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The Human *onc* Gene *c-myc*: Structure, Expression, and Amplification in the Human Promyelocytic Leukemia Cell Line HL-60*

R. Dalla Favera, E. Westin, E. P. Gelmann, S. Martinotti, M. Bregni, F. Wong-Staal, and R. C. Gallo

Substantial evidence indicates that retroviral transforming (*v-onc*) genes originated by means of recombination between a present nontransforming virus and normal cellular sequences (Duesberg et al., this volume). These sequences, called cellular *onc* gene, are highly conserved during evolution, suggesting that they may code for protein products which are essential for cell growth or tissue differentiation. As these normal cellular genes are homologous to viral- transforming genes, their potential role in tumorigenesis is of great interest. As an alternative to direct transformation by a viral *onc* gene, abnormal activation of a cellular *onc* gene may cause transformation. Two models have been proposed for such a mechanism. First, high levels of expression of a cellular *onc* gene may be caused by the insertion nearby of a viral promoter [12, 15, 16, 17] or by alteration of the physiological promoter by a mutagenic agent such as a chemical carcinogen. Secondly, a cellular *onc* gene may be relocated in a transcriptionally active region of the genome as a consequence of chromosomal rearrangements [2, 5, 6,13]. In this chapter we review the evidence for a possible third mechanism for *onc* gene activation in neoplastic cells, that of gene amplification. The human homologue, *c-myc*, of the transforming gene of avian myelocytomatosis virus (MC29), which is expressed at relatively high levels in the human promyelocytic leukemia cell line HL-60, is stably amplified in the genome of these cells [7]. Amplification was also detected in primary, uncultured leukemic cells from the same individual, suggesting that the *c-myc* amplification may have been involved in the leukemic transformation in this case.

A. Genomic Organization of Human *c-myc* Sequences

The avian myelocytomatosis virus genome (MC29) contains an *onc* gene, *v-myc*, coding for a DNA-binding nuclear protein which is responsible for the transforming ability of the virus (Moelling et al., this volume) [9]. A recombinant plasmid (pMC0) containing the entire *v-myc* gene was derived from an integrated provirus clone (Papas et al., this volume) [14]. Hybridization of pMC0 to normal human genomic DNA indicated that multiple regions in the human genome contain sequences related to *v-myc* [8]. In order to establish the genomic organization of these sequences a human recombinant DNA library was screened using pMC0 as a probe, and five recombinant phages were isolated (λ-LMC-3-4-12-26-41) [8]. Restriction enzyme analysis of λ-LMC-12 and 41 determined that these two clones share approximately 17 kb DNA where restriction sites are conserved, suggesting that they represent the same genomic segment (Fig. 1). The 8.2-kb *Hind*III-*Eco*RI fragment was isolated by preparative electrophoresis and analyzed by hybridization of Southern blots to *v-myc* probes (Fig. 1 B). Hybridization of pMC0 to *Cla*I-*Sst*-I digests (Fig. 1 C) shows that

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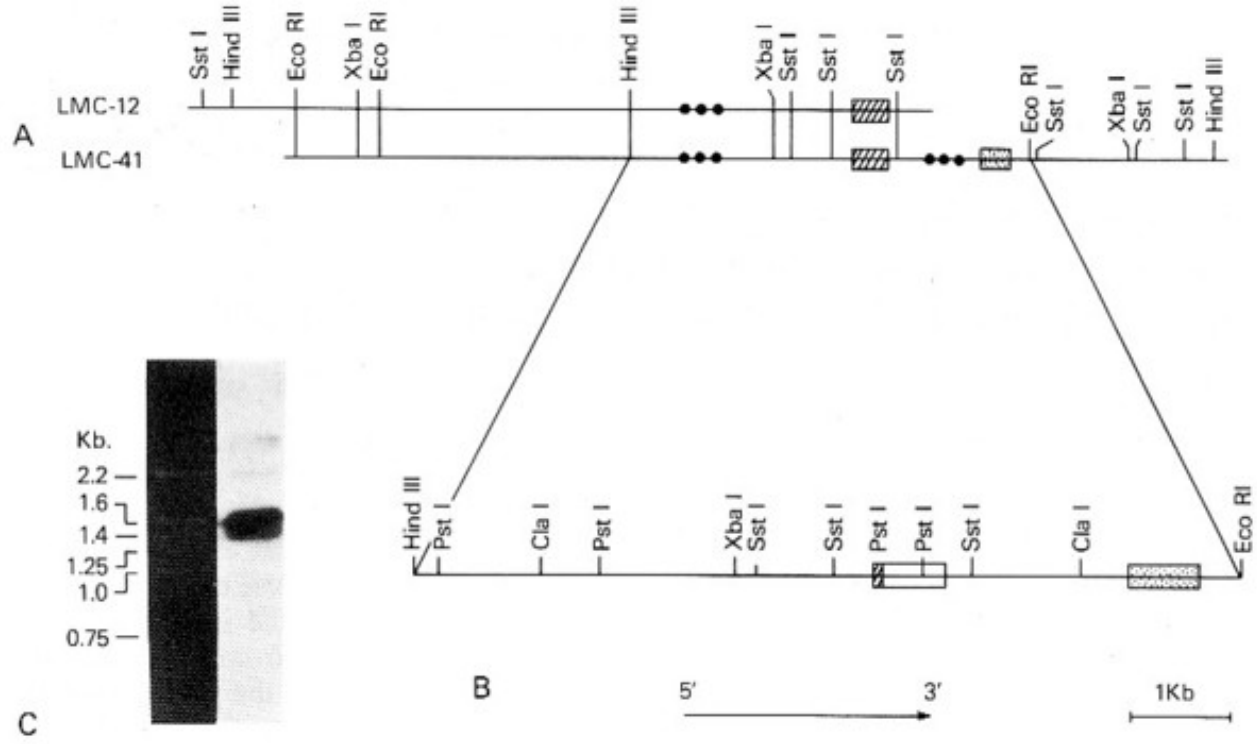

Fig. 1A–C. Genomic organization of the *c-myc* gene. **A** Restriction map of λ-LMC-41 and 12 DNA inserts. **B** Detailed restriction map of the 8.2-kb *Hind-Eco*RI fragment. *Cross-hatched box,* hybridizing to a 5′ probe fragment; *white box,* pMCO hybridizing fragments; *stippled box,* hybridizing fragment to a 3′ probe; ●●●, fragments containing *Alu* repets. **C** Right, ethidium bromide staining of DNA fragments generated by *Sst*I-*Cla*I cleavage of the fragment shown in **B** *Left,* hybridization to pMCO showing the two hybridizing fragments [8]

the region of hybridization is not continuous. A 1.0-kb *Cla*I-*Sst*-I fragment does not contain *c-myc* sequences. This fragment, which probably represents an intron in the *c-myc* gene, contains sequences related to the Alu family of repeats (data not shown).

In order to investigate further this structure, heteroduplex studies were performed. DNA from the recombinant λ-LMC phages were used to form heteroduplex molecules with phage DNA containing the MC29 provirus (Fig. 2). In agreement with the restriction enzyme data, *v-myc*-homologous sequences in λ-LMC-41 are interrupted by a nonhybridizing segment. Heteroduplex measurements of the *v-myc* hybridizing region of the λ-LMC-41 insert match the length of the viral *onc* gene, 1.56 kb (T. S. Papas, personal communication). These data suggest that the 19-kb region of clone λ-LMC-41 contains the active functional *c-myc* gene. Furthermore this structure is analogous to one of the single-copy chicken *c-myc* genes [18], some of whose restriction sites have been conserved in the human gene (T. S. Papas, personal communication).

Restriction maps of λ-LMC-3, 4, and 26 DNA (Fig. 3) show that these clones represent nonoverlapping, although possibly contiguous, regions of the human genome. Unlike the hybridization analysis of λ-LMC-41, we were unable to detect nonhybridizing intervening regions in λ-LMC-3, 4, or 26. Heteroduplex formation with λ-MC29 DNA showed for these single DNA clones an uninterrupted region of homology ranging from 0.2 to 0.4 kb (Fig. 2). As seen in Fig. 3, these sequences are homologous to the central portion of *v-myc,* lacking 5′ and 3′ homologous sequences. Moreover, these incomplete sequences are more divergent from the viral sequences than the complete gene since stringent washing conditions abolished the hybridization with λ-LMC-3, 4, and 26 without diminishing the intensity of the signal in λ-LMC-12 and 41. These sequences may represent parts of different functional genes which are partially homologous with the *c-myc* gene. However, as described below, only one species of mRNA has been detected in several different human tissues tested [10, 20], and higher levels of his

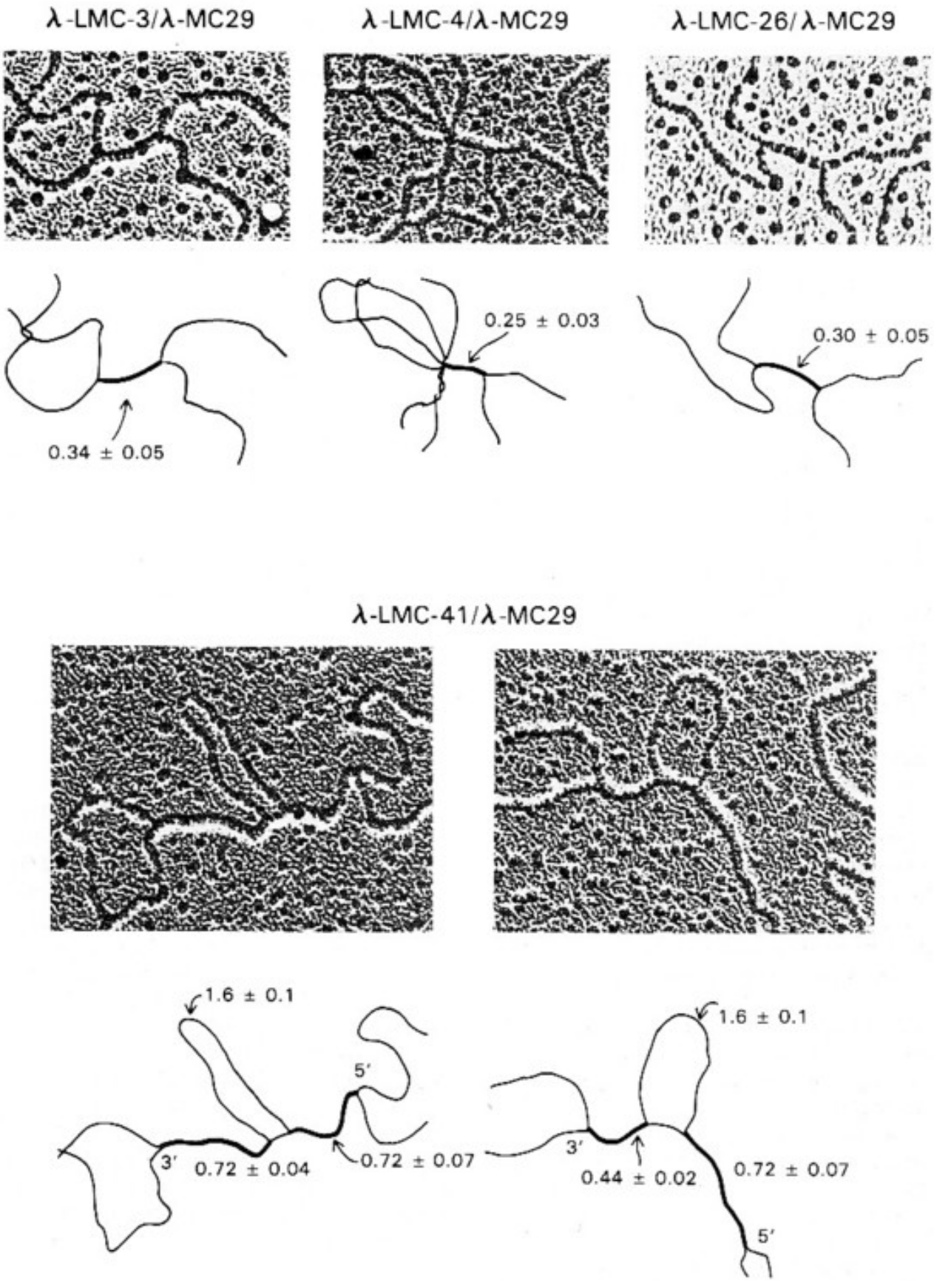

Fig. 2. Heteroduplex analysis of different human *c-myc* clones. The *top three panels* show the regions of the heteroduplex molecules containing the pseudogene hybridization. The *lower two panels* have the two kinds of molecules seen in the λ-LMC-41/λ-MC29 heteroduplex. Measurements are in kilobases [8] × 155,000

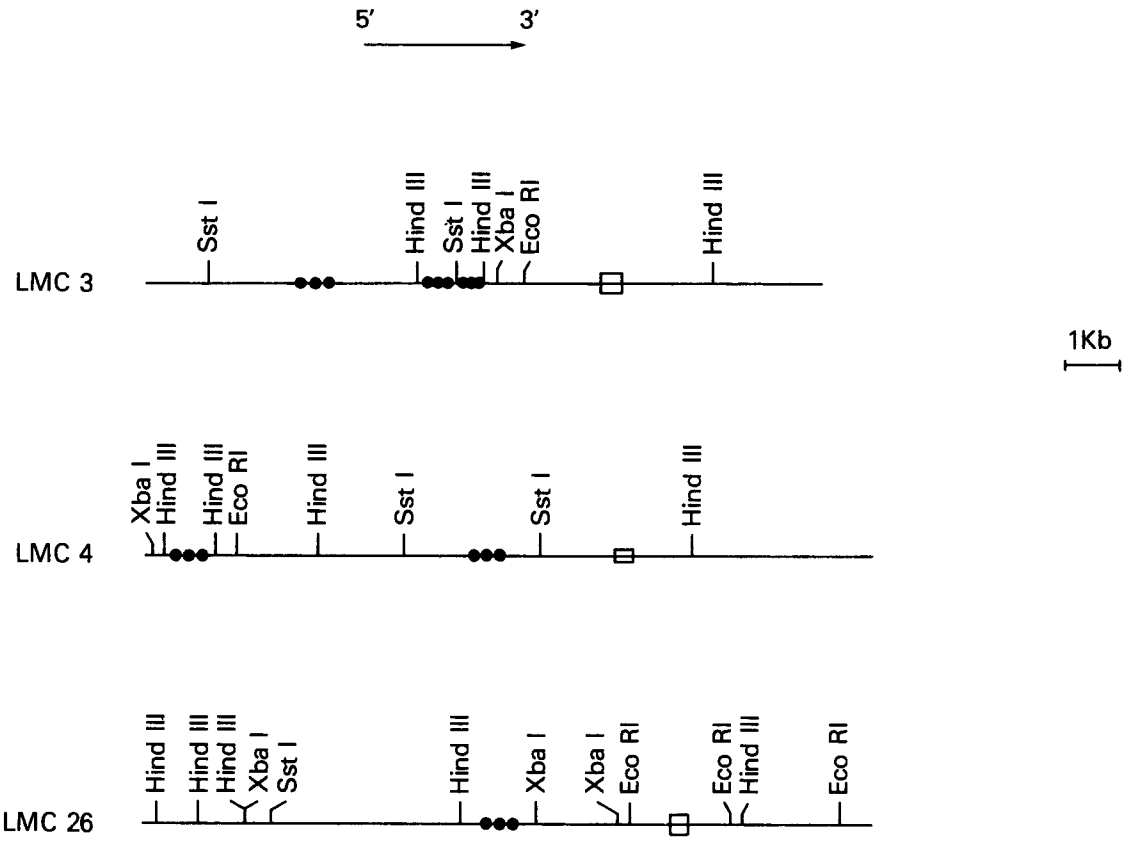

Fig. 3. Restriction maps of clones containing incomplete *c-myc* sequences. Symbols are as in Fig. 4 [8]

mRNA correlate with the amplification of the *c-myc* locus as cloned in λ-LMC-41 [7]. These data suggest that the additional, incomplete *c-myc* sequences may not be functional and may represent pseudogenes.

B. Expression of the *c-myc* Gene in Human Hematopoietic Cells

As detailed elsewhere in this volume (Wong-Staal et al., this volume) [20] the *myc* gene is transcribed into a single 2.7 kb mRNA transcript in all hematopoietic cells examined, where less then a two- to three-fold variation in the mRNA levels was detected. A single exception is HL-60, a human promyelocytic leukemic cell line [3], which expressed *myc*-related sequences at approximately a ten fold greater level compared with other cell lines. Because HL-60 has the unique capacity to differentiate into more mature myeloid cells after induction with several chemical agents, most notably Me_2SO and retinoic acid [1], we wished to determine whether expression of cellular *onc* genes is modulated as a function of myeloid cell differentiation. Figure 4A shows hybridization of the *myc* probe to RNA from untreated HL-60 cells and HL-60 treated with Me_2SO or retinoic acid. Expression of *c-myc* was reduced 80%–90% in HL-60 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 hat persist in culture.

In contrast to *c-myc*, the *c-abl* gene remained expressed at similar levels in the differentiated and undifferentiated cells (Fig. 4B). 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.

C. *C-myc* Amplification in HL-60 DNA

To investigate whether any structural alterations of the *c-myc* gene or adjacent regulatory regions could account for increased levels of *c-myc* expression which were present in HL-60, we analyzed the

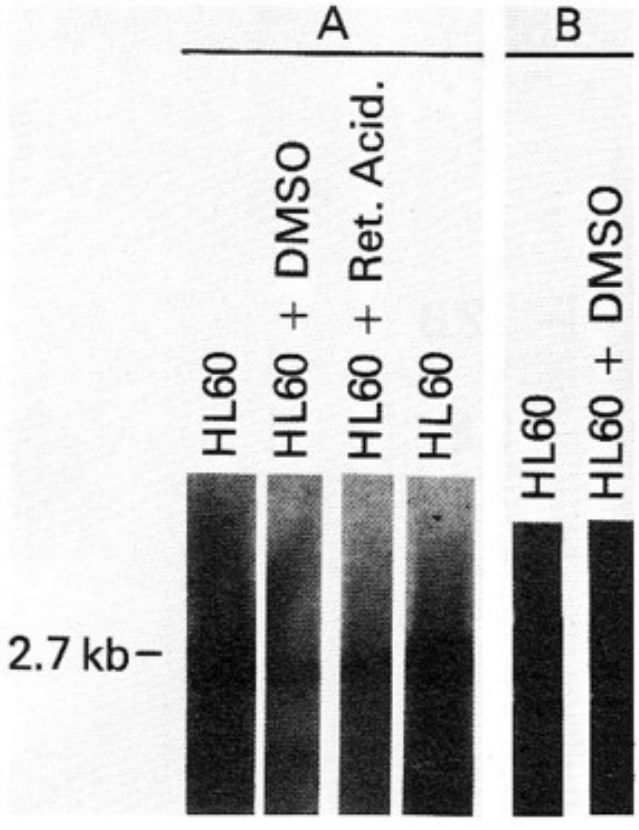

Fig. 4 A, B. Hybridization of *myc* and *abl* probes to RNA from HL-60 induced to differentiate with various agents. Degree of differentiation was judged by the percentage of cells able to reduce the dye nitroblue tetrazolium (NBT), which is a histochemical marker of more mature myeloid cells, and by light microscopic examination of Giemsa-stined cells. **A** Hybridization of *myc* probe to two independent isolates of RNA. Lanes: *a* and *d,* from uninduced HL-60 (<2% of cells were NBT positive); *b,* RNA from HL-60 induced to differentiate with Me_2SO (87% NBT positive); *c,* RNA from HL-60 cells differentiated to 40% NBT-positive cells by using retinoic acid. **B** Hybridization of *able* probe to HL-60 (<2% NBT positive) RNA (lane a) and to RNA from HL-60 induced to 87% NBT positive with Me_2SO (lane b) [20]

structure of *c-myc* sequences in HL-60 by Southern blot hybridization. *Eco*RI digests of DNA from primary and cultured HL-60 cells, as well as from other normal and leukemic DNAs, were hybridized to a probe (pMCO) which contains the entire *v-myc* gene. This probe detects all *c-myc*-related sequences in the human genome, including the pseudogenes [8]. Fig. 5 shows that the 12.8-kb band, corresponding to the functional gene, was markedly increased in the two HL-60 samples, confirming the results shown in Fig. 1. One of the two DNA samples (Fig. 5) was isolated from primary uncultured HL-60 cells obtained from the patient's peripheral blood. This result proves that the amplification event was not a result of cell culture. In contrast, the intensity of the 5.8-kb band, which corresponds to one of the *c-myc* pseudogenes, is uniform in all the samples, suggesting that just the functional gene, but not the pseudogenes, is amplified. Moreover, this band serves a an internal control in proving that the difference in intensity of the 12.8-kb band is not due to experimental artifacts such as irregular DNA transfer or nonuniform hybridization across the nitrocellulose filter. In addition, a 4.6-kb fragment appears in the two HL-60 DNA samples which is not visible in the other samples. The origin of this fragment cannot be explained at the present time. We suggest that it could represent either an unidentified pseudogene which would be part of the amplification unit or a new fragment generated during the *c-myc* amplification event in HL-60 cells. Finally experiments were also performed to quantitate the amplification of *c-myc* in HL-60. For this purpose 30 μg *Sst*I digested HL-60 DNA was sequentially diluted as indicated in Fig. 6, and the intensity of the hybridization band was compared with the one obtained with 30 μg normal human lymphocyte DNA. Hybridization to a probe derived from another human *onc* gene, *c-sis,* which is a single copy gene [4], was used as control in the same experiment. Whereas the intensity of the *c-sis* band is comparable in normal spleen and undiluted HL-60 DNA, a 16- to 32-fold dilution is necessary to bring the *c-myc* band to the same levels as the control DNA. These data indicate that the *c-myc* gene is amplified between 16- and 32-fold in the HL-60 genome.

D. Summary and Conclusions

We have studied the genomic organization of *c-myc* sequences in the human genome. Analysis of different recombinat clones suggest the presence of at least one complete gene and several related sequences which may represent either distantly related genes or pseudogenes. Transcripts from the *c-myc* gene are detectable in a variety of human hematopoietic and non-

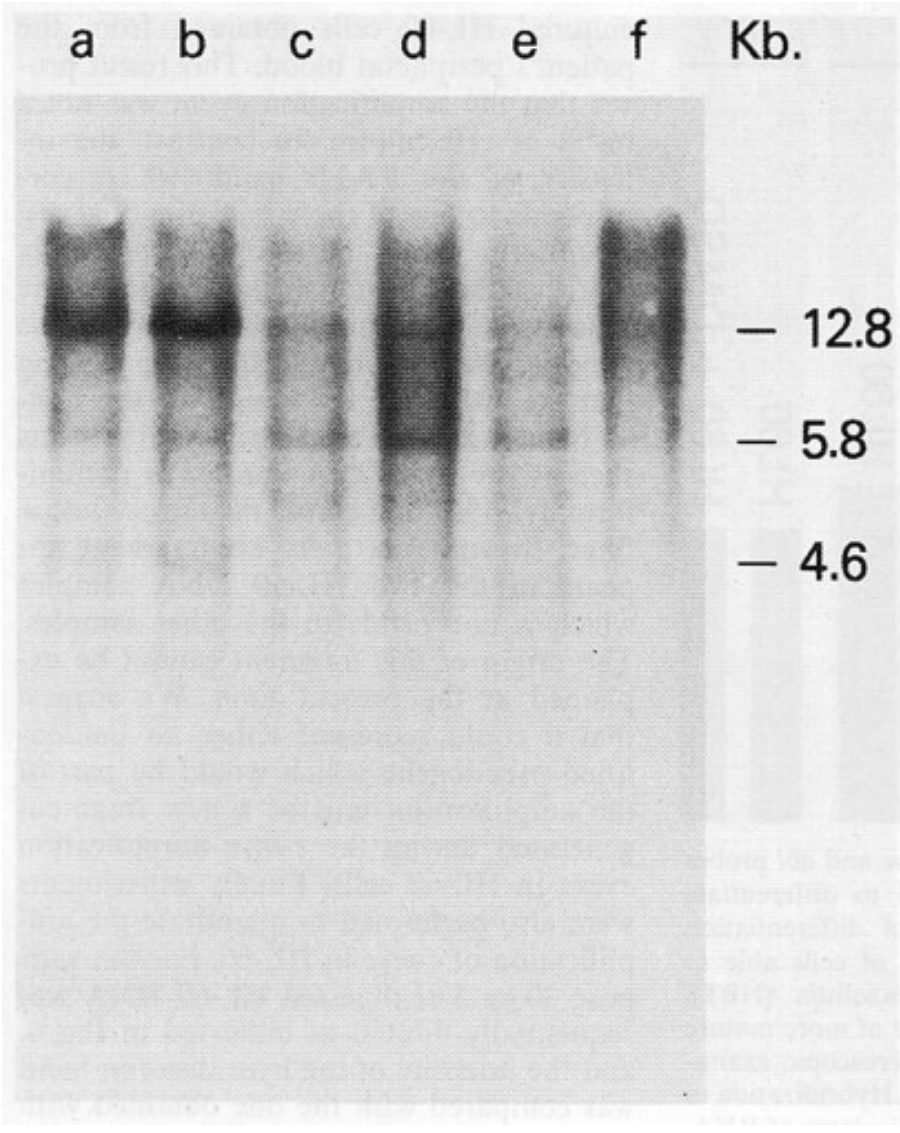

Fig. 5. Hybridization of HL-60 and other human DNAs to *v-myc* probe (pMCO). Thirty micrograms of DNA from different normal and leukemic cells were digested with *Eco*RI and hybridized to a *v-myc* probe (pMCO): *as,* HL-60 p. 70; *b,* uncultured leukemic HL-60; *c,* normal human spleen; *d,* peripheral blood AML cells; *e,* Molt 4 cell line; *f,* peripheral blood normal human lymphocytes [7]

hematopoietic cells, and increased levels of *myc* messenger RNA have been occasionally detected in some neoplastic tissues. The highest levels have been detected in the cell line HL-60 derived from neoplastic cells from a patient with acute promyelocytic leukemia.

Our data indicate that the *c-myc onc* gene is amplified in the genome of the HL-60 cell line, as well as in the original uncultured leukemic leukocytes which were obtained from the peripheral blood of the patient prior to any chemotherapeutic treatment [11]. The levels of *c-myc* amplification do not seem to vary during prolonged cell culture or after indicution to differentiation when the *c-myc* gene is no longer expressed. This result cannot be simply explained by amplification of specific chromosome(s) in these cells since they are hypodiploid, and no hyperdiploidy of individual chromosomes was present [11]. Since tissues other than the leukemic cells are not available from the patient we cannot determine whether *c-myc* was amplified in the other nonleukemic cells of the same individual. The amplification event may have occurred at the germ line level or may represent a normal event during myeloid differentiation analogous to the developmental amplifications of chorion genes during oogenesis in *Drosophila,* or actine genes during myogenesis in chickens (for review see Schimke [19]). Alternatively if *c-myc* amplification occurred just in the leukemic clone, it may

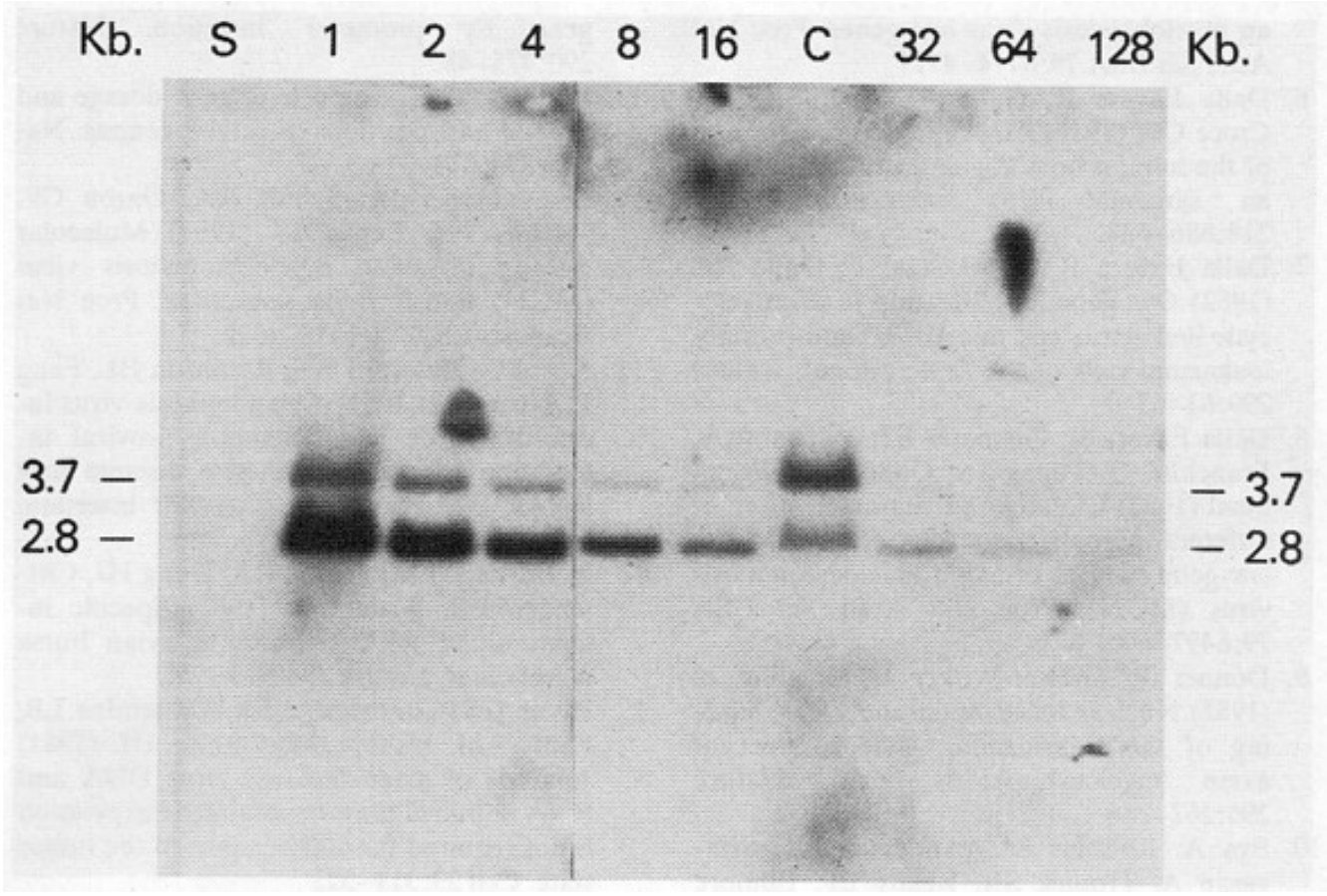

Fig. 6. Estimate of *c-myc* copy number in HL-60 DNA. For this Southern blot diution experiment all DNAs, as listed below, were digested with *Sst*I and simultaneously hybridized to pMC41-3RC and to a human *c-sis* probe (pL335). *C-myc* and *c-sis* hybridization bands in 30 μg DNA from normal human lymphocytes (lane *C*) were used as standards and their intensity compared with that obtained with the same amount of DNA from uncultured HL-60 cells (lane *1*) and serial dilutions of the latter with equal amounts of *Sst*I-digested salmon sperm DNA. The reciprocal of the dilution factor is shown above the lanes. Salmon sperm DNA was chosen as diluting DNA since *c-myc or c-sis* related sequences are not detectable under these conditions of hyridization, as shown in lane *S* (30 μg). The 2.8-kb *c-myc* band in lane *C* is intermediate in intensity between the bands in lane *16* and *32*. The 3.7-kb *c-sis* band closely matches the one in undiluted HL-60 DNA. Therefore, the *c-myc* copy number is between 16- and 32-fold higher in HL-60 compared with the normal standard. In contrast, the unamplified *c-sis* gene is present as a single copy in both genomes [7]

have been involved in the pathogenesis of leukemia in this case. However, the *c-myc* gene is not amplified in a few other cases of acute promyelocytic leukemia tested, suggesting that different mechanisms, including activation of different cellular *onc* genes, may lead to transformation even within similar neoplastic diseases. The validity of the *onc* gene amplification model can be tested in several different tumors expressing high levels of any of the known cellular *onc* genes.

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