Chromosomal Localization of Three Endogenous Retrovirus Loci Associated with Virus Production in White Leghorn Chickens

ALLAN TEREBA,^{1*} LYMAN B. CRITTENDEN,² AND SUSAN M. ASTRIN³

Division of Virology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101¹; U.S. Department of Agriculture, Science and Education Administration, Agricultural Research, Regional Poultry Research Laboratory, East Lansing, Michigan 48823²; and The Institute for Cancer Research, The Fox Chase Cancer Institute, Philadelphia, Pennsylvania 19111³

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Proposed mechanisms for the generation of endogenous retrovirus loci have been examined by determining the chromosomal distribution of these loci by means of in situ hybridization. Unlike the clustering on chromosome 1 of five endogenous retrovirus loci associated with the $gs^- chf^-$ phenotype A. Tereba and S. M. Astrin, submitted for publication), three loci associated with endogenous retrovirus production (V^+ phenotype) were located on three separate chromosomes. *ev2*, which codes for the prototype endogenous RAV-0 genome in line 7₂ chickens, was localized near the middle of the long arm of chromosome 2. *ev7*, coding for a noninfectious, inducible genome present in line 15_B chickens, was located near the end of the long arm of the Z chromosome. A third V^+ locus, designated *ev14*, was detected near the middle of chromosome 3. This arrangement of V^+ loci is consistent with an integration mechanism employing randomly distributed integration sites in the chicken genome. In addition, these data provide evidence suggesting that the $gs^- chf^-$ -associated loci may have been generated by a different mechanism.

Most White Leghorn chickens contain DNA sequences that share homology with the genomes of currently circulating avian leukosis viruses (6, 20, 26, 37, 39). The origin of these sequences and the mechanism by which they have entered the chicken genome, however, have not been determined. Weiss and Biggs (41) showed that the genome of the wild red jungle fowl (*Gallus gallus*), the proposed progenitor of the domestic chicken (10), contains a genetically transmitted endogenous retrovirus genome similar to the ones found in domestic chickens, and Frisby et al. (11) demonstrated that other species of jungle fowl lack detectable retrovirus sequences. On the basis of this information, the introduction of these viral sequences into *Gallus gallus* has occurred within the last million years.

Assuming that evolutionarily recent exogenous infections were the source of endogenous retrovirus sequences, it would be logical to suppose that these closely related viruses used the same mechanism of integration as current laboratory strains. Data obtained by analyzing retrovirus-specific sequences in restriction endonuclease-digested DNA of laboratory infected cells indicates hundreds, perhaps thousands, of integration sites (16, 28, 40). This result is indicative

of a mechanism requiring either no specific recognition sequence or only a short sequence present by chance hundreds of times in the host genome. When uninfected chicken DNA was examined in a similar manner many different patterns were observed, which indicated that integration sites were present at many chromosomal locations (1, 5, 15, 34).

However, our examination of the chromosomal position of several chicken endogenous retrovirus loci by using in situ hybridization has revealed a very nonrandom distribution (35; Tereba and Astrin, submitted for publication). All five loci in White Leghorn chickens that are associated with the nonexpressed $gs^- chf^-$ phenotype were localized to distinct regions on chromosome 1. The probability of this occurring by a random integration mechanism is less than 2×10^{-5} . These results led us to propose that either the generation of the $gs^- chf^-$ -associated retrovirus loci utilized a mechanism involving very specific sites of integration, or the loci were generated from a preexisting locus by a process of intrachromosomal transposition.

To determine whether other endogenous retrovirus loci in White Leghorn chickens display this highly nonrandom distribution, we ex-

amined the chromosomal locations of three endogenous retrovirus loci associated with virus production (V^+ phenotype). We report here that, unlike the nonexpressed $gs^- chf^-$ -associated loci, the V^+ -associated loci are located on several different chromosomes. This arrangement is consistent with an integration mechanism similar to that used by currently circulating retroviruses.

MATERIALS AND METHODS

Cells. Primary fibroblast cultures were prepared from 11-day-old line 7₂ and 15_B White Leghorn embryos raised at the U.S. Regional Poultry Research Laboratory (East Lansing, Mich.) and from embryo 9893, which had a C/E phenotype (Heisdorf and Nelson Farms, Redmond, Wash.) as described by Rubin (27).

Reverse transcriptase assays. A 50- μ l sample of culture medium that had been in contact with cells for 24 h was analyzed for reverse transcriptase activity by using a polyribonucleic acid-oligodeoxythymidylic acid template primer as described by Tereba and Murti (36). A kinetic analysis was performed on medium from each cell type to ensure that linear kinetic data were employed in calculating specific activities.

Characterization of DNA. DNA was prepared from cultured cells by using phenol as described by Varmus et al. (38). The digestion of cell DNA with restriction endonucleases *Sst*I (Bethesda Research Laboratory, Rockville, Md.) and *Bam*HI (New England BioLabs, Beverly, Mass.) has been described by Astrin (1). After digestion, the DNA fragments were electrophoresed in 1% agarose gels (18) and transferred to nitrocellulose filters (Millipore HAWP00010) by the method of Southern (32) as modified by Ketner and Kelley (18). The transferred DNA fragments were hybridized with ³²P-labeled Rous-associated virus type 2 RNA and then autoradiographed (1).

Metaphase chromosome preparation and in situ hybridization. Metaphase chromosome preparations were prepared from colcemid-treated fibroblast cultures that had been grown on microscope slides (34, 35). In situ hybridization was performed using a probe containing RNA from the Prague strain of Rous sarcoma virus, subgroup C (PR-RSV-C) that had been attached to ¹²⁵I-labeled high-molecular-weight sea urchin DNA via polybromodeoxyuridylic acid-polyadenylic acid hybrids. The synthesis and characteristics of this probe have been fully described (35). Hybridization reactions were performed at 40°C in 50- μ l volumes that contained 40% formamide, 0.6 M NaCl-0.06 M sodium citrate (4 $\times$ SSC), and 1 to 2 ng of probe. The reactions were stopped after 40 h, washed, and prepared for autoradiography as described by Tereba et al. (35). Autoradiograms were exposed for 1 month. The background was calculated assuming a Poisson distribution of background grains over the metaphase chromosomes and took into account the size and number of chromosomes observed (34).

RESULTS

Virus expression. Although the sponta-

neous release of endogenous retroviruses from several lines of White Leghorn chickens can be detected, the amount and biological properties of the viruses that are released can differ significantly. In some cases these differences may be the result of host restriction mechanisms (9), but in other cases the differences have been attributed to the presence of different genetic loci (1-5, 24; L. B. Crittenden and S. M. Astrin, *Virology*, in press). To obtain as large a variation as possible in our examination of retrovirus loci associated with virus production (V^+ phenotype), we selected three distinct V^+ lines that can be readily distinguished from each other. Fibroblasts from lines 7₂ and 15_B (maintained at the U.S. Regional Poultry Research Laboratory in East Lansing, Mich.) have been extensively studied with respect to virus production and host range of released virus and differ significantly in several respects (7, 8, 22, 23, 25, 39). Embryo 9893 (Heisdorf and Nelson Farms) was characterized as having a V^+ phenotype during a routine examination of released reverse transcriptase activity from chicken embryo fibroblasts.

The amount of reverse transcriptase activity released by cells appears to correlate directly with the number of released virus particles (36). Therefore, as a convenient way to compare virus production, we examined the amount of activity released by each of the three cell types. Table 1 shows that no significant activity was detected from line 7₂. This is consistent with the low level of infectious units released (10^2 to 10^3 /ml; 22). Line 15_B and embryo 9893, on the other hand, released significant, and approximately equal, amounts of reverse transcriptase. This activity, however, was only 5% of that released by PR-RSV-A-infected chicken cells.

Although the polymerase activity was low compared to that in the PR-RSV-A-infected

TABLE 1. Reverse transcriptase activity released by various cell lines

Cell type	Polymerase activity ^a		
	cpm ^b	Reaction time (h) ^c	pmol/h
7 ₂	247	20	0.0005
15 _B	6,948	1	0.25
9893	38,697	6	0.23
C/E (PR-A)	136,334	1	4.96

^a In 50 μ l of growth medium that had been in contact with cells for 24 h.

^b A background of 465 cpm (growth medium) has been subtracted.

^c Reaction times were selected to obtain maximal sensitivity under linear nonsaturating conditions.

cells, it was somewhat higher than previously reported for uninduced line 15_B cells (24). It is thus possible that these cells have been reinfected by released endogenous viruses, causing an elevation in virus production. These new integration sites would not be observed by our restriction endonuclease or in situ hybridization analysis, however, since each cell would have these new integrated loci at different positions in the chicken genome (1).

Although similar in amount, the virus from line 15_B is biologically distinct from virus released by embryo 9893. The virus from line 7₂ and embryo 9893 can complement the glycoprotein-defective Bryan High titer strain, can infect Japanese quail cells (39; our unpublished data), and can give interference patterns consistent with having E subgroup glycoproteins. In addition, the reverse transcriptases from embryo 9893 and line 7₂ viruses (grown in susceptible cells) are fairly stable in our polymerase assays, producing linear kinetics for at least 6 to 12 h (unpublished data). The virus obtained from line 15_B will neither complement the Bryan strain nor infect Japanese quail cells (22). Its polymerase also appears to be unstable, showing linear kinetics for no more than 1 h (data not shown). A more detailed characterization of the biological and biochemical properties of embryo 9893 and its released virus will be published separately.

Identification of distinct loci. The variation of the properties of the viruses produced by the three cell types indicated the presence of three distinct retrovirus loci. To confirm this, we analyzed retrovirus-specific fragments of restriction endonuclease-digested cell DNA. This approach, which assumes that distinct patterns represent distinct genetic loci, has been successful in proving that the two loci defined as *ev2* and *ev7* (1) are the loci responsible for virus production in lines 7₂ and 15_B, respectively (3,

24). To determine whether a new and distinct locus could be assigned to the V⁺ phenotype of embryo 9893, we examined virus-specific fragments from cell DNA that was digested with the restriction endonucleases *Sst*I and *Bam*HI (Fig. 1). For comparison, DNA from lines 7₂ and 15_B are included also and demonstrate the presence of *ev1* in each of these lines in addition to the specific V⁺ loci. Only one distinct fragment at the position of *ev1* (9.2 kilobases or 5.8×10^6 daltons) was observed when *Sst*I was used to digest embryo 9893 DNA. However, when *Bam*HI, which cuts within the viral genome, was used, four fragments were observed. The three lower-molecular-weight fragments are common to all DNA containing *ev1*. The larger *Bam*HI fragment (10 kilobases or 6.3×10^6 daltons), along with the *Sst*I digest pattern, does not correspond to any known locus and, thus, has been designated *ev14*. Since viral proteins have not been detected in cells containing only *ev1* (1), we suggest that *ev14* is responsible for the V⁺ phenotype observed in embryo 9893.

Chromosomal localization of viral loci.

Using a sensitive in situ hybridization technique, we have previously localized *ev1*, 4, 5, 8, and 13 on specific regions of chromosome 1 (34; Tereba and Astrin, submitted for publication). Although low levels of mRNA transcribed from some of these loci can be detected (13), no protein products have been observed. This is in contrast to the virus production of the cells being examined in this study. Due to this difference, it was of interest to determine whether these V⁺ loci also were located on chromosome 1.

To determine the location of the virus loci, metaphase chromosomes from each cell type were hybridized to a probe consisting of PR-RSV-C RNA that had been attached to ¹²⁵I-labeled high-molecular-weight sea urchin DNA (35). This probe would be expected to hybridize to both the virus loci as well as to the endoge-

FIG. 1. Restriction endonuclease digestion of DNAs from three embryos and identification of endogenous viral loci. A marker of *Eco*RI-cleaved, ³²P-labeled λ DNA mixed with *Hind*III-cleaved ³²P-labeled simian virus 40 DNA is shown at the left. Molecular weights are shown in kilobase pairs. The DNA fragments representing *ev1*, *ev2*, and *ev7* have been designated in accordance with previous work (1, 2). The *Bam*HI fragments marked with an asterisk are internal fragments of the viral genome.

nous *sarc* gene present on the chromosome group 10-12 in all chickens (34, 35). Figure 2 shows a representative autoradiogram of a metaphase chromosome spread and demonstrates the ease of identifying the larger macrochromosomes. The in situ hybridization results from line 7₂ are presented in Table 2. After subtracting the background, only chromosomes 1, 2, and 10-12 contained a statistically significant number of grains. Previous studies have demonstrated that *ev1* is on chromosome 1, thus accounting for hybridization on this chromosome. Since the *sarc* gene is on the chromosome group 10-12, by elimination, *ev2* must be located on chromosome 2. This provides the first evidence that an endogenous retrovirus locus can be located on a chromosome other than chromosome 1.

The location of *ev7* in line 15_B was determined next. The data in Table 3 again show three

chromosomes containing a statistically significant number of grains. As before, chromosome groups 1 and 10-12 hybridized, demonstrating the presence of *ev1* and *sarc*, respectively. In addition, the sex chromosome Z hybridized, demonstrating the location of *ev7*. These results have been confirmed by animal mating studies (E. Smith and L. B. Crittenden, unpublished data) and help to confirm the reliability of our in situ hybridization approach.

To examine further the distribution of the V⁺ loci, the location of *ev14* was determined. Table 4, showing the data from embryo 9893, demonstrated the hybridization of three chromosomes, 1, 3, and 10-12. Due to the less efficient hybridization in this experiment, the excess grains over chromosome 1 were statistically significant only at the 98% level (2% chance of random occurrence). However, when these findings are coupled with the known presence of *ev1* in this

FIG. 2. Metaphase chromosome spread hybridized in situ with a virus-specific probe. Metaphase chromosomes from line 15_B were hybridized in situ with a ¹²⁵I-labeled probe containing PR-RSV-C RNA and autoradiographed for 1 month. (A) Photomicrograph showing chromosomes in focus. (B) Same field as in (A) with grains in focus and using Kodak filters 35 and 36 to suppress chromosome color. The arrow points to multiple grain over *ev7*.

TABLE 2. Grain distribution over metaphase chromosomes from line 7₂ hybridized with a probe containing PR-RSV-C RNA^a

Chromosome	Total grains ^b	Back-ground grains ^c	Grains above back-ground	Probability of random occurrence ^d
1	90	58	32	<0.0001
2	68	42	26	<0.0001
3	31	32	-1	>0.1
4	26	22	4	>0.1
Z	20	19	1	>0.1
6	15	19	-4	>0.1
7-9	33	31	2	>0.1
10-12	38	19	19	<0.0001
Micro	48	50	-2	>0.1

^a Line 7₂ contains *ev1* and 2 as determined by analysis of virus-specific restriction endonuclease-digested cell DNA.

^b From 77 chromosome spreads.

^c Determined as described in the text.

^d Assuming a Poisson distribution of background grains over the metaphase chromosomes.

TABLE 3. Grain distribution over metaphase chromosomes from line 15_B hybridized with probe containing PR-RSV-C RNA^a

Chromosome	Total grains ^b	Back-ground grains ^c	Grains above back-ground	Probability of random occurrence ^d
1	183	149	34	<0.006
2	122	108	14	>0.1
3	65	78	-13	>0.1
4	59	55	4	>0.1
Z	84	41	43	<0.0001
6	41	45	-4	>0.1
7-9,w	80	86	-6	>0.1
10-12	85	45	40	<0.0001
Micro	130	125	5	>0.1

^a Line 15_B contains *ev1* and 7 as determined by analysis of virus-specific restriction endonuclease-digested cell DNA.

^b From 192 chromosome spreads.

^c Background was determined as described in the text.

^d Assuming a Poisson distribution of background grains over the metaphase chromosomes.

embryo, the expected distribution of grains over the other nonhybridized chromosomes (greater than 30% probability of random occurrence), and the expected equal number of grains over each of the viral loci and *sarc* gene (35; Tereba and Astrin, submitted for publication), we believe that *ev1* was detected in this experiment. Also, as expected, the chromosome group 10-12, containing the *sarc* gene, hybridized. By elimination, this placed *ev14* on chromosome 3 and increased the likelihood that additional V⁺ loci as yet unexamined would be scattered through-

out the genome.

Arrangement of virus loci on chromosomes 1, 2, 3, and Z. The localization of the V⁺ loci on different chromosomes provided important new information on the distribution of endogenous retrovirus loci. To determine more precisely the chromosome region at which each virus locus resided, we analyzed the grain distribution on chromosome 1 for each of the three embryos tested. Figure 3 shows that one peak was detected in each case and occurred at a mean position of 0.26 (range, 0.25 to 0.26). This is identical to the position determined for *ev1* (34) and confirms the accuracy of the technique. The location of *ev1* on a photomicrograph of chromosome 1 is shown in Fig. 3D.

The distribution of grains over chromosome 2 from line 7₂ is shown in Fig. 4. One peak was observed at position 0.3, which is near the middle of the long arm. This helps to confirm the presence of *ev2* on chromosome 2. Figure 5 shows the grain distribution over the sex chromosome Z. A broad peak of grains was present at 0.4 ± 0.1 , which is towards the end of the longer arm of this metacentric chromosome. The resolution of grains was less than on chromosomes 1 and 2, partly because of the Z chromosome's smaller size. A detailed examination of the grains from each chromosome spread revealed many multiple grains at the end of the chromosome (data not shown). However, a significant number of grains also were noted near the centromere. Although this possible second site of hybridization may contain a small region of homology with our probe, we have elected to interpret the data as indicating only one site near the end of

TABLE 4. Grain distribution over metaphase chromosomes from embryo 9893 hybridized with a probe containing PR-RSV-C RNA^a

Chromosome	Total grains ^b	Back-ground grains ^c	Grains above back-ground	Probability of random occurrence ^d
1	172	144	28	<0.02
2	97	104	-7	>0.3
3	97	74	23	<0.008
4	60	55	5	>0.3
Z	47	43	4	>0.3
6	46	41	5	>0.3
7-9	79	75	4	>0.3
10-12	76	45	31	<0.0001
Micro	105	115	-10	>0.3

^a Embryo 9893 contains *ev1* and 14 as determined by analysis of virus-specific restriction endonuclease-digested cell DNA.

^b From 150 chromosome spreads.

^c Determined as described in the text.

^d Assuming a Poisson distribution of background grains over the metaphase chromosomes.

FIG. 3. Localization of *ev1* on chromosome 1. The distribution of silver grains on chromosome 1 from (A) line 7_a, (B) line 15_B, and (C) embryo 9893 was determined from autoradiographs of metaphase chromosome spreads hybridized in situ with a [¹²⁵I]DNA probe containing PR-RSV-C RNA. The length of chromosome 1 is defined as 1 unit, and the distance is measured from the centromere, with the long arm given positive values and the short arm given negative values. The dashed line (---) indicates the background level of silver grains. (D) The position of *ev1* (0.26) is indicated by an arrow on a photomicrograph of chromosome 1.

the chromosome. The presence of only one site is consistent with our restriction endonuclease analysis of line 15_B DNA. Figure 6 shows the grain distribution on chromosome 3 of embryo 9893. Again, the resolution was not as great as with *ev1* and 2. This is due to the smaller size of chromosome 3 and to the higher background levels observed in this hybridization reaction. However, one broad peak was observed at 0.6 ± 0.1 on this acrocentric chromosome, thus indicating the position of *ev14*.

DISCUSSION

The chromosomal distribution of endogenous chicken retrovirus loci associated with the

nonexpressed *gs*⁻ *chf*⁻ phenotype was examined previously (34; Tereba and Astrin, submitted for publication). The results showed that all five loci examined (*ev1*, 4, 5, 8, and 13) were located on chromosome 1 and occurred at regularly spaced intervals of approximately 1.8×10^7 base pairs. This led to the supposition that either very specific cell sequences were used as recognition sites for integration of virus genomes after independent infections, or the multiple loci were generated by a series of transpositions restricted to chromosome 1, but not necessarily involving specific recognition sites.

Our present data on three loci associated with the virus-producing V⁺ phenotype are in sharp contrast to this earlier finding. Each locus was associated with a different chromosome, and none of these loci was associated with chromosome 1. These new data demonstrate that endogenous retrovirus loci do occur on several distinct chicken chromosomes. Although the number of loci examined was small, the distri-

FIG. 4. Localization of *ev2* on chromosome 2. The distribution of silver grains on chromosome 2 from line 7_a was determined from autoradiographs of metaphase chromosome spreads hybridized in situ with a [¹²⁵I]DNA probe containing PR-RSV-C RNA. The dashed line (---) indicates the background level of silver grains. The length of chromosome 2 is defined as 1 unit, with the long arm having positive values and the short arm having negative values. The arrow indicates the probable position (0.3) of *ev2*.

FIG. 5. Localization of *ev7* on chromosome Z. The distribution of silver grains on chromosome Z from line 15_B was determined from autoradiographs of metaphase chromosome spreads hybridized in situ with a [¹²⁵I]DNA probe containing PR-RSV-C RNA. The dashed line (---) indicates the background level of silver grains. The length of chromosome Z is defined as 1 unit, with the long arm having positive values and the short arm having negative values. The arrow indicates the probable position (0.4) of *ev7*.

FIG. 6. Localization of *ev14* on chromosome 3. The distribution of silver grains on chromosome 3 of embryo 9893 was determined from autoradiographs of metaphase chromosome spreads hybridized *in situ* with a [125 I]DNA probe containing PR-RSV-C RNA. The dashed line (---) indicates the background level of silver grains. The length of chromosome 3 is defined as 1 unit, with the origin at the end containing the centromere. The arrow indicates the probable position (0.6) of *ev14*.

bution of these V^+ loci is compatible with a random distribution among the chromosomes and is more in keeping with data obtained on the integration of laboratory strains of retroviruses (16, 28, 40).

This dichotomy of results raises some important new questions regarding the generation of endogenous retrovirus loci. The division of the two groups of loci is correlated with phenotypic expression of virus protein and not transcriptional levels (there is more *ev1* mRNA present than *ev2* mRNA; 13). Thus, selective pressures which eliminated chickens with virus loci in critical chromosomal locations are unlikely to account for the difference in distribution of these two groups within the genome. Therefore, the results must be explained either by the existence of two distinct groups of viruses that used different and specific recognition sequences for integration, or by a combination of mechanisms involving both virus integration (either random or specific) and transposition of existing loci to new locations in a manner similar to the transposition of the bacteriophage Mu.

As an example, the gs^- chf^- -associated loci could form a group that used a specific integration sequence. This is required to explain the highly nonrandom clustering of these related loci. The integration sequences would have to be long enough to occur only a few times by chance and, for unknown reasons, would occur only on chromosome 1. The V^+ loci would have to form another group and could utilize very short or completely random integration sequences, as suggested for current exogenous retroviruses. Although the specific integration hypothesis is in conflict with most data concerning exogenous retrovirus integration (40), the results bear a striking resemblance to the apparent specific integration of baboon endogenous virus on human chromosome 6 (19).

Alternatively, two separate mechanisms could

have been used to generate the virus loci. The V^+ -associated loci, along with one of the gs^- chf^- -associated loci, probably *ev1*, could have been generated by independent exogenous infections by using random integration involving either no particular recognition sequence or short specific cell sequences which are randomly distributed throughout the cell genome. Then, by a process of intrachromosomal transposition, the gs^- chf^- -associated locus could have been inserted at different regions of chromosome 1 in a manner similar to Mu transposition (29). The process of moving genes within the same chromosome does have a precedent in yeast cells (12, 14). This two-step mechanism of generating endogenous loci would be consistent with the multiple integration sites of laboratory strains of avian sarcoma viruses, indicating a lack of long recognition sequences (16, 28), and with the transposition-like structure of retroviruses (16, 17, 28, 30, 33). Thus, the number of endogenous loci present in a species may be an overestimation of the number of successful infections involving the germinal tissue.

In addition to providing information about the generation of endogenous retrovirus loci, the localization of *ev2*, 7, and 14 to chromosomes 2, Z, and 3, respectively, provides the means to associate and orient genetic linkage groups (31) with specific chromosomes. The virus loci also will provide simple markers (in the form of specific restriction endonuclease fragments) to test the linkage of many unmapped genetic loci. Eventually, this should provide a greater understanding about the organization and development of genes in a genome that has remained remarkably unchanged in millions of years (21).

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