recombinants between Pr-RSV-B and RAV-0 were prepared. RAV-0-producing cells were infected with Pr-RSV-B, the progeny were grown on T/BD cells, and env^E src^+ recombinants were isolated as described in Materials and Methods. All clones were tested for biological purity by their failure to infect C/E cells and recloned as necessary to obtain only subgroup E virus. Twenty-three recombinants were isolated in this manner, each from a different initial infection of line 100 cells by Pr-RSV-B. Fingerprints of RNase T_1 digests of the genome RNA from the parental viruses are shown in Fig. 1, and those from two of the recombinants are shown in Fig. 2A and B. It is apparent from comparison with the parental genomes that these were recombinant viruses. Both contained the *src* oligonucleotides 5, 6A, and 6B from Pr-RSV-B and the env^E set of oligonucleotides (308 through 02) from RAV-0

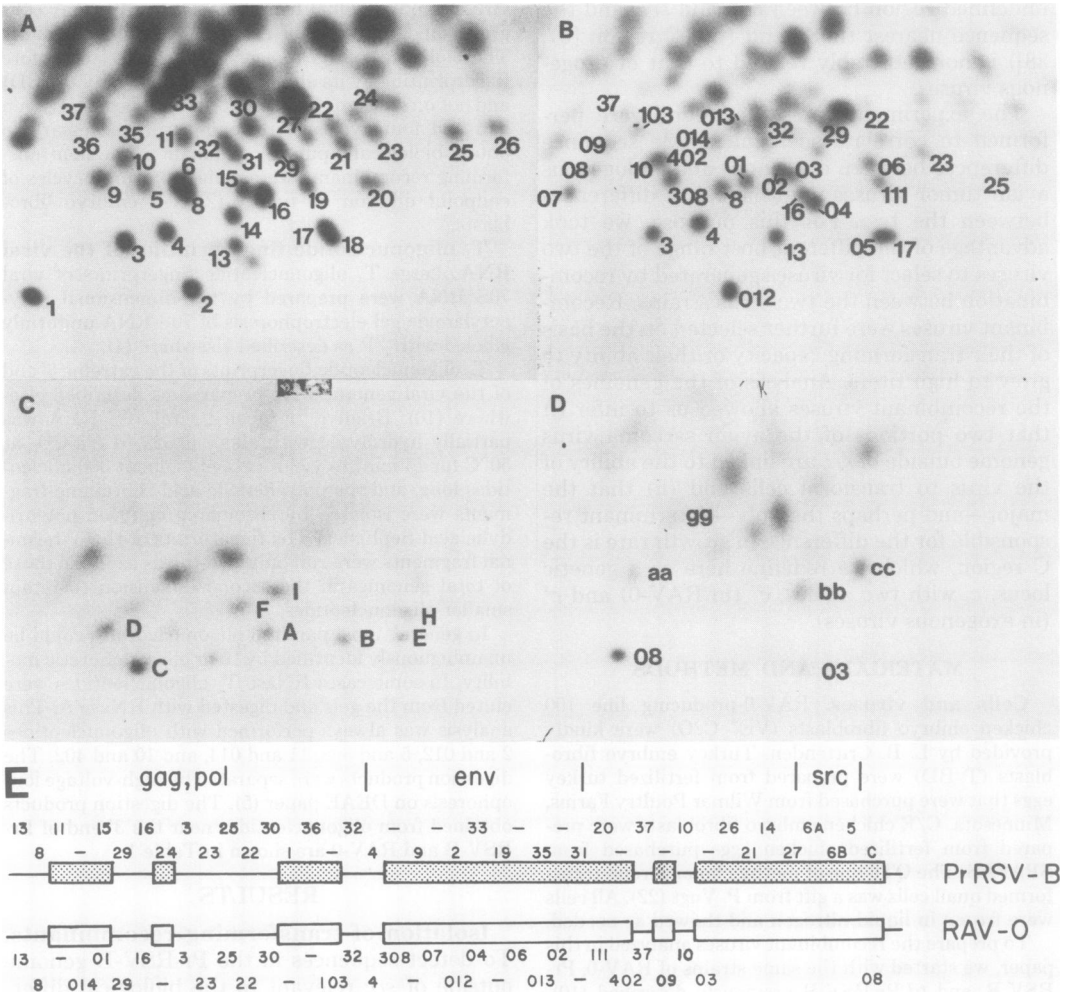

FIG. 1. *Fingerprints of genome RNA of Pr-RSV-B and RAV-0. ^{32}P-labeled 70S RNA from Pr-RSV-B (A) and RAV-0 (B) was digested with RNase T_1 and fingerprinted as described elsewhere (4). Fingerprints of the extreme 3′ end (C region) of the Pr-RSV-B (C) and RAV-0 (D) genomes are also shown. These fingerprints were run only two-thirds the usual distance in the second dimension to retain smaller oligonucleotides. The T_1 oligonucleotide maps of the two viruses are shown in E. Here and in the remaining figures Pr-RSV-B-specific regions are shown by shaded boxes, RAV-0-specific regions are shown by open boxes, and indistinguishable regions are shown by horizontal lines.*

and lacked the *env*^{B} set (9 through 35) from Pr-RSV-B. In addition, both recombinants contained the Pr-RSV-B 5′ oligonucleotide 15 and not the corresponding RAV-0 oligonucleotide 01. Oligonucleotides 26, 21, 14, and 27, which map between *env* and *src* in the Pr-RSV-B genome, were also inherited by both of these transforming recombinants. Both recombinant genomes were also lacking the RAV-0 oligonucleotide 08, which is very near the polyadenylic acid at the 3′ end. Since the Pr-RSV-B genome contains no large oligonucleotides in this region (10), fingerprints of 3′-terminal fragments of both genomes were prepared (Fig. 2C and D). In each case, the fingerprint obtained was identical to that of the same region of the Pr-RSV-B genome (Fig. 1C) and contained no unique oligonucleotides in common with RAV-0 (Fig. 1D). The Pr-RSV-B 3′-proximal sequence is closely related to that found in other exogenous viruses and has been designated the C region (37, 38). Oligonucleotide C is a marker for the exogenous virus C region. The corresponding region in RAV-0 (and other endogenous avian oncoviruses) is characterized by oligonucleotide 08. We shall differentiate between these two allelic regions by referring to the Pr-RSV-B type as c^x and the RAV-0 as c^n. Thus the genotype of these two recombinants

was *gag, pol, env^E, src$^+$, c^x*. This general pattern of inheritance was found for all recombinants examined.

TABLE 1. *Partial sequence analysis of RNase T_1-resistant oligonucleotides derived from the extreme 3' end of RAV-0 and Pr-RSV-B genomes*[a]

Oligonucleotide	Composition
RAV-0	
03	$3A_2U$, 2AU, AC, 5C, 6U, AG
08	A_3U, A_3C, AU, AC, 6C, G
aa	A_3U, AC, U, A_2G
bb	2AU, AC, 2C, U, G
cc	2AU, 2U, A_2G
gg	A_2C, 2AC, C, U, G
Pr-RSV-B	
A	A_2C, AU, C, 2U, G
B	AU, 2C, 3U, AG
C	A_3C, A_2U, AU, AC, G
D	A_2C, AC, C, U, G
E	AU, 3U, A_2G
F	A_2U, 2C, U, G
H	2AU, U, G
I	AU, U, C, AG

[a] RNase T_1-resistant oligonucleotides were eluted from the gel (Fig. 1C and D) and digested with RNase A. The digestion products were separated by high-voltage ionophoresis on DEAE paper.

The T_1 oligonucleotide maps of 23 transforming subgroup E recombinants between Pr-RSV-B and RAV-0 are shown in Fig. 3. Of the 23 recombinants isolated, 14 were judged to be pure since all oligonucleotides present were in equimolar yield (Fig. 3A). Nine of the recombinants were not pure clones since they contained some oligonucleotides in low yield relative to others. These nonpure viruses, designated MRE (Fig. 3B), were judged to be mixtures of recombinants, in which the parental viruses had been eliminated from the population. We base this conclusion on their ability to transform only T/BD and not C/E cells, and (with the possible exception of MRE-5, which had a trace amount of oligonucleotide 08) the complete absence of at least one RAV-0 oligonucleotide from the virus population. To test this point directly, MRE-1 was subcloned by focus assay on T/BD cells to select subgroup E transforming viruses and by soft agar cloning of infected QT6 cells to isolate subgroup E nontransforming viruses. Five transforming and two nontransforming clones were analyzed. One of each is shown in Fig. 4. All transforming and nontransforming viruses isolated were recombinants in various portions of the genome. In another experiment (data not shown) MRE-2 and MRE-4 were subcloned by

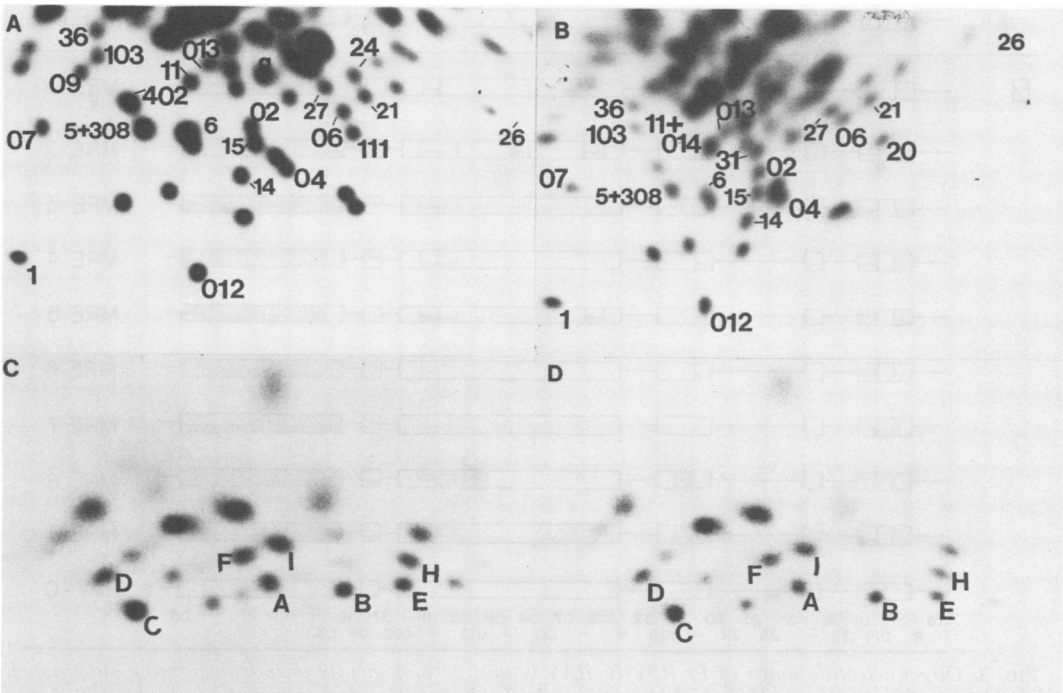

FIG. 2. *Genomes and the C region of two transforming subgroup E recombinant viruses. Fingerprints of 70S [^{32}P]RNA of TRE-7 (A) and TRE-4 (B) and the c regions of TRE-8 (C) and TRE-12 (D) were prepared as described in the legend to Fig. 1. All oligonucleotides not common to both parental genomes are numbered.*

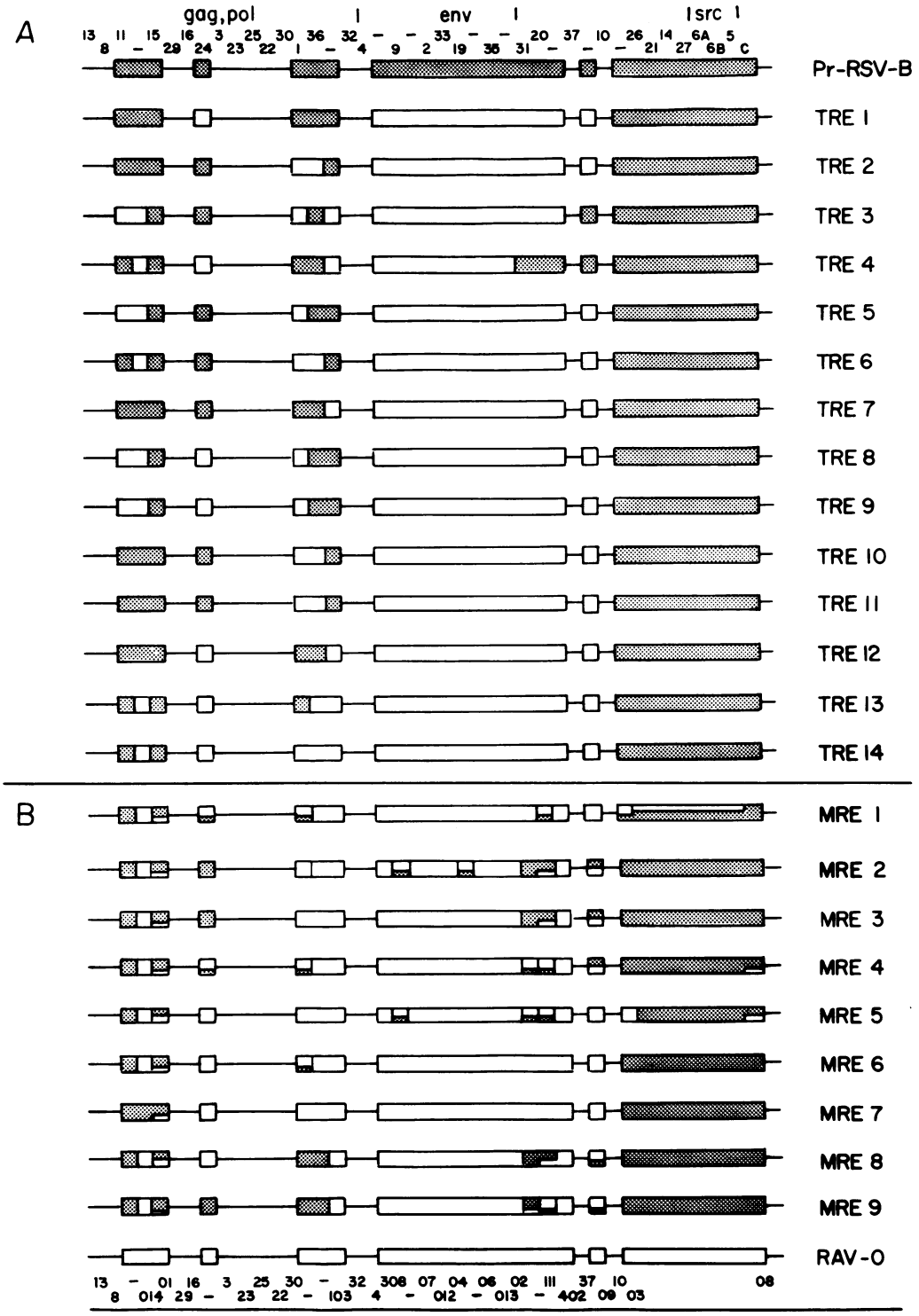

FIG. 3. *Oligonucleotide maps of Pr-RSV-B, RAV-0, and 23 biologically pure trnasforming subgroup E recombinants. Fourteen recombinants that were pure by T_1 oligonucleotide fingerprinting are shown in A and designated TRE, whereas nine biologically pure "clones" that by T_1 oligonucleotide fingerprinting were found to be mixtures of recombinants are shown in B and designated MRE. Areas of the genome where the two parental viruses are identical are depicted as lines. Shaded boxes indicate Pr-RSV-B-specific regions. Partially shaded boxes in the MRE series indicate regions where oligonucleotides specific for both parents were present.*

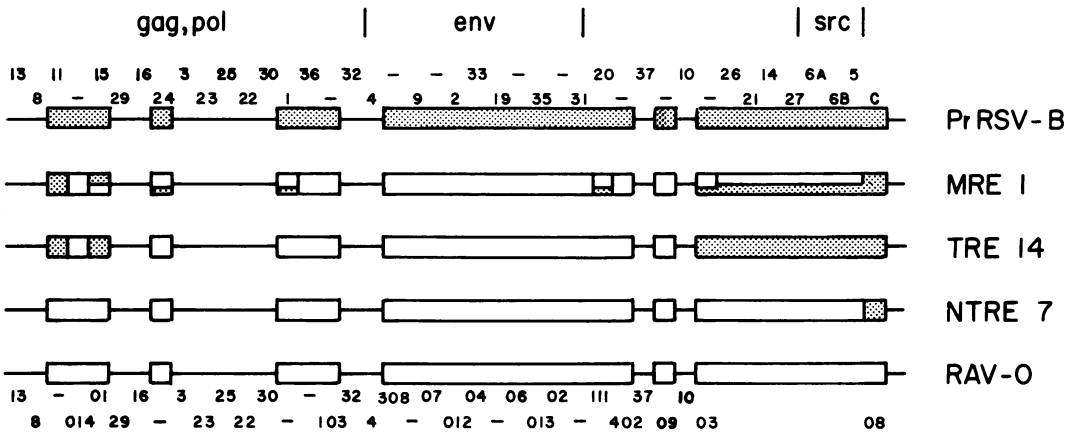

FIG. 4. *Oligonucleotide maps of a transforming and a nontransforming recombinant clone obtained by subcloning of recombinant mixture MRE-1. TRE-14 was obtained by focus formation on T/BD cells, and NTRE-7 was obtained by cloning infected QT6 cells in soft agar.*

infecting T/BD cells and isolating colonies of transformed cells in soft agar. Forty-six clones were isolated and analyzed. These clones had various combinations of the oligonucleotides that were present in the original mixtures. The MRE recombinants illustrate the difficulty of considering a recombinant virus to be a pure species only on the basis of its biological characteristics.

Patterns of inheritance of parental sequences. Figure 5 shows the frequency of appearance of Pr-RSV-B-specific oligonucleotides in all the 14 pure recombinants. With the exception of oligonucleotide 15, oligonucleotides that map within the 5′ half, including the *gag* and *pol* region of the Pr-RSV-B and RAV-0 genomes (oligonucleotides 13 to 32) appeared to be interchangeable in a random fashion. As can be seen from Fig. 3, all crossover sites were used with approximately equal frequency. We conclude that closely neighboring regions of the viral genome are not linked if no selection is applied. Therefore, we are confident that any apparent linkage must have been a consequence of selective effects.

All the recombinants were selected for subgroup E envelope. As expected, examination of their fingerprints (Fig. 3) revealed a block of information between *pol* and *src* that always came from the RAV-0 parent. These results allow us to locate the subgroup E coding region between oligonucleotides 308 and 02 and subgroup B between oligonucleotides 9 and 35, in good agreement with previous reports (18, 38).

An interesting exception to this general rule was seen with one of the nontransforming recombinants (NTRE-4) described in the next section. This virus infected C/E and T/BD cells with equal efficiency and had only a small portion of the *env* gene derived from the RAV-0 parent. This virus is representative of a number of extended host range viruses that we have isolated and will discuss in a separate report (P. N. Tsichlis, K. Conklin, and J. M. Coffin, Proc. Natl. Acad. Sci. U.S.A., in press.

The selection of the env^E and *src* genes in all recombinants was expected from previous results. In addition to these genes, however, we found three additional regions derived from the Pr-RSV-B genome in all transforming recombinants (Fig. 3A). The most surprising of these was a small region near the 5′ end of the genome marked by the presence of Pr-RSV-B oligonucleotide 15 and the absence of the allelic RAV-0 oligonucleotide 01. The relative electrophoretic mobility and RNase A digestion products of these two oligonucleotides are consistent with only a single C → U difference between these oligonucleotides (10). In addition to its presence in all pure transforming recombinants, all the mixed recombinants (Fig. 3B) contained a large majority of genomes with oligonucleotide 15 relative to those with oligonucleotide 01 (Fig. 3B). Also, recloning of MRE-1 by focus assay yielded only transforming recombinants with oligonucleotide 15 (see Fig. 4).

Examination of Fig. 3 also shows the presence in all pure transforming recombinants of sequences on either side of the *src* gene, including the block of oligonucleotides from 26 through 27, and the c^x region. Among the mixed recombinants, only two contained detectable genomes with c^n from RAV-0, and eight out of nine contained the sequence marked by oligonucleotides 26 through 27 in full yield, implying that 90% or more of the genomes contained this region.

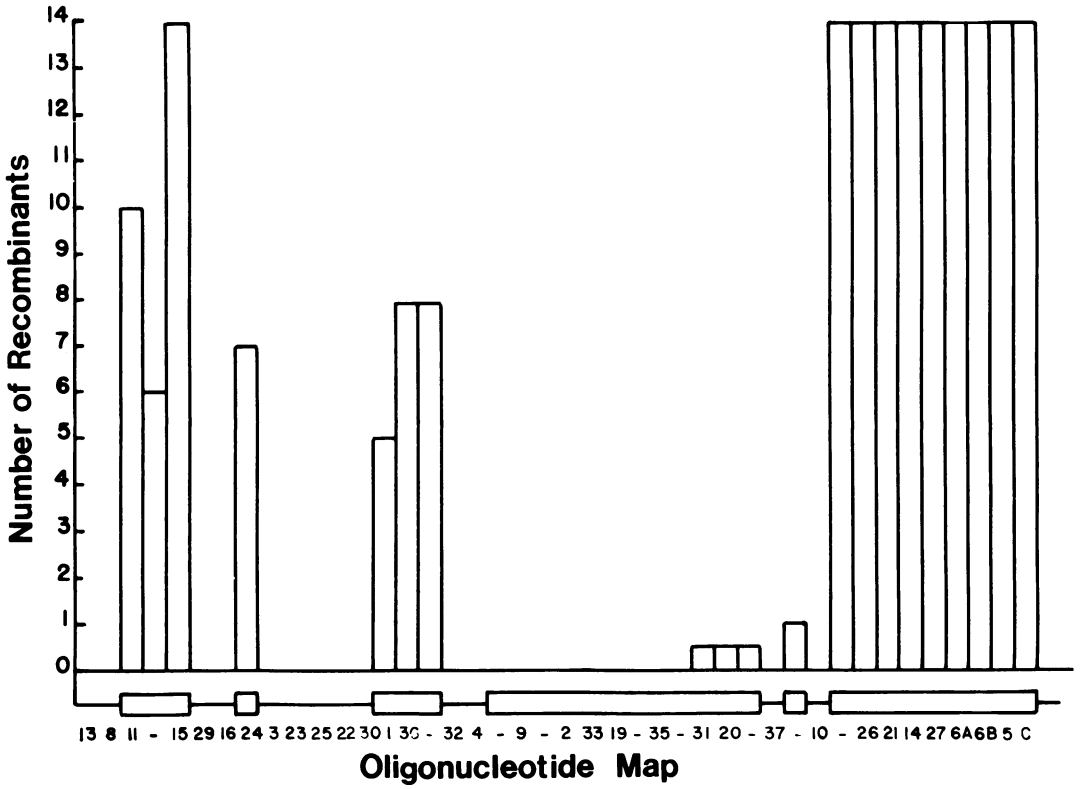

FIG. 5. *Frequency of appearance of parental oligonucleotides in the 14 pure clones of transforming recombinants. Only the Pr-RSV-B oligonucleotides are shown.*

Again, all transforming virus recloned from MRE-1 contained oligonucleotides 26 through 27.

The constant inheritance of these three regions of the Pr-RSV-B genome was in marked contrast to the random inheritance of unselected portions of the genome and strongly suggested some functional significance of these regions. Such a functional significance could have been related to the ability of the virus to grow, the ability of the virus to transform cells, or the inability of the two virus genomes to recombine within these regions due to the absence of homology. To decide among these alternatives, the nontransforming subgroup E recombinants between *td*-Pr-RSV-B and RAV-0 described in the next section were prepared and analyzed.

Isolation of nontransforming recombinants. Line 100 cells were infected with *td*-Pr-RSV-B, and recombinant progeny were isolated from T/BD cells infected with limiting dilutions of the mixed virus. Six recombinants were isolated, four of which were judged to be pure. The fingerprints of both the total genome RNA and the C region of two recombinants is shown in Fig. 6. One of them (NTRE-3) inherited the block of oligonucleotides 26 through 27, and the other (NTRE-2) was missing oligonucleotides 26, 21, and 14. Both acquired the c^x region from the *td*-Pr-RSV-B parent and only NTRE-3 inherited oligonucleotide 15. Examination of the T_1 oligonucleotide maps of all the nontransforming recombinants (Fig. 7) showed that one more virus (NTRE-4) was missing oligonucleotide 26 and that the inheritance of oligonucleotide 15 was random. All the nontransforming recombinants inherited the c^x region from the *td*-RSV-B parent. These findings allowed us to draw the following conclusions. (i) Recombination between Pr-RSV-B and RAV-0 can take place within the part of the genome specified by oligonucleotides 26 through 27. (ii) This segment of the Pr-RSV-B genome and the one near the 5′ end marked by oligonucleotide 15 are functionally linked to the *src* gene. Their role could be related either to the efficiency of transformation or to the growth of transforming viruses. It is also conceivable that at least a segment of the sequence specified by oligonucleotides 26 through 27 could be part of the structural gene

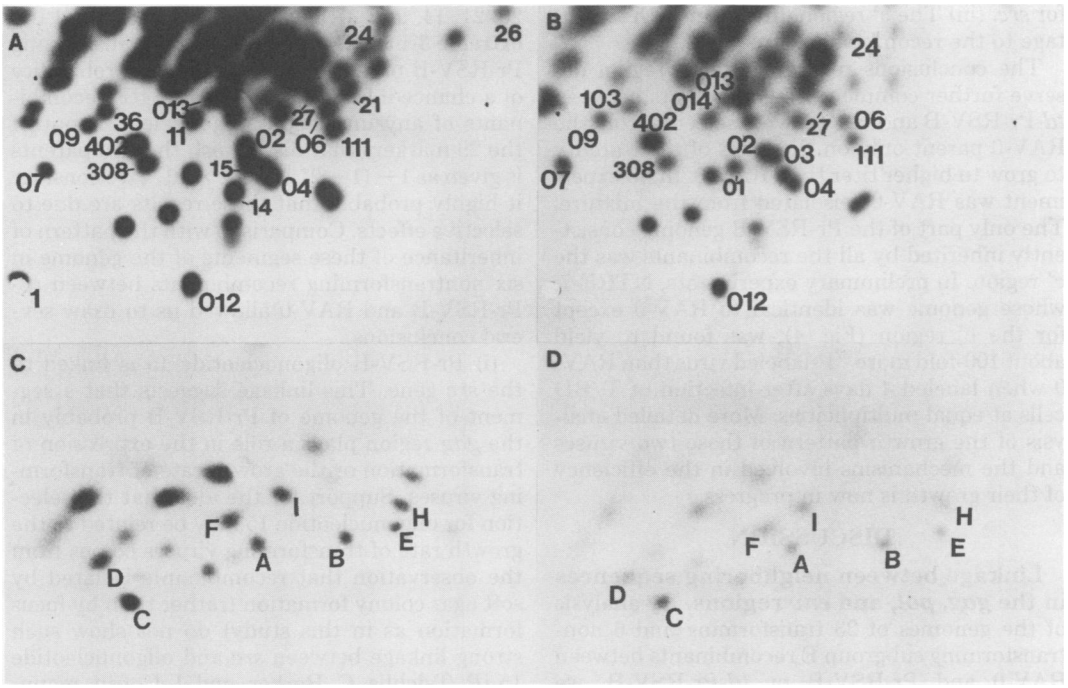

FIG. 6. *Fingerprints of the genomes of two (NTRE) nontransforming recombinant viruses. A and C show the total 70S RNA and C region of NTRE-3; B and D show NTRE-2. All conditions were described in the legend to Fig. 1.*

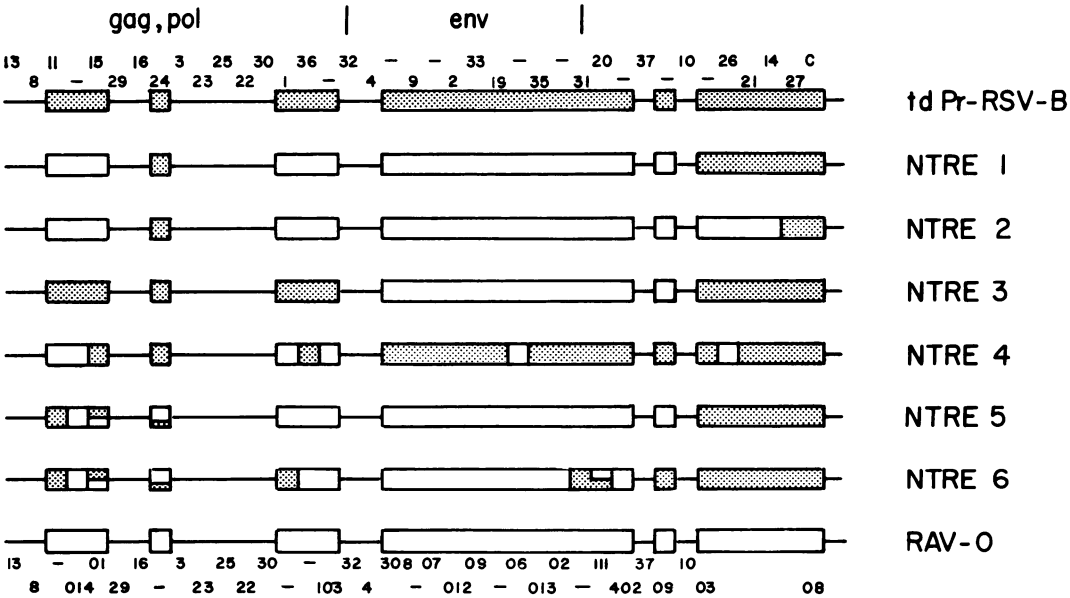

FIG. 7. *Oligonucleotide maps of nontransforming recombinants from crosses between td-PR-RSV-B and RAV-0. Four of these recombinants are pure by T_1 oligonucleotide fingerprinting and two are not.*

for *src*. (iii) The c^x region offers a growth advantage to the recombinant virus.

The conclusions regarding the C region deserve further comment. Recombinants between *td*-Pr-RSV-B and RAV-0 were selected from the RAV-0 parent only on the basis of their ability to grow to higher titer than RAV-0. In no experiment was RAV-0 reisolated from the mixture. The only part of the Pr-RSV-B genome consistently inherited by all the recombinants was the c^x region. In preliminary experiments, NTRE-7, whose genome was identical to RAV-0 except for the c^x region (Fig. 4), was found to yield about 100-fold more ^{32}P-labeled virus than RAV-0 when labeled 4 days after infection of T/BD cells at equal multiplicities. More detailed analysis of the growth pattern of these two viruses and the mechanisms involved in the efficiency of their growth is now in progress.

DISCUSSION

Linkage between neighboring sequences in the *gag*, *pol*, and *env* regions. By analysis of the genomes of 23 transforming and 6 nontransforming subgroup E recombinants between RAV-0 and Pr-RSV-B or *td*-Pr-RSV-B, we could find no linkage between neighboring oligonucleotides within the *gag* and *pol* genes. It is particularly striking that the inheritance of the region immediately to the left of the selected env^E gene was random from either parent (Fig. 3 and 5). Implications of the extraordinarily high frequency of recombination implied by these results have been discussed elsewhere (8). For the purpose of this discussion, these results give us confidence that any nonrandom inheritance patterns observed were due to the effects of selection and not to genetic linkage.

As previously reported (18, 38), a strong linkage was observed between neighboring oligonucleotides in the *env* region, such that all subgroup E recombinants had RAV-0 oligonucleotides 308 through 02 with no internal crossovers. Considering that there is substantial homology between the parental genomes in this region (10) and some of the mixed recombinants contain genomes with crossovers inside *env* (Fig. 3), we interpret this linkage as not being due to a lack of recombination within the *env* gene. Rather, it is likely that such recombination leads to a genome coding a nonfunctional glycoprotein due to mismatch of amino acid sequences which must interact in a specific way.

Linkage of oligonucleotides to the *src* gene. Inheritance of the extreme 3′ end of the virus. All 23 transforming Pr-RSV-B × RAV-0 recombinants examined inherited oligonucleotide 15 in the *gag* region, oligonucleotide 26, 21, 14, and 27 between *env* and *src* and the extreme 3′ end (C region) from the transforming Pr-RSV-B parent (Fig. 3). Since the probability of a chance inheritance by all 14 TRE recombinants of any unselected oligonucleotide out of the 29 markers that distinguish the two parents is given as $1 - (1 - 2^{-14})^{29} \sim 2 \times 10^{-3}$, we consider it highly probable that these results are due to selective effects. Comparison with the pattern of inheritance of these segments of the genome in six nontransforming recombinants between *td*-Pr-RSV-B and RAV-0 allowed us to draw several conclusions.

(i) Pr-RSV-B oligonucleotide 15 is linked to the *src* gene. This linkage suggests that a segment of the genome of Pr-RSV-B probably in the *gag* region plays a role in the expression of transformation or the growth rate of transforming viruses. Support for the idea that the selection for oligonucleotide 15 may be related to the growth rate of transforming viruses comes from the observation that recombinants isolated by soft agar colony formation (rather than by focus formation as in this study) do not show such strong linkage between *src* and oligonucleotide 15 (P. Tsichlis, C. Barker, and J. Coffin, manuscript in preparation). Location of oligonucleotide 15 within the T_1 oligonucleotide map of the virus indicates that it may represent part of the viral genome that codes for p19. It is of interest that Hayman and Vogt (14) have shown a close linkage between p19 and the *src* gene in recombinants between RSV and subgroup A leukosis viruses. This linkage was considered to be evidence for the involvement of a circular intermediate in the recombination process. This explanation is probably incorrect in view of the random inheritance of oligonucleotide 15 in nontransforming recombinants and the presence of crossovers on either side of oligonucleotide 15 in many recombinants.

Our observations may be related to those of Shaikh et al. who reported that some recombinants similar to the ones presented here (except isolated by colony formation) have p19 proteins with an electrophoretic mobility distinct from that of either parent (30). Taken together, these results suggest a strain-specific interaction between *gag* proteins and sequences elsewhere in the genome (or their protein products).

Thus, selection of a gene from a particular parent would select the matching part of the *gag* gene from the same parent. Such an interaction could be at the level of virion assembly or protein or mRNA processing. For example, p19 has been shown to be an RNA binding protein, and it is conceivable that it might play a role in the splicing of the *src* message (19). In such a case,

if the RAV-0 p19 did not match the relevant *src* sequence, then the result might be inefficient or overefficient splicing leading to either poorly transforming or poorly replicating virus.

(ii) A set of oligonucleotides between *env* and *src* is linked to the *src* gene. The fact that in two of the nontransforming recombinants a portion of this set of oligonucleotides was not found indicates both that recombination can take place within this region and that inheritance of this sequence does not offer a selective advantage to the virus in terms of growth (all the nontransforming recombinants are good producers). This region could be either part of the structural gene for *src* or a control sequence regulating the expression of transformation. The presence of this sequence in all *td* mutants implies that it may have a dual role both in replication and transformation and that the corresponding RAV-0 sequence is suited only to replication. It should be noted that previously we could not detect any RAV-0 sequence related to this region (10), and oligonucleotide 03 is the only possible candidate for a RAV-0 sequence allelic to this portion of the Pr-RSV-B genome. Our results are therefore also consistent with the possibility that the functional analog of this sequence is located elsewhere in the RAV-0 genome and that its loss in some NTRE recombinants is the result of deletion.

(iii) The extreme 3′ end (C region) of the exogenous Pr-RSV-B or *td*-Pr-RSV-B parent offers a selective growth advantage to the virus. An indication of the importance of the C region was the inheritance of the c^x allele by all transforming and nontransforming recombinants. Only this region of the genome was inherited by all the virus progeny whether or not recombinants were selected for transformation. The selective disadvantage of viruses with the c^n region to viruses with the c^x region must be quite high, since we have isolated more than 80 recombinants of the type described here and all have the c^x region. In addition, we have previously shown an identical pattern of inheritance in crosses between RAV-0 and the avian myelocytomatosis virus MC-29 (36). The amount of virus produced by turkey cells infected by any of these clones was similar to that produced by C/E cells infected with Pr-RSV-B and at least 10- to 100-fold higher than that produced by RAV-0-infected cells. The isolation of the rapidly growing virus NTRE-7 (Fig. 4) which is identical to RAV-0 except for the c^x region inherited from the exogenous parent should allow us to directly test the role of the C region in the efficiency of virus replication. Based on the association of a genotype and phenotype in this part of the genome, these results allow us to define the C region as a genetic locus with (at least) two alleles, each of which confers a characteristic phenotype on the virus.

Role of the C region in replication and transformation. The mechanism by which the C region affects the ability of the virus to grow is not completely understood. The region responsible for the growth difference between RAV-0 and Pr-RSV-B must be quite short, since the crossover in NTRE-7 is between oligonucleotides 08 and 03 and therefore within the first 300 to 400 nucleotides from the 3′ end (10). We therefore consider it improbable that a protein is encoded by this region. Instead, it is more likely that the C region is more directly involved in the regulation of viral replication, quite possibly at the level of transcription of the provirus into progeny RNA. The finding by Hsu et al. (15) and Shank et al. (32) that the C region is repeated at the left end of the proviral DNA outside of the region transcribed into RNA suggests that this region may contain a "promoter" for viral RNA synthesis. An efficient promoter as in Pr-RSV-B and other exogenous viruses would allow a higher rate of synthesis of viral RNA (and therefore virions) than an inefficient promoter as in RAV-0. The recombinants described here will allow us to directly test this possibility. If the C region plays the role of promoter for RNA synthesis, it is conceivable that it might affect the ability of a src^+ virus to cause transformation by control of the amount of *src* message synthesized in virus-infected cells. To study this possibility, which might be responsible at least in part for the selection of src^+, c^x recombinants, it would be necessary to isolate an src^+ recombinant virus that has inherited the c^n region from RAV-0.

Nontransforming avian oncoviruses, like *td*-Pr-RSV-B, cause leukemia (and other neoplastic disease) in chickens with a long latent period (3, 25). RAV-0 causes no disease, even in highly susceptible chickens (25; L. B. Crittenden, personal communication). The set of recombinants that we have generated will provide us with the means to identify directly the portion of the virus genome responsible for this difference. Suggestive evidence currently implicates the c^x region, since a number of RAV-60 strains (env^E c^x recombinants between exogenous avian tumor viruses and defective endogenous viruses) induce leukemia with an efficiency equal to exogenous viruses (H. L. Robinson, M. N. Pearson, D. W. DeSimone, P. N. Tsichlis, and J. M. Coffin, Cold Spring Harbor Symp. Quant. Biol., in press). Thus, the nonpathogenicity of endogenous virus is probably unrelated to the enve-

lope glycoprotein. Recent observations have also suggested a possible correlation between 3'-proximal oligonucleotides and leukemogenicity in murine leukemia viruses (our unpublished data; N. Hopkins, personal communication). The incubation period for development of leukemia by this class of viruses is long, and the leukemia seems to be monoclonal (7). These findings, coupled with the observation that the virus produced by such tumor cells is usually identical by fingerprinting to the virus originally injected (Robinson et al., in press), indicate that the virus does not carry a transforming gene and at least in the majority of cases it does not acquire a cellular transforming gene by recombination with endogenous genetic material. If the hypothesis that the viral C region plays the role of promoter for viral RNA synthesis is correct, then the possibility exists that leukemogenesis by this class of viruses is the result of chance integration of the provirus to the left of a potentially transforming cellular gene and induction of a high level of mRNA synthesis from this gene by the provirus C region. Such an induction would also occur with RAV-0, but at too low a level to result in transformation.

Ample evidence has suggested that avian sarcoma viruses arose by recombination of viral and endogenous cellular genetic information (13, 39). The endogenous "sarc" sequence present in the genome of eucaryotic cells (34, 35) is transcribed at a low rate into mRNA (32, 33) and translated into a protein with molecular weight and antigenic determinants similar to the protein product of the viral *src* gene (6, 11, 24). Why the product of this endogenous sequence does not transform cells is not known, but it is likely that the transition of the nonfunctional (nontransforming) endogenous sarc into the functional (transforming) viral *src* may be a consequence of either mutations in *src* or recombination with viral sequences which alter the gene product or the rate of transcription of mRNA. Our research illustrates three other regions of the viral genome of potential importance in this transition and indicates that the acquired *src* gene has come under a rather complex set of viral controls which seems to be essential for its function or for replication of the src^+ virus.

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LITERATURE CITED

1. **Beard, J. W., D. G. Sharp, E. A. Eckert, D. Beard, and E. B. Mommaerts.** 1952. Properties of the virus of the fowl erythromyeloblastic disease. Proc. Second Natl. Cancer Conf. **2**:1396–1411.
2. **Begg, A. M.** 1927. A filterable endothelioma of the fowl. Lancet **i**:912–915.
3. **Biggs, P. M., B. S. Milne, T. Graf, and H. Bauer.** 1973. Oncogenicity of nontransforming mutants of avian sarcoma viruses. J. Gen. Virol. **18**:399–403.
4. **Billeter, M. A., J. T. Parsons, and J. M. Coffin.** 1974. The nucleotide sequence complexity of avian tumor virus RNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3560–3564.
5. **Brownlee, G. G.** 1972. Determination of sequences in RNA. North Holland Publishing, Co., Amsterdam.
6. **Brugge, J. S. and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
7. **Canaani, E., and S. A. Aaronson.** 1979. Restriction enzyme analysis of mouse cellular type C viral DNA: emergence of new viral sequences in spontaneous AKR/J lymphomas. Proc. Natl. Acad. Sci. U.S.A. **76**:1677–1681.
8. **Coffin, J. M.** 1979. Structure, replication and recombination of retrovirus genomes: some unifying hypotheses. J. Gen. Virol. **42**:1–26.
9. **Coffin, J. M., and M. A. Billeter.** 1976. A physical map of the Rous sarcoma virus genome. J. Mol. Biol. **100**: 293–318.
10. **Coffin, J. M., M. Champion, and F. Chabot.** 1978. Nucleotide sequence relationship between the genomes of an endogenous and an exogenous avian tumor virus. J. Virol. **28**:972–991.
11. **Collett, M. S., J. S. Brugge, and R. L. Erikson.** 1978. Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. Cell **15**:1363–1369.
12. **Engelbreth-Holm, J., and A. Rothe-Meyer.** 1932. Bericht über neue erfahrungen mit einem Stamm Hühner-Erythroleukose. Acta Pathol. Microb. Scand. **9**:293–312.
13. **Hanafusa, H., C. C. Halpern, D. L. Buchhagen, and S. Kawai.** 1977. Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J. Exp. Med. **146**:1735–1747.
14. **Hayman, M. J., and P. K. Vogt.** 1976. Subgroup specific antigenic determinants of avian RNA tumor virus structural proteins: analysis of virus recombinants. Virology **73**:372–380.
15. **Hsu, T. W., J. L. Sabran, G. E. Mark, R. V. Guntaka, and J. M. Taylor.** 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virol **28**:810–818.
16. **Hunter, E.** 1978. The mechanism for genetic recombination in the avian retroviruses. Curr. Top. Microbiol. Immunol. **79**:295–309.
17. **Ivanov, X., Z. Mladenov, S. Nedalkov, T. G. Todorov, and M. Yakimov.** 1964. Experimental investigations into avian leukosis V transmission, hematology and morphology of avian myelocytomatosis. Bull. Inst. Pathol. Comp. Anim. Acad. Bulg. Sci. **10**:5–38.
18. **Joho, R. H., M. A. Billeter, and C. Weisman.** 1975. Mapping of biological function of RNA of avian tumor viruses: location of regions required for transformation and determination of host range. Proc. Natl. Acad. Sci. U.S.A. **72**:4772–4776.
19. **Leis, J. P., J. McGinnis, and R. W. Green.** 1978. Rous sarcoma virus P19 binds to specific double-stranded regions of viral RNA: effect of P19 on cleavage of viral RNA by RNase III. Virology **84**:87–98.
20. **Linial, M., and P. E. Neiman.** 1976. Infection of chick cells by subgroup E viruses. Virology **73**:508–520.

21. **Luria, S. E., J. E. Darnell, D. Baltimore, and A. Campbell.** 1978. General virology, 3rd ed., p. 190. John Wiley and Sons, New York.
22. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. G. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11**:95–103.
23. **Neiman, P. E., S. Das, D. MacDonnel, and C. McMillin-Helsel.** 1977. Organization of shared and unshared sequences in the genomes of chicken endogenous and sarcoma viruses. Cell **11**:321–329.
24. **Oppermann, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop.** 1979. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (src). Proc. Natl. Acad. Sci. U.S.A. **76**:1804–1808.
25. **Purchase, H. G., W. Okazaki, P. K. Vogt, H. Hanafusa, B. R. Burmester, and L. B. Crittenden.** 1977. Oncogenicity of avian leukosis viruses of different subgroups and of mutants of sarcoma viruses. Infect. Immun. **15**:423–428.
26. **Robinson, H. L.** 1978. Inheritance and expression of chicken genes that are related to avian leukosis sarcoma virus genes. Curr. Top. Microbiol. Immunol. **83**:1–36.
27. **Robinson, H. L.** 1976. Intracellular restriction on the growth of induced subgroup E avian type C viruses in chicken cells. J. Virol. **18**:856–866.
28. **Rous, P.** 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med. **13**: 397–411.
29. **Rubin, H., and P. K. Vogt.** 1962. An avian leukosis virus associated with stocks of Rous sarcoma virus. Virology **17**:184–194.
30. **Shaikh, R., M. Linial, S. Brown, A. Sen, and R. Eisenman.** 1979. Recombinant avian oncoviruses. II. Alterations in the gag proteins and evidence for intragenic recombination. Virology **92**:463–481.
31. **Shank, P. R., S. Hughes, H. J. Kung, J. E. Majors, N. Quintrell, R. V. Guntaka, J. M. Bishop, and H. E. Varmus.** 1978. Mapping unintegrated forms of avian sarcoma virus DNA: both termini of linear DNA bear a 300 nucleotide sequence present once or twice in two species of circular DNA. Cell **15**:1383–1395.
32. **Spector, D. H., B. Baker, H. E. Varmus, and J. M. Bishop.** 1978. Characteristics of cellular RNA related to the transforming gene of avian sarcoma viruses. Cell **13**:381–386.
33. **Spector, D. H., K. Smith, T. Padgett, P. McCombe, D. Roulland-Dussoix, C. Moscovici, H. E. Varmus, and J. M. Bishop.** 1978. Uninfected avian cells contain RNA related to the transforming gene of avian sarcoma viruses. Cell **13**:371–379.
34. **Spector, D., H. Varmus, and J. M. Bishop.** 1978. Nucleotide sequences related to the transforming genes of avian sarcoma virus are present in DNA of uninfected vertebrates. Proc. Natl. Acad. Sci. U.S.A. **75**:4102–4106.
35. **Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt.** 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature (London) **260**:170–172.
36. **Tsichlis, P. N., and J. M. Coffin.** 1979. Recombination between the defective component of an acute leukemia virus and Rous associated virus O, an endogenous virus of chickens. Proc. Natl. Acad. Sci. U.S.A. **76**:3001–3005.
37. **Wang, L.-H., P. H. Duesberg, K. Beemon, and P. K. Vogt.** 1975. Mapping RNase-T_1 resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation-defective viruses are at the poly(A) end. J. Virol. **16**:1051–1070.
38. **Wang, L.-H., P. H. Duesberg, S. Kawai, and H. Hanafusa.** 1976. Location of envelope-specific and sarcoma-specific oligonucleotides on RNA of Schmidt-Ruppin Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **73**: 447–451.
39. **Wang, L.-H., C. C. Halpern, M. Nadel, and H. Hanafusa.** 1978. Recombination between viral and cellular sequences generates transforming sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **75**:5812–5816.