Structure and Expression of *c-rel*, the Cellular Homolog to the Oncogene of Reticuloendotheliosis Virus Strain T

IRVIN S. Y. CHEN,[†] KIRK C. WILHELMSSEN, AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received 21 June 1982/Accepted 27 August 1982

The cellular homolog of the *onc* sequences in the avian retrovirus reticuloendotheliosis virus strain T (*v-rel*) was studied by molecular cloning and nucleic acid hybridization. In contrast to *v-rel* sequences, which are 1.4 kilobase pairs long, the cellular homolog, *c-rel*, from line 15B chickens is at least 25 kilobase pairs long, with multiple apparent introns. A 4.0-kilobase polyadenylic acid-containing RNA transcript is the primary species of *c-rel* RNA present in uninfected chicken cells. Sequences at the 3' end of this *c-rel* RNA are not present in *v-rel*.

Reticuloendotheliosis virus strain T (REV-T) is a highly oncogenic avian retrovirus that causes rapidly fatal leukemia in newly hatched chicks and turkey poults (27). Like the genomes of other highly oncogenic avian retroviruses, the genome of REV-T contains sequences (*v-rel*) that are not necessary for viral replication (6, 7, 13, 14). The *v-rel* sequences are necessary for the transforming properties of REV-T (7a). These sequences are related to sequences in the genomes of uninfected chickens and turkeys (*c-rel*) (7, 31). The *c-rel* sequences in turkeys are believed to be the progenitors of the *v-rel* sequences.

Previous studies have indicated that the cellular homolog of *v-rel* is arranged differently than *v-rel* of REV-T (7). Multiple large DNA fragments having homology to *v-rel* have been detected in cellular DNA, indicating either that *c-rel* is distributed over a large region of cellular DNA or that *c-rel* represents a small gene family. These previous studies were extended by obtaining molecular clones of *c-rel* from chicken DNA and comparing the structure of *c-rel* with the structure of *v-rel*. Our results show that, rather than being duplicated, *c-rel* is distributed over a large contiguous region of chicken DNA. In addition, a comparison of *c-rel* mRNA and *v-rel* DNA showed that at least part of a 3' *c-rel* exon is not present in *v-rel*. (See Addendum in Proof.)

MATERIALS AND METHODS

Cells. The general sources and procedures for obtaining and propagating avian fibroblastic cells have been described previously (12).

[†] Present address: Division of Hematology and Oncology, School of Medicine, University of California at Los Angeles, Los Angeles, CA 90024.

Sources of DNAs. Line 15B chicken DNA was extracted from line 15B chicken embryo fibroblasts as previously described (7).

Clone 2-20-4 is a variant REV-T proviral clone in Charon 4A (7).

pREV-T 3 is a circularly permuted molecular clone of REV-T unintegrated circular DNA in pBR322 (7a). Infectious virus that can transform chicken spleen cells in culture can be recovered from this DNA.

Ch4A REV-T 52B is a molecular clone of infectious REV-T proviral DNA (Wilhelmsen, unpublished data).

Clones propagated in pBR322 were grown in *Escherichia coli* strain HB101 as previously described (24). Clones propagated in the bacteriophage vectors Charon 4A (5) and λ 1059 (16) were grown in *E. coli* strains DP50 supF and C600, respectively.

Source of RNA. Chicken spleens were obtained from 2- to 3-week-old White Leghorn $\times$ New Hampshire Red chickens (University of Wisconsin, Madison). The spleens were stored at -70°C . For extraction of RNA, the spleens were ground to a fine powder with a mortar and pestle over dry ice. The tissue was lysed rapidly with urea lysis buffer (15), and total cellular RNA was purified by centrifugation in CsCl (22). Polyadenylic acid containing RNA was purified by using oligodeoxythymidylic acid cellulose (2).

Preparation of molecular clones. A recombinant DNA library in Charon 4A constructed from a partial *EcoRI* digest of line 15B chicken DNA (generously supplied by Ed Fritsch, Michigan State University, East Lansing), was screened by hybridization to the middle *v-rel* probe (see Fig. 1). Several positive clones were obtained. A preliminary analysis showed that all of the clones were identical except for the orientation of the chicken DNA insertion with respect to Charon 4A; therefore, only one of these clones, λ Ch 4A *c-rel* 1, was characterized in detail.

A recombinant DNA library of line 15B chicken DNA in λ 1059 was constructed by using *BamHI* chicken DNA fragments selected on an NaCl gradient to be between 10 and 25 kilobase pairs (kbp) long. The λ 1059 *c-rel* 3 and λ 1059 *c-rel* 4 recombinant clones

were obtained by using DNA fragments derived from the *c-rel* insertion in λ Ch 4A *c-rel* 1 as hybridization probes. The *Hind*III DNA fragment at 16.3 to 18.9 kbp and the *Hind*III-*Eco*RI DNA fragment at 21.6 to 23.6 kbp were subcloned from λ Ch 4A *c-rel* 1 into pBR322 (see Fig. 2). The chicken DNA insertions were purified from pBR322 DNA and used as hybridization probes.

Another recombinant DNA library of line 15B chicken DNA was constructed in λ 1059 by using *Bgl*II DNA fragments selected on an NaCl gradient to be between 9.0 and 20 kbp long. The λ 1059 *c-rel* 5 recombinant clone was obtained by using the *Bam*HI-*Eco*RI DNA fragment (4.5 to 6.5 kbp [see Fig. 2]) subcloned from λ 1059 *c-rel* 4 into pBR322 as a hybridization probe to screen this library.

Subclones of the *c-rel* clones in pBR322 were prepared as previously described (24).

Gel electrophoresis, nucleic acid transfer to nitrocellulose, and molecular hybridization. Gel electrophoresis, transfer to nitrocellulose, and molecular hybridization of DNA have been described previously (7). The relative amounts of hybridization were determined by densitometry tracing of autoradiograms with a Joyce-Loebl microdensitometer.

RNA was denatured with glyoxal, subjected to electrophoresis, and transferred to nitrocellulose as described by Thomas (28). Molecular size markers were visualized by soaking the gel in a solution containing 0.02% methylene blue, 0.02% sodium acetate, and 0.02% acetic acid. RNA treated in this manner does not hybridize efficiently. Therefore, the lanes containing molecular size markers were cut from the remainder of the gel.

The RNA immobilized on a filter was hybridized at 42°C for 24 to 36 h in a solution containing 50% deionized formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 1 $\times$ Denhardt solution, 0.1% sodium dodecyl sulfate, and 50 μ g of sonicated salmon sperm DNA per ml (28).

Hybridization probes (32 P]DNA or 32 P]cDNA) were prepared as previously described (7).

RESULTS

Previous experiments in which a limited region of *v-rel* was used as a probe indicated that *c-rel* in different avian species is structurally more complex than *v-rel* in REV-T (7); that is, there is either a single complex *c-rel* gene or a *c-rel* gene family in chicken DNA. To differentiate between these two possibilities, a series of DNA subclones that included all 1.4 kbp of *v-rel* were made from different regions of REV-T and were used as hybridization probes in the experiments described below (Fig. 1). (The sequences in the 3' *v-rel* probe related to the helper virus, reticuloendotheliosis virus strain A, do not hybridize to uninfected chicken DNA [unpublished data].)

Restriction enzyme analysis of *c-rel* in chicken DNA. The *v-rel* hybridization probes hybridized strongly to several DNA fragments in chicken DNA digested with either *Eco*RI, *Bam*HI, or *Hind*III. After *Eco*RI digestion of chicken DNA,

5.5- and 1.9-kbp DNA fragments hybridized with the 5' *v-rel* (*Hind*III-*Eco*RI) probe, 21.0- and 9.0-kbp fragments hybridized with the middle *v-rel* probe, and 10.0- and 9.0-kbp fragments hybridized with the 3' *v-rel* probe (Fig. 1). With the exception of the 9.0-kbp DNA fragment, the DNA fragments detected by the different *v-rel* probes were different in size. The combined size of the five different DNA fragments was approximately 47 kbp. Similarly, *Bam*HI digestion of chicken DNA revealed 9.7- and 3.1-kbp *c-rel* DNA fragments with the 5' *v-rel* probe, 21.0- and 12.0-kbp fragments with the middle *v-rel* probe, and 21.0- and 5.0-kbp fragments with the 3' *v-rel* probe (Fig. 1). As described above for *Eco*RI digestion, five DNA fragments of different sizes were detected. The combined size of these fragments was approximately 50 kbp. *Hind*III digestion of chicken DNA also revealed the following *c-rel* DNA fragments: 4.0- and 0.5-kbp fragments with 5' *v-rel*; 6.5-, 2.5-, 1.7-, and 1.3-kbp fragments with middle *v-rel*; and 12.0- and 6.5-kbp fragments with 3' *v-rel* (Fig. 1). Six of the DNA fragments were different in size. The combined size of these fragments was approximately 28 kbp.

With the exceptions of the 9.0-kbp *Eco*RI DNA fragment, the 21.0-kbp *Bam*HI DNA fragment, and the 6.5-kbp *Hind*III DNA fragment, none of the DNA fragments generated by digestion with the three enzymes hybridized with more than one of the nonoverlapping *v-rel* probes. Therefore, each different *c-rel* DNA fragment could not represent a different member of a *c-rel* gene family. Thus, these results indicate either that at least five regions of homology to *v-rel* are distributed over approximately 28 to 50 kbp or more of chicken DNA or, alternatively, that different parts of *v-rel* are homologous to unlinked regions in chicken DNA.

Molecular cloning of *c-rel* (chicken). To determine more precisely the arrangement of the regions in chicken DNA homologous to *v-rel*, molecular clones of *c-rel* were obtained from chicken DNA. Four lambda recombinant clones were obtained by plaque hybridization from recombinant DNA libraries of line 15B chicken DNA (see above). Detailed restriction endonuclease cleavage maps of the chicken DNA insertions were made and compared with each other (Fig. 2). A total of 40 kbp of contiguous chicken DNA was cloned in overlapping DNA fragments of the four recombinant lambda clones.

Several lines of evidence indicate that the recombinant clones did not suffer gross alterations as a result of the cloning process. First, the regions of overlap between the clones were identical with respect to the positions of the restriction enzyme cleavage sites. Second, subclones were constructed from various regions of

FIG. 1. Hybridization of *v-rel* probes to line 15B chicken cellular DNA. Chicken DNA was prepared as described in the text. DNA (20 μ g) was digested with either *EcoRI*, *BamHI*, or *HindIII* and was subjected to electrophoresis in a 0.8% agarose gel. Chicken sequences homologous to each of the indicated *v-rel* probes were detected by hybridization as described in the text. The size of each of the DNA fragments is shown to the left of each autoradiogram. The positions of the restriction enzyme cleavage sites in REV-T DNA are shown below the autoradiograms. Only the *BamHI* site within *v-rel* is indicated. 5' and 3' refer to the orientation of the viral DNA with respect to the viral RNA. The numbers indicate the distances from the 5' end of viral DNA. The sources of the hybridization probes from REV-T DNA are indicated by the bars below the map of REV-T. The hybridization probes were made from subcloned DNA fragments of REV-T which were purified free from the plasmid vector before radioactive labeling. The sources of the REV-T DNA in each of the subclones were as follows: 5' *v-rel* (*HindIII*-*EcoRI*), infectious REV-T proviral recombinant clone (Charon 4A REV-T 52B) (Wilhelmsen, unpublished data); middle *v-rel*, variant REV-T proviral clone 2-20-4 (7); 3' *v-rel*, infectious REV-T unintegrated circular DNA clone (pREV-T 3) (7a). The break in the middle *v-rel* probe indicates a deletion of about 50 base pairs (7).

the clones and were used as hybridization probes with restriction enzyme-digested chicken DNA. In all cases, except when the DNA contained highly repetitive sequences (see below), the subclones hybridized to a DNA fragment in restriction enzyme-digested chicken DNA of the same size as the subcloned DNA fragment (data not shown). Third, cloned DNA fragments that contained regions homologous to *v-rel* were identical in size to the fragments detected by *v-rel* probes in chicken DNA (see below). Therefore, the *c-rel* clones represent an accurate copy of the *c-rel* region of chicken DNA.

The positions of regions of the clones having homology to *v-rel* were determined by hybridization, using [³²P]cDNA made to REV-T RNA. Seven regions of homology were located in the following fragments: a *Bgl*III-*Eco*RI DNA fragment at -1.0 to 1.0 kbp, a *Hind*III DNA fragment at 1.5 to 1.7 kbp, an *Eco*RI-*Hind*III DNA fragment at 6.5 to 6.9 kbp, a *Cl*aI-*Hind*III DNA fragment at 18.7 to 19.0 kbp, a *Hind*III-*Cl*aI DNA fragment at 19.0 to 20.6 kbp; a *Bgl*III-*Eco*RI DNA fragment at 22.6 to 23.6 kbp, and an *Eco*RI-*Hind*III DNA fragment at 23.5 to 28 kbp (Fig. 2). (See Addendum in Proof.) These results were confirmed by an electron microscopic heteroduplex analysis (Wilhelmsen, manuscript in preparation).

A comparison of the *c-rel* DNA fragments detected by hybridization of *v-rel* to chicken DNA (Fig. 1) and the cloned region of *c-rel* DNA indicated that the majority of the *c-rel* DNA fragments detected in cellular DNA were present in the clones. All of the fragments detected by the 5' *v-rel* (*Hind*III-*Eco*RI) and middle *v-rel* probes were present in the cloned region of *c-rel*. However, not all of the fragments detected by the 3' *v-rel* probe were present in the cloned region of *c-rel*. Among the DNA fragments detected by the 3' *v-rel* probe in chicken cellular DNA (Fig. 1), the 9.0-kbp *Eco*RI DNA fragment, the 21.0-kbp *Bam*HI DNA fragment, and the 6.5-kbp *Hind*III DNA fragment were present in the cloned region corresponding to the rightmost region of homology in *c-rel*. The other DNA fragments detected in chicken cellular DNA (a 10.0-kbp *Eco*RI DNA fragment, a 5.0-kbp *Bam*HI DNA fragment, and a 12.0-kbp *Hind*III DNA fragment) were not present in the cloned region of *c-rel*. Hybridization to these fragments was relatively weak, but reproducible.

Experiments designed to determine the locations or origins of these additional regions of *c-rel* were unsuccessful. These regions did not hybridize to the helper virus-related sequences present in the 3' *v-rel* probe (Fig. 1 shows the source of the 3' *v-rel* probe [data not shown]). Therefore, these sequences are *rel* specific. These sequences may be linked to the cloned

c-rel region, in which case they would be separated from the other regions of homology by at least 9 and 12 kbp at the left and right, respectively. Alternatively, they are not linked or represent weak homology to sequences distantly related to 3' *v-rel*.

Only one *Eco*RI fragment in turkey DNA was detected with the 3' *v-rel* probe, and the region homologous to *v-rel* in this turkey DNA fragment (which has been cloned) was conserved between chicken and turkey DNAs (Wilhelmsen, manuscript in preparation). This result most likely indicates that the 10-kbp *Eco*RI fragment in chicken DNA that was detected with the 3' *v-rel* probe is not linked to *c-rel* and is only distantly related to the 3' *v-rel* sequences.

Thus, these results demonstrate that the majority of the regions of *c-rel* that hybridize to *v-rel* are arranged contiguously in a single region of the chicken genome. The total size of this *c-rel* region is about 25 kbp. Seven regions separable by restriction enzyme digestion are homologous to *v-rel*. These seven regions of homology are separated by DNA sequences ranging in size from about 2 to 12 kbp. A DNA electron microscopic heteroduplex analysis indicated that the regions of homology at 23.0 and 26.0 kbp are not contiguous (Wilhelmsen, manuscript in preparation). The regions of homology separated by a single restriction enzyme cleavage site at 18.8 to 19.1 kbp may be contiguous. Therefore, *c-rel* has a minimum of six discontinuous regions of homology to *v-rel*.

Although the organization of *c-rel* is quite different from that of *v-rel*, the regions of *c-rel* homologous to *v-rel* appear to be arranged in the same orientation as the corresponding regions of *v-rel*; that is, 5' *v-rel*, middle *v-rel*, and 3' *v-rel* hybridize to DNA fragments from the left, middle, and right of *c-rel*, respectively (Fig. 2). (Note that 5' *v-rel* [*Hind*III-*Hind*III] contains approximately 0.1 kbp more of *v-rel* to the 3' side than 5' *v-rel* [*Hind*III-*Eco*RI]. This additional 0.1 kbp hybridizes to the next two leftmost fragments of *c-rel* having homology to *v-rel*.) Therefore, the left side of *c-rel* was designated the 5' end with respect to *v-rel*.

The single *Hind*III site in *v-rel* is located at 3.6 kbp in REV-T (Fig. 1). About 0.6 kbp of *v-rel* is located 5' to this *Hind*III site. This 0.6 kbp of *v-rel* (the total size of *v-rel* is 1.4 kbp) is homologous to four discontinuous regions in *c-rel* located 5' to the *Hind*III site at 19.0 kbp (Fig. 2, homology to 5' *v-rel* [*Hind*III-*Hind*III]). Therefore, the average size of these homologous regions in *c-rel* is less than 0.2 kbp. (This size was confirmed by an electron microscopic heteroduplex analysis [Wilhelmsen, manuscript in preparation].) Two of these regions of homology, at 7.0 and 19.0 kbp, are separated by a large region of chicken DNA about 12.0 kbp long.

FIG. 2. Restriction endonuclease cleavage map of the *c-rel* region of line 15B chickens. The complete restriction endonuclease cleavage map of *c-rel* is shown in the center. The four recombinant DNA clones of *c-rel* were obtained as described in the text. The chicken DNA insertions of each clone are indicated above the map of *c-rel*. The orientations of lambda arms are indicated (λ L or λ R) for each clone. The region that was not cloned extends to the left of the *Bgl*III site at ~ 1.0 kbp. The positions of the restriction enzyme cleavage sites were determined by identifying the unique patterns of fragments derived from digestion of the DNAs with different enzymes, singly and in combination. The regions of overlap between different clones aided in orientation of the *c-rel* clones relative to each other. The positions of the multiple *Hind*III sites between 7.0 and 16.6 kbp were mapped by partially digesting λ 1059 *c-rel* 4 DNA with *Hind*III, completely digesting with *Bam*HI, and subcloning the DNA to agarose gel electrophoresis. Hybridization with a subcloned *Hind*III DNA fragment (16.5 to 19.0 kbp; equivalent to end labeling) resulted in a pattern of hybridization which allowed determination of the position of sequential *Hind*III sites to the left of the *Bam*HI site at 16.5 kbp. The *Bam*HI, *Hind*III, and

EcoRI sites of the region to the left which was not cloned were determined by digestion of chicken cellular DNA and hybridization with 5' *v-rel* (data not shown). The DNA fragments of *c-rel* that contained sequences with homology to *v-rel* (solid boxes centered between restriction enzyme cleavage sites, except at 24 kbp, where the region of homology was mapped more precisely [Fig. 3]) were determined by hybridization with [³²P]cDNA_{REV-T}. The approximate relative amount of homology in each DNA fragment (indicated by the lengths of the solid boxes) was determined by densitometry tracings of autoradiograph exposures. The positions of some restriction enzyme cleavage sites and some regions of homology to *v-rel* were confirmed by subcloning the following DNA fragments: *BamHI-EcoRI* DNA fragment (4.5 to 6.5 kbp) *EcoRI-HindIII* DNA fragment (6.5 to 6.9 kbp); *HindIII* DNA fragment (6.9 to 10.7 kbp); *HindIII* DNA fragment (15.0 to 16.3 kbp); *HindIII* DNA fragment (16.3 to 19.0 kbp); *HindIII* DNA fragment (19.0 to 20.5); *HindIII-EcoRI* DNA fragment (21.6 to 23.5 kbp); *BamHI-EcoRI* DNA fragment (17.0 to 23.5 kbp); *EcoRI-HindIII* DNA fragment (23.5 to 28.0 kbp); *HindIII-BamHI* DNA fragment (35.7 to 37.6 kbp); and *EcoRI-BamHI* DNA fragment (36.7 to 37.6 kbp). The DNA fragments containing highly repetitive chicken DNA are indicated by r. The *v-rel* probes 5' *v-rel* (*HindIII-EcoRI*), middle *v-rel*, and 3' *v-rel* are described in the legend to Fig. 1. The 5' *v-rel* (*HindIII-HindIII*) probe included all of the DNA of the 5' *v-rel* (*HindIII-EcoRI*) probe plus an additional 0.1 kbp farther 3' in *v-rel*, up to the *HindIII* site at 3.6 kbp (see map of REV-T in Fig. 1). The DNA fragments of *c-rel* having homology to the different *v-rel* probes are indicated below the map of *c-rel*. (The homology between the 5' *v-rel* [*HindIII-HindIII*] DNA and the *c-rel* DNA at -1.0 to 2.2 kbp was not directly tested.) The orientation of *c-rel* with respect to *v-rel* in REV-T is indicated by the designation of 5' and 3' ends. Map position 0.0 was defined as the 5'-most sequence of *c-rel* having homology to *v-rel*.

A total of 0.8 kbp of *v-rel* is present 3' to the *HindIII* site in *v-rel*. The sequences in this region, which comprises more than one-half of *v-rel*, are homologous to regions within the 3' one-fifth of *c-rel*. Most of the homology is present within two regions of *c-rel*, which are located at 23.0 and 24.5 kbp. A DNA electron microscopic heteroduplex analysis confirmed this arrangement of homologous regions (Wilhelmsen, manuscript in preparation).

c-rel DNA fragments in the cloned region containing highly repetitive chicken DNA sequences were determined by hybridization, using radioactively labeled chicken DNA. Four repetitive regions were located at about 15.0, 20.0, 36.0, and 38.0 kbp (Fig. 2, regions r).

Expression of *c-rel* in chicken spleen cells. The structure of *c-rel* resembles that of a cellular gene having exons and introns. The large regions of *c-rel* that separate the regions homologous to *v-rel* may be intervening sequences with respect to *c-rel* mRNA. In that case, *c-rel* mRNA would be colinear to *v-rel* sequences in REV-T. Alternatively, the regions of homology to *v-rel* may differ from the exons for *c-rel*. To determine whether any of the regions in *c-rel* DNA that are not homologous to *v-rel* DNA are present in *c-rel* mRNA, RNA preparations from chicken cells were hybridized with DNA probes from different regions of cloned *v-rel* and *c-rel*.

Preliminary experiments showed that the levels of RNA homologous to *v-rel* in chicken hemopoietic tissue were two- to threefold higher than the levels in muscle or fibroblasts (data not shown). Levels of RNA similar to the level in hemopoietic tissue were present in a Marek's virus-transformed cell line, RP-4 (30), and a lymphoid leukemia cell line, RP-13 (30). A 4.0-kilobase (kb) polyadenylic acid-containing RNA species was detected in chicken spleen cells by using the 5' *v-rel* and middle *v-rel* hybridization probes (Fig. 3). The 3' *v-rel* hybridization probe did not detect any RNA species, possibly because of the small region (less than 250 base pairs) of *v-rel* in this probe. Occasionally, in some RNA preparations a larger species (6.0 kb) was detected in addition to the 4.0-kb species. However, the 4.0-kb species was the only species consistently detected in several different preparations of RNA. Presumably, the 4.0-kb species is the mRNA for *c-rel*. The 6.0-kb species may be a precursor RNA.

Since the *v-rel* region in REV-T is 1.4 kbp long, it is apparent that the combined size of *c-rel* exons (about 4.0 kbp, as determined from the size of *c-rel* mRNA) is about 2.6 kbp larger than *v-rel*. To determine the positions of the exons in *c-rel* DNA that are not represented in *v-rel*, chicken polyadenylic acid-containing RNA was hybridized with DNA probes from dif-

FIG. 3. Hybridization of *v-rel* and *c-rel* DNA fragments to chicken spleen polyadenylic acid-containing RNA. Polyadenylic acid-containing RNA was prepared from chicken spleen cells as described in the text. RNA (25 μ g) was subjected to gel electrophoresis in parallel lanes (except lanes i and j, which were from a separate gel). Specific RNA species were detected by hybridization with the specific DNA probes indicated. The source of the DNA probe for each lane is indicated on the restriction enzyme cleavage site maps of REV-T and *c-rel* below the autoradiographs. The three *v-rel* probes correspond to the 5' *v-rel* (*Hind*III-*Eco*RI), middle *v-rel*, and 3' *v-rel* probes described in the legend to Fig. 1. The map of REV-T is shown as a permuted sequence about the *Sall* site that is 0.86 kbp from the 5' end of viral DNA (Fig. 1). Only part of the cloned region of *c-rel* is shown in the map of *c-rel*. The positions of the *Xba*I sites were determined only for the *Eco*RI-*Hind*III DNA fragment at 23.5 to 28.5 kbp. r indicates the presence of repetitive chicken DNA. S, *Sall*; H, *Hind*III; R, *Eco*RI; B, *Bam*HI; X, *Xba*I.

ferent regions of *c-rel* (Fig. 3). Some of these DNA probes contained sequences homologous to *v-rel*. Others were entirely nonhomologous to *v-rel*.

No detectable hybridization to RNA was observed with any of the *c-rel* DNA fragments that were from 5.0 to 10.7 kbp on the *c-rel* map (Fig. 3). Neither fragments which contained homology to *v-rel* nor fragments from apparent intervening regions hybridized. This result indicates that regions of homology to *c-rel* mRNA, if present at all, are too small to give detectable hybridization. (The lack of hybridization obtained with fragments having homology to *v-rel* might have been a result of the small size of the regions of homology to *v-rel*.)

The *c-rel* *Hind*III-*Hind*III, *Hind*III-*Eco*RI, and *Eco*RI-*Hind*III DNA fragments at 16.3 to 19.0, 21.6 to 23.5, and 23.5 to 28.0 kbp, respectively, hybridized to a 4.0-kb RNA species.

(DNA fragments at 15.0 to 16.3 and 19.0 to 20.5 kbp were not tested due to the presence of repetitive sequences.) These DNA fragments contained relatively large regions of homology to *v-rel*. Therefore, the hybridization to *c-rel* mRNA could be attributed to this region of homology. However, the intensity of hybridization with the *Eco*RI-*Hind*III DNA fragment (23.6 to 28.0 kbp) was much greater than expected based on the relative amount of homology to *v-rel* (data not shown), indicating possible homology between regions of this fragment not homologous to *v-rel*.

This possibility was tested by further dividing the *Eco*RI-*Hind*III DNA fragment into regions without homology to *v-rel*. Two *Xba*I sites (25.0 and 27.2 kbp) were mapped in this *Eco*RI-*Hind*III DNA fragment. All of the homology to *v-rel* was located between the *Eco*RI site and the next *Xba*I site to the right (23.5 to 25.0 kbp) (Fig.

3). Regions of *c-rel* farther to the right of the *Xba*I site at 25.0 kbp did not hybridize to *v-rel*, delimiting the 3' end of *v-rel* (data not shown). When the 25.0- to 27.2-kbp *Xba*I fragment was used for hybridization to chicken RNA, a 4.0-kb RNA species, which was the same size as the species detected by *v-rel* and other regions of *c-rel*, was detected (Fig. 3). Thus, this 3' region of *c-rel*, although not present in *v-rel*, was transcribed into *c-rel* mRNA and, therefore, represents an exon of *c-rel* not present in *v-rel*.

DISCUSSION

Structural relationship between *c-rel* and *v-rel*. The structural relationship between *c-rel* in line 15B chickens and *v-rel* in REV-T is similar to the relationships between most *v-onc* genes and their cellular *c-onc* homologs (8). *v-rel* is 1.4 kbp long (Wilhelmsen, unpublished data), whereas in *c-rel* the regions of homology to *v-rel* span about 25 kbp of chicken DNA. A minimum of six regions of homology to *v-rel* are separated by five apparent introns. The largest apparent intron is about 12.0 kbp long.

Despite the dissimilarity in size between *c-rel* and *v-rel*, the regions in *c-rel* that are homologous to *v-rel* appear to be in the same order as in *v-rel*. Similar observations have also been made for the order of other *v-onc* genes and their respective *c-onc* counterparts (3, 9–11, 18–21, 23, 26, 29).

In addition, there may be some restriction enzyme cleavage sites in common between *v-rel* and *c-rel*. The *Hind*III site in *c-rel* at 19.0 kbp is likely to be conserved in *v-rel* at 3.6 kbp (on the map of REV-T), since sequences 5' and 3' of the *Hind*III site in *v-rel* are homologous to sequences 5' and 3', respectively, of the *Hind*III site in *c-rel*. A DNA heteroduplex analysis is being performed to confirm this possibility. The *Eco*RI site at 6.5 kbp and the *Cla*I site at 18.7 kbp in *c-rel* may also be present in *v-rel*, since these sites are present at analogous positions in the two sequences.

However, several enzyme sites in *v-rel* are not present in *c-rel*. The *Bam*HI site at 4.1 kbp and the *Eco*RI site at 4.3 kbp (on the map of REV-T in Fig. 1) in *v-rel* are not present in *c-rel*, since *c-rel* contains no *Bam*HI or *Eco*RI sites at the corresponding positions.

v-rel was probably derived from *c-rel* in turkeys (7, 31). Therefore, the restriction enzyme cleavage site differences between chicken *c-rel* and *v-rel* could represent differences between chicken *c-rel* and turkey *c-rel*. However, these enzyme sites in *v-rel* are also not present in *c-rel* from turkeys (Wilhelmsen and Temin, manuscript in preparation), indicating divergence between the *c-rel* and *v-rel* sequences rather than divergence of *c-rel* sequences in chickens and

turkeys. Alternatively, the sites could have been created by the splicing of small exons. DNA sequencing will distinguish between these possibilities.

Most *c-onc* sequences appear to be conserved in the genomes of many vertebrate species that are widely divergent from the species from which the oncogenic virus originated. The organization of *c-rel* in turkeys is very similar to the organization described here for chickens with regard to the distribution and organization of regions of homology to *v-rel* (Wilhelmsen, manuscript in preparation). In addition, the structure of *c-rel* is also similar in other avian species (7). However, unlike most other *c-onc* sequences, *c-rel* appears not to be highly conserved in nonavian species (7, 31).

One particularly interesting relationship is that between *c-rel* and *c-abl*, the cellular counterpart of the murine Abelson leukemia virus oncogene. There is no nucleic acid homology between *v-abl* and *v-rel* (7, 31), yet Abelson virus and REV-T cause lymphoid neoplasia in mice and chickens (1, 27), respectively, and the structures of the cellular genes are remarkably similar (3). Both genes are distributed over more than 20 kbp of DNA, with multiple large apparent introns. In both genes, a major portion of the homology between the *v-onc* and *c-onc* sequences is located at the 3' end. This similarity of structure between *c-abl* and *c-rel* and the similarity of function between *v-abl* and *v-rel* may indicate a common function for *c-rel* and *c-abl* in chickens and mice, respectively.

Expression of *c-rel* in chicken cells. RNA sequences homologous to *v-rel* are present at low levels in all chicken tissues which have been tested (data not shown). However, the levels of RNA are slightly higher (two- to threefold) in hemopoietic tissue than in other tissues. The significance of this observation is unclear, although it may be related to the postulated role of the expression of *c-onc* sequences in normal differentiation (4).

The mRNA of *c-rel* appears to be 4.0 kb long. This mRNA is about 2.6 kb larger than *v-rel*. We identified an exon at the 3' end of *c-rel* which is not present in *v-rel* and may represent the DNA transcribed to give the additional 2.6 kb. Many eucaryotic mRNAs have 3' untranslated regions (17), and this region of *c-rel* may represent such a sequence. There also could be regions 5' to the *c-rel* sequences which are transcribed. It is possible that these additional sequences are involved in the control of *c-rel* expression or, alternatively, they may encode additional peptides. The sequences 3' to *c-rel* are also likely to contain signals for the termination of *c-rel* RNA transcription. Since termination signals prevent the formation of infectious virus (25), removal of

such sequences was probably necessary for the formation of infectious REV-T. Experiments are in progress to determine whether these sequences have any effect on the transforming potential of REV-T by constructing a recombinant virus that contains *v-rel* plus the additional sequences from the 3' end of *c-rel*.

The large regions in *c-rel* separating regions of homology to *v-rel* do not appear to be expressed as stable RNA, since we were unable to detect RNA transcripts by using DNA probes from parts of these regions. However, the possible existence of RNA transcripts having only small regions of homology to our probes or RNA transcripts from other regions not represented in our probes cannot be excluded.

In summary, the evolution of the oncogenic virus REV-T from *c-rel* involved many steps. From the experiments described here, the minimum events required to generate *v-rel* from *c-rel* involved recombination between helper virus and *c-rel*, splicing of apparent large introns, perhaps analogous to splicing of α -globin introns in retrovirus construction (25a), base pair mutations, and deletion of at least part of an exon from *c-rel*.

ACKNOWLEDGMENTS

We thank Virginia Goiffon, Susan Hellenbrand, and Hwey Yang for excellent technical assistance and M. Emerman, J. Mertz, C. Miller, R. Risser, T. Schedl, and B. Sugden for helpful comments on the manuscript.

This investigation was supported by Public Health Service research grants CA-07175 and CA-22443 from the National Cancer Institute. K.C.W. was supported by Public Health Service training grant CA-09135 from the National Cancer Institute. H.M.T. is an American Cancer Society Research Professor.

ADDENDUM IN PROOF

Since the submission of this manuscript, we have learned that the 1.9-kbp *EcoRI* fragment detected in genomic chicken DNA sequences with the 5' *v-rel* probe (see Fig. 1) also hybridizes to a *c-rel HindIII/HindIII* fragment (1.7 to 2.4 kbp in Fig. 2). This result indicates that a portion of *c-rel* is duplicated in the chicken genome. Furthermore, we have learned that the *BglIII* to *EcoRI* portion of λ 1059 *c-rel* 5' (-1.0 to 1.0 kbp in the *c-rel* map, Fig. 2) does not represent a portion of the 1.9-kbp *EcoRI* fragment detected in genomic chicken sequences. We believe these results indicate that there is a second locus in chicken DNA with a partial duplication of the *c-rel* sequences.

LITERATURE CITED

1. Abelson, H. T., and L. S. Rabstein. 1970. Influence of prednisolone on Moloney leukemogenic virus in BALB/c mice. *Cancer Res.* 30:2208-2212.
2. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. U.S.A.* 69:1408-1412.
3. Baltimore, D., J. Wang, S. Goff, and C. Queen. 1982. Origin, structure and function of the transforming region of the Abelson murine leukemia virus. *J. Cell. Biochem. (Suppl.)* 6:189.
4. Bishop, J. M. 1981. Enemies within: the genesis of retrovirus oncogenes. *Cell* 23:5-6.
5. Blattner, F. R., B. G. Williams, A. E. Blechl, K. Denniston-Thompson, H. E. Faber, L. A. Furlong, D. J. Grunwald, D. O. Kiefer, D. O. Moore, J. W. Schumm, E. I. Sheldon, and O. Smithies. 1977. Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. *Science* 196:161-169.
6. Breitman, M. L., M. M. C. Lai, and P. K. Vogt. 1980. The genomic RNA of avian reticuloendotheliosis virus REV. *Virology* 100:450-461.
7. Chen, I. S. Y., T. W. Mak, J. J. O'Rear, and H. M. Temin. 1981. Characterization of reticuloendotheliosis virus strain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. *J. Virol.* 40:800-811.
- 7a. Chen, I. S. Y., and H. M. Temin. 1982. Substitution of 5' helper virus sequences into non-*rel* portion of reticuloendotheliosis virus strain T suppresses transformation of chicken spleen cells. *Cell* 31:111-120.
8. Coffin, J. M. 1982. Structure of the retroviral genome, p. 261-368. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *The molecular biology of RNA tumor viruses*, 2nd ed. RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. Dalla Favera, R., E. P. Gelmann, R. C. Gallo, and F. Wong-Staal. 1981. A human *onc* gene homologous to the transforming gene (*v-sis*) of simian sarcoma virus. *Nature (London)* 292:31-35.
10. Ellis, R. W., D. DeFeo, T. Y. Shih, M. A. Gonda, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick. 1981. The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. *Nature (London)* 292:506-511.
11. Franchini, G., J. Even, C. J. Sherr, and F. Wong-Staal. 1981. *onc* sequences (*v-fes*) of Snyder-Theilen feline sarcoma virus are derived from noncontiguous regions of a cat cellular gene (*c-fes*). *Nature (London)* 290:154-157.
12. Fritsch, E., and H. M. Temin. 1977. Formation and structure of infectious DNA of spleen necrosis virus. *J. Virol.* 21:119-130.
13. Gonda, M. A., N. R. Rice, and R. V. Gilden. 1980. Avian reticuloendotheliosis virus: characterization of the high-molecular-weight viral RNA in transforming and helper virus populations. *J. Virol.* 34:743-751.
14. Hoelzer, J. D., R. B. Lewis, C. R. Wasmuth, and H. R. Bose. 1980. Hematopoietic cell transformation by avian reticuloendotheliosis: characterization of the genetic defect. *Virology* 100:462-474.
15. Holmes, D. S., and J. Bonner. 1973. Preparation, molecular weight, base composition and secondary structure of giant nuclear ribonucleic acid. *Biochemistry* 12:2330-2338.
16. Karn, J., S. Brenner, L. Barnett, and G. Cesareni. 1980. Novel bacteriophage λ cloning vector. *Proc. Natl. Acad. Sci. U.S.A.* 77:5172-5176.
17. Nurnberg, J. H., R. J. Kaufman, A. C. Y. Chang, S. N. Cohen, and R. T. Schimke. 1980. Structure and genomic organization of the mouse dihydrofolate reductase gene. *Cell* 19:355-364.
18. Oskarsson, M., W. L. McClements, D. G. Blair, J. V. Maizel, and G. F. Vande Woude. 1980. Properties of a normal mouse cell DNA sequence (*sarc*) homologous to the *src* sequence of Moloney sarcoma virus. *Science* 207:1222-1224.
19. Parker, R. C., H. E. Varmus, and J. M. Bishop. 1981. Cellular homologue (*c-src*) of the transforming gene of Rous sarcoma virus: isolation, mapping, and transcriptional analysis of *c-src* and flanking regions. *Proc. Natl. Acad. Sci. U.S.A.* 78:5842-5846.
20. Perbal, B., and M. A. Baluda. 1982. Avian myeloblastosis virus transforming gene is related to unique chicken DNA

- regions separated by at least one intervening sequence. *J. Virol.* **41**:250-257.
21. Robins, T., K. Bister, C. Garon, T. Papas, and P. Duesberg. 1982. Structural relationship between a normal chicken DNA locus and the transforming gene of the avian acute leukemia virus MC29. *J. Virol.* **41**:635-642.
22. Ross, J. 1976. A precursor of globin messenger RNA. *J. Mol. Biol.* **106**:403-420.
23. Shalloway, D., A. D. Zelenetz, and G. M. Cooper. 1981. Molecular cloning and characterization of the chicken gene homologous to the transforming gene of Rous sarcoma virus. *Cell* **24**:531-541.
24. Shimotohno, K., S. Mizutani, and H. M. Temin. 1980. Sequence of retrovirus provirus resembles that of bacterial transposable elements. *Nature (London)* **285**:550-554.
25. Shimotohno, K., and H. M. Temin. 1981. Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell* **26**:67-77.
- 25a. Shimotohno, K., and H. M. Temin. 1982. Loss of intervening sequences in genomic mouse α -globin DNA inserted in an infectious retrovirus vector. *Nature (London)* **299**:265-268.
27. Theilen, G. H., R. F. Zeigel, and M. J. Twiehaus. 1966. Biological studies with RE virus (strain T) that induces reticuloendotheliosis in turkeys, chicken, and Japanese quail. *J. Natl. Cancer Inst.* **37**:731-743.
28. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* **77**:5201-5205.
29. Vennstrom, B., and J. M. Bishop. 1982. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell* **28**:135-143.
30. Witter, R. L. 1979. A proposed method for designating avian cell lines and transplantable tumours. *Avian Pathol.* **8**:487-498.
31. Wong, T. C., and M. M. C. Lai. 1981. Avian reticuloendotheliosis virus contains a new class of oncogene of turkey origin. *Virology* **111**:289-293.