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Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells

(human *onc* genes/hematopoietic cells/RNA gel blotting/type C RNA tumor viruses/human leukemia)

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ABSTRACT **Total cellular poly(A)-enriched RNA from a variety of fresh human leukemic blood cells and hematopoietic cell lines was analyzed for homology with molecularly cloned DNA probes containing the *onc* sequence of Abelson murine leukemia virus (Ab-MuLV), Harvey murine sarcoma virus (Ha-MuSV), simian sarcoma virus (SSV), and avian myelocytomatosis virus strain MC29. Results with the fresh blood cells paralleled those obtained with the cell lines. With Ab-MuLV and Ha-MuSV, multiple RNA bands were visualized in all cell types examined without significant variation in the relative intensities of the bands. When SSV was used as the probe, expression of related *onc* sequences was absent in all of the hematopoietic cell types examined except for one neoplastic T-cell line (HUT 102), which produces the human T-cell leukemia (lymphoma) retrovirus HTLV. In this cell line, a single band (4.2 kilobases) was observed. With MC29 as the probe, a single band of 2.7 kilobases was visualized in all cell types examined with only a 1- to 2-fold variation in intensity of hybridization. An exception was the promyelocytic cell line, HL60, which expressed approximately 10-fold more MC29-related *onc* sequences. With induction of differentiation of HL60 with either dimethyl sulfoxide or retinoic acid, a marked diminution in the amount of the MC29-related, but not the Ab-MuLV-related, *onc* message was observed.**

The transforming genes of retroviruses are derived from normal cellular genes (*c-onc*) that are conserved among vertebrates (1, 2). The function(s) of these cellular gene products is not clearly defined. However, there is evidence that virus-induced transformation is correlated with enhanced levels of expression of these genes (3–5). Using molecularly cloned DNA probes containing viral *onc* sequences, we and others (6–10) have shown that there are genetic loci in human DNA corresponding to each viral *onc* gene. No homology was detected between human DNA and helper virus-derived sequences under similar hybridization conditions. To study the possible role of these *onc* gene homologues in normal and neoplastic growth and differentiation of human cells, we studied their expression in various human hematopoietic cells. We chose the hematopoietic cell system for analysis because extensive marker analyses suggest that human leukemic cells reflect normal cells at particular stages in hematopoiesis (11). Furthermore, numerous established cell lines with well-defined marker characteristics and fresh uncultured blood cells from patients with different types of leukemia are readily available. Thus, they provide useful systems for studying *onc* gene expression as a function of differentiation, as well as growth and neoplastic transformation, in hematopoietic cells.

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In this study, we examined both fresh blood cells and human cell lines of myeloid, lymphoid, and erythroid origin at various stages of differentiation for the expression of cellular genes homologous to the transforming genes of avian myelocytomatosis virus strain MC29, Abelson murine leukemia virus (Ab-MuLV), Harvey murine sarcoma virus (Ha-MuSV), and simian sarcoma virus (SSV). MC29 induces myelocytomatosis and, less frequently, sarcomas and carcinomas *in vivo* and transforms macrophage-like cells *in vitro* (12). Ab-MuLV causes lymphosarcomas in mice and transforms fibroblasts and cells of the B-lymphocyte series (13). Ha-MuSV induces erythroleukemias and fibrosarcomas in mice (14). Finally, SSV is known to induce sarcomas in newborn primates and transforms fibroblasts specifically (15). In accordance with recent convention, the cell-derived *onc* sequences of MC29, Ab-MuLV, Ha-MuSV, and SSV are designated *myc*, *abl*, Harvey-*ras*, and *sis*, respectively (16). Nick-translated DNA probes of defined *onc*-containing segments from molecularly cloned viral genomes were hybridized to poly(A)-containing RNAs by using the RNA gel blotting techniques (17, 18). The results obtained with each probe differ with respect to their cell specificity and number or size of mRNA species.

MATERIALS AND METHODS

Source of Cells. Permanent human leukemia and lymphoma cell lines were selected that show a stable marker profile of their original tumor cell and that represent the spectrum of lymphoid, myeloid, and erythroid cell types at various stages in hematopoiesis. Many of the marker characteristics of these cell lines have been described (11). B-cell lines that were used included Daudi, Raji, NC-37, and IM-9 (19–22). Immature T-cell lines included CCRF-CEM, MOLT 4, and KM-3 (23–25). KG-1, an immature myeloid cell line from a patient with acute myelogenous leukemia (AML) (26), and K562, a cell line derived from a patient with chronic myelogenous leukemia (27), were also studied. HL60, a human promyelocytic leukemia cell line (28), was studied in its promyelocytic form and after induction of differentiation with dimethyl sulfoxide (Me_2SO) and retinoic acid (29, 30). HUT 78 and HUT 102, two mature cutaneous T-cell lymphoma lines established with human T-cell growth factor (TCGF) (31, 32), but presently maintained as TCGF-independent cells that produce TCGF autonomously (33, 34), were also studied. Fresh peripheral leukemic and normal cells from the blood of patients with AML and acute lymphoid leukemia

Abbreviations: MC29, avian myelocytomatosis virus strain MC29; Ha-MuSV, Harvey murine sarcoma virus; Ab-MuLV, Abelson murine leukemia virus; SSV, simian sarcoma virus; AML, acute myelogenous leukemia; TCGF, T-cell growth factor; Me_2SO, dimethyl sulfoxide; EBV, Epstein–Barr virus; kb, kilobase(s); ALV, avian leukosis virus.

and from normal volunteers were obtained by using a cell separator.

Preparation of RNA. Total cellular RNA was extracted as described with a modified guanidine hydrochloride extraction technique (35, 36). Briefly, 10^9 cells were pelleted and solubilized in 10 ml of 8 M guanidine hydrochloride/10 mM sodium acetate, pH 5.0, and then were homogenized with 20–30 strokes of a loose-fitting pestle in a Dounce homogenizer. RNA was precipitated with 1/2 volume of ethanol at −20°C for 30 min. After centrifugation, the RNA pellet was redissolved and then precipitated with 1/2 volume of ethanol three additional times, with the dissolution each time in a decreasing volume of the solution described above buffered with 20 mM EDTA (pH 7.5). The final precipitate was redissolved in 5 ml of 20 mM EDTA (pH 7.5) and extracted with an equal volume of chloroform/isobutanol, 4:1 (vol/vol). The RNA then was precipitated overnight at −20°C in 3 M sodium acetate (pH 6.0), dissolved in 0.2 M sodium acetate (pH 5.0), and reprecipitated with 2 volumes of ethanol at −20°C. The final RNA precipitate was redissolved in water, and the total cellular poly(A)-containing RNA was selected by affinity chromatography over oligo(dT)-cellulose (37). The same RNA preparations were used in hybridization experiments against different probes. The yield of poly(A)-containing RNA per cell varied 2- to 3-fold; however, results have not been normalized for this variation. In the case of HL60 and HL60 induced to differentiate, similar amounts of poly(A)-containing material were obtained per cell.

RNA Gel Blotting Procedure. Poly(A)-selected RNA (5 μg per lane) was electrophoresed in 1% agarose gels containing 5 mM methyl mercury, transferred to nitrocellulose with 3 M NaCl/0.3 M sodium citrate, pH 7, and hybridized as described by Thomas (18) except that the competitor used to prevent absorption of nonspecific counts during hybridization was *Escherichia coli* DNA (250 μg/ml) rather than salmon sperm DNA. Filters were washed four times in 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% $NaDodSO_4$ at room temperature for 5 min each and then two times in 0.15 M NaCl/0.015 M sodium citrate, pH 7/0.1% $NaDodSO_4$ at 42°C for 15 min each.

Derivation of *onc* Gene Probes and Nick Translation. The cloning of the genomes of MC29, Ab-MuLV, Ha-MuSV, and SSV have been described (38–41). Fig. 1 shows simplified physical maps of the molecular clones and indicates the regions of the *onc* genes and segments used as probes for this study. The *onc*-containing DNA's were nick-translated as described (42), with specific activities of 2–4 × 10^8 cpm/μg of input DNA.

RESULTS

Sensitivity of the RNA Blotting and Hybridization Procedure. To estimate the threshold of detection, we fractionated on gel varying amounts of total cellular RNA from an Ab-MuLV-transformed nonproducer cell line that contains ≈350 copies of Ab-MuLV per cell (unpublished data). This was blot-hybridized to nick-translated *abl* DNA. A single 5.8-kilobase (kb) species of Ab-MuLV mRNA was detected with as little as 0.25 μg of total cellular RNA applied to the gel (Fig. 2). Selection of poly(A)-containing RNA on oligo(dT)-cellulose gave a 15- to 20-fold enrichment of this and other mRNA (data not shown). For subsequent experiments, we routinely used 5 μg of poly(A)-selected RNA in each lane—equivalent to 100 μg of total cellular RNA, or 400-fold more than the minimum amount of RNA from the Ab-MuLV-transformed cells required for detection. Therefore, the limit of detection in our assay is approximately one copy per cell. One should take this only as a rough guide, however, because of variability in the efficiencies of transfer from the gel and binding to nitrocellulose filters, in the proportion

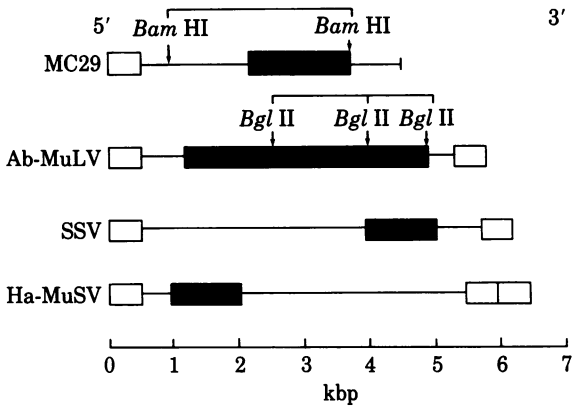

FIG. 1. Simplified maps of the transforming retroviral genomes used to detect *onc*-related expression in various human hematopoietic cells. The white boxes represent the long terminal repeats and the black boxes the viral *onc*-genes. Host cellular flanking sequences, if present, and cloning vector sequences are not shown. DNA probes were derived from subgenomic fragments of MC29 and Ab-MuLV. The *Bam* HI–*Bam* HI fragment of MC29 was subcloned into pBR322 and used as the probe directly, whereas the 5′ *Bgl* II–*Bgl* II portion of Ab-MuLV was isolated as a fragment after agarose gel electrophoresis and was used in this form for nick translation. The SSV and Ha-MuSV cloned in pBR322 were used in their entirety as probes. kbp, Kilobase pairs.

of *onc* sequences in the mRNA, and in the degree of homology of the human RNA transcripts to the viral probes. Nonetheless, we assume that, if expression of a gene is not detectable, no more than a few copies of its message are present per cell.

Wide Distribution of Expression of Multiple mRNA Species of the *c-abl* and Harvey-*ras* Genes in Human Hematopoietic Cells. Table 1 summarizes the different cell lines and fresh leukemic cells used in this study, including cells at different stages of differentiation in the myeloid, lymphoid, and erythroid series. The *abl* and Harvey-*ras* probes detected multiple-size species of mRNA, generally at low levels of expression based on band intensity. The *abl* probe detected at least four differently sized transcripts of 7.2, 6.4, 3.8, and 2.0 kb (Fig. 3). The Harvey-*ras* probe detected at least two faintly hybridizing transcripts of 6.5 and 5.8 kb and perhaps lower molecular weight species as well (not shown). There was a narrow range of variation in the intensity of these bands from one sample to another.

Wide Distribution of Expression of a Single mRNA Species of *c-myc* in Human Hematopoietic Cells. The *myc* probe detected only a single transcript of 2.7 kb in all hematopoietic cells

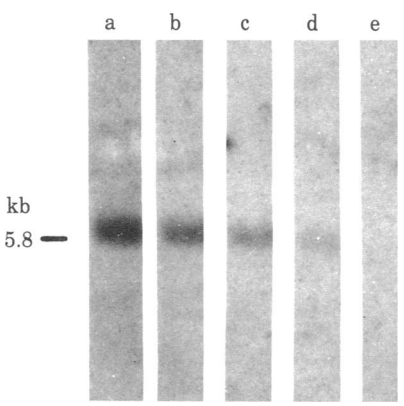

FIG. 2. Sensitivity of the RNA blotting procedure. Varying amounts of total cellular RNA from Ab-MuLV NIH 3T3 transformed nonproducer cells were blot-hybridized to nick-translated Ab-MuLV probe to determine sensitivity of the blotting procedure. Lanes: a, 2 μg; b, 1 μg; c, 0.5 μg; d, 0.25 μg; e, 0.1 μg.

Table 1. Classification of fresh human hematopoietic cells and cell lines tested for expression of cellular *onc*-related sequences

Cell type	Cell lines	Fresh cells
Myeloid	KG-1 (myeloblast)	AML (myeloblasts)
	HL60 (promyelocyte)	(4 patients)
		CML blast crisis
		(1 patient)
Erythroid	K562 (Erythroid precursor)	
Lymphoid		
T cells	CEM (immature)	T-cell ALL (immature T-cells)
	MOLT 4 (immature)	(1 patient)
	KM-3 (immature)	
	HUT 78 (mature)	
	HUT 102 (mature)	
B cells	Raji (Burkitt lymphoma)	Normal, peripheral blood lymphocytes*
		(1 normal donor)
	Daudi (Burkitt lymphoma)	(1 patient)
	Normal, EBV-transformed	Lectin-stimulated lymphocytes*
	NC37	(1 normal donor)
	IM-9	
	C.R.B.	

ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia.
* Examined only with probes of *sis* and *myc*.

examined. Representative data are shown in Fig. 4. With one exception, there is less than a 2- to 3-fold variation in the signal intensity of this band across a spectrum of hematopoietic cells at different stages of differentiation, including (as a control) normal peripheral blood lymphocytes. A single exception is HL60, a human promyelocytic leukemic cell line, which expressed *myc*-related sequences at approximately a 10-fold greater level compared with other cell lines.

Expression of the *c-myc* Gene Was Not Detected in Differentiated HL60 Cells. The human promyelocytic leukemia cell line, HL60, expresses RNA transcripts of the *myc*, *abl*, and Harvey-*ras* genes. Because HL60 has the unique capacity to differentiate into more mature myeloid cells after induction

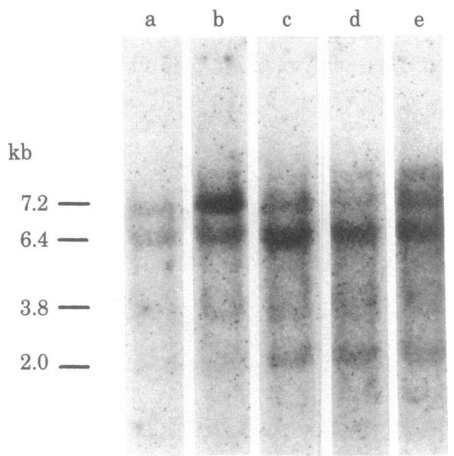

FIG. 3. Hybridization of poly(A)-selected cellular mRNA to the *abl* probe. The RNA was from the following cells: peripheral blood of a patient with AML (lane a); Molt 4, an immature T-cell line (lane b); HL60, a promyelocytic leukemia cell line (lane c); C.R.B. cells, an Epstein–Barr virus (EBV)-transformed B-cell line derived from the same patient that yielded the HUT 102 T-cell line (lane d); and KG-1, a myeloblast cell line (lane e).

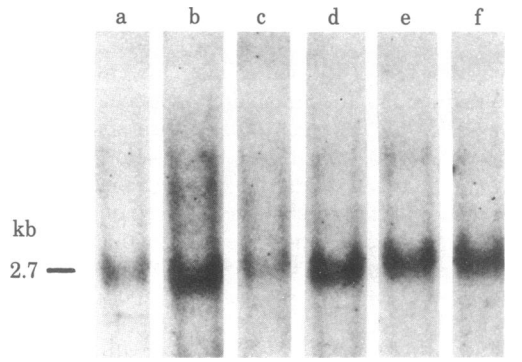

FIG. 4. Hybridization of RNA to the *myc* probe. The RNA was from fresh AML cells (lane a); T-cell lymphoma cell lines HUT 78 and HUT 102 (lanes b and c, respectively); normal EBV-transformed B-cell lymphoblast line IM-9 (lane d); and Burkitt lymphoma cell lines Raji and Daudi (lanes e and f, respectively).

with several chemical agents, most notably Me_2SO (29) and retinoic acid (30), we wished to determine if expression of cellular *onc* genes is modulated as a function of myeloid cell differentiation. Fig. 5*A* shows hybridization of the *myc* probe to RNA from untreated HL60 cells and HL60 treated with Me_2SO or retinoic acid. Expression of *c-myc* was reduced 80–90% in HL60 cells after induction with either Me_2SO or retinoic acid. It seems likely that this gene was not expressed at all in the differentiated cell and that the residual band is due to the small population of undifferentiated cells that persist in culture. Consistent with this idea is the fact that the intensity of the residual band correlates with the percentage of the undifferentiated cells remaining in the cell population analyzed (87% mature cells with the less intense band and 40% mature cells with the more intense band; see Fig. 5*A*).

In contrast to *c-myc*, the *c-abl* gene remained expressed at similar levels in the differentiated and undifferentiated cells (Fig. 5*B*). These results also mitigate against the possibility that the apparent decrease in expression of *c-myc* is due to generalized RNA degradation in the mature granulocytic cells.

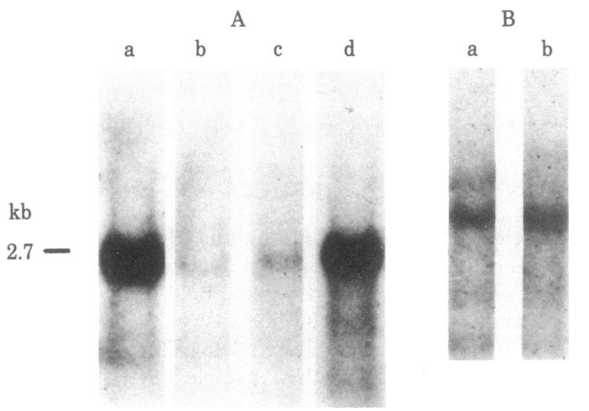

FIG. 5. Hybridization of *myc* and *abl* probes to RNA from HL60 induced to differentiate with various agents. Degree of differentiation was judged by the percentage of cells able to reduce the dye nitro blue tetrazolium (NBT), which is a histochemical marker of more mature myeloid cells (43), and by light microscopic examination of Giemsa-stained cells (20). (*A*) Hybridization of *myc* probe to two independent isolates of RNA. Lanes: a and d, from uninduced HL60 (<2% of cells were NBT positive); b, RNA from HL60 induced to differentiate with Me_2SO (87% NBT positive); and c, RNA from HL60 cells differentiated to 40% NBT-positive cells by using retinoic acid. (*B*) Hybridization of *abl* probe to HL60 (<2% NBT positive) RNA (lane a) and to RNA from HL60 induced to 87% NBT positive with Me_2SO (lane b).

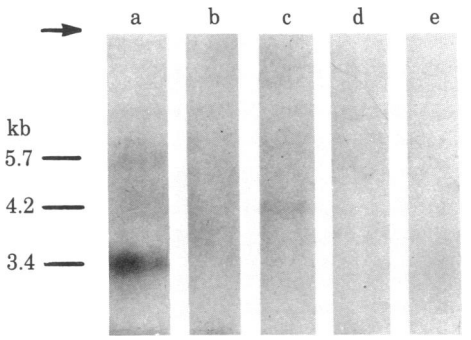

FIG. 6. Hybridization of RNA to the *sis* probe. Lanes: a, RNA from an SSV-transformed nonproducer NRK cell line; b and c, RNA from two T-cell lymphoma cell lines, HUT 78 and HUT 102, respectively; and d and e, RNA from two Burkitt lymphoma cell lines Raji and Daudi.

Limited Expression of the *c-sis* Gene in Human Hematopoietic Cells. In sharp contrast to the wide expression of the *c-myc*, *c-abl*, and Harvey-*ras* genes, we were not able to detect expression of the human *c-sis* gene in a variety of hematopoietic cells. The only sample in which a transcript was detected is a human T-cell lymphoma cell line (HUT 102). A closely analogous cell line, HUT 78, other cell lines, and fresh leukemic cells were negative (Fig. 6). The size of the transcript (4.2 kb) in HUT 102 differed from the mRNA of transformed nonproducer cells but is identical in size to *c-sis* gene transcripts in human fibrosarcoma cell lines not infected with SSV (43). Because HUT 102 is a primary tumor line producing the type C human T-cell leukemia (lymphoma) retrovirus designated HTLV (33), which has been clearly distinguished from known animal retroviruses (44–46), it should be determined whether integration of this provirus correlates with activation of *c-sis*, which is usually not transcribed in hematopoietic cells.

DISCUSSION

The identification of genetic loci in human DNA homologous to viral *onc* gene raises the questions of whether they are functional genetic elements and whether they play a role in normal or neoplastic cell growth. Our present study shows that the human homologues of the *onc* genes of MC29, Ab-MuLV, Ha-MuSV, and SSV are expressed in some or all human hematopoietic cells tested. The *abl* and Harvey-*ras* genes are transcribed into multiple transcript sizes, possibly by differences in splicing or transcription of more than one genetic locus homologous to these probes. However, the transcripts are present in all cells examined at relatively constant levels. A parallel study (43) examining human solid tumor cell lines and normal fibroblasts with the same *abl* probe and the viral *onc* gene of BALB-MuSV, which is closely related to Harvey-*ras* (47), similarly revealed widespread transcription of multiple species of mRNA. These results are consistent with the interpretation that these genes may have an important role in normal cellular functions. It has been shown that a mouse erythroid precursor cell line expressed high levels of a p21 protein assumed to be the product of *c*-Harvey-*ras* (48), thus suggesting a correspondence of the pathogeneity of *v*-Harvey-*ras* (erythroleukemia) with the *in vivo* tissue specificity for expression of the cellular gene. Analogous findings were not obtained in our studies of human cells. A cell line (K562) that has some erythroid precursor features (49, 50) was found to express only minimal levels of *c*-Harvey-*ras*.

The *myc* gene has been implicated in B-cell lymphomas induced by the avian leukosis virus in a mechanism termed "downstream promotion" (4, 5). Our results show that although this gene is expressed in human neoplastic B cells, it is also expressed at similar levels in hematopoietic cells of other lineages, including normal peripheral blood lymphocytes and in solid tumor cells (43). The highest level of expression of *c-myc* transcript is found in the promyelocytic leukemia cell line, HL60. However, absolute levels of variation cannot be accurately determined at the present time and will have to await future studies using a totally homologous probe of the cloned human *myc* gene. Of interest is the finding that *c-myc* transcription seemed to be turned off after HL60 cells were induced to differentiate with Me_2SO or retinoic acid into more mature phagocytic, nondividing myeloid cells (metamyelocytes and granulocytes). This result is not due to nonspecific degradation of the RNA because expression of *c-abl* was similar in both undifferentiated and differentiated cells. The significance of this finding is unknown. However the lack of expression did not appear to be related simply to lack of cell proliferation in terminally differentiated HL60 cells because normal nondividing peripheral blood lymphocytes expressed *c-myc* mRNA at comparable levels to most of the human cell lines.

Of the four *onc* genes examined, the *sis* gene shows the strictest regulation of expression. It is not expressed in any hematopoietic cell examined except HUT 102, a primary T-cell lymphoma cell line producing a unique human T-cell leukemia retrovirus, HTLV (32). Among human solid tumors, the *sis* gene is transcribed in five out of six sarcoma cell lines and in three of five glioblastoma cell lines but not in any carcinoma or melanoma cell lines analyzed (43). Thus, based on our available information, this gene may be relatively specific for certain types of tumors. Another *onc* gene that is also expressed in restricted cell types is the gene analogue of avian myeloblastosis virus. This gene is transcribed only in immature myeloid, erythroid, and T-lymphoid cells, but not in the mature hematopoietic cells or any nonhematopoietic cells (51). Thus, this gene could be involved in hematopoietic cell differentiation.

The viral and cellular counterpart *onc* gene products have been shown to be structurally and functionally similar (3, 48, 52). Where the viral *onc* gene products are not linked to *gag* determinants, their sizes are usually similar to the cellular analogues (3, 48). However, the present study and those of Eva *et al.* (43) and Westin *et al.* (51) have shown that the sizes of the human *c-onc* transcripts are in most cases greater than the size of the *v-onc* genes themselves, suggesting simultaneous transcription of adjacent cellular sequences (flanking or intervening). The exact alignment of these cellular transcripts with the viral and cellular *onc* genes remains to be determined. The sizes of the transcripts are conserved among all cell types and perhaps among different species as well because the human *c-myc* mRNA (2.7 kb) is similar in size to the avian *c-myc* mRNA (53). These results suggest a conserved organization of the *c-onc* genes of different species or specific processing events leading to the final gene products, or both.

The high degree of conservation of cellular *onc* genes supports the idea that these genes are important in normal cells, but there is only fragmentary evidence for expression of *c-onc* genes in normal animal cells. RNA homologous to the *onc*-specific sequences of Rous sarcoma virus (54, 55), MC29 (56), avian erythroblastosis, and avian myeloblastosis virus (57) has been detected in tissues of several avian species. However, attempts to detect transcripts of the cellular analogues of Moloney sarcoma virus (58) and feline sarcoma virus (59) have been unsuccessful. Our results indicate that some *onc* genes are expressed in all neoplastic and normal cells examined and, thus, are likely to have functions that are important to normal cells. Others are expressed only in some cell types or certain stages of maturation and may play a role in cellular differentiation.

Several studies have implicated derepression of cellular *onc* genes as a crucial event in retrovirus-induced tumorigenesis. In B-cell lymphomas of chickens induced by two distinct viruses, avian leukosis virus (ALV) and avian syncytia virus, the exogenous proviruses are integrated in the vicinity of *c-myc*; presumably as a result of this, expression of *c-myc* is greatly enhanced (5, 60–62). Less frequently, ALV can induce erythroblastosis, and in these tumors the ALV provirus is integrated next to *c-erb*, a cellular gene homologous to the transforming gene of avian erythroblastosis virus (H. J. Kung, personal communication). However, there is as yet no evidence for activation of *onc* genes in nonviral induced tumors in animal systems. Our results also failed to show a correlation of high-level expression of the *onc* genes examined with specific types of leukemias. Thus, the exact role of these *onc* genes in normal and neoplastic cellular processes remains to be determined.

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