Oncogene Expression in Reticuloendotheliosis Virus-Transformed Lymphoid Cell Lines and Avian Tissues

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The genome of reticuloendotheliosis virus (REV-T) contains a unique oncogene, designated *v-rel*, which is inserted into the *env* region. Employing a cloned *rel* DNA probe, a single 2.9- to 3.0-kilobase *v-rel* mRNA was identified in poly(A)⁺ RNA from REV-T-transformed lymphoid cell lines. A 4.0-kilobase *rel*-specific transcript corresponding to the cellular homolog of the *v-rel* oncogene was identified in MSB-1 cells, a herpesvirus-transformed lymphoid cell line. Cytodot hybridization was used to quantitate the levels of *rel*, *c-rel*, *c-myc*, *c-myb*, *c-abl*, *c-fms*, *c-Ha-ras*, *c-Ki-ras*, *c-src*, *c-yes*, *c-mos*, and *c-sis* mRNA in REV-T-transformed cells. The levels of *rel* transcription in REV-T-transformed cells were elevated only two to eightfold over levels found in the transformed immature avian lymphoid cell line MSB-1. The relatively modest levels of *rel* transcription in REV-T-transformed cells and the significant differences between the lengths of the *v-rel* and *c-rel* mRNA suggest that REV-T transformation is the result of the production of an altered *rel* protein. The *c-rel* proto-oncogene is expressed in all avian hematopoietic tissues but is not expressed at significant levels in brain and muscle. The transcription of other proto-oncogenes is not enhanced in REV-T-transformed lymphoid cell lines.

Reticuloendotheliosis virus (REV-T) is a replication-defective, type C retrovirus which transforms hematopoietic cells and fibroblasts in vitro (9, 10, 16, 17). REV-T-transformed hematopoietic cells have infinite growth potential and lymphoblastoid morphology, and they express both low levels of terminal-deoxynucleotidyl-transferase activity and B cell determinants (3, 21, 26). Unlike most retrovirus-transformed cells, REV-T-transformed nonvirus-producing lymphoid cells are tumorigenic (2, 15, 21). REV-T exists as a complex with a replication-competent helper virus, reticuloendotheliosis-associated virus (REV-A) (4, 16). Like other avian acute leukemia viruses, the genome of REV-T contains deletions of portions of the *gag*, *pol*, and *env* genes and has acquired helper virus unrelated sequences (4, 5, 7, 12, 17, 22, 27). It appears likely that REV-A acquired a turkey proto-oncogene (*c-rel*), which by further alterations became REV-T with the unique 1.4-kilobase (kb) oncogene *v-rel* (33-35). The *v-rel* oncogene is distinct from other known oncogenes (5, 28, 33, 35). The *v-rel* protein, however, remains to be identified. The nucleotide sequence of the *v-rel* oncogene (28, 33) has revealed that *v-rel* is inserted into the *env* gene of the helper virus.

The *v-rel* sequence, when translated, would encode a protein with helper-derived amino acids at both the amino and carboxy termini. Furthermore, the sequence suggests that *v-rel* protein translation initiates within *env* sequences and is translated from a spliced subgenomic messenger RNA. This study demonstrates the presence of a 2.9- to 3.0-kb mRNA species that hybridized to cloned *v-rel* DNA in cells transformed by REV-T. This mRNA was readily distinguishable from the 4.0-kb *c-rel* mRNA which is also present in REV-T-transformed cells. The levels of various proto-oncogene mRNAs were determined in REV-T-transformed cells. Though the levels of many proto-oncogene mRNAs appeared to increase when compared with those in normal bone marrow cells, no increased mRNA levels of any proto-oncogene other than *rel* could be

found to be specifically associated with transformation by REV-T. The levels of *c-rel* expression were also determined for various normal chicken tissues and organs.

To identify the *v-rel* transcript, polyadenylated RNA was isolated from various REV-T-transformed cell lines, MSB-1 cells, and REV-T (REV-A) virions (1, 17, 29). Poly(A)⁺ RNA was selected by twofold oligo(dT) cellulose chromatography (25). RNA species were separated by denaturing formaldehyde gel electrophoresis (20), blotted onto Genescreen, and hybridized to the denatured nick-translated (23) *rel*-specific plasmid *prel* (2×10^7 cpm; specific activity, 5×10^8 cpm/ μ g). Hybridizations were carried out in the presence of 50% formamide-5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate-0.2% polyvinylpyrrolidone-0.2% Ficoll-0.2% bovine serum albumin at 42°C for 48 h. Blots were washed sequentially in 2× SSC for 5 min, 2× SSC plus 0.5% sodium dodecyl sulfate for 10 min, and 0.1× SSC for 1 to 5 min, all at room temperature. Blots were dried and exposed to Kodak X-Omat XAR film at -70°C for 1 to 5 days. Virions of REV-T (REV-A) contained a single species of RNA which hybridized to cloned *v-rel* DNA (Fig. 1). This RNA (5.7 kb) corresponded to the length of REV-T genomic RNA (4, 12, 17). The MSB-1 cells provided a convenient source of *c-rel* mRNA and contained a single 4.0-kb *rel*-specific mRNA. The *c-rel* mRNA was found to be of a similar length in chicken splenic RNA by Chèn et al. (6). In an REV-T-transformed, virus-producing cell line (RECC-UT1) and in nonvirus-producing cell lines (UTC41 and UT370), three distinct species of RNA were found that would hybridize to the molecularly cloned *v-rel* DNA. REV-T genomic RNA (5.7 kb) and a 4.0-kb *c-rel* mRNA were present in all REV-T-transformed cell lines. A third *rel*-specific mRNA of 2.9 to 3.0 kb was detected in all REV-T-transformed cell lines and is presumed to be the subgenomic *v-rel* mRNA. A 2.9- to 3.0-kb mRNA was also detected by hybridization with REV-T cloned DNA that includes the viral long terminal repeat and leader regions. The relative levels of expression of these three mRNA species were determined by

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FIG. 1. Northern blot hybridizations used in the identification of *rel*-specific mRNA species in REV-T-transformed cell lines. Poly(A)⁺ RNA was fractionated by formaldehyde gel electrophoresis (20). The separated RNA samples (20 to 50 μ g) were blotted and immobilized onto Genescreen (30). Blots were hybridized to nick-translated cloned *rel* DNA. The length measurements of mRNA were based on a linear regression line generated from the electrophoretic migration of *Gallus domesticus* 28S rRNA (4,626 bp) and 18S rRNA (1,800 bp) and of *Escherichia coli* 26S rRNA (3,100 bp) and 16S rRNA (1,520 bp). Relative amounts of various RNA species present were determined by densitometric measurements of the autoradiograph negatives. Lanes: virion, RNA from virions of REV-T and REV-A; MSB-1, a herpesvirus-transformed cell line; RECC-UT370 and RECC-UTC41, REV-T-transformed nonvirus-producing cell lines; RECC-UT1, REV-T-transformed virus-producing cell line.

densitometric scanning of the lanes of the autoradiograms of Northern blots. The genomic REV-T RNA represented 45 to 75% of the total amount of *rel*-specific RNA in these cell lines. The *c-rel* mRNA was found at variable levels in REV-T-transformed cells (5 to 40%). Finally, the presumptive *v-rel* mRNA of 2.9 to 3.0 kb represented 20 to 35% of the RNA which hybridized to cloned *v-rel* DNA. Comparison of the relative levels of REV-T genomic RNA, *c-rel* mRNA, or subgenomic *v-rel* mRNA appearing in virus-producing and nonvirus-producing cell lines indicated that their expression was independent of the presence of the helper virus (Fig. 1 and Table 1).

Injection of REV-T-transformed nonvirus-producing cells into histocompatible chickens induces lethal reticuloendotheliosis (20, 24). Therefore, similar target cells are transformed both in vivo and in vitro. This system provides the unique opportunity to study the process of transformation in vitro. Oncogenesis is generally considered a multistep process (19). It was of interest, therefore, to examine the expression of other known proto-oncogenes in REV-T-transformed cells to determine if any other oncogenes are involved in REV-T transformation or tumorigenicity. Because REV-T transforms very immature lymphoid cells which are present in low levels in hematopoietic tissue, it was not possible to compare the level of oncogene expression in target cells before transformation. Therefore, the level of proto-oncogene expression in MSB-1 cells was measured because these are lymphoid cells with growth characteristics similar to REV-T-transformed lymphoid cell lines. Bone marrow cells were also used as the basis for comparison. Cytodot analysis (32) was used to quantitate the levels of several proto-oncogenes (*rel*, *c-rel*, *c-myc*, *c-myb*, *c-abl*, *c-src*, *c-mos*, *c-yes*, *c-sis*, *c-fms*, *c-Ha-ras*, and *c-Ki-ras*). Cytoplasmic RNA from equivalent numbers of cells (10^7) was serially diluted and applied to the hybridization membrane. Immobilized RNA was hybridized to various nick-translated plasmids containing oncogene DNAs. The type of data generated by cytodot analysis is shown in Fig. 2. Quantitation of the relative amounts of the various RNA species was determined by scanning densitometry of the autoradiographs. The areas beneath the peaks representing the individual dilutions were calculated. The background level of nonspecific hybridization was subtracted from the total of each peak. A summary of the quantitation of the relative levels of transcription of various oncogenes in REV-T-transformed cell lines (both virus producing and nonvirus producing) is contained in Table 1. As shown in Fig. 2 and Table 1, REV-T-transformed cell lines contained 32- to 128-fold more *rel*-specific RNA than uninfected bone marrow cells. The levels of *rel*-specific RNA in uninfected and REV-A-infected bone marrow cells were comparable, indicating that REV-A infection does not influence the transcription of *c-rel*. However, approximately 25% of the *rel*-specific poly(A)⁺ RNA obtained from these cell lines represented *v-rel* subgenomic mRNA, and an average of 20% corresponded to *c-rel* mRNA. The remaining 55% of the *rel*-specific RNA in these REV-T-transformed cell lines corresponded to REV-T genomic RNA. Furthermore, MSB-1 cells had levels of *c-rel* mRNA that were elevated 8- to 16-fold over the levels detected in bone marrow cells. Therefore, REV-T-transformed cell lines expressed only 5- to 30-fold more *v-rel* mRNA than the *c-rel* mRNA levels in uninfected bone marrow cells. The combined levels of *v-rel* and *c-rel* mRNA in REV-T-transformed cells were only two- to eightfold greater than the levels of *c-rel* mRNA in MSB-1 cells.

Oncogenes generally associated with lymphoid neoplasms include *rel*, *myc*, *myb*, and *abl* (31). The *c-myc* oncogene was found to be transcribed at levels 32- to 64-fold higher in REV-T-transformed cell lines than in normal bone marrow cells. However, MSB-1 cells were found to contain levels of *c-myc* mRNA comparable to those of REV-T-transformed cell lines (Fig. 2 and Table 1). The *c-myb* oncogene mRNA levels in REV-T-transformed cell lines were 16- to 64-fold higher than levels found in uninfected bone marrow cells; however, they were only two- to fourfold higher than levels found in MSB-1 cells. The *c-abl* transcript levels were 4- to 128-fold higher than levels in bone marrow cells and were

TABLE 1. Oncogene expression in REV-T-transformed cell lines^a

Sample ^b	Level of mRNA expression ^c											
	<i>rel</i>	<i>c-rel</i>	<i>c-myc</i>	<i>c-src</i>	<i>c-myb</i>	<i>c-abl</i>	<i>c-mos</i>	<i>c-sis</i>	<i>c-fms</i>	<i>c-yes</i>	<i>c-Ha-ras</i>	<i>c-Ki-ras</i>
MSB (NP) ^d	8-16	16	32	1-4	32	32	8	ND ^e	1-2	1	8	8
RECC-UTC41 (NP)	32	8	32	1-2	64	64	16	ND	16	8	8	16
UT370 (NP)	64-128	16	64	1-4	64	128	32	ND	16	ND	8	32
UT371 (NP)	64-128	16	64	1-4	64	64	16	ND	16	ND	8	32
UT1 (P) ^f	64-128	32	64	1-8	64	64	16	ND	16	1	8	32
UTBB5 (P)	32-64	16	32	1-4	64	64	8	ND	16	1	8	16
UT377 (P)	128	16	64	1-8	64	128	8	ND	16	ND	8	32
UT211 (P)	16	8	32	None ^g	16	8	8	1	ND	1	8	8
UT024 (P)	32	4	32	None	16	8	32	2	ND	4	2	4
UT011 (P)	32	32	32	None	16	8	None	4	ND	4	2	8
UT023 (P)	64	16	32	None	16	8	None	1	ND	4	2	8
UT013 (P)	64	16	32	None	16	8	8	1	ND	4	2	8
UT022 (P)	8	ND	32	None	16	8	8	1	ND	4	2	8
UT021 (P)	16	16	32	None	16	4	16	1	ND	4	2	8

^a Cytodot hybridization was used to quantitate the relative levels of mRNA species in various cell lines.

^b Cell lines UT1, UTC41, UT370, UT371, UT377, and UTBB5 are REV-T-transformed cell lines that have been maintained in culture from 2 to 10 years. Cell lines UT211, UT024, UT011, UT023, UT013, UT022, and UT021 were derived by in vitro transformation of chicken spleen cells (16) and were expanded in suspension culture to 10⁸ cells (approximately 3 months) and then analyzed.

^c Serially diluted (1:2) cytoplasmic RNA from an equivalent number of cells (10⁷) was compared by using the level of proto-oncogene expression in 10⁷ normal bone marrow cells from 2-week-old chickens. Equivalence of each sample was determined by duplicate cell counts verified by DNA determination with an acceptable variance of 10 to 20%. Values represent the average of at least two experiments, and where inexplicable variation occurred, the range of expression is given.

^d NP, Nonvirus producing.

^e ND, Not done.

^f P, Virus producing.

^g None, no detectable levels of mRNA.

two- to fourfold higher than levels in MSB-1 cells. Other proto-oncogenes such as *c-src*, *c-fms*, *c-mos*, *c-sis*, *c-yes*, *c-Ha-ras*, and *c-Ki-ras* are generally associated with sarcomas and other solid tumors (6). The transcription of these oncogenes generally was found to be elevated in REV-T-transformed cells when compared with uninfected bone marrow cells. These proto-oncogenes were, however, only slightly elevated (twofold) if at all when compared with the levels of these cellular transcripts in MSB-1 cells. The levels of *rel* transcription in newly established (3 months in culture) cell lines (UT211, UT024, UT011, UT023, UT013, UT022, UT021) were comparable or somewhat lower than the levels of *rel* in the more established (2 to 10 years in culture) cell lines (UTC41, UT1, UT370, UT371, UTBB5, UT377). In general, the mRNA levels of the various other cellular oncogenes tended to be lower in the more recently established cell lines.

The levels of *c-rel* transcription in organs and tissues of normal, uninfected chickens were quantitated (Fig. 3). The *c-rel* mRNA was found to be expressed in all hematopoietic organs. The bursa of Fabricius was found to contain the highest levels of *c-rel* mRNA, followed by liver, bone marrow, spleen, and thymus. Nonhematopoietic organs

FIG. 2. Example of data generated measuring oncogene expression in REV-T-transformed cell lines. Cytoplasmic RNA from each cell line was prepared from 10⁷ cells (32). Samples were serially diluted (1:2) and then filtered and immobilized onto Genescreen. Hybridizations were carried out as previously described to nick-translated plasmid DNA as indicated. Normal bone marrow cells (10⁷) served as the standard for comparison. Deoxyribose determination (11) was used to verify the equivalence of each sample. Controls on each dot blot included *E. coli* tRNA or rRNA as a measure of the nonspecific binding of cDNA probes. Treatment of samples with NaOH or RNase eliminated hybridization. Similar results were obtained with both purified total cytoplasmic RNA (29) and poly(A)⁺-selected mRNA (1).

FIG. 3. Expression of *c-rel* in the organs of uninfected chickens. The organs of 2-week-old chickens were disrupted in 7.2 M guanidine hydrochloride in a Waring blender. The cytoplasmic RNA was isolated by the procedure of Strohman et al. (29). The concentration and purity of the RNA were determined spectrophotometrically. Equivalent amounts of RNA (25 μ g) were serially diluted 1:2 beginning with 12.5 μ g of RNA. Samples were filtered onto the Genescreen hybridization membrane. The membrane was baked at 80°C and then hybridized to a denatured, nick-translated *rel*-specific probe (2×10^7 cpm; specific activity, 5×10^8 cpm/ μ g). Relative amounts of *c-rel*-specific RNA present in each sample were determined by densitometric measurements taken of the autoradiographic negatives. Bone marrow (10^7) cells were used as the standard of comparison.

such as muscle and brain were found to contain very low levels of *c-rel* mRNA.

REV-T-transformed cells contain a 2.9- to 3.0-kb *rel*-specific RNA which corresponds in size to the mRNA predicted from the *v-rel* nucleotide sequence. A 4.0-kb *c-rel* mRNA was also identified in MSB-1 cells. Quantitation of the levels of *v-rel* mRNA in REV-T-transformed cells has revealed only a two- to eightfold increase over *c-rel* mRNA levels in MSB-1 cells. Transformation by REV-T may be the result of qualitative differences between the *v-rel* and *c-rel* proteins. These qualitative changes may be reflected in alterations in the regulation, function, or specificity of the *v-rel* protein.

REV-T-transformed cell lines were found to have elevated mRNA levels of a number of other proto-oncogenes. Proto-oncogenes generally associated with lymphoid neoplasias (*rel*, *myc*, *myb*, and *abl*) were found to be consistently expressed at higher levels than oncogenes generally associated with solid neoplasias (*src*, *Ha-ras*, *Ki-ras*, *sis*, *yes*, *fms*, and *mos*). The level of mRNA expression of *c-rel*, *c-myc*, *c-myb*, and *c-abl* in REV-T-transformed lymphoid cells was comparable to the level detected in MSB-1 cells. Transformation of lymphoid cells by REV-T does not involve the increased transcription of other proto-oncogenes.

Although the *c-rel* proto-oncogene was found to be expressed in all hematopoietic tissues, its expression was found to be highest in the bursa. In general, the expression of a proto-oncogene in a specific cell type has not been found to correlate with the susceptibility of that cell to transformation by a virus carrying the activated forms of that oncogene (8, 14). REV-T, however, transforms a very immature B cell *in vivo* and *in vitro*, and *c-rel* is transcribed at the highest levels in the bursa where B-cell differentiation is thought to occur (18). Sequences homologous to the *c-rel* gene of vertebrates are present in *Drosophila melanogaster* DNA, and *c-rel* transcripts can be detected in adult flies (S. Takata, personal communication). The expression of a *rel*-related sequence in adult *Drosophila* suggests that the *rel* gene is involved in a vital cellular process.

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