Characterization of a Novel Promoter Insertion in the *c-rel* Locus

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Avian leukosis virus (ALV)-induced neoplasias are commonly found associated with integrations of proviral DNA in proximity to the *myc* gene. However, studies suggest that other genetic events are necessary for the complete neoplastic phenotype. A cell line (HP46) derived from an ALV-induced tumor has been analyzed and found to contain, in addition to an alteration in the *myc* gene, a promoter insertion in the *c-rel* locus. Both loci expressed large amounts of mRNA coding for their respective proteins. Several *rel*-related transcripts were expressed in the HP46 line, and four *rel*-related proteins of lower molecular weight than the wild-type p68^{c-rel} product were detected. At least two of these transcripts contained U5 long terminal repeat sequences on the 5' end of the RNA. Structural data suggest that the messages may have evolved by an alternative splicing mechanism. This is the first example of a promoter insertion in the *c-rel* locus, a gene whose viral counterpart *v-rel* is responsible for the induction of lymphoid tumors.

Cellular transformation and tumor formation are complex phenomena associated with multiple genetic alterations. These alterations can be elicited by a variety of agents, including retroviruses, which can initiate tumor formation by two predominant mechanisms. Retroviruses containing oncogenes (acute retroviruses) are able to transform cells and initiate tumor formation in vivo within a short latency period. Alternatively, retroviruses without oncogene sequences (nonacute retroviruses) initiate neoplastic disease by inserting proviral DNA in the host genome and altering the expression of adjacent cellular genes. Both mechanisms have been demonstrated in the induction of lymphoid tumors in chickens (for review, see reference 10).

One of the most potent acute retroviruses is reticuloendotheliosis virus strain T (REV-T), which causes fatal lymphomas within days after infection (25). Tumor induction by REV-T is the direct result of the expression of its oncogene *v-rel* (4), a recombinant between viral envelope and cellular *rel* sequences (5, 35, 42). The *c-rel* gene, encoding a protein of 68 kilodaltons (kDa), is expressed at highest levels in lymphoid tissue—particularly in bursa, spleen, and thymus (20, 21, 34). Overexpression of the *c-rel* gene is apparently insufficient to cause transformation (3, 17), suggesting that the mutations found in *v-rel* are necessary to convert *c-rel* to a fully oncogenic form. The function of both the viral and cellular *rel* proteins is unknown; however, several observations on the *rel* protein may give insight into its function. For example, the *v-rel* protein has been demonstrated to transactivate gene expression from a variety of promoters, perhaps by indirect mechanisms (11, 12, 17). Additionally, *rel* has an associated serine-threonine kinase activity (27, 40). Recently, both viral and cellular *rel* proteins have been demonstrated to associate with at least three other uncharacterized proteins, and thus it is possible that these cellular gene products are mediating the functions described (21, 33, 39). The *rel* genes are not highly related to other identified oncogenes; however, they are homologous to the *Drosophila melanogaster* gene *dorsal*, a gene required for proper dorsal-ventral axis formation in the developing embryo (29, 30, 36, 37).

The second mechanism by which avian retroviruses initiate neoplastic disease was elucidated by analysis of tumors resulting from infection with the nonacute avian leukosis viruses (for review, see G. Peters, in D. M. Glover and B. D. Hames, ed., *Frontiers in Molecular Biology*, in press). Unlike acute retroviruses, these viruses do not carry oncogenes. However, like all other retroviruses, they have the ability to integrate into host chromosomes. As proviral sequences contain both transcriptional enhancer and promoter elements, integration events can potentially alter gene expression and regulation. Analysis of B-cell lymphomas induced by avian leukosis virus (ALV) showed that integration near the *c-myc* locus occurred at a very high frequency, leading to high levels of transcription from this cellular oncogene (13, 14, 28). The diseases initiated by these agents usually have an extended latency, promoting the idea that secondary mutations are necessary for disease progression. For example, integration at the *myc* locus is thought to represent an early event, resulting in the proliferation of a bursal stem cell (22, 38). This hyperplastic state progresses at a later stage to a frank neoplasia characterized by tumorigenic cells that are often clonally derived. The nature of the events required for progression to the neoplastic state is unknown, but it is possible that other oncogenes cooperate with the primary deregulated gene (*myc*) to potentiate the fully transformed phenotype. Several examples have been described in which two loci (e.g., *c-myc* and *pim-1*; *c-myc* and *c-bic*; *int-1* and *int-2*) are disrupted by promoter insertions, reinforcing the idea that multiple gene products might act in concert to generate the transformed phenotype (4, 7, 32).

In this article we describe a cell line (HP46) which we demonstrate contains a novel promoter insertion in the *c-rel* locus and additionally an altered *myc* locus. Although lymphoid tumors arise at high frequency in chickens infected with retroviruses containing the *v-rel* oncogene, a deregulated *c-rel* locus has not been demonstrated previously in lymphoid tumors initiated by nonacute viruses. We present data indicating that an ALV insertion has deregulated the expression of the *c-rel* locus in both a qualitative and quantitative manner. High levels of altered *rel*-specific RNA are expressed in this cell line, leading to the synthesis of high levels of novel *rel*-related proteins. In addition, *myc*-specific

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mRNA and protein are overexpressed. The finding of overexpression of both *rel* and *myc* in this cell line suggests synergy between these two oncogenes in the development of this lymphoid tumor.

MATERIALS AND METHODS

Cells. The HP46 and HP50 cell lines were derived from line 151 birds inoculated intra-abdominally at day 1 with 3×10^4 infectious units of HPRS (Houghton Poultry Research Station) F42 A subgroup lymphoid leukosis virus. Moribund birds were killed 7 months after infection. HP46 and HP50 cells were isolated from tumor-containing liver and spleen, respectively. The REV-T-transformed lymphoid cell line NPB4 was derived as described previously (2). HP46, HP50, and NPB4 cell lines were all grown in Dulbecco modified Eagle (DME) medium supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum, 100 U of penicillin per ml, and 100 μ g of streptomycin (GIBCO Laboratories) per ml at 37°C. To obtain normal B cells, bursae were removed from 4- to 7-day-old chickens (Spafas, Norwalk, Conn.; specific pathogen free), forced through a fine-wire mesh, and washed with DME medium. To enrich for lymphocytes, this mixture was then placed on a Ficoll-Paque (Pharmacia) cushion and centrifuged at $500 \times g$ for 10 min. Cells at the interface were then isolated and washed with phosphate-buffered saline. These cells were subsequently used for RNA extraction. Chicken embryo fibroblasts (CEF) were prepared and cultured as described previously (9).

Nucleic acid extraction and analysis. Total cellular RNA (bursae and CEF) or cytoplasmic RNA (HP46 and HP50) was extracted from cells essentially as described before (19) and run on denaturing formaldehyde gels (5 μ g per lane) or poly(A) selected for use in cDNA library construction. Probes used in Northern (RNA blot) and Southern analyses were as follows: *rel*, *XbaI-NruI* fragment from the plasmid pREV-T (ATCC no. 45011), which encompasses the whole *v-rel* gene; *myc*, *PstI* fragment from the plasmid *pmycPst-1*, which contains the viral *myc* gene; long terminal repeat (LTR), U5 and primer-binding site (*EcoRI-SacI* fragment) from the vector pRCAS, which contains U5 and primer-binding sequences (4, 9, 16). High-molecular-weight DNA extractions and Northern and Southern blots were performed as described before (19) except that prehybridization and hybridization were carried out in $3 \times$ SSC–50% formamide– $5 \times$ Denhardt solution–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0)–0.2% sodium dodecyl sulfate (SDS)–100 μ g of salmon sperm DNA per ml ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate; $1 \times$ Denhardt solution is 0.01% polyvinylpyrrolidone–0.01% Ficoll–0.01% bovine serum albumin). Blots were washed at 55 to 65°C in $1 \times$ SSC–0.1% SDS, followed by another 30-min wash in $0.1 \times$ SSC–0.1% SDS.

cDNA library construction and sequencing. cDNA was constructed by using Statagene's ZAP cDNA Synthesis kit and packaged by using Statagene's Gigapack 2 Gold packaging extracts. *rel*-hybridizing cDNA clones were sequenced with Pharmacia's T7 sequencing kit. Libraries were screened by using the *v-rel* fragment (*XbaI-NruI*) or 5' *v-rel* fragment (*XbaI-HindIII*) of pREV-T. All protocols were performed as recommended by the manufacturers.

Immunoprecipitation. Equal numbers of cells (10^6) were labeled and immunoprecipitated as described previously (21). Antibody reactive to the *rel* protein was made by immunizing rabbits with bacterially expressed Rel-TrpE

fusion protein (the *BamHI-PstI* fragment from the *v-rel* gene cloned into a bacterial expression vector containing the *trpE* gene, pJH12, kindly provided by Richard Schwartz [31]). Monoclonal antibody M4 against avian immunoglobulin M (IgM) was kindly provided by M. D. Cooper (6). Protein analysis was performed by SDS-polyacrylamide gel electrophoresis as described previously (18).

RESULTS

Derivation and characterization of HP46 and HP50 cell lines. The cell lines HP46 and HP50 were derived from line 151 chickens infected at day 1 with the ALV F42 (Houghton Poultry Research Station strain, subgroup A). Chickens were injected intra-abdominally with 3×10^4 infectious units of F42 virus and were reared in isolation. Birds which appeared ill were killed 7 months after infection and autopsied to determine whether tumors were present. In the case of birds HP46 and HP50, tumors were found in the liver and spleen, respectively. Cells isolated from these organs were adapted to cell culture and characterized in more detail.

In order to determine whether the cells isolated from the HP46 and HP50 birds were of lymphoid origin, the cells were analyzed by immunofluorescence with antibodies that were reactive to B and T cells (23, 26). Both HP46 and HP50 cells were found to be reactive with B-cell-specific antiserum but not to T-cell-specific antiserum. These results suggested that the HP46 and HP50 cells are of a B-lymphoid lineage. Preliminary evidence, however, suggests that these cells do not have rearranged immunoglobulin genes (J. C. Weill, personal communication), nor was it possible to detect IgM expression by immunoprecipitation of the cells with a monoclonal antibody against avian IgM, M4 (data not shown). This suggests that HP46 and HP50 cells are early lymphoid cells or that they represent another cell type aberrantly expressing epitopes that are reactive to the bursal cell antiserum. Further analyses are under way to phenotypically characterize these cells more thoroughly.

Immunoprecipitations. In the course of screening a variety of cell lines for the level of expression of the *c-rel* gene, HP46 cells were labeled with [35 S]methionine and lysates were immunoprecipitated with polyclonal antisera directed toward the *v-rel* oncogene (see Materials and Methods). As a positive control, a REV-T-transformed cell line expressing the *v-rel* oncogene, NPB4 cells, was immunoprecipitated with anti-*rel* antibody. As can be seen in Fig. 1, a predominant 59-kDa protein, corresponding to $p59^{v-rel}$, was precipitated from NPB4 cells by immune serum. Additionally, $p68^{c-rel}$ was found at relatively low levels compared with $p59^{v-rel}$ (21, 34). Proteins of 36, 115, and 124 kDa were also coimmunoprecipitated and have previously been shown to exist in a high-molecular-weight complex with $p68^{c-rel}$ and $p59^{v-rel}$ (21). In contrast, in the HP46 cell line, proteins of approximately 50, 56, 58, and 62 kDa were observed upon precipitation with the anti-Rel antibody. Immunoprecipitation of these four proteins could be blocked by the fusion protein used to raise this anti-Rel serum, but not by another fusion protein containing a nonoverlapping Rel epitope (31) (data not shown). In order to determine the relationship of these proteins to one another, partial proteolytic cleavage with V8 protease was carried out on the four Rel-related proteins from HP46 cells. The HP46 proteins were found to be highly related to one another, sharing several proteolytic cleavage products (data not shown). In addition, they were clearly immunologically related to Rel.

Interestingly, it was not possible to detect the normal *c-rel*

FIG. 1. Immunoprecipitation of cell lines with *rel* antiserum. Cell lines indicated (HP46 and NPB4) were immunoprecipitated with antiserum (lanes 1) directed towards the Rel protein (lanes 2 and 4). Immunoprecipitation with preimmune serum (P) is indicated in the odd-numbered lanes (lanes 1 and 3). Samples were run on 7.5% SDS-polyacrylamide gels. Numbers at the sides of the lanes indicate approximate molecular mass (in kilodaltons).

product in the HP46 cell line, a result that has been confirmed by Western immunoblot analysis (data not shown) and Northern blot analysis (see below). Several other proteins were seen in the immunoprecipitation of the HP46 cells with anti-Rel antibody. One was the 36-kDa protein found routinely in immunoprecipitates of HP46 cells and comigrated with the p36 protein found previously to be in complex with p59^{v-rel}. The other two smaller proteins were not seen reproducibly and most likely represent proteolytic cleavage products recognized by the antibody. In the higher-molecular-weight range, proteins which were slightly larger than the p115-p124 complex proteins were also detected at very low levels. We are currently characterizing the interactions of the HP46 proteins with other cellular proteins in more detail. Thus, it appears that the HP46 proteins precipitated by the anti-Rel antibody are different in size than the normal *c-rel* product and are coprecipitated with only one of the three proteins (p36) normally associated with p68^{c-rel}.

These results, taken together, suggested to us that the *c-rel* locus in the HP46 cell line was altered in some fashion, since aberrant expression of several structurally similar, Rel-related proteins was observed. Alterations in cellular oncogene loci have been shown to arise by amplification, rearrangement, or, in the case of ALV infection, by integration of the ALV provirus in the vicinity of a cellular oncogene locus. Therefore, we wanted to examine the structure of this locus and its transcripts to determine the nature of the alteration at the *c-rel* locus in the HP46 cell line.

Southern blot analysis. Alterations in cellular oncogene loci can frequently be detected by performing Southern blot analysis of genomic DNA derived from tumor cells and normal control cells. To determine whether the *rel* locus was altered in HP46 cells, high-molecular-weight DNA from HP46 cells, HP50 cells (a cell line derived from a bird from the same experiment which had been adapted to culture in a manner equivalent to HP46 cells), and normal line 151 CEF was isolated and digested with *Eco*RI. Following gel electrophoresis and transfer to nitrocellulose, the filters were hybridized to three specific probes (see Materials and Methods). A *rel* probe which included the entire *v-rel* gene was

FIG. 2. Genomic Southern analysis of high-molecular-weight DNA isolated from HP46, HP50, and line 151 CEF. Genomic DNA digested with *Eco*RI was hybridized to a *rel* probe (lanes 1 to 3), LTR probe (lanes 4 to 6), or *myc* probe (lanes 7 to 9) (see Materials and Methods for description of probes used). Sizes of lambda *Hind*III markers are indicated on the side of the panel (in kilobase pairs).

used to detect alterations in the *rel* locus. Because ALV is found integrated near the *c-myc* gene at high frequency in ALV-induced lymphomas, the digests were also analyzed with a *myc* probe. Finally, a probe specific for the LTR region (U5 and primer-binding site) was used in order to determine whether any of the alterations were associated with viral sequences. Namely, we wanted to determine whether a proviral integration had occurred in the *c-rel* locus.

As can be seen in Fig. 2, compared with normal line 151 CEF DNA (lanes 3 and 9), HP50 (lanes 2 and 8) appeared to have unaltered *rel* and *myc* loci. In contrast, the DNA from the HP46 line contained novel *rel*- and *myc*-hybridizing fragments, as shown by the presence of a 3.2-kilobase (kb) band in the blot hybridized to the *rel* probe (Fig. 2, lane 1) and the 2.9-kb band which hybridized to *myc* (Fig. 2, lane 7). These alterations were not present in DNA from HP50 cells, suggesting that they were specific to the HP46 cell line.

In order to determine whether these novel *rel* and *myc* fragments also hybridized to LTR sequences, which would be an indication of an ALV integration in the *rel* and *myc* loci, parallel digests of HP46, HP50, and line 151 DNAs run on the same gel as the DNAs probed with *rel* and *myc* were hybridized with the LTR probe. As can be seen in Fig. 2 (lanes 4, 5, and 6), there were numerous exogenous and endogenous proviruses in each of the cell types analyzed. However, the novel *rel*-hybridizing fragment of 3.2 kb from HP46 cells comigrated with an LTR-hybridizing fragment (lane 4), suggesting that this fragment might represent a junction fragment containing viral sequences and *c-rel* sequences. Further studies are in progress to map the precise location of the provirus with respect to the *c-rel* locus (see Discussion). None of the LTR-positive fragments comigrated with the novel 2.9-kb *myc*-hybridizing fragment.

This Southern blot analysis indicated structural alterations in both the *myc* and *rel* loci. In particular, the novel *rel*-hybridizing fragment of 3.2 kb in the HP46 cells appeared to be associated with viral LTR sequences, which is indicative of a proviral integration in the *c-rel* locus. In order to

FIG. 3. Northern analysis of cell lines, normal bursa, and CEF RNA. (A) Total cellular RNA (bursae and CEF) or cytoplasmic RNA (HP46 and HP50) (5 μ g) was run on a denaturing formaldehyde gel and hybridized with a *rel* probe (lanes 2 to 5) or a *myc* probe (lanes 6 to 9). HP46 RNA was also hybridized to an LTR probe (lane 1). See Materials and Methods for details of the specificity of the probes used. (B) Methylene blue stain of 18S RNA to indicate that equivalent quantities of RNA were loaded on the gel (5 μ g).

investigate the effect of such an insertion on the transcriptional activity of the locus, Northern blot analysis was carried out.

Northern blot analysis. Retroviral LTR sequences contain strong promoter and enhancer sequences. Therefore, it is not unusual to find enhanced transcription of cellular genes adjacent to integrated proviruses (14). These integrations alter normal expression and regulation of genes and might be critical in converting proto-oncogenes to their activated, oncogenic form. It was possible that the altered expression of *rel* indicated by immunoprecipitation of the four Rel-related proteins was the result of such an event. To examine this possibility further, Northern blot analysis was performed on RNA from HP46, HP50, and normal bursal lymphoid cells. This allowed us to determine both the sizes and levels of *c-rel* mRNAs expressed in normal avian B-lymphoid cells and provided a standard for comparison of the cell lines. To determine whether altered messages or elevated quantities of *rel*- or *myc*-specific RNA were present in the cell lines, RNA isolated from HP46, HP50, normal CEF, and normal bursal cells was run on a denaturing formaldehyde gel and blotted onto nitrocellulose. Blots were subsequently hybridized to *rel*- or *myc*-specific probes as described previously (see Materials and Methods). The HP50, normal CEF, and normal bursal cells contained comparable levels of a *rel*-hybridizing message of approximately 4 kb (Fig. 3A, lanes 2, 4, and 5), as expected (5).

In contrast, the HP46 cell line displayed several *rel*-related RNA species migrating at different positions than normal *c-rel* RNA (Fig. 3A, lanes 2 and 3). These species were also

present at greatly elevated quantities relative to normal bursal cells. Figure 3B shows a methylene blue stain of 18S rRNA on the nitrocellulose filters hybridized with the indicated probes to demonstrate that equivalent amounts of RNA were loaded in each lane. These results suggested that an alteration of the transcriptional regulation of the *c-rel* gene had occurred in the HP46 cell line, resulting in overexpression of aberrant *rel* transcripts.

Because Southern blotting demonstrated an alteration in the *myc* locus of the HP46 cell line, a Northern analysis was performed with a *v-myc* probe (Fig. 3, lanes 6, 7, 8, and 9). Again, HP46 cells (lane 7) displayed a large increase in the levels of a 2.2-kb *myc* mRNA that migrated slightly faster than the *c-myc* RNA found in bursal cells, HP50 cells, and normal CEF (*myc* expression in bursal cells was barely detectable).

In other systems, alteration of *c-onc* loci by promoter insertion sometimes results in the transcription of hybrid RNA species that contain LTR sequences fused to cellular sequences, implying that transcription has initiated within proviral DNA and extended into adjacent cellular regions (13, 14). To determine whether the altered transcripts detected in the HP46 cell lines contained viral sequences, RNA was hybridized to an LTR probe that contained U5 and primer-binding site sequences. As can be seen in Fig. 3, lane 1, the LTR probe hybridized to a number of RNA species, as predicted, since Southern blot analysis with the LTR probe had shown the presence of multiple proviruses in the HP46 cell line. However, some of these bands comigrated with bands hybridizing to a *rel*-specific probe, reinforcing the notion that integration of an ALV provirus had occurred in the HP46 cell line at the *c-rel* locus.

cDNA cloning and analysis. The data presented above strongly suggested a promoter insertion in the *c-rel* locus in the HP46 cell line. In particular, hybrid mRNAs encoded by cellular loci interrupted by proviral integrations sometimes carry characteristic LTR sequences at the 5' end. To demonstrate conclusively that a promoter insertion had indeed occurred in the *c-rel* locus of HP46 cells and to examine the structure of the altered mRNA species, we constructed a cDNA library from RNA extracted from the HP46 cell line as described in Materials and Methods. The cDNA library in the vector lambda Zap II was screened with a 5' *v-rel*-specific probe (the *Xba*I-*Hind*III fragment from the *rel*-containing plasmid pREVT). Two positively hybridizing clones (pBS46.A1 and pBS46.44) were subsequently chosen for sequence analysis. These clones, pBS46.A1 and pBS46.44, had insert sizes of 1.6 and 1.8 kb, respectively.

The sequences of the 5' ends of these two clones, indicated in Fig. 4A, are compared with the *c-rel* sequence provided by Capobianco et al. (3) and the LTR sequence from Rous sarcoma virus (41). The borders between R and U5 as well as U5 and the primer-binding site are indicated by arrows. Both pBS46.A1 and pBS46.44 contained LTR sequence at their 5' ends. Clone pBS46.A1 began in the U5 region, while pBS46.44 began within R. It is unlikely that either clone has a complete 5' end, since the cap site for RNA transcribed from the LTR sequences is at the border of the R region and U3 (41). This is most likely the result of incomplete first-strand synthesis (reverse transcription) in the cDNA library construction.

Up to the primer-binding site, both clones were highly homologous to the viral LTR, with only a few nucleotide changes. These differences may represent the variation between the LTR of the F42 ALV used to infect the line 151 birds and the Rous sarcoma virus LTR depicted here. Two

FIG. 4. Structure and sequence of 5' ends of two cDNA clones obtained from HP46 library. (A) 5' sequence comparison between Rous sarcoma virus (RSV) LTR, pBS46.A1, pBS46.44, and *c-rel* sequences. Arrows above LTR sequence indicate domains of LTR (i.e., either repeat, U5, or primer-binding site [P.B.S.] regions). Arrows below *c-rel* sequence indicate exon boundaries. Capital letters in either LTR or *c-rel* rows are indicative of sequence identity between the two clones (pBS46.A1 and pBS46.44) and LTR or *c-rel* sequences. Dotted lines represent continuation of sequences. Absence of sequence in a row represents lack of homologous sequences between that row and clone pBS46.44 or pBS46.A1. The underlined ATG is the presumed initiating methionine codon. Shaded area is a 53-nucleotide region which is not homologous to published *c-rel* or LTR sequence. The untranslated region of *c-rel* is indicated by boldface letters. The diagonal lines demonstrate a presumptive splice. (B) Schematic representation of clones pBS46.A1 and pBS46.44. R and U5 are sequences found to be homologous to Rous sarcoma virus R and U5 LTR sequences. Boxed exons (exons 0 and 4) are representative of sequences homologous to exons of the *c-rel* locus. Box containing ATG is a presumptive upstream exon (exon -1) containing the putative initiating methionine codon. The border of this exon is undefined, as indicated by the broken line. Diagonal lines are indicative of a presumed splice. Straight, unbroken lines are sequences that do not have strong homology to other sequences in the data base. This figure is not drawn to scale.

nucleotides before the border between U5 and the primer-binding site, the homology between pBS46.A1, pBS46.44, and the Rous sarcoma virus LTR dropped off sharply. Each clone contained a stretch of 53 nucleotides (the shaded region in Fig. 4A) that were not homologous to viral sequences, known *c-rel* sequences, or any other sequence in the GenBank data base. The origin of these sequences is not clear, but they may represent unidentified nontranslated regions of the *c-rel* gene, upstream regulatory sequences, or intron sequences.

A comparison of pBS46.A1 and pBS46.44 with the chicken *c-rel* sequence (3) is also shown in Fig. 4A. The exon boundaries (indicated by arrows) were defined by an analysis of *c-rel* genomic clones from a turkey (42). This study showed that exon 0 encoded 5' sequences of *v-rel*. Subsequent isolation of *c-rel* cDNA clones demonstrated that *c-rel* contains additional 5' sequence that is not present in *v-rel* or exon 0. These additional sequences have not been identified in genomic clones and therefore have not been assigned to an exon(s). Thus, for purposes of discussion, we have desig-

nated these *c-rel* sequences exon -1. The 5' border of exon -1, however, remains undefined.

Both pBS46.A1 and pBS46.44 were almost completely homologous with chicken *c-rel* through the untranslated region (bold sequence in Fig. 4) and the start of *c-rel* (the proposed initiating ATG codon is underlined). While clone pBS46.44 appeared to continue through exon 0, clone pBS46.A1 differed significantly. As stated, clone pBS46.A1 contained the untranslated region of *c-rel*, including the initiating methionine codon, but downstream sequence was then homologous to exon 4. This is presumably the result of splicing, since the sequence broke precisely at the end of exon -1 and started precisely at the beginning of exon 4, with exons 0, 1, 2, and 3 missing. The splice is indicated on Fig. 4A by the diagonal lines, and borders of the exons were designated as described above. Interestingly, the reading frame remained intact over this novel junction. The clone pBS46.A1 may represent one of the smaller *rel* messages identified by Northern blotting and thus could encode one of the smaller proteins immunoprecipitated from the HP46 cell line with anti-*rel* antibody. Figure 4B is a schematic representation of the 5' ends of these two clones.

Neither pBS46.A1 nor pBS46.44 was a full-length clone (they did not have complete 3' ends), and both appeared to have been primed from internal regions (data not shown). Further characterization is required to determine their complete structures. Additionally full-length clones representing the other mRNAs present in the HP46 cell line must be identified to fully characterize the transcriptional products from this locus. These results definitively demonstrate that a promoter insertion has occurred in the *c-rel* locus in the HP46 cell line which generates at least two hybrid transcripts which contain both LTR sequence and *c-rel* sequence. At least one of the mRNAs (as represented in pBS46.A1) appears to be the product of altered splicing, since sequence from exons 0, 1, 2, and 3 was missing from this mRNA.

DISCUSSION

In this article we demonstrate that the integration of an ALV provirus has occurred within the *c-rel* locus in the cell line HP46. This cell line was derived from a bird which had been injected at day 1 with the F42 strain of ALV and developed lymphoid tumors after long latency. In most previously reported analyses of ALV-induced lymphomas, promoter insertion within the *c-myc* locus has been shown (13, 14). Indeed, the *c-myc* locus has also sustained an alteration which results in the transcriptional activation of the *c-myc* gene, although the nature of this alteration is unclear at present. While integrations have been observed in loci such as *c-bic* and *c-pim* (7, 32) in combination with *c-myc*, this is the first report of an integration in the *c-rel* locus. This is obviously a very small sample size, and of four L15I lymphoid tumors examined, only HP46 contained a *c-rel* proviral insertion (data not shown).

The proviral insertion within the *c-rel* locus had a profound effect on the transcriptional activity of the locus. Not only was there an increased amount of RNA transcribed from this locus; there were multiple transcripts, none of which corresponded in size to the normal *c-rel* transcript. Analysis of two cDNA clones which represent at least two of these transcripts demonstrated conclusively that their synthesis initiated within viral LTR sequences and extended into *c-rel* sequence. The homology to viral sequences only extended to the U5 and R regions. This arrangement implies that these RNA species were transcribed from a 3' LTR,

since transcripts initiating from within the 5' LTR usually contain the portions of primer-binding site and *gag* sequences that are 5' to the viral splice donor site. Sequences homologous to the primer-binding site or to *gag* were not present in either pBS46.A1 or pBS46.44. Further mapping of the genomic locus will be required to determine precisely the position of the provirus with respect to the *c-rel* gene. However, the sequence analysis presented here indicates that the provirus is 5' of *c-rel* coding exons.

The structure of the mRNA transcripts represented in pBS46.A1 and pBS46.44 suggests that both transcripts initiate within the same sequence. Both clones share the 53 base pairs of unknown sequence (the shaded area in Fig. 4), the untranslated region of *c-rel* (the bold letters in Fig. 4), and the initiating methionine codon from *c-rel* found in exon -1. However, in contrast to pBS46.44, which continues through exon 0, pBS46.A1 lacks exons 0, 1, 2, and 3 but has sequences corresponding to the start of exon 4 now abutted to the exon -1 sequence. This result suggests that enhanced and altered transcription is occurring within the *c-rel* locus in the HP46 cell line. It is thus possible that the transcript represented by pBS46.A1, and perhaps the other *rel*-related transcripts in HP46 cells, is generated by aberrant splicing induced by proviral insertion.

As mentioned above, a transcript corresponding in size to the normal *c-rel* transcript was not identified, nor was the protein product p68^{*c-rel*} found. It is possible, although unlikely, that both alleles of the *rel* gene are altered or have promoter insertions in the same relative positions. There is evidence for promoter insertion in both alleles of the p53 gene in an erythroleukemic cell line (15). Alternatively, it is possible that the normal locus has been silenced by an allelic exclusion mechanism such as has been described for the *c-myc* gene in various Burkitt's lymphoma cell lines (8).

The induction of lymphoid leukosis by ALV is a complex process that appears to require many steps. It is thought that an early step in the progression of the disease is the activation of the *c-myc* locus by integration of an ALV provirus in the locus. Additional genetic alterations are then thought to be required in order to bring about the fully transformed phenotype. It is clear that there is a deregulated *c-myc* locus in the HP46 tumor, and, additionally, there is overexpression of altered forms of the *c-rel* gene product.

The relative contributions of the alterations in *c-myc* and *c-rel* to the genesis and progression of the disease are not clear. However, this finding suggests that synergy between the two oncogenes may be important for generating the transformed phenotype. Further experiments to assess the frequency of proviral integration in the *c-rel* locus in concert with alterations of the *c-myc* locus will be needed, as will experiments addressing the level of cooperativity between *rel* and *myc* in bringing about the transformation of avian lymphoid cells.

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LITERATURE CITED

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